Facing a Physiological Crossroad: Response of an Intertidal Sculpin to a Multi-stressor Challenge

by

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Abstract

As we move into the Anthropocene, organisms inhabiting marine environments will continue to face growing challenges associated with changes in ocean pH (ocean acidification), dissolved oxygen (dead zones) and temperature. These factors, in combination with naturally variable environments such as the rocky intertidal, may create extreme physiological challenges for organisms that are already performing near their biological limits. Although numerous studies have examined the impacts of climate related stressors on intertidal animals, little is known about the underlying physiological mechanisms driving adaptation to ocean acidification and how this may alter organism interactions, particularly in marine vertebrates. Therefore, we have investigated the effects of decreased ocean pH on the hypoxia response of an intertidal sculpin, Clinocottus analis. We used both whole animal and biochemical based analyses to examine how the energetic demands associated with acclimation to low pH environments may impact the fish’s reliance on facultative air breathing in low oxygen environments. Our study demonstrated that acclimation to OA results in elevated routine metabolic rates (RMR) and acid base regulatory capacity (Na⁺/K⁺ ATPase activity). These in turn had down stream effects that resulted in decreased hypoxia tolerance (i.e. elevated Pcrit). Furthermore, we present evidence that these fish may be living near their physiological capacity when challenged by ocean acidification. This serves as a reminder that the susceptibility of teleost fish to changes in ocean pH may be underestimated, particularly when considering the multiple stressors that many experience in their natural environments.

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Introduction

Anthropogenically driven global climate change is fundamentally altering ocean environments at an alarming rate (Doney et al. 2009). As atmospheric carbon dioxide concentrations continue to rise above 400µatm, the invariably linked consequences of a warmer planet and more acidic oceans are becoming more evident. In the past 50 years alone, average global temperatures have increased by ~0.6°C (Walther et al. 2002) while oceanic carbon dioxide sequestration has occurred at a rate 100 times greater than anytime in the past 650K years (Siegenthaler et al. 2005). The intergovernmental panel on climate change (IPCC) predicts that by the year 2100, our oceans could decrease in pH by 0.3 to 0.4 units, corresponding to a partial pressure of CO₂ (pCO₂) upwards of 1000µatm (IPCC 2013). Indeed as we monitor these broad scale environmental changes, it becomes apparent that marine organisms are, and will continue to be faced with physiological stressors associated with both increased sea surface temperatures (ocean warming) as well as declining ocean pH (ocean acidification, OA) (Hughes 2000, Fabry et al. 2008).

Regional differences in environmental conditions will likely affect the impact of climate related stressors on marine biota. For example, ocean acidification may exacerbate high pCO₂ conditions associated with upwelling in coastal oceans (Hales et al. 2005, Hauri et al. 2009, Thomsen et al. 2010, Gruber 2011). Similarly, OA will likely act in combination with other environmental variables (e.g., temperature, salinity, etc.) that are naturally variable in the rocky intertidal to create extreme physiological challenges for organisms that may already be performing near their biological limits. Numerous studies have demonstrated that within the intertidal, small changes in environmental conditions could have consequences for fitness and community composition, while also causing distributional shifts as the adaptive capacities of
these organisms are pushed to their limits (Helmuth et al. 2006, Tomanek 2008). Subsequently, it becomes important to investigate the physiological and biochemical pathways challenged by the exacerbating effects of climate change in intertidal organisms in order to understand whole organism and ecosystem consequences.

Responses to environmental stressors such as temperature have been well documented in marine systems. However, relatively little is known about the underlying physiological mechanisms driving adaptation to ocean acidification, particularly in vertebrates (Kroeker et al. 2010). Teleost fishes for example, are known to be sensitive to changes in temperature (Pörtner et al. 2007, Pankhurst et al. 2011) but traditionally have been thought resilient to intermediate levels of increasing carbon dioxide (e.g. Ishimatsu 2005, 2008). One likely reason for this insensitivity is because of their well-established acid/base regulatory capacity (Ishimatsu 2005). However, the maintenance of cellular homeostasis comes at a significant energetic cost (Kültz 2005, Hochachka and Somero 2014). For example, under increasing environmental temperatures, maintaining rates of protein synthesis, enzyme kinetics, and membrane permeability within physiological norms are just a few of the many challenges faced in the cell that, when altered, can lead to whole organism consequences (Kültz 2005, Tomanek 2008). Similarly, as nearly all cellular functions rely on strict homeostasis of intracellular pH, fishes must expend large amounts of energy regulating ion concentrations. The largest portion of pH maintenance is done via chloride cells in the gill epithelium (Clairborne et al. 2002). This balance is maintained through the direct, electro neutral transfer of $H^+$ and $HCO_3^-$ for $Na^+$ and $Cl^-$ respectively. These chloride cells use $Na^+/K^+$-ATPase (NKA) pumps in concert with carbonic anhydrase, a ubiquitous enzyme found in vertebrates, to catalyze the hydrolysis reaction of $CO_2$ and enhance the exchange of $Na^+$ and $Cl^-$ for $H^+$ and $HCO_3^-$ (Gilmour and Perry 2009). At higher carbon
dioxide concentrations, NKA pumps can be up-regulated in response to excess H\(^+\) ions in the cell (Ishimatsu 2005, Deigweiher et al. 2008). Although this mechanism is highly successful at buffering changes in intracellular pH, it accounts for as much as 20% of the cells energy demand under non-stressed conditions (Clairborne et al. 2002). The high costs of these basal functions are thought to translate into differences in species distribution, behavior and ultimately underlie trade-offs in fitness, especially in intertidal organisms (Somero 2002, Lang et al. 2009). Indeed, shifts in energy allocation have been previously observed in teleost fish in response to hypercapnic conditions where acid/base balance can consume upwards of 40% of the total cellular energy budget (Deigweiher et al. 2009). Although this study used severe hypercapnia (~10,000 ppm \(\text{CO}_2\)), it is likely that ecologically relevant changes in \(p\text{CO}_2\), combined with additional stresses such as hypoxia or ocean warming, will result in energy shifts and create fitness trade-offs in teleost fishes.

Intertidal sculpins in the genus Clinocottus, such as the Woolly sculpin (Clinocottus analis), are common to the coast of central California. These fishes, like most intertidal organisms, experience a variety of abiotic stressors brought about by low tide, including depleted oxygen content (hypoxia) in tide pools with a high degree of respiration (Yoshiyama 1981). When oxygen becomes limiting, sculpins may use behavioral mechanisms, such as facultative aerial respiration, to satisfy metabolic needs (Martin 1991, Watters and Cech 2003). With an increased demand for oxygen to maintain cellular function in more acidic waters, intertidal sculpins capable of aerial respiration may exit hiding spots in their tide pools for longer periods of time. This behavior, however, has a tradeoff, as sculpins face increased temperature, desiccation and predation near the surface and outside of the water. Conversely, in response to deoxygenation, oxygen expensive cellular machinery such as \(\text{Na}^+/\text{K}^+\)-ATPase pumps may be
down-regulated (Bogdanova et al. 2005) in order to conserve oxygen and avoid exiting their tide pool. By doing so however, sculpins may fail to defend against the accumulation of $H^+$ and become vulnerable to acidosis. This trade-off leads to an interesting juxtaposition when these behavioral and physiological responses to hypoxia are considered in conjunction with the projected decreases in ocean pH associated with OA. The acclimatory response of these fish to future $pCO_2$ levels should involve a significant up-regulation of the acid/base regulatory pathway and incur an elevated energetic debt. This long-term acclimatory response however would also be expected to impair the sculpin’s ability to physiologically compensate for low oxygen environments via down-regulation of energetically expensive pathways as described above. Instead, long-term declines in pH may require the sculpin to rely on behavioral alterations such as facultative respiration to offset the energy demands, which could increase predation risk for sculpins in these ecosystems.

Here we investigated the potential for ocean acidification to alter the routine metabolic rate, critical oxygen tension ($P_{\text{crit}}$, a marker for hypoxia tolerance), and acid base regulatory capacity in the gill of $C. analis$. In addition, we attempted to gain insight into potential behavioral changes in these fish by monitoring the oxygen tension at which point fish first displayed facultative aerial respiration. Today, only a handful of studies have examined the effects of ocean acidification on teleost physiology and none, that we are aware of, have been conducted on fishes living within the intertidal zone. Furthermore, even fewer have quantified acid base regulatory capacity or were able to draw insight from changes to whole animal physiology, which are relevant to ecologically important behaviors such as hypoxia avoidance. Our understanding of how global climate change may impact the rocky intertidal community is limited by an incomplete understanding of pH effects on intertidal fishes, which act as important
members of the intertidal food web. We hypothesize that the decreases in ocean pH associated with ocean acidification would challenge these animals physiologically as evidenced by increases in basal metabolism, decreases in hypoxia tolerance, and increased activity of NKA pumps as an indicator of acid/base regulatory capacity.

Methods

Collection of Study Specimens

Woolly sculpins, *Clinocottus analis*, commonly found in tide pools along the California coast, were collected from Horseshoe Cove (38.316, -123.071) and Shell Beach (38.417, -123.107) near Bodega Bay, CA. Specimens from the mid-intertidal pools were caught during low tide on three different dates, July 28th (n=12), October 1st (n=12) and November 23rd (n=14), 2015 using dip nets. All fish were transported in fresh aerated seawater to holding aquaria at Sonoma State University where they were maintained under ambient seawater conditions (~14 °C, 400µatm pCO₂) for at least one week prior to the start of experimentation. Fish were fed to satiation every other day using a diet of frozen mysis and brine shrimp. All fish were handled according to guidelines approved by the Sonoma State University Institutional Animal Care and Use Committee (IACUC, protocol # 2015-51).

Experimental Design

After acclimation to the aquaria for at least 7 days, fish were randomly distributed to one of sixteen 10-L experimental tanks that consisted of a control treatment that mimicked the ambient seawater conditions near Horseshoe Cove (~400 µatm pCO₂, 14 °C) or an elevated CO₂ treatment (~1100 µatm pCO₂, 14 °C) that was consistent with predicted future ocean conditions (IPCC 2013). Fish were placed in experimental tanks for a period of 7 days (n=12 per treatment)
or 28 days (n=7 per treatment) to examine the effects of ocean acidification on the routine metabolic rate (RMR), critical oxygen tension \( (P_{\text{crit}}) \) and \( \text{Na}^+ / \text{K}^+ \) ATPase enzyme activity. It was noticed prior to experimentation that \( C. \text{analis} \) individuals could be territorial. Thus to prevent behavioral altercations, no more than two fish were housed per aquarium, and all fish were separated by a physical barrier.

**Manipulation of Seawater Conditions**

Treatment \( p\text{CO}_2 \) levels were maintained using a \( p\text{CO}_2 \) generating system, described by Fangue *et al.* (2010) and further modified for the use on fish by Enzor *et al.* (2013). Briefly, atmospheric air was dried using a drying column of drierite and scrubbed of \( \text{CO}_2 \) using Sodasorb®. The \( \text{CO}_2 \) free air was then mixed with pure \( \text{CO}_2 \) in precise quantities via digital mass flow controllers (Sierra Instruments, Monterey, CA, USA) and pumped into the sumps of experimental aquaria using venturi injectors. Two 45-gallon header tanks were also equilibrated with appropriate \( \text{CO}_2 \) mixtures and were used to perform weekly water changes. Experimental tanks were sampled daily for \( \text{pH}_T \), temperature, salinity, \( p\text{CO}_2 \), and every 72hrs for total alkalinity (\( T_A \)), during the acclimation period (Table 1). For \( p\text{CO}_2 \) analysis, we followed the standard operating procedure as described in the Best Practices Guide (Riebesell *et al.* 2010) for the spectrophotometric determination of \( \text{pH} \) using m-Cresol Purple and measurement of total alkalinity via acid titration using a computer-controlled T50 Titrator (Mettler Toledo, Columbus, OH, USA). Temperature was measured with a calibrated digital thermocouple (Omega Engineering Inc., Stamford, CT, USA) and salinity was measured using a YSI 3100 conductivity meter (Yellow Springs, OH, USA). The program \( \text{CO}_2 \) calc (Robbins *et al.* 2010), using the constants of Mehrbach *et al.* (1973) as refit by Dickson and Millero (1987), were used to calculate all other carbonate parameters. Additionally, water quality assessments were taken by
testing for ammonia, nitrite and nitrate levels every 72hrs. No detectable increase in nitrogenous waste was measured throughout each experiment.

**Evaluation of RMR and \( P_{\text{crit}} \)**

To establish routine metabolic rates (RMR), oxygen consumption rates were measured using an intermittent respirometry system (Loligo systems, Denmark) described in Enzor *et al.* (2013). Fish were housed in 500mL respirometry chambers fitted with internal acrylic sleeves (35-55mm internal diameter) to restrict movement. These chambers were placed in covered 12-L trays receiving a continuous flow of recirculating seawater (~115L total vol.) held at treatment temperature (14°C) and control (~400 µatm) pCO\(_2\). Fish were allowed to acclimate to the chambers overnight (10-12hrs) with flush pumps running before metabolic rates were determined. Oxygen consumption was recorded over a 12 min period, followed by a 5 min flush period allowing re-oxygenated seawater back into the chamber. Respiration rates (\( \dot{M}O_2 \)) were measured according to Enzor *et al.* 2013. Oxygen consumption measurements were collected continuously over a three-hour period with mean \( \dot{M}O_2 \) values determined once no discernable chamber effect could be observed as indicated by no significant changes in oxygen consumption over a 1h period and \( r^2 \) values of >0.95 for the slope describing the rate of oxygen consumption for each measurement during this same time period.

Following the determination of RMR, we next evaluated how acclimation to elevated pCO\(_2\) affected the hypoxia tolerance of *C. analis*. Critical oxygen tension (\( P_{\text{crit}} \)) was measured utilizing the same intermittent respirometry system. Using a solenoid controlled by a galvanic O\(_2\) probe, pure nitrogen was gradually bubbled into the sump tank. Respiration rates were measured as described above as dissolved oxygen levels were lowered from 8 mg/L to severe hypoxia (<0.5mg/L) over the course of ~3hrs. MO\(_2\) measurements continued on a 12 min record cycle.
followed by a 5 min flush cycle, until a discernable decrease in \( \dot{MO}_2 \) from RMR was noticed. At this point, 5 additional flush cycles were conducted to ensure accurate calculations for \( P_{\text{crit}} \). Upon completion of metabolic measurements, fish were humanely euthanized by immersion in MS-222 (Sigma Aldrich, St. Luis MO, USA). Gill tissues were excised, immediately frozen in liquid nitrogen and stored at -80°C until used for biochemical analysis.

*Spectrophotometric determination of Na\(^+\)/K\(^+\) ATPase enzyme activity*

Total Na\(^+\)/K\(^+\) ATPase (NKA) enzyme activity was quantified from gill tissue using protocols which were optimized from McCormick (1993). Briefly, gill tissue (~50mg) was homogenized in 200µL homogenization buffer (50mm Imidazole, 20mm Na\(_2\)EDTA, 300mm sucrose, 0.2mm PMSF, and 5mm BME, pH = 7.0), followed by centrifugation of the homogenate at 2000 rpm for 2 min. Supernatant was transferred to a new 1.5ml microcentrifuge tube where 2µL of a 5µg/µL alamethicin stock solution was added for improved membrane permeability, decreasing the risk of vesicle formation around enzymes. Samples were then incubated at the experimental temperature (14°C) in a water bath for 20min. ATPase activity was measured spectrophotometrically via the consumption of NADH in the presence or absence of the inhibitor ouabain. Uninhibited reactions were started by adding 15µL of protein extract to 100µL of MilliQ H\(_2\)O and 2mL assay cocktail (19 parts buffer – 148mM NaCl, 23.7mM KCl, 7.74mM MgCl\(_2\), 35.5mM Imidazole/Cl, 0.59mM EGTA, 0.47mM KCN, pH = 7 and 1 part substrate solution - 22.5mM Na\(_2\)ATP, 6.75mM Na\(_2\)NADH, 45mM Phosphoenolpyruvate) in a 3mL quartz cuvette. The cuvette was inverted to mix and the rate of NADH disappearance was monitored at 340nm. Inhibited reactions only differed in that 100µL of 11.25mM ouabain octahydrate was used instead of MilliQ H\(_2\)O. Total Na\(^+\)/K\(^+\) ATPase enzyme activity was
calculated by subtracting the rate of NADH disappearance in the inhibited reaction from the activity rate of the uninhibited reaction.

Statistics

We used a combination of one-way analysis of variance (ANOVA) and covariance (ANCOVA) models to test for the effects of treatment and acclimation time on our responses. Since mass has the potential to affect RMR (Clarke and Johnston 1999) and thus $P_{\text{crit}}$ (Richards 2011), variation in the size of individual fish was controlled for by using body mass as a covariate. Prior to analyses, we ensured our data met normality and homoscedasticity assumptions. Specifically, we utilized an ANCOVA model to test for the effect of $pCO_2$ on routine metabolic rates at the 7 and 28-day acclimation times, using mass as covariate. In addition, we ran an ANCOVA model to test for the effect of acclimation time on routine metabolic rates within $pCO_2$ treatment groups, using mass as a covariate. Similarly, an ANCOVA model was also used in the evaluation of critical oxygen tension ($P_{\text{crit}}$), with an effect of RMR or acclimation time and mass as a covariate. One-way ANOVA was used in the evaluation of $pCO_2$ and acclimation time on $\text{Na}^+$/K$^+$ ATPase enzyme activity. Finally, since fish were collected at three different times over a 5-month period, we used an ANCOVA, with mass as a covariate, to test for the effect of collection date to rule out physiological differences related to seasonality. All statistical analyses were performed using the JMP (v11) statistical analysis software.

Results

Seawater Chemistry
Temperature, salinity and total alkalinity remained constant over the course of both experiments with only pH and $pCO_2$ varying based on desired treatment levels (Table 1). Within both the 7 and 28 day acclimation groups, control and high $pCO_2$ treatments significantly differed (students t-test $p < 0.05$). Between acclimation times, only control $pCO_2$ and pH differed ($p < 0.05$). This difference however was less than 70 µatm $pCO_2$ or 0.06 pH units, well within natural variation of present day ocean conditions.

**Routine Metabolic Rate (RMR)**

Within both the 7 and 28 day acclimation groups, *C. analis* acclimated to high $pCO_2$ showed significant elevations in RMR (25.6% and 48.7% respectively) compared to their control counterparts (ANCOVA; 7 d - $F_{(1,21)} = 4.56$, $p = 0.047$; 28 d - $F_{(1,11)} = 20.002$, $p = 0.0009$) (Fig.1A). Interestingly, we found that RMR differed in the control treatments when compared between acclimation times (ANCOVA; control, $F_{(1,16)} = 6.18$, $p = 0.024$). Specifically, we observed a 52.9% decrease in RMR of 28 d acclimated fish (Fig. 1B). Alternatively, RMR in the high treatments remained the same at 7 and 28 days (ANCOVA; high, $F_{(1,16)} = 0.67$, $p = 0.424$). In addition we also confirmed that mass effected RMR at both 7 and 28 days (ANCOVA; 7 d – $F_{(1,21)} = 24.0045$, $p < 0.0001$; 28 d – $F_{(1,11)} = 16.0189$, $p = 0.0021$). Lastly, we found no effect of collection date on RMR (ANCOVA; $F_{(2,34)} = 2.358$ $p = 0.1099$).

**Hypoxia Tolerance ($P_{crit}$)**

We observed no significant changes in $P_{crit}$ between treatments as an effect of RMR at 7 days, however an increase in $P_{crit}$ was found at 28 days (ANCOVA; 28 d - $F_{(1,11)} = 9.70$ $p = 0.0098$). While *C. analis* were no less hypoxia tolerant at day 7, by day 28 their tolerance decreased, evidenced by a 33.6% increase in $P_{crit}$ (Fig. 2A). In addition, critical oxygen tension followed a pattern similar to RMR between acclimation times. Within the control treatment, $P_{crit}$
decreased by 58% across acclimation time (ANCOVA; control, $F_{(1,16)} = 6.91$ $p = 0.0182$), while no discernable variation was observed within the high treatment (ANCOVA; high, $F_{(1,16)} = 0.32$ $p = 0.580$)(Fig. 2B). Mass had no effect on $P_{\text{crit}}$ at 7 or 28 days (ANCOVA; 7 d, $F_{(1,21)} = 1.52$ $p = 0.231$; 28 d, $F_{(1,11)} = 1.30$ $p = 0.278$).

$Na^+/K^+$ ATPase Enzyme Activity

$Na^+/K^+$ ATPase total enzymatic activity was quantified in gill tissue as a proxy for acid base regulatory capacity in *C. analis*. We found that acclimation to a high $pCO_2$ environment had no effect on fish acclimated for 7 days (ANOVA; $F_{(1,22)} = 0.68$, $p = 0.419$), however, fish acclimated to high $pCO_2$ for 28 days showed a significant increase in activity (ANOVA; $F_{(1,12)} = 6.73$, $p = 0.024$) (Fig. 3A). The variability in response between fish acclimated to the same experimental treatment for 7 and 28 days coincided with responses observed in RMR and $P_{\text{crit}}$. Within the control $pCO_2$ treatment, $Na^+/K^+$ ATPase activity decreased 35.6% by day 28 (ANOVA; $F_{(1,17)} = 6.23$ $p = 0.023$), however, $Na^+/K^+$ ATPase activity remained constant in the high $pCO_2$ treatment (ANOVA; $F_{(1,17)} = 0.85$ $p = 0.369$)(Fig. 3B).

Discussion

Single stressor studies for many marine animals, including fish, have generated a framework for understanding physiological and behavioral responses to the environment (Cooper *et al.* 2002, Kurihara 2008, Munday *et al.* 2009). However, the ecological relevance of results from these single stressor studies may be limited, as it may be more common for organisms in the wild to experience shifts in multiple environmental parameters concomitantly, rather than a single parameter at a time. While studies exploring the effects of multiple stressors on fish are less common, results of the few studies that have been conducted on other taxa suggest that the
physiological response of a species to one environmental stressor can be contrary to the response to other stressors (Steckbauer et al. 2015). Such opposing responses raise the possibility that organisms may respond suboptimally under scenarios when multiple stressors change concomitantly (Gobler and Baumann 2016).

In this study, we set out to examine the vulnerability of C. analis to elevated pCO$_2$ levels (ocean acidification) in conjunction with hypoxia stress. Intertidal organisms that regularly experience steep gradients in pCO$_2$ likely have evolved greater capacities for offsetting the deleterious effects of short-term acidification (Seibel and Walsh, 2001, 2003). It is uncertain, however, if those physiological adaptations will translate into a greater tolerance for chronic exposure to elevated pCO$_2$. Complexities in topography, biodiversity/density, and local upwelling regimes within the rocky intertidal zone further confound this problem and point to the need for experimental studies that utilize exposure regimes more complex than static pCO$_2$ manipulation. However, to date, no studies have investigated the amplified effects of multiple stressors on the behavior or physiology of intertidal sculpins, even though research examining how these fishes respond physiologically to ocean acidification in an ecologically relevant context have the potential to provide crucial insights for predicting how acidification will impact rocky intertidal communities.

Our study examined how intertidal Woolly sculpins were affected by exposure to differing levels of acidification coupled with environmental hypoxia. Both of these environmental variables are expected to be important stressors for intertidal sculpins experiencing a changing climate. Our results indicate there are increased metabolic costs associated with living in a more acidic ocean, which in turn have consequences for hypoxia tolerance, as well as acid base regulation. More specifically, the ability of sculpins to (1) avoid
hypoxia through the down-regulation of oxygen demanding cellular machinery, directly conflicts with (2) the defense against increasing carbon dioxide (acidity) stress via the up-regulation of oxygen demanding Na\(^+\)/K\(^+\) ATPase pumps. Perhaps most strikingly however, is the fact that this study serves to highlight the degree of stress intertidal fishes are already surviving under, providing further support for the idea these organisms are likely living near their physiological limits.

*Hypercapnia and Metabolic Rate*

Previous studies have shown that physiological compensation to hypercapnia, either through acid base regulation or ventilation, results in increased energetic debt for the organism (Perry and Gilmour 2002, Evans *et al.* 2005, Perry and Gilmour 2006). Heuer and Grosell (2014) point-out that similar metabolic studies are highly limited and inconsistent in their results, but often operate under the assumption that fishes with capable acid base regulatory mechanisms should increase basal metabolism when faced with hypercapnia. Indeed, the metabolic rates of marine animals are highly variable, with debates flexing around the importance of anatomical features, ecological function and environmental constraints (Seibel and Drazen 2007). For *C. analis*, routine metabolic rate (RMR) was significantly higher in fish acclimated to elevated \(p\text{CO}_2\) in both the short (7-day) and longer-term (28-day) experiments, with the highest measured RMR coming after 28 days of acclimation to a \(p\text{CO}_2\) of \(~1100\) µatm. Metabolic responses consistent with our results have also been found in other fish species, including species from tropical (Munday *et al.* 2009) and polar (Enzor *et al.* 2013) ecosystems, where either total aerobic scope was decreased or RMR was increased. Our results suggest that within the intertidal, environmental constraints, most likely associated with fluctuating abiotic conditions
including hypercapnia, at least in part could significantly drive metabolic responses within *C. analis*.

While the RMR of *C. analis* acclimated to high pCO$_2$ was significantly higher than fish acclimated to the control treatment (~400 µatm), we observed no differences across time points within the high pCO$_2$ acclimation groups. This differs from the metabolic response observed in Antarctic notothenioids which initially display a sharp increase in RMR after 7 days of acclimation to elevated pCO$_2$, yet quickly return to basal ŌO$_2$ levels within 28 days of acclimation (Enzor *et al.* 2013). Unexpectedly, ŌO$_2$ values for *C. analis* acclimated to ~400 µatm pCO$_2$ showed a significant decline (~52.9%) after 28 days in experimental conditions compared to the RMR of fish acclimated to the control treatment for only 7 days. We postulate that placing these animals in a static experimental environment alleviated high levels of stress routinely encountered in the intertidal due to the absence of tidal or daily cycles, thus reducing the metabolic costs typically associated with life in a tide pool. Despite being periodically replenished with ambient seawater, tide pools in the marine intertidal zone are known to display extreme swings in abiotic parameters such as dissolved oxygen concentration, pH, salinity and temperature (Truchot and Duhamel-Jouve 1980, Morris and Taylor 1983, Bridges *et al.* 1984). It is plausible that the likelihood of experiencing these extreme environmental shifts is high enough to force these fish to maintain cellular defense mechanisms at a relatively high constitutive level. Osmoregulation and the heat shock response (HSR) are critical cellular defense mechanisms routinely employed by organisms that exist in highly variable environments (Kültz 2005, Fangue *et al.* 2006, Evans and Somero 2008, Somero 2010, Tomanek 2010), both of which can come at significant energetic costs to the organism (Goolish and Burton 1989, Iwama *et al.* 1998, McAllen and Taylor 2001, Kidder *et al.* 2006, Tomanek and Zuzow 2010). Relaxation of these
and other costly cellular defense mechanisms could explain the observed drop in RMR when significant environmental variation is removed. For example, field studies involving *Mytilus californianus* have shown key indications of both aerobic and anaerobic capacity which are elevated in populations routinely exposed to variation in pH and dissolved oxygen as a result of periodic upwelling. These same metabolic indicators are routinely lower in populations that do not experience significant variations in these abiotic parameters (Dahlhoff and Menge 1996). Furthermore, wave exposed regions within the rocky intertidal that provide relief from thermal stress are characterized by mussels with lower indicators of cellular stress (heat shock proteins) and increased RNA/DNA ratios (a proxy for growth potential) (Dahlhoff and Menge 1996, Dahlhoff *et al.* 2001, 2002).

By removing these natural environmental stressors in our control treatments we would expect to see indications of the relaxation of energetic constraints, particularly when examining the downstream effects of RMR on hypoxia tolerance, the role of NKA activity in acid base regulatory capacity, and the behavioral responses to hypoxia. For instance, some fish species like *C. analis* can meet metabolic oxygen needs during bouts of hypoxia through behavioral responses such as aerial surface respiration (Congleton 1980, Watters and Cech 2003). However, we were unable to observe these behavioral responses in *C. analis* individuals that had been acclimated to the control treatments even when exposed to severe anoxia for extended periods (data not shown). The absence of these behaviors suggests the control treatments never exerted a metabolic cost large enough to elicit the need to perform aerial surface respiration even under a low oxygen environment.

*Hypoxia Tolerance and Acid/Base Regulation*
Similar to our observations with respect to metabolic rate, *C. analis* did not exhibit any differences in hypoxia tolerance ($P_{\text{crit}}$) or $\text{Na}^+/\text{K}^+$ ATPase activity between treatments after 7 days of acclimation to elevated $p\text{CO}_2$. Although similar acid/base regulatory studies are absent among intertidal fish, others have observed increases in NKA for marine teleosts when challenged with hypercapnia (Deigweiher *et al.* 2009, 2010). Thus, our findings confirm the aforementioned idea that intertidal animals, particularly vertebrate fish, likely have the capacity to offset short-term bouts of acidification or hypercapnia. This comes as no surprise since seasonal upwelling, which acts as a selective pressure along the California coast, often brings these animals in contact with corrosive waters (Feely *et al.* 2008). Our data, however, do suggest that longer-term acclimation to high $p\text{CO}_2$ may pose a significant physiological cost to *C. analis* since we found a significant difference in hypoxia tolerance and acid base regulatory capacity between control and high $p\text{CO}_2$ treatment groups after 28 days of acclimation.

As seen with measurements of RMR, we again saw no difference in total acid base regulatory capacity or hypoxia tolerance in fish acclimated to elevated $p\text{CO}_2$ across time points. However, *C. analis* acclimated to control treatments showed significantly lower NKA levels and were more hypoxia tolerant in 28-day acclimated groups compared to 7-day acclimated groups. These data further support the idea that fish acclimated to the high $p\text{CO}_2$ treatment display physiological capacities that reflect the basal requirements of life in the intertidal. More specifically, our data confirm that acclimation to future OA scenarios sets increased demands on acid base balance in these fish via the NKA pump and that this in turn, comes at a metabolic cost that is equivalent to the maintenance costs associated with the current natural variation that occurs in rocky tide pools. For *C. analis*, it is unclear if the lack of change in NKA capacity over 28 days suggests these fish have reached their maximum capacity to respond. However, even if
these fish maintain sufficient capacity to further elevate NKA activity, they are likely to see further reductions in hypoxia tolerance.

Our data suggest that increases in $p$CO$_2$ are compensated, in part, through changes in acid/base regulatory capacity and this may indirectly impact hypoxia tolerance in these fish. RMR has been previously shown to directly impact $P_{\text{crit}}$ (Richards 2011). As such, the increased metabolic rate necessary to maintain NKA capacity under high $p$CO$_2$ has resulted in a reduction in hypoxia tolerance. This reduced tolerance is increasingly problematic when considering the exacerbating effect of OA on natural fluctuations in tide pool carbon dioxide levels. For example, Truchot (1988) demonstrates that at nighttime, when respiration rates are high, $p$CO$_2$ levels in tide pools can exceed 3,000 µatm. As global $p$CO$_2$ averages rise, it is likely that tide pools with little photosynthetic activity could reach similar values during day time hours, creating increased oxygen demands on these systems. Field observations (data not shown) confirm many $C. \text{analis}$ reside in high intertidal pools where algal cover is limited, thus they may find themselves continuously challenged to meet the metabolic demands of life in a highly variable environment.

**Conclusions**

The rocky intertidal is widely understood to be one of the most abiotically challenging environments on the planet (Denny and Wethey 2001, Tomanek and Helmuth 2002). Organisms native to these habitats are therefore under extreme selective pressures, which demand physiological mechanisms capable of offsetting the deleterious effects of a harsh existence. Furthermore, numerous studies have demonstrated that many invertebrate species are functioning near maximal physiological capacity (Hofmann and Todgham 2010, Somero 2010). We have
provided the first evidence suggesting an intertidal vertebrate, the Woolly sculpin, may similarly be operating near the edge of its tolerance when challenged by ocean acidification.

As we evaluate the ability of marine species to respond to environmental stress associated with climate change, it becomes increasingly important to consider the effects of multiple naturally occurring concomitant stressors. Our study highlights two naturally occurring stressors, hypoxia and OA, that may require trade-offs between hypoxia tolerance and acid-base regulation capacity. It is likely that climate change will further exacerbate these natural stressors, emphasizing the need for scientific studies to address these challenges. To our knowledge, no studies have looked at the effects of these interacting climate change related stressors on intertidal fish. And thus this study provides critical insight into an important component of rocky intertidal ecosystems.

Moving forward, this study highlights an increasing need for investigations into the synergistic effects of climate change on our coastal fish as the world’s oceans continue to warm and low oxygen environments further expand within the California Current Large Marine Ecosystem (Di Lorenzo et al. 2005, Bograd et al. 2008). In a climate change context, many marine species have been broadly studied (Pörtner et al. 2005, Brennand et al. 2010, Edmunds et al. 2012, Harvey et al. 2013, Gaitán-Espitia et al. 2014) but only recently have fishes received similar consideration (Munday et al. 2009, Enzor et al. 2013, Ferrari et al. 2015). In the end, similar to our study, the limited data available from these studies points to a critical need to consider the broader effects that climate change may have on overall condition of these animals. It has become evident that even though an organism may possess the physiological tools and capacity to withstand predicted changes in ocean chemistry, that physiological plasticity likely comes at a significant cost that will have impacts on behavior and or energy allocation, which
may alter ecosystem dynamics. Lastly, these studies have helped to highlight that our current understanding of the susceptibility of teleost fish to changes in ocean pH may be underestimated. Consequently, future conservation strategies must consider multi-stress experiments when assessing the impacts of global climate change on species distributions and population dynamics.
Table 1. Mean measurements of temperature, salinity, pH, $p\text{CO}_2$, and total alkalinity ±SD over the course of the experiment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Duration</th>
<th>Temperature (°C)</th>
<th>Salinity (ppt)</th>
<th>pH</th>
<th>$p\text{CO}_2$ (µatm)</th>
<th>Total Alkalinity (µmol/kg sol'n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7 days</td>
<td>13.79 ± 0.74</td>
<td>33.46 ± 0.69</td>
<td>8.042 ± 0.034</td>
<td>406.15 ± 34.04</td>
<td>2286.51 ± 150.41</td>
</tr>
<tr>
<td>High</td>
<td>7 days</td>
<td>13.70 ± 0.80</td>
<td>33.73 ± 0.80</td>
<td>7.656 ± 0.055</td>
<td>1105.51 ± 152.72</td>
<td>2305.92 ± 41.29</td>
</tr>
<tr>
<td>Control</td>
<td>28 days</td>
<td>13.86 ± 0.32</td>
<td>33.48 ± 0.15</td>
<td>7.982 ± 0.045</td>
<td>475.05 ± 51.44</td>
<td>2273.10 ± 35.07</td>
</tr>
<tr>
<td>High</td>
<td>28 days</td>
<td>13.82 ± 0.31</td>
<td>33.53 ± 0.23</td>
<td>7.644 ± 0.034</td>
<td>1118.59 ± 93.20</td>
<td>2271.60 ± 22.85</td>
</tr>
</tbody>
</table>
Figure 1. (A) The routine metabolic rate (RMR) of *C. analis* when evaluating the effect of pCO$_2$ treatment at both acclimation times, 7 and 28 days, and controlling for the mass of individual fish. Values are least squared means ± SE. Asterisks indicated statistical significance. (B) The routine metabolic rate (RMR) of *C. analis* when evaluating the effect of acclimation time in control and high pCO$_2$ treatments, and controlling for the mass of individual fish. Values are least squared means ± SE. Asterisks indicate statistical significance.
Figure 2. (A) The hypoxia tolerance ($P_{crit}$) of *C. analis* when evaluating the effect of RMR at both acclimation times, 7 and 28 days, and controlling for the mass of individual fish. Values are least squared means ± SE. Asterisks indicate statistical significance. (B) The hypoxia tolerance ($P_{crit}$) of *C. analis* when evaluating the effect of acclimation time in control and high pCO$_2$ treatments, and controlling for the mass of individual fish. Values are least squared means ± SE. Asterisks indicated statistical significance.
Figure 3 (A) The total Na\(^+\)/K\(^+\) ATPase activity when evaluating the effect of pCO\(_2\) treatments at both acclimation times, 7 and 28 days. Values are adjusted means ± SE. Asterisks indicated statistical significance. (B) The total Na\(^+\)/K\(^+\) ATPase activity when evaluating the effect of acclimation time in both control and high pCO\(_2\) treatments. Values are adjusted means ± SE. Asterisks indicated statistical significance.
References


