

# Impaired learning of predators and lower prey survival under elevated CO<sub>2</sub>: a consequence of neurotransmitter interference

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## 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<sub>2</sub> levels impaired the ability of damselfish to learn the identity of predators. These effects stem from impaired neurotransmitter function; impaired learning under elevated CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub>, as a result of impaired learning, could have a major influence on population recruitment.

**Keywords:** CO<sub>2</sub>, GABA-A receptors, global change, learning, neurotransmitter, ocean acidification, predator recognition, survival

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## Introduction

Burning of fossil fuels, production of cement and large-scale land-use changes have resulted in the concentration of carbon dioxide (CO<sub>2</sub>) in the atmosphere rising at an unprecedented rate (Raupach *et al.*, 2007; Peters *et al.*, 2012). Atmospheric CO<sub>2</sub> 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<sub>2</sub> emissions trajectory is maintained atmospheric CO<sub>2</sub> could exceed 900 ppm by the end of the century (Meinshausen *et al.*, 2011). The amount of CO<sub>2</sub> dissolved in the surface ocean is increasing in line with atmospheric CO<sub>2</sub> because of the equilibration of gas partial pressures at the air–sea interface (Doney, 2010). About 30% of the excess CO<sub>2</sub> 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<sub>2</sub> at the ocean surface is higher ocean pCO<sub>2</sub>, 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<sub>2</sub> causes fish to respond inappropriately to homing odours (Munday *et al.*, 2009) and cues associated with predation, including predators odours and alarm cues (Dixon *et al.*, 2010; Ferrari *et al.*, 2011a). Fish exposed

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to elevated CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub>, led Nilsson *et al.* (2012) to test whether CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> levels in fish is likely to induce ion/pH-regulatory changes involving chloride (Cl<sup>-</sup>) and bicarbonate (HCO<sub>3</sub><sup>-</sup>) (Brauner & Baker, 2009; Esbaugh *et al.*, 2012). The GABA-A receptor is an ion channel with conductance for Cl<sup>-</sup> and HCO<sub>3</sub><sup>-</sup>, and its involvement suggests that the gradients of these anions have been altered in some neurons of fish exposed to high CO<sub>2</sub> (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 known 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<sub>2</sub> conditions has on learning in larval damselfish (Ferrari *et al.*, 2012a). Therefore, we exposed larval damselfish to projected near-future CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> levels) or 987 µatm CO<sub>2</sub> (end of century CO<sub>2</sub> projection, Table 1). Fish were fed four times a day with live *Artemia* nauplii and were kept under elevated CO<sub>2</sub> 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<sub>2</sub> levels, indicating that alterations in behaviour and learning are not due to a sudden exposure to CO<sub>2</sub> (Dixon *et al.*, 2010; Munday *et al.*, 2010). Furthermore, juvenile damselfish are naturally exposed to a substantive change in CO<sub>2</sub> conditions. They recruit from the open ocean, where CO<sub>2</sub> conditions are relatively stable, to the coral reef where pCO<sub>2</sub> can fluctuate on a daily basis due to 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<sub>2</sub> on reefs can range from 431 to 622 µatm on a daily basis (Shamberger *et al.*, 2011).

CO<sub>2</sub> treatments were established by dosing with 100% CO<sub>2</sub> 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 <sub>NBS</sub>	Total alkalinity (µmol kg <sup>-1</sup> SW)	pCO <sub>2</sub> (µatm)
Control	27.7 (±0.1)	35.2	8.15 (±0.01)	2273 (±14)	440 (±19)
Elevated-CO <sub>2</sub>	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<sub>2</sub> to achieve the desired pH (elevated-CO<sub>2</sub> treatment). A pH-controller (Aqua Medic, Germany) attached to the CO<sub>2</sub> treatment header tank maintained pH at the desired level. A water pump in the sump rapidly dissolved CO<sub>2</sub> into the seawater and also served as a vigorous stirrer. Control or elevated-CO<sub>2</sub> seawater was supplied to 35-L aquaria housing larval fish at 720 ml min<sup>-1</sup>. Seawater pH<sub>NBS</sub> (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 Titrand, 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<sub>4</sub>.

### 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<sup>-1</sup>. 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<sub>2</sub>

The goal of our first experiment was to test whether altered function of GABA-A receptors was responsible for the effects of elevated CO<sub>2</sub> on predator learning in damselfish. The subtlety of our experimental design comes from differentiating the effect of CO<sub>2</sub> on learning *per se* (the acquisition of new information) from the effects of CO<sub>2</sub> on the behavioural response of the fish. We used a 2 × 2 × 2 × 2 design, whereby we crossed 2 levels of CO<sub>2</sub> 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<sub>2</sub> do not learn to respond to predator odours (Ferrari *et al.*, 2012a). Consequently, we predicted that control CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> on learning, then treatment with the GABA-A antagonist, gabazine, should reverse the CO<sub>2</sub>-induced learning impairment and gabazine-treated fish exposed to elevated CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> on behaviour. We know that it takes 2–4 days for fish risk assessment to recover to normal following exposure to elevated CO<sub>2</sub> (Munday *et al.*, 2010). Thus, we tested for learned recognition of the predator 1 day postconditioning (when the CO<sub>2</sub> was still in effect) and also 5 days post CO<sub>2</sub> (when the effect of CO<sub>2</sub> 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<sub>2</sub>).

Fish that were exposed to control and elevated CO<sub>2</sub> 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<sup>-1</sup>) 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<sub>2</sub> 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<sub>2</sub>: 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<sub>2</sub> on predator learning, as evident by survival for fish released into the wild. Fish from the 987 µatm CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> treatment and conditioning to ensure that the CO<sub>2</sub> 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).



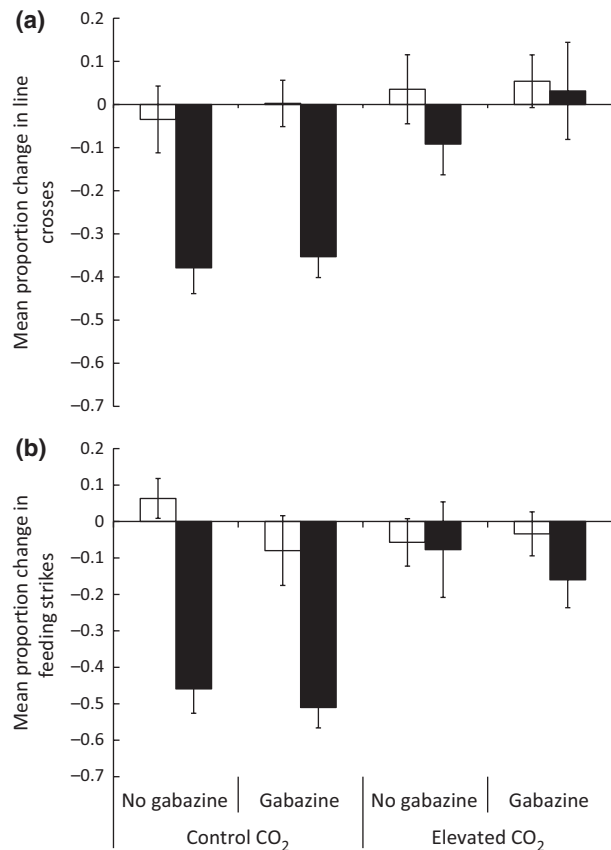
### Statistical analyses

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

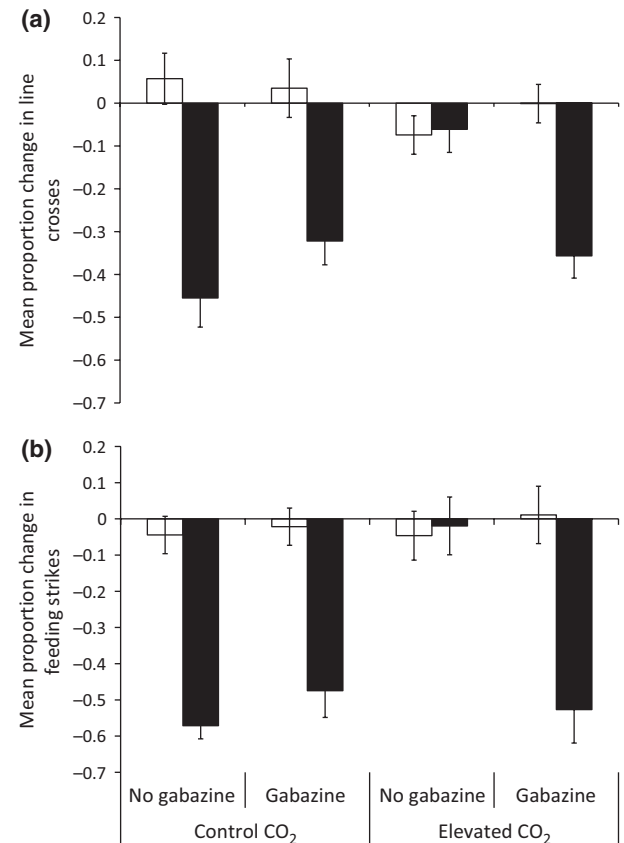
When the fish were tested 1 day postgabazine treatment, we found a CO<sub>2</sub> × Cue interaction on the response of the fish (Pillai's Trace,  $F_{2,82} = 5.6$ ,  $P = 0.004$ ; Fig. 1). For



**Fig. 1** Mean ( $\pm$ SE) proportion change in line crosses (a) and feeding strikes, (b) for fish exposed to control or elevated levels of CO<sub>2</sub> 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.

the control CO<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> treatment.

When the fish were tested 5 days postgabazine treatment, we found a 3-way interaction between CO<sub>2</sub>, 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,

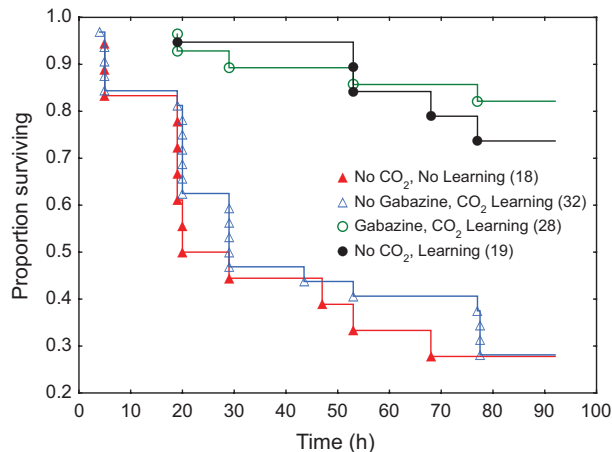


**Fig. 2** Mean ( $\pm$ SE) proportion change in line crosses (a) and feeding strikes, (b) for fish exposed to control or elevated levels of CO<sub>2</sub> and then held in jars containing a solution of gabazine or a seawater control prior to being taught to recognize a moonwrasse as a predator. White 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  $\times$  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  $\text{CO}_2$  group, however, exhibited a gabazine by cue interaction ( $F_{2,40} = 7.9$ ,  $P = 0.001$ ). Fish not exposed to gabazine did not respond 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  $\text{CO}_2$  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  $\text{CO}_2$  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



**Fig. 3** Survival of damselfish under elevated or current day levels of  $\text{CO}_2$ , exposed or not to gabazine when they were (or not) taught the identity of 3 common predators. Kaplan–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.

reduced the  $\text{CO}_2$  effect sufficiently to allow associative learning through chemical cues.

### Discussion

The results of our study provide compelling evidence that levels of  $\text{CO}_2$  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  $\text{CO}_2$  on predator learning. The effect of elevated  $\text{CO}_2$  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  $\text{CO}_2$  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  $\text{CO}_2$  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  $\text{CO}_2$  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  $\text{CO}_2$  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  $\text{CO}_2$  had worn off, fish returned to normal activity, and we could see that the gabazine treatment had indeed reversed the effects of  $\text{CO}_2$  during the learning session.

The fact that the specific GABA-A receptor antagonist gabazine has such potent effects on behavioural functions altered by elevated  $\text{CO}_2$  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  $\text{Cl}^-$  and  $\text{HCO}_3^-$  – precisely the

two ions most likely to be affected by elevated CO<sub>2</sub> 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<sup>-</sup> and/or HCO<sub>3</sub><sup>-</sup> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub> 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<sub>2</sub>, 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<sub>2</sub>. Therefore, any behavioural deficiencies of predators under elevated CO<sub>2</sub> 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<sub>2</sub> 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.

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