Expression of genes involved in brain GABAergic neurotransmission in three-spined stickleback exposed to near-future CO₂

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Change in the activity of the main inhibitory receptor, GABAₐ, has been suggested to be a general mechanism behind the behavioural alterations reported in ocean acidification studies on fish. It has been proposed that regulatory acid–base mechanisms in response to high CO₂ alter the neuronal Cl⁻ and HCO₃⁻ gradients that are important for GABAₐ receptor function. Here, we report a comprehensive analysis of gene expression of GABAₐ receptor subunits and of genes involved in GABAergic transmission in the brain of fish exposed to near-future CO₂. Altogether, 56 mRNA transcripts were quantified in brains of three-spined stickleback (Gasterosteus aculeatus) kept in control pCO₂ (333 ± 30 μatm CO₂) or at high pCO₂ levels (991 ± 57 μatm) for 43 days. The gene expression analysis included GABAₐ receptor subunits (α₁–6, β₁–3, γ₁–3, δ, π and ρ₁–3), enzymes and transporters involved in GABA metabolism (GAD₁–2, GABAT and GAT₁–3), GABAₐ receptor-associated proteins (GABARAP and GABARAPL), ion cotransporters (KCC₁–4, NKCC₁, ClC₂₁–3, AE3 and NDAE) and carbonic anhydrase (CAII). Exposure to high CO₂ had only minor effects on the expression of genes involved in GABAergic neurotransmission. There were significant increases in the mRNA levels of α family subunits of the GABAₐ receptor, with a more pronounced expression of α₁₂, α₃, α₄ and α₆b. No changes were detected in the expression of other GABAₐ subunits or in genes related to receptor turnover, GABA metabolism or ion transport. Although the minor changes seen for mRNA levels might reflect compensatory mechanisms in the high-CO₂ conditions, these were apparently insufficient to restore normal neural function, because the behavioural changes persisted within the time frame studied.

Key words: GABAₐ receptor, GABAergic system, ion cotransporters, ocean acidification, quantitative polymerase chain reaction, three-spined stickleback

Introduction

The ongoing increase of CO₂ levels in the atmosphere and the resultant changes in the ocean chemistry are leading to what is commonly referred to as ocean acidification. In their most recent assessment report, the Intergovernmental Panel on Climate Change (IPCC) predicted an increase in the atmospheric CO₂ concentration from the present level of 400 μatm to 800–1150 μatm within this century (Collins et al., 2013). These changes in the atmosphere can then lead
Numerous studies on ocean acidification have reported alterations in behaviour and sensory responses in both tropical and temperate fish after sustained exposure to predicted near-future CO2 levels. The sensory systems affected include olfaction, hearing and vision (Munday et al., 2009; Dixson et al., 2010; Ferrari et al., 2011; Simpson et al., 2011; Forsgren et al., 2013; Chung et al., 2014; Rossi et al., 2016). Other neural challenges detected involve brain lateralization (Domenici et al., 2012; Nilsson et al., 2012; Jutfelt et al., 2013; Lai et al., 2015), learning (Ferrari et al., 2012), anxiety (Hamilton et al., 2014), boldness and activity (Munday et al., 2010; Jutfelt et al., 2013). Nonetheless, a few studies on temperate species (Atlantic cod, Gadus morhua, and Atlantic silversides, Menidia menidia) find resilience against elevated ambient CO2, which can be related to adaptations in species experiencing a strong variation in the partial pressure of CO2 (pCO2) in their current habitat (Murray et al., 2014; Jutfelt and Hedgärde, 2015).

Studies using an antagonist (gabazine) or an agonist (muscimol) of the γ-aminobutyric acid receptor A (GABAA receptor) have indicated that an altered function of this inhibitory neurotransmitter receptor underlies these behavioural abnormalities. In particular, gabazine has been found to restore much of the altered behaviours (Nilsson et al., 2012; Chivers et al., 2014; Chung et al., 2014; Hamilton et al., 2014; Lai et al., 2015). The GABAA receptor is an ion channel with conductance for Cl− and HCO3−, and these are the same two ions that are involved in pH regulation in fish exposed to elevated CO2. Thus, when fish are exposed to high CO2 levels, the reduction in blood pH is countered by accumulation of HCO3− in blood and tissues (Ishimatsu et al., 2008; Brauner and Baker, 2009), accompanied by a release of H+ and Cl− over the gills into the ambient water. This led Nilsson et al. (2012) to suggest that pH-regulatory changes in fish exposed to high CO2 alter the gradients of Cl− and HCO3− over neuronal membranes in a way that renders some GABAA receptors depolarizing (i.e. excitatory) rather than hyperpolarizing (i.e. inhibitory).

The GABAA receptor is the major inhibitory neurotransmitter receptor in the vertebrate brain, and ~30% of all synapses respond to GABA (Bloom and Iversen, 1971; Kaila, 1994; Somogyi et al., 1996; Sieghart and Sperk, 2002). It is expressed throughout the central nervous system, and its role has been linked to important processes such as brain development, neural migration and excitability, network interaction in the cerebral cortex, memory, learning, cognition, vigilance and behaviour (Sieghart and Sperk, 2002; Makkar et al., 2010; Luscher et al., 2011).

The GABAA receptor is a ligand-gated ion channel composed by pentameric assemblies of subunits, arranged to form a central selective anion channel (Bormann et al., 1987). To date, a total of 19 genes have been found to encode for GABAA receptor subunits in mammals, namely α1–6, β1–3, γ1–3, δ, π, ε, θ and ρ1–3 (reviewed by Farrant and Kaila, 2007). However, information on GABAA receptor composition in fish is very scarce. An immunohistochemistry analysis has confirmed a widespread distribution of the receptor in Atlantic salmon (Salmo salar) brain (Anzelius et al., 1995). Ellesen et al. (2008) surveyed mRNA transcripts of GABAA subunits in the anoxia-tolerant crucian carp (Carassius carassius), quantifying the effect of anoxia on the mRNA expression of subunits α1–6, β2, γ2 and δ1–2. Cocco et al. (2016) recently profiled the expression of GABA subunits in zebrafish (Danio rerio) brain, showing α1, β2, γ2 and δ to be the most prominently expressed subunits.

The combination of different subunits in the pentameric GABAA receptor can give rise to diverse receptor subtypes, with distinct physiological and pharmacological properties (Herd et al., 2007). Generally, a combination of the two most highly expressed subunits, α and β, is sufficient to form a functional GABAA receptor, while the presence of a third subunit is also often observed (reviewed by Farrant and Kaila, 2007). Indeed, the most predominant GABAA receptor stoichiometry among mammals is a heteromeric receptor composed of two α, two β and one γ subunit, with the most common combination being α1, β2 and γ2 subunits (Fritschi et al., 1992; McKernan and Whiting, 1996; Pirker et al., 2000; Sieghart and Sperk, 2002; Whiting, 2003; Benke et al., 2004). In other GABAA receptors, the γ subunit is replaced by δ, π or ε (forming αβδ, αβπ or αβε), whereas the θ subunit might replace the β subunit (Sieghart and Sperk, 2002; reviewed by Farrant and Kaila, 2007).

Activation of the receptor takes place when two GABA molecules bind to the extracellular domains between the α and β subunit, triggering a rapid conformational change in the transmembrane region that allows movement of Cl− and HCO3− through the channel (Bormann et al., 1987). Intracellular and extracellular [Cl−] and [HCO3−] are important for setting the ECl reversal potential. In most mature mammalian neurons, GABAA receptor activation reduces the excitatory neurotransmission through membrane hyperpolarization caused by a net influx of negatively charged Cl− ions into the neuron, with a smaller component of HCO3− flowing out (reviewed by Farrant and Nusser, 2005). However, in the fetal mammalian brain, and in some conditions of neuronal overactivity, such as in epilepsy, anion gradients are reversed as a result of increased intracellular [Cl−] and/or intracellular [HCO3−] linked to a different or altered expression of ion transporters. The Cl− gradient across neuronal membranes has been shown to depend largely on two ion-exchange mechanisms (Delpeire, 2000). The K+–Cl− cotransporters (KCC) are responsible for K+–coupled Cl− outward transport in central neurons. In contrast, the Na+–K+–2Cl− cotransporter (NKCC) family is responsible for transporting Cl− into cells through a Na+–K+–coupled Cl− inward transport. The high intracellular [Cl−] that makes GABAA receptors excitatory in developing
fetal brains has been linked to an upregulation of NKCC1 mRNA expression, whereas the low intracellular [Cl\(^-\)] in mature neurons is attributable to an upregulation of KCC2 mRNA expression (Delpire, 2000).

Additional mechanisms may influence anion gradients across neural membranes. One is the voltage-gated Cl\(^-\) channel 2 (CIC2), which has an important role in determining the intracellular [Cl\(^-\)] through chloride excursion in neurons expressing inhibitory GABA\(_A\) receptors and directly reducing excitability (Rätté and Prescott, 2011). The anion exchanger 3 (AE3) affects both [Cl\(^-\)] and [HCO\(_3^+\)] by exchanging Cl\(^-\) and HCO\(_3^-\) over cell membranes, while the Na\(^+\)-driven anion exchanger (NDAE) can influence intracellular [Cl\(^-\)] through a Na\(^+\)-coupled HCO\(_3^-\) outward transport (Romero et al., 2000; Casey et al., 2009). Finally, intracellular [HCO\(_3^-\)] is influenced by the rate of hydration of intracellular CO\(_2\) through the action of carbonic anhydrases (CA; Lindskog, 1997).

The function of the GABAAergic transmission is also affected by the timing of GABA release and clearance in the extracellular space. Extracellular GABA in not subject to enzymatic degradation, but its turnover relies on diffusion and uptake by specific GABA transporters, GAT1–3 (reviewed by Scimemi, 2014). GABA is consequently processed in the neurons by GABA aminotransferase (GABAT) and glutamate decarboxylases (GAD1–2, also known as GAD67 and GAD65; Delpire, 2000). The clustering, targeting and degradation of the GABA\(_A\) receptor in the post-synaptic area is regulated by GABA\(_A\) receptor-associated proteins (GABARAP and GABARAPL; Nemos et al., 2003). Both proteins belong to a microtubule-associated protein family.

Interestingly, in some fish the behavioural dysfunctions observed in hypercapnia set only in after several days of exposure to high CO\(_2\) and then persist for several days after normal CO\(_2\) levels have been restored (Munday et al., 2010). This led Lai et al. (2015) to propose that gene transcription may be involved. This could include the expression of GABA\(_A\) receptor genes and the genes encoding for proteins responsible for establishing Cl\(^-\) and HCO\(_3^-\) ion gradients over neuronal membranes.

The three-spined stickleback (Gasterosteus aculeatus) should provide a good model for investigating the effects of CO\(_2\) on gene expression because its genome has been sequenced and annotated (Kingsley, 2003). Importantly, sustained high-CO\(_2\) exposure has been shown to alter three-spined stickleback behaviour (Jutfelt et al., 2013; Näslund et al., 2015), and this impairment can be reversed by treatment with the GABA\(_A\) antagonist gabazine (Lai et al., 2015). Thus, as in other fishes, the neural effects of high-CO\(_2\) exposure on three-spined stickleback appear to depend on altered GABA\(_A\) receptor function.

We hypothesized that the proposed ion disturbances leading to altered GABA\(_A\) receptor function in brains of hypercapnic fish lead to alterations in the expression of genes related to the function of these systems. Consequently, we have quantified the mRNA transcription levels of 56 genes involved in GABAergic transmission and anion regulation in brains of three-spined stickleback exposed to present and predicted future CO\(_2\) levels. Our analysis included the expression of 28 genes encoding for the GABA\(_A\) receptor subunits in three-spined stickleback, six for GABA transporters (GAT1–3), GABA aminotransferase (GABAT), three for glutamate decarboxylases (GAD1–2), three for GABA\(_A\) receptor-associated protein and protein-like (GABARAP and GABARAPL), 14 for ion cotransporters (KCCs, NKCCs, CIC2s, AE3 and NDAE) and two for carbonic anhydrases (CAII and CAVII).

### Materials and methods

#### Experimental animals

One hundred marine female three-spine sticklebacks weighing 1.24 ± 0.07 g were caught in Fiskebäckskil, Sweden, during July–August 2012 and were randomly distributed into ten 25 litre glass aquaria of 10 individuals each in Sven Lovén Centre for Marine Sciences, Kristineberg, Sweden. The aquaria were constantly supplied with water at 17.6 ± 1.2°C (SD) and salinity 24.2 ± 3.4 PSU (SD). Chemical parameters such as salinity, oxygen saturation, temperature and pCO\(_2\) were measured daily, and alkalinity was measured weekly. Further details are given by Jutfelt et al. (2013), who published behavioural data from the same groups of fish.

The fish were divided into two experimental groups (distributed in duplicate aquaria for each group), where one group was exposed to increased pCO\(_2\) (991.3 ± 56.6 µatm), while the other served as a control and was exposed to present-day CO\(_2\) levels (333.0 ± 30.0 µatm pCO\(_2\); Jutfelt et al., 2013). Fish were kept in a 14 h–10 h light–dark cycle and fed ad libitum twice daily with frozen Artemia nauplii. The exposures lasted for 43 days. Upon termination of exposure and behavioural studies, 12 individuals weighing 2.06 ± 0.14 g from the control group and 12 individuals weighing 1.58 ± 0.18 g from the CO\(_2\) group were killed using an overdose of 2-phenoxycethanol in seawater. For the gene expression analysis, the whole brains were rapidly dissected, snap-frozen in liquid nitrogen and stored at −80°C until further use. Prior to downstream experiments, samples were transferred on dry ice to the Department of Biosciences, University of Oslo, Norway.

Animal experiments were carried out in accordance with national regulations and were approved by the ethical committee on animal experiments of Gothenburg, Sweden (ethical permit: Fredrik Jutfelt 100-2010 and 151-2011).

#### Quantification of mRNA expression using qPCR

**RNA extraction and cDNA synthesis**

Total RNA was extracted from brains using 15 µl/mg TRiZol® reagent (Invitrogen, Carlsbad, CA, USA). A NanoDrop 2000
UV-Vis Spectrophotometer (Thermo Fisher Scientific, Rockland, DE, USA) and a 2100 BioAnalyzer with RNA 6000 Nano Lab Chip Kit (Agilent Technologies, Palo, Alto, CA, USA) were used to assess the quantity and quality of the extracted total RNA. Prior to cDNA synthesis, 1 μg of total RNA was treated with TURBO DNase using TURBO DNase-free kit (Ambion Applied Biosystems, Foster City, CA, USA) to avoid any remnants of genomic DNA. Subsequently, cDNA was synthesized in duplicate from each total RNA sample using SuperScript III reverse transcriptase (Invitrogen) and oligo(dT)18 in a total reaction volume of 20 μl. All procedures were carried out in accordance with the manufacturer’s protocols.

**Real-time RT-PCR primer design**

To our knowledge, expression analyses of the GABA<sub>α</sub> subunits or genes linked with the GABA<sub>α</sub> activity studied here have previously not been described in stickleback. Therefore, a total of 56 gene-specific real-time rt-PCR (qPCR) primer pairs were designed from stickleback gene sequences retrieved from the Ensembl database (http://www.ensembl.org/index.html; see Table 1 for accession numbers). For each transcript, a minimum of three primer pairs were initially designed for each nucleotide sequence using Primer3 (http://primer3.ut.ee) and synthesized by ThermoScientific (Ulm, Germany). Emphasis was put on designing primers spanning exon–exon junctions to avoid amplification of any remnant genomic DNA. All primers were analysed for crossing point (Cp) values, primer efficiencies (E) and melting peaks, and their products were sequenced by GATC (Cologne, Germany), ensuring amplification of a single amplicon. The primer pairs showing the highest efficiency, lowest crossing point value and a single melting peak curve were selected for qPCR and are listed in Table 1.

For genes with known paralogues or splice variants, efforts were made to design transcript-specific primers when possible, in order to discriminate between closely related transcripts. A comparison aiming at determining identities between genes was carried out using a global alignment (NCBI-Needleman–Wunsch Global Align Nucleotide Sequences; blast.ncbi.nlm.nih.gov), which is a sequence alignment method based on the Needleman–Wunsch algorithm (Needleman and Wunsch, 1969) used to find the best optimal alignment along two sequences (Table S1).

Thirty-five gene sequences for GABA<sub>α</sub> receptor subunits were retrieved from the Ensemble stickleback database. Diverse paralogue sequences exist in the GABA<sub>α</sub> subunit families, except for the δ subunit, which has only one known gene variant (see Table 1A). The majority of these sequences showed a distant relationship (identities ranging from 37 to 78%; Table S1A–E), and qPCR primers were directed at conserved regions. In contrast, paralogues belonging to the subunits β3, γ2 and ρ1 showed a close identity (51–100%), and qPCR primers were directed at poorly conserved regions and analysed as single transcripts (Table S1B, C and E). Among all paralogues, Ensembl presents alternative splice variants for β2<sub>1</sub>, β3<sub>1</sub> and β3<sub>2</sub> (Table 1A). In the γ family, γ2 is the only subunit that splices for three alternative transcripts (Table 1A), and for the ρ subunits, two alternative variants are known to be present for ρ1<sub>1</sub> and ρ3a: ρ1<sub>2</sub> and ρ3<sub>a</sub>, ρ3<sub>a</sub> (Table 1A). Altogether, a total of 28 qPCR primers were designed for the gene expression analysis of subunits (some sequences showed too much similarity to allow for the design of specific primers).

For the genes involved in GABA turnover, 11 different gene paralogues were found to encode for GAT, three for GAD, one for GABAT, two for GABARAP and four for GABARAPL. We designed a total of 13 qPCR primers, of which some will work for more than one transcript. Moreover, four different genes encoding for CIC<sub>2</sub>, seven for KCCs and four for NKCC1 are found in the stickleback genome, whereas AE3, NDAE and CAII have only one variant (Table 1). A total of 15 qPCR primers were designed for the ion cotransporter analysis. Effort was made to design primers able to detect CAVII, but we were unsuccessful in detecting this transcript.

As for the GABA<sub>α</sub> subunits, splice variants are present for some of the members of GAT and GAD families (Table 1B). The gene GAT1 splices for two splice variants (Table 1B). The other transporters, GAT2<sub>1</sub>, GAT2<sub>3</sub> and GAT3, splice into two (GAT2<sub>1</sub> and GAT2<sub>3</sub>), three (GAT2<sub>3a</sub>, GAT2<sub>3ii</sub> and GAT2<sub>3iii</sub>) and two variants (GAT3<sub>1</sub> and GAT3<sub>3</sub>), respectively (Table 1B). The GABARAPL2 gene encodes for three splice variants, GABARAPL2<sub>i</sub> and GABARAPL2<sub>ii</sub> (Table 1B). In the KCC family, two KCC<sub>4b</sub> splice variants are known, KCC<sub>4b1</sub> and KCC<sub>4b2</sub> (Table 1C). Likewise for CIC<sub>2</sub>, there are two alternative splice variants (CIC<sub>2</sub> and CIC<sub>2ii</sub>; Table 1C). Two NKCC1 paralogues exist (NKCC1<sub>1</sub> and NKCC1<sub>2</sub>), both having alternative splice variants (Table 1C).

**Quantitative PCR**

Quantitative PCR was carried out in duplicates using 1:30 diluted cDNA (3 μl), LightCycler 480 SYBR Green I Master Mix (5 μl; Roche Diagnostics, Basel, Switzerland), primers (1 μl, 5 μM) and nuclease-free water (1 μl; Ambion Applied Biosystems). The reaction mix and samples were loaded onto 384 multwell plates (Roche Diagnostics) using an Agilent Bravo robot (Agilent Technologies, USA) The following qPCR program was used: (i) 95°C for 10 min; (ii) 95°C for 10 s; (iii) 60°C for 10 s; (iv) 72°C for 13 s; and (v) repeat steps (ii) to (iv) 42 times. A melting curve analysis was performed for each amplicon after the qPCR program. Ubiquitin (ubc) and ribosomal protein L13A (rpl13A) were used as reference genes for normalization, as they have previously been demonstrated to be the most stably expressed genes in the three-spined stickleback (Hibbeler et al., 2008) (Table 1). The geometric average of their expression was used to normalize the data sets, because this method has been shown to be a prerequisite for an accurate qPCR expression analysis to determine the possibility of studying small expression differences (Vandesompele et al., 2002; Hellemans et al., 2007).
Table 1: Primer sequences for qPCR in three-spined stickleback

| Gene                                      | GenBank ID       | Primers for real-time PCR | E (±)          | Cp (±)          |
|-------------------------------------------|------------------|----------------------------|----------------|----------------|
| Ubiquitin                                 | ENSGACG000000008021 | ubc F AGACGGGCATAGCACCTG  | 1.894 ± 0.001  | 22.17 ± 0.06   |
|                                           |                  | R CAGGACAAGGAAGGCATCC     |                |                |
| Ribosomal protein L13A                    | ENSGACT00000012382| rpl13A F CACCTGGTCAAATTGACATG | 1.897 ± 0.006  | 21.66 ± 0.16   |
| GABAα1 (1of2)                             | ENSGACT00000027474 | α1 F GCCAGAGTGGATTCTGGT  | 1.896 ± 0.003  | 25.62 ± 0.11   |
|                                           |                  | R GGACGGACTCTCTGTTGAGC    |                |                |
| GABAα1 (2of2)                             | ENSGACT00000027475 | α12 F GCTATGACAATCGCCTCAGG | 1.878 ± 0.000  | 26.75 ± 0.17   |
| GABAα2                                    | ENSGACT00000024778 | α2 F GAGGATTTCCCATAGGACTT | 1.910 ± 0.002  | 28.14 ± 0.21   |
| GABAα3                                    | ENSGACT00000026865 | α3 F CACCTGGACATCAGTGCTA | 1.846 ± 0.002  | 32.11 ± 0.27   |
| GABAα4                                    | ENSGACT00000024781 | α4 F TTTGAGACCATCCTGGAC  | 1.882 ± 0.001  | 31.19 ± 0.19   |
| GABAα5 (1of2)                             | ENSGACT00000018222 | α51 F TCCGCTCAATCAATAACCA | 1.865 ± 0.003  | 31.73 ± 0.22   |
|                                           |                  | R CGGCATGTAGGTCTGGATGA    |                |                |
| GABAα5 (2of2)                             | ENSGACT00000019800 | α52 F ATGCCATATCCGGTGCTAGAG | 1.877 ± 0.001  | 28.92 ± 0.13   |
| GABAβ1                                    | ENSGACT00000017426 | β1 F GGGCGGAAAAACATTGAACCT | 1.885 ± 0.005  | 27.08 ± 0.17   |
| GABAβ2 (1of2)                             | ENSGACT00000024053 | β21 F AAGATGAGACCCGACCCCAA | 1.906 ± 0.000  | 27.42 ± 0.14   |
|                                           |                  | R TGCTGGCTATGCTCTAATAGC   |                |                |
| GABAβ2 (2of2)                             | ENSGACT00000024054 | β21i F ATCCGGGAAACCCTGAATAAAA | 1.894 ± 0.003  | 27.68 ± 0.14   |
|                                           |                  | R CGTCCGACGTTCCCTCCCTCA   |                |                |
| GABAβ3 (1of2)                             | ENSGACT00000018209 | β31at F AGGGATACGACATCGCTG | 1.811 ± 0.002  | 34.34 ± 0.20   |
|                                           |                  | R CGTACGGTAGATCTTGTGCC    |                |                |
| GABAβ3 (2of2)                             | ENSGACT00000019821 | β32at F AGCTACGTCGACTGGGCTCT | 1.853 ± 0.000  | 28.37 ± 0.04   |
|                                           |                  | R GGTGGATCTGGGTTGATCC     |                |                |
| GABAγ1                                    | ENSGACT00000026662 | γ1 F ATCAATTACCAGCTGCGCAGAG | 1.820 ± 0.000  | 29.71 ± 0.09   |

(Continued)
| Gene        | GenBank ID                  | Primers for real-time PCR                     |
|------------|-----------------------------|-----------------------------------------------|
| GABAγ2     | (i) ENSGACT00000027471      | γ2at F GAACAAACAAAGAGGCAAA 1.874 ± 0.001 29.59 ± 0.10 |
|            | (ii) ENSGACT00000027472    | R GGCACAAGTTTGGTCATCTG                         |
|            | (iii) ENSGACT00000027473   | F GCTGTCTCCTTCTTCACTC 1.820 ± 0.003 33.08 ± 0.11 |
| GABAγ3     | (1of2) ENSGACT0000001822    | γ31 F GCTGTCTGTCCTTCTCACCT 1.820 ± 0.003 33.08 ± 0.11 |
|            | (ii) ENSGACT0000001978      | R GGCACAATGTTGGTCATCTG                         |
|            | (iii) ENSGACT0000001979     | F GCTGTCTGTCCTTCTCACCT 1.820 ± 0.003 33.08 ± 0.11 |
| GABAδ      |                                | δ F CTGGAGCTTCCAGGATCCTGA 1.917 ± 0.003 25.02 ± 0.16 |
| GABAπ      | (1of2) ENSGACT00000003740   | π1 F TTCTGCCTCCACCACCATCT 1.835 ± 0.002 32.40 ± 0.31 |
|            | (ii) ENSGACT00000003741     | R TTGGTTGCCCTCGAAACCAAG                         |
| GABAρ1     | (1of2) ENSGACT0000001217    | ρ11 F GTCACTGTTACCGCCATGTG 1.830 ± 0.002 29.3 ± 0.15 |
|            | (ii) ENSGACT0000001618      | R TGGTGGTGTGGAATTTCTGA                         |
| GABAρ2a    | (1of2) ENSGACT0000001615    | ρ2a1 F CACTAAAGTCTGGGGTCCGA 1.896 ± 0.008 33.61 ± 0.03 |
|            | (ii) ENSGACT0000001616      | R TTGGTGGTGTGGAATTTCTGA                         |
| GABAρ2a    | (2of2) ENSGACT0000001727    | ρ2a2 F GCAATGAATCTGGCCACCTGA 1.888 ± 0.001 32.5 ± 0.09 |
|            |                                | R GGATGAGGAACTGGGGAGCA                         |
| GABAρ3a    | (1of2) ENSGACT00000027380   | ρ3a1 F AGGACGGGGCGATTCCTG 1.904 ± 0.003 22.08 ± 0.01 |
|            | (ii) ENSGACT00000027381     | R CAGGACAAGGAAAGCATTCC                         |
| Ubiquitin  |                                | F AGACGGGGCATAGCACCTGC 1.904 ± 0.003 22.08 ± 0.01 |
|            |                                | R CAGGACAAGGAAAGCATTCC                         |
| Ribosomal protein L13A | ENSGACT00000012382 | rpl13A F CACCTTGTCACCTGACCTG 1.902 ± 0.010 22.07 ± 0.26 |
| GAT11      | ENSGACG00000009684           | GAT11 F AGAGTACGTGTTCCGG 1.879 ± 0.000 25.19 ± 0.04 |
| GAT12      | (i) ENSGACT00000020044      | GAT12 F AGAGTACGTGTTCCGG 1.879 ± 0.000 25.19 ± 0.04 |
|            | (ii) ENSGACT00000020046     | R ATAGGTTCGCTGCGTGTCATCTG 1.894 ± 0.001 32.9 ± 0.28 |
| GAT21      | (i) ENSGACT00000004780      | GAT21 F AGAGTACGTGTTCCGG 1.879 ± 0.000 25.19 ± 0.04 |
|            | (ii) ENSGACT00000004778     | R ATAGGTTCGCTGCGTGTCATCTG 1.894 ± 0.001 32.9 ± 0.28 |
| GAT22      | ENSGACT000000025159         | GAT22 F AGAGTACGTGTTCCGG 1.879 ± 0.000 25.19 ± 0.04 |
|            |                                | R ATAGGTTCGCTGCGTGTCATCTG 1.894 ± 0.001 32.9 ± 0.28 |

(B)
Table 1: continued

| Gene         | GenBank ID                      | Primers for real-time PCR |
|--------------|--------------------------------|---------------------------|
|              |                                |                           |
| GAT2a        | (i) ENSGACT00000001890 GAT2a  | R AATCGCATAACCCCACCAGG    |
|              | (ii) ENSGACT00000001897 GAT2a | F GGTCTGGAGCCCCCTCGTAAC  |
|              | (iii) ENSGACT00000001899 GAT2a| R GAGAGTATCCCACTGCAAGG    |
| GAT3         | (i) ENSGACT00000009625 GAT3   | F GCGGAGTGTGTTGCTGTTT     |
|              | (ii) ENSGACT00000009632 GAT3  | R CCAGTCAGGGTAGGCTGAC     |
| GABAT        | (i) ENSGACT0000006245 GABAT   | F TGTCGATCAAAGCAGCTCG     |
|              | (ii) ENSGACT0000006238 GABAT  | R CATGGTCAAGCCCGGAGCA     |
| GAD1a        | ENSGACT00000006685 GAD1a      | F GGGACACCTTGAAGTACGGA    |
|              |                                | R CATGTCCTCAAGCTGGTGG     |
| GAD1b        | ENSGACT00000017175 GAD1b      | F CCATTGGGTITGAGCAGGCA    |
|              |                                | R ATCATCTTGTGCCCGGTTCA    |
| GAD2         | ENSGACT0000006820 GAD2        | F ACCTCTTTCGCCATAACCG     |
|              |                                | R ATCATCTTGTGCCCGGTTCA    |
| GABARAP      | (i) ENSGACG000000025686 GABARAP| F ATACTCTTCGCCCTCTCCGACC |
|              | (ii) ENSGACG000000025685 GABARAP| R CGCTCTCATCACTGTAAGCA   |
| GABARAPL1    | ENSGACG000000013851 GABARAPL1 | F AGGTGAGGAGAGCAGAAGGA    |
|              |                                | R GGGGAAGGGAGTTGTTGCA     |
| GABARAPL2    | (i) ENSGACG00000002829 GABARAPL2| F AAGTACCTGGTGCCCTCTGA   |
|              | (ii) ENSGACG00000002816 GABARAPL2| R GTTTCGTCAGCCTGGCAT    |
|              | (iii) ENSGACG00000002836 GABARAPL2| R GTTTCGTCAGCCTGGCAT    |
| (C) Ubiquitin| ENSGACG00000008021 ubc        | F AGACGGGCATAGCACCTGC     |
|              |                                | R CAGGAAAGAAGCAGTACC     |
| Ribosomal protein L13A | ENSGACT00000021382 rpl13A   | F CACCTCTGTCACCTGAGCAGT   |
|              |                                | R TCCCTCGGCCCCCTAGCAG     |
| KCC1         | ENSGACT0000022002 KCC1        | F ACAAGGAGAGGCTCAGTG     |
|              |                                | R TCATGCTTAGGAAAGCACGC    |
| KCC2a        | ENSGACT00000007272 KCC2a      | F AGACGCAAGACCTGAGACGC    |
|              |                                | R GCACGCTAGACTGTTGCTA     |
| KCC2b        | ENSGACT00000003694 KCC2b      | F AGAACATCCTCAGCTACGC     |
|              |                                | R GCACGCTAGACTGTTGCTA     |
| KCC3         | ENSGACT00000025029 KCC3       | F GCCGCTCAGCCTGATATCC    |
|              |                                | R GCACGCTAGACTGTTGCTA     |
| KCC4a        | ENSGACT0000019164 KCC4a       | F GCAAAGAATCGACCCATT     |
|              |                                | R CACACAGCATCAGACGTA      |
| KCC4b        | (i) ENSGACT0000001353 KCC4b  | F AAAGACACAGGCGAGGAGCA    |
|              | (ii) ENSGACT0000001355 KCC4b | R CATGAGGATGTGGTGTCAG     |

(Continued)
The Cp values and priming efficiencies for each reaction were calculated using the second derivative maximum method (Roche Lightcycler 480; Rasmussen, 2001) and the LinRegPCR software (Ruijter et al., 2009), respectively. Subsequently, relative mRNA expression levels were calculated using the following formula:

\[ \text{Expression of target gene} = \left( \frac{E_{ga}^{\Delta\text{C}_{FPCR}}}{E_{tar}^{\Delta\text{C}_{PCP}}} \right) \]

Where \( ga \) is the geometric average of the two reference genes; \( tar \) is the gene of interest, \( E \) is priming efficiency and \( Cp \) is the crossing point.

Given that duplicate cDNA syntheses were performed, and each of these were analysed in duplicates in the qPCR analyses, four data points were present for each original sample for each primer pair used, and their means were used in the mRNA expression calculations.

**Statistical analysis**

All statistical analyses were performed using GraphPad Prism (GraphPad Software; version 6.0d; Mac OS X). Normality and homogeneity of variance were assessed using the D’Agostino & Pearson omnibus normality test and \( F \)-test. According to function, data were grouped into seven families as follows: (i) GABA\(_{\alpha}\) subunits; (ii) GABA\(_{\beta}\) subunits; (iii) GABA\(_{\gamma}\) subunits; (iv) GABA\(_{\delta, \pi, \rho}\) subunits; (v) GAT, GAD and GABAT; (vi) GABARAP and GABARAPL; and (vii) KCC, NKCC, ClC2, AE3, NDAE and CAVII. Two-way analysis of variance (ANOVA) followed by the Sidak post hoc test was used to examine differences in expression between the genes within the families and between the two treatment groups. A value of \( P < 0.05 \) was considered significant. All data are presented as means ± SEM, unless otherwise stated.

**Results**

As listed in Table 1A, GABA\(_{\alpha}\) subunits can be regrouped into six families: \( \alpha(1-6) \), \( \beta(1-3) \), \( \gamma(1-3) \), \( \delta, \pi \) and \( \rho(1-3) \). In contrast to mammals, no genes encoding for \( \epsilon \) and \( \theta \) subunits are present in the stickleback genome. All GABA\(_{\alpha}\) paralogues retrieved on the Ensembl database were found to be expressed in the three-spined stickleback brain (Fig. 1A–D). Expression within each gene family was analysed using two-way ANOVA, with subunit and \( \text{CO}_2 \) treatment as the two factors.
variables. Not surprisingly, the mRNA transcripts levels differed significantly for the different subunits (Fig. 1; two-way ANOVA, P < 0.001). The most highly expressed GABA_A subunits in the three-spined stickleback belonged to the α, β, γ and δ families (Fig. 1A–D), and within these families the expression was dominated by α11 and α12, α6b, β1, γ1 (there was only a single isoform for the δ subunit). Among the ρ subunits, ρ11 was the most abundant (Fig. 1D). The π subunits showed the lowest expression levels (Fig. 1D). Exposure of three-spined stickleback to elevated pCO2 (~990 μatm) resulted in significantly altered expression levels for relatively few of the GABA_A subunits investigated. The α family

Figure 1: Messenger RNA expression levels of GABA_A receptor subunits. Data were normalized to the geometric average of the reference genes ribosomal protein L13A (rpl13A) and ubiquitin (ubc) and grouped into four families as follows: α subunits (A); β subunits (B); γ subunits (C) and δ, π and ρ subunits (D). Each family was analysed by two-way ANOVA followed by Sidak post hoc test. Open and filled columns represent three-spined sticklebacks exposed to control water (n = 12) and high-CO2 water (n = 12) for 43 days. Values are shown as means + SEM.
subunits, which are composed of nine different isoforms, showed a significantly higher expression in high-CO$_2$ fish compared with control fish (Fig. 1A; two-way ANOVA, $P = 0.0165$). All $\alpha$ subunits showed a numerically higher mean expression in the high-CO$_2$ group, with increased mRNA transcription levels of 24, 48, 50 and 25% for the $\alpha_{12}, \alpha_3, \alpha_4$ and $\alpha_6b$, respectively, although the post hoc test failed to identify significant treatment effects for any individual isoform (Fig. 1A; Sidak post hoc test, $P > 0.05$). The high-CO$_2$ exposure did not significantly affect the expression of the $\beta$ (two-way ANOVA, $P = 0.6639$), $\gamma$ (two-way ANOVA, $P = 0.1861$), $\delta$, $\pi$ or $\rho$ subunits (two-way ANOVA, $P = 0.7189$; Fig. 1B–D).

Of the genes involved in GABA turnover included in Ensembl Genome Browser (GAT, GAD, GABAT, GABARAP and GABARAPL), all were found to be expressed in three-spined stickleback brain (Figs 2 and 3). Within families, there were significant differences in the mRNA abundance of the paralogue members (two-way ANOVA, $P < 0.001$). In the control group, the GAT1 paralogues (GAT11 and GAT12at) were more abundant than GAT21–3 (Fig. 2). In the GAD family, GAD1b displayed higher mRNA expression levels than the GAD1a and GAD2 transcripts (Fig. 2). GABARAP was almost four times more highly expressed than GABARAPL1 and eight times more highly expressed than GABARAPL2 (Fig. 3).

![Figure 2: Messenger RNA expression levels of GAT, GABAT and GAD genes. Data were normalized to the geometric average of the reference genes ribosomal protein L13A (rpl13A) and ubiquitin (ubc). Data were analysed by two-way ANOVA followed by Sidak post hoc test. Open and filled columns represent three-spined sticklebacks exposed to control water ($n = 12$) and high-CO$_2$ water ($n = 12$) for 43 days. Values are shown as means $\pm$ SEM.](https://academic.oup.com/conphys/article-abstract/4/1/cow068/2753368)

![Figure 3: Messenger RNA expression levels of GABARAP and GABARAPL genes. Data were normalized to the geometric average of the reference genes ribosomal protein L13A (rpl13A) and ubiquitin (ubc). Data were analysed by two-way ANOVA followed by Sidak post hoc test. Open and filled columns represent three-spined sticklebacks exposed to control water ($n = 12$) and high-CO$_2$ water ($n = 12$) for 43 days. Values are shown as means $\pm$ SEM.](https://academic.oup.com/conphys/article-abstract/4/1/cow068/2753368)
In our experiment, none of the transporters or enzymes involved in GABA metabolism displayed significant alterations in expression in response to high-CO2 treatment (Figs 2 and 3; two-way ANOVA, P > 0.05).

Of the genes involved in ion transport, the expression level of the transcripts differed between the members of the gene families (two-way ANOVA, P < 0.0001; Fig. 4). KCC2a, ClC2a, and NKCC1i were the most highly expressed transcripts among the ion transporters (Fig. 4). Exposure to high CO2 did not cause significant changes in mRNA expression levels for the ion transporter transcripts in high-CO2-treated fish compared with control fish (Fig. 4; two-way ANOVA, P > 0.05).

**Discussion**

Changes in the function of GABA_A receptors caused by altered ion gradients have been suggested as a general mechanism behind the behavioural disturbances seen in CO2-exposed fish (Nilsson et al., 2012; Chivers et al., 2014; Chung et al., 2014; Hamilton et al., 2014; Lai et al., 2015). Exposure to high CO2 levels triggers acid–base adjustments in fish involving altered levels of Cl^- and HCO3^- (Brauner and Baker, 2009), which have been suggested to change neuronal membrane gradients of these ions, switching some GABA_A receptors from being inhibitory to excitatory (Nilsson et al., 2012). Based on the scarce data available, calculations of GABA_A equilibrium potentials of neurons in fish exposed to near-future pCO2 are consistent in showing a possibility for a shift in the GABA_A receptor equilibrium potential from causing hyperpolarization to depolarization (Heuer and Grosell, 2014; Nilsson and Lefèvre, 2016; Heuer et al., 2016; Regan et al., 2016). Here, we hypothesized that an increase in CO2 levels in the marine environment, triggering acid–base regulatory mechanisms in fish, could lead to changes in the expression of genes involved in regulating GABA_A receptor function and neuronal ion distribution. Such molecular changes could be adaptive. However, the persistence of the behavioural disturbances reported in some experiments, and a lack of transgenerational acclimation (Welch et al., 2014), suggest that possible molecular responses are insufficient, or even maladaptive.

The present study is the first comprehensive expression analysis focused on genes involved in GABAergic transmission in fish exposed to elevated CO2. The fish in this study were the same individuals as those previously examined behaviourally (Jutfelt et al., 2013), where the behavioural alterations, including reduced exploratory behaviour and lateralization, were characterized and found to persist for the whole experimental period. Of the 28 GABA_A receptor subunits examined herein, there was a significant effect of the high-CO2 treatment on the mRNA expression level for the α family subunits, all showing a tendency to be more highly expressed in the CO2 group. This could suggest some subunit rearrangement of GABA_A receptors in this group, assuming that gene expression is reflected in

**Figure 4:** Messenger RNA expression levels of KCCs, NKCC1, CIC2, NDAE, AE3 ion cotransporters and CAII enzyme. Data were normalized to the geometric average of the reference genes ribosomal protein 13A (rpL13A) and ubiquitin (ubc). Data were analysed by two-way ANOVA followed by Sidak post hoc test. Open and filled columns represent three-spined sticklebacks exposed to control water (n = 12) and high-CO2 water (n = 12) for 43 days. Values are shown as means ± SEM.
protein expression. Changes in the GABA<sub>A</sub> receptor composition are often suggested to induce a change in the receptor function (reviewed by Farrant and Kaila, 2007). The α subunits play important roles on desensitization and deactivation of the receptor, because the GABA binding is presumed to take place at the α-β interfaces (Böhme et al., 2004). In the present experiment, changes at the mRNA expression level of the α subunit family after exposure to high CO<sub>2</sub> might indicate possible compensatory mechanisms used by three-spined stickleback to restore proper GABA<sub>A</sub> receptor function, but further investigations are required. However, if adaptive, the changes are apparently not sufficient, because the behavioural alterations detected in the same individuals by Jutfelt et al. (2013) remained, and if anything increased, during the 43 day exposure period. Also, we cannot exclude the possibility that the changes detected in this study are maladaptive rather than adaptive and contribute to the behavioural alterations.

As mentioned, the three dominant subunits that make up the mammalian receptor are α, β and γ (reviewed by Farrant and Kaila, 2007), and our data show that these subunit families are also highly expressed in three-spined stickleback, but it is striking that the δ subunit is also expressed at a level similar to the most highly expressed α subunits (Fig. 1). Although the mammalian receptors are dominated by subunits α1 β2 and γ2 (Pirker et al., 2000), the most predominantly expressed subunits in three-spined stickleback were found to be α1, α6b, β1, γ1 and δ. Receptors that comprise γ2 in association with α1, α2 or α3 subunits are normally localized to post-synaptic membranes, where they mediate a phasic inhibition involving a rapid and brief inhibitory postsynaptic potential in response to GABA in the synaptic cleft (reviewed by Farrant and Nusser, 2005). In contrast, GABA leaking out of the synapse can activate extrasynaptic GABA<sub>A</sub> receptors made up of δ, α4 or α6 subunits, and these are mainly responsible for slower, but longer-lasting inhibitory postsynaptic potentials, causing tonic inhibition (reviewed by Farrant and Nusser, 2005). Consequently, the high expression of the extrasynaptic δ and the α6b subunits might indicate a more important role of extrasynaptic GABA<sub>A</sub> receptors in three-spined stickleback compared with mammals. Interestingly, previous studies on crucian carp (Carassius carassius) and zebrafish (Danio rerio) brain found a dominating expression of the δ subunits (Ellefsen et al., 2008; Cocco et al., 2016). In light of this high expression of δ subunits, it is tempting to suggest that extrasynaptic GABA<sub>A</sub> receptors causing tonic inhibition play more important roles in fish than in mammals. In contrast to both crucian carp and zebrafish, three-spined stickleback express alternative splice variants for β2, β31, β32, ρ12 and ρ3. The only common subunit that exhibits alternative splicing in all three species is γ2 (Ellefsen et al., 2008; Cocco et al., 2016). The presence of numerous splice transcripts in three-spined stickleback brain could mean that GABA<sub>A</sub> receptor isoforms are particularly diverse in this species, and fishes differ in the degree to which alternative splicing is used to modulate GABA<sub>A</sub> receptor function (see also Cresko et al., 2003).

Perhaps surprisingly, exposure to high CO<sub>2</sub> did not result in significant changes in the mRNA expression levels for ion cotransporters. Heuer et al. (2016) showed that the increase in plasma partial pressure of CO<sub>2</sub> (in millimetres of mercury) in spiny damselfish (Acanthochromis polyacanthus) kept in high-CO<sub>2</sub> conditions was accompanied by increases in intracellular and extracellular HCO<sub>3</sub><sup>-</sup> concentrations, with an assumed decrease in intracellular Cl<sup>-</sup> (Heuer et al., 2016). Based on their measurements, they calculated a positive deviation in the E<sub>GABA<sub>A</sub></sub> resting potential in fish exposed to 1900 µatm CO<sub>2</sub>, consistent with a shift in the GABA action towards depolarization (excitation) rather than hyperpolarization (inhibition). Such alterations could be compensated for by changes in the expression of the Cl<sup>-</sup> transporters NKCC1 and KCC2 in fish exposed to high CO<sub>2</sub>. As mentioned, these transporters are known to play important roles in setting the reversal potential for Cl<sup>-</sup> (E<sub>Cl</sub>) in the mammalian central nervous system, leading to a shift of the GABA<sub>A</sub> receptor function from excitatory in immature neurons to inhibitory in mature neurons (Delpire, 2000). In any case, this does not appear to happen in CO<sub>2</sub>-exposed stickleback in the present conditions, as we found no significant changes in the expression of NKCC1 and KCC2, or in other Cl<sup>-</sup> and HCO<sub>3</sub><sup>-</sup> transporters.

Our gene expression results are in agreement with the recent findings of Schunter et al. (2016) on juvenile spiny damselfish (Acanthochromis polyacanthus). In a transcriptome and proteome analysis, they investigated the molecular responses of offspring of CO<sub>2</sub>-tolerant and CO<sub>2</sub>-sensitive parents reared in control or high-CO<sub>2</sub> conditions. The main molecular differences between the two groups were found among genes involved in circadian rhythm control, such as bmal1, clock, per1 and nr1d1 (Schunter et al., 2016). In contrast, the GABA<sub>A</sub> receptor genes were expressed at similar levels across treatments. The only possible change seen in the GABAergic system was at the protein level of an enzyme that may participate in GABA synthesis, aldehyde dehydrogenase 9 member 1 (A9A1), which were more highly expressed in the offspring of the CO<sub>2</sub>-tolerant parents (Schunter et al., 2016).

Conclusions and perspectives

In general, the present findings show that exposure of three-spined stickleback to elevated CO<sub>2</sub> resulted in only few and minor changes in the expression of genes involved in GABAergic neurotransmission in the brain. If these few adjustments reflect compensatory mechanisms they are apparently not sufficient, because the behavioural dysfunctions remained during the course of the 43 day high-CO<sub>2</sub> exposure (Jutfelt et al., 2013). Thus, the present results, together with results reporting that aberrant behaviours displayed by fish exposed to elevated pCO<sub>2</sub> are persistent and not reduced even by transgenerational acclimation (Welch et al., 2014), lead to the worrying conclusion that fish might be incapable of adaptive responses to these new conditions. Given that globally sustained pCO<sub>2</sub> levels >500 µatm have
probably not occurred on earth during the last 30 million years (Beerling and Royer, 2011), we may have to face the conclusion that many present-day fishes do not possess the genes and mechanisms necessary to cope with the projected near-future elevation of CO2 levels.

**Supplementary material**

Supplementary material is available at Conservation Physiology online.

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**References**

Anzelius M, Ekström P, Möhler H, Richards JG (1995) Immunocytochemical localization of GABA_A receptor β1/β3 subunits in the brain of Atlantic salmon (Salmo salar L.). J Chem Neuroanat 8: 207–221.

Beerling DJ, Royer DL (2011) Convergent cenozoic CO2 history. Nat Geosci 4: 418–420.

Benke D, Fakitsas P, Roggenmoser C, Michel C, Rudolph U, Mohler H (2004) Analysis of the presence and abundance of GABA_A receptors containing two different types of α subunits in murine brain using point-mutated α subunits. J Biol Chem 279: 43654–43660.

Bloom FE, Iversen (1971) Localizing 3H-GABA in nerve terminals of rat cerebral cortex by electron microscopic autoradiography. Nature 229: 628–630.

Böhme I, Rabe H, Lüddens H (2004) Four amino acids in the α subunits determine the γ-aminobutyric acid sensitivities of GABA_A receptor subtypes. J Biol Chem 279: 35193–35200.

Bormann J, Hamill OP, Sakmann B (1987) Mechanism of anion permeation through channels gated by glycine and γ-aminobutyric acid in mouse cultured spinal neurones. J Physiol 385: 243–286.

Brauner CJ, Baker DW (2009) Patterns of acid-base regulation during exposure to hypercarbia in fishes. In ML Glass, SC Wood, eds, Cardio-Respiratory Control in Vertebrates. Springer, Berlin, pp 43–63.

Casey JR, Sly WS, Shah GN, Alvarez BV (2009) Bicarbonate homeostasis in excitable tissues: role of AE3 CI−/HCO_3− exchanger and carbonic anhydrase XIV interaction. Am J Physiol Cell Physiol 297: C1091–C1102.

Chivers DP, McCormik MJ, Nilsson GE, Munday PL, Watson SA, Meekan M, Mitcheel MD, Corkill KC, Ferrari MCO (2014) Impaired learning of predators and lower prey survival under elevated CO2: a consequence of neurotransmitter interference. Glob Chang Biol 20: 515–522.

Chung WS, Marshall NJ, Watson SA, Munday PL, Nilsson GE (2014) Ocean acidification slows retinal function in a damaseelfish through interference with GABA_A receptors. J Biol 217: 323–326.

Ciais P, Sabine C, Bala G, Bopp L, Brovkin V, Canadell JG, Chhabra A, DeFries R, Galloway J, Heimann M et al. (2013) Carbon and other biogeochemical cycles. In TF Stocker, D Qin, G-K Plattner, M Tignor, SK Allen, J Boschung, A Nauels, Y Xia, V Bex, PM Midgley, eds, Climate Change 2013: the Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, UK and New York, NY, USA.

Corrao A, Rönnberg AMC, Jin Z, André GI, Vossen LE, Bhandage AK, Thörnqvist PO, Birminn B, Winberg S (2016) Characterization of the γ-aminobutyric acid signalling system in the zebrafish (Danio rerio Hamilton) central nervous system by reverse transcription-quantitative polymerase chain reaction. Neuroscience in press. doi:10.1016/j.neuroscience.2016.07.018.

Collins M, Knutti R, Arblaster J, Dufresne T, Fichefet P, Friedlingstein X, Fanning CP, Qin D, Tignor, SK Allen, J Boschung, A Nauels, Y Xia, V Bex, PM Midgley, eds, Climate Change 2013: the Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, UK and New York, NY, USA.

Cresko WA, Yan YL, Baltrus DA, Amores A, Singer A, Rodrigues-Mari A, Postlethwait JH (2003) Genome duplication, subfunction partitioning, and lineage divergence: Sox9 in stickleback and zebrafish. Dev Dyn 228: 480–489.

Delpire E (2000) Cation-chloride cotransporters in neuronal communication. News Physiol Sci 15: 309–312.

Dixon DL, Munday PL, Jones GP (2010) Ocean acidification disrupts the innate ability of fish to detect predator olfactory cues. Ecol Lett 13: 68–75.

Domenici P, Allan B, McCormick MI, Munday PL (2012) Elevated carbon dioxide affects behavioural lateralization in a coral reef fish. Biol Lett 8: 78–81.

Doney SC, Fabry VJ, Feely RA, Kleypas JA (2009) Ocean acidification: the other CO2 problem. Ann Rev Mar Sci 1: 169–192.

Ellefson S, Stenslakken KO, Fagernes CE, Kristensen TA, Nilsson GE (2008) Expression of genes involved in GABAergic neurotransmission in anoxic crucian carp brain (Carassius carassius). Physiol Genomics 36: 61–68.

Farrant M, Kaila K (2007) The cellular, molecular and ionic basis of GABA_A receptor signaling. In J Biol Chem 282: 21718–21726.

Farrant M, Kaila K (2007) The cellular, molecular and ionic basis of GABA_A receptor signaling. In J Biol Chem 282: 21718–21726.

Farrant M, Nusser Z (2005) Variation on an inhibitory theme: phasic and tonic activation of GABA_A receptors. Nat Rev 6: 215–229.
Ferrari MCO, McCormick MI, Munday PL, Meekan MG, Dixson DL, Lonnstedt Ö, Chivers DP (2011) Putting prey and predator into the CO2 equation—qualitative and quantitative effects of ocean acidification on predator–prey interactions. Ecol Lett 14: 1134–1148.

Ferrari MCO, Manassa RP, Dixson DL, Munday PL, McCormick MI, Meekan MG, Sih A, Chivers DP (2012) Effects of ocean acidification on learning in coral reef fishes. PLoS One 7: e31478.

Forsgren E, Dupont S, Jutfelt F, Amundsen T (2013) Elevated CO2 affects embryonic development and larval phototaxis in a temperate marine fish. Ecol Evol 3: 3637–3646.

Fritschy JM, Benke D, Mertens S, Oertel WH, Bachi T, Möhler H (1992) Five subtypes of type A γ-aminobutyric acid receptors identified in neurons by double and triple immunofluorescence staining with subunit-specific antibodies. Proc Natl Acad Sci USA 89: 6726–6730.

Hamilton TJ, Holcombe A, Tresguerres M (2014) CO2-induced ocean acidification increases anxiety in Rockfish via alteration of GABA_A receptor functioning. Proc Biol Sci 281: 20132509.

Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J (2008) Fishes in high CO2 acidification—General research considerations. Proc Natl Acad Sci USA 105: 14506–14507.

Herd MB, Belelli D, Lambert JJ (2007) Neurosteroid modulation of synaptic and extrasynaptic GABA_A receptors. Pharmacol Ther 116: 20–34.

Heuer RM, Grosell M (2014) Physiologica impacts of elevated carbon dioxide and ocean acidification on fish. Am J Physiol Regul Integr Comp Physiol 307: R1061–R1084.

Heuer RM, Welch MJ, Rummer JL, Munday PL, Grosell M (2016) Altered brain gradients following compensation for elevated CO2 are linked to behavioural alteration in a coral reef fish. Nat Sci Rep 6: 33216.

Hibbeler S, Scharsack JP, Becker S (2008) Housekeeping genes for quantitative expression studies in the three-spined stickleback Gasterosteus aculeatus. BMC Mol Biol 9: 18.

Ishimatsu A, Hayashi M, Kikkawa T (2008) Fishes in high CO2 acidification—General research considerations. Proc Natl Acad Sci USA 105: 14506–14507.

Jutfelt F, Hedgärde M (2015) Juvenile Atlantic cod behaviour appears robust to near-future CO2 levels. Front Zool 12: 20.

Jutfelt F, Souza KB, Vuylsteeke A, Sturve J (2013) Behaviour disturbance in a temperate fish exposed to sustained high-CO2 levels. PLoS One 8: e65825.

Kaila K (1994) Ionic basis of GABA_A receptor channel function in the nervous system Prog Neurobiol 42: 489–537.

Lai F, Jutfelt F, Nilsson G (2015) Altered neurotransmitter function in CO2-exposed stickleback (Gasterosteus aculeatus): a temperate model species for ocean acidification research. Conserv Physiol 3: cov018; doi:10.1093/conphys/cov018.

Lindskog S (1997) Structure and mechanism of carbonic anhydrase. Pharmacol Ther 74: 1–20.

Luscher B, Fuchs T, Kilpatrick CL (2011) GABA_A receptor trafficking-mediated plasticity of inhibitory synapses. Neuron 70: 385–409.

McKernan RM, Whiting PJ (1996) Which GABA_A receptor subtypes really occur in the brain? Trends Neurosci 19: 139–143.

Makkar SR, Zhang SQ, Cranney J (2010) Behavioral and neural analysis of GABA in the acquisition, consolidation, reconsolidation, and extinction of fear memory. Neuropsychopharmacology 35: 1625–1652.

Munday PL, Dixson DL, Donelson JM, Jones GP, Pratchett MS, Devitsina GV, Daving KB (2009) Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. Proc Natl Acad Sci USA 106: 1848–1852.

Munday PL, Dixson DL, McCormick MI, Meekan M, Ferrari MCO, Chivers DP (2010) Replenishment of fish populations is threatened by ocean acidification. Proc Natl Acad Sci USA 107: 12930–12934.

Murray CS, Malvezzi A, Gobler CJ, Baumann H (2014) Offspring sensitivity to ocean acidification changes seasonally in a coastal marine fish. Mar Ecol Prog Ser 504: 1–11.

Nåslund J, Lindström E, Lai F, Jutfelt F (2015) Behavioural responses to simulated bird attacks in marine three-spined sticklebacks after exposure to high CO2 levels. Mar Freshwater Res 66: 877–885.

Needleman SB, Wunsch CD (1969) A general method applicable to the search for similarities in the amino acid sequence of two proteins. J Mol Biol 48: 443–453.

Nemos C, Mnsuy V, Vernier-Magning S, Fraichard A, Jouvenot M, Delage-Mourroux R (2003) Expression of gec1/GABARAPL1 versus GABARAP mRNAs in human: predominance of gec1/GABARAPL1 in the central nervous system. Mol Brain Res 119: 216–219.

Nilsson GE, Lefevre S (2016) Physiological challenges to fishes in a warmer and acidified future. Physiology 31: 409–417.

Nilsson GE, Dixson DL, Domenici P, McCormick MI, Saresen C, Watson SA, Munday PL (2012) Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. Nat Clim Chang 2: 201–204.

Pirker S, Schwarzer C, Wieselthal A, Sieghart W, Sperk G (2000) GABA_A receptors: immunocytochemical distribution of 13 subunits in the adult rat brain. Neuroscience 101: 815–850.

Rasmussen R (2001) Quantiﬁcation of the light cycler instrument. In S Meuer, K Nakagawara, eds, Rapid Cycle Real-time PCR: Methods and Applications. Springer, Heidelberg, pp 21–34.

Ratte S, Prescott SA (2011) CIC-2 channels regulate neuronal excitability, not intracellular chloride levels. J Neurosci 31: 15838–15843.

Regan MD, Turko AJ, Heras J, Andersen MK, Lefevre S, Wang T, Bayley M, Brauner C, Huang DTT, Phuong NT et al. (2016) Ambient CO2 fish behaviour and altered GABAergic neurotransmission: exploring the mechanism of CO2-disrupted behaviour by...
taking a hypercapnia dweller down to low CO₂ levels. J Exp Biol 219: 109–118.

Romero MF, Henry D, Nelson S, Harte PJ, Dillon AK, Sciortino CM (2000) Cloning and characterization of Na⁺-driven anion exchanger (NDAE1). J Biol Chem 32: 24552–24559.

Rossi T, Nagelkerken I, Pistevos JCA, Connell SD (2016) Lost at sea: ocean acidification undermines larval fish orientation via altered hearing and marine soundscape modification. Biol Lett 12: 20150937.

Ruijter JM, Hoogaars WMH, Karlen Y, Bakker O, Van de Hoff MJB, Moorman FM (2009) Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res 37: e45.

Scimemi A (2014) Structure, function, and plasticity of GABA transporters. Front Cell Neurosci 8: 161.

Sieghart W, Sperk G (2002) Subunit composition, distribution and function of GABA-A receptor subtypes. Curr Top Med Chem 2: 795–816.

Simpson SD, Munday PL, Wittenrich ML, Manassa R, Dixson D, Gagliano M, Yan HY (2011) Ocean acidification erodes crucial auditory behaviour in a marine fish. Biol Lett 7: 917–920.

Somogyi P, Fritschy JM, Benke D, Roberts JD, Sieghart W (1996) The gamma 2 subunit of the GABAₐ receptor is concentrated in synaptic junctions containing the alpha 1 and beta 2/3 subunits in hippocampus, cerebellum and globus pallidus. Neuropharmacology 35: 1425–1444.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3: RESEARCH0034.

Welch MJ, Watson SA, Welsh JQ, McCormick MI, Munday PL (2014) Effects of elevated CO₂ on fish behaviour undiminished by transgenerational acclimation. Nat Clim Change 4: 1086–1089.

Whiting PJ (2003) GABA-A receptor subtypes in the brain: a paradigm for CNS drug discovery? Drug Discov Today 8: 445–450.