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## Original Article

# The early life stages of the orange-spotted grouper, *Epinephelus coioides*, exhibit robustness to hypercapnia

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Ocean acidification (OA) and other climate change-induced environmental alterations are resulting in unprecedented rates of environmental degradation. This environmental change is generally thought to be too fast for adaptation using evolutionary process dependent on natural selection, and thus, resilience may be related to the presence of existing tolerant genotypes and species. Estuaries undergo natural partial pressure carbon dioxide ($pCO_2$) fluctuations, with levels regularly exceeding predicted end of the century values. In this study, we use the estuarine orange-spotted grouper (*Epinephelus coioides*) to explore the intrinsic resilience to elevated $pCO_2$. Our sensitivity endpoints included: survival, heart rate, growth, and yolk consumption. Furthermore, we attempted to determine whether their acid–base regulatory machinery was plastic in response to elevated $pCO_2$ by analysing the gene expression of key transporters and ionocyte density. Survival was not significantly altered by exposure to elevated $pCO_2$. Interestingly, the heart rate was significantly elevated at both 1500 and 3100 µatm exposure. However, other metrics of energetic consumption, such as yolk consumption and growth, were not significantly altered. Furthermore, we found no changes in gene expression in *vha*, *nhe3*, and *nbc*, as well as ionocyte density at elevated $pCO_2$. Overall, these results support the hypothesis that estuarine species are resilient to the impacts of OA.

**Keywords:** development, estuarine, hypercapnia, ocean acidification, teleost.

## Introduction

Since the beginning of the industrial revolution, anthropogenic carbon dioxide ($CO_2$) emissions have had measurable impacts on the oceanic carbonate chemistry—colloquially known as ocean acidification (OA). Models project that partial pressure $CO_2$ ($pCO_2$) could increase to 1000 µatm by the of the century, which would represent a 250% increase in $pCO_2$ from present oceanic levels (Caldeira and Wickett, 2003; Solomon *et al.*, 2007; Doney *et al.*, 2009; McNeil and Sasse, 2016). This increase in $pCO_2$ would result in a 0.3–0.4 units reduction in pH (Orr *et al.*, 2005). Studies on the impacts of elevated $pCO_2$ exposure on marine teleosts have found a plethora of negative outcomes across a variety of endpoints (Heuer and Grosell, 2014; Esbaugh, 2018), including impaired sensory systems (Munday *et al.*, 2009b; Nilsson *et al.*, 2012; Williams *et al.*, 2019), alterations in aerobic scope (Munday *et al.*, 2009a; Couturier *et al.*, 2013), ionoregulatory physiology (Esbaugh *et al.*, 2012; Heuer *et al.*, 2012; Strobel *et al.*, 2012), increased tissue damage (Frommel *et al.*, 2012; Chambers *et al.*, 2014; Frommel *et al.*, 2014), and diminished growth and survival (Baumann *et al.*, 2012). Recent studies have concluded that the negative effects of OA may be species specific, with a large number of articles measuring a variety of endpoints reporting no effects on fish (Munday *et al.*, 2016; Lonthair *et al.*, 2017; Baumann, 2019).

The cumulative effects of OA are thought to stem from a compensated respiratory acidosis that results from the altered $pCO_2$ gradients between the water and blood. In fact, previous studies on have demonstrated elevated plasma $HCO_3^-$ and $pCO_2$ coincident with normal pH in response to exposure to OA relevant $pCO_2$ levels in marine fish species (Esbaugh *et al.*, 2012; Heuer *et al.*, 2012; Strobel *et al.*, 2012; Esbaugh *et al.*, 2016). The metabolic compensation pathways in teleosts generally

involve apical excretion of $H^+$ and basolateral re-uptake of $HCO_3^-$ at the gills (Hwang *et al.*, 2011). More specifically, the process occurs in a single branchial cell type called the ionocyte, whereby apical $H^+$ transport occurs through $Na^+/H^+$ exchangers (NHE2 and NHE3) and/or the V-type $H^+$ ATPase (VHA) (Marshall and Grosell, 2006). The basolateral re-uptake of $HCO_3^-$ is thought to occur mainly through the electrogenic $Na^+/HCO_3^-$ cotransporter (NBC), which operates in the efflux direction due to the favourable electrochemical gradients (Hirata *et al.*, 2003). It is important to note that other transporters play a role in acid-secretion mechanisms in other saltwater-acclimated and estuarine species (Takei *et al.*, 2014; Liu *et al.*, 2016).

One major concern of exposure to elevated $pCO_2$ is increased metabolic cost and potential metabolic reallocation that results from the increased transport of acid–base equivalents in larval teleosts. Previous work often uses additional metabolic costs as an explanation for physiological responses to OA (Stumpp *et al.*, 2011, 2012). Furthermore, recent work on sea urchin larvae exposed to elevated $pCO_2$ did not exhibit alterations in size, metabolic rate, biochemical content, and gene expression but did exhibit a metabolic reallocation, specifically in protein synthesis and ion transport (Pan *et al.*, 2015). This alteration in energy allocation is critical for understanding the significance of sublethal stress, because individuals with maintenance costs less sensitive to environmental stressors are more likely to survive (Pan *et al.*, 2015).

Recent studies on OA have moved beyond defining the detrimental effects of elevated $pCO_2$ on marine life to exploring the potential for resilience in marine systems. This is especially important for economic resources such as fish, and OA is predicted to have severe effects on fish populations (see reviews Hofmann and Todgham, 2010; Munday *et al.*, 2010; Kelly and Hofmann, 2013; Pfister *et al.*, 2014). The resilience of marine fish species to the long-term environmental degradation caused by OA is dependent on a number of factors. Evolutionary processes such as the rise in novel beneficial mutations that can accumulate in a population through natural selection are unlikely to facilitate resilience in species with generation times of months to years, although recent work has shown that rapid evolution may be possible (Torda *et al.*, 2017; Ryu *et al.*, 2018; Catullo *et al.*, 2019). Thus, resilience to OA and climate change may depend on the presence of existing tolerant genotypes in a population, and the ability of individuals to alter their physiology to suit new environmental conditions; a process known as phenotypic plasticity (Bell, 2013; Gonzalez *et al.*, 2013; Pespeni *et al.*, 2013). Previous studies have demonstrated resilient responses to OA among a variety of marine teleosts from across a variety of ecosystems, including species from the Antarctic, estuaries, and coastal upwelling (Davis *et al.*, 2016; Munday *et al.*, 2016; Lonthair *et al.*, 2017; Baumann, 2019). These studies have found that various endpoints are not altered by elevated exposure to OA, including survival, larval morphometrics, starvation rate, heart rate, enzymatic activity, and behaviour (Davis *et al.*, 2016; Munday *et al.*, 2016; Allmon and Esbaugh, 2017; Lonthair *et al.*, 2017; Baumann *et al.*, 2018).

On this background, the current study sought to examine whether a sub-tropical species which migrates into an estuarine environment exhibits resilience to OA. We first experimentally assessed the sensitivity of a fast-developing economically important teleost species, the orange-spotted grouper (*Epinephelus coioides*). Orange-spotted grouper, also known as the estuary cod, are native to the Indo-Pacific and are critical species for aquaculture in the Asia-Pacific region (Zhou *et al.*, 2011). We chose orange-spotted grouper because of their dependency on the tropical estuarine environment (Sheaves, 1993). We then tested whether this species exhibits plasticity of the acid–base regulatory machinery by measuring the gene expression of key exchangers in the mechanism. We hypothesized that orange-spotted grouper, owing to their estuarine-dependent life history, will be tolerant to the impacts of elevated $pCO_2$ across a range of physiological, sublethal, and lethal endpoints.

## Methods

### Lethal and sublethal impacts

All embryos were produced via common strip-spawning methods by an unknown number of captive orange-spotted grouper broodstock at the Academia Sinica, Institute of Cellular and Organismic Biology (ICOB) Marine Research Station (Jiaoxi, Taiwan), and transported to the Academia Sinica, ICOB (Taipei City, Taiwan), where all tests were initiated within 12 hpf, with hatching occurring within 24 hpf. For both development and $pCO_2$ exposure experiments, analyses were completed on a minimum of two different spawning events with different parental pairings, although broodstock size is unknown. Seawater was filter sterilized using a Millipore ExpressPlus 0.22-μm filter, and salinity was corrected with deionized water to eliminate the potential bacterial growth during testing. $pCO_2$ levels were achieved via methods outlined in Chapter 2, page 44, and Chapter 4, page 83, of Riebesell et al. (2010). Previous work in our laboratory on larval species has shown that bubbling $CO_2$ can cause high mortality, so the method of adding HCl acid and $HCO_3$ was chosen for these experiments, even though adding acid and bicarbonate does have its limitations we felt that the pros outweighed the cons (Lonthair *et al.*, 2017). Both development and $pCO_2$ exposure tests were completed in 24–1-l vacuum-sealed containers. Furthermore, animal care and experimentation were completed in accordance with IACUC-approved protocols through the University of Texas at Austin.

Water quality analysis [temperature, salinity, $pH_{NBS}$, and titratable alkalinity (TA)] was completed on the sterilized seawater to determine the necessary amount of hydrochloric acid and sodium bicarbonate needed to reach the desired $pCO_2$ level. Sterilized seawater is the same as the control water used in the associated experiments, with no additives. The three $pCO_2$ exposures included a control treatment (650 μatm), a medium $pCO_2$ level (1500 μatm), and a predicted high coastal-upwelling $pCO_2$ level (3100 μatm) (Cai *et al.*, 2011; Lonthair *et al.*, 2017). A final water sample was collected and analysed at the conclusion of the test to calculate the $pCO_2$ using the CO2SYS software package developed by Lewis and Wallace (1998) (Table 1). Calculation preferences that were used in the software package include: $CO_2$ constant—K1, K2 from Mehrbach *et al.* (1973)-refit by Dickson and Millero (1987); KHSO4—Dickson (1990); pH scale—NBS scale (mol/kg $H_2O$); total Boron—Uppstrom (1974); and air-sea flux—Wanninkhof (2014). Temperature and salinity were measured using a standard thermometer and refractometer. pH was measured with a combination of pH electrode, calibrated immediately before use (NBS scale),

**Table 1.** Mean (±1 SEM) temperature, salinity, pH, total alkalinity, and $pCO_2$ from a subset of experiments with orange-spotted grouper (*Epinephelus coioides*) embryos and early life stages

| Experiment | Treatment | Temp. (°C) | Salinity | $pH_{NBS}$ | *TA* (μmol/kg) | $pCO_2$ (μatm) |
|---|---|---|---|---|---|---|
| Survival and morphological experiments | Control ($n = 7$) | 26.8 ± 0.1 | 33.2 ± 0.2 | 8.04 ± 0.01 | 2498 ± 16 | 656 ± 18 |
| | Medium $pCO_2$ ($n = 7$) | 27.0 ± 0.1 | 33.5 ± 0.3 | 7.72 ± 0.02 | 2519 ± 85 | 1545 ± 84 |
| | High $pCO_2$ ($n = 7$) | 27.0 ± 0.1 | 33.2 ± 0.3 | 7.43 ± 0.01 | 2498 ± 23 | 3143 ± 101 |
| qPCR—24 h exposure | Control ($n = 8$) | 26.8 ± 0.2 | 33.7 ± 0.3 | 8.05 ± 0.01 | 2536 ± 21 | 692 ± 26 |
| | Medium $pCO_2$ ($n = 8$) | 26.9 ± 0.1 | 34 ± 0.4 | 7.71 ± 0.03 | 2501 ± 28 | 1778 ± 129 |
| | High $pCO_2$ ($n = 8$) | 27.0 ± 0.2 | 34 ± 0.3 | 7.39 ± 0.01 | 2520 ± 23 | 3509 ± 101 |
| qPCR—48 h exposure | Control ($n = 8$) | 26.9 ± 0.2 | 33.7 ± 0.2 | 8.04 ± 0.02 | 2463 ± 13 | 613 ± 31 |
| | Medium $pCO_2$ ($n = 8$) | 27.0 ± 0 | 33 ± 0.2 | 7.74 ± 0.04 | 2482 ± 16 | 1239 ± 120 |
| | High $pCO_2$ ($n = 8$) | 27.1 ± 0.1 | 32.4 ± 0.3 | 7.46 ± 0.02 | 2426 ± 22 | 2982 ± 105 |

attached to an Orion Star A121 pH meter (Thermo Scientific). Importantly, $pH_{NBS}$ measurements are known to be uncertain up to 0.05 $pH_{NBS}$ units for seawater measurement, which could lead to uncertainty in estimated $pCO_2$ (Riebesell et al., 2010). However, work has shown that, with careful use, these issues are minimized and that $pH_{NBS}$ is an appropriate method in biological $CO_2$ manipulation experiments measuring differences over 100 μatm (Watson *et al.*, 2017). TA was calculated via pH and total $CO_2$, which was measured using a Corning 965 $CO_2$ analyser. Water quality measures were monitored from a subset of replicates at the end of all embryonic incubation experiments, and $pCO_2$ exposures were maintained throughout experiments (Table 1).

For survival assays, 20 embryos were incubated in a 1-l vacuum-sealed container, with four replicates at each $pCO_2$ treatment per spawn (minimum of two spawns). Survival was assessed at 60 hpf. This time point was chosen because the closed containers lack available food sources, and thus, survival measurements after the transition to exogenous feeding, which occurs at ∼72 hpf, would be the result of starvation. At the end of 48 h of exposure, unhatched and dead larval fish were removed and surviving larvae were anaesthetized using a buffered MS-222 solution (250 mg $l^{-1}$) and counted. In some cases, we observed a hatch rate of zero in a control replicate; this necessitated us to remove the corresponding $CO_2$ treatments from data analysis. A hatch rate of 0 in the control replicate would indicate that embryos were of poor quality and if included may create a bias and impact the results of the $pCO_2$ treatments.

A second series of morphometric analyses were performed on the animals from the survival assay to assess total length, yolk consumption, and heart rate at 48 hpf. Each treatment consisted of four experimental replicates and three larvae were sampled per replicate. Heart rate was analysed as described by Incardona *et al.* (2014). With only one beaker at a time being measured, so that all animals were anaesthetized for the same period of time. For all images, two to three larvae were mounted on 3% methylcellulose in sea water, which allowed for rapid processing of images and videos. Replicates were processed in random order, with controls being analysed throughout the imaging process. Videos and images were randomly numbered by a third-party laboratory member to remove any potential bias during analysis. Heart rate was manually determined from video, while images were analysed for total length and relative yolk sac area using the ImageJ free software programme. Figure 1 illustrates measurement methods of total length and yolk sac area.

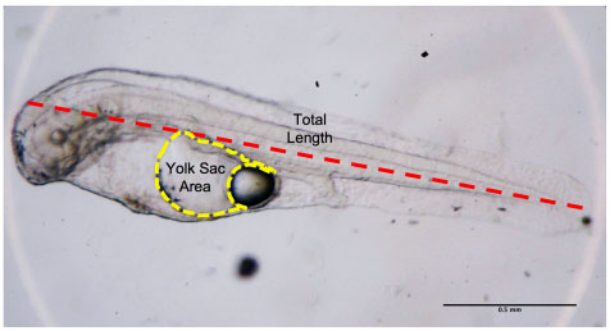


**Figure 1.** Inverted microscope image of 60 hpf orange-spotted grouper (*Epinephelus coioides*). Overlaying illustration details methods used in ImageJ to determine total length (mm) and yolk sac area ($mm^2$) at varying $pCO_2$ conditions.

**Table 2.** List of primers used for real-time PCR

| Gene | Accession # | Orientation | Sequence |
|---|---|---|---|
| *ef1-a* | #KU885470.1 | L | CTTCAACATCAAGAACGTGTCC |
| | | R | CATTAATCTGACCAGGGTGGTT |
| *nhe3* | #MN511303 | L | TATCATGGTGTTTGGAGAGTCG |
| | | R | ATTAATTTTGGGTCCTCCCAGT |
| *nbc* | #MN511305 | L | TGAACGACATTTCTGACAAACC |
| | | R | CCGAGCAAGATGAATAAAAACC |
| *vha* | #MN511304 | L | CTAAGAAGACGGCCTGTGAGTT |
| | | R | CTGGATCATCTCCTCTGGGTAG |

All sequences are 5′–3′, and reverse primers are reverse compliments of the genetic sequence.

## Acid–base regulatory plasticity

Real-time polymerase chain reaction (PCR) primers were developed for *ef1α*, *nbc*, *vha* (B subunit), and *nhe3*. Full-length sequences for *ef1α*, *nbc*, *vha*, and *nhe3* were identified from an in-house gill transcriptome. The identified sequences were then verified against the NCBI database using a standard Blast search. Primer pairs were identified using Primer3Plus software package. All primers and GenBank accession numbers for related sequences are found in Table 2.

Embryo exposures were performed as described above. A total of 20 embryos were incubated in a 1-l vacuum-sealed container, with four replicates at each $pCO_2$ treatment per spawn (minimum of two spawns). All surviving larvae were collected at 12, 36, and 60 hpf under both control conditions and after exposure to elevated $pCO_2$ levels. Samples were flash frozen and stored in −80°C, until further processing was required. Total RNA

isolation was performed using QIAzol (Qiagen) according to manufacturer's protocols and quantified using an ND-1000 spectrophotometer (Thermo Scientific). Total RNA was treated for potential DNA contamination by incubating with DNase 1 (Roche), according to manufacturer's protocols. complementary DNA (cDNA) synthesis was performed on 1 µg of total RNA using SuperScript IV reverse transcriptase (Invitrogen Live Technologies), according to manufacturer's protocols. For all cDNA synthesis runs, no reverse transcriptase controls were performed to test for genomic DNA contamination. Samples were diluted tenfold using nuclease free water and stored at −20°C until quantitative polymerase chain reaction (qPCR) analysis.

qPCR analysis was performed using the Maxima SYBR Green kit (Thermo Scientific). Reactions were prepared according to the manufacturer's protocols with the exception that a 12.5-µl total reaction volume was used. All reactions were processed using an MX3000P qPCR machine (Stratagene) with accompanying software. A serial dilution was used for standard curves to determine the reaction efficiency of each primer pair. PCR efficiencies ranged from 70% to 97.4% with an $R^2 \geq 0.97$. For all genes, negative and no reverse transcriptase control reactions were performed. The CT values for each sample were used to assess the relative abundance of each gene in relation to the control gene *ef1α* using the delta–delta CT method.

## Immunofluorescence methods

Samples were collected following the survival experiments described above and fixed overnight in Z-fix at 4°C. Samples were then washed one time with 100% methanol and then transferred to 100% methanol and stored at −20°C; this procedure constituted chill permeabilization of the sample. Samples were then washed with phosphate buffered saline (PBS) 1% triton X (PBST) four times for 5 min followed by 1 h in blocking buffer (PBST with 5% foetal calf serum) at room temperature. Blocking buffer was removed, and samples were then incubated with primary antibodies for Na+/K+ ATPase (NKA) (in 1:100 dilution in blocking buffer) at 4°C overnight on a rocker. The primary polyclonal rabbit antibody for NKA (sc-28800) was obtained from the Santa Cruz Biotechnology, and its effectiveness was verified using a western blot (Allmon and Esbaugh, 2017). Following primary incubation, samples were washed in blocking buffer three times for 5 min then incubated with secondary antibodies—goat anti-rabbit Alexa Flour 488 (1:500)—in the dark for 6 h at 4°C on a rocker. Samples were then washed with blocking buffer four times for 5 min and mounted using Vectashield hard-mount with DAPI and stored in the dark at 4°C until imaged. Imaging was completed using a Nikon C2+ confocal microscope system with a Nikon Eclipse Ti-E inverted microscope and utilizing NIS-Element imaging software for image acquisition, processing, and analysis. Images were randomly numbered to remove any potential bias during analysis. Ionocyte density was determined by creating a 0.0625-mm$^2$ box over the yolk sac area and manually counting the number of ionocytes using the ImageJ free software programme. Figure 2 illustrates the immunofluorescence ionocyte density count methods.

## Statistical methods

Survival and total length data across $pCO_2$ exposures passed the Shapiro–Wilk normality test and was assessed using a one-way analysis of variance (ANOVA). Heart rate across $pCO_2$ exposures was assessed via a Kruskal–Wallis one-way ANOVA on ranks due to failure of the Shapiro–Wilk normality test. Gene expression data across development passed the Shapiro–Wilk normality test after natural log transformation and was assessed using a one-way ANOVA and Holm–Sidak *post hoc* test against control values. Gene expression data across $pCO_2$ exposures passed the Shapiro–Wilk normality test after natural log transformation and was assessed using a one-way ANOVA. Ionocyte density across $pCO_2$ exposures passed the Shapiro–Wilk normality test after natural log transformation and assessed using a one-way ANOVA. All statistical measurements were completed using SigmaPlot (Systat Software, San Jose, CA, USA).

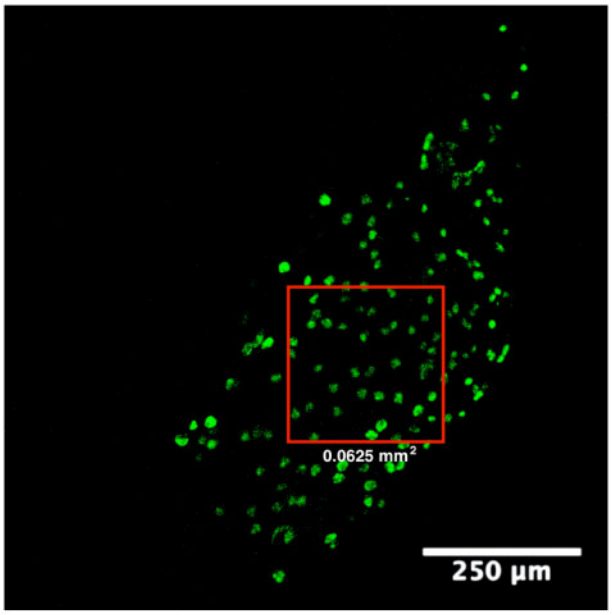


**Figure 2.** Confocal microscopy image of ionocyte density using an antibody for NKA in 60 hpf orange-spotted grouper (*Epinephelus coioides*). The 0.0625 mm$^2$ illustrates methods used to complete counts of number of ionocytes at varying $pCO_2$ conditions.

# Results

## Sensitivity experiments

### *Lethal and sublethal impacts*

Survival was not impacted by 48 h exposure to increased $pCO_2$ levels (Figure 3). Heart rate was significantly elevated by 48 h exposure to elevated $pCO_2$ levels at both 1500 and 3100 µatm ($p \leq 0.05$; Kruskal–Wallis ANOVA on ranks) (Figure 4a). The control heart rate in mean ± standard error of mean (SEM) was 134 ± 14 beats per minute (bpm) at 600 µatm, the 1500 µatm heart rate was 174 ± 10 bpm with, while the 3100 µatm heart rate was 190 ± 7 bpm, an increase of 40 bpm (30% increase) and 56 bpm (42% increase), respectively. No effects were observed in response to 48 h exposure to elevated $pCO_2$ levels in both total length (Figure 4b) and yolk size (Figure 4c).

### *Acid–base regulatory pathway plasticity*

Gene expression for *nbc* and *vha* exhibited significant up-regulation as a result of development at 60 h post-fertilization when compared with 12 h post-fertilization ($p \leq 0.05$; ANOVA) (Figure 5a and c). In contrast, *nhe3* exhibited no alterations in

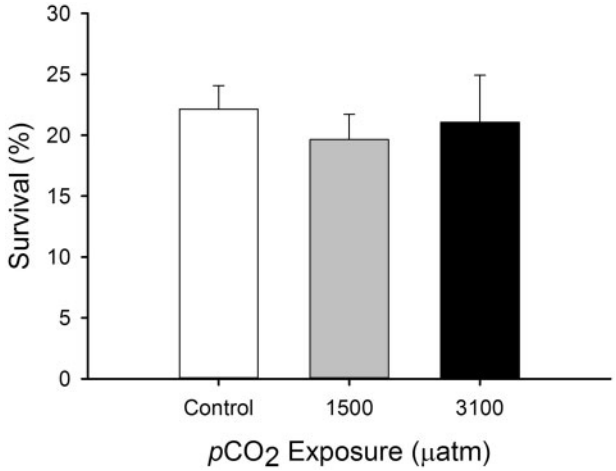


**Figure 3.** Mean (±SEM) survival of orange-spotted grouper (*Epinephelus coioides*) after 48 h exposure to control, 1500 µatm, and 3100 µatm $pCO_2$. There were no significant differences between groups (ANOVA; $N = 7$ per treatment).

relative messenger RNA (mRNA gene expression as development progressed (Figure 5b). Interestingly, at two time points, 24 and 48 h, elevated $pCO_2$ exposure at both 1500 and 3100 µatm had no effect on a variety of the transporters that are thought to play a critical role in acid–base regulation in marine teleosts, including *nbc*, *nhe3*, and *vha* (Figure 6). Furthermore, ionocyte density was not altered as a result of exposure to increased levels of $pCO_2$ (Figure 7).

## Discussion

Determining species resilience is a crucial aspect of understanding the impacts that OA and other climate change-induced environmental changes will have on future marine ecosystems, specifically within the discussion of evolutionary rescue. Evolutionary rescue is described as when genetic adaptation allows for a population to recover from deteriorating population effects, which were initiated by environmental change and would have otherwise caused a local extinction (Gonzalez *et al.*, 2013). An important facet of evolutionary rescue is that a subset of individuals are resilient to the changing environment and have the appropriate phenotypic solutions. Thus, identifying species and ecosystems with tolerant traits that can defend against the physiological stresses of environmental degradation is critical. Here, we provide evidence that a fast growing and economically important estuarine-dependent species, the orange-spotted grouper, exhibits no clear detrimental effects of OA when exposed during the most sensitive early life stages. These findings are consistent with the hypothesis that fish endemic to coastal and upwelling regions that commonly experience elevated $CO_2$ may have a level of intrinsic resilience (Baumann, 2019) and corroborates a case study on highly $CO_2$-sensitive offshore fish species (Murray *et al.*, 2019). It should be noted that the influence of parental $CO_2$ exposure on the $CO_2$ tolerance observed here in orange-spotted grouper is unknown as brood stock rearing conditions were not monitored. Prior work has shown that high $CO_2$ conditions may contribute to $CO_2$ tolerance in embryos in some species (Snyder *et al.*, 2018).

Early work on the impacts of OA on early life stage fish has emphasized survival as a critical endpoint, because larval survival and recruitment represents a crucial population bottleneck.

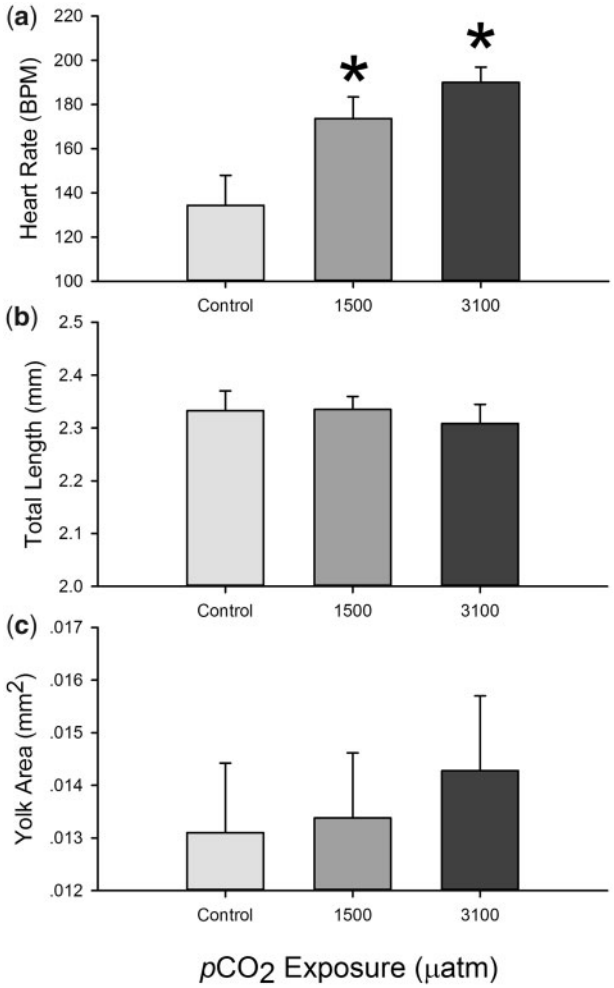


**Figure 4.** Mean (±SEM) (a) heart rate, (b) total length, and (c) yolk area of orange-spotted grouper (*Epinephelus coioides*) after 48 h exposure to control, 1500 µatm, and 3100 µatm $pCO_2$. Asterisk indicated statistically significant difference from control (ANOVA; $p < 0.05$; $N = 19$–21 larvae per treatment).

Baumann *et al.* (2012) is a foundational study in the field, which found a 73% reduction in the survival of *Menidia beryllina* at elevated $pCO_2$. More recent studies have found that other species exhibit reduced survival but none with such extreme sensitivity as *M. beryllina*, which likely indicates that *M. beryllina* is an outlier (Miller *et al.*, 2012; Chambers *et al.*, 2014; Lonthair *et al.*, 2017). Conversely, other studies have shown that a number of species exhibit tolerance to high $pCO_2$ (Esbaugh, 2018; Baumann, 2019) Furthermore, while a significant portion of the populations is unable to tolerate OA, there are tolerant individuals present in all species tested (Esbaugh, 2018).

While larval survival is easy to interpret and has clear population level outcomes, it is also important to consider sublethal effects of OA that may indicate a poor prognosis for fish in later life. Following on our hypothesis that OA may place an additional energetic burden on endogenous feeding life stages, we sought to use two morphological traits that may indicate changing energy burdens: size at age and yolk sac area. Interestingly, there was no effect of elevated $CO_2$ on either endpoint, which argues against our hypothesis and supports the premise that this species may have a level

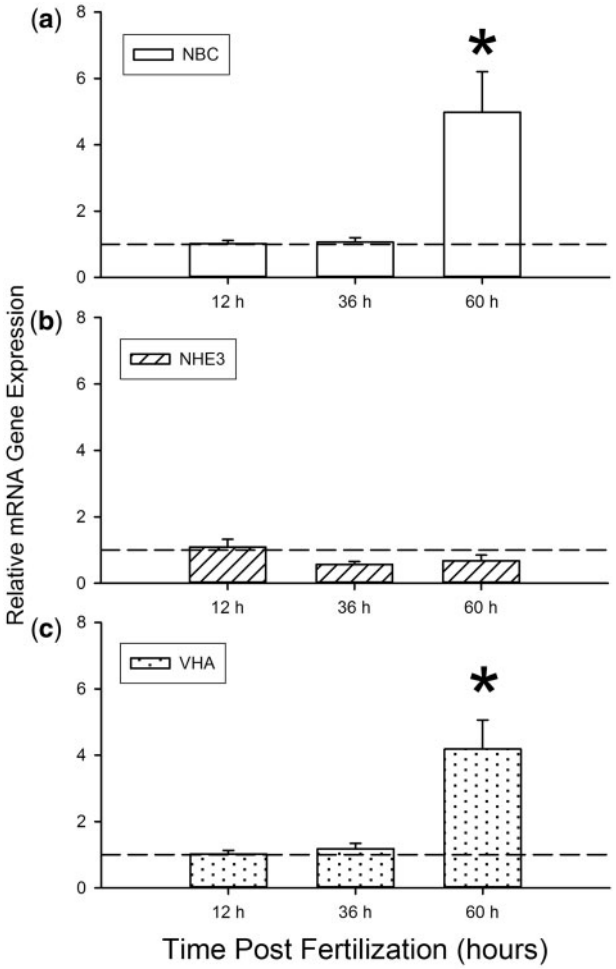


**Figure 5.** Whole animal gene expression of $H^+$ excretion pathways: (a) *nbc*, (b) *nhe3*, and (c) *vha*, during development in orange-spotted grouper (*Epinephelus coioides*). All values are relative to housekeeping gene *ef1a*. Values set relative to control values denoted by dashed lines at 1.0. Significant differences from initial time point (12 h) denoted by an asterisk (ANOVA; $p < 0.05$; $N = 8$ per time point). All values are mean ± SEM survival.

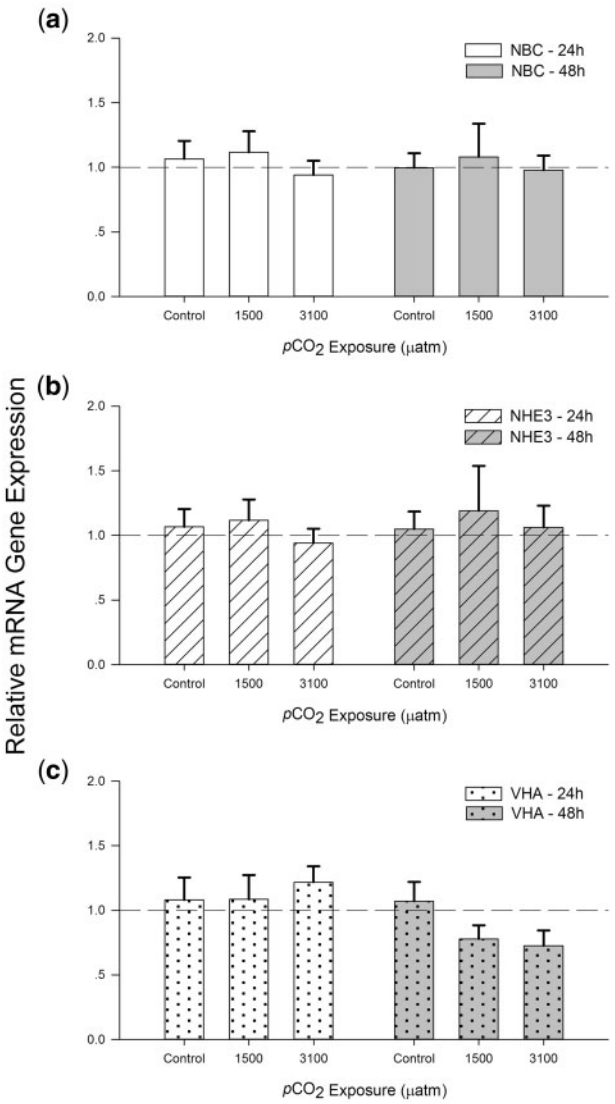


**Figure 6.** Whole animal gene expression of $H^+$ excretion pathways: (a) *nbc*, (b) *nhe3*, and (c) *vha*, during 24 and 48 h exposure to control, 1500 µatm, and 3100 µatm in orange-spotted grouper (*Epinephelus coioides*). All values are relative to housekeeping gene *ef1a*. Values set relative to control values denoted by dashed lines at 1.0. There were no significant differences between groups (ANOVA; $N = 8$ per treatment). All values are mean ± SEM survival.

of intrinsic resilience. Previous work has highlighted that the impacts of OA on embryonic and larval growth can be variable depending on the model species. Some studies have found species exhibiting detrimental decreases in growth (Miller *et al.*, 2012; Frommel *et al.*, 2016), no effects on growth (Bignami *et al.*, 2013; Frommel *et al.*, 2013), and even increases in growth and size at age (Bignami *et al.*, 2014; Chambers *et al.*, 2014). This indicates that energy utilization may vary considerably between species when exposed to elevated $pCO_2$, with some species allocating a larger portion of energy to maintaining growth.

Plasticity in the acid–base regulatory machinery is a critical metric to understand resilience of OA in a teleost species. Our study indicates that orange-spotted grouper maintains acid–base regulatory transporters in a high enough abundance to correct for OA without a transcriptional change. We saw no alterations in gene expression in any of the acid–base regulatory transporters that we measured, including: *nhe3*, *vha*, and *nbc*. Other studies have found similar results via quick stabilization of net whole body titratable acid flux following $pCO_2$ exposure (Edwards *et al.*, 2005; Allmon and Esbaugh, 2017) and via the lack of plasticity in acid–base transport gene expression, enzyme activity, and protein abundance (Esbaugh *et al.*, 2012; Michael *et al.*, 2016; Allmon and Esbaugh, 2017). Furthermore, when measuring ionocyte density as a result of exposure to elevated $pCO_2$, we found no significant changes. This further supports the argument that the orange-spotted grouper maintain high enough levels of acid–base regulatory machinery at even the earliest life stages.

In contrast to the previously discussed data, the evidence provided from heart rate is consistent with the hypothesis that OA will result in elevated energetic costs of survival in orange-spotted grouper. Our data demonstrate that heart rate increases by ~40% when exposed to elevated $CO_2$ (3100 µatm). This trend is consistent with prior work (Lonthair *et al.* 2017); however, the

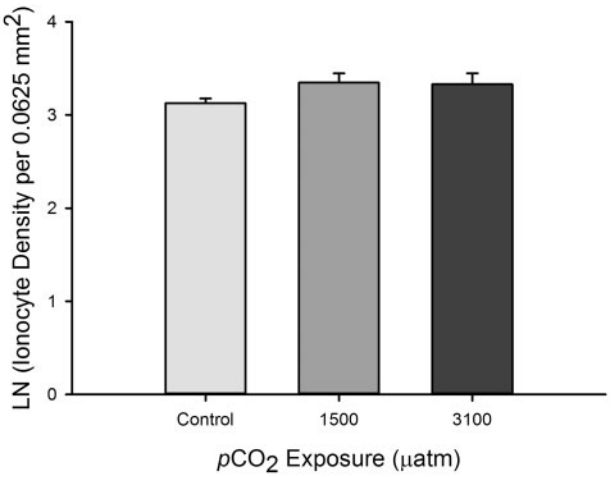


**Figure 7.** Natural log of the mean ionocyte density per 0.0625 $mm^2$ area (±1 SEM) after 48 h exposure to control, 1500 µatm, and 3100 µatm $pCO_2$ in orange-spotted grouper (*Epinephelus coioides*). There were no significant differences between $pCO_2$ (ANOVA; $N = 8–12$ per treatment).

magnitude of the response in grouper is much greater than previously described for other species. The significance of this finding is rooted in two physiological concepts. The first is that heart rate is an effective proxy for metabolic rate, whereby higher heart rates indicate greater energy utilization (Green, 2011). The second is that larval fishes generally have limited metabolic scope (Killen *et al.*, 2007), which is the difference between the baseline costs of living and the maximum capacity of the system. This would suggest that the energetic costs of increased heart rate will remove available energy from other functions, such as future growth or activity. The significance of such metabolic reallocation is highlighted in Pan *et al.* (2015), which showed that sea urchin larvae expend increased energy on protein synthesis and ion transport under OA conditions, despite no evidence of OA induced effects on size or gene expression (Pan *et al.*, 2015). Our data may suggest that grouper are undergoing similar metabolic reallocations to maintain the growth rate in the early life stage despite higher metabolic rates. While this would presumably aid the fish in reducing early life predation risk, the long-term cost of such metabolic reallocation is unknown.

It is also important to consider the physiologically advantages of elevated heart rate to early life stage orange-spotted grouper. Adult teleosts control convective fluid movement across and through their respiratory epithelium in response to respiratory stress, such as reduced oxygen or elevated $CO_2$ [see review in Gilmour and Perry (2007)]. In fact, OA relevant $pCO_2$ exposures resulted in significant elevation in ventilatory parameters, which significantly reduces the magnitude of the metabolic compensation response in juvenile life stages (Ern and Esbaugh, 2016). Yet as such a benefit would seem unlikely in the current study given that early life fishes use cutaneous gas exchange with little role for convective fluid movement (Rombough, 2002; Rombough, 2007; Fu *et al.*, 2010). It instead seems likely that the increased heart rate is a by-product of the developing sensory system related to cardiorespiratory control (Vulesevic and Perry, 2006; Miller *et al.*, 2014).

In conclusion, our study has shown that the estuarine-dependent orange-spotted grouper show no clear detrimental effects of OA exposure in the most sensitive life stage. This is consistent with the hypothesis that species that have evolved in habitats with natural fluctuations in $pCO_2$ may have intrinsic resilience to the impact of OA. While these results are encouraging for the long-term prospects of orange-spotted grouper, it is important to recognize that our conclusions are limited to the early life stages as we were unable to complete extended grow-out studies due to the severe drop in survival that occurs at first feeding. Furthermore, we cannot inform on the potential behavioural effects of OA that may occur in later life stages, nor the potential implication of elevated temperature and reduced oxygen as additive stressors, both of which can exacerbate concerns regarding the baseline energetic cost of living in the future oceans.

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