



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

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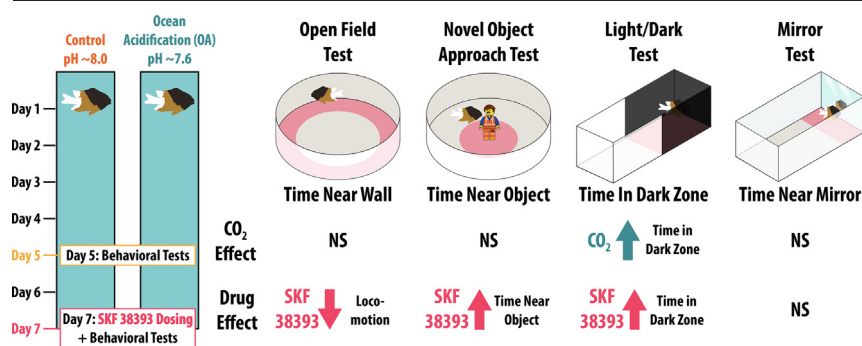
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HIGHLIGHTS

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

GRAPHICAL ABSTRACT



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ABSTRACT

We investigated whether CO₂-induced ocean acidification (OA) affects dopamine receptor-dependent behavior in bicolor damselfish (*Stegastes partitus*). Damselfish were kept in aquaria receiving flow through control (pH ~ 8.03; pCO₂ ~ 384 μatm) or OA (pH ~ 7.64; CO₂ ~ 1100 μatm) seawater at a rate of 1 L min⁻¹. Despite this relatively fast flow rate, fish respiration further acidified the seawater in both control (pH ~7.88; pCO₂ ~ 595 μatm) and OA (pH ~7.55; pCO₂ ~ 1450 μatm) fish-holding aquaria. After five days of exposure, damselfish locomotion, boldness, anxiety, and aggression were assessed using a battery of behavioral tests using automated video analysis. Two days later, these tests were repeated following application of the dopamine D₁ receptor agonist SKF 38393. OA-exposure induced ceiling anxiety levels that were significantly higher than in control damselfish, and SKF 38393 increased anxiety in control damselfish to a level not significantly different than that of OA-exposed damselfish. Additionally, SKF 38393 decreased locomotion and increased boldness in control damselfish but had no effect in OA-exposed damselfish, suggesting an alteration in activity of dopaminergic pathways that regulate behavior under OA conditions. These results indicate that changes in dopamine D₁ receptor function affects fish behavior during exposure to OA. However, subsequent measurements of seawater sampled using syringes during the daytime (~3–4 pm local time) from crevasses in coral reef colonies, which are used as shelter by damselfish, revealed an average pH of 7.73 ± 0.03 and

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pCO₂ of 925.8 ± 62.2 μ atm; levels which are comparable to Representative Concentration Pathway (RCP) 8.5 predicted end-of-century mean OA levels in the open ocean. Further studies considering the immediate environmental conditions experienced by fish as well as individual variability and effect size are required to understand potential implications of the observed OA-induced behavioral effects on damselfish fitness in the wild.

1. Introduction

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

To date, the leading mechanism to explain OA-induced fish behavior alteration involves neuronal GABA_A receptors that are activated by the neurotransmitter gamma-aminobutyric acid (GABA). This “GABA_A receptor hypothesis” proposes that blood acid-base regulation during exposure to OA results in a change in the concentrations of bicarbonate (HCO₃⁻) and chloride (Cl⁻) ions in the internal fluids of the fish, which depolarizes the neuronal plasma membrane and shifts the action of GABA from inhibitory to excitatory (Nilsson et al., 2012; reviewed in Tresguerres and Hamilton, 2017). Other neuronal mechanisms have been proposed (Tresguerres and Hamilton, 2017; Heuer et al., 2019); however, the “GABA_A receptor hypothesis” remains the only mechanism supported by experimental data (Nilsson et al., 2012; Hamilton et al., 2014a).

Unlike ionotropic GABA_A receptors which form an ion channel pore in the cell membrane, dopamine receptors are G-protein coupled receptors (GPCRs) meaning they initiate intracellular transduction via G-proteins (Beaulieu and Gainetdinov, 2011). However, dopamine and GABA_A receptors display complex interactions both in mammals (Michaeli and Yaka, 2010; Diaz et al., 2011) and in fish (Hoerbelt et al., 2015). Interestingly, some dopaminergic neurons are inhibited downstream of GABA_A receptor activation (Tepper and Lee, 2007; Garbutt and van Kammen, 1983), raising the possibility that OA-induced altered GABA_A receptor function results in hyperdopaminergic activity. Supporting this possibility, dopamine receptors can regulate fish boldness (Hamilton et al., 2018; Oliveri and Levin, 2019; Thörnqvist et al., 2019), and in separate studies, OA exposure altered fish boldness as well (Jutfelt and Hedgärde, 2015; Tix et al., 2017). Adding to the complexity, concentrations of dopamine in the midbrain of client fish (*Naso elegans*) are increased with OA, however they are also decreased in the forebrain and hindbrain and decreased in the cleaner fish (*Labroides dimidiatus*) in all brain areas (Paula et al., 2019). Species-specific brain changes are likely to occur with many environmental perturbations, necessitating the study of whether these will result in significant behavioral and ecological impacts.

The bicolor damselfish (*Stegastes partitus*; Poey, 1869) inhabits shallow patch reefs throughout the Caribbean Sea, the Florida Keys, and the Bahamas (Allen, 1991), and are a model reef fish species for population ecology studies (Goldstein et al., 2016). Damselfish are abundantly found in Punta Caracol, a coastal shallow reef in the Bocas Del Toro province, in the Caribbean coast of Panama. Similar to other damselfishes, *S. partitus* is extremely territorial (Allen, 1991; Schmale, 1981) and it is often

observed lurking inside or in the vicinity of small crevasses on the reef (Fig. 1), which theoretically affords more effective sheltering from predators (Nemeth, 1998).

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

2. Material and methods

2.1. Damselfish collection

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

2.2. Aquarium conditions and experimental exposures

The STRI-Bocas aquarium system provided flow-through seawater at a rate of 1 L min⁻¹ with natural temperature (29.5 ± 0.5 °C), light, and photoperiod (12:12 h). Each damselfish was placed into an individual 3.5 L tank (to prevent conspecific aggression) that contained a piece of white 10 × 2.54 cm polyvinyl chloride piping (to serve as shelter). Damselfish were fed once daily with food pellets (Omega One sinking mini-pellets, OmegaSea Ltd., USA) after 15.00 h.

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

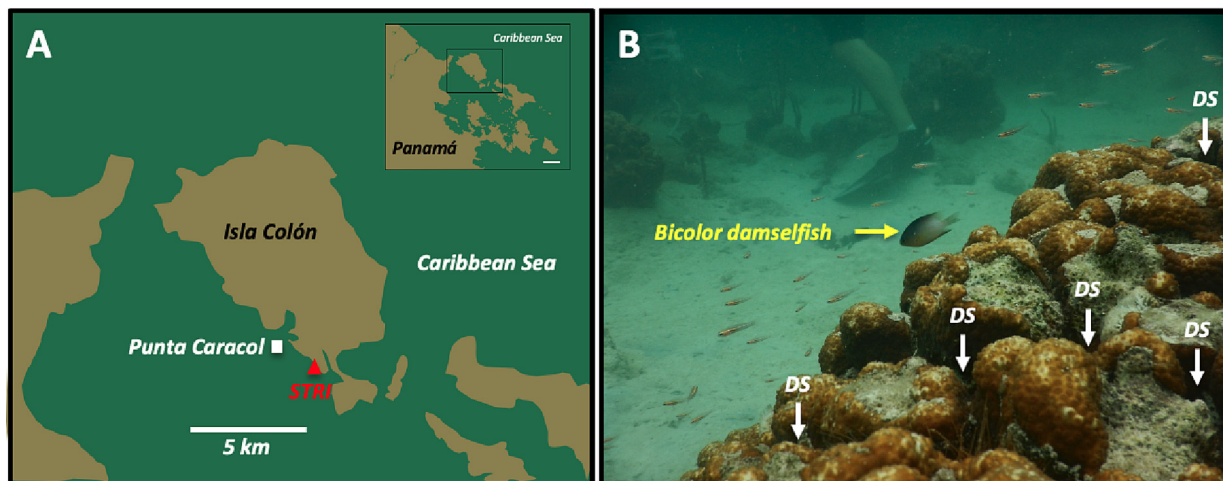


Fig. 1. Study site and natural habitat of the bicolor damselfish. A. Eastern part of Bocas del Toro province in the Panamanian Caribbean. Punta Caracol: coral reef site where the damselfish were collected. STRI: Smithsonian Tropical Research Institute where behavioral testing was conducted. B. A bicolor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

laboratory of A. Dickson at Scripps Institution of Oceanography (SIO). Precision of TA ($\pm 1.6 \mu\text{mol kg}^{-1}$, $n = 15$) was determined with respect to certified reference material provided by the laboratory of A. Dickson. Seawater temperature, salinity and TA were used to calculate pH and pCO_2 using CO2SYS (v.2.1) with dissociation constants from Mehrbach et al. (1973) as refitted by Dickson and Millero (1987) (Table 1). Five additional discrete seawater samples were collected while SCUBA diving (2–3 m depth) at Punta Caracol reef between 15:00 and 16:00 h on November 14, 2016: four were from four crevasses (“damselfish shelter”) on separate *O. franksi* coral patches, and the remaining was environmental seawater sampled over a sandy patch approximately 5 m away from the nearest coral colony. Seawater was drawn into 60 mL syringes connected to 30 cm-long surgical tubing and treated with HgCl_2 immediately upon return to the boat following best practices (Dickson and Millero, 1987). These samples were processed identical to aquarium samples described above.

2.3. Behavioral testing

Behavioral testing took place on days 5 and 7 of exposures between 9:00 and 15:00 h. While still in their individual tank, damselfish were transported to a modified intermodal container that served as the behavioral testing room. The behavioral arenas were filled to 7 cm height with seawater from their respective treatment (control or OA). Seawater was replaced before testing each fish, which prevented blind execution of behavioral testing. This was compensated by blocking the view of the researcher during the tests with white corrugated plastic surrounding the arenas and by video recording of each trial and automatized behavioral analyses as described below. Arenas were rotated every second trial to compensate for potential residual visual and confounding auditory signals. After 30 min of acclimation to the testing room, damselfish were gently netted from their tank and placed into an arena for the open field and novel object tests.

Damselfish were then transferred via net to the light/dark test arena, and finally transferred via net to the mirror/aggression arena. Each test lasted 5 min (300 s) and were run in succession. Damselfish were then immediately returned to their original tank in the flow-through aquarium. Two days later, each damselfish underwent the identical behavioral test battery after 30-min exposure to 10 mg/L SKF 38393 (Abcam, Eugene, OR) as previously described (Hamilton et al., 2017).

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

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

2.4. Open field test

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

2.5. Novel object approach test

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

Table 1

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

	Sumps		Fish Tank		Field (3 m)	
	Control	OA	Control	OA	SW_{env}	Damselfish Shelter
pH	8.03 ± 0.01	7.64 ± 0.01	7.88 ± 0.01	7.55 ± 0.02	8.03	7.73 ± 0.03
Alkalinity ($\mu\text{mol kgSW}^{-1}$)	2202.1 ± 2.0	2208.7 ± 2.1	2195.8 ± 2.3	2222.9 ± 1.3	2443.9	2251.1 ± 27.3
pCO_2 (μatm)	384.1 ± 12.2	1132.5 ± 30.0	595.4 ± 22.2	1453.7 ± 71.7	400.2	925.8 ± 62.2

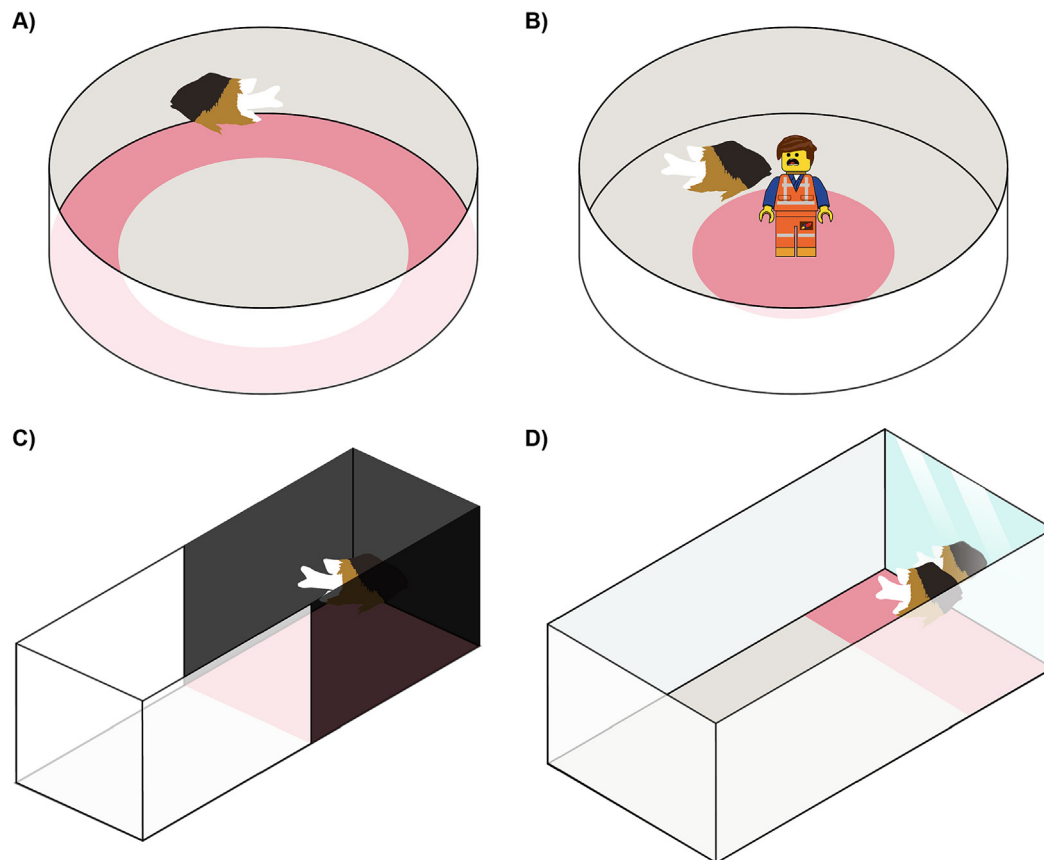


Fig. 2. Arenas used in the various behavioral tests. A) Open field arena to test thigmotaxis; B) novel-object approach arena to test boldness; C) light/dark preference arena to test scototaxis; and D) mirror aggression test. The areas in each arena relevant for the respective behavioral test (i.e. thigmotaxis, boldness, scototaxis, aggression) are highlighted in pink. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.6. Light/dark preference test

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

2.7. Mirror aggression test

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

2.8. SKF 38393 treatment

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

2.9. Statistical analysis

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

3. Results

3.1. Open field test

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

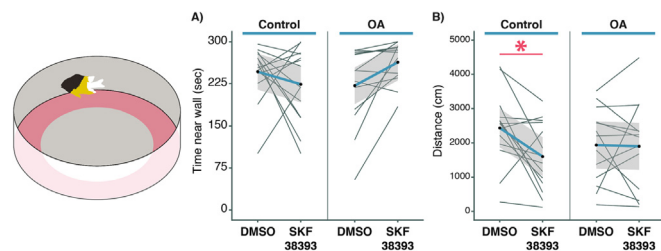


Fig. 3. Open field test. Effects of exposure (Control, ocean acidification [OA]) and drug (DMSO, SKF 38393) on A) time near wall and B) locomotion. $\alpha = 0.025$. Red asterisk = significant drug effect. Grey area denotes 95 % confidence interval. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

thigmotaxis between control and OA-exposed fish were not statistically significant ($p = 0.4306$; Fig. 3A).

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

3.2. Novel object approach test

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

There were no differences in average locomotion between control and OA-exposed damselfish, and locomotion was not significantly affected by SKF 38393 ($p = 0.0313$; Fig. 4B). But similar to the open field test, SKF 38393 induced seemingly differential effects in control (a general decrease

in locomotion) versus OA-exposed fish (a general increase in locomotion) in the novel approach test.

3.3. Light/dark preference test

This test is used to measure scototaxis (“preference for the dark”), which is the most sensitive proxy for anxiety-like behavior in fish (Maximino et al., 2010). Both control and OA-exposed fish spent more than half of the time in the area with dark walls, (all $p < 0.0002$); however, scototaxis was significantly more pronounced in OA-exposed fish ($p = 0.0115$; Fig. 5A). SKF 38393 significantly increased scototaxis but only in control damselfish ($p = 0.0067$) as OA-exposed damselfish already were at a near-ceiling scototaxis level and it could not become any higher (Fig. 5A). The effects of SKF 38393 were consistent at the individual level, inducing stronger effects on damselfish that originally displayed less scototaxis, and mild to no effects on fish that originally had strong scototaxis.

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

3.4. Mirror aggression test

This test takes advantage of the propensity of certain fishes to react aggressively towards their reflected image, which they perceive as an opponent (Desjardins and Fernald, 2010). There were no significant differences in aggressive behavior between control and OA-exposed fish ($p = 0.8773$; Fig. 6A), no significant differences in locomotion in the mirror test ($p = 0.1855$; Fig. 6B), and no significant effect of SKF 38393 on aggression ($p = 0.2292$; Fig. 6A) or locomotion ($p = 0.4632$; Fig. 6B). However, SKF 38393 induced opposite trends in control and OA-exposed fish, with average decreases in aggression and locomotion in the former and the opposite trends in the latter.

3.5. CO₂ and pH levels in natural damselfish shelters

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

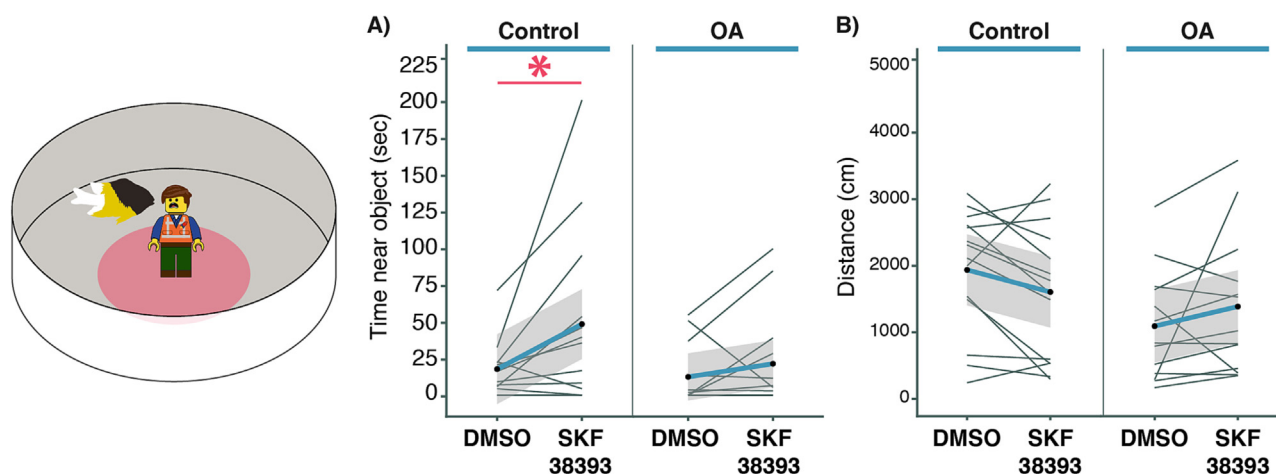


Fig. 4. Novel object approach test. Effects of exposure (Control, ocean acidification [OA]) and drug (DMSO, SKF 38393) on A) time near novel object and B) locomotion. $\alpha = 0.025$. Red asterisk = significant drug effect. Grey area denotes 95 % confidence interval. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

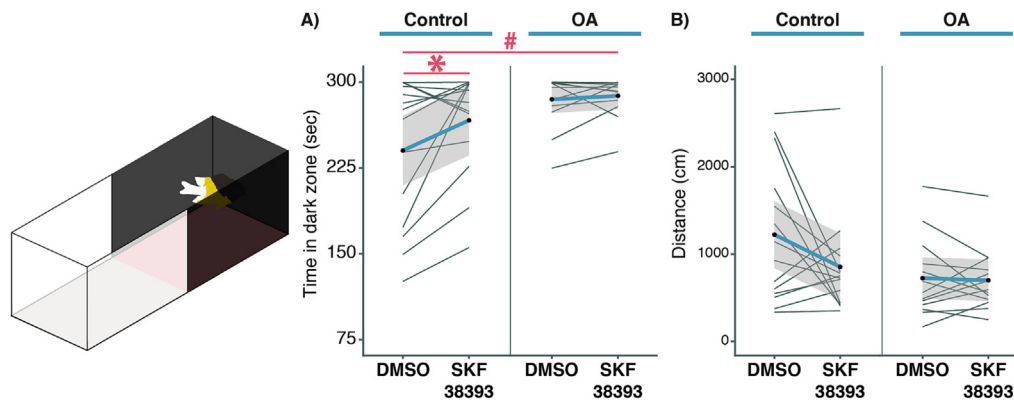


Fig. 5. Light/dark preference test. Effects of exposure (Control, ocean acidification [OA]) and drug (DMSO, SKF 38393) on A) time in dark zone and B) locomotion. $\alpha = 0.025$. # = significant CO_2 effect. * = significant drug effect. Grey area denotes 95 % confidence interval.

this possibility, we took discrete water samples from four reef crevasses. Upon return to the USA, seawater chemistry measurements revealed that the potential damselfish shelters had an average pH of 7.73 ± 0.03 and pCO_2 of $925.8 \pm 62.2 \mu\text{atm}$ based on early afternoon samples, which were markedly different from the surrounding seawater at the same depth (pH 8.03, pCO_2 400.2 μatm ; Table 1).

4. Discussion

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

Our experiments revealed increased scototaxis to ceiling levels in control damselfish upon treatment with the D_1 -receptor agonist SKF 38393 and ceiling scototaxis levels in OA-exposed damselfish (Fig. 4). Furthermore, the

anxiogenic effect of OA-exposure seen in damselfish in the current study was qualitatively identical to that reported for rockfish using the same scototaxis test in a previous study (Hamilton et al., 2014a). Interestingly, pharmacological stimulation of D_1 -receptors in control damselfish (with SKF 38393) induced the same anxiogenic effect as inhibition of GABA_A receptors (with gabazine) in control rockfish. Altogether, this points out to a mechanism that connects GABA_A and dopamine signaling that is altered during OA exposure in a manner that anxiety is increased.

The neuronal mechanisms that determine anxiety are complex. Recognizing this complexity is essential for our ability to design future experiments to identify the mechanism(s) that could potentially be affected during OA exposure. The brain of fishes shares many similarities with that of mammals (Porter and Mueller, 2020) including an amygdala-like structure with neurons that release glutamate to other brain regions and modulate anxiety: increased glutamate release is anxiogenic and decreased release is anxiolytic. These glutamatergic neurons are negatively modulated by the activity of local circuit GABA_A neurons, and thus GABA_A receptor activity is anxiolytic (Fig. 7A). This is the fundamental mechanism of action of anti-anxiety medication that stimulate GABA_A receptors such as benzodiazepines and barbiturates (Levinthal and Hamilton, 2021). In addition, glutamate release by the amygdala is stimulated by dopaminergic innervation from other brain structures, and in turn, those dopaminergic neurons are under the negative regulation of local circuit GABA_A neurons (Fig. 7B) (and thus local GABA_A receptor activity in these dopamine-releasing brain structures decreases anxiety as well). The reported anxiogenic effects of the GABA_A receptor antagonist, gabazine (Hamilton et al., 2014a), are likely to result from release of inhibition of glutamatergic neurons in the amygdala and of dopaminergic neurons elsewhere in the brain (Fig. 7C).

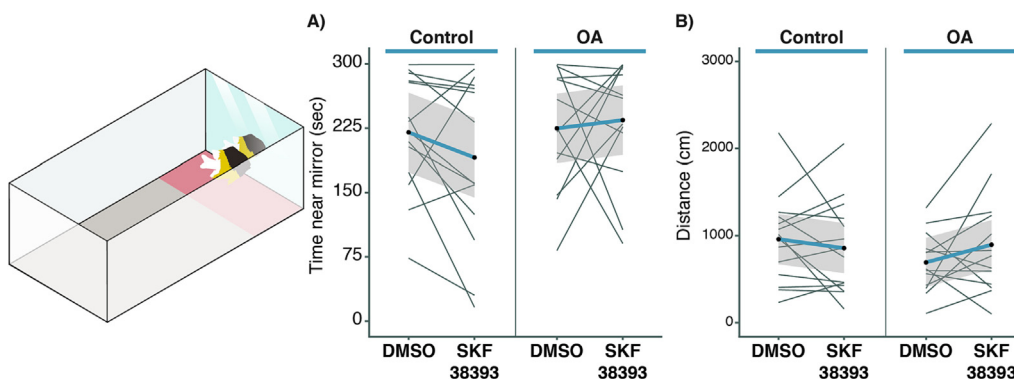


Fig. 6. Mirror aggression test. Effects of exposure (Control, ocean acidification [OA]) and drug (DMSO, SKF 38393) on A) time near mirror and B) locomotion. $\alpha = 0.025$. Grey area denotes 95 % confidence interval.

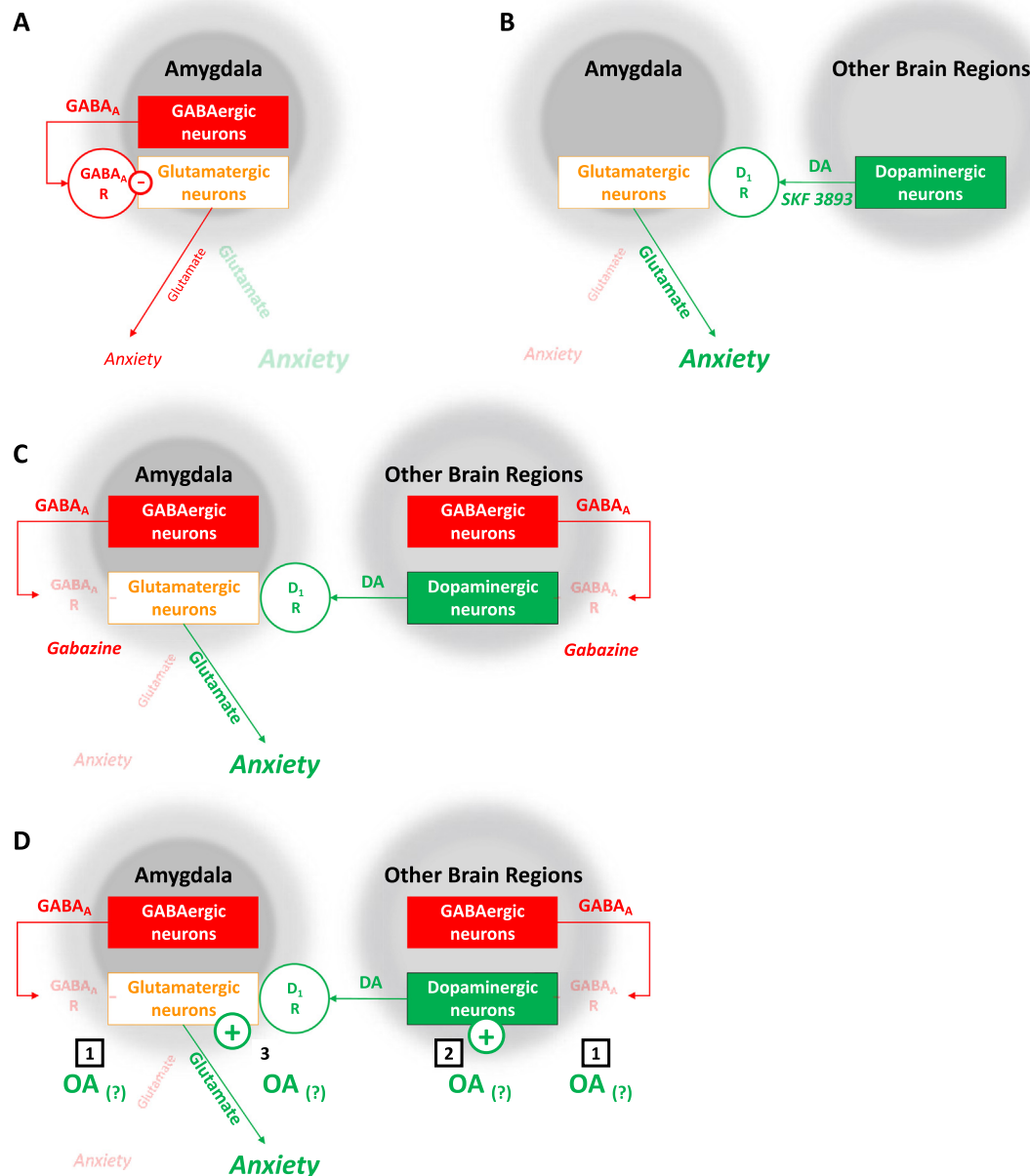


Fig. 7. Potential neurological mechanisms underlying the effect of ocean acidification on fish behavior. A) Under basal conditions, local GABA_A in the amygdala negatively modulates glutamate output and decreases anxiety. B) Dopamine from other brain regions activates D₁ receptor in the amygdala, which positively modulates glutamate and increases anxiety. C) In addition to local dopamine in the amygdala, dopamine release is negatively modulated by local GABA_A receptors. As a result, the GABA_A receptor antagonist gabazine increases anxiety directly by releasing inhibition on glutamatergic neurons in the amygdala and indirectly by releasing inhibition on dopaminergic neurons in other brain regions. D) The effects of ocean acidification on fish behavior may result from alteration of one or many of these anxiogenic pathways, namely (1) inhibition of GABA_A receptors in the amygdala or other brain regions, (2) stimulation of dopaminergic neurons in other brain regions, (3) stimulation of amygdala glutamatergic neurons.

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

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

Interestingly, OA-exposure induced a trend for decreased locomotion in all tests, although the differences were not statistically significant. Furthermore, SKF 38393 tended to induce differential responses in locomotion

between control and OA-exposed damselfish in all tests, an effect that was most evident in the open field test (significantly decreased locomotion in controls, unchanged in OA-exposed, Fig. 3B). These results highlight that the behavioral tests used in this study rarely yield “all-or-nothing” results and that different tests may be more sensitive to detect specific behaviors. For example, the differential effects of effects of SKF 38393 on locomotion were evident in all tests, but it only reached statistical significance in the open field test (which is specifically design to test locomotion (Royce, 1977)). Similarly, both scototaxis and thigmotaxis can be used as proxies for anxiety; with variable sensitivities depending on the fish species and here, damselfish being more sensitive to the light/dark test. Additionally, the different behavioral responses can have different OA thresholds and that increased OA levels do not necessarily induce linear or additive behavioral responses (Hamilton et al., 2021a).

Another interesting point is that CO₂ and pH levels of coral reef seawater do not necessarily match those at the surface open ocean (Andersson and Mackenzie, 2012). These regional and temporal CO₂/pH variability is increasingly being recognized, and extends to different reef substrates such as sand, soft coral and hard coral (Hannan et al., 2020). Taking these concepts to the laboratory, cyclic variation in CO₂ to resemble diel fluctuations reduces the behavioral impact of OA (Jarrold et al., 2017). Our results suggest that biological activity during experimentation may affect the microhabitat immediately experienced by reef organisms to an even greater extent. Spot measurements throughout our exposures revealed the damselfish-holding tanks had decreased pH compared to the sumps despite a relatively fast seawater flow of 1 L min⁻¹, which could only be due to CO₂ generated by damselfish respiration. This observation was later corroborated by the discrete water chemistry measurements (Table 1). Similarly, measurements of crevasses on coral colonies revealed higher pCO₂ and lower pH than the adjacent water column; the conditions in these crevasses (~925 µatm, pH ~7.73) were comparable to predicted OA values used in our experiments. Importantly, the crevasse water samples were taken in the afternoon, and the crevasses did not house damselfish at the time of water sampling. Thus, it is likely that this microenvironment experiences even more extreme CO₂/pH levels when housing a damselfish as a result of respiration (as observed in our tanks), and especially at night due where photosynthesis is not available to remove CO₂. As damselfish move between their shelters and the open reef during foraging and other routine activities, they would not be exposed to such pronounced CO₂/pH levels continuously. However, the routine usage of crevasses (Nemeth, 1998; Fig. 1) might be associated with a robust ability to regulate blood acid-base homeostasis in bicolor damselfish, perhaps affording them resilience to future OA conditions. Future research is necessary to characterize the CO₂/pH in these and other reef microenvironments in more detail and the blood acid-base regulatory mechanisms of associated coral reef fishes.

5. Conclusions

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

Data availability

Data is uploaded on Dryad.

Declaration of competing interest

The authors declare no competing interests.

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CRediT authorship contribution statement

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2023.162860>.

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Supplemental Material

Supplemental Material 1) Statistical details of linear mixed-effects models fit by restricted maximum likelihood (REML). Alpha = 0.025. **Bolded** = statistically significant.

Open Field Test: Time Near Wall - linear mixed-effects models fit by REML

asin(sqrt(time near wall)) ~ treatment + drug + treatment:drug

	Value	Std.Error	DF	t-value	p-value
TreatmentOA	-0.0979417	0.0960062	25	-1.02016	0.3174
DrugSKF	-0.0694245	0.08664885	25	-0.801216	0.4306
TreatmentOA:DrugSKF	0.2706143	0.12487428	25	2.167094	0.0400
	Correlation:				
	(Intr)	TrtmOA	DrgAft		
TreatmentOA	-0.694				
DrugSKF	-0.650	0.451			
TreatmentOA:DrugSKF	0.451	-0.650	-0.694		
Marginal R ²	0.08747564				
Conditional R ²	0.22809850				

Open Field Test: Locomotion - linear mixed-effects models fit by REML

locomotion ~ treatment + drug + treatment:drug

	Value	Std.Error	DF	t-value	p-value
TreatmentOA	-500.2051	429.9618	25	-1.163371	0.2557
DrugSKF	-837.8604	311.093	25	-2.69328	0.0125
TreatmentOA:DrugSKF	800.6424	448.3327	25	1.785822	0.0863
	Correlation:				
	(Intr)	TrtmOA	DrgAft		
TreatmentOA	-0.694				
DrugSKF	-0.521	0.362			
TreatmentOA:DrugSKF	0.362	-0.521	-0.694		
Marginal R ²	0.07113019				
Conditional R ²	0.49502960				

Open Field Test: Locomotion - least square means

locomotion ~ treatment + drug + treatment:drug

Contrast	Value	Std.Error	DF	t-value	p-value
CtrlDMSO – OADMSO	500.2	430	25	1.163	0.2557
CtrlDMSO – CtrlSKF	837.9	311	25	2.693	0.0125
CtrlDMSO - OASKF	537.4	430	25	1.25	0.2229
OADMSO - CtrlSKF	337.7	430	25	0.785	0.4396
OADMSO - OASKF	37.2	323	25	0.115	0.9091

CtrlSKF – OASKF	-300.4	430	25	-0.699	0.4912
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Novel Object Approach Test: Time near Novel Object - linear mixed-effects models fit by REML

asin(sqrt(time near novel object)) ~ treatment + drug + treatment:drug

	Value	Std.Error	DF	t-value	p-value
TreatmentOA	-0.0507404	0.04410738	25	-1.150382	0.2609
DrugSKF	0.06636321	0.0264325	25	2.510667	0.0189
TreatmentOA:DrugSKF	-0.03189	0.03809328	25	-0.837156	0.4104
	Correlation:				
	(Intr)	TrtmOA	DrgAft		
TreatmentOA	-0.694				
DrugSKF	-0.432	0.300			
TreatmentOA:DrugSKF	0.300	-0.432	-0.694		
Marginal R ²	0.12413320				
Conditional R ²	0.67335020				

Novel Object Approach Test: Time near Novel Object - least square means

asin(sqrt(time near novel object)) ~ treatment + drug + treatment:drug

Contrast	Value	Std.Error	DF	t-value	p-value
CtrlDMSO – OADMSO	0.0507	0.0441	25	1.15	0.2609
CtrlDMSO – CtrlSKF	-0.0664	0.0264	25	-2.511	0.0189
CtrlDMSO - OASKF	0.0163	0.0441	25	0.369	0.7154
OADMSO - CtrlSKF	-0.1171	0.0441	25	-2.655	0.0136
OADMSO - OASKF	-0.0345	0.0274	25	-1.257	0.2205
CtrlSKF – OASKF	0.0826	0.0441	25	1.873	0.0728

Novel Object Approach Test: Locomotion - linear mixed-effects models fit by REML

locomotion ~ treatment + drug + treatment:drug

	Value	Std.Error	DF	t-value	p-value
TreatmentOA	-845.8614	370.6386	25	-2.282173	0.0313
DrugSKF	-332.8806	207.6104	25	-1.603391	0.1214
TreatmentOA:DrugSKF	627.5997	299.1985	25	2.097603	0.0462
	Correlation:				
	(Intr)	TrtmOA	DrgAft		
TreatmentOA	-0.694				
DrugSKF	-0.404	0.280			
TreatmentOA:DrugSKF	0.280	-0.404	-0.694		
Marginal R ²	0.09508189				
Conditional R ²	0.70515300				

Light/Dark Test: Time in Dark Zone - linear mixed-effects models fit by REML

time in dark zone ~ treatment + drug + treatment:drug

	Value	Std.Error	DF	t-value	p-value
TreatmentOA	0.1485392	0.05445127	25	2.727929	0.0115
DrugSKF	0.0874286	0.02959573	25	2.954094	0.0067
TreatmentOA:DrugSKF	-0.0771927	0.04265198	25	-1.809826	0.0824
	Correlation:				
	(Intr)	TrtmOA	DrgAft		
TreatmentOA	-0.694				
DrugSKF	-0.392	0.272			
TreatmentOA:DrugSKF	0.272	-0.392	-0.694		
Marginal R ²	0.17012260				
Conditional R ²	0.74540700				

Novel Object Approach Test: Time in Dark Zone - least square means

time in dark zone ~ treatment + drug + treatment:drug

Contrast	Value	Std.Error	DF	t-value	p-value
CtrlDMSO – OADMSO	-0.1485	0.0545	25	-2.728	0.0115
CtrlDMSO – CtrlSKF	-0.0874	0.0296	25	-2.954	0.0067
CtrlDMSO - OASKF	-0.1588	0.0545	25	-2.916	0.0074
OADMSO - CtrlSKF	0.0611	0.0545	25	1.122	0.2724
OADMSO - OASKF	-0.0102	0.0307	25	-0.333	0.7417
CtrlSKF – OASKF	-0.0713	0.0545	25	-1.31	0.2020

Light/Dark Test: Locomotion - linear mixed-effects models fit by REML

locomotion ~ treatment + drug + treatment:drug

	Value	Std.Error	DF	t-value	p-value
TreatmentOA	-496.6463	222.7133	25	-2.22998	0.0350
DrugSKF	-368.4831	161.4488	25	-2.282353	0.0312
TreatmentOA:DrugSKF	343.6785	232.6725	25	1.477091	0.1521
	Correlation:				
	(Intr)	TrtmOA	DrgAft		
TreatmentOA	-0.694				
DrugSKF	-0.522	0.362			
TreatmentOA:DrugSKF	0.362	-0.522	-0.694		
Marginal R ²	0.11826240				
Conditional R ²	0.51882060				

Mirror Test: Time Near Mirror - linear mixed-effects models fit by REML

time near mirror ~ treatment + drug + treatment:drug

	Value	Std.Error	DF	t-value	p-value
TreatmentOA	0.0156425	0.10025423	25	0.156028	0.8773
DrugSKF	-0.0980476	0.07954891	25	-1.232545	0.2292
TreatmentOA:DrugSKF	0.1301604	0.11464218	25	1.135363	0.2670
	Correlation:				
	(Intr)	TrtmOA	DrgAft		
TreatmentOA	-0.694				
DrugSKF	-0.572	0.397			
TreatmentOA:DrugSKF	0.397	-0.572	-0.694		
Marginal R ²	0.04312816				
Conditional R ²	0.37438480				

Mirror Test: Locomotion - linear mixed-effects models fit by REML

locomotion ~ treatment + drug + treatment:drug

	Value	Std.Error	DF	t-value	p-value
TreatmentOA	-266.8159	195.9846	25	-1.361413	0.1855
DrugSKF	-103.0196	138.2804	25	-0.745005	0.4632
TreatmentOA:DrugSKF	306.3895	199.2833	25	1.537457	0.1367
	Correlation:				
	(Intr)	TrtmOA	DrgAft		
TreatmentOA	-0.694				
DrugSKF	-0.508	0.353			
TreatmentOA:DrugSKF	0.353	-0.508	-0.694		
Marginal R ²	0.03631941				
Conditional R ²	0.50180290				