



Active in the sac: damselfish embryos use innate recognition of odours to learn predation risk before hatching



Jennifer Ann Atherton*, Mark Ian McCormick

College of Marine and Environmental Sciences and ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, Queensland, Australia

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Predation-induced mortality rates of aquatic species are much higher in larvae than in adults. Consequently, the ability of an organism to recognize relevant predators as early as possible could increase its chance of survival, especially in areas with high predator diversity. Heart rates of embryonic cinnamon clownfish, *Amphiprion melanopus*, were monitored to assess their reaction to damage-released conspecific alarm cues. These cues were then combined with a predator odour in a conditioning trial to establish whether the embryos were capable of learning a predatory threat. Results showed that *A. melanopus* embryos were not only able to detect and react to conspecific chemical alarm cues, but were also capable of using this information to learn about predation risk before they hatched. This recognition could lead to a number of antipredator behavioural adaptations, such as modifications of habitat choice at settlement, and could affect development and behaviour in postembryonic individuals, all of which may increase their chance of survival.

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Predation is one of the major driving forces in population and community dynamics (Pettorelli, Coulson, Durant, & Gaillard, 2011). The predators that pose the greatest threat to a prey species can vary greatly with ontogeny and habitat (Holmes & McCormick, 2010; Mitchell & McCormick, 2013; Sih, Ziemba, & Harding, 2000). The ability to identify the degree of threat can help individuals to avoid wasting energy on costly antipredator behaviours, energy that could otherwise be used for other fitness-related activities, such as foraging (Brown & Smith, 1996; Houston, McNamara, & Hutchinson, 1993). Recognition of predators can be innate (Hawkins, Magurran, & Armstrong, 2004) or learned through association using visual and/or chemical cues from predation events (Ferrari, Wisenden, & Chivers, 2010).

Chemical alarm cues (CACs) are odours that are released when the upper epidermis of an animal is damaged. Recognition of such cues is innate and, when combined with predator odours, these cues can provide information on relevant threats (Smith, 1992). Research suggests that the epidermal cells containing these alarm cues serve a primarily immune function, and their use as warning signals is a secondary and incidental advantage (Chivers et al.,

2007). None the less, both laboratory and field studies have verified the use of CACs in learning predation risk in a wide range of aquatic taxa, including amphibians and fishes (Ferrari, Manek, & Chivers, 2010; Ferrari, Wisenden, et al., 2010; Manassa, Dixon, McCormick, & Chivers, 2013; Manassa, McCormick, & Chivers, 2013). This learning can occur both directly, by witnessing a predation event, or indirectly, through social learning (e.g. observing an individual's antipredator response to a threat odour; Griffin, 2004). Furthermore, recent research has shown that individuals are able to use alarm cues of conspecifics as well as heterospecifics, and this interspecific learning is also possible in juvenile fishes (Manassa, McCormick, et al., 2013). After acquiring knowledge about one threat, fishes are able to generalize this information to identify and avoid closely related predators (Ferrari, Gonzalo, Messier, & Chivers, 2007; Mitchell, McCormick, Ferrari, & Chivers, 2013). Most of the research into associative learning of predators using alarm cues has focused on juveniles and adults, especially in fishes, but very little is known about the sensory and learning capabilities of the early developmental stages, which are most vulnerable to predation.

It is widely accepted that, in the marine environment, settlement-stage fish larvae are naïve to all predators, owing to their complex life cycles and pelagic larval stage. Yet, a growing body of research has demonstrated that the predator environment experienced during embryogenesis can induce developmental and

* Correspondence: J. A. Atherton, College of Marine and Environmental Sciences and ARC Centre of Excellence for Coral Reef Studies, James Cook University, Townsville, Queensland 4811, Australia.

E-mail address: jennifer.atherton@my.jcu.edu.au (J. A. Atherton).

behavioural changes, increasing the chance of survival after hatching (Bernard, 2004). For example, common frogs, *Rana temporaria*, that experience predators during embryogenesis are shorter with deeper tails, which improves their swimming ability and predator avoidance capabilities as a tadpole (Laurila, Crochet, & Merila, 2001). Additionally, recognition of predators in early life stages can help individuals differentiate between suitable settlement habitats in coral reef fishes (Vail & McCormick, 2011).

Innate recognition of predators by embryos can also reduce prey mortality through premature hatching. Some frog species have been found to use either vibrational cues (Warkentin, 1995a, 1995b) or chemical cues (Chivers et al., 2001) to hatch early and escape predation from snakes and leeches, respectively. However, although this allows for immediate escape from a predator, there could be subsequent costs, such as an increased chance of mortality in the less developed premature hatchers (Kusch & Chivers, 2004; Warkentin, 1995a, 1995b). Some embryos have also been found to possess the ability to learn predation risk while still in the egg, showing reductions in activity and boldness posthatching (Mathis, Ferrari, Vindel, Messier, & Chivers, 2008; Nelson, Alemadi, & Wisenden, 2013). There are costs and benefits associated with both innate and learnt recognition of predators, yet a study on the San Marco salamander, *Eurycea nana*, suggests that some animals could use the combination of the two to exhibit an antipredator response equivalent to the level of threat (Epp & Gabor, 2008).

While the role of CACs in associative learning of predation risk has been widely studied, little is known of its importance to pre-settlement life stages. Tropical reefs are one of the most biodiverse ecosystems in the world, where prey species are often faced with a large variety of predatory threats, from different species with different foraging tactics (Heinlein, Stier, & Steele, 2010). Many species lay benthic eggs that are guarded by the parents until hatching, prior to their larval phase. This early association with the parental reef gives them the opportunity to learn about appropriate settlement habitats (Arvedlund, McCormick, Fautin, & Boldsoe, 1999; Dixson et al., 2014), but also the potential to learn about predators that may be relevant to the parental habitat. This information is particularly pertinent as recent research has also shown that a large proportion of offspring return to their natal reef after a 1–5-week larval phase, and at times settle to sites only metres away from their parents (Berumen et al., 2012). Hence, there may be an evolutionary driver for fishes to be able to learn about relevant predators as early as possible to avoid being preyed upon. The goal of the present research was to establish the capacity of clownfish, *Amphiprion melanopus*, embryos to learn predatory threat. This was achieved by first identifying whether the embryos could detect and react to relevant CACs in the latter stages of their embryonic development. Second, an associative learning experiment was carried out, whereby embryos were conditioned to recognize a predator odour as a threat, to see whether they were able to acquire relevant predator information before they hatch.

METHODS

Study Species

Amphiprion melanopus is an anemone-associated clownfish species, of the family Pomacentridae, distributed on coral reefs throughout the Western Pacific (Allen, 1991). On average, embryogenesis (egg development) usually lasts for 8 days in this species depending on the temperature, during which time the offspring are cared for by their parents (Green, 2004). Once they have hatched as transparent larvae, offspring remain in the pelagic environment for 15–21 days before settling on the reef as juveniles

(Doherty, Planes, & Mather, 1995). *Amphiprion melanopus* serves as a good study species for developmental experiments because it reproduces successfully in captivity.

Animal Housing

All research was carried out, and animals housed, at the Marine and Aquaculture Research Facilities Unit (MARFU) at James Cook University (JCU), Townsville, Australia. Five adult breeding pairs of *A. melanopus* were kept outside in 70-litre tubs, with half a terracotta pot serving as shelter and a substrate for laying clutches on. These fishes were obtained from an existing broodstock at MARFU, JCU, but were originally wild caught as adults from coral reefs off the coast of Cairns, Queensland, Australia. The tanks were on a constant flow-through system with well-aerated water, which was maintained at 27 °C with a salinity of 35 ppm. Fish were fed pelleted food daily and tanks were then checked for clutches, with the day of spawning being classed as day 1 postfertilization. The eggs were left with their parents until day 6 postfertilization when they were carefully removed from the terracotta pot using a scalpel and collected in a 1-litre beaker. The clutch was kept in this beaker overnight, bubbling in very well-aerated water maintained at 27 °C using a flow-through bath.

Cue Preparation

CACs were made by crushing 10 *A. melanopus* embryos, from the same clutch as the test embryos, in a petri dish, and rinsing them with 5 ml of sea water. This was then filtered through filter paper to remove any particulate matter, leaving only sea water infused with alarm cue. We used 1 ml per trial and made fresh embryo alarm cues as required throughout the experiments, to prevent degradation of cues.

Predator odours were prepared using the dottyback, *Pseudochromis fuscus*, a well-known opportunistic predator, which preys on both eggs and juvenile damselfish (Emslie & Jones, 2001; Feeney et al., 2012). A 10 cm (total length) *P. fuscus* individual was starved for 24 h prior to the experiment to limit the amount of dietary cues used in trials. It was then placed in 10 litres of well-aerated sea water for 12 h before any cues were collected. Water was removed for each set of five trials, to reduce the chance of degradation of cues. Once trials had been run for the day, a full tank water change was carried out to reset the concentration of the odour used in the trials carried out the next day (day 8 postfertilization).

Embryonic Detection of CACs

A time series trial was carried out to examine whether or not *A. melanopus* embryos are able to detect and respond to alarm cues. On day 5 postfertilization, embryos were carefully removed from the clutch and kept in 1-litre, well-aerated beakers overnight at the same temperature as the parental tanks. On day 6, embryos were tested with either sea water (SW) or an embryo CAC stimulus ($N = 15$ embryos per cue). This was carried out by placing an embryo in a small container of 10 ml of sea water, under a dissecting microscope, and allowing it to acclimatize for 2 min. The baseline heart rate (beats/30 s), was then measured using a stopwatch and a tally counter. The cue (1 ml) was then added to the sea water and the heart rate was remeasured, to allow the calculation of any stimulus-induced changes in heart rate. On day 7, embryos from the same clutch were retested with a CAC stimulus to assess whether there was a difference in reaction to the odour with a further 24 h development. This experiment was repeated using four different clutches produced by four different breeding pairs.

Associative Learning in Embryos

Day 7 treatment trials

Embryos from each clutch were split across one of three treatments on day 7 postfertilization: (1) sea water control (SW), (2) predator odour (PO) or (3) predator odour combined with a CAC (PO+CAC). A subsample of 15 embryos was taken to assess the change in heart rate induced by each of the three test cues. Heart rate (per 30 s) was recorded before and after stimulus introduction, and the percentage difference was calculated for each embryo, using the same methods outlined in the previous experiment. The proportions of the three cues were as follows: (1) 2 ml of sea water, as a control; (2) 2 ml of predator odour; (3) 1 ml of predator odour and 1 ml of an embryo CAC.

A further 30 embryos from each clutch were treated per cue to account for any potential overnight mortality. Thus, there were a total of 45 embryos for each treatment, kept in well-aerated beakers in water baths overnight, to be retested on day 8 postfertilization. This experiment was repeated for four separate clutches, produced by four different breeding pairs of *A. melanopus*.

Day 8 test trials

On the following day (day 8 postfertilization), 15 embryos from each of the three treatments were tested with 1 ml of predator odour. The same procedure as carried out on day 7 was followed to establish each embryo's reaction, in terms of its change in heart rate induced by the cue. Therefore, the three sets of treatments undergone by the subsamples of embryos were: (1) sea water control, then predator odour; (2) predator odour, then predator odour again; (3) predator odour with CAC, followed by predator odour alone. This allowed us to assess whether *A. melanopus* embryos are able to use CACs to learn, through conditioning, to recognize predation threat.

Statistical Analyses

Residual analysis found that data met the assumptions of ANOVA. The model used for the pilot trial tested cue and clutch and

the interaction between them as fixed factors. Similarly, the model for the learning experiment looked at the effects of the fixed parameters clutch, day and treatment, and the interactions among them. Tukey's HSD post hoc tests were used to see where the significant differences lay in both data sets.

Ethical Note

All research was approved by the James Cook University Animal Ethics Committee under the permit A1871.

RESULTS

Embryonic Detection of Chemical Alarm Cues

The ANOVA showed a significant difference between the increases in heart rate induced by SW and CACs on days 6 and 7 postfertilization (Appendix Table A1). Furthermore, clutch did not have a significant impact on mean heart rate increase (Appendix Table A1). Introduction of an SW control on day 6 induced a negligible change in heart rate, which was significantly different from those induced by both CAC treatments (SW day 6 = -0.07% ; Tukey's HSD: $P < 0.0001$; Fig. 1). Embryos exhibited a greater reaction to a conspecific CAC (in terms of an increase in heart rate) on day 7 of development, compared to day 6 (day 6 = $+6.60\%$, day 7 = $+12.22\%$; Tukey's HSD: $P < 0.0001$).

To show that it was not the introduction of a cue on consecutive days that induced a stress response (increase in heart rate), a pilot study testing embryos with SW cues on both day 7 and day 8 was conducted. The SW cue caused a 0.18% increase in heart rate on day 7, followed by a 0.10% increase on day 8. An ANOVA showed this to be a nonsignificant difference (Appendix Table A2).

Associative Learning in Embryos

An ANOVA showed the interaction between day and treatment cue was significant (Appendix Table A3). All other factors, and

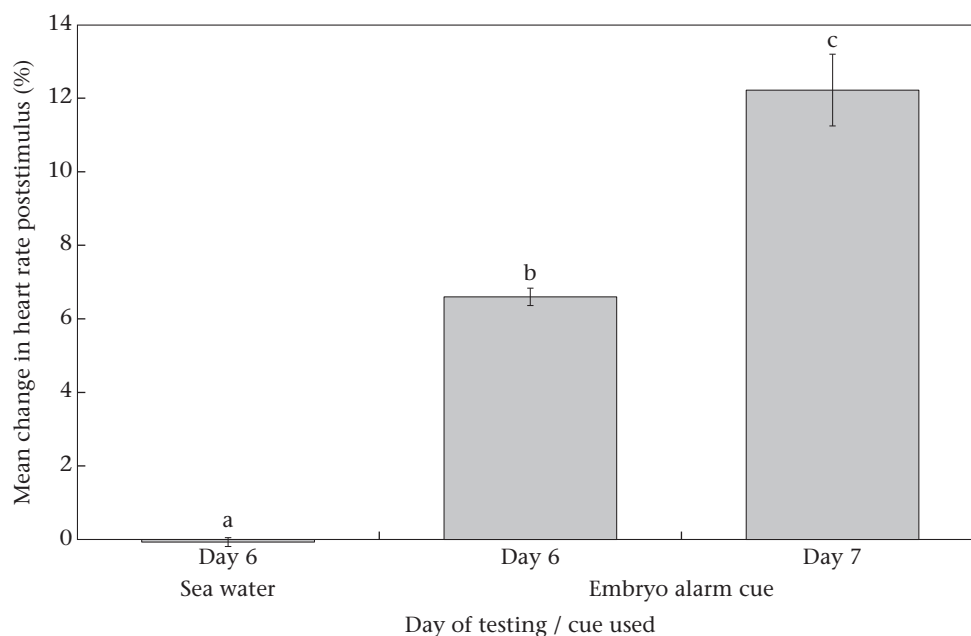


Figure 1. Comparison of the mean percentage change in heart rate \pm SE induced by a within-clutch embryo alarm cue on days 6 and 7 of postfertilization development in *A. melanopus* embryos, compared to a sea water control ($N = 60$ per treatment). The letters above the bars represent Tukey's HSD groupings of means.

interactions between effects, of clutch, day and treatment cue were not significant (Appendix Table A3).

When treated with a PO combined with a CAC during the initial conditioning, embryos showed an increase in heart rate of +7.91%. This increase was significantly higher than that produced by PO (Tukey's HSD: $P < 0.0001$) or SW conditioning ($P < 0.0001$). When the PO+CAC-conditioned fish were retested with just a PO on day 8, the conditioned embryos showed a very similar increase in heart rate as they did on day 7 (+8.07%; $P = 0.999$; Fig. 2), suggesting they had learnt that PO represented a threat.

Embryos tested with PO, both initially as a treatment on days 7 and 8 (after SW conditioning on day 7), showed very similar increases in heart rates after the introduction of the cue (PO day 7 = +4.10%, SW day 7-PO day 8 = +4.09%; Tukey's HSD: $P = 1.000$). The increase in heart rate induced by PO on day 7 was significantly larger than that of the SW control, which elicited very little response (SW day 7 = +0.29%; Tukey's HSD: $P < 0.0001$).

Embryos that were treated with a PO on day 7, and then retested with PO on day 8, showed a significantly reduced increase in heart rate with cue introduction (PO day 7 = +4.10%; PO day 7-PO day 8 = +1.81%; $P = 0.020$). However, although a slight increase in mean heart rate was still induced by the day 8 PO test cue, the change in heart rate was not statistically different from that of the SW control (Tukey's HSD: $P = 0.291$).

DISCUSSION

Embryonic Detection of CACs

Embryos of clownfish were able to detect and react to CACs of conspecifics. The increase in heart rate induced by the presence of a CAC suggests recognition is innate and can occur immediately after olfactory development. Furthermore, the two-fold increase in reaction from day 6 to day 7 suggests that olfactory senses develop rapidly in the latter stages of embryogenesis. This is supported by research carried out by Arvedlund, Larsen, and Winsor (2000) who found that olfactory development starts on day 6 postfertilization,

but embryonic olfaction is unlikely to be fully functional until day 7. Kavanagh and Alford (2003) also found that olfactory development occurred more quickly in an anemonefish than in other pomacentrids (black axil chromis, *Chromis atripectoralis*, ambon damsel, *Pomacentrus amboinensis*, and spiny chromis, *Acanthochromis polyacanthus*). It has been suggested that this rapid olfactory development may allow clownfish to imprint on their host anemone (Arvedlund & Nielsen, 1996).

Associative Learning in Embryos

The clownfish embryos were able to use their innate recognition of CACs to learn to recognize a correlate of predation risk. A positive correlation between increases in heart rate and antipredator behaviours has been reported in a number of species of fish (Hojesjo, Johnsson, & Axelsson, 1999; Johnsson, Hojesjo, & Fleming, 2001; Metcalfe, Huntingford, & Thorpe, 1987). With this in mind, it is likely that the increase in heart rate exhibited by the conditioned *A. melanopus* embryos could later translate to increased predator avoidance posthatching, through enhanced recognition of risk. The learning capability of embryos has only been identified in a few other aquatic species: wood frogs, *Lithobates sylvaticus* (Ferrari, Manek, et al., 2010), ringed salamanders, *Ambystoma annulatum* (Ferrari, Manek, et al., 2010; Ferrari, Wisenden, et al., 2010), convict cichlids, *Amatitlania nigrofasciatus* (Nelson et al., 2013) and rainbowfish, *Melanotaenia duboulaui* (Oulton, Haviland, & Brown, 2013). Individuals with knowledge of relevant predatory threats experience higher survival rates (Lonnstedt, McCormick, Meekan, Ferrari, & Chivers, 2012; Mirza & Chivers, 2001; Polo-Cavia & Gomez-Mestre, 2014). In spite of this, it is widely thought that new coral reef fish recruits are naïve to predators at the settlement stage. As coral reefs have high biodiversity, juveniles are subject to intense predation pressure from a suite of predators once they have hatched (Almany & Webster, 2006). Mitchell, McCormick, Ferrari, and Chivers (2011b) showed settlement-stage juvenile lemon damselfish, *Pomacentrus moluccensis*, were able to quickly learn a number of novel predators using chemical stimuli, and exhibit

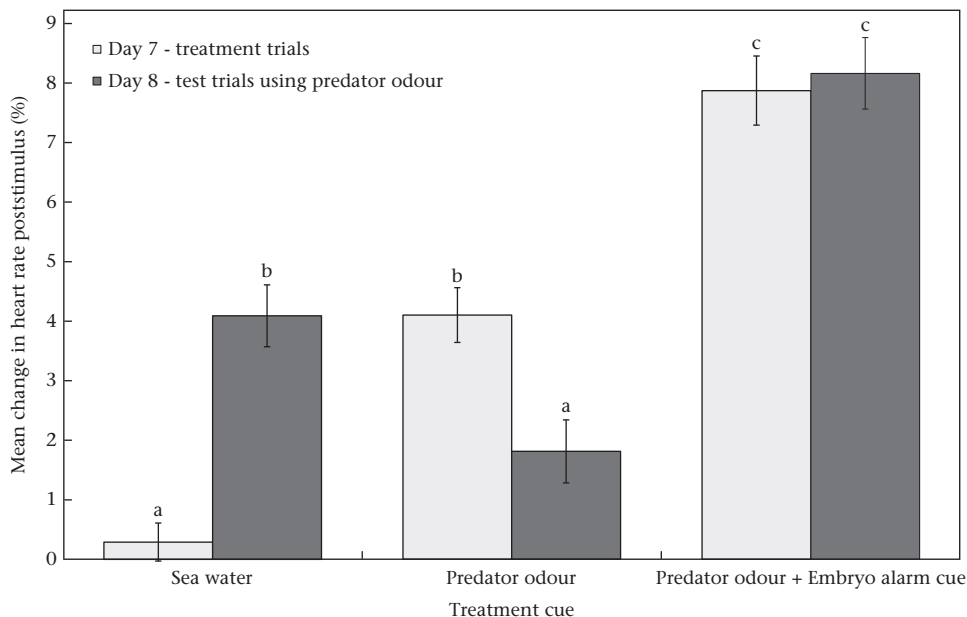


Figure 2. Mean percentage change in heart rate \pm SE after cue introduction. The white bars indicate the reaction to one of three treatment cues on day 7 of development: sea water, predator odour or predator odour combined with a within-clutch embryo chemical alarm cue. The dark grey bars indicate the increase in heart rate induced by the introduction of the predator odour to embryos from each of the three conditioning treatments, on day 8 of development. The letters above the bars represent Tukey's HSD groupings of means ($N = 60$ per column, except the dark grey bar in predator odour treatment, where $N = 53$).

antipredator responses to odours after a single conditioning event. Our study suggests that some species of damselfish are capable of doing this at the embryonic stage and may in fact already have some knowledge of relevant predatory threats when they hatch.

Research suggests that some species of fish may have innate predator recognition (Dixon, Munday, & Jones, 2010; Kempster, Hart, & Collin, 2013; Oulton et al., 2013). The increase in embryo heart rate induced by the predator cue in our experiment could be interpreted as such (Oulton et al., 2013). However, there is also the possibility that this reaction could be neophobic, i.e. a 'fear' response induced by an unfamiliar cue (Brown, Chivers, Elvidge, Jackson, & Ferrari, 2014). Embryos that were treated with a predator odour twice reacted less to the cue on the second exposure. This suggests their first reaction could be a neophobic response, as without the reinforcement of a predation event/alarm cue, the perceived threat level of the predator odour decreased. Berejikian, Tezak, and LaRae (2003) studied naïve, hatchery-reared chinook salmon, *Oncorhynchus tshawytscha*, and found an innate antipredator response, which was significantly amplified by a conditioning event with an alarm cue. Thus, while it may be possible that some fishes possess innate predator recognition, it is likely that the continuation of antipredator behaviours to such cues would cease if not associated with an alarm cue (Mitchell, McCormick, Ferrari, & Chivers, 2011a).

Once an individual has learnt to recognize a threat, this information could be used to avoid predation during subsequent ontogenetic stages. Hepper and Waldman (1992) showed that a preference for a chemical stimulus experienced as an embryo lasted through the larval stage, metamorphosis and into the juvenile stage in two frog species. This finding was supported by more recent studies, in which embryonic conditioning with alarm cues prompted increased antipredator behaviours posthatching in wood frogs, ringed salamanders (Mathis et al., 2008) and convict cichlids (Nelson et al., 2013). Research has shown that levels of dispersal in clownfish are a lot lower than was first thought, with high levels of self-recruitment to parental reefs (Jones, Planes, & Thorrold, 2005). Therefore, predators experienced by individuals in the embryonic stage are likely to be similar and relevant to those experienced by many settlement-stage larvae and juveniles. Indeed, many of the key predators on newly settled juvenile fishes also eat embryos from benthic egg clutches, such as the moonwrasse, *Thalassoma lunare*, and dottyback (McCormick & Meekan, 2007). Hence, the chance of mortality through predation is likely to be decreased in settlement-stage fishes that have prior knowledge of the predators in their microhabitat.

Our findings indicate that embryonic anemonefish have functional olfactory receptors capable of detecting chemical stimuli important in early life stages. Numerous studies have shown clownfish innately imprint on certain host species of anemones, which strongly influences their selection of settlement habitats (Arvedlund & Nielsen, 1996; Arvedlund et al., 1999; Arvedlund et al., 2000; Dixon et al., 2008). It has already been shown, in a number of taxa, that juveniles use chemical recognition to avoid settling in habitats in which predators are present (i.e. barnacles: Johnson & Strathman, 1989; plaice, *Pleuronectes platessa*: Wennhage & Gibson, 1998; salamanders: Mathis et al., 2008; fishes: Vail & McCormick, 2011). Therefore, it is possible that the rapid development of olfaction and early learning capabilities of clownfish could increase survival through informed habitat choice and avoidance of predators. Dixon (2012) recently examined the combined effect of predator and host/nonhost anemone (species-specific) odours on habitat selection in three species of clownfish, and showed that larvae strongly prefer host anemones, especially in the absence of predators, demonstrating the importance of odour identification and categorization.

Conclusion

Our findings suggest that anemonefish embryos have refined olfactory capabilities before they hatch and can use chemical stimuli to learn predatory threats during embryogenesis. This could aid survival through predator avoidance by selecting safer habitats at settlement and refining predator recognition to prevent wasting energy on antipredator behaviours. Further research is needed to fully assess the extent of the embryonic sensory and learning capabilities of fishes and the impact they have on subsequent life stages.

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Appendix

Table A1

A two-factor ANOVA comparing the change in heart rate induced by the type of cue used (sea water or conspecific alarm cue) and the day of treatment (day 6 or 7), and the clutch from which the embryos were sourced

Effect	df	MS	F	P
Clutch	3	9.781	0.587	0.624
Cue & Day	2	2270.034	136.323	<0.0001
Clutch* Cue & Day	6	25.734	1.545	0.166
Error	168	16.652		

Table A2

A two-factor fixed ANOVA comparing the change in heart rate induced by a sea water cue on day 7, followed by a repeated sea water cue on day 8, and the clutch from which the embryos were sourced

Effect	df	MS	F	P
Clutch	2	0.012	0.001	0.999
Day	1	0.084	0.006	0.939
Clutch* Day	2	2.748	0.190	0.828
Error	54	14.468		

Table A3

A three-factor ANOVA investigating the changes in heart rates in response to the treatment cue used (sea water, predator odour or predator odour + conspecific alarm cue) during and after a conditioning event, while taking into account the source of the clutch

Effect	df	MS	F	P
Clutch	3	6.620	0.419	0.739
Day	1	30.762	1.948	0.164
Treatment cue	2	1172.841	74.254	<0.0001
Clutch* Day	3	3.131	0.198	0.896
Clutch* Treatment cue	6	3.686	0.233	0.966
Day* Treatment cue	2	263.134	16.659	<0.0001
Clutch* Day* Treatment cue	6	3.299	0.209	0.974
Error	329	15.795		