

Antarctic emerald rockcod have the capacity to compensate for warming when uncoupled from CO₂-acidification

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Abstract

Increases in atmospheric CO₂ levels and associated ocean changes are expected to have dramatic impacts on marine ecosystems. Although the Southern Ocean is experiencing some of the fastest rates of change, few studies have explored how Antarctic fishes may be affected by co-occurring ocean changes, and even fewer have examined early life stages. To date, no studies have characterized potential trade-offs in physiology and behavior in response to projected multiple climate change stressors (ocean acidification and warming) on Antarctic fishes. We exposed juvenile emerald rockcod *Trematomus bernacchii* to three PCO₂ treatments (~450, ~850, and ~1,200 μatm PCO₂) at two temperatures (−1 or 2°C). After 2, 7, 14, and 28 days, metrics of physiological performance including cardiorespiratory function (heart rate [f_H] and ventilation rate [f_V]), metabolic rate ($\dot{M}O_2$), and cellular enzyme activity were measured. Behavioral responses, including scototaxis, activity, exploration, and escape response were assessed after 7 and 14 days. Elevated PCO₂ independently had little impact on either physiology or behavior in juvenile rockcod, whereas warming resulted in significant changes across acclimation time. After 14 days, f_H , f_V and $\dot{M}O_2$ significantly increased with warming, but not with elevated PCO₂. Increased physiological costs were accompanied by behavioral alterations including increased dark zone preference up to 14%, reduced activity by 12%, as well as reduced escape time suggesting potential trade-offs in energetics. After 28 days, juvenile rockcod demonstrated a degree of temperature compensation as f_V , $\dot{M}O_2$, and cellular metabolism significantly decreased following the peak at 14 days; however, temperature compensation was only evident in the absence of elevated PCO₂. Sustained increases in f_V and $\dot{M}O_2$ after 28 days exposure to elevated PCO₂ indicate additive (f_V) and synergistic ($\dot{M}O_2$) interactions occurred in combination with warming. Stressor-induced energetic trade-offs in physiology and behavior may be an important mechanism leading to vulnerability of Antarctic fishes to future ocean change.

KEYWORDS

behavior, cardiorespiratory physiology, climate change, metabolism, ocean acidification, polar, temperature, *Trematomus bernacchii*

1 | INTRODUCTION

High-latitude, polar oceans are predicted to undergo some of the greatest changes in pH and temperature over the next century (IPCC, 2013; McNeil & Matear, 2008). Open ocean seawater pH may decrease as much as 0.3–0.5 units, whereas temperature is projected to increase 2–4°C (IPCC, 2013; McNeil, Tagliabue, & Sweeney, 2010) due to the continued increase in carbon dioxide (CO₂) emissions into the atmosphere, ultimately driving ocean warming and CO₂-acidification of seawater (ocean acidification, OA; Gattuso et al., 2014; IPCC, 2013). Within the Southern Ocean specifically, some of the fastest rates of change have been recorded (Turner et al., 2013; Vaughan et al., 2003). The Antarctic Peninsula has experienced increases in air temperature of 3.4–5.7°C per century, whereas McMurdo Sound in the Ross Sea has shown trends of 2.5–3.3°C per century acquired from datasets ranging from 1957 to 2001 (Vaughan et al., 2003). Simulated models project that the duration of low pH events in winter may increase in McMurdo Sound, thus exposing marine organisms to deleterious levels of pH for longer periods (Kapsenberg, Kelley, Shaw, Martz, & Hofmann, 2015). There are numerous studies investigating the effects of high temperature on the physiology of adult Antarctic fishes (Beers & Jayasundara, 2015; Bilyk & DeVries, 2011; Egginton & Campbell, 2016; Peck, Morley, Richard, & Clark, 2014; Sandersfeld, Davison, Lamare, Knust, & Richter, 2015; Sandersfeld, Mark, & Knust, 2017; Seebacher, Davison, Lowe, & Franklin, 2005; Somero & Hochachka, 1968; Weinstein & Somero, 1998); however, there are considerably fewer studies focused on how Antarctic fishes may respond to elevated PCO₂, and the effects of elevated PCO₂ and temperature concurrently, with the focus on adult fishes (Enzor, Hunter, & Place, 2017; Enzor & Place, 2014; Enzor, Zippay, & Place, 2013; Strobel, Graeve, Pörtner, & Mark, 2013; Strobel, Leo, Pörtner, & Mark, 2013; Strobel et al., 2012) and only one study on an early life stage (i.e., embryos, Flynn, Bjelde, Miller, & Todgham, 2015). Since sensitivity to environmental stressors can vary across ontogeny (Hamdoun & Epel, 2007; Pörtner & Peck, 2010), we cannot predict species vulnerability to ocean changes of elevated PCO₂ and temperature by only evaluating sensitivity to change in adults.

Antarctic fishes have evolved under a stable, cold environment for millions of years (Eastman, 1993) and as a result are predicted to be highly sensitive to changes in ocean conditions, particularly ocean warming. Studies assessing the physiological impacts of warming on Antarctic fishes in the Nototheniidae family have shown changes to cardiorespiratory and metabolic performance (Egginton & Campbell, 2016; Enzor et al., 2013, 2017; Franklin, Davison, & Seebacher, 2007; Jayasundara, Healy, & Somero, 2013; Seebacher et al., 2005); however, the degree of vulnerability and acclimation capacity may be species-specific (Egginton & Campbell, 2016; Franklin et al., 2007; Jayasundara et al., 2013; Robinson & Davison, 2008a,b; Seebacher et al., 2005). For example, studies of the emerald rockcod, *Trematomus bernacchii*, a dominant benthic species by biomass in the Ross Sea (Vacchi, La Mesa, & Greco, 2000), have demonstrated impacts of warming across several levels of organization, including

limitations in cardiac performance (Jayasundara et al., 2013), sustained elevated metabolic costs (Enzor et al., 2013, 2017), aerobic to anaerobic fuel switching (Enzor et al., 2017; Jayasundara et al., 2013), mitochondria break temperatures (Weinstein & Somero, 1998), a twofold increase in brain oxygen (O₂) consumption (Somero & DeVries, 1967), and up to an 85% reduction in growth (Sandersfeld et al., 2015). In comparison, the bald notothen *Pagothenia borchgrevinki* has exhibited less sensitivity to warming, demonstrating partial compensation for increased metabolic rates (Enzor et al., 2013, 2017) and a greater capacity to cope marked by modifications in cardiorespiratory and metabolic adjustments (Franklin et al., 2007; Robinson & Davison, 2008a,b; Seebacher et al., 2005). Differential responses to warming may be explained by varied ecological niches of these species. *Trematomus bernacchii* are more benthic, ranging from shallow to deeper waters (50–400 m), compared to *P. borchgrevinki* that live just under the annual sea-ice potentially exposing them to more environmental variability and influencing a greater capacity to acclimate (Eastman, 1993). Although notothen species have showed varying responses to warming alone, the simultaneous addition of elevated CO₂ (i.e., multiple stressor) elicited more similar physiological responses (Enzor et al., 2013, 2017; Strobel et al., 2012; Strobel, Graeve, et al., 2013; Strobel, Leo, et al., 2013). When exposed to elevated PCO₂ concurrently with warming, both species (*T. bernacchii* and *P. borchgrevinki*) exhibited significant increases in metabolic rates (Enzor et al., 2013) and mRNA expression of genes involved in the cellular stress response (Huth & Place, 2016a,b). These studies support the hypothesis that exposure to CO₂-acidification may exacerbate the effects of temperature stress (Pörtner, 2008) in adult Antarctic notothen. To date, no studies have explored the impacts of elevated temperature and PCO₂ on juvenile stages of Antarctic fishes, which may have different sensitivities than adults to environmental change. Our previous work has demonstrated that the effects of high PCO₂ on oxygen consumption rates in juvenile *T. bernacchii* (no change; Davis, Miller, Flynn, & Todgham, 2016) are not consistent with what has been found in adults (increased; Enzor et al., 2013), suggesting stage-specific responses to future ocean changes. It remains unknown if juvenile *T. bernacchii* exposed to increased temperature and PCO₂ concurrently would have similar synergistic effects to a multiple stressor scenario as seen in the adults (Enzor et al., 2013).

Energetic demands of early life stages are predominantly driven by development and growth, such that environmental changes may create tradeoffs in energy allocation to stress response mechanisms (Ishimatsu, Hayashi, & Kikkawa, 2008; Pankhurst & Munday, 2011; Pörtner, Lucassen, & Storch, 2005), leaving early life stages more sensitive to stress. Although juvenile *T. bernacchii* did not change cardiac performance and metabolic rate in response to elevated PCO₂, they did hyperventilate likely to excrete CO₂ and alleviate blood acidosis (Davis et al., 2016). Hyperventilation, while a sufficient buffering mechanism of hypercapnia in fishes, is also energetically expensive (Cameron & Cech, 1970; Dejours, 1981). It remains unclear if the addition of increased temperature would potentially create a mismatch in energy supply and the energy demands

necessary to cope with both stressors concurrently or if tradeoffs in basic maintenance and stress response mechanisms would instead occur. Of the few studies assessing the interacting effects of increased temperature and PCO_2 on early life stages of fishes (Bignami, Sponaugle, Hauff, & Cowen, 2016; Dahlke et al., 2017; Flynn et al., 2015; Pimentel et al., 2016), the results are contrasting, suggesting the two factors may interact differently dependent on life stage and species.

Although understanding physiological sensitivity to acute and chronic exposures of CO_2 -acidification and elevated temperature is essential to predicting vulnerability to environmental change, often the first response of an organism to environmental change is to alter behavior (Nagelkerken & Munday, 2015). Biotic interactions are strongly driven by abiotic influences such that species may experience “trade-offs” between mounting stress tolerance mechanisms and behaviors involving interactions with other species through prey capture, predator avoidance or competition (Dunson & Travis, 1991; Gilman, Urban, Tewksbury, Gilchrist, & Holt, 2010). Elevations in PCO_2 can have significant effects on a variety of behaviors in fishes such as habitat detection (Devine, Munday, & Jones, 2012a), predator and prey detection (Cripps, Munday, & McCormick, 2011; Ferrari, McCormick, et al., 2011; Ferrari et al., 2015; Munday et al., 2013; Sundin & Jutfelt, 2016), lateralization (Domenici, Allan, McCormick, & Munday, 2012; Jutfelt, de Souza, Vuylsteke, & Sturve, 2013), anxiety (Hamilton, Holcombe, & Tresguerres, 2014); and activity (Munday et al., 2013). In contrast, other studies have shown early life stages of fish to be more robust to CO_2 -acidification with no changes in swimming and foraging behaviors (Maneja et al., 2013, 2015), response to predation cues (Jutfelt et al., 2013; Sundin et al., 2017), and activity and social behaviors (Duteil et al., 2016). Elevated temperature also results in varied behavioral responses. Some studies show warming increased activity, boldness, aggression (Biro, Beckmann, & Stamps, 2010; Ojanguren & Braña, 2000), and altered group dynamics (e.g., near neighbor distances and physical contacts; Colchen, Teletchea, Fontaine, & Pasquet, 2017), whereas other studies have shown warming decreased swimming speeds (Johansen & Jones, 2011), routine activity (Colchen et al., 2017; Nowicki, Miller, & Munday, 2012), and foraging activity (Nowicki et al., 2012). Similar to physiological responses, it appears that fish behavior responses to CO_2 -acidification and warming are both species and life stage-specific. Only a few studies have begun to link physiological and behavioral responses to elevated temperature and/or CO_2 -acidification (Hamilton et al., 2017; Johansen & Jones, 2011; Lonthair, Ern, & Esbaugh, 2017; Ou et al., 2015; Pimentel et al., 2016), and only a single study has examined behavior and physiology to increased temperature in an Antarctic fish (Evans, Williams, Vacchi, Brimble, & DeVries, 2012).

The objectives of this study were (i) to examine how acclimation to increased temperature and PCO_2 impacted the physiological performance of juvenile emerald rockcod, *T. bernacchii*, (ii) to characterize changes in movement, anxiety, and boldness behaviors in response to elevated temperature and PCO_2 , and (iii) to determine if

potential trade-offs occurred between physiological performance and behavior. To accomplish these goals, juvenile *T. bernacchii* were exposed to elevated temperature and PCO_2 and physiological and behavior parameters were quantified. We examined changes in cardiorespiratory phenotypes, mass-specific metabolic rates ($\dot{M}O_2$), and cellular enzyme activities after 2, 7, 14, and 28 days of exposure as well as scototaxis, activity and boldness behaviors at 7 and 14 days. On the basis of previous findings (Davis et al., 2016), we predicted limited effects of elevated PCO_2 alone on juvenile rockcod; however, we predicted that increased physiological costs would be incurred under increased temperature acclimation resulting in increased energetic demands when elevated temperature was combined with elevated PCO_2 . Behaviorally, we predicted that rockcod would exhibit similar alterations in anxiety and activity behaviors as in temperate and tropical fish species (Hamilton et al., 2014; Jutfelt et al., 2013; Munday et al., 2013) in response to elevated PCO_2 and temperature, with greater impacts driven by elevated PCO_2 . To our knowledge, no studies have characterized the impacts of multiple stressors (i.e., elevated PCO_2 and temperature) on physiology and behavior of Antarctic fishes. Determining if changes in behavior due to physiological consequences is critical to better characterize the potential energetic trade-offs juvenile Antarctic rockcod *T. bernacchii* may face under future ocean conditions.

2 | MATERIALS AND METHODS

2.1 | Experimental design

Six experimental treatments were implemented (a 2 by 3 factorial design) as described in Flynn et al. (2015); 2 temperatures (-1.0 and $2^\circ C$) by 3 CO_2 -acidified seawater treatments (450, 850, and 1,200 $\mu atm PCO_2$). Experimental treatments were repeated twice; once for the physiology-based experiment extending over a 4-week period (Phase I), and once for the behavior experiment that lasted 2 weeks (Phase II). Two temperatures were selected; $-1.0^\circ C$ ($-0.8^\circ C$ warmer than ambient Ross Sea temperature) and $2^\circ C$ (a $+3^\circ C$ increase from the control) simulating a Representative Concentration Pathway scenario (RCP8.5; IPCC, 2013). Three experimental CO_2 -acidification treatments were selected based on evidence that juvenile rockcod were tolerant to lower levels of elevated PCO_2 (Davis et al., 2016) and more recent climate change scenarios predicted for 2100 (Gattuso et al., 2014; IPCC, 2013), where 450 $\mu atm PCO_2$ represents *Ambient* seawater, 850 $\mu atm PCO_2$ a *Moderate* scenario (RCP6.0) and 1,200 $\mu atm PCO_2$ a *High* scenario prediction (RCP8.5; see actual experimental values in Table 1). Temperature was maintained by manipulating the flow-rate of incoming McMurdo Sound seawater into the tanks that served as water baths for the fish culture buckets, whereas the CO_2 -acidified treatments were attained and kept stable by delivering PCO_2 gas to each treatment as described in detail in Flynn et al. (2015) and Davis et al. (2016). Each treatment combination (i.e., temperature x PCO_2) was replicated in triplicate and conditions remained stable for the duration of each experiment.

Treatment	Temperature (°C)	pH (total scale)	PCO ₂ (µatm)	Alkalinity (µmol/kg)	Salinity (ppt)
Phase I—Physiology					
Ambient	-1.0 ± 0.1	7.98 ± 0.03	458 ± 30	2355.1 ± 1.8	34.2 ± 0.1
PCO ₂	2.2 ± 0.1	7.99 ± 0.03	468 ± 31	2356.4 ± 2.2	34.2 ± 0.1
Moderate	-1.0 ± 0.1	7.75 ± 0.03	822 ± 51	2356.1 ± 2.3	34.2 ± 0.1
PCO ₂	2.2 ± 0.1	7.70 ± 0.01	958 ± 33	2356.7 ± 2.0	34.2 ± 0.1
High PCO ₂	-1.0 ± 0.1	7.58 ± 0.01	1233 ± 40	2355.9 ± 1.9	34.2 ± 0.1
	2.2 ± 0.1	7.56 ± 0.02	1323 ± 64	2355.4 ± 2.9	34.2 ± 0.1
Phase II—Behavior					
Ambient	-0.9 ± 0.1	8.00 ± 0.01	438 ± 16	2353.5 ± 2.1	34.1 ± 0.1
PCO ₂	2.2 ± 0.1	8.01 ± 0.02	434 ± 16	2354.2 ± 3.9	34.2 ± 0.1
Moderate	-0.9 ± 0.1	7.73 ± 0.01	846 ± 19	2354.5 ± 1.3	34.1 ± 0.1
PCO ₂	2.2 ± 0.1	7.71 ± 0.02	906 ± 43	2352.7 ± 2.1	34.2 ± 0.1
High PCO ₂	-0.9 ± 0.1	7.59 ± 0.02	1193 ± 44	2355.5 ± 2.7	34.2 ± 0.1
	2.2 ± 0.1	7.57 ± 0.01	1283 ± 39	2353.9 ± 4.3	34.2 ± 0.1

Phase I experiments were conducted over a 4-week period, whereas the seawater chemistry for Phase II experiments were conducted over a 2-week period.

2.2 | Seawater chemistry

Temperature in the water bath tanks were monitored every 30 min using submerged HOBO data loggers (Onset, Bourne, MA, USA), whereas temperature of each culture bucket was recorded once daily using a handheld thermocouple (HH81A, Omega, Stamford, CT, USA). Total pH was measured every other day from each culture bucket and PCO₂ gas mixing reservoir bucket using m-cresol dye (Sigma-Aldrich, St. Louis, MO, USA) in a UV spectrophotometer (Shimadzu, Columbia, MD, USA). Total alkalinity was measured every 4 days using open-cell titration (T50 titrator, Mettler Toledo Inc., Columbus, OH, USA) of the PCO₂ gas mixing reservoir buckets (certified reference material [CRM] and titrant from the Dickson Laboratory, Scripps Institute, La Jolla, CA, USA). Salinity was extremely stable and measured in the titration samples using a conductivity meter (YSI 3100, Yellow Springs, OH, USA). PCO₂ values were then calculated for each culture bucket using total pH, temperature, alkalinity, and salinity in R (package *seacarb*; v2.4.10; Gattuso et al., 2015; R Development Core Team, 2013) and are summarized in Table 1.

2.3 | Fish collection and maintenance

Juvenile *T. bernacchii* (standard length 41.7 ± 1.5 mm, mass 709.8 ± 104.6 mg, mean ± SD, see Figure S1 for size regression) were collected from Cape Evans Ice Wall, Ross Island in McMurdo Sound, Antarctica (77°38.407'S, 166°31.068'E) by scientific SCUBA divers in October 2014 for Phase I of the study (Physiology). Juvenile *T. bernacchii* (standard length 40.9 ± 1.4 mm, mass: 624.4 ± 78.3 mg, mean ± SD) were collected from Arrival Heights, Ross Island in McMurdo Sound, Antarctica (77°50.528'S, 166°38.176'E) by scientific SCUBA divers in November 2014 for

TABLE 1 Average (±SD) seawater treatment conditions for both physiology and behavior experiments

Phase II of the study (Behavior). Fish were transported back to the A.P. Crary Science and Engineering Center at McMurdo Station within 3 hr of collection (temperature upon arrival was -1.2°C). Fish were then counted, visually assessed for injury and held for 1 week in recirculating seawater tanks (34 ppt and -1.4°C) until the start of experiments. Fish were assumed to be in their second year of age as described by previous studies (Davis et al., 2016; La Mesa, Vacchi, Arneri, Giannetti, & Greco, 1996; North, 1991), and sex was not able to be determined. During Phase I (Physiology), 42 fish were randomly selected and placed in each 19 L triplicate culture bucket ($n = 126$ for each PCO₂ treatment at each temperature; 756 fish total). The addition of fish (experiment start) for each temperature treatment was staggered by a day to ensure all physiological measurements across PCO₂ levels could be conducted at the same acclimation time points. Fish were held in the temperature × PCO₂ treatments from 48 hr to 4 weeks providing insight into both short-term and longer term acclimatory changes. Specifically, $n = 9$ per PCO₂ × temperature treatment (with 3 individuals sampled from each replicate bucket at 2, 7, 14, and 28 days) were sampled for each of the physiological parameters described below. During Phase II (Behavior), sixteen fish were randomly selected and placed in each triplicate bucket ($n = 48$ for each PCO₂ treatment at each temperature; 288 fish total). Similar to Phase I, each temperature treatment was staggered by a day to ensure all fish behavior trials at a given temperature could be measured within a single day. Fish were held in the temperature × PCO₂ treatments for 7 and 14 days at which time behavioral response trials took place ($n = 10$ –11 per treatment combination at each time point). For the duration of the experiment fish were fed twice daily frozen plankton (0.025 g/fish, Hikari Bio-Pure Ocean Plankton, Hayward, CA, USA) and food was adjusted over time for the number of fish/replicate bucket. 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).

2.4 | Cardiorespiratory physiology

Heart rate (f_H) and ventilation rate (f_V) were measured and analyzed as in Davis et al. (2016) with slight modifications. Within one trial, three fish, one from each PCO_2 treatment were placed in separate 75 ml chambers with their respective treatment water. Chambers were held at -1 or 2°C and videos of the translucent, ventral side of each fish were recorded for 15 min. One fish was observed at a time, whereas the other two fish remained in an insulated water bath at their respective temperatures. Fish were allowed 15–20 min recovery before video recording began. Nine trials were conducted for each temperature treatment ($n = 9$ per PCO_2 , and $n = 27$ per temperature and time point [2, 7, 14, 28 days]). At 28 days, additional recordings were conducted on fish remaining and were included in the final analyses (High PCO_2 : $n = 12$ [-1°C], $n = 12$ [2°C]; Moderate PCO_2 : $n = 13$ [-1°C], $n = 10$ [2°C]). After each video trial, the three fish were euthanized in 0.3% tricane methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA, USA), and measured for length and weight. Heart rate and ventilation rate were analyzed using VLC media player (VideoLan, v2.0.9). For each video recording three 1 min sections were analyzed. Sections analyzed were selected for complete inactivity and/or most rested state if following time of minimal activity. In general, activity in the chambers was minimal; however, 14 of 216 recordings were deemed unusable as the fish were not at rest. f_H and f_V were measured by counting the number of heart beats/min (bpm) and operculum movements, or breaths/min (brpm). The three 1 min sections for f_H and f_V were averaged for a single value for each individual. Preliminary trials conducted in Davis et al. (2016) yielded anesthetic could not be used due to continued reductions in f_H and f_V , and a 15-min recovery period was required for f_H and f_V to level out before video recording began after which f_H and f_V remained stable over a 60 min period.

2.5 | Metabolic rate

Oxygen consumption rate was measured as a proxy for metabolic rate ($\dot{M}O_2$) as described in Davis et al. (2016). Oxygen consumption rate was measured for 9 fish from each PCO_2 treatment and temperature ($n = 3$ /triplicate, $n = 54$ per time point [2, 7, 14, 28 days]). After each $\dot{M}O_2$ trial, fish were euthanized in MS-222 and measured for length and weight. Preliminary trials measuring oxygen consumption over 2 hr showed that during the first 20 min, the rate of oxygen depletion was rapid, likely reflecting handling stress. As a result, the first 20 min was removed from analyses. The rates of oxygen consumption were relatively similar for the remaining 1 hr and 40 min and therefore we chose to run every fish for 1 hr, analyzing 20, 2-min regressions for each fish and report the 20th quantile regression values (see figure S1 in Davis et al., 2016). Using this shortened trial time, we were able to accommodate measuring 9 fish

per pCO_2 treatment per day ($n = 27$ /day) at one temperature, with another 27 fish run the subsequent day for the other temperature. $\dot{M}O_2$ was quantified and analyzed using methods described in Davis et al. (2016) and modified from Chabot, Steffensen, and Farrell (2016). Final $\dot{M}O_2$ values presented as the 20th quantile values (lower estimates) as $\mu\text{mol } O_2 \text{ hr}^{-1} \text{ g}^{-1}$.

2.6 | Aerobic and anaerobic enzyme activity

Skeletal muscle tissue was extracted from 9 fish in each treatment ($n = 3$ /replicate bucket) at 2, 7, 14, and 28 days, frozen in liquid nitrogen, stored at -80°C , and transported to University of California Davis, where all assays took place. The muscle tissue, while predominately white muscle, also contained red muscle fibers that were not able to be excluded from these small juvenile fish (Davis et al., 2016). Metabolic enzymes citrate synthase (CS), cytochrome c oxidase (COX), and lactate dehydrogenase (LDH) were quantified. Sample preparation was as follows; muscle tissue was diluted in 1:9 weight to volumes of 50 mM potassium phosphate buffer (pH = 7.5) and was homogenized on ice using a handheld pestle for 20 s, followed by 10 passes with a PowerGen homogenizer. Homogenates were centrifuged for 10 min at 1,000 g at 4°C . The supernatants were transferred to a new tube and kept on ice. Fresh supernatant was assayed for COX activity, whereas 50 μl was removed in duplicate and frozen at -20°C for later CS and LDH assays. For each enzyme assay, tissue samples were measured in triplicate, background activity rates (no substrate) were determined (CS and LDH) and enzyme activity rates are presented as $\mu\text{mol hr}^{-1} \text{ g}$ fresh muscle weight⁻¹. All assays were conducted at 5°C in a spectrophotometer (Synergy HT, BioTek).

COX activity, a measure of activity of a transmembrane protein (complex IV) in the mitochondrial electron transport chain was measured within 90 min of homogenization as described in Dalziel, Ou, and Schulte (2012). Cytochrome c (0.1 mM) was reduced with sodium dithionite in assay buffer made fresh each day as described in Spinazzi, Casarin, Pertegato, Salviati, and Angelini (2012). CS, an enzyme in the citric acid cycle in the mitochondrial matrix, was measured as in Davis et al. (2016) with slight modifications. Assay buffer (50 mM Imidazole/HCl, pH = 8.2, 0.2 mM DNTB, 0.3 mM Acetyl Co-enzyme A) was added to each tissue homogenate and read for background activity. Oxaloacetate (1 mM) was added to each well and read immediately for 10 min. LDH enzyme activity, a measure of glycolytic anaerobic potential, was quantified by monitoring the conversion of NADH to NAD^+ and associated decrease in absorbance at 340 nm. NADH (0.15 mM) was mixed with the assay buffer (52.5 mM imidazole/HCl at pH 7.5 at RT), added to the tissue homogenate and read for a background reading. 2.65 mM sodium pyruvate was added to each well and immediately assayed for activity for 10 min. The ratio of COX to CS was calculated to evaluate mitochondrial adjustments and oxidative capacity in response to elevated PCO_2 and temperature (Strobel, Leo, et al., 2013). The ratio of LDH to CS was calculated to assess metabolic potential (aerobic and anaerobic) and potential switching in ATP generating pathways in

response to elevated PCO_2 and temperature (Hamilton et al., 2017; Jayasundara et al., 2013).

2.7 | Behavioral response testing

All behavior testing took place in Phase II of experiments (see Table 1 for seawater conditions). Scototaxic testing was conducted only after 14 days exposed to elevated PCO_2 and temperature (there was a camera memory card failure at 7 days), whereas novel object testing (including activity and emergence time) was conducted after both 7 and 14 days.

Scototaxic testing, also known as light/dark preference testing (dark simulating a sheltered area), was conducted as in Maximino, de Brito, Dias, Gouveia, and Morato (2010) and Hamilton et al. (2014) with modifications. Individual fish were placed on the center line of a 5 L (20 cm in diameter) round bucket that was split into a light zone (white bucket surface) and a dark zone (black-lined surface). Upon fish release the bucket lid was immediately put in place with a video camera (GoPro, v.Hero2) affixed to the lid. For each trial the bucket was filled with 6.5 cm of PCO_2 /temperature treatment water, and the dark and light zones were rotated 180° each trial to control for any potential aquarium room lighting bias. Trials began 1–5 s after fish were released on the center-line and video recorded for 15 min. One fish from each PCO_2 /temperature treatment was run individually until $n = 11$ fish from each treatment ($n = 3$ –4/replicate bucket) were tested. Videos were analyzed with JWWatcher (v1.0), quantifying the time in each zone (light vs. dark).

Novel object testing, total activity, and boldness testing were conducted similar to Munday et al. (2013) and Ou et al. (2015). Fish were placed in a 5 L (20 cm diameter) round bucket with a 3×3 cm lined grid on the bottom (~one body length of the fish) and a novel object in the center of the arena. The novel object was a 4 by 5.5 cm long dark tube sealed at one end that the fish could enter if desired. Each bucket was filled with 6.5 cm of the respective PCO_2 /temperature treatment water. The bucket lid was affixed with a GoPro video camera (v.Hero3, silver) and immediately set in place at the start of each trial. Each trial was recorded for 15 min with the first 5 min eliminated for handling. One fish was recorded at a given time (order of treatments randomized) until $n = 11$ from each PCO_2 /temperature treatment ($n = 3$ –4/replicate bucket) was completed at 7 and 14 days. The video recordings were analyzed with EthoVision tracking software (Noldus EthoVision XT 11.5) for (i) time spent active (i.e., swimming), (ii) time spent in the center zone exploring the novel object, and (iii) maximum distance from the object center point. Time spent near the object was determined by calculating the time spent within three postexperimentally assigned concentric circles around the object as described in Ou et al. (2015). The center zone was defined as the area within one body length of the object, the middle zone was greater than one body length from the object, and the thigmotaxic zone was defined as the outermost zone where the fish exhibited edge-searching and/or escape behaviors. Following each 15 min trial, fish were chased for 1 min and then placed inside the object. Fish were then held within the object with a paddle for

30 s and released. The time to emergence from the object was recorded for up to 120 s at which time the trial was ended; fish that never emerged were assigned a maximum score of 120 s (McDonald, Rands, Hill, Elder, & Ioannou, 2016). Time to emergence was recorded to characterize boldness.

Detailed postvideo recording analyses revealed that some behavior assays were in fact conducted on an additional juvenile species, *Trematomus pennellii*. Due to similar morphological characteristics, some fish included in the behavior analyses were actually *T. pennellii*, and not *T. bernacchii*. To confirm all fish identities, the caudal section of each fish was DNA barcoded (following Ivanova, Zemplak, Hanner, & Hebert, 2007). *Trematomus pennellii* were removed from the behavior dataset, leaving $n = 5$ –9 (scototaxis) and $n = 7$ –10 (activity) for behavior analyses of *T. bernacchii* for PCO_2 /temperature treatments.

2.8 | Statistical analyses

All statistical analyses were conducted in R (v3.1.3) with an alpha-value set at .05. Data were tested for parametric assumptions using visual inspections of Q–Q plots, residuals vs. fitted values, and residuals vs. factor levels including PCO_2 treatment, temperature, and acclimation time. When data did not meet parametric assumptions, data were log transformed. A three-way analysis of variance (ANOVA) was performed on all physiology datasets with a TukeyHSD (honest significant difference) post hoc test conducted if F -values were significant. Initial models included fish nested within their appropriate replicate culture buckets; however, with no significant effects, culture bucket was removed and models were reduced to the simplest form. The dependent variables were heart rate, ventilation rate, MO_2 (transformed), and enzyme activities including citrate synthase (transformed), cytochrome c oxidase, and lactate dehydrogenase (transformed), whereas the independent statistical parameters were PCO_2 level, temperature, and acclimation time. Scototaxic testing was analyzed with a two-way ANOVA with time spent in the dark zone as the dependent variable, and PCO_2 level and temperature as the independent factors. Activity and novel object testing parameters were initially plotted with a Principal Component Analyses (PCA) having numerous repeated behavior metrics for each individual. The PCA showed overlapping vectors for time spent active (s), distance traveled (cm), velocity (m/s), and rotational changes, whereas time in the center zone (near the object), and time to emergence from the object were orthogonal (Figure S2). Of the similar activity measures in the PCA only time active (swimming) was analyzed with a three-way ANOVA, with PCO_2 level, temperature, and acclimation time as the independent factors, and a post hoc TukeyHSD test followed. Time spent in the center zone near the object was transformed (square root) and a three-way ANOVA was conducted. Time to emergence from the object (e.g., boldness) had skewed normality and potential censoring (those that never emerged considered infinite), and hence violated linear regression models. Therefore, emergence curves were compared between PCO_2 and temperature treatments within each acclimation time (7 and 14 days)

separately using the *Survival* package in R (v2.37; Therneau, 2015). Within each time point, six treatment combination (PCO_2 /temperature) curves were fit showing the proportion of fish remaining in the cover (object) at any given time. To determine significant differences between emergence curves a nonparametric log-rank test was conducted (Chi-squared test in the *Survival* package). Results are presented as means \pm SEM.

3 | RESULTS

3.1 | Cardiorespiratory physiology

Within each PCO_2 treatment (Ambient, Moderate, and High PCO_2), f_H was significantly elevated with warming ($F_{1,191} = 333.551$, $p < .0001$, Figure 1a) compared to -1°C (TukeyHSD; $p < .0001$ for all PCO_2 treatments). Overall f_H at -1°C was 33.0 ± 0.1 and 39.1 ± 0.1 bpm at 2°C , increasing linearly roughly 6 bpm with warming in every PCO_2 treatment. Heart rate increased across acclimation time (independent of temperature and PCO_2 treatment; $F_{3,191} = 9.837$, $p < .0001$), with the highest f_H after 14 days (TukeyHSD; $p < .001$) of acclimation compared to f_H after 2 and 7 days, and only a subtle decrease after 28 days remaining elevated from initial f_H at 2 days ($p < .001$). PCO_2 had no effect ($F_{2,191} = 0.457$, $p = .623$) on f_H and there were no significant interactions between any of the main effects.

Similar to f_H , f_V significantly increased with warming ($F_{1,191} = 137.638$, $p < .0001$); however, within both temperature

treatments (-1 and 2°C), f_V also increased with elevated PCO_2 ($F_{2,191} = 9.979$, $p < .0001$, Figure 1b). f_V of fish in High PCO_2 was significantly higher than under Ambient PCO_2 (TukeyHSD; $p < .0001$), whereas f_V in Ambient and Moderate PCO_2 ($p = .060$) and Moderate and High PCO_2 treatments ($p = .074$) were more similar. The main effect of acclimation time ($F_{3,191} = 3.699$, $p = .013$) on f_V was dependent on temperature (significant interaction, $F_{6,191} = 3.171$, $p = .026$), such that fish acclimated to -1°C had similar f_V across all acclimation time points (TukeyHSD; $p > .05$), whereas fish acclimated to 2°C had similar f_V at 2, 7, and 14 days ($p > .05$) followed by a significant decrease in f_V at 28 days compared to 14 days ($p < .001$).

3.2 | Metabolic rate

Mass-specific metabolic rate ($\dot{M}O_2$) of juvenile rockcod significantly increased with increased temperature ($F_{1,202} = 25.083$, $p < .0001$; Figure 2) from 1.7 ± 0.1 at -1°C to 2.6 ± 0.1 $\mu\text{mol O}_2 \text{ hr}^{-1} \text{ g}^{-1}$ at 2°C ; however, a near significant three-way interaction ($F_{6,202} = 2.132$, $p = .051$) indicated the effect of temperature was assessed more accurately in relationship with PCO_2 treatment ($F_{2,202} = 0.417$, $p = .659$) and acclimation time ($F_{3,202} = 5.267$, $p = .002$). Fish under Ambient CO_2 showed more similar $\dot{M}O_2$ at 2 and -1°C (TukeyHSD; $p = .734$, Figure 2a) compared to fish under Moderate ($p < .05$) and High CO_2 ($p < .001$) where warming significantly increased $\dot{M}O_2$ (Figure 2b,c). Notable changes in $\dot{M}O_2$ in response to elevated PCO_2 and warming were also seen across

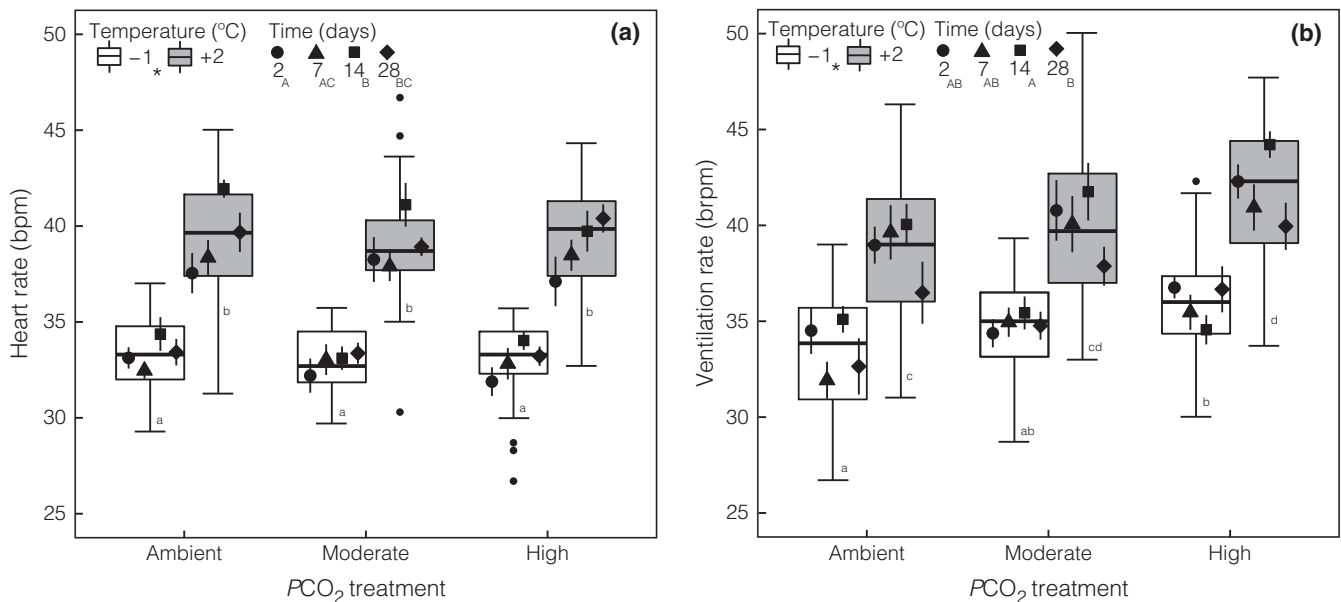


FIGURE 1 Heart rate (a, beats per minute [bpm]) and ventilation rate (b, breaths per minute, [brpm]) of *Trematomus bernacchii* acclimated to CO_2 -acidified seawater and warming across acclimation time. Data are presented as boxplots ($n = 32$ – 38 per box) with -1°C (white) and 2°C (gray). The box represents the interquartile range (IQR) and the whiskers extend 1.5 times IQR. Within each temperature/ PCO_2 boxplot are four symbols representing the mean \pm SEM of heart rate and ventilation rate following 2 (circle), 7 (triangle), 14 (square), and 28 (diamond) days of acclimation ($n = 6$ – 13 per point). Asterisks signify a significant effect of temperature ($p < .0001$). Lowercase letters represent significant differences ($p < .05$) among PCO_2 treatments and temperature, and uppercase letters represent a difference by acclimation time ($p < .05$)

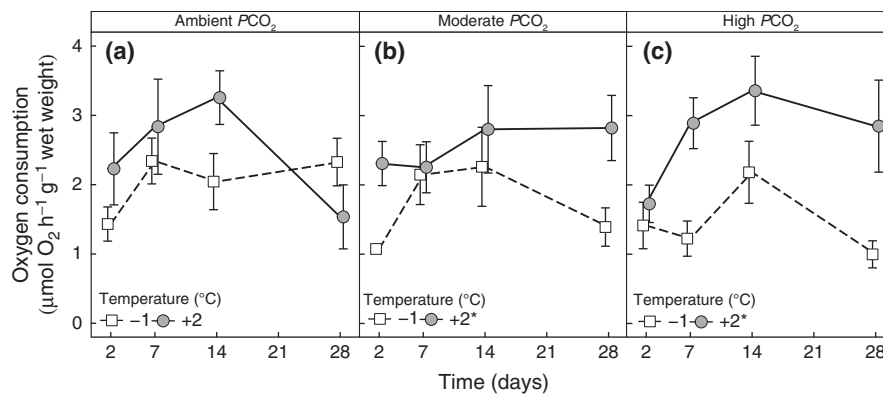


FIGURE 2 Mass-specific metabolic rate of *Trematomus bernacchii* measured by oxygen consumption. Each panel represents a single PCO_2 treatment (a) Ambient, (b) Moderate, and (c) High, with white squares representing fish acclimated to $-1^\circ C$ and gray circles representing fish acclimated to $2^\circ C$ (mean \pm SEM, $n = 9$ individuals for each data point). Asterisks signify a significant effect of warming on oxygen consumption within each PCO_2 treatment (TukeyHSD; $p < .05$)

acclimation time, specifically at 28 days. $\dot{M}O_2$ for fish under $2^\circ C$, Ambient PCO_2 conditions at 28 days was $1.5 \pm 0.5 \mu mol O_2 hr^{-1} g^{-1}$ compared to $1.4 \pm 0.2 \mu mol O_2 hr^{-1} g^{-1}$ near the start of the experiment (day 2, Ambient PCO_2 , $-1^\circ C$) showing thermal compensation (Figure 2a); however, fish under elevated temperature and PCO_2 conditions at 28 days demonstrated no thermal compensation as $\dot{M}O_2$ remained elevated (Figure 2b,c).

3.3 | Enzyme activities

COX activity showed no main effect of PCO_2 treatment ($F_{2,192} = 0.537$, $p = .585$), temperature ($F_{1,192} = 2.556$, $p = .112$) or acclimation time ($F_{3,192} = 2.248$, $p = .079$), and there were no significant interactions between any of the independent variables ($p > .05$, Figure 3a–c). A significant interaction ($F_{2,192} = 3.336$; $p = .038$) between the main effects of PCO_2 treatment ($F_{2,192} = 0.394$, $p = .675$) and temperature ($F_{1,192} = 2.070$, $p = .152$) on CS activity showed within $-1^\circ C$, overall CS activity of fish in the Ambient PCO_2 treatment was significantly greater than Moderate PCO_2 (TukeyHSD; $p < .05$) and High PCO_2 ($p < .05$). However, under $2^\circ C$, CS activity in the Ambient PCO_2 treatment was significantly lower than Moderate PCO_2 ($p < .05$) and High PCO_2 ($p < .05$), suggestive of thermal compensation (Figure 3d–f). Acclimation time had no effect ($F_{3,192} = 1.161$, $p = .326$) on CS activity and there were no other significant interactions between any of the main effects. A similar significant interaction ($F_{2,192} = 5.031$, $p = .007$) between PCO_2 treatment ($F_{2,192} = 1.230$, $p = .295$) and temperature ($F_{1,192} = 0.466$, $p = .466$) was detected in LDH activity (Figure 3g–i). Within $-1^\circ C$, the Moderate PCO_2 fish had a different LDH response (TukeyHSD; $p < .05$) than fish in Ambient and High PCO_2 , but within $2^\circ C$, overall LDH activity was different in each PCO_2 treatment ($p < .05$). There was a significant effect of acclimation time ($F_{3,192} = 4.056$, $p = .008$) on LDH activity such that overall activity at 7 days ($p = .026$) and 14 days ($p = .051$) was greater than activity at 28 days. Ratios of COX:CS and LDH:CS activity showed no effect of PCO_2 treatment, temperature, or acclimation time on ratios ($p > .05$; see Table S1 for data values).

3.4 | Behavior assays

Scototaxis or light/dark preference was significantly altered by temperature (two-way ANOVA, $F_{1,37} = 5.305$, $p = .027$). In general, fish under ambient temperatures ($-1^\circ C$) across all PCO_2 treatments showed little preference for the dark zone ($53 \pm 5\%$ Ambient, $54 \pm 6\%$ Moderate, and $46 \pm 2\%$ in High PCO_2); however, warming ($2^\circ C$) significantly increased fish preference for the dark zone (Figure 4). For example, under Ambient PCO_2 warming increased dark zone preference by 12% (65% time spent in dark). Warming with Moderate PCO_2 increased dark preference by 8% (60% time spent in dark), and warming with High PCO_2 increased dark zone preference by 14% (61% time spent in dark zone). PCO_2 did not alter scototaxis ($F_{2,37} = 0.526$, $p = .595$) and there was no interaction between temperature and PCO_2 ($p > .05$).

Fish activity level significantly decreased with temperature ($F_{1,86} = 4.960$, $p = .028$, Figure 5a,b) independent of PCO_2 ($F_{2,86} = 3.055$, $p = .052$) and acclimation time ($F_{1,86} = 1.168$, $p = .282$). Fish acclimated to $-1^\circ C$ were more active spending on average 493 ± 18 s time swimming out of 600 s (82%), whereas fish acclimated to $2^\circ C$ decreased activity by 12% spending 419 ± 29 s swimming (70%). There were no significant interactions among PCO_2 , temperature, or acclimation time ($p > .05$).

The novel object test (conducted in the same test arena as activity) showed there was no significant effect of PCO_2 ($F_{2,86} = 1.063$, $p = .350$), temperature ($F_{1,86} = 0.480$, $p = .490$), acclimation time ($F_{1,86} = 1.332$, $p = .252$) on the amount of time fish spent in the center zone (within one body length of the object; Figure S3), and there were no significant interactions between any of the independent variables ($p > .05$). Average time spent near the novel object was 147 ± 12 s out of the 600 s trial ($-25 \pm 2\%$). Proportional to the time spent exploring the object was the time spent away from the object. Overall, fish spent $75 \pm 2\%$ time away from the object, with $22 \pm 2\%$ (across all PCO_2 treatments) of that time as thigmotactic (i.e., swimming along the arena walls exhibiting escape behaviors; Figure S4).

Boldness after 7 days of acclimation showed a significant difference between the curves (Figure 6a) generated for time to emerge

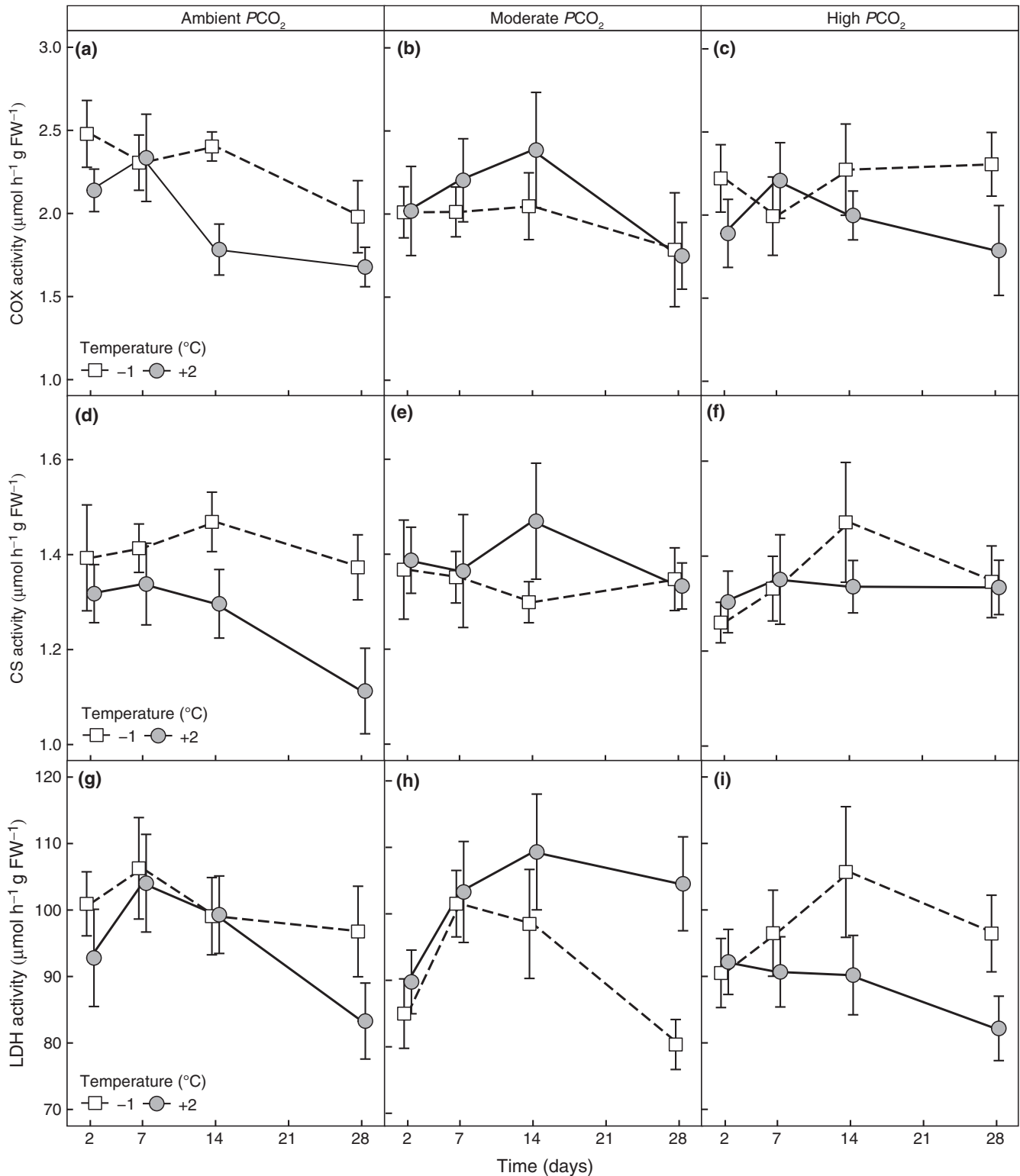


FIGURE 3 Aerobic and anaerobic enzyme activity of muscle tissue ($\mu\text{mol hr}^{-1} \text{g FW}^{-1}$) of juvenile *Trematomus bernacchii* under different PCO_2 and temperature treatments across time. Each column of panels represents a single PCO_2 treatment (Ambient, Moderate, and High) with aerobic enzymes (a–c) cytochrome c oxidase (COX), and (d–f) citrate synthase (CS) activity, and anaerobic enzyme (g–i) lactate dehydrogenase (LDH) represented across all treatments. Within each PCO_2 and enzyme panel, white squares represent fish acclimated to -1°C and gray circles represent fish acclimated to 2°C (mean \pm SEM, $n = 9$ individuals for each data point). Enzyme activities were measured at 5°C

from the object among the PCO_2 /temperature treatments (nonparametric log-ranked test; $\chi^2_1 = 11.4$, $df = 5$, $p = .044$). After 120 s (the trial end) $\sim 75\%$ of fish in Moderate $\text{PCO}_2/2^\circ\text{C}$ never emerged from

the object, compared to 20% in High $\text{PCO}_2/2^\circ\text{C}$ and 15% in Ambient $\text{PCO}_2/2^\circ\text{C}$ treatments. At ambient temperatures (-1°C) fish emerged from the object more rapidly with 100% emergence by 50 s in

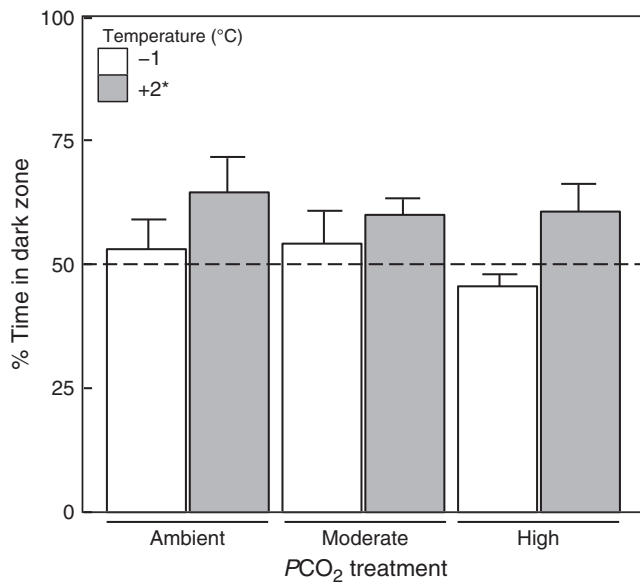


FIGURE 4 Scototaxis, light/dark preference testing of *Trematomus bernacchii* after 14 days. Each bar represents the mean \pm SEM of each PCO_2 /temperature treatment with white bars representing fish acclimated to $-1^\circ C$ ($n = 8$ Ambient, $n = 8$ Moderate, $n = 6$ High PCO_2) and gray bars representing fish acclimated to $2^\circ C$ ($n = 7$ Ambient, $n = 9$ Moderate, $n = 5$ High PCO_2). The black dashed line at 50% represents no preference for the light or dark zone. Asterisks represent a significant effect of temperature ($p < .05$)

Moderate PCO_2 / $-1^\circ C$, and 100% emergence by 90 s in Ambient PCO_2 / $-1^\circ C$. Approximately 38% of fish under High PCO_2 / $-1^\circ C$ had not emerged after the trial conclusion. After 14 days, there was no difference in emergence curves for PCO_2 /temperature treatments ($\chi^2_1 = 3.6$, $df = 5$, $p = .609$), and all PCO_2 /temperature treatments had fish remaining in the object (never emerged) after the trial ended (Figure 6b).

4 | DISCUSSION

Here we demonstrate, for the first time, the combined effects of CO_2 -acidification and warming on the physiology and behavior of an

Antarctic fish. As predicted, several aspects of cardiorespiratory physiology including heart rate (f_H), ventilation rate (f_V), and metabolic rate ($\dot{M}O_2$) significantly increased with warming. Most noteworthy, juvenile *Trematomus bernacchii* demonstrated a degree of thermal compensation and temperature acclimation that was much quicker than observed in the adults under Ambient PCO_2 (Enzor et al., 2013, 2017; Jayasundara et al., 2013; Sandersfeld et al., 2015). The addition of elevated PCO_2 , however, appeared to create an interacting additive effect on hyperventilation, and a potential synergistic effect on $\dot{M}O_2$ (i.e., lower capacity for temperature acclimation when exposed to elevated PCO_2). In addition, we predicted that elevated PCO_2 would have a greater impact on fish behavior than elevated temperature; however, elevated PCO_2 had no effect on activity and anxiety behaviors in juvenile emerald rockcod. In contrast, elevated temperature as a single stressor increased dark zone preference in fish, suggesting increased anxiety, whereas decreasing total fish activity and boldness. The highest physiological rates (i.e., energetic costs) seen at 14 days including $\dot{M}O_2$, f_H , and f_V in response to temperature corresponded with reduced swimming activity, boldness, and anxiety at 14 days, providing some initial evidence of a potential trade-off between altering behavior and recruiting energetically demanding stress tolerance mechanisms (Careau & Garland, 2012; Roche, Careau, & Binning, 2016).

Organismal-level mechanisms for oxygen (O_2) supply and delivery to the tissues, including gill ventilation and cardiac performance, have been hypothesized to be the limiting step in determining acclimation capacity and tolerance to changing environmental conditions (Farrell, Eliason, Sandblom, & Clark, 2009; Pörtner & Farrell, 2008). In this study, f_V , an index of O_2 uptake and indirectly O_2 supply (Ern & Esbaugh, 2016), was significantly elevated by increased PCO_2 and temperature (Figure 1b), whereas f_H , a mechanism for O_2 delivery to the body tissues was increased by temperature but independent of PCO_2 (Figure 1a). Changes in cardiorespiratory mechanisms are a common response of fishes when making metabolic adjustments to environmental changes such as O_2 levels, reduced pH and increased temperature (Franklin, Farrell, Altimiras, & Axelsson, 2013; Heuer & Grosell, 2014; Ishimatsu et al., 2008; Penney, Nash, & Gamperl, 2014; Pörtner & Peck, 2010; Robinson, Egginton, & Davison, 2011; Sandblom et al., 2016). Elevated f_V and f_H likely increased O_2 supply

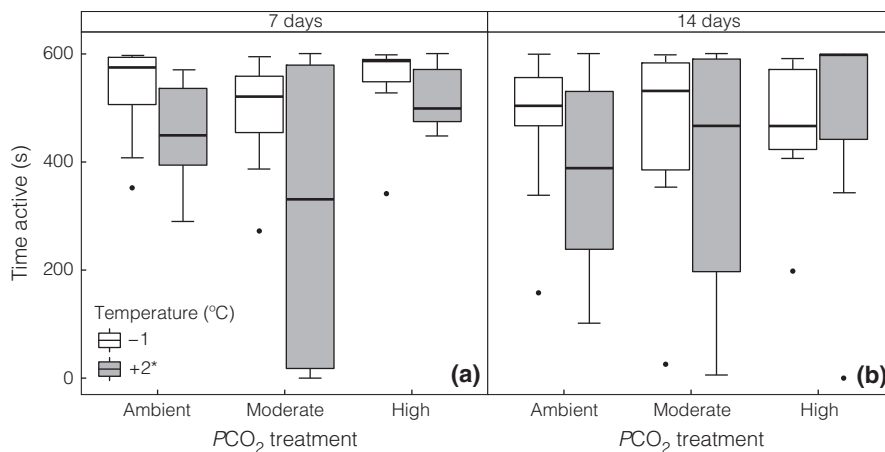


FIGURE 5 Total time active (i.e., swimming) of *Trematomus bernacchii*. Each image represents activity after 7 (a) or 14 (b) days of acclimation. Activity data are presented as boxplots ($n = 7$ – 10 per box) with fish acclimated to $-1^\circ C$ represented in white and fish acclimated to $2^\circ C$ in gray. The box represents the interquartile range (IQR), the center line is the median, and the whiskers extend 1.5 times IQR. Asterisks signify a significant effect of temperature ($p < .05$)

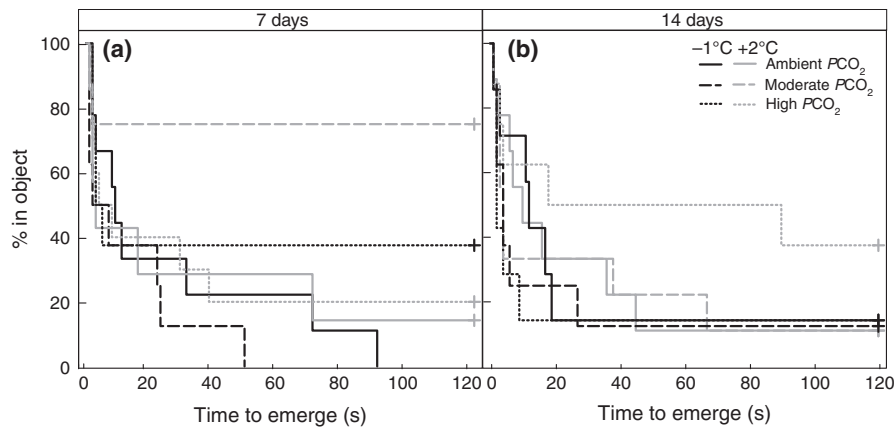


FIGURE 6 Time to emergence (a proxy for boldness) of *Trematomus bernacchii* from an object is shown as the cumulative proportion remaining in the object across time (Kaplan–Meier estimate). Each figure represents boldness of fish after 7 (a) or 14 (b) days of acclimation. Fish acclimated to -1°C are represented in black, whereas fish acclimated 2°C are represented in gray. Different line-types describe CO_2 -acidified seawater treatments, Ambient PCO_2 (solid), Moderate PCO_2 (long dash), and High PCO_2 (dotted) with $n = 7\text{--}10$ per fish per line. Fitted PCO_2 /temperature curves at 7 days are significantly different ($p < .05$), where curves at 14 days are similar

to facilitate the increased metabolic costs under elevated temperature and PCO_2 with $\dot{\text{M}}\text{O}_2$ of fish significantly increasing with elevated temperature. The Antarctic notothen *Notothenia coriiceps* exhibited a similar cardiorespiratory response to juvenile *T. bernacchii* in this study when exposed to warming (5°C) for 6 weeks (Egginton & Campbell, 2016) with f_{H} remaining elevated across acclimation time and thus showing no compensation for increased temperature. Conversely, adult *T. bernacchii* acclimated for 2 weeks at 2°C had no change in f_{H} or f_{V} compared to control conditions at -1°C (Jayasundara et al., 2013), suggesting cardiorespiratory responses to warming may have stage-specific limitations. Juvenile *T. bernacchii* f_{H} was not affected by Moderate or High levels of PCO_2 ($\sim 900\text{--}1,300$ μatm PCO_2), similar to a previous study on this species exposed to different elevations in PCO_2 (650 and 1,000 μatm PCO_2 ; Davis et al., 2016). Ventilation rate of juvenile fish increased significantly in a dose-dependent fashion in response to PCO_2 , with the additive effect of elevated PCO_2 intensified by warming. Hyperventilation has been previously exhibited in this species in response to elevated PCO_2 (Davis et al., 2016). A similar dose-dependent response to increasing levels of PCO_2 (1,000 and 5,000 μatm PCO_2) in ventilation amplitude, stroke volume and minute volume has been shown in red drum (Ern & Esbaugh, 2016; Esbaugh, Ern, Nordi, & Johnson, 2016). Of note, after 28 days of acclimation f_{V} decreased under warming within each PCO_2 treatment, showing partial compensation, yet the linear effect of elevated PCO_2 on f_{V} persisted (Figure 1b). These results suggest the additive effect of elevated PCO_2 on f_{V} (not present in f_{H}) likely served as an acid-base balance mechanism to excrete CO_2 . Adjustments in f_{H} and f_{V} suggest juvenile rockcod have a degree of cardiorespiratory plasticity that can support the additional energetic costs (seen in $\dot{\text{M}}\text{O}_2$) induced by exposures to elevated temperature and PCO_2 and likely also serve as a mechanism of acid-base regulation.

Several physiological measures suggest that juvenile Antarctic rockcod have some capacity to compensate for a 2°C ocean

temperature (a 3°C increase from the control) if given sufficient time to acclimate. For example, f_{V} increased over acute time scales in response to elevated temperature and PCO_2 ; however, after 28 days of acclimation, f_{V} significantly decreased within each PCO_2 level showing partial thermal compensation (Figure 1b). A similar trend was exhibited in whole organism $\dot{\text{M}}\text{O}_2$. While $\dot{\text{M}}\text{O}_2$ increased in juvenile fish acclimated to 2°C after 14 days, after 28 days fish under Ambient PCO_2 conditions showed temperature compensation with $\dot{\text{M}}\text{O}_2$ decreasing significantly and returning to $\dot{\text{M}}\text{O}_2$ rates at -1°C (Ambient PCO_2 , 2 days, see Figure 2a). Cellular aerobic and anaerobic enzyme activity provide additional evidence that juvenile rockcod have the capacity to compensate for a $+3^{\circ}\text{C}$ increase in temperature, when uncoupled from changes in PCO_2 . For example, enzyme activity of CS and LDH in control fish (e.g., Ambient PCO_2 , -1°C) remained relatively constant across acclimation time; however, when exposed to an increase in temperature (Ambient PCO_2 , 2°C) both CS and LDH enzyme activities decreased. The lowest enzyme activity rates in Ambient PCO_2 , 2°C were seen after 28 days, showing a degree of temperature compensation (Figure 3a,d,g).

The capacity to acclimate to warming was diminished when warming was coupled with elevated PCO_2 . When juvenile rockcod were exposed to elevated temperature accompanied by elevated PCO_2 similar degrees of thermal compensation of $\dot{\text{M}}\text{O}_2$ were not observed. Elevated $\dot{\text{M}}\text{O}_2$ was sustained for the duration of the experiment rather than returning to baseline levels as seen in the fish only exposed to warming (Figure 2b,c). Further evidence of a reduced metabolic capacity to acclimate was reflected in CS enzyme activity, whereas response kinetics of COX and LDH activity varied under exposures to multiple stressors. CS activity of fish exposed to warming alone (i.e., Ambient $\text{PCO}_2/2^{\circ}\text{C}$) decreased over acclimation time and had significantly lower activity than fish exposed to warming and PCO_2 together (i.e., Moderate and High $\text{PCO}_2/2^{\circ}\text{C}$). Decreased muscle CS activity in fish exposed to warming suggests that fish were acclimating to warming and could maintain adequate

ATP production with reduced CS activity at warmer temperatures (Strobel, Leo, et al., 2013; Windisch, Kathöver, Pörtner, Frickenhaus, & Lucassen, 2011). The maintained CS activity under multiple stressors (Moderate/High PCO_2 and warming) suggests the need for sustained aerobic ATP production to support other physiological processes and maintenance costs (Enzor et al., 2017; Huth & Place, 2013). It remains unclear what physiological trade-offs juvenile rockcod may exhibit over longer periods (>4 weeks) under the multiple stressors of increased temperature and PCO_2 and if reductions in growth and energy consumption would be observed as in older life stages (Enzor et al., 2017; Sandersfeld et al., 2015).

Behavior alteration is an integrated, ecologically relevant response to environmental stress in fishes (Rand, 1985), as behavior responds to internal signals from physiological and biochemical processes as well as external signals from the environment (Kane, Salierno, & Brewer, 2005). Linking physiological markers with behavioral outcomes allows for better understanding of individual responses as well as potential community-level impacts of ocean warming and acidification. Anxiety and activity behaviors of Antarctic juvenile rockcod were not significantly altered by exposure to elevated PCO_2 . This was unexpected as much of the work to date in temperate and tropical fishes has described significant behavioral changes in response to elevated PCO_2 (reviewed in Nagelkerken & Munday, 2015). Previous studies on activity and movement behaviors have shown that elevated PCO_2 altered scototaxis (Hamilton et al., 2014), exploratory and thigmotaxis behavior (Jutfelt et al., 2013; Munday et al., 2010; Ou et al., 2015), activity (Cripps et al., 2011; Devine, Munday, & Jones, 2012b; Munday et al., 2009, 2010, 2013), and boldness (Jutfelt et al., 2013; Munday et al., 2013; Rossi et al., 2015). A more recent study in red drum *Sciaenops ocellatus* reported that anxiety (measured by scototaxis dark preference) was not increased by elevated PCO_2 (Lonthair et al., 2017). In this study increased temperature had a stronger effect on altering the behavior of juvenile rockcod. Warming increased fish dark zone preference by 8%–14% (i.e., anxiety [Figure 4]) and warming increased inactivity (i.e., resting periods) from 12% to 30% after 7 days, and from 24% to 31% after 14 days (Figure 5). In addition to reduced activity, juvenile rockcod exposed to increased temperature were less bold. While most fish emerged from the object within the first 20–40 s, fish acclimated to elevated temperatures remained in the object for longer periods of time, with several never emerging for the duration of the 120 s trial (Figure 6). Similar to juvenile rockcod, rainbow wrasse *Coris julis* and adult coral trout *Plectropomus leopardus* exhibited reduced activity with increasing temperatures by displaying longer resting periods (Johansen, Messmer, Coker, Hoey, & Pratchett, 2014; Milazzo, Mirto, Domenici, & Gristina, 2013). The natural PCO_2 environment of juvenile rockcod in this study has been shown to be more variable than temperature (Kapsenberg et al., 2015) and may explain why these juvenile *T. bernacchii* were relatively robust to elevations in PCO_2 , but exhibited physiological and behavioral alterations in response to increased temperature.

Reduced activity under warming may be an energy-conserving behavior to compensate for the increased energetic demands of

being reared under elevated temperatures. Juvenile rockcod had a significantly higher $\dot{M}O_2$ and increased f_H and f_V in response to elevated temperature acclimation (2°C) following 14 days of acclimation (Phase I), and this corresponded with decreased activity in fish in the behavior trials (Phase II). Our results provide initial evidence that juvenile rockcod may be experiencing a “trade-off” between costly metabolic processes to cope with warming and behavior at 2 weeks. A mechanistic behavioral compensation model described by Careau and Garland (2012) links personality types and energetics, correlating energy expenditure mechanisms such as basal metabolic rates and activity levels. If an organism has a fixed energy-budget, any energy demanding activity may be altered and/or reduced in efforts to meet the energetic budget (Careau & Garland, 2012). In support of this hypothesis, aerobic scope in five species of damselfishes decreased by 24%–65% after 3 weeks acclimation to a 3°C increase in temperature (Johansen & Jones, 2011). This decrease was matched with a loss in swimming performance including maximum swimming speed (decreased ~21%–28%) and gait-transition speed (i.e., ~33%–51% reduction in transition speed from pectoral fin usage to pectoral and caudal fin swimming; Johansen & Jones, 2011). Juvenile rockcod may be reducing energy allocation to locomotory activity to ensure enough energy is available to meet additional metabolic requirements of elevated temperatures. Ecologically, increased inactivity may increase susceptibility of fishes to predation as well as interfere with foraging. In the current experiment, increased inactivity corresponded with an increased preference for dark areas (e.g., scototaxis results and delayed time to emerge from dark object) and although speculative, this behavior might be linked with cryptic coloration (i.e., camouflage) to reduce detection by predators (Fuiman & Magurran, 1994; Maximino et al., 2010). Color morphs of juvenile rockcod in the laboratory appeared plastic with pigmentation alteration occurring within minutes of a new environment such as a tank change or the dark light/zones of the scototaxis trials (personal observations). Studies testing both physiological-behavior trade-offs and the crypsis linked to inactivity hypotheses are needed. Furthermore, while juvenile rockcod showed a degree of temperature compensation in physiological performance after 28 days under warming alone, given the shorter, 2-week acclimation of the behavior trial it remains unknown if behavioral responses to warming would also have been compensated for after 28 days or if trade-offs would persist. Connecting how warm-induced alterations (reductions) in activity and boldness may interfere with foraging and food consumption (Nowicki et al., 2012), as well as antipredator responses (Ferrari, Dixson, et al., 2011) may serve as critical next steps in forecasting ecological impacts of future ocean conditions.

In conclusion, this study showed that slight alterations in cardiorespiratory phenotypes coupled with metabolic rate compensation and depression of cellular enzyme activities may give juvenile emerald rockcod *T. bernacchii* sufficient capacity to compensate for a +3°C temperature change after 28 days. However, when warming was combined with elevated PCO_2 a reduced metabolic capacity to acclimate to warming both at the whole organism and cellular level provide evidence that under realistic climate change scenarios of

coupled ocean warming and acidification, fish will likely be vulnerable to projected future ocean conditions. The greatest physiological energetic costs measured at 14 days in response to warming were correlated with significant alterations in behavior at 14 days, including reduced activity and boldness and increased preference for a dark-sheltered zone, suggesting potential trade-offs in energy allocation between behavior and physiology. Studies linking physiological performance and behavior are warranted to fully understand the scope of impact multiple stressor ocean changes may have on overall fitness of fishes through changes in energy allocation and behavior. Lastly, this study highlights the need to conduct experimental studies across longer acclimation periods and ontogeny. Studies with shorter acclimation periods to future climate changes may not capture the time needed to acclimate. If this study had concluded after just 14 days of exposure to warming and CO₂-acidification, the conclusions drawn would be different, as we would have missed juvenile *T. bernacchii*'s ability to compensate for warming and the interactive effects of warming and CO₂-acidification.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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