1 **Effects of elevated CO$_2$ on predator avoidance behaviour by reef fishes is**

2 **not altered by experimental test water**

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23 **Abstract**

24 Pioneering studies into the effects of elevated CO$_2$ on the behaviour of reef fishes often tested

25 high-CO$_2$ reared fish using control water in the test arena. While subsequent studies using

26 rearing treatment water (control or high CO$_2$) in the test arena have confirmed the effects of

27 high CO$_2$ on a range of reef fish behaviours, a further investigation into the use of different

28 test water in the experimental arena is warranted. Here, we used a fully factorial design to test

29 the effect of rearing treatment water (control or high CO$_2$) and experimental test water

30 (control or high CO$_2$) on antipredator responses of larval reef fishes. We tested antipredator

31 behaviour in larval clownfish *Amphiprion percula* and ambon damselfish *Pomacentrus*

32 *amboinensis*, two species that have been used in previous high CO$_2$ experiments. Specifically

33 we tested if: 1) using control or high CO$_2$ water in a two channel flume influenced the

34   response of larval clownfish to predator odour, and 2) using control or high CO_2 water in the

35   test arena influenced the escape response of larval damselfish to a startle stimulus. Finally, 3)

36   because the effects of high CO_2 on fish behaviour appear to be caused by altered function of

37   the GABA-A neurotransmitter we tested if antipredator behaviours were restored in

38   clownfish treated with a GABA antagonist (gabazine) in high CO_2 water. Larval clownfish

39   reared from hatching in control water (496 µatm) strongly avoided predator cue whereas

40   larval clownfish reared from hatching in high CO_2 (1022 µatm) were attracted to the predator

41   cue, as has been reported in previous studies. There was no effect of testing fish using control

42   or high CO_2 water in the flume. Larval damselfish reared for 4 days in high CO_2 (1051 µatm)

43   exhibited a slower response to a startle stimulus, slower escape speed and a shorter escape

44   distance compared with fish reared in control conditions (464 µatm). There was no effect of

45   test water on escape responses. Treatment of high-CO_2 reared clownfish with 4 mg l^{-1}

46   gabazine in high CO_2 seawater restored the normal response to predator odour, as has been

47   previously reported with fish tested in control water. Our results show that using control

48   water in the experimental trials did not influence the results of previous studies on

49   antipredator behaviour of reef fishes and also supports the results of novel experiments

50   conducted in natural reef habitat at ambient CO_2 levels.

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53   **Introduction**

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55   Rising concentrations of atmospheric carbon dioxide (CO_2) have caused an increased uptake

56   of CO_2 by the ocean, leading to a decline in seawater pH and changes in the relative

57   concentration of carbonate and bicarbonate ions, a process called ocean acidification (Sabine

58   et al., 2004: Doney, 2010). The partial pressure of carbon dioxide (pCO_2) at the ocean surface

59   is increasing at the same rate at the atmosphere (Doney, 2010) and thus marine species will

60   need to deal with rising CO_2 levels in addition to declining pH and other changes in ocean

61   chemistry (Pörtner et al., 2004). Recent models suggest an amplification of seasonal cycles in

62   ocean pCO_2 as atmospheric CO_2 continues to rise, such that surface ocean pCO_2 will reach

63   1000 µatm in summer for atmospheric CO_2 concentrations that exceed 650 parts per million.

64   Hypercapnic conditions (>1000 µatm CO_2) are projected occur in up to half the surface ocean

65   by 2100 on the current CO_2 emissions trajectory (McNeil & Sasse, 2016).

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67   Elevated CO_2 levels can have a variety of effects on the physiology, life history and

68   behaviour of marine organisms (Pörtner et al., 2004). Recent studies have demonstrated that

69   high CO_2 levels can fundamentally alter the behaviour of marine fishes and some

70   invertebrates (reviewed by Briffa et al., 2012; Clements & Hunt, 2014; Heuer & Grosell,

71   2014; Nagelkerken & Munday, 2016). Of particular concern is an impaired response to the

72   threat of predation, because it may increase mortality rates in natural populations (Munday et

73   al., 2010; Ferrari et al., 2011a; Chivers et al., 2014a). Larval and juvenile reef fish exhibit an

74   innate aversion to predator odour and chemical alarm cues from injured conspecifics because

75   they indicate a heightened risk of predation (Holmes et al., 2010; Dixson et al., 2012).

76   Individuals typically respond to these olfactory cues by reducing activity and seeking shelter

77   (Ferrari et al., 2010). However, larval reef fish that have been reared at near-future CO_2 levels

78   do not reduce activity or stop feeding in the presence of chemical alarm cues (Ferrari et al.,

79   2011a; Lönnstedt et al., 2013) and even become attracted to predator odour and chemical

80   alarm cues when presented in a two channel flume (Dixson et al., 2010; Welch et al., 2014).

81   Furthermore, juvenile fish exposed to high CO_2 lose their ability for associative learning

82   (Ferrari et al., 2012), a process that enables fine-tuning of risk assessment by the association

83   of chemical alarms cues with the identity of specific predators. Finally, high CO_2 affects the

84   kinematics of predator-prey interactions, with juvenile prey exposed to elevated CO_2

85   allowing predators to get closer before responding (Allan et al., 2013) and having reduced

86   escape speeds and distances compared with fish reared at current-day CO_2 levels (Allan et al.,

87   2013, 2014). These changes in behaviour alter the outcome of predator-prey interactions,

88   leading to significantly increased rates of mortality of small juveniles in mesocosm

89   experiments (Ferrari et al., 2011b) and in fish transplanted to natural coral reef habitat

90   (Munday et al., 2010; 2012; Chivers et al., 2014a).

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92   Many of the pioneering studies on the effects of elevated CO_2 on reef fish behaviour (e.g.

93   Munday et al., 2009, Dixson et al., 2010; Ferrari et al., 2011a,b; Cripps et al., 2011) involved

94   rearing fishes in control and elevated CO_2 conditions and then testing their responses to

95   olfactory cues in control water only. This method was chosen in these early experiments

96   owing to logistical constraints and because pilot experiments showed that the response of

97   high CO_2 exposed fish to predator cue did not differ if the cue was presented in either control

98   or high CO_2 water (Dixson et al., 2010; Munday et al., 2010). Moreover, studies with

99   freshwater fishes had shown that a pH reduction of 0.5 units in freshwater (<pH6.5)

100   irreversibly alters the structure of chemical alarm cues and can dramatically affect prey

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101   antipredator responses (Brown et al., 2002; Leduc et al., 2004a,b; reviewed in Leduc et al.,

102   2013). Consequently, there was concern that testing marine fish in CO_2-acidified water (albeit

103   still above pH7.5) could confound the interpretation of any effects of high CO_2 on the

104   behavioural response of the fish with a diminished efficacy of the olfactory cue itself.

105   Indeed, subsequent studies have shown that chemical alarm cues from reef fishes degrade

106   much more quickly in high CO_2 water than they do in current-day conditions (Chivers et al.,

107   2014b); therefore, experiments testing olfactory responses of marine species to high CO_2

108   need to take this into account. Finally, small-scale laboratory experiments often have limited

109   ecological relevance and it is challenging to extrapolate their findings to natural communities

110   and ecologically relevant spatial scales (Naglekerken & Munday, 2016). Consequently, it was

111   critical to test the effects of exposure to high CO_2 in natural habitats in the field. This

112   involved transplanting larval fish that had been reared in either control or high CO_2 seawater

113   back into natural coral reef habitat and monitoring their behaviour (Munday et al., 2010,

114   2012; McCormick et al., 2013).

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116   Since these initial studies, a number of other experiments have been conducted in which the

117   respective treatment seawater in which the fish were reared (control or high CO_2) has been

118   used in the experimental trials, confirming the results of earlier studies (e.g. Allan et al.,

119   2014; Dixson et al., 2014; Welch et al., 2014; Nagelkerken et al., 2015) and/or revealing

120   other behavioural effects of high CO_2, such as an impaired response to auditory cues

121   (Simpson et al., 2011). While previous pilot studies found no effect of the test water on the

122   response of high-CO_2 fish in behavioural assays (Dixson et al., 2010; Munday et al., 2010)

123   and subsequent studies conducted in the respective treatment water have produced

124   confirmatory results (Allan et al., 2014; Welch et al., 2014; Dixson et al., 2015), a more

125   thorough validation study of the test water used in antipredator experiments is warranted.

126   Here, we compared the use of control versus high CO_2 seawater (called test water from

127   hereafter) in experimental trials designed to test the antipredator responses of larval reef

128   fishes reared in high CO_2. We conducted antipredator experiments for two species that have

129   been widely used in previous high CO_2 experiments, the clownfish *Amphiprion percula* and

130   the ambon damselfish *Pomacentrus amboinensis*. Specifically we tested if: 1) using control

131   or high CO_2 water in a two channel flume influenced the response of larval clownfish to

132   predator odour in fish reared in either control or high CO_2, and 2) using control or high CO_2

133   water in the test arena influenced the kinematic response of larval damselfish to a startle

134   stimulus in fish reared in either control or high CO_2. Finally, because the effects of high CO_2

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135   on fish behaviour appear to be due to altered function of GABA-A neurotransmitter receptors

136   (Nilsson et al., 2012; Hamilton et al., 2013; Heuer & Grosell, 2014) we tested if antipredator

137   behaviours were restored in clownfish treated with a GABA antagonist (gabazine) in high

138   CO_2 water. Previous studies have demonstrated that abnormal antipredator behaviour of

139   clownfish and damselfish reared in high CO_2 conditions is reversed following treatment with

140   gabazine (Nilsson et al., 2012; Chivers et al., 2014a). However, these previous studies

141   involved high CO_2 fish that were treated with gabazine in control water and then

142   behaviourally tested in control water. In this experiment we therefore conducted the same

143   gabazine treatments as previously used, except the drug was administered and behaviour was

144   tested in high CO_2 water.

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146   **Methods**

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148   The experiment with larval clownfish was conducted at James Cook University's (JCU)

149   experimental aquarium facility in Townsville, Australia, where previous high CO_2

150   experiments with this species have been conducted. The experiment with larval ambon

151   damselfish was conducted at Lizard Island Research Station on the northern Great Barrier

152   Reef, where previous high CO_2 experiments with this species have been conducted. Both

153   experiments were conducted during February 2016. Because our goal was to assess the use of

154   control seawater in experimental trials of previous experiments (e.g. Dixson et al., 2010;

155   Munday et al., 2010, 2012) we used the same methods as previous experiments, except that

156   we applied a fully factorial design where fish reared in control and high CO_2 conditions were

157   behaviourally tested in both control and high CO_2 water.

158

159   *Response to predator cue*

160   Two clutches of larval clownfish from different parental pairs maintained at JCU were reared

161   in control and high CO_2 conditions from hatching until settlement (11-12 days post hatching)

162   using standard practices (Wittenrich, 2007). Briefly, larvae were reared in 100 l incubator

163   tanks supplied with a continuous flow of treatment seawater. Water temperature was

164   maintained at 29 °C. Photoperiod was 12 hours light and 12 hours dark. A non-viable

165   microalgae blend (Nano 3600 – Reed Mariculture™) was drip-fed into the incubator tanks

166   during daylight hours to reduce light levels and enhance larval feeding during the first four

167   days. Larvae were fed live rotifers (15 per ml) for the first four days and then transitioned

168   onto artemia (5 per ml). Fish were not fed on the morning of testing. Each clutch was divided

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169   at hatching so that half the clutch was reared in control seawater (496 ± 55 S.D. μatm CO_2)

170   and half reared in the high CO_2 treatment (1022 ± 37 S.D. μatm CO_2). Two 10,000 L

171   recirculating aquarium systems supplied seawater to the incubator tanks, one supplied control

172   seawater and the other high CO_2 seawater. To achieve the desired CO_2 level in the high CO_2

173   treatment, seawater was dosed with CO_2 to a set pH in a 3000 L sump using an Aqua Medic

174   AT Control System (Aqua Medic, Germany). The pCO_2 in rearing tanks was measured by

175   nondispersive infrared (NDIR) following the method described by Hari et al. (2008). Air in a

176   closed loop was circulated by a small pump (1 l min^{-1} flow rate) through a coil of thin-walled

177   (membrane thickness ϕ 0.4 mm, outer ϕ 3.8 mm) medical grade silicone tubing placed in the

178   tank. CO_2 in the closed loop was then measured at 1 minute intervals with a Vaisala GMP343

179   infrared CO_2 probe (accuracy ± 5 ppm CO_2 + 2% of reading over the range of experimental

180   manipulations). Each reading (N=9 in control and N= 9 in high CO_2) lasted 1 hour to ensure

181   complete equilibration of CO_2 between tank seawater and the closed loop of air. The average

182   CO_2 for the final ten minutes was calculated for each reading.

183

184   The response of settlement stage clownfish to predator cue was tested in a two-channel flume

185   specifically designed for testing olfactory preferences of larval fish (Gerlach et al., 2007).

186   Larvae were given the choice between a stream of seawater containing predator cue versus a

187   stream of seawater without the predator cue. Both streams of water were either control or

188   high CO_2 test water for a given trial. Water from the respective paired sources was gravity

189   fed into the flume at 100 ml min^{-1}, maintained by a flow meter and dye test with every water

190   exchange. Water was exchanged after every fish, with random alternation between control

191   and high CO_2 test water. For each trial, a single fish was placed in the centre of the

192   downstream end of the flume and allotted a two-minute acclimation period. The position of

193   the fish at 5-second intervals was recorded for the next two minutes. A one-minute rest period

194   followed during which the water sources were switched to eliminate potential side

195   preference. After the water switch, the fish was re-centred in the downstream end of the

196   flume and the entire acclimation (2 minutes) and test period (2 minutes) were repeated. The

197   temperature of the test water was kept within 1 °C of the water temperature in the rearing

198   tanks. Three trials where the difference in water temperature between the flume and the

199   rearing tanks exceeded 1 °C were discarded. The experimenter was blinded to the rearing

200   treatment of the fish.

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202   Predator cues were obtained from the common coral-cod, *Cephalopholis miniatus*. Five cod
203   collected from the Great Barrier Reef (supplied by Cairns Marine, Cairns QLD) were
204   maintained in 60 L aquariums supplied with a continuous flow of seawater from a different
205   seawater system to that used to rear the clownfish. Each cod was fed one pellet of
206   commercial fish food (Marine Food, Fish Fuel Co™ – 1 cube per fish) each evening. Water
207   temperature was maintained at 29 °C. To obtain predator cues for the experiment, the water
208   was drained from two predator tanks and refilled with water from the same system used to
209   rear the clownfish. To match the methods used in previous experiments, water to the predator
210   tank was turned off and predator cue water was collected after a 2 hours soak time. Predator
211   tanks were aerated to maintain oxygen content and water-bathed to maintain a stable
212   temperature. One of the two predator tanks was dosed with CO_2 at the end of the 2 hour soak
213   period, immediately before cue water was extracted. CO_2 was dosed at low pressure by hand,
214   with gentle stirring, until the pH matched that of the high CO_2 rearing treatment. The other
215   predator tank was also gently stirred but was not dosed with CO_2. The individual cod used for
216   predator cues was alternated within and between days. An identical procedure was followed
217   with two tanks that did not house a predator to obtain seawater without predator cue to use in
218   the flume.

219

220   *Gabazine treatment*

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

231

232   **Kinematics of escape response**

233   Settlement stage larval *Pomacentrus amboinensis* were collected using light traps in the
234   Lizard Island Lagoon and transferred to an environmentally-controlled aquarium facility at
235   Lizard Island Research Station. Larval fish were assigned randomly to four replicate control

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236   $(464 \pm 32$ S.D. µatm $CO_2)$ or four replicate high-CO_2 $(1051 \pm 18$ S.D. µatm $CO_2)$ aquaria.

237   Twenty five *P. amboinensis* were housed in each 32 l (38Lx28Wx30H cm) aquarium. Water

238   temperature was maintained at approximately 29.5 °C. Fish were kept for 4 days in treatment

239   conditions and fed *Artemia* sp. twice daily *ad libitum*. Fish were not fed on the morning of

240   testing. Each aquarium was supplied with control or elevated-CO_2 seawater at 750 ml.min^{-1}.

241   Elevated-CO_2 seawater was achieved by dosing with CO_2 to a set pH, following standard

242   techniques (Gattuso et al., 2010). Seawater was pumped from the ocean into 32 l header tanks

243   where it was diffused with ambient air (control) or 100% CO_2 to achieve the desired pH

244   (elevated-CO_2 treatment). A pH-controller (Aqua Medic, Germany) attached to the CO_2

245   treatment header tank maintained pH at the desired level. Duplicate control and high CO_2

246   systems supplied seawater to two replicate aquaria in each system. The pCO_2 in rearing tanks

247   was measured by NDIR as described above (N= 9 in control and N= 5 in high CO_2).

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249   After rearing for four days in control or high CO_2 conditions, the escape response of the

250   damselfish was tested in both control and high CO_2 water (i.e. a fully crossed design).

251   Individual fish were placed into the testing arena, which consisted of a transparent circular

252   acrylic arena (diameter 200 mm) within a larger plastic tank (585 x 420 x 330 mm; 60 L)

253   filled to a depth of 100 mm with either control or high CO_2 water. A shallow water depth was

254   used to limit vertical displacement of the fish during escape response trials. Water

255   temperature in the experimental arena was 29°C - 30°C and the water treatment used was

256   alternated after every 2nd trial. The arena was illuminated by LED strip lighting (750 lumens)

257   placed above the water surface on the outside of the tank. Five minutes after being released

258   into the testing arena, an escape response was elicited by the release of a tapered metal

259   weight from above the water surface. This was accomplished by turning off an electromagnet

260   to which the metal weight was attached. The metal weight was controlled by a piece of

261   fishing line that was long enough such that the tapered tip only just touched the surface of the

262   water. In order to provide a sudden stimulation and allow quantification of escape latency, the

263   stimulus was released through a white PVC tube (diameter 40 mm, length 550 mm)

264   suspended above the experimental arena, with the bottom edge at a distance of 10 mm above

265   the water level. Fish were only startled when they moved to the middle portion of the tank,

266   allowing an individual to move an equal distance in any direction and standardising for fish

267   position relative to the stimulus. Escape responses were recorded at 480 frames per second

268   (Casio EX-ZR1000) as a silhouette from below obtained through pointing the camera at a

269   mirror angled at 45˚. To minimise visual disturbances, black sheeting surrounded the front of

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270   the mirror so that any movement within the room was undetected by the fish. Treatment

271   water in the testing arena was changed every 2nd trial to minimise CO_2 loss in the high CO_2

272   treatment water. The rate of CO_2 loss from the testing arena was found to be negligible within

273   this time frame. The experimenter was blinded to the rearing treatment of the fish.

274   From the videos, we quantified latency to initiate an escape, escape distance, escape speed

275   and maximum speed. A 1 cm line was drawn in the centre of the inner arena to enable

276   calibration for video analysis. All videos were analysed with the observer blind to the

277   treatments.

278

279   *Kinematic variables*

280   Kinematic variables associated with the fast-start response were analysed using the image-

281   analysis software Image-J, with a manual tracking plug-in. The centre of mass (CoM) of each

282   fish was tracked for the duration of the response. The following kinematic variables were

283   measured:

284   1. Latency to respond (sec) was measured as the time interval between the stimulus

285   onset and the first detectable movement leading to the escape of the animal.

286   2. Escape distance (m) is a measure of the total distance covered by the fish during the

287   first two flips of the tail (the first two axial bends, i.e. stages 1 and 2 defined based on

288   Domenici and Blake (1997), which is the period considered crucial for avoiding

289   ambush predator attacks (Webb, 1976).

290   3. Escape speed (m s^{-1}) was measured as the distance covered within a fixed time (first

291   24 milliseconds after initial response) which corresponds to the average duration of

292   the first two tail flips (the first two axial bends, i.e. stages 1 and 2 based on Domenici

293   & Blake, 1997). This period is considered crucial for avoiding predator ambush

294   attacks (Webb, 1976).

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

296   during stage 1 and stage 2.

297

298   **Data analysis**

299   Two way ANOVA was used to compare percent time that larval clownfish spent in predator

300   cue in the flume. Percentage data was logit-transformed for analysis (Warton & Hui, 2001).

301   Treatment water (control or high CO_2) and test water (control or high CO_2) were the fixed

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302   factors. For the gabazine experiment, the percentage time that larval clownfish treated with

303   gabazine spent in predator cue in the flume was compared with control fish using a t-test on

304   logit-transformed data. A linear mixed effects model (LME) was used to compare escape

305   responses of larval damselfish. Latency, escape distance, escape speed and maximum speed

306   were each tested with treatment and test water as fixed factors. Tank was included in each

307   model as a random effect to account for the subsampling of fish from replicate tanks within

308   each CO_2 treatment. The normality, linearity and homoscedasticity of residuals of the models

309   were verified by visual inspection of residual-fit plots. Latency data was log10 transformed

310   before analysis to improve the distribution of the data. ANOVA was conducted with

311   Statistica (version x) and LMEs were fitted using the 'nlme' package in the R software

312   package (Pinheiro et al. 2013).

313

314   **Results**

315

316   There was a highly significant effect of treatment water on the percentage time that larval

317   clownfish spent in the water stream containing predator odour ($F_{1,57}$=6.256, p = 0.015), but

318   no effect of test water ($F_{1,57}$=0.0003, p = 0.973). Laval clownfish reared in control water

319   strongly avoided predator odour, spending <9% of the time in that water stream (Figure 1). In

320   contrast, larval clownfish reared in high CO_2 water were strongly attracted to predator odour,

321   spending nearly 80% of the time in that water stream (Figure 1).

322

323   As with the response to predator odour, treatment water had a significant effect on escape

324   responses of larval damselfish, but there was no effect of test water. Latency to respond

325   significantly increased in fish reared in elevated CO_2, with fish displaying latencies that were

326   30% slower compared with fish reared in control water (Figure 2a: $F_{1.14}$= 5.971, p = 0.018).

327   There was no effect of test water on latency to respond (p = 0.7). Escape distance following

328   stimulation was significantly shorter for fish reared in high CO_2 compared with control water

329   (Figure 2b: $F_{1,14}$= 6.034, p = 0.02) and there was no effect of test water (p = 0.8). Response

330   speed and maximum speed responded similarly with both kinematic variables declining in

331   fish reared in high CO_2 compared to fish reared in control water (Figure 2c; $F_{1,14}$= 8.678, p =

332   0.01 and Figure 2d; $F_{1,14}$ = 9.015, p = 0.009). Again, there was no effect of test water (p = 0.7

333   and p = 0.5).

334

335   Gabazine reversed the effects of rearing in high CO_2 (Figure 1). Fish reared in high CO_2 and

336   then treated with gabazine exhibited a strong avoidance of predator cue, with the response

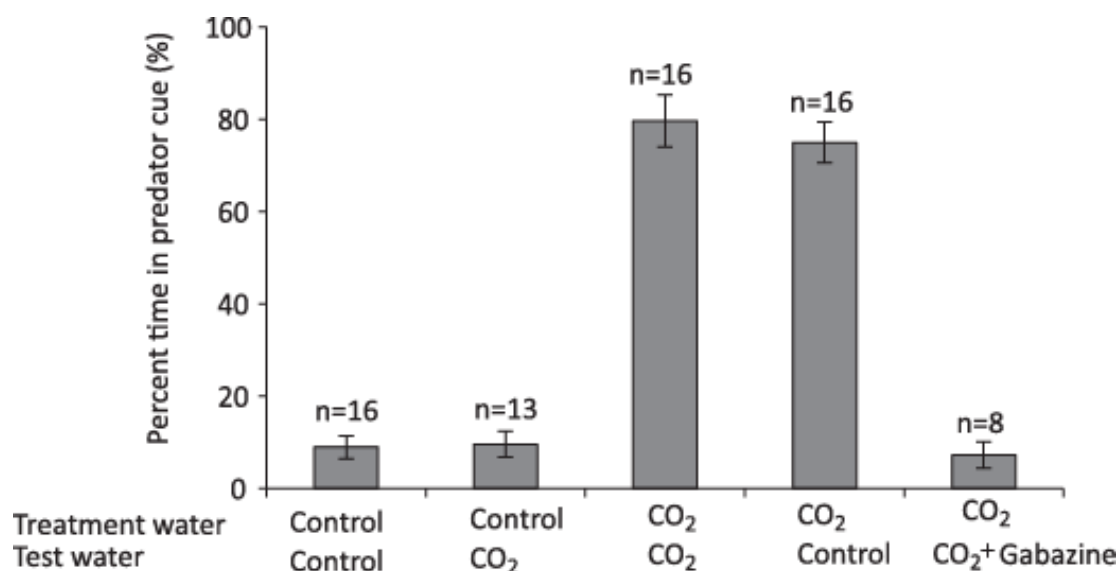337   almost identical to fish reared and tested in control water (p=0.96).

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341   Figure 1. The effect of rearing treatment water (control or elevated CO_2) and test water used

342   in a two-channel flume (control or elevated CO_2) on the olfactory response of larval *A.*

343   *percula* to predator cue. One stream of water in the flume contained seawater with predator

344   cue and the other stream of water had untreated seawater. Shown is the percent time (mean ±

345   s.e.) that fish from each experimental combination spent in the water stream containing

346   predator cue. The final group shows fish that were treated with 4 mg l^{-1} gabazine in high CO_2

347   seawater for 30 minutes before testing in the flume. Sample size shown above the bars.
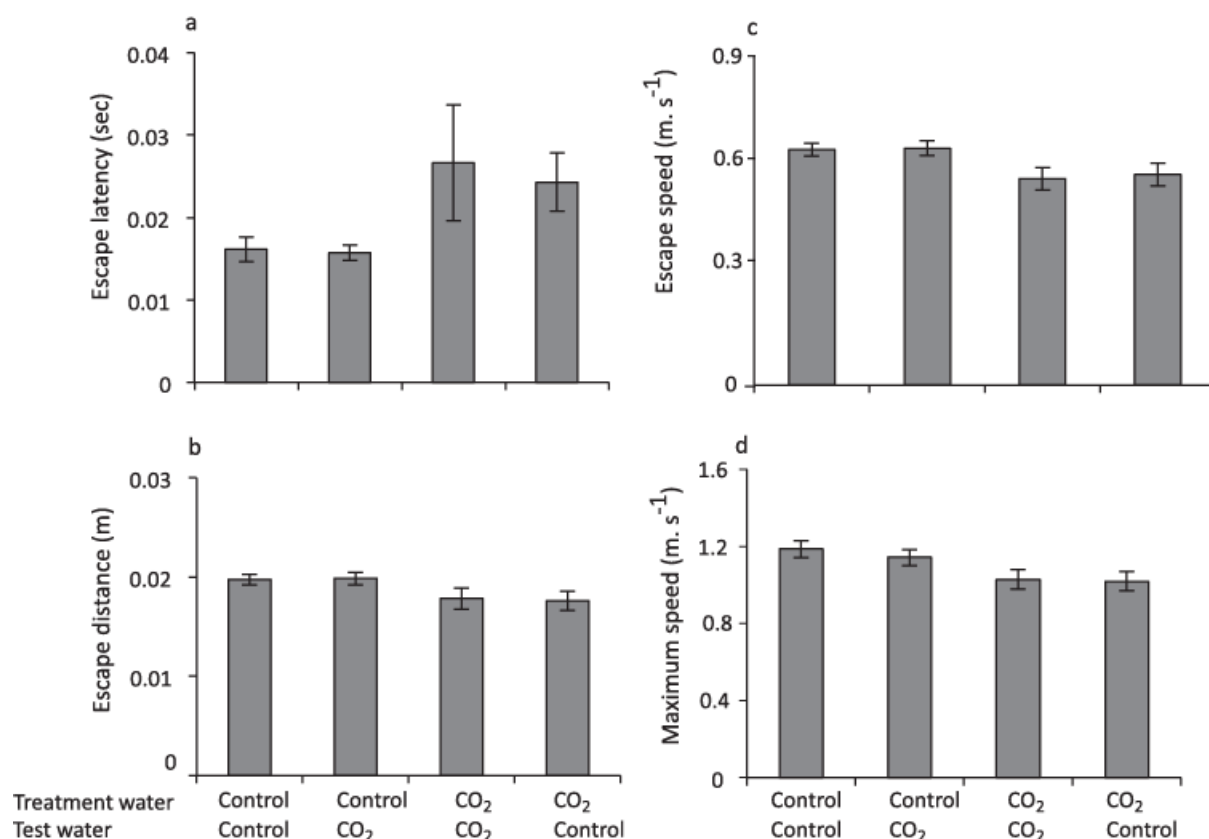
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Figure 2. The effect of rearing treatment (control or high CO_2) and test water used in the experimental trial (control or high CO_2) on the escape performance of larval *P. amboinensis*. Variables displayed are: (*a*) latency, (*b*) distance, (*c*) 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.

Discussion

Previous studies have demonstrated that CO_2 levels projected to occur in the surface ocean by the end of this century (700-1000 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

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368   in high CO_2 exhibited the same responses to predator odour in a two channel flume and the

369   same kinematic responses to a startle stimulus regardless of whether high CO_2 or control

370   water was used in the experimental trial. Similarly, there was no difference in the behavioural

371   response of fish reared in control seawater when tested in either high CO_2 or control water.

372   These results demonstrate that the choice of test water has not influenced the results of

373   previous laboratory studies into the effects of high CO_2 on antipredator behaviour.

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375   Our results are consistent with previous pilot studies showing that the test water (control or

376   high CO_2) used in a two-channel flume did not alter the behavioural response of larval fishes

377   to predator odour (Dixson et al., 2010; Munday et al., 2010). Furthermore, our study extends

378   these findings to the kinematics of predator avoidance, by showing that the type of water used

379   in the test arena does not affect vital components of the escape response, including latency to

380   respond, escape speed and distance travelled. Together these results show that antipredator

381   behaviours of larval fishes that have been reared in high CO_2 are not compromised by a

382   return to control seawater for experimental testing. These findings are also relevant to field-

383   based studies that have tested the effects of high CO_2 on reef fishes in their natural habitat

384   (e.g. Munday et al., 2010, 2012; Devine et al., 2012, 2013; Ferrari et al., 2011a; McCormick

385   et al., 2013; Chivers et al., 2014a). Such studies are critical for predicting the impacts of high

386   CO_2 on reef fish populations; however, they involve rearing larval fish for 4-5 days in high

387   CO_2 conditions before transferring them to ambient CO_2 conditions in the field. These studies

388   have demonstrated mortality rates from predation are much higher in juvenile fish that have

389   been exposed to high CO_2 compared with fish exposed to ambient control conditions

390   (Munday et al., 2010, 2012; Ferrari et al., 2011a; Chivers et al., 2014a). Our results indicate

391   that the results of such studies are unlikely to have been affected by a change in antipredator

392   behaviour due to transplanting fish from high CO_2 conditions in the laboratory to ambient

393   CO_2 conditions in their natural habitat.

394

395   In contrast to the absence of a test water effect on antipredator behaviours, we found that

396   rearing treatment water had a highly significant effect on antipredator behaviour. Consistent

397   with previous studies (Dixson et al., 2010; Munday et al., 2010), larval clownfish reared in

398   high CO_2 exhibited a reversal from strongly avoiding predator cue (<10% of time in predator

399   cue in the flume) to a strong attraction to predator cue (nearly 80% of time in predator cue in

400   the flume). Similarly, larval damselfish exposed to high CO_2 for 4 days exhibited a slower

401   response to a threat stimulus, a slower escape speed and shorted distance travelled compared

13

402   with fish kept in ambient control water, as has been reported previously (Allan et al., 2013,

403   2014). Thus, our results provide further confirmatory evidence for the effects of high CO_2 on

404   antipredator behaviour in larval reef fishes. The differences in escape speed and escape

405   distance between high CO_2 and control fish were relatively small in the escape response trial.

406   However, when combined with a much longer latency to respond (>50% increase in time to

407   respond) these changes in kinematic responses would likely have a detrimental effect on the

408   probability of a prey fish escaping a predator attack.

409

410   Altered behaviour of fish in a high CO_2 environment appears to be due to the effects of acid-

411   base regulation on the function of GABA-A receptors (Nilsson et al., 2012; Hamilton et al.,

412   2013; Heuer & Grosell, 2014). Fish prevent plasma and tissue acidosis in high CO_2 by

413   accumulating HCO_3^- in exchange for Cl^- (Brauner & Baker 2009). The GABA-A receptor is

414   a gated ion channel that conducts HCO_3^- and Cl^- when activated by the neurotransmitter

415   GABA. The altered concentration of HCO_3^- and Cl^- in fish exposed to high CO_2 could

416   decrease or reverse the flow of these ions through the GABA-A receptor, causing a reversal

417   in receptor polarization (Heuer & Grosell, 2014). Evidence for the role of GABA-A receptors

418   in behavioural impairment of fish in high CO_2 has come from studies showing that treatment

419   with gabazine, a GABA antagonist, reverses the effects of high CO_2 on behaviour (Nilsson et

420   al., 2012; Chivers et al., 2014a; Chung et al., 2014, Lai et al., 2015; Ou et al., 2015; Regan et

421   al., 2016). In the first of these studies, Nilsson et al. (2012) found that gabazine reversed the

422   impaired response to predator odour exhibited by clownfish that had been reared in high CO_2.

423   However, in that study gabazine was administered in control seawater and high-CO_2 reared

424   fish were tested in control water. Therefore, the effects of gabazine in restoring the response

425   of larval fishes to predator odour has not been previously tested in high-CO_2 conditions. Here

426   we show that treatment with 4 mg l^{-1} gabazine completely reverses the abnormal behavioural

427   of high-CO_2 fish to predator odour, restoring their natural aversion to predator odour. This

428   contributes to a large number of independent studies pointing to a key role of GABA-A

429   receptors in the behavioural impairment of fish exposed to high CO_2.

430

431   While we found no effects of using control or high CO_2 water in experimental trials to test

432   the effects of high CO_2 on fish behaviour, Sundin and Jutfelt (2016) reported differences in

433   relative lateralization of juvenile goldsinny wrasse when tested in control water versus high

434   CO_2 water after 21 days in high CO_2. Their study did not include the reverse treatment, where

435   control reared fish were tested in high CO_2 water, and there is the potential for learning

436   effects in control fish because the same fish were tested on three different occasions, at days

437   9, 19 and 21. Nevertheless, that study does suggest that the response of some behavioural

438   traits could be sensitive to differences in the test water used in behavioural trials. Further

439   experiments with a broader range of behavioural traits and using experiments that are not

440   complicated by the repeated testing of the same individuals are required to test this

441   possibility.

442

443   Pioneering studies into the effects of high CO_2 on fish behaviour used control seawater in

444   experimental trials due to logistical constraints and concerns about the possible effects of low

445   pH on olfactory cues. Furthermore, pilot studies indicated that there was no difference in the

446   behaviour of fish that had been exposed to high CO_2 when tested in control or high CO_2

447   water. Our results confirm the results of those initial studies by showing that test water

448   (control of high CO_2) had no effect on the antipredator responses exhibited by fish reared in

449   either control or high CO_2 conditions. Nevertheless, we do not advocate the continued use of

450   control seawater for testing the behavioural effects of high CO_2 in marine organisms.

451   Methods for treating seawater to replicate future pCO_2 levels are now sufficiently validated

452   that it is possible to treat most experimental arenas with the respective treatment water that

453   the animals have been reared in, and there may be some behavioural traits that are sensitive to

454   different test water in the experimental trials. Therefore, most reliable and unambiguous

455   results will likely come from using the respective treatment water in experimental trials

456   testing the effects of high CO_2 on the behaviour of marine organisms.

457

458   **Acknowledgments**

463

464   **References**

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