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SHORT COMMUNICATION

Ocean acidification slows retinal function in a damselfish through interference with $GABA_A$ receptors

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ABSTRACT

Vision is one of the most efficient senses used by animals to catch prey and avoid predators. Therefore, any deficiency in the visual system could have important consequences for individual performance. We examined the effect of CO_2 levels projected to occur by the end of this century on retinal responses in a damselfish, by determining the threshold of its flicker electroretinogram (fERG). The maximal flicker frequency of the retina was reduced by continuous exposure to elevated CO_2, potentially impairing the capacity of fish to react to fast events. This effect was rapidly counteracted by treatment with a GABA antagonist (gabazine), indicating that $GABA_A$ receptor function is disrupted by elevated CO_2. In addition to demonstrating the effects of elevated CO_2 on fast flicker fusion of marine fishes, our results show that the fish retina could be a model system to study the effects of high CO_2 on neural processing.

KEY WORDS: Flicker fusion frequency, Electroretinogram, Carbon dioxide, Vision, Coral reef

INTRODUCTION

CO_2 levels in the surface ocean are rising in line with rising atmospheric CO_2 (Doney, 2010). It has recently been shown that projected near-future CO_2 levels can impair sensory systems and alter the behaviour of marine fishes (Munday et al., 2009; Munday et al., 2012; Jutfelt et al., 2013). Behavioural changes include increased boldness and activity (Munday et al., 2010; Munday et al., 2013; Jutfelt et al., 2013), loss of behavioural lateralization (Domenici et al., 2012; Jutfelt et al., 2013), altered auditory preferences (Simpson et al., 2011) and impaired olfactory function (Munday et al., 2009; Dixson et al., 2010; Ferrari et al., 2011). The underlying reason for sensory impairment and behavioural changes in fish exposed to elevated CO_2 appears to be a disruption to neurotransmitter function, probably caused by changes to ion gradients over neuronal membranes (Nilsson et al., 2012). Fish regulate acid–base relevant ions, primarily bicarbonate (HCO_3^-) and chloride (Cl^-), to maintain blood and tissue pH when exposed to high CO_2 (Ishimatsu et al., 2008). Experimental evidence suggests that this leads to a disruption of $GABA_A$ receptors, which are Cl^- and HCO_3^- channels gated by the neurotransmitter GABA (gamma-amino butyric acid). Indeed, the sensory and behavioural alterations caused by high-CO_2 exposure are virtually abolished by a moderate dose of the $GABA_A$ receptor blocker gabazine (Nilsson et al., 2012). Normally, $GABA_A$ receptors act by hyperpolarizing neuronal membranes due to the inflow of Cl^-, causing neuronal inhibition. It has been hypothesized that during high-CO_2 exposure, the transmembrane gradients of Cl^- and HCO_3^- are altered in some neurons, thereby affecting $GABA_A$ function. Given the ubiquity of $GABA_A$ receptors in animal nervous systems, it is likely that exposure to elevated CO_2 could affect a wide variety of behavioural functions and activities in marine organisms.

To date, research on sensory impairment of fishes at elevated CO_2 levels has concentrated mainly on the effects to olfactory discrimination (e.g. Munday et al., 2009; Dixson et al., 2010; Ferrari et al., 2011), and to some extent on auditory preferences (Simpson et al., 2011). Recently, Ferrari and colleagues (Ferrari et al., 2012) found that visual risk assessment was altered in juvenile fish exposed to 850 μatm CO_2. When presented with the sight of a large novel reef fish, of sufficient size to be a predator, juvenile damselfish that had been reared at high CO_2 exhibited reduced antipredator responses and lacked the typical signalling behaviour (bobbing) seen in juvenile damselfishes exposed to a threatening situation (Ferrari et al., 2012). This suggests that the function of the visual system is affected by high CO_2. Such alterations to vision-mediated behaviour could involve processing at the retinal level or in higher brain centres.

In this study, we focused on the possibility that visual function at the retinal level is affected by high-CO_2 exposure. The rapidity of the response of animals to visual stimuli may be correlated with fast flicker fusion (FFF). A visual system viewing a flickering light source has a critical flicker fusion (CFF) threshold, above which the flicker becomes too fast for the system to follow (Fritsches et al., 2005), and the light appears continuous to the animal, and not flashing. The CFF threshold varies between animals, and is often related to lifestyle and illumination level, being fast in rapidly moving organisms in bright light and slow in nocturnal slow-movers (Horodysky et al., 2008; Smolka et al., 2013). It is therefore likely that reduced CFF could impair the capacity to react to fast events such as prey capture and predator avoidance.

Here we examined the effect of elevated CO_2 on retinal function in the spiny damselfish, *Acanthochromis polyacanthus* (Bleeker 1855) by determining the CFF threshold of its electroretinogram (ERG). This method records the electrical light response of the retina using a non-invasive electrode placed on the eye. We show that continuous exposure to the CO_2 level projected to occur in the surface ocean by the end of this century (944 μatm) reduces the CFF threshold (i.e. reduces the speed of the light response) and that this effect can be effectively counteracted by gabazine treatment, indicating an involvement of $GABA_A$ receptor function.

RESULTS AND DISCUSSION

The CFF of damselfish exposed to elevated CO_2 (78.6±3.9 Hz, mean ± s.e.m.) was significantly decreased compared with that of the

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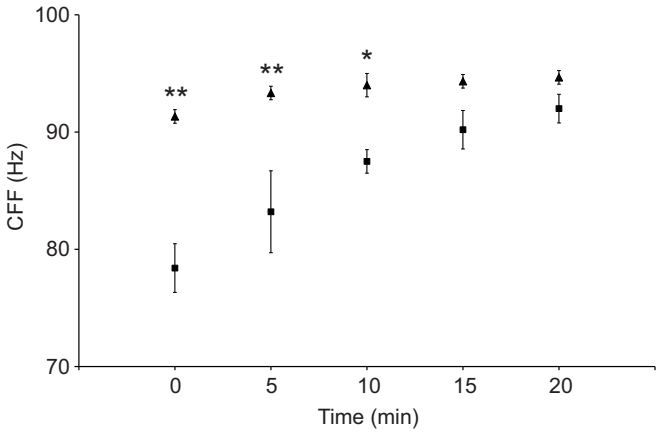

Fig. 1. The suppressed critical flicker fusion (CFF) threshold in elevated-CO_2 treated fish is restored by gabazine treatment. Graph shows CFF of control (triangles) and elevated-CO_2 (squares) treated *Acanthochromis polyacanthus*. Gabazine was introduced into the respiratory water of both groups at time zero. The CFF of the control group was unaffected by gabazine, resulting in a stable phase at 93.5±1.4 Hz. The resolution of the high-CO_2 fishes recovered to the control level after 15 min of gabazine treatment. Student's *t*-test, **P*<0.05, ***P*<0.01. Error bars are s.e.m.

control group (89.0±1.6 Hz) (t_{10}=5.28, P<0.0004). There was a significant interaction between time interval and CO_2 treatment in gabazine-treated fish ($F_{4,20}$=35.64, P<0.0001). The CFF values of fish from the elevated-CO_2 group were significantly less than those of fish from the control group at 0, 5 and 10 min; however, the value increased through time to reach approximately the same level as the control group after 15–20 min (Fig. 1). The CFF of the control group did not change significantly through time. There appeared to be a slight increase in CFF of the control group after the first 5 min, when gabazine was first administered, but there was no further increase through time, indicating that gabazine itself had negligible effect on CFF.

Our results show that the ability or the fish retina to react to fast visual stimuli is reduced after exposure to CO_2 levels projected to occur in the ocean by the end of this century. Moreover, the underlying mechanism appears to involve altered $GABA_A$ receptor function, as the CFF threshold could be restored by treatment with the $GABA_A$ receptor antagonist gabazine, at a dose that has previously been found to restore impaired olfactory preference and lateralization in reef fish exposed to high CO_2 (Nilsson et al., 2012). $GABA_A$ receptors have been found to be intimately coupled to retinal signal processing and direction selectivity at the ganglion cell level, including the control of the fast flicker response in the fish retina (Mora-Ferrer and Neumeyer, 2009). As $GABA_A$ receptors have been linked to neural dysfunction of high-CO_2 exposed fish, it is not unexpected that retinal function is affected by elevated CO_2 levels.

The CFF threshold of fish correlates with their lifestyle (Horodysky et al., 2008) and a high CFF is likely to reflect the need to react to fast events in their habitat, at the cost of reduced performance at low light conditions. Pelagic fish CFF varies from around 25 Hz in species living in deeper water, to 80 Hz in the surface-dwelling yellow-fin tuna or the dolphin fish (Fritsches et al., 2005). This fits well with the CFF of about 90 Hz we found in *A. polyacanthus*, which lives in a well-lit complex environment in close proximity to predators.

Predatory events in the ocean may also be very rapid; it is therefore of concern that spiny damselfish exposed to a near-future CO_2 level slow their retinal response, with CFF dropping from around 90 Hz to less than 80 Hz. This decrease in visual speed might result in reduced reaction times, for example to a rapidly approaching predator, a possibility supported by experiments showing that prey fish exposed to similar levels of CO_2 to those used in our experiments have a reduced perception and response to predation threat (Ferrari et al., 2012; Allan et al., 2013). While behavioural responses to visual stimuli may be different to those measured electrophysiologically at the level of the retina, behavioural responses would probably be slower than the initial physiological signal, and in the light of the ubiquitous presence of $GABA_A$ receptors in the nervous system, it is possible that CO_2 exposure may lead to additional reductions in reaction time due to disturbances at higher levels of neural processing (Domenici et al., 2012).

Our results add to the increasing evidence that elevated CO_2 can affect critical sensory processes in marine fishes and that the underlying mechanism is associated with the function of $GABA_A$ receptors. An important aspect of the present result is that the fish retina could be used as a relatively simple model system to study the effects of high CO_2 on neural processing. Indeed, isolated fish retina preparations have long been used as models for neurophysiological research (e.g. Hankins and Ruddock, 1984). A better understanding of how and why high-CO_2 exposure affects $GABA_A$ receptor function, and possibly other neural components, would increase the power to predict which physiological processes are likely to be affected, and which organisms are most at risk, from future rises in ocean CO_2 levels.

MATERIALS AND METHODS

Animals and experimental treatments

Small *A. polyacanthus*, between 55 and 80 mm standard length (SL), were collected from the lagoon at Lizard Island (14°40′08″S; 145°27′34″E), Great Barrier Reef, Australia, using barrier nets. Fish were distributed among eight aquaria supplied with a constant flow of seawater at ambient summer temperature (28–30°C) and fed to satiation twice daily with INVE aquaculture pellets (Dendermonde, Belgium). Four of the aquaria were supplied with seawater at present-day CO_2 levels (466 µatm) and four with elevated CO_2-equilibrated (944 µatm) seawater, as described below. The elevated CO_2 treatment is consistent with projected CO_2 levels in the atmosphere and surface ocean at year 2100 on a business-as-usual carbon emissions trajectory (RCP 8.5) (Meinshausen et al., 2011). Fish were maintained at control and elevated CO_2 for 6–7 days prior to experimentation, which is sufficient to induce the full range of sensory and behavioural impairment in reef fish (Munday et al., 2010; Munday et al., 2012; Ferrari et al., 2012). All animal care and experimental protocols complied with ethics regulations of James Cook University and University of Queensland.

Seawater manipulation

Elevated CO_2 levels were achieved by CO_2-dosing seawater in a 60 l header tank to a set pH_{NBS} (pH calibrated in National Bureau of Standards buffers) to match the required CO_2 level. A pH controller (Aqua Medic GmbH, Bissendorf, Germany) delivered CO_2 into a power-head pump at the bottom of the header tank if the pH rose above the set point. Individual aquaria received CO_2-equilibrated seawater from the header tank at ~1000 ml min^{-1}. The pH_{NBS} of each aquarium was monitored regularly to ensure it remained within ±0.05 of the desired level. Control aquaria received seawater from a 60 l header tank diffused with ambient air. The temperature in each aquarium was measured twice daily. Seawater total alkalinity and pH_{NBS} for CO_2 calculations were measured from replicate water samples of control and high CO_2 water taken throughout the experiment. Total alkalinity was estimated by Gran titration using certified reference materials (Dr A. Dickson, Scripps Institution of Oceanography). Carbonate chemistry values are shown in Table 1.

Table 1. Seawater carbonate chemistry parameters for control and elevated CO_2 treatments

Treatment	Temperature (°C)	Salinity	pH_{NBS}	Total alkalinity (μmol kg^{-1} SW)	pCO_2 (μatm)
Control	29.6±0.1	34.5	8.13±0.01	2269±9	466±15
High CO_2	29.6±0.1	34.5	7.87±0.01	2257±4	944±19

Data are means ± s.e.m.
Average seawater pCO_2 was calculated in CO2SYS using the constants K_1, K_2 from Mehrbach et al. (Mehrbach et al., 1973) refitted by Dickson and Millero (Dickson and Millero, 1987), and Dickson (Dickson, 1990) for HSO_4^-.

Experiments

Flicker ERG (fERG) was used to test the temporal visual resolution of control and elevated-CO_2-exposed fish (SL 63.0±3.4 mm, mean ± s.d.). Fish that had been in experimental treatments for 6–7 days were anaesthetized using 20 ppm clove oil and pithed prior to the fERG measurement. ERGs were recorded from the pithed fish, which was restrained in a horizontal position on a sponge attached to a plastic board and held firmly in place using silicone bandages. The fish was placed in a seawater bath maintained at 29±1°C. Moistened tissue paper was placed on the upper side of the fish and only one eye was exposed into the air for ERG recording, as described below. Fish were maintained with constant gill irrigation using seawater at the same CO_2 level as their experimental treatment (control or elevated CO_2) flowing at 0.1 l min^{-1}. Five elevated CO_2 individuals and five control individuals were tested.

A second experiment tested the potential role of $GABA_A$ receptors in the temporal visual resolution of high-CO_2-exposed fish. The procedure described above was used, except control and high-CO_2-exposed damselfish (SL 74.3±5.8 mm) were treated with gabazine (Sigma Chemical Co., St Louis, MO, USA) throughout the fERG procedure. Gabazine is a fast-acting and highly selective antagonist to the $GABA_A$ receptor (Ueno et al., 1997). From the start of the measurements the fish were ventilated with seawater containing 4 mg l^{-1} gabazine at a flow rate of 0.1 l min^{-1}. The visual resolution of fish from the two treatment groups was tested with fERG every 5 min for a total of 20 min. Five individuals from the elevated-CO_2 group and three control individuals were tested in this experiment.

ERG setup

A white LED lamp was placed 30 cm above the test eye. The LED was connected to a PowerLab ML 866 module (ADinstruments, Colorado Springs, CO, USA) from which stimulus presentations were controlled by the built-in functional generator to produce flickering stimuli (30 square pulses) using the software LabChart Pro 7 (v7.2.5; ADinstruments). Each pulse possessed 5 ms power-on duration, rendering a constant photon flux per flash. The irradiance of the lamp was calibrated with a USB4000 spectrometer (Ocean Optics, Dunedin, FL, USA). The light intensity was controlled to emit 10^{14} photons cm^{-2} ms^{-1}. Teflon-coated chlorided 0.5 mm silver wire (Ag–$AgCl_2$) electrodes were used to record the whole-eyeball corneal ERGs. The recording electrode was placed on the corneal surface so that it had contact at the edge of the pupil. The reference electrode was placed on fatty tissue inside the orbit. Liquid conductive gel (EcoGel 200, Mississauga, ON, Canada) was added to the tip of the recording electrode. The system was grounded to the water of the experimental chamber. ERG signals were amplified with a DP301 amplifier (Warner Instruments, Hamden, CT, USA) using a 1000 gain passed through a 1 Hz high-pass and 1 kHz low-pass filter. The amplified ERG signals were further filtered with the software's electronic notch filter to remove periodic electrical noise using LabChart Pro 7 with the Powerlab ML 866 module. The sampling frequency was set at 4 kHz.

Electrophysiological procedure

The electrical response of the whole eye was measured as the frequency of a flickering light was increased. The threshold of the FFF was determined by a standardized method (Fritsches et al., 2005), and in physiological terms is when the eye's response no longer follows the modulation of the light (supplementary material Fig. S1). Measurements were carried out during the daytime on light-adapted fish immobilized as described elsewhere (Horodysky et al., 2008).

The flicker rate was started at 10 Hz and the flash frequency gradually increased until CFF. CFF is defined as the point where the modulated ERG wavelets are no longer following the flickering light. The flickering stimuli were presented for 3–10 s (depending on the number and length of sweeps used). Data were recorded across 30–100 sweeps for every flicker rate. Two methods were used to determine the CFF threshold. First, the ERG waveforms were visually inspected to determine whether they remained in phase with the flickering stimuli. This process was restricted to the lower frequency test (less than 65 Hz). When the flicker frequency approached the CFF threshold, visual inspection was insufficient to determine whether the wavelets remained in phase with the flickering light. The CFF threshold was therefore determined by analysing the power spectrum as described by Fritsches and colleagues (Fritsches et al., 2005). The power at the stimulus frequency was compared with the standard deviation of the power of a neighbouring frequency section. The criterion for CFF was defined as the highest frequency (tested in 1 Hz steps) at which the power of the signal was at least five times larger than the power of the noise. At higher frequencies the power of the response signal was indistinct compared with the power of the noise.

Statistics

A *t*-test was used to determine whether CFF values differed between control fish and those in the elevated-CO_2 group. Repeated measures ANOVA was used to compare CFF values of individuals in the elevated CO_2 group against the control group over the 20 min duration of the gabazine experiment. Dunn's multiple comparisons were then used to test the time intervals at which the mean CFF of the two groups differed.

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Competing interests

The authors declare no competing financial interests.

Author contributions

The study was conceived and designed by P.L.M., N.J.M. and G.E.N. Experiments and measurements were executed by W.-S.C., G.E.N., S.-A.W. and P.L.M. All authors participated in the analysis, interpretation and writing process.

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Supplementary material

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