Altered neurotransmitter function in CO\textsubscript{2}-exposed stickleback (\textit{Gasterosteus aculeatus}): a temperate model species for ocean acidification research

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Studies on the consequences of ocean acidification for the marine ecosystem have revealed behavioural changes in coral reef fishes exposed to sustained near-future CO\textsubscript{2} levels. The changes have been linked to altered function of GABAergic neurotransmitter systems, because the behavioural alterations can be reversed rapidly by treatment with the GABA\textsubscript{A} receptor antagonist gabazine. Characterization of the molecular mechanisms involved would be greatly aided if these can be examined in a well-characterized model organism with a sequenced genome. It was recently shown that CO\textsubscript{2}-induced behavioural alterations are not confined to tropical species, but also affect the three-spined stickleback, although an involvement of the GABA\textsubscript{A} receptor was not examined. Here, we show that loss of lateralization in the stickleback can be restored rapidly and completely by gabazine treatment. This points towards a worrying universality of disturbed GABA\textsubscript{A} function after high-CO\textsubscript{2} exposure in fishes from tropical to temperate marine habitats. Importantly, the stickleback is a model species with a sequenced and annotated genome, which greatly facilitates future studies on underlying molecular mechanisms.

Key words: \gamma\textsuperscript{-}Aminobutyric acid, brain, global change, hypercapnia, lateralization, temperate fish

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Introduction

Burning of fossil fuel continues to increase the atmospheric CO\textsubscript{2} level, widely affecting the ocean chemistry in a process commonly referred to as ocean acidification. According to the fifth assessment report from the Intergovernmental Panel on Climate Change in 2013, the atmospheric CO\textsubscript{2} level may reach 800–1150 µatm within this century (Collins \textit{et al}., 2013), and the resultant increase in oceanic CO\textsubscript{2} levels is now recognized as a serious threat to the marine ecosystem.

Initial experimental studies on the effects of these near-future CO\textsubscript{2} levels on fish behaviour have focused on tropical species, suggested to be more sensitive to carbon chemistry changes owing to their high metabolic rate and therefore high CO\textsubscript{2} exchange with water (Nilsson \textit{et al}., 2012). These studies show that sustained exposure to such near-future CO\textsubscript{2} levels causes an array of sensory and behavioural alterations in coral reef damselfishes. The alterations observed include reversed olfactory and auditory preferences (Munday \textit{et al}., 2009; Dixon \textit{et al}., 2010; Simpson \textit{et al}., 2011), loss of behavioural lateralization (Domenici \textit{et al}., 2012), loss of learning (Ferrari \textit{et al}., 2012), increased boldness and activity (Munday \textit{et al}., 2010) and reduced temporal resolution in the retinal response to light (Chung \textit{et al}., 2014).
It has recently become clear that the behaviour of temperate fish also may be affected by elevated CO$_2$. It was found that the three-spined stickleback (Gasterosteus aculeatus) displays decreased behavioural lateralization, learning, boldness and curiosity when exposed to 990 μatm CO$_2$ (Jutfelt et al., 2013). Moreover, the Californian rockfish (Sebastes diploproa) shows increased anxiety when exposed to 1125 μatm CO$_2$ (Hamilton et al., 2014).

For the coral reef fish, the neural mechanisms causing behavioural abnormalities have been found to involve altered function of the γ-aminobutyric acid (GABA) neurotransmitter, because normal behaviour can be restored effectively by a moderate dose of the specific GABA$_A$-receptor antagonist gabazine (SR-95531; Nilsson et al., 2012; Chivers et al., 2014; Chung et al., 2014). Furthermore, Hamilton et al. (2014) suggested that the increase of anxiety in the Californian rockfish is linked to altered function of the GABA$_A$ receptor.

γ-Aminobutyric acid is the major inhibitory neurotransmitter in vertebrate brains. The GABA$_A$ receptor is an ion channel with conductance for Cl$^-$ and HCO$_3^-$ (Bormann et al., 1987). A net inflow of these negatively charged ions into the postsynaptic neuron will cause hyperpolarization of the neuronal membrane that counteracts depolarizing excitatory input. When exposed to elevated CO$_2$, fish regulate their acid–base balance to avoid acidosis by accumulating HCO$_3^-$, accompanied by a release of H$^+$ and Cl$^-$ to the water (Ishimatsu et al., 2008; Brauner and Baker, 2009). It is likely that such ion- regulatory mechanisms lead to altered transmembrane Cl$^-$ and HCO$_3^-$ gradients in the brain during high-CO$_2$ exposure, which in turn cause a reversal of GABA$_A$-receptor function (Nilsson et al., 2012). It is also likely that the ion-regulatory changes and/or changes in GABA$_A$-receptor function involve complex long-term perturbations, such as altered gene expression, because the behavioural dysfunctions are manifested only after several days of high-CO$_2$ exposure and persist for several days after normal CO$_2$ levels have been restored (Munday et al., 2012).

Our ability to characterize the physiological and molecular mechanisms linking elevated CO$_2$ to altered GABA$_A$-receptor function would be greatly enhanced if studies can be done in a model species with a sequenced genome and well-characterized behaviour, life history and physiology. Clearly, the three-spined stickleback is such a species if it can be shown that the behavioural effects of high-CO$_2$ displayed by this fish are linked to the GABA$_A$ receptor. Consequently, the aim of this study was to test whether the loss of behavioural lateralization seen in high-CO$_2$-exposed stickleback can be reversed by treatment with the specific GABA$_A$-receptor antagonist, gabazine.

**Materials and methods**

**Ethics statement**

The experiment was conducted in accordance with Swedish law and regulations and approved by the Ethical Committee on Animal Experiments in Gothenburg, Sweden (ethical permit numbers 100-2010 and 151-2011).

**Experimental animals**

Adult marine three-spined stickleback (G. aculeatus) were collected using a hand-trawl in the Gullmars Fjord (58° 15.781’ N, 11° 29.815’ E) on the Swedish west coast and kept at the nearby Sven Lovén Centre for Marine Sciences (Kristineberg), University of Gothenburg (Sweden). After being sedated using 2-phenoxyethanol in seawater (0.5 ml l$^{-1}$), fish were tagged using subcutaneous implants of fluorescent elastomer tags (Northwest Marine Tech. Inc., Shaw Island, WA, USA), allowing us to keep track of individual variables measured during the experiment.

Fish were divided into eight groups and transferred to eight 80 l aquaria with either control or high-CO$_2$ water. Initially, four fish were put into each aquarium. During experiments, the fish were kept with a 14 h–10 h light–dark cycle and fed twice a day *ad libitum* with frozen *Artemia* nauplii (Kordon Golden Gate, Hayward, CA, USA). At the beginning of the experiment, fish weight was 1.30 ± 0.230 g and standard length 46.50 ± 2.219 mm (mean ± SD). Subsequent weight and length measures are given in Supplementary Figs S1 and S2.

**Manipulation of CO$_2$**

The eight aquaria (four for each treatment group) were continuously supplied with water from four header tanks, two providing aerated control water and two providing high-CO$_2$ water. The header tanks were supplied with surface seawater pumped from the fjord. Water from the header tanks was gravity-fed through silicone tubing to each holding aquarium at a rate of ~1 l min$^{-1}$. In the high-CO$_2$ tanks, the level of CO$_2$ was regulated by keeping the pH stable near 7.7 with a pH stat (Aqua Medic, Bissendorf, Germany) connected to a solenoid valve controlling the bubbling of the water with CO$_2$. Temperature, pH and partial pressure of CO$_2$ (pCO$_2$) were measured daily from a randomly chosen aquarium from each exposure group. The pCO$_2$ was measured with an infra-red CO$_2$ probe connected to a submerged CO$_2$-permeable membrane (GM70 CARBOCAP; Vaisala, Vantaa, Finland) equipped with an aspiration pump, as described by Munday et al. (2012). Alkalinity was measured every third day by taking a sample of 25 ml from one treatment and one control aquarium. These samples were filtered through a 2 μm filter and analysed in a titration machine (SI Analytics, Mainz, Germany) by stepwise addition of hydrogen chloride. Water salinity measures were provided by the field station’s monitoring of surface water. The measured pCO$_2$ in the treatment aquarium was 992.1 ± 119.3 μatm CO$_2$ and the pH was 7.69 ± 0.057. The control aquarium had a pCO$_2$ of 442.4 ± 71.0 μatm CO$_2$ and a pH of 8.02 ± 0.052 (mean ± SD; Table 1).

**Behavioural lateralization test**

After 40 days of treatment, a double T-chamber was used to evaluate the effect of acidified water on behavioural asymmetry. The chamber measured 50 cm × 50 cm, with a runway 9 cm wide (Fig. 1) and was filled to high 5 cm of water supplied from the holding aquarium from which the fish was taken.
The three-spined stickleback kept in elevated CO₂ demonstrated a complete loss of lateralization. Thus, after 40 days in the experimental aquaria, the high-CO₂ treatment group had a significantly lower \( L_a \) than the control group (Fig. 2b). The \( L_a \) was 33.3 ± 7.2 in control fish (exposed to 442 µatm CO₂) as a result of a significant individual preference for either turning left or right in the lateralization test (but with no population bias because the number of left- and right-turners were approximately equal). In contrast, fish exposed to 992 µatm CO₂ for 40 days showed an \( L_a \) of 13.8 ± 5.0. Also, the \( L_a \) was differently distributed among fish exposed to control conditions and high CO₂ (\( F = 4.15, P = 0.0213 \); Fig. 2a).

After 50 days in the experimental tanks, the fish were tested for lateralization again (Fig. 3), but this time in two subsequent runs, i.e. before and after treatment with gabazine (4 mg l⁻¹ gabazine in seawater for 30 min). In the first run, control fish \( (L_a = 36.3 ± 5.3) \) and high-CO₂ fish \( (L_a = 16.7 ± 3.7) \) showed virtually the same degree of lateralization as they did on day 40, with the suppressive effect of high-CO₂ exposure on lateralization being retained (two-way ANOVA, \( P = 0.0269 \)). In the second run, the lateralization in the high-CO₂ group was completely restored by the gabazine treatment and, as a result, there was no difference between the control group treated with gabazine.
gabazine \((L_a = 34.5 \pm 5.6)\) and the high-CO\(_2\) group treated with gabazine \((L_a = 34.2 \pm 6.3;\) two-way ANOVA, \(P = 0.2229)\).

Lengths and weights over the experimental period did not differ between control fish and fish exposed to elevated CO\(_2\) (two-way ANOVA, \(P = 0.797\) and 0.884, respectively; see Supplementary data). During the 50 day experiment, five control fish and four high-CO\(_2\)-exposed fish died from unknown causes.

**Discussion**

The result of the gabazine treatment shows that altered GABA\(_A\) receptor function is underlying the behavioural abnormalities displayed by stickleback after exposure to high-CO\(_2\) levels, exactly like previous findings in coral reef damselfish (Nilsson et al., 2012) and, recently, in rockfish (Hamilton et al., 2014).

Previous studies on coral reef damselfish (reviewed by Munday et al., 2012) have shown that the behavioural effects of high-CO\(_2\) treatment set in only after several days of sustained exposure and persist for several days after the high-CO\(_2\) exposure has ended, suggesting that alterations in gene expression may be involved. This could include changes in expression of genes for proteins involved in ion and pH regulation as well as the GABA\(_A\) receptor itself. Exposure to high-CO\(_2\) water has been found to reduce the response of the retina to fast stimuli, probably by altering the function of GABA\(_A\) receptors (Chung et al., 2014), and one may speculate that if other retinal functions are also altered this could contribute the loss of behavioural lateralization observed in this study as well as in previous studies on other species.

Most importantly, the present study suggests that the stickleback will provide us with a very useful model that opens up an avenue of experimental studies, including the examination of underlying molecular changes. For instance, transcriptomic studies will be aided by the annotated genome of this species, and we can also envision transgenerational studies, because this species can be bred readily in aquarium populations.

The three-spined stickleback is regarded as fairly tolerant of various environments, thriving in both freshwater and seawater (Pottinger et al., 2002; Östlund-Nilsson et al., 2006; Barrett et al., 2011), suggesting an extraordinary ion-regulatory plasticity. Nevertheless, its neural functions are apparently sensitive to sustained exposure to predicted future CO\(_2\) levels, as shown by the complete loss of behavioural lateralization in the present study, in the study by Näslund et al. (2015) and in other behavioural tests by Jutfelt et al. (2013).

Behavioural lateralization is thought to be a consequence of hemispheric specialization in the brain, allowing animals to carry out parallel processing and simultaneous responses to different stimuli (Bisazza et al., 1998; Dadda et al., 2012). In fish, the left optic lobe is connected to the right eye and vice versa, leading to a corresponding specialization of the eyes. If the right eye is, for example, focused on observing potential threats, pointing the head to the right and turning to the right allows the right eye to observe possible threats coming from behind. At the same time, the left eye can be focused on a different task, such as acquiring resources. Improved schooling performance, escape response, orientation and cognitive tasks are other suggested advantages of brain asymmetry (Bisazza and Dadda, 2005; Sovrano et al., 2005; Vallortigara and Rogers, 2005; Dadda et al., 2010). Loss of lateralization may therefore possibly affect fish fitness and survival.
Initially, it was hypothesized that highly active fish with a need for rapid oxygen uptake, and therefore large respiratory surface areas, would be more at risk for being affected by increases in the water CO₂ level (Nilsson et al., 2012). The reason for this is that a large respiratory surface area should also lead to a fast release of CO₂ from blood to water, approaching an equilibrium with the ambient water, making the blood carbonate system more sensitive to ambient changes in CO₂. Indeed, fish in general have relatively low internal levels of CO₂ in comparison to air-breathing animals owing to the high solubility of CO₂ in water. Consequently, few would have regarded the stickleback as particularly vulnerable, because it is not a ‘high-performance’ species with a particularly high rate of gas exchange with the environment and because it is able to tolerate a wide range of environmental conditions. A worrying aspect of the present study is therefore that it indicates that altered GABA_A-receptor function in response to sustained environmental hypercapnia may affect virtually any fish, at least in marine habitats.

At present, we do not know to what degree natural selection will be able to counteract neural effects of elevated CO₂ in the relatively short period during which oceanic CO₂ levels have been projected to reach close to 1000 µatm. What the present study shows is that individual acclimation is unlikely to be of help, because the behavioural change was in no way reduced after 50 days in the high-CO₂ environment.

**Supplementary material**

Supplementary material is available at Conservation Physiology online.
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Author contributions

The study was conceived and designed by G.E.N. and F.J. Experiments and measurements were executed by F.L. All authors contributed to writing the paper.

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