

THE COMBINED EFFECTS OF CO<sub>2</sub>-INDUCED ACIDIFICATION AND WARMING ON  
BEHAVIORAL LATERALIZATION IN JAPANESE RICEFISH (*ORYZIAS LATIPES*)

by

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### DEDICATIONS

I dedicate this to mother Earth, and its Creator, who saw that “it was very good.”

### ACKNOWLEDGMENTS

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## ABSTRACT

Future projections from the Intergovernmental Panel on Climate Change Report forecast an increase in anthropogenic atmospheric CO<sub>2</sub> emissions, thereby exacerbating ocean acidification. Studies on CO<sub>2</sub>-induced acidification report evidence of its deleterious effect on behavioral alterations in marine fish species. One such disturbance affects behavioral lateralization, a function of brain asymmetry and a critical component to schooling performance. Though research has divulged the adverse behavioral effects of ocean acidification on stenohaline marine fishes, euryhaline models have not been explored to such extent. This study explored the combined effects of projected levels of pCO<sub>2</sub> (~1300 ppm) and warming (+3.0°C) on behavioral lateralization in a euryhaline teleost, the adult Japanese ricefish (*Oryzias latipes*). After just five days of the treatment, CO<sub>2</sub>-treated fish exhibited significantly lower individual-level lateralization indices than that of fish in control CO<sub>2</sub> (~300 ppm) and temperature, as well as no statistical difference to that of a random simulation. The implications are far-reaching even for highly efficient osmoregulatory fishes, in that coordination and schooling performance may be hampered at end-of-century conditions, thereby reducing fish population fitness.

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## CHAPTER 1: INTRODUCTION

Future projections from the Intergovernmental Panel on Climate Change (IPCC, 2014) Report forecast an increase in anthropogenic atmospheric CO<sub>2</sub> emissions, which will further exacerbate ocean acidification (IPCC, 2014). As excess CO<sub>2</sub> acidifies the ocean chemistry, marine fish populations have been hampered due to changes in predator-prey interactions and habitat and resource availability, which leads to modified biodiversity and interspecific interactions (Nagelkerken *et al.*, 2016). Stunted effects of CO<sub>2</sub>-induced acidification on fish development and survival have also been observed, as aggravated organ degradation and reduced larval growth in herring larvae were observed at projected *p*CO<sub>2</sub> levels (Frommel *et al.*, 2014); mortality rates of Atlantic cod were doubled at end-of-century CO<sub>2</sub> levels (Stiasny *et al.*, 2016); even in estuarial and anadromous species, CO<sub>2</sub>-induced acidification led to a significant decline in survival and larval growth rates, as well as embryonic growth (Baumann *et al.*, 2012; Ou *et al.*, 2015). In freshwater development, pink salmon experienced reduced yolk sac absorption, production efficiencies, fork length reduction, and anxiety in CO<sub>2</sub> conditions ranging from 1600-2000  $\mu$ atm (Ou *et al.*, 2015).

**Mechanism of High CO<sub>2</sub>**

As much a concern is the effect of high-CO<sub>2</sub> levels on behavioral change in marine fish, which is one of the underlying factors behind species survival and function, as well as interspecific relationships; these and other environmental factors may lead to shifts in population dynamics (Nagelkerken *et al.*, 2016). Sensory functions such as olfaction, visual and auditory performance, and behavioral lateralization all have been targets of manipulation induced by elevated CO<sub>2</sub> (Munday *et al.*, 2009; Domenici *et al.*, 2014; Nilsson *et al.*, 2012; Chung *et al.*,

2014; Simpson *et al.*, 2011). The same studies exploring these sensory functions have divulged on the role of the primary inhibitory neurotransmitter receptor in vertebrates,  $\gamma$ -Aminobutyric acid-A (GABA<sub>A</sub>), in altering physiological pathways in marine fish. The research conducted by Nilsson *et al.* (2012) proposed the mechanism: excess CO<sub>2</sub> induces manipulation of HCO<sub>3</sub><sup>-</sup> and Cl<sup>-</sup> ion gradients, thus altering GABA<sub>A</sub> receptor function. In normal CO<sub>2</sub>-level environments, fish open their GABA receptors, leading to an inflow of Cl<sup>-</sup> ions which triggers hyperpolarization and an inhibition of neural activity. However, in high-CO<sub>2</sub> water, an accumulation of HCO<sub>3</sub><sup>-</sup> ions activates acid-base ion regulation, which triggers an outflow of Cl<sup>-</sup> and HCO<sub>3</sub><sup>-</sup>, inducing instead depolarization and an activation of GABA<sub>A</sub> receptors leading to excitation of neural activity. This change of ion regulation results in alteration of neural responses eventually expressed in behavioral and sensory changes. The study used juvenile clownfish and damselfish to study carbon dioxide's impact on olfactory and lateralization responses, respectively, subjecting them to normal CO<sub>2</sub> (~450 ppm) and high CO<sub>2</sub> conditions (~900 ppm). A severe impairment of behavioral responses was observed in fish subjected in high CO<sub>2</sub> treatments. However, when both treatments were later placed in water containing gabazine, a highly specific antagonist of the GABA<sub>A</sub> receptor known to reverse its function, there was observed a significant reversal of behavior in only the high CO<sub>2</sub>-treatments' response. This highly suggests that elevated CO<sub>2</sub> manipulates GABA<sub>A</sub> receptor function.

Another study (Lai *et al.*, 2015) explored the effects of high-CO<sub>2</sub> on the GABA<sub>A</sub>-receptor function of a temperate marine fish species (three-spined stickleback, *Gasterosteus aculeatus*). This extended the research conducted by Nilsson *et. al* (2012) which focused only on tropical coral reef fish species (2012). The experimental setup mirrored the study that Nilsson *et. al* underwent, using damselfish as the subject to test for lateralization alterations due to CO<sub>2</sub>

exposure (2012). Two treatments of high-CO<sub>2</sub> and control water tanks were used for the stickleback and later subjected to subsequent tests to analyze for behavioral asymmetry. Similar results to the Nilsson *et. al* (2012) experiment were collected, as significant lateralization was observed in the control treatment, but almost a complete loss in that of high-CO<sub>2</sub>. Lateralization alterations were sustained even after 50 days in the tanks, after which a gabazine treatment (a receptor antagonist to GABA<sub>A</sub>) was induced. The observed capacity of a gabazine treatment, a receptor antagonist specific to GABA<sub>A</sub> receptors, reverting behavioral alterations in marine fishes supports GABA<sub>A</sub> receptor function as the underlying cause of CO<sub>2</sub>-causing behavioral disturbances.

Elevated temperatures are coupled with projections of exacerbated CO<sub>2</sub>-induced acidification. According to one Representative Concentration Pathway prediction (RCP8.5), ocean surface temperatures are expected to increase by as much as 3.7°C and global surface ocean pH to decrease near 0.31 units, near 7.75, by the end of the century (IPCC, 2014). Under the combined effect of both low pH and high temperatures, reef fish have exhibited loss of behavioral lateralization (Domenici *et al.*, 2014), which is a powerful indicator of brain function in response to external stimuli (Domenici *et al.*, 2011). In the same study, the sole effect of a 2.9°C increase led to a lower relative lateralization (L<sub>R</sub>) value (L<sub>R</sub> = 9.5±4.43) for damselfish than the control temperature (L<sub>R</sub> = 26.9±4.04); however, coupling both elevated CO<sub>2</sub> and temperature, Domenici *et al.* found the L<sub>R</sub> value (L<sub>R</sub> = -5.8±5.96) to have insignificant directional bias (2014).

### **Behavioral lateralization**

Lateralization of the brain, or asymmetry of the nervous system, confers behavioral biases at the motor, perceptual, and functional level (Frasnelli, 2013). It was primitively believed that humans were only capable of such a function. However, most vertebrates exhibit lateralization, and even among invertebrates (Frasnelli, 2013). That such a neurological feature is ubiquitous among numerous taxa suggests that animals with such capabilities are advantageous over those that do not. Thus, lateralization is vital to biological fitness (Frasnelli, 2013). Studying lateralization in fishes demands importance, as it has been observed across a wide range of fish species (Bisazza *et al.*, 2000) and is linked to better coordination and school cohesion (Bisazza & Dadda, 2005), as well as social behavior (Bisazza *et al.*, 2000).

### **Objectives**

Thus, in the face of ocean warming and acidification worsening, and its behavior-altering impact on fishes, we are exploring the effects of projected temperature and CO<sub>2</sub>-induced pH levels on behavioral lateralization of a euryhaline teleost, *Oryzias latipes*. This species of ricefish has intermediate adaptability to seawater (Miyanishi *et al.*, 2016) due in part to its genetic hyperosmotic tolerance (Myosho *et al.*, 2018) and epigenetic hypoosmoregulatory ability (Miyanishi *et al.*, 2016). Since elevated *p*CO<sub>2</sub> has been known to manipulate osmoregulation in a wide range of fish species (Kreiss *et al.*, 2015), the unique osmoregulatory faculties in *Oryzias latipes* may lead to a different response to ocean acidification compared with stenohaline teleosts.

## **CHAPTER 2: METHODS**

### **Organism**

This research will study the medaka, or Japanese ricefish, *Oryzias latipes*, var. “Youkihi,” a hardy euryhaline species that can adapt to both freshwater and brackish environments. This teleost species was shipped from an aquarium company (Aquatic Arts, Indianapolis, IN) in two schools of 13 fish. A mortality of five fish reduced the sample size to 21.

### **Seawater preparation**

Seawater to ricefish were acclimated and prepared by manually mixing 70g of sea salt (Instant Ocean® Aquarium Salt) with 2 L of reverse-osmosis-purified water (LiquaGen Water Technology, Huntington Beach, CA) in 2000 mL beakers; two 32 L tanks for both control and CO<sub>2</sub> treatments were set up.

### **Animal Care & Maintenance**

The Japanese ricefish were shipped in freshwater and acclimated to seawater tanks over 2 weeks, transferring the schools from diluted seawater 20 ppt to full-strength seawater of 35 ppt salinity, as recommended for medaka seawater adaptation (Miyanishi *et al.*, 2016). Aquarium power filters (Tetra® Whisper) continuously circulated seawater through filter bags. Air pumps connected to airstones via tubing provided an oxygen saturation above 93%. Brine shrimp flakes (O.S.O ®) were fed sufficiently twice a day to each tank.

### **Monitored Parameters**

Several parameters besides pH and water temperature, namely salinity, alkalinity, and dissolved oxygen saturation (DO), were continually monitored and measured in both treatments to ensure healthy upkeep of the environment (**Figure 1**). Salinity was measured by a salinity checker (TekcoPlus®) and kept on average between ~37-39 ppt in both treatments. Alkalinity was computed using an accurate handheld alkalinity colorimeter (Hanna® Instruments); the

parameters fluctuated considerably across control and CO<sub>2</sub> treatments: a difference greater than 87 ppm CaCO<sub>3</sub> was observed. DO was measured using an optical DO probe (Vernier Software & Technology, LLC) to check for healthy, oxygen-saturated water. DO was maintained close to full saturation (~92-94%; **Figure 1**), as has been recommended for small-bodied fish such as zebrafish (Lawrence, 2007). Salinity and alkalinity measurements were also used to calculate *p*CO<sub>2</sub> using CO2calc software (Robbins *et al.*, 2010).

| CONTROL            | PARAMETERS                          | ELEVATED CO <sub>2</sub> |
|--------------------|-------------------------------------|--------------------------|
| 11                 | Sample size (n)                     | 10                       |
| 26.05°C (±0.09)    | Temperature (°C)                    | 29.05°C (±0.07)          |
| 295.32 ppm (±46.9) | <i>p</i> CO <sub>2</sub> (ppm)      | 1325.91* ppm (±151.87)   |
| 8.267 (±0.03)      | pH <sub>NBS</sub>                   | 7.844 (±0.03)            |
| 36.95 ppt (±0.32)  | Salinity (ppt)                      | 39.21 ppt (±0.60)        |
| 213.0 ppm (±3.46)  | Alkalinity (ppm CaCO <sub>3</sub> ) | >300 ppm (±0)            |
| 92.694 % (±1.90)   | Dissolved Oxygen (%)                | 94.125 % (±2.57)         |

**Figure 1. Seawater parameters in control and CO<sub>2</sub> treatments.** Parameter (mean ± SEM) values were collected over the duration of the treatment (five days). Temperature, pH, salinity, alkalinity, and dissolved oxygen were measure directly. The *p*CO<sub>2</sub> treatments were calculated using CO2calc. \*Because alkalinity values were rendered above 300 ppm beyond the measuring range, *p*CO<sub>2</sub> values are greater than presented.

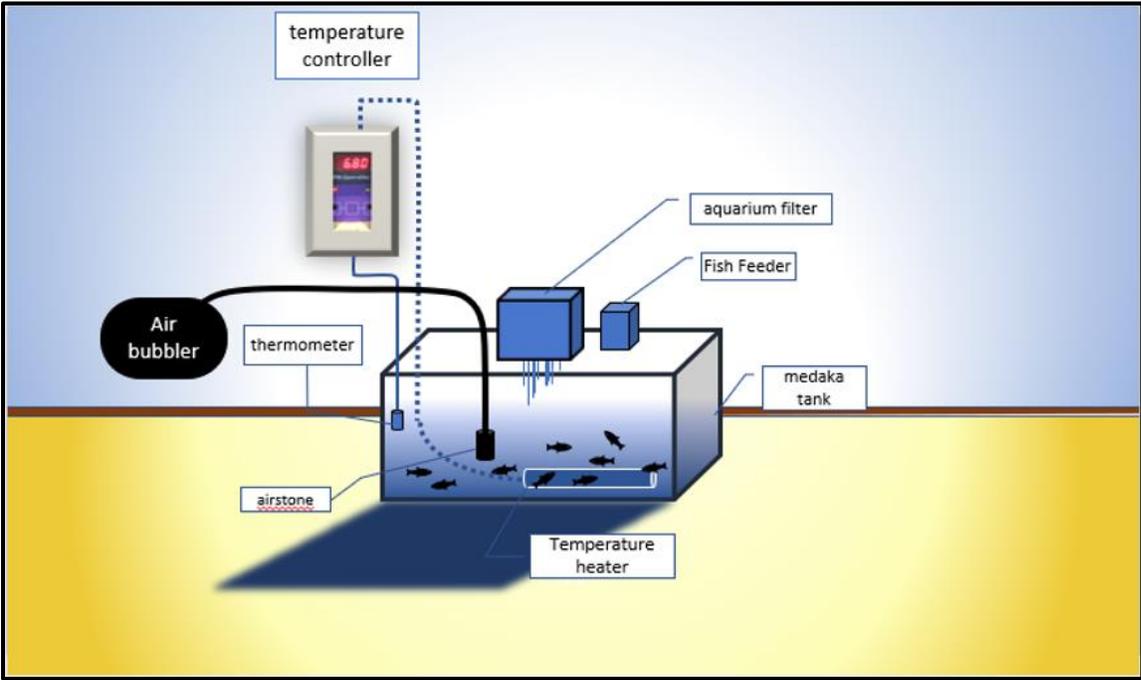
### Control and CO<sub>2</sub> Treatments

Two 32L tanks were designated the control and CO<sub>2</sub> treatment. The manipulated tank achieved a low pH at ~7.8, roughly 0.4 units lower than the controlled pH at ~8.3. The pH in the CO<sub>2</sub> treatment was monitored by dissolved CO<sub>2</sub> diffusion connected to a solenoid which controlled the flow of pure CO<sub>2</sub> bubbling from a CO<sub>2</sub> cylinder tank (**Figure 4**). The power source solenoid was moderated by a pH controller (WEIPRO®) which kept pH value within range via a pH

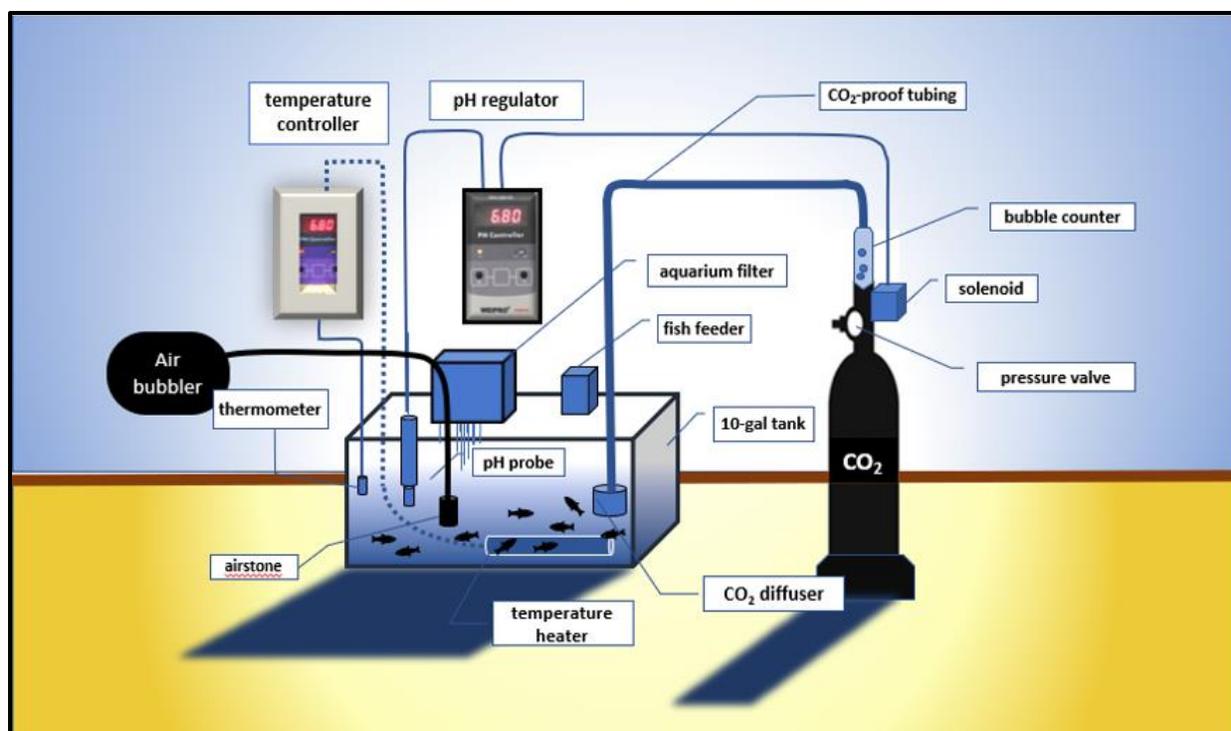
probe continuously kept within seawater. Control pH values were kept relatively consistent to 8.3 without need of moderated CO<sub>2</sub> diffusion (**Figure 3**). Temperatures were monitored by temperature controllers (Finnex®) connected to thermometers and heaters in both tanks; values differed by 3°C (26°C for control and 29°C for CO<sub>2</sub> treatment). After data were collected for pH, temperature, alkalinity, and salinity, measurements were inputted in CO2calc software (Robbins *et al.*, 2010), an accurate alternative of CO2SYS (Pierrot *et al.*, 2006), using Mehrbach *et al.* (1973) CO<sub>2</sub> constants refit by Dickson and Millero (1987), KHSO<sub>4</sub> constants from Dickson (1990) and total Boron constants from Uppstrom (1974). The mean control pCO<sub>2</sub> value (~300 ppm) is around 100 ppm from the current partial pressure of CO<sub>2</sub> in oceans which measures ~400 ppm (Baumann *et al.*, 2012). The ~1300 ppm pCO<sub>2</sub> from the elevated CO<sub>2</sub> treatment was kept consistent with the range of predicted surface ocean pCO<sub>2</sub> levels of 851- 1370 ppm at the end of the century (IPCC, 2014). Thus, the temperature differences, pH and CO<sub>2</sub> values in both treatments attempt to simulate both the current and predicted (IPCC, 2014) scenarios in 2100. The fish were exposed to the treatment for 5 days; a study carried out by Munday *et al.*, (2010) revealed that clownfish larvae within an 850 ppm CO<sub>2</sub> treatment revealed altered behavior within just 2 days.

| Sample Results  |   |
|---|---|
| Input Conditions  | Computed Constants  |
| <b>Physical Parameters</b><br>Salinity: 39.800<br>Temperature (°C): 29.000<br>Pressure (dbars): 0.000<br>Density (kg m <sup>-3</sup> ): 1025.676  | <b>Carbonate</b><br>TA (μmol/kgSW): 3000.000<br>TCO <sub>2</sub> (μmol/kgSW): 2782.930<br>pH (chosen scale): 7.850<br>fCO <sub>2</sub> water (μatm): 1264.392<br>pCO <sub>2</sub> water (μatm): 1268.246<br>CO <sub>3</sub> (μmol/kgSW): 181.773  |
| <b>Nutrient Data</b><br>Total P (μmol/kgSW): 0.000<br>Total Si (μmol/kgSW): 0.000   | <b>Calcium</b><br>Calculated (μmol/kgSW): 11695.025<br>User Value:  |
| <b>Air-Sea CO<sub>2</sub> Flux</b><br>Flux (mmol/m <sup>2</sup> /d):  | <b>Auxiliary Results</b><br>HCO <sub>3</sub> (μmol/kgSW): 2569.361<br>CO <sub>2</sub> (μmol/kgSW): 31.797<br>B Alk (μmol/kgSW): 62.375<br>OH (μmol/kgSW): 4.733<br>P Alk (μmol/kgSW): 0.000<br>Si Alk (μmol/kgSW): 0.000<br>Revelle: 12.687<br>xCO <sub>2</sub> (dry @ 1 atm) (ppm): 1319.263 |
| <b>Calculation Preferences</b><br>CO <sub>2</sub> Constant: K1, K2 from Mehrbach et al., 1973 refit by Dickson and Millero, 1987<br>pH Scale: NBS scale (mol/ka-H2O)      KHSO <sub>4</sub> : Dickson, 1990 |   |

**Figure 2. Sample interface of CO<sub>2</sub>calc computation.** A reading taken from the fifth day of treatment (March 27, 2019). Of the physical parameters, only salinity (ppt), temperature (°C), and pressure (0 dbars) was required. Of the carbonate values, total alkalinity (TA) (μmol/kg SW) and pH (NBS scale) were factored.



**Figure 3. Control treatment setup.** A graphic of the control treatment. A 32 L tank contained 11 medaka, replete with an automatic fish feeder (fed twice a day), continuous aquarium filter, air bubbler diffusing oxygenated air, and a temperature controller regulating via a thermometer and heater.



**Figure 4. CO<sub>2</sub> treatment setup.** A graphic of the CO<sub>2</sub> treatment. All equipment from the control setup (Figure 3) were identical to this setup. A pH regulator was connected to a pH probe which detected pH; values were kept within 0.1 units by turning on or off the solenoid that allowed for CO<sub>2</sub> bubbling from the CO<sub>2</sub> cylinder. A bubble counter indicated the CO<sub>2</sub> flow rate through the tubing, which contained a CO<sub>2</sub> diffuser that atomized the pure CO<sub>2</sub> gas.

### Test for Lateralization

A confirmed method for assessing left-right behavioral lateralization is the detour test. Numerous studies have implemented the double T-chamber, or T-maze runway, to conduct the detour test for left-right lateralization (Bisazza *et al.*, 1998; Bisazza *et al.*, 2000; Domenici *et al.*, 2011; Domenici *et al.*, 2014; Jutfelt *et al.*, 2013). The constructed runway closely mirrored the double T-chamber in Domenici *et al.* (2014) with a few disparities in dimensions (**Figure 5**). The adult ricefish were introduced to one end of the chamber for a period to allow for acclimation. The water was ~4 cm deep. After, the fish would be gently prodded with a fishing net and ladle into the 4-cm-wide runway. At the end of the channel, a full right or left turn at the 15-cm wide barrier would be recorded by observation. Ten consecutive runs were conducted for each fish of

both treatments; each of the two sides of the tank were alternately used to account for any asymmetry of the tank, as conducted by Domenici *et al.* (2014).

### Calculating Relative and Absolute Lateralization

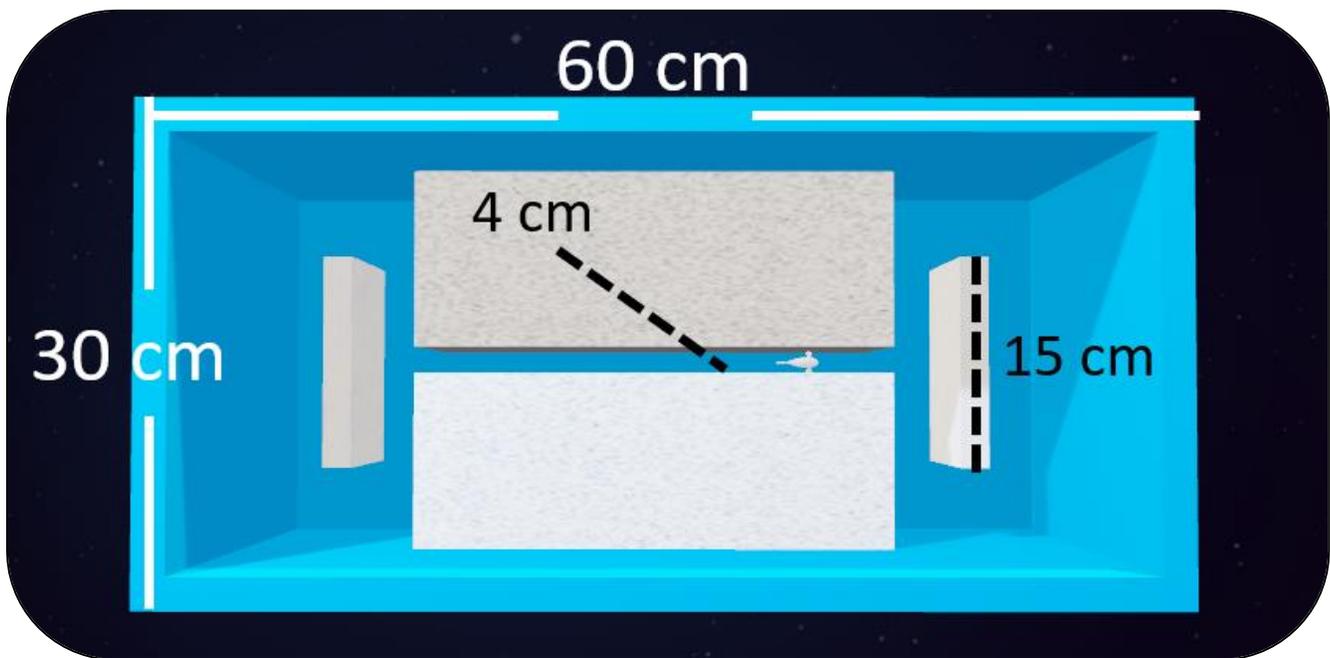
After 10 runs were observed for all control and CO<sub>2</sub>-treated fish (n = 21), the relative lateralization index (L<sub>R</sub>) was computed for each specimen to give the mean L<sub>R</sub> for each treatment. The formula for relative lateralization index is given as:

$$\frac{R - L}{(R + L)} * 100 = L_R$$

The *R* stands for the amount of right turns, while *L* the left turns, out of the total 10 turns (*R* + *L*) each individual made (Domenici *et al.*, 2014). L<sub>R</sub> was used to compute for population-level lateralization, which is indicative of a collective preference for the left or right turn (Domenici *et al.*, 2014). Negative indices would indicate a population-level preference for left turning, and positive for right turning. For individual-level lateralization, which assesses the degree of preference of each specimen regardless of right or left direction, the absolute lateralization index (L<sub>A</sub>) was implemented using this formula:

$$\frac{|R - L|}{(R + L)} * 100 = L_A$$

The absolute value of left subtracted from right turns dismisses specific right- or left-turn bias, indicating directional bias at the individual level. Thus, an L<sub>A</sub> of 100 would indicate maximum left-right lateralization (turn right or left all ten times), whereas an L<sub>A</sub> of 0 would suggest no bias (five right and five left turns).



**Figure 5. Three-dimensional graphic of double T-chamber.** A drawn-to-scale top view of the 30 x 60 cm tank. The 15-cm-wide barriers and side partitions consisted of polystyrene foam secured to the glass tank with hot glue.

### Data Analyses

Along with the control and CO<sub>2</sub> treatment groups, a random simulation (RS) of 10 turns was generated on an online random number generator (Haahr, 2019) to compare L<sub>A</sub> and L<sub>R</sub> results of both treatments to that of the RS. Such a comparison will indicate whether turning preferences are any different from random expectation (Domenici *et al.*, 2011). For comparing the L<sub>R</sub> of the control and CO<sub>2</sub> group to the RS, two-tailed one-sample *t*-tests were used. A one-way ANOVA (with CO<sub>2</sub> and temperature as the combined factor) and a Tukey's post-hoc test was applied for comparison of these three groups' L<sub>R</sub> values. The same ANOVA and post-hoc tests were conducted for L<sub>A</sub> values. Levene's tests were analyzed for homogeneity of variances. All statistical tests were performed using SPSS.

## CHAPTER 3: RESULTS

ANOVA and post-hoc tests for absolute lateralization revealed that  $L_A$  values were affected by the combined effect of elevated  $CO_2$  and temperature from the  $CO_2$  treatment ( $F_{1,29} = 5.05$ ,  $p < 0.05$ ) (**Figure 7a**). Mean  $L_A$  values ( $\pm$  s.e.) were  $40 \pm 7.14$  for control,  $18 \pm 5.54$  for  $CO_2$  treatment, and  $20 \pm 2.70$  for RS (**Figure 6a**). There was a significant difference between the control and  $CO_2$  treatment ( $p < 0.05$ ), as well as between control and RS ( $p < 0.05$ ). The  $CO_2$  treatment found no significant difference compared against RS ( $p > 0.5$ ), revealing that  $L_A$  values of fish subjected in elevated  $CO_2$  and temperature levels were no different from random selection (**Figure 7b**).

Mean  $L_R$  values ( $\pm$  s.e.) were  $-18.18 \pm 13.33$  for control,  $2 \pm 8.14$  for  $CO_2$  treatment, and  $5.45 \pm 6.66$  for RS (**Table 1, 2, 3; Figure 6b**). Concerning population-level lateralization, no significant right or left bias was indicated from the one-sample  $t$ -tests (mean test value of RS: 5.4545; see **Table 3**): all  $p$ -values were greater than 0.1 and 0.5 for control and  $CO_2$  groups, respectively. (Control,  $t_{(10)} = -1.77$ ,  $p = 0.107$ ;  $CO_2$ ,  $t_{(9)} = -0.425$ ,  $p = 0.681$ ; **Figure 8a, 8b**). The one-way ANOVA revealed no significant interaction between control,  $CO_2$ , and random simulation  $L_R$  values at the population level ( $F_{2,29} = 1.695$ ,  $p = 30.201$ ; **Figure 7c**). Tukey's post-hoc also indicates no statistical difference between control and  $CO_2$  treatment ( $p > 0.1$ ), control and RS ( $p > 0.1$ ) and  $CO_2$  and RS ( $p > 0.5$ ; **Figure 7d**). Levene's test for homogeneity of variances report that population variances were unequal for  $L_R$  means ( $F_{2,29} = 5.918$ ,  $p = 0.007$ ) and  $L_A$  means ( $F_{2,29} = 5.473$ ,  $p = 0.01$ ), though sample sizes are roughly equal (control,  $n = 11$ ;  $CO_2$ ,  $n = 10$ ).

Thus, while the ricefish population did not exhibit any collective right-left lateralization in normal CO<sub>2</sub> and temperature conditions, the individuals did exhibit a loss of directional bias in either direction when exposed to end-of-century acidification and warming.

| Trial   | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| <b>Right/Left Turns</b>   | 4/6 | 1/9 | 3/7 | 7/3 | 2/8 | 2/8 | 2/8 | 7/3 | 2/8 | 6/4 | 5/5 |
| <b>L<sub>R</sub></b>  | -20 | -80 | -40 | 40  | -60 | -60 | -60 | 40  | 20  | 20  | 0   |
| <b>Mean L<sub>R</sub> (for population-level lateralization) = -18.18 ±13.33</b> |     |     |     |     |     |     |     |     |     |     |     |
| <b>Mean L<sub>A</sub> (for individual lateralization) = 40 ±7.14</b>            |     |     |     |     |     |     |     |     |     |     |     |

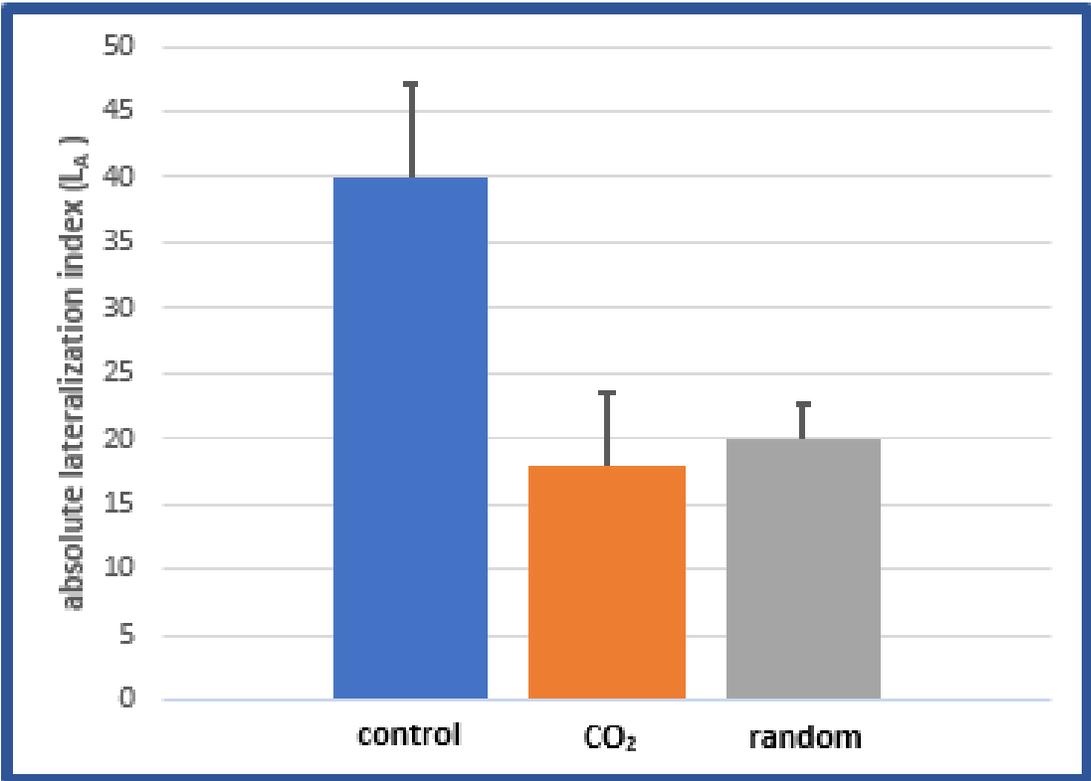
**Table 1: Control Treatment: Lateralization indices.** Only the last trialed fish exhibited an index of 0. More fish showed a left-turn bias (6), resulting in a negative mean L<sub>R</sub>.

| Trial   | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| <b>Right/Left Turns</b>   | 5/5 | 3/7 | 5/5 | 3/7 | 7/3 | 5/5 | 6/4 | 6/4 | 6/4 | 5/5 |
| <b>L<sub>R</sub></b>  | 0   | -40 | 0   | -40 | 40  | 0   | 20  | 20  | 20  | 0   |
| <b>Mean L<sub>R</sub> (for population-level lateralization) = 2 ±8.14</b> |     |     |     |     |     |     |     |     |     |     |
| <b>Mean L<sub>A</sub> (for individual lateralization) = 18±5.54</b>       |     |     |     |     |     |     |     |     |     |     |

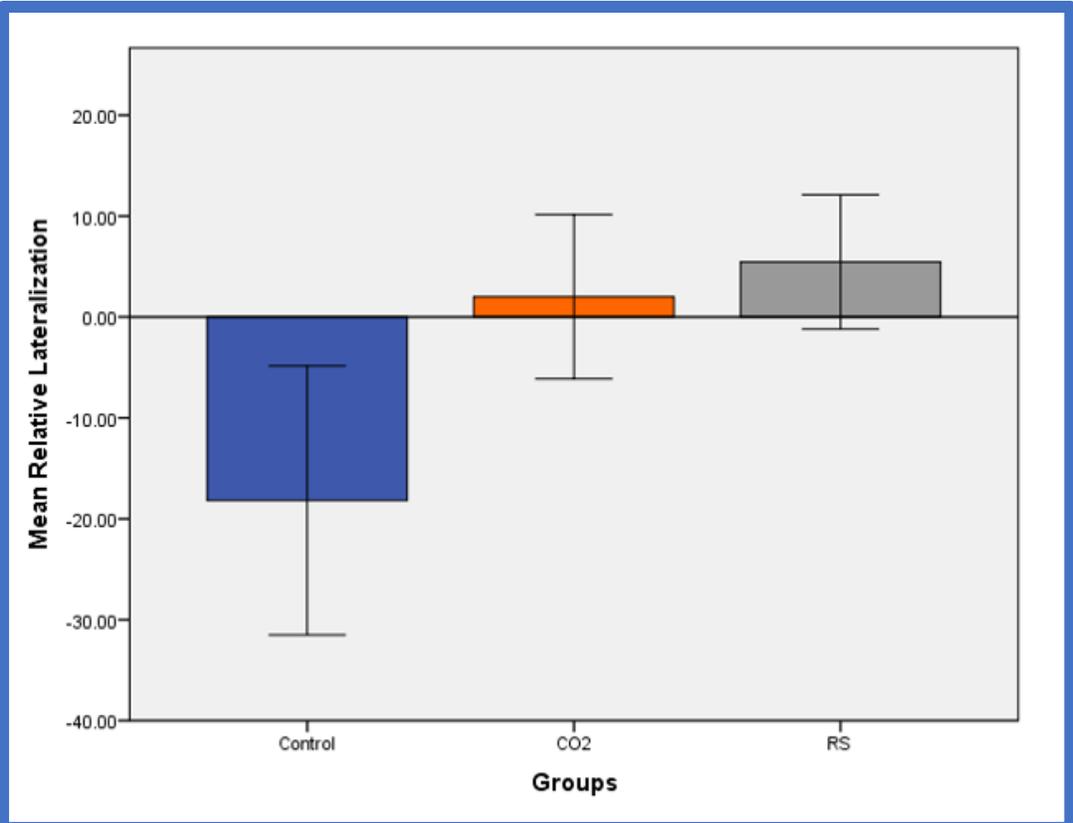
**Table 2: Elevated CO<sub>2</sub> Treatment: Lateralization indices.** Four of the ten fish exhibited no lateralization (L<sub>A</sub> = 0). An overall positive mean L<sub>R</sub> resulted from the 100 runs.

| RS Trial  | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  |
|---|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| <b>Right/Left Turns</b>   | 7/3 | 5/5 | 4/6 | 6/4 | 6/4 | 4/6 | 6/4 | 6/4 | 6/4 | 4/6 | 4/6 |
| <b>L<sub>R</sub></b>  | 40  | 0   | -20 | 20  | 20  | -20 | 20  | 20  | 20  | -20 | -20 |
| <b>Mean L<sub>R</sub> (for population-level lateralization) = 5.454 ±6.66</b> |     |     |     |     |     |     |     |     |     |     |     |
| <b>Mean L<sub>A</sub> (for individual lateralization) = 20 ±2.70</b>          |     |     |     |     |     |     |     |     |     |     |     |

**Table 3: Random Simulation of L<sub>R</sub>.** The RS was generated from random.org. Ten trials were inputted. Both mean L<sub>R</sub> and L<sub>A</sub> averaged within control and CO<sub>2</sub> treatments.



**Figure 6a.** Mean L<sub>A</sub> values ( $\pm$  s.e.). Control (blue):  $40 \pm 7.14$ , CO<sub>2</sub> treatment (orange):  $18 \pm 5.54$  for CO<sub>2</sub> treatment, and random simulation (grey):  $20 \pm 2.70$ . Error bars represent standard error of the means. L<sub>A</sub> values in the CO<sub>2</sub> treatment closely resemble indices generated by chance.



**Figure 6b. Mean  $L_R$  values ( $\pm$  s.e.).** Control (blue):  $-18.18 \pm 13.33$ , CO<sub>2</sub> treatment (orange):  $2 \pm 8.14$  for CO<sub>2</sub> treatment, and random simulation (grey):  $5.454 \pm 6.66$ . Error bars represent standard error of the means.  $L_R$  seemed to have shifted from left- (as mean control  $L_R$  is negative) to right-bias, resembling that of the randomly generated  $L_R$  values, but  $t$ -tests reveal no statistically significant biases in any treatment.

| ANOVA          |                |    |             |       |      |
|----------------|----------------|----|-------------|-------|------|
| Lateralization | Sum of Squares | df | Mean Square | F     | Sig. |
| Between Groups | 3190.000       | 2  | 1595.000    | 5.050 | .013 |
| Within Groups  | 9160.000       | 29 | 315.862     |       |      |
| Total          | 12350.000      | 31 |             |       |      |

**Figure 7a. ANOVA results for  $L_A$ .** For control, CO<sub>2</sub> treatment, and RS groups. F-statistic (df = 29) and  $p$ -value ( $p < 0.05$ ) are highlighted in yellow.

| ANOVA - Multiple Comparisons       |                 |                       |            |       |                         |             |
|------------------------------------|-----------------|-----------------------|------------|-------|-------------------------|-------------|
| Dependent Variable: Lateralization |                 |                       |            |       |                         |             |
| Tukey HSD                          |                 |                       |            |       |                         |             |
| (I) Groups                         | (J) Groups      | Mean Difference (I-J) | Std. Error | Sig.  | 95% Confidence Interval |             |
|                                    |                 |                       |            |       | Lower Bound             | Upper Bound |
| Control                            | Random          | 20.00000*             | 7.57822    | 0.034 | 1.2845                  | 38.7155     |
|                                    | CO <sub>2</sub> | 22.00000*             | 7.76537    | 0.022 | 2.8223                  | 41.1777     |
| Random                             | Control         | -20.00000*            | 7.57822    | 0.034 | -38.7155                | -1.2845     |
|                                    | CO <sub>2</sub> | 2.00000               | 7.76537    | 0.964 | -17.1777                | 21.1777     |
| CO <sub>2</sub>                    | Control         | -22.00000*            | 7.76537    | 0.022 | -41.1777                | -2.8223     |
|                                    | Random          | -2.00000              | 7.76537    | 0.964 | -21.1777                | 17.1777     |

\*. The mean difference is significant at the 0.05 level.

**Figure 7b. Tukey's post-hoc test for  $L_A$ .** Comparisons of mean  $L_A$  values between control group and CO<sub>2</sub> and RS groups reveal a  $p$ -value  $< 0.05$  (highlighted in yellow). Post-hoc

comparison between random and CO<sub>2</sub> groups demonstrate no significant difference in mean L<sub>A</sub> (0.964,  $p < 0.5$ , also highlighted in yellow).

| ANOVA                   |                |    |             |       |      |
|-------------------------|----------------|----|-------------|-------|------|
| Relative Lateralization |                |    |             |       |      |
|                         | Sum of Squares | df | Mean Square | F     | Sig. |
| Between Groups          | 3553.636       | 2  | 1776.818    | 1.695 | .201 |
| Within Groups           | 30396.364      | 29 | 1048.150    |       |      |
| Total                   | 33950.000      | 31 |             |       |      |

**Figure 7c. ANOVA results for L<sub>R</sub>.** For control, CO<sub>2</sub> treatment, and RS groups. F-statistic (df = 29) and  $p$ -value ( $p > 0.1$ ) are highlighted in yellow.

| ANOVA - Multiple Comparisons                |           |                       |            |       |                         |             |
|---|-----------|-----------------------|------------|-------|-------------------------|-------------|
| Dependent Variable: Relative Lateralization |           |                       |            |       |                         |             |
| Tukey HSD                                   |           |                       |            |       |                         |             |
| (I) Group                                   | (J) Group | Mean Difference (I-J) | Std. Error | Sig.  | 95% Confidence Interval |             |
|   |           |                       |            |       | Lower Bound             | Upper Bound |
| RS  | Control   | 23.63636              | 13.80481   | 0.218 | -10.4567                | 57.7294     |
|   | CO2       | 3.45455               | 14.14572   | 0.968 | -31.4804                | 38.3895     |
| Control                                     | RS        | -23.63636             | 13.80481   | .218  | -57.7294                | 10.4567     |
|   | CO2       | -20.18182             | 14.14572   | 0.341 | -55.1168                | 14.7531     |
| CO2   | RS        | -3.45455              | 14.14572   | .968  | -38.3895                | 31.4804     |
|   | Control   | 20.18182              | 14.14572   | .341  | -14.7531                | 55.1168     |

**Figure 7d. Tukey’s Post-hoc test for L<sub>R</sub>.** Comparisons of mean L<sub>R</sub> values between control group and CO<sub>2</sub> and RS groups reveal a  $p$ -value greater than 0.1 for RS vs. control and control vs. CO<sub>2</sub> groups, and particularly high  $p$ -value above 0.5 for CO<sub>2</sub> and RS (all  $p$ -values highlighted in yellow).

| One-Sample Test     |        |    |                 |                 |   |        |
|---------------------|--------|----|-----------------|-----------------|---|--------|
| Test Value = 5.4545 |        |    |                 |                 |   |        |
|                     | t      | df | Sig. (2-tailed) | Mean Difference | 95% Confidence Interval of the Difference |        |
|                     |        |    |                 |                 | Lower                                     | Upper  |
| Control             | -1.772 | 10 | .107            | -23.63632       | -53.3510                                  | 6.0783 |

**Figure 8a. Control vs. RS  $t$ -test of L<sub>R</sub>.** The  $p$ -value of 0.107 is greater than 0.1 when  $t = -1.772$  at  $df = 10$ . This reveals that control L<sub>R</sub> values have no significant difference to chance selection (test value mean = 5.4545) at 95% confidence interval.

| One-Sample Test                           |       |    |                 |                 |          |         |
|---|-------|----|-----------------|-----------------|----------|---------|
| Test Value = 5.4545                       |       |    |                 |                 |          |         |
| 95% Confidence Interval of the Difference |       |    |                 |                 |          |         |
|   | t     | df | Sig. (2-tailed) | Mean Difference | Lower    | Upper   |
| CO2                                       | -.425 | 9  | .681            | -3.45450        | -21.8633 | 14.9543 |

**Figure 8b. CO<sub>2</sub> vs. RS *t*-test.** The *p*-value of 0.681 is greater than 0.5 when  $t = -0.425$  at  $df = 9$ . This reveals that CO<sub>2</sub> L<sub>R</sub> values have no significant difference to chance selection (test value mean = 5.4545) at 95% confidence interval.

#### CHAPTER 4: DISCUSSION

The findings of this study show that elevated  $p\text{CO}_2$  and water temperatures have a behavior-altering effect on individual-level lateralization in a euryhaline species. We are the first to use *Oryzias latipes* as a euryhaline model to test for behavioral lateralization. Not only did L<sub>A</sub> significantly lessen in acidic and warm seawater, but the extent to which end-of-century surface ocean conditions dampened individual lateralization in ricefish leveled it to that of indiscriminate decision-making (post-hoc test showed  $p$ -value = 0.964 compared between CO<sub>2</sub> and random simulation groups).

While population-level lateralization was not exhibited in ricefish, nor was there any significant difference in L<sub>R</sub> between control and CO<sub>2</sub> treatments, L<sub>A</sub> values suggest far-reaching implications on fish population fitness. Studies have linked higher prevalence of behavioral lateralization to better coordination and school cohesion (Bisazza & Dadda, 2005), as well as social behavior (Bisazza et al., 2000). Lateralization is a prevalent feature across numerous fish species (even including other vertebrates [Bisazza *et al.*, 2000] and invertebrates [Frasnelli, 2013]), suggesting that behavioral lateralization is phylogenetically ancient (Bisazza *et al.*,

2000). Thus, population survival for many species, especially for shoaling or schooling fish like *Oryzias latipes*, may be at risk at end-of-century ocean conditions.

The treatment lasted only five days in which behavioral effects were already observed, but further studies need to be conducted across generations to further investigate adaptation to high-CO<sub>2</sub> and high temperatures. Particularly for euryhaline species such as Japanese ricefish, their gill ionocytes are in abundance and replete with complex functions, which allow for enhanced ion regulation (Miyanishi *et al.*, 2016). Elevated *p*CO<sub>2</sub> has been known to manipulate osmoregulation in a wide range of fish species (Kreiss *et al.*, 2015). Supported findings also report hypercapnic conditions inducing fish to undergo changes in acid-base regulation, particularly in decreasing plasma Cl<sup>-</sup> concentrations and increasing plasma bicarbonate ion concentrations, shifting ion gradients (Ishimatsu *et al.*, 2008). Despite heightened osmoregulatory adaptability, these species still experience behavior shifts in high-CO<sub>2</sub> conditions coupled with warming, as the results display. This is suggestive that euryhaline models still do not carry the mechanism to overcome the CO<sub>2</sub>-induced GABAergic responses due to ion gradient alterations (Heuer *et al.*, 2016). Continued research should explore the behavior-altering mechanism at the biomolecular level.

The lack of *in situ* research on seawater-dwelling species still gives us insufficient knowledge on how fish populations will thrive at end-of-century conditions in the ecological context. Behavioral and physiological responses to increased CO<sub>2</sub> has been highly suggested to be species specific (Baumann *et al.*, 2012; Pimentel *et al.*, 2016). In some species such as the Atlantic cod, larvae were largely unaffected in swimming behavior at *p*CO<sub>2</sub> levels as high as 4200  $\mu$ atm (Maneja *et al.*, 2013). Adaptability to high-CO<sub>2</sub> is expected to differ in efficiency across species dwelling in various regional areas. Coastal and estuarial waters temporally

fluctuate in its  $p\text{CO}_2$  to as high as 2,500 atm, to which freshwater and marine species are subjected (Reum *et al.*, 2014; Evans *et al.*, 2011; Hales *et al.*, 2011, Frieder *et al.*, 2012 as cited in Ou *et al.*, 2015). Fish living within more  $p\text{CO}_2$ -steady environments may be more susceptible to varying shifts of dissolved  $\text{CO}_2$ .

Ocean warming may also have a behavioral impact on fish species. Not as extensive research has been examined on the behavioral effects of temperature as on  $\text{CO}_2$ -induced acidification. Previous studies have divulged on the capacity of high temperatures to cause changes in behavioral lateralization, metabolic and respiratory rate, but little is known concerning its neurological impact on behavioral decisioning (Domenici *et al.*, 2014; Kreiss *et al.*, 2015; Nilsson *et al.*, 2009). Future studies should inspect temperature as the sole variable on behavioral lateralization.

### **Limitations**

The small sample size ( $n = 21$ ) may have dampened the statistical strength and increased standard error of the means. This was due in part to mortality, the causes which are unclear, but most probably unrelated to  $p\text{CO}_2$  and temperature levels as death occurred in both treatments. Parameters such as salinity and alkalinity levels were not as accurate and consistent which possibly made  $p\text{CO}_2$  calculations uncalibrated. Moreover, limited tanks and sample size did not allow for separating  $\text{CO}_2$  levels and temperatures as independent variables of behavioral disturbance as Domenici *et al.* set up (2014).

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