Abstract

While ocean acidification is predicted to threaten marine biodiversity, the processes that directly impact species persistence are not well understood. For marine species, early life history stages are inherently vulnerable to predators and an innate ability to detect predators can be critical for survival. However, whether or not acidification inhibits predator detection is unknown. Here, we show that newly hatched larvae of the marine fish *Amphiprion percula* innately detect predators using olfactory cues and this ability is retained through to settlement. Aquarium-reared larvae, not previously exposed to predators, were able to distinguish between the olfactory cues of predatory and non-predatory species. However, when eggs and larvae were exposed to seawater simulating ocean acidification (pH 7.8 and 1000 p.p.m. CO$_2$) settlement-stage larvae became strongly attracted to the smell of predators and the ability to discriminate between predators and non-predators was lost. Newly hatched larvae were unaffected by CO$_2$ exposure and were still able to distinguish between predatory and non-predatory fish. If this impairment of olfactory preferences in settlement-stage larvae translates to higher mortality as a result of increased predation risk, there could be direct consequences for the replenishment and the sustainability of marine populations.

Keywords

*Amphiprion percula*, innate behaviour, ocean acidification, olfactory cues, predator recognition.

INTRODUCTION

Concern that ocean acidification will severely impact on the biodiversity of marine ecosystems has escalated (Orr et al. 2005; Hoegh-Guldberg et al. 2007; Fabry et al. 2008; Hall-Spencer et al. 2008). Average ocean pH has already declined by 0.1 units since pre-industrial times because of absorption of additional carbon dioxide (CO$_2$) from the atmosphere. Ocean pH is predicted to decline another 0.3–0.4 units by 2100 (The Royal Society 2005; Meehl et al. 2007), with some locations showing an even greater than predicted rate of decline (Wootton et al. 2008). To date, most concerns about the effects of ocean acidification have centered on the likely impacts on calcifying organisms (Kleypas et al. 1996; Hall-Spencer et al. 2008; Kuffner et al. 2008). A range of effects, including the dissolution of calcifying plankton (Orr et al. 2005; Moy et al. 2009), reduced growth and shell thickness in gastropods and echinoderms (Shirayama & Thornton 2005; Bibby et al. 2007) and declining growth of reef-building corals (Langdon & Atkinson 2005; De’ath et al. 2009) have been documented. Recently, the effects of acidification have been extended to other marine organisms (Rosa & Seibel 2008), including fishes, where acidification impairs critical sensory mechanisms (Munday et al. 2009a). Although there are many scenarios that could potentially link acidification to increased mortality of marine species, few direct links have been established.

The ability to detect and avoid predators is one of the most important mechanisms to ensure survival, particularly at vulnerable juvenile stages (Lima & Dill 1990). Consequently, many learned (Brown 2003; Kelley & Magurran 2003) and innate (Hawkins et al. 2004) mechanisms for sensing the presence of predators and avoiding them have evolved. Under high predation risk, innate predator recognition can be critical; if an individual fails to detect a predator when first encountered, it may not get a second chance. For most marine organisms, periods of extreme predation risk occur at critical early life-history transitions,
such as hatching and when settling to benthic habitat at the end of the pelagic larval phase (Caley et al. 1996; Almany & Webster 2006; Freitas et al. 2008). On coral reefs, for example, newly hatched larvae departing from the reef to open water and late-stage larvae returning to reefs to settle must navigate a ‘wall of predator mouths’ (Hamner et al. 1988; Leis & Carson-Ewart 1998). The ability to innately recognize predators during these important life-history transitions should increase both the immediate and future prospects of survival.

Predator recognition and avoidance in aquatic ecosystems often involves detection of olfactory cues from predators (Wisenden 2000; Kelley & Magurran 2003). A range of sensory mechanisms may be used by larval reef fishes to detect and avoid predators (Chivers et al. 2001), including vision and mechanoreception, but olfaction is likely to be especially important during settlement. Reef fish larvae typically settle at night when visual predator recognition is likely to be less effective. Furthermore, many reef fish settle around the new moon (Valles et al. 2009), when light levels for visual predator detection are at their lowest. The well-developed olfactory system of settlement-stage fishes (Munday et al. 2009a), and the use of olfactory cues to locate settlement habitat by many species (Arvedlund et al. 1999; Arvedlund & Takemura 2006; Gerlach et al. 2007), point to the importance of olfaction during the settlement process. However, it has recently been shown that the ability of fish larvae to discriminate between the olfactory cues of different habitat types at settlement is impaired at the level of ocean acidification predicted to occur c. 2100 on a business-as-usual scenario of CO2 emissions (Munday et al. 2009a). It is unknown if ocean acidification could affect predator recognition behaviour in a similar way, although this would potentially have far-reaching consequences for populations of prey species.

We tested if larvae of the orange clownfish (*Amphiprion percula*) have the innate capacity to discriminate between the olfactory cues produced by predators and non-predators, both at hatching and at the end of their pelagic larval stage. We then tested if ocean acidification could disrupt the recognition of predator olfactory cues. Clownfish were reared from hatching until the end of their larval phase in control seawater or in seawater where the pH had been reduced by 0.35 units by bubbling additional CO2 (equivalent to 1000 p.p.m. atmospheric CO2). This simulated ocean pH and CO2 levels that could occur c. 2100 because of present and future CO2 emissions under the SRES A2 scenario (Meehl et al. 2007). Predator-naive larvae reared in control and acidified water were tested in a flume chamber for their ability to respond to olfactory cues of predatory species and for their ability to distinguish between the olfactory cues of predatory and non-predatory species.

**MATERIAL AND METHODS**

**Study species and general protocol**

*Amphiprion percula* were reared in a 70 000 L recirculating seawater system at James Cook University’s experimental marine aquarium facility. Larvae were offspring of 21 breeding pairs of *A. percula* collected from the Great Barrier Reef, Australia, and kept at the experimental facility for 3–5 years. Pairs were maintained in separate 70 L aquariums and fed twice daily to satiation with INVE Aquaculture Nutrition 12/20 pellets. Breeding pairs laid eggs clutches on the underside of a terracotta pot placed in their aquarium. On the night of hatching (6–8 days post-laying) egg clutches were transferred from the parental aquarium to a 70-L larval rearing aquarium. Readiness to hatch was identified by the appearance of the embryos. After hatching, larvae were reared in a semi-closed system, where each aquarium had no water exchange during the day and was slowly flushed with filtered, UV-sterilized, seawater each night. This cycle ensured that larvae could feed ad libitum throughout the day and that any unconsumed food was removed each night. Larvae were fed rotifers (*Brachionurus* sp.) at 5 individuals ml⁻¹ each morning for the first 3 days. *Artemia nauplii* were added at 1 individual ml⁻¹ each morning beginning at day 3. The ratio of *Artemia* nauplii to rotifiers was increased each day until larvae were fed only 5 individuals of *Artemia* nauplii ml⁻¹ from day 8. Larvae were reared until they were competent to settle at 11 days post-hatching (Almany et al. 2007).

A small rockcod, *Cephalopholis cyanostigma*, and a dottyback, *Pseudochromis fuscus*, were chosen as the predators species for olfactory trials. These are common and widely distributed predatory fishes that are known to target newly settled fish (Stewart & Jones 2001; Beukers-Stewart & Jones 2004). Two herbivorous reef fishes of similar size to the predators, a surgeonfish, *Acanthurus pyroferus*, and a rabbitfish, *Siganus corallinus*, were chosen as the non-predators for olfactory trials. Predatory and non-predatory species were housed in separate 70 L aquariums that were isolated from the larvae. Predators were fed every second day one portion of manufactured pre-packaged frozen fish food (Fish Dinner; Fish Fuel Co., The Barton, Australia). The non-predators were fed every day the green algae *Caulerpa lentillifera*. Chemical cues were collected from predators and non-predators by turning off water flow to their aquariums for 2 h and then removing 20 L of water.

The response of newly hatched and settlement-stage larvae to olfactory cues of predators and non-predators was tested in a two-channel flume chamber (Gerlach et al. 2007), where larvae were given the choice of two streams of water containing different olfactory cues. Specifically, larvae were given the choice of water streams in the flume chamber containing olfactory cues from: (1) untreated water vs.
untreated water (blank control); (2) C. cyanostigma (predator 1) vs. untreated water; (3) P. fuscus (predator 2) vs. untreated water; (4) A. pyroferus (non-predator 1) vs. untreated water; (5) S. corallinus (non-predator 2) vs. untreated water; (6) C. cyanostigma vs. A. pyroferus (predator 1 vs. non-predator 1) and P. fuscus vs. S. corallinus (predator 2 vs. non-predator 2). All trials were conducted on larvae from a minimum of three parental groups. For each parental group at least 15 randomly selected larvae were tested for each combination of olfactory cues at hatching and at settlement (11 days post-hatching). This was repeated for larvae reared in control water and larvae reared in acidified water.

**CO₂ manipulation**

Clownfish were reared in either control seawater (pH = 8.15 ± 0.07) or CO₂-acidified seawater, from the day that eggs were laid until the larvae had reached settlement at 11 days post-hatching. To simulate ocean acidification the pH of treatment seawater was adjusted to 7.8 ± 0.05 in both the breeding aquariums and the larval rearing aquariums as described by Munday et al. (2009a). Briefly, an electronic pH-controller (Tunze, Aquariumtechnik, Germany) was attached to each aquarium to maintain pH at 7.8 by CO₂ injection. The pH controller was connected to a laboratory-grade glass pH probe in the aquarium and to an electronic solenoid connected to a cylinder of CO₂. The solenoid injected a slow stream of CO₂ into a diffuser (Red Sea CO₂ Reaktor 500, Red Sea Co., Houston, USA) at the bottom of the aquarium whenever the pH of the aquarium seawater rose above 7.8. pH was maintained within ± 0.05 units of the desired level and there was no detectable gradient in seawater pH within the aquarium. The equivalent atmospheric concentration of CO₂ was estimated to be 1050 p.p.m. (Munday et al. 2009b), which is consistent with other studies that have used CO₂ to reduce seawater pH by Springwood, 0.3–0.4 units (Havenhand et al. 2008; Rosa & Seibel 2008).

The pH of each aquarium was independently validated each day using a WP80 pH meter (TPS, Springwood, Australia) calibrated daily with fresh pH buffers (Merck, Darmstadt, Germany). CO₂ was only injected to adjust pH when eggs or larvae were present. The average pH of unmanipulated seawater was 8.15, which is similar to mean ocean pH (The Royal Society 2005). Water temperature was maintained at 30 °C ± 0.6 (SD) using electric heaters. Oxygen levels were maintained above 90% saturation by the mixing action of the diffuser pump.

**Olfactory choice tests**

A two channel choice flume (13 cm × 4 cm) developed by Gerlach et al. (2007) was used to assess the ability of larval _A. percula_ to discriminate between water containing different odour stimuli. This apparatus was designed to conduct pairwise choice experiments, with fish able to freely choose between water from two different sources. Water from the two different sources was gravity fed into the choice flume, which is partitioned along half of its length. Fish were released at the downstream end of the flume where they were free to move to either side or swim towards the preferred water source. Using the protocols outlined in Gerlach et al. (2007), a constant gravity driven flow of 100 mL min⁻¹ per channel was maintained throughout all trials. Flow rates were measured using a flow meter and dye tests were conducted at each water change to ensure that the two flow channels exhibited parallel water flow, with no turbulence or eddies. Preliminary trials (n = 20) showed that larval behaviour was not affected by using either acidified water or control water in the flume. Larvae spent 49.8% of time on the right side of the chamber in control water and 50.4% of the time on the right side in the acidified water. Therefore, all olfactory trials were conducted using control water.

Larvae were tested within 24 h of hatching or when they were competent to settle at 11 days post-hatching. For each trial, a single fish was placed into the centre of the downstream end of the choice flume and acclimated to the two water choices for 2 min. Fish which did not swim actively during the acclimation period were discarded. Two per cent of fish tested were discarded from both control and low pH treatments. At the end of the acclimation period, the position of the fish in the chamber was recorded at five second intervals for a 2-min period. This was followed by a 1-min rest period, during which the water sources were switched, providing a control for potential side preferences that were not associated with the water source. Following the switch of watersources, the entire test including the acclimation period, was repeated. Each fish was used only once.

**Statistical analysis**

Kolmogorov–Smirnov tests were used to compare: (1) the proportion of time that individuals spent in the stream of water containing the olfactory cue verses the proportion of time that individuals spent on one side of the chamber when no cues were present (i.e. results from the blank control); (2) the proportion of time that individuals spent in the stream of water containing the olfactory cue produced by a predator when presented simultaneously with the olfactory cue produced by a non-predator verses the proportion of time that individuals spent on one side of the chamber when no cues were present; and (3) the proportion of time that individuals spent in the stream of water containing the olfactory cue when reared in control water vs. the proportion of time that individuals spent in that stream of...
water when reared in acidified water. There was no statistically significant difference between the three parental groups used for each olfactory comparison, either for larvae reared in control or acidified water (Kolmogorov–Smirnov test $P > 0.10$ for all comparisons). Therefore, parental groups were pooled for each analysis.

**RESULTS**

In blank controls, where there was no olfactory cue added to either water stream in the flume chamber, larvae spent equal amounts of time on each side of the chamber (mean per cent time on one side of the chamber $49.9 \pm 0.13$ SE). Furthermore, there was no effect of rearing conditions (acidified or control water) on the behaviour of larvae in the blank controls for either newly hatched or settlement-stage larvae ($P > 0.10$ for both comparisons). These results indicate that the larvae behaved as expected in the flume chamber when both water streams contained unmanipulated water.

Newly hatched clownfish larvae from control water in that control water innately recognized the chemical cues of predators and were able to discriminate between chemical cues produced by a predatory and a non-predatory species. Newly hatched larvae remained in untreated water over 90% of the time when presented with the choice of seawater containing olfactory cues from either of two predator species. Newly hatched larvae also avoided water containing olfactory cues from the two non-predator species when presented in combination with untreated seawater (Fig. 1a; $P < 0.001$ for both comparisons). However, when olfactory cues of a non-predator and predator were presented simultaneously, the larvae spent over 88% of the time in the non-predator water stream (Fig. 1a; $P < 0.001$ for both comparisons), indicating that they could distinguish between predators and non-predators.

The ability to detect olfactory cues produced by both predators and non-predators was retained in settlement-stage larvae. All settlement-stage larvae presented with seawater containing chemical cues of a predator vs. untreated seawater remained in the untreated seawater 100% of the time (Fig. 1b; $P < 0.001$ for both tests). In contrast, settlement-stage larvae displayed no preference or avoidance of seawater containing olfactory cues from a non-predator when tested against untreated seawater, with larvae spending approximately equal time on either side of the flume chamber (Fig. 1b; $P > 0.1$ for both tests). However, when larvae were simultaneously presented with water containing chemical cues of a predator and a non-predator, they exhibited a strong preference for the non-predator water stream (Fig. 1b; $P < 0.001$ for both tests), spending 100% of the time in the water stream containing olfactory cues from the non-predator.

Exposure to acidified water affected the olfactory preferences of settlement-stage larvae, but not newly hatched larvae. Newly hatched *A. percula* larvae reared from the egg stage in CO2-acidified water exhibited similar preferences to newly hatched larvae from control seawater for all comparisons (Figs 1a and 2a; $P > 0.1$). Newly hatched larvae from acidified water avoided water containing chemical cues from a predator when presented against untreated seawater (Fig. 2a; $P < 0.001$). They also avoided chemical cues produced by non-predator species when presented against untreated seawater (Fig. 2a; $P < 0.001$), but were attracted to the water stream containing olfactory cues of the non-predator species when simultaneously presented with chemical cues from a predator (Fig. 2a; $P < 0.001$). This indicates that the olfactory preferences of larvae were not affected by exposure of the eggs to high CO2 and low pH during embryogenesis.

In contrast, settlement-stage larvae reared in acidified water exhibited significantly different preferences to larvae reared in control seawater for all comparisons (Fig. 2b; $P < 0.001$ for all comparisons). Larvae reared in acidified water always chose the stream of water containing an olfactory cue over untreated water, regardless of the source of the odour. All larvae reared in CO2 acidified water and presented with chemical cues from the same predator...
These results suggest that clownfish larvae have an innate ability to detect predators using olfactory cues and to differentiate between the chemical cues of predatory and non-predatory species, but this ability is lost when larvae are exposed to seawater that has been acidified with an atmospheric equivalent of \( \approx 1000 \text{ p.p.m. CO}_2 \) (pH 7.8). Avoiding predation is critical for individual survival; however, settlement-stage larvae changed from complete avoidance of the olfactory cues from predators in control water to complete attraction to these cues in acidified water. This means that larvae might exhibit a fatal attraction to predators at CO\(_2\) and pH levels that could occur in our oceans by 2100 on a business-as-usual scenario of greenhouse gas emissions. Potentially, such a dramatic loss or reversal of predator-avoidance behaviour could greatly increase mortality rates of settling larvae, which might lead to decreased population replenishment and subsequent population decline. If other species exhibit similar dramatic changes in predator avoidance behaviour in acidified water and these behavioural changes translate to increased mortality rates, the effects on marine biodiversity could be profound and extend far beyond those impacts already reported for calcifying organisms.

Although numerous studies have shown the importance of olfactory cues in predator recognition by marine fishes (Brown 2003; McCormick & Manassa 2008), the relative importance of vision and olfaction to newly settled larvae in avoiding predation is largely unknown. It is possible that olfaction helps keep larvae and juveniles a safe distance from predators, while vision is more important in escaping a predatory attack. A serious concern is that under acidified conditions newly settled larvae may be exposed to a greater risk of a predatory attack if attraction to the smell of predators draws them closer to predatory fish. Furthermore, reef fish larvae usually settle at night, often around the new moon, when visual acuity is at a minimum. Therefore, the ability to detect the presence of predators by olfaction may be especially important during settlement to reef habitat. Further experiments are now required to determine if the attraction to olfactory cues produced by a predator for larval fish reared in acidified water leads to increased mortality under natural conditions.

The potential for marine organisms to adapt to rapid ocean acidification remains largely unknown (Fabry et al. 2008; Munday et al. 2008). The complete reversal in preference of chemical cues by settlement-stage larvae in our experiments and the absence of any variation among individuals in their behavioural responses in acidified water suggests that there is limited opportunity for adaptation to this threat by the selection of less affected individuals, at least at the levels of acidification used here (\( \approx 1000 \text{ p.p.m. CO}_2 \) and 7.8 pH). Whether the dramatic shift in behaviour exhibited by larvae reared in acidified water could be mitigated by adaptation to a slow increase in CO\(_2\) over many generations remains to be seen. It will be important for future studies to test if the behavioural responses to ocean
acidification increase gradually with increasing CO₂ levels, or if the switch from avoidance to preference of predator cues occurs abruptly around a tipping point. These different outcomes would have important implications for the potential for adaptation of larval behaviour to ocean acidification.

The physiological mechanism responsible for changes to olfactory preferences by larvae reared in acidified water has yet to be determined. However, Munday et al. (2009a) found no abnormalities in the morphology of the olfactory organs of larval clownfishes reared in acidified water. Consequently, it is likely that the inability to distinguish between important olfactory cues is caused by disruption to the transfer of chemosensory signals across the olfactory epithelium, or within the neuro-sensory system, rather than any effect on development of the olfactory system itself. If this is the physiological mechanism responsible for the behavioural changes observed, we expect that the ontogenetic timing of changes to the behavioural preferences exhibited by larvae is likely to be related to the level of ocean acidification that they experience, with earlier induction at more extreme levels of acidification. Finally, because CO₂ and pH covaried in our experiments, as they would under naturally occurring ocean acidification, we were not able separate the specific effects of CO₂ and pH on the sensory system. Future research could examine: (1) the physiological mechanisms responsible for disruption to olfactory preferences of larvae exposed to acidified conditions, (2) the relative contribution caused by respiration and photosynthesis of reef organisms to natural variation in pH and CO₂ close to the reef matrix, we were not able separate the specific effects of CO₂ and pH on the sensory system. Future research could examine: (1) the physiological mechanisms responsible for disruption to olfactory preferences of larvae exposed to acidified conditions, (2) the relative contribution caused by respiration and photosynthesis of reef organisms to natural variation in pH and CO₂ close to the reef matrix. The neuro-sensory system, rather than any effect on development of the olfactory system itself. If this is the physiological mechanism responsible for the behavioural changes observed, we expect that the ontogenetic timing of changes to the behaviour of marine organisms can fine-tune these responses so that juveniles are more capable of avoiding threats and learning after settlement might fine-tune these responses so that juveniles are more capable of avoiding threats (McCormick & Holmes 2006) and so that they do not respond unnecessarily to species that are unlikely to target them as prey. Whether ocean acidification could affect learning behaviour of post-settlement fishes is unknown, but deserves consideration.

The presence of an innate ability for predator recognition through olfactory cues underlines the importance of predator avoidance for survival of larvae and newly settled juveniles that are naive to predators. Any mechanism that disrupts the ability to detect predators, or even worse causes larvae to be attracted to predators, could increase mortality rates and lead to population declines. Further research is required to confirm that attraction to predator odour can increase the risk of predation, or if other sensory mechanisms (e.g. vision or mechanoreception) can compensate for impairment of the olfactory system. However, even if other sensory mechanisms can help larvae escape a predatory attack, the increased proximity to predators caused by the failure to respond to the presence of predator odour may still lead to higher rates of mortality.

Our results and another recent report (Munday et al. 2009b) indicate that ocean acidification could have previously unrecognized effects on the behaviour of marine species. In particular, critical behavioural decisions during the transition between larval and juvenile life phases could be impaired, leading to reduced replenishment of benthic populations and impacts on marine connectivity (Munday et al. 2009b). Most reef organisms have pelagic larvae that must avoid predators and locate suitable adult habitat to successfully recruit to the benthic population. Our results
suggest that ocean acidification could affect the number of individuals that successfully negotiate this transition, with potentially significant implications for marine biodiversity.

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