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Perspective

Neural effects of elevated CO_2 in fish may be amplified by a vicious cycle

Celia Schunter[1], Timothy Ravasi[2], Philip L. Munday[3] and Göran E. Nilsson[4,*]

[1]Swire Institute of Marine Science and School of Biological Sciences, The University of Hong Kong, Pokfulam, Hong Kong
[2]Marine Climate Change Unit, Okinawa Institute of Science and Technology Graduate University, Tancha, Onna-son, Okinawa 904-0495, Japan
[3]ARC Centre of Excellence for Coral Reef Studies, James Cook University, 1 James Cook Dr, Townsville, Queensland 4814, Australia
[4]Section for Physiology and Cell Biology, Department of Biosciences, University of Oslo, Blindernveien 31, 0316 Oslo, Norway

***Corresponding author:** Section for Physiology and Cell Biology, Department of Biosciences, University of Oslo, 0316 Oslo, Norway. Email: g.e.nilsson@ibv.uio.no

Maladaptive behavioural disturbances have been reported in some fishes and aquatic invertebrates exposed to projected future CO_2 levels. These disturbances have been linked to altered ion gradients and neurotransmitter function in the brain. Still, it seems surprising that the relatively small ionic changes induced by near-future CO_2 levels can have such profound neural effects. Based on recent transcriptomics data, we propose that a vicious cycle can be triggered that amplifies the initial disturbance, explaining how small pH regulatory adjustments in response to ocean acidification can lead to major behavioural alterations in fish and other water-breathing animals. The proposed cycle is initiated by a reversal of the function of some inhibitory $GABA_A$ receptors in the direction of neural excitation and then amplified by adjustments in gene expression aimed at suppressing the excitation but in reality increasing it. In addition, the increased metabolic production of CO_2 by overexcited neurons will feed into the cycle by elevating intracellular bicarbonate levels that will lead to increased excitatory ion fluxes through $GABA_A$ receptors. We also discuss the possibility that an initiation of a vicious cycle could be one of the several factors underlying the differences in neural sensitivity to elevated CO_2 displayed by fishes.

Key words: Ocean acidification, hypercapnia, GABA, behaviour, fish, brain

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Introduction

Since the first reports of behavioural disturbances in fish exposed to elevated CO_2 (hypercapnia) (Munday *et al.*, 2009), a steady stream of reports has shown an array of behavioural and sensory functions being altered by environmental hypercapnia in aquatic animals (Nagelkerken and Munday, 2016; Cattano *et al.*, 2018). These impairments, which affect olfactory preferences (by reversing them), lateralization, hearing, vision, learning, physical activity and boldness, occur at elevated CO_2 levels projected for the next 50 to 100 years and are exhibited not only in fish but also in some invertebrates, including molluscs and crustaceans (Watson *et al.*, 2014; Jellison *et al.*, 2016; Ren *et al.*, 2018). However, there is also a clear intraspecific variation in the response to elevated CO_2, as we will discuss later.

In order to respond to hypercapnia and defend blood and tissue pH, fish accumulate HCO_3^- and Na^+ while excreting Cl^- and H^+ (reviewed by Heuer and Grosell, 2014). This occurs both over the gills to defend blood and whole body pH and on the tissue level to protect intracellular pH, and several fish species have been found to preferentially regulate intracellular brain pH when exposed to environmental

hypercapnia (Harter *et al.*, 2014; Heuer *et al.*, 2016; Shartau *et al.*, 2016). As a result, the gradients of HCO_3^- and Cl^- over cell membranes are likely to be altered, and Nilsson *et al.*, (2012) suggested that the neuronal disturbances underlying the behavioural impairments are likely linked to an alteration in the gradients of Cl^- and HCO_3^- over neuronal membranes induced by the pH regulatory adjustments. More specifically, it was suggested that these altered gradients can cause a reversal of the function of $GABA_A$ receptors in the brain (Nilsson *et al.*, 2012). The $GABA_A$ receptor is the major inhibitory neurotransmitter receptor in vertebrates as well as many invertebrates (Tsang *et al.*, 2007). It is an ion channel with permeability for Cl^- and HCO_3^-, and it is about 2–5 times more permeable to Cl^- than to HCO_3^- dependent on brain region and species (Farrant and Kaila, 2007). The electrochemical driving forces for Cl^- and HCO_3^- are usually such that Cl^- will move inwards while HCO_3^- will move outwards (Staley *et al.*, 1995; Farrant and Kaila, 2007), and the opening of this ion channel normally leads to a net influx of negative charge carried by Cl^- over the cell membrane, causing hyperpolarization and therefore inhibition of neuronal activity. However, if intracellular levels of HCO_3^- increase and/or extracellular Cl^- decrease, the activity of this receptor can turn from inhibitory to excitatory by causing a net outflow of negative charge primarily driven by HCO_3^- (Staley *et al.*, 1995; Farrant and Kaila, 2007; Do-Young *et al.*, 2009).

Evidence for an involvement of the $GABA_A$ receptor has relied on pharmacological suppression of the receptor with gabazine, a treatment that Nilsson *et al.*, (2012) and subsequent studies found to reverse hypercapnia-induced behavioural disturbances in both fish (e.g. Chivers *et al.*, 2014; Chung *et al.*, 2014; Lai *et al.*, 2015; Lopes *et al.*, 2016; Regan *et al.*, 2016) and invertebrates (e.g. Watson *et al.*, 2014). To date, a reversal of the function of $GABA_A$ receptors remains the only well-founded mechanistic explanation for the behavioural alterations seen in animals exposed to near-future CO_2 levels (Tresguerres and Hamilton, 2017). Although measured changes in tissue Cl^- and HCO_3^- at 1900 µatm are supportive of the $GABA_A$ hypothesis (Heuer *et al.*, 2016), it has surprised many physiologists that the relatively small ionic changes expected in response to projected future pCO_2 can have such dramatic effects of neural functions (typically seen around 1000 µatm or even lower). It is of course possible that other neural mechanisms are involved in the behavioural alterations seen in fish exposed to elevated CO_2. For example, Tresguerres and Hamilton (2017) made the hypothetical suggestion that 'a theoretical OA-induced decrease in glutamate release could be "restored" by gabazine, and this could be erroneously interpreted as $GABA_A$ receptor reversal'. However, no plausible mechanistic link between elevated CO_2 and altered neural function has been proposed for such alternative explanations. Glutamate receptors, for example, are linked to Ca^{2+} and/or Na^+ channels, so unlike the $GABA_A$ receptor that gates Cl^- and HCO_3^- fluxes, there is no obvious reason why they would be affected by pH regulatory responses to elevated CO_2. Similarly, altered electrical responses of the olfactory organ seen during acute high-CO_2 exposure in sea bass (*Dicentrarchus labrax*) (Porteus *et al.*, 2018) cannot explain altered behaviours in high-CO_2-exposed fish when the actual behavioural trials are carried out in low-CO_2 control water (subsequent to the high-CO_2 exposure) (e.g. Munday *et al.*, 2009, 2016, Dixson *et al.*, 2010); and a range of other behaviours and sensory systems not associated with olfaction are also affected by elevated CO_2 (Heuer and Grosell, 2014; Nagelkerken and Munday, 2016). Moreover, the behavioural disturbances linger on for several days after multiday exposure to elevated CO_2, as, for example, in studies where CO_2-treated fish have been put back into their marine habitat (Munday *et al.*, 2010; Chivers *et al.*, 2014). Indeed, the persistence of the effects, together with the fact that it takes several days for the behavioural disturbances to set in (Munday *et al.*, 2010), point at the possibility that processes such as altered gene transcription also could be coming into play.

Evidence from the transcriptome

We recently carried out a comprehensive brain transcriptome study on the effect of acute (4 days), developmental (from hatching to 5 months) and transgenerational exposure to hypercapnia (pCO_2 of 740 µatm) in spiny damselfish (*Acanthochromis polyacanthus*) (Schunter *et al.*, 2018). Importantly, this study provided a strong second line of evidence for an involvement of the $GABA_A$ receptor, because a majority of genes linked to the function of this receptor were found to be altered (Fig. 1), particularly after acute and developmental hypercapnia treatment. Based on this transcriptome study, and an analysis of the direction of change of the various components of GABA signalling, we here elaborate on the possibility that altered gene expression leads to a vicious cycle that amplifies the initial disturbance in $GABA_A$ receptor function.

Normal function of neural circuits depends on an interplay between excitatory and inhibitory input, and the inhibitory input is generally provided by GABA-releasing neurons affecting $GABA_A$ receptors on other neurons (Farrant and Kaila, 2007). Inhibitory and excitatory input has to be balanced by intrinsic regulatory mechanisms. The balancing of excitatory and inhibitory activities has been found to involve adjustments on the transcriptional (mRNA) level, although post-translational changes may also be involved. It has, for example, been repeatedly shown that an overall increase in excitation will lead to transcriptional upregulation of mRNA levels for the different genes making up the subunits of the pentameric $GABA_A$ receptor (Uusi-Oukari and Korpi, 2010; Drexel *et al.*, 2013; Yu *et al.*, 2017). Such changes would strive to increase the inhibitory input in overexcited circuits, thereby restoring normal activity levels.

These are exactly the transcriptome signal we find in the brains of spiny damselfish exposed to elevated CO_2 for 4 days and 5 months (Schunter *et al.*, 2018), suggesting that neural

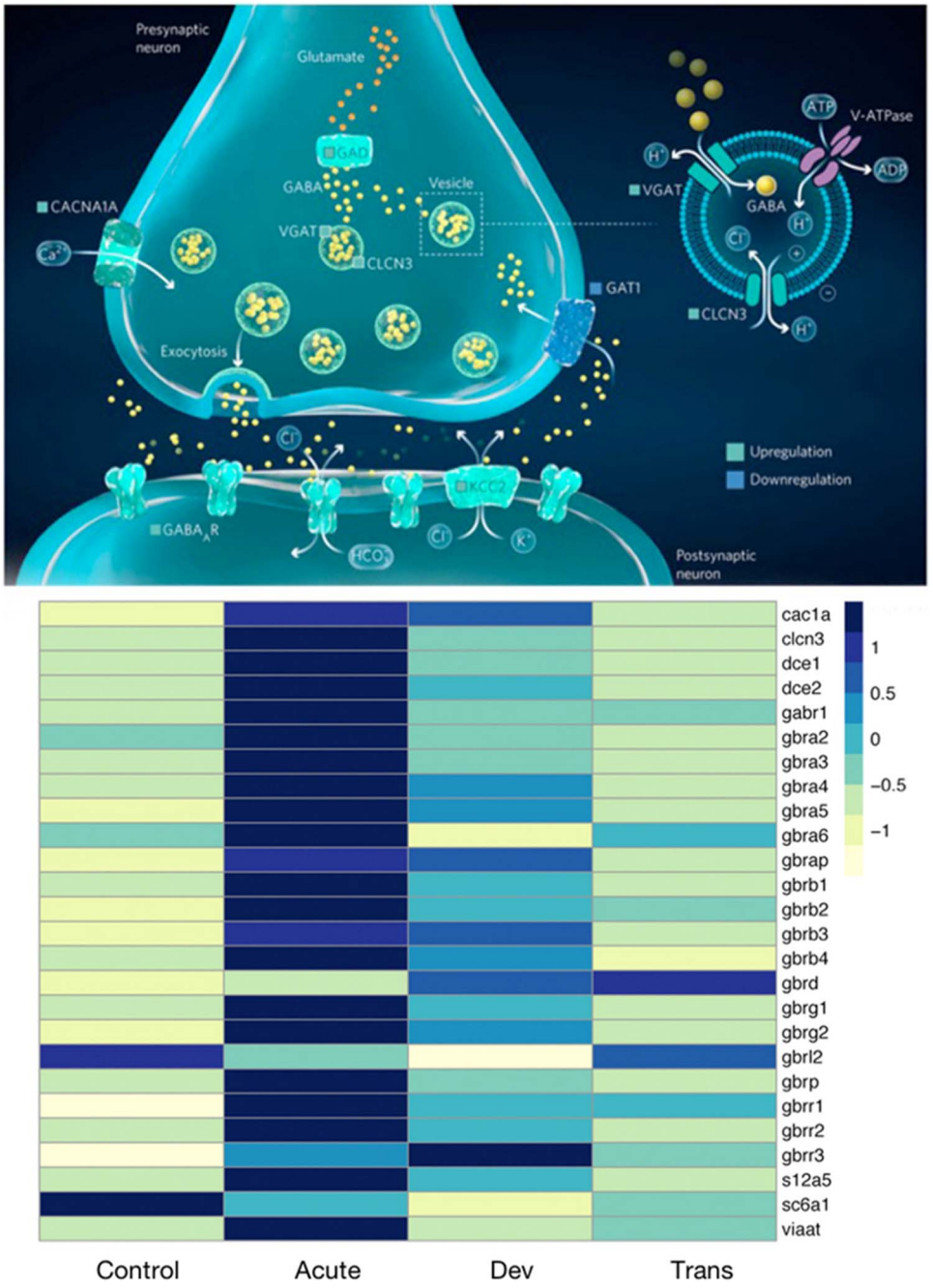

Figure 1: (a) The GABA (γ-aminobutyric acid) signalling pathway in the synapse including differential expression of genes after exposure to elevated CO_2. GAD = glutamate decarboxylase 1; VGAT = GABA and glycine transporter; CLCN3 = chloride voltage-gated channel 3; KCC2 = neuronal K Cl co-transporter; GAT1 = GABA transporter 1; CACNA1A = brain calcium channel 1; GABAAR = $GABA_A$ receptor subunits α,β and γ. From Schunter *et al.* (2018). (b) Heatmap of relative expression levels in *A. polyacanthus* brain after different exposures to elevated CO_2. Acute: 4-day exposure at the age of 5 months, Developmental (Dev): Exposure since hatching for 5 months, Trans: Transgenerational exposure including parental and 5 months of offspring elevated CO_2 exposure. Data from Schunter *et al.* (2018) where experimental details are given.

excitation caused by $GABA_A$ receptors that have become excitatory triggers regulatory changes in gene expression that strive to restore inhibition by elevating $GABA_A$ receptor activity. Indeed, in addition to elevated $GABA_A$ receptor expression, we also saw other changes that would serve the same function, including elevated expression of genes responsible for synthesizing GABA (glutamate decarboxylases) and moving it into synaptic vesicles (VGAT and CLCN3).

Furthermore, we found reduced expression of mRNA for the transporter protein GAT1 responsible for removing extracellular GABA from synapses (Fig. 1, Supplementary Table 1). Thus, virtually all GABA-associated changes appeared to be aimed at increasing GABAergic signalling.

A vicious cycle

Unfortunately, these transcriptional changes are likely to make things worse if $GABA_A$ receptors have become excitatory due to altered ion gradients. This is where we suggest that a vicious cycle is triggered, where regulatory processes aimed at increasing inhibition instead cause more excitation (Fig. 2). Importantly, the cycle could be further amplified by the resultant increase in neural metabolic activity. Electrical activity is the main energy consumer in the brain (Lutz *et al.*, 2003), and any increase in excitatory signalling in neural circuits will translate into increased energy metabolism and thereby metabolic production of CO_2 inside neurons. Indeed, this strong link between electrical activity and metabolism in brain tissue is the reason why techniques such as PET scanning and functional MRI can be used to detect changes in brain activity. Because neurons contain high levels of carbonic anhydrase (Ruusuvuori and Kaila, 2014), which catalyses the conversion of CO_2 and H_2O into HCO_3^-, it is likely that intracellular HCO_3^- levels will rise when neurons become overactive and produce more metabolically derived CO_2. An elevation of intracellular HCO_3^-, which will flow out of $GABA_A$ receptors and counteract or overwhelm the influx of Cl^- (Staley *et al.*, 1995; Farrant and Kaila, 2007; Do-Young *et al.*, 2009), would further drive $GABA_A$ receptors in the direction of excitation. Indeed, this could in itself drive a vicious cycle also in the absence of changes in GABA-related gene expression. Interestingly, both Schunter *et al.* (2018) and Williams *et al.* (2018) found increased expression of glucose transporters in the brain of fish exposed to elevated CO_2, which is indicative of increased neural metabolism.

There are two other studies on transcriptional changes in fish brains in response to elevated CO_2. Both show changes in the direction of increased GABAergic signalling although not as many of the components appear altered as in the spiny damselfish. Thus, a study on stickleback (*Gasterosteus aculeatus*) behaviourally affected by elevated CO_2 found elevated mRNA levels of $GABA_A$ alpha-subunits, which are major subunits present in all $GABA_A$ receptors (Lai *et al.*, 2016). Another recent study showed changes in GABA-related gene expression (mRNA) in the olfactory bulb of ocean-phase coho salmon (*Oncorhynchus kisutch*) exposed to elevated CO_2 (Williams *et al.*, 2018). In the latter study, stimulated GABAergic signalling was indicated by reduced mRNA for a transporter responsible for GABA reuptake (as also seen in the spiny damselfish), while $GABA_A$ receptor subunits appeared unaffected. Reduced GABA reuptake would lead to elevated extracellular GABA levels that will activate $GABA_A$ receptors. However, Williams *et al.* (2018) found that $GABA_B$ receptor expression was upregulated. An upregulation of $GABA_B$ receptors could help dampen a vicious cycle in this species as $GABA_B$ receptors are not anion channels but act in an inhibitory way by opening K^+ channels that are less likely to be affected by pH regulatory responses to elevated CO_2.

Obviously, more studies will be needed to show if these transcriptional responses are as widespread among fish, and possibly invertebrates, as the behavioural alterations caused by environmental hypercapnia. It may be that a vicious cycle fuelled by altered gene expression and increased metabolic CO_2 production is more developed in some species or life stages than others and that this correlates with their neural sensitivity to elevated CO_2 exposure.

Notably, the pattern of altered gene expression that we found was absent or largely subdued in spiny damselfish where both the parents and offspring were exposed to elevated CO_2 levels (Schunter *et al.*, 2016, 2018). Because behavioural disturbances have been found to persist even after such transgenerational CO_2 exposure (Welch *et al.*, 2014), it is tempting to speculate that the altered GABAergic function has at this stage entered a chronic phase that is no longer reflected at the level of mRNA expression. At this stage, a vicious cycle could still be driven by elevated intracellular levels of HCO_3^- derived from increased neural excitation and metabolism.

Variation among species

The large number of studies that have now been conducted into behavioural effects of elevated CO_2 in fish show that there is considerable interspecific variation in sensitivity (Ferrari *et al.*, 2011; Schmidt *et al.*, 2017a) and some species do not seem to be affected at all (Maneja *et al.*, 2013; Jutfelt and Hedgärde, 2015; Heinrich *et al.*, 2016, Kwan *et al.*, 2017).

One would expect that the alteration of ion gradients needs to reach a threshold for triggering a self-amplifying vicious cycle altering GABAergic function. Nilsson *et al.* (2012) suggested that species and life stages with high metabolic rates, and therefore large respiratory surface areas and high rates of gas exchange, could be particularly prone to have their tissue ion gradients altered by elevated CO_2 levels. The rapid CO_2 flux over a large respiratory surface area could bring the internal CO_2 levels closer to those of the water, making the animal more affected by any changes in water pCO_2. Indeed, tropical coral reef fish larvae, where the behavioural effects of elevated CO_2 were first observed, display extremely high rates of gas exchange (Nilsson *et al.*, 2007). One could argue that the high rate of metabolic CO_2 production in highly active fishes also would lead to high internal levels of CO_2 and HCO_3^-. However, because of the high solubility of CO_2 in water, it is more easily released over the gills than O_2 is taken up. Indeed, one of the lowest blood CO_2 levels recorded in fish has been found in mackerel (*Scomber scombrus*), especially when swimming at high speed (i.e. at

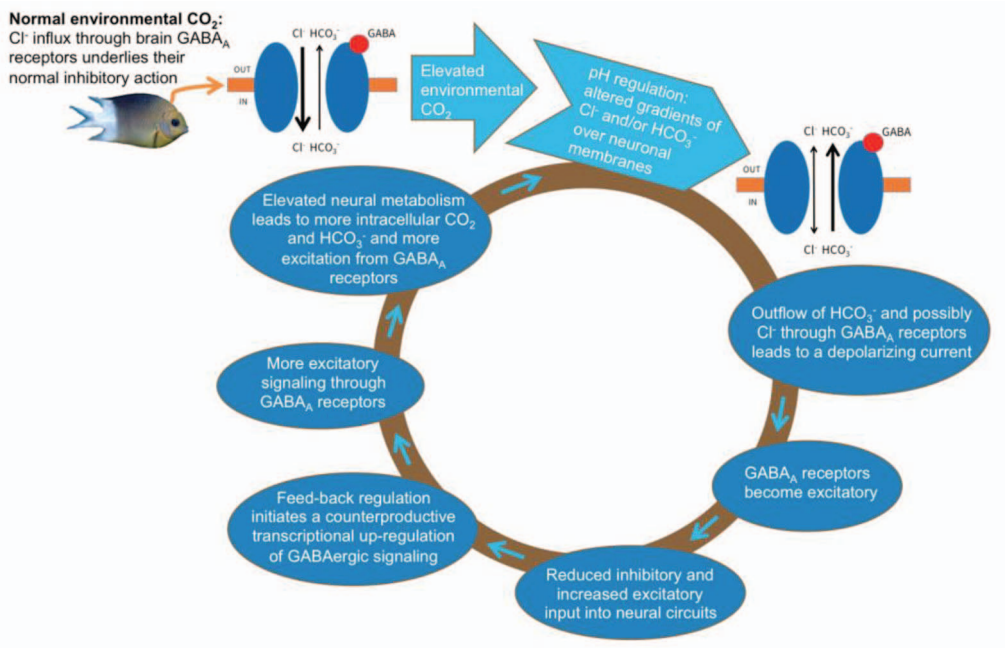

Figure 2: The proposed vicious cycle by which a relatively modest increase in water pCO_2 can create significant neural and thereby behavioural impairments in fish. pH regulatory mechanisms that compensate for the elevated CO_2 lead to altered neuronal gradients of Cl^- and HCO_3^-. The cycle is initially triggered by these altered membrane ion gradients that turn $GABA_A$ receptors from acting inhibitory (hyperpolarizing) to excitatory (depolarizing). The resultant loss of inhibitory input causes overactivity in neuronal circuits that leads to transcriptomal changes striving to boost inhibitory GABA signalling. Unfortunately, this becomes counterproductive since $GABA_A$ receptors have become excitatory, and the result is even more excitatory overactivity. The increase in electric activity leads to higher energy demands and therefore increased metabolic production of intracellular CO_2, which is turned into HCO_3^- by intracellular carbonic anhydrase, thereby raising intracellular [HCO_3^-] that will further increase a depolarizing current through $GABA_A$ receptors.

high metabolic CO_2 production rates) where blood pCO_2 approached 1 mm Hg (Boutilier *et al.*, 1984). However, other factors are likely to be involved as closely related species may show clear differences in sensitivity to elevated CO_2 (Ferrari *et al.*, 2011; Schmidt *et al.*, 2017a). One factor that could underlie species and life stage differences is adaptation to naturally occurring variation in pCO_2 in the habitats, allowing for resilience to any CO_2-induced alterations in ion gradients and $GABA_A$ receptor function. Indeed, many of the species exhibiting behavioural tolerance to elevated CO_2 occupy habitats that periodically experience naturally high pCO_2 (Heinrich *et al.*, 2016, Kwan *et al.*, 2017). For example, studies have indicated that Atlantic cod (*Gadus morhua*) are not behaviourally affected by elevated CO_2 (Jutfelt and Hedgärde, 2015; Maneja *et al.*, 2013; Schmidt *et al.*, 2017a). By contrast, behavioural lateralization was affected by elevated CO_2 in polar cod (*Boreogadus saida*) (Schmidt *et al.*, 2017a). A possible explanation for this difference is that Atlantic cod are adapted to a large natural variation in pCO_2 in the habitats they commonly occupy and thus have different GABAergic responses to high CO_2 (Schmidt *et al.*, 2017b). Another possible factor here could involve differences in the acid–base regulatory capacity between the species, where those that show a low regulatory response to acidification, and therefore essentially maintain internal HCO_3^- concentrations and gradients, will retain normal GABAergic function. However, failure to defend internal pH could bring other problems. For example, it is noteworthy that Atlantic cod is one of the few species of fish for which projected future CO_2 levels have been found to directly impact larval development (Frommel *et al.*, 2012) and greatly increase mortality (Stiasny *et al.*, 2016). A weak acid–base regulatory response in larval Atlantic cod could potentially explain both their high mortality in high CO_2 compared with other fishes and the absence of behavioural effects, because the changes in Cl^- and HCO_3^- could be too small to induce a significant change in GABAergic function.

Conclusions and directions for the future

To conclude, the vicious cycle that we describe here can explain why relatively small initial disturbances in neuronal ion gradients after a few days become manifested in neural dysfunction severe enough to cause an array of behavioural alterations. A self-amplifying cycle that involves changes in gene expression and ultimately protein synthesis may take some time to be fully expressed, which can explain why the behavioural disturbances are not seen until after 2–3 days

of high-CO_2 exposure and lingers on for several days after the exposure has ended (Munday *et al*., 2010). Moreover, if a sustained increase in water pCO_2 is needed to trigger and/or sustain the vicious cycle, then this could also explain recent findings showing that daily cycling of water pCO_2 has less behavioural effects in fish than a maintained high CO_2 level (Jarrold *et al*., 2017).

The variation between species and life stages in CO_2 responses should prove useful for testing hypotheses about the relationship between altered behaviours and changes in GABAergic function caused by elevated CO_2. More comparative studies on more species and life stages would of course be most welcome as they would allow us to better identify and examine the patterns and processes that determine the neural sensitivity to elevated CO_2. Experimentally, finding signs of the vicious circle we propose here could involve transcriptomic and proteomic studies. It would be desirable if these could be linked to measurements of ion and pH regulatory variables in blood and brain tissue, although this will be difficult in small individuals. Future studies could adopt new technologies that allow a more fine-scale approach, such as single-cell transcriptomics to examine the responses of specific cell types in discrete brain regions rather than whole brain tissue. Direct electrophysiological recordings of effects of elevated CO_2 on neural functions, which have only rarely been done (Chung *et al*., 2014; Porteus *et al*., 2018; Williams *et al*., 2018), will also bring us closer to understanding the mechanisms involved.

Understanding the physiological mechanisms by which elevated CO_2 affects fish behaviour, and how this links to variation in responses among species and habitats, will help establish where and when future ocean acidification conditions could impact fish populations. For iconic and/or high value species, such as salmon (Ou *et al*., 2015; Williams *et al*., 2018), this knowledge might even be used in adaptive management practices to limit exposure to the precise conditions that induce behavioural effects. A detailed physiological understanding might also be exploited in aquaculture to manipulate CO_2 levels such that any effects on behaviour are optimal for production.

Supplementary material

Supplementary material is available at *Conservation Physiology* online.

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