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Effects of ocean acidification on dopamine-mediated behavioral responses of a coral reef damselfish

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Peer reviewed

1 **Effects of ocean acidification on dopamine-mediated behavioral responses of a coral**
2 **reef damselfish**

3

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24

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28

29 **Highlights:**

- 30 • CO₂-induced ocean acidification (OA) altered dopamine-mediated fish behavior.
- 31 • The dopamine D₁-receptor agonist SKF 38393 increased anxiety in control fish.
- 32 • OA-exposed fish exhibited maximally measurable anxiety levels.
- 33 • CO₂/pH measured in reef crevasses used as fish shelters were similar to OA tested
34 here.
- 35 • The implications of OA on fish fitness should be assessed through future studies.

36

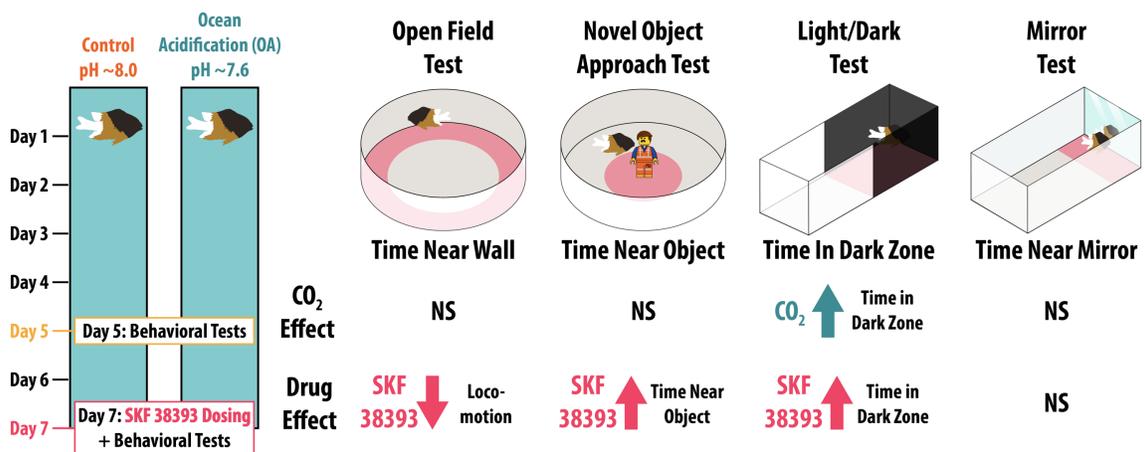
37 **Abstract:**

38 We investigated whether CO₂-induced ocean acidification (OA) affects dopamine
39 receptor-dependent behavior in bicolor damselfish (*Stegastes partitus*). Damselfish were
40 kept in aquaria receiving flow through control (pH~8.03; pCO₂~384 μatm) or OA
41 (pH~7.64; CO₂~1,100 μatm) seawater at a rate of 1 L min⁻¹. Despite this relatively fast
42 flow rate, fish respiration further acidified the seawater in both control (pH ~7.88; pCO₂
43 ~595 μatm) and OA (pH ~7.55; pCO₂~1,450 μatm) fish-holding aquaria. After five days
44 of exposure, damselfish locomotion, boldness, anxiety, and aggression were assessed
45 using a battery of behavioral tests using automated video analysis. Two days later, these
46 tests were repeated following application of the dopamine D₁ receptor agonist SKF
47 38393. OA-exposure induced ceiling anxiety levels that were significantly higher than in
48 control damselfish, and SKF 38393 increased anxiety in control damselfish to a level not

49 significantly different than that of OA-exposed damselfish. Additionally, SKF 38393
 50 decreased locomotion and increased boldness in control damselfish but had no effect in
 51 OA-exposed damselfish, suggesting an alteration in activity of dopaminergic pathways
 52 that regulate behavior under OA conditions. These results indicate that changes in
 53 dopamine D₁ receptor function affects fish behavior during exposure to OA. However,
 54 subsequent measurements of seawater sampled using syringes during the daytime (~3-4
 55 pm local time) from crevasses in coral reef colonies, which are used as shelter by
 56 damselfish, revealed an average pH of 7.73 ± 0.03 and pCO₂ of $925.8 \pm 62.2 \mu\text{atm}$; levels
 57 which are comparable to Representative Concentration Pathway (RCP) 8.5 predicted end-
 58 of-century mean OA levels in the open ocean. Further studies considering the immediate
 59 environmental conditions experienced by fish as well as individual variability and effect
 60 size are required to understand potential implications of the observed OA-induced
 61 behavioral effects on damselfish fitness in the wild.

62

63 **Mandatory Graphical Abstract**



64

65

66 **Key words: Dopamine receptor, hypercapnia, carbon dioxide, CO₂, ocean**
67 **acidification, scototaxis, *Stegastes partitus***

68

69 **Introduction**

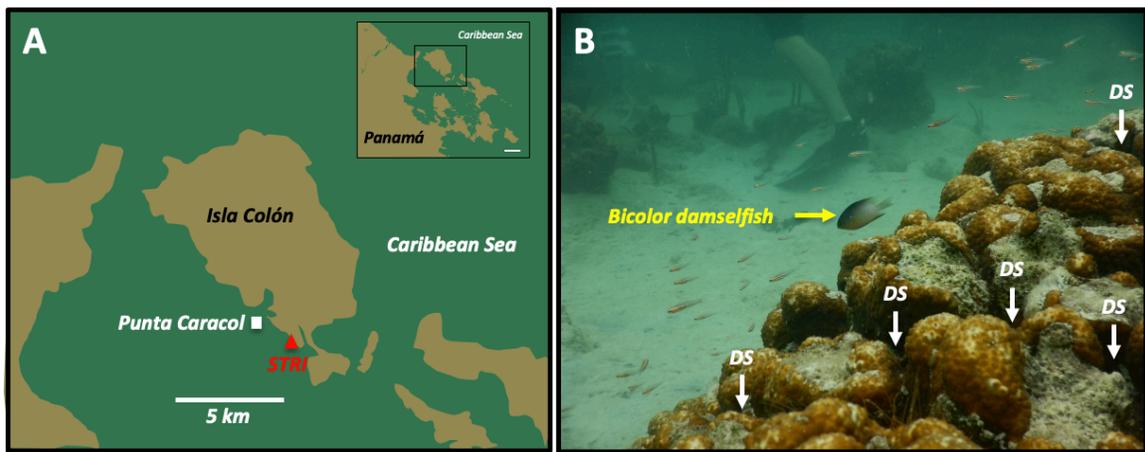
70 Ocean acidification (OA) is a global phenomenon whereby increased absorption of
71 human-generated (anthropogenic) atmospheric CO₂ into the ocean causes a decrease in
72 seawater pH. Average pCO₂ in the open surface ocean is predicted to increase from
73 current levels of ~400 uatm to ~1100 uatm with associated pH decreases from ~8.10 to
74 ~7.60 by the year 2100 under IPCC AR6 Shared Socioeconomic Pathway SSP5-8.5
75 (IPCC 2021, Arias et al 2021, Lee et al 2021). These changes are much more rapid than
76 any natural geological fluctuations of pH in the global ocean, raising concerns about the
77 fate of marine organisms under future OA conditions. Among these concerns are the
78 effects of OA on fish physiology and behavior (reviewed in Esbaugh 2018). While the
79 effect sizes (Clark et al 2020; Clements et al 2022), environmental relevance (Hamilton et
80 al 2021a), and potential mitigating factors such as natural environmental variability
81 (Jarrold et al., 2017) are under scrutiny, it is clear that OA-exposure can affect fish
82 behavior to some extent (e.g. Hamilton et al 2014; Clark et al 2020a,b; Munday et al.,
83 2020a,b).

84 To date, the leading mechanism to explain OA-induced fish behavior alteration
85 involves neuronal GABA_A receptors that are activated by the neurotransmitter gamma-
86 aminobutyric acid (GABA). This “GABA_A receptor hypothesis” proposes that blood
87 acid-base regulation during exposure to OA results in a change in the concentrations of
88 bicarbonate (HCO₃⁻) and chloride (Cl⁻) ions in the internal fluids of the fish, which

89 depolarizes the neuronal plasma membrane and shifts the action of GABA from
90 inhibitory to excitatory (Nilsson et al., 2012; reviewed in Tresguerres and Hamilton,
91 2017). Other neuronal mechanisms have been proposed (Tresguerres and Hamilton, 2017,
92 Heuer et al 2019); however, the “GABA_A receptor hypothesis” remains the only
93 mechanism supported by experimental data (Nilsson et al., 2012; Hamilton et al., 2014).

94 Unlike ionotropic GABA_A receptors which form an ion channel pore in the cell
95 membrane, dopamine receptors are G-protein coupled receptors (GPCRs) meaning they
96 initiate intracellular transduction via G-proteins (Beaulieu & Gainetdinov, 2011).
97 However, dopamine and GABA_A receptors display complex interactions both in
98 mammals (Michaeli and Yaka, 2009; Diaz et al., 2011) and in fish (Hoerbelt et al., 2015).
99 Interestingly, some dopaminergic neurons are inhibited downstream of GABA_A receptor
100 activation (Tepper and Lee, 2007; Garbutt and van Kammen, 1983), raising the
101 possibility that OA-induced altered GABA_A receptor function results in
102 hyperdopaminergic activity. Supporting this possibility, dopamine receptors can regulate
103 fish boldness (Hamilton et al., 2018, Oliveri and Levin, 2019, Thörnqvist et al. 2019), and
104 in separate studies, OA exposure altered fish boldness as well (Jutfelt et al 2015, Tix et
105 al., 2017). Adding to the complexity, concentrations of dopamine in the midbrain of
106 client fish (*Naso elegans*) are increased with OA, however they are also decreased in the
107 forebrain and hindbrain and decreased in the cleaner fish (*Labroides dimidiatus*) in all
108 brain areas (Paula et al., 2019). Species-specific brain changes are likely to occur with
109 many environmental perturbations, necessitating the study of whether these will result in
110 significant behavioural and ecological impacts.

111 The bicolor damselfish (*Stegastes partitus*; Poey, 1868) inhabits shallow patch reefs
112 throughout the Caribbean Sea, the Florida Keys, and the Bahamas (Allen, 1991), and are
113 a model reef fish species for population ecology studies (Goldstein et al., 2016).
114 Damselfish are abundantly found in Punta Caracol, a coastal shallow reef in the Bocas
115 Del Toro province, in the Caribbean coast of Panama. Similar to other damselfishes, *S.*
116 *partitus* is extremely territorial (Allen, 1991; Schmale, 1981) and it is often observed
117 lurking inside or in the vicinity of small crevasses on the reef (Fig. 1), which theoretically
118 affords more effective sheltering from predators (Nemeth, 1998).



119
120 **Figure 1. Study site and natural habitat of the bicolor damselfish.** A. Eastern part of
121 Bocas del Toro province in the Panamanian Caribbean. Punta Caracol: coral reef site
122 where the damselfish were collected. STRI: Smithsonian Tropical Research Institute
123 where behavioral testing was conducted. B. A bicolor damselfish (*Stegastes partitus*) on
124 an *Orbicella franksi* coral patch at Punta Caracol. Notice the complex substate
125 architecture and the numerous crevasses that serve as shelter for damselfish and other reef
126 organisms. DS= damselfish shelter.
127

128 Here, we investigated the effects of OA on the behavior of bicolor damselfish and the
129 potential involvement of dopaminergic activity. We selected tests for behaviors with
130 known connections to dopaminergic activity and with relevance to the behavior of
131 damselfish in the wild (i.e. open field test, novel object approach test, light/dark test,
132 mirror-approach test). To specifically investigate the involvement of D₁-receptors, we
133 used the agonist SKF 38393. We established baseline behavior of each fish on day 5 of
134 control or OA-exposure, and repeated experiments again on day 7 following exposure to
135 SKF 38393 while keeping track of the identity of each fish.

136

137 **Material and Methods**

138 *Damselfish collection*

139 *S. partitus* (n=27) were caught from Punta Caracol reef near Bocas del Toro (Panama)
140 in November, 2016; experiments were approved by the Institutional Animal Care and Use
141 Committee at STRI (protocol 2016-1020-2019-A1). Damselfish were captured while
142 SCUBA diving using clove oil (100 mg L⁻¹) as an immobilizing agent and hand nets.
143 Each captured damselfish was placed in an individual container underwater, taken to the
144 boat, and transported to the Smithsonian Tropical Research Institute Bocas del Toro
145 Research Station (STRI-Bocas) located about 5 km away.

146

147 *Aquarium conditions and experimental exposures*

148 The STRI-Bocas aquarium system provided flow-through seawater at a rate of 1 L
149 min⁻¹ with natural temperature (29.5 ± 0.5 °C), light, and photoperiod (12:12 h). Each
150 damselfish was placed into an individual 3.5 l tank (to prevent conspecific aggression)

151 that contained a piece of white 10 x 2.54 cm polyvinyl chloride piping (to serve as
152 shelter). Damselfish were fed once daily with food pellets (Omega One sinking mini-
153 pellets, OmegaSea Ltd., USA) after 15.00 h.

154 Damselfish were acclimated to the STRI-Bocas aquarium for at least 10 days prior to
155 random allocation to control (n=14) or OA (n=13) groups. Average length and weight
156 were 5.16 ± 0.14 cm and 3.08 ± 0.24 g respectively, with no significant differences
157 between fish from control and OA treatments. OA-like conditions were generated in four
158 200 L sumps receiving flowing seawater and equipped with a pH-stat system (IKS,
159 Karlsbad, Germany) that continuously measured pH and injected CO₂ to achieve the
160 target level (Kwan et al., 2017; Hamilton et al., 2014). The IKS system additionally
161 monitored temperature every 1 min. Each sump supplied OA-seawater to three 3.5 L
162 tanks, each housing an individual fish. The control treatment received unmodified
163 seawater from the STRI aquarium system. Seawater pH was additionally monitored with
164 a handheld sensor (HACH HQ40d with pH probe PHC101) at least twice per day.
165 Salinity (33.6 ppt) and dissolved oxygen (>86 % saturation) were measured on days 1, 4,
166 5, 6, and 7 using a handheld multimeter (YSI Pro2030, Xylem Inc., Washington D.C.,
167 USA). Discrete seawater samples were taken for Total Alkalinity (TA) measurements in
168 250 ml borosilicate glass bottles on 11/13/2016 and 11/14/2016 around 17:00 in every
169 sump as well as in four randomly selected control and four OA tanks. Samples were
170 immediately poisoned with 100 μ L of saturated HgCl₂ solution. TA samples were
171 analyzed in the Scripps Coastal and Open Ocean Biogeochemistry lab within 12 months
172 of collection. TA was measured via a potentiometric open-cell acid titration system
173 developed by the laboratory of A. Dickson at Scripps Institution of Oceanography (SIO).

174 Precision of TA ($\pm 1.6 \mu\text{mol kg}^{-1}$, $n=15$) was determined with respect to certified
 175 reference material provided by the laboratory of A. Dickson. Seawater temperature,
 176 salinity and TA were used to calculate pH and $p\text{CO}_2$ using CO2SYS (v.2.1) with
 177 dissociation constants from Mehrbach et al (1973) as refitted by Dickson and
 178 Millero (1987) (Table 1). Five additional discrete seawater samples were collected while
 179 SCUBA diving (2-3m depth) at Punta Caracol reef between 15.00-16.00 h on November
 180 14, 2016: four were from four crevasses (“damselfish shelter”) on separate *O. franski*
 181 coral patches, and the remaining was environmental seawater sampled over a sandy patch
 182 approximately 5m away from the nearest coral colony. Seawater was drawn into 60 mL
 183 syringes connected to 30 cm-long surgical tubing and treated with HgCl_2 immediately
 184 upon return to the boat following best practices (Dickson et al., 2007). These samples
 185 were processed identical to aquarium samples described above.

186

187 **Table 1. Chemistry of discrete seawater samples in experimental sumps, animal**
 188 **tanks, and field.** Values are mean \pm S.E.M from four independent samples except for
 189 environmental seawater (SW_{env}) ($n=1$).

	Sumps		Fish Tank		Field (3m)	
	Control	OA	Control	OA	SW_{env}	Damselfish Shelter
pH	8.03 \pm 0.01	7.64 \pm 0.01	7.88 \pm 0.01	7.55 \pm 0.02	8.03	7.73 \pm 0.03
Alkalinity ($\mu\text{mol kgSW}^{-1}$)	2202.1 \pm 2.0	2208.7 \pm 2.1	2195.8 \pm 2.3	2222.9 \pm 1.3	2443.9	2251.1 \pm 27.3
$p\text{CO}_2$ (μatm)	384.1 \pm 12.2	1132.5 \pm 30.0	595.4 \pm 22.2	1453.7 \pm 71.7	400.2	925.8 \pm 62.2

190

191

192 *Behavioral testing*

193 Behavioral testing took place on days 5 and 7 of exposures between 9:00 and 15:00 h.
194 While still in their individual tank, damselfish were transported to a modified intermodal
195 container that served as the behavioral testing room. The behavioral arenas were filled to
196 7 cm height with seawater from their respective treatment (control or OA). Seawater was
197 replaced before testing each fish, which prevented blind execution of behavioral testing.
198 This was compensated by blocking the view of the researcher during the tests with white
199 corrugated plastic surrounding the arenas and by video recording of each trial and
200 automatized behavioral analyses as described below. Arenas were rotated every second
201 trial to compensate for potential residual visual and confounding auditory signals. After
202 30 min of acclimation to the testing room, damselfish were gently netted from their tank
203 and placed into an arena for the open field and novel object tests. Damselfish were then
204 transferred via net to the light/dark test arena, and finally transferred via net to the
205 mirror/aggression arena. Each test lasted 5 min (300 sec) and were run in succession.
206 Damselfish were then immediately returned to their original tank in the flow-through
207 aquarium. Two days later, each damselfish underwent the identical behavioral test battery
208 after 30-min exposure to 10 mg/L SKF 38393 (Abcam, Eugene, OR) as previously
209 described (Hamilton et al., 2017).

210 Damselfish behavior was recorded using a FireWire 400 Colour Industrial Camera
211 with a Tamron CCTV lens (2.8–12mm, f/1.4) positioned 1 m above the arena. The
212 recordings were used to unbiasedly quantify damselfish behavior *ex post facto* using
213 EthoVision motion tracking software system (v.10, Noldus, Leesburg, VA, USA) and are
214 freely available on dryad (doi:10.5061/dryad.7m0cfxpws).

215 Locomotion was assessed based on the total distance the fish moved during each test.
216 Thigmotaxis, boldness, scototaxis, and aggression behaviors were estimated as follows:

217

218 *Open Field Test*

219 The open field test arena consisted of a white plastic circular container (27.5 cm in
220 diameter; Fig. 2A). Damselfish were placed in the center of the arena and allowed to
221 swim freely. Thigmotaxis (the tendency to remain close to the walls, also known as
222 “wall-hugging” behavior) was assessed based on the time fish spent in the zone located 5
223 cm closest to the wall.

224

225

226 *Novel object approach test*

227 The novel object approach test was initiated by placing a multicolored 7 cm tall
228 LEGO® figurine (Fig. 2B) into the center of the arena immediately after the end of the
229 open field test. Boldness behavior was estimated based on the time the damselfish spent
230 in the 5 cm diameter area around the novel object (Dean et al., 2020, Hamilton et al.,
231 2021a,b).

232

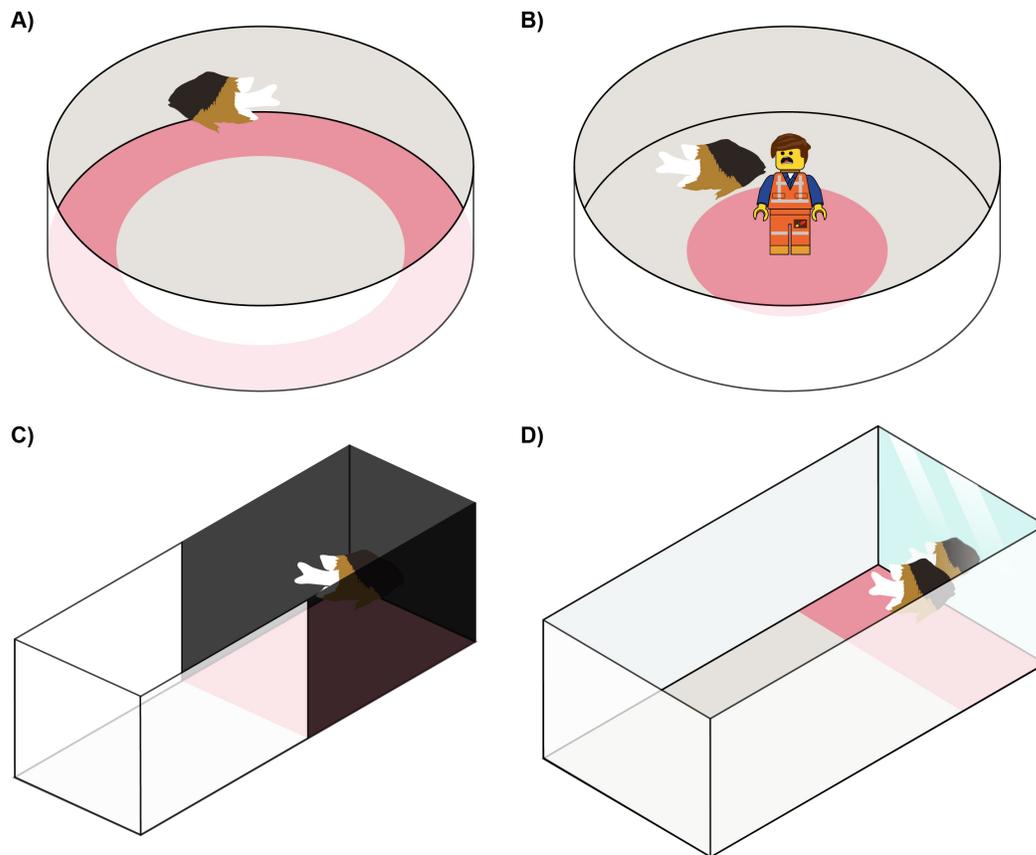
233 *Light/dark preference test*

234 This test (also known as the scototaxis test) took place in a rectangular arena (32 x 13
235 cm) lined with white and black plastic such that half of the arena had black white walls
236 and the other half had white walls (Fig. 2C). Anxiety-like behavior was assessed based on
237 the time the damselfish spent in the dark zone of the arena (Holcombe et al., 2013,
238 Hamilton et al., 2014a,b).

239

240 *Mirror aggression test*

241 This test was conducted in a rectangular arena (32 x 22 cm) with a mirror at one end
242 (Fig. 2D). Aggression behavior was assessed based on the time the damselfish spent in
243 the zone within 5 cm from the mirror (da Silva-Pinto et al., 2020).



244

245

246 **Figure 2. Arenas used in the various behavioral tests. A)** Open field arena to test
247 thigmotaxis; **B)** novel-object approach arena to test boldness; **C)** light/dark preference
248 arena to test scototaxis; and **D)** mirror aggression test. The areas in each arena relevant
249 for the respective behavioral test (i.e. thigmotaxis, boldness, scototaxis, aggression) are
250 highlighted in pink.

251

252 ***SKF 38393 treatment***

253 Exposure to the selective D₁ receptor agonist SKF 38393 (Sibley et al., 1982;
254 Abcam, Eugene, OR) was performed as previously described (Hamilton et al., 2017).
255 Briefly, SKF 38393 was dissolved in fresh water and DMSO (Sigma Aldrich, St. Louis,
256 MO, USA) and mixed into 500 mL of OA or control seawater to a final concentration of
257 10 mg/L (30 µM). An equivalent amount of DMSO was administered as vehicle control.
258 Fish were carefully netted from their housing tank and placed in the dosing container for
259 30 minutes, which was surrounded by corrugated plastic that blocked visual stimuli and
260 prevented the fish from leaping out. Immediately after dosing, fish were placed into the
261 open field arena for the behavioral test battery as described above.

262

263 ***Statistical analysis***

264 Statistical tests were performed in R (version 4.0.3; R Development Core Team,
265 2013). Behavioral parameters were analyzed using linear mixed-effects model fit by
266 restricted maximum likelihood (REML) using the package *nlme* (Pinheiro et al., 2014),
267 with “CO₂ treatment” and “SKF 38393” as factors, and “individuals” as random effects.
268 Post-hoc tests were conducted using least-square means using the package *emmeans*
269 (Lenth et al., 2021). Additional statistical details for the analyses are reported in
270 Supplemental Material 1. Shapiro-Wilks and Levene’s test were used to test the
271 assumption of normality and homoscedasticity, respectively. The arcsine-square root
272 transformation was used on proportion datasets that failed to meet assumptions of
273 normality. Bonferroni Correction was used to limit Type I error, thus an alpha of 0.025

274 was used in our behavior analyses (two parameters tested per behavioral test). Values are
275 reported as mean \pm s.e.m.

276

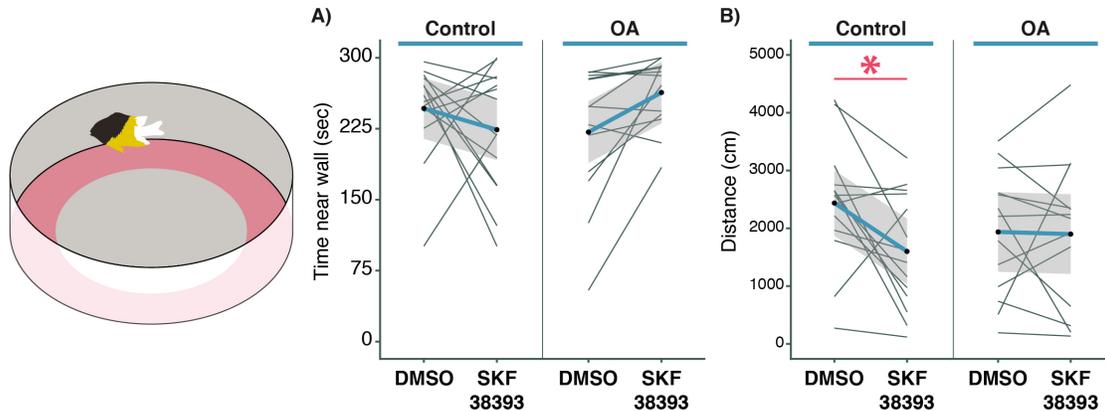
277 **Results**

278 **Open Field Test**

279 The open field test is routinely used to quantify fish thigmotaxis and locomotion (Prut
280 and Belzung, 2003). There were no significant differences in average thigmotaxis
281 between control and OA-exposed damselfish ($p=0.3174$; Fig. 3A). Interestingly, SKF
282 38393 induced opposite thigmotaxis average tendencies in control and OA-exposed
283 damselfish that were driven by a strong decrease effect on seven control fish compared to
284 only one OA-exposed fish in which thigmotaxis was only mildly decreased. However, the
285 responses of the other damselfish to SKF 38393 were diverse (i.e. they did not change or
286 change to varied degrees) and as a result the trends in thigmotaxis between control and
287 OA-exposed fish were not statistically significant ($p=0.4306$; Fig. 3A).

288 There were no differences in average locomotion between control and OA-exposed
289 damselfish ($p=0.2557$); however, SKF 38393 induced differential effects whereby it
290 significantly decreased average locomotion in control damselfish ($p=0.0125$) without
291 significantly affecting OA-exposed damselfish ($p=0.9091$; Fig. 3B). Examination of
292 individual fish revealed a strong SKF 38393-induced decrease effect in nine control
293 damselfish compared to only three OA-exposed damselfish. The other damselfish had
294 varied responses to SKF 38393, including a much milder decreases, no change, or an
295 increase in locomotion (Fig. 3B).

296



297

298 **Figure 3. Open field test.** Effects of exposure (Control, ocean acidification [OA]) and
 299 drug (DMSO, SKF 38393) on **A)** time near wall and **B)** locomotion. $\alpha = 0.025$. Red
 300 asterisk = significant drug effect. Grey area denotes 95% confidence interval.

301

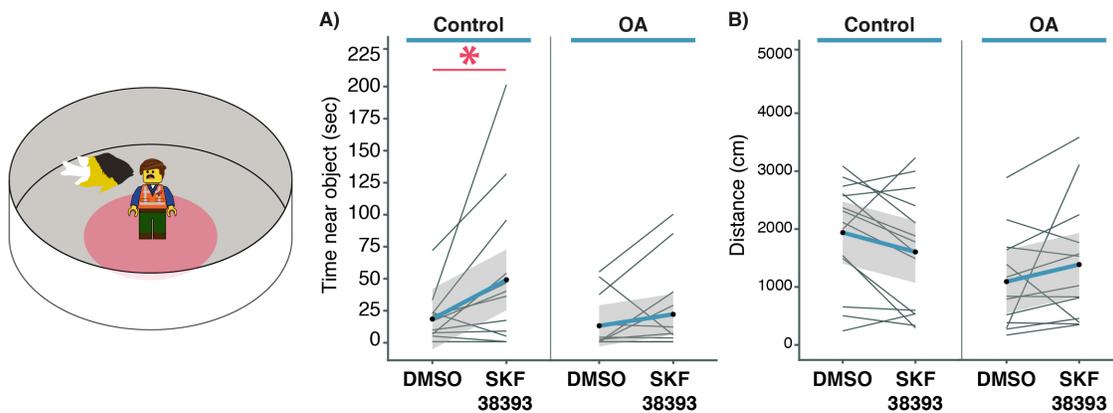
302

303 **Novel Object Approach Test**

304 This test examines the level of ‘exploratory behavior’ or ‘boldness’ of a fish (Toms et
 305 al., 2010). Average boldness behavior was not significantly different between control and
 306 OA-exposed fish ($p=0.2609$); however, SKF 38393 increased average boldness only in
 307 control fish ($p=0.0189$; Fig. 4A). This effect was fairly homogenous in control
 308 damselfish, as 10 out of the 13 specimens displayed increased boldness, one had no
 309 change, and two experienced a very mild decrease. In contrast, SKF 38393 induced a
 310 relatively milder increase in boldness in OA-exposed damselfish in only five out of 12
 311 specimens while it had no effect on five others and induced a decrease in boldness in the
 312 remaining two.

313 There were no differences in average locomotion between control and OA-exposed
 314 damselfish, and locomotion was not significantly affected by SKF 38393 ($p=0.0313$; Fig.

315 4B). But similar to the open field test, SKF 38393 induced seemingly differential effects
 316 in control (a general decrease in locomotion) versus OA-exposed fish (a general increase
 317 in locomotion) in the novel approach test.
 318



319
 320 **Figure 4. Novel object approach test.** Effects of exposure (Control, ocean acidification
 321 [OA]) and drug (DMSO, SKF 38393) on **A)** time near novel object and **B)** locomotion. α
 322 = 0.025. Red asterisk = significant drug effect. Grey area denotes 95% confidence
 323 interval.

324

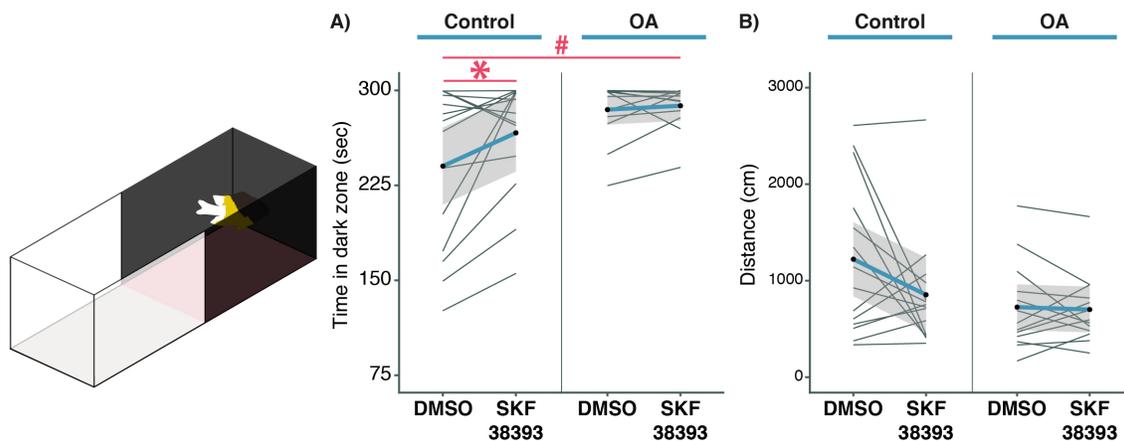
325 Light/dark preference Test

326 This test is used to measure scototaxis (“preference for the dark”), which is the most
 327 sensitive proxy for anxiety-like behavior in fish (Maximino et al., 2010). Both control
 328 and OA-exposed fish spent more than half of the time in the area with dark walls, (all
 329 $p < 0.0002$); however, scototaxis was significantly more pronounced in OA-exposed fish
 330 ($p = 0.0115$; Fig. 5A). SKF 38393 significantly increased scototaxis but only in control
 331 damselfish ($p = 0.0067$) as OA-exposed damselfish already were at a near-ceiling
 332 scototaxis level and it could not become any higher (Fig. 5A). The effects of SKF 38393

333 were consistent at the individual level, inducing stronger effects on damselfish that
334 originally displayed less scototaxis, and mild to no effects on fish that originally had
335 strong scototaxis.

336 Damselfish locomotion in the light/dark test was not significantly affected by OA
337 exposure ($p=0.035$) or SKF 38393 treatment ($p=0.0312$; Fig. 5B). However, again there
338 was a trend for differential effects of SKF 38393 in control versus OA-exposed fish, with
339 a more pronounced decrease in movement in the control group.

340



341

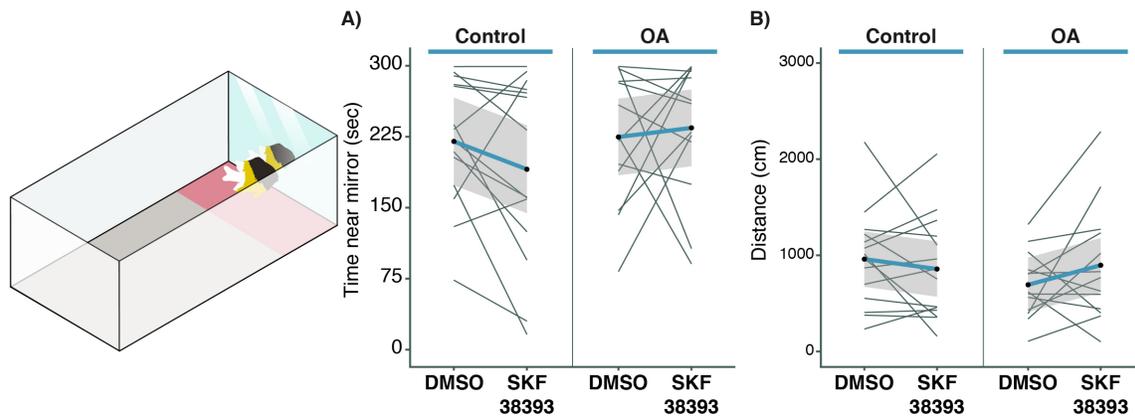
342 **Figure 5. Light/dark preference test.** Effects of exposure (Control, ocean acidification
343 [OA]) and drug (DMSO, SKF 38393) on **A**) time in dark zone and **B**) locomotion. $\alpha =$
344 0.025. # = significant CO₂ effect. * = significant drug effect. Grey area denotes 95%
345 confidence interval.

346

347 **Mirror aggression Test**

348 This test takes advantage of the propensity of certain fishes to react aggressively
349 towards their reflected image, which they perceive as an opponent (Desjardins and
350 Fernald, 2010). There were no significant differences in aggressive behavior between

351 control and OA-exposed fish ($p=0.8773$; Fig. 6A), no significant differences in
 352 locomotion in the mirror test ($p=0.1855$; Fig. 6B), and no significant effect of SKF 38393
 353 on aggression ($p=0.2292$; Fig. 6A) or locomotion ($p=0.4632$; Fig. 6B). However, SKF
 354 38393 induced opposite trends in control and OA-exposed fish, with average decreases in
 355 aggression and locomotion in the former and the opposite trends in the latter.
 356



357
 358 **Figure 6. Mirror aggression test.** Effects of exposure (Control, ocean acidification
 359 [OA]) and drug (DMSO, SKF 38393) on **A)** time near mirror and **B)** locomotion. $\alpha =$
 360 0.025. Grey area denotes 95% confidence interval.

361
 362 **CO₂ and pH levels in natural damselfish shelters**

363 During routine monitoring of pH levels in the aquarium system we noticed that
 364 the seawater pH in tanks housing damselfish tended to be more acidic compared to the
 365 flowing seawater (control) or sumps (OA) suggesting the influence of damselfish
 366 respiration. This prompted us to ask whether the reef crevasses that damselfish typically
 367 use for shelter (Fig. 1) may have different pH/CO₂ levels than the surrounding seawater
 368 resulting from the respiration of reef-associated organisms. To investigate this possibility,

369 we took discrete water samples from four reef crevasses. Upon return to the USA,
370 seawater chemistry measurements revealed that the potential damselfish shelters had an
371 average pH of 7.73 ± 0.03 and $p\text{CO}_2$ of $925.8 \pm 62.2 \mu\text{atm}$ based on early afternoon
372 samples, which were markedly different from the surrounding seawater at the same depth
373 (pH 8.03, $p\text{CO}_2$ 400.2 μatm ; Table 1).

374

375 **Discussion**

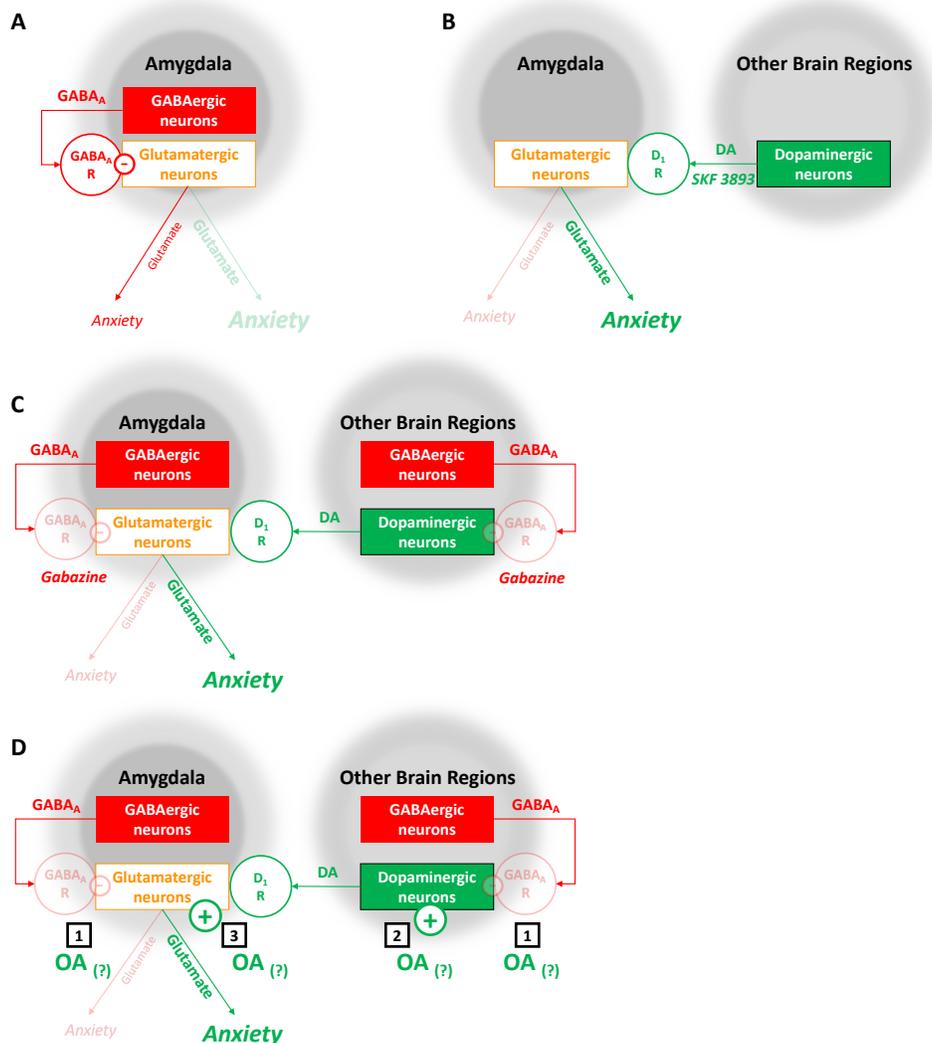
376 When these experiments were performed in 2016, some scientific studies had
377 reported significant negative effects on the behavior of coral reef fishes and expressed
378 concern about far-reaching consequences for fish populations, ecosystems, and fisheries
379 (Munday et al., 2009; Munday et al., 2010; Munday et al., 2012; Nilsson et al., 2012;
380 Chivers et al., 2014). With the aim of investigating these concerns, we conducted
381 behavioral experiments at the STRI-Bocas field station; which is ideally located in a coral
382 reef region and has excellent aquarium facilities. Recent developments (Clark et al 2020;
383 Clements et al 2022) have abridged those initial dooming concerns; however, abundant
384 evidence indicates that exposure to elevated CO_2 levels does alter fish behavior, albeit
385 with more nuanced effects (reviewed in Tresguerres and Hamilton 2017, Esbaugh 2018).
386 Identifying the magnitude of these effects, the underlying mechanisms, and potential
387 implications for fish in the wild thus remains a worthy area of investigation. Furthermore,
388 the vast majority of research about the effect of OA on fish behavior has been conducted
389 with Indo-Pacific fish species, and thus it is important to also study species from other
390 regions.

391 Our experiments revealed increased scototaxis to ceiling levels in control damselfish
392 upon treatment with the D₁-receptor agonist SKF 38393 and ceiling scototaxis levels in
393 OA-exposed damselfish (Fig. 4). Furthermore, the anxiogenic effect of OA-exposure seen
394 in damselfish in the current study was qualitatively identical to that reported for rockfish
395 using the same scototaxis test in a previous study (Hamilton et al 2014). Interestingly,
396 pharmacological stimulation of D₁-receptors in control damselfish (with SKF 38393)
397 induced the same anxiogenic effect as inhibition of GABA_A receptors (with gabazine) in
398 control rockfish. Altogether, this points out to a mechanism that connects GABA_A and
399 dopamine signaling that is altered during OA exposure in a manner that anxiety is
400 increased.

401 The neuronal mechanisms that determine anxiety are complex. Recognizing this
402 complexity is essential for our ability to design future experiments to identify the
403 mechanism(s) that could potentially be affected during OA exposure. The brain of fishes
404 shares many similarities with that of mammals (Porter and Mueller 2020) including an
405 amygdala-like structure with neurons that release glutamate to other brain regions and
406 modulate anxiety: increased glutamate release is anxiogenic and decreased release is
407 anxiolytic. These glutamatergic neurons are negatively modulated by the activity of local
408 circuit GABAergic neurons, and thus GABA_A receptor activity is anxiolytic (Fig. 7A).
409 This is the fundamental mechanism of action of anti-anxiety medication that stimulate
410 GABA_A receptors such as benzodiazepines and barbiturates (Levinthal and Hamilton,
411 2019). In addition, glutamate release by the amygdala is stimulated by dopaminergic
412 innervation from other brain structures, and in turn, those dopaminergic neurons are
413 under the negative regulation of local circuit GABAergic neurons (Fig. 7B) (and thus

414 local GABA_A receptor activity in these dopamine-releasing brain structures decreases
415 anxiety as well). The reported anxiogenic effects of the GABA_A receptor antagonist,
416 gabazine (Hamilton et al 2014), are likely to result from release of inhibition of
417 glutamatergic neurons in the amygdala and of dopaminergic neurons elsewhere in the
418 brain (Fig. 7C).

419 This opens several possibilities for the mechanism that leads to increased anxiety in
420 OA-exposed damselfish (Fig. 7D). It could be due to altered GABA_A receptor
421 functionality in the amygdala, in dopamine releasing areas, or in both (Fig. 7D₁). In
422 addition, the effect of OA may bypass ionotropic GABA_A receptors and affect the D1
423 GPCRs and their G-protein mediated transduction mechanisms (Fig. 7D₂). OA may also
424 affect the amygdala glutamatergic neurons through some other unidentified pathway
425 running in parallel (Fig. 7D₃). Finally, dopamine play roles in peripheral systems
426 including the retina (Missale et al., 1998; Djamgoz & Wagner, 1992), so an effect on
427 vision cannot be ruled out. Importantly, these possibilities are not mutually exclusive, and
428 require experimental verification.



429

430 **Figure 7. Potential neurological mechanisms underlying the effect of ocean**
 431 **acidification on fish behavior. A)** Under basal conditions, local GABA_A in the
 432 amygdala negatively modulates glutamate output and decreases anxiety. **B)** Dopamine
 433 from other brain regions activate D₁ receptor in the amygdala, which positively
 434 modulates glutamate and increases anxiety. **C)** In addition to local circuits in the
 435 amygdala, dopamine release is negatively modulated by local GABA_A receptors. As a
 436 result, the GABA_A receptor antagonist gabazine increases anxiety directly by releasing

437 inhibition on glutaminergic neurons in the amygdala and indirectly by releasing inhibition
438 on dopaminergic neurons in other brain regions. **D)** The effects of ocean acidification on
439 fish behavior may result from alteration of one or many of these anxiogenic pathways,
440 namely (1) inhibition of GABA_A receptors in the amygdala or other brain regions, (2)
441 stimulation of dopaminergic neurons in other brain regions, (3) stimulation of amygdala
442 glutaminergic neurons.

443

444 Although exposure to OA did not significantly affect damselfish thigmotaxis,
445 boldness or aggression, the differential effects of SKF 38393 further supported that OA-
446 exposure altered D₁-receptor mediated behavior. Specifically, SKF 38393 induced a trend
447 for decreased thigmotaxis in control damselfish but for decreased thigmotaxis on OA-
448 exposed damselfish (Fig. 3A), it significantly increased boldness in control damselfish
449 only (Fig. 4A), and induced a trend for decreased aggression in control damselfish but
450 increased aggression in OA-exposed damselfish (Fig. 6A).

451 Interestingly, OA-exposure induced a trend for decreased locomotion in all tests,
452 although the differences were not statistically significant. Furthermore, SKF 38393
453 tended to induce differential responses in locomotion between control and OA-exposed
454 damselfish in all tests, an effect that was most evident in the open field test (significantly
455 decreased locomotion in controls, unchanged in OA-exposed, Fig. 3B). These results
456 highlight that the behavioral tests used in this study rarely yield “all-or-nothing” results
457 and that different tests may be more sensitive to detect specific behaviors. For example,
458 the differential effects of effects of SKF 38393 on locomotion were evident in all tests,
459 but it only reached statistical significance in the open field test (which is specifically

460 design to test locomotion (Royce, 1977)). Similarly, both scototaxis and thigmotaxis can
461 be used as proxies for anxiety; with variable sensitivities depending on the fish species
462 and here, damselfish being more sensitive to the light/dark test. Additionally, the different
463 behavioral responses can have different OA thresholds and that increased OA levels do
464 not necessarily induce linear or additive behavioral responses (Hamilton et al 2021a).

465

466 Another interesting point is that CO₂ and pH levels of coral reef seawater do not
467 necessarily match those at the surface open ocean (Andersson and Mackenzie, 2012).
468 These regional and temporal CO₂/pH variability is increasingly being recognized, and
469 extends to different reef substrates such as sand, soft coral and hard coral (Hannan et al
470 2020). Taking these concepts to the laboratory, cyclic variation in CO₂ to resemble diel
471 fluctuations reduces the behavioral impact of OA (Jarrold et al., 2017). Our results
472 suggest that biological activity during experimentation may affect the microhabitat
473 immediately experienced by reef organisms to an even greater extent. Spot measurements
474 throughout our exposures revealed the damselfish-holding tanks had decreased pH
475 compared to the sumps despite a relatively fast seawater flow of 1 L min⁻¹, which could
476 only be due to CO₂ generated by damselfish respiration. This observation was later
477 corroborated by the discrete water chemistry measurements (Table 1). Similarly,
478 measurements of crevasses on coral colonies revealed higher pCO₂ and lower pH than the
479 adjacent water column; the conditions in these crevasses (~925 μatm, pH ~7.73) were
480 comparable to predicted OA values used in our experiments. Importantly, the crevasse
481 water samples were taken in the afternoon, and the crevasses did not house damselfish at
482 the time of water sampling. Thus, it is likely that this microenvironment experiences even

483 more extreme CO₂/pH levels when housing a damselfish as a result of respiration (as
484 observed in our tanks), and especially at night due where photosynthesis is not available
485 to remove CO₂. As damselfish move between their shelters and the open reef during
486 foraging and other routine activities, they would not be exposed to such pronounced
487 CO₂/pH levels continuously. However, the routine usage of crevasses (Nemeth, 1998;
488 Figure 1) might be associated with a robust ability to regulate blood acid-base
489 homeostasis in bicolor damselfish, perhaps affording them resilience to future OA
490 conditions. Future research is necessary to characterize the CO₂/pH in these and other
491 reef microenvironments in more detail and the blood acid-base regulatory mechanisms of
492 associated coral reef fishes.

493 **Conclusions**

494 The current study presents evidence that exposure to OA alters damselfish anxiety
495 and locomotion behaviors as well as D₁-receptor functioning. However, assessing the
496 relevance of observed behavioral test results to damselfish fitness in the real world is not
497 straightforward, in part because the damselfish immediate environment may experience
498 CO₂/pH levels that are more similar to experimental OA treatments than to the bulk
499 seawater. This raises several questions including: what are “control” CO₂/pH conditions
500 and behavior for damselfish? Do increased preference for dark areas and decreased
501 locomotion have an adaptive value for damselfish in their natural coral reef crevasse
502 shelters, such as affording them better protection from predators? (c.f. Nemeth, 1998). To
503 what extent will OA affect CO₂/pH conditions in the damselfish shelters? And given that
504 anxiety is already at a ceiling during our experimental conditions, can we expect OA to
505 have any further effects? Finally, we would like to highlight that any predictions about

506 effects of OA on organisms ultimately hinges on our understanding of the natural
507 environment and fundamental biology under “control” conditions.

508

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515

516 **Authors' contributions**

517 TJH., MT and DIK. Conceived and executed the study. TJH collected the behavioral
518 data. TJH., MT, BF, and GTK analyzed the data. TC performed seawater chemistry
519 analyses. TJH and MT wrote the manuscript. All authors edited the manuscript and
520 approved the final version.

521

522 **Competing Interests**

523 The authors declare no competing interests.

524

525

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