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SYMPOSIUM

Predation in High CO_2 Waters: Prey Fish from High-Risk Environments are Less Susceptible to Ocean Acidification

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Synopsis Most studies investigating the effects of anthropogenic environmental stressors do so in conditions that are often optimal for their test subjects, ignoring natural stressors such as competition or predation. As such, the quantitative results from such studies may often underestimate the lethality of certain toxic compounds. A well-known example of this concept is illustrated by the marked increase in the lethality of pesticides when larval amphibians are concurrently exposed to the odor of potential predators. Here, we investigated the interaction between background levels of environmental predation risk (high vs. low) and ocean acidification (ambient vs. elevated CO_2) in 2 × 2 design. Wild-caught juvenile damselfish, *Pomacentrus amboinensis*, were exposed in the laboratory to the different risk and CO_2 conditions for 4 days and released onto coral reef patches. Using a well-established field assay, we monitored the *in situ* behavior and mortality of the damselfish for 2 days. We predicted that juvenile fish exposed to elevated CO_2 and high-risk conditions would display more severe behavioral impairments and increased mortality compared to fish exposed to elevated CO_2 maintained under low-risk conditions. As expected, elevated CO_2 exposure led to impaired antipredator responses and increased mortality in low-risk fish compared to ambient CO_2 controls. However, we failed to find an effect of elevated CO_2 on the behavior and survival of the high-risk fish. We hypothesized that the results may stem from either a behavioral compensation or a physiological response to high risk. Our results provide insights into the interactive nature of environmental and natural stressors and advance our understanding of the predicted effect of ocean acidification on aquatic ecosystems.

Introduction

Almost all organisms live in an environment that has been altered in some ways by human activities, whether these alterations take the form of habitat degradation, the addition or removal of species via introduction or extinction, pollution or climate change (Brook et al. 2008). While a number of studies have shown the ways in which most of these stressors affect individuals, populations, communities, or ecosystems, we have comparatively little knowledge on the way most stressors may interact to interfere or enhance each other's effects (Clements and Rohr 2009; Kroeker et al. 2013; Manciocco et al. 2014; Ferrari et al. 2015d).

In aquatic ecotoxicology, it is common to examine the action of individual pollutants on the physiology and survival of fish and aquatic insects, as these endpoints are often used as indicators of the general impact of the pollutant on biodiversity (Martinez-Haro et al. 2015) and may inform legislative bodies setting environmental quality guidelines. However, the effects of particular pollutants can be highly

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dependent on environmental conditions and natural stressors, such as predation risk, may render pesticides far more deadly (Relyea and Mills 2001; Relyea 2003). In particular, these studies highlighted that the perceived risk of predation, in the form of predator smells, was enough to turn a sub-lethal dose of pesticide into a lethal one, presumably via the added stress of perceived risk (Sih et al. 2004).

Predation risk is a natural and omnipresent stressor for the vast majority of species (Lima and Dill 1990). Predation is a strong selective force shaping the behavior, morphology, and life-history of prey (Ferrari et al. 2010); however, it is rarely included as an interactive stressor in environmental studies. Given that most toxicology studies are performed in optimal conditions for their test individuals, such as those outlined in the Organization for Economic Co-operation and Development (OECD) guidelines and other protocols (Ankley et al. 2010), laboratory-derived results on the lethality and individual-level effects of particular stressors may represent a "best-case" scenario. In the natural world, individuals are subject to the pressures of competition (food, mate, or otherwise) and predation which may magnify the effects of anthropogenic stressors. For marine organisms, one such stressor that is predicted to increase dramatically in the coming decades is ocean acidification, which is caused by the uptake of additional anthropogenic carbon dioxide (CO_2) by the ocean. Understanding the mechanisms by which ocean acidification may affect marine organisms and its interacting effects with other stressors is important to predicting how aquatic ecosystems will be affected by climate change in the near future (Kroeker et al. 2013; Gaylord et al. 2015; Nagelkerken and Munday 2015; Nilsson and Lefevre 2016).

Ocean chemistry is changing faster than any time during the last 65 million years (Ridgwell and Schmidt 2010), and possibly the last 300 million years (Hönisch et al. 2012). One-third of all anthropogenic CO_2 emissions released into the atmosphere since the beginning of the industrial revolution has been absorbed by the oceans (Sabine et al. 2004), lowering the pH of the open ocean by 0.1 units (Hoegh-Guldberg et al. 2014). At the same time, the pCO_2 of the ocean is rising at the same rate as the atmospheric CO_2 (Doney 2010). Under current CO_2 emission scenarios (Representative Concentration Pathways, RCP 8.5), atmospheric CO_2 levels are projected to exceed 900 ppm by the end of this century (Collins et al. 2013), from current day levels of 400 ppm. Ocean acidification is known to affect the survival, growth, physiology, and calcification of marine organisms (Heuer and Grosell 2014) as well as the behavior of fishes and some invertebrates (Briffa et al. 2012; Clements and Hunt 2015; Nagelkerken and Munday 2015). Of particular concern is that increased levels of CO_2 are associated with marine organisms displaying a reduced and/or loss of antipredator response to visual and chemical cues from predators (Dixson et al. 2010; Ferrari et al. 2011a, 2011b; Manríquez et al. 2013; Chivers et al. 2014; Jellison et al. 2016), which could have significant effects on population success and community structure.

This study aimed to investigate the interactive effects of predation risk and CO_2 levels on the behavior and survival of juvenile coral reef fishes in the field. Settlement-stage juvenile damselfish were exposed to either a low- or high-risk environment, under ambient or elevated CO_2 conditions, in a 2 $\times$ 2 design. Fish were then released in the wild and their *in situ* behavior was recorded and mortality monitored for 2 days. If predation risk represents an additional stressor that enhances the detrimental effects of elevated CO_2, we predicted that fish exposed to high-risk and elevated CO_2 conditions would show more severe impairment of their antipredator behaviors and a reduced survival compared to fish exposed to low-risk and elevated CO_2 conditions.

Methods

Test species

Settlement-stage juveniles of the Ambon damselfish, *Pomacentrus amboinensis*, were collected overnight using light traps moored in open water of Lizard Island (14'40°S, 145'28°E), in the northern Great Barrier Reef, Australia in November 2013. Fish were captured ~100 m away from the reef and hence were naïve to the specific predators that awaited them upon settlement, including numerous species of wrasses, lizardfish, and dottybacks. This species naturally settles on reef environments where juveniles are exposed to a diverse range of predators.

Experimental design

The fish were maintained under high- and low-risk conditions for 7 days. On Day 4, the CO_2 treatment was superimposed onto the risk treatment, to produce a 2 $\times$ 2 design whereby fish were maintained under low or high risk, while being exposed to ambient or elevated CO_2 conditions.

Risk background: We manipulated the background level of risk for individual prey fish using previously established protocols (Ferrari et al. 2015b). Following

capture, juvenile damselfish were taken to the laboratory and placed in groups of five in a series of 24 5-L flow-through plastic aquaria with a flow rate of ~3 L/h. The fish were fed *ad libitum* with newly-hatched brine shrimp (*Artemia* sp.) three times per day and were allowed to acclimate for 24 h before starting the experimental treatment. Fish were then exposed to high- or low-risk conditions by introducing, upstream of the fish, a solution of injured conspecific cues (hereafter, alarm cue—high risk) or a seawater control (low risk) into the tanks three times per day for 7 days. Half the fish (12 randomly selected tanks) received the high risk treatment while the fish in the remaining half (12 tanks) received the low risk treatment. The alarm cue solution was prepared minutes prior to being used by making six vertical cuts on each side of 10, freshly euthanized (cold-shocked and pithed), donor conspecifics (13–14 mm SL) using a scalpel and then rinsing these fish in 60 mL of seawater. We injected 5 mL of this alarm cue solution into the conditioning tanks using a hose attached to a 5-mL syringe, giving a concentration of 2 cuts/L when injected into the tanks (Ferrari et al. 2015b). The timing of the three injections occurred randomly between 0800 and 1800 h with a minimum of 1.5 h between successive injections.

CO_2 treatment: On Day 4, the fish were assigned randomly to six replicate ambient-CO_2 (427 µatm pCO_2) or six replicate elevated-CO_2 (798 µatm pCO_2) 18-L aquaria, to obtain three tanks per CO_2 × risk combination. Each aquarium was supplied with ambient or elevated-CO_2 seawater at 1.12 L/min. Elevated-CO_2 seawater was achieved by dosing with CO_2 to a set pH. Seawater was pumped from the ocean into 60-L header tanks where it was diffused with ambient air (control) or 100% CO_2 to achieve the desired pH (elevated-CO_2 treatment). A pH-controller (Aqua Medic, Germany) attached to the CO_2 treatment header tank maintained pH at the desired level. Seawater pH_{NBS} (HQ40d, Hach, Colorado, USA) and temperature (C22, Comark, Norwich, UK) were recorded daily in each aquarium and seawater CO_2 confirmed with a portable CO_2 equilibrator and infra-red sensor (GMP343, Vaisala, Helsinki, Finland) (Munday et al. 2014b). Water samples were analyzed for total alkalinity by Gran titration (888 Titrando, Metrohm, Switzerland) to within 0.4% of certified reference material (Prof. A. Dickson, Scripps Institution of Oceanography). Carbonate chemistry parameters (Table 1) were calculated in CO2SYS (Pierrot et al. 2006) using the constants K1, K2 from Mehrbach et al. (1973) refit by Dickson and Millero (1987) for $KHSO_4$. Salinity data were obtained from moorings around Lizard Island, which form part of the Australian National Mooring Network Integrated Marine Observing System (IMOS) operated by the Australian Institute of Marine Science (AIMS). Water parameters are presented in Table 1. During this period, the risk regime continued. The volume of cues injected in the larger tanks was altered to obtain a final concentration of 2 cuts/L, as during the previous phase.

As in previous studies, working on the short-terms effects of elevated CO_2, the exposure to higher pCO_2 was rapid. However, previous studies have shown that pomacentrid larvae exposed to elevated CO_2 over a few days showed identical behavioral impairment as larvae raised under the same CO_2 levels from hatching (Munday et al. 2010b, 2014a), indicating that alterations in behavior were not due to a sudden CO_2 exposure. Furthermore, larval fish naturally experience a change in CO_2 environments as they transit from the open ocean to coral reef habitats and naturally experience fluctuating CO_2 concentrations on a daily basis (Ohde and van Woesik 1999; Shamberger et al. 2011; Shaw et al. 2013). This life history transition from the open ocean to coral reef habitat is also when the population is subject to a severe predation-induced bottleneck, which means this transition is likely to be the point in time when most of the CO_2-tolerance phenotypic selection will occur. Thus, testing survival in the field is an excellent and easily measured proxy for fitness. While we could not continue the CO_2 treatment in the field, the effects of CO_2 are observable up to 2 days post-treatment (Munday et al. 2010b). In addition, recent evidence would suggest that the absence of elevated CO_2 in test water does not affect experimental outcomes of CO_2 effects (Munday et al. 2016).

Behavioral and survival assay

We followed well-established assays to measure behavior and survival (Munday et al. 2010a; Ferrari et al. 2015c). Prior to their release, the fish were tagged with an elastomer, photographed, and placed in labeled 1-L plastic bags filled with seawater. Tagging has been found not to affect growth or mortality of Ambon damselfish juveniles (Hoey and McCormick 2006). All the fish were tagged with the same color elastomer in the same body location, and used, if needed, to discriminate our experimental fish from non-experimental ones that could have been forced to settle on the patches. The bags were kept in a water bath of flowing seawater pumped directly from the ocean (to minimize temperature fluctuations) until deployment in the field. To reduce transport and handling stress, fish in bags

Table 1 Seawater carbonate chemistry for each treatment

Treatment	Temperature (°C)	Salinity (ppt)	pH_{NBS}	Total alkalinity (μmol/kg SW)	pCO_2 (μatm)
Control	29.0 (±0.2)	35.4	8.16 (±0.01)	2287.4 (±4.3)	426.9 (±7.2)
Elevated-CO_2	29.0 (±0.2)	35.4	7.93 (±0.02)	2275.3 (±12.0)	798.2 (±51.2)

Note: Values are means (± SE).

were transported to the field site in a 60-L bin of seawater under subdued light conditions.

Individual Ambon damselfish, randomly selected, were released onto patch reefs (25 × 20 × 20 cm) made up of a 50:50 mixture of live and dead bushy hard coral (*Pocillopora damicornis*) that were cleared of all resident fishes and invertebrates. The random allocation of fish with respect to treatment ensured there was no temporal or spatial confounding with our results. A small acclimation cage was then placed over the patch reef to prevent predation for 30 min while the fish was habituating to the new conditions (note that the mesh size is large enough for the fish to leave, but too small for predators to enter and attack the focal fish). This period ensured that the fish explored its new habitat and resumed feeding at a rate similar to that observed for wild fish. After this habituation period, the cage was removed and a scuba diver, located ∼2 m away from the patch reef, recorded the behavior of each individual for a period of 3 min with the aid of a magnifying glass. A 3-min observation period has been found to obtain a representative quantification of behavior for Ambon damselfish (McCormick 2009). Three aspects of activity and behavior were assessed: (1) bite rate, regardless of success; (2) maximum distance ventured from the habitat patch; and (3) boldness. Boldness was assessed from the fish's overall behavior over the observation period and scored on an ordinal scale from 0 to 3 (in 0.5 increments), where 0 was hiding in hole and seldom emerging, 1 was retreating to hole when scared and taking >5 s to re-emerge, weakly or tentatively striking at food, 2 was shying to shelter of patch when scared but quickly emerging and resuming purposeful strikes at food, and 3 is not hiding when scared, exploring around the coral patch, and striking aggressively at food. When fish displayed behaviors that appeared intermediate between categories, they were given intermediate scores (e.g., 2.5). At the end of the observation period, the fish was approached with a pencil and the fish's reaction and latency to emerge from shelter were also taken into account in the determination of the boldness assessment. Following the end of the observation period, the fish were monitored twice daily for survival. A missing fish was considered consumed by a predator. Indeed, previous studies (Munday et al. 2010b) have shown that fish do not leave their patch reefs (100% survival observed when the acclimation cage is left for the duration of the observation). The observer was blind to the treatment during behavioral observations and survival surveys. One hundred fish were released and observed ($N = 23$–27/treatment).

Statistical analysis

Survival curves for fish within each treatment were calculated and plotted using the Kaplan–Meier method, a nonparametric estimator of survival that incorporates incomplete (censored) observations, such as those cases where fish had not died by the end of the census period. Survival (up to 52 h) of *P. amboinensis* among the four treatments (2 risk levels × 2 CO_2 levels) was compared using a Kaplan–Meier multiple-sample survival analysis, which uses a Mantel–Cox proportional hazard model, followed by Tukey pairwise comparisons.

We performed a two-way Multivariate Analysis of Variance (MANOVA) investigating the effect of risk (low vs. high) and CO_2 (ambient vs. elevated) on the behavioral data. We used a multivariate approach to account for the interdependency of the three behaviors. Data met parametric assumptions.

Results

The survival analysis indicated differences in survival among treatment groups (Mantel–Cox $\chi^2_3=25.5$, $P<0.001$, Fig. 1). As expected under low-risk conditions, exposure to increased CO_2 led to increased mortality *in situ* ($\chi^2_1=6.9$, $P=0.009$). Surprisingly, however, no effect of CO_2 was detected on the survival of high risk fish ($\chi^2_1=1.1$, $P=0.3$).

The behavioral analyses supported the patterns observed for survival. The MANOVA revealed a significant interaction between risk and CO_2 (Pillai's Trace: $F_{3,93}=4.6$, $P=0.005$). This interaction is explained by the fact that elevated-CO_2 conditions significantly increased the risk-taking propensity of low-risk fish (Pillai's Trace: $F_{3,48}=3.2$, $P=0.031$), that increased their distance ventured, feeding rate, and boldness. However, no such effect was detected for high-risk fish (Pillai's Trace: $F_{3,43}=2.2$, $P=0.19$, Fig. 2).

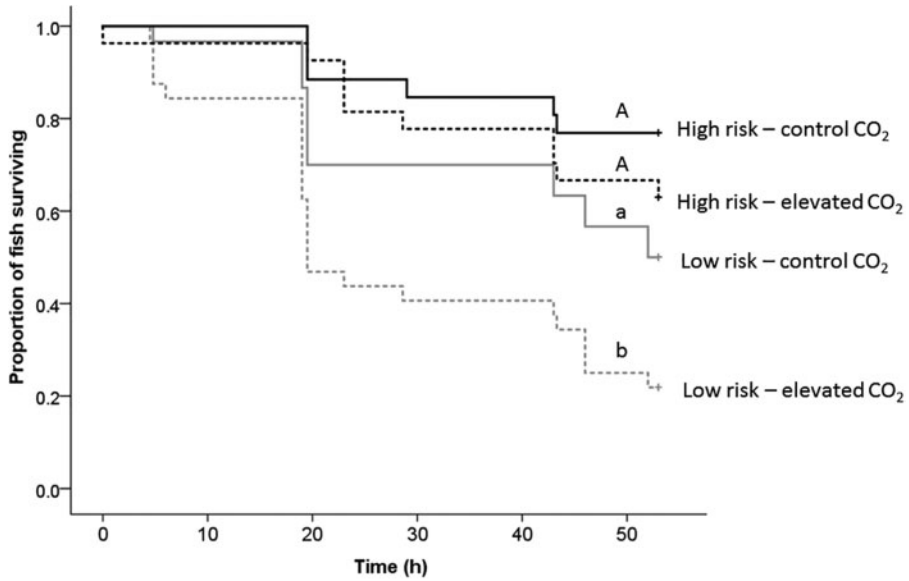

Fig. 1 *In situ* survival curves (up to 52 h) of juvenile *P. amboinensis* exposed to high- (black) or low- (gray) risk conditions, while being exposed to control (solid) or elevated CO_2 (dashed) conditions for 4 days. Letters indicate statistical significance at $\alpha = 0.05$.

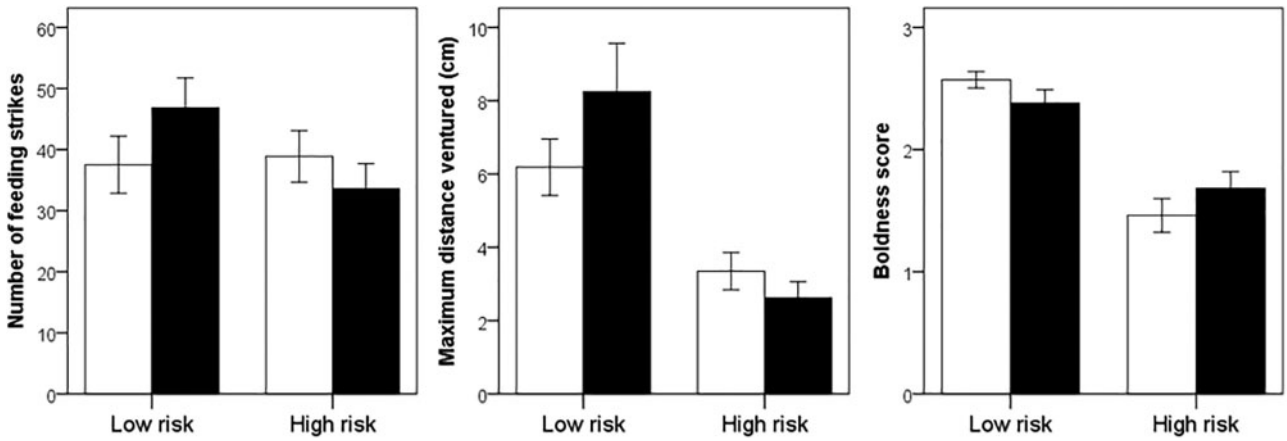

Fig. 2 Mean (±SE) number of feeding strikes (left), maximum distance ventured (center), and boldness score (right) for *P. amboinensis* exposed to low-risk or high-risk conditions while being maintained under ambient (empty bars) or elevated (solid bars) CO_2 conditions for 4 days. Observations were performed *in situ*.

Discussion

Contrary to our predictions, our results provide supporting evidence that predation risk interacts with CO_2, not in a synergistic manner, but rather in an antagonistic one. When exposed to elevated CO_2 conditions, fish maintained in low-risk control conditions displayed the previously documented alterations in *in situ* behavior, including increased boldness, distance ventured from the reef, and increased number of feeding bites (McCormick et al. 2013), and the associated increase in mortality rate (Munday et al. 2010a; Ferrari et al. 2011b). Surprisingly, fish maintained under high-risk conditions appeared to be immune to the detrimental effects of CO_2, as we did not observe a difference between the behavior and survival of the fish exposed to ambient versus elevated CO_2 conditions. Our results are consistent with other studies that have found an antagonistic effect between elevated CO_2 and other environmental stressors. For example, Domenici et al. (2014) found that elevated temperature had an opposing effect on behavioral lateralization when combined with elevated CO_2. Similarly, elevated temperature had an opposing effect on prey selectivity compared with elevated CO_2 in predatory reef fishes (Ferrari et al. 2015d) and temperature and elevated CO_2 had antagonistic effects on the ability of sharks to locate their prey (Pistevos et al. 2017).

In this case, however, it was the perceived environment (high or low risk), not the physical environment, that interacted antagonistically with elevated CO_2.

Frequent and sustained exposures to odors from injured conspecifics should undoubtedly create a stressful environment for juvenile damselfish. In fact, such conditions have been known to cause profound phenotypic changes in aquatic prey species. Fish and larval amphibians exposed to such odors are known to display a neophobic phenotype, whereby prey show fear responses to novel stimuli. In damselfish in particular, such conditions have been associated with changes in prey behavioral lateralization and altered metabolic rate, whereby high-risk prey do not increase their resting metabolic rate, but return to resting levels faster than their low-risk counterparts after a standardized disturbance. In fact, survival studies have shown that high-risk prey may have increased survival when exposed to their natural predators (Ferrari et al. 2015c), although this pattern might not be maintained when prey are exposed to evolutionary novel species (Ferrari et al. 2015a).

From a mechanistic point-of-view, the effect of elevated CO_2 on behavior appears to be mediated by interference from acid–base regulation in a high CO_2 environment with the function of the GABA-A neurotransmitter receptor (Nilsson et al. 2012; Chivers et al. 2014; Regan et al. 2016). GABA-A is the major inhibitory neurotransmitter in the vertebrate brain. The GABA-A receptors control an ion channel with conductivity for bicarbonate and chloride. Changes in the concentration of these two ions to prevent acidosis in a high CO_2 environment are sufficient to alter the polarization of the receptor membrane, and therefore alter the function of the neurotransmitter (Heuer and Grosell 2014; Heuer et al. 2016). Our results suggest that exposure to a high risk environment mitigate these effects.

How would exposure to risk mediate these abovementioned effects? First, it is unknown whether the observed behavior and survival outcomes reflect a change in fish physiology, or simply behavioral compensation. Indeed, if exposure to risk led to the overexpression of beneficial antipredator responses such as neophobia, then our results may simply reflect the counteracting effects of CO_2-mediated increased boldness with risk-mediated increased wariness. On the other hand, previous studies have shown that the metabolic profile of prey might be impacted by exposures to high-risk conditions (Ferrari et al. 2015b). Whether these metabolic changes lead to an improved ability by high-risk prey to normalize membrane potential and consequently, be less vulnerable to increased CO_2 effects is unknown. Given that elevated CO_2 conditions are known to prevent learned predator recognition, future studies could investigate the ability of high-risk prey under elevated CO_2 conditions to learn to recognize novel predators. If this cognitive task is restored in high-risk fish compared to low-risk fish, the results would support a physiological compensation by the fish. If, on the other hand, high-risk fish are still unable to learn to recognize novel predators, then these results would support the behavioral compensation hypothesis.

To conclude, regardless of which mechanism is responsible for the observed pattern, our results provide evidence that prey from different risk environments may differ in their susceptibility to a major anthropogenic stressor. From an ecological viewpoint, it means that spatial and temporal variation in predation risk across locations and life-stages may represent a predictor of CO_2 susceptibility. Future work should investigate whether these high-risk mitigation effects are applicable to other species and other life-stages.

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