

SCIENTIFIC REPORTS



OPEN

Elevated CO₂ increases energetic cost and ion movement in the marine fish intestine

Rachael M. Heuer[†] & Martin Grosell

Received: 28 June 2016
Accepted: 14 September 2016
Published: 29 September 2016

Energetic costs associated with ion and acid-base regulation in response to ocean acidification have been predicted to decrease the energy available to fish for basic life processes. However, the low cost of ion regulation (6–15% of standard metabolic rate) and inherent variation associated with whole-animal metabolic rate measurements have made it difficult to consistently demonstrate such a cost. Here we aimed to gain resolution in assessing the energetic demand associated with acid-base regulation by examining ion movement and O₂ consumption rates of isolated intestinal tissue from Gulf toadfish acclimated to control or 1900 μatm CO₂ (projected for year 2300). The active marine fish intestine absorbs ions from ingested seawater in exchange for HCO₃[−] to maintain water balance. We demonstrate that CO₂ exposure causes a 13% increase of intestinal HCO₃[−] secretion that the animal does not appear to regulate. Isolated tissue from CO₂-exposed toadfish also exhibited an 8% higher O₂ consumption rate than tissue from controls. These findings show that compensation for CO₂ leads to a seemingly maladaptive persistent base (HCO₃[−]) loss that incurs an energetic expense at the tissue level. Sustained increases to baseline metabolic rate could lead to energetic reallocations away from other life processes at the whole-animal level.

Although fish respond effectively and restore blood pH during CO₂ induced acid-base balance disturbance, recent studies have noted potential impacts of ocean acidification across a range of areas including neurosensory disruptions, increased otolith growth, altered mitochondrial function, and changes to metabolic rate^{1–9}. A suggested unifying hypothesis is that the compensatory response induced during CO₂ exposure to correct blood pH may have negative downstream consequences or induce tradeoffs^{1,4,10}. This pH compensation is associated with a sustained increase of extra and intracellular concentration of HCO₃[−] in response to the elevated partial pressure of CO₂ (pCO₂)^{11–15}. Assuming the rate of adaptation does not keep pace with a rapidly acidifying ocean, fish in future oceans will require consistent and elevated levels of ion exchange to sustain elevated HCO₃[−] and normal pH, a process that is anticipated to add to the cost of basic maintenance of homeostasis, most often quantified as standard metabolic rate (the minimum O₂ consumption rate of a resting animal in the post-absorptive state¹⁶).

The gill, intestine, and kidney are the organs involved with acid-base balance and osmoregulation in marine fish^{13,17}. Estimates vary widely, but metabolic cost of ion regulation has been proposed to range from 1–30% or 6–15% of whole-animal standard metabolic rate (reviewed in ref. 18,19). More targeted estimates of ion transport using specific isolated organs suggest that the gills and intestine account for ~4%²⁰ and 5.6%²¹ of standard metabolic rate, respectively, which combined would account for about 10% of whole-animal standard metabolic rate. Interestingly, despite the need for HCO₃[−] retention during a CO₂-induced acidosis, toadfish experience ~34% increase in HCO₃[−] ion loss through the intestine when exposed to near-future CO₂ scenarios (1900 μatm CO₂)²², a trend also apparent at higher CO₂ levels²³. Although the intestine is a metabolically active tissue central to water balance and survival in seawater, the functional consequence of this relatively high HCO₃[−] loss during CO₂ acclimation has not been examined. Elevated HCO₃[−] secretion may also stimulate an increase in fish CaCO₃ production and release to the marine environment, potentially altering the marine carbon cycle²⁴. Since teleosts are the largest vertebrate group with a vital role in oceanic food webs, it is important to understand how impacts to the intestine during future ocean acidification scenarios could affect both fish and their surrounding environment. The first goal of the present study was to determine if the intestine of a marine teleost, an organ known to

University of Miami- Rosenstiel School of Marine and Atmospheric Science, 4600 Rickenbacker Causeway, Miami, FL 33149, USA. [†]Present address: University of North Texas, Department of Biological Sciences, 1511 West Sycamore, Denton, TX 76203, USA. Correspondence and requests for materials should be addressed to R.M.H. (email: rheuer@rsmas.miami.edu)

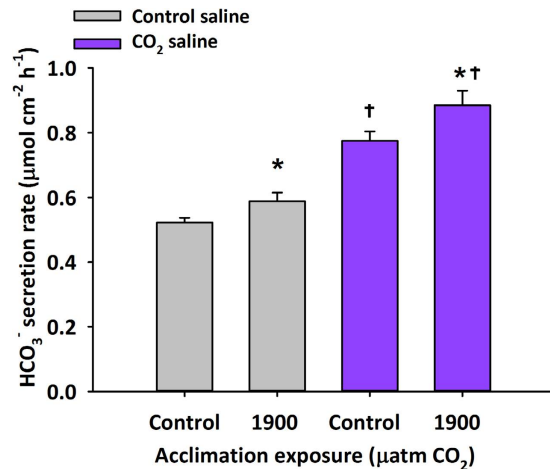


Figure 1. Bicarbonate secretion rates of isolated anterior intestine from control and CO₂ acclimated toadfish. Effect of blood-side saline composition and acclimation exposure on HCO₃⁻ secretion rates (means ± s.e.m.) of isolated anterior intestinal tissue obtained from gulf toadfish acclimated to control (~440 µatm CO₂, n = 9) or ~1900 µatm CO₂ (n = 10) for 2–4 weeks. Tissues from either control or 1900 µatm CO₂ acclimated fish mounted in this dual chambered Ussing/pH-stat system were bathed on either side by salines that mimicked *in vivo* ionic composition. Each tissue received two blood-side (serosal) saline treatments, control and CO₂ saline, representative of HCO₃⁻ and pCO₂ previously measured in toadfish blood following control and 1900 µatm CO₂ exposure (Supplementary Table 3). Two-way ANOVA; Acclimation exposure: P < 0.004, Saline: P < 0.001, Acclimation exposure × saline: P < 0.475. *Significant acclimation effect, †Significant saline effect.

show phenotypic plasticity in other ion regulatory challenges²⁵, would dynamically regulate intestinal function to reduce CO₂-induced intestinal HCO₃⁻ loss in favor of whole-body HCO₃⁻ retention. We predicted that tissue from CO₂-acclimated toadfish would show reduced bicarbonate secretion rates. After seeing a stimulation rather than a reduction of bicarbonate secretion rates, a second goal was to test the hypothesis that an increase in intestinal ion transport that occurs in response to elevated CO₂ would be associated with an increased tissue metabolic demand. Experiments were conducted at 1900 µatm CO₂, a level currently seen in certain coastal and upwelling zones^{26,27} and predicted globally in year 2300²⁸.

Results

Bicarbonate secretion rates of isolated tissue. Contrary to expectations, anterior intestinal tissue from CO₂ acclimated toadfish exhibited significantly increased HCO₃⁻ secretion rates (µmol cm⁻² h⁻¹) when compared to control tissue under identical conditions (Fig. 1). This result indicated prior acclimation to CO₂ does not suppress but stimulates intestinal HCO₃⁻ transport by around 13%. In addition, within both the control and CO₂ acclimated fish, HCO₃⁻ secretion rates using serosal salines mimicking plasma conditions at 1900 µatm CO₂ were significantly higher than HCO₃⁻ secretion rates under control serosal salines (Two-way ANOVA, Fish treatment P < 0.009, Saline P < 0.001, Fish treatment × saline P < 0.490, Fig. 1). TEP and conductance remained stable during experiments and were in agreement with earlier reports (Supplementary Table 1)^{21,29}.

Oxygen consumption rates of isolated tissue. Under both saline compositions, tissue from 1900 µatm CO₂ acclimated toadfish showed a significant 8% increase in oxygen consumption rate compared to tissue from control acclimated fish. In contrast to HCO₃⁻ secretion rates, oxygen consumption rates of isolated tissue from both control and 1900 µatm CO₂ exposed fish showed no effect of saline composition (Two-way ANOVA, Fish treatment P < 0.033, Saline P < 0.769, Fish treatment × saline P value < 0.509, Fig. 2). A similar relationship was observed when data was corrected for body mass (Supplementary Fig. 1).

Discussion

These results indicate that the marine fish intestine has a higher metabolic demand at 1900 µatm CO₂ than at present-day ambient conditions (~400 µatm CO₂), that is likely attributed to an increase in intestinal HCO₃⁻ loss from the body. This elevated CO₂ level is predicted for year 2300 and is currently seen in upwelling coastal areas²⁶. Similar to other fish experiencing elevated CO₂ in a marine environment, gulf toadfish have been shown to defend blood pH following exposure to 1900 µatm CO₂ by sustaining elevated levels of HCO₃⁻ in the face of higher pCO₂ in the blood¹¹. However, this compensation was associated with an increased intestinal HCO₃⁻ loss that was presumed to be associated with an activation of existing ionoregulatory transport pathways^{11,22}. These pathways involve the movement of plasma HCO₃⁻ into the intestinal lumen in exchange for Cl⁻ and are critical for maintaining water balance³⁰. However, from a whole-animal acid-base balance perspective, HCO₃⁻ loss during CO₂ compensation counteracts the need to retain HCO₃⁻ and defend pH, suggesting that fish faced with longer term acclimation to CO₂ must dynamically downregulate pathways involved in intestinal HCO₃⁻ secretion. Contrary to our initial hypothesis, comparison of HCO₃⁻ secretion rates of intestinal tissue from CO₂ and

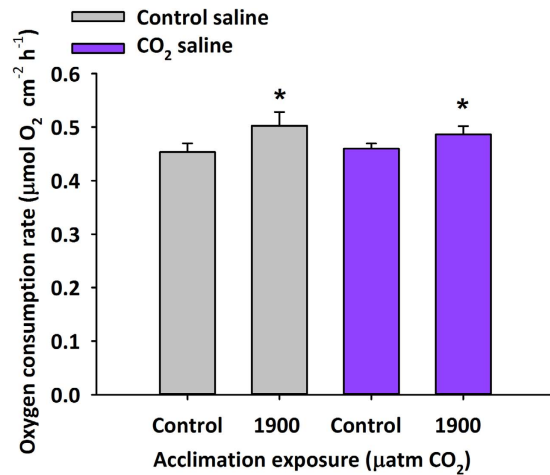


Figure 2. Oxygen consumption of isolated anterior tissue from control and CO₂ acclimated toadfish.

Effect of blood-side saline composition and acclimation exposure on oxygen consumption rates (means ± s.e.m.) of isolated anterior intestinal tissue taken from toadfish acclimated to control (~440 µatm CO₂; n = 12) or ~1900 µatm CO₂ (n = 8) for 2–4 weeks. Tissues from either control or 1900 µatm CO₂ acclimated fish mounted in this dual-chambered epithelial respirometer were bathed on either side by salines designed to mimic *in vivo* ionic composition. Each tissue received two blood-side (serosal) saline treatments, control saline and CO₂ saline, that were representative of HCO₃⁻ and previously measured in toadfish blood following acclimation at control and 1900 µatm CO₂ (Supplementary Table 3). Two-way ANOVA; Acclimation exposure: P < 0.033, Saline: P < 0.769, Acclimation exposure × saline: P < 0.509. *Significant acclimation effect.

control acclimated fish under identical saline conditions revealed that CO₂ exposure leads to a stimulation, rather than a downregulation of HCO₃⁻ transport pathways.

We propose that the 13% increase in HCO₃⁻ loss from CO₂ acclimated fish reflects an increased energetic demand, and thus increased CO₂ production, in intestinal tissue. This suggestion is supported by an 8% increase in O₂ consumption in tissue from CO₂-acclimated fish. A detailed mechanistic explanation of this proposed response can be seen in Fig. 3. Sustained elevated plasma HCO₃⁻ following CO₂ compensation leads to an increase in HCO₃⁻ movement from the blood into the intestinal cell that is paired with the movement of Na⁺ through basolateral NBC1, a Na⁺-HCO₃⁻ co-transporter³¹. The sustained Na⁺ influx must be compensated for by Na⁺ extrusion through the Na⁺-K⁺ ATPase (NKA). Increased NKA activity leads to an increased ATP and thus O₂ demand. Meeting this demand results in increased endogenous CO₂ production available for hydration via intracellular carbonic anhydrase (CAc) to form additional HCO₃⁻. Secretion of this excess HCO₃⁻ through apical SLC26a6 anion exchange likely accounts for the stimulation of HCO₃⁻ loss during CO₂ exposure. Hydration of endogenous CO₂ also results in formation of protons that must be eliminated from the intestinal cell and could put additional demand on the gill. This proton extrusion by the intestine may occur via Na⁺-dependent or independent pathways³⁰, both of which are energy demanding and could contribute to the observed elevation of O₂ consumption.

It is likely that the 8% increase in intestinal tissue O₂ consumption would not be observable by measurements of whole-animal metabolic rate. Difficulties in picking up small differences in organismal metabolic rate may explain some of the varying results from studies examining the effects of ocean acidification on fish standard metabolic rate¹. In addition, there appears to be considerable intra- and inter-species variation in the response to elevated CO₂ and there is inherent difficulty in comparing measurements using different methodologies^{32,33}. As demonstrated in the present study and by others^{5,34–37}, increased resolution and mechanistic insight into energetic tradeoffs and/or apparent consequences of ocean acidification may be obtained by integrating techniques and methods that examine multiple levels of organization. Altered mitochondrial capacity⁵, shifts in energetic budgets^{34,38,39}, increased expression/activity of gill and intestinal ionoregulatory genes and proteins¹⁵, increased ventilation⁴⁰, and increased protein turnover³⁸ have all been noted during ocean acidification-relevant CO₂ exposures with little impact to whole-animal measurements. The importance of integrating multiple techniques is apparent in a recent study on CO₂ exposure (1,200 and 2,200 µatm CO₂) in Atlantic cod exhibiting increased intestinal NKA mRNA and protein concentration, while exhibiting no change in NKA protein activity⁴¹. While the present study of isolated intestinal tissue precluded normal hormonal cascades or feedback mechanisms, it offered the advantage of careful control of blood-side (serosal) saline conditions and made it possible to identify mechanistic differences in tissue function that were impossible to observe in previous *in vivo* work²². One other caveat to note is that air, rather than custom CO₂/O₂ gas mixtures were used during respirometry experiments. Earlier work on this preparation revealed that tissue metabolic rate is not limited by O₂ above 75% air saturation²¹. Thus, tissue was not O₂-limited in the present study since saturation remained above 80%.

The marine fish intestine is fine-tuned to changes in plasma HCO₃⁻ to aid in water uptake and to handle the alkaline tide associated with digesting a meal^{21,31}. Although the functional consequence of stimulated intestinal HCO₃⁻ loss remains to be fully elucidated, the increase in O₂ consumption leads us to conclude that this

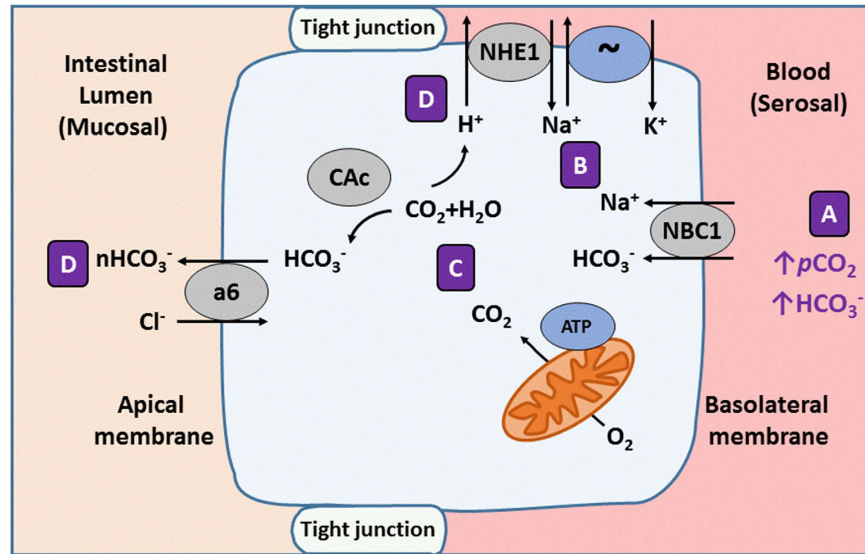


Figure 3. Proposed impacts of 1900 μatm CO_2 on intestinal transport physiology in a marine teleost.

Compensation for a CO_2 -induced acidosis increases HCO_3^- and $p\text{CO}_2$ in extracellular fluids¹¹. (A) Elevated serosal HCO_3^- during CO_2 exposure stimulates transport via $\text{Na}^+:\text{HCO}_3^-$ co-transporter, NBC1, leading to both an increase influx of HCO_3^- and Na^+ across the basolateral membrane. (B) Sustained influx of Na^+ via NBC1 likely leads to a demand for increased Na^+ extrusion via the energy-demanding $\text{Na}^+ \text{K}^+$ ATPase (~). (C) The metabolic demand and increased O_2 consumption associated with handling an increased Na^+ influx would generate additional endogenous CO_2 , increasing substrate for intracellular CAC hydration. (D) Intracellular HCO_3^- generated via this process likely accounts for the observed stimulation of bicarbonate secretion via SLC26a6 (a6) in isolated tissue from fish acclimated to 1900 μatm CO_2 compared to control fish under serosal salines with identical bicarbonate concentrations. Protons generated in via carbonic anhydrase in step C would likely be extruded from the cell via NHE1 and add to the increase in intracellular Na^+ . For a more detailed overview of marine fish intestinal transport processes see review³⁰.

CO_2 -induced response is potentially maladaptive but persists for protection of osmoregulatory and digestive functions. Support for this conclusion comes from a recent study, on Atlantic cod, demonstrating that high levels of CO_2 (9200 μatm) lengthen the time needed to digest a meal⁴². Interestingly, reduced digestive efficiency has also been noted in the sea urchin, albeit through a different mechanism, reduced stomach pH. These urchins exhibited a behavioural adjustment, to counteract this reduced efficiency. Fish may also adjust feeding behaviour in such instances, but broad behavioural impairments across various species with CO_2 exposure^{4,8} may impact such processes and should be further investigated. An increased metabolic demand from ion transport processes, as seen in the present study, may detract from energy available for digestive functions in the intestine, slowing the process of digestion. Calculations using estimates of toadfish standard metabolic rate⁴³ suggest that the energetic cost of CO_2 acclimation in the intestine would account $\sim 0.5\%$ of whole animal O_2 consumption²¹. Albeit small, any factor that promotes an energy reallocation or increases standard metabolic rate could exacerbate already narrow metabolic constraints⁴⁴ or possibly interact with projected temperature elevations to increase overall impact^{36,39,41,45}.

The demand to secrete HCO_3^- in the intestine to maintain water balance is well-conserved across marine teleosts³⁰. Increased intestinal HCO_3^- secretion with CO_2 exposure as reported here has also been demonstrated at higher CO_2 levels in the plainfin midshipmen ($\sim 50,000$ μatm CO_2)⁴⁶, in the toadfish (5000–20,000 μatm CO_2)²³, and suggested from gene expression and/or protein assays in the Japanese ricefish (7000 μatm CO_2)³⁴ and the Atlantic cod (1,200 and 2,200 μatm CO_2)⁴¹. These studies suggest that increased intestinal HCO_3^- secretion and metabolic demand during CO_2 exposure could be a ubiquitous response to elevated CO_2 throughout marine bony fishes. Finally, intestinal HCO_3^- secretion results in formation and excretion of CaCO_3 by marine fish which amounts to at least 3–15% of the marine inorganic CaCO_3 production²⁴. Although a study on the toadfish reported unaltered CaCO_3 excretion rates²² at 1900 μatm a more recent study on toadfish²³ and an earlier study on midshipmen⁴⁶ may suggest otherwise. Thus, increased intestinal HCO_3^- loss at elevated CO_2 in other species may impact the magnitude of this globally important calcification process, a possibility worthy of further study. In this context, it is important to consider an alternative scenario in which the increase stimulation of HCO_3^- secretion during CO_2 exposure serves to assist the animal in buffering ingested acidified seawater, making conditions more favorable for carbonate precipitation and continued water absorption. However, this cannot be the sole purpose of the increase HCO_3^- secretion, as the extra protons ingested in a given amount of 1900 μatm CO_2 water is several orders of magnitude lower than the increase in HCO_3^- secretion due to acclimation. These calculations support the idea that HCO_3^- loss during CO_2 exposure is not beneficial for the animal.

Surprisingly few studies have examined the impact of sustained global increases of carbon dioxide on acid-base and osmoregulatory processes in marine fish. While the presents study reports relatively small increases in bicarbonate secretion and oxygen consumption in the intestine, it is important to remember that these changes

likely require reallocations or adjustments in an organism that may already be facing other downstream impacts of CO₂. Although compensation for elevated CO₂ in marine fish typically occurs within days¹¹, it cannot be ruled out that longer acclimation periods, transgenerational effects, and/or adaptation may affect the dynamics of acid-base balance. Furthermore, interspecies differences associated with the cost of ionoregulatory demands are likely⁴⁰.

Methods

Animal collection and care. Gulf toadfish (*Opsanus beta*) were obtained from local shrimpers as by-catch in Biscayne Bay and acclimated to flow-through, aerated, sand-filtered seawater from Bear Cut, FL (22–25 °C, 32–35 ppt) in the laboratory at the University of Miami for at least two weeks prior to experimentation. During this period, fish were fed squid twice weekly. Once introduced to experimental tanks, toadfish (HCO₃⁻ secretion mass range: 21.7–30.9 g, O₂ consumption mass range: 28.1–48.6 g) were fed 5% of their body weight weekly. Food was withheld at least 4 days prior to experimentation, a time period previously demonstrated to ensure that confounding effects of specific dynamic action (SDA) would not be a factor for HCO₃⁻ secretion or oxygen consumption measurements²¹. Fish were sacrificed using a lethal dose of 0.2 g/L MS-222 buffered with 0.3 g/L NaHCO₃. All general animal care and animal sacrifice protocols were carried out in accordance with relevant guidelines for experiments on teleost vertebrates provided by University of Miami IACUC (Institutional Animal Care and Use Committee). All experimental protocols were approved by University of Miami Institutional Animal Care and Use Committee. (IACUC 13-225). IACUC is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AALAC). Toadfish were collected with the approval and in accordance with guidelines outlined by the Florida Fish and Wildlife Conservation Commission (Permit SAL-12-0729 SR).

General experimental procedures. Toadfish were acclimated to either control or CO₂ (~440 and ~1900 μatm CO₂, respectively, Supplementary Table 2) for 2–4 weeks, a time period previously deemed sufficient to elicit a CO₂ compensatory response in the toadfish¹¹. Throughout the methods section, reference to CO₂ refers to the 1900 μatm CO₂ level, with respect to both treatment exposure and saline. Following acclimation, anterior intestinal tissue was dissected and immediately placed between two half-chambers where the tissue was bathed on either side by salines representative of *in vivo* conditions. Gut lumen-side (mucosal) salines were identical throughout all treatments, however, two different blood-side (serosal) salines (“control” or “CO₂”) were used to mimic measured values at control or 1900 μatm CO₂ (Supplementary Table 3)¹¹. These Ussing chambers were combined with pH-stat titration to allow for measurement of HCO₃⁻ secretion rates from the blood-side to the gut-side saline²⁹. In addition, the O₂ consumption rate of isolated intestinal segments were measured using a custom-built epithelial respirometer²¹. Each mounted tissue was treated with both the control and the CO₂ serosal saline, allowing for examination of the effect of prior CO₂ acclimation along with the effect of blood-side saline conditions that mimicked *in vivo* measured blood chemistry. If dynamic regulation was occurring in tissue from fish exposed to CO₂ to reduce HCO₃⁻ loss, reduced HCO₃⁻ secretion rates would be expected in tissue from CO₂ acclimated fish.

Seawater manipulation. Stable CO₂ levels were achieved using a Loligo pH-based negative feedback system (Loligo Systems, Tjele, Denmark) as previously described^{11,22}. In this system, a standard curve was generated based on the relationship between known gas standards and seawater pH. Once a pH setpoint corresponding to 1900 μatm CO₂ was calculated following calibration and measurement of ambient seawater, 100% CO₂ was gassed directly into flow-through tanks via solenoid valves controlled by pH electrodes (Sentix H, wtw Germany) and a pCO₂/pH DAQ-M digital relay instrument connected to CapCTRL software (Loligo Systems, Tjele, Denmark) to achieve the desired pCO₂ setpoint. pCO₂ levels typically stayed within 4–10 percent of setpoint values. Independent pH measurements were taken multiple times a week using a separate pH electrode (pH_{NBS}, PHC3005, Radiometer, France). Target CO₂ levels were confirmed with measurements of seawater TCO₂ using a Corning 965 CO₂ Analyzer (Corning 965, Corning Diagnostics, UK). TA (titratable alkalinity) and pCO₂ levels were calculated from measured pH_{NBS} and TCO₂ measurements into CO2SYS software⁴⁷. These calculations confirmed target pCO₂ values for control (ambient--439 μatm CO₂) and 1900 (1878 μatm CO₂) were reached. Water chemistry parameters including temperature and salinity are reported in Supplementary Table 2.

Electrophysiological measurements. In the Ussing chamber systems (Physiological Instruments, San Diego, CA, USA), current and voltage electrodes attached to an amplifier measured transepithelial potential (TEP, mV) differences between a baseline 0 μA current clamp and a 3 second pulse of 50 μA that was applied every 60s. Measurements were logged using Acknowledge software (v. 3.8.1, BIOPAC Systems). TEP values are reported with a luminal reference of 0 mV and conductance was calculated using Ohm’s law (Supplementary Table 1).

***In vitro* Ussing chamber/pH stat electrophysiological and bicarbonate secretion measurements.** Simultaneous measurement of electrophysiological parameters and bicarbonate secretion of isolated tissue was achieved using Ussing chamber systems (Physiological Instruments, San Diego, CA, USA) combined with automated pH-stat titration (TIM854/856 Titralab and Titramaster software v.5.1.0, Radiometer, Copenhagen, Denmark)²⁹. Following acclimation in either control or CO₂ treatment tanks (1900 μatm CO₂, 2–4 weeks; Supplementary Table 2), anterior intestine segments were mounted on tissue holders designed to expose 0.71 cm² of tissue to two half-chambers (1.6 mL) in the Ussing chamber system (P2400, Physiological Instruments). In this setup, isolated tissue was bathed in pre-gassed serosal (blood-side) or mucosal (gut-side) salines continuously mixed by air-lift gassing and held at 25 °C using a recirculating water bath. Mucosal saline composition remained unaltered throughout all experiments (Supplementary Table 3) and was gassed with 100% O₂. “Control” and “CO₂” serosal salines were designed to mimic *in vivo* HCO₃⁻ and pCO₂ levels previously measured in the plasma

of toadfish during exposure to control and 1900 μatm CO_2 (3.3 and 6.3 mM HCO_3^- , 0.225 and 0.462% CO_2^{11} , respectively; Supplementary Table 3).

A pH electrode (PHC4000-8, Radiometer, Denmark) and an acid-dispensing microburette were submerged into the mucosal half-chamber. The rate of acid titrant addition and the titrant concentration (0.005 N HCl) needed to hold the mucosal saline at a constant pH of 7.8 were used to calculate the epithelial HCO_3^- secretion rate. Electrophysiological measurements were taken simultaneously with bicarbonate secretion rates (Supplementary Table 1). Once tissue preparations were considered stable based upon steady bicarbonate secretion and electrophysiological parameters, a minimum 30-minute measurement interval was recorded prior to a saline change. Each isolated intestinal tissue received both control and CO_2 serosal saline treatments. Although tissues have been demonstrated to be viable for at least 5 hours in prior studies using this species and setup²⁹, the order of serosal salines applied were randomized. During a saline change, the first serosal saline was carefully removed with a syringe, the half chamber was rinsed, and replaced by the second serosal saline treatment. Measurements post-saline change were continued until stable levels were recorded for a minimum of 30 minutes.

In vitro oxygen consumption measurements. Following the same acclimation procedures outlined for bicarbonate secretion experiments, the oxygen consumption rate of isolated anterior intestine was measured using a custom-designed epithelial respirometer (Loligo Systems, Tjele, Denmark). Intestinal tissue was mounted so that 0.87 cm^2 of tissue was exposed to two half-chambers (2.80 mL), mucosal saline on the gut side and one of two serosal saline treatments (described above) on the blood-side. All salines were pre-gassed with air (100% O_2 saturation) rather than custom O_2 mixes to make measurements of oxygen consumption rates comparable to whole-animal measurements. Saline HCO_3^- concentrations were kept the same in the control and CO_2 serosal salines (Supplementary Table 3).

Salines in half chambers were continuously mixed by micromagnetic glass-coated Teflon stir bars (Loligo Systems), and a Teflon tissue mount ensured that the system was gas-tight²¹. Oxygen measurements were conducted using a fiber-optic cable secured to the outside wall of either glass half-chamber that illuminated a fiber-optic sensor spot glued to the inside wall of each respective side. Each cable was connected to a separate single-channel oxygen meter (Fibox 3) used in conjunction with Oxy-View software (PST3-V6.02; PreSens, Regensburg, Germany). Prior to daily experiments, calibrations were performed with salines pre-gassed with air for 100% air saturation and gassed with N_2 conditions representing no oxygen saturation.

Intermittent-flow respirometry was performed to determine oxygen consumption rates of isolated tissue by flushing and replacing salines using a manual gravity-fed system. Flush cycles guaranteed complete saline replacement during open cycles and time intervals during closed measurements were limited to ~20 minutes, to ensure that air saturation of tissue did not drop below 80%, since values below 75% were previously shown to restrict this tissue²¹. Previous use of this respirometry system has shown negligible rates of gas back-flux with the atmosphere or across chambers and a constant O_2 consumption rate at air saturation above 75%²¹. Tissue O_2 consumption was estimated from the mucosal and serosal O_2 depletion rates.

Statistical analysis. Two-way ANOVAs were used to analyze bicarbonate secretion (Fig. 1), oxygen consumption (Fig. 2, Supplementary Fig. 1), and electrophysiological measurements (Supplementary Table 1) with treatment exposure (acclimation) and saline exposure as independent variables. Significance for all tests was determined at $P < 0.05$ for all tests and all data are presented as means \pm s.e.m. unless specifically noted otherwise.

References

- Heuer, R. M. & Grosell, M. Physiological impacts of elevated carbon dioxide and ocean acidification on fish. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **307**, R1061–R1084 (2014).
- Munday, P. L., McCormick, M. I. & Nilsson, G. E. Impact of global warming and rising CO_2 levels on coral reef fishes: what hope for the future? *J. Exp. Biol.* **215**, 3865–3873 (2012).
- Rummer, J. L. *et al.* Elevated CO_2 enhances aerobic scope of a coral reef fish. *Conserv. Physiol.* **1**, 1–7 (2013).
- Nilsson, G. E. *et al.* Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. *Nature Clim. Change* **2**, 201–204 (2012).
- Strobel, A. *et al.* Metabolic shifts in the Antarctic fish *Notothenia rossii* in response to rising temperature and PCO_2 . *Front. Zool.* **9**, 28–28 (2012).
- Checkley, D. M. Jr. *et al.* Elevated CO_2 enhances otolith growth in young fish. *Science* **324**, 1683 (2009).
- Couturier, C. S., Stecyk, J. A. W., Rummer, J. L., Munday, P. L. & Nilsson, G. E. Species-specific effects of near-future CO_2 on the respiratory performance of two tropical prey fish and their predator. *Comp. Biochem. Physiol.* **166A**, 482–489 (2013).
- Munday, P. L. *et al.* Replenishment of fish populations is threatened by ocean acidification. *Proc. Natl. Acad. Sci. USA* **107**, 12930–12934 (2010).
- Heuer, R. M., Welch, M. J., Rummer, J. L., Munday, P. L. & Grosell, M. Altered brain ion gradients following compensation for elevated CO_2 are linked to behavioural alternations in a coral reef fish. *Scientific Reports* **6**, 33216, doi: 10.1038/srep33216 (2016).
- Portner, H. O., Langenbuch, M. & Reipschlag, A. Biological impact of elevated ocean CO_2 concentrations: Lessons from animal physiology and earth history. *J. Oceanogr.* **60**, 705–718 (2004).
- Esbaugh, A. J., Heuer, R. & Grosell, M. Impacts of ocean acidification on respiratory gas exchange and acid-base balance in a marine teleost, *Opsanus beta*. *J. Comp. Physiol. B* **182**, 921–934 (2012).
- Toews, D. P., Holeton, G. F. & Heisler, N. Regulation of the acid-base status during environmental hypercapnia in the marine teleost fish *Conger conger*. *J. Exp. Biol.* **107**, 9–20 (1983).
- Claiborne, J. B., Edwards, S. L. & Morrison-Shetlar, A. I. Acid-base regulation in fishes: Cellular and molecular mechanisms. *J. Exp. Zool.* **293**, 302–319 (2002).
- Brauner, C. J. & Baker, D. W. In *Cardio-Respiratory Control in Vertebrates: Comparative and Evolutionary Aspects* (ed M.L. and S.C. Wood Glass) Ch. Patterns of Acid-Base Regulation during Exposure to Hypercarbia in Fishes 43–63 (Springer, 2009).
- Esbaugh, A., Ern, R., Nordi, W. & Johnson, A. Respiratory plasticity is insufficient to alleviate blood acid-base disturbances after acclimation to ocean acidification in the estuarine red drum, *Sciaenops ocellatus*. *J. Comp. Physiol. B* 97–109 (2015).
- Brett, J. & Groves, T. In *Fish physiology* Vol. 8 Ch. Physiological energetics 279–352 (1979).

17. Perry, S. F. & Gilmour, K. M. Acid-base balance and CO₂ excretion in fish: Unanswered questions and emerging models. *Respir. Physiol. and Neurobiol.* **154**, 199–215 (2006).
18. Ishimatsu, A., Hayashi, M. & Kikkawa, T. Fishes in high-CO₂, acidified oceans. *Mar. Ecol. Prog. Ser.* **373**, 295–302 (2008).
19. Ern, R., Huong, D., Cong, N., Bayley, M. & Wang, T. Effect of salinity on oxygen consumption in fishes: a review. *J. Fish Biol.* **84**, 1210–1220 (2014).
20. Morgan, J. D. & Iwama, G. K. Energy cost of NaCl transport in isolated gills of cutthroat trout. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **277**, R631–R639 (1999).
21. Taylor, J. & Grosell, M. The intestinal response to feeding in seawater gulf toadfish, *Opsanus beta*, includes elevated base secretion and increased epithelial oxygen consumption. *J. Exp. Biol.* **212**, 3873–3881 (2009).
22. Heuer, R. M., Esbaugh, A. J. & Grosell, M. Ocean acidification leads to counterproductive intestinal base loss in the gulf toadfish (*Opsanus beta*). *Physiol. Biochem. Zool.* **85**, 450–459 (2012).
23. Heuer, R. M. *et al.* Changes to intestinal transport physiology and carbonate production at various carbon dioxide (CO₂) levels in a marine teleost, the gulf toadfish (*Opsanus beta*). *Physiol. Biochem. Zool.* **89**, 402–416, doi: 10.1086/688235 (2016).
24. Wilson, R. W. *et al.* Contribution of fish to the marine inorganic carbon cycle. *Science* **323**, 359–362 (2009).
25. Genz, J., Taylor, J. R. & Grosell, M. Effects of salinity on intestinal bicarbonate secretion and compensatory regulation of acid-base balance in *Opsanus beta*. *J. Exp. Biol.* **211**, 2327–2335 (2008).
26. Feely, R. A., Sabine, C. L., Hernandez-Ayon, J. M., Janson, D. & Hales, B. Evidence for upwelling of corrosive “acidified” water onto the continental shelf. *Science* **320**, 1490–1492 (2008).
27. Cai, W. J. *et al.* Acidification of subsurface coastal waters enhanced by eutrophication. *Nat Geosci* **4**, 766–770, doi: 10.1038/Ngeo1297 (2011).
28. Caldeira, K. & Wickett, M. E. Anthropogenic carbon and ocean pH. *Nature* **425**, 365–365, doi: 10.1038/425365a (2003).
29. Grosell, M. & Genz, J. Ouabain-sensitive bicarbonate secretion and acid absorption by the marine teleost fish intestine play a role in osmoregulation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **291**, R1145–R1156 (2006).
30. Grosell, M. Intestinal anion exchange in marine teleosts is involved in osmoregulation and contributes to the oceanic inorganic carbon cycle. *Acta Physiol.* **202**, 421–434 (2011).
31. Taylor, J. R., Mager, E. M. & Grosell, M. Basolateral NBCe1 plays a rate-limiting role in transepithelial intestinal HCO₃⁻ secretion, contributing to marine fish osmoregulation. *J. Exp. Biol.* **213**, 459–468 (2010).
32. Roche, D. G., Binning, S. A., Bosiger, Y., Johansen, J. L. & Rummer, J. L. Finding the best estimates of metabolic rates in a coral reef fish. *J. Exp. Biol.* **216**, 2103–2110 (2013).
33. Clark, T. D., Sandblom, E. & Jutfelt, F. Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. *J. Exp. Biol.* **216**, 2771–2782 (2013).
34. Tseng, Y. C. *et al.* CO₂-driven seawater acidification differentially affects development and molecular plasticity along life history of fish (*Oryzias latipes*). *Comp. Biochem. Physiol. A* **165**, 1190–1130 (2013).
35. Stapp, L., Kreiss, C., Pörtner, H. & Lannig, G. Differential impacts of elevated CO₂ and acidosis on the energy budget of gill and liver cells from Atlantic cod, *Gadus morhua*. *Comp. Biochem. Physiol. A* **187**, 160–167 (2015).
36. Kreiss, C. M., Michael, K., Bock, C., Lucassen, M. & Pörtner, H.-O. Impact of long-term moderate hypercapnia and elevated temperature on the energy budget of isolated gills of Atlantic cod (*Gadus morhua*). *Comp. Biochem. Physiol. A* **182**, 102–112 (2015).
37. Stumpp, M. *et al.* Acidified seawater impacts sea urchin larvae pH regulatory systems relevant for calcification. *Proc. Natl. Acad. Sci. USA* **109**, 18192–18197 (2012).
38. Pan, T.-C. F., Applebaum, S. L. & Manahan, D. T. Experimental ocean acidification alters the allocation of metabolic energy. *Proc. Natl. Acad. Sci. USA* **112**, 4696–4701 (2015).
39. Strobel, A., Leo, E., Pörtner, H. O. & Mark, F. C. Elevated temperature and PCO₂ shift metabolic pathways in differentially oxidative tissues of *Notothenia rossii*. *J. Comp. Physiol. B* **166**, 48–57 (2013).
40. Ern, R. & Esbaugh, A. J. Hyperventilation and blood acid–base balance in hypercapnia exposed red drum (*Sciaenops ocellatus*). *J. Comp. Physiol. B* **447**–460 (2016).
41. Hu, M. Y.-A. *et al.* Temperature modulates the effects of ocean acidification on intestinal ion transport in Atlantic cod, *Gadus morhua*. *Front. in Physiol.* **7**, 198 (2016).
42. Tirsgaard, B., Moran, D. & Steffensen, J. F. Prolonged SDA and reduced digestive efficiency under elevated CO₂ may explain reduced growth in Atlantic cod (*Gadus morhua*). *Aquatic Tox.* **158**, 171–180 (2015).
43. Gilmour, K. *et al.* Nitrogen excretion and the cardiorespiratory physiology of the gulf toadfish, *Opsanus beta*. *Physiol. Zool.* **71**, 492–505 (1998).
44. Deutsch, C., Ferrel, A., Seibel, B., Pörtner, H.-O. & Huey, R. B. Climate change tightens a metabolic constraint on marine habitats. *Science* **348**, 1132–1135 (2015).
45. Enzor, L. A., Zippay, M. L. & Place, S. P. High latitude fish in a high CO₂ world: Synergistic effects of elevated temperature and carbon dioxide on the metabolic rates of Antarctic notothenioids. *Comp. Biochem. Physiol. A* **164**, 154–161 (2013).
46. Perry, S. F. *et al.* Acid-base regulation in the plainfin midshipman (*Porichthys notatus*): an aglomerular marine teleost. *J. Comp. Physiol. B* **180**, 1213–1225 (2010).
47. Pierrot, D., Lewis, E. & Wallace, D. M. S. Excel program developed for CO₂ system calculations. ORNL/CDIAC-105a. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, US Department of Energy, Oak Ridge, Tennessee (2006).

Acknowledgements

R.M. Heuer was supported by a National Science Foundation Graduate Research Fellowship (DGIE-0951782) and the University of Miami Koczy Fellowship and M. Grosell is a Maytag professor of Ichthyology and is supported by a National Science Foundation award (1146695).

Author Contributions

R.M.H. and M.G. designed the study. R.M.H. performed the experiments. R.M.H. and M.G. analyzed the data; R.M.H. wrote the paper. M.G. provided comments and feedback and secured funding.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Heuer, R. M. and Grosell, M. Elevated CO₂ increases energetic cost and ion movement in the marine fish intestine. *Sci. Rep.* **6**, 34480; doi: 10.1038/srep34480 (2016).



This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>

© The Author(s) 2016