

10 **Abstract**

11 Coral reef organisms are exposed to both an increasing magnitude of $p\text{CO}_2$ and natural
12 fluctuations on a diel scale. For coral reef fishes, one of the most profound effects of ocean
13 acidification is the impact on ecologically important behaviors. Previous behavioral research has
14 primarily been conducted under static $p\text{CO}_2$ conditions and have recently come under criticism.
15 Recent studies have provided evidence that the negative impacts on behavior may be reduced
16 under more environmentally realistic, fluctuating conditions. We investigated the impact of both
17 present and future day, static (500 and 1000 μatm) and diel fluctuating (500 \pm 200 and 1000 \pm
18 200 μatm) $p\text{CO}_2$ on the lateralization and chemosensory behavior of juvenile anemonefish,
19 *Amphiprion percula*. Our static experimental comparisons support previous findings that under
20 elevated $p\text{CO}_2$, fish become un-lateralized and lose the ability to discriminate olfactory cues.
21 Diel-fluctuating $p\text{CO}_2$ may aid in mitigating the severity of some behavioral abnormalities such
22 as the chemosensory response, where a preference for predator cues was significantly reduced
23 under a future diel-fluctuating $p\text{CO}_2$ regime. This research aids in ground truthing earlier
24 findings and contributes to our growing knowledge of the role of fluctuating conditions.

25

26 **1. Introduction**

27 Anthropogenic climate change is rapidly altering Earth's oceans, leading to the phenomenon of
28 ocean acidification (OA). The burning of fossil fuels from human activities is exhausting
29 excessive concentrations of carbon dioxide (CO_2) into the atmosphere at an unprecedented rate
30 (Kerr 2010). The ocean sequesters atmospheric CO_2 by uptaking over one third of anthropogenic
31 CO_2 emissions (Sabine et al. 2004; Rhein et al. 2013). The additional input of atmospheric CO_2
32 reacts with seawater to acidify the oceans (Hoegh-Guldberg et al. 2007; Doney et al. 2009).

33 From the pre-industrial era, global ocean pH has decreased by at least 0.1 units (Wootton et al.
34 2008). Under “business as usual” conditions, it is forecasted that ocean pH will decrease by up to
35 0.43 units by the end of the century (RCP8.5 scenario; IPCC 2014). If these future projections
36 hold true, changes in ocean carbonate chemistry will be unlike any past event (Hönisch et al.
37 2012).

38 Ocean acidification has been shown to affect the sensory system, physiology, and behavior of
39 marine fishes (Heuer and Grosell 2014; Ashur et al. 2017; Cattano et al. 2018). Some of the most
40 notable effects of OA on coral reef fishes are on ecologically important behaviors (Nagelkerken
41 and Munday 2016). These behavioral changes include disruption to olfactory preferences
42 (Munday et al. 2009; Dixson et al. 2010; Ferrari et al. 2012a), reduced prey detection (Cripps et
43 al. 2011), decreased learning ability (Ferrari et al. 2012b), reduced behavioral lateralization
44 (Domenici et al. 2012, 2014; Nilsson et al. 2012), increased activity and boldness (Munday et al.
45 2010, 2014; Nilsson et al. 2012; Nagelkerken and Munday 2016), reduced hearing and vision
46 (Simpson et al. 2011; Chung et al. 2014), and altered reaction times, escape speeds and distances
47 (Allan et al. 2013, 2017; Munday et al. 2016). Furthermore, elevated CO₂ exposure can affect
48 coral reef fish settlement behavior (Devine et al. 2012), shoaling behavior (Nadler et al. 2016),
49 habitat preference (Devine and Munday 2013; Nagelkerken and Munday 2016; Goldenburg et al.
50 2018), and the replenishment of fish stocks (Munday et al. 2010).

51 The effect of OA on coral reef fish juveniles is not ubiquitous across all species, even within the
52 same genus, with some being more tolerant to increased CO₂ than others (Ferrari et al. 2011;
53 McCormick et al. 2013). An analysis of the OA literature shows large variation in the sensitivity
54 of behavioral responses to OA, at habitat and environmental scales, as well as interspecific and

55 intraspecific scales (McCormick et al. 2013; Clements and Hunt 2015; Schunter et al. 2016;
56 Vargas et al. 2017; Cattano et al. 2018; Munday et al. 2019, Munday et al. 2020).

57 The impacts of future climate change scenarios on coral reef fish behavior are not yet completely
58 understood. Stressors such as OA occur under fluctuating regimes and in synergy with other
59 stressors. Marine organisms experience a range of pH variability on both temporal and spatial
60 scales dependent on their environment, and these fluctuations are expected to be exacerbated
61 under future climate change conditions (Hofmann et al. 2011; Johnson et al. 2013; Shaw et al.
62 2013; Kapsenberg et al. 2015; McNeil and Matsumoto 2019). Coral reefs experience diel $p\text{CO}_2$
63 fluctuations due to a range of processes including biological reef metabolism (Waldbusser and
64 Salisbury 2014), as well as seasonal variability, ranging greater than 200 μatm at times
65 (Shamberger et al. 2011; Price et al. 2012; Albright et al. 2013; Duarte et al. 2013; Kline et al.
66 2015). Furthermore, fish seldom remain in one location and will likely experience a wide range
67 of pH variability over their life history due to movement around and off the reef, and ecosystem
68 structure and function can vary across different locations on reefs due to variability in pH (Price
69 et al. 2012; Shaw et al. 2013). There is evidence to suggest that temporary movement to another
70 water source (e.g. from an area of low pH to an area of higher pH) will not immediately
71 influence the behavioral changes resulting from elevated CO_2 (Munday et al. 2016).

72 The majority of previous studies have assessed behavioral changes using static OA treatment
73 conditions, however, considering the importance of better reflecting the natural environment, a
74 greater understanding of the impacts fluctuating conditions have on behavior is required. Little
75 research has been conducted on the impact fluctuating stressors may have on the behavior of
76 coral reef fish. Recent studies have shown that diel pH fluctuations may offset or reduce the
77 severity of behavioral changes when compared to static future OA conditions (Ou et al. 2015;

78 Jarrold et al. 2017; Jarrold and Munday 2018) suggesting that the findings of earlier research
79 (conducted under static OA conditions) may have overestimated the degree of behavioral
80 impairment.

81 Key behavioral traits exhibited by coral reef fishes such as behavioral lateralization and
82 chemosensory response have primarily been studied under static OA conditions (Munday et al.
83 2009, 2010, 2013; Dixson et al. 2010; Cripps et al. 2011; Ferrari et al. 2011, 2012a, 2012b;
84 Devine et al. 2012; Domenici et al. 2012, 2014; Nilsson et al. 2012; Devine and Munday 2013;
85 Welch et al. 2014). These behaviors play important ecological roles and are often crucial for
86 survival. The inability to generalize OA findings has resulted in a challenge to the understanding
87 of the behavioral impacts of OA on coral reef fishes, with differing results collected on
88 alternative species, life history stages, methods and testing apparatus being used (Munday et al.
89 2020). These discrepancies make it important to revisit and accurately replicate previous studies
90 to determine if earlier findings hold true, while still advancing the research field through the
91 inclusion of diel fluctuations. Technological advancements have now made it possible to test
92 these behavioral traits under more realistic and biologically relevant environmental conditions. It
93 is largely unknown if, and to what degree, the brief release from low pH in the evenings will
94 offset the negative effects of OA. The objectives of this research were to assess the impacts
95 future climate change conditions will have on the behavior of juvenile coral reef fish,
96 *Amphiprion percula*, under both static conditions (to revisit and replicate previous findings) and
97 under more ecologically relevant conditions (i.e. fluctuating conditions). Two experimental trials
98 were conducted investigating: 1) behavioral lateralization; 2) the chemosensory response.

99 **2. Materials and Methods**

100 2.1. Study Species:

101 A total of 169 chemically naïve, laboratory bred juvenile *Amphiprion percula* were sourced from
102 Sustainable Aquatics (Jefferson City, TN). All fish were bred from wild stock parents that were
103 randomized to include the offspring of three parental groups to account for any genetic
104 differences. Fish were 18 weeks old at the commencement of the experiment. Juvenile *A. percula*
105 were fed 0.8 mm pellets (Sustainable Aquatics) and *Artemia* sp. nauplii daily in the morning of
106 the first two weeks, before transitioning to a pellet-only diet during behavioral trials. During
107 behavioral trials, fish were fed at the end of the trial period on test days. Fish were maintained on
108 a 12:12 hr light:dark cycle.

109

110 2.2. Experimental Design and Protocol:

111 The impact of static and fluctuating OA on behavioral changes was assessed using current day
112 static temperature (28.5 °C) with a 2 × 2 cross-factorial design. Treatment groups included: 1)
113 static present-day control conditions; 2) static future-day acidification conditions; 3) fluctuating
114 present-day control conditions; and 4) fluctuating future-day acidification conditions. Each
115 treatment group consisted of 5 replicate 20 L aquariums (26 cm × 26 cm × 31 cm) holding 7-9 *A.*
116 *percula*. Juveniles were habituated to their treatment conditions for 15 days prior to
117 commencement of behavioral trials. A habituation timeframe of 4-7 days has proven to be
118 sufficient to impair a range of behavioral responses (Munday et al. 2010; Devine and Munday
119 2013; Chivers et al. 2014).

120 Aquaria were separated into one of two large re-circulating systems (111 cm × 248 cm × 22 cm),
121 each holding two treatment groups in a water bath. Natural seawater was used and sourced from

122 the Indian River Inlet (Delaware, USA). A header sump (680 L) pumped water into each of the
123 aquaria at an adjusted flow rate (75 mL min^{-1}). Four APEX Fusion systems (Neptune Systems)
124 were used to independently control temperature and pH_{NBS} based on programmed set points. CO_2
125 was regulated and monitored using an APEX computer system. This system injects additional
126 CO_2 when pH levels exceed the desired computer set point by opening a solenoid connected to a
127 precision needle valve. A steady slow stream of CO_2 was bubbled into the tank through an air
128 stone until the pH has dropped below the set maximum. CO_2 -stripped air (achieved using a soda
129 lime filter) was also bubbled into each aquarium system at a controlled flow rate to raise and
130 maintain pH at desired values. Both control and future day treatments used the same methods,
131 however pH set points for each varied. Temperature was monitored at 2 min intervals using
132 Neptune temperature probes controlled by the APEX system and adjusted with 200 W heaters
133 (ViaAqua) placed in each tank. When the temperature probe read below the desired set point,
134 power was turned on to the tank's heater, and the heater was then turned off when the
135 temperature reached the set point.

136 Both pH and temperature were independently tested twice daily using a handheld Mettler Toledo
137 probe (SevenGo Duo pro SG68 pH meter). Salinity was tested twice daily using a handheld
138 refractometer (Fisherbrand Salinity Refractometer). Partial water changes were performed every
139 second day to control for salinity. Water quality testing (ammonia, nitrate, nitrite) was conducted
140 twice weekly.

141

142 2.3. Carbonate Chemistry:

143 Juvenile *A. percula* were treated for 15 days (4th - 19th Nov 2019) in either static present day (500
144 μatm), static future day (1000 μatm), fluctuating present day (1000 \pm 200 μatm), or fluctuating
145 future day (500 \pm 200 μatm) CO₂ treatments at a current day static temperature of 28.5 °C (Table
146 1). Treatment continued throughout the next 15 days during the behavioral trial period (20th Nov
147 - 4th Dec 2019). The control pH levels were based on the averages of five present-day reef
148 systems (Fig. 1; Hofmann et al. 2011; Albright et al. 2013). The targeted static future day
149 treatment values (\sim 1000 μatm) were based on commonly used forecast open ocean values for the
150 end of the century (Kroeker et al. 2013; IPCC 2014). Coral reef $p\text{CO}_2$ fluctuations can typically
151 range between \pm 50-150 μatm on a diel scale (Albright et al. 2013; Kline et al. 2015). The
152 magnitude in range of fluctuations is forecast to increase in the future (McNeil and Sasse 2016),
153 and this experiment aimed for a range of \pm 200 μatm , as seen in Jarrold et al. (2017). Fluctuating
154 treatment groups had hourly shifts in pH_{NBS} of 0.01 - 0.02 units to reflect diel fluctuations that
155 occur on a natural reef (Fig. 1). Future day treatment levels used the control levels as a baseline
156 and were adjusted to include the forecast values expected to occur in 2100 (IPCC 2014), where
157 fluctuations were extrapolated to 0.3 units lower than control levels. The pH_{NBS} set points were
158 determined through prior experimentation and programmed into the APEX system to correspond
159 with target $p\text{CO}_2$ values. Temperature levels were based on summer values recorded by Albright
160 et al. (2013). Throughout the experimental period mean temperature was 28.54 ± 0.02 °C, and
161 mean salinity was 34.43 ± 0.06 ppm. To determine the carbonate chemistry of each treatment
162 group, dissolved inorganic carbon (DIC) and pH samples were taken each week. The certified
163 reference materials provided by the laboratory of Dr. A Dickson (San Diego, CA, USA) with
164 known DIC were used to validate DIC measurements, and pH was measured
165 spectrophotometrically (Dickson et al. 2007). DIC and spectrophotometric pH were used to

166 calculate $p\text{CO}_2$ with the CO2SYS software (Pierrot et al. 2006) using constants K1 and K2
167 (Mehrbach et al. 1973) and refit by Dickson and Millero (1987). Measurements for the
168 fluctuating treatments were taken midweek at multiple times to correspond with the highest,
169 median, and lowest expected $p\text{CO}_2$ values (Table 1.).

170

171 2.4. Behavioral Trials:

172 Two experimental trials measuring ecologically relevant behaviors were conducted investigating
173 1) behavioral lateralization, and 2) chemosensory response. Trials were run sequentially, and
174 each trial was conducted once from a haphazardly selected sample of juvenile fish from each
175 treatment group. Each individual fish was only tested once per experimental trial. All behavioral
176 trials were undertaken between 08:00 - 17:00. Additional sensory stimuli were minimized
177 throughout all trials.

178 Behavioral lateralization, which reflects the tendency for fish to have a left or right turning
179 preference, was evaluated using a detour test following similar methods to Domenici et al.
180 (2012). A two-way T-maze was used to measure the turning direction of an individual. Water
181 from the respective treatment conditions of each fish was used to fill the maze to a depth of 4 cm.
182 A single fish was placed at one end of the T-maze where it could explore the maze and habituate
183 for 3 min (n=30). Following the habituation period, the fish was gently coaxed with a plastic rod
184 (no closer than two body lengths away from the fish) to the middle of the runway, then through
185 the maze to the end of the runway where it was confronted with a turning choice of either left or
186 right. Ten consecutive runs were recorded for each fish, and the score of the turning direction
187 and the degree of lateralization was obtained. Direction choice was determined as the first

188 direction chosen when the fish exited the runway. The observer was kept blind to the treatment
189 during the trial.

190 An Atema two-channel choice flume (Atema et al. 2002) was used to test the chemosensory
191 mediated behavior towards predator chemical signals by juvenile *A. percula*, following the
192 methods of Gerlach et al. (2007). Chemical cues for testing were generated by soaking either a
193 single predator (*Cephalopholis cyanostigma*) or a single non-predator (*Zebrasoma falvescens*) in
194 a closed, aerated 10 L seawater system for 2 hr. The seawater used was from the same source as
195 the seawater in the fish aquaria, which had not been treated during the soak period. The predator
196 or non-predator was then removed from the water immediately following the soak. Cue water
197 was then placed in the system to treat the water to the relevant temperature and pH associated
198 with the fish being tested. This process ranged from 10 - 30 min depending on the treatment
199 conditions. To run the two-channel choice flume, water from two sources (either predator, non-
200 predator, or untreated control water) were gravity fed into the flume (13 cm × 4 cm) at 100 mL
201 min⁻¹. Water velocity was controlled using two flow meters, ensuring water was delivered at
202 equal rates to prevent mixing of the water masses and to achieve laminar flow for the cues. Each
203 individual fish was isolated in a small 200 mL beaker of associated treatment water for 10 min,
204 and then gently placed downstream in the flume and given a 2 min habituation period where it
205 was free to swim throughout the chamber. At the conclusion of the habituation period, the fish's
206 position on either the right or left side was recorded at 5-second intervals for 2 min. The water
207 sources were then switched to discount a side preference, and the flume was then given a 1 min
208 flushing period before the entire 2 min habituation period and 2 min testing period were
209 repeated. Dye tests were conducted before and after trials and regularly throughout to ensure
210 laminar flow with no areas of turbulence or eddies. Juvenile fish were haphazardly selected from

211 each treatment group (n=20). Three runs were conducted in the same order for each fish: 1) non-
212 predator vs. untreated, 2) predator vs. untreated, 3) non-predator vs. predator. All trials were run
213 double blinded, with both fish treatment group and chemical cues blind to the observer.

214

215 2.5. Statistical Analysis

216 To assess both the turning preference of the fish and the strength of lateralization, relative
217 lateralization (L_R) and absolute lateralization (L_A) were calculated using established methods
218 (Bisazza et al. 1998). To compare differences between treatment groups, a Kruskal-Wallis test
219 was performed on both relative and absolute lateralization, as the static present day data set was
220 not normal (D'Agostino & Pearson omnibus normality test, $p = 0.0336$). Additionally,
221 lateralization was also assessed at both the population-level using a generalized linear random-
222 effects model (GLMM; using the lme4 package in R), and at the individual-level using a chi-
223 square test, following previously used methods (Roche et al. 2020). Shapiro-Wilk normality tests
224 were performed on the chemosensory response data. To assess if there was a significant
225 preference towards a specific cue within a treatment group per chemosensory response trial, one
226 sample t -tests were conducted comparing the mean and variance against an expected value of
227 50%. To compare if there were differences between treatment groups, data was first transformed
228 using the arcsine (square root) transformation. A one-way ANOVA with Tukey's posthoc test
229 was run on each of the three different chemosensory trials.

230

231 **3. Results**

232 3.1. Behavioral lateralization:

233 All treatment groups exhibited a mean left turning preference (Fig. 2A), where fish from the
234 static future day (SFD) and fluctuating future day (FFD) treatments displayed a higher degree of
235 mean relative lateralization (L_R) (SFD: -9.33 ± 6.49 , FFD: -6.67 ± 6.80). However, there were no
236 significant differences between treatments for L_R (Kruskal-Wallis, $p = 0.9254$), and no treatment
237 group was lateralized at the population level (GLMM, $p > 0.05$ for all cases).

238 There were significant differences between treatments for absolute lateralization (L_A) (Kruskal-
239 Wallis, $p < 0.05$). Fish from the static present day (SPD) and fluctuating present day (FPD)
240 controls exhibited the highest mean values of L_A (SPD: 44 ± 4.54 , FPD: 46 ± 5.27) (Fig. 2B). In
241 contrast, fish from the static future day and fluctuating future day treatments displayed lower
242 mean L_A values (SFD: 28 ± 4.25 , FFD: 29.33 ± 4.26). Fish from both present day controls
243 remained individually lateralized (χ^2 , SPD: $p < 0.00001$, FPD: $p < 0.000001$), whereas fish from
244 future day treatments lost their individual lateralization (χ^2 , SFD: $p = 0.1571$; FFD: $p = 0.0797$).
245 Fish treated with static and fluctuating future day conditions displayed significant differences in
246 L_A with fish from the static present day control (Kruskal-Wallis, $p < 0.05$ in both cases), but not
247 with fish from the fluctuating present day control (Kruskal-Wallis, SFD: $p = 0.0673$; FFD: $p =$
248 0.0544). There were no significant differences in L_A between both static and fluctuating present
249 day (Kruskal-Wallis, $p = 0.8624$) and static and fluctuating future day (Kruskal-Wallis, $p = 1$)
250 treatments.

251

252 3.2. Chemosensory response:

253 Fish treated with static present day, fluctuating present day, and fluctuating future day seawater
254 displayed no preference for the chemical cues produced by the non-predator (*Zebrafoma*

255 *falvescens*) when tested against untreated seawater (t -test, $p > 0.05$), spending between 45.4 -
256 54.3% (SPD: $54.3 \pm 2.2\%$, FPD: $45.4 \pm 2.1\%$, FFD: $49.2 \pm 5.0\%$) time in the non-predator
257 chemical cues (Fig. 3A; Table 2.). Fish in the static future day treatment group showed a
258 significant preference toward the untreated control water (SFD: $54.1 \pm 3.2\%$; t -test, $p = 0.042$).
259 There were no significant differences between treatment groups for time spent in cue (ANOVA,
260 $p > 0.05$; Table 3.).

261 When the predator chemical cue (*Cephalopholis cyanostigma*) was tested against untreated
262 seawater, fish held in future day conditions displayed a preference for the predator chemical
263 cues, spending between 53.7 - 63.9% (SFD: $63.9 \pm 3.3\%$, FFD: $53.7 \pm 2.5\%$) of their time in the
264 predator chemical cue (Fig. 3B; Table 2.). A statistically significant preference between predator
265 cue and control water was identified for the static future day treatment (t -test, $p < 0.001$) but not
266 for the fluctuating future day treatment (t -test, $p = 0.158$). Significant differences in the time
267 spent in predator cue between treatments were identified (ANOVA, $p < 1 \times 10^{-8}$; Table 3). The
268 preferences observed in the future day treatments significantly differed in comparison to present
269 day treatments groups, where fish from the static future day treatment spent significantly more
270 time in the predator cue compared to the static (ANOVA, $p < 1 \times 10^{-5}$) and fluctuating (ANOVA,
271 $p < 1 \times 10^{-6}$) present day treatments. The same was true for fish in the fluctuating future day
272 treatment compared to the static (ANOVA, $p < 0.001$) and fluctuating (ANOVA, $p < 1 \times 10^{-4}$)
273 present day treatment groups. There was no significant difference in time spent in predator cue
274 between fish from the fluctuating future day treatment and static future day treatment (ANOVA,
275 $p > 0.05$; Fig. 3B). Fish from both present day treatments (static and fluctuating) spent
276 significantly more time in the untreated water over the predator cue (t -test, SPD: $p < 0.001$; FPD:
277 $p < 1 \times 10^{-6}$), spending 68.5 - 72.6% of their time in the untreated water (SPD: $68.5 \pm 4.3\%$, FPD:

278 72.6 ± 3.6%). No significant difference in percent time spent in the predator cue between present
279 day treatments was found (ANOVA, $p = 0.888$).

280 When comparing the predator chemical cues to the non-predator chemical cues simultaneously
281 (i.e. *C. cyanostigma* vs. *A. pyroperus*), a significant preference for the predator chemical cue
282 was displayed by fish treated in static future day conditions (t -test, $p < 1 \times 10^{-5}$; Fig. 3C),
283 spending almost three times longer in this cue. The CO₂ treatment of the fish resulted in
284 significantly different responses to the predator cue (ANOVA, $p < 1 \times 10^{-11}$; Table 3.). Fish from
285 the static future day treatment spent significantly more time in the predator cue than all other
286 treatments (ANOVA, SPD: $p < 1 \times 10^{-7}$; FPD: $p < 1 \times 10^{-7}$; FFD: $p < 0.001$). Fish treated under
287 fluctuating future day conditions displayed no significant preferences for the predator or non-
288 predator (t -test, $p = 0.6111$). This was a significantly greater amount of time in comparison to the
289 static and fluctuating present day treatments (ANOVA, $p < 0.01$ in both cases), but notably
290 significantly less time in comparison to the static future day treatment (ANOVA, $p < 0.001$; Fig.
291 3C). Fish from both present day treatments spent a significant amount of time in the chemical
292 cues produced by the non-predator over the predator (t -test, SPD: $p < 1 \times 10^{-7}$; FPD: $p < 1 \times 10^{-6}$),
293 but this did not significantly differ between the two groups (ANOVA, $p = 0.9999$).

294 **4. Discussion**

295 This study supports most previous research assessing the impact of ocean acidification on
296 juvenile coral reef fish behavior. In both behavioral trials conducted, fish in the future day
297 treatments exhibited behavioral changes that are likely deleterious in comparison to fish in
298 present day treatments. When provided with the option of a predator cue compared to either
299 untreated control water or non-predator cue, fish from the static future day treatment spent
300 significantly more time in the predator cue compared to fish treated with present day conditions.

301 Diel $p\text{CO}_2$ fluctuations had varying impacts on juvenile *A. percula* behavior depending on
302 whether fish were treated under present day or future day conditions. When comparing
303 fluctuating conditions with static conditions for fish treated in future day $p\text{CO}_2$, fluctuations
304 reduced the attraction (i.e. amount of time) toward the predator cue. Fish from the fluctuating
305 future day treatment had no significant preference between the non-predator and predator cues,
306 where under static future day conditions they preferred the predator cue, thus indicating that
307 fluctuating conditions may help mitigate the degree of negative behavioral impairment on
308 chemosensory response. In contrast, fluctuations did not reduce the amount of time fish from
309 present day treatments spent in the predator cue. These results suggest that natural diel
310 fluctuations may help mitigate negative behavioral impairment in the future, but under present
311 day conditions there is no apparent influence on behaviors tested in this study.

312 The results from our behavioral lateralization trials support those of previous findings, where fish
313 from both future day treatments (static and fluctuating) became un-lateralized, while fish from
314 present day conditions remained individually lateralized (Domenici et al. 2012, 2014). However,
315 fluctuating conditions did not appear to mitigate or offset this behavioral change, suggesting
316 fluctuating conditions may not aid in behavioral lateralization abnormalities. Contrasting this,

317 Jarrold et al. (2017) found that fluctuating future day conditions did reduce the negative
318 behavioral impairment of *A. percula* becoming un-lateralized in their study. Given the small
319 number of studies conducted in this field at the time of writing, more research is required to
320 garner a better picture of how behavioral lateralization may be impacted under more realistic,
321 future day conditions. Fish from all treatment groups displayed a left turning preference,
322 differing from previous findings of other juvenile coral reef damselfish that have showed varied
323 results (Domenici et al. 2012, 2014; Jarrold et al. 2017). Recent research has suggested that
324 behavioral lateralization, through the methods of a detour test, is not repeatable in fishes (Roche
325 et al. 2020). When five different species of fish were run multiple times, there was no
326 repeatability in results. Although we did not repeat behavioral lateralization trials multiple times,
327 given the high degree of both interspecific and intraspecific sensitivity to OA, this suggestion
328 may not hold true to all fishes. For example, a recent study found that behavioral lateralization is
329 repeatable across contexts (McLean and Morell 2020). In this study, relative lateralization was
330 repeatable for male and female adult *Poecilia reticulata*, and absolute lateralization was
331 repeatable for males. It is also possible that fish could learn the detour maze or become
332 unthreatened when placed in the same situation repeatedly. Future day $p\text{CO}_2$ conditions impacted
333 the ability of juvenile coral reef fish to discriminate between different cue sources, as fish from
334 the present day treatments showed an attraction toward the non-predator cue when given the
335 option between a predator and non-predator cue. A higher percent of time spent in the predator
336 cue from fish treated with future day $p\text{CO}_2$ conditions is likely due to a loss of discriminatory
337 ability rather than an attraction toward the predator cue, supporting previously reported findings
338 of attraction toward unfamiliar settlement cues and predator cues (Munday et al. 2009, 2016;
339 Dixson et al. 2010; Nilsson et al. 2012).

340 Changes in chemosensory discrimination may also result in higher mortality rates (Munday et al.
341 2014). However, our results indicate that naturally occurring diel $p\text{CO}_2$ fluctuations may, to a
342 degree, mitigate the impact of these negative behavioral impairments. Jarrold et al. (2017) found
343 similar reductions in time spent in predator cues under fluctuating conditions for two different
344 species of juvenile coral reef fish, and other studies have found similar results (Jarrold and
345 Munday 2018). Although fish in fluctuating future day conditions spent less time in the predator
346 cue than fish in static future day conditions, their time spent was still higher than fish from both
347 present day treatments, indicating that although the effect is reduced, it is still an issue of future
348 concern. Given no significant differences between fluctuating and static conditions for present
349 day treatments, fluctuations are more likely to play a crucial role in the future.

350 While our results support the overall conclusions of most behavioral studies investigating OA
351 impacts on coral reef fishes (Munday et al. 2019), our results differ in the magnitude of these
352 behavioral impairments. For example, the first chemosensory response study to report on
353 predator detection found that under static future day conditions, settlement stage fish spent all of
354 their time in predator cue, whereas those in static present day control conditions displayed a
355 higher predator avoidance and spent no time in predator cue (Dixson et al. 2010). In contrast,
356 when fish in our study were given the option between predator and non-predator cue, fish from
357 the static future day treatment and static present day control spent 72.5% and 26.5% of time in
358 predator cue, respectively. As the same focal species (*Amphiprion percula*), testing apparatus,
359 and methods were used, a direct comparison can be made, potentially highlighting the role of
360 juvenile age in response towards cues. Fish tested in Dixson et al. (2010) were treated during the
361 egg and larval stage and tested at settlement (11 days post hatch), whereas the *A. percula* tested
362 here were treated only during a two week period of the juvenile stage and tested at 20 weeks post

363 hatch. Sensitivity to OA may likely be greater at earlier stages of ontogeny. As OA can affect
364 larval processes, settlement and metamorphosis, early life stages represent a critical “bottleneck”
365 period, meaning behaviors such as those tested in this study may be even more essential for
366 survival at a younger age (Almany et al. 2006; Espinel-Velasco et al. 2018). Furthermore, our
367 results suggest that fluctuations appear to provide little, if any, behavioral benefits under present
368 day conditions. As coral reefs are generally not a homogenous environment, $p\text{CO}_2$ ranges and
369 fluctuations may vary both spatially across and within reefs, and temporarily, accounting for
370 different behavioral and biological responses (Duarte et al. 2013; Boyd et al. 2016; Vargas et al.
371 2017).

372 As continual exposure to OA over time and movement from high to lower levels of $p\text{CO}_2$ does
373 not appear to reduce the negative behavioral impacts (Munday et al. 2014, 2016), fluctuating
374 conditions and organismal adaptivity should be researched on a local scale, with a key emphasis
375 on finetuning the different degrees of fluctuations and the important roles they play (Wahl et al.
376 2016). Given the variability and complexity of coral reefs, it remains largely unknown how $p\text{CO}_2$
377 fluctuations may impact behavior, and on a larger scale, ecosystem structure and function
378 (Queirós et al. 2014; Goldenburg et al. 2018).

379

380 **5. Conclusion**

381 The results found here underscore and expand on previous research that has assessed behavioral
382 abnormalities of *Amphiprion percula* and other coral reef fishes under static future day $p\text{CO}_2$
383 conditions (Munday et al. 2009, 2010; Dixson et al. 2010; Domenici et al. 2012; Ferrari et al.
384 2012a; Nilsson et al. 2012; Allan et al. 2013; Chivers et al. 2014). Furthermore, this study adds

385 to the small yet growing literature suggesting that naturally occurring diel fluctuating $p\text{CO}_2$
386 conditions may help mitigate or reduce OA-induced behavioral abnormalities under future
387 climate change regimes.

388

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396

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616 **Tables**

617 **Table 1.** Mean values for seawater parameters ± 1 SD throughout the entirety of the experimental
 618 duration. Average values of $p\text{CO}_2$ and pH_T for fluctuating treatments are from samples taken at different
 619 times of the day to reflect the middle, minimum, and maximum values. Range of $p\text{CO}_2$ and pH_T represent
 620 the average range of all replicates within a treatment between the minimum and maximum values.
 621 Temperature values provided are from a portable Mettler Toledo probe.

Parameter	$p\text{CO}_2$ treatment			
	Static present day (500 μatm)	Static future day (1000 μatm)	Fluctuating present day (500 \pm 200 μatm)	Fluctuating future day (1000 \pm 200 μatm)
Average pH_T	7.98 \pm 0.02	7.77 \pm 0.02	8.03 \pm 0.04	7.74 \pm 0.02
Min. pH_T	-	-	7.95 \pm 0.04	7.67 \pm 0.03
Max. pH_T	-	-	8.07 \pm 0.04	7.81 \pm 0.02
pH_T range	-	-	0.13 \pm 0.02	0.14 \pm 0.02
Average $p\text{CO}_2$ (μatm)	512 \pm 33	918 \pm 49	458 \pm 46	989 \pm 54
Min. $p\text{CO}_2$ (μatm)	-	-	408 \pm 45	826 \pm 40
Max. $p\text{CO}_2$ (μatm)	-	-	566 \pm 80	1161 \pm 61
$p\text{CO}_2$ range (μatm)	-	-	158 \pm 46	335 \pm 53
TA ($\mu\text{mol kg}^{-1}$)	2880 \pm 63	2470 \pm 63	2494 \pm 35	2481 \pm 33
Min. TA ($\mu\text{mol kg}^{-1}$)	-	-	2493 \pm 86	2476 \pm 98
Max. TA ($\mu\text{mol kg}^{-1}$)	-	-	2539 \pm 49	2512 \pm 23
Temperature ($^{\circ}\text{C}$)	28.5 \pm 0.2	28.6 \pm 0.1	28.4 \pm 0.1	28.5 \pm 0.2
Salinity	34.4 \pm 0.5	34.4 \pm 0.5	34.4 \pm 0.5	34.4 \pm 0.5

622

623

624 **Table 2.** Comparison of percent time (\pm SE) fish from each treatment group spent in either chemical cue
 625 presented in each of the three chemosensory trials (n=30). The p-values represent one-sample *t*-tests
 626 conducted on percent time spent in cue against an expected value of 50%. Significance values are
 627 reflected with an asterisk and represent a preference toward the cue tested (left side chemical cue
 628 comparison column).

Treatment	Fluctuation	Chemical cue comparison (%)		p-value
		Non-predator	Untreated seawater	
Present Day	Static	54.27 \pm 2.16	45.73 \pm 2.16	0.062
Present Day	Fluctuating	45.94 \pm 3.22	54.06 \pm 3.22	0.222
Future Day	Static	45.42 \pm 2.10	54.58 \pm 2.10	0.042 *
Future Day	Fluctuating	49.17 \pm 4.95	50.83 \pm 4.95	0.860
		Predator	Untreated seawater	
Present Day	Static	31.46 \pm 4.29	68.54 \pm 4.29	3.702 $\times 10^{-4}$ *
Present Day	Fluctuating	63.85 \pm 3.33	36.15 \pm 3.33	5.849 $\times 10^{-6}$ *
Future Day	Static	27.40 \pm 3.64	72.60 \pm 3.64	5.409 $\times 10^{-4}$ *
Future Day	Fluctuating	53.65 \pm 2.48	46.35 \pm 2.48	0.158
		Predator	Non-predator	
Present Day	Static	26.46 \pm 2.78	73.54 \pm 2.78	6.935 $\times 10^{-8}$ *
Present Day	Fluctuating	72.50 \pm 3.41	27.50 \pm 3.41	6.752 $\times 10^{-7}$ *
Future Day	Static	26.35 \pm 3.25	73.65 \pm 3.25	2.6 $\times 10^{-6}$ *
Future Day	Fluctuating	49.06 \pm 3.57	50.94 \pm 3.57	0.611

629

630

631 **Table 3.** Comparison of percent time (\pm SE) fish from each treatment group spent in either chemical cue
 632 presented in each of the three chemosensory trials (n=30). The p-values represent comparisons between
 633 treatment groups in each chemical cue (conducted via ANOVA), where: SPD = Static Present Day; SFD
 634 = Static Future Day; FPD = Fluctuating Present Day; and FFD = Fluctuating Future Day. Asterisks reflect
 635 significant differences.

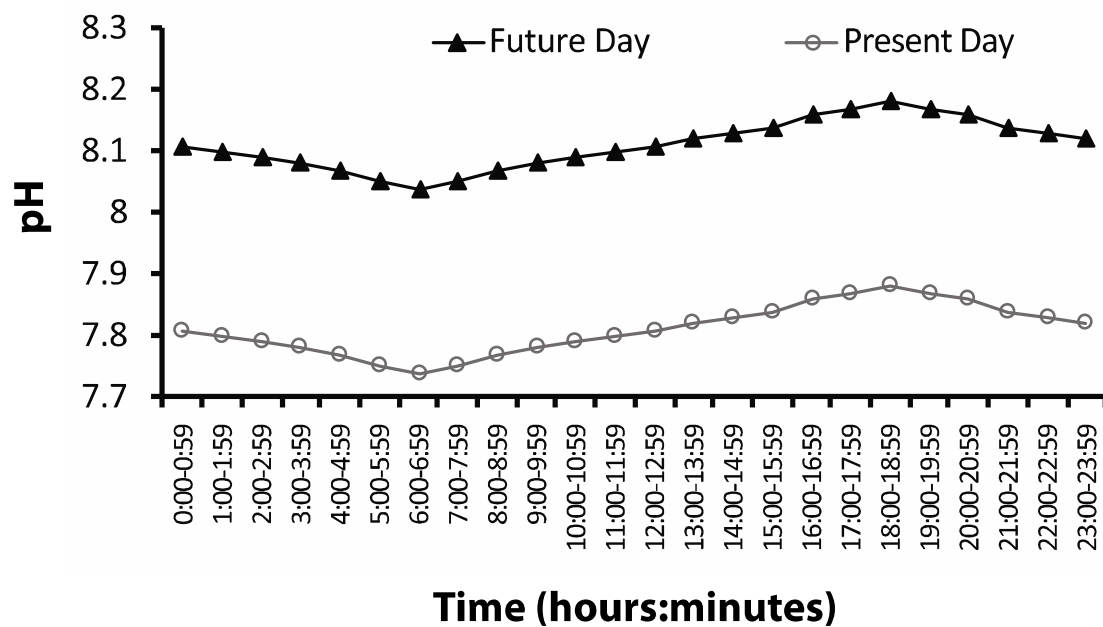
Treatment	Fluctuation	Chemical cue comparison (%)		p-value
		Non-predator	Untreated seawater	
Present Day	Static	54.27 \pm 2.16	45.73 \pm 2.16	SPD-SFD: 0.493 SPD-FPD: 0.417 SPD-FFD: 0.736
Future Day	Static	45.94 \pm 3.22	54.06 \pm 3.22	SFD-FPD: 0.999 SFD-FFD: 0.979 FPD-FFD: 0.954
Present Day	Fluctuating	45.42 \pm 2.10	54.58 \pm 2.10	
Future Day	Fluctuating	49.17 \pm 4.95	50.83 \pm 4.95	
		Predator	Untreated seawater	
Present Day	Static	31.46 \pm 4.29	68.54 \pm 4.29	SPD-SFD: 1.5×10^{-6} * SPD-FPD: 0.888 SPD-FFD: 8.359×10^{-4} *
Future Day	Static	63.85 \pm 3.33	36.15 \pm 3.33	SFD-FPD: 1×10^{-7} * SFD-FFD: 0.353 FPD-FFD: 6.21×10^{-5} *
Present Day	Fluctuating	27.40 \pm 3.64	72.60 \pm 3.64	
Future Day	Fluctuating	53.65 \pm 2.48	46.35 \pm 2.48	
		Predator	Non-predator	
Present Day	Static	26.46 \pm 2.78	73.54 \pm 2.78	SPD-SFD: $<1 \times 10^{-7}$ * SPD-FPD: 0.999 SPD-FFD: 0.003 * SFD-FPD: $<1 \times 10^{-7}$ * SFD-FFD: 1.296×10^{-4} *
Future Day	Static	72.50 \pm 3.41	27.50 \pm 3.41	FPD-FFD: 0.004 *
Present Day	Fluctuating	26.35 \pm 3.25	73.65 \pm 3.25	
Future Day	Fluctuating	49.06 \pm 3.57	50.94 \pm 3.57	

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639 **Figures**



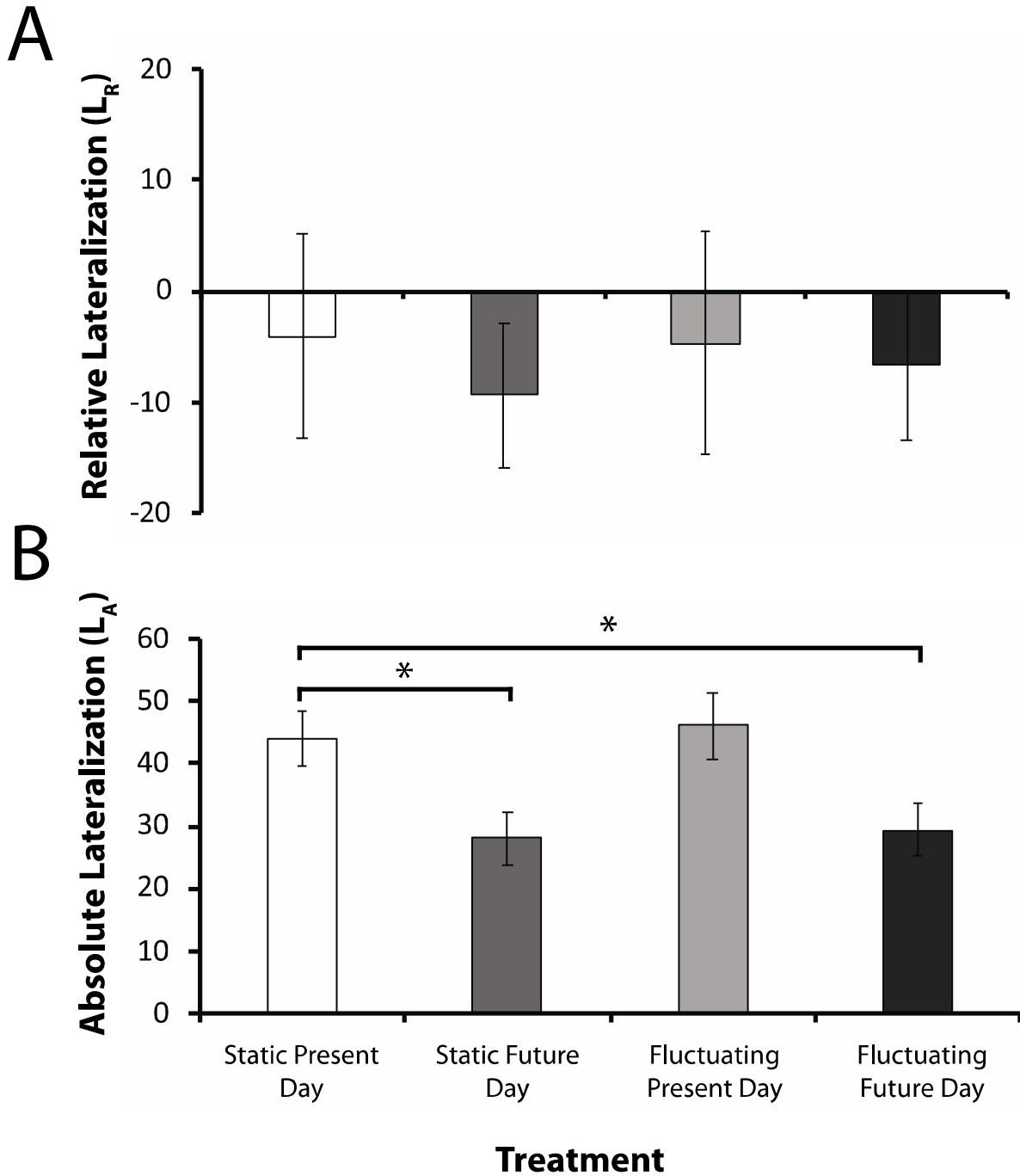
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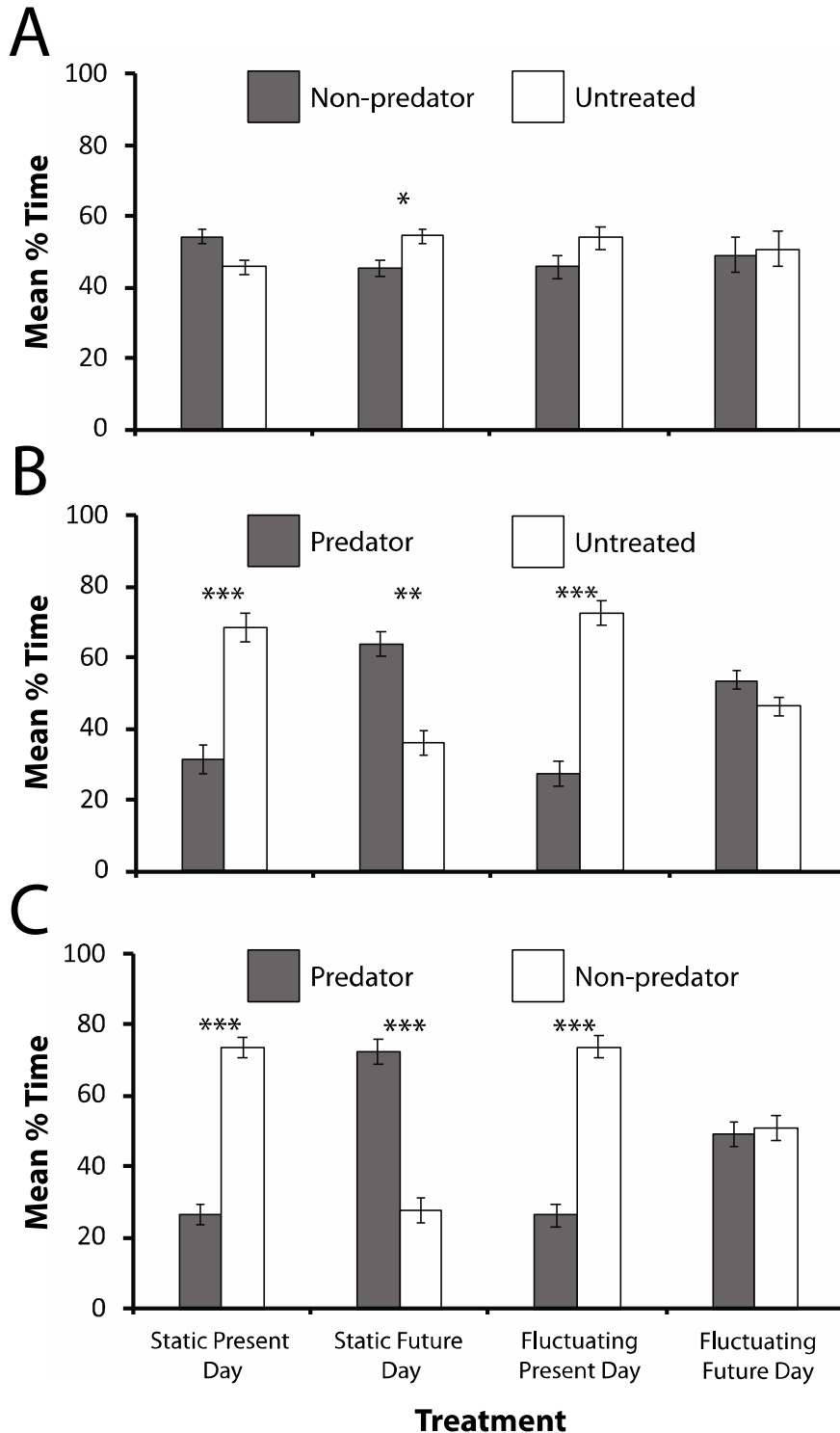
642 **Fig 1.** Target diel pH fluctuations for present day control (black, closed triangles) and future

643 day treatment (gray, opened circles) tanks. APEX probe error ± 0.02 .

644



645
646 **Figure 2. A)** Relative lateralization (L_R) of juvenile *Amphiprion percula* (mean \pm S.E.). Positive and
647 negative values on the y-axis indicate either a right or left group turning preference, respectively. **B)**
648 Absolute lateralization (L_A) of juvenile *Amphiprion percula* (mean \pm S.E.). Significant differences ($p <$
649 0.05) between groups are represented by an asterisk. A total of 30 fish from each $p\text{CO}_2$ treatment group
650 were used in the detour test.



651
 652 **Figure 3.** Mean percentage of time (\pm S.E.) juvenile *Amphiprion percula* spent in different chemical cues
 653 presented in a two-channel choice flume. A total of 20 fish from each $p\text{CO}_2$ treatment group were used
 654 per chemical cue trial with a choice of: **A**) non-predator (Tang) and untreated (control), **B**) predator (Cod)
 655 and untreated (control), and **C**) predator (Cod) and non-predator (Tang). Significant differences between
 656 chemical cue preference within treatment groups are represented by asterisks, where: * = $p < 0.05$; ** = p
 657 < 0.001 ; *** = $p < 0.00001$.