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RESEARCH ARTICLE

Juvenile Antarctic rockcod (*Trematomus bernacchii*) are physiologically robust to CO_2-acidified seawater

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ABSTRACT

To date, numerous studies have shown negative impacts of CO_2-acidified seawater (i.e. ocean acidification, OA) on marine organisms, including calcifying invertebrates and fishes; however, limited research has been conducted on the physiological effects of OA on polar fishes and even less on the impact of OA on early developmental stages of polar fishes. We evaluated aspects of aerobic metabolism and cardiorespiratory physiology of juvenile emerald rockcod, *Trematomus bernacchii*, an abundant fish in the Ross Sea, Antarctica, to elevated partial pressure of carbon dioxide (P_{CO_2}) [420 (ambient), 650 (moderate) and 1050 (high) μatm P_{CO_2}] over a 1 month period. We examined cardiorespiratory physiology, including heart rate, stroke volume, cardiac output and ventilation rate, whole organism metabolism via oxygen consumption rate and sub-organismal aerobic capacity by citrate synthase enzyme activity. Juvenile fish showed an increase in ventilation rate under high P_{CO_2} compared with ambient P_{CO_2}, whereas cardiac performance, oxygen consumption and citrate synthase activity were not significantly affected by elevated P_{CO_2}. Acclimation time had a significant effect on ventilation rate, stroke volume, cardiac output and citrate synthase activity, such that all metrics increased over the 4 week exposure period. These results suggest that juvenile emerald rockcod are robust to near-future increases in OA and may have the capacity to adjust for future increases in P_{CO_2} by increasing acid-base compensation through increased ventilation.

KEY WORDS: Antarctica, Cardiorespiratory physiology, Early life stages, Notothenioid, Ocean acidification

INTRODUCTION

As levels of atmospheric carbon dioxide (CO_2) rise as a result of increased anthropogenic emissions of CO_2, the world's oceans are concurrently absorbing CO_2 and becoming more acidic. Increased dissolved CO_2 in the ocean alters the chemical equilibrium, decreasing carbonate ion concentration and pH (Feely et al., 2004) in a process known as ocean acidification (OA) (Sabine et al., 2004; Caldeira and Wickett, 2005; Orr et al., 2005; Meehl et al., 2007; Gattuso et al., 2014). In the past 200 years, the ocean has absorbed over 50% of the anthropogenically produced CO_2 and open-ocean pH has decreased by 0.1 units (Sabine et al., 2004). Global climate change (GCC) models predict that by the next century, ocean pH will decline by an additional 0.2–0.4 units (Ciais et al., 2013; Gattuso et al., 2014) and an accumulating body of literature suggests that OA will have negative consequences for marine organisms across different ecosystems (Kroeker et al., 2013). In order to predict the impact of OA on marine organisms it is important to understand whether contemporary individuals currently exhibit the capacity to tolerate projected OA scenarios and possess the mechanisms necessary to respond to elevated partial pressures of carbon dioxide (P_{CO_2}).

High-latitude oceans are projected to undergo the greatest decreases in pH, from 0.3 to 0.5 units by the end of the century (Ciais et al., 2013) because of the increased solubility of gases in colder waters. Polar ecosystems support organisms that are adapted to cold, stable environments (Eastman, 1993) and as a result, are predicted to be more sensitive than lower-latitude systems to changes in ocean conditions associated with GCC (Barnes and Peck, 2008; Fabry et al., 2009; Barnes et al., 2009). The physical effects of GCC in polar oceans have already been documented and are of particular concern. Increases in sea and air temperatures along the Antarctic Peninsula are occurring at the fastest rates on Earth (Meredith and King, 2005), alongside a net decrease in ocean pH, and predicted rapid changes in ocean P_{CO_2} could create highly unfavorable conditions for marine organisms by 2050 (McNeil and Matear, 2008; McNeil et al., 2010).

Real-time monitoring of near-shore Antarctic habitats has shown that P_{CO_2} is quite variable (Kapsenberg et al., 2015), with several Antarctic habitats projected by 2100 to experience yearly cycles of harmful, low pH during the Antarctic winter (Kapsenberg et al., 2015). Such results suggest that in polar regions the effects of GCC may manifest in greater P_{CO_2} variability. Potential biological effects of elevated P_{CO_2} have already been observed in several laboratory studies on Antarctic calcifying marine organisms, including morphological changes with reduced growth (Yu et al., 2013), developmental malformations (Byrne et al., 2013) and the dissolution of shells (Orr et al., 2005; McClintock et al., 2009; Bednaršek et al., 2012). Physiological alterations in oxygen consumption rates, heat shock proteins and enzymes involved in shell growth have also been observed (Cummings et al., 2011). Although more recent studies have expanded the list of taxa vulnerable to OA to include non-calcifying organisms such as fishes, these OA studies in fishes have primarily focused on tropical (Dixson et al., 2010; Nowicki et al., 2012; Pimentel et al., 2014a) and temperate species (Hurst et al., 2012; Hamilton et al., 2014; see also review by Heuer and Grosell, 2014). Few studies have been conducted on the impact of increased P_{CO_2} on polar fishes (Strobel et al., 2012, 2013; Enzor et al., 2013; Enzor and Place, 2014) and even fewer on early developmental stages of polar fishes (Flynn et al., 2015).

Although knowledge of the impact of OA on fishes has grown, large gaps remain in understanding the effects of OA on fish physiology. Physiological and biochemical processes are largely pH dependent, such that changes in blood and tissue pH due to increased environmental P_{CO_2} can have negative effects on critical

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List of symbols and abbreviations

CO	cardiac output
CS	citrate synthase
EDV	end diastolic volume
ESV	end systolic volume
f_H	heart rate
f_V	ventilation rate
GCC	global climate change
$\dot{M}_{O_2}$	metabolic rate
OA	ocean acidification
P_{CO_2}	partial pressure of carbon dioxide
V_S	stroke volume

processes (Ishimatsu et al., 2005, 2008; Heuer and Grosell, 2014), and these effects may vary with acclimation time (Pörtner et al., 2004). For instance, short-term acute effects of elevated P_{CO_2} on physiology might include alterations in cardiorespiratory phenotypes, acid-base balance, blood circulation and the nervous system, whereas longer acclimation periods can reduce growth and reproduction (Ishimatsu et al., 2008; Pörtner et al., 2004; Esbaugh et al., 2012; Heuer and Grosell, 2014). Adult fishes are suggested to be more tolerant to OA because of their well-developed ion-regulatory mechanisms (Melzner et al., 2009; although some studies have shown otherwise: e.g. Devine et al., 2012), with early life-history considered to be especially sensitive to environmental change (Ishimatsu et al., 2008; Melzner et al., 2009; Pankhurst and Munday, 2011).

Several studies have shown that the early developmental stages of fishes are indeed vulnerable to elevated P_{CO_2} including changes in metabolism and swimming duration (Pimentel et al., 2014a), swimming speed (Bignami et al., 2013), sensory perception and behavior (see reviews by Munday et al., 2012; Clements and Hunt, 2015), morphological deformations (Chambers et al., 2014; Pimentel et al., 2014b; Mu et al., 2015), and survival and growth (Kikkawa et al., 2003; Baumann et al., 2011; Frommel et al., 2011, 2014). Other studies have shown that the early stages of fishes are relatively robust, remaining unaffected by elevated P_{CO_2} (Hurst et al., 2012; Frommel et al., 2013), suggesting that sensitivity to OA is both species and life-stage specific. Even for tolerant fish species, it remains uncertain whether the additional energetic costs of coping with elevations in P_{CO_2} will have negative fitness costs, and hence, longer-term studies of the consequences of OA must be conducted.

In the current study, we examined the effect of elevated P_{CO_2} (OA) on the juvenile emerald rockcod *Trematomus bernacchii* Boulenger 1902, one of the most abundant notothenioid fishes in the Ross Sea, Antarctica (Vacchi et al., 2000). Several studies have characterized the physiological response of adult *T. bernacchii* to various environmental changes, including temperature (Todgham et al., 2007; Buckley and Somero, 2009: Jayasundara et al., 2013; Sandersfeld et al., 2015), hypoxia (Davison et al., 1994) and toxicants (Regoli et al., 2005; Borghesi et al., 2008), with recent work on the effects of elevated P_{CO_2} (Enzor et al., 2013; Enzor and Place, 2014). There remains no information on physiological performance of earlier life-history stages in this species. The emerald rockcod is a benthic notothen with a broad depth range of roughly 50–400 m (Eastman, 1993), with life-history stages inhabiting different mean depths (North, 1991). The juveniles in McMurdo Sound have been observed to settle into shallower anchor ice and crevasses to avoid predation (<20 m). Since juveniles and adults vary in the habitat use and have different energetic demands (e.g. development and growth versus reproduction), it is possible that different life-history stages may have different sensitivities to elevated P_{CO_2}. Here, we investigated both acute and longer-term (1–4 weeks) effects of exposure to elevated P_{CO_2} on the aerobic metabolism of juvenile *T. bernacchii*. On the basis of previous OA studies on early life stages of temperate and tropical fishes, the sensitivity of adult Antarctic fishes to P_{CO_2}, and the lack of any physiological studies on the capacity of juvenile *T. bernacchii* to respond to environmental change, we predicted that these younger stages of fish would be more sensitive to elevated P_{CO_2}. We hypothesized that elevated P_{CO_2} would increase physiological costs and energetic demands of juvenile *T. bernacchii*.

Environmental stressors, such as increased temperature, hypoxia and very high levels of P_{CO_2} (e.g. 8000–50,000 µatm P_{CO_2}; Cech and Crocker, 2002; Lee et al., 2003), have been shown to alter the cardiorespiratory physiology of fishes, which, in turn, can negatively affect oxygen circulation and delivery to body tissues (Mark et al., 2002; Pörtner and Farrell, 2008). Previous research has shown that adult Antarctic species, including *T. bernacchii*, *T. newnesi*, *T. hansoni* and *Pagothenia borchgrevinki*, increase aerobic metabolism when acclimated to elevated P_{CO_2}, probably to meet increased energetic demands (Enzor et al., 2013). We assessed juvenile cardiorespiratory physiology by examining heart rate, stroke volume, cardiac output and ventilation rate in response to elevated P_{CO_2} to provide insight into oxygen supply and circulation mechanisms. Changes in aerobic metabolism, measured as oxygen consumption rate and citrate synthase activity in the current study, provide insight into the capacity of fishes to supply enough energy to support any increased physiological costs associated with OA, such as an increase in basic cellular maintenance mechanisms. Investigating how juveniles of an abundant Antarctic fish species are impacted by OA, and whether younger stages of fish have the physiological strategies to compensate for increased levels of P_{CO_2} by adjustments of cardiorespiratory mechanisms, will provide much needed information on stage-specific vulnerability of polar fishes to OA.

MATERIALS AND METHODS

Experimental P_{CO_2} system and seawater chemistry

Three experimental P_{CO_2} treatments were selected based on the climate change scenarios predicted for 2100, where 420 µatm represents ambient seawater (Matson et al., 2011; Hofmann et al., 2011), 650 µatm P_{CO_2} represents a moderate scenario, and 1050 µatm P_{CO_2} represents a high, worst case scenario prediction (Meehl et al., 2007). These three P_{CO_2} treatments were simulated and maintained using a gas-delivery CO_2 system as described in Fangue et al. (2010) with modifications as described in Flynn et al. (2015). Briefly, CO_2 and air (moisture and CO_2 removed) were mixed using mass-flow controller valves (Sierra Instruments, Monterey, CA, USA). Mixed gas was then delivered to a venturi injector to be vigorously bubbled with seawater continuously drawn from McMurdo Sound, creating a 19 l equilibrated reservoir bucket for each P_{CO_2} treatment. Nine 19 l culture buckets, with three replicates per P_{CO_2} treatment, were continuously dripped with reservoir treatment water (and water in the reservoirs replaced) at a rate of 16 l h^{-1}. In addition, appropriate mixed P_{CO_2} gas was directly bubbled into each culture bucket at 1.02 l min^{-1}.

Seawater chemistry was closely monitored throughout the experiment. Total alkalinity was measured every 4 days in the gas-mixing reservoirs for the duration of the 28 day experiment using open-cell titration (T50 titrator, Mettler Toledo, Columbus, OH, USA) with a certified reference material (CRM) standard and

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0.1 mol l^{-1} HCl in seawater titrant (Dickson Laboratory, Scripps Institute, La Jolla, CA, USA) (SOP 3b, Dickson et al., 2007). Total pH was measured spectrophotometrically (Shimadzu) for each reservoir and culture bucket every other day using m-cresol dye (SOP 6b, Dickson et al., 2007). Temperature of the circulating tank and culture buckets was recorded daily using both a handheld thermocouple (HH81A, Omega) and submerged HOBO data loggers recording tank temperature every 30 min (Onset, Bourne, MA, USA). Salinity was measured for each seawater sample used to measure total alkalinity (YSI 3100 conductivity meter, Yellow Springs, OH, USA). Seawater P_{CO_2} was calculated using the seawater carbonate assessment package SeaCarb (Lavigne et al., 2011) in R (v3.0.3, R Development Core Team), with inputs of total alkalinity, pH, temperature and salinity. Experimental P_{CO_2} treatment values are presented in Table 1.

Fish collection and maintenance

Once the experimental P_{CO_2} treatments were stabilized, juvenile emerald rockcod *Trematomus bernacchii* (standard length=38.9±0.1 mm, mass=541±8 mg, mean±s.e.m.) were collected from 1 to 10 m water depths by SCUBA in late October to early November 2013 from Cape Evans Ice Wall, McMurdo Sound, Ross Sea, Antarctica (77°38′23.8″S, 166°31′09.7″E). Fish were transferred to an aerated, insulated cooler (−1.8°C) and transported to the A.P. Crary Science and Engineering Center at McMurdo Station within 3 h of collection (−1.2°C upon arrival). Fish were then counted, visually assessed for injury and placed into a flow-through circulating seawater tank at −1.0±0.2°C, and held at these conditions until the start of the experiment (5–9 days depending on collection date). Fish were assumed to be roughly in their second year of age based on a combination of previous findings that Antarctic fish larvae metamorphose into juveniles around 30 mm from 6 to 12 months of age (North, 1991), and we observed a single ring from the central nucleus of the otolith (La Mesa et al., 1996). The sex of fish at this juvenile life-history stage could not be determined. Thirty-nine fish were randomly selected and placed in each triplicate bucket, N=117 for each P_{CO_2} treatment. Fish in each P_{CO_2} treatment bucket were fed frozen brine shrimp once daily (~5 brine shrimp μl^{-1}; San Francisco strain, *Artemia franciscana*, Brine Shrimp Direct, Oghen, UT, USA) as described for similar sized fish in Chambers et al. (2014). Every day, each culture bucket was siphoned to remove feces and maintain water quality. Nitrogenous waste levels in culture buckets were monitored by checking nitrate, nitrite and ammonia levels (Aquarium Pharmaceuticals, Mars, McLean, VA, USA) daily during the first week of the experiment, followed by monitoring ammonia levels in culture buckets every 2 days thereafter. Fish were held in P_{CO_2} treatment conditions up to 4 weeks and sub-sampled immediately before the start of the experiment (Pre-exp) and following 1, 7, 14 and 28 days under different P_{CO_2} conditions to provide insight into possible short-term physiological changes and longer-term acclimatory changes. Owing to limited previous work on the identification of juvenile *T. bernacchii* and the presence of juveniles with slightly different coloration patterns in the experiment, DNA barcoding was performed to confirm the juveniles in this study were *T. bernacchii* (following Ivanova et al., 2007). The research project was conducted in accordance with US federal animal welfare laws by approval by the University of California Davis Institutional Animal Care and Use Committee (protocol no. 18248).

Cardiorespiratory physiology

Video recordings of fish in their respective P_{CO_2} treatments were used to determine ventilation rate (f_V), heart rate (f_H), stroke volume (V_S) and cardiac output (CO). For each experimental trial, three fish, one from each P_{CO_2} treatment, were placed in separate 75 ml flasks. In an environmental room held at −1°C, each flask was secured horizontally above the video camera and recorded from their ventral side for 15 min. From the ventral side of the fish, the heart could easily be visualized through the translucent body wall. This process was repeated until three fish from each triplicate bucket had been recorded (N=9 per P_{CO_2} treatment, n=27 per time point). At the 4 week sampling time, additional video recordings were made on remaining fish, which were included in the cardiorespiratory measurements (28 days: ambient N=10, moderate N=14, high N=9). Following video recordings, fish were quickly killed in a lethal dose of 0.3% tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA, USA) for length and weight measurements and DNA barcoding. Video recordings were analyzed using VLC media player (VideoLan, v2.0.9). Several preliminary trials were conducted to determine: (1) if vial volume and the amount of time for recording altered physiological metrics including f_V and f_H, and (2) if fish should be anesthetized during the trial (data not shown). Both f_V and f_H remained similar (stable and low) when measured every 5–10 min over a 1 h period. Both f_V and f_H were similar (stable and low) in the smaller flasks compared with a larger flask size (75 ml vs 250 ml) across time and smaller flasks were used for all subsequent analyses. A low dose of MS-222 anesthetic as in Mirkovic and Rombough (1998) continued to decrease f_V and f_H over time, and therefore was not used.

Ventilation

Ventilation rate (f_V) was measured as a proxy for minute volume, a parameter that can provide insight into ventilatory acid-base compensation. f_V was determined by counting the number of opercular movements, or breaths, per minute (breaths min^{-1}) in three 1 min video sequences. Values were then averaged to produce a single f_V value per fish. If a 1 min section could not be obtained because of subtle fish movement, a 30 s sequence was analyzed and multiplied by two for the breaths min^{-1} value.

Table 1. Experimental seawater chemistry for ambient, moderate and high P_{CO_2} treatments

Parameters	Incoming seawater	Ambient	Moderate	High
Temperature (°C)	−0.99±0.21	−0.93±0.04	−0.92±0.03	−0.91±0.02
Salinity (ppt)	33.7±0.3	33.7±0.3	33.7±0.3	33.7±0.3
pH (total scale)	7.962±0.004	8.010±0.003	7.844±0.007	7.641±0.006
P_{CO_2} (μatm)	485±6	430±3	649±11	1065±14
Alkalinity (μmol kg^{-1})	2351.4±2.5	2351.1±2.8	2352.1±1.9	2352.3±2.7
DIC (μmol kg^{-1})	2265.8±3.5	2249.2±1.1	2303.0±2.0	2363.0±1.9
Ω aragonite (calc.)	1.16±0.01	1.28±0.01	0.91±0.01	0.58±0.01

Temperature, salinity, pH and alkalinity were directly measured, whereas P_{CO_2}, DIC and aragonite were calculated using SeaCarb (Lavigne et al., 2011). Values are presented as mean±s.d.

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Cardiac performance

Heart rate (f_H; beats min^{-1}) was analyzed using the same three video sequences as in f_V. Heartbeats were counted for each 1 min video sequence and a mean was calculated from three video sequences. Stroke volume (V_S) was determined by slowing the video sequences by 50% and capturing five still frame pairs of the heart at its maximal and minimal dimension, representing end-diastolic volume (EDV) and end-systolic volume (ESV), respectively. EDV and ESV images were converted to a tri-color scheme to enhance contrast between tissue and blood in the heart and analyzed in ImageJ (1.47v, Java 1.6.0_65). Following Mirkovic and Rombough (1998), the heart was modeled as a prolate spheroid such that V, the estimated heart volume (i.e. EDV or ESV) is calculated using:

$$V = \frac{4}{3}\,\pi a b^2, \quad (1)$$

where a is one-half the length of the major axis (width) of heart and b is one-half the minor axis (length) of the heart. Major and minor axes were determined following Mirkovic and Rombough (1998) and Jacob et al. (2002) with slight modifications. The outline of blood flow within the heart was traced, fitted with an ellipse, and the major and minor axes lengths were determined using photo calibrated to a micrometer standard (mm) taken during the real-time video recordings. Each of the five image pairs was traced five times to reduce error and averaged. V_S was calculated as the difference between the EDV and ESV, corrected for mass of the fish, and presented as mass-specific V_S (µl g^{-1} $beat^{-1}$). Cardiac output (CO) was calculated as $f_H \times V_S$ and presented as mass-specific CO (µl g^{-1} min^{-1}). Sample sizes for V_S and CO for some groups were reduced (N=4–10) as a result of the position of fish in the vials. When fish were not resting flat against the bottom of the vial, images of the heart were skewed, which made it difficult to analyze heart volume.

Metabolic rate

Routine mass-specific metabolic rate ($\dot{M}_{O_2}$) was indirectly determined by measuring oxygen consumption of fish in their respective P_{CO_2} treatment (−1.35±0.05°C) using closed-chamber respirometry. The rate of oxygen consumption was measured by recording decreases in oxygen saturation using a fiber optic oxygen meter and oxygen sensor spots (Witrox 4, Loligo Systems, Denmark; accuracy of 0.4% at 20.9% O_2) fixed within 150 ml chambers. At 7, 14 and 28 days, fish from each P_{CO_2} treatment were measured simultaneously, at their acclimation P_{CO_2}, until three fish from each triplicate bucket were measured (N=9 per P_{CO_2} treatment; N=27 per time point). Oxygen consumption was recorded continuously (every 1 s) for 1 h and the chambers were manually mixed every 5 min with a paddle affixed to the top of the chamber. A control chamber was run for each P_{CO_2} treatment to account for any background biological activity in the seawater. Routine $\dot{M}_{O_2}$ was also measured for 11 fish immediately before the start of the experiment (pre-exp). Following $\dot{M}_{O_2}$ trials, fish were quickly killed with a lethal-dose of 0.3% MS-222, measured for length and weight, and the caudal section was sampled for DNA barcoding to confirm fish species. We recognize that our methods for measuring oxygen consumption do not follow standard procedures, as the consensus in the field is to allow 24–48 h of rest for fish in the $\dot{M}_{O_2}$ chambers to eliminate capture and handling stress, which has been shown to elevate $\dot{M}_{O_2}$ (Holeton, 1974; Clark et al., 2013). Unfortunately, due to logistical limitations and challenges associated with the extreme cold at an isolated field station, we were constrained to using closed-chamber $\dot{M}_{O_2}$ measurements. Preliminary trials were conducted showing no change in fish $\dot{M}_{O_2}$ over 2 h (see Fig. S1); therefore, 1 h trials were run to maximize the number of fish that could be included at each sampling time.

Because fish activity could not be monitored during measurements and fish could not be acclimated for long periods within the respirometry chambers, quantifying metabolic rates that best approximated routine values required a unique quantile-analysis approach as described in Chabot et al. (2016). The first 20 min of data showed elevated $\dot{M}_{O_2}$ compared with the last 40 min, which is probably the result of handling stress and was therefore excluded (see Fig. S1). The remaining 40 min of data was divided into 20 non-overlapping 2 min regions, over which individual linear regressions were fit. The result was 20 individual estimates of metabolic rate per fish. Within this distribution of metabolic rates, some are likely to include periods when the fish was active (those in the right tail of the distribution) and some when the fish was sedentary (those in the left tail). Recognizing that there is a distribution of metabolic rates for each individual fish and that activity was not monitored, quantiles (a range of P-values referred to as $q_{0.1}$, $q_{0.2}$, $q_{0.5}$, $q_{0.8}$ in this paper) were calculated (see Chabot et al., 2016). With the left tail of the distribution characterizing the lowest metabolic rates, $q_{0.2}$ of the distribution (the 20th quantile) was used as the estimate of each individual's routine metabolic rate. This metric was chosen because it is sensitive to the distribution of metabolic rates and serves as a reasonable compromise between too few data points in the tail (such as with $q_{0.05}$ or $q_{0.1}$, potentially assuming no spontaneous activity and/or stress) and too many points in the tail (such as $q_{0.8}$, potentially incorporating metabolic rates during which the fish was active). Comparative quantile values have been used previously, such as $q_{0.15}$ in halibut and shrimp (Dupont-Prinet et al., 2013a,b) and $q_{0.25}$ in snakes (Dorcas et al., 2004). Calculated $\dot{M}_{O_2}$ is presented as µmol O_2 h^{-1} g^{-1} wet weight.

Citrate synthase enzyme assay

At 1, 7, 14 and 28 days, three fish from each P_{CO_2} triplicate bucket (N=9 per P_{CO_2} treatment, per sampling time) were removed and killed in 0.3% MS-222. Muscle tissue and the tail caudal section were sampled, snap frozen in liquid nitrogen, and shipped to University of California, Davis, for citrate synthase enzyme activity assays. *T. bernacchii* muscle contains a mixture of muscle fibers but is predominantly white muscle with smaller oxidative (red) and intermediate fibers (pink) intermixed within the upper and middle trunk and tail sections of muscle tissue (Davison and MacDonald, 1985). Muscle fibers could not be identified or separated in the juvenile fish and hence a single cross-section of muscle tissue from the lower trunk area was made for each fish for measuring citrate synthase enzyme activity.

Citrate synthase (CS), a key enzyme in the tricarboxylic acid cycle that provides a measure of aerobic potential, was measured in whole muscle tissue as in Flynn et al. (2015) modified from Jayasundara et al. (2013). CS activity was monitored in a BioTek Synergy HT spectrophotometer at 25°C and measured as the maximum rate of increase in absorbance at 412 nm, caused by the production of a coenzyme A-SH (sulfhydryl group) monitored by DNTB. CS enzyme activity was calculated by subtracting the background activity (negative control) from the CS enzyme activity (positive reaction) for each sample and quantified using the molar extinction coefficient of DTNB (14.15 ml $µmol^{-1}$ cm^{-1}). Protein concentrations of each tissue homogenate were determined using the bicinchoninic acid method (Smith et al., 1985) with bovine serum albumin as the protein standard (Thermo Fisher Scientific).

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CS enzyme activity was then calculated per mg of protein and expressed as µmol min^{-1} mg $protein^{-1}$.

Statistical analyses

Statistical analyses were conducted in R (v3.0.3, R Development Core Team). All individual fish were nested within their replicate culture bucket to check for an effect of 'bucket'. Each model test showed no effect of culture bucket, and hence 'bucket' was not kept in the statistical analyses. Data were analyzed for normality and homogeneity of variances of residuals for the assumptions of an analysis of variance (ANOVA). Both visual inspections and Shapiro–Wilks for normality tests and Levene's test for variances were conducted, data were transformed (square-root transformation) if the assumptions were not met, and a two-way ANOVA was run with significant F-values followed up with a Tukey HSD test. The alpha value was set at 0.05. Ventilation rate, heart rate, stroke volume, cardiac output, metabolic rate (transformed) and citrate synthase (transformed) were the dependent parameters used in the statistical tests, whereas P_{CO_2} treatment and acclimation time were independent variables. Measurements taken immediately before the start of the experiment are presented visually in figures as a pre-experimental (Pre-exp) value, but these data were not included in statistical analyses. Results are presented as means±s.e.m. unless noted otherwise.

RESULTS

Ventilation

Fish ventilation rate (f_V) was significantly affected by P_{CO_2} treatment (Fig. 1; two-way ANOVA, $F_{2,78}$=6.311, P<0.01) and acclimation time ($F_{2,78}$=5.657, P<0.01); however, there was no interaction between P_{CO_2} treatment and acclimation time ($F_{4,78}$=2.337, P=0.062). Overall f_V was greater in the high P_{CO_2} compared with the ambient P_{CO_2} treatment (Tukey HSD, P=0.0019) by 4 opercula beats (i.e. breaths). Ventilation rate in the ambient (34±0.7 breaths min^{-1}) and moderate (36±0.5 breaths min^{-1}) P_{CO_2} treatments did not differ significantly (P=0.095) nor did f_V between moderate and high (38±0.6 breaths min^{-1}) P_{CO_2} treatments (P=0.266). Furthermore, f_V of all fish at 28 days of acclimation increased significantly (38±0.4 breaths min^{-1}) in comparison to fish acclimated to P_{CO_2} treatments for 14 days (35±0.7 breaths min^{-1}, P=0.017) and 7 days (35±0.6 breaths min^{-1}, P=0.012). Ventilation rates were similar at 7 and 14 days (P=0.993).

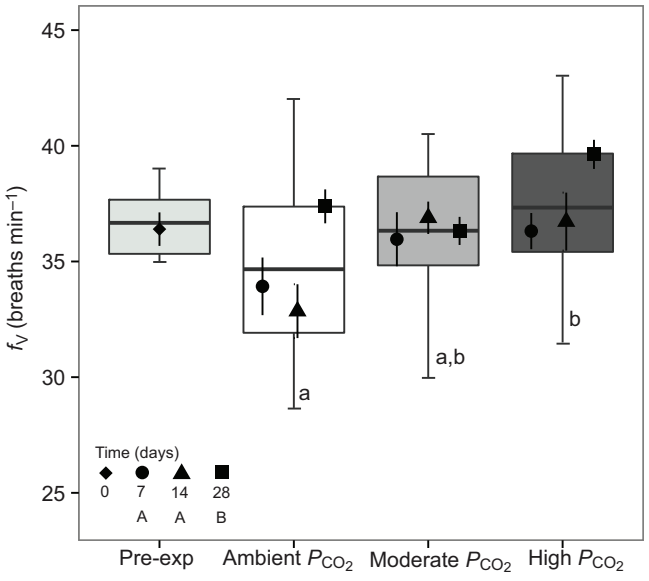

Fig. 1. Ventilation rate (f_V) in breaths per minute of *Trematomus bernacchii* across different P_{CO_2} treatments. The line on the box plot represents the median, the box represents the inter-quartile range (IQR) and the whiskers extend 1.5 times IQR. Pre-experiment (pre-exp, N=9), ambient P_{CO_2} (N=28), moderate P_{CO_2} (N=32) and high P_{CO_2} (N=27). Different shapes within the boxplot are means±s.e.m. for each experimental day within each P_{CO_2} treatment; time 0 (N=9), 7 days (N=9), 14 days (N=9) and 28 days (ambient, N=10; moderate, N=14; high, N=9). The sample size (N=9) was attained by measuring f_V of three fish from each of the three replicate buckets per P_{CO_2} treatment. Different letters represent a significant difference between P_{CO_2} treatments (lowercase) and acclimation time (uppercase) independently (ANOVA, P<0.05), and 87 fish in total were used.

Cardiac performance

Fish heart rates (f_H) were not affected by P_{CO_2} treatment (Fig. 2A; two-way ANOVA, $F_{2,78}$=0.884, P=0.417) or acclimation time ($F_{2,78}$=1.812, P=0.170). In addition, there was no interaction between P_{CO_2} treatment and acclimation time ($F_{4,78}$=0.898, P=0.470) on f_H. On average, f_H was 35±2 beats min^{-1} (mean±s.d.).

Acclimation time had a significant effect on stroke volume (V_S) (Fig. 2B; two-way ANOVA, $F_{2,51}$=14.69, P<0.0001), with no effect of P_{CO_2} treatment ($F_{2,51}$=1.063, P=0.353) and no interaction between P_{CO_2} treatment and time ($F_{4,51}$=0.292, P=0.882). V_S significantly increased from 11.6±0.7 µl g^{-1} $beat^{-1}$ at 7 days to 15.4±0.5 µl g^{-1} $beat^{-1}$ after 14 days (Tukey HSD, P<0.001) and 16.1±0.6 µl g^{-1} $beat^{-1}$ after 28 days (P<0.0001), but stroke volume was similar at 14 and 28 days (P=0.689).

Similar to stroke volume, mass-specific cardiac output (CO) significantly increased over acclimation time (Fig. 2C; two-way ANOVA, $F_{2,51}$=15.768, P<0.0001); however, fish experienced no effect of P_{CO_2} treatment ($F_{2,51}$=0.845, P=0.435) or an interaction between P_{CO_2} treatment and time ($F_{4,51}$=0.158, P=0.959). Cardiac output significantly increased from 405±25 µl g^{-1} min^{-1} at 7 days to 549±22 µl g^{-1} min^{-1} after 14 days (Tukey HSD, P<0.001). After 28 days of acclimation, CO was 582±21 µl g^{-1} min^{-1}, also significantly greater than at 7 days (P<0.0001).

Metabolic rate

Mass-specific metabolic rate ($\dot{M}_{O_2}$) measured by oxygen consumption of juvenile fish showed no effect of P_{CO_2} treatment (Fig. 3; two-way ANOVA, $F_{2,72}$=2.462, P=0.092) or acclimation time ($F_{2,72}$=2.503, P=0.089). There was also no interaction between P_{CO_2} treatment and time ($F_{4,72}$=0.567, P=0.687). The average $\dot{M}_{O_2}$ for juvenile *T. bernacchii* from all treatments and time points was 2.6±0.1 µmol O_2 h^{-1} g^{-1}.

Citrate synthase enzyme activity

Cellular aerobic potential, measured by citrate synthase activity in muscle tissue, showed a significant effect of acclimation time (Fig. 4; two-way ANOVA, $F_{3,96}$=5.616, P=0.001), but no effect of P_{CO_2} treatment ($F_{2,96}$=0.876, P=0.419) or an interaction between P_{CO_2} and acclimation time ($F_{6,96}$=1.583, P=0.16). In general, CS enzyme activity significantly increased over time from 0.68±0.06 µmol min^{-1} mg $protein^{-1}$ at day 1 compared with 1.06±0.08 µmol min^{-1} mg $protein^{-1}$ after 28 days acclimation (P<0.001), with no significant differences detected at other time points. Although there were no overall significant effects of P_{CO_2} on CS activity over time, fish in moderate P_{CO_2} did increase CS activity from 0.59±0.10 µmol min^{-1} mg $protein^{-1}$ at 1 day to 1.23±0.13 µmol min^{-1} mg $protein^{-1}$ at 28 days.

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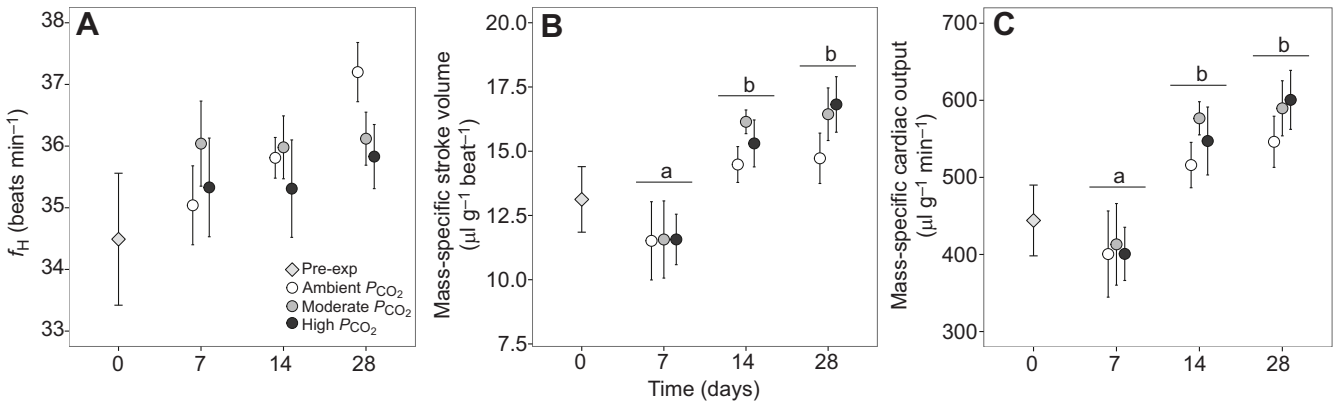

Fig. 2. Cardiac performance metrics of juvenile *Trematomus bernacchii* exposed to different P_{CO_2} treatments. Data in each panel are presented as means±s.e.m. (A) Heart rate (f_H) in each P_{CO_2} treatment over time [N=9 per point, except at 28 days (ambient, N=10; moderate, N=14)] for 87 fish. The minimum sample size (N=9) was attained from measurements of three fish from each of the three replicate buckets per P_{CO_2} treatment. (B) Mass-specific stroke volume (V_S). (C) Mass-specific cardiac output (CO) for 60 fish. For B and C, sample sizes were reduced at 7, 14 and 28 days, such that in each P_{CO_2} treatment sample sizes were N=5, 4 and 6 (ambient), N=6, 5 and 10 (moderate) and N=8, 8 and 8 (high), respectively. Different letters above lines indicate a significant difference by acclimation time (ANOVA, P<0.05).

DISCUSSION

Although early stages of fishes are predicted to be more vulnerable to the physical drivers of GCC compared with adults (Pörtner et al., 2004; Ishimatsu et al., 2004), juvenile *T. bernacchii* appear to be physiologically robust to increased P_{CO_2} predicted by GCC emission scenarios (Meehl et al., 2007). Small changes at the whole-organism level in terms of increased ventilation rate (f_V) suggest increased minute volume may contribute to acid-base compensation and is likely to be a sufficient strategy for dealing with shifts in environmental P_{CO_2}. The juvenile rockcod collected for this study were estimated to be in their second year of age (at least >1 year) with functional gills and kidneys, important for acid-base regulation and gas-exchange in teleosts (see review by Evans et al., 2005; Lawrence et al., 2015). This marked development of the juvenile life-history stage might explain why heart rate (f_H), stroke volume (V_S), cardiac output (CO), oxygen consumption ($\dot{M}_{O_2}$) and citrate synthase (CS) enzyme activity all remained largely unaffected by elevated P_{CO_2}. Most studies characterizing the effects of elevated P_{CO_2} or OA on early life-history stages of fishes such as larvae and juveniles have been conducted in temperate (Hurst et al., 2012; Hamilton et al., 2014) and tropical species (Munday et al., 2009; Dixson et al., 2010; Nowicki et al., 2012; Pimentel et al., 2014a). This is the first study to characterize the impact of OA on a juvenile Antarctic fish and to describe the physiology of juvenile rockcod, *Trematomus bernacchii*, a dominant fish (by biomass) in the Ross Sea, Antarctica (Vacchi et al., 2000).

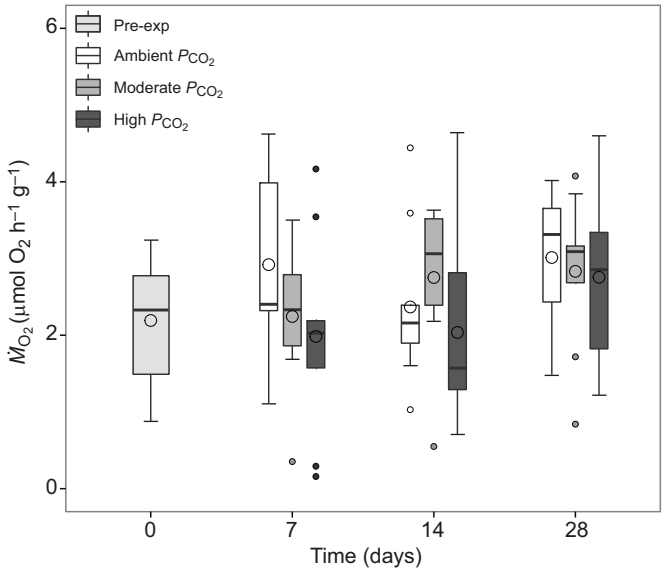

Fig. 3. Mass-specific metabolic rates ($\dot{M}_{O_2}$) measured by oxygen consumption of juvenile emerald rockcod in each P_{CO_2} treatment. Pre-experimental (N=10) data serve as a reference at time 0; $\dot{M}_{O_2}$ values are shown for ambient P_{CO_2} (N=9, except at 28 days, where N=8), moderate P_{CO_2} (N=9) and high P_{CO_2} (N=9) treatments (N=90 fish in total). The open circle within the boxplots represents the mean and the line represents the median. The box represents the inter-quartile range (IQR), with the whiskers extending to 1.5 times the IQR. Points beyond the whiskers are outliers but were included in the data analyses.

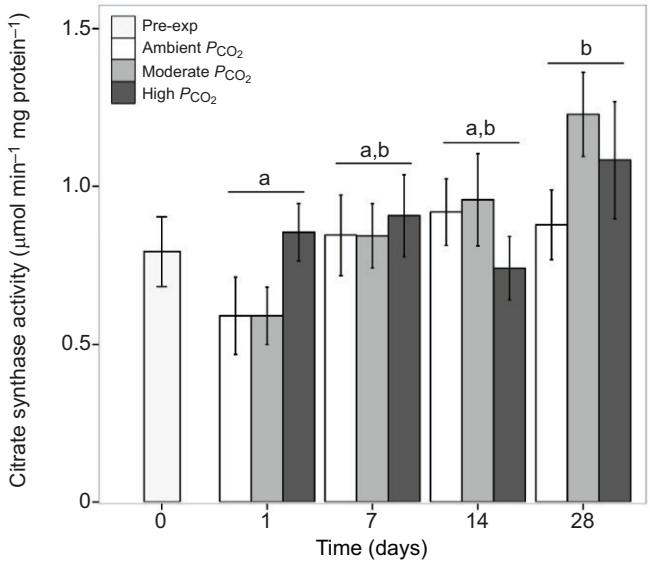

Fig. 4. Citrate synthase enzyme activity in muscle tissue of juvenile *Trematomus bernacchii*. Bars indicate the mean enzyme activity ±s.e.m. for fish in ambient P_{CO_2} (N=9), moderate P_{CO_2} (N=9) and high P_{CO_2} (N=9, except at 28 days, where N=8) over a 28 day period. Pre-experimental CS activity is plotted as a reference (N=9) for a total of 116 fish used for citrate synthase analyses. Statistical letters indicate a significant difference in citrate synthase activity by acclimation time (ANOVA, P<0.05).

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Elevated P_{CO_2} had a significant effect on f_V in emerald rockcod juveniles such that fish under high P_{CO_2} had overall greater f_V than fish in current ambient P_{CO_2} conditions (Fig. 1). It is common for fishes to alter f_V in response to environmental changes (Janssen and Randall, 1975; Randall et al., 1976; Smith and Jones, 1982), and fish primarily use metabolic adjustments to cope with acid-base disturbances and changing oxygen levels (Ishimatsu et al., 2004, 2005; Heuer and Grosell, 2014). Fish have specialized neuroepithelial cells (NECs) in their gill arches, positioned to sense both partial pressure of gases (e.g. P_{CO_2} and P_{O_2}) in the blood and in the external environment (Bailly et al., 1992; Zaccone et al., 1994; Perry et al., 2009). Increases in external P_{CO_2} stimulate these gill chemoreceptors and initiate a cardiorespiratory response, such as hyperventilation (Burleson and Smatresk, 2000; Perry and Abdallah, 2012; Heuer and Grosell, 2014) to minimize the effects of elevated P_{CO_2} on blood pH (Gilmour, 2001; Perry and Abdallah, 2012). Numerous studies on teleost fishes have shown hyperventilation in response to hypercapnia (Sundin et al., 2000; Perry and McKendry, 2001; Perry and Reid, 2002, also see review by Gilmour, 2001); but typically these studies exposed fishes to substantially higher levels of CO_2 (e.g. 10,500–48,000 µatm).

Commonly, hyperventilation is coupled with other physiological adjustments (Perry and Abdallah, 2012); however, adjustments in aerobic metabolism and cardiac output were not observed in this study, because only f_V was significantly affected by increased P_{CO_2}. Increased ventilation can be a mechanism to increase oxygen supply to offset metabolic costs or oxygen demand associated with coping with environmental change (Frederich and Pörtner, 2000; Mark et al., 2002). $\dot{M}_{O_2}$ in the current study was not increased in response to elevated P_{CO_2} (Fig. 3), suggesting fish did not have an increased oxygen demand under conditions of OA. The mismatch between $\dot{M}_{O_2}$ and f_V could be explained by differences in gas solubility and diffusion of CO_2 and O_2 in seawater. At 0°C, CO_2 is about ∼38 times more soluble in seawater than O_2 and diffuses roughly 1.2 times faster (Denny, 1993). The differences in gas solubility and diffusion rate between CO_2 and O_2 coupled with no evidence of increased aerobic metabolism suggests hyperventilation or an increase in ventilatory minute volume is probably a sufficient mechanism to off-load/excrete CO_2 quickly, contributing to acid-base compensation without increasing uptake of O_2. Respiratory plasticity in response to OA (1000 µatm) has been described in the estuarine red drum *Sciaenops ocellatus*: although $\dot{M}_{O_2}$ was unaffected by acclimation to elevated P_{CO_2}, the capacity for CO_2 excretion increased as marked by an increase in CO_2 channel proteins and a significant reduction in the branchial distance over which diffusion can occur (Esbaugh et al., 2015). The respiratory physiology of fishes does facilitate excretion of CO_2 from the countercurrent exchange from water to blood and unidirectional water flow across the gills to maintain stable blood pH (Heuer and Grosell, 2014). However, more common mechanisms for acid-base balance in fish include the secretion of H^+ and the retention and absorption of HCO_3^- through different ion exchanges and *de novo* synthesis of HCO_3^- by the kidney (Wright, 1995; Wood et al., 1999; Lawrence et al., 2015), facilitated by carbonic anhydrase (Gilmour and Perry, 2009; Perry et al., 2010; Esbaugh et al., 2012; Tseng et al., 2013). Future studies could quantify plasma HCO_3^- and P_{CO_2} and analyze the activity or mRNA expression of these enzymes and transporters to provide additional insight into any acid-base imbalances that might have occurred in response to elevated P_{CO_2} (Melzner et al., 2009; Perry et al., 2010; Esbaugh et al., 2012; Lawrence et al., 2015).

Although the $\dot{M}_{O_2}$ of juveniles was unaffected by P_{CO_2}, elevated P_{CO_2} of similar magnitudes to the current study (∼950–1025 µatm; Enzor et al., 2013) have previously been shown to increase $\dot{M}_{O_2}$ in adult *T. bernacchii* following a 1 week exposure. Following 2 weeks of acclimation to elevated P_{CO_2}, the $\dot{M}_{O_2}$ of adult *T. bernacchii* returned to basal levels and remained stable for up to 4 weeks (Enzor et al., 2013). Other closely related adult notothenioid species including *T. hansoni* and *P. borchgrevinki*, however, showed no effect of elevated P_{CO_2} on $\dot{M}_{O_2}$ after acclimation for 1 or 4 weeks (Enzor et al., 2013). It is possible that juvenile *T. bernacchii* increased their $\dot{M}_{O_2}$ in the short term (i.e. within days) but were able to compensate more quickly than adults, such that by 1 week of exposure to elevated P_{CO_2}, $\dot{M}_{O_2}$ was already reduced to basal levels. As we did not measure immediate, short-term responses (f_H, f_V and $\dot{M}_{O_2}$) to elevated P_{CO_2}, it is not possible to know whether juvenile rockcod make acute adjustments to elevated P_{CO_2}. Alternatively, some studies have shown tolerance decreases with ontogenetic stage because of increasing physiological performance capacity of juveniles and sub-adults at a smaller size, whereas adults may become oxygen limited with increasing demand and insufficient supply (for review, see Pörtner and Peck, 2010). For example, temperature tolerance of juveniles as the North Sea sole (Rijnsdorp et al., 2009), California anchovy (Brewer, 1976) and delta smelt (Komoroske et al., 2014) is greater compared with the adult of each species. It is of note that there was high variability in $\dot{M}_{O_2}$ of juvenile *T. bernacchii* under all P_{CO_2} treatments, which was not as apparent in the adult *T. bernacchii* exposed to elevated P_{CO_2} in Enzor et al. (2013). Steffensen (2002) showed that over the course of a single day, oxygen consumption rates of adult *T. bernacchii* held under ambient temperatures of −1°C were highly variable, ranging from 17 to 85 mg O_2 kg^{-1} h^{-1} and so differences might reflect the time of day measurements were made. It is noteworthy that the current environmental CO_2 levels of the shallow, benthic habitats where both the adults (Enzor et al., 2013) and juveniles were collected are similar and relatively benign compared with experimental CO_2 levels (Matson et al., 2014). Therefore, it is not likely that differences in environmental P_{CO_2} exposure have led to differences in sensitivity between adults and juveniles.

In addition to whole-organism measurements of aerobic metabolism, sub-organismal aerobic potential, measured as citrate synthase (CS) enzyme activity in muscle tissue, was also unaffected by elevated P_{CO_2} (Fig. 4). This finding provides additional evidence that juvenile emerald rockcod are not adjusting their capacity for aerobic metabolism in response to elevated P_{CO_2}. It is important to note that CS activity is tissue specific in several fishes (Michaelidis et al., 2007; Strobel et al., 2013) and was only measured in muscle tissue in the current study. In the Antarctic notothenioid *Notothenia rossii* from the western Antarctic peninsula, CS activity was unaffected by P_{CO_2} (0.2 kPa or ∼1975 µatm) in cardiac, liver and white muscle tissue, whereas CS activity in red muscle increased (Strobel et al., 2013). In the Mediterranean seabream *Sparus aurata* acclimation to increased P_{CO_2} significantly decreased CS activity in red and white muscle but increased in cardiac tissue (Michaelidis et al., 2007). Concurrently, although aerobic potential decreased in the sea bream, activity of the anaerobic enzymes pyruvate kinase and lactate dehydrogenase increased, demonstrating a switch in metabolic pathways in response to elevated P_{CO_2} (Michaelidis et al., 2007). Although anaerobic metabolites were not measured in this study, aerobic metrics of $\dot{M}_{O_2}$, f_H, and CS in response to elevated P_{CO_2} provide indirect evidence to suggest that these juveniles were not relying heavily on anaerobic pathways to fuel energetic demands of exposure to elevated P_{CO_2}. Antarctic fishes have been shown to

express 20–50% more mitochondrial proteins than temperate fishes (Johnston et al., 1998). High mitochondrial densities might allow for sustained aerobic capacity in response to changing environmental parameters such as P_{CO_2}. Furthermore, O_2 is not limiting in the Antarctic and it is therefore unlikely that these fishes need to rely on anaerobic pathways.

Pörtner and colleagues (2004) have suggested that hypercapnic limitations might be defined by cardiac and circulatory system failure at extremely high levels of CO_2, rather than ventilation capacity being the limiting mechanism (Ishimatsu et al., 2004); however, we found no evidence of adjustments in heart rate (f_H, Fig. 2A), stroke volume (V_S, Fig. 2B) or cardiac output (CO, Fig. 2C) in juvenile emerald rockcod in response to predicted P_{CO_2} levels (650 and 1050 µatm). Previous studies have shown that fishes adjust f_H, V_S and CO in response to elevated P_{CO_2}, with the response varying across species (Perry et al., 1999; Crocker et al., 2000; McKendry et al., 2001; Lee et al., 2003). Most of these studies characterized responses after an acute exposure to elevated P_{CO_2} of much higher magnitudes (e.g. 8000–26,000 µatm), whereas we assessed cardiac performance over 1–4 weeks of acclimation and found no effects of elevated P_{CO_2} predicted for OA scenarios. While it is more common for temperate fishes to regulate CO by modifying V_S, rather than f_H, Antarctic fishes have been found to increase f_H with increased oxygen uptake, not V_S (Farrell, 1991; Axelsson et al., 1992). Egginton et al. (2006) have suggested that f_H is a good predictor of $\dot{M}_{O_2}$ in adult *T. bernacchii* and *P. borchgrevinki*, because both exhibit a linear relationship between f_H and $\dot{M}_{O_2}$ (Egginton et al., 2006; Campbell et al., 2009). In the current study, there was no change in either f_H or $\dot{M}_{O_2}$ in juvenile *T. bernacchii* in response to elevated P_{CO_2}.

Of interesting note in this study was that juvenile *T. bernacchii* showed a significant response to acclimation time, independent of P_{CO_2} treatment. As time held under laboratory conditions (i.e. captivity) increased, aerobic performance parameters including f_V, V_S, CO and CS activity also increased (Fig. 1, Fig. 2B,C and Fig. 4). Acclimation time effects may be confounded by subtle changes in development over the 4 week experimental period; however, growth appeared to be minimal. On average, fish length increased ∼1% whereas wet mass increased ∼3% over the 4 week period (data not shown). Although it is challenging to unravel the specific cause underlying acclimation time effects, wild fish during development may undergo confinement stress with an increase in the cortisol stress hormone, a well-documented occurrence in teleost fishes (Barton and Iwama, 1991; Bonga, 1997). Furthermore, previous studies have shown that laboratory conditions, such as tank color and densities of fish held together, can affect behavior and physiology (Brown et al., 1992; Pavlidis et al., 2013; Hasenbein et al., 2016). For example, when held in lower densities, Arctic char (*Salvalinus alpinus*) exhibited altered swimming behavior and more aggressive interactions (Brown et al., 1992). Whereas crowding increased stress in zebrafish, lower densities altered the social environment as hierarchies were formed (Pavlidis et al., 2013). More negative interactions between fish are suggested to increase energetic demands (Brown et al., 1992). Stress markers were also significantly greater in late larval delta smelt (*Hypomesus transpacificus*) held at lower densities of 7 and 14 individuals compared with higher densities of 28 and 49 (Hasenbein et al., 2016). Here, 39 fish were initially placed in each P_{CO_2} triplicate bucket, after which nine fish were sequentially removed for sampling metrics. After 1 and 2 weeks of sampling there were 24 and 15 fish remaining in each bucket, respectively, for the duration of the 4 week period. We did observe some dominance behavior while feeding, such as tail biting or chasing and hence, suggestive that juvenile emerald rockcod in low densities might experience comparable alterations in behavior as demonstrated by zebrafish and Arctic char. If greater stress and energy demands increased from 2 to 4 weeks, changes in f_V (O_2 extraction) and CO marked by stroke volume (O_2 circulation) might represent mechanisms to maintain organismal and sub-organismal oxygen demands. As this is the first laboratory study of juvenile *T. bernacchii*, more research is needed to understand how laboratory factors affect fish behavior and physiology.

This study demonstrates that juvenile *T. bernacchii* appear to be robust to elevated P_{CO_2} levels that are predicted to occur over the next century. However, as the current experiment only lasted 4 weeks, research with more chronic exposures to elevated P_{CO_2} are needed to thoroughly assess the vulnerability of these fishes – a difficult undertaking with field seasons limited to the austral summers in McMurdo Sound, Antarctica. Results from the current study suggest that the physiological responses of juvenile emerald rockcod do differ from the adult *T. bernacchii* such that juvenile fish appear to have a greater capacity to buffer elevated P_{CO_2} as seen by relatively stable cardiovascular physiology and aerobic metabolism. In addition to elevated P_{CO_2}, temperature is predicted to increase by 2–4°C. While several studies have demonstrated that adult *T. bernacchii* have the capacity for thermal acclimation and the ability to increase thermal tolerance limits (Bilyk and DeVries, 2011; Bilyk et al., 2012), few studies have investigated how both elevated P_{CO_2} and temperature will impact Antarctic fishes (although see Enzor et al., 2013; Enzor and Place, 2014; Strobel et al., 2012, 2013) and with only a single study on early developmental stages of Antarctic dragonfish (Flynn et al., 2015). Stressors can interact in non-linear ways, making it difficult to predict how fish will respond to multiple stressors from single stressor experiments (Todgham and Stillman, 2013). More research is warranted to understand the potential combined and synergistic effects of elevated P_{CO_2} and temperature on physiological performance and behavior of juvenile fishes.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
B.E.D., N.A.M. and A.E.T. designed the study. B.E.D., E.E.F. and N.A.M. maintained the CO_2 system and measured seawater chemistry. B.E.D., N.A.M. and A.E.T. carried out the physiological measurements of fish during experimentation at McMurdo station. B.E.D. carried out all analyses at UC Davis and drafted the manuscript. B.E.D. and A.E.T. revised the manuscript critically with edits from E.E.F. and N.A.M.

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