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CO₂-induced ocean acidification increases anxiety in Rockfish via alteration of GABAA receptor functioning

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The average surface pH of the ocean is dropping at a rapid rate due to the dissolution of anthropogenic CO₂, raising concerns for marine life. Additionally, some coastal areas periodically experience upwelling of CO₂-enriched water with reduced pH. Previous research has demonstrated ocean acidification (OA)-induced changes in behavioural and sensory systems including olfaction, which is due to altered function of neural gamma-aminobutyric acid type A (GABAₐ) receptors. Here, we used a camera-based tracking software system to examine whether OA-dependent changes in GABAₐ receptors affect anxiety in juvenile Californian rockfish (Sebastes diploproa). Anxiety was estimated using behavioural tests that measure light/dark preference (scototaxis) and proximity to an object. After one week in OA conditions projected for the next century in the California shore (1125 ± 100 m atm, pH 7.75), anxiety was significantly increased relative to controls (483 ± 40 µatm CO₂, pH 8.1). The GABAₐ-receptor agonist muscimol, but not the antagonist gabazine, caused a significant increase in anxiety consistent with altered Cl⁻ flux in OA-exposed fish. OA-exposed fish remained more anxious even after 7 days back in control seawater; however, they resumed their normal behaviour by day 12. These results show that OA could severely alter rockfish behaviour; however, this effect is reversible.

1. Introduction

A large proportion of anthropogenic CO₂ produced by burning of fossil fuels is readily absorbed by the ocean, lowering oceanic pH in a process called ocean acidification (OA) [1]. Worldwide, the average ocean pH has already decreased by more than 0.1 pH units, which represents a 30% increase in the concentration of hydrogen ions (H⁺). These unprecedented changes in ocean CO₂ partial pressure (pCO₂) and pH levels are predicted to severely impact marine organisms [2]. To date, calcifying phytoplankton and invertebrates such as corals and mollusks have received the most attention due to the potential impact of acidification on carbonate shell integrity and formation [3,4]. By contrast, fish were initially believed to be safe from the effects of OA, as early studies demonstrated a lack of mortality under extremely high CO₂ levels (greater than 10 000 µatm) [5]. However, an increasing body of evidence suggests that OA-relevant CO₂/pH levels induce various sublethal effects in fish, including otolith over-growth [6,7], a shift in behavioural lateralization [8,9], alterations in olfaction that affect detection of cues from substrates, parents [10], prey [11] and predators [8,12,13], and impaired learning [9,14]. Because the gamma-aminobutyric acid type A (GABAₐ) receptor antagonist, gabazine, restores proper discrimination of predator odour, learning of predatory cues [14] and behavioural lateralization in OA-exposed fish [8], at least some of the deleterious effects of OA in fish seem to be related to altered neurotransmitter function. If this model is correct, an alteration of GABA function is bound to have several other profound effects on behaviour owing to the ubiquitous distribution of GABA receptors in the brain.
Activation of the ligand-gated GABA$_A$ receptor is the major inhibitory mechanism in the central nervous system of vertebrate animals, and is involved in reducing anxiety in humans [15], rodents [16] and fish [16,17]. GABA$_A$ receptors are the main target for pharmaceuticals that decrease anxiety (anxiolytics) such as benzodiazepines, which have been found in surface waters at concentrations that can alter food intake, social behaviour and activity levels in fish [18]. The opening of ionotropic GABA$_A$ receptors typically results in a shunting/hyperpolarizing effect on neural excitability via an influx of Cl$^-$ ions from the extracellular to intracellular space. However, the concentration of intracellular Cl$^-$ ions in some immature neurons is higher compared with mature neurons, causing the flow of Cl$^-$ ions to reverse and move outward through GABA$_A$ receptors, thus resulting in depolarization [19]. Similarly, the compensatory response of fish to hypercapnic acidosis involves accumulating HCO$_3^-$ in plasma to buffer pH [20,21], which leads to a decrease in plasma Cl$^-$ concentration (to maintain charge balance). The resulting altered Cl$^-$ concentration gradient between cells and plasma is thought to turn some GABA$_A$ receptors from inhibitory to excitatory, which, if correct, will have profound effects on anxiety.

To date, OA research on behaviour has predominantly been investigated in Australian fish that live close to or on coral reefs, a notable exception being a recent study on three-spined stickleback, a temperate fish [9]. Non-reef fish that live near kelp forest ecosystems are candidates to be especially vulnerable because it has recently been demonstrated that upwelling events can significantly affect seawater chemistry [22]. Short-term fluctuations in ocean dynamics can create localized areas of low pH (approx. 7.8) and higher than normal pCO$_2$ that can reach as high as 820 μatm at shallow depths (7 m) and as high as 1016 μatm at greater depths (17 m) for at least 5–7 days [22]. Therefore, the California coast already regularly experiences CO$_2$-acidified water and will be exposed to more extreme OA conditions earlier than in other locations [23,24].

The aim of this study was to investigate whether CO$_2$ OA alters anxiety in a Californian fish, and whether (consistent with studies on reef fish) OA affects GABA$_A$-receptor function. In addition to the GABA$_A$-receptor antagonist gabazine, we used the GABA$_A$-receptor agonist muscimol to verify the mechanism proposed by Nilsson et al. [8]. Finally, we explored whether any potential effects of OA on rockfish behaviour were reversible after placing rockfish back into normal seawater.

2. Material and methods

Juvenile rockfish (Sebastes diploproa; n = 30; 4.5–6 cm; 1.5–3.2 g) were caught from kelp drifting off the shores of San Diego–La Jolla and maintained in the Scripps Institution of Oceanography flow-through seawater system (where the seawater for the experimental aquarium was pumped from), which is impossible to measure prior to light/dark testing). Data were analysed using GraphPad PRISM v. 4.0B (La Jolla, CA).

(a) Behavioural testing

All testing took place between 9.00 and 18.00. The light/dark arena was 9.5 cm wide by 55 cm long and 9.5 cm deep, and had a white plastic floor; water level was 6.5 cm. The walls of the arena were lined with white or black non-reflective waterproof paper affixed to the sides of the arena with Velcro. Prior to each trial, the arena was rotated 180° to control for potential subtle differences in lighting and refilled with fresh control or high-CO$_2$ seawater. Experimental scototaxic (light/dark preference) testing was performed as previously described [30]. Fish were released into the centre of the arena facing parallel to the long axis to prevent biasing to the light or dark zone. Trials began 1–5 s after fish were released into the arena and lasted 15 min, consistent with other light/dark testing in fish [30–32]. The shelter test arena was 31 cm in diameter and refilled with 10 cm of water prior to each trial with control or high-CO$_2$ seawater. The object in the centre was a 5 cm tall Lego figurine constructed of a variety of colours to avoid any innate colour preference of the fish. Time spent near the object was quantified with a VISION XT motion tracking software. Time spent in zones (light or dark preference) was calculated for each calculated value of pCO$_2$ of 26 μatm. All pCO$_2$ values are reported at average A$_T$. Using these values, CO$_2$ in control and experimental seawater was 483 ± 40 μatm and 1125 ± 100 μatm (mean ± s.e.m.), respectively.

(b) Drug administration

Gabazine (4 μg 1$^{-1}, 10.9 μM$) and muscimol (1 mg 1$^{-1}, 8.8 μM$) were purchased from Sigma (St Louis, MO) and dissolved in 500 ml of seawater (high-CO$_2$ seawater for the CO$_2$ group).
Heat, sonication and gentle stirring were used to dissolve the compounds. Fish were placed in the drug solution for 30 min, and then placed in the experimental arena filled with control or high-CO2 water. Gabazine and muscimol were applied to Ctrl 1 and OA 1 groups on days 9 and 10, respectively. Gabazine is a specific GABA_A receptor antagonist [34] that has become a popular tool to study the effects of OA on fish behaviour [8,14]. Muscimol is a specific agonist of GABA_A receptors [35], including fish GABA_A receptors [36–38]. To the best of our knowledge, this is the first study to use muscimol to characterize the effects of OA on fish behaviour.

(c) Statistical analyses
Normality was tested with either the D’Agostino and Pearson omnibus test or the Kolmogorov–Smirnov test depending on the sample size. Time in the dark zone was analysed with one-sample t-tests or Wilcoxon signed-rank tests to assess statistically significant differences, as commonly used in these types of study [11,30,32,39]. Two-tailed paired and unpaired t-tests and one-way ANOVAs were used for parametric data, and Mann–Whitney U-tests and Kruskal–Wallis tests with Dunn’s multiple comparison post hoc tests for data that were not normally distributed. An α-level of p < 0.05 and 95% confidence intervals were used for assessing statistical significance in all tests. Data were analysed using GraphPad PrisM v. 4.0B. Data are presented as mean ± S.E.M.

3. Results
In our first set of experiments, we tested for scototaxis (light/dark preference), which is a validated behavioural test for anxiety in fish [31]. Control rockfish (n = 15) housed in seawater with present-day CO2 levels for 7 days (483 ± 40 µatm) showed no significant preference for either the light (413 ± 100 s) or dark zone (455 ± 96 s; paired t-test, p = 0.8314; figure 1a). However, rockfish that were exposed to high seawater CO2 (1125 ± 100 µatm) (n = 15) for 7 days demonstrated a significant preference for the dark zone, indicative of increased anxiety (light: 150 ± 53 s; dark: 750 ± 53 s; Mann–Whitney test, p < 0.0001; figure 1a). There were no significant differences in distance moved (control: 1066 ± 122 cm, n = 15; CO2: 1420 ± 304 cm, n = 15; Mann–Whitney test, p = 0.6783; electronic supplementary material, table S1), nor immobility (control: 517 ± 32 s, n = 15; CO2: 485 ± 53 s, n = 15; Mann–Whitney test, p = 0.6924; electronic supplementary material, table S1) between the control and OA groups, suggesting that there were no changes in basic locomotion caused by OA. Next, we administered gabazine to both control and OA fish prior to a second scototaxic test in the same fish (control: n = 7, CO2: n = 8). Because decreased GABA_A receptor activity is correlated with an increase in anxiety in many species, we predicted that gabazine would have a pronounced effect on the control group and not the OA group. Indeed, figure 1 demonstrates that the administration of gabazine resulted in a significant dark preference in the control group (light: 156 ± 124 s; dark: 744 ± 144 s; Mann–Whitney test, p = 0.002) but did not change the dark preference of the OA group (figure 1b).

To examine whether the OA-induced increase in anxiety is reversible, 10-day OA-exposed rockfish were placed into normal seawater and tested for light/dark preference. After 7 days of recovery, we calculated dark preference and still found a robust preference for dark (light: 112 ± 29 s, dark: 788 ± 29 s; n = 7, p < 0.0001; electronic supplementary material, figure S1). However, after 12 days in normal seawater there was a significant shift in behaviour with the previously OA-exposed rockfish no longer displaying dark preference (light: 360 ± 78 s, dark: 540 ± 78 s; n = 7, p = 0.2932; electronic supplementary material, figure S1), suggesting the increase in anxiety is reversible.

To further support the theory that OA is shifting anion flux through GABA_A receptors, we tested whether activation of GABA_A receptors induced a differential effect on the behaviour of control and OA-exposed fish. However, testing this hypothesis required a method that can detect both an
Figure 2. Muscimol produces opposing effects on behaviour. (a) Control and OA-exposed fish were placed in the shelter test arena and their location was recorded for 600 s. Two days later, muscimol (1 mg l⁻¹) was administered for 30 min prior to a second shelter test. The red lines illustrate the movement of one representative fish from each treatment over the trial. (b) There was no difference in time spent near the object for control (180 ± 80 s, n = 7) versus the OA-exposed fish (302 ± 82 s, n = 8; p > 0.05). Muscimol induced a significant difference in time spent near the object (control: 164 ± 98 s, n = 7; OA: 503 ± 60 s, n = 7; p < 0.05; Kruskal–Wallis test (p = 0.0382) with Dunn’s Multiple Comparison post hoc test). Values are mean ± s.e.m. *p < 0.05.

4. Discussion

In this study, we increased CO₂ to levels projected for the start of the twenty-second century and measured the impact on anxiety behaviour in juvenile rockfish. As fish exposed to high CO₂ (1125 ± 100 μatm) demonstrated increased anxiety compared with controls (483 ± 40 μatm), we applied GABA₂-receptor modulators to test the model that OA alters Cl⁻ flux through these receptors (figure 3; see also [8]). Consistent with OA-induced GABA₂-receptor-mediated alteration of olfactory ability and lateralization [8], antagonizing GABA₂ receptors increased anxiety in control rockfish but had no effect on OA-exposed rockfish. Activation of GABA₂ receptors decreased anxiety in controls, but increased anxiety in OA-exposed rockfish.

Until now, anxiety testing in fish has predominantly been performed on freshwater goldfish and zebrafish. Common tests of anxiety in fish include the novel tank test, open field test, light/dark test, predator avoidance task and novel object approach tests [17,31,42]. To date, these tests have not been used with marine fish; however, a recent study has demonstrated a decrease in boldness in sticklebacks (Gasterosteus aculeatus) in a novel object approach test after 20 days of OA exposure [10]. Here, we have shown that control rockfish have no innate preference in the light/dark test under our experimental conditions. This is different from the strong preference for the dark area observed in zebrafish (Danio rerio), tetra (Paracheirodon axelrodi), swordtail (Xiphophorus helleri) and guppy (Poecilia reticulata) [31], but similar to mosquitofish (Gambusia holbrooki) behaviour. Our observed lack of dark preference in control rockfish may be related to the fluctuation in light levels in kelp forests, which is the natural habitat of juvenile rockfish.

The light/dark test has been validated as an appropriate test for anxiety, because pharmacological compounds that reduce anxiety (anxiolytics) have profound effects on location preference. For example, anxiolytics cause zebrafish to switch their preference from the dark to the light zone [43]. In our study, we used the selective GABA₂ antagonist gabazine as an anxiety-generating (anxiogenic) compound, which caused control rockfish to prefer the dark zone, thus establishing
that, for rockfish, dark preference in the light/dark test is indicative of increased anxiety. As OA-exposed rockfish display a strong preference for the dark zone, we conclude that high CO₂ levels result in increased anxiety. Gabazine had no further effect, indicating that the already anxious CO₂ group had reached a ceiling level of anxiety. When tested for recovery in normal seawater the OA-exposed group returned to control levels after 12 days. Any potential habituation to the light/dark test was not investigated in this study; however, previous work in zebrafish suggests that habituation does not occur in the light/dark test even after 5 consecutive days of testing [44]. As we administered the light/dark test on a minimal schedule and fish were rarely handled, and the change in dark preference was robust between recovery days 7 and 12, habituation to the test was unlikely to occur. It is more probable that there was a change in GABAₐ-receptor functioning that explains the absence of dark preference with recovery.

Until now, the role of GABAₐ in OA-induced altered behaviour has been exclusively inferred using the GABAₐ antagonist gabazine [8,14]. However, experimentation with pharmacological compounds can never rule out undesired effects on unspecific targets. As genetic manipulation (e.g. gene knockdown or silencing) is not yet feasible in marine fish, we opted to confirm the involvement of GABAₐ receptors by applying a second, structurally different drug. If OA results in a shift in the equilibrium potential for Cl⁻ (ECl) and reversal of Cl⁻ influx across GABAₐ receptors, the GABAₐ-receptor agonist muscimol should produce opposing effects in OA-exposed compared with control fish (figure 3). The effect of muscimol was tested using the ‘shelter test’, which is designed to measure the amount of time that the fish spends near an object located in the centre of the arena. Both control and experimental fish had the same baseline behaviour, which allowed us to test the hypothesis that activation of GABAₐ receptors leads to opposing behaviours in OA-exposed and control fish. Control fish moved away from the object upon muscimol administration; as muscimol is an anxiolytic, this behaviour is indicative of reduced anxiety in rockfish. On the other hand, OA-exposed fish moved closer to the object, consistent with the anxiogenic effects of OA shown in figure 1. These results clearly indicate that the activation of GABAₐ receptors results in normal anxiolytic action in control fish, but anxiogenesis in OA-exposed fish. All together, these results are consistent with Cl⁻ influx through GABAₐ receptors in control fish, but Cl⁻ efflux in OA-exposed fish (figure 3).

In previous studies [8,14], gabazine restored the OA-induced effects on impaired olfactory discrimination, unlike our results, in which gabazine has an effect only on control fish. Increased activity of olfactory neural circuitry because of a shifted ECl would result in a depolarizing action on GABAₐ receptors leading to impaired olfactory signalling downstream to brain areas that regulate the escape response. The underlying mechanisms are not known in fish but the impaired response may be because of decreased tonic inhibition that mediates olfactory discrimination in the olfactory bulb, as described in mice [45]. Gabazine could then act to suppress the overactivity, resulting in a normal escape response.

GABAₐ receptors are constantly active in the mammalian amygdala, maintaining a tonic state of neuronal inhibition that keeps anxiety at a low behavioural level [46]. Conversely, prolonged antagonism of GABAₐ receptors in this brain area...
removes this tonic inhibition and results in a chronic increased anxiety state [46]. Because the same behavioural response takes place in control rockfish after gabazine administration in our study, we hypothesize that the fear associated with the light area is regulated by brain areas that are analogous to the mammalian amygdala. Therefore, reversal of function of GABA_A receptors via OA or pharmacological antagonism (gabazine) will induce an anxiogenic state (figure 1). This explains why gabazine does not have any further anxiogenic effect on OA-exposed rockfish.

A clear picture is emerging whereby alterations in GABA_A receptor function have profound impacts on fish behaviour. OA has been reported to affect behaviours triggered by olfactory cues such as predator–prey interactions [8,10,11] and homing ability [10]. In addition, contamination of freshwater with wastewater effluents containing the anxiolytic drug oxazepam increases boldness of exposed fish [18]. The apparent shift in GABA_A receptor function due to OA found here in rockfish results in increased anxiety, which has an opposite behavioural effect compared with pollution caused by oxazepam, yet both are a danger to wild fish populations. The proposed mechanism (figure 3) is evident after 7 days of OA exposure; however, the onset and long-lasting consequences are unknown. When OA-exposed rockfish were placed back into seawater containing the present-day CO2 level, they still demonstrated increased anxiety 7 days later, but not 12 days later. Thus, the impact of OA on rockfish anxiety seems to be reversible. This is similar to previous studies on reef fish, which showed restoring of predator avoidance 2 days after placing damselfish larvae in normal seawater [13]. Differences in the time OA-induced changes return to normal could be due to sensitivity or net Cl− flux through various subtypes of GABA_A receptors involved, with more sustained effects on anxiety relative to olfactory circuitry. Species-specific and life stage differences, as well as magnitude of OA, are other potential explanations.

Short-term fluctuations in ocean dynamics, such as upwellings or currents that are characteristic of the southern California near-shore environment, result in low pH and high CO2 levels that are similar to our experimental OA condition. For example, while the pH of seawater is normally approximately 8.05 units and pCO2 is approximately 400 μatm, they can reach approximately 7.70 pH units and 1016 μatm for several days [22] (approx. 5 days in that particular study, but as long as 11 days in other surveys that estimated upwelling from dissolved O2 [47]). These values are very similar to our control (pH: 8.10; pCO2: 483 μatm) and experimental (pH: 7.75; pCO2: 1125 μatm) conditions and duration of the exposure (7 days). Therefore, it is possible that rockfish off the coast of southern California commonly experience increased anxiety during upwelling events.

GABA_A receptors are present in the nervous system of vertebrates and invertebrates, and therefore the effect of OA on anxiety could be widespread. Interestingly, during development GABA receptors are expressed prior to the main excitatory neurotransmitter, glutamate. Giant depolarizing potentials are generated by GABA_A receptors because of the high intracellular Cl− concentration present at birth, but as glutamatergic innervation increases, the intracellular Cl− concentration decreases and GABA then becomes inhibitory [41]. The excitatory action of GABA during development has been identified in every animal studied, including rats, mice, rabbits, frogs, kittens and ferrets [41]. Evolutionarily, it can be assumed that this development-induced shift in excitatory to inhibitory action of GABA_A receptors is also present in fish, although the exact timing during development is unknown. Prior to this shift, the OA-induced shift in ECl− is unlikely to induce any major effects on behaviour. However, as development proceeds and GABA_A receptors become inhibitory, there may be profound consequences of OA on brain physiology. As GABA_A receptors are ubiquitous and act physiologically to dampen excitatory circuitry throughout the brain, there are a multitude of potential neurological effects beyond what has been studied here. For example, in addition to the increased anxiety reported here, epileptic seizures may occur, as reported in the brain of adult mammals containing low GABA levels [41]. All of these effects would greatly influence population dynamics and other ecological interactions (e.g. predator–prey, dispersal). However, it is also possible that longer exposures to OA result in compensatory responses such as GABA_A-receptor internalization or decreased GABA release in juvenile and adult fish, which would ameliorate the anxiolytic effect of OA on fish behaviour.

In summary, prolonged exposure to OA has detrimental effects on the neurophysiology of marine fish, resulting in increased anxiety behaviour. Future studies need to investigate the extent of OA-induced effects both physiologically and behaviourally, how these effects influence fish fitness in the wild, and whether fish are capable of adapting to OA.

All experiments were approved by the SIO-UCSD animal care committee under protocol no. S10320 in compliance with the IACUC guidelines for the care and use of experimental animals.

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References


Table S1: Distance travelled and immobility in the light/dark test. Distance travelled and immobility were calculated for the duration of the 900 second trial in each experimental condition. There were no significant differences between control and OA-exposed groups for distance travelled (Kruskal-Wallis test, $p = 0.1369$) or immobility (One-way ANOVA: $F(4, 47) = 0.8799$, $p = 0.4832$). Values are mean ± s.e.m.

<table>
<thead>
<tr>
<th>Light/Dark</th>
<th>Light/Dark + Gabazine</th>
<th>Light/Dark Dark Recovery 12 days</th>
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<tr>
<td></td>
<td>Distance travelled (cm)</td>
<td>Immobility (s)</td>
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<tr>
<td>Control</td>
<td>1066 ± 122</td>
<td>517 ± 32</td>
</tr>
<tr>
<td>OA</td>
<td>1420 ± 304</td>
<td>485 ± 53</td>
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Table S2: Distance travelled and immobility in the shelter test. Distance travelled and immobility were calculated for the duration of the 600 second trial in each experimental condition. There were no significant differences between control and OA-exposed groups for distance travelled (Kruskal-Wallis test, $p = 0.2624$) or for immobility (Kruskal-Wallis test, $p = 0.5965$). Values are mean ± s.e.m.

<table>
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<th>Shelter Test + Muscimol</th>
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<td>Immobility (s)</td>
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<tr>
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<td>386 ± 55</td>
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<tr>
<td>OA</td>
<td>526 ± 99</td>
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Figure S1

![Bar chart showing time in dark zone (s) for REC-7 and REC-12. The chart indicates a significant difference (***).](image-url)