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RESEARCH ARTICLE

Ambient CO_2, fish behaviour and altered GABAergic neurotransmission: exploring the mechanism of CO_2-altered behaviour by taking a hypercapnia dweller down to low CO_2 levels

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ABSTRACT

Recent studies suggest that projected rises of aquatic CO_2 levels cause acid–base regulatory responses in fishes that lead to altered GABAergic neurotransmission and disrupted behaviour, threatening fitness and population survival. It is thought that changes in Cl^- and HCO_3^- gradients across neural membranes interfere with the function of GABA-gated anion channels ($GABA_A$ receptors). So far, such alterations have been revealed experimentally by exposing species living in low-CO_2 environments, like many oceanic habitats, to high levels of CO_2 (hypercapnia). To examine the generality of this phenomenon, we set out to study the opposite situation, hypothesizing that fishes living in typically hypercapnic environments also display behavioural alterations if exposed to low CO_2 levels. This would indicate that ion regulation in the fish brain is fine-tuned to the prevailing CO_2 conditions. We quantified pH regulatory variables and behavioural responses of *Pangasianodon hypophthalmus*, a fish native to the hypercapnic Mekong River, acclimated to high-CO_2 (3.1 kPa) or low-CO_2 (0.04 kPa) water. We found that brain and blood pH was actively regulated and that the low-CO_2 fish displayed significantly higher activity levels, which were reduced after treatment with gabazine, a $GABA_A$ receptor blocker. This indicates an involvement of the $GABA_A$ receptor and altered Cl^- and HCO_3^- ion gradients. Indeed, Goldman calculations suggest that low levels of environmental CO_2 may cause significant changes in neural ion gradients in *P. hypophthalmus*. Taken together, the results suggest that brain ion regulation in fishes is fine-tuned to the prevailing ambient CO_2 conditions and is prone to disruption if these conditions change.

KEY WORDS: *Pangasianodon hypophthalmus*, Acid–base regulation, Carbon dioxide, Climate change, Gabazine

INTRODUCTION

As a result of anthropogenic CO_2 release, current atmospheric CO_2 levels have risen to approximately 0.04 kPa (~400 µatm) – 40% higher than levels in pre-industrial times – and may reach 0.10 kPa (~1000 µatm) by the end of the 21st century (IPCC, 2013). The world's water bodies absorb large amounts of this CO_2 and so considerable research efforts are being directed towards understanding how this elevated P_{CO_2} (hypercapnia) might affect aquatic organisms, with particular focus on those thought to have evolved in relatively stable CO_2 and pH conditions.

Although not immediately lethal (Doney et al., 2009; Brauner and Baker, 2009), the projected hypercapnic scenarios have been linked to striking effects on the behaviour of some marine fish species in ways that are likely to impair fitness and population survival. These effects include reversed olfactory and auditory preferences (Munday et al., 2009; Dixson et al., 2010; Simpson et al., 2011), loss of behavioural lateralization (Domenici et al., 2012; Jutfelt et al., 2013), loss of learning (Ferrari et al., 2012; Jutfelt et al., 2013), increased boldness and activity (Munday et al., 2010), reduced temporal resolution of vision (Chung et al., 2014) and increased anxiety (Hamilton et al., 2014). The physiological basis for these behavioural changes is currently thought to stem from the changes in extracellular ion concentrations that occur as a result of acid–base regulation interfering with neurotransmitter function (Nilsson et al., 2012). Typically, fishes alleviate respiratory acidosis during hypercapnia by increasing plasma HCO_3^- levels in exchange for Cl^- (Ishimatsu et al., 2008; Brauner and Baker, 2009) and the associated redistribution of ions across cell membranes seems to interfere with $GABA_A$ receptor function. The GABA-gated ion channels conduct Cl^- and HCO_3^- fluxes (Bormann et al., 1987) when activated by the inhibitory neurotransmitter GABA, and normally reduce neural excitability by hyperpolarizing the cell through an inflow of Cl^-. However, in hypercapnia, when new gradients of HCO_3^- and/or Cl^- are established across neural membranes, $GABA_A$ receptor activation could result in a net outflow of these anions, depolarizing the membrane (Nilsson et al., 2012; Heuer and Grosell, 2014). Evidence for this mechanism stems from the observation that gabazine, a specific blocker of the $GABA_A$ receptor, effectively reverses the disruptive effects of high levels of CO_2 on lateralization, olfactory preferences, learning and vision in fishes (Nilsson et al., 2012; Chivers et al., 2014; Chung et al., 2014, Lai et al., 2015). Similarly, muscimol, a $GABA_A$ receptor agonist, causes increased anxiety in Californian rockfish exposed to high CO_2 concentrations (Hamilton et al., 2014). The widely conserved function of $GABA_A$ receptors suggests that a wide range of aquatic animals, including invertebrates, may be susceptible to similar behavioural abnormalities (e.g. Watson et al., 2014).

Tropical freshwater systems with frequent hypoxia and organic loading can become extremely hypercapnic, with P_{CO_2} levels reaching as high as 8.7 kPa (Willmer, 1934; Ultsch, 1987; Furch and Junk, 1997) and inhabitants clearly adapt to these hypercapnic

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conditions. The Mekong River, with its high organic loading, is one such environment. Mean P_{CO_2} across the entire river is 0.11 kPa (Li et al., 2013), 2.7-fold higher than atmospheric levels. This value varies both spatially and temporally, from 0.023 kPa in the river's upper reaches in the flood season, to 0.61 kPa in its lower reaches in the dry season (Li et al., 2013). Overall, most regions of the Mekong are hypercapnic for most of the year (Li et al., 2013), yet the river still harbours enormous biodiversity (Valbo-Jørgensen et al., 2009), including ∼850 fish species (Hortle, 2009; Gephart et al., 2010). These fishes are probably thriving in their hypercapnic environment, so they are unlikely to display the maladaptive behavioural abnormalities observed in the hypercapnia-exposed marine fish. Moreover, in Vietnam, some fishes like the facultative air-breathing striped catfish *Pangasianodon* (formerly *Pangasius*) *hypophthalmus* (Sauvage 1878) are extensively cultured in ponds with no aeration and low water exchange and hence are hypoxic (Lefevre et al., 2011, 2014; Damsgaard et al., 2015), with P_{CO_2} of up to 4.5 kPa (Damsgaard et al., 2015). Despite the harsh conditions, *P. hypophthalmus* – and the entire aquaculture industry surrounding them – continue to grow at a remarkable rate (Phan et al., 2009; De Silva and Phuong, 2011).

Given the neural and behavioural abnormalities induced by mild hypercapnia (∼0.1 kPa) in some marine fish and recently in a temperate freshwater fish (Ou et al., 2015), it is relevant to investigate whether variation in CO_2 levels exerts similar effects in hypercapnia-tolerant fish such as *P. hypophthalmus*. We hypothesized that a fish species native to a regularly hypercapnic environment (such as *P. hypophthalmus*) would have $GABA_A$ receptors that are functional at atypically high blood [HCO_3^-] and low [Cl^-]. The mechanisms that may enable this [e.g. regulation of brain intracellular pH (pH_i)] could alter ion concentrations in such a way that might even leave *P. hypophthalmus* vulnerable to reductions in ambient P_{CO_2}. If this is the case, $GABA_A$-mediated behavioural abnormalities would be predicted when *P. hypophthalmus* is exposed to relatively low-CO_2 environments.

We addressed this by scoring various ecologically relevant behavioural traits of *P. hypophthalmus* at two P_{CO_2} levels. The high-CO_2 group (3.1 kPa) can be regarded as the control group as the fish are likely to have been raised under such conditions (Damsgaard et al., 2015), whereas the low-CO_2 group was acclimated to 0.04 kPa P_{CO_2} for 2 weeks. We used established behavioural tests: an empty tank trial to measure routine activity (Munday et al., 2010), a novel object trial to assess responsive behaviour (Jutfelt et al., 2013), a predator trial to mimic predator avoidance (Munday et al., 2010; Dixson et al., 2010) and a conspecifics trial to assess social behaviour (Munday et al., 2009). The experiments were performed before and after gabazine treatment (Nilsson et al., 2012) to determine whether $GABA_A$ receptors are involved in any behavioural abnormalities. Our hypothesis predicted behavioural abnormalities in the normocapnia-exposed *P. hypophthalmus* consistent with those shown in normocapnia-acclimated fishes when exposed to relatively high-CO_2 environments: increased boldness and activity (Munday et al., 2010), an attraction to predators (Munday et al., 2010; Dixson et al., 2010) and a lack of preference for time spent near unrelated conspecifics (Munday et al., 2009). Furthermore, gabazine was predicted to attenuate these behavioural abnormalities. In addition to the behaviour trials, we measured blood pH, blood P_{CO_2}, blood [HCO_3^-] and brain pH_i of the fish to understand how these were affected by the different CO_2 exposures and to allow us to calculate theoretical effects of a range of CO_2 levels on neural membrane potential using the Goldman equation.

MATERIALS AND METHODS

Experimental animals

Pangasianodon hypophthalmus (∼5 months old; 13.9±5.11 g (mean±s.d.), 9.1–25.8 g; sex unknown because of juvenile life stage) were obtained from an aquaculture pond near Can Tho, Vietnam. Seven days prior to the start of the experiments, animals were transferred to 5000 litre fibreglass holding tanks (∼100 fish per tank) that were maintained at either high CO_2 (3.1 kPa P_{CO_2}; pH 5.8±0.2) or low CO_2 (0.04 kPa P_{CO_2}; pH 7.2±0.1) at normoxic levels. The aquarium facility was semi enclosed, allowing for natural light conditions (∼11.5 h light:12.5 h dark in December). Aquatic P_{CO_2} was continuously measured using a G10ps CO_2 probe and regulated by monitoring pH with a K01svpld probe, both connected to an Oxyguard Pacific System (Oxyguard International A/S, Farum, Denmark) that added CO_2 through a solenoid valve if pH increased above a threshold corresponding to the desired P_{CO_2} of 3.1 kPa for the high-CO_2 treatment. P_{CO_2} for the low-CO_2 treatment was achieved by recirculating water through a CO_2-diffusing tower where water was fully equilibrated with the air (Oxyguard International), yielding a P_{CO_2} of 0.04 kPa (below the limit of detection of the G10ps CO_2 probe; ∼0.1 kPa P_{CO_2}). Previous studies have shown that behavioural effects of changes in ambient CO_2 manifest within 4 days of exposure and that longer exposure periods do not further alter behavioural responses (Munday et al., 2010), suggesting that our CO_2 acclimation period was sufficient. All animals were exposed to the same ambient temperatures of 27–29°C for the entire acclimation and experimental period of 2 weeks. Fish were fed *ad libitum* with commercially available catfish pellets.

Behavioural analyses

The behaviour trials used a total of 48 fish comprising six treatment groups: low-CO_2 (N=12), low-CO_2 with 4 mg l^{-1} gabazine treatment (N=6), low-CO_2 with 8 mg l^{-1} gabazine treatment (N=6), high-CO_2 (N=12), high-CO_2 with 4 mg l^{-1} gabazine treatment (N=6) and high-CO_2 with 8 mg l^{-1} gabazine treatment (N=6). The behaviour trials were conducted in a series of three identical rectangular grey fibreglass arenas (66 cm×232 cm×50 cm, 18 cm water depth) filled with water from the acclimation tanks so that fish were acclimated and tested at the same P_{CO_2}. Four trials measuring ecologically relevant behaviours were performed on each fish in the same order: an empty tank trial (to measure routine activity), a novel object trial (to measure boldness), a predator trial (to measure predator avoidance) and a conspecific trial (to measure sociality). Each arena had a grid of squares (26 cm×26 cm) marked on the bottom, and activity was measured as the number of line crossings over time. Behaviour was recorded visually by two observers that were out of the focal fish's sight and the observers' roles were consistent across all trials and all experimental fish.

Individual fish were selected randomly from the acclimation tanks and within ∼30 s were gently transferred to the first experimental arena in a small plastic container so that the fish were never removed from water. Fish were transferred between arenas in the same way and were always released in the centre of the tank. First, routine activity (measured as line crossings) was measured for 5 min in an empty tank. After the 5 min period, a novel object (clay brick) was gently placed on the lengthwise midline of the arena, two squares (52 cm) from the experimental fish. Activity and proximity to the novel object (time spent within one grid line (26 cm) of the object) were recorded for 5 min.

Immediately following the novel object test, the fish was carefully transferred to the centre of a second arena for the predator sensitivity trial. In this arena, 31 cm of each end was fenced off with plastic mesh and a large snakehead (*Channa striata*; 37 cm, 740 g), a known predator of *P. hypophthalmus*, was placed behind the mesh in a randomly determined end of the arena. The mesh was permeable to the chemical cues of the *C. striata* and *P. hypophthalmus*, allowing both fish to confirm the other's presence visually and chemically. The arena was divided lengthwise into thirds to allow an observer to record the proportion of time the focal *P. hypophthalmus* spent near the predator, in the middle, or far away from the predator. Activity and location were recorded for 5 min. Only the zone closest to the predator (two grid squares, 52 cm) was used for the analysis.

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Finally, the experimental fish was transferred into a third arena for the sociality test. This arena was set up in the same manner as that used for the predator-avoidance trials, except a group of five *P. hypophthalmus* were present behind one of the mesh screens instead of a predator (random side). Activity and location were again recorded for 5 min. As with the predator trial, only the zone closest to the conspecifics (two grid squares, 52 cm) was used in the analysis.

Gabazine treatment

Gabazine targets $GABA_A$ receptors and reduces their anion conductivity through specific binding and allosteric interaction (Ueno et al., 1997). Gabazine is known to be free of the non-GABAergic calcium-dependent potassium channel effects of bicuculline, an older and more widely used $GABA_A$ antagonist (Heaulme et al., 1986; Hamann et al., 1988). Because gabazine is the most specific $GABA_A$ antagonist available, it comes with the lowest likelihood of off-target effects.

Gabazine treatment followed the procedure described by Nilsson et al. (2012), placing the fish in a bucket with 3 litres of water containing gabazine (4 or 8 mg l^{-1}; Sigma-Aldrich, St Louis, MO, USA) for 30 min. The two doses of gabazine were used to ensure it remained effectual in the latter 10 min of the behaviour trials. Although data for all six trials are presented in Fig. 1, the effect of gabazine appeared to diminish over the 20 min experiment and so the subsequent figures and analyses used the 4 mg l^{-1} treatment group to represent the gabazine-treated fish in the initial 10 min (empty tank and novel object trials) and the 8 mg l^{-1} treatment group to represent the gabazine-treated fish in the final 10 min (predator and conspecific trials) of the behaviour trials.

Tissue analyses

Fish were euthanized immediately following the final behaviour trial with a swift blow to the head, whereupon ~0.3 ml of blood was sampled via caudal puncture using a heparinized syringe. The brain was then excised and immediately frozen in liquid nitrogen. Blood pH, P_{CO_2} and HCO_3^- were measured using an iStat Systems portable clinical analyser. All measurements were temperature corrected to 27°C according to Harter et al. (2014). These values were further corrected according to Damsgaard et al. (2015).

Brain intracellular pH (pH_i) was measured using the methods of Pörtner et al. (1990) and Baker et al. (2009), where tissue was ground to a fine powder under liquid nitrogen using a mortar and pestle. Approximately 0.1 g of the powder was then transferred to a 1.5 ml centrifuge tube containing 0.8 ml of metabolic inhibitor cocktail comprising 150 mmol l^{-1} potassium fluoride (KF) and 6 mmol l^{-1} nitrilotriacetic sodium (Na_2NTA). The solution was immediately vortexed for 30 s and centrifuged at 3000 ***g*** for 45 s and the resulting supernatant represented the intracellular medium (cytosol) of the tissue sample. pH_i measurements were made on 0.2 ml volumes of this supernatant (in duplicate) using a Radiometer PHM 84 (Copenhagen, Denmark) pH meter connected to a radiometer SaS gK2401C (Radiometer Analytical, Lyons, France) pH electrode.

Statistical analyses

Activity data (line crossings) were analysed using two-way repeated-measures ANOVAs with treatment (gabazine and CO_2) and trials as the factors. One analysis was performed for the first two behaviour trials (empty tank and novel object) in which gabazine-treated fish received a 4 mg l^{-1} dose. A second analysis was performed for the latter behaviour trials (predator and conspecific exposures) in which gabazine-treated fish received an 8 mg l^{-1} dose. Two-way repeated-measures ANOVA was also used to analyse the proportion of time fish spent near the *C. striata* predator and group of conspecifics in the final two trials. All other data were analysed using one-way ANOVAs, and *post hoc* Holm–Šidák tests were used to test for differences between treatment groups. Non-proportional data were square root transformed when necessary to better meet the assumptions of normal distribution and equal variance. Proportional data were logit transformed when necessary (Warton and Hui, 2011). Despite the relatively small and uneven sample sizes, statistical power was generally high (>0.7) with the exception of the comparisons of time spent adjacent to predator (0.10) and blood pH (0.24). SigmaPlot 11 (Systat Software, San Jose, CA, USA) was used for all analyses (critical α=0.05). Throughout the text, values are given as means±s.e.m.

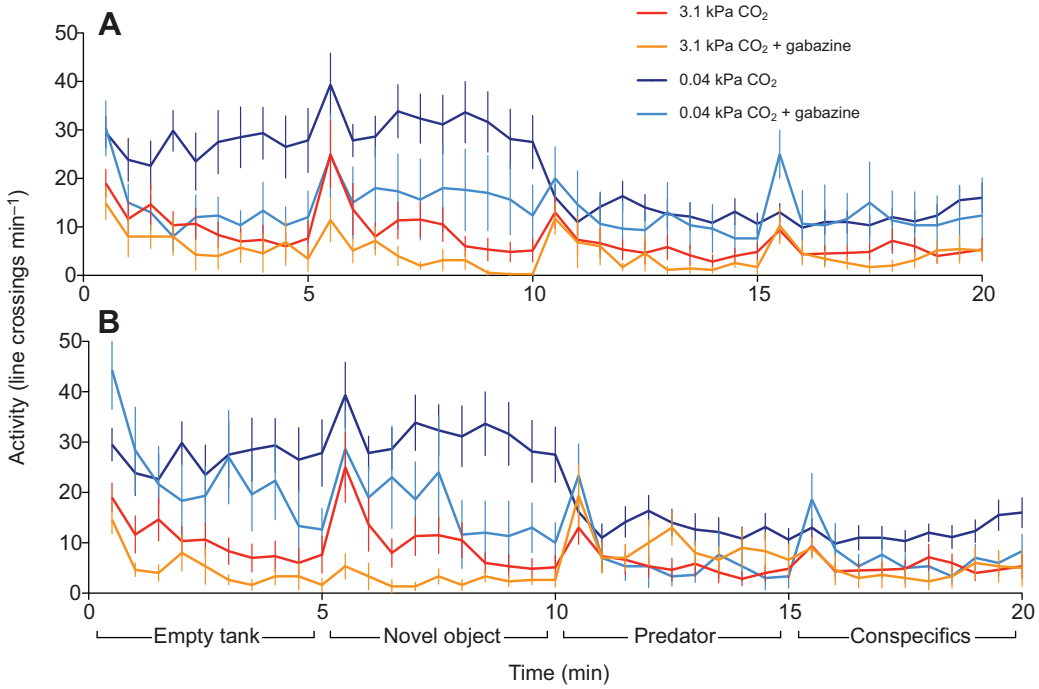

Fig. 1. Activity of *P. hypophthalmus* in high-CO_2 (3.1 kPa) and low-CO_2 (0.04 kPa) water. The data points represent average line crossings per min over the course of four behaviour trials with and without treatment with gabazine at 4 mg l^{-1} (A) or 8 mg l^{-1} (B) in response to an empty tank, a novel object (a clay brick), a natural predator (snakehead, *C. striata*) held behind mesh at one end of the tank and a group of five unrelated size-matched conspecific fish held behind mesh at one end of the tank. *N*=12 for the non-gabazine treatments, and *N*=6 for each gabazine treatment. Error bars represent s.e.m.

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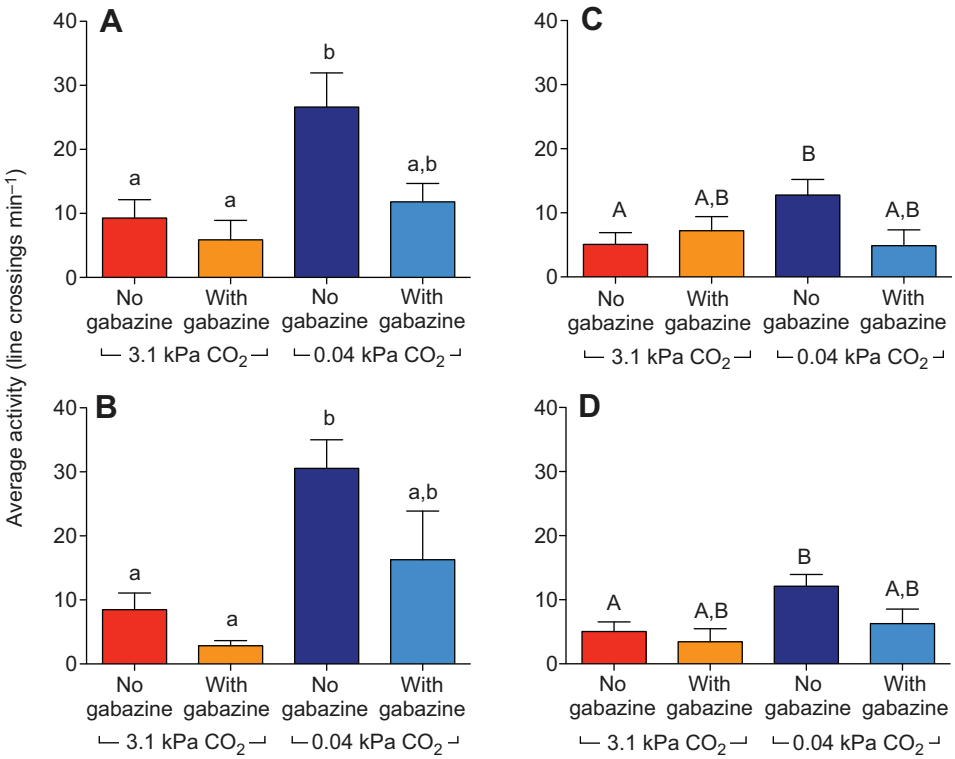

Fig. 2. Mean activity of *P. hypophthalmus* in high-CO_2 and low-CO_2 water, with and without treatment with gabazine. Measurements were made in an empty tank (A) and in the presence of a novel object (B), a natural predator (C) and a group of conspecifics (D). The values represent the mean activity (line crossings min^{-1}) of each fish over the duration of each 5 min behaviour trial, not including the initial 30 s value. *N*=12 for the non-gabazine treatments and *N*=6 for the gabazine treatments. Because the gabazine effect wore off over time, the initial two trials (empty tank and novel object) include data from fish initially treated with 4 mg l^{-1} gabazine, while the final two trials (predator and group of conspecifics) include data from fish initially treated with 8 mg l^{-1} gabazine (see text for further explanation). Error bars represent s.e.m. Different lowercase letters denote significant overall differences between treatment groups across the empty tank (A) and novel object (B) trials ($P<0.05$, two-way repeated-measures ANOVA). Different uppercase letters indicate significant overall differences between treatment groups across the predator (C) and conspecific (D) trials ($P<0.05$, separate two-way repeated-measures ANOVA).

RESULTS

Behaviour

Generally, the low-CO_2 fish were more active than the high-CO_2 fish across all four behaviour trials (Fig. 1). The two-way repeated-measures ANOVA for the empty tank and novel object trials revealed a significant overall difference between high- and low-CO_2 fish untreated with gabazine (treatment $P<0.05$, both trial and trial by treatment interaction $P>0.05$; Fig. 2A,B). The same trend held for the two-way repeated-measures ANOVA run on the predator and conspecifics trials, with a significant overall difference between high- and low-CO_2 fish and a significant difference in activity between trials regardless of treatment, but no significant trial by treatment interaction (Fig. 2C,D).

Exposure to 4 mg l^{-1} gabazine reduced activity levels of the low-CO_2 fish by approximately 50% in the first two behaviour trials (empty tank and novel object) to levels not statistically different than those of the high-CO_2 fish ($P>0.05$; Fig. 2A,B). During the subsequent 10 min, the effect of this low gabazine dose had apparently worn off and activity no longer differed from untreated low-CO_2 fish (*t*-test $P=0.61$ and $P=0.91$ for predator and conspecific trials, respectively; Fig. 1). As gabazine is rapidly taken up over the gills, it is also likely to depurate by this route and thus the low gabazine dose probably wore off after 10 min. This led us to test the effect of a double dose. When treated with the higher gabazine dose (8 mg l^{-1}), the low-CO_2 fish showed slightly higher activity during the first two behaviour trials (empty tank and novel object) relative to the low-CO_2 fish treated with 4 mg l^{-1} gabazine, consistent with what has previously been shown for high doses of GABA receptor blockers (Turski et al., 1985). However, during the final two behaviour trials (predator and conspecifics), when gabazine levels in the 8 mg l^{-1}-treated fish were likely to have fallen closer to those of the 4 mg l^{-1}-treated fish during the first 10 min, activity of the low-CO_2 gabazine-treated fish was reduced by approximately 50% relative to the non-treated fish, to levels not statistically different than those of the high-CO_2 fish ($P>0.05$; Fig. 2C,D). Because the effect of gabazine appeared to diminish over the 20 min experiment, we have represented gabazine-treated fish using the 4 mg l^{-1} dosage when averaging behavioural traits during the initial 10 min (empty tank and novel object trials) and the 8 mg l^{-1} dosage when averaging behavioural traits during the final 10 min (predator and conspecifics trials). Neither of the gabazine treatments had any significant effect on the activity levels of the high-CO_2 fish (all $P>0.05$; Figs 1 and 2).

The high-CO_2 fish spent an average of 3.3±1.4% of their time in close proximity (within 26 cm) to the clay brick in the novel object trial, whereas the low-CO_2 fish spent significantly more time (21.0±2.8%) in close proximity to the novel object (ANOVA, $P<0.05$;

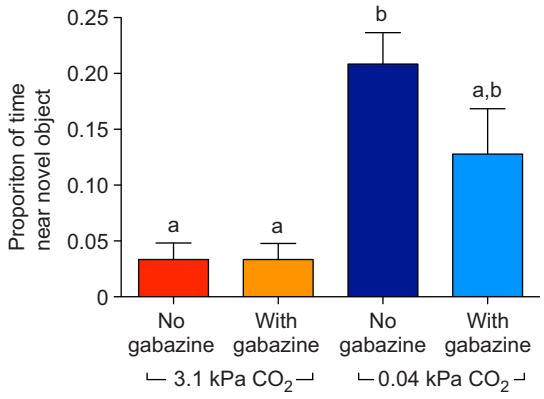

Fig. 3. The mean proportion of a 300 s period that *P. hypophthalmus* spent within 26 cm (one grid line) of a novel object when in high-CO_2 and low-CO_2 water, with and without gabazine. *N*=12 for the non-gabazine treatments and *N*=6 for the gabazine treatments (4 mg l^{-1}). Error bars represent s.e.m. Different letters denote significant differences between treatment groups ($P<0.05$, one-way ANOVA).

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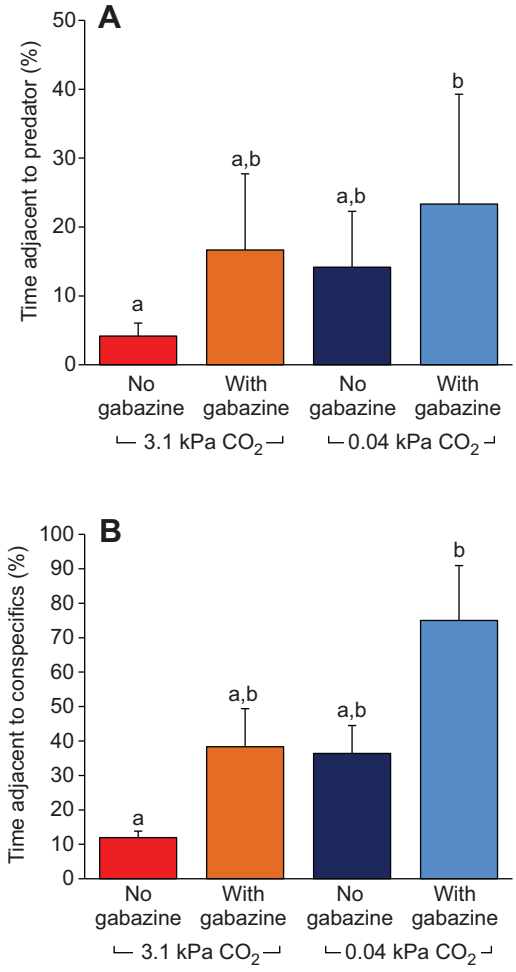

Fig. 4. The mean percentage of 300 s *P. hypophthalmus* spent within 52 cm (two grid lines) of a natural predator or a group of unrelated conspecifics. The natural predator (*C. striata*; A) and the conspecifics (B) were held behind mesh at one end of their respective tanks, while *P. hypophthalmus* was allowed to move freely throughout the tank. For both figures, N=12 for the non-gabazine treatments and N=6 for the gabazine (8 mg l^{-1}) treatments. Error bars represent s.e.m. Different letters denote significant overall differences between treatment groups (P<0.05, two-way repeated-measures ANOVA).

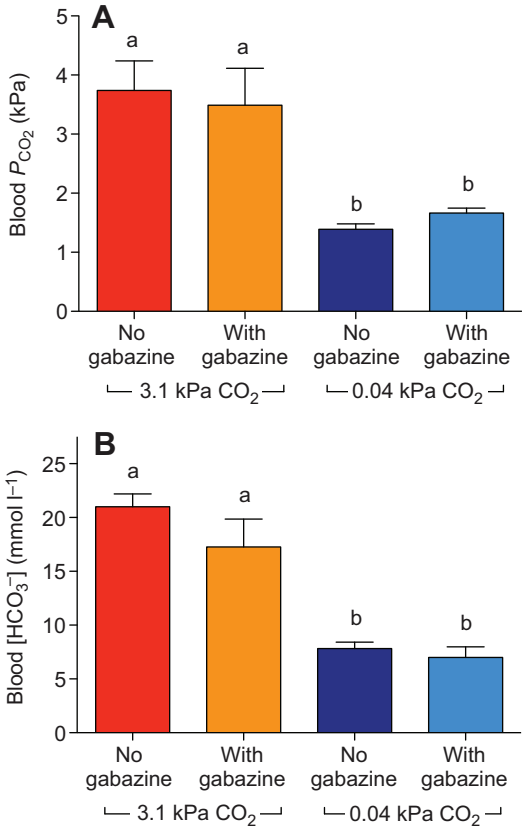

Fig. 5. Mean P_{CO_2} and $[HCO_3^-]$ of *P. hypophthalmus* blood when in high-CO_2 and low-CO_2 water, with and without gabazine. P_{CO_2} (A) and $[HCO_3^-]$ (B) measurements. The 8 mg l^{-1} dose is presented because the measurements were made following the behaviour experiments when 4 mg l^{-1} gabazine dose had worn off. The measurements were made using iStat System's portable clinical analyser, corrected for temperature and species according to Harter et al. (2014) and Damsgaard et al. (2015). For both figures, N=12 for the non-gabazine treatments and N=6 for the gabazine treatments. Error bars represent s.e.m. Different letters represent significant differences between groups (P<0.05, 1-way ANOVA).

Fig. 3). When treated with gabazine, the proportion of time spent by the low-CO_2 fish near the brick was reduced to a value similar to the high-CO_2 fish (12.8±4.1%; P>0.05; Fig. 3). Gabazine had no effect on the time the high-CO_2 fish spent close to the brick (3.3±1.5%; P>0.05; Fig. 3).

Overall, *P. hypophthalmus* spent very little time (4–23%) near the predatory *C. striata* (within two grid squares, or 52 cm) and this aversion was unaffected by CO_2 alone (P>0.05; Fig. 4A). However, fish acclimated to high CO_2 levels spent significantly less time near the predator (within two grid squares, or 52 cm) than low-CO_2 fish treated with gabazine (P<0.05; Fig. 4A). The same trend was observed in the sociality test (Fig. 4B).

Tissue analyses

The high-CO_2 fish had higher P_{CO_2} and $[HCO_3^-]$ in the blood than the low-CO_2 fish (P<0.05; Fig. 5A,B). Blood pH values of the high- and low-CO_2 fish were not statistically different (pH 7.50±0.05 and 7.48±0.03, respectively), whereas treatment with gabazine slightly reduced these values in both the high-CO_2 (pH 7.44±0.06) and low-CO_2 fish (pH 7.34±0.04; Fig. 6A). A similar trend was observed with brain pH_i: values of the high- and low-CO_2 fish were not statistically different (pH 6.81±0.02 and 6.79±0.04, respectively), whereas treatment with gabazine slightly reduced these values in the high-CO_2 fish (pH 6.76±0.03; P>0.05) and significantly reduced them in the low-CO_2 fish (pH 6.64±0.02; P<0.05; Fig. 6B).

DISCUSSION

We hypothesized that a fish species native to a regularly hypercapnic environment would have $GABA_A$ receptors that remain functional at atypically high blood $[HCO_3^-]$ and low $[Cl^-]$, and thus would display $GABA_A$-mediated behavioural abnormalities when exposed to a relatively low-CO_2 environment. Mechanistically, these effects should be similar to those previously seen in coral reef fish that are adapted to a low-CO_2 habitat and are unable to respond appropriately to chronic hypercapnia. In both instances, neural ion gradients could change in ways that render $GABA_A$ receptors depolarizing (excitatory) rather than hyperpolarizing (inhibitory). The underlying mechanisms could involve an inability of reef fish to regulate brain pH_i, resulting in maintained Cl^- levels intracellularly while these fall extracellularly (leading to a depolarizing gradient). By contrast, in the hypercapnia-adapted *P. hypophthalmus*, well-developed brain pH_i regulation

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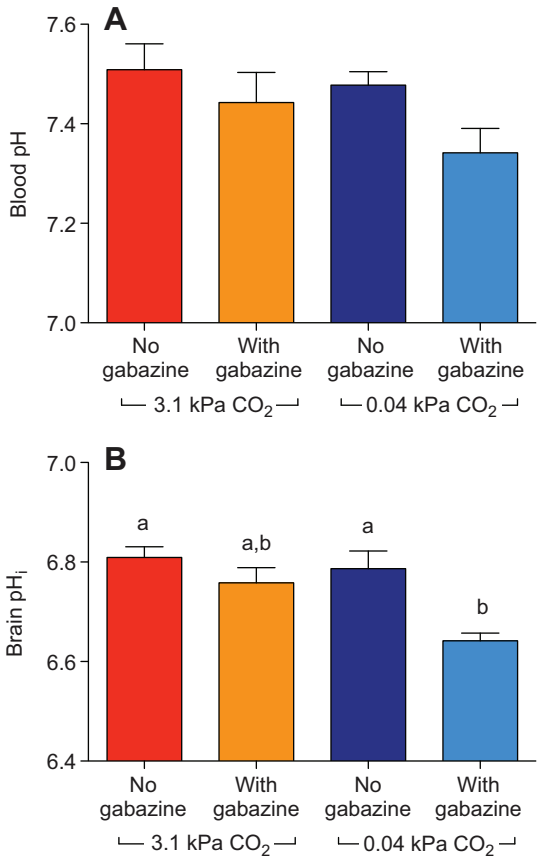

Fig. 6. Mean blood pH and intracellular brain pH of *P. hypophthalmus* when in high-CO_2 and low-CO_2 water, with and without gabazine. Blood pH (A) and intracellular brain pH (pH_i) (B). The 8 mg l^{-1} dose is presented because the measurements were made following the behaviour experiments when 4 mg l^{-1} gabazine dose had worn off. See text for details of measurements. For both figures, N=12 for the non-gabazine treatments and N=6 for the gabazine treatments. Error bars represent s.e.m. Different letters represent significant differences between groups (P<0.05, one-way ANOVA).

involving atypical Cl^-:HCO_3^- exchange ratios in the blood (Damsgaard et al., 2015) could lead to depolarizing $GABA_A$ receptors; these scenarios are discussed in depth below. Consistent with some of our predictions, we observed similar behavioural alterations in normocapnia-acclimated *P. hypophthalmus* as found previously in normocapnia-acclimated reef fishes when exposed to relatively high-CO_2 environments: increased boldness and activity (Munday et al., 2010), and more time spent in the presence of a predator (Munday et al., 2010; Dixson et al., 2010). Furthermore, the $GABA_A$ receptor antagonist gabazine attenuated these abnormalities in behaviour.

As predicted, the low-CO_2 *P. hypophthalmus* displayed significantly higher activity levels than the high-CO_2 control fish (Figs 1 and 2) and the elevated activity levels of the low-CO_2 fish were attenuated by gabazine (Figs 1 and 2). This implies an involvement of $GABA_A$ receptors and an alteration of the neural Cl^- and HCO_3^- ion gradients gated by these receptors. Likewise, the low-CO_2 fish spent six times longer in close proximity to a novel object placed in their tank – a measure of boldness – than the high-CO_2 fish (Fig. 3). An alternative interpretation of these results might be that the low-CO_2 fish were not bold with respect to the object's presence, but rather indifferent, whereas the high-CO_2 fish were timid. This interpretation is supported by the fact that 'close proximity' was defined as a radius of 26 cm (one grid square) around the object, an area one quarter the size of the tank's area. If the fish was indifferent to the presence of the object, one might predict that it would be found in the quarter surrounding the object 25% of the time; this agrees well with the value for the low-CO_2 fish of 21.0±2.8%. In any case, when the low-CO_2 fish were treated with gabazine, this period of time was reduced by 40% to a level not statistically different from that of high-CO_2 fish (Fig. 3), again implying the involvement of $GABA_A$ receptors.

Contrary to our expectations, low-CO_2 fish were not attracted to the predatory *C. striata*. Indeed, the low- and high-CO_2 fish spent statistically similar amounts of time near the *C. striata* (Fig. 4A). These trends were unaffected by gabazine treatment and are therefore contrary to previous results that have demonstrated a clear role of $GABA_A$ receptors in the olfactory sensing of predators (e.g. Nilsson et al., 2012). These inconsistencies may be caused by the use of different species, different CO_2 exposures (e.g. high-to-low CO_2 in our study versus low-to-high CO_2 in other studies) or different methodologies. In our study, the fish could use at least three cues (vision, olfaction, hearing) to detect the predator, whereas previous studies (e.g. Munday et al., 2010; Nilsson et al., 2012) tested predator avoidance based only on chemical cues. It may be that any sensory impairment caused by the altered CO_2 environment is compensated for by the use of more than one sensory modality (Rauschecker and Kniepert, 1994; Chapman et al., 2010).

Regulation of brain pH_i

P. hypophthalmus regulates brain pH_i when faced with drastic changes in environmental CO_2 levels (Fig. 6B). This is not surprising given that extracellular pH (pH_e) was also regulated over the duration of CO_2 exposure (Damsgaard et al., 2015) and is consistent with other CO_2-tolerant fishes (Brauner and Baker, 2009) exposed to short-term hypercapnia [white sturgeon (Baker et al., 2009); Amazonian catfish (Brauner et al., 2004)] and long-term hypercapnia [gilthead seabream (Michaelidis et al., 2007)].

What benefits come with brain pH_i regulation, and how might $GABA_A$-mediated behaviour be affected by it? Defending brain pH_i maintains the routine excitability of brain cells that may otherwise be increased or decreased depending on the magnitude of hypercapnia (Siesjö et al., 1972). The primary mechanism of active pH_i regulation is believed to involve the equimolar exchange of intracellular Cl^- for extracelluar HCO_3^- (Brauner and Baker, 2009), where intracellular [Cl^-] and [HCO_3^-] decrease and increase, respectively. In turn, this increases extracellular [Cl^-], and since $GABA_A$ channels are approximately five times more conductive to Cl^- than to HCO_3^-, at least in mammals (Farrant and Kaila, 2007), the net result is an increased likelihood of a neuroinhibiting hyperpolarization of the brain cell upon $GABA_A$ receptor activation. Therefore, the relatively low activity levels observed in our high-CO_2 fish may be partially explained by putative increases in extracellular [Cl^-] resulting from the active regulation of brain pH_i, which itself also helps to preserve routine neural excitability.

Neural ion gradients and Goldman calculations

The observation that a moderate dose of gabazine reduced the activity levels of the low-CO_2 fish relative to those of the high-CO_2 fish suggests that $GABA_A$ receptors are involved in this behavioural response. $GABA_A$ channels are conductive to Cl^- and HCO_3^-, and environmental CO_2 levels influence these ion concentrations in both the blood and the cytosol (Ishimatsu et al., 2008; Brauner and Baker, 2009; Damsgaard et al., 2015). This is related to acid–base

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regulatory mechanisms, and during this regulation in *P. hypophthalmus* exposed to 3 kPa CO_2, $[Cl^-]$ decreases by 13 mmol l^{-1} with an increase in $[HCO_3^-]$ of 20 mmol l^{-1} (Damsgaard et al., 2015). We did not measure $[Cl^-]$ in either compartment of our fish, but our measurements of blood $[HCO_3^-]$ during hypercapnia are in line with this (Fig. 5B). Combining our P_{CO_2} measurements (Fig. 5A) with our brain pH_i measurements (Fig. 6B), Henderson–Hasselbach calculations estimate intracellular $[HCO_3^-]$ of the brain to increase from approximately 1.53± 0.23 mmol l^{-1} at 0.04 kPa P_{CO_2} to 4.32±0.77 mmol l^{-1} at 3.1 kPa P_{CO_2}. And as *P. hypophthalmus* brain cells appear to actively regulate pH_i under high CO_2 (Fig. 6B), the increase in intracellular $[HCO_3^-]$ is probably matched by an equimolar decrease in intracellular $[Cl^-]$. It is possible that $GABA_A$ receptors of hypercapnia-adapted fishes have evolved to function under shifted neural anionic set points and that deviating from these set points could result in a reversal of $GABA_A$ receptor function.

We used these ion concentrations and the Goldman equation to examine possible $GABA_A$-mediated effects on neural polarization; if these shifted anion gradients do affect activity levels via $GABA_A$ channels on the neural membranes, then we would predict the high-CO_2 neural gradients to produce hyperpolarizing (i.e. neuroinhibitory) $GABA_A$ currents and the low-CO_2 gradients to produce depolarizing (i.e. neuroexcitatory) $GABA_A$ currents, at least in some neural circuits. We calculated the reversal potential for $GABA_A$ (E_{GABA_A}) using the equation:

$$E_{GABA_A} = \frac{RT}{F} \ln \frac{P_{Cl} - [Cl^-]_i + P_{HCO_3^-} - [HCO_3^-]_i}{P_{Cl} - [Cl^-]_o + P_{HCO_3^-} - [HCO_3^-]_o}, \quad (1)$$

where R is the ideal gas constant (8.315 J K^{-1} mol^{-1}), T is temperature (300.15 K), F is Faraday's constant (96,485 Coulombs mol^{-1}) and P is the relative HCO_3^-/Cl^- permeability of the $GABA_A$ channel. This relative permeability varies from 0.2 to 0.6 in mammals (Farrant and Kaila, 2007) and values of 0.3 and 0.4 have previously been applied to fish (e.g. Heuer and Grosell, 2014). Blood $[HCO_3^-]$ ($[HCO_3^-]_o$) in our high- and low-CO_2 fish (Fig. 5B), and the corresponding P_{CO_2} values (Fig. 5A) and brain pH_i (Fig. 6B), yields an intracellular $[HCO_3^-]$ ($[HCO_3^-]_i$) of 4.32 and 1.53 mmol l^{-1} for the high and low-CO_2 fish, respectively. Blood $[Cl^-]$ ($[Cl^-]_o$) of the low-CO_2 fish was assumed to be 110 mmol l^{-1} (Damsgaard et al., 2015) and we considered two scenarios for the high-CO_2 fish: one with the typical equimolar (1:1) exchange of blood Cl^- for HCO_3^- (e.g. Brauner and Baker, 2009) and another with a 1:2 exchange of blood Cl^- for HCO_3^-, as observed by Damsgaard et al. (2015) in *P. hypophthalmus*. Such a ratio might be related to hypercapnia tolerance or could even be unique to *P. hypophthalmus*. Intracellular $[Cl^-]$ ($[Cl^-]_i$) in the low-CO_2 fish was estimated to be 8.0 mmol l^{-1} based on Delpire and Staley (2014) and 5.2 mmol l^{-1} in the high-CO_2 fish based on an equimolar exchange of intracellular Cl^- for HCO_3^- resulting from active pH_i regulation of the brain. Finally, we assumed the commonly used resting neural membrane potential of −70 mV (Moyes and Schulte, 2006). All values are summarized in Table 1.

We plotted a linear trend line between the high- and low-CO_2 values for each ion concentration and used its equation to determine ion concentrations under 20 different P_{CO_2} conditions, ranging from 0 to 4 kPa. These ion concentrations were then used together in the Goldman equation to calculate E_{GABA_A} reversal potentials as a function of P_{CO_2} at each of the 20 P_{CO_2} conditions. We did this for $P_{HCO_3^-/Cl^-}$ ratios of 0.3 and 0.4, and for hypercapnic blood Cl^-:HCO_3^- exchange ratios of 1:1 and 1:2 (Fig. 7).

The results of the Goldman calculations (Fig. 7) agree with both our hypothesis and our behavioural observations. The neural anion gradients of high-CO_2 fish result in a hyperpolarizing $GABA_A$ reversal potential when assuming either 0.3 or 0.4 $P_{HCO_3^-/Cl^-}$ permeability ratios, while the neural anion gradients of low-CO_2 fish result in a depolarizing $GABA_A$ reversal potential assuming either 0.3 or 0.4 $P_{HCO_3^-/Cl^-}$ permeability ratios. The P_{CO_2} at which the membrane potential changes from hyperpolarizing to depolarizing varies with both $P_{HCO_3^-/Cl^-}$ permeability ratio and blood Cl^-:HCO_3^- exchange ratios (Fig. 7). A hyperpolarizing membrane potential is retained over a larger P_{CO_2} range when the $P_{HCO_3^-/Cl^-}$ permeability ratio of $GABA_A$ is lower and when the exchange ratio of blood Cl^-:HCO_3^- is at 1:2, with the latter ratio seemingly playing a greater role. Therefore, a possible effect of the unusual 1:2 anion exchange ratio in *P. hypophthalmus* may be an avoidance of hyperactivity in the high CO_2 levels that are typical of its natural environment. In any case, our calculations of E_{GABA_A} reversal potentials agree with our behavioural findings, where high-CO_2 fish exhibit inhibitory membrane potentials and relatively low activity, and low-CO_2 fish exhibit excitatory membrane potentials and relatively high activity. By reducing the activity of $GABA_A$ channels, gabazine effectively counteracts this $GABA_A$-mediated excitation and subsequently reduces the activity levels of the low-CO_2 fish.

For comparison, we can attempt to estimate the E_{GABA_A} reversal potential for a 'typical' hypercapnia-intolerant reef fish when exposed to climate change-relevant high-CO_2 (0.1 kPa). Here, we assume that the reef fish does not display *P. hypophthalmus*' 1:2

Table 1. Values used for Goldman calculations of the reversal potential for $GABA_A$ (E_{GABA_A}) in *P. hypophthalmus* and a hypercapnia-intolerant reef fish under high- and low-CO_2 conditions

	Hypercapnia-tolerant *P. hypophthalmus*		Hypercapnia-intolerant reef fish	
	3.1 kPa P_{CO_2}	0.04 kPa P_{CO_2}	0.1 kPa P_{CO_2}	0.04 kPa P_{CO_2}
$P_{HCO_3^-/Cl^-}$	0.3 or 0.4	0.3 or 0.4	0.3 or 0.4	0.3 or 0.4
$[HCO_3^-]_o$	21	7.82	6.3	3.3
$[HCO_3^-]_i$	4.32	1.53	5	1.8
$[Cl^-]_o$	103.4	110	147	150
$[Cl^-]_i$	5.2	8	9	9
E_{GABA_A}	−73.5 or −72.3 (inhibitory)	−67.2 or −67.0 (excitatory)	−68.9 or −67.8 (excitatory)	−71.8 or −71.4 (inhibitory)

The $[Cl^-]_o$ value for the high-CO_2 *P. hypophthalmus* is based on a 1:2 exchange ratio of blood Cl^- for HCO_3^-. A hypercapnia-intolerant reef fish is assumed to be exchanging blood Cl^- for HCO_3^- at the common 1:1 ratio. See text for information on how these values were estimated. Assuming a resting membrane potential of −70 mV, the calculated values for E_{GABA_A} reveal reversed directions of the electrochemical gradient for Cl^- and HCO_3^- over the membrane and as a result $GABA_A$ receptors would change from being inhibitory (E_{GABA_A} more negative than −70 mV) to excitatory (E_{GABA_A} more positive than −70 mV).
$P_{HCO_3^-/Cl^-}$ is the permeability of GABA channels for HCO_3^- relative to Cl^-. All concentration values are expressed as mmol l^{-1}. E_{GABA_A} is expressed as mV.

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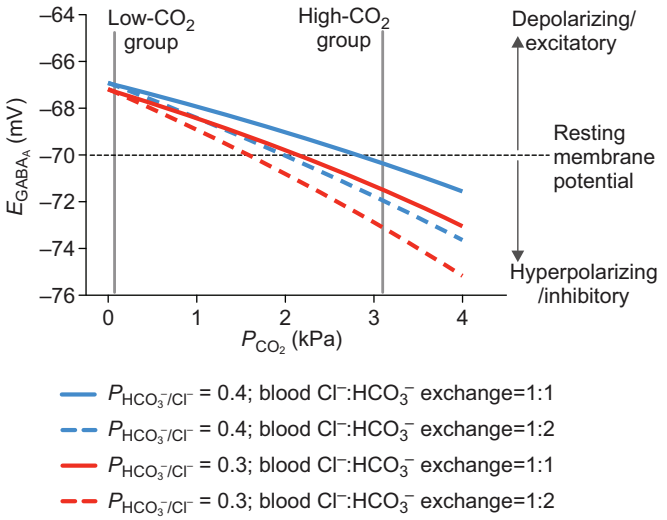

Fig. 7. Results of Goldman calculations of the reversal potential for the $GABA_A$ channel (E_{GABA_A}). Measurements were made for a range of CO_2 conditions (0–4 kPa), at $P_{HCO_3^-/Cl^-}$ ratios of 0.3 (red lines) and 0.4 (blue lines) and at hypercapnic Cl^-:HCO_3^- exchange ratios of 1:1 (solid lines) and 1:2 (dotted lines). Resting membrane potential is assumed to be −70 mV. Hyperpolarizing/inhibitory reversal potentials are less than −70 mV and depolarizing/excitatory potentials are greater than −70 mV. See text for further information.

exchange of blood Cl^- for HCO_3^-, which, when combined with the fish's seawater environment, influences intracellular and extracellular ion concentrations (detailed in Table 1) in a way that yields depolarizing (excitatory) E_{GABA_A} reversal potentials (−68.9 or −67.8 mV for 0.3 and 0.4 $P_{HCO_3^-/Cl^-}$ ratios, respectively) when used in the Goldman equation. In contrast, a typical reef fish under normocapnic CO_2 conditions (0.04 kPa) produces hyperpolarizing (inhibitory) E_{GABA_A} reversal potentials (−71.8 and −71.4 mV for 0.3 and 0.4 $P_{HCO_3^-/Cl^-}$ ratios, respectively; Table 1; $[HCO_3^-]_o$ from Esbaugh et al., 2012; $[HCO_3^-]_i$ from Henderson–Hasselbach calculations; $[Cl^-]_o$ from Marshall and Grosell, 2006; $[Cl^-]_i$ from Delpire and Staley, 2014). These calculations not only support the results of previous studies on reef fishes that have shown excitatory behaviour under high-CO_2 conditions but also suggest that some of *P. hypophthalmus*' mechanisms of hypercapnia tolerance (i.e. brain pH_i regulation, 1:2 exchange ratio of blood Cl^- for HCO_3^-) help preserve the neuroinhibiting effect of GABA under hypercapnia.

Alternative explanations

An alternative explanation for our activity results involves the anaesthetic effect of CO_2 (Fish, 1943; Bell, 1964). That is, the high activity of the low-CO_2 fish may only be 'high' relative to the activity of the high-CO_2 group, whose behaviour may have been influenced by the anaesthetic effect of CO_2. Although it is possible for CO_2 to have an anaesthetic effect on fishes at a P_{CO_2} as low as 2.4 kPa [e.g. rainbow trout (Iwama et al., 1989)], hypercapnia-tolerant species tend to require a much higher P_{CO_2} [e.g. 0.89–4.44 kPa pCO_2 for common carp (Yoshikawa et al., 1988, 1991)]. P_{CO_2} levels higher than 3.1 kPa would probably be required to elicit an anaesthetic effect in *P. hypophthalmus*. Furthermore, other aspects of water chemistry, such as P_{O_2}, have been shown to strongly influence anaesthetic effect of CO_2 on fish (Bernier and Randall, 1998). For example, an anaesthetizing P_{CO_2} in one study (Iwama et al., 1989) may have no anaesthetic effect on the same species (rainbow trout) when it is not coupled with hyperoxia (Bernier and Randall, 1998). We did not hyperoxygenate our water, which may have mitigated any anaesthetic effect the CO_2 may have otherwise had. Finally, our qualitative observations of the fish provided no evidence of anaesthesia. We therefore find it unlikely that our high-CO_2 fish were under CO_2-induced anaesthesia.

Conclusions

As a species native and presumably adapted to typically hypercapnic environments, *P. hypophthalmus* allowed for a novel test of the involvement of $GABA_A$ channels in the behavioural response of fishes to high levels of environmental CO_2 (assuming the hatchery-derived individuals used in our study were representative of wild *P. hypophthalmus*). We hypothesized that *P. hypophthalmus* would have GABA receptors that are functional at atypically high blood $[HCO_3^-]$ and low $[Cl^-]$, and thus would be predicted to display $GABA_A$-mediated behaviour abnormalities when exposed to a relatively low-CO_2 environment. The increased activity levels of normocapnia-exposed fish, together with the reversal of this hyperactivity when treated with the $GABA_A$ receptor blocker gabazine, support this hypothesis. Further support is provided by our theoretical calculations of E_{GABA_A} reversal potentials, suggesting the hyperpolarizing $GABA_A$ reversal potentials of hypercapnic *P. hypophthalmus* are indeed reversed to depolarizing potentials when the fish are in normocapnic environments.

In an increasingly hypercapnic world, we can look to native species of today's hypercapnic environments for some insight into how other species might cope with increases in environmental CO_2 and acidity. Our results suggest that *P. hypophthalmus* retains functional $GABA_A$ receptor activity in high-CO_2 environments and consequently avoids the CO_2-induced behavioural alterations observed in numerous normocapnia-native reef fishes (Munday et al., 2012). Assuming that *P. hypophthalmus* or its ancestors were at one point native to normocapnic environments, this would suggest that fish are indeed capable of adapting to increases in environmental CO_2 in a way that mitigates the adverse behaviours observed in recent studies (Welch et al., 2014; but see Munday et al., 2014). However, the ability of natural selection to adapt species to hypercapnia will of course depend on the presence of relevant gene variants in the gene pool and on the rate at which environmental CO_2 levels increase, and there is a considerable risk that the present rate is too high to allow adaptation through natural selection.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

The study was conceived by S.L., G.E.N. and M.B. M.D.R., A.J.T., J.H., M.K.A. and G.E.N. designed and carried out the experiments and analysed the results. C.B. supervised the tissue analyses. D.T.T.H., N.T.P., M.B., C.B. and T.W. provided the animals, research facilities and equipment. M.D.R. wrote the first draft of the manuscript and all authors contributed to the final version.

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