

Kin recognition in embryonic damselfishes

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Predator-induced mortality rates are highest in early life stages; therefore, early recognition of threats can greatly increase survival chances. Some species of coral reef fishes have been frequently found to recruit back to their natal reefs; in this instance, there is a high chance of juveniles encountering their siblings, among other kin, after hatching. Kin recognition plays an important ecological role in that it allows individuals to protect genetically similar relatives, and hence increase their inclusive fitness. By observing changes in heart rates, we demonstrated that embryos of two damselfish species, *Acanthochromis polyacanthus* and *Amphiprion melanopus*, not only possess recognition of kin and damage-released alarm odours, but also react to them in a graded manner. Such refined olfactory capabilities in embryonic stage organisms (seven and eleven days after fertilisation) suggest identification of threats may provide survival advantages post-hatching, such as the informed choice of low risk habitats at settlement. To our knowledge this is the first time that kin recognition has been identified in embryos of any species.

Kin recognition is the ability of organisms to distinguish their relatives from other conspecifics and has been identified in a wide range of taxa. Examples include amphibians (Blaustein and Waldman 1992), birds (Krause et al. 2012), fish (Frommen et al. 2007), insects (Whitehorn et al. 2009), mammals (Mateo 2003), and reptiles (Léna and Fraipont 1998). Kin identification allows individuals to select mates that are not close relatives and consequently avoid inbreeding, and enables differential behavioural interactions that promote fitness of kin, thereby increasing inclusive fitness (Frommen et al. 2007). The theory of inclusive fitness (also referred to as kin selection theory) was proposed by Hamilton (1964) and suggests that an individual's fitness, in terms of their ability to reproduce and pass on their genes, is also contributed to by the success of genetic relatives and their shared genes, providing an explanation for cooperative behaviours among kin.

Many factors affect the presence and extent of kin recognition in a species, as well as the mechanisms through which it is achieved (Waldman 1988). For example, schooling fish species and amphibious species that form aggregations show a greater propensity for kin recognition, as they are more likely to encounter relatives throughout their lives (Carreno et al. 1996, Arnold 2000). Kin recognition may also assist individuals in avoiding predation (FitzGerald and Morrisette 1992, Griffiths et al. 2004) through the production of warning signals. The evolution of some alarm calls (i.e. the act of warning others of local threats) has been proposed to be driven by kin selection (Charnov and Krebs 1975, Sherman 1977), though this is by no means the only

mechanism influencing the evolution of alarm odours (Chivers et al. 2007, Lönnstedt and McCormick 2015). When living in close proximity to kin, the involuntary release of alarm or disturbance cues can alert family members to local predatory threats and improve their chance of evading capture, which concurrently increases the inclusive fitness of the individual that passively released the cue (Smith 1986, Blaustein 1988, Hettyey et al. 2015, Wisenden 2015). Chemical alarm odours are passively released when an aquatic organism is attacked and the upper epidermis is damaged (Chivers and Smith 1998), or blood is released (Barreto et al. 2013); both of which are honest and reliable indicators of a nearby threat. As the predators that pose a threat to prey species can vary greatly with both life history and habitat (Wilbur 1980), it can be imperative to survival to be able to not only recognise alarm odours, but also know which are relevant (Hill and Weissberg 2014). Many species have demonstrated recognition of both conspecific and heterospecific odours, an ability which often confers a survival advantage (Brown et al. 2011). Meuthen et al. (2014) investigated the antipredator responses of the cichlid, *Pelvicachromis taeniatus*, induced by both kin and non-kin alarm odours and found no difference in the magnitude of the reaction. However, Mitchell et al. (2012) found the magnitude of the antipredator behaviour of a damselfish species, elicited by alarm odours, directly related to the phylogenetic relatedness of the donor. Studies on gastropods (Dalesman et al. 2007) and amphibians (Schoeppner and Relyea 2005) demonstrated a similar ability to respond to cues in a threat sensitive manner based on phylogenetic proximity. Research suggests that individuals

often react in a graded manner to alarm odours, depending on the level of relevant risk they represent (i.e. higher concentration of the odour, and odours from the same ontogenetic stage represent a greater threat; Ferrari and Chivers 2010, Mitchell and McCormick 2013).

Embryos have also been shown to possess advanced olfactory capabilities. Not only can they detect and react to risk odours, but some can associatively learn risk from odours and respond to them in a threat sensitive manner (Mathis et al. 2008, Ferrari and Chivers 2010, Oulton et al. 2013, Atherton and McCormick 2015, Atherton et al. unpubl.). In some amphibian species, embryos have demonstrated the ability to use chemosensory cues denoting risk as a trigger for premature hatching, allowing them to escape predation (Warkentin 2011). However, it is currently unknown whether embryos are capable of differentially prioritising information from kin with respect to threat relevance. Such information should be advantageous as it provides the most relevant information on threats in the immediate vicinity of the individual.

The present research sought to determine if embryonic fishes could recognise kin using damage-released odours, and whether they responded differentially to odours from donors of varying levels of relatedness and phylogenetic proximity. Changes in the heart rate of two species of coral reef damselfish, *Amphiprion melanopus* and *Acanthochromis polyacanthus*, were assessed in response to conspecific odours originating from three sources: 1) kin – siblings from the same clutch; 2) kin previous – offspring from the same parents, but from a different clutch; and 3) non-kin – conspecifics from a different breeding pair. Additionally, we tested the reaction of embryos from both species to two different heterospecific cues. Both species were tested against cues from a phylogenetically different damselfish *Chrysiptera cyanea*, and *A. melanopus* and *Ac. polyacanthus* were tested against cues from embryos of the other species, representing a phylogenetically closer heterospecific (Cooper et al. 2009). We predicted that kin recognition would be present in both study species, but expected it to be more prevalent in *Ac. polyacanthus* due to the high level of association with genetic relatives immediately after hatching. Additionally, as a number of studies have demonstrated the well-developed olfactory capabilities of embryos (Arvedlund et al. 2000), we anticipated that both species of damselfish would be able to distinguish between chemical cues of varying relevance.

Methods

Study species

The present study compared the embryonic recognition of chemical alarm odours in two damselfish (Pomacentridae) species, one of which has a conventional dispersive larval phase (the cinnamon clownfish *Amphiprion melanopus*), while the other species is the only damselfish to brood its young (the spiny chromis *Acanthochromis polyacanthus*). *Amphiprion melanopus* and *Ac. polyacanthus* are both demersal spawners which lay their eggs in caves, and kin recognition is potentially relevant to both species. For those species with a pelagic larval phase, studies have found a high propor-

tion of larvae returning (~50%), not only to their natal reef, but within metres of their parents at settlement (Jones 2015, Salles et al. 2016).

Acanthochromis polyacanthus is the only reef associated damselfish to lack a pelagic larval stage and juveniles stay with the parents for up to three months after hatching (Kavanagh 2000) and then disperse locally to varying extents (Miller-Sims et al. 2008). Embryogenesis usually lasts 9–11 days, and *Ac. polyacanthus* eggs are very large compared to those of other damselfish (approximately 4 mm). They hatch during the day, and are considered to be settled at hatching (Kavanagh 2000). These early interactions amongst kin may be particularly important in affecting early survival when juveniles are most vulnerable to predators (Almany and Webster 2006).

The clownfish, *A. melanopus*, is an anemone-associated species and has a relatively short pelagic larval stage for a damselfish, at just 8–14 days (Bay et al. 2006a). The eggs are approximately 2.3 mm in length and develop for an average of 7.5 days before hatching in the evening as transparent larvae, and dispersing into the pelagic environment (Green 2004).

The blue devil damselfish, *Chrysiptera cyanea*, was used as an additional donor of damage-released odours. *Chrysiptera cyanea* is a rubble-associated species that lives in a different habitat within the coral reef (high current rubble patches on the reef flat) compared to both of the former species. They have similar life-history characteristics to *A. melanopus*, starting as a member of a benthic-laid egg mass, guarded by the parents for four days (Gronell 1989), prior to hatching and undergoing a pelagic larval phase, which can last up to 24–30 days (Gopakumar et al. 2009).

Animal housing

All animals used were housed, and experiments were carried out, at the Marine and Aquaculture Research Facilities Unit, James Cook Univ., Australia. Adult breeding pairs of both *Ac. polyacanthus* (seven pairs) and *A. melanopus* (three pairs) were kept in well-aerated 70-l tubs of seawater, on a constant flow-through seawater system. The temperature was maintained at 28°C throughout the breeding season, with a salinity of 35 ppm. The holding tanks were situated outside, so the fish were kept under a normal summer diurnal light cycle (light:dark, 12.5:11.5 h). Half of a terracotta pot was placed in each tank for the breeding pairs to use as shelter and to provide a surface for the females to lay their eggs on. Adults were fed pelleted food twice per day, at which point all tanks were also checked for egg clutches.

When a clutch of eggs was produced, it was left with its parents until two days prior to hatching; this occurred at day six after fertilisation for *A. melanopus* and ten days post-fertilisation for *Ac. polyacanthus*. Embryos were collected from their parental tanks by gently cutting the tissue adhering the clutch to the terracotta pot with a scalpel. Each clutch of embryos was kept in a separate well-aerated 1-l tub, in a shared water bath with flow through water, to maintain the temperature at that of the parental tanks. Water changes (25%) were carried out twice daily, using water from the parental tanks and the light cycle in the laboratory was set to mirror that of the outside parental tanks.

Stimulus preparation

Embryonic heart rates were recorded in response to a seawater control, or one of five damage-released odours from embryos that included: 1) embryos of kin, 2) kin from a previous clutch, 3) non-kin conspecifics, 4) *A. melanopus* or *Ac. polyacanthus* (the opposite species to the recipient), and 5) *C. cyanea*. The seawater for the control cue trials was sourced from the respective parental tanks. All odours derived from *Ac. polyacanthus* embryos were created by crushing five embryos in a petri dish, and then adding 5 ml of seawater. This solution was then passed through filter paper, removing any particulate matter to leave the odour-infused seawater, which provided enough for five replicate trials. The same method for odour production was used for all *A. melanopus* cues, the only difference being that ten embryos were used instead of five to produce 5 ml of alarm odour. This methodological difference was necessary for the standardisation of cue concentrations between species, as *Ac. polyacanthus* embryos are almost twice the size of those of *A. melanopus* (yielding a standard concentration of 7.6 ± 0.14 mg embryo per ml seawater [mean \pm SE]; Supplementary material Appendix 1 Table A1).

While the concentration of cues may seem high, they were consistently high for each test odour. This allowed for identification of whether discrimination between alarm odours and kin recognition were possible in these species, by eliciting the strongest likely response. We also believe that the concentrations of alarm odours used in the current study are ecologically relevant for two reasons. Firstly, when egg predators (e.g. the common reef fishes *Pseudochromis fuscus* or *Thalassoma lunare*; Emslie and Jones 2001) attack a benthic clutch of eggs, their bite disrupts multiple eggs at once. Secondly, research on the way chemical cues diffuse and are transported through naturally turbulent seawater suggests that chemicals do not necessarily evenly disperse throughout the water body, but rather travel in packets, so the concentration metres away from the source may, at times, be similar to those at the source (Weissburg 2000).

Chrysiptera cyanea eggs are very small in comparison to the other two damselfish species (approximately 1.3 mm in length). Thus, the quantity of eggs used to make each set of five *C. cyanea* alarm odours was determined by weighing five *Ac. polyacanthus* embryos and ten *A. melanopus* embryos and using the mean as a guide for collecting a standardised weight of *C. cyanea* embryos (Supplementary material Appendix 1 Table A1). Once weighed, the *C. cyanea* embryos were promptly placed into vials and frozen in liquid nitrogen. A pilot trial was carried out which showed that a very similar magnitude of heart rate increase was induced by damage-released odours produced by both fresh and frozen (and defrosted) embryos (Supplementary material Appendix 2 Table A2, Fig. A1). All embryos, from all three species, which were used to create alarm odours, were snap frozen in liquid nitrogen (-80°C) and stored for at least 1 hour, but no more than 14 days, before being defrosted and used in trials. This facilitated the measurement of the response of embryos to alarm odours produced from embryos from the same parents, but also from a previous clutch ('kin previous').

Experimental procedure

Trials were carried out on the day of hatching for *A. melanopus* and the day before hatching for *Ac. polyacanthus*. Change in heart rate was determined in response to one of the six olfactory cues previously outlined for both species. For each trial, one embryo was carefully placed in a small container of 10 ml of seawater, which was then placed under a dissecting microscope illuminated with an optic fibre cold light. This kept any heat from the light bulb well away from the observed embryos, and the temperature during observations was maintained at that of the water controlled holding tubs and parental tanks (28°C). The embryo was allowed to acclimatise for 2 min, after which the heart rate was directly recorded for a continuous 30 s by the same researcher to maintain consistency among trials. Mean baseline heart rate was 100 beats per 30 s for *A. melanopus* and 70 beats per 30 s for *Ac. polyacanthus*. The researcher then slowly injected 1 ml of one of the six recently prepared test cues into the container, and the heart rate was recorded for another 30 s. Each embryo was randomly assigned to, and tested with, only one of the six test odours. For *A. melanopus*, embryos were tested from five clutches produced by three breeding pairs (two pairs produced two clutches, and one pair produced one clutch), and for *Ac. polyacanthus*, embryos were tested from eight clutches obtained from seven breeding pairs (all pairs produced one clutch, except one which produced two clutches). Fifteen embryos from each clutch were tested per trial odour, for as many odours as possible, and for each species (Supplementary material Appendix 3 Table A3).

Changes in heart rate have been identified as a viable behavioural proxy for quantifying reactions to predatory threats, as increases or decreases in heart rate often simultaneously accompany, or precede, antipredator behaviours (Höjesjö et al. 1999). Fluctuations in basal heart rates and tachycardic responses to threats have been shown to induce phenotypic plasticity in traits that infer a survival advantage (Holopainen et al. 1996). Changes in heart rate are also thought to be a valid means to assess threat recognition in embryos which are unable to respond with any other observable behaviour (Oulton et al. 2013, Atherton and McCormick 2015).

Statistical analyses

Using Statistica 13, a fixed one-factor ANOVA model was run to determine if there were significant differences in baseline (i.e. before any treatment odours were introduced) heart rates between clutches, for each species. Both sets of baseline heart rate (HR) raw data met the assumptions of ANOVA.

To determine whether the different odours affected heart rates, the change in heart rate (difference between HR before and HR after the introduction of an odour) was used as the raw data for both study species (*A. melanopus* and *Ac. polyacanthus*). Proportional change data are commonly used in behavioural studies to reduce the impact of the high levels of individual variability seen in behavioural traits. Residual analysis indicated that residuals were normally distributed and the residual variances were homogeneous for both species. Two linear mixed effects (LME) models using

a restricted maximum likelihood approach were used. For each species, an intercept-only LME model was used that included 'Odour' as a fixed effect and 'Clutch' as a random effect. Tukey's HSD post hoc tests were used to determine the nature of significant differences found by LME analyses.

Data deposition

Data available from the Dryad Digital Repository: <<http://dx.doi.org/10.5061/dryad.tm335>> (Atherton and McCormick 2017).

Results

There were significant differences between the baseline heart rates among clutches for both *Amphiprion melanopus* ($F_{1,327} = 57.20$, $p < 0.001$) and *Acanthochromis polyacanthus* ($F_{1,423} = 33.35$, $p < 0.001$). These differences were largely driven by naturally lower heart rates in one of the clutches produced by the *A. melanopus* pairs and two clutches from *Ac. polyacanthus* pairs.

There was a significant difference between the changes in heart rates induced by the six test odours for both species (Table 1). In *A. melanopus*, the alarm odours derived from both direct kin (+10.50%) and kin from a previous clutch (+9.13%) caused an increase in heart rate that was significantly higher than all other odours (Tukey's HSD: $p < 0.05$ for all interactions; Fig. 1). The same significantly larger reaction to both types of kin alarm odours (kin = +11.32%; kin previous = +10.02%) occurred in *Ac. polyacanthus* (Tukey's HSD: $p < 0.01$ for all pairwise comparisons; Fig. 2). For both species, the introduction of a seawater control cue induced negligible increases in heart rate (*A. melanopus* = +0.06%; *Ac. polyacanthus* = +0.08%), which contrasted markedly to their reaction to alarm odours derived from conspecifics (Tukey's HSD: < 0.001 for all; Fig. 1, 2).

The introduction of a conspecific, non-kin odour resulted in a significantly lower increase in heart rate compared to conspecific kin alarm odour in both *A. melanopus* (+6.33%; Tukey's HSD: $p < 0.05$) and *Ac. polyacanthus* (+5.85%; Tukey's HSD: $p < 0.01$). The mean initial heart rates for embryos exposed to kin and non-kin odours for *A. melanopus* were 104 beats per 30s (SD = 11.86) and 97 (SD = 12.63), respectively. For *Ac. polyacanthus*, the mean initial heart rates (per 30 s) in embryos exposed to kin and non-kin cues were 71 (SD = 14.52) and 73 (SD = 11.46), respectively. Heterospecific cues (either *A. melanopus* / *Ac. polyacanthus*, or *C. cyanea*) induced increases in heart rates for both species; however, these increases were significantly lower than

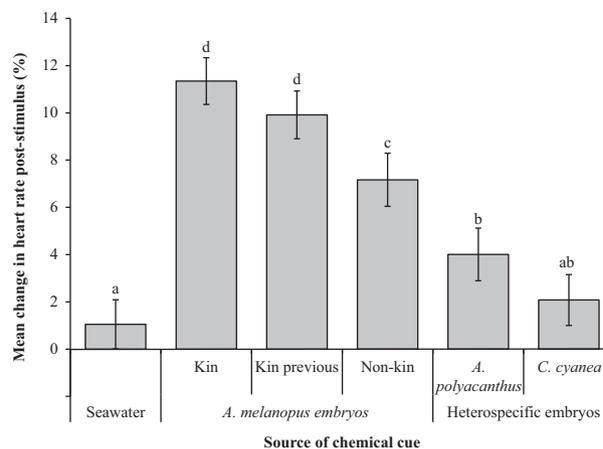


Figure 1. Mean percentage change in heart rate (\pm SE) of *Amphiprion melanopus* embryos after introduction of one of six chemical cues (saltwater, or conspecific alarm odours from: direct kin, kin from a previous clutch, or non-kin; or heterospecific alarm odours from: *Acanthochromis polyacanthus*, or *Chrysiptera cyanea*). Letters indicate Tukey's groupings of means ($n = 75$ for seawater and kin and kin alarm odours, and $n = 45$ for the remaining four alarm odours).

the reactions elicited by any of the three conspecific odours (Tukey's HSD groupings on Fig. 1, 2).

Discussion

Embryos of both species of damselfish (*Amphiprion melanopus* and *Acanthochromis polyacanthus*) were able to differentiate between kin and other non-related conspecifics, and heterospecifics, using olfactory cues. The ability of juveniles and adults to recognise their kin using olfactory cues alone has been identified in a limited number of taxa, including freshwater fishes (three-spined sticklebacks, *Gasterosteus aculeatus*; Mehlis et al. 2008) and birds (zebra finches, *Taeniopygia guttata*; Krause et al. 2012), but never before has it been documented at the embryonic stage. There have also been instances of species where kin recognition does not appear to occur (e.g. a cichlid, Meuthen et al. 2014). Blaustein et al. (1984) demonstrated that kin identification lasted through metamorphosis, with both tadpoles and froglets of the species *Rana cascadae* showing a preference to associate with siblings. If kin recognition can carry-over across ontogenetic stages in species with a pelagic larval phase, then it could contribute to the informed selection of low risk and preferential habitats at settlement (Dixson 2012). By passively releasing odours

Table 1. Results of two, linear mixed effects ANOVAs (with clutch included as a random factor) investigating the comparison of the mean changes in heart rates induced by one of six chemical alarm odours, in two damselfish species: *Amphiprion melanopus* and *Acanthochromis polyacanthus*.

Species	Effect	df	MS	F	p
<i>Amphiprion melanopus</i>	Odour	5	1155.967	68.535	<0.0001
	Residual	324	16.867		
<i>Acanthochromis polyacanthus</i>	Odour	5	1843.822	72.670	<0.0001
	Residual	424	25.373		

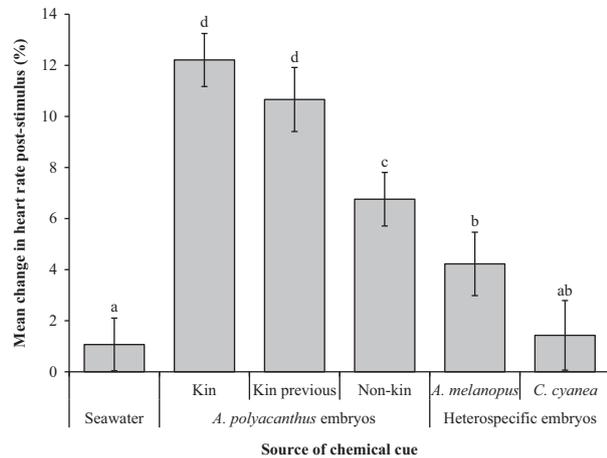


Figure 2. Mean percentage change in heart rate (\pm SE) of *Acanthochromis polyacanthus* embryos after introduction of one of six chemical cues (saltwater, conspecific alarm odours from: direct kin, kin from a previous clutch, or non-kin; or heterospecific alarm odours from: *Amphiprion melanopus*, or *Chrysiptera cyanea*). Letters denote Tukey's groupings of means (left to right, $n = 120, 120, 30, 75, 55$ and 30 , respectively).

that are indicative of risk, which may be more readily recognised by genetic relatives, individuals may enhance the survival chances of their kin; thus, increasing their own inclusive fitness (Hamilton 1964). However, further research is required to determine if the reactions observed in the present study are an example of such a phenomenon.

There was a significant difference between the reactions of embryos to kin and non-kin damage odours for both species of damselfish, despite the two species having very different life history characteristics. *Acanthochromis polyacanthus* lacks a pelagic larval stage, has a long egg duration, and remains with its parents and siblings for a number of months post-hatching; whereas, *A. melanopus* disperses into the pelagic environment as larvae for 8-14 days before settling on the reef (Bay et al. 2006a). This suggests that kin recognition may be a widespread mechanism in fish species that possess a benthic embryo phase. Research suggests that the prevalence of kin recognition in a species is dependent on the likelihood of encountering relatives in their environment (Carreno et al. 1996, Arnold 2000). Due to the brooding period of juvenile *Ac. polyacanthus*, this species spends an extended period of time with their siblings and parents. Furthermore, despite its pelagic larval phase, *A. melanopus*, among other anemonefish and coral reef fish species, often recruit back to their natal reefs (Green et al. 2015); sometimes even settling only metres away from their parents (Jones 2015). This is supported by Bay et al. (2006b), whose study demonstrated that pelagic larval duration is not a reliable indicator of genetic differentiation within and between populations. Thus, juvenile *A. melanopus* are still likely to encounter relatives, meaning kin recognition could be pertinent to coral reef fish species with high levels of self-recruitment.

High sibling association has been found in some other species of reef fishes with pelagic larval stages, which also have lower levels of self-recruitment than our study species. For example, groups of siblings were found in marine gobies *Coryphopterus personatus* (Selwyn et al. 2016),

three-spot Dascyllus *Dascyllus trimaculatus* (Buston et al. 2009) and humbug Dascyllus *D. aruanus* (Bernardi et al. 2012). Selwyn et al. (2016) proposed two possible mechanisms through which this could occur. The first is that some larvae remain with their kin throughout the pelagic larval duration, which can be up to a month long in the case of both *Dascyllus* species (Buston et al. 2009, Bernardi et al. 2012). Alternatively, larvae may not be dispersing very far, and instead, remaining in the reef habitat where there is better food availability (Selwyn et al. 2016), which could also explain the high levels of recruitment to natal reefs found in other species (Berumen et al. 2012). These findings, in conjunction with the results of the present study, suggest that kin recognition and association may be more important in coral reef fishes than previously realised. However, given the high levels of variability in both pelagic larval durations and dispersal distances in reef ecosystems (Jones et al. 2009, Green et al. 2015), kin association may not be present in, or adaptive for, all coral reef fish species. This highlights the importance of research into the prevalence and impact of kin recognition in the population dynamics of coral reef fish species.

Kin recognition can promote both short and long term advantages to individuals, though the evolutionary driver is thought to be the protection their gene pool and increasing their inclusive fitness (Hamilton 1964). Griffiths et al. (2004) found increased foraging rates (long term benefit) and more rapid responses to predatory threats (immediate survival advantage) in brown trout that associated with familiar individuals. Similarly, Schneider and Bilde (2008) found increased growth and foraging efficiency in the spider *Stegodyphus lineatus* when associating with kin; and three-spined sticklebacks spent more time with kin members when predators were present (FitzGerald and Morrissette 1992). Thus, kin recognition and cooperation may decrease the amount of pressure from competition and territorial aggression, allowing for more energy and attention to be focused on foraging and predator avoidance (Gerlach et al. 2007). Waldman (1982) suggested that the release of alarm cues is an altruistic act, in that it usually incurs predator-induced mortality, and would only increase a prey individual's inclusive fitness if relatives were nearby to benefit from its release; i.e. kin recognition could be a potential contributing driver for the evolution of alarm cues (for an alternative hypothesis see Lönnstedt and McCormick 2015).

While kin recognition has been identified in many taxa, and some survival benefits suggested, many studies have not demonstrated an adaptive advantage conferred by the recognition of genetic relatives (Blaustein et al. 1991). In the present study, the introduction of alarm odours did not induce premature hatching escape response, as has been observed in some amphibian species (Warkentin 2011). As such, reacting to threat cues during the embryonic stage could have maladaptive consequences for survival in subsequent life history stages, due to the metabolic and immune function costs incurred by increased levels of stress (Slos et al. 2009, Jarvis 2010). Also, the specificity in the level of response displayed by the embryos in both species of damselfish contradicts what one may expect, considering how often prey species eavesdrop on conspecific and heterospecific risk cues

(Brown et al. 2011). Thus, the apparent kin recognition-driven responses could be an epiphenomenal result of an alternative form of recognition (Grafen 1990). For example, Pfennig (1990) demonstrated that, regardless of the source of the cue, *Scaphiopus multiplicatus* tadpoles showed a preference for cues they learned in early life stages; i.e. even though tadpoles favoured unfamiliar siblings over unfamiliar non-siblings, they showed a preference for unfamiliar non-siblings reared on familiar food over unfamiliar siblings reared on unfamiliar food.

The magnitude of response by embryos of both species of damselfish to alarm odours was dependent on the phylogenetic proximity of the cue donor, consistent with studies that examined the relative sensitivities of adults to different donor cues (Cooper et al. 2009, Mitchell et al. 2012). Moreover, while there seemed to be recognition of cues from the more phylogenetically distant *Chrysiptera cyanea*, neither *A. melanopus* nor *Ac. polyacanthus* appeared to recognise it as a sufficient indicator of a relevant threat, with the responses not statistically differing from those induced by the seawater control. Studies on the freshwater gastropod *Lymnaea stagnalis* found similar graded responses to alarm odours from heterospecifics of varying phylogenetic distance (Dalesman et al. 2007). In contrast, three-spined sticklebacks showed no discrimination between threat cues sourced from conspecifics or heterospecifics, responding to them equally (FitzGerald and Morrissette 1992). Dalesman and Rundle (2010) suggested that the capacity for heterospecific alarm cue recognition is likely dependent on the degree of cohabitation between the species, as well as their relatedness. This is supported by the work of Chivers et al. (1995), who showed fathead minnows *Pimephales promelas* associated both conspecific and sympatric heterospecific alarm cues with danger and subsequently avoid areas marked with both cues. A combination of phylogenetic relatedness and habitat may explain our current findings, as *C. cyanea* typically live in a different habitat on coral reefs to that of the two focal species.

A number of potential mechanisms have been proposed for how organisms are able to distinguish among alarm odours, based on their relevance and the level of threat they indicate. While some consider kin recognition to be 'innate', via a mechanism known as self-referent phenotype matching (Mateo and Johnston 2000, Thünken et al. 2014), research suggests there may also be an imprinting and/or learning component involved (Waldman 1981, Mateo 2004, Frommen et al. 2007, Hain and Neff 2007, Gerlach et al. 2008). This may explain the slight difference observed in reactions for both species to kin and kin-previous cues in the present study, as this comparison also tests embryonic responses to familiar and unfamiliar kin cues. Hence, in the context of this study, it is possible that it is a combination of both some form of self-referent recognition and imprinting that are the controlling mechanisms. Similarly, anemonefish have been shown to possess refined olfactory capabilities allowing them to successfully imprint on specific species of host anemones, which then bias their choice of settlement habitat after their pelagic larval stage (Arvedlund et al. 2000). Recognition alleles and phenotype matching have also been proposed as potential methods for the recognition of kin via genetic and/or epigenetic mechanisms (Hepper 1986). With

regards to the capacity of organisms to differentiate between conspecific and heterospecific cues, Mirza and Chivers (2001) surmised that cues must either be: 1) identical and contain other chemical components that make them distinguishable; or 2) similar enough to be recognisable by both species, but vary in their overall composition. Irrespective of the mechanism, threat sensitive reactions by embryos to damage-released odours, demonstrated in both this study and those by Atherton et al. (unpubl.) and Ferrari and Chivers (2010), suggest they hold important survival advantages in early life stages when predation rates are often highest.

Conclusion

Our findings demonstrate that the embryos of two species of damselfish with very different life history strategies have a highly sophisticated capacity to discriminate between odours that may be of ecological importance. Not only could they detect damage released odours and determine the species of the donor, but they have the capacity to discriminate between odours from related and non-related donors. Moreover they can differentiate between alarm odours from their own clutch and previous clutches from the same parents. Future research directions should include identifying the prevalence of kin recognition and kin association in juvenile and adult populations of coral reef damselfishes, and determining whether it confers fitness and survival advantages. Of interest is whether the level of kin recognition is associated with the level of self-recruitment to natal reefs, as such recognition will be important to avoid inbreeding fitness depression. Kin recognition is understudied in marine organisms with complex life cycles, due to historically-assumed poor retention of offspring close to the parents. The surprisingly high level of return to natal habitats at the end of the larval phase in marine fishes found in the last two decades (Jones 2015) underscores the potential relevance of inclusive fitness hypotheses to these complex ecosystems and further research will provide valuable insight into the population dynamics of marine fishes.

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Supplementary material (available online as Appendix oik-03597 at <www.oikosjournal.org/appendix/oik-03597>). Appendix 1–3.