Effects of elevated CO_2 on predator avoidance behaviour by reef fishes is not altered by experimental test water

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ABSTRACT

Pioneering studies into the effects of elevated CO_2 on the behaviour of reef fishes often tested high-CO_2 reared fish using control water in the test arena. While subsequent studies using rearing treatment water (control or high CO_2) in the test arena have confirmed the effects of high CO_2 on a range of reef fish behaviours, a further investigation into the use of different test water in the experimental arena is warranted. Here, we used a fully factorial design to test the effect of rearing treatment water (control or high CO_2) and experimental test water (control or high CO_2) on antipredator responses of larval reef fishes. We tested antipredator behaviour in larval clownfish *Amphiprion percula* and ambon damselfish *Pomacentrus amboinensis*, two species that have been used in previous high CO2 experiments. Specifically, we tested if: (1) using control or high CO_2 water in a two channel flume influenced the response of larval clownfish to predator odour; and (2) using control or high CO_2 water in the test arena influenced the escape response of larval damselfish to a startle stimulus. Finally, (3) because the effects of high CO_2 on fish behaviour appear to be caused by altered function of the GABA-A neurotransmitter we tested if antipredator behaviours were restored in clownfish treated with a GABA antagonist (gabazine) in high CO_2 water. Larval clownfish reared from hatching in control water (496 μatm) strongly avoided predator cue whereas larval clownfish reared from hatching in high CO_2 (1,022 μatm) were attracted to the predator cue, as has been reported in previous studies. There was no effect on fish responses of using either control or high CO_2 water in the flume. Larval damselfish reared for four days in high CO_2 (1,051 μatm) exhibited a slower response to a startle stimulus and slower escape speed compared with fish reared in control conditions (464 μatm). There was no effect of test water on escape responses. Treatment of high-CO_2 reared clownfish with 4 mg l^{-1} gabazine in high CO_2 seawater restored the normal response to predator odour, as has been previously reported with fish tested in control water. Our results show that using control water in the experimental trials did not influence the results of previous studies on antipredator behaviour of reef fishes and also supports the results of novel experiments conducted in natural reef habitat at ambient CO_2 levels.

Subjects Animal Behavior, Aquaculture, Fisheries and Fish Science, Marine Biology
Keywords Ocean acidification, Carbon dioxide, Antipredator behaviour, Larval fish, GABA

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INTRODUCTION

Rising concentrations of atmospheric carbon dioxide (CO_2) have caused an increased uptake of CO_2 by the ocean, leading to a decline in seawater pH and changes in the relative concentration of carbonate and bicarbonate ions, a process called ocean acidification (*Sabine et al., 2004*; *Doney, 2010*). The partial pressure of carbon dioxide (pCO_2) at the ocean surface is increasing at the same rate at the atmosphere (*Doney, 2010*) and thus marine species will need to deal with rising CO_2 levels in addition to declining pH and other changes in ocean chemistry (*Portner, Langenbuch & Reipschlager, 2004*). Recent models suggest an amplification of seasonal cycles in ocean pCO_2 as atmospheric CO_2 continues to rise, such that surface ocean pCO_2 will reach 1,000 μatm in summer for atmospheric CO_2 concentrations that exceed 650 parts per million. Hypercapnic conditions (>1,000 μatm CO_2) are projected to occur in up to half the surface ocean by 2100 on the current CO_2 emissions trajectory (*McNeil & Sasse, 2016*).

Elevated CO_2 levels can have a variety of effects on the physiology, life history and behaviour of marine organisms (*Portner, Langenbuch & Reipschlager, 2004*). Recent studies have demonstrated that high CO_2 levels can fundamentally alter the behaviour of marine fishes and some invertebrates (reviewed by *Briffa et al., 2012*; *Clements & Hunt, 2015*; *Heuer & Grosell, 2014*; *Nagelkerken & Munday, 2016*). Of particular concern is an impaired response to the threat of predation, because it may increase mortality rates in natural populations (*Munday et al., 2010*; *Ferrari et al., 2011a*; *Chivers et al., 2014*). Larval and juvenile reef fish can exhibit an innate aversion to chemical cues released by predators and chemical alarm cues from injured conspecifics, because they indicate a heightened risk of predation (*Holmes & McCormick, 2010*; *Dixson, Pratchett & Munday, 2012*). Individuals typically respond to these chemical cues by reducing activity and seeking shelter (*Ferrari, Wisenden & Chivers, 2010*). However, larval reef fish that have been reared at near-future CO_2 levels do not reduce activity or stop feeding in the presence of chemical alarm cues (*Ferrari et al., 2011a*; *Lönnstedt et al., 2013*) and even become attracted to chemical cues from a predator (called predator odour from herein) and chemical alarm cues when presented in a two channel flume (*Dixson, Munday & Jones, 2010*; *Welch et al., 2014*). Furthermore, juvenile fish exposed to high CO_2 lose their ability for associative learning (*Ferrari et al., 2012*), a process that enables fine-tuning of risk assessment by the association of chemical alarms cues with the identity of specific predators. Finally, high CO_2 affects the kinematics of predator–prey interactions, with juvenile prey exposed to elevated CO_2 allowing predators to get closer before responding (*Allan et al., 2013*) and exhibiting reduced escape speeds and distances compared with fish reared at current-day CO_2 levels (*Allan et al., 2013*; *Allan et al., 2014*). These changes in behaviour alter the outcome of predator–prey interactions, leading to significantly increased rates of mortality of small juveniles in mesocosm experiments (*Ferrari et al., 2011b*) and in fish transplanted to natural coral reef habitat (*Munday et al., 2010*; *Munday et al., 2012*; *Chivers et al., 2014*).

Many of the pioneering studies on the effects of elevated CO_2 on reef fish behaviour (e.g., *Munday et al., 2009*; *Dixson, Munday & Jones, 2010*; *Ferrari et al., 2011a*; *Ferrari et al.,2011b*; *Cripps, Munday & McCormick, 2011*) involved rearing fishes in control and

elevated CO_2 conditions and then testing their responses to chemical cues in control water only. This method was chosen in these early experiments owing to logistical constraints and because pilot experiments showed that the response of high-CO_2 exposed fish to predator cue did not differ if the cue was presented in either control or high CO_2 water (*Dixson, Munday & Jones, 2010*; *Munday et al., 2010*). Furthermore, studies with freshwater fishes had shown that a pH reduction of 0.5 units in freshwater (<pH 6.5) irreversibly alters the structure of chemical alarm cues and can dramatically affect prey antipredator responses (*Brown et al., 2002*; *Leduc et al., 2004*; *Leduc, Kelly & Brown, 2004*; reviewed in *Leduc et al., 2013*). Consequently, there was concern that testing marine fish in CO_2-acidified water (albeit still above pH 7.5) could confound the interpretation of any effects of high CO_2 on the behavioural response of the fish with a diminished efficacy of the chemical cue itself. Testing in control water prevented this potential problem. Finally, small-scale laboratory experiments often have limited ecological relevance and it is challenging to extrapolate their findings to natural communities and ecologically relevant spatial scales (*Nagelkerken & Munday, 2016*). Consequently, it was critical to test the effects of exposure to high CO_2 in natural habitats in the field. This involved transplanting larval fish that had been reared in either control or high CO_2 seawater back into natural coral reef habitat and monitoring their behaviour (*Munday et al., 2010*; *Munday et al., 2012*; *McCormick, Watson & Munday, 2013*).

Since these initial studies, a number of other experiments have been conducted in which the respective treatment seawater in which the fish were reared (control or high CO_2) has been used in the experimental trials, confirming the results of earlier studies (e.g., *Allan et al., 2014*; *Dixson et al., 2015*; *Welch et al., 2014*; *Nagelkerken et al., 2016*). Nevertheless, a more thorough validation study of the test water used in previous antipredator experiments is warranted. Here, we compared the use of control versus high CO_2 seawater (called test water from hereafter) in experimental trials designed to test the antipredator responses of larval reef fishes reared in high CO_2. We conducted antipredator experiments for two species that have been widely used in previous high CO_2 experiments, the clownfish *Amphiprion percula* and the ambon damselfish *Pomacentrus amboinensis*. Specifically, we tested if: (1) using control or high CO_2 water in a two channel flume influenced the response of larval clownfish to predator odour in clownfish reared in either control or high CO_2; and (2) using control or high CO_2 water in the test arena influenced the kinematic response of larval damselfish to a startle stimulus in fish reared in either control or high CO_2. Finally, (3) because the effects of high CO_2 on fish behaviour appear to be due to altered function of GABA-A neurotransmitter receptors (*Nilsson et al., 2012*; *Hamilton, Holcombe & Tresguerres, 2014*; *Heuer & Grosell, 2014*) we tested if antipredator behaviours were restored in clownfish treated with a GABA antagonist (gabazine) in high CO_2 water. Previous studies have demonstrated that abnormal antipredator behaviour of clownfish and damselfish reared in high CO_2 conditions is reversed following treatment with gabazine (*Nilsson et al., 2012*; *Chivers et al., 2014*). However, these previous studies involved high CO_2 fish that were treated with gabazine in control water and then behaviourally tested in control water. In this experiment we therefore conducted the same gabazine treatments as

previously used, except the drug was administered and behaviour was tested in high CO_2 water.

MATERIALS AND METHODS

The experiment with larval clownfish was conducted at James Cook University's (JCU) experimental aquarium facility in Townsville, Australia, where previous high CO_2 experiments with this species have been conducted. The experiment with larval ambon damselfish was conducted at Lizard Island Research Station (LIRS) on the northern Great Barrier Reef, where previous high CO_2 experiments with this species have been conducted. Both experiments were conducted during February 2016. Because our goal was to assess the use of control seawater in experimental trials of previous experiments (e.g., *Dixson, Munday & Jones, 2010*; *Munday et al., 2010*; *Munday et al., 2012*) we used the same methods as previous experiments, except that we applied a fully factorial design where fish reared in control and high CO_2 conditions were behaviourally tested in both control and high CO_2 water. Control water was the ambient in the experimental facility at JCU (496 ± 55 S.D. μatm CO_2) and at LIRS where water is pumped directly from the Lizard Island lagoon (464 ± 32 μatm CO_2). The high CO_2 conditions (1,022 ± 37 and 1,051 ± 18 μatm CO_2, respectively) matched projections for CO_2 levels in open ocean by the end of this century (*McNeil & Sasse, 2016*).

Response to predator cue

Two clutches of larval clownfish from different parental pairs maintained in ambient seawater conditions at JCU were reared in control and high CO_2 conditions from hatching until settlement (12 days post hatching) using standard practices (*Wittenrich, 2007*). Briefly, larvae were reared in 100 l incubator tanks supplied with a continuous flow of treatment seawater. Water temperature was maintained at 29 °C. Photoperiod was 12 hours light and 12 hours dark. A non-viable microalgae blend (Nano 3600–Reed Mariculture^{TM}) was drip-fed into the incubator tanks during daylight hours to reduce light levels and enhance larval feeding during the first four days. Larvae were fed live rotifers (15 per ml) for the first four days and then transitioned onto *Artemia* (5 per ml). Fish were not fed on the morning of testing. Each clutch was divided at hatching so that half the clutch was reared in control seawater (496 ± 55 S.D. μatm CO_2) and half reared in the high CO_2 treatment (1,022 ± 37 S.D. μatm CO_2) for 12–15 days. Two 10,000 L recirculating aquarium systems supplied seawater to the incubator tanks, one supplied control seawater and the other high CO_2 seawater. To achieve the desired CO_2 level in the high CO_2 treatment, seawater was dosed with CO_2 to a set pH in a 3,000 L sump using an Aqua Medic AT Control System (Aqua Medic, Bissendorf, Germany). The pCO_2 in rearing tanks was measured by nondispersive infrared (NDIR) following the method described by *Hari et al. (2008)*. Air in a closed loop was circulated by a small pump (1 l min^{-1} flow rate) through a coil of thin-walled (membrane thickness 0.4 mm, outer ø 3.8 mm) medical grade silicone tubing placed in the tank. CO_2 in the closed loop was then measured at 1 min intervals with a Vaisala GMP343 infrared CO_2 probe (accuracy ± 5 ppm CO_2 + 2% of reading over the range of experimental manipulations). Each reading ($N = 9$ in control and $N = 9$ in

high CO_2) lasted 1 hour to ensure complete equilibration of CO_2 between tank seawater and the closed loop of air. The average CO_2 for the final ten minutes was calculated for each reading. Due to the short duration of the experiment, and because we were primarily concerned about quantifying pCO_2, we did not perform alkalinity titrations to paramterize other components of the carbonate system.

The response of settlement stage clownfish (12–15 days old) to predator cue was tested in a two-channel flume (13 cm × 4 cm internal dimensions to the taper) specifically designed for testing the preferences of larval fish to different chemical cues (*Gerlach et al., 2007*). Larvae were given the choice between a stream of seawater containing predator cue versus a stream of seawater without the predator cue. Both streams of water were either control or high CO_2 test water for a given trial. Water from the respective paired sources was gravity fed into the flume at 100 ml min^{-1}, maintained by a flow meter. Water was exchanged after every fish, with random alternation between the use of control and high CO_2 test water in the flume. A dye test was conducted with every water exchange to ensure laminar flow and no mixing of the water streams. For each trial, a single fish was placed in the centre of the downstream end of the flume and allotted a two-minute acclimation period. The position of the fish at 5-second intervals was recorded for the next two minutes. A one-minute rest period followed, during which the water sources were switched to eliminate the effect of any potential side preference by the fish. After the water switch, the fish was re-centred in the downstream end of the flume and the acclimation (2 min) and test period (2 min) were repeated with the predator water on the opposite side of the flume. The time spent in the water stream containing the predator cue was summed for the two 2-minute test periods for each fish. The temperature of the test water was kept within 1 °C of the water temperature in the rearing tanks. Three trials where the difference in water temperature between the flume and the rearing tanks exceeded 1 °C were discarded to avoid any effect of temperature shock on behaviour. The experimenter was blinded to the rearing treatment of the fish, but it was not logistically possible for the experimenter to also be blinded to the water treatments in the flume.

Predator cues were obtained from the common coral-cod, *Cephalopholis miniatus*. Five cod collected from the Great Barrier Reef (supplied by Cairns Marine, Cairns QLD) were maintained in 60 L aquaria supplied with a continuous flow of seawater from a different seawater system to that used to rear the clownfish. Each cod was fed one cube of commercial fish food (Marine Food, Fish Fuel Co^{TM}) each evening. Water temperature was maintained at 29 °C. To obtain predator cues for the experiment, the water was drained from two predator tanks and refilled with water from the same system used to rear the clownfish. To match the methods used in previous experiments, water to the predator tank was turned off and predator cue water was collected after a 2 hour soak time. Predator tanks were aerated to maintain oxygen content and water-bathed to maintain a stable temperature. One of the two predator tanks was dosed with CO_2 at the end of the 2 hour soak period, immediately before cue water was extracted. CO_2 was dosed at low pressure by hand, with gentle stirring, until the pH matched that of the high CO_2 rearing treatment. The other predator tank was also gently stirred but was not dosed with CO_2. The individual cod used for predator cues was alternated within and between days. An identical procedure was

followed with two tanks that did not house a predator to obtain seawater without predator cue to use in the flume.

Gabazine treatment

The gabazine treatment followed the flume methodology described above, except that fish were treated with 4 mg l^{-1} gabazine for 30 min immediately before testing in the flume. Eight larval clownfish were randomly selected from the high CO_2 treatments and placed in 40 ml of high CO_2 water containing gabazine. The fish were gently removed from the gabazine after 30 min and transferred to the flume using a small net. Their response to predator odour was tested in high CO_2 water only. Previous experiments demonstrating that gabazine restores antipredator responses in larval fishes reared in high CO_2 water have been conducted in control water (*Nilsson et al., 2012*; *Chivers et al., 2014*). This experiment only tested if a similar effect was observed when gabazine was administered and fish tested in high CO_2 water.

Kinematics of escape response

Settlement stage larval *Pomacentrus amboinensis* were collected using light traps in the Lizard Island lagoon and transferred to an environmentally-controlled aquarium facility at LIRS. Settlement stage *P. amboinensis* at Lizard Island have an average age of 15–23 days old (*Kerrigan, 1996*). Larval fish were assigned randomly to four replicate control (464 ± 32 S.D. μatm CO_2) or four replicate high-CO_2 (1,051 ± 18 S.D. μatm CO_2) aquaria. Twenty five *P. amboinensis* were housed in each 32 l (38L × 28W × 30H cm) aquarium. Water temperature was maintained at approximately 29.5 °C. Fish were kept for 4 days in treatment conditions and fed *Artemia* sp. twice daily *ad libitum*. Fish were not fed on the morning of testing. Each aquarium was supplied with control or elevated-CO_2 seawater at 750 ml min^{-1}. Elevated-CO_2 seawater was achieved by dosing with CO_2 to a set pH, following standard techniques (*Gattuso et al., 2010*). Seawater was pumped from the ocean into 32 l header tanks where it was diffused with ambient air (control) or 100% 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. Duplicate control and high CO_2 systems supplied seawater to two replicate aquaria in each system. The pCO_2 in rearing tanks was measured by NDIR as described above ($N = 9$ in control and $N = 5$ in high CO_2).

After rearing for four days in control or high CO_2 conditions, the escape response of the damselfish was tested in both control and high CO_2 water (i.e., a fully crossed design). Individual fish were placed into the testing arena (Fig. S1), which consisted of a transparent circular acrylic arena (ø 200 mm) within a larger plastic tank (585 × 420 × 330 mm; 60 L) filled to a depth of 100 mm with either control or high CO_2 water. A shallow water depth was used to limit vertical displacement of the fish during escape response trials. Water temperature in the experimental arena was 29 °C–30 °C and the water treatment used was alternated after every 2nd trial. The arena was illuminated by LED strip lighting (750 lumens) placed above the water surface on the outside of the tank. Five minutes after being released into the testing arena, an escape response was elicited by the release of a

tapered metal weight from above the water surface. This was accomplished by turning off an electromagnet to which the metal weight was attached. A length of fishing line was attached to the weight so that it stopped when the tapered tip of the weight only just touched the surface of the water after release from the magnet. In order to provide a sudden stimulation and allow quantification of escape latency, the stimulus was released through a white PVC tube (ø 40 mm, length 550 mm) suspended above the experimental arena, with the bottom edge of the pipe at a distance of 10 mm above the water level. The tube hides the weight from view so there is no visual stimulation before the mechanical stimulation occurs. Fish were startled when they moved to the middle portion of the tank. Escape responses were recorded at 480 frames per second (Casio EX-ZR1000) as a silhouette from below obtained through pointing the camera at a mirror angled at 45°. To minimise visual disturbances, black sheeting surrounded the front of the mirror so that any movement within the room was undetected by the fish. Treatment water in the testing arena was changed every 2nd trial to minimise CO_2 loss in the high CO_2 treatment water. The rate of CO_2 loss from the testing arena was found to be negligible within this time frame. The escape response trials were conducted over two consecutive days. The experimenter was blinded to the rearing treatment of the fish when conducting the trials.

From the videos, we quantified latency to initiate an escape, escape distance, escape speed and maximum speed. A 1 cm line was drawn in the centre of the inner arena to enable distance calibration for video analysis. The moment the stimulus weight hit the water was benchmarked by the first detectable water disturbance in the video. The distance of each fish from the point the stimulus first hit the water was also quantified from the video. All videos were analysed with the observer blinded to the treatments.

Kinematic variables

Kinematic variables associated with the fast-start response were analysed using the image-analysis software Image-J, with a manual tracking plug-in. The centre of mass (CoM) of each fish was tracked for the duration of the response. The following kinematic variables were measured:

(1) Latency to respond (s) was measured as the time interval between the stimulus onset and the first detectable movement leading to the escape of the animal.

(2) Escape distance (m) is a measure of the total distance covered by the fish during the first two flips of the tail (the first two axial bends, i.e., stages 1 and 2 defined based on *Domenici & Blake (1997)*, which is the period considered crucial for avoiding ambush predator attacks (*Webb, 1976*).

(3) Escape speed (m s^{-1}) was measured as the distance covered within a fixed time (first 24 ms after initial response) which corresponds to the average duration of the first two tail flips (the first two axial bends, i.e., stages 1 and 2 based on (*Domenici & Blake, 1997*). This period is considered crucial for avoiding predator ambush attacks (*Webb, 1976*).

(4) Maximum speed (m s^{-1}) was measured as the maximum speed achieved at any time during stage 1 and stage 2.

DATA ANALYSIS

Two-way ANOVA was used to compare percent time that larval clownfish spent in predator cue in the flume. Percentage data was logit-transformed for analysis (*Warton & Hui, 2011*). Treatment water (control or high CO_2) and test water (control or high CO_2) were the fixed factors. ANOVA was conducted with Statistica (version 13). For the gabazine experiment, the percentage time that larval clownfish treated with gabazine spent in predator cue in the flume was compared with control fish using a *t*-test on logit-transformed data. Linear mixed effects models (LME) were used to compare escape responses of larval damselfish. Latency, escape distance, escape speed and maximum speed were each tested separately. Treatment water (control or high CO_2), test water (control or high CO_2) and day (1 or 2) were fixed factors in the model. The distance of each fish from the point the stimulus hit the water was also included as a fixed effect in the model to account for any variation associated with differences in the location of fish in the chamber when the stimulus occurred. Tank was included in each model as a random effect to account for the subsampling of fish from replicate tanks within each CO_2 treatment. Best-fit model selection based on AIC values was automated using 'MuMIn' (*Bartoñ, 2015*) within the 'nlme' package in the R software package (*Pinheiro et al., 2013*). The normality, linearity and homoscedasticity of residuals of the models were verified by visual inspection of residual-fit plots. Latency data was log10 transformed before analysis to improve the distribution of the data and a heterogeneous variance structure was used in the model for latency (but not other variables). R code is available in Supplementary Information 1.

This research was conducted in accordance with James Cook University animal ethics guidelines and permits A1961, A1973 and A2080. Fish were collected under permit G12/35117.1 from the Great Barrier Reef Marine Park Authority.

RESULTS

There was a highly significant effect of treatment water on the percentage time that larval clownfish spent in the water stream containing predator odour ($F_{1,57} = 125.91, p < 0.001$), but no effect of test water ($F_{1,57} = 0.20, p = 0.66$) and no interaction ($F_{1,57} = 0.06, p = 0.81$). Laval clownfish reared in control water strongly avoided predator odour, spending <9% of the time in that water stream (Fig. 1). In contrast, larval clownfish reared in high CO_2 water were strongly attracted to predator odour, spending nearly 80% of the time in that water stream (Fig. 1).

As with the response to predator odour, treatment water had a significant effect on escape responses of larval damselfish, and there was no effect of test water. Distance to stimulus did not affect any of the response variables and was not retained in any of the final models. Latency to respond significantly increased in fish reared in elevated CO_2 ($F_{1,6} = 7.76, p = 0.032$), with fish displaying latencies that were 30% longer compared with fish reared in control water (Fig.2A). There was no effect of test water or day of testing on latency to respond and these factors were not retained in the final model. Escape distance following stimulation was not significantly different for fish reared in high CO_2 compared with control water ($F_{1,5} = 6.15, p = 0.056$) although there was a clear tendency for a shorter

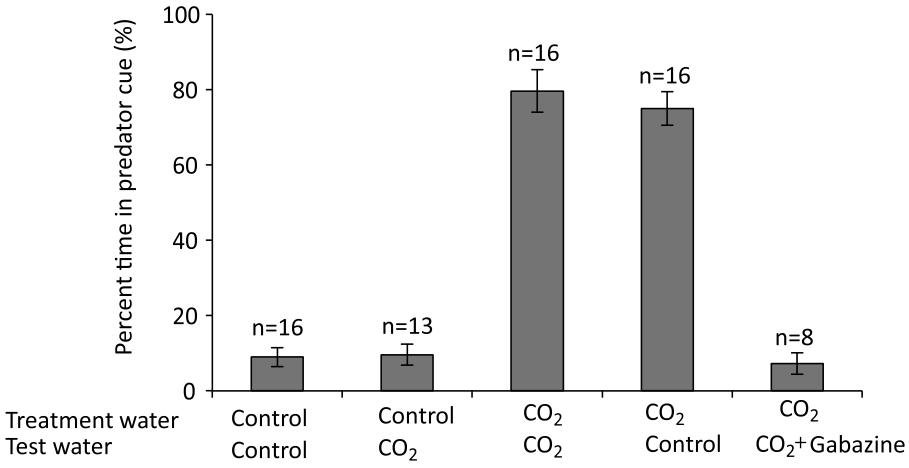

Figure 1 The effect of rearing treatment water (control or high CO_2) and test water used in a two-channel flume (control or high CO_2) on the olfactory response of larval *A. percula* to predator cue. One stream of water in the flume contained seawater with predator cue and the other stream of water had untreated seawater. Shown is the percent time (mean ± s.e.) that fish from each experimental combination spent in the water stream containing predator cue. The final group shows fish that were treated with 4 mg l^{-1} gabazine in high CO_2 seawater for 30 minutes before testing in the flume. Sample size shown above the bars.

escape distance in high CO_2 fish (Fig. 2B). Day was retained in the final model, but it was not statistically significant ($F_{1,5} = 5.15, p = 0.07$). There was no effect of test water on escape distance and it was not retained in the final model. Escape speed and maximum speed responded similarly with both kinematic variables declining significantly in fish reared in high CO_2 compared to fish reared in control water (Fig. 2C; $F_{1,5} = 6.81, p = 0.048$ and Fig. 2D; $F_{1,6} = 9.016, p = 0.0239$). Day was retained in the final model for escape speed, but it was not statistically significant ($F_{1,5} = 3.64, p = 0.115$). Day was not retained in the final model for maximum speed. Again, there was no effect of test water for either escape speed of maximum speed and it was not retained in the final model.

Gabazine reversed the effects of rearing in high CO_2 (Fig. 1). Fish reared in high CO_2 and then treated with gabazine exhibited a strong avoidance of predator cue, with the response almost identical to fish reared and tested in control water ($t_{22} = 0.04, p = 0.96$).

DISCUSSION

Previous studies have demonstrated that CO_2 levels projected to occur in the surface ocean by the end of this century (700–1,000 μatm) impair predator avoidance behaviours in larval reef fishes (reviewed in *Nagelkerken & Munday, 2016*). However, some of these previous studies have tested the behaviour of high-CO_2 reared fish using control water in the experimental trials, which could potentially influence the results if there is an immediate physiological response associated with the transfer of fish from high CO_2 rearing water to control test water. Here we show that the results of these earlier studies are robust and that antipredator behaviour is not affected by using control water in the experimental trials. Larval fish reared in high CO_2 exhibited the same responses to predator odour in a two channel flume and the same kinematic responses to a startle stimulus regardless of

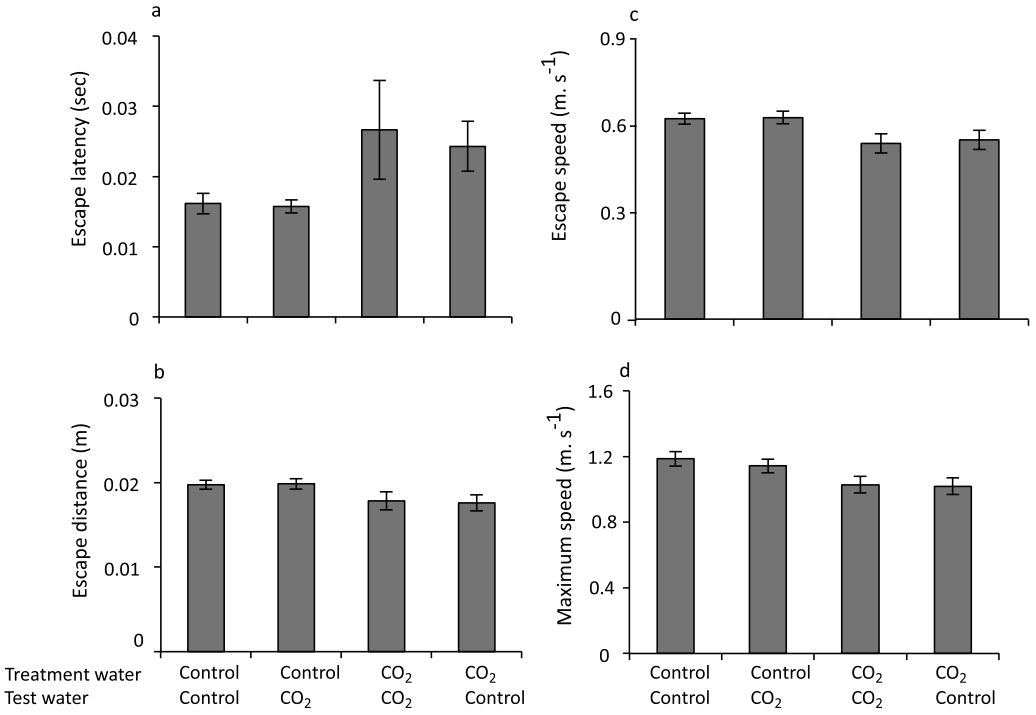

Figure 2 The effect of rearing treatment (control or high CO_2) and test water used in the experimental trial (control or high CO_2) on the escape performance of larval *P. amboinensis*. Variables displayed are: (A) latency, (B) distance, (C) mean speed, and (D) maximum speed. Errors are standard errors. $N = 26, 28, 26, 28$ for control - control, control - high CO_2, high CO_2 - high CO_2 and high CO_2 - control, respectively.

whether high CO_2 or control water was used in the experimental trial. Similarly, there was no difference in the behavioural response of fish reared in control seawater when tested in either high CO_2 or control water. These results demonstrate that the choice of test water has not influenced the results of previous laboratory studies into the effects of high CO_2 on antipredator behaviour.

Our results are consistent with preliminary observations carried out in previous studies showing that the test water (control or high CO_2) used in a two-channel flume did not alter the behavioural response of larval fishes to predator odour (*Dixson, Munday & Jones, 2010*; *Munday et al., 2010*). Furthermore, our study extends these findings to the kinematics of predator avoidance, by showing that the type of water used in the test arena does not affect vital components of the escape response, including latency to respond, escape speed and maximum speed. Together these results show that antipredator behaviours of larval fishes that have been reared in high CO_2 are not compromised by a return to control seawater for experimental testing. These findings are also relevant to field-based studies that have tested the effects of high CO_2 on reef fishes in their natural habitat (e.g., *Munday et al., 2010*; *Munday et al., 2012*; *Devine, Munday & Jones, 2012*; *Devine & Munday, 2013*; *Ferrari et al., 2011a*; *McCormick, Watson & Munday, 2013*). Such studies are critical for predicting the impacts of high CO_2 on reef fish populations; however, they involve rearing larval fish for 4–5 days in high CO_2 conditions before transferring them to ambient CO_2 conditions

in the field. These studies have demonstrated mortality rates from predation are higher in juvenile fish that have been exposed to high CO_2 compared with fish exposed to ambient control conditions (*Munday et al., 2010*; *Munday et al., 2012*; *Ferrari et al., 2011a*). Our results indicate that such studies are unlikely to have been affected by any immediate effect on antipredator behaviour caused by transplanting fish from high CO_2 conditions in the laboratory to ambient CO_2 conditions in their natural habitat. Nevertheless, fish in the field studies also remained in control conditions for a much longer period of time (several days) compared with the laboratory tests conducted here (minutes) and further experiments of longer duration would be required to ascertain any differences in control and high CO_2 conditions that might accrue over a longer period of time.

In contrast to the absence of a test water effect on antipredator behaviours, we found that rearing treatment water had a highly significant effect on antipredator behaviour. Consistent with previous studies (*Dixson, Munday & Jones, 2010*; *Munday et al., 2010*), larval clownfish reared in high CO_2 exhibited a reversal from strongly avoiding predator cue (<10% of time in predator cue in the flume) to a strong attraction to predator cue (nearly 80% of time in predator cue in the flume). Similarly, larval damselfish exposed to high CO_2 for 4 days exhibited a slower response to a threat stimulus and a slower escape speed compared with fish kept in ambient control water. There was also a tendency for a shorter distance travelled. These findings are consistent with changes in escape responses that have has been reported previously (*Allan et al., 2013*; *Allan et al., 2014*) and provide further confirmatory evidence for the effects of high CO_2 on antipredator behaviour in larval reef fishes. The differences in escape speed between high CO_2 and control fish were relatively small; however, when combined with a much longer latency to respond (>30% increase in time to respond) these changes in kinematic responses would likely have a detrimental effect on the probability of a prey fish escaping a predator attack.

Altered behaviour of fish in a high CO_2 environment appears to be due to the effects of acid-base regulation on the function of GABA-A receptors (*Nilsson et al., 2012*; *Hamilton, Holcombe & Tresguerres, 2014*; *Heuer & Grosell, 2014*). Fish prevent plasma and tissue acidosis in a high CO_2 environment by accumulating HCO_3^- in exchange for Cl^- (*Brauner & Baker, 2009*). The GABA-A receptor is a gated ion channel that conducts HCO_3^- and Cl^- when activated by the neurotransmitter GABA. The altered concentration of HCO_3^- and Cl^- in fish exposed to high CO_2 could decrease or reverse the flow of these ions through the GABA-A receptor, causing a reversal in receptor polarization (*Heuer & Grosell, 2014*). Evidence for the role of GABA-A receptors in behavioural impairment of fish in high CO_2 has come from studies showing that treatment with gabazine, a GABA antagonist, reverses the effects of high CO_2 on behaviour (*Nilsson et al., 2012*; *Chivers et al., 2014*; *Chung et al., 2014*; *Lai, Jutfelt & Nilsson, 2015*; *Ou et al., 2015*; *Regan et al., 2016*). In the first of these studies, *Nilsson et al. (2012)* found that gabazine reversed the impaired response to predator odour exhibited by clownfish that had been reared in high CO_2. However, in that study gabazine was administered in control seawater and high-CO_2 reared fish were tested in control water. Therefore, the effects of gabazine in restoring the response of larval fishes to predator odour has not been previously tested in high-CO_2 conditions. Here we show that treatment with 4 mg l^{-1} gabazine completely reverses the abnormal behavioural of

high-CO_2 fish to predator odour, restoring their natural aversion to predator odour. This contributes to a large number of independent studies pointing to a key role of GABA-A receptors in the behavioural impairment of fish exposed to high CO_2.

While we found no effects of using control or high CO_2 water in experimental trials to test the effects of high CO_2 on fish behaviour, *Sundin & Jutfelt (2016)* reported differences in relative lateralization of juvenile goldsinny wrasse when tested in control water versus high CO_2 water after 21 days in high CO_2. Their study did not include the reverse treatment, where control reared fish were tested in high CO_2 water, and there is the potential for learning effects in control fish because the same fish were tested on three different occasions, at days 9, 19 and 21. Nevertheless, that study does suggest that the response of some behavioural traits could be sensitive to differences in the test water used in behavioural trials. Further experiments with a broader range of behavioural traits and using experiments that are not complicated by the repeated testing of the same individuals are required to test this possibility. Furthermore, there are likely to be substantial species-specific differences in the response of fish to control or high CO_2 water. For example, *Jutfelt & Hedgärde (2013)* found that juvenile Atlantic cod were able to distinguish between low and high CO_2 water and exhibited a strong preference for low CO_2 water. Here, we only tested two species of damselfish, but other species might exhibit different responses. Indeed, there is considerable interspecific variation in the sensitivity of damselfishes to high CO_2 (*Ferrari et al., 2011a*), therefore, it is possible that there may also be species-specific differences in their antipredator responses depending on the test water used in the experimental arena.

Pioneering studies into the effects of high CO_2 on fish behaviour used control seawater in experimental trials due to logistical constraints and concerns about the possible effects of low pH on olfactory cues. Our results confirm the results of those initial studies by showing that test water (control of high CO_2) had no effect on the antipredator responses exhibited by fish reared in either control or high CO_2 conditions. Nevertheless, we do not advocate the continued use of control seawater for testing the behavioural effects of high CO_2 in marine organisms. Methods for treating seawater to replicate future pCO_2 levels are now sufficiently validated that it is possible to treat most experimental arenas with the respective treatment water that the animals have been reared in, and there may be some behavioural traits that are sensitive to different test water in the experimental trials. The most reliable and unambiguous results will likely come from using the respective treatment water in experimental trials testing the effects of high CO_2 on the behaviour of marine organisms. Future studies also need to consider the potential for adaptation of behavioural responses to projected future CO_2 levels (*Sunday et al., 2014*). Individual variation in behavioural tolerance to higher CO_2 levels (*Munday et al., 2012*) could provide the opportunity for selection of more tolerant genotypes and improved performance in a future high CO_2 environment.

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Competing Interests

The authors declare there are no competing interestes

Author Contributions

- Philip L. Munday conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, wrote the paper, prepared figures and/or tables, reviewed drafts of the paper.
- Megan J. Welch conceived and designed the experiments, performed the experiments, reviewed drafts of the paper.
- Bridie J.M. Allan conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, reviewed drafts of the paper.
- Sue-Ann Watson conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, reviewed drafts of the paper.
- Shannon J. McMahon performed the experiments, reviewed drafts of the paper.
- Mark I. McCormick conceived and designed the experiments, contributed reagents/materials/analysis tools, reviewed drafts of the paper.

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The following information was supplied relating to ethical approvals (i.e., approving body and any reference numbers):

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REFERENCES

Allan BJM, Domenici P, McCormick MI, Watson SA, Munday PL. 2013. Elevated CO_2 affects predator–prey interactions through altered performance. *PLoS ONE* **8**:e58520 DOI 10.1371/journal.pone.0058520.

Allan BJM, Miller GM, McCormick MI, Domenici P, Munday PL. 2014. Parental effects improve escape performance of juvenile reef fish in a high-CO_2 world. *Proceedings of The Royal Society B: Biological Sciences* **281**:2013–2179 DOI 10.1098/rspb.2013.2179.

Bartoñ K. 2015. MuMIn: Multi-Model Inference. R package version 1.15.6. *Available at http://CRAN>R-project.org/package=MuMIn*.

Brauner CJ, Baker DW. 2009. Patterns of acid–base regulation during exposure to hypercarbia in fishes. In: Glass ML, Wood SC, eds. *Cardio-respiratory control in vertebrates*. Berlin, Heidelberg: Springer, 43–63.

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 DOI 10.1016/j.marpolbul.2012.05.032.

Brown GE, Adrian JC, Lewis MG, Tower JM. 2002. The effects of reduced pH on chemical alarm signalling in ostariophysan fishes. *Canadian Journal of Fisheries and Aquatic Sciences* **59**:1331–1338 DOI 10.1139/f02-104.

Chivers DP, McCormick MI, Nilsson GE, Munday PL, Watson SA, Meekan MG, Mitchell MD, Corkill KC, Ferrari MCO. 2014. Impaired learning of predators and lower prey survival under elevated CO_2: a consequence of neurotransmitter interference. *Global Change Biology* **20**:515–522 DOI 10.1111/gcb.12291.

Chung WS, Marshall NJ, Watson SA, Munday PL, Nilsson GE. 2014. Ocean acidification slows retinal function in a damselfish through interference with $GABA_A$ receptors. *Journal of Experimental Biology* **217**:323–326 DOI 10.1242/jeb.092478.

Clements JC, Hunt HL. 2015. Marine animal behaviour in a high CO_2 ocean. *Marine Ecology Progress Series* **536**:259–279 DOI 10.3354/meps11426.

Cripps IL, Munday PL, McCormick MI. 2011. Ocean acidification affects prey detection by a predatory reef fish. *PLoS ONE* **6**:e22736 DOI 10.1371/journal.pone.0022736.

Devine BM, Munday PL. 2013. Habitat preferences of coral-associated fishes are altered by short-term exposure to elevated CO_2. *Marine Biology* **160**:1955–1962 DOI 10.1007/s00227-012-2051-1.

Devine BM, Munday PL, Jones GP. 2012. Homing ability of adult cardinalfish is affected by elevated carbon dioxide. *Oecologia* **168**:269–276 DOI 10.1007/s00442-011-2081-2.

Dixson DL, Jennings AR, Atema J, Munday PL. 2015. Odor tracking in sharks is reduced under future ocean acidification conditions. *Global Change Biology* **21**:1454–1462 DOI 10.1111/gcb.12678.

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 DOI 10.1111/j.1461-0248.2009.01400.x.

Dixson DL, Pratchett MS, Munday PL. 2012. Reef fishes innately distinguish predators based on olfactory cues associated with recent prey items rather than individual species. *Animal Behaviour* **84**:45–51 DOI 10.1016/j.anbehav.2012.04.001.

Domenici P, Blake RW. 1997. Fish fast-start kinematics and performance. *Journal of Experimental Biology* **200**:1165–1178.

Doney SC. 2010. The growing human footprint on coastal and open-ocean biogeochemistry. *Science* **328**:1512–1516 DOI 10.1126/science.1185198.

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 DOI 10.1111/j.1365-2486.2011.02439.x.

Ferrari MCO, Manassa RP, Dixson DL, Munday PL, McCormick MI, Meekan M, Sih A, Chivers D. 2012. Effects of ocean acidification on learning in coral reef fishes. *PLoS ONE* **7**:e31478 DOI 10.1371/journal.pone.0031478.

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 DOI 10.1111/j.1461-0248.2011.01683.x.

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 DOI 10.1139/Z10-029.

Gattuso JP, Gao K, Lee K, Rost B, Schulz KG. 2010. Approaches and tools to manipulate the carbonate chemistry. In: Riebesell U, Fabry VJ, Hansson L, Gattuso JP, eds. *Guide to best practices for ocean acidification research and data reporting*. Luxembourg: Publications Office of the European Union, pp. 41–52.

Gerlach G, Atema J, Kingsford MJ, Black KP, Miller-Sims V. 2007. Smelling home can prevent dispersal of reef fish larvae. *Proceedings of The National Academy of Sciences of The United States of America* **104**:858–863 DOI 10.1073/pnas.0606777104.

Hamilton TJ, Holcombe A, Tresguerres M. 2014. CO_2-induced ocean acidification increases anxiety in Rockfish via alteration of $GABA_A$ receptor functioning. *Proceedings of The Royal Society B: Biological Sciences* **281**:20132509 DOI 10.1098/rspb.2013.2509.

Hari P, Pumpanen J, Huotari J, Kolari P, Grace J, Vesala T, Ojala A. 2008. High-frequency measurements of productivity of planktonic algae using rugged nondispersive infrared carbon dioxide probes. *Limnology and Oceanography-Methods* **6**:347–354 DOI 10.4319/lom.2008.6.347.

Heuer RM, Grosell M. 2014. Physiological impacts of elevated carbon dioxide and ocean acidification on fish. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology* **307**:R1061–R1084 DOI 10.1152/ajpregu.00064.2014.

Holmes TH, McCormick MI. 2010. Smell, learn and live: the role of chemical alarm cues in predator learning during early life history in a marine fish. *Behavioural Processes* **83**:299–305 DOI 10.1016/j.beproc.2010.01.013.

Jutfelt F, Hedgärde M. 2013. Atlantic cod actively avoid CO_2 and predator odour, even after long-term CO2 exposure. *Frontiers in Zoology* **10**:81 DOI 10.1186/1742-9994-10-81.

Kerrigan BA. 1996. Temporal patterns in the size and condition of settlement in two tropical reef fishes (Pomacentridae: *Pomacentrus amboinensis* and *P. nagasakiensis*). *Marine Ecology Progress Series* **135**:27–41 DOI 10.3354/meps135027.

Lai F, Jutfelt F, Nilsson GE. 2015. Altered neurotransmitter function in CO_2-exposed stickleback (*Gasterosteus aculeatus*): a temperate model species for ocean acidification research. *Conservation Physiology* **3**:cov018 DOI 10.1093/conphys/cov018.

Leduc A, Ferrari MCO, Kelly JM, Brown GE. 2004. Learning to recognize novel predators under weakly acidic conditions: the effects of reduced pH on acquired predator recognition by juvenile rainbow trout. *Chemoecology* **14**:107–112 DOI 10.1007/s00049-003-0268-7.

Leduc A, Kelly JM, Brown GE. 2004. Detection of conspecific alarm cues by juvenile salmonids under neutral and weakly acidic conditions: laboratory and field tests. *Oecologia* **139**:318–324 DOI 10.1007/s00442-004-1492-8.

Leduc A, Munday PL, Brown GE, Ferrari MCO. 2013. Effects of acidification on olfactory-mediated behaviour in freshwater and marine ecosystems: a synthesis. *Philosophical Transactions of The Royal Society B* **368**:20120447 DOI 10.1098/rstb.2012.0447.

Lönnstedt OM, Munday PL, McCormick MI, Ferrari MCO, Chivers DP. 2013. Ocean acidification and responses to predators: can sensory redundancy reduce the apparent impacts of elevated CO_2 on fish? *Ecology and Evolution* **3**:3565–3575 DOI 10.1002/ece3.684.

McCormick MI, Watson SA, Munday PL. 2013. Ocean acidification reverses competition for space as habitats degrade. *Scientific Reports* **3**:03280 DOI 10.1038/srep03280.

McNeil BI, Sasse TP. 2016. Future ocean hypercapnia driven by anthropogenic amplification of the natural CO_2 cycle. *Nature* **529**:383–386 DOI 10.1038/nature16156.

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 DOI 10.1073/pnas.0809996106.

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 DOI 10.1073/pnas.1004519107.

Munday PL, McCormick MI, Meekan M, Dixson DL, Watson S-A, Ferrari MCO, Chivers D. 2012. Selective mortality associated with variation in CO_2 tolerance in a marine fish. *Ocean Acidification* **1**:1–5 DOI 10.2478/oac-2012-0001.

Nagelkerken I, Munday PL. 2016. Animal behaviour shapes the ecological effects of ocean acidification and warming: moving from individual to community-level responses. *Global Change Biology* **22**:974–989 DOI 10.1111/gcb.13167.

Nagelkerken I, Russell BD, Gillanders BM, Connell SD. 2016. Ocean acidification alters fish populations indirectly through habitat modification. *Nature Climate Change* **6**:89–93 DOI 10.1038/nclimate2757.

Nilsson GE, Dixson DL, Domenici P, McCormick MI, Sorensen C, Watson SA, Munday PL. 2012. Near-future carbon dioxide levels alter fish behaviour by interfering with neurotransmitter function. *Nature Climate Change* **2**:201–204 DOI 10.1038/nclimate1352.

Ou M, Hamilton TJ, Eom J, Lyall EM, Gallup J, Jiang A, Lee J, Close DA, Yun S-S, Brauner CJ. 2015. Responses of pink salmon to CO_2-induced aquatic acidification. *Nature Climate Change* **5**:950–955 DOI 10.1038/nclimate2694.

Pinheiro J, Bates D, Debroy S, Sarkar D. 2013. nlme: Linear and Nonlinear Mixed Effects Models. R Package Version 3.1-113.

Portner HO, Langenbuch M, Reipschlager A. 2004. Biological impact of elevated ocean CO_2 concentrations: lessons from animal physiology and earth history. *Journal of Oceanography* **60**:705–718 DOI 10.1007/s10872-004-5763-0.

Regan MD, Turko AJ, Heras J, Andersen MK, Lefevre S, Wang T, Bayley M, Brauner CJ, Huong DTT, Phuong NT, Nilsson GE. 2016. Ambient CO_2, fish behaviour and altered GABAergic neurotransmission: exploring the mechanism of CO_2-altered behaviour by taking a hypercapnia dweller down to low CO_2 levels. *Journal of Experimental Biology* **219**:109–118 DOI 10.1242/jeb.131375.

Sabine CL, Feely RA, Gruber N, Key RM, Lee K, Bullister JL, Wanninkhof R, Wong CS, Wallace DWR, Tilbrook B, Millero FJ, Peng TH, Kozyr A, Ono T, Rios AF. 2004. The oceanic sink for anthropogenic CO_2. *Science* **305**:367–371 DOI 10.1126/science.1097403.

Sunday JM, Calosi P, Dupont S, Munday PL, Stillman JH, Reusch TBH. 2014. Evolution in an acidifying ocean. *Trends in Ecology and Evolution* **29**:117–125 DOI 10.1016/j.tree.2013.11.001.

Sundin J, Jutfelt F. 2016. 9-28 d of exposure to elevated pCO_2 reduces avoidance of predator odour but had no effect on behavioural lateralization or swimming activity in a temperate wrasse (*Ctenolabrus rupestris*). *ICES Journal of Marine Science* **73**:620–632 DOI 10.1093/icesjms/fsv101.

Warton DI, Hui F.KC. 2011. The arcsine is asinine: the analysis of proportions in ecology. *Ecology* **92**:3–10 DOI 10.1890/10-0340.1.

Webb PW. 1976. The effect of size on the fast-start performance of rainbow trout *Salmo cairdneri*, and a consideration of piscivorous predator–prey interactions. *Journal of Experimental Biology* **65**:157–177.

Welch MJ, Watson SA, Welsh JQ, McCormick MI, Munday PL. 2014. Effects of elevated CO_2 on fish behaviour undiminished by transgenerational acclimation. *Nature Climate Change* **4**:1086–1089 DOI 10.1038/nclimate2400.

Wittenrich ML. 2007. *Breeders guide to marine aquarium fishes.* Neptune City: T.F.H. Publications.