

Exposure to elevated carbon dioxide does not impair short-term swimming behaviour or shelter-seeking in a predatory coral-reef fish

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Adult bluespotted rockcod *Cephalopholis cyanostigma*, a coral-reef grouper, were acclimated to either ambient (mean \pm S.D. 41.12 ± 2.13 Pa;) or high pCO₂ (95.75 ± 11.75 Pa) conditions in a laboratory for 8–9 days, then released at the water surface directly above a reef (depth *c.* 5 m) and followed on video camera (for 191 ± 21 s) by scuba divers until they sought cover in the reef. No differences were detected between groups in any of the six measured variables, which included the time fish spent immobile after release, tail beat frequency during swimming and the time required to locate and enter the protective shelter of the reef.

Keywords: aquatic acidification; conservation behaviour; Great Barrier Reef; hypercapnia.

Teleosts have a well-documented physiological capacity to maintain a stable internal pH in the face of changes in environmental pH (Fromm, 1980; Ishimatsu *et al.*, 2004). For that reason, it has long been thought that ocean acidification (OA; the anthropogenic acidification of the oceans *via* diffusion of increased atmospheric CO₂) would not pose direct problems for marine fishes for at least the next century (Heuer & Grosell, 2014). However, a series of papers on larval and juvenile coral-reef fishes reported that fishes were behaviourally impaired when exposed for 4 days to end-of-century levels of CO₂ (partial pressure of CO₂, pCO₂ *c.* 101.32 Pa) (Munday *et al.*, 2009; Dixson *et al.*, 2010; Nilsson *et al.*, 2012). Conducted under laboratory conditions, these initial studies primarily suggested that high CO₂ could disrupt the innate abilities of fish to sense or appropriately respond to chemosensory cues, perhaps most alarmingly with fish being strongly attracted to predator odours rather than avoiding them (Dixson *et al.*, 2010; Munday *et al.*, 2010). Interest in the effects of OA on fishes subsequently grew rapidly, with studies (primarily laboratory-based) reporting a range of impacts including deleterious effects on behavioural lateralization (Domenici *et al.*, 2014), increased activity levels (Munday *et al.*, 2013), avoidance of prey cues (Dixson *et al.*, 2015), increased boldness (Munday *et al.*, 2013), impaired hearing (Simpson *et al.*, 2011) and impaired visual risk assessment (Ferrari *et al.*, 2012).

Previous research suggested that the OA-associated behavioural and sensory impairments in fish were retained for a period of days after the fish were returned to ambient (control) water, including when they were released into their natural environment following ≥ 4 days of laboratory exposure to high CO₂ (Devine *et al.*, 2012; Devine & Munday, 2013; Munday *et al.*, 2013). Some studies have suggested that fish acclimated to OA conditions may have difficulty locating appropriate habitat or avoiding predators once released into the wild (*i.e.* returned to

ambient control water; Briffa *et al.*, 2012; McCormick *et al.*, 2013), corroborating the reports of impaired vision, hearing and detection of chemosensory cues (*e.g.* predators and habitats), senses that are each likely to have some role in fish finding suitable habitat. The literature makes it apparent that CO₂ induced behavioural impairment affects a range of species and life stages, yet predatory species, in particular, have received relatively little attention (but see Munday *et al.*, 2013; Dixon *et al.*, 2014; Heinrich *et al.*, 2016) despite their importance in maintaining ecosystem function and biodiversity (Myers *et al.*, 2007; Estes *et al.*, 2011; Atwood *et al.*, 2013). In one of the few examples, juveniles of a common coral-reef teleost piscivore, the coral trout grouper *Plectropomus leopardus* (Lacépède 1802), displayed some of the most dramatic behavioural effects of CO₂ exposure that have been reported. Exposure to 97.27 Pa CO₂ for 4 days caused the fish to become strongly (> 90%) attracted to predator cues, increase their swimming activity by two orders of magnitude and decrease their shelter use 10 fold, suggesting that groupers (Epinephelinae) could be the most susceptible teleost family to be tested thus far (Munday *et al.* 2013).

The objective of this study was to enhance our understanding of the potential effects of elevated CO₂ on fishes by examining the behaviour of a coral-reef mesopredator *Cephalopholis cyanostigma* (Valenciennes 1828) when returned to the reef following an acute 8–9 day exposure to elevated CO₂. *Cephalopholis cyanostigma* is a widely distributed grouper that has not been examined in an OA context except for having been used as a model predator in experiments focused on other fishes (*e.g.* to generate predator chemical cues; Dixon *et al.*, 2010; Munday *et al.*, 2010). Based on previous reports, it was hypothesized that following re-release into their natural environment, *C. cyanostigma* acclimated to high CO₂ would exhibit clear evidence of

increased swimming activity and signs of behavioural impairment including a reduced ability to locate and enter protective reef shelter.

Between 26 August 2014 and 1 September 2014, adult *C. cyanostigma* ($n = 21$; mean fork length, $L_F = 262$ mm, range = 235–290 mm) were captured from 5–20 m deep coral reefs around Lizard Island (14° 40' 43.7" S; 145° 26' 54.4" E) by snorkel fishing using hand lines baited with chopped pilchards (*Sardinops* spp.) on size 8/0 hooks. Once brought to the vessel, fish were transported in a container filled with ambient seawater back to Lizard Island Research Station (LIRS). Once at LIRS, fish were immersed in freshwater for *c.* 2 min as a precautionary anti-parasite treatment and transferred into one of two treatment tanks (80 × 50 × 40 cm deep, 160 l). Both treatment tanks were supplied with a continuous flow-through of fresh seawater from four well-aerated header tanks. Two of the header tanks were regularly diffused with CO₂ gas to achieve elevated pCO₂ and reduced pH (simulated OA). This was achieved by the use of a CO₂ dosing system (pH stat Computers, Aqua Medic; www.aqua-medic.de), which monitored pH using a pH probe and automatically diffused CO₂ into the header tank *via* a solenoid valve attached to a cylinder of compressed CO₂ gas. This system achieved a mean ± s.d. pCO₂ of 95.75 ± 11.75 Pa (*cf.* 41.14 ± 2.13 Pa in the control treatment), based on 1–4 daily measurements of pCO₂ made with a handheld meter (GM70, Vaisala; www.vaisala.com) connected to a submerged gas-permeable polytetrafluoroethylene (PFTE) probe (Qubit systems; www.qubitbiology.com). Multiple sections of PVC pipe were placed in each exposure tank to provide shelter in which the fish readily hid and fish were fed chopped *Sardinops* spp. *ad libitum* every 2 days. Water temperatures were 23.3 ± 1.2° C (mean ± s.d.) over this period (monitored with iButton thermal loggers set to record temperature every 10 min; Maxim Integrated Products

Inc.; www.maximintegrated.com). Food was withheld for 20–24 h prior to fish being transported back to the field for release and behavioural observations.

On September 4 and September 9 2014, fish were transported in water-filled containers (control water) by boat to a field location near LIRS for release (between 0900 and 1100 hours). In each case, fish were in their exposure treatments for 8–9 days prior to release. The release site was *c.* 5 m deep and surrounded by an open sandy bottom with the exception of a *c.* 1m diameter coral structure in the immediate vicinity of the boat (1–2 m away laterally). The boat was kept in position using both bow and stern anchors. Fish were released at the surface one at a time [Fig. 1(a); alternating between fish from the two acclimation treatments], after which two scuba divers, each equipped with underwater video cameras (camera 1 = Nikon J3 with a Nikkor 10-30 lens in a Nikon WP-N2 underwater housing; www.nikon.com; camera 2 = Hero3, GoPro Inc.; www.shop.gopro.com) followed it for 191 ± 21 s (mean \pm s.d.). The coral structure below the boat provided an obvious patch of habitat and shelter towards which the fish could navigate [Fig. 1(b)]. The time the fish were followed on camera was deemed sufficient for the fish to locate and enter the protective shelter of the nearby reef structure (Supporting Information Video S1). After each *c.* 3 min recording, fish were manually chased away by the divers to the next nearest (and much larger) reef *c.* 8 m away) to ensure that the shelter space within the coral structure did not become crowded with conspecifics. Videos recorded by camera 1 were manually scored (observer blinded to treatment) using the software Observer XT 10.5 (Noldus Information Technology; www.noldus.com). Six variables (five behaviours + ventilation rate) were quantified that were subsequently used in statistical analyses: time spent immobile under-adjacent to the boat in a vulnerable position upon release (s); overall time spent immobile [*i.e.* not swimming, *e.g.* Fig. 1(c)]; time (s) required to reach the ocean floor from the release point;

time (s) required to enter protective reef shelter; median tail-beat frequency (beats min^{-1}) during swimming; median ventilation rate (opercular beats min^{-1}) while immobile. For the latter two variables, we calculated a median value for the fish and used it in subsequent analyses only if five or more such events were time-stamped in the analyses for that individual (an event = two successive tail or opercular beats, from which a frequency could be calculated based on the duration between the two). There were enough data points (*e.g.* tailbeat-to-tailbeat time gaps from which to calculate a rate) to meet such criteria to use data from $n = 14$ and $n = 15$ fish in total for ventilation rate and tail-beat frequency, respectively. These two variables were compared between the two exposure treatments using a generalized linear model (GLM; Gaussian distribution, *i.e.* a basic linear model). For non-normally distributed data, the most appropriate distribution to use in a GLM was assessed based on visual inspection of the raw data, visual assessments of model residuals and the use of a dispersion statistic for models fitted with different distributions. For time required for fish to reach the ocean floor, a GLM with a Poisson distribution was used (single factor = pCO_2 exposure treatment). Negative binomial GLMs were used for time spent immobile adjacent to the boat upon release and for time taken to enter shelter. Overall time spent immobile (s) was transformed into a proportion of the total duration of the fish's behavioural trial and compared between exposure treatments with a quasi-binomial GLM. Model diagnostics followed procedures described in Zuur *et al.* (2010) and Zuur & Ieno (2016) as applicable and α was set to 0.05.

Upon release, 13 of 21 fish (across treatments) immediately oriented themselves and began swimming towards the ocean floor [Fig. 2(a)], with the longest time spent immobile upon release being 3.3 s with a median of 0 s [no difference between exposure treatments; $t_{1,20} = -0.66$, $P > 0.05$; Fig. 2(a)]. There was no difference between exposure treatments in the time

required for fish to reach the ocean floor [$Z_{20,1} = -1.16$, $P > 0.05$, overall median = 8.3 s; Fig. 2(b)] or in the time the fish took to locate and enter shelter within the nearby reef structure [$t_{1,20} = 0.28$, $P > 0.05$, overall median = 18.5 s; Fig. 2(c)]. Once fish reached a secure position within the reef structure, most of them remained there for the duration of the behavioural trial, such that all fish were immobile for the majority of the trial [median proportion of time spent immobile = 0.9; Fig. 2(d)] with no effect of prior CO₂ exposure ($t_{1,20} = 0.16$, $P > 0.05$). This behaviour of remaining relatively stationary was not markedly different from how this species typically behaves when observed on reefs given that it is a sit-and-wait ambush predator (the authors' pers. obs.). However, it is possible that the presence of the divers (typically *c.* 1–2 m away) discouraged the fish from venturing out of the shelter within the 3 min observation period. There was also no effect of treatment on the tail-beat frequency used by fish to reach the bottom and to enter shelter [$t_{1,14} = -0.58$, $P > 0.05$, overall mean = 3.19 tailbeats s⁻¹; Fig. 2(e)]. Ventilation rate could be easily assessed from video recordings once fish were in an immobile position; fish averaged 20.4 opercular beats min⁻¹ with no difference between treatments [$t_{1,13} = -1.26$, $P > 0.05$; Fig. 2(f)].

This study provides some evidence that *C. cyanostigma* may suffer negligible behavioural effects when exposed to simulated OA conditions. Reports in other teleosts suggest that behavioural changes under OA conditions are caused by changes to Cl⁻ and HCO₃⁻ gradients across neuronal membranes, which in turn have effects on functioning of the neurotransmitter γ -aminobutyric acid (GABA)-A (Nilsson *et al.*, 2012; Tresguerres & Hamilton, 2017). This neurotransmitter is widely conserved among animals. Thus, although the observations made here were relatively acute, effects of exposure to high CO₂ were expected, especially in light of the large effect sizes reported in some of the previous studies (Dixson *et al.*, 2010; Munday *et al.*,

2013). Nevertheless, there is a growing number of studies that have failed to detect any statistically significant behavioural changes in coral-reef fish acclimated to high CO₂ (Bender *et al.*, 2015; Heinrich *et al.*, 2016; Sundin *et al.*, 2017). The measurements made in this study primarily involved the rapidity with which fish could orient themselves upon release at the surface, identify a nearby shelter and swim towards and enter that shelter. These metrics differ somewhat from those typically measured in this context (fish and OA), which have included things like avoidance or reaction to chemosensory cues (Dixson *et al.*, 2010; Ou *et al.*, 2015; Sundin & Jutfelt, 2016), or changes to activity levels or boldness (Hamilton *et al.*, 2013; Munday *et al.*, 2013), both of which have been implicated in lower survival for such fishes when released to the reef (McCormick *et al.*, 2013; Ferrari *et al.*, 2015). Yet, the measurements from this study share similarities with some other studies that have released fish back onto the reef following short-term exposure to elevated CO₂ (Devine *et al.*, 2012; Devine & Munday, 2013). An important caveat with the present study and some of the previous work in this field is that the fish were likely stressed by transport, handling and by being held in captivity (*i.e.* elevated circulating cortisol), which might be expected to affect the behavioural responses of fish in both the control and treatment groups.

Clearly, further research is required to determine the generality and repeatability of CO₂-associated behavioural and sensory impairments in coral-reef fishes. The effects of CO₂ on fish behaviour are of fundamental interest to fish biology and may have some useful applications (Kates *et al.*, 2012; Donaldson *et al.*, 2016). More broadly, in the context of OA, there is a need for experiments with longer acclimation times (Welch *et al.*, 2014; Sundin *et al.*, 2017) and that are designed to assess the effects of ecologically relevant combinations of elevated temperature and CO₂ (Ferrari *et al.*, 2015), given that these two factors are changing in tandem in the real

world (Hughes *et al.*, 2017). Ultimately, a more important driver of changes to fish communities (*i.e.* rather than potential changes to fish behaviour) is likely to be changes in coral habitat caused by warming and acidification (Booth & Beretta, 2002; Wilson *et al.*, 2006). Nevertheless, it is our hope that with more rigorous future experiments, including mesocosm experiments (Alsterberg *et al.*, 2013), an ecosystem perspective will emerge on the likely changes to coral reefs and associated fish communities over the next 50–100 years and perhaps also about the efficacy of mitigation options (Ilyina *et al.*, 2013; Feng *et al.*, 2016). The findings in the present study suggested that basic functioning of vision, swimming and decision making that were required by fish to locate and enter shelter upon release from the surface were not meaningfully affected by exposure to high CO₂. The difference in effect sizes among different studies in this field is perplexing and the larger question of how fish behaviour might be affected by future ocean acidification remains unclear. Further efforts are needed in what is still a relatively new area of research to clarify the apparent context-specificity of the effects of CO₂ on fish behaviour, with a focus on independent replication of existing studies and more effort to understand the effects of multiple drivers of ecosystem change.

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Experimental procedures were approved by the James Cook University Animal Ethics Committee in association with the Australian Institute of Marine Science (A1924).

Supporting Information

Supporting information can be found in the online version of this paper.

VIDEO S1. An exemplary behavioural trial (from camera 2) showing swimming behaviour and shelter seeking in a bluespotted rock cod *Cephalopholis cyanostigma* for 3 min after release into the field.

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FIGURE CAPTIONS

FIG. 1. Screen captures from videos recorded during behavioural trials with *Cephalopholis cyanostigma*, showing (a) the fish during its initial descent from the release point at the stern of the boat, (b) the fish approaching the small coral structure that was positioned near the boat and (c) the fish in position within the reef shelter.

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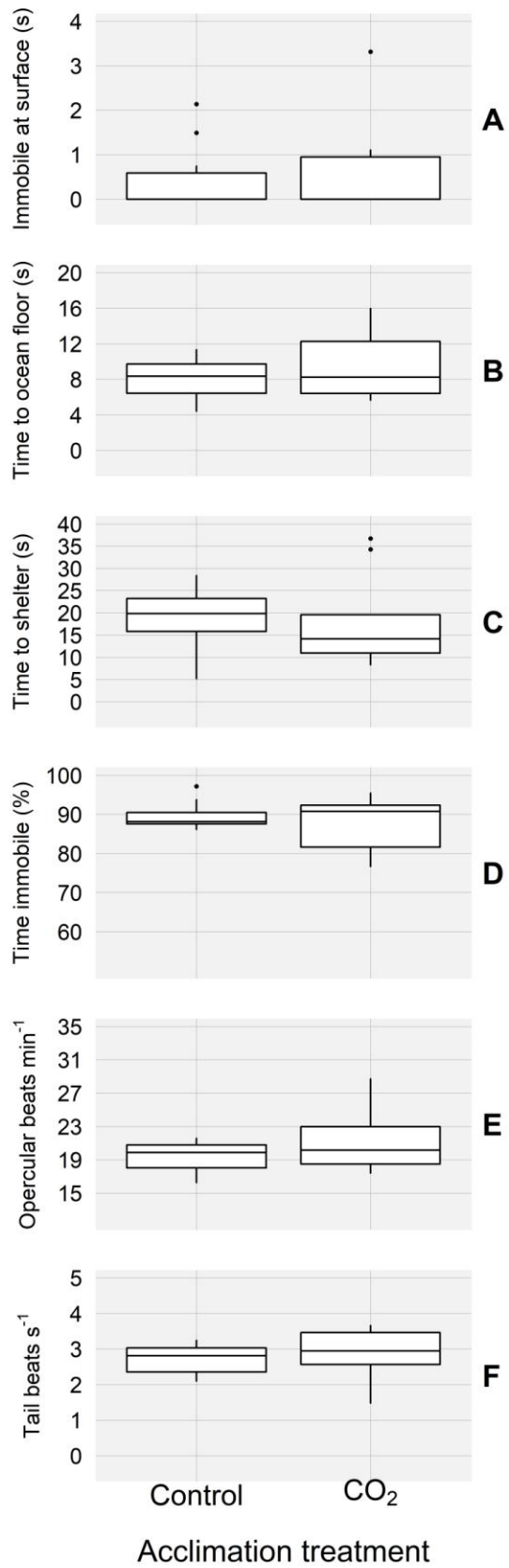
1 Change A, B, C to (a), (b), (c).



FIG. 2. Boxplot comparisons of post-release measurements between *Cephalopholis cyanostigma* acclimated to control water ($n = 10$) and water with elevated CO_2 ($n = 11$). (a) Time immobile at the surface upon release; (b) time taken for fish to reach the ocean floor after release; (c) duration from release to entering shelter within the reef structure; (d) overall percentage of the behavioural observation period spent immobile; (e) median opercular beat frequency in fish after they reached the reef shelter; (f) median tailbeat frequency in fish during swimming. The lower and upper ends of the box plots represent the 1st and 3rd quartiles, the horizontal line within each boxplot is the median, and the upper and lower whiskers are either the $1.5\times$ the interquartile range or the most extreme value (whichever is closer to the median).

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- 1 Change A, B, C etc to (a), (b), (c) etc.
- 2 Y-axes of (e) and (f): change min^{-1} to (min^{-1}) and s^{-1} to (s^{-1}) .



SUPPORTING INFORMATION

VIDEO S1. An exemplary behavioural trial (from camera 2) showing swimming behaviour and shelter seeking in a bluespotted rock cod *Cephalopholis cyanostigma* for 3 min after release into the field.