J Comp Physiol B (2012) 182:921–934
DOI 10.1007/s00360-012-0668-5

ORIGINAL PAPER

# Impacts of ocean acidification on respiratory gas exchange and acid–base balance in a marine teleost, *Opsanus beta*

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Received: 16 January 2012 / Revised: 11 April 2012 / Accepted: 14 April 2012 / Published online: 12 May 2012


**Abstract** The oceanic carbonate system is changing rapidly due to rising atmospheric $CO_2$, with current levels expected to rise to between 750 and 1,000 μatm by 2100, and over 1,900 μatm by year 2300. The effects of elevated $CO_2$ on marine calcifying organisms have been extensively studied; however, effects of imminent $CO_2$ levels on teleost acid–base and respiratory physiology have yet to be examined. Examination of these physiological processes, using a paired experimental design, showed that 24 h exposure to 1,000 and 1,900 μatm $CO_2$ resulted in a characteristic compensated respiratory acidosis response in the gulf toadfish (*Opsanus beta*). Time course experiments showed the onset of acidosis occurred after 15 min of exposure to 1,900 and 1,000 μatm $CO_2$, with full compensation by 2 and 4 h, respectively. 1,900-μatm exposure also resulted in significantly increased intracellular white muscle pH after 24 h. No effect of 1,900 μatm was observed on branchial acid flux; however, exposure to hypercapnia and $HCO_3^-$ free seawater compromised compensation. This suggests branchial $HCO_3^-$ uptake rather than acid extrusion is part of the compensatory response to low-level hypercapnia. Exposure to 1,900 μatm resulted in downregulation in branchial carbonic anhydrase and slc4a2 expression, as well as decreased $Na^+/K^+$ ATPase activity after 24 h of exposure. Infusion of bovine carbonic anhydrase had no effect on blood acid–base status during 1,900 μatm exposures, but eliminated the respiratory impacts of 1,000 μatm $CO_2$. The results of the current study clearly show that predicted near-future $CO_2$ levels impact respiratory gas transport and acid–base balance. While the full physiological impacts of increased blood $HCO_3^-$ are not known, it seems likely that chronically elevated blood $HCO_3^-$ levels could compromise several physiological systems and furthermore may explain recent reports of increased otolith growth during exposure to elevated $CO_2$.

**Keywords** Climate change · Hypercapnia · pH balance · Carbon dioxide · Gill $HCO_3^-$ uptake

Communicated by I.D. Hume.

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## Introduction

The earth's oceanic carbonate system (partial pressure of $CO_2$, pH and alkalinity) is currently changing 100-times faster than at any point in the past 650,000 years (Caldeira and Wickett 2003; Siegenthaler et al. 2005). In fact, current oceanic $CO_2$ levels represent an increase of 100 μatm (30 %) over pre-industrial levels, equating to an approximate 0.1 pH drop. At the current rate of change, atmospheric $CO_2$ levels are expected to reach between 750 and 1,000 μatm by the end of the century, and 1,900 μatm by 2300, yielding pH reductions of 0.3–0.4 and 0.7–0.8 units, respectively (Caldeira and Wickett 2003). In addition, current day, regional $CO_2$ levels in upwelling zones have been reported to reach levels up to 2,300 μatm (Cai 2011; Feely et al. 2008, 2010; Thomsen et al. 2010). These unprecedented changes in the marine environment pose potentially dramatic challenges for marine organisms and ecosystems. Marine calcifying organisms likely comprise the most at-risk animal group due to effects that ocean acidification has on the $CaCO_3$ saturation state. Shell

formation and maintenance both become more challenging and more energetically costly as saturation state declines (Fabry 2008; Guinotte and Fabry 2008; Orr et al. 2005). Consequently, the effects of ocean acidification on these organisms have been frequently studied leading to reports of significant deleterious effects on coral and crustose coralline algae bleaching and productivity (Albright et al. 2010; Anthony et al. 2008), coral recruitment (Albright et al. 2010), bivalve growth and shell formation (Miller et al. 2009; Talmage and Gobler 2010). Alternatively, high $PCO_2$ levels may aid calcification in coccolithophores, which have increased in size by 40 % over the past 220 years (Iglesias-Rodriguez et al. 2008).

While the general physiological response of fish to hypercapnia (high environmental $CO_2$) is well established (see reviews Brauner and Baker 2009; Perry and Gilmour 2006), only recently have studies begun to focus on environmentally realistic and near future $CO_2$ levels. Such $CO_2$ levels affect larval survival and growth of *Medinia beryllina* (Baumann et al. 2012) and cause tissue damage in larval *Gadus morhua* (Frommel et al. 2012). Further effects for reef fish species include olfactory disturbances that may impede habitat selection (Dixson et al. 2010; Munday et al. 2009b), auditory effects (Simpson et al. 2011), and predator–prey interactions (Ferrari et al. 2011a, b; Munday et al. 2010). This group of effects are related to inhibited neurotransmitter function (Nilsson et al. 2012) and are predicted to have significant impacts on reef fish populations and recruitment (Munday et al. 2010). Furthermore, otolith growth in white sea bass was enhanced at approximately 1,000 and 2,500 μatm (Checkley et al. 2009). As of yet, no work has carefully examined the potential effects that near-future hypercapnia levels may have on respiratory gas exchange and acid–base balance. Generally, studies involving hypercapnia and fish have focused on physiological mechanisms related to acid–base balance and respiratory gas exchange and therefore have typically involved very high $CO_2$ levels of 10,000–50,000 μatm (e.g. Baker et al. 2009; Brauner et al. 2004; Gilmour and Perry 1994; Perry et al. 2010; Tzaneva et al. 2011). These levels result in $CO_2$ diffusion into the animal leading to an acidosis in the plasma and intracellular environment. However, fish have been shown to be tolerant of such acidosis owing to a variety of protective mechanisms (see reviews Claiborne et al. 2002; Perry and Gilmour 2006). Generally, fish protect extracellular fluid pH status by exchanging acid–base equivalents with the environment via branchial mitochondrial-rich cells; tissue intracellular pH is protected in a qualitatively similar pattern (Cameron and Kormanik 1982). With respect to acid equivalents, protons are excreted apically via either a sodium–proton exchange protein (NHE) or a V-type $H^+$ ATPase. Both of these mechanisms occur in freshwater fish while marine animals predominantly use NHEs (Claiborne et al. 2002), likely due to favorable thermodynamic gradients (Potts 1994). Freshwater fish transport $HCO_3^-$ across the basolateral membrane by a sodium bicarbonate co-transport protein (nbc) (Perry et al. 2003). Little is known about basolateral $HCO_3^-$ transport in the gills of marine fish, but nbc is expressed in the gills of gulf toadfish (Taylor et al. 2010) and shows upregulation in response to hypersalinity transfer, presumably to compensate for the associated plasma acidosis.

The current study used three experimental series to examine the effects of low-level hypercapnia on the blood acid–base chemistry of a marine teleost, the gulf toadfish (*Opsanus beta*). In the first experimental series, we tested the hypothesis that acute exposure to predicted future levels of atmospheric $CO_2$ (560, 750, 1,000 and 1,900 μatm) will have significant impacts on the blood acid–base chemistry of toadfish. In series two we investigated the mechanisms of branchial acid–base compensation initiated by low-level hypercapnia, by comparing acid–base flux measurements, gene expression of relevant proteins, and the effects of $HCO_3^-$ free seawater for control animals and those exposed to 1,900 μatm $CO_2$. For the third experimental series, we tested the hypothesis that respiratory acidoses caused by low-level hypercapnia result from a decreased outward gradient of $CO_2$ (1,000 μatm $CO_2$) and are indirectly the result of apparent diffusion limitations of $CO_2$ during branchial capillary transit.

## Methods

### Experimental animals

Adult gulf toadfish ($N = 73$; 30–121 g) were obtained from commercial fisherman in Biscayne Bay, Florida, USA. Once obtained, animals were sorted by size and transferred to 40 l aquaria (>50 g fish, 8–10 per tank; <50 g fish, 20 per tank) supplied with flow through aerated, circulating filtered seawater (22–25 °C) at Rosenstiel School of Marine and Atmospheric Science, University of Miami. After initial transfer to the holding facility fish were treated with Malachite Green (Aquatic Ecosystems) to prevent ectoparasite infection (McDonald et al. 2003). Each tank was supplied with short lengths of polyvinylchloride tubing for use as shelters, and animals were fed twice a week with frozen squid. Food was withheld for at least 48 h prior to experimentation. All experiments were performed using protocols approved by the University of Miami Institutional Animal Care and Use Committee.

### Hypercapnia exposure system

Exposure $CO_2$ concentrations were controlled using a commercially available $PCO_2$/pH feedback controller (DAQ-S; Loligo Systems Inc.) connected to a wtw pH 3310 meter and SenTix 41 pH electrode (Loligo Systems Inc) and controlled using CapCTRL software (Loligo Systems Inc). This automated system used measured pH data and an input $PCO_2$–pH standard curve to determine water $PCO_2$. When chamber $PCO_2$ dropped below the set point, as determined by pH measurements, the system would add pure $CO_2$ gas via an airstone until the $PCO_2$ was returned to the desired level. To prevent excessive fluctuations in $PCO_2$ and over exposure, the $CO_2$ gas rates were manually set to the lowest possible levels that still allowed the desired $PCO_2$ level to be obtained. The $PCO_2$–pH standard curve was generated by equilibrating seawater with normal air or custom $CO_2$–$O_2$ mixed gas (3,090 ppm) and measuring the resulting pH. Periodic measurements of total $CO_2$ were also taken to verify $PCO_2$ concentrations using $CO_2$SYS. A header tank design was not used for exposures because it resulted in excessive variance in water $CO_2$, but it should be noted that equilibrated tanks of either size showed very little within-tank variation in pH/$PCO_2$, as detected using a second pH meter/electrode.

### Surgical procedures

Gulf toadfish ($N = 42$) were affixed with caudal artery catheters to assess the effects of varying levels of hypercapnia on blood acid–base status. This procedure involved anesthetizing the animal using MS-222 (0.5 g/l) buffered with $NaHCO_3$ (1 g/l) and implanting a heparin-saline (0.9 % NaCl) filled catheter (PE-50) into the caudal artery, as outlined by Wood et al. (1997). Animals were allowed to recover for 24–48 h prior to experimentation. Toadfish were equipped with a rectal collection sac to isolate the branchial contribution to acid–base balance, according to the method described by Genz et al. (2008). After surgery, animals were placed directly in exposure tanks, as collection of gut contents would begin immediately after the collection sac was equipped.

### Series I: the effects of low-level hypercapnia on blood acid–base chemistry

Cannulated animals were placed in individual numbered holding tanks (approximately 750 ml) with numerous 2 cm holes to allow for water flow. These holding tanks were placed within larger exposure tanks. Both 30- and 6-l plastic containers with flow-through sand-filtered seawater and aeration were used as exposure tanks. The tank size was determined on the basis of the number of animals exposed at a given time, with the exception of 560 µatm experiments that were all performed in the 6-l tank due to the finer control necessary to reliably maintain the exposure levels. One experimental series consisted of an initial blood sample through the arterial catheter followed by a 24-h exposure period to a given $CO_2$ after which a final blood sample was taken. The tested $CO_2$ exposure treatments were 380 (control), 560, 750, 1,000 and 1,900 µatm $CO_2$. Each cannulated animal was exposed to control conditions and only one of the above $CO_2$ treatments. A second time trial experimental series was performed at 1,000 and 1,900 µatm $CO_2$ and consisted of 0-, 0.25-, 0.5-, 1-, 2-, 4-, 8-, 24-, and 48-h sampling points. A 200-µl blood sample was taken for all sampling points and analyzed for blood pH and plasma total $CO_2$ (see analytical methods). For time trial experiments involving nine sampling points, the removed red blood cells were isolated, resuspended in saline, and re-injected into the animal via the catheter, to prevent confounding effects related to possible anemia. At the termination of experiments, the animals were euthanized by spinal transection.

### Series II: branchial contributions to acid–base compensation during low-level hypercapnia exposure

To isolate the branchial contribution to acid–base compensation in toadfish, animals were first equipped with a rectal collection sac, as described above. The branchial acid–base flux was determined on individual animals in 1.5 l exposure containers, and $PCO_2$ was controlled as described above. The animal was allowed to recover for 4 h during which the exposure containers received seawater flow. After recovery, seawater flow was stopped and a 15-ml initial water sample was taken. After 24 h, a final sample was taken followed by a 4-h chamber flush period, after which another initial sample was taken. Experiments lasted 72 h in total with exact flux times, animal mass and chamber volumes recorded. Both $HCO_3^-$ and $NH_3$ concentrations (see analytical techniques) were determined for all initial and final samples and acid–base equivalent flux rates were calculated for each interval. After 72 h animals were euthanized by an overdose of MS-222 followed by a spinal transection; all intestinal and rectal fluids were collected for other research purposes.

To investigate branchial gene expression and enzyme activity changes in response to low-level hypercapnia, toadfish ($N = 31$; 30–40 g) were placed in a 30-l tank with flowing seawater and allowed to acclimate for 24 h. Control animals ($N = 8$) were sampled for gill and muscle tissue, after which the 1,900-µatm $CO_2$ exposure was initiated. Subsequent samples were taken at 8 h ($N = 8$), 24 h ($N = 8$) and 72 h ($N = 7$) post-exposure. This time window has previously demonstrated gene expression changes in teleost gills and intestinal tissue following environmental

manipulation (Guffey et al. 2011; Ivanis et al. 2008; Sattin et al. 2010; Taylor et al. 2010). Animals were euthanized with an overdose of MS-222, after which muscle tissue (approximately 100 mg) was excised from the dorsal tail region, freeze clamped using liquid nitrogen and stored at −80 °C. The gills were perfused by making a ventral incision to expose the heart and aorta. A 23-gauge needle equipped to a 10-ml syringe filled with heparin-saline was passed through the ventricle and into the aorta after which the atrium was severed. The syringe was slowly depressed to clear the body of blood; successful perfusion was determined based on gill color, and if deemed incomplete was repeated. The procedure took approximately 5 min after which the gill arches were sampled for mRNA and ATPase activity, frozen in liquid nitrogen and subsequently stored at −80 °C. Samples for ATPase activity were frozen in 100 µl of SEI buffer (described in analytical techniques).

For a final set of experiments, animals were exposed to 1,900 µatm $CO_2$ while being held in nominally $HCO_3^-$-free water ($N = 6$). The $HCO_3^-$ free water (469 mM NaCl, 28.2 mM $MgSO_4$, 10.3 mM KCl, 24.6 mM $MgCl_2$) was buffered with 3 mM HEPES free acid and the pH was set to 8.1 using NaOH. A separate $PCO_2$/pH standard curve was created with this water, as described above, which was qualitatively similar to regular seawater in the magnitude of pH change. Additionally, $CO_2$ gassed water was allowed to return to normal under standard aeration to ensure that hypercapnia did not permanently alter pH and thereby give overestimates of $PCO_2$. Post-experiment a 50-ml aliquot of water was analyzed for $HCO_3^-$ by double endpoint titration. Remaining experimental procedures were as described for series I.

### Series III: the effects of carbonic anhydrase infusion on the response to low-level hypercapnia

A third experimental series examined the effects of carbonic anhydrase infusion on blood acid–base chemistry after low-level hypercapnia exposure. Animals were equipped with a caudal artery catheter, as described above, and allowed to recover for 24 h. An initial blood sample of 200 µl was taken and analyzed as in series one, after which 5 mg/kg of bovine carbonic anhydrase was infused through the catheter. Volumes ranged from 113 to 176 µl per animal. The animals were allowed to equilibrate for 1 h after which the $PCO_2$ exposure was initiated. A final blood sample was taken 24 h after exposure and analyzed as described above. An additional 500 µl of blood was removed for analysis of plasma CA activity. Experiments were performed at both 1,000 µatm (outward $CO_2$ diffusion gradient; $N = 4$) and 1,900 µatm (neutral $CO_2$ gradient; $N = 4$), and the response was analyzed in relation to the response of series one animals.

### Molecular techniques

Total RNA was extracted from toadfish gill tissues using RNA Stat-60 reagent (Tel-test Inc, TX, USA) according to manufacturer guidelines, with homogenization performed using motor driven tissue homogenizer. Total RNA was quantified using an ND-1,000 (Thermo Fisher Scientific, DE, USA) spectrophotometer at a wavelength of 260 nm. Prior to cDNA synthesis, a subsample of RNA was DNase-treated with amplification grade DNase I (Invitrogen, CA, USA; manufacturer specifications) to remove potential DNA contamination. Subsequent cDNA synthesis was performed using RevertAid MULV reverse transcriptase (Fermentas, MD, USA), according to manufacturer's specifications. Real-time PCR was performed on an Mx3000P real-time PCR system (Stratagene, CA, USA.) using the Brilliant SYBR green master mix kit (Stratagene, CA, USA.; 12.5 µl reactions). The gene-specific primers are listed in Table 1; new primer sets (slc4a2, slc26a3) were designed using the Fastpcr freeware program. Both the thermocycler set-up and reaction composition were

**Table 1** List of real-time PCR primers used for branchial gene expression analysis

| Gene | Accession # | Orientation | Sequence |
|---|---|---|---|
| EF1α | n.a.[c] | F | AGG TCA TCA TCC TGA ACC AC |
| | | R | GTT GTC CTC AAG CTT CTT GC |
| CAc[a] | GQ443599.1 | F | TCA CTT GAA GGA TGC ACG GAC |
| | | R | GAA GAC CGA CAT GGT TAT CGC |
| slc4a2 | EU016214.1 | F | AGA ACG CTG AGG CCA CAG TT |
| | | R | AGG ACT GAC TCA AGC TCC ACT GC |
| slc26a3 | EU016213.1 | F | TGT CAG CAD TGG ACT CGT CG |
| | | R | AGA TGT GTC TGG AGG TCC CCA G |
| slc26a6[b] | EF529734.1 | F | CTC TCA CTT ATT TAT ACT GTG GTG |
| | | R | GCA GGT TAT GTC TGT AAC ATG C |
| nbc[c] | FJ463158.1 | F | ACC AAA GTT TCT GGG TGT CAG AGA GCA |
| | | R | ACA GCA CAG GCA TAG GGA TGA ACT TTA |

All sequences listed 5′–3′ with the reverse primer sequences listed as the reverse compliment of the gene sequence

[a] Sattin et al. (2010))

[b] Grosell et al. (2009)

[c] Taylor et al. (2010)

performed according to manufacturer guidelines, and dissociation curves were used to assess the primer specificity of each reaction. The PCR efficiency of each primer pair was calculated using a cDNA standard curve. PCR efficiencies ranged from 85 to 103 % with an $R^2 \geq 0.98$. Relative mRNA expression was calculated using the delta–delta ct method using elongation factor 1α (EF1α) as an internal control and the fasted treatment as the relative control (Pfaffl 2001). Successful DNase treatment was verified using a no-reverse transcriptase control for each tissue set.

### Analytical techniques

Blood pH was analyzed using a custom-built gastight, thermostated sleeve equipped with a pH electrode (Radiometer) attached to a MeterLab portable pH meter (Radiometer). Plasma total $CO_2$ was determined using a Corning total $CO_2$ analyzer; red blood cells and plasma were separated by low-speed centrifugation for 1 min using a bench top centrifuge (VWR). Plasma $PCO_2$ was calculated from total $CO_2$ and pH according to the Henderson-Hasselbach equation using the appropriate toadfish plasma constants (Boutilier et al. 1984). Levels of $HCO_3^-$ were assessed using a double-endpoint titration procedure (Hill 1973) as previously described (Genz et al. 2008). Ammonia was determined using colorimetric assays (Verdouw et al. 1978). $Na^+/K^+$ ATPase and V-type ATPase enzyme levels were assessed as previously described (Lin and Randall 1993; McCormick and Bern 1989) and normalized to total protein as determined by the Bradford assay. Plasma CA levels were assessed using the delta pH method (Henry 1991). Intracellular muscle pH was determined using the homogenate method (Portner et al. 1990) and the gastight pH apparatus described above.

### Statistical analysis

Paired student's *t* tests were used for all comparisons on the effects of varying levels of hypercapnia on blood acid–base properties. Time trial analysis of blood gas properties at 1,000 and 1,900 µatm $CO_2$ were assessed by repeated measures analysis of variance (ANOVA). The effects of CA injection and $HCO_3^-$ free water on the hypercapnia response were assessed using two-way repeated measures ANOVA with $CO_2$ status as one factor and the experimental treatment as the other. Effects of hypercapnia on NKA and V-type ATPase levels, muscle $pH_i$ and gene expression were assessed using ANOVA analyses. Branchial acid–base flux data were assessed using an unpaired student's *t* test. The level of significance for all statistical analyses was $P < 0.05$. Also note that power tests were performed on all negative results in an effort to assess whether increased sample size would affect the outcome. The number of animals required to meet statistical significance at a power level of 0.8 at a constant mean difference and standard deviation was deemed excessive.

## Results

### Series I: The effects of low-level hypercapnia on blood acid–base chemistry

The $PCO_2$/pH feedback controller system was successfully able to control 560-, 750-, 1,000- and 1,900-µatm exposure treatments. In general, the measured $PCO_2$ exposures did not exceed the desired set point by more than 5 % for the 560-µatm treatment, 10 % for the 750- and 1,000-µatm treatments and 15 % for the 1,900-µatm treatment. The duration between $CO_2$ bursts was variable between treatments, but was generally less than 90 s. These treatments resulted in seawater pH decreases of approximately 0.15, 0.2, 0.4 and 0.6 for 560, 750, 1,000 and 1,900 µatm, respectively.

Initial experiments tested the effects of 24-h exposure at 560-, 750-, 1,000- and 1,900-µatm $CO_2$ on the blood acid–base chemistry of gulf toadfish (Fig. 1). The 750- ($N = 4$), 1,000- ($N = 6$), and 1,900 ($N = 6$)-µatm exposures all had significantly higher plasma $HCO_3^-$ and $PCO_2$ at 24 h of exposure compared with control measurements. Neither the 560-µatm exposure ($N = 5$) nor the control exposure (380 µatm; $N = 3$) had significant effects on any blood acid–base parameter (paired *t* test); pH was unchanged by 24 h of exposure to any treatment. The mean plasma $HCO_3^-$ concentration appears to increase in a dose-dependent manner with environmental $CO_2$. It should be noted that control plasma $HCO_3^-$ values were significantly lower in the 1,000-µatm treatment than the 560- and 1,900-µatm treatments, likely owing to intrinsic differences between the groups of fish used. No differences were detected between control groups for plasma $PCO_2$ or blood pH. Intracellular $pH_i$ measurements were performed on white muscle samples from toadfish exposed to 1,900 µatm for 8 h and 24 h, as well as unexposed animals (Fig. 2). Exposure resulted in significantly increased $pH_i$ at the 24-h time point compared with unexposed animals, while no change was observed at the 8-h time point.

A time course experiment was performed to more fully characterize the blood acid–base disturbance at both 1,000 and 1,900 µatm $CO_2$ (Fig. 3; $N = 4$). Significant decreases in blood pH relative to initial samples were observed as early as 15 min after the initiation of hypercapnia at both

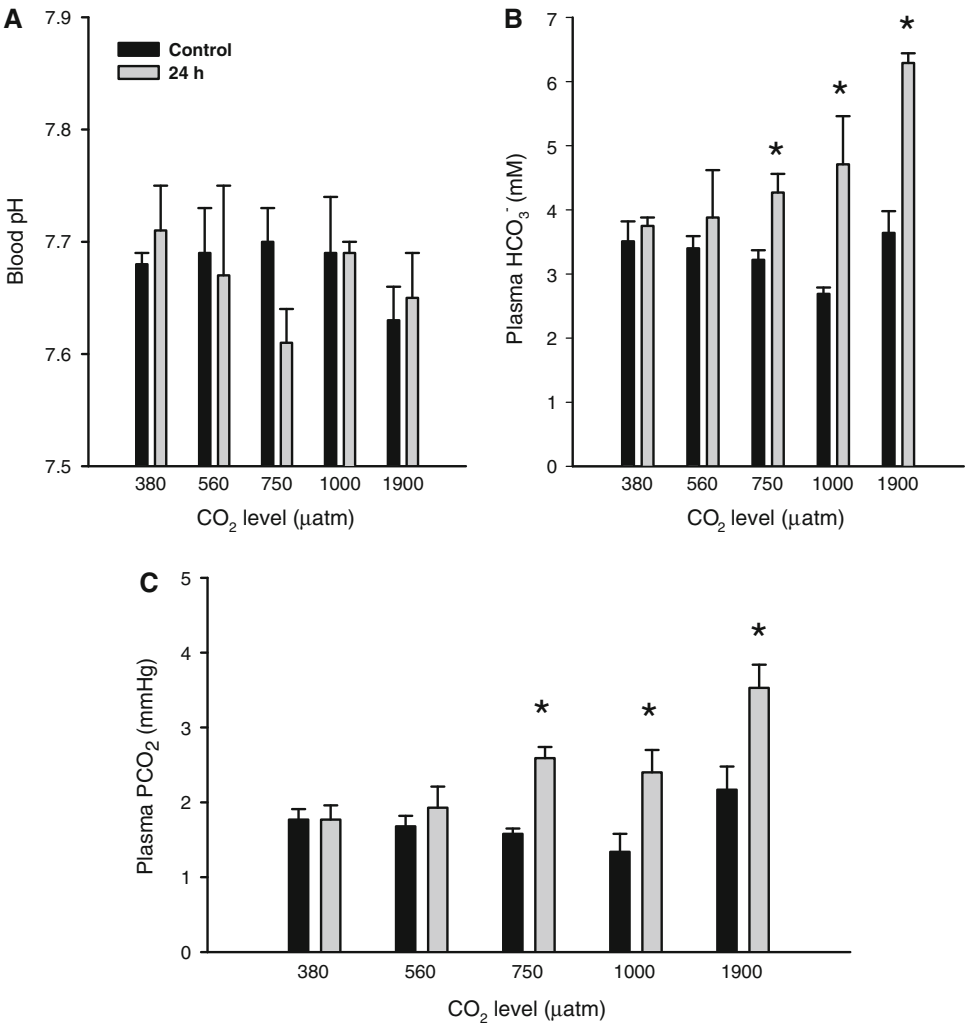


**Fig. 1** The effects of 24-h exposure to low-level hypercapnia on **a** blood pH, **b** plasma $HCO_3^-$ and **c** plasma $PCO_2$ in the gulf toadfish, *Opsanus beta*. *Values* represent mean ± SEM, $N = 3$, 5, 4, 6 and 6 for 380, 560, 750, 1,000 and 1,900 µatm $CO_2$, respectively. A significant difference between control (*black*) and 24-h (*gray*) blood variables is denoted by an *asterisk* (paired *t* test, $P \leq 0.05$)

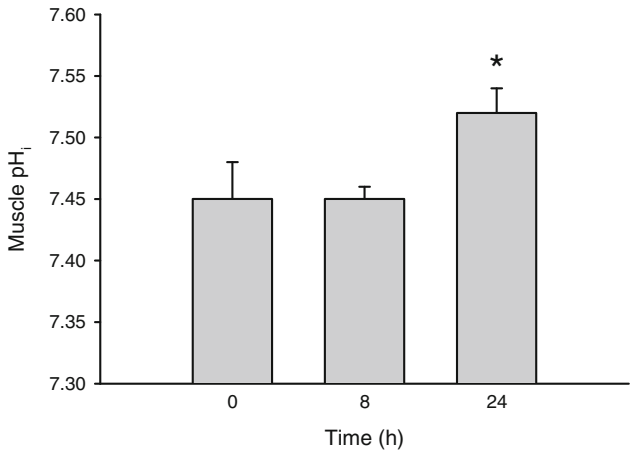


**Fig. 2** The effects of 1,900 µatm $CO_2$ on intracellular white muscle pH ($pH_i$) in the gulf toadfish, *Opsanus beta*. *Values* represent mean ± SEM, $N = 7$. A significant difference from time 0 values is denoted by an *asterisk* (ANOVA, $P \leq 0.05$)

1,000 and 1,900 µatm (Fig. 3a). Blood pH returned to pre-hypercapnia levels by 2 and 4 h in the 1,900- and 1,000-µatm treatments, respectively. The plasma $PCO_2$ increased by 15 min and remained elevated for the duration of the time trial in the 1,900-µatm treatment (Fig. 3b). In the 1,000-µatm treatment a significant difference was not detected until 8 h after exposure was initiated. Plasma $HCO_3^-$ began to increase by 1 h post-exposure in the 1,900-µatm treatment and reached statistical significance by 4 h, remaining elevated for the duration of the exposure (Fig. 3c). The 1,000-µatm treatment showed a peak response in $HCO_3^-$ concentration at 8 h post-exposure. When displayed as $PCO_2$/pH/$HCO_3^-$ diagrams (Fig. 3d, e) the 1,900-µatm data show a response typical of a respiratory acidosis followed by compensation. While the 1,000-µatm data show a similar response over the first 8-h period, the 24- and 48-h data suggest a secondary acidosis in this treatment.

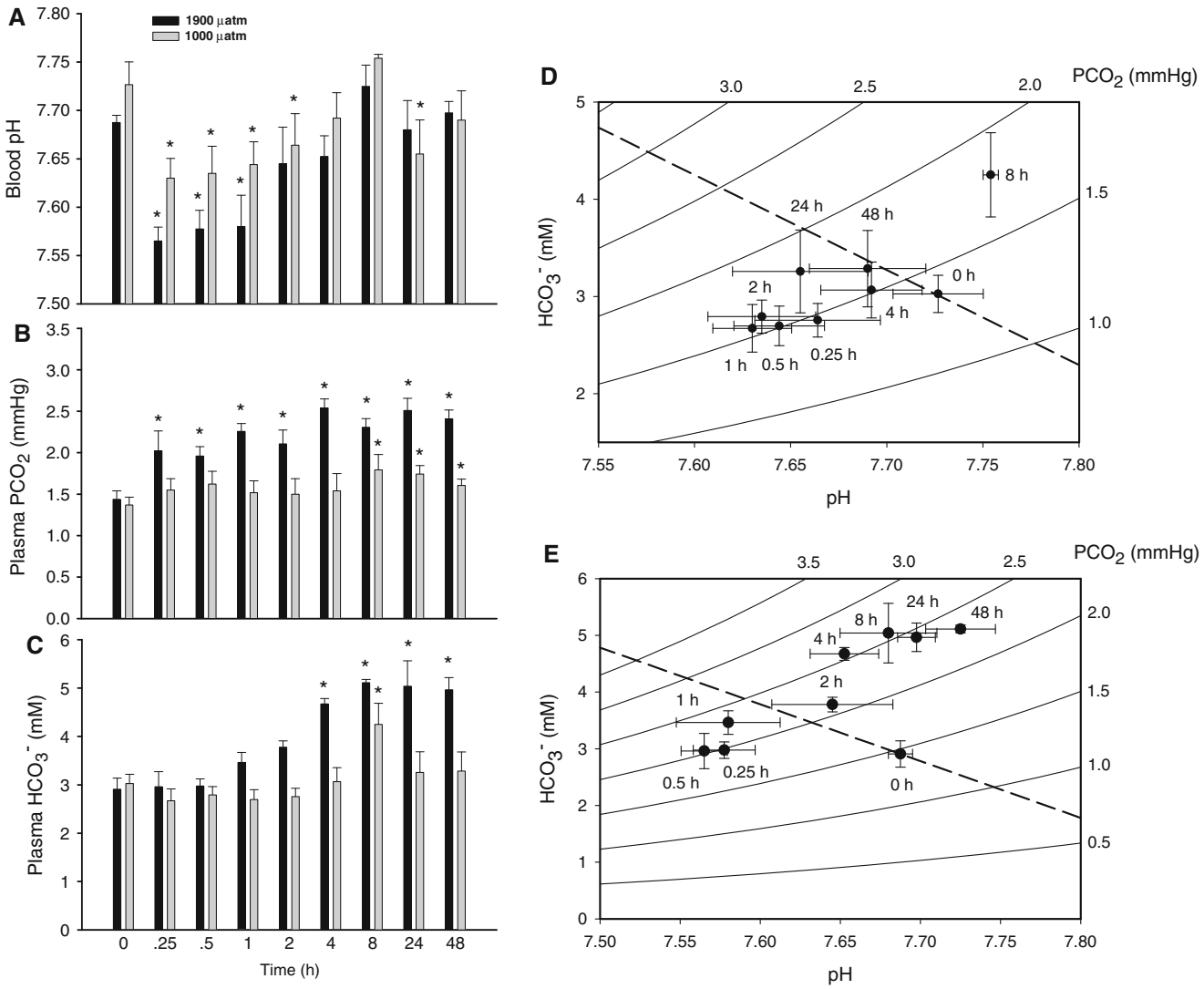


**Fig. 3** A time course of the effects of 1,000 and 1,900 µatm $CO_2$ on **a** blood pH, **b** plasma $PCO_2$ and **c** plasma $HCO_3^-$ in the gulf toadfish, *Opsanus beta*, and expressed as pH/$HCO_3^-$/$PCO_2$ diagrams (D = 1,000 µatm, E = 1,900 µatm, *dashed line* estimated blood buffer line). *Values* represent mean ± SEM, $N = 4$. **a**, **b** and **c**: A significant difference from control values is denoted by an *asterisk* (repeated measures ANOVA, $P \leq 0.05$)

## Series II: branchial contributions to acid–base compensation during low-level hypercapnia exposure

The branchial contribution to acid–base flux during 1,900 µatm $CO_2$ exposures was assessed after equipping animals with rectal collection sacs, which were implanted to remove intestinal contributions to measured acid–base compensation. There were no detectable differences between titratable acid efflux (69 ± 30 and 4.9 ± 44.8), ammonia efflux (18 ± 6.5 and 17.9 ± 4.5) or total acid efflux (85.3 ± 36.8 and 22.8 ± 45.3) between control and the 1,900-µatm-exposed fish, respectively. Further study of the mechanistic pathway for acid–base compensation was performed by exposing animals to hypercapnia in $HCO_3^-$ free (nominally) seawater (Fig. 4). After exposure to 1,900 µatm $CO_2$ for 24 h, toadfish in $HCO_3^-$ free seawater ($N = 6$) were unable to compensate for blood acidosis in a similar manner to animals in normal seawater ($N = 10$). Toadfish exposed to hypercapnia in $HCO_3^-$ free water had significantly lower blood pH after exposure. Plasma $HCO_3^-$ was unaffected by $CO_2$ exposure in $HCO_3^-$ free water, while showing a significant increase in normal seawater. Plasma $PCO_2$ was significantly increased in both $HCO_3^-$ free and control seawater; however, $HCO_3^-$ free exposed animals had a lower increase as compared with control water animals.

Branchial gene expression of known $HCO_3^-$ transporters, including slc26a3, slc26a6, slc4a2 and the sodium bicarbonate co-transporter (nbc), as well as cytoplasmic carbonic anhydrase (CA) was examined at 0, 8, 24 and 72 h post-exposure to 1,900 µatm $CO_2$. A significant

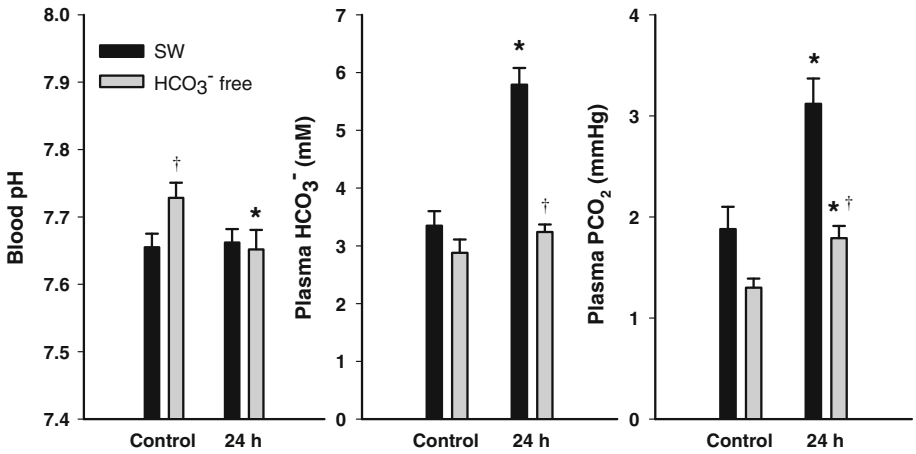


**Fig. 4** The effects of $HCO_3^-$ free seawater on the blood acid–base response to 24-h exposure to 1,900 µatm $CO_2$ in the gulf toadfish, *Opsanus beta*. Significant effects of hypercapnia within a treatment are denoted by an *asterisk*, while a significant difference between treatments is denoted by a *dagger* ($N = 10$ and 6 for control and $HCO_3^-$ free seawater, respectively; 2-way repeated measures ANOVA, $P \leq 0.05$)

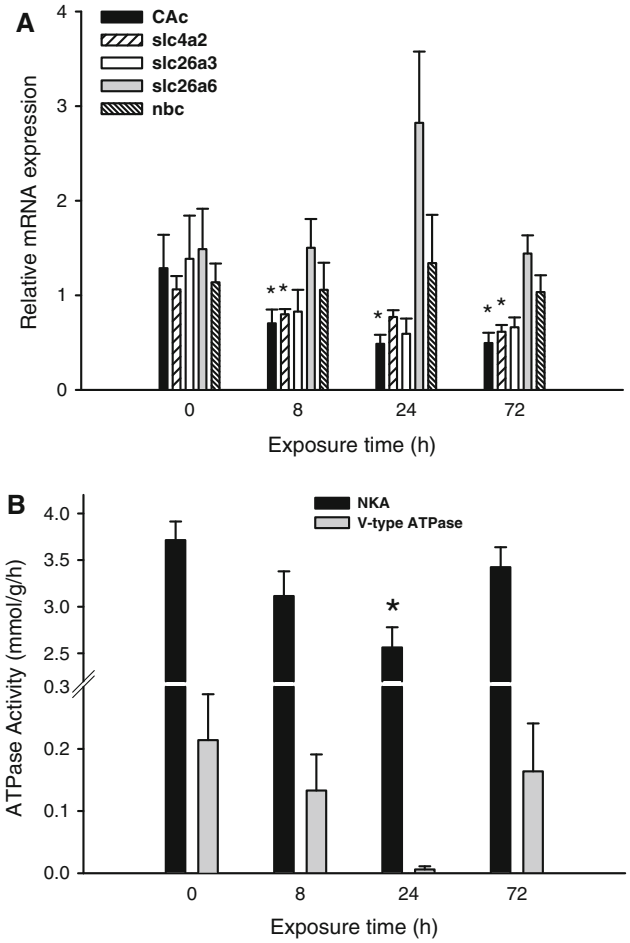


**Fig. 5** The effects of exposure to 1,900 µatm $CO_2$ on **a** branchial gene expression, and **b** $Na^+/K^+$ (NKA) and V-type $H^+$ATPase enzyme activity. Significant differences versus control samples are denoted by an *asterisk* (ANOVA, $N = 7-8$)

decrease in CA expression was observed at 8, 24 and 72 h post-exposure, while slc4a2 had decreased expression at 8 and 72 h post-exposure. No effects on gene expression were observed for nbc, slc26a3 or slc26a6. $Na^+/K^+$ ATPase enzyme activity was significantly decreased at 24 h relative to the 0 h time point and returned to control levels by 72 h of exposure (Fig. 5b). Although not statistically significant, V-type $H^+$ ATPase enzyme activity showed a similar trend (Fig. 5b).

### Series III: the effects of carbonic anhydrase infusion on the response to low-level hypercapnia

Experiments examining the effects of 5 mg/kg bovine CA infusion into the blood were performed at both 1,000 and 1,900 µatm ($N = 4$ each; Fig. 6). The blood acid–base profile of CA infused animals after 24-h exposure to 1,900 µatm $CO_2$ conformed to trends of non-CA infused animals for all three blood parameters (Fig. 6a). In contrast, infusion of CA in combination with 24-h exposure to 1,000 µatm resulted in significantly elevated blood pH relative to both paired pre-exposure measurements and non-infused toadfish post-exposure measurements. Furthermore, plasma $PCO_2$ was not significantly elevated after hypercapnia exposure in CA infused animals; plasma $HCO_3^-$ conformed to control animal trends with a significant increase after 24 h hypercapnia exposure. The amount of CA in the plasma of injected toadfish after 24 h was 250 ± 120 and 932 ± 191 enzyme units for the 1,900- and 1,000-µatm treatments, respectively. Non-injected animals contained only 60 ± 23 enzyme units, where a single enzyme unit is defined as a doubling of the uncatalyzed rate.

## Discussion

The current study has demonstrated that acute exposure to $CO_2$ levels relevant for present time upwelling zones and near-future climate change result in a respiratory acidosis in toadfish and compensation for this respiratory acidosis depends on seawater $HCO_3^-$ uptake. We have also demonstrated that the less favorable partial pressure gradients observed at 1,000 µatm can be compensated for through

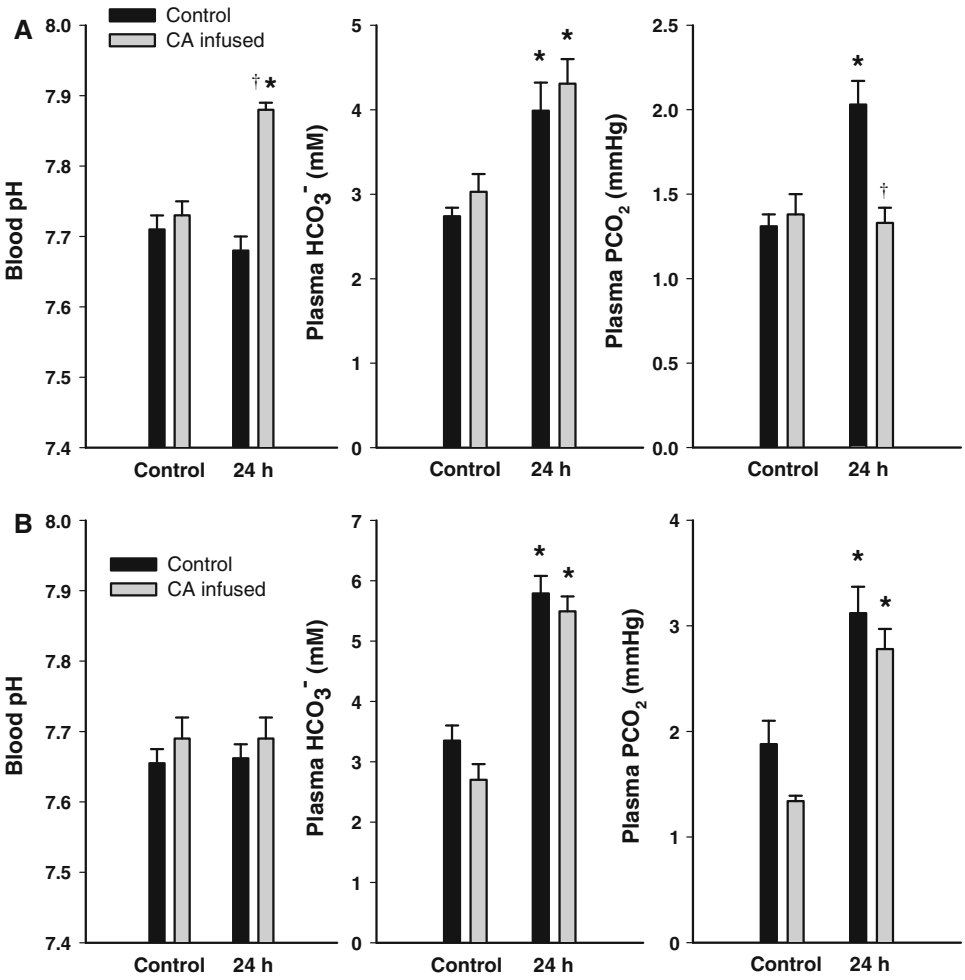


**Fig. 6** The effects of carbonic anhydrase (CA) infusion (5 mg/kg) on the blood acid–base response to 24-h exposure to **a** 1,000 μatm and **b** 1,900 μatm $CO_2$ in the gulf toadfish, *Opsanus beta*. Significant effects of hypercapnia within a treatment are denoted by an *asterisk*, while a significant differences between treatments is denoted by a *dagger* ($N = 10$ and 4 for control and CA infusion, respectively; 2 way repeated measures ANOVA, $P \leq 0.05$)

CA infusion into the blood, which acts to lower the chemical equilibrium constraints governing $CO_2$ excretion.

Hypercapnia as an experimental treatment has been extensively studied, both in freshwater and marine fish. However, the majority of studies in this area have utilized hypercapnia as a means to study mechanisms of acid–base and ion transport, or cardiorespiratory physiology, and have used environmentally unrealistic $CO_2$ levels ranging from 1 to 5 % to highlight physiological effects and mechanisms. Only recently has focus turned to the possible impacts of environmentally relevant $CO_2$ levels related to climate change and ocean acidification or current regionally elevated $CO_2$ levels, with documented impacts of 1,000 μatm $CO_2$ on embryo survival and growth in *M. beryllina* (*Baumann* et al. 2012), olfactory responses and aerobic scope in several reef fish species (Dixson et al. 2010; Munday et al. 2009a, b), as well as otolith growth in white sea bass (Checkley et al. 2009) and tissue damage in larval Atlantic cod (Frommel et al. 2012). The results of the current study further support the hypothesis that predicted future levels of atmospheric $CO_2$, levels that are estimated to occur within the next 100 years will pose physiological challenges to marine teleosts.

Exposure to 24-h hypercapnia at levels of 750-, 1,000- and 1,900 μatm $CO_2$ resulted in significantly increased plasma $HCO_3^-$ and $PCO_2$. It seems likely that the increase in $HCO_3^-$ observed at 750 μatm is the result of blood non-$HCO_3^-$ buffering, assuming a non-$HCO_3^-$ buffer value of 8–10 as is typical of teleosts (Tufts and Perry 1998). However, the findings at 1,000 and 1,900 μatm are consistent with the long-established response to hypercapnia of respiratory acidosis followed by metabolic compensation owing to raised plasma $HCO_3^-$ (Lloyd and White 1967). This pattern is further supported by time trial experiments at both 1,000 and 1,900 μatm $CO_2$, where acidosis occurred in both treatments within 15 min followed by progressively increased $HCO_3^-$ concentrations until the acidosis was alleviated. Interestingly, while blood acid–base chemistry parameters remained stable at 1,900 μatm after compensation (after 8 h), there was more substantial variation in the 1,000-μatm treatment. More specifically, plasma $HCO_3^-$ peaked at 8 h and subsequently fell at 24 and 48 h

of exposure; plasma pH was significantly depressed at 24 h. This suggests an incidence of secondary respiratory acidosis at these time points, likely indicating that after initial compensation toadfish stopped retaining $HCO_3^-$ until plasma pH again began to decrease. Of greater interest, however, is that low levels of hypercapnia affect blood acid–base chemistry in an apparent dose–response pattern, whereby higher levels of hypercapnia resulted in larger changes in plasma $HCO_3^-$ and $PCO_2$ (Fig. 1). This pattern has been previously documented for higher levels of hypercapnia in a number of species (Baker et al. 2009; Brauner et al. 2004; McKenzie et al. 2002, 2003; Perry et al. 2010), but this is the first report of such low levels of hypercapnia having similar effects.

Exposure to 1,900 μatm $CO_2$ also resulted in elevated white muscle intracellular pH ($pH_i$). Similar results for muscle $pH_i$ have been observed for higher levels of hypercapnia in heart, brain and liver of the white sturgeon (Baker et al. 2009), although not in the white muscle. The increased muscle $pH_i$ likely stems from metabolic compensation to an initial acidosis similar to that observed in the plasma. In fact, 48 h of hypercapnia (1.5 kPa; ≈ 15,000 μatm) in white sturgeon increased white muscle intracellular $HCO_3^-$ threefold (Baker et al. 2009), while that of 24 h resulted in a twofold $HCO_3^-$ increase in the white muscle of the armored catfish (Brauner et al. 2004); $pH_i$ was unaffected in both species. It is somewhat surprising that muscle $pH_i$ was affected at such low levels of hypercapnia; however, because $pH_i$ is typically closer to the $CO_2$ equilibrium constant ($pK_I$) than plasma pH, a small increase in $HCO_3^-$ will have a greater impact on muscle $pH_i$. Conversely, a smaller intracellular $PCO_2$ increase will also have a more profound impact. The increased muscle $pH_i$ may reflect active regulation by the muscle via $HCO_3^-$ uptake from the extracellular fluid in response to intracellular acidosis, or it could also reflect passive $HCO_3^-$ uptake facilitated by the greater extracellular concentrations resulting from extracellular pH compensation.

A typical response to plasma acidosis is the net efflux of acid equivalents into the environment (Claiborne et al. 2002). However, no change in branchial titratable acid excretion, ammonia excretion or net acid excretion was observed over a 72-h exposure to 1,900 μatm $CO_2$. Although the current study took effort to eliminate the intestinal contributions of acid–base balance (reviewed by Taylor et al. 2011), no effort was made to prevent renal contributions. However, gulf toadfish are aglomerular, so renal contributions to acid–base balance are likely negligible, as previously demonstrated for hypersalinity-induced acidosis (Genz et al. 2011). As such these results are likely representative of branchial flux. It may seem surprising that no net efflux of acid equivalents was detected given the clear compensatory responses described above; however, the variability in these measurements likely made detecting small differences difficult in the non-paired experiments.

As described earlier, marine teleosts are known to compensate for blood acidosis by excreting protons through the combined action of apical NHE, basolateral NBC and intracellular CA found in the mitochondrial rich cells of the branchial epithelium (Claiborne et al. 2002; Perry and Gilmour 2006). Interestingly, exposure to 1,900 μatm $CO_2$ in nominally $HCO_3^-$ free seawater (182 μM) prevented metabolic compensation by toadfish, as shown by the persistent acidosis and unchanged plasma $HCO_3^-$ in combination with a significantly increased $PCO_2$ (Fig. 4). While the increased $PCO_2$ resulting from 1,900 μatm exposure is not equivalent between the seawater and $HCO_3^-$ free treatments, the $HCO_3^-$ free blood parameters are qualitatively similar to those described for the pre-compensation time points (15 min) of the time trial experiment (Fig. 3). It seems likely that the higher $PCO_2$ in control seawater results from an inability to excrete the $CO_2$ that is generated during pH compensation after $HCO_3^-$ uptake. Overall, these results suggest that toadfish, in contrast to hypotheses, rely on environmental $HCO_3^-$ uptake to drive pH compensation.

It is not easy to reconcile $HCO_3^-$ uptake from seawater with current models of seawater branchial ion transport (Fig. 7). To further examine this phenomenon the effects of low-level hypercapnia on the mRNA expression of a suite of $HCO_3^-$ transporters in the gill were examined. While time points for transcriptional studies can often be difficult to identify, studies of osmoregulatory and acid–base disturbances have routinely reported transcriptional responses at 24 h post-exposure (e.g. Gilmour et al. 2011; Ivanis et al. 2008). The expression of cytoplasmic CA, the cytoplasmic isoform found in the gills of teleosts (Esbaugh et al. 2005; Sattin et al. 2010), was also examined. Both cytoplasmic CA and slc4a2 were slightly but significantly down regulated in response to 1,900 μatm hypercapnia (Fig. 5a). The slc4a2 anion exchanger is an electroneutral transporter, presumably in the apical membrane that would only function in the $Cl^-$ uptake/$HCO_3^-$ excretion direction in seawater (Romero et al. 2004), so down regulation in response to an acidosis is obviously beneficial. The role of branchial CA is to hydrate $CO_2$ to provide $H^+$ and $HCO_3^-$ for transport processes (Gilmour and Perry 2009), which in the case of an acidosis involves excreting $H^+$ apically and reabsorbing $HCO_3^-$ across the basolateral membrane. Interestingly, down regulation of CA supports the observation that compensation is due to $HCO_3^-$ uptake from the environment, since CA activity would have no benefit to such a pathway and may only interfere with the transcellular movement of $HCO_3^-$ from the environment to the plasma.

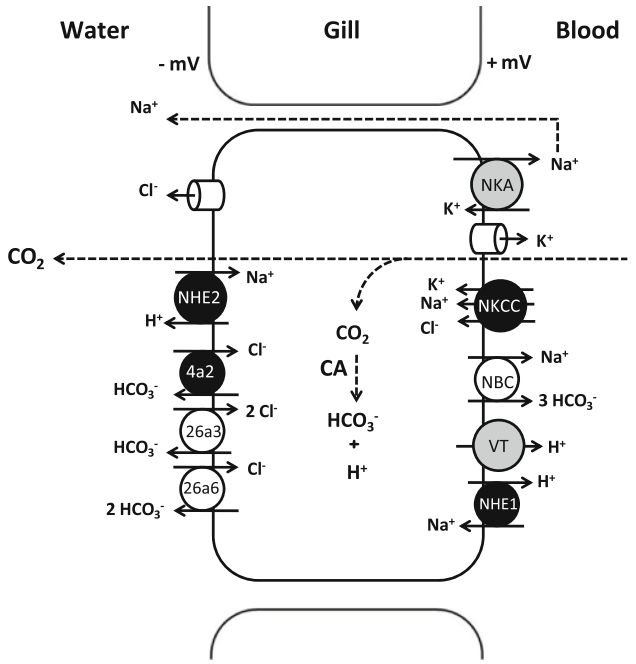


**Fig. 7** Mechanistic model of branchial ion transport in gulf toadfish, *Opsanus beta*. During an acidosis both intracellular carbonic anhydrase (CA) and slc4a2 expression are down regulated, and $Na^+$ $K^+$ ATPase (NKA) activity is reduced. Decreased basolateral NKA activity could act to increase cytoplasmic $Na^+$ to aid in $HCO_3^-$ uptake via NBC and reduce $H^+$ uptake through NHE1. Downregulation of slc4a2 would prevent $HCO_3^-$ loss, while down regulated CA would prevent $CO_2$ trapping supporting the finding that compensation is the result of environmental $HCO_3^-$ uptake. *Gray circles* are ATPases, *black circles* are electroneutral transporters and *white circles* are electrogenic transporters

No significant effects of 1,900 µatm $CO_2$ were observed on gene expression of the available branchial electrogenic $HCO_3^-$ transporters (Fig. 5a); however, examination of the enzyme activity of NKA revealed a significant decrease after 24 h of exposure. This reduction returned to control levels by 72 h of exposure, and a similar but not significant pattern was observed for V-type ATPase activity. Such decreases in NKA and V-type ATPase were observed for mRNA expression in Atlantic salmon exposed to 1 and 3 days of 20 matm $CO_2$ (Seidelin et al. 2001), and exposure to 10 matm $CO_2$ also resulted in decreased expression of the a1a subunit of NKA in eelpout (Deigweiher et al. 2008). Although these results do not give clear indication of the mechanisms of $HCO_3^-$ uptake, they do provide insight into basolateral aspects of transport. It is relatively well established that basolateral $HCO_3^-$ transport occurs via nbc (Marshall 2002; Perry et al. 2003; Perry and Gilmour 2006; Sussman et al. 2009), where NHE1 is also localized to move protons into the plasma. It seems likely that lower NKA activity at the basolateral membrane could lessen the $Na^+$ gradient by elevating cytoplasmic $Na^+$ levels. This would reduce proton movement back into the plasma via NHE1 and aid the gradient for $HCO_3^-$ transport via nbc. It is important to note that lowered NKA activity could have detrimental effects on $Na^+$ efflux (Marshall 2002; Marshall and Grosell 2006). This trade off may explain why NKA activity returns to control levels by 72 h after the onset of exposure and may also suggest that an alternate pathway—such as the NHE pathway—could become more dominant during chronic exposures. Although it cannot be excluded that the time points chosen failed to capture early and transient expression changes of some of the selected genes, changes in mRNA expression in teleost gill and intestinal tissue following environmental manipulations are often reported to occur at the time points chosen in the present study (e.g. Gilmour et al. 2011; Guffey et al. 2011; Ivanis et al. 2008; Sattin et al. 2010; Taylor et al. 2010).

Unlike previous studies examining hypercapnia the levels used in the current study largely represent outward $CO_2$ diffusion gradients (Table 2). On this basis the current findings are surprising and suggest that $CO_2$ diffusion is limited under these circumstances owing to the decreased partial pressure gradients. It is important to remember that even under normal conditions $CO_2$ transport in fish is constrained by an *apparent* diffusion limitation, which is imposed by access to red blood cell CA via AE1 anion exchange (Esbaugh and Tufts 2006; Perry 1986; Perry et al. 2009; Tufts and Perry 1998). This is simply because the vast majority of the blood total $CO_2$ load exists as plasma $HCO_3^-$, which must be transported into the red blood cell through AE1 where it is hydrated by CA to form $CO_2$ before subsequently diffusing across the gill (see reviews Perry et al. 2009; Tufts and Perry 1998). A number of studies have previously shown that this *apparent* diffusion limitation caused by chemical equilibrium constraints during capillary transit can be alleviated by CA infusion into the blood stream (Desforges et al. 2001, 2002; Julio et al. 2000), thereby removing the rate limiting step to chemical equilibration.

We sought to investigate whether the respiratory acidosis caused by low-level hypercapnia was caused by a

**Table 2** The partial pressure of carbon dioxide ($PCO_2$) in seawater equilibrated to various environmental carbon dioxide levels and mean pre-exposed *Opsanus beta* plasma

| Sample | $PCO_2$ (mmHg) |
|---|---|
| Toadfish plasma | 1.53 ± 0.06 |
| Seawater 380 µatm | 0.29 |
| Seawater 560 µatm | 0.43 |
| Seawater 750 µatm | 0.57 |
| Seawater 1,000 µatm | 0.76 |
| Seawater 1,900 µatm | 1.45 |

true diffusion limitation, or an *apparent* diffusion limitation. In other words, would decreasing the chemical equilibrium constraints during capillary transit prevent the respiratory acidosis. To investigate this, toadfish were infused with 5 mg/kg of CA via a caudal artery catheter and exposed to either 1,900 µatm $CO_2$ (Fig. 6a) or 1,000 µatm $CO_2$. We predicted that CA infusion would prevent the respiratory acidosis at 1,000 µatm due to the outward diffusion gradient of $CO_2$, while having little to no effect during exposure to 1,900 µatm. Previous experiments have shown that 5–10 mg/kg doses of bovine CA cause respiratory alkalosis within 1 h post infusion (Desforges et al. 2001; Wood and Munger 1994). As predicted, the infusion of CA had no effect relative to controls on the blood acid–base parameters during exposure to 1,900 µatm $CO_2$. In contrast, exposure to 1,000 µatm $CO_2$ (Fig. 6b) in combination with CA infusion resulted in a significantly different response in both plasma pH and $PCO_2$. This response, as predicted, is consistent with CA infusion ameliorating the chemical equilibrium constraints that limit $CO_2$ diffusion. The observed respiratory acidosis is therefore the net result of a lower outward $CO_2$ diffusion gradient combined with chemical equilibrium constraints that limit the mobilization of plasma $HCO_3^-$ during capillary transit. This is similar to the results of Julio et al. (2000), which showed that increased arterial $PCO_2$ stemming from reduced gill surface area was corrected by CA infusion. To our knowledge this is the first study to show that $CO_2$ chemical equilibrium constraints can impact excretion when confronted with only marginally less favorable diffusion gradients.

It is noteworthy that plasma $HCO_3^-$ also increased with CA infusion, suggesting some degree of metabolic compensation. This is corroborated by the increased pH, since a steady $PCO_2$ would result in constant pH provided $HCO_3^-$ was also stable. These results may be indicative of physiological sensing of elevated $PCO_2$ by externally oriented chemoreceptors, which could ultimately initiate $HCO_3^-$ uptake pathways. Similar findings have been reported for the cardiovascular effects of hypercapnia on rainbow trout (Perry and McKendry 2001); however, to our knowledge the connection between $CO_2$ sensing and cellular acid–base compensation mechanisms has yet to be made.

## Significance

Although the idea of hypercapnia as a physiological stress is not new, the current results are the first to demonstrate significant impacts on acid–base and respiratory physiology of low-level hypercapnia, levels currently found regionally and are predicted globally within 50 years. However, the potential concern to this group of animals is not likely to stem directly from these systems. Instead, the potential concerns lie in the downstream impacts of chronic exposure, most notably the energetic cost and tradeoffs associated with defending acid–base status in the presence of elevated $CO_2$. For example, red blood cell and tissue $pH_i$ must be protected to prevent potential effects on oxygen uptake and other biochemical pathways. Acid–base disturbances can also impact cardiorespiratory variables such as ventilation rate, blood pressure and heart rate (Gilmour and Perry 2006). Furthermore, because otolith growth is proportional to $HCO_3^-$ concentrations of the medium surrounding the saccular epithelium (Tohse and Mugiya 2001), it seems likely that elevated plasma $HCO_3^-$ stemming from pH regulation may account for the otherwise counter-intuitive observations of increased otolith growth in white sea bass at 1,000 and 2,500 µatm (Checkley et al. 2009). Furthermore, chronically elevated plasma $HCO_3^-$ is likely to dramatically affect gastrointestinal $HCO_3^-$ excretion, as well as $Cl^-$, $Na^+$ and water uptake owing to the kinetics of the rate limiting basolateral nbc (Taylor et al. 2010)—effects that are currently under investigation. Admittedly, over time fish may adapt to a low-level hypercapnia environment and therefore not rely on acid–base compensation processes. Such adaptation could involve morphological changes in the gill to alter $CO_2$ permeability, or as theoretically demonstrated here, fish may alter the $CO_2$ chemical equilibrium constraints in the blood that lead to an acidosis. Such adjustments could include increased haematocrit, increased red blood cell anion exchange capacity or decreased branchial diffusion distance. Clearly, the physiological implications of imminent levels of hypercapnia require further study.

**Acknowledgments** Financial support for this work was provided by a Natural Science Foundation grant to M. Grosell (IOS-0,743,903 and 1,146,695).

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