No effect of ocean acidification on individual-level variation in behaviour and susceptibility to predation in a Great Barrier Reef damselfish

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Keywords

Behavioural traits, coral reef fish, open field test, personality, predator-prey interaction, novel object test, repeatability, survival analysis

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Abstract

1) Ocean acidification, caused by rising carbon dioxide (CO_2) in the atmosphere, has been reported to negatively impact a wide variety of behaviours in fishes, including activity, exploration, and predator avoidance.
2) These effects have been documented at the population level, but many animal species naturally show large and repeatable individual-level differences in behaviour. How environmental stressors, such as ocean acidification, affect behavioural variation at the individual level remains largely unknown but is critically important to understand adaptation given natural selection operates on variation at the individual rather than population level.
3) Using a statistical approach allowing variation in means and variation in variance to be modeled within a single framework, we quantified individual-level differences across five behaviours in the coral reef damselfish *Pomacentrus amboinensis* (emergence time, activity level, time spent sheltering, thigmotaxis, novel object inspection). We measured behaviour in a novel environment assay, twice before (CO_2 ~450 µatm) and twice following acclimation to predicted end-of-century ocean acidification conditions (~1,100 µatm).
4) Following behavioural assays, we tested individual survival in a live predation experiment. We used predatory rock cod, *Cephalopholis microprion,* acclimated to the same CO_2 treatments as Ambon damsel and examined predictors of survival probability.
5) All behaviours in damselfish were moderately and significantly repeatable, with no marked differences in repeatability estimates between the ambient CO_2 and elevated CO_2 treatment groups. Exposure to end-of-century ocean acidification conditions had no effect on any of

the five behaviours measured, both in terms of group means and residual (within-individual) variance.

6) The probability of survival in the predation trials was similar for damselfish in the elevated and ambient CO_2 treatment groups. Smaller damselfish as well as those that spent a greater amount of time inspecting a novel object (i.e., bolder individuals) had a lower probability of survival regardless of their CO_2 treatment.
7) Our results challenge assumptions about the impacts of ocean acidification on coral reef fish behaviour and susceptibility to predation, both at the population and individual level. They also provide support for a trade-off between boldness and predation risk in fish.

Introduction

The partial pressure of carbon dioxide (CO_2) in the oceans has increased by ~45% over the past century, driving a process known as ocean acidification (OA) (Lüthi *et al.* 2008; Hönisch *et al.* 2012). This environmental change shows no signs of stopping: forecasts predict that oceanic CO_2 levels at the beginning of the next century will reach those not seen in the last 30 million years (Lüthi *et al.* 2008). Among the range of detrimental impacts reported for animals under ocean acidification, altered species interactions — including between predators and their prey — have received considerable attention (Draper *et al.* 2019). For example, lab-based experiments have suggested that end-of-century levels of CO_2 cause coral reef fishes to become highly attracted to the chemical cues of their predators (Dixson *et al.* 2010; Munday *et al.* 2010), implying increased predation risk under these scenarios (but see Clark et al. 2020, which questions the repeatability of this finding). Indeed, small demersal fishes exposed to end-of-century CO_2 and then returned to their natural coral reefs were reported to disappear faster than control individuals (Munday *et al.*

2010; Munday *et al.* 2012). The exact fate of these fish was not recorded, but suggestions for their disappearance included predator attraction, increased activity/boldness, and poorer homing/sheltering ability, all leading to increased predation (Munday *et al.* 2010; Briffa *et al.* 2012; Devine *et al.* 2012; Devine *et al.* 2013). However, a limited number of studies have directly tested the hypothesis that ocean acidification leads to decreased prey survival during a predator interaction. Interestingly, the few laboratory studies on predator-prey interactions between coral reef fishes following exposure to end-of-century levels of CO_2 suggest that the performance of both predators and prey may be altered (e.g. Allan *et al.* 2013; Allan *et al.* 2017). Conversely, and contrary to early findings (e.g. Dixson *et al.* 2010; Munday *et al.* 2010), there is an emerging consensus that ocean acidification may have only negligible effects on coral reef fish behaviour (Clark *et al.* 2020; Clements *et al.* 2022). This uncertainty makes it difficult to predict the impact of future ocean acidification on predator-prey relationships on coral reefs.

Many experiments exploring fish behaviour following exposure to ocean acidification report extremely low inter-individual variability in behavioural responses (e.g. Dixson *et al.* 2010; Munday *et al.* 2010; Clements *et al.* 2022). This is particularly alarming given that the resilience of a species to environmental perturbations depends on variation in individual responses (Tuomainen *et al.* 2011). Phenotypic variation is an inherent biological trait that forms the basis for natural selection and enables an adaptive response to stressors across generations.

Individual behaviours that are repeatable across time and contexts are often referred to as animal “personality” (Wolf *et al.* 2012; Carter *et al.* 2013; Roche *et al.* 2016), and interest in understanding the implications of these individual-level differences on trait evolution has grown over the last 20 years. Behavioural differences influence both the ways in which individuals interact with their environment as well as the outcome of biotic interactions such as predation, competition, parasitism, cooperation and mate-choice (Wolf *et al.* 2012; Roche *et al.* 2016; Dubois *et al.* 2022; Gradito *et al.* 2024). For example, individuals that are consistently ‘bold’ may expend more energy on activity, increasing their susceptibility to predation but also providing greater foraging opportunities compared with ‘shy’ individuals that remain in hiding for longer durations. Thus, in order to understand how a species might respond to environmental stressors, including ocean acidification, variability in individual responses should be explicitly considered rather than overlooked.

Focusing on individual-level variation as opposed to population mean responses to environmental perturbations has major conservation implications by allowing us to understand the traits responsible for conferring resilience to stressful conditions (Browman 2016; Killen *et al.* 2016). As such, behavioural ecologists are increasingly adopting a reaction norm approach to the study of behavioural variation in order to examine how behaviour and phenotypic plasticity are correlated and/or under selection in a given environment (Dingemanse *et al.* 2010; Dingemanse *et al.* 2013; Roche *et al.* 2016; Fig. 1). Hypothetically, a population-level increase in a behaviour (e.g., activity level) due to elevated CO_2 exposure can occur from all individuals increasing their activity score (Fig. 1 A1), or only some individuals exhibiting a marked increase in activity (Fig. 1 A2, A3). In this example, individuals that do not show a change in activity after elevated CO_2 exposure may be considered tolerant. Identifying tolerant individuals and their associated behaviours is a critical step in predicting how species will respond and adapt to future ocean acidification conditions.

To address several of the knowledge gaps highlighted above, we studied individual-level behavioural differences and reaction norms in the Ambon damselfish, *Pomacentrus amboinensis*, exposed to current day, ambient CO_2 conditions and elevated CO_2 conditions approximating end-of-century levels of ocean acidification. We also performed a live predation experiment with the same individuals to explicitly test the effect of ocean acidification on fitness while considering the influence of individual behavioural differences on the outcome of predator-prey interactions. Ours is the first study with the explicit aim of understanding how individual-level behavioural variation affects resilience and susceptibility to ocean acidification while incorporating a direct test of the fitness consequences of any CO_2-induced behavioural changes. The approaches used here enable us to address key outstanding questions in this field of research: (1) Are individual-level behavioural differences in Ambon damselfish repeatable?; (2) How does ocean acidification affect population- and individual-level differences in behaviour?; (3) Do effects of ocean acidification on behaviour lead to higher predation rates?; and (4) Do behavioural traits predict susceptibility to predation? We hypothesized that the repeatability of the behaviours we examined would depend on CO_2 treatment and that exposure to elevated CO_2 would induce behavioural differences compared with individuals in the ambient CO_2 treatment at both the population and individual levels. We also hypothesized that behavioural traits and CO_2 exposure would impact survival in a predation trial with bolder fish acclimated to elevated CO_2 being the most likely to be eaten.

Materials and methods

Fish collection and husbandry

Animals were collected and cared for under Marine Parks Permit no. G13/35909.1 issued by the Australian Government's Great Barrier Reef Marine Park Authority. All experiments were approved by the Animal Experimentation Ethics Committee at James Cook University (permit no.: A1924) according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, 7th edition 2007.

All experiments were conducted between January 12th and February 1st, 2016. Post-settlement juvenile *P. amboinensis* were collected by SCUBA divers on January 12-13 from lagoon reefs at Lizard Island on the northern Great Barrier Reef, Australia (14°40’ S; 145° 28’ E) using hand nets and a barrier net (10 mm stretch monofilament). Fish were transported within 90 minutes of capture to the aquarium facilities at the Lizard Island Research Station (LIRS) in 20 L buckets containing seawater aerated with battery-operated pumps. At LIRS, the fish were placed in 30 L holding tanks receiving seawater at ~ 750 ml min^{-1} from one of two flow-through 32 L header tanks (receiving seawater pumped directly from the reef) diffused with ambient air. At 24-48 h post-capture, fish were weighed (Mettler Toledo PL602-S, d = 0.01 g) and measured for standard length in a water-filled plastic bag to the nearest 0.1 mm with digital callipers. Seventy-four *P. amboinensis* (33.1 ± 2.5 mm; 1.43 ± 0.35 g; mean ± SD) were then randomly placed individually in 1 L flow-through plastic aquaria (17 × 12 × 7 cm, L × W × H) using the randomisation tool in Excel. These aquaria were supplied with seawater pumped directly from the reef. Each aquarium contained a 4 cm long white PVC pipe for shelter. The outer sides of each plastic aquarium were covered in black tape to prevent visual interaction and disturbance. Fish were fed daily with 0.5 ml of a commercial fish flake-saltwater slurry (TetraMin Tropical Flakes, Tetra, Blacksburg, VA). All animals fed readily by day three post-capture. Fish were habituated in their aquaria for three days prior to the start of the experiment. Seawater temperature in the aquaria was 29.5 ± 1˚C. Behavioural assessments for Experiment 1 (below) were performed on each individual twice under ambient CO_2 conditions, then half the fish were acclimated to elevated CO_2 for 4-6 days and half were maintained at ambient CO_2 conditions for the same length of time before behavioural assessments were performed another two times on all individuals. Predation experiments for Experiment 2 (below) were conducted 1-4 days following the last (4^{th}) behavioural trial.

Experiment 1: behavioural trials before and after CO_2 exposure

We examined behavioural differences among *P. amboinensis* using a novel environment assay. This assay is a modified version of the open-field test in which an individual is introduced into an unfamiliar environment, where the environment also included a novel object (Carter *et al.* 2013; Roche *et al.* 2016). Each assay lasted 25 min and was repeated twice (two days between trials) on all fish at ambient CO_2 (458 ± 17.9 µatm; mean ± standard deviation) and then two more times (two days between trials) after half the fish were acclimated to elevated CO_2 or maintained at ambient CO_2 conditions (see CO_2 exposure below and Fig. S1). All trials were conducted between 9:00-16:00, and the time of day was randomized across replicates to control for diurnal patterns in activity. Each trial was filmed (top view) with a digital camera (GoPro Hero 3+ black; GoPro, San Mateo USA). The stock lens of the camera was replaced with a commercially available lens to avoid image distortion (4.14 mm f/3.0 86° HFOV 5MP GP41430; Peau Productions Inc., San Diego, USA). We presented a note with the fish ID and trial number at the start of each video (Clark *et al.* 2016). Fish were transferred from their individual aquaria to the experimental arena using their water-filled shelter (the experimenter blocked both ends of the PVC shelter with the palm of their hands). Fish either entered their shelter when the experimenter approached their aquaria or were carefully placed into the shelter by the experimenter. This procedure minimized air exposure and reduced handling stress prior to introduction into the experimental arena. In instances where a fish was accidentally air exposed, we noted the handling time to account for differences in handling stress prior to the start of the experiment.

The experimental arena (38 × 28 × 30 cm, L × W × H) had white, opaque sides and was devoid of structure other than a novel object affixed in the centre. Four different novel objects were used (one object was used per arena at any given trial), each measuring approximately 2 cm in diameter and 8 cm in height. The object was changed when the assay was repeated, and the order of objects was randomized between fish and trials such that individual fish were presented with each object only once. The experimenter positioned the shelter and the fish at a fixed, pre-determined location in the arena and exited the room for the duration of the trial to minimize disturbance. The fish was then free to exit its shelter and explore its surroundings over the 25-minute duration of the trial. Water height (seawater pumped directly from the reef) was maintained at 7 cm and the arena was emptied and rinsed with water between each trial. At the end of each trial, the fish was returned to its individual aquarium with its shelter.

To avoid observer bias, we extracted behavioural data from the videos using the automated tracking software ViewPoint (ZebraLab, Lyon, France). For each trial, we recorded five behavioural measurements: 1) emergence time (the time at which the fish's entire body first exited the shelter in seconds); 2) activity level (the distance covered during the trial [25 min] after emergence in mm per minute), 3) sheltering (the time spent in the vicinity of the shelter [within 3 cm; approximately 1 BL] in seconds); 4) thigmotaxis (the time spent within 3 cm [approximately 1 BL] of the arena walls in seconds); and 5) novel object inspection (time spent within 5 cm i.e. <2 body lengths, of the novel object) in seconds.

CO_2 exposure

Once all individuals had repeated the behavioural assay twice under ambient CO_2, half of the fish (n = 37) were maintained in ambient water at present-day pCO_2 (ambient; 458 ± 17.9 μatm; mean

± SD) and the other half (n = 37) were exposed to end-of-century pCO_2 levels (elevated; 1104 ± 175 μatm; mean ± SD) for 4-6 days. This exposure time was chosen to be consistent with studies reporting altered behaviour in coral reef fishes and reports that longer exposure durations (10 or 25 days) do not alter the response any further (e.g. Munday *et al.* 2010; Allan *et al.* 2013; Munday *et al.* 2013). Control aquaria received seawater from one of two flow-through 32 L header tanks diffused with ambient air and filled at ~ 750 ml min^{-1} from seawater pumped from the reef. CO_2-treatment aquaria received water from one of two additional aerated header tanks (32 L, flow-through filled at ~ 750 ml min^{-1}) in which the pCO_2 level was gradually increased over 24 h using pH stat computers (Aqua Medic GmbH, Bissendorf, Germany) connected to solenoid valves regulating administration of 100% CO_2. We monitored pCO_2 in the header tanks and holding tanks multiple times daily using a handheld CO_2 meter (Vaisala GMT 222, Finland) connected to an aspiration pump (Vaisala GM 70, Finland) and a submerged gas-permeable PFTE probe (Qubit Systems, Kingston, Canada) following (Hari *et al.* 2008; Jutfelt *et al.* 2013). The Vaisala CO_2 meter was factory calibrated prior to experiments. The experimental design and CO_2-dosing system thus followed best practices for ocean acidification research (Reibesell *et al.* 2011; Cornwall *et al.* 2015).

Experiment 2: predation trials

We collected 22 dot-head rock cod (*Cephalopholis microprion*; hereafter rock cod) between January 12th and 17th 2016 from lagoon reefs nearby LIRS using hook-and-line. Rock cod are common predators of small fishes on the Great Barrier Reef (Vail *et al.* 2011). They were transported to the aquarium facility in 20 L aerated buckets within two hours of capture and housed in large (~300 l) flow-through aquaria. We selected 14 individuals that began feeding within 12h of capture and moved them to individual 32 L white opaque plastic aquaria (38 × 28 × 30 cm, L × W × H). Each aquarium contained a 12.5 cm long pvc pipe (55 mm diameter) providing shelter for the rock cod at one end of the tank. At the other end, we positioned six pieces of half pvc pipe (55 mm diameter) glued together to create a three-dimensional refuge for *P. amboinensis*. This allowed us to standardize the refuge size and location across all aquaria. The predator shelter and prey refuge were affixed to the bottom of the tank with silicone. To habituate rock cod in these aquaria, we fed them pieces of cut, defrosted pilchard during four days prior to the experiments. During this time, seven rock cod were kept in present-day control water (508 ± 48.4 μatm; mean ± SD) and seven were exposed to end-of-century $p\text{CO}_2$ levels (1229 ± 252 μatm; mean ± SD) with seawater temperature at approximately 29.5 ± 1˚C. CO_2 dosing and measurements followed the procedures as detailed in the *CO_2 Exposure* section above. Two header tanks were used for the control fish and four for the CO_2 fish.

Predator trials commenced when experiment 1 had been completed (January 28 – February 1). Twenty minutes before the onset of a predation trial, we introduced an opaque partition in the middle an aquarium containing a rock cod to restrict *C. microprion* to the area of the tank containing the shelter. One *P. amboinensis* from experiment 1 was introduced to the half of the tank, using its PVC shelter as in Experiment 1, and allowed a 20-minute exploration period. We then lifted the partition, and allowed the predator and prey to interact for 60 min. We filmed interactions with the same cameras as in Experiment 1 a digital camera mounted above the aquarium (GoPro Hero 3+ black fitted with a commercial lens). Prey that were not consumed within 60 min were returned to their holding aquarium. Control and CO_2-treated *P. amboinensis* were paired with a predator receiving the same experimental treatment using stratified randomization so that each predator received approximately the same number of prey, which spanned a range of behavioural scores. Each predator was tested between 5 and 6 times with different prey individuals. Each predator was tested only once in a given day and all predators were fed one piece of pilchard daily, in the evening.

Statistical analysis

Experiment 1: behavioural trials before and after CO_2 exposure

We checked the distributions of each of the five behavioural measurements (Fig. S2) and determined appropriate transformations to comply with the normality assumption of model residuals. We used the R package *lme4* (Bates *et al.* 2015) to fit five general Linear Mixed-effects Models (LMM), one for each behavioural measurement. We specified the following fixed effects in each model: treatment (binary variable: ambient vs. elevated CO_2); trial number (ordinal variable from 1 to 4 with trials 3 and 4 corresponding to elevated CO_2 exposure for the treatment group); the interaction between treatment and trial number; and the standard length of fish (continuous variable). The random effects for these models included trial number as random slopes and fish identity as random intercepts. Activity level and thigmotaxis (time spent close to the wall) were square-root-transformed; latency to approach a novel object was log-transformed after adding 0.5; time to emergence from the shelter was log-transformed; time spent in shelter was not transformed (Fig. S2).

We used the *brms* package (Bürkner 2017) to fit double-hierarchical general linear mixed-effects model (DHGLM), one for each of the five behavioural measurements. A DHGLMs comprises two parts, allowing variation in means and variation in variances to be modelled within a single framework (Cleasby *et al.* 2015; O'Dea *et al.* 2022). The mean component of the DGHLMs was identical to the LMMs above, and the variation component included the fixed effects treatment, trial, and their interaction, as well as the random effect fish identity. The DGHLM allowed us to investigate the effect of CO_2 treatment on both mean and residual variance (or within-individual variance), and to compute an estimate of population-wide variation in predictability (CV_P, the coefficient of variation in predictability) (Cleasby *et al.* 2015). Predictability refers to the level of variability in an individual's behaviour in a given environment (i.e., within-individual variation in behaviour), in contrast to repeatability, which is a measure of the consistency in how individuals differ in their average phenotypes (i.e., between-individual variation in behaviour; see below). CV_P represents standardized variation in within-individual variance among individuals and can serve as a standardized effect size for comparisons across traits, groups, or studies.

We used the *rptR* package (Stoffel *et al.* 2017) with 10,000 bootstrapping iterations to calculate three estimates of repeatability (*R*) for each of the five behavioural measurements: one for all fish, irrespective of treatment group (N=73); one for fish in the ambient CO_2 treatment group (N=37); and one for fish in the elevated CO_2 treatment group (N=36). In total, we computed 15 *R* estimates. *R* ranges between 0 and 1 and is the proportion of the total phenotypic variance attributable to differences between individuals; it provides a standardized measure of the consistency of phenotypes across time or contexts (Nakagawa *et al.* 2010; Roche *et al.* 2016). We note that *R* estimates can be obtained from DGHLMs, but these models could not provide separate estimates for the ambient and elevated CO_2 treatment groups in our study (see Cleasby *et al.* 2015). The *rptR* package uses mixed models from the *lme4* package to obtain the repeatability of behavioural measurements; we obtained 'adjusted' *R* estimates by fitting trial number as a fixed effect to control for time-related changes in behaviour (Mitchell *et al.* 2019). We tested whether *R* differed between the ambient and elevated CO_2 treatment groups for each behavioural measurement by calculating contrasts between the two groups (five contrasts in total). When the

95% confidence interval (CI) for a given contrast excluded zero, we considered the two *R* estimates to be statistically different.

Experiment 2: predation trials

We examined differences in the survival of elevated CO_2-exposed (N=34) and ambient CO_2 (N=34) Ambon damsel in predation trials with a Cox Proportional Hazards model, using the *survival* package (Therneau 2020). Five Ambon damsel used in *Experiment 1* were excluded from this analysis due to issues during the predation trials (i.e., camera malfunction, manipulation error). Seven explanatory variables were included in the model: CO_2 treatment, prey body size, and the mean of each of the five behavioural measurements recorded four times per Ambon damsel in *Experiment 1*. Predator identity was specified as a cluster (i.e., random) variable to account for repeated measurements. We examined the assumption of proportional hazards using cox.zph() in the package *survival* and *ggcoxdiagnostics()* in the package *survminer* (Therneau 2020).

We examined differences in the number of predatory attacks resulting in capture for fish in the elevated CO_2 (N=30) and ambient CO_2 (N=28) groups with a Generalized Linear Mixed-effects Model (GLMM), specifying a Poisson error distribution. Fixed and random effects in the model were the same as in the survival analysis. Marginal ($R^2_{GLMM(m)}$) and conditional ($R^2_{GLMM(c)}$) R-squared values were computed with *r.squaredGLMM()* in the package *MuMIn* (Barton 2016). We examined model assumptions with a qqplot of residuals and other diagnostic plots of residuals using *simulateResiduals()* in the package *DHARMa* (Hartig 2018).

The assumptions of both models were met although the was an indication overdispersion and slight quantile deviations for the GLMM (Dispersion test *P*=0.040). All analyses were done in R 4.2.2 (R Core Team 2023). We used the packages *ggplot2* (Wickham 2016), *tidybayes* (Kay 2024), *emmeans* (Lenth 2025), and *survminer* (Therneau 2020) for data visualization.

Results

Experiment 1: behavioural trials before and after CO_2 exposure

Exposure to projected end-of-century CO_2-induced aquatic acidification had no effect on any of the five behaviours measured in *P. amboinensis,* both in terms of group means (i.e., population-level) (Fig. 2, Table 1) and residual (i.e., within-individual) variance (Fig. 3, Table 1). Thigmotaxis decreased, and time spent near the novel object increased with trial number for fish in both the ambient CO_2 and elevated CO_2 groups, indicating habituation to the experimental setup and/or procedure in both treatment groups (Fig. 2, Table 1). No such population-level trends were observed for emergence time, activity level, or time spent sheltering (Fig. 2, Table 1). Intra-individual variability decreased with trial number for all five behaviours for fish in both the ambient CO_2 and elevated CO_2 groups (Fig. 3), indicating some degree of habituation to the setup/manipulation.

All behaviours were moderately and significantly repeatable, with no marked differences in repeatability estimates between CO_2 groups (Table 2). There was substantial variability in predictability (i.e., within-individual variance) for all five behavioural measurements for both groups (CV_p; see Table 1).

Experiment 2: predation trials

The probability of survival in a predation trial was similar for Ambon damsel in the elevated CO_2 and ambient groups (Fig. 4), with fish in both treatments having a similar hazard of death (ambient

CO_2 treatment hazard ratio relative to elevated CO_2 treatment group = 0.88, 95% CI = 0.43–1.78, *P*=0.719; Table 3). Body size, emergence time, activity level, time sheltering and thigmotaxis were unrelated to survival probability (Table 3). Ambon damsel that spent a greater amount of time inspecting a novel object had a lower probability of survival (hazard ratio = 2.66, 95% CI = 1.36–5.20, *P*=0.004; Table 3).

The number of predatory attacks leading to capture was similar for fish in the elevated CO_2 (mean ± SD: 5.96 ± 3.18) and ambient CO_2 groups (mean ± SD: 5.39 ± 3.86) (GLMM estimate ± SE: -0.040 ± 0.182, *P*=0.827; Table S1, Fig. S3). Predators required more attacks to capture large Ambon damsel (estimate ± SE: 0.147 ± 0.067, *P*=0.027) as well as Ambon damsel that spent more time near a shelter (estimate ± SE: 0.490 ± 0.243, *P*=0.044) and in the vicinity of the novel object during the repeated behavioural trials (estimate ± SE: 0.386 ± 0.137, *P*=0.005) (Table S1). The proportion of the total variance explained by the model's fixed effects ($R^2_{GLMM(m)}$) was 0.19 and the proportion of the variance explained by fixed and random effects ($R^2_{GLMM(m)}$) was 0.35.

Discussion

Studies on the impacts of end-of-century ocean acidification on coral reef fish behaviour are largely based on comparisons between treatment means rather than individual-level effects. Although it has been argued that some individuals are potentially more tolerant to elevated CO_2 exposure than others (Tresguerres *et al.* 2017; Esbaugh 2018), the extent to which individual behaviour affects responses to ocean acidification has never been tested.

We investigated the repeatability, individual- and population-level differences in five commonly measured behaviours as well as survival outcomes during a predation trial in the context of ocean acidification. All individuals habituated to the experimental set-up as evidenced by decreased intra-individual variability over the course of the four behaviourial trials (Fig. 3). Habituation to experimental arenas is common when repeatedly measuring behaviour, especially in laboratory settings when the timing between assays is short (Martin *et al.* 2008; Blumstein 2016). Nevertheless, we found that all five behaviours measured were significantly repeatable in both treatments, indicating that our assays measured consistent behavioural differences in Ambon damsels. Our repeatability estimates (0.278 – 0.497; Table 2) are relatively high considering that when measured in the laboratory and on ectotherms, estimates tend to be lower than when measured in the field and/or on endotherms (Bell *et al.* 2009). Contrary to widespread assumptions, this behavioural consistency was not affected by CO_2 treatment: both control and elevated CO_2 groups had similar estimates of repeatability in all behaviours, and population reaction-norms followed similar trends in both groups even after CO_2 exposure (scenario B1 in Fig. 1). This suggests that individual behavioural traits are not differentially impacted by CO_2 exposure, providing evidence refuting the suggestion that some individual phenotypes are more tolerant or susceptible to elevated CO_2 exposure than others.

When population-level differences in behaviours were considered, exposure to projected end-of-century CO_2 had no effect on any of the five behaviours measured in Ambon damselfish. Our results are in line with Clark et al. (2020) suggesting that CO_2 exposure does not meaningfully alter coral reef fish behaviour. This contrasts with earlier literature showing large effects of CO_2 exposure on many aspects of fish behaviour (reviewed in Briffa *et al.* 2012; Clements *et al.* 2015; Clements *et al.* 2022; Clark *et al.* 2024). A lack of a CO_2 treatment effect is perhaps not surprising since fishes have a well-developed acid-base regulatory system, which allows them to maintain tissue pH under acidified conditions (Ishimatsu *et al.* 2005). In fact, more than a century of research has shown that fish physiology is generally tolerant to elevated CO_2 levels including those that exceed climate change projections (Clark *et al.* 2024). That behavioural responses are similarly resilient is consistent with this idea, and there is growing empirical evidence of a decline effect in results testing for behavioural differences between control individuals and individuals exposed to ocean acidification (Clements *et al.* 2022; Clements *et al.* 2023). The results from our carefully designed study provide robust evidence that fish behaviour is not altered following exposure to elevated CO_2 whether individual or population-level effects are considered. Although ocean acidification may indeed have negative impacts on some marine life, especially calcifying organisms (Kroeker *et al.* 2010; Clark *et al.* 2024), our results highlight the growing agreement around the behavioural resilience of fishes to ocean acidification, as well as the value of transparent and reproducible research practices in achieving consensus around controversial research topics (O'Dea *et al.* 2021; Roche *et al.* 2022).

CO_2 treatment had no effect on the probability of prey fish surviving in a predation trial; Ambon damsel from both treatment groups had a similar hazard of death (Fig 4). The number of predatory attacks leading to capture was also similar between treatment groups. These results are consistent with several other studies suggesting that CO_2 exposure has limited effects on predator-prey interactions in coral reef fishes (Allan *et al.* 2017; Ferrari *et al.* 2017; McCormick *et al.* 2018), refuting early assertions, based on indirect evidence, that ocean acidification dramatically increases prey susceptibility to predation (e.g. Munday *et al.* 2010; Devine *et al.* 2013). Indeed, Allan et al. (2013) found that when both the predator and prey had been exposed to elevated CO_2 (as in our study), the capture rate was similar to that of the control group (where both predators and prey were exposed to present-day CO_2). Similarly, a study exploring the effect of elevated CO_2 on predation in freshwater fishes found no effect of CO_2 treatment on predator success or time to survival in prey (Midway *et al.* 2017). Our results further highlight the importance of behavioural tests on interacting animals when assessing the impact of anthropogenic stressors such as ocean acidification on complex ecological interactions such as predation.

Although elevated CO_2 had no impact on survival outcomes during predation trials in our study, we did observe small differences in the survival of Ambon damsel based on individual traits. We found that predators required more attacks to capture large Ambon damsel, which may be due to minor differences in mouth gape requirements of the predators and/or higher absolute burst swimming speeds in larger versus smaller Ambon damsel conspecifics (Taylor *et al.* 1985). While emergence time, activity level, time spent sheltering, and thigmotaxis behaviours were not related to individual survival, Ambon damsels that spent a greater amount of time inspecting the novel object had a lower probability of survival. This trend was independent of CO_2 treatment. Our results are consistent with the notion that bolder individuals face higher predation risk than shy individuals (Hulthén *et al.* 2017). However, as four out of five behaviours tested had no relationship with survival, the role of individual behavioural traits in predicting predation risk should be interpreted with caution. Furthermore, although we predicted higher mortality in bolder fish from the elevated CO_2 treatment group compared to the control group, the effect of behaviour on survival outcome was independent of CO_2 exposure. This result runs counter to those of several studies that have linked elevated CO_2 to increased boldness in fishes (e.g. Ferrari *et al.* 2011; Munday *et al.* 2014; Ferrari *et al.* 2017; Mitchell *et al.* 2022). Again, our results align with more recent studies demonstrating negligible effects of ocean acidification on fish behaviour (Clark *et al.* 2020; Clements *et al.* 2022; Clements *et al.* 2023; Clark *et al.* 2024) and do not support the notion of differences in individual tolerance or susceptibility to ocean acidification within a fish population.

Conclusion

Ocean acidification presents a growing threat to marine ecosystems globally as a result of anthropogenic activities. Concerns for how this change in water chemistry will impact aquatic organisms is warranted and an important area of continued research. Yet, there are limited resources to understand the complexities of ocean acidification impacts on marine life and thus researchers should focus their time and effort on species, lifestages, interactions and physiological processes that are most likely to suffer negative consequences as a result of ocean acidification in order to inform decision making and conservation strategies (Buxton *et al.* 2021). Based on our results, as well as the growing body of evidence from different species and study systems, we suggest that fish behaviour is not likely to be meaningfully impacted by near-future ocean acidification levels. Thus, future research should prioritize studying how other aspects of elevated atmospheric CO_2 impact marine fishes, including the rapid rise in the temperature of the global oceans.

Author contributions

DGR, BSR, JS, TDC and SAB conceived the study and designed methodology; DGR, BSR and SAB collected the data; TDC processed the data; DGR and SN analysed the data; DGR and SAB led the writing of the manuscript with substantial input from JS and TDC. All authors contributed critically to the drafts and gave final approval for publication. Our study brings together authors from a number of different countries, including two scientists based in Australia where the study was carried out.

Data and code availability

The data and analytical code to reproduce the results of the study are publicly available on the Open Science Framework and were shared with the editor and reviewers on manuscript submission: https://doi.org/10.17605/OSF.IO/CZVSF.

Conflicts of Interest

None to declare.

Acknowledgements

We thank the staff of the Lizard Island Research Station for logistical support. This study was supported by grants from Swedish Research Council Formas (Dnr: 2013-947), Magnus Bergvalls stiftelse, Stiftelsen Lars Hiertas minne, IRIS stipendiet, Wallenbergstiftelsen, Inez Johanssons stiftelse and Sederholms resestipendium to JS, the Fonds de Recherche du Québec Nature et Technologies (SAB, DGR), Discovery Grants from the Natural Sciences and Engineering Research Council of Canada (SAB, BSR), and an Australian Research Council Future Fellowship to TDC (FT180100154) funded by the Australian Government.

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TABLES

Table 1. Estimates and 95% confidence intervals (CI) for predictors included in five double hierarchical linear mixed-effects models (DHGLMs) assessing how end-of-century CO_2-induced aquatic acidification affects five behavioural measurements in the Ambon damselfish (*Pomacentrus amboinensis*): activity level (Activity), emergence time (Emergence), time spent inspecting a novel object (Novel), time sheltering (Sheltering), time close to the arena walls (Thigmotaxis). Predictors 1-4 relate to the mean component of the DHGLMs and predictors 5-7 relate to the dispersion component (i.e., residual variances for individuals). Estimates in bold have a 95% credible interval non-overlapping zero.

Predictor	**Activity (95% CI)**	**Emergence (95% CI)**	**Novel (95% CI)**	**Sheltering (95% CI)**	**Thigmotaxis (95% CI)**
Intercept	57.74	2.45	0.70	32.17	3.55
	(53.40, 61.90)	(1.74, 3.18)	(0.39, 1.02)	(27.69, 36.69)	(3.17, 3.92)
sigma_Intercept	2.37	0.73	-0.44	2.41	0.23
	(2.10, 2.63)	(0.50, 0.95)	(-0.66, -0.22)	(2.19, 2.62)	0.01, 0.43)

1. groupcontrol	1.05	0.09	0.27	-1.37	-0.48
	(-4.65, 7.00)	-0.92, 1.08)	(-0.17, 0.73)	(-7.76, 4.90)	(-1.01, 0.04)
2. trial	-0.96	-0.13	**0.51**	2.53	**-0.77**
	(-6.60, 4.83)	(-0.80, 0.56)	(0.18, 0.83)	(-3.08, 8.17)	(-1.31, -0.21)
3. SL	0.24	0.6	0.19	-5.09	0.16
	(-5.60, 6.13)	(-0.40, 1.61)	(-0.24, 0.63)	(-11.48, 1.46)	(-0.36, 0.66)
4. groupcontrol:trial	3.42	**-1.64**	0.24	-4.5	0.30
	(-4.51, 11.30)	(-2.54, -0.70)	(-0.21, 0.69)	(-12.29, 3.09)	(-0.48, 1.07)
5. sigma_groupcontrol	0.01	-0.13	0.06	-0.06	-0.12
	(-0.34, 0.37)	(-0.43, 0.19)	(-0.24, 0.36)	(-0.35, 0.22)	(-0.39, 0.15)
6. sigma_trial	**-0.56**	-0.2	-0.24	-0.22	-0.24
	(-1.01, -0.11)	(-0.58, 0.15)	(-0.69, 0.23)	(-0.66, 0.26)	(-0.64, 0.16)
7.	0.15	0.27	0.13	-0.34	-0.23
sigma_groupcontrol:trial	(-0.48, 0.80)	(-0.25, 0.82)	(-0.5, 0.78)	(-1.01, 0.28)	(-0.78, 0.31)

Table 2. Estimates of repeatability (*R*) and variation in predictability (CVp) for five behavioural traits measured experimentally four times in the Ambon damselfish, *Pomacentrus amboinensis*. Behaviour was assessed in all fish under ambient CO_2 conditions twice, and thereafter twice more after having exposed half of the fish to elevated CO_2. The difference in *R* between the ambient CO_2 group (control) and elevated CO_2 treatment group for each behaviour is presented as a contrast (Contrast). 95% confidence intervals are indicated in parentheses. The behaviours measured were activity level (Activity), emergence time (Emergence), time spent inspecting a novel object (Novel), time sheltering (Sheltering), and time close to the arena walls (Thigmotaxis).

Behaviour	**Overall *R* (95% CI)**	**Control *R* (95% CI)**	**Treatment *R* (95% CI)**	**Contrast (95% CI)**	**CVp (95% CI)**
Activity	0.278 (0.149, 0.402)	0.255 (0.068, 0.427)	0.303 (0.113, 0.472)	0.046 (-0.211, 0.302)	0.805 (0.625, 0.997)
Emergence	0.298 (0.169, 0.424)	0.317 (0.124, 0.488)	0.303 (0.118, 0.478)	-0.015 (-0.269, 0.247)	0.754 (0.533, 0.967)
Novel object	0.497 (0.363, 0.605)	0.568 (0.390, 0.703)	0.405 (0.210, 0.571)	-0.162 (-0.403, 0.082)	0.636 (0.324, 0.856)

Shelter	0.381 (0.247, 0.502)	0.427 (0.230, 0.588)	0.340 (0.147, 0.509)	-0.085 (-0.335, 0.174)	0.545 (0.202, 0.775)
Thigmotaxis	0.245 (0.118, 0.368)	0.243 (0.066, 0.413)	0.225 (0.043, 0.398)	-0.020 (-0.271, 0.229)	0.568 (0.175, 0.805)

Table 3. Hazard ratios (HR) for fixed-effects predictors in a Cox Proportional Hazards model examining differences in survival probability for CO_2-exposed (N=34) and control (N=34) Ambon damsel (*Pomacentrus amboinensis*) (prey). A hazard ratio lower than 1 indicates reduced hazard of death whereas a hazard ratio greater than 1 indicates an increased hazard of death. 95% confidence intervals, Z scores, and p-values are indicated. The predator (*Cephalopholis microprion*) was exposed to the same conditions of water chemistry as the prey.

Predictor	**HR**	**95% CI**	**Z**	***P***
Prey standard length	1.11	0.87, 1.42	0.833	0.405
Treatment_control	0.88	0.43, 1.78	-0.360	0.719
Emergence time	1.11	0.84, 1.47	0.746	0.456
Activity level	1.36	0.87, 2.14	1.349	0.177
Time sheltering	2.99	0.90, 9.96	1.785	0.074
Thigmotaxis	1.60	0.69, 3.71	1.084	0.278
Novel object	2.66	1.36, 5.20	2.871	0.004

FIGURES

Figure 1. Examples of hypothetical behavioural reaction norms from exposure to high CO_2. On average, individuals might exhibit either (A) an increase or (B) no change in a given behavioural trait (e.g. activity, anxiety). Different combinations of individual-level responses can underpin population-level patterns, where individuals might or might not respond similarly to high CO_2 exposure. A population-level increase in behaviour can result from (A1) all individuals increasing their behaviour or (A2-A3) some individuals exhibiting a sharp increase in behaviour and others, no change in behaviour. A lack of population-level change in behaviour can result from: (B1) the behaviour of all individuals remaining unchanged; (B2) differences in the behaviour of individuals accentuating; or (B3) no consistent changes in behaviour across individuals.

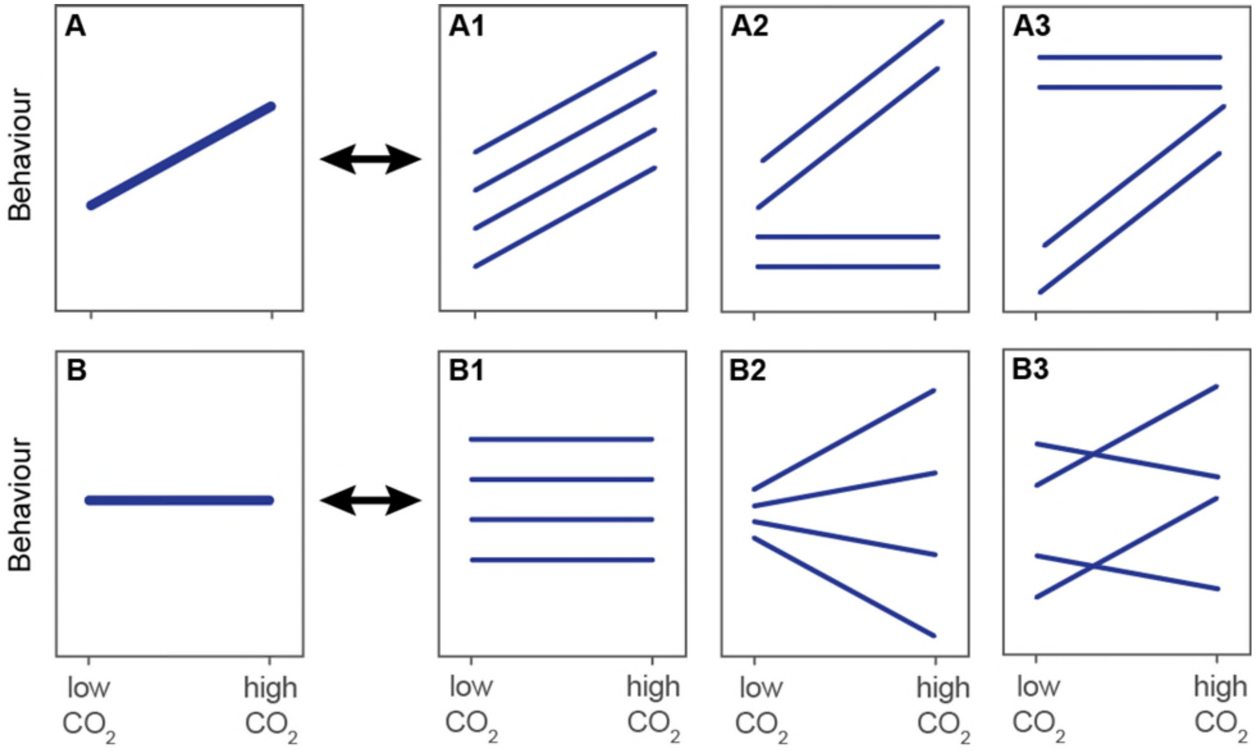


Figure 2. Reaction norm plots of 5 behavioural measurements recorded across 4 trials for Ambon damselfish (*P. amboinensis*) exposed to present-day levels of dissolved CO_2 (Ambient CO_2; n=36) and projected end-of-century CO_2-induced aquatic acidification (Elevated CO_2, n=37): (A) emergence, (B) activity, (C) time sheltering, (D) thigmotaxis and (E) inspection of a novel object. Fish in the control group were exposed to present-day CO_2 levels in all 4 trials, whereas fish in the CO_2 group were exposed to present-day CO_2 levels in trials 1-2 and projected end-of-century CO_2 levels in trials 3-4. Data points and coloured lines represent individual fish; the black line is the population-level (i.e., mean) response with the 95% CI credible interval indicated by dotted lines.

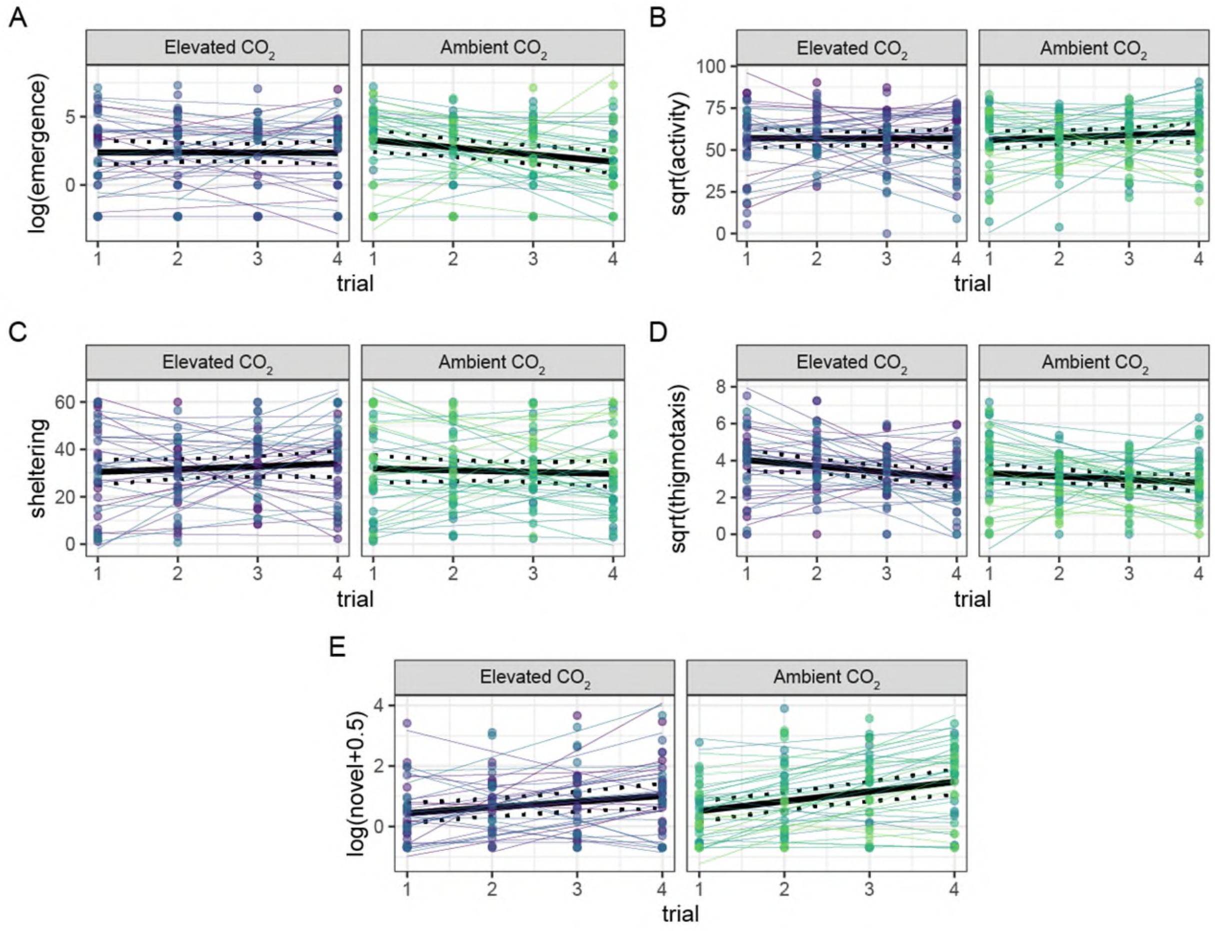


Figure 3. Intra-individual variation (residual variance for individual fish) in 5 behavioural measurements across 4 trials in Ambon damselfish (*P. amboinensis*) exposed to present-day levels of dissolved CO_2 (Ambient CO_2, n=36) and projected end-of-century CO2-induced aquatic acidification (Elevated CO_2, n=37): (A) activity, (B) time sheltering, (C) thigmotaxis, (D) inspection of a novel object, and (E) time to emergence from the shelter. Fish in the control group were exposed to present-day CO_2 levels in all 4 trials, whereas fish in the CO_2 group were exposed to present-day CO_2 levels in trials 1 and 2, and projected end-of-century CO_2 levels in trials 3 and 4.

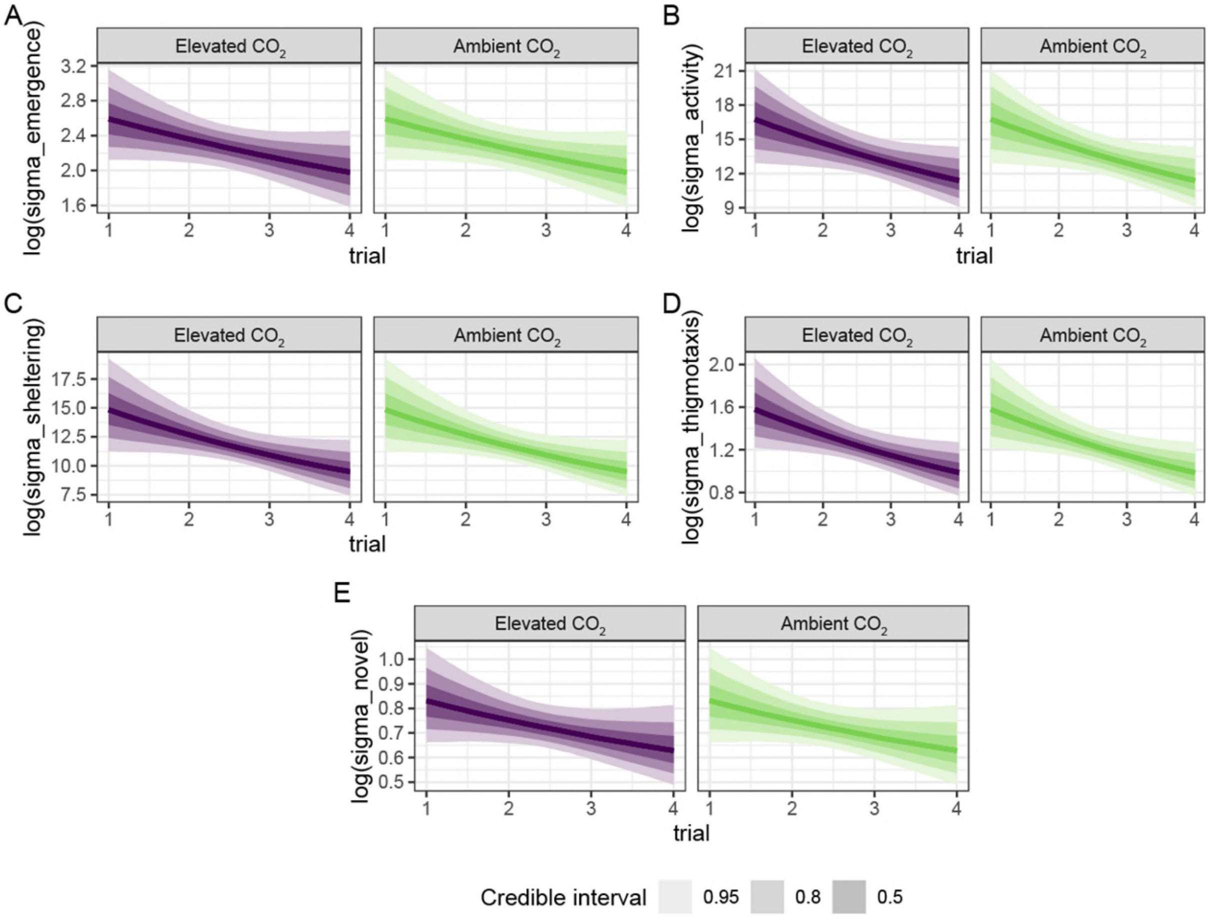
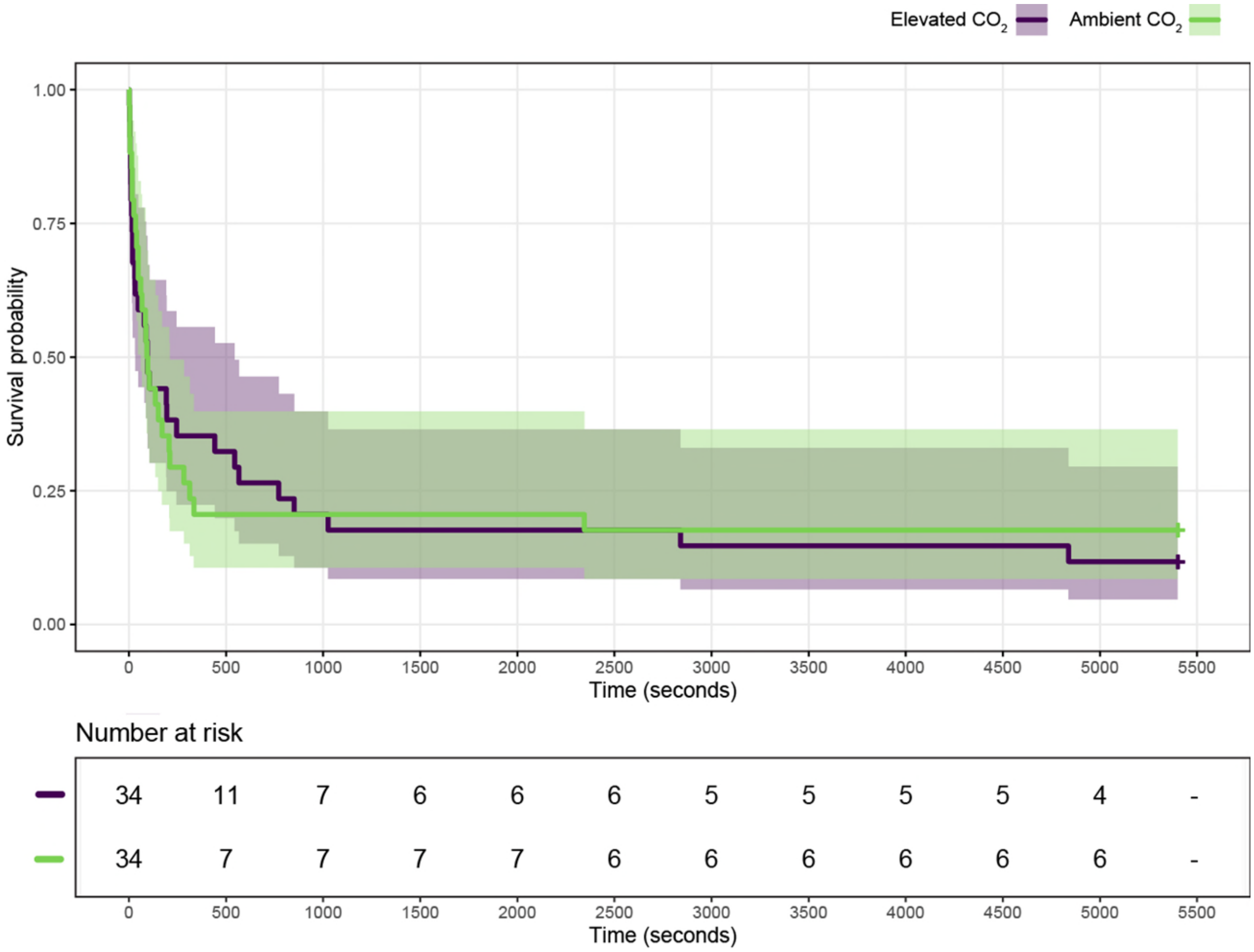


Figure 4. Kaplan Meier survival curves for Ambon damselfish (*P. amboinensis*) exposed to present-day levels of dissolved CO_2 (Ambient CO_2, n=34) and projected end-of-century CO_2-induced aquatic acidification (Elevated CO_2, n=34). Fish were subjected to a 90 min predation trial involving a predator, the rock cod *Cephalopholis microprion*, exposed to the same conditions of water chemistry.



SUPPLEMENTARY MATERIAL

Table S1. Summary table with estimates, standard errors (SE), Z and *P* values for fixed effects included in a Poisson generalized linear mixed-effects model examining differences in number of predatory attacks that resulted in the capture of Ambon damsel (*Pomacentrus amboinensis*) in ambient CO_2 (N=28) and elevated CO_2 conditions (N=30). The sample size excludes Ambon damsel that were not captured. Predators (*Cephalopholis microprion*) were exposed to the same conditions of water chemistry as their prey. Predator identity was included as a random effect.

Explanatory variable	**Estimate**	**SE**	**Z**	***P***
Prey standard length	0.147	0.067	2.210	0.027
Mean emergence time	0.017	0.075	0.223	0.823
Mean time active	0.168	0.108	1.555	0.120
Mean time in/near shelter	0.490	0.243	2.012	0.044
Mean time displaying thigmotaxis	0.260	0.146	1.785	0.074
Mean time near novel object	0.386	0.137	2.815	0.005
Treatment_control	-0.040	0.182	-0.219	0.827

Figure S1. Graphical representation of the experimental timeline used to examine the effect of exposure to end-of-century CO_2 levels on personality and susceptibility to predation in *Pomacentrus amboinensis*. Fish personality was assessed by repeatedly measuring behaviour in the same fish in a novel environment assay on specific days (orange arrows) during 4 days exposure to present day, ambient, CO_2 (control, n=74) followed the next day by treatment with either present day CO_2 or elevated CO_2 for 6 days (n=37 each). At the end of the CO_2 treatments, each fish was exposed to a predator-prey trial (using *Cephalopholis microprion* as predator) under the same CO_2 treatment.

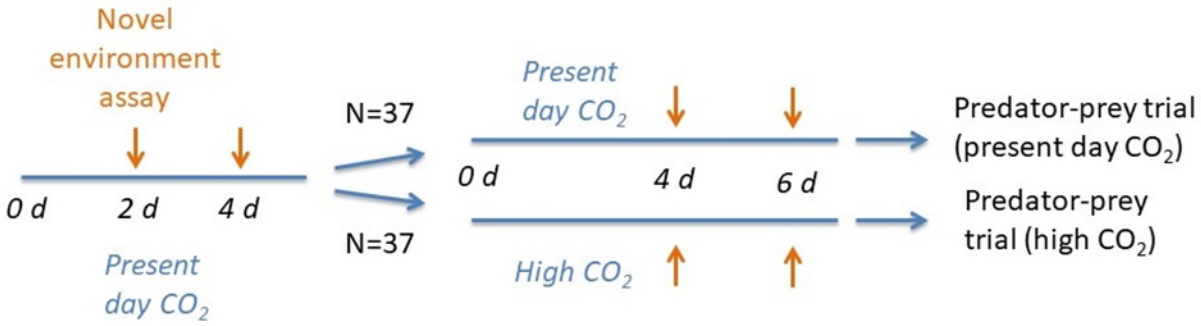


Figure S2. The distribution of model residuals (m_) after the following transformations: time to emergence from the shelter (emergT) was log-transformed; activity levels (activity) and thigmotaxis (thigmotaxis) were square-root-transformed; time spent sheltering (shelter) was not transformed; and time near a novel (novel) object was log-transformed after adding 0.5.

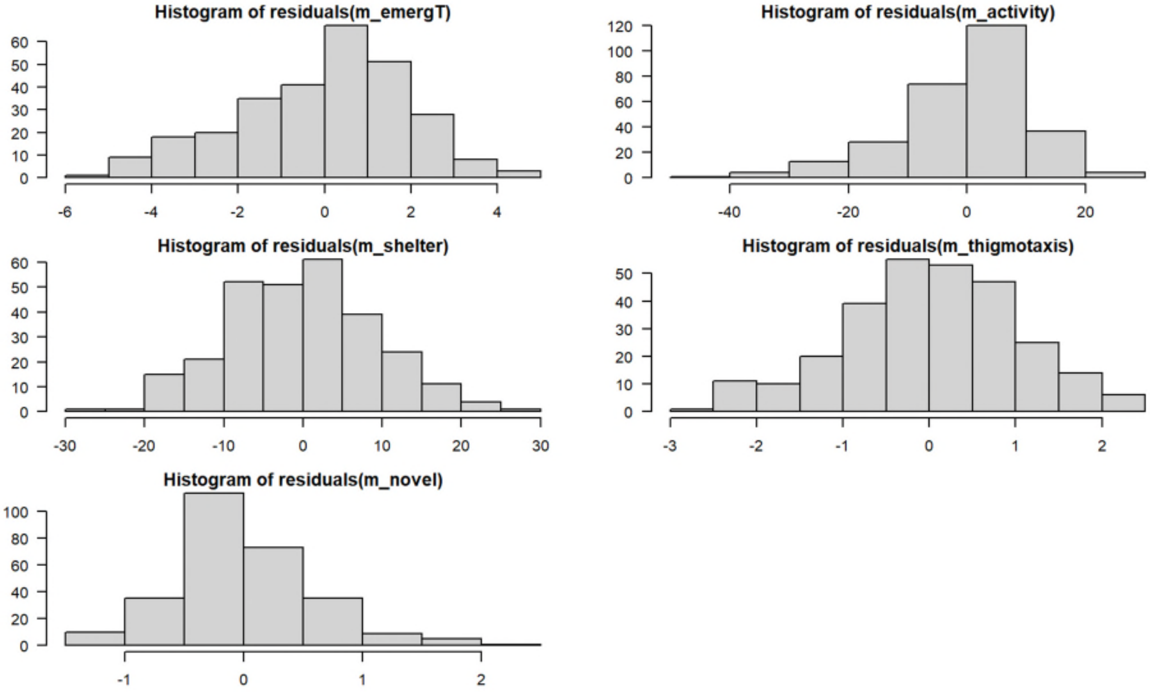


Figure S3. The number of predatory attacks by dot-head rock cod (*Cephalopholis microprion*) resulting in the capture of Ambon damsel (*Pomacentrus amboinensis*) in ambient CO_2 (N=28) and elevated CO_2 (N=30) treatment groups. Ambon damsel that were not captured are not shown.