Global Change Biology

Global Change Biology (2014) **20**, 515–522, doi: 10.1111/gcb.12291

Impaired learning of predators and lower prey survival under elevated CO_2: a consequence of neurotransmitter interference

DOUGLAS P. CHIVERS*, MARK I. MCCORMICK†, GÖRAN E. NILSSON‡, PHILIP L. MUNDAY†, SUE-ANN WATSON†, MARK G. MEEKAN§, MATTHEW D. MITCHELL†, KATHERINE C. CORKILL† and MAUD C. O. FERRARI¶

Department of Biology, University of Saskatchewan, Saskatoon, SK S7N 5E2, Canada, †ARC Centre of Excellence for Coral Reef Studies, School of Marine and Tropical Biology, James Cook University, Townsville, QLD 4811, Australia, ‡Programme for Physiology and Neurobiology, Department of Biosciences, University of Oslo, Oslo, NO 0316, Norway, §Australian Institute of Marine Science, UWA Ocean Sciences Centre (MO96), Crawley, WA Australia, ¶Department of Biomedical Sciences, WCVM, University of Saskatchewan, Saskatoon, SK S7W 5B4, Canada

Abstract

Ocean acidification is one of the most pressing environmental concerns of our time, and not surprisingly, we have seen a recent explosion of research into the physiological impacts and ecological consequences of changes in ocean chemistry. We are gaining considerable insights from this work, but further advances require greater integration across disciplines. Here, we showed that projected near-future CO_2 levels impaired the ability of damselfish to learn the identity of predators. These effects stem from impaired neurotransmitter function; impaired learning under elevated CO_2 was reversed when fish were treated with gabazine, an antagonist of the GABA-A receptor – a major inhibitory neurotransmitter receptor in the brain of vertebrates. The effects of CO_2 on learning and the link to neurotransmitter interference were manifested as major differences in survival for fish released into the wild. Lower survival under elevated CO_2, as a result of impaired learning, could have a major influence on population recruitment.

Keywords: CO_2, GABA-A receptors, global change, learning, neurotransmitter, ocean acidification, predator recognition, survival

Received 15 May 2013 and accepted 30 May 2013

Introduction

Burning of fossil fuels, production of cement and large-scale land-use changes have resulted in the concentration of carbon dioxide (CO_2) in the atmosphere rising at an unprecedented rate (Raupach *et al.*, 2007; Peters *et al.*, 2012). Atmospheric CO_2 now exceeds 395 ppm (Dlugokencky & Tans, 2013), higher than any time in the past 800 000 years (Luthi *et al.*, 2008). If the current CO_2 emissions trajectory is maintained atmospheric CO_2 could exceed 900 ppm by the end of the century (Meinshausen *et al.*, 2011). The amount of CO_2 dissolved in the surface ocean is increasing in line with atmospheric CO_2 because of the equilibration of gas partial pressures at the air–sea interface (Doney, 2010). About 30% of the excess CO_2 produced since the industrial revolution has been absorbed by the oceans (Sabine *et al.*, 2004). Carbon dioxide reacts with water to generate carbonic acid, bicarbonate and hydrogen ions, which increases the acidity of the water, a process known as ocean acidification. Moreover, increasing hydrogen ions bond with carbonate ions to form more bicarbonate, leading to a reduction in carbonate-ion saturation (Orr *et al.*, 2005; Fabry *et al.*, 2008). The net effect of the increased uptake of atmospheric CO_2 at the ocean surface is higher ocean pCO_2, reduced seawater pH and a change in the concentration of carbonate and bicarbonate ions.

Many fundamental biological processes, including metabolism, growth, calcification and reproduction are known to change when ocean chemistry changes (Fabry *et al.*, 2008; Widdicombe & Spicer, 2008; Doney *et al.*, 2009; Kroeker *et al.*, 2010; Briffa *et al.*, 2012) and a diversity of taxa are affected by ocean acidification (Fabry *et al.*, 2008; Kroeker *et al.*, 2010; Barry, 2011). In general, fishes have received less attention than other taxa, but damselfish, common on reefs around the world, are rapidly becoming a model system to study such effects. Recent studies show that exposure to elevated CO_2 causes fish to respond inappropriately to homing odours (Munday *et al.*, 2009) and cues associated with predation, including predators odours and alarm cues (Dixson *et al.*, 2010; Ferrari *et al.*, 2011a). Fish exposed

Correspondence: Douglas P. Chivers, tel. + 306 966 4419, fax + 306 966 4461, e-mail: doug.chivers@usask.ca

to elevated CO_2 levels also have impaired responses to the sight of potential predators (Ferrari *et al.*, 2012b). They have reduced auditory abilities (Simpson *et al.*, 2011), reduced behavioural lateralization (Domenici *et al.*, 2012) and are unable to learn to recognize novel predators (Ferrari *et al.*, 2012a). Impaired responses to important information about predators have far reaching consequences for survival and recruitment (Munday *et al.*, 2010) and ultimately community structure (Ferrari *et al.*, 2011b). Munday *et al.* (2010) and Ferrari *et al.* (2011a) both found that larval fish raised in seawater enriched with CO_2 at levels predicted for the end of this century had a five to sevenfold increase in predation-related mortality in the first few days after settlement.

The fact that such a diversity of behavioural responses is influenced by exposure to elevated CO_2, led Nilsson *et al.* (2012) to test whether CO_2 affected the function of neurotransmitters. They showed that abnormal olfactory preferences and loss of behavioural lateralization exhibited by larval coral reef fish exposed to elevated CO_2 could be rapidly reversed by treatment with gabazine, an antagonist of the GABA-A receptor – a major inhibitory neurotransmitter receptor in the brain of vertebrates. Sustained exposure to elevated CO_2 levels in fish is likely to induce ion/pH-regulatory changes involving chloride (Cl^-) and bicarbonate (HCO_3^-) (Brauner & Baker, 2009; Esbaugh *et al.*, 2012). The GABA-A receptor is an ion channel with conductance for Cl^- and HCO_3^-, and its involvement suggests that the gradients of these anions have been altered in some neurons of fish exposed to high CO_2 (Nilsson *et al.*, 2012). Dependent on the magnitude of intra- and extracellular changes, the resultant alterations of ion gradients could either potentiate the receptor function or reverse its action, making it excitatory (if the channel opening led to an efflux of anions) rather than inhibitory (due to the normal influx of anions).

It is presently not know how widespread the GABA-A receptor dysfunction is in the fish brain, and our goal here was to examine if it affects higher cognitive functions by testing whether gabazine can reverse the strong detrimental effect that sustained high CO_2 conditions has on learning in larval damselfish (Ferrari *et al.*, 2012a). Therefore, we exposed larval damselfish to projected near-future CO_2 levels and treated some with gabazine prior to putting the fish through a well-established Pavlovian predator-learning protocol (Chivers & Smith, 1994, 1998; Ferrari *et al.*, 2010). We then conducted a direct test of fitness by measuring survival rates in the wild of fish from the different CO_2 and gabazine treatment groups. It is crucial to demonstrate that effects observed under laboratory conditions do, in fact, influence an individual's fitness, in our case, measured as survival of the fish *in situ*. Thus, in our second experiment, we assessed whether CO_2 exposed fish, that had their GABA-A receptors function restored with gabazine prior to learning, had higher survival when released onto patch reefs than those taught without gabazine treatment.

Materials and methods

Fish collection and CO_2 treatment

Our experiments took place in the laboratory facilities and reefs surrounding the Lizard Island Research Station (14°40′S, 145°28′E), Great Barrier Reef, Australia, in November and December 2012. Juvenile ambon damselfish (*Pomacentrus amboinensis*, 16–21 days old) were caught overnight using light traps (Meekan *et al.*, 2001) moored approximately 100 m off the fringing reef. These traps collect young fish at the end of their pelagic phase, immediately prior to their settlement to the reef. Fishes caught in the traps were brought back to the station just after dawn, sorted by species, and small groups of *P. amboinensis* were transferred into a series of 35-L aquaria at 440 (corresponding to present-day control CO_2 levels) or 987 μatm CO_2 (end of century CO_2 projection, Table 1). Fish were fed four times a day with live *Artemia* nauplii and were kept under elevated CO_2 conditions for 4 days prior to the start of the experiment. Four days is sufficient time for damselfish larvae to show impaired behavioural responses to predation cues (Munday *et al.*, 2010) and is sufficient time to impair learned predator recognition (Ferrari *et al.*, 2012a). Identical behavioural impairment is seen in fishes raised from hatching under the same CO_2 levels, indicating that alterations in behaviour and learning are not due to a sudden exposure to CO_2 (Dixson *et al.*, 2010; Munday *et al.*, 2010). Furthermore, juvenile damselfish are naturally exposed to a substantive change in CO_2 conditions. They recruit from the open ocean, where CO_2 conditions are relatively stable, to the coral reef where pCO_2 can fluctuate on a daily basis due the net effects of photosynthesis, respiration and calcification (Ohde & Van Woesik, 1999; Gagliano *et al.*, 2010; Shamberger *et al.*, 2011; Shaw *et al.*, 2013). The fluctuation in pCO_2 on reefs can range from 431 to 622 μatm on a daily basis (Shamberger *et al.*, 2011).

CO_2 treatments were established by dosing with 100% CO_2 to a set pH, following standard techniques (Gattuso *et al.*,

Table 1 Water chemistry parameters (mean ± SE) for each treatment

Treatment	Temperature (°C)	Salinity	pH_{NBS}	Total alkalinity (μmol kg^{-1} SW)	pCO_2 (μatm)
Control	27.7 (±0.1)	35.2	8.15 (±0.01)	2273 (±14)	440 (±19)
Elevated-CO_2	27.7 (±0.1)	35.2	7.85 (±0.01)	2265 (±5)	987 (±16)

2010). Seawater was pumped from the ocean into 2 × 60 l header tanks where it was diffused with ambient air (control) or CO_2 to achieve the desired pH (elevated-CO_2 treatment). A pH-controller (Aqua Medic, Germany) attached to the CO_2 treatment header tank maintained pH at the desired level. A water pump in the sump rapidly dissolved CO_2 into the seawater and also served as a vigorous stirrer. Control or elevated-CO_2 seawater was supplied to 35-L aquaria housing larval fish at 720 ml min^{-1}. Seawater pH_{NBS} (HQ40d, Hach, Colorado, US) and temperature (C22, Comark, Norwich, UK) were recorded daily in each aquarium. Water samples were analysed for total alkalinity by Gran titration (888 Titrando, Metrohm, Switzerland) to within 1% of certified reference material (Prof. A. Dickson, Scripps Institution of Oceanography). Carbonate chemistry parameters (Table 1) were calculated in CO2SYS (Pierrot *et al.* 2006) using the constants K1, K2 from Mehrbach *et al.* (1973) refit by Dickson & Millero (1987) and Dickson for $KHSO_4$.

Predator odour and alarm cue preparation

Six moonwrasse (*Thalassoma lunare*), four lizardfish (*Synodus dermatogenys*) and four dottybacks (*Pseudochromis fuscus*) were used as predators in our experiments because these predator species were common at the site where monitoring of survival was to be conducted. They were captured using hand nets and anaesthetic clove oil mixed with alcohol and seawater (Munday & Wilson, 1997). Predators were placed together by species in 20-l flow-through containers and supplied with a water flow of approximately 1 l-min^{-1}. Predators were fed ad libitum with a diet of commercial bait squid (*Uroteuthis noctiluca)* for a minimum of 3 days prior to collecting predator odours. We turned off the water for 2 h and used the resulting tank water as the predator odour in our experiments.

Alarm cues were prepared in batches by killing 3 donor fish by cold shock and then making a series of 6 vertical cuts along both sides of each fish. Afterwards, the three fish were rinsed in 15 ml of seawater and the resulting solution (36 cuts in 15 ml of water) was used as our standard alarm cue stimulus. In all cases, alarm cues were used within a few minutes of being prepared to ensure that the cues remained active.

Experiment 1: A laboratory test of the role of GABA-A receptors in impaired predator learning for fish exposed to elevated CO_2

The goal of our first experiment was to test whether altered function of GABA-A receptors was responsible for the effects of elevated CO_2 on predator learning in damselfish. The subtlety of our experimental design comes from differentiating the effect of CO_2 on learning *per se* (the acquisition of new information) from the effects of CO_2 on the behavioural response of the fish. We used a 2 × 2 × 2 × 2 design, whereby we crossed 2 levels of CO_2 exposure (440 and 987 μatm) with 2 treatments (placing the fish in a jar with or without gabazine in seawater) and tested the fish at two times (1 and 5 days postconditioning) for each of 2 cues (seawater or predator odour). Like many fishes, damselfishes innately respond to conspecific alarm cues (Ferrari *et al.*, 2011a), but often need to learn to respond to the odours of different predators (Mitchell *et al.*, 2011a; Lonnstedt *et al.*, 2012). Damselfishes that are exposed to predator odours paired with alarm cue normally learn to respond to predator odours with an antipredator response and retain such responses for at least a week (Larson & McCormick, 2005; Mitchell *et al.*, 2011a; Ferrari *et al.*, 2012a; Lonnstedt *et al.*, 2012); however, fish exposed to elevated CO_2 do not learn to respond to predator odours (Ferrari *et al.*, 2012a). Consequently, we predicted that control CO_2 fish would show a response to the predator odour (but not seawater) 1 day and 5 days postconditioning (learning phase), while those exposed to elevated CO_2 would not respond on either day 1 or day 5. If altered function of GABA-A receptors was responsible for the effects of high CO_2 on learning, then treatment with the GABA-A antagonist, gabazine, should reverse the CO_2-induced learning impairment and gabazine-treated fish exposed to elevated CO_2 should show an antipredator response to predator odour. However, given that gabazine is taken up quickly over the gills, it will also wash out quite quickly (Nilsson *et al.*, 2012). This means that gabazine may only provide a short-term CO_2 reversal. In this case, the fish could successfully learn to recognize the predator during conditioning (i.e. shortly after the gabazine treatment), but fail to display an appropriate antipredator response 1 day later due to the recurrent effect of CO_2 on behaviour. We know that it takes 2–4 days for fish risk assessment to recover to normal following exposure to elevated CO_2 (Munday *et al.*, 2010). Thus, we tested for learned recognition of the predator 1 day postconditioning (when the CO_2 was still in effect) and also 5 days post CO_2 (when the effect of CO_2 has worn off). This ensured that we distinguished between the failure to learn predator cues (due to failed conditioning) and failure to respond to predator cues (due to a masking effect of CO_2).

Fish that were exposed to control and elevated CO_2 were removed from their holding tank after 4 days and held in aerated 100 ml jars containing seawater or a gabazine solution (4 mg l^{-1}) in groups of 6 for 30 min. Afterwards, fish were placed individually into 1-l plastic trays in clean seawater and allowed to acclimate for 15 min. They were then conditioned to recognize the odour of moonwrasse, by exposing them to 1 ml of the alarm cue solution paired with 5 ml of moonwrasse odour. This is a well-established method of training fish to recognize unknown predators (Chivers & Smith, 1994; Brown & Smith, 1998; Lonnstedt *et al.*, 2012). If damselfish learn to recognize the predator based on this pairing they should subsequently respond to the predator odour when it is presented alone. Damselfish were exposed to the predator odour/alarm cue solution for 5 min after which they were removed and placed into 20 l testing tanks for recognition tests the following day or were placed into 5 l holding tanks and tested 5 days postconditioning. Fish were tested for their response to predator odour or a control of seawater on both of the testing days.

Learning was assessed using a well-established protocol (Ferrari *et al.*, 2012a). A day prior to testing individual fish were placed into plastic 20-l flow-through tanks (32 × 16 × 16 cm) with a substratum of sand, a small piece of dead coral

(*Pocillopora damicornis)* as a shelter, an airstone and a 1.5 m long injection tube used to introduce predator odour or water into the tank. Each tank had a 4 × 4 cm grid drawn on the side of the test tank to help the observer record the activity of the fish during the experiment. The tanks were covered on three sides with black plastic to avoid visual transfer of information from surrounding tanks. A black plastic curtain was hung in front of the tanks to minimize disturbance to the fish by the movement of the observer. One h after adding fish to the conditioning tanks and again, 1 h prior to testing, the fish were fed *ad libitum* with *Artemia* larvae.

At the beginning of each trial, fish were fed 2.5 ml of food (seawater containing approximately 250 *Artemia* larvae/ml) to remove the possibility of a 'feeding frenzy' effect at the start of the bioassay. Prestimulus observations began 5 min later, when another 2.5 ml of food was injected into the tank. During the prestimulus observation period, we measured two behaviours: (i) the total number of feeding strike displayed by the fish, regardless of whether they were successful at capturing a food item or not; (ii) the total number of lines the fish crossed during the observation period, using the grid drawn on the side of the tank. A line was counted as crossed when the entire body of the fish crossed a line. At the end of this 5-min prestimulus observation period, 30 ml of moonwrasse odour or 30 ml of seawater were introduced into the tank followed by 2.5 ml of food. The behaviour of the fish was then observed for another 5 min. Prey fishes exposed to risk typically reduce feeding and activity, consequently if fish recognize the predator odour we should observe a reduction in feeding and activity from the prestimulus baseline. We tested between 10 and 14 fish in each of the 16 treatments. The experimenter was blind to the treatments during the observations.

Statistical analyses

For both behaviours, data were computed to obtain a proportion change in behaviour from the prestimulus baseline (post–pre)/pre), which were then used as raw data in the analysis. Due to their dependence, both behaviours were analysed simultaneously using a MANOVA approach. Due to the complexity of the design, we ran the two testing days separately (day 1 and day 5 postgabazine treatment). For each day, we performed the 2 × 2 × 2 MANOVA testing the effect of CO_2 treatment (control vs. elevated), gabazine treatment (gabazine vs. no gabazine) and test cue (water vs. predator odour) on the behavioural response of the fish.

Experiment 2: The effect of restoring GABA-A receptor function on predator learning in fish exposed to elevated CO_2: a test of survival

The goal of this experiment was to test whether restoration of GABA-A receptor function would reverse the negative effects of CO_2 on predator learning, as evident by survival for fish released into the wild. Fish from the 987 μatm CO_2 treatment that were taught to recognize a series of three common reef predators in the laboratory after being treated with gabazine or sham treatment. If gabazine reversed the effect of CO_2 on learning then gabazine-treated fish should survive longer when stocked onto patch reefs. Two additional treatments were added to the survival analysis. Control fish (held in present day CO_2 conditions) that were taught to recognize the predator odour in the absence of gabazine served as a positive control, while control fish that did not have the opportunity to learn the predator odour and were not exposed to gabazine served as the negative control.

We followed a similar protocol as experiment 1 to train fish to recognize predators. Fish were held in small groups in 100 ml aerated jars and were treated with gabazine (or a control of seawater) for 30 min as described for the previous experiment. Afterwards, they were removed and placed into a series of 32 20-l conditioning tanks. After a 15 min acclimation period, they were exposed to 30 ml of moonwrasse odour paired with 5 ml of alarm cue. At the same time, we placed a plastic bag containing a moonwrasse into the tank for 1 min. This training procedure allowed the prey the opportunity to learn both the sight and odour signature of the predator (Lonnstedt *et al*., 2012). The same procedure was repeated using dottybacks and lizardfish as predators to ensure the damselfish had the opportunity to learn three of the main predators likely to be encountered on the reef. The flow-through water system remained on during and after the predator training to flush the cues from the tank. Fish in the negative control treatment went through the same procedure except we introduced seawater and an empty bag instead of predator odour, alarm cues and the live predators. The field trial took place at least 5 days after the CO_2 treatment and conditioning to ensure that the CO_2 effect had completely worn off.

Ambon damselfish naturally settle on patch reef habitats near the continuous reef. In this habitat, juveniles are exposed to a diverse range of predators that use a variety of feeding modes from ambush (lizardfish) to pursuit (dottybacks and wrasse). These fishes can be observed to prey on juveniles that venture too far from shelter. Our field experiment consisted of an array of patch reefs (ca. 18 × 15 × 18 cm) composed of pieces of healthy and dead bushy hard coral, *Pocillopora damicornis,* placed 3 m apart on a sandflat at the backreef of the Lizard Island fringing reef.

On the days of the field trials, damselfish were placed into individually labelled 1 l plastic bags of seawater, photographed against a 1 cm grid for size measurements and kept in a water bath of flowing seawater until deployment in the field. To reduce transport and handling stress, fish in bags were transported to the field site in a 60 l bin of seawater (to reduce temperature fluctuations) under subdued light conditions. A single fish was carefully released on each patch reef. Patches were cleared of any other fishes or invertebrates prior to release. A small wire cage (ca. 30 × 30 × 30 cm, 12 mm mesh size) was placed over the patch to allow fish to acclimate to their new surroundings while being protected from predators. Cages were removed 40–60 min after release of the fish between 7:00 hours and 9:00 hours. The presence of experimental fish on the patch reefs was monitored twice a day (ca. 8:00 hours and 17:00 hours) for 4 days. Missing fish were presumed dead as newly recruited juveniles are highly sedentary and previous studies using tagged fish have indicated negligible migration from similar patch reefs (Hoey & McCormick, 2004; McCormick, 2009; Lonnstedt *et al*., 2012).

Statistical analyses

Survival (up to 92 h) of damselfish among the 4 treatments was compared using multiple-sample Survival Analysis, which uses a Cox's proportional hazard model (complete observations 46, censored 51; Statistica 9.0). Survival curves for fish within each treatment were calculated and plotted using the Kaplan–Meier product–limit method. The Kaplan–Meier method is a nonparametric estimator of survival that incorporates incomplete (censored) observations, such as those cases where fish had not died by the end of the census period. Differences in fish survival between particular pairs of treatments were compared using Cox-Mantel test with a Cox-F statistic.

Results

Experiment 1

When the fish were tested 1 day postgabazine treatment, we found a CO_2 × Cue interaction on the response of the fish (Pillai's Trace, $F_{2,82} = 5.6$, $P = 0.004$; Fig. 1). For the control CO_2 fish, the analysis revealed an effect of cue ($F_{2,41} = 24.0$, $P < 0.001$), but no effect of gabazine ($F_{2,41} = 1.0$, $P = 0.37$) and no interaction between CO_2 and gabazine ($F_{2,41} = 0.6$, $P = 0.52$) on their behavioural response. This indicates that control fish displayed an antipredator response when exposed to the moonwrasse odour, regardless of the gabazine treatment. The antipredator response was evident as reduced activity (Fig. 1a) and reduced feeding (Fig 1b). In contrast, fish in the elevated CO_2 group did not respond differently to the cues, as shown by the lack of effect of cue ($F_{2,40} = 0.3$, $P = 0.75$), gabazine ($F_{2,40} = 0.1$, $P = 0.95$) and cue × gabazine interaction ($F_{2,40} = 0.6$, $P = 0.57$). Thus, testing the fish at this time did not reveal an effect of gabazine on learning deficiency induced by high-CO_2 treatment.

When the fish were tested 5 days postgabazine treatment, we found a 3-way interaction between CO_2, gabazine and cue (Pillai's Trace: $F_{2,84} = 7.2$, $P = 0.001$) on the behaviour of the fish (Fig. 2). The control fish,

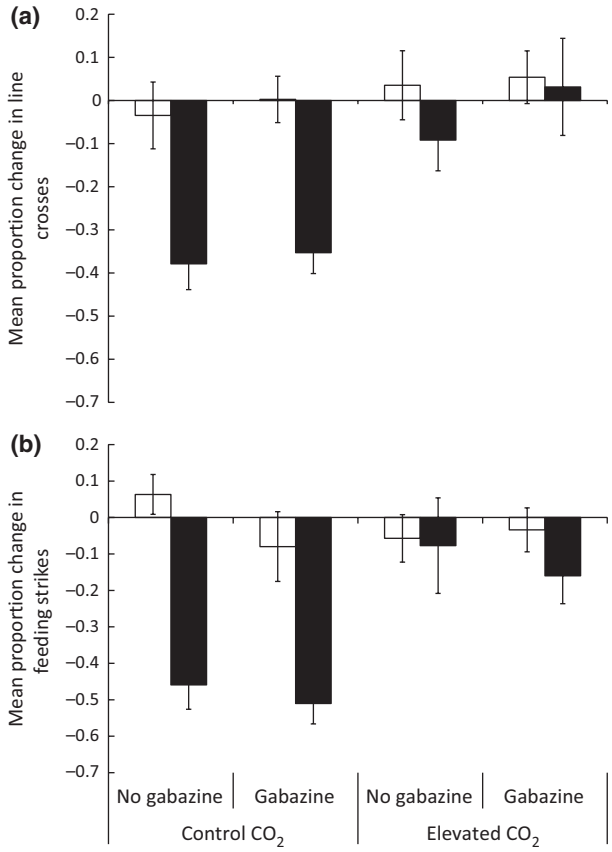

Fig. 1 Mean (±SE) proportion change in line crosses (a) and feeding strikes, (b) for fish exposed to control or elevated levels of CO_2 and then held in jars containing a solution of gabazine or a seawater control prior to being taught to recognize a moonwrasse as a predator. White bars represent the response of the fish to the introduction of control water and black bars represent the response to moonwrasse odour 1 day following the learning trial.

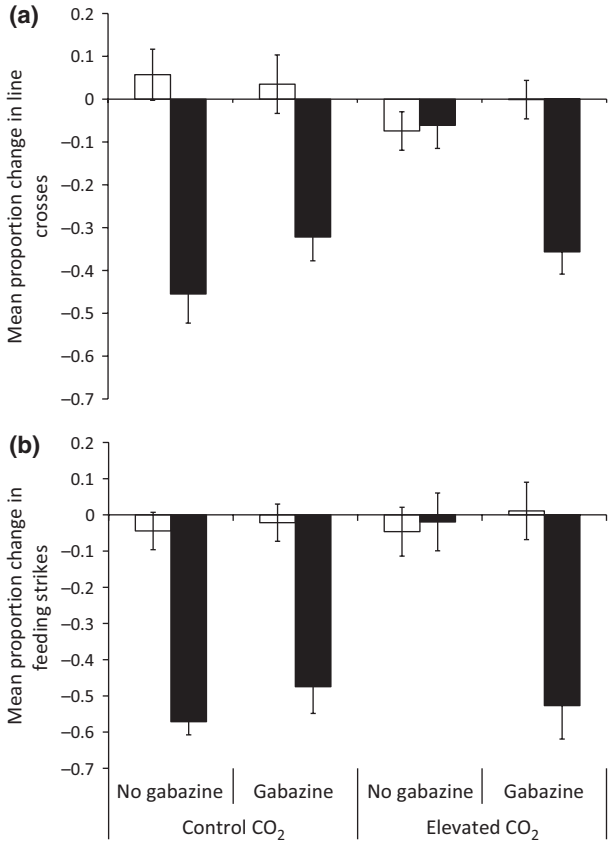

Fig. 2 Mean (±SE) proportion change in line crosses (a) and feeding strikes, (b) for fish exposed to control or elevated levels of CO_2 and then held in jars containing a solution of gabazine or a seawater control prior to being taught to recognize a moonwrasse as a predator. White bars represent the response of the fish to the introduction of control water and black bars represent the response to moonwrasse odour 5 days following the learning trial.

once again, were affected by cue ($F_{2,43} = 31.9$, $P < 0.001$), but not by gabazine ($F_{2,84} = 0.9$, $P = 0.40$) or gabazine × cue ($F_{2,84} = 0.9$, $P = 0.41$). Thus, the control fish responded to the odour of moonwrasse with an antipredator response, regardless of gabazine treatment. The fish in the elevated CO_2 group, however, exhibited a gabazine by cue interaction ($F_{2,40} = 7.9$, $P = 0.001$). Fish not exposed to gabazine did not response differently to water and predator odour, indicating a failure to learn to recognize the predator odour. However, fish treated with gabazine prior to the learning session responded to the predator 5 days later, indicating that the presence of gabazine had allowed learning to occur in those fish during the learning session.

Experiment 2

There was a significant difference in the survival trajectories among the four treatments ($\chi^2 = 26.76$, df = 3, $P < 0.0001$; Fig. 3). The trajectories fell into two pairs that did not differ from one another (Cox–Mantel tests $P > 0.5$). Fish that had been under high CO_2 but had received gabazine treatment prior to being taught the identity of the three predators and those fish that learnt the predators under control conditions had equal highest survival (Fig. 3). Control fish that had not been taught, and those fish exposed to high CO_2 but not exposed to gabazine prior to being taught, had the lowest survival, with about 50% of fish dying within 24 h (Fig. 3). This suggests that the treatment with gabazine reduced the CO_2 effect sufficiently to allow associative learning through chemical cues.

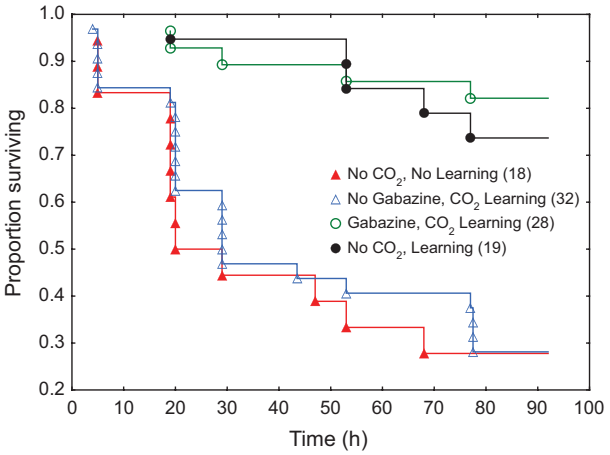

Fig. 3 Survival of damselfish under elevated or current day levels of CO_2, exposed or not to gabazine when they were (or not) taught the identity of 3 common predators. Kapalin–Meier survival trajectories illustrate the different survivals on patches of live healthy *Pocillopora damicornis* hard coral. Individuals were placed on the corals as solitary individuals. Numbers in brackets represent the total number of trials undertaken for each treatment.

Discussion

The results of our study provide compelling evidence that levels of CO_2 predicted to occur by the end of the century, dramatically impair the ability of damselfish to learn to recognize predators. This result is in accordance with previous laboratory findings (Ferrari *et al.*, 2012b); however, here we showed that this has a direct fitness consequence through dramatically lower survival in the wild. This is the first field demonstration of the negative effects of CO_2 on predator learning. The effect of elevated CO_2 on the ability of fish to learn predators was reversed when the fish were treated with gabazine, an antagonist of the GABA-A receptor. This was evident in both our behavioural assay in the laboratory and in our survival assay in the wild. Our work provides the first example of how CO_2 impairment at the neuronal level leads to changes in learning and eventually to a measurable survival difference in the wild.

Previous studies have shown it takes juvenile damselfish at least 2 or 3 days to recover from the effects of elevated CO_2 when they are returned to control seawater (Munday *et al.*, 2010). During this time, they do not respond appropriately to ecologically relevant stimuli and cannot learn to recognize predators (Ferrari *et al.*, 2012a). In our experiment, fish received a waterborne exposure to gabazine for 30 min immediately after being removed from the CO_2 treatment and were allowed to recover in fresh seawater for 15 min prior to a 5 min learning procedure. When the fish were tested the following day, there was no evidence that gabazine reversed the effects of CO_2 on learning. Thus, at this point fish treated with gabazine could not demonstrate that they had learnt to respond to the predator. However, when fish were tested 5 days later, the learning was obvious. This indicates that the reversing effect of gabazine on neurotransmitter function was not long lasting, and that the effects had worn off before we tested the fish in the learning trial the next day. However, by day five, the effects CO_2 had worn off, fish returned to normal activity, and we could see that the gabazine treatment had indeed reversed the effects of CO_2 during the learning session.

The fact that the specific GABA-A receptor antagonist gabazine has such potent effects on behavioural functions altered by elevated CO_2 exposure clearly implicates an involvement of this major inhibitory neurotransmitter receptor. This is also supported by the fact that the GABA-A receptor is an ion channel with specific conductance for Cl^- and HCO_3^- – precisely the

two ions most likely to be affected by elevated CO_2 levels in fish (Ishimatsu *et al*., 2008; Brauner & Baker, 2009). Unfortunately, at present we do not know the exact nature of the underlying changes in intracellular and extracellular Cl^- and/or HCO_3^- levels because the small size of the fish used in our study precluded measurements of blood and tissue ion gradients. Alterations of the gradients of these ions over neuronal membranes could either potentiate the GABA-A receptor function or reverse its action. Moreover, the finding that several days of sustained high-CO_2 exposure was needed to alter fish behaviour, and that these alterations remains for several days after the exposure, suggest that mechanism such as altered gene expression may be involved. Clearly, further studies are needed to clarify the exact nature of the neural perturbations that affect fish exposed to near-future CO_2 levels.

Juvenile damselfish that recruit to the reef are naive to the suite of predators that await them. There is immense pressure to rapidly learn the identity of the local predators (Mitchell *et al*., 2011a), and to be able differentiate predators from nonpredators (Mitchell *et al*., 2011b) particularly given that predators may remove more than 60% of new recruits in the first 2 days after settlement (Almany & Webster, 2006). In our experiment, impaired learning under elevated CO_2 increased the rate of mortality approximately 2.5–3 times. This could create a significant population bottleneck and has the potential to alter the structure of coral reef communities, especially as the magnitude of effect of elevated CO_2 on juvenile behaviour appears to differ among species (Ferrari *et al*., 2011a). While the predators in our field experiment were not exposed to high CO_2, Ferrari *et al*. (2011b) found that the mortality rate of small juvenile damselfishes from predation was still significantly increased when both predator and prey species had been exposed to elevated CO_2. Therefore, any behavioural deficiencies of predators under elevated CO_2 do not appear to compensate for the impaired learning ability of the prey.

Ocean acidification has the potential to dramatically change our world's oceans. If we are to maximize our understanding of how changes in ocean chemistry affect the communities and species that compose them, then much of our research focus should be on questions that integrate what we know from a diversity of disciplines. Our work linking CO_2 changes at the neurotransmitter level to changes in behaviour and learning and ultimately survival in the wild, could provide a template for similar integrative studies. Future studies will also need to integrate an evolutionary perspective (Munday *et al*., 2012) because only with an understanding of the potential for adaptation can we predict how ecological impacts of ocean acidification will interact with evolutionary changes that could occur over coming decades.

Acknowledgements

We thank the Natural Sciences and Engineering Research Council of Canada, the University of Oslo, the Australian Research Council (ARC) and the ARC Centre of Excellence for Coral Reef Studies for funding this study. DC, MF, MIM, PM, GN conceived the project; DC, MF, MIM collected and analysed data; DC and MF wrote the first draft of the ms; PM and SW provided water chemistry parameters and technical assistance with the CO_2 system; MGM, MDM, KC provided assistance with the implementation of the experiment. All authors contributed to the final version of the manuscript.

References

Almany GR, Webster MS (2006) The predation gauntlet: early post-settlement mortality in reef fishes. *Coral Reefs*, **25**, 19–22.

Barry JP, Widdicombe S, Hall-Spencer JM (eds) (2011) *Effects of Ocean Acidification on Marine Biodiversity and Ecosystem Function*. Oxford University Press, Oxford.

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

Briffa M, De La Haye K, Munday PL (2012) High CO_2 and marine animal behaviour: potential mechanisms and ecological consequences. *Marine Pollution Bulletin*, **64**, 1519–1528.

Brown GE, Smith JF (1998) Acquired predator recognition in juvenile rainbow trout (*Oncorhynchus mykiss*): conditioning hatchery-reared fish to recognize chemical cues of a predator. *Canadian Journal of Fisheries and Aquatic Sciences*, **55**, 611–617.

Chivers DP, Smith RJF (1994) The role of experience and chemical alarm signaling in predator recognition by fathead minnows, *Pimephales promelas*. *Journal of Fish Biology*, **44**, 273–285.

Chivers DP, Smith RJF (1998) Chemical alarm signalling in aquatic predator-prey systems: a review and prospectus. *Ecoscience*, **5**, 338–352.

Dickson AG, Millero FJ (1987) A comparison of the equilibrium constants for the dissociation of carbonic acid in seawater media. *Deep-Sea Research*, **34**, 1733–1743.

Dixson DL, Munday PL, Jones GP (2010) Ocean acidification disrupts the innate ability of fish to detect predator olfactory cues. *Ecology Letters*, **13**, 68–75.

Dlugokencky E, Tans P (2013) Trends in atmospheric carbon dioxide. Available at: http://www.esrl.noaa.gov/gmd/ccgg/trends/ (accessed 6 May 2013).

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

Doney SC (2010) The growing human footprint on coastal and open-ocean biogeochemistry. *Science*, **328**, 1512–1516.

Doney SC, Fabry VJ, Feely RA, Kleypas JA (2009) Ocean acidification: the other CO_2 problem. *Annual Review of Marine Science*, **1**, 169–192.

Esbaugh AJ, Heuer R, Grosell M (2012) Impacts of ocean acidification on respiratory gas exchange and acid-base balance in a marine teleost, Opsanus beta. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology*, **182**, 921–934.

Fabry VJ, Seibel BA, Feely RA, Orr JC (2008) Impacts of ocean acidification on marine fauna and ecosystem processes. *Ices Journal of Marine Science*, **65**, 414–432.

Ferrari MCO, Wisenden BD, Chivers DP (2010) Chemical ecology of predator-prey interactions in aquatic ecosystems: a review and prospectus. *Canadian Journal of Zoology*, **88**, 698–724.

Ferrari MCO, Dixson DL, Munday PL, McCormick MI, Meekan MG, Sih A, Chivers DP (2011a) Intrageneric variation in antipredator responses of coral reef fishes affected by ocean acidification: implications for climate change projections on marine communities. *Global Change Biology*, **17**, 2980–2986.

Ferrari MCO, McCormick MI, Munday PL, Meekan MG, Dixson DL, Lonnstedt O, Chivers DP (2011b) Putting prey and predator into the CO_2 equation - qualitative and quantitative effects of ocean acidification on predator-prey interactions. *Ecology Letters*, **14**, 1143–1148.

Ferrari MCO, Manassa RP, Dixson DL *et al*. (2012a) Effects of ocean acidification on learning in coral reef fishes. *PLoS ONE*, **7**, e31478.

Ferrari MCO, McCormick MI, Munday PL, Meekan MG, Dixson DL, Lonnstedt O, Chivers DP (2012b) Effects of ocean acidification on visual risk assessment in coral reef fishes. *Functional Ecology*, **26**, 553–558.

Gagliano M, McCormick MI, Moore JA, Depczynski M (2010) The basics of acidification: baseline variability of pH on Australian coral reefs. *Marine Biology*, **157**, 1849–1856.

Gattuso J-P, Gao K, Lee K, Rost B, Schulz KG (2010) Approaches and tools to manipulate the carbonate chemistry. In: *Guide to Best Practices for Ocean Acidification Research and Data Reporting*, (eds Riebesell U, Fabry VJ, Hansson L, Gattuso J-P), pp. 41–52. European Union, Luxembourg.

Hoey AS, McCormick MI (2004) Selective predation for low body condition at the larval-juvenile transition of a coral reef fish. *Oecologia*, **139**, 23–29.

Ishimatsu A, Hayashi M, Kikkawa T (2008) Fishes in high-CO_2, acidified oceans. *Marine Ecology-Progress Series*, **373**, 295–302.

Kroeker KJ, Kordas RL, Crim RN, Singh GG (2010) Meta-analysis reveals negative yet variable effects of ocean acidification on marine organisms. *Ecology Letters*, **13**, 1419–1434.

Larson JK, McCormick MI (2005) The role of chemical alarm signals in facilitating learned recognition of novel chemical cues in a coral reef fish. *Animal Behaviour*, **69**, 51–57.

Lonnstedt OM, McCormick MI, Meekan MG, Ferrari MCO, Chivers DP (2012) Learn and live: predator experience and feeding history determines prey behaviour and survival. *Proceedings of the Royal Society B-Biological Sciences*, **279**, 2091–2098.

Luthi D, Le Floch M, Bereiter B *et al*. (2008) High-resolution carbon dioxide concentration record 650,000-800,000 years before present. *Nature*, **453**, 379–382.

McCormick MI (2009) Behaviourally mediated phenotypic selection in a disturbed coral reef environment. *PLoS ONE*, **4**, 1–8.

Meekan MG, Wilson SG, Halford A, Retzel A (2001) A comparison of catches of fishes and invertebrates by two light trap designs, in tropical NW Australia. *Marine Biology*, **139**, 373–381.

Meinshausen M, Smith SJ, Calvin K *et al*. (2011) The RCP greenhouse gas concentrations and their extensions from 1765 to 2300. *Climatic Change*, **109**, 213–241.

Mehrbach C, Culberson CH, Hawley JE, Pytkowicz RM (1973) Measurements of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnology and Oceanography*, **18**, 897–907.

Mitchell MD, McCormick MI, Ferrari MCO, Chivers DP (2011a) Coral reef fishes rapidly learn to identify multiple unknown predators upon recruitment to the reefs. *PLoS ONE*, **6**, e15764.

Mitchell MD, McCormick MI, Ferrari MCO, Chivers DP (2011b) Friend or foe? The role of latent inhibition in predator and non-predator labelling by coral reef fishes. *Animal Cognition*, **14**, 707–714.

Munday PL, Wilson SK (1997) Comparative efficacy of clove oil and other chemicals in anaesthetization of Pomacentrus amboinensis, a coral reef fish. *Journal of Fish Biology*, **51**, 931–938.

Munday PL, Dixson DL, Donelson JM, Jones GP, Pratchett MS, Devitsina GV, Doving KB (2009) Ocean acidification impairs olfactory discrimination and homing ability of a marine fish. *Proceedings of the National Academy of Sciences of the United States of America*, **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. *Proceedings of the National Academy of Sciences of the United States of America*, **107**, 12930–12934.

Munday PL, McCormick MI, Nilsson GE (2012) Impact of global warming and rising CO_2 levels on coral reef fishes: what hope for the future? *Journal of Experimental Biology*, **215**, 3865–3873.

Nilsson GE, Dixson DL, Domenici P, McCormick MI, Sorensen C, Watson S-A, Munday PL (2012) Near-future CO_2 levels alter fish behaviour by interfering with neurotransmitter function. *Nature Climate Change*, **2**, 201–204.

Pierrot D, Lewis E, Wallace DWR (2006) MS Excel program developed for CO_2 system calculations. In: *ORNL/CDIAC-105a*. Carbon Dioxide Information Analysis Center, Oak Ridge National Laboratory, US. Department of Energy, Oak Ridge, TN, USA.

Ohde S, Van Woesik R (1999) Carbon dioxide flux and metabolic processes of a coral reef, Okinawa. *Bulletin of Marine Science*, **65**, 559–576.

Orr JC, Fabry VJ, Aumont O *et al*. (2005) Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature*, **437**, 681–686.

Peters GP, Marland G, Le Quere C, Boden T, Canadell JG, Raupach MR (2012) CORRESPONDENCE: Rapid growth in CO_2 emissions after the 2008–2009 global financial crisis. *Nature Climate Change*, **2**, 2–4.

Raupach MR, Marland G, Ciais P, Le Quere C, Canadell JG, Klepper G, Field CB (2007) Global and regional drivers of accelerating CO_2 emissions. *Proceedings of the National Academy of Sciences of the United States of America*, **104**, 10288–10293.

Sabine CL, Feely RA, Gruber N, Key RM, Lee KS, Al E (2004) The oceanic sink for anthropogenic CO_2. *Science*, **305**, 367–371.

Shamberger KEF, Feely RA, Sabine CL *et al*. (2011) Calcification and organic production on a Hawaiian coral reef. *Marine Chemistry*, **127**, 64–75.

Shaw EC, Mcneil BI, Tilbrook B, Matear R, Bates ML (2013) Anthropogenic changes to seawater buffer capacity combined with natural reef metabolism induce extreme future coral reef CO_2 conditions. *Global Change Biology*, **19**, 1632–1641.

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

Widdicombe S, Spicer JI (2008) Predicting the impact of ocean acidification on benthic biodiversity: what can animal physiology tell us? *Journal of Experimental Marine Biology and Ecology*, **366**, 187–197.