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JAMES COOK UNIVERSITY AUSTRALIA

Smelling home: The use of olfactory cues for settlement site selection by coral reef fish larvae

PhD thesis submitted by

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in August 2011

For the degree of Doctor of Philosophy

School of Marine and Tropical Biology

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The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *National Statement on Ethics Conduct in Research Involving Human (*1999*),* the *Joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997), the *James Cook University Policy on Experimentation Ethics*. *Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee (approval number A1264, A1598, A1335, and A1435).

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STATEMENT ON THE CONTRIBUTIONS OF OTHERS

The chapters of this thesis are also manuscripts that have been published, submitted or are in preparation for submission. Several researchers have made contributions to these manuscripts and it is necessary to recognise their contributions. This work is a result of collaborations with my supervisors Prof. Philip Munday, Prof Geoffrey Jones, and Dr. Morgan Pratchett, who provided intellectual and editorial guidance throughout the project. Research was funding was provided by the Australian Research Council, James Cook University, and the ARC Centre of Excellence for Coral Reef Studies. In addition I received a stipend from James Cook University's Postgraduate Research Scholarship and the American Australian Association.

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ABSTRACT

Most coral reef fish larvae hatch with well-developed sensory systems, and are thought to utilize a range of sensory cues for navigation in the pelagic environment and in settlement site selection. While visual, auditory and olfactory cues are all known to be important, our understanding of how, when and where each sense is used is incomplete. Olfaction is believed to be particularly important in habitat selection. However, reef fish larvae may respond to a range of different olfactory cues at different developmental stages, with olfaction involved in everything from navigation towards reefs to choice of specific settlement sites. This thesis combined novel laboratory and field-based research techniques to explore the use of olfactory cues for orientation and settlement site selection in coral reef fish larvae, using anemonefish as a model group.

It is known that olfactory cues are used in selection of specific microhabitats by coral reef fishes, but olfaction may also be relevant on larger spatial scales, to orientate towards specific reefs. In **Chapter 2**, I tested whether newly recruited anemonefish (*Amphiprion percula*) can distinguish between different reef habitats using olfactory cues and assessed whether attraction to certain cues is consistent with field-based settlement patterns. *A. percula* recruits displayed strong preferences for olfactory cues in water collected adjacent to reefs with vegetated islands compared with submerged reefs where no islands were present. These fishes also showed a strong preference for water treated with the olfactory cues of leaves from rainforest plants. The two anemones utilized by *Amphiprion percula* are found almost exclusively on reefs associated with vegetated islands, therefore leaves could provide a good olfactory cue to locate appropriate reefs on which to settle.

Strong preferences for terrestrial leaf odour and the ability of *A. percula* to distinguish between reefs suggests that the use of terrestrial borne olfactory cues for island reef identification may be significant to a variety of island-dwelling reef fishes. In **Chapter 3**, I tested the generality of these findings for other fishes that are found predominantly on reefs associated with vegetated islands. Eight island-associated species, from 3 families (Chaetodontidae, Pomacentridae and Labridae) were tested for island odour preferences and the use of terrestrial cues in island identification. Juveniles of all species displayed a preference for both island reef cues and terrestrial leaf litter, highlighting the potential importance of terrestrial cues in the island-reef ecosystem. The strong preferences for these cues across a wide range of fishes highlights the need for integrated terrestrial and marine management for the conservation of coral reef fishes.

Although settlement-stage reef fish larvae possess olfactory preferences involved in settlement site selection, it is not known at what stage the ability to distinguish olfactory cues becomes established. In **Chapter 4**, the responses to specific olfactory cues were tested throughout the entire larval stage for two species of anemonefish (*A. percula* and *Amphiprion melanopus*). A clear preference for untreated water over reef-based cues occurred during the first three days post-hatching. However, approximately halfway through the larval phase, at seven days post-hatching, larvae displayed a preference for reef-based olfactory cues, which strengthened until nine days post-hatching. These results indicate that reef fish larvae are capable of distinguishing olfactory cues almost immediately after hatching, but their responses to reef-based cues changes during development; initially newly hatched larvae are repelled by reef-based cues, which would tend to take them into the pelagic environment. These findings suggest that olfaction may be important in both dispersal and settlement of larval reef fish.

Recent studies show that many reef fishes settle on natal reefs, which may be due to imprinting on specific reef cues during early development. In **Chapter 5**, I examined the olfactory preferences of imprinted larval anemonefish in the laboratory and then tested the effect that innate preferences have on imprinting in the natural system. Laboratory reared larval *A. percula* exhibited the ability to imprint on chemical cues after a 12-hour window of exposure. Chemical cues which larvae displayed a strong innate preference to (e.g. host anemones) resulted in only a slight increase in olfactory preference after imprinting. In the field, larvae with the opportunity to imprint on the olfactory cues of one of two host-anemones did not display a significantly increase in the probability of returning to that species of host anemone once settlement stage was reached. The capacity for anemonefish to imprint on different chemical stimuli suggests it may be important in nature. While strong settlement preferences are exhibited, which may be refined by imprinting, settlement site selection ends up being largely governed by availability.

In the natural system, settlement-stage larvae are exposed to a magnitude of different odour cues and must respond appropriately to the information received. In **Chapter 6**, I determined the olfactory decision-making ability of three species of anemonefish, testing whether predation risk moderates settlement site selection. Settlement preferences, in all species, were conditional to the choices available, with larvae always choosing the host anemone or low-risk option compared with a non-host anemone species or high-risk areas indicated by the olfactory cues of a predator. These results demonstrate that olfactory cues can be used by larval reef fishes for complex risk assessment during settlement site selection. However, locating the correct habitat appears to be the most important factor when selecting a settlement site.

Although emphasis is placed on selecting the correct habitat, the importance of predator recognition cannot be discounted**.** It has recently been demonstrated that the ability of fish larvae to discriminate between olfactory cues of settlement habitat is impaired by elevated levels of dissolved CO_2. In **Chapter 7**, eggs and larval *A. percula* were reared in water treated with increased CO_2 levels. I tested the effect elevated CO_2 has on predator recognition in both newly hatched and settlement-stage coral reef fish larvae. Larvae, regardless of age, normally displayed an innate avoidance of odour cues indicating the presence of a predator. Newly hatched larvae exposed to the elevated CO_2 treatment exhibited expected patterns of olfactory discrimination. However, settlement-stage larvae treated with elevated CO_2 were incapable of discriminating between olfactory cues of predators and non-predators. The disruption of larval behaviour could have profound effects on population replenishment and connectivity patterns of many marine species.

This thesis provides strong evidence for the importance of olfaction in the decisions made by reef fish larvae, and highlights the complexity and sophistication of this remarkable sensory mechanism. Future research into the way chemical cues interact with other important sensory information will significantly improve our understanding of the critical factors that give coral reef fish larvae the ability to find their way home.

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CHAPTER 1: **General Introduction**

Habitat selection is one of the most important decisions all animals must make as it can have both immediate and long-term consequences for survival and reproduction (Thorpe, 1945; Klopfer, 1963; Morris, 2003; Radovic and Mikuska, 2009; Webley *et al.,* 2009; Schulte and Koehler, 2010; Mestre and Lubin, 2011). Animals vary in the degree to which juveniles remain at or return to natal sites, or disperse to other locations; leading to complex patterns of habitat selection that may change with ontogeny (Robbins *et al.,* 2009; Costello, 2010; Gienapp and Merilae, 2011; le Roux *et al.,* 2011; Oro *et al.*, 2011). Whether in search of home or a new habitat, juveniles may use a range of innate sensory abilities or learned behaviours to maximize chances of finding the best place to live (Klopfer, 1963; Wecker, 1963 etc.). In general, the relative importance of innate versus learned behaviours, and the roles of different sensory mechanisms in locating habitats are poorly understood. As natural habitats are increasingly being lost or degraded, there is an increased need to understand the mechanisms of finding, and the consequences of living in, different habitats.

In most marine animals, habitat selection occurs in a brief window of time early in life. Marine species typically have a life history characterized by a pelagic larval stage followed by a relatively sedentary adult stage (Brothers *et al.,* 1983; Davis, 1987; Leis, 1991; Kingsford *et al.,* 2002; Scheltema, 1986; Shulman and Bermingham, 1995). In marine fishes, the pelagic life-stage typically begins immediately after (or just before) embryogenesis (Petersen *et al.,* 2001), lasting days, weeks or months depending on the species. At the conclusion of the larval period, larvae are faced with the challenge of

locating appropriate settlement habitat, and making the dangerous ecological transition from a pelagic to a benthic existence (Kaufman *et al.,* 1992). The mechanism behind how marine fish larvae navigate through the pelagic environment, eventually locating a suitable benthic settlement site, remains largely unknown.

Due to the limited ability of recently fertilized embryos to detect environmental cues (Egner and Mann, 2005) as well as the limited capacity for movement and orientation thought to occur in early stage larvae (Hogan and Mora, 2005), the dispersal of marine fishes has largely been considered to be a passive process, whereby patterns of dispersal or local retention are an outcome of hydrodynamic processes (Roberts and Hawkins, 1997; Fisher and Wilson, 2004; Kobayashi, 2006). However, it is now understood that some larval fishes possess exceptional sensory and swimming capabilities, especially during the latter portion of the pelagic period (Wolanski and Hamner, 1988; Doherty and McIlwain, 1996; Leis *et al.*, 1996; Stobutzki and Bellwood, 1998; Montgomery *et al.,* 2001; Mora and Sale, 2002, Kingsford *et al.*, 2002). This suggests that pre-settlement fishes have some ability to control the extent of dispersal and their settlement location (Leis, 1986). Nonetheless, orientation in the featureless pelagic environment is challenging, and whether this transition from a pelagic existence to benthic habitat is opportunistic or self-determined and which cues are involved in the process has yet to be adequately resolved.

Senses allow organisms to perceive their environment and this information is then processed to make informed decisions. It is widely accepted that there are five basic senses - vision, taste, sound, touch and smell. Organisms potentially use all of these senses during habitat selection, although the dependency on each sense may vary with

species. It can be assumed that the senses most utilized for habitat choices would be highly developed so that an optimal location could be selected. For most organisms, it is unknown which senses contribute to locating habitats. In the pelagic environment, sensory cues provide larvae with important directional information. This information coupled with the ability to vertically distribute within the water column allows larvae to actively exploit different current speeds and trajectories, which in turn can impact their final settlement site (Paris *et al.*, 2007; Irisson *et al.,* 2010). Visual, auditory and olfactory cues are thought to be the most useful in settlement site selection for larval fish (Myrberg and Fuiman, 2002; Tolimieri *et al.*, 2004; Leis *et al.*, 2003). Fish larvae have well-developed sensory organs for all three cue types and both laboratory and field studies have indicated their use for orientation. In a review of larval sensory abilities, Kingsford *et al.* (2002) suggested auditory cues are most likely useful at large spatial scales (between 10-100 km), followed by chemical cues at intermediate scales (1-10 km) and visual cues at small spatial scales (only 0.1 km). However the specific cues, the dependency on each cue and the distance cues may be utilized still remains speculative. This information is crucial to a proper understanding of the relatively elusive time period when larvae are dispersing.

Responses to sensory cues can take two main forms, innate or learned. In an innate response the behaviour is inflexible, meaning, regardless of the environment or situation the response will remain. An organism receives a stimulus through one of its senses and a behaviour is automatically preformed, regardless of the situation. Innate behaviours are inherited; specific genes construct the discrete neural structures that underlie certain specie-specific behaviours, and these can be modified through mutations

of the genetic blueprint and as a result behavioural evolution is possible (Tierney, 1986). For example, honeybees display an innate preference for specific flower colours. The evolution of this preference is easily explained by the correlation between innately preferred colour and high nectar production (Giurfa *et al.*, 1995; Gumbert, 2000). Innate colour recognition has also been found to effect avian behaviour with birds innately avoiding colours associated with aposematic prey (Guilford, 1990; Rowe & Guilford, 1999). Extensive research on innate recognition has been conducted in terrestrial and freshwater taxa, especially regarding innate predator recognition (Epp & Gabor, 2008; Kindermann *et al.*, 2009; Gall & Mathis, 2010). The alternative to an innate behavioural response is a learned response. One form of learning called imprinting, may be especially useful in habitat recognition by dispersing juveniles. While acquired recognition, or imprinting, of a particular object is best known in birds; imprinting has also been demonstrated in coral reef fishes. Imprinting occurs when exposure to an object during a sensitive period early in life causes a change in the animal's behaviour towards that object at a later time. Although there are many types of imprinting recognized, ecological imprinting is concerned with the establishment of food and habitat preferences (Immelman, 1975). The criteria for ecological imprinting follows the same criteria for classical imprinting, which includes: (1) it can take place only during a restricted time of an individual's life, the sensitive period; (2) it is irreversible; (3) it involves the learning of supra-individual, or species-specific characteristics and; (4) it may be completed at a time when the appropriate reaction itself is not yet preformed (Lorenz, 1935).

Marine waters vary significantly in chemical composition on both large and small scales. Multiple sources of chemical cues that could be used for navigational information by larvae exist in the seemingly featureless pelagic environment (Kingsford *et al.*, 2002). Controlled by physics, chemistry and biology, chemical signals have a restricted lifetime. Once released into the environment, signals can decline below detectable levels as a result of mixing, diffusion, absorption, photolysis, chemical transformation and the uptake and breakdown by organisms (Atema, 1995). Small molecules or those emitted from a small source, dissipate as a result of molecular diffusion quickly, while larger molecules or compounds released from a larger source, can remain in the water column for days, weeks, or months (e.g. an oil slick from a whale carcass, terrestrial runoff during a wet season) (Atema, 1995). Olfactory cues appear to be particularly important in navigation (Atema, 1995; Williamson *et al.*, 2004; Mitamura *et al.*, 2005) and settlement site selection (Sweatman, 1988; Lecchini *et al.*, 2005 a,b; Horner *et al.*, 2006) for marine species. However, the use of olfaction for orientation is location dependent. Olfactory cues rely on the physical movement of water to disperse them throughout the environment; therefore a fish upstream from the olfactory source will be unable to sense the cue, however downstream the cue could be received. This is demonstrated in field studies where larvae chose settlement sites upstream of a current, ignoring sites, which were downstream (Elliott *et al.*, 1995; Lecchini *et al.*, 2005b).

Larval fish possess the necessary sensory morphology for chemotaxis and behave in ways that indicate chemotaxis is used for orientation (Atema *et al.*, 2002; Kingsford *et al.*, 2002; Arvedlund and Takemura, 2006; Gerlach *et al.*, 2007; Lara, 2008). For larval fish, chemical cues are best known to play a role in the recognition of settlement habitats

(Sweatman, 1983; Elliott *et al.*, 1995; Munday *et al.*, 2001; Atema *et al.*, 2002), food (Dempsey, 1978; Knutsen, 1992; Døving *et al.*, 1994; Batty and Hoyt, 1995; Kolkovski *et al.*, 1997), conspecifics (Sweatman, 1983; Sweatman, 1988; Ben-Tzvi *et al.*, 2010) and predators (Dixson *et al.*, 2010). The use of chemical cues in microhabitat selection has been demonstrated in a number of coral reef fish species. For example, Sweatman (1988) showed that not only are the settling damselfish *Dascyllus aruanus* attracted to the olfactory cues of conspecifics, but also that other species use the same chemical cues to avoid settlement sites occupied by *D. aruanus*. Corals also have been shown to produce important chemical cues that are detectable by settling larvae of associated fish species (Ben-Tzvi *et al.*, 2010). *Chromis viridis* chose settlement sites based on the olfactory cues of corals known to have positive settlement histories, indicating that settlement site selection could be based on water-borne cues originating from the coral itself, and that these varied among individual coral colonies (Ben-Tzvi *et al.*, 2010). Microhabitat selection in anemonefish is well documented with numerous studies showing an attraction of larvae to species-specific anemone odour (Elliot *et al.*, 1995; Arvedlund and Nielsen, 1996; Arvedlund *et al.*, 1999; Dixson *et al.*, 2008).

Specific organisms often characterize settlement habitats, for example corals are the major component defining a coral reef. Consequently, it is likely that specific chemical signatures of habitat types could be used for broader-scale habitat selection. Atema *et al*. (2002) showed that apogonid larvae were attracted to the olfactory cues of the location to which they were acclimated (lagoon water or ocean water). This suggests that larvae may be able to relocate reefs once settlement stage is reached through active retention within the reef odour plume (Atema *et al.*, 2002). Furthermore, Gerlach *et al.*

(2007) found that apoginid and pomacentrid larvae were able to distinguish between the chemical cues emitted from the reef they were collected from compared with the cues emitted from other reefs within a ten km radius of the "home" location. This indicates that chemical cues may be useful for settlement on a much larger scale than previously expected with reefs themselves showing distinct chemical signatures which larvae are able to recognize and respond to.

Although never proven, the use of chemical cues in natal settlement site recognition has been hypothesized, particularly where a greater proportion of recruits return home to natal reefs than would be expected if larvae were passively drifting (Almany *et al.*, 2007, Jones *et al.*, 2005). Natal settlement site recognition through olfactory cues requires the ability for larvae to recognize and respond to the odour associated with the reefs in which they were produced, possibly through imprinting. Chemical imprinting in coral reef fishes has been shown to occur under laboratory conditions, but this mechanism of learning has not been demonstrated in the natural environment. If imprinting were occurring in the wild, the sensitive phase would need to occur by either chemical cues passively diffusing across the egg membrane or immediately after hatching before larvae are exported away from their natal location. Imprinting in zebrafish occurs within a 24 hour window six days post-fertilization, 2 days after hatching (Gerlach *et al.*, 2008). Many fish have strong microhabitat associations and these microhabitats are known to have a complex influence on recruitment patterns (Sale *et al.*, 1984; McCormick and Makey, 1997). Imprinting is one mechanism that could help to explain site attachments in some species (Arvedlund and Nielsen, 1996;

Arvedlund *et al.*, 1999). While the possibility of olfactory imprinting exists, the influence it plays in the natural system remains unknown.

Although larval olfactory capabilities during the later portion of the larval phase have been researched in laboratory settings (Arvedlund *et al.*, 1999; Arvedlund and Takemura, 2006; Gerlach *et al.*, 2007; Dixson *et al.*, 2008; Dixson *et al.*, 2010; Atema *et al.*, 2002; Wright *et al.*, 2005; Wright *et al.*, 2008), relatively few studies have been conducted in the field (Sweatman, 1988; Elliott *et al.*, 1995; Lecchini *et al.*, 2005 a,b; Ben-Tzvi *et al.*, 2010). Sensory information available to larvae for behavioural choices is poorly understood; with little known about the specific chemical cue and concentrations required for larvae to recognize and respond to these cues. Fish larvae are fragile, exhibiting high levels of mortality as a result of stress, improper handling techniques, specific food requirements and the need for pristine water quality; causing only a limited number of marine fish species capable of captive breeding, so research thus far has primarily been conducted on larvae during the latter part of their larval period. Scientists can now say with certainty, that late-stage larvae of a few species have well-developed olfactory systems that are capable of distinguishing between different associated habitats (e.g. live coral, conspecifics, symbionts), and that this appears to influence the location of their final settlement site. However, very little is known about chemotaxis throughout the entire larval stage. The few studies that have examined early larvae have primarily documented morphological development (Arvedlund *et al.*, 1999; Lara, 2008) rather than behaviour and ability. Although, morphological variation was found among species and with ontogenetic development, this variation has yet to be correlated with olfactory ability or behaviour. Research is now required to identify the chemical cues used and the

behavioural responses that their recognition generates. Because the larval stage of demersal fishes can last from weeks to months in the water column, a full understanding of marine connectivity will not be achieved until researchers chart the ontogenetic development of olfactory abilities throughout the entire larval period.

Regardless of how larval fish actually detect reefs and then distinguish among reef habitats, the ultimate fate of fish larvae is largely dictated by the appropriate selection of specific microhabitats. Microhabitat recognition is a complex process, with multiple factors needing to be assessed. Such factors include whether or not to settle with conspecifics, which species of habitat structure to choose and what threat of predation is associated with each habitat assessed. Many studies of microhabitat recognition focus on just one aspect of habitat selection at a time (Arvedlend *et al.*, 1999; Arvedlund and Takemura, 2006; Booth, 1992; Brolund *et al.*, 2003; Ben-Tzvi *et al.*, 2010). Although it is useful to isolate the importance of the different habitat components, in the natural environment, larvae are required to make a more complex decision, weighing all of the variables associated with each settlement option. The capacity of larvae to make complex hierarchical decisions has not been tested, and this is a necessary aspect of the settlement process that needs to be assessed experimentally.

The importance and influence of predation in the recruitment process has led to the evolution of many learned (Brown, 2003; Kelley and Magurran, 2003) and innate (Hawkins *et al.*, 2004) mechanisms for sensing predators and avoiding them. 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. Predator recognition and avoidance in aquatic ecosystems often involves detection of olfactory cues from

predators (Wisenden, 2000; Kelley and Magurran, 2003). A range of sensory mechanisms may be used by larval reef fish to avoid predator detection (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 larvae and the use of olfactory cues to locate settlement habitat by many species, point to the importance of olfaction during the settlement process as well as the potential for complex settlement decisions to be based on the information received through the olfactory system.

Anthropogenic induced changes to the environment threaten many natural processes and ecosystems, often by altering the chemical composition of the ocean (Holdgate, 1994). This can occur by adding additional non-natural elements, as is the case in terrestrial runoff or shifting chemical equations to cause altered levels of naturally present compounds, such as in ocean acidification. Human activities such as land-use changes and the combustion of fossil fuels have caused an increase in the amount of CO_2 in the atmosphere. The concentration of CO_2 at the ocean surface is at approximate equilibrium with the atmosphere, therefore, as CO_2 levels increase in the atmosphere they also increase in the ocean (Doney, 2010), with potentially serious implications for many marine species (Portner *et al.*, 2005; Fabry *et al.*, 2008; Doney *et al.*, 2009). Research has just begun to look into the effects that increased ocean CO_2 levels could have on olfactory-mediated behaviour of reef fishes (Munday *et al.*, 2009; 2010; Dixson *et al.*,

2010), with some startling results. One recent study has demonstrated that increased CO_2 can affect the critical sensory mechanisms in larval coral reef fishes. Munday *et al.* (2009) revealed that larval anemonefish reared at1000ppm CO_2 (a level expected to occur by 2100 on a business as usual scenario) were unable to identify olfactory cues necessary for settlement site location. As discussed above, olfactory cues are relied upon by coral reef fish for survival techniques such as locating substrate, food, potential mates, and avoiding predators, therefore olfactory impairment may have far reaching effects. Although there are many scenarios that could potentially link increased CO_2 to the increased mortality of marine species, few direct links have yet been established.

The overall objective of this thesis is to test the importance of olfactory cues for settlement site selection by coral reef fish larvae. To address this question, I have conducted six independent, but related studies investigating detailed response at various ontogenetic stages from hatching to settlement using anemonefishes as a model group.

Anemonefish are characterized by the symbiotic relationship they form with host sea-anemones. As a result of their highly specific habitat requirements, anemonefish are an ideal species for studying habitat selection. There are 28 species of anemonefish all residing in the Indo-Pacific region and inhabiting up to ten different species of host sea-anemones. This symbiotic relationship is species specific with each anemonefish species utilizing between one and up to all ten sea-anemone species, with the average anemonefish only able to associate with three sea-anemone species (Fautin and Allen, 1992). The highly specific nature of the anemonefishes' habitat requirements at settlement and the importance of recruitment for the maintenance of the adult population, have focused research on the problems of host recognition by newly settling juveniles

(Miyagawa and Hidaka, 1980; Murata *et al.*, 1986; Miyagawa, 1989; Konno *et al.*, 1990; Elliott *et al.*, 1995; Arvelund and Nielsen, 1996; Arvedlund *et al.*, 1999). Due to their high degree of specificity, not only do anemonefish larvae need to locate a suitable reef habitat, but they must also locate a reef that supports the species-specific host sea-anemone required. A considerable amount of physiological research has been conducted on anemonefishes, showing a well-developed olfactory system (Lara, 2008). This physiology combined with previous research mentioned above (i.e. imprinting, and host identification through olfactory cues) indicates that the olfactory sense may be heavily relied upon in settlement site location on both a macro and micro level. Anemonefish have also been the focus of recent ground breaking research in connectivity patterns of reef fishes (Almany *et al.*, 2007; Jones *et al.*, 2005). The large established knowledge base on anemonefish physiology, connectivity and sensory capabilities combined with well established breeding protocols and relative ease with which these species can be reared in captivity, make these species idea candidates to determine the importance olfactory cues play in settlement site selection for coral reef fish larvae.

Chapter 2 explores if olfactory cues are useful in settlement site selection at a much larger scale than previously identified, and if so, which unique olfactory cues are useful in distinguishing between different reefs for settlement site recognition. This study was conducted using the orange anemonefish *Amphiprion percula*, in Papua New Guinea, where there is a strong correlation between the associated host anemones it inhabits and the reefs surrounding vegetated offshore islands. I aim to determine if olfactory cues are useful for distinguishing reefs where settlement habitats are located and which olfactory cues may be used in identifying these reefs.

Chapter 3 tests if the habitat settlement cues identified as important for the orange anemonefish in Chapter 2 are also used in a range of other reef fish species. This study aims to determine if the use of the olfactory cues produced by terrestrial leaf litter is a common settlement site identifier for other reef fish species associated with offshore vegetated islands. Recruits of eight different species, from three reef fish families, were tested for the olfactory preference of terrestrial leaf litter.

Understanding the role imprinting plays in olfactory cue recognition in the natural environment and the effect innate preferences have on the imprinting process is important. Chapter 4 aims to determine the capacity for imprinting to olfactory settlement cues by the orange anemonefish *A. percula.* The role imprinting plays in the field was then tested using DNA parentage analysis that enabled a comparison to be made between the host anemone odour which the eggs were exposed to during development and potentially imprinted on, to the host anemone species they settled on.

Throughout larval development, the behavioural response evoked by different olfactory cues may change. For example, newly hatched larvae are exposed to the same chemical cues when being exported away from the reef as settlement-stage larvae returning back to the reef, however, the response to these cues may be very different between the two developmental stages. Chapter 5 identifies the ontogenetic development of olfactory cue use. Throughout the pelagic larval duration the preference for settlement cues were tested on two species of anemonefish. This is the first study to investigate the ontogeny of behavioural responses to olfactory cues in reef fishes.

During the settlement process larvae have multiple factors which need to be weighed to determine the ideal settlement habitat available, for example, the influence

correct habitat selection and the role predator odour plays in site selection. Chapter 6 explores the ability of coral reef fish larvae to process multiple olfactory cues and the effect predation plays in settlement site selection. The ability of coral reef fish larvae to make conditional settlement choices based on the options available is important in optimizing search time with an ideal location.

Lastly, Chapter 7 determines the impact the anthropogenic change of ocean acidification will have on the olfactory system through predator recognition. Ocean acidification has already proven to disrupt the ability of coral reef fish larvae to distinguish between olfactory cues. Predator recognition is a critical component in the settlement process and if disrupted could lead to dire consequences for the replenishment and sustainability of marine populations.

CHAPTER 2: **Coral reef fish smell leaves to find island homes** †

† Dixson DL, Jones GP, Munday PL, Planes S, Pratchett MS, Srinivasan S, Syms C, Thorrold SR (2008) Coral reef fish smell leaves to find island homes. *Proceedings of the Royal Society of London Series B* 275:2831-2839

2.1 ABSTRACT

Recent studies have shown that some coral reef fish larvae return to natal reefs, while others disperse to distant reefs. However, the sensory mechanisms used to find settlement sites are poorly understood. One hypothesis is that larvae use olfactory cues to navigate home or find other suitable reef habitats. Here we show a strong association between the clownfish *Amphiprion percula* and coral reefs surrounding offshore islands in Papua New Guinea. Host anemones and *A. percula* are particularly abundant in shallow water beneath overhanging rainforest vegetation. A series of experiments were carried out using paired-choice flumes to evaluate the potential role of water-borne olfactory cues in finding islands. Recently settled *A. percula* exhibited strong preferences for: (i) water from reefs with islands over water from reefs without islands; (ii) water collected near islands over water collected offshore; and (iii) water treated with either anemones or leaves from rainforest vegetation. Laboratory reared-juveniles exhibited the

same positive responses to anemones and rainforest vegetation, suggesting that olfactory preferences are innate rather than learned. We hypothesize that *A. percula* use a suite of olfactory stimuli to locate vegetated islands, which may explain the high levels of self-recruitment on island reefs. This previously unrecognized link between coral reefs and island vegetation argues for the integrated management of these pristine tropical habitats.

2.2 INTRODUCTION

The replenishment and persistence of animal populations are contingent upon dispersing individuals finding and becoming established in a suitable habitat (Rosenzweig, 1981; Morris, 2003). Habitat selection by dispersing offspring is critical for their future survival and reproduction, and may involve both innate and acquired preferences for their natal habitat (Davis and Stamps, 2004; Mabry and Stamps, 2008). The majority of marine organisms begin life as larvae that may disperse among isolated adult populations (Scheltema, 1986; Caley *et al.*, 1996; Pechenik, 1999). The ability to find suitable habitat after a period in open water represents a significant challenge to marine larvae and the mechanisms by which this is achieved are poorly understood. Both passive larval dispersal and behavioural decisions based on a variety of sensory modalities and environmental cues have been implicated (Butman, 1987; Pawlik, 1992; Rodriguez *et al.*, 1993), but their relative importance is uncertain.

Theory suggests that the persistence of coral reef fish populations requires not only significant connectivity among sub-populations, but also that some juveniles return

to natal sub-populations (Armsworth, 2002; Hastings and Botsford, 2006). Recent empirical research has confirmed a high level of self-recruitment within and connectivity among isolated reef habitats (Jones *et al.*, 1999, 2005; Swearer *et al.*, 1999; Cowen *et al.*, 2000, 2006; Almany *et al.*, 2007; Patterson and Swearer, 2007). However, the means by which coral reef fish larvae detect, orient towards and settle onto reefs, either home or away, remains a mystery. In addition, the roles of acquired or innate habitat preferences have seldom been investigated.

Recent advances in our understanding of coral reef fish larvae indicate well-developed sensory and swimming capabilities (Stobutzki and Bellwood, 1997; Kingsford *et al.*, 2002; Lecchini *et al.*, 2005 a,b; Leis, 2006). Evidence suggests that both reef sounds (Leis *et al.*, 2003; Simpson *et al.*, 2005; Montgomery *et al.*, 2006) and olfactory cues (Atema *et al.*, 2002; Arvedlund and Takemura, 2006; Døving *et al.*, 2006; Gerlach *et al.*, 2007) could be used as means to locate and orient towards home or a suitable habitat. For example, Gerlach *et al.*, (2007) showed that settling larvae are not only capable of olfactory discrimination among reefs, but also prefer the water-borne odours of their home reefs. However, the source of the chemical cues that larvae respond to has not been identified.

It is well known that coral reef fish larvae can use olfactory cues to identify suitable settlement sites once they are in the vicinity of reef habitat (Sweatman, 1988; Lecchini *et al.*, 2005 a,b, 2007; Gerlach *et al.*, 2007). Settling larvae have been shown to respond to a variety of olfactory signals, including those that can be traced to resident conspecifics (Sweatman, 1988; Booth, 1992; Lecchini *et al.*, 2005a,b; Døving *et al.*, 2006), coral tissues (Lecchini *et al.*, 2005a,b, 2007) or symbiotic partners such as

anemones (Elliott *et al.*, 1995; Arvedlund and Nielsen, 1996; Arvedlund *et al.*, 1999). In the case of anemones, the specific chemical cues that induce the symbiosis have been isolated (Murata *et al.*, 1986). There is also some evidence that larvae can exhibit habitats selection without prior experience (Elliott *et al.*, 1995; Arvedlund and Takemura, 2006), while early natal experiences may also influence habitat choices at later developmental stages (Arvedlund and Nielsen, 1996). However, in general, the chemical cues that larvae use for finding suitable habitats or returning home remain poorly understood.

Recent work in Papua New Guinea has shown that a high proportion of juvenile clownfish (Amphiprion spp.) recruit to their natal populations (Jones *et al.*, 2005; Almany *et al.*, 2007). Many of these populations appear to be associated with the coral reef habitat that surrounds vegetated islands (Jones *et al.*, 2005; Almany *et al.*, 2007). Some individuals also appear to successfully disperse tens of kilometres between reefs with islands. However, the mechanisms by which larvae discriminate between reefs with and without islands, or identify home islands have not been investigated.

The overall aim of this study was to investigate the role olfactory cues potentially play for the orange clownfish (*Amphiprion percula*) larvae when attempting to locate island homes. First, we examined the extent to which this species is restricted to the fringing reefs surrounding islands in Kimbe Bay, Papua New Guinea. We then conducted a series of field experiments, using the two-channel choice flumes developed by Gerlach *et al*. (2007), to test the ability of settled juveniles to discriminate odours from water surrounding islands or treated with either anemones of rainforest leaves. We hypothesize that juveniles would prefer water from reefs with islands over water from

reefs without islands; would prefer water from near islands over offshore water; and would positively respond to water containing chemical cues from anemones or island vegetation. Finally, to test weather natal experiences influences habitat choices or whether larvae exhibited innate preferences, the ability of naive laboratory-reared larvae to respond to cues from anemone and vegetation was also investigated.

2.3 MATERIALS AND METHODS

2.3.1 Study location and species

The study was carried out in Kimbe Bay (5°12.530 S, 150° 22.801 E) on the island of New Britain, Papua New Guinea (Fig. 2.1). The focal clownfish species, *A. percula*, inhabits two host anemone species, *Heteractus magnifica* and *Stichodactyla gigantea*, in this region (Ollerton *et al.*, 2007). Field observations and experiments on *A. percula* were conducted over two 10 day intervals (20 February-2 March, and 18-28 April 2007). The study encompasses size locations on the western side of Kimbe Bay, including three reefs with islands covered with tropical vegetation (Kapepa Island, Kimbe Island, and Tuare Island) and three emergent reefs without islands (Margett's Reef, May Reef, and South Bay Reef; Fig. 2.1). Supplementary laboratory experiments were conducted at James Cook University Marine Aquarium Facility in Townsville Australia

Figure 2.1 Satellite image showing the locations of Kimbe Bay (New Britain, Papua New Guinea) and the six study locations within western Kimbe Bay. Three locations were fringing reefs surrounding small islands covered with rainforest vegetation (Kimbe Island, Kapepa Island and Tuare Island) and three were emergent reefs with no islands (Margett's Reef, May Reef, South Bay Reef)

2.3.2 Association with island reefs

Amphiprion percula were counted in four randomly placed 50x4 m visual transects at each of the six study sites. The number of the host anemones of each species (*H. magnifica* and *S. gigantea*), and the number of *A. percula* individuals on each anemone were counted on each transect. All of the transects on reefs with islands were laid out 5 m from the parallel to the shoreline. On reefs with no islands, transects were laid out 5 m from the inner edge of the reef. A nested ANOVA was used to analyse the

square root-transformed anemone transect data, to determine if there was a difference in the abundance of *A. percula* between reefs with and without islands, and among reefs nested within the categories.

A small boat was used to conduct visual transects to assess the abundance of floating leaf litter on two reefs with islands (Kimbe Island and Tuare Island) and without islands (May Reef and South Bay Reef). In addition, on the two reefs with islands, leaves were counted at four different distances from the shoreline (within 5 m of shoreline, reef crest, 500 m offshore and 1 km offshore). At each location and distance from shore, 10 replicate 100x4 m transects were counted by driving the boat in a straight line parallel to the shoreline and counting the number of floating leaves in a 4 m wide path. Distance offshore was maintained using a hand-held GPS unit.

A detailed map was made of the locations of the two anemone types at the primary study location (Kimbe Island) to determine their proximity to the island and their distribution among reef habitat types.

2.3.3 General protocol for field olfactory choice tests

Field olfactory choice tests were conducted aboard a dive vessel moored near the study locations. To assess the ability of juveniles *A. percula* to actively choose between water potentially containing different odour stimuli, we used a two-channel choice flume (13cm x 4 cm) developed by Gerlach *et al.* (2007). This apparatus was deigned to conduct pairwise choice experiments, with fish able to freely choose between water flowing from two different sources. The water from the two different sources is gravity-

fed from buckets and flows through tubes into the choice flume that is partitioned along half of its length. Fish are released at the downstream end where they are free to move to either side to 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. The flow rates were measured using a flow metre and dye tests were conducted at each water change to ensure that the rate remained the same for the entire study. The dye tests ensure that the two flow channels exhibited distinct and parallel water flow, with no turbulence or eddies.

Newly settled juveniles *A. percula* were collected from anemones using small hand nets and placed in 11 individual plastic bags that were two-thirds filled with water. The fish were retained in the bag for a maximum of 24-hours before experiments were conducted, although the majority of the trials took place within 4 hours of collection. For each trial, a single fish was placed into the centre of the downstream end of the choice flume and given five minutes to acclimatize to the two water choices. During this period, the fish were able to swim throughout the chamber. At the end of the acclimation period, the position of the fish on each side of the chamber was recorded at five second intervals for a two minute period. This was followed by a one minute 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 water sources, the entire test, including the acclimation period, was repeated and the total time the juveniles were associated with each water source was recorded. Each juvenile was subjected to a single trial, and a minimum of 20 and a maximum of 28 replicates were used for each

test. The few fish that did not swim against the water flow during the acclimation period were removed from the study.

Water samples for all tests were stored in buckets for a maximum of two days; however, when possible, recruits were tested in water collected within 24 hours. Gerlach *et al.* (2007) showed in a previous experiment that the age of the water did not affect the choice behaviour. All water was stored in the same location and shaded to ensure similar temperatures throughout the experiment. Chi-squared tests were conducted to detect statistically significant water choices. For each set of experiments, a trial was scored as showing a preference for the water source containing the test stimulus if the fish spent more than 50 per cent of its time on that side of the chamber. A trial was scored as not showing a preference for the test stimulus in the fish that spent 50 per cent or less of its time on the side of the chamber with the water source containing the test stimulus. The total number of trials in which the fish showed a preference for the test stimulus compared with the total number of trials in which the fish did not show a preference were then compared using a χ^2-test and given the null expectation of a 1:1 distribution.

2.3.4 Field experiment 1: olfactory discrimination between water samples from reefs with and without islands

Pairwise choice experiments were conducted to test whether juveniles could discriminate water from reefs surrounding islands from water collected at reefs without islands. Beach water (collected from 1 m off the shoreline) was used in the island trials. On non-island reefs, water was collected from the middle of the reef top at its shallowest

point. Two separate comparisons were made among island and non-island locations: Kimbe Island versus May Reef (n=28) and Tuare Island verse South Bay Reef (n=22).

2.3.5 Field experiment 2: olfactory discrimination between water samples from different distances from island reefs

Juveniles were given pairwise choices between water from three different positions associated with island reefs: (1) beach water (collected within 1 m off the shoreline), (ii) reef crest water (collected from near the outer edge of the reef crest), and (iii) offshore water (collected 1 km from the island, measured using a GPS). All three combinations were tested for replicate juveniles collected from each of two islands: Kimbe Island (n-24) and Tuare Island (n=22).

2.3.6 Field experiment 3: olfactory discrimination between water samples treated and not treated with either the anemone *S. gigantea* or leaves from rainforest trees that overhang water.

Juveniles were given pairwise choices between untreated offshore water and offshore water treated with either anemones or rainforest leaves. The anemone trials were conducted to ensure that juveniles were making realistic choices and not just responding to novel chemical cues. Previous studies had shown a strong orientation towards anemones and an ability to detect anemone odours over short distances (e.g.

Elliott *et al.,* 1995). For these trials a single whole anemone was added to the treatment water source.

Rainforest leaves were tested owing to the clear association between *A. percula* and leaf litter near islands. The potential response to chemical signals from leaves were tested by adding broken leaves from five common shoreline trees native to Kimbe Island. For each trial, enough leaf matter was added to offshore water samples to cover the surface of the water bucket (9.6 l) and allowed to stand for two hours before each trial. Leaves from the five different species were tested both individually (n=20) as well as for a mixture of all the five species (n=24).

2.3.7 Laboratory experiment: olfactory discrimination by naive laboratory reared larvae

To test whether the olfactory preferences observed for field caught settlers were acquired or innate, larvae reared in the laboratory without prior exposure to the stimuli were subjected to similarly paired choice experiments. Larvae were raised from a brood stock of 21 adult breeding pairs of *A. percula* maintained in 70 litre tanks in a closed seawater system at the James Cook University Marine Aquarium Facility for two years prior to experimentation. To test weather naive juveniles could discriminate water samples treated with leaves, larvae from different clutches were raised in indoor rearing chambers for 11 days, at which stage the larvae exhibited positive attraction to the sides of the rearing chamber consistent with settlement behaviour. Artificial seawater (Red Sea Brand) assumed to contain no biological cues was used for controls and to establish

the treatments. At no stage were adults, embryos or larvae allowed to come into contact with terrestrial organic matter, prior to the experimental manipulations. In a manner similar to the field trials, reared larvae were given a choice between water sources treated and not treated with anemone to test whether laboratory-reared juveniles could make realistic choices consistently with well-known symbiotic association between *A. percula* and anemones. To prepare the treatment water, an anemone was placed in a water bucket (9.6 L) for two hours and then removed prior to sensory tests being conducted. The same anemone was used for all the trials.

To test responses to leaves, the water samples were treated with leaves from a common coastal rainforest tree (*Xanthostemon chrysanthus*: Myrtaceae). If olfactory preference for tropical trees were innate, we predicted that the naive larvae would be positively attracted to the stream of water that had been treated with leaves of *X. chrysanthus*. To ensure that leaves were not simply attracted to stimuli from any organic source introduced into the test apparatus, we also tested the response of larvae to water samples treated with leaves from a swamp based tree (*Melaleuca nervosa*: Myrtacaea). *M. nervosa* leaves contains pungent oils that, we expected, would normally be avoided by the larvae. Each treatment source was treated with 20 grams of broken leaves (approximately the same amount as used in the field trials). The leaves were cut into 1 cm squared pieces, soaked in 10 litre of seawater for two hours, and then removed from the water before trials were conducted. A total of 36 and 30 *A. percula* larvae were tested for their responses to *X. chryanthus* and *M. nervosa*-treated water, respectively. These larvae were offspring from five adult breeding pairs and seven different clutches from each pair.

2.4 RESULTS

2.4.1 Association with vegetated islands

Amphiprion percula and the anemones they occupied, exhibited a strong association with the fringing reefs surrounding the islands (Fig. 2.2). *Amphiprion percula* were found at densities of approximately 6-12 per 200 m^2 in transects close to islands. A nested ANOVA indicated significantly higher densities near islands ($F_{1,4}$=27.3, $p<0.001$) and no significant variation among reefs with islands ($F_{4,18}$=0.37, p=0.82). The *A. percula* were rarely observed on emergent reefs without islands (Fig. 2.2).

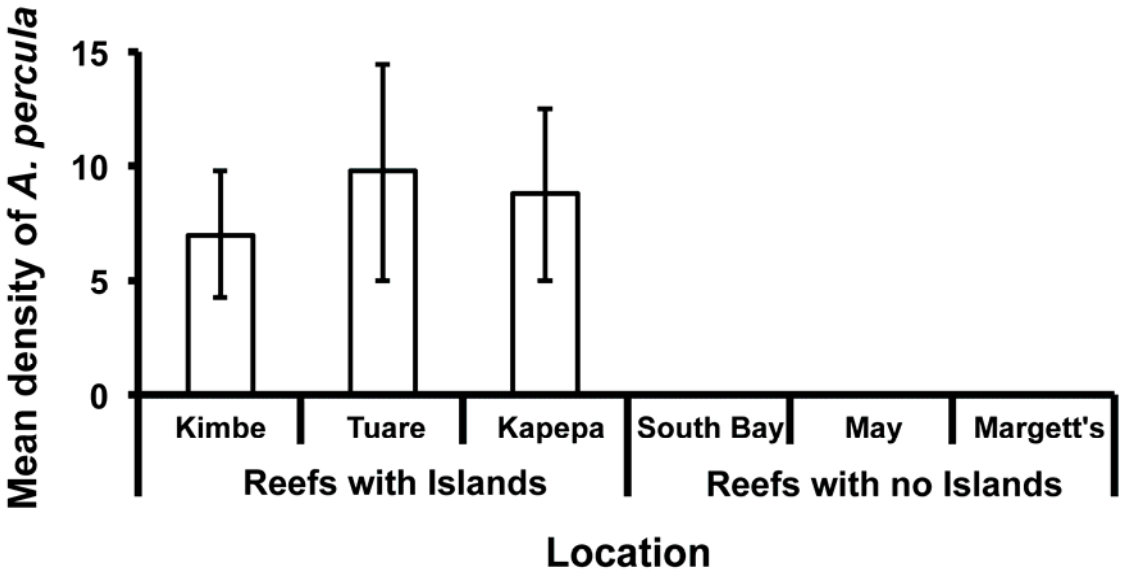

Figure 2.2 Mean density per 200m^2 of *A. percula* counted along four replicated 50 x 4 m visual transects at each of six different locations. Reefs are grouped into those with and without vegetated islands.

Floating leaves were commonly observed in the beach zone and on reef flats near islands (Fig. 2.3) and were completely absent from reefs without islands. On reefs with islands, the densities of floating leaves declined with distance offshore, and no leaves were observed in the random transects 1 km offshore.

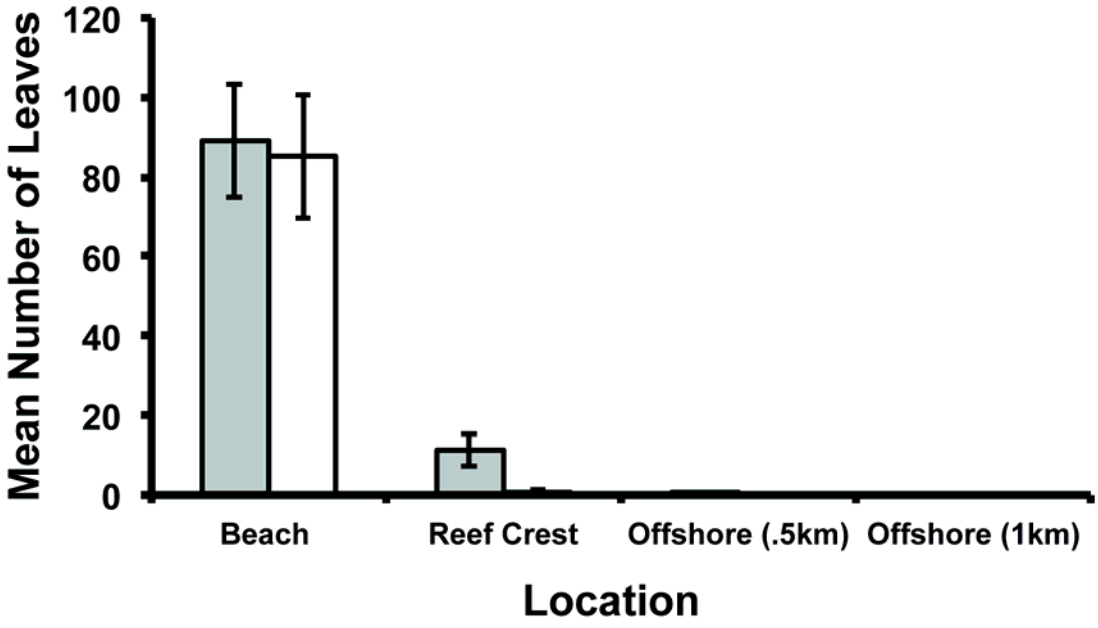

Figure 2.3 Mean density per 400m^2 of rainforest leaves at four different distances (beach, reef crest, 0.5 and 1 km) from the two islands (Kimbe Island (filled bar) and Tuare Island (open bar)). On two reefs without islands (May Reef, South Bay Reef), no leaves were recorded. Error bars are standard errors

On the island reefs, the two anemones occupied by *A. percula* were not randomly distributed with respect to the position of the island. At Kimbe Island, *S. gigantea* was always located within 10m off of the shoreline (Fig. 2.4), where it was commonly observed beneath overhanging vegetation and in close proximity to leaf litter. The other anemone *H. magnifica* was more commonly associated with the perimeter of small lagoons, usually 10-30 m from the shoreline, but not on the substantial areas of reef flat or slope further offshore (Fig. 2.4).

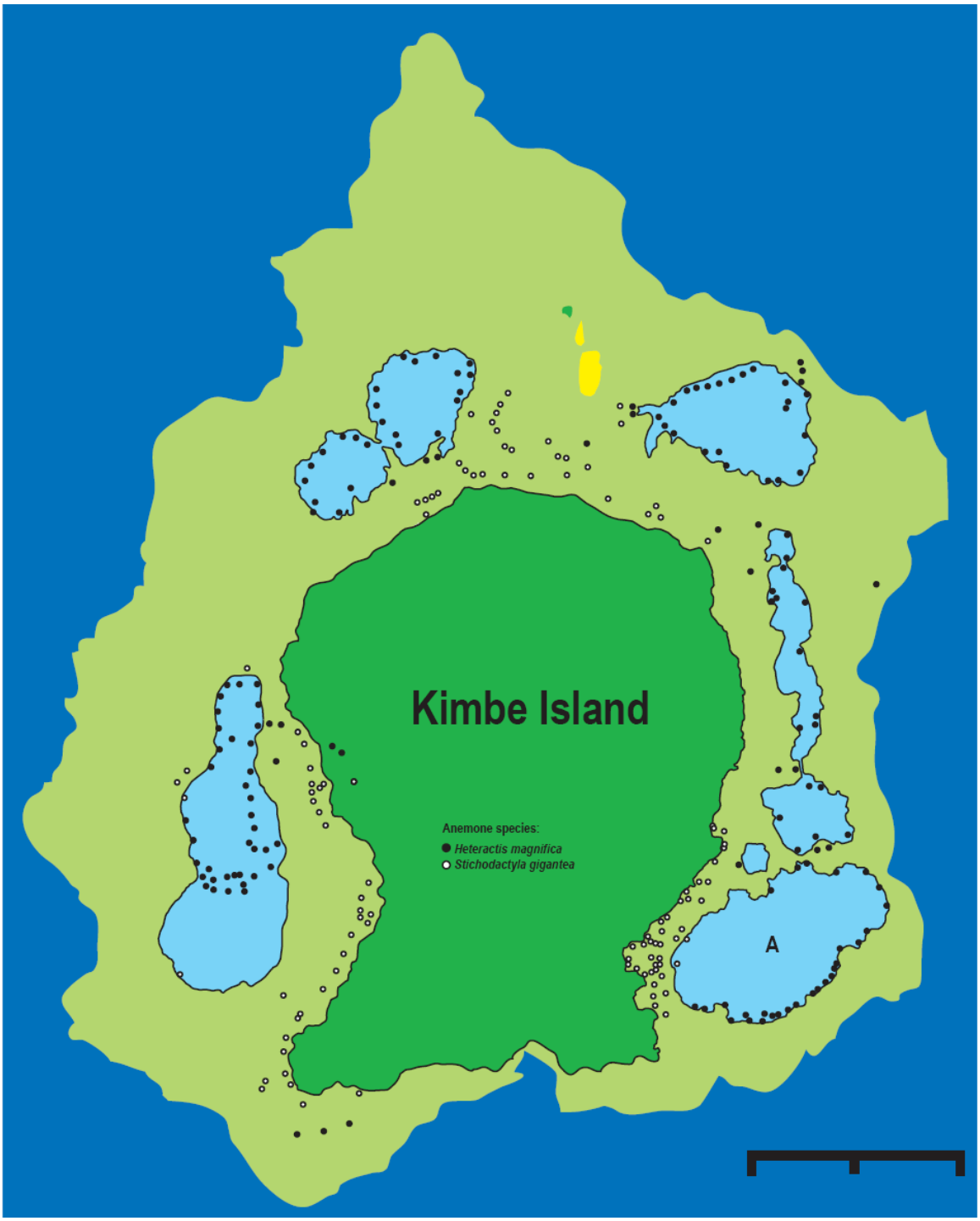

Figure 2.4 Map of Kimbe Island drawn from an IKONOS-2 satellite image with a resolution of 1m (see Almany *et al.*, 2007). The locations of all the anemones of *H. magnifica* (filled circles) and *S. gigantea* (open circles) occupied by the groups of *A. percula* are marked

2.4.2 Field experiment 1: olfactory discrimination between water samples from reefs with and without islands

Juvenile *A. percula* exhibited a strong preference for water samples taken from reefs with islands (Table 2.1a). Juveniles spent over 99 per cent of their time in the choice flume on the side of the island water samples and less than one per cent of their time associated with water from reefs with no islands. Both the choice of Tuare Island water over South Bay Reef water and that of Kimbe Island water over May Reef water were statistically significant (Table 2.1a).

2.4.3 Field experiment 2: olfactory discrimination between water samples from different distances from island reefs

For the two island reefs investigated, juvenile *A. percula* consistently showed a strong preference for beach water, regardless of whether the choice was between beach water and reef crest water, or between beach water and offshore water (Table 2.1b). The average time in association with beach water was always greater than 90 per cent, and in all the cases, the preference for beach water was statistically significant. The juveniles exhibited no significant discrimination between offshore and reef crest water samples, spending close to 50 per cent of their time in the side of the flow channel of each water source.

2.4.4 Field experiment 3: olfactory discrimination between water samples treated and not treated with either the anemone *S. gigantea* or leaves from rainforest trees overhanging water

A strong preference was always exhibited for offshore water sources treated with the anemone *S. gigantea* (Table 2.1c). When given a choice between treated and untreated water, juveniles pent over 90 per cent of their time associated with the anemone-treated water.

The strong field preference for water treated with rainforest leaves was also apparent (Table 2.1c). Given the choice between offshore water treated with rainforest leaves and untreated offshore water, the juveniles spent between 90 to 95 per cent of their time in the treated water flume. The choice was highly significant for each of the five rainforest species tested and the mixed leaf treatment.

2.4.5 Laboratory experiment: olfactory discrimination by naive laboratory-reared larvae

Laboratory-reared larvae were strongly attracted to chemical cues from anemones, indicating that 11-day-old larvae that have not settled on reefs are capable of olfactory responses in the water flow chamber. These larvae had no developmental experience with water treated with vegetation. However, when given a choice between artificial seawater and the same water treated with leaves from a rainforest plant, hey spent on

average 96 per cent of their time in the flow channel receiving leaf-treated water. The preference for rainforest-leaf-treated water was statistically significant and numerically as strong as the attraction to anemone water (Table 2.1c). All the larvae tested exhibited 100 per cent avoidance for water-treated with olfactory cues from the plant *M. nervosa*, confirming that they were not attracted to inappropriate stimuli in the test chamber.

Table 2.1 Results of pairwise olfactory choice experiments on field-collected juvenile *A. percula*, including the choices made between (a) water from reefs with and without islands, (b) water from different distances away from islands and (c) water with and without anemones and rainforest leaves. In addition, (d) shows the pairwise trials for laboratory-reared juveniles and their choices between water with and without anemones and leaves. Data are mean per centages of time spent in water flowing from the two sources ± SE. Statistic tests are χ^2-tests on the number of trails where larvae exhibited a preference for one water source in the test chamber. n, sample size; p, probability of the data given the null hypothesis that there is no choice.

Pairwise test	Choice 1, mean % time ± SE		Choice 2, mean % time ± SE		χ^2	n	p
(a) Field experiment 1: reefs with and without islands	Tuare Is.	99.6 ± 0.04	South Bay Reef	0.6 ± 0.04	28.0	28	<0.001
	Kimbe Is.	99.5 ± 0.1	May Reef	0.5 ± 0.1	22.0	22	<0.001
(b) Field experiment 2: distance from island	Tuare Is. beach	98.0 ± 0.1	Tuare Is. offshore	2.0 ± 0.1	22.0	22	<0.001
	Tuare Is. beach	95.0 ± 0.1	Tuare Is. crest	5.0 ± 0.1	22.0	22	<0.001
	Tuare Is. crest	55.5 ± 0.6	Tuare Is. offshore	44.5 ± 0.6	0.17	22	0.683
	Kimbe Is. beach	93.0 ± 0.4	Kimbe Is. offshore	7.0 ± 0.4	24.0	24	<0.001
	Kimbe Is. beach	97.2 ± 0.8	Kimbe Is. crest	2.8 ± 0.8	20.2	24	<0.001
	Kimbe Is. crest	57.0 ± 0.8	Kimbe Is. offshore	43.0 ± 0.8	0.0	24	1
(c) Field experiment 3: response to anemones and leaves	Anemone	91.0 ± 0.7	No anemone	9.0 ± 0.7	18.2	22	<0.001
	Mixed leaves	89.5 ± 0.7	No leaves	10.5 ± 0.7	20.2	24	<0.005
	Leaves sp. 1	90.0 ± 0.6	No leaves	10.0 ± 0.6	20.0	20	<0.001
	Leaves sp. 2	92.0 ± 0.4	No leaves	8.0 ± 0.4	20.0	20	<0.001
	Leaves sp. 3	90.0 ± 0.7	No leaves	10.0 ± 0.7	16.2	20	<0.001
	Leaves sp. 4	92.0 ± 0.6	No leaves	8.0 ± 0.6	20.0	20	<0.001
	Leaves sp. 5	94.0 ± 0.4	No leaves	6.0 ± 0.4	20.0	20	<0.001
(d) Laboratory experiment: response of naïve larvae to anemones and leaves	Anemone	98.0 ± 0.2	No anemone	2.0 ± 0.2	24.0	24	<0.001
	Rainforest leaves	96.0 ± 0.2	No leaves	4.0 ± 0.2	36.0	36	<0.001
	Melaleuca nervosa leaves	0 ± 0	No leaves	100.0 ± 0	30.0	30	<0.001

2.5 DISCUSSION

The clownfish *A. percula* clearly has a close association with coral reefs surrounding vegetated islands. Both clownfish and host anemone numbers are high on island reefs and sparse on other emergent reefs. Within islands reef systems, numbers are the greatest immediately adjacent to the islands themselves, where they are often found beneath overhanging vegetation. Given the strong association between host anemones and island reefs, *A. percula* larvae can clearly maximize their chance of finding a suitable settlement site by being able to locate and orient towards islands. The islands themselves are a potential source of many olfactory water-borne cues that would not be emanating from reefs without islands. Elevated levels of organic material from the lush, tropical, rainiest vegetation could clearly extend some distance from the islands. The experimental data presented here strongly suggests that *A. percula* has an innate olfactory attraction to rainforest vegetation, and once detected, could use this stimulus to find suitable habitat.

One of the striking features of our results is the strength of the preferences with the juveniles spending in excess of 90 per cent of their time in the flow channel of their choice. They exhibited a strong preference for water coming from reefs surrounding vegetated islands compared with that from reefs without islands. They exhibited a clear preference for beach water in the flow chamber, when presented with a choice between this and reef crest or offshore water options. Both the native and field-experienced juveniles exhibited equally strong preference for both anemones and rainforest leaves. Furthermore, the naive larvae demonstrated an ability to distinguish between appropriate

and inappropriate cues, by avoiding water treated with leaves from a swamp-dwelling tree that contained pungent oils. Given the potential difficulty of finding coral reef habitats after a pelagic larval phase, it is likely that *A. percula* uses a suite of sensory cues to find island reefs and settlement sites. Our results indicate that leaves from terrestrial plants are one of the cues that guide larvae towards suitable settlement sites.

The high densities of anemones and *A. percula* on island reefs are almost certainly part of the sensory cocktail used to find a place to settle. Reef fish are clearly capable of responding to chemicals signals from anemones (Elliott *et al.*, 1995; Arvedlund and Nielsen, 1996; Arvedlund *et al.*, 1999) and conspecifics (Sweatman, 1988; Booth, 1992; Lecchini *et al.*, 2005b). However, olfactory cues from these sources may be the most important once larvae are in close proximity to reefs. It is likely that the terrestrial signals can be detected at greater densities, given that leaves and organic debris clearly float considerable distances away from reefs. The cues would greatly increase the 'island mass' effect (*sensu* Gilmartin and Revelante, 1974), essentially making islands a bigger target for larvae searching for a suitable habitat. Although the juveniles could not distinguish between offshore and reef crest water in our study, we have noted larvae mounds of organic debris in island wakes. Elevated ichthyoplankton densities in islands wakes and oceanographic features associated with islands are well known (e.g. Leis, 1986; Wolanski and Hamner, 1988; Heywood *et al.*, 1990; Boehlert *et al.*, 1992). *Amphiprion percula* larvae that enter island wakes carrying leaves would have a clear olfactory signal that indicated the proximity of an island.

The high levels of natal or self-recruitment on island reefs raise further questions about how larvae find island homes (Jones *et al.*, 1999, 2005; Almany *et al.*, 2007). For

example, Almany *et al.* (2007) have shown that after a planktonic period of 10-12 days, up to 60 per cent of the juvenile *A. percula* settling at Kimbe Island were the offspring of resident adults. How these juveniles found their way back to the islands is unknown, but their ability to respond to olfactory stimuli from islands is clearly implicated. It is possible that larvae use terrestrial cues to remain in sensory contact with home islands in order to find a suitable habitat. Given that juvenile fishes can potentially discriminate home reefs from others (Gerlach *et al.*, 2007), it is also possible that *A. percula* larvae are homing to their natal island in response to specific vegetation cues. While our experiments suggest that larvae have an innate ability to respond to rainforest vegetation, they may also be capable of imprinting on chemical signals experienced during the embryonic stage (Arvedlund and Nielsen, 1996). Alternatively, a general orientation towards vegetated islands, combined with the sparse distribution of reefs surrounding islands in the study site area, could lead to a high probability of natal recruitment, even in the absence of a strict homing mechanism. Further work is required to distinguish among these competing hypotheses.

The study of Almany *et al.*, (2007) also suggests that a significant proportion of juveniles settling at Kimbe Island travel considerable distances in excess of 20 km among reefs. In Kimbe Bay, island reefs tend to be small and account for less than one per cent of the marine habitat (see figure 2 and Beger *et al.*, 2003). Given that fish larvae possess limited physical ability and sensory mechanisms early in development (Egner and Mann, 2005; Hogan and Mora, 2005), it is likely that they are initially transported away from islands by prevailing currents. However, successful larval migration between such small

island targets argues for remarkable sensory and swimming abilities at later developmental stages.

The specific chemical cues that *A. percula* respond to and the distance over which they can be detected require further investigation. The larvae of many benthic marine organisms either select or avoid chemical signals from marine pants when undergoing metamorphosis or settlement (Pawlik, 1992; Forward *et al.*, 2001; Steinberg and de Nys, 2002). Tannins and related compounds produced by marine algae and seagrasses have been implicated (Arnold and Targett, 2002). However, there are no other published examples of marine organisms responding to chemical stimuli from terrestrial plants, and the chemical cues themselves are unknown.

The results of our study have important implications for the management of island reef ecosystems. The iconic clownfish *A. percula* (aka Nemo) provides clear examples of a strong link between coral reefs and rainforest habitats. Offshore islands are often targets of marine protected area status because they harbour a diversity of habitats and species, and are the focal point of human activities (Fernandes *et al.*, 2005). Calls for integrated management of terrestrial and marine habitats (Allison *et al.*, 1998; Jameson *et al.*, 2002; Aronson and Precht, 2006) are vital in island environments. Deforestation and development of offshore islands could have an obvious detrimental effect on the ability of these clownfish to find suitable habitat and therefore persist on tropical island reefs. Islands in remote oceanic environments are often associated with increased numbers of endemic species (e.g Jones *et al.*, 2002; Allen, 2007). Mechanisms for homing are likely to be even more important for the persistence of such species.

In conclusion, we hypothesize that *A. percula* larvae can use olfactory cues from rainforest vegetation for finding island reefs. There are many further questions to be answered including what the chemical cues are, how far from reefs they can be detected and what developmental stage larvae can respond to them. However, our results demonstrate an important and previously unrecognized link between coral reefs and tropical forests, which may be significant for a variety of island-dwelling reef fishes. The increased knowledge of how larvae navigate to suitable adult habitats contributes directly to management actions that aim to sustain viable marine populations. Any factors that disrupt this process will have immediate and devastating effects on the ecology of marine organisms.

CHAPTER 3: **Terrestrial chemical cues aid island-associated coral reef fish larvae in locating settlement habitat**[†]

[†] Dixson DL, Jones GP, Munday PL, Planes S, Pratchett MS, Srinivasan M, Planes, S, Thorrold SR (2011) Terrestrial chemical cues help coral reef fish larvae locate settlement habitat surrounding islands. *Ecology and Evolution* In press. (doi: 10.1002/ece.3.53)

3.1 ABSTRACT

Understanding the degree of connectivity between coastal and island landscapes and nearby coral reefs is vital to the integrated management of terrestrial and marine environments in the tropics. Coral reef fishes are capable of navigating to appropriate settlement habitats following their pelagic larval phase, but the mechanisms by which they do this are unclear. The importance of olfactory cues in settlement-site selection has been demonstrated, and there is increasing evidence that chemical cues from terrestrial sources may be important for some species. Here we test the olfactory preferences of eight island-associated coral reef fish recruits to discern the capacity for terrestrial cue

recognition that may aid in settlement-site selection. A series of pairwise choice experiments were used to evaluate the potential role that terrestrial, water-borne olfactory cues play in island-reef recognition. Olfactory stimuli tested included near-shore water, terrestrial rainforest leaf litter, and olfactory cues collected from different reef types (reefs surrounding vegetated islands, and reefs with no islands present). All eight species demonstrated high levels of olfactory discrimination and responded positively towards olfactory cues indicating the presence of a vegetated island. We hypothesize that although these fish use a suite of cues for settlement site recognition, one mechanism in locating their island/reef habitat is through the olfactory cues produced by vegetated islands. This research highlights the role terrestrial olfactory cues play in large-scale settlement site selection and suggests a high degree of ecosystem connectivity.

3.2 INTRODUCTION

The importance of integrated management of terrestrial and marine environments has been highlighted by numerous studies (Allison *et al.*, 1998; Jameson *et al.*, 2002; Aronson & Precht, 2006; Dixson *et al.*, 2008); however, reserve networks are often designed in either terrestrial or marine ecosystems independently, ignoring interactions between the two (Beck, 2003). There are many examples of how one ecosystem can be jeopardized as a result of anthropogenic activities in another (Stoms *et al.*, 2005). With nearly half of the world's population residing within 150 km of a coastline, the need for effective integrated management of coastal ecosystems is vital (Cohen *et al.*, 1999).

Coastal ecosystems, including terrestrial, freshwater and marine environments, are connected by the important exchange of materials, energy and organisms (Reiners and Driese, 2001; Stoms *et al.*, 2005). For example, coastal mangroves have been identified as an important nursery habitat for many coral reef fish species, including species of commercial interest (Nagelkerken *et al.*, 2000), however 30-60% of the world's mangroves have already been lost from human development (Ellison, 2008; Upadhyay *et al.*, 2002; Valiela *et al.*, 2001). At the same time, many coral reefs have been degraded by sedimentation and eutrophication from coastal development and agriculture, causing losses in diversity and abundance of reef species (McCulloch *et al.*, 2003; Jones *et al.*, 2004; Hughes *et al.*, 2010).

The ability of dispersing individuals to locate suitable habitat is critical for their future survival and reproduction, which in turn influences the replenishment and persistence of adult populations (Morris, 2003; Rosenzweig, 1981). Most marine organisms begin life as pelagic larvae and, depending on the species, spend days to months in the pelagic environment. Regardless of larval duration, suitable adult habitat must be located at the conclusion of the larval stage. Settlement-stage larvae are thought to use a variety of settlement cues to locate suitable habitats, for example chemical cues given off by living substrates (e.g. algae Steinberg and de Nyes, 2002; Williamson *et al.*, 2000; coral Ben-Tzvi *et al.*, 2010) and conspecifics (e.g. barnacles Thiyagarajan, 2010; fish Atema *et al.*, 2002; Kingsford *et al.*, 2002; Lecchini *et al.*, 2005b). To date, most research on the chemical cues used by fish larvae for orientation and habitat selection has focused on chemicals produced by reef organisms. However, Dixson *et al.*, (2008) showed that settlement-stage larvae of the anemonefish, *Amphiprion percula*, were

positively attracted to the odour of terrestrial leaf litter, most likely because their symbiotic anemones are associated with reefs surrounding vegetated islands. The positive response of *A. percula* to the odour cues of terrestrial rainforest vegetation was found to be an innate response. It was suggested that the ability for terrestrial cues, such as leaf litter, to be exported away from island-based reefs allows detection at greater distances than traditional settlement cues such as anemone or coral odour.

Offshore islands are important ecosystems within the marine environment, often harbouring a diversity of habitats and species, and are the focal point of human activity (Fernandes *et al.*, 2007). Islands in remote locations typically display large numbers of endemic species (Allen *et al.*, 2007; Jones *et al.*, 2002), as well as high levels of self-recruitment within populations (Almany *et al.*, 2007). Consequently, mechanisms by which larvae locate suitable habitats are likely to be especially important to the persistence of such species. A better understanding of the mechanisms utilized by island-associated species at settlement will help to determine how populations on isolated islands are maintained. Understanding the degree to which the terrestrial landscape and the nearby coral reefs are connected is vital in achieving this goal.

The mechanisms that reef fish larvae use to find their way through the pelagic environment, and ultimately to locate a suitable demersal settlement site, remains poorly understood (see Leis *et al.,* 2011). Most fish larvae begin the pelagic stage with limited locomotory abilities and with incompletely developed sensory organs, although they conclude the pelagic phase as competent swimmers (Stobutski and Bellwood, 1998; Fisher and Bellwood, 2002) with highly developed sensory systems (Kingsford *et al.*, 2002). The physical capabilities larvae display during the latter portion of the larval

period has led biologists to conclude that larval behaviour influences settlement-site selection at a variety of spatial scales (Atema *et al.*, 1988; Cowen and Sponaugle, 2009; Kingsford *et al.*, 2002; Leis, 2007; Montgomery *et al.*, 2006). The larvae of coral reef fishes possess the necessary sensory morphology for detecting chemical cues and have been demonstrated to use chemical cues for orientation and habitat selection (Atema *et al.*, 2002; Kingsford *et al.*, 2002; Lechinni *et al.,* 2005b). It is thought that the primary sense used in detecting chemical cues is olfaction (Leis *et al.*, 2011). Olfactory cues have been shown to play an important role for settling larval fish in the recognition of microhabitats (Arvedlund *et al.*, 1999; Elliott *et al.*, 1995; Lechinni *et al.*, 2005b; Nangelkerken *et al.*, 2008; Sweatman, 1983), food (Batty and Hoyt, 1995; Dempsey, 1978; Døving *et al.*, 1994; Knutsen, 1992; Kolkovski *et al.*, 1997), conspecifics (Ben-Tzvi *et al.*, 2010; Sweatman, 1983, 1988) and predator avoidance (Dixson *et al.*, 2010). Olfactory cues may also be important for orientation and navigation at much larger scales. For example, Gerlach *et al.*, (2007) demonstrated that larvae are able to distinguish between reefs within a 10 km radius, indicating not only that olfactory cues may be useful over a greater distance than previously recognized but also that individual reefs have distinct characteristic odours that larvae are able to identify and respond to.

The aim of this study was to investigate the role that terrestrial olfactory cues play in settlement-site selection among a variety of reef fishes associated with coral reefs adjacent to islands. Using similar methods as described in Dixson *et al.* (2008), we first examined the extent to which eight species from three reef fish families are restricted to the fringing reefs surrounding islands in Kimbe Bay, Papua New Guinea. We hypothesize that species with strong island-reef association will use cues produced by the

terrestrial island mass for reef identification. Specifically, we predicted that olfactory cues are used to identify between island and non-island reefs and that at least one of the olfactory cues utilized to distinguish between reef types is a product of the terrestrial system. A series of pairwise choice tests were conducted to test the ability of newly settled island-associated recruits of each species to discriminate between different olfactory cues in the water column. Olfactory trials aimed to determine if newly settled recruits were able to distinguish between: (1) water taken from a reef surrounding an island compared with water from a reef where no island was present; (2) all combinations of water taken from different distances from the island source; and lastly (3) water treated with terrestrial rainforest vegetation compared to untreated offshore water. We hypothesize that juveniles will prefer island water compared to water from reefs without islands, that they will prefer water from near islands over offshore water and that they will favour water containing chemical cues from island vegetation over untreated water.

3.3 MATERIALS AND METHODS

3.3.1 Study location and species

The study was carried out in Kimbe Bay (5°20'S, 150°15' E) on the island of New Britain, Papua New Guinea. The study encompassed eight locations on the western side of Kimbe Bay, including five reefs surrounding vegetated islands (Kapepa Island, Kimbe Island, Ban Ban Island, Garove Island and Tuare Island) and five emergent reefs without

islands (Margett's Reef, May Reef, South Bay Reef, no named Reef 1, and no named Reef 2).

Eight reef fish species that displayed strong island association were chosen as study species, including: two species of butterflyfishes (Chaetodontidae) *Chaetodon vagabundus* and *C. rafflesii*; four species of damselfishes (Pomacentridae), *Amphiprion percula*, *Pomacentrus simsiang*, *Dischistodus prosopotaenia*, and *Dascyllus melanurus*; and two species of wrasses (Labridae), *Halichoeres chloropterus* and *H. argus*. All study species were collected from reefs using clove oil and hand nets as settled recruits. Fish were held in 1L plastic bags following collection and were tested for olfactory cue preferences within 24 hours of collection.

3.3.2 Association with island reefs

To assess the importance of island based chemical cues, island-associated coral reef fish recruits were needed. To estimate densities of the study species at island and non-island locations, fish were surveyed along four randomly placed 50x4 meter transects at each of six sites; three reefs not associated with islands (Margett's Reef, May Reef and South Bay Reef) and three reefs that surround offshore islands (Kimbe Island, Tuare Island, Kapepa Island). Within each transect, the number of individuals of each fish species was recorded. Transects on reefs surrounding islands were placed 5 meters from and parallel to the shoreline. On reefs with no islands, transects were placed 5 meters from the leeward side of the reef. A one-way ANOVA was used to compare fish

densities between reefs with and without islands. This information ensures that the species used associate strongly with the island habitat.

3.3.3 General protocol for field olfactory choice tests

A two-channel choice flume (13cm x 4cm) developed by Gerlach *et al.* (2007) was used to assess the ability of newly settled fishes 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 flowing from two different sources. Water from the two different sources was gravity-fed into the choice flume, which was 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 toward 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 using flow meters. Each trial consisted of a two-minute acclimation period, followed by a two-minute testing period where the position of the fish, on either the right or left side of the chamber was recoded at five-second intervals. Then, there was a one-minute rest period and the water sources were switched from one side to the other, a measure to ensure a side preference was not being displayed. The two-minute acclimation period and two-minute testing period were then repeated. Dye tests were conducted at each water change to ensure that the two flow channels exhibited parallel water flow with no areas of turbulence or eddies.

All trials were conducted on a minimum of 15 newly settled individuals from each species. Each individual was only tested once. Kolmogorov-Smirnov tests were used to compare the proportion of time that individuals spent in the stream of water containing an olfactory cue compared to the proportion of time that individuals spent on one side of the chamber when no cues were present (i.e. offshore water), the results from the blank control trial.

3.3.4 Experiment 1: olfactory discrimination between water samples from reefs with and without islands

Pairwise choice experiments were conducted to test whether juveniles could discriminate between water collected at reefs surrounding islands and water collected at reefs with no islands (i.e. submerged reefs separated by >10 km from the nearest island). On reefs surrounding islands, water was collected 1 m from the shoreline. On reefs without islands, water was collected from the centre of the reef flat at its shallowest point. Water was collected from at least two different island and non-island locations for each fish species, to ensure olfactory preferences displayed were a result of general olfactory preferences rather than a response to a specific location.

3.3.5 Experiment 2: olfactory discrimination from water sampled at different distances away from island shoreline

To determine if olfactory cues are used for distinguishing between different habitat locations within reefs that surround islands, juveniles were given pairwise choices between water from three different positions associated with island reefs: (i) beach water (collected within 1 m off the shoreline), (ii) reef crest water (collected from near the outer edge of the reef crest), and (iii) offshore water (collected 1 km from the reef crest, measured using a GPS). All possible combinations of the three water sources were tested against each other.

3.3.6 Experiment 3: olfactory discrimination between water samples treated with terrestrial cues

To test if island-associated species are attracted to the chemical cues of tropical plants, juveniles were given pairwise choices between offshore water treated with terrestrial leaf litter and untreated offshore water. Offshore water was collected 1 km from the reef crest of the island and was chosen as it was assumed to contain less background terrestrial cues than water from near the island. Beach almond (*Terminalia catappa*) was chosen as the terrestrial chemical cue; this species elicited a positive respond from *A. percula* (Dixson *et al.*, 2008) and is a common shoreline plant on all offshore islands visited. For each trial, 20g of leaves leaf matter was added to offshore water samples (9L) and allowed to stand for two hours before experimental use.

Table 3.1 Results of island association and pairwise olfactory choice experiments on field-collected juvenile coral reef fish. Choice results include the choices made in experiment 1 (comparing water from reefs with and without islands), experiment 2 (comparing water collected at 3 different distances away from an island), and experiment 3 (comparing water treated with leaves to untreated offshore water). The percentages are the mean % of time spent in water flowing from each of the two sources ±SE. The p-values indicate the probability that the preferences are significantly different to the blank trial run for each species (Kolmogorov-Smirnov tests)

Species	Experiment	Water source 1, mean % time spent ±SE	Water source 2, mean % time spent ±SE	n	p
Cheatodon vagabundus	1	Tuare Island 93% ±0.54	South Bay Reef 7% ±0.54	20	<0.001
		Kimbe Island 94% ±0.49	May Reef 6% ±0.49	23	<0.001
	2	Beach 91% ±0.35	Offshore 9% ±0.35	25	<0.001
		Reef Crest 92% ±0.48	Offshore 8% ±0.48	22	<0.001
		Beach 93% ±0.42	Reef Crest 7% ±0.42	25	<0.001
	3	Leaf 86% ±0.56	Offshore 14% ±0.56	16	<0.001
Cheatodon rafflesii	1	Banban Island 94% ±0.41	No name reef 2 6% ±0.41	21	<0.001
		Kimbe Island 95%±0.19	May Reef 5%±0.19	15	<0.001
	2	Beach 93% ±0.30	Offshore 7%±0.30	15	<0.001
		Reef Crest 89% ±0.35	Offshore 11% ±0.35	15	<0.001
		Beach 93% ±0.42	Reef Crest 7% ±0.42	15	<0.001
	3	Leaf 92%±0.40	Offshore 8%±0.40	15	<0.001
Pomacentrus simsiang	1	Garove Island 95% ±0.38	No name reef 1 5% ±0.38	15	<0.001
		Banban Island 93% ±0.73	No name reef 2 7% ±0.73	15	<0.001
	2	Beach 95% ±0.38	Offshore 5% ±0.38	15	<0.001
		Reef Crest 87% ±0.53	Offshore 13% ±0.53	15	<0.001
		Beach 91% ±0.37	Reef Crest 9% ±0.37	15	<0.001
	3	Leaf 90% ±0.42	Offshore 10% ±0.42	15	<0.001
Dischistodus prosopotaenia	1	Banban Island 92% ±0.24	No name reef 2 8% ±0.24	15	<0.001
		Garove Island 94% ±0.27	No name reef 1 6% ±0.27	15	<0.001
	2	Beach 88% ±0.52	Offshore 12% ±0.52	15	<0.001
		Reef Crest 87% ±0.65	Offshore 13% ±0.65	15	<0.001
		Beach 88% ±0.58	Reef Crest 8% ±0.58	15	<0.001
	3	Leaf 79% ±0.46	Offshore 21% ±0.46	15	<0.001
Dascyllus melanurus	1	Banban Island 93% ±0.51	No name reef 2 7% ±0.51	15	<0.001
		Garove Island 93% ±0.56	No name reef 1 7% ±0.56	15	<0.001
	2	Beach 92% ±0.51	Offshore 8% ±0.51	15	<0.001
		Reef Crest 88% ±0.51	Offshore 12% ±0.51	15	<0.001
		Beach 90% ±0.34	Reef Crest 10% ±0.34	15	<0.001
	3	Leaf 88% ±0.50	Offshore 12% ±0.50	20	<0.001
Amphiprion percula	1	Tuare Island 99% ±0.18	South Bay Reef 1% ±0.18	30	<0.001
		Kimbe Island 97% ±0.23	May Reef 3% ±0.23	30	<0.001
	2	Beach 97% ±0.26	Offshore 3% ±0.26	30	<0.001
		Reef Crest 95% ±0.41	Offshore 5% ±0.41	30	<0.001
		Beach 97% ±0.19	Reef Crest 3% ±0.19	30	<0.001
Halichoeres argus	1	Tuare Island 92% ±0.20	South Bay Reef 8% ±0.20	15	<0.001
		Kimbe Island 92% ±0.28	May Reef 8% ±0.28	15	<0.001
	2	Beach 96% ±0.36	Offshore 4% ±0.36	15	<0.001
		Reef Crest 84% ±0.43	Offshore 16% ±0.43	15	<0.001
		Beach 94% ±0.45	Reef Crest 6% ±0.45	15	<0.001

	3	Leaf 94% ±0.23	Offshore 6% ±0.23	15	<0.001
Halichoeres chloropterus	1	Tuare Island 95% ±0.20	South Bay Reef 5% ±0.20	15	<0.001
		Kimbe Island 93% ±0.54	May Reef 7% ±0.54	15	<0.001
	2	Beach 96% ±0.35	Offshore 4% ±0.35	15	<0.001
		Reef Crest 88% ±0.40	Offshore 12% ±0.40	15	<0.001
		Beach 96% ±0.40	Reef Crest 6% ±0.40	15	<0.001
	3	Leaf 99% ±0.17	Offshore 1% ±0.17	15	<0.001

3.4 RESULTS

3.4.1 Association with island reefs

All eight study species exhibited a strong association with the fringing reefs surrounding islands. For each species, the mean density was significantly higher on reefs surrounding islands than on reefs where no island was present (ANOVA $F_{1,14}$=13.413, p<0.002, Fig. 3.1). All four species of pomacentrids (*Pomacentrus simsiang, Dischistodus prosopotaenia, Dascyllus melanurus* and *Amphiprion percula*) were only recorded on reefs that surrounded islands, with no individuals found on reefs where no islands were present. *D. melanurus* displayed the strongest island association with a mean density of 42.5 individuals on reefs surrounding vegetated islands. Both Chaetodonids showed strong islands associations, with *Chaetodon vagabundus* exhibiting five times higher density at island locations opposed to reefs with no islands and *C. rafflesii* had a mean density of 15.1 individuals on reefs surrounding vegetated islands compared to 0.9 individuals on reefs with no islands present. The labrids surveyed displayed similar levels of association. *Halichoeres chloropterus* showed the weakest

island association of the eight species in this study; however, when individual mean density on reefs surrounding islands was three times higher than the individual mean density on reefs with no islands, *H. argus* increased their mean density between the two locations by 3.6 times.

3.4.2 Experiment 1: olfactory discrimination between water samples from reefs with and without islands

All island-associated species spent a significantly greater amount of time in the olfactory cues from water collected from reefs surrounding islands over water collected from reefs where no island was present (Table 3.1). This indicates that these species are all able to discriminate between olfactory cues, as both water sources would contain different olfactory compounds. All species displayed at least a 92% preference for the olfactory cues collected from reefs surrounding islands over the olfactory cues collected from reefs alone. The strongest island preference was shown by *A. percula*, which preferred the island water between 97-99% of the time while both *Halichoeres argus* and *Dischistodus prosopotaenia* exhibited the weakest preference for island water, still spending 92% of their time in the island cue.

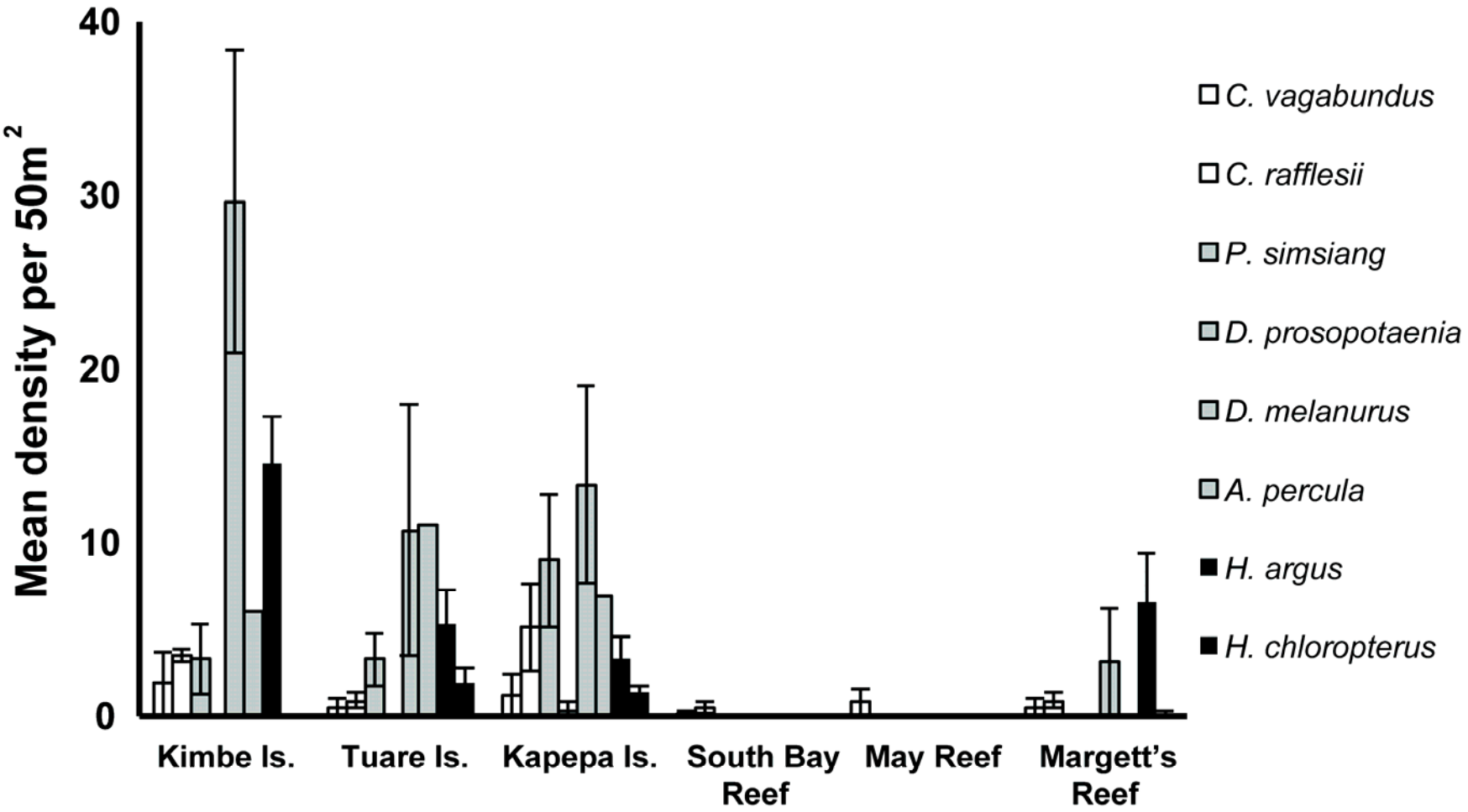

Figure 3.1 Mean density per 200 m^2 of study species ± SE (open bars= cheatodontids, grey bars= pomacentrids, black bars=labrids) counted along four replicate 50x4 m visual transects at each of six different locations. Reefs are grouped into those with and without vegetated islands

3.4.3 Experiment 2: olfactory discrimination from water sampled at different distances away from island shoreline

All eight species were able to discriminate between water collected at different distances from the reef. Juveniles spent a disproportionate amount of time (>84%) in water collected from both the beach and reef crest location over the water collected 1 km offshore (Table 3.1). Juveniles were also able to discriminate between the reef crest water and beach water, showing a strong preference for the beach water (Table 3.1). Beach water was preferred by all species, when compared against either reef crest water or offshore water, with juveniles spending greater than 90% of their time in the beach water. Reef crest water was preferred over offshore water by all species, with juveniles spending at least 84% of their time in the reef crest water olfactory cue.

3.4.4 Experiment 3: olfactory discrimination between water samples treated with terrestrial cues

All eight study species exhibited significant discrimination between offshore water treated with leaves of a common rainforest plant, *T. catappa* versus untreated offshore water; responding positively to the olfactory cue from terrestrial leaf litter. Each species spent >79% (and up to 99%) of observations in offshore water treated with leaf litter compared to untreated offshore water (Table 3.1). The weakest response (79% of

time in offshore water treated with leaf litter) was found in *Dischistodus prosopotaenia*, and the strongest response (99%) was found in *Halichoeres chloropterus*.

3.5 DISCUSSION

All 8 fish species had significantly higher densities on reefs surrounding islands compared with reefs with no islands present. Some species were found exclusively at the island reef location; therefore species used in this study are strongly associated to the island location and require a means for island reef identification. All species could discriminate between water from reefs surrounding islands and non-island reefs; indicating that they are able to use olfactory cues to identify island reefs. Each species was also able to discriminate between water taken at different distances from an island, preferring the beach location, presumably due to a higher concentration of distinct olfactory cues arising from island itself. All 8 species were also capable of detecting a difference between offshore water treated with terrestrial leaf litter and untreated offshore water. In all trials, fish showed a strong preference for water containing terrestrial cues, or the greatest concentration of terrestrial cues. One of the striking features of our results is the strength and consistency of the preferences within and amongst the species. In Experiment 1, all juveniles, regardless of species, spent over 92% of their time in the water from reefs surrounding islands. A clear preference was also shown by all species for beach water over offshore water, as well as for reef crest water compared to offshore water. Variation in cue preference within species was extremely low. Perhaps most

remarkable, is the strength of attraction to the olfactory cues produced by terrestrial leaf litter. In this experiment, the strength of the preference varied the most among species, with preferences ranging from 79% to 99%, but one of the species, *Halichoeres chloropterus*, displayed the strongest preference (99%) seen among all 3 experiments.

While our experiments were conducted on newly settled fish, which were not naïve to the island cues, it is likely that these responses are innate rather than learned. In a similar study on *Amphiprion percula*, Dixson *et al.,* (2008) compared the olfactory preference of newly settled individuals collected from the field with the preferences of naïve larvae reared in culture. The naïve larvae displayed the same preferences for rainforest leaf litter as the field-caught fish, despite the laboratory reared larvae never having encountered that odour previously. Due to the difficulty in rearing the species used in the present experiment, we were unable to conduct naïve trials. It is likely, however, that other island-associated species would have similar innate olfactory preferences for island cues displayed by *A. percula*.

Reef fish are capable of responding to chemical cues from anemones (Arvedlund *et al.*, 1999; Arvedlund and Nielsen, 1996; Elliott *et al.*, 1995), conspecifics (Booth, 1992; Lecchini *et al.,* 2005b; Sweatman, 1988), and live coral (Ben-Tzvi *et al.*, 2010; Lecchini *et al.* 2005b). However, the chemical cues from these sources may be of the greatest importance once the larvae are already within the reef matrix. The chemical signals produced by terrestrial leaf litter may be detected over a greater distance, allowing island-associated fish to use this cue before coming into contact with the reef itself. The strength of attraction supports the island mass effect (sensu Gilmartin and Revelante, 1974), which predicts that the export of leaf litter causes island habitats to be a bigger

target than their actual size for larvae that respond to these terrestrial cues. The use of olfactory cues in habitat selection has also been shown among seagrass-associated fishes (Nagelkerken *et al.*, 2002). For example, the Spangled emperor, *Lethrinus nebulosus*, uses olfactory cues produced by seagrasses to distinguish this habitat from rubble (Arvedlund and Takemura, 2006). Results from hydrodynamic modelling have also shown that collective chemical cues from habitats extend significant distances into oceanic environments. For example, the lagoons of coral reefs contain high concentrations of mucous and dissolved organic compounds from corals and other associated organisms (Davies and Hughes, 1983). This material can be transported out of the lagoon in turbid plumes that are tens of meters to kilometres long (Booth *et al.*, 2000). Water from the continental shelf of the Great Barrier Reef and its associated lagoons can generate a chemical gradient detectable by larvae in the Coral Sea (Wolanski, 1994). While identifying specific chemical cues remains difficult, research has shown that chemical cues carrying useful information are able to disperse great distances from their source with the potential to be used by navigating larvae.

Conservation plans are typically designed to encompass single ecosystems. However, as this study suggests, ecosystems do not function independently from one another, further supporting the need for integrated management policies between the terrestrial and the marine environments. Although the earlier work on *A. percula* (Dixson *et al.*, 2008) has already suggested that terrestrial cues are important, an even stronger link can now be made between coral reefs and rainforest habitat with the addition of seven other island-associated species using the same terrestrial signals. Connectivity among ecosystems has been demonstrated in terms of the transfer of energy and nutrients

from one ecosystem to another, as well as in terms of life history movements and the movements of adults (reviewed by Beger *et al.*, 2010). For example, extensive mangrove habitat in the Caribbean has been shown to positively influence the biomass of fishes on coral reefs (Mumby *et al.*, 2004), and is also important as a nursery habitat for a number of commercially important coral reef species (Nagelkerken *et al.*, 2000, 2002, 2008). Some coastal species such as the coconut crab (*Birgus latro*) have a pelagic larval stage, which uses the oceanic environment for biological dispersal and must locate suitable coastal habitat at the conclusion of their larval stage (Lavery *et al.*, 1996). Our study demonstrates a unique terrestrial-marine link in a group of coral reef species for which such a link would not be expected.

Coastal environments, including vegetated islands, are often the focal point for human activity whether it is recreational or agricultural; both resulting in significant loss of native rainforest vegetation. This study has demonstrated the importance of native vegetation through chemical cues in the recognition of appropriate reef habitat by a number of coral reef fish species. Removal of native cues could potentially affect patterns of connectivity if larvae are relying on the use of terrestrial cues to locate reef habitat. Although formulating management plans that include multiple ecosystems adds complexity and cost to an already complicated process, the connectivity between different ecosystems cannot be ignored.

This study suggests that the rainforest vegetation is an important chemical cue for location of suitable settlement sites among island-associated coral reef fish. There is still a significant amount of further research required, including identifying the chemical compounds involved and at what spatial scales they are detected. Nonetheless, our

results demonstrate the broad importance of the link between the coral reef and rainforest ecosystem.

CHAPTER 4: **Ontogenetic changes in responses to settlement cues by Anemonefish**[†]

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4.1 ABSTRACT

Population connectivity for most marine species is dictated by dispersals during the pelagic larval stage. Although reef fish larvae are known to display behavioural adaptations that influence settlement site selection, little is know about the development of behavioural preferences through the larval phase. Whether larvae are attracted to the same sensory cues throughout their larval phase, or exhibit distinct ontogenetic shifts in sensory preferences is unknown. Here, we demonstrate an ontogenetic shift in olfactory cue preferences for two species of anemonefish, a process that could aid in understanding both patterns of dispersal and settlement. Aquarium-bred naïve Amphiprion percula and A. melanopus larvae were tested for olfactory preferences of relevant reef-associated chemical cues through the 11-day pelagic larval stage. Age post hatching had a significant effect on the preference for olfactory cues from host anemones and live corals for both species. Preferences of olfactory cues from tropical plants of A. percula,

increased by approximately ninefold between hatching and settlement, with a. percula larvae sowing a fivefold increased in preference for the olfactory cue produced by the grass species. Larval age had no effect on the olfactory preference for untreated seawater over the swamp-based tree Melaleuca nervosa, which was always avoided compared with blank seawater. These results indicate that reef fish larvae are capable of utilizing olfactory cues early in the larval stage and may be predisposed to disperse away from reefs, with innate olfactory preferences drawing newly hatched larvae into the pelagic environment. Towards the end of the larval phase, larvae become attracted to the olfactory cues of appropriate habitats, which may assist them in identification of and navigation towards suitable settlement sites.

4.2 INTRODUCTION

Most marine organisms are relatively sedentary as adults, dispersing only during the larval phase (Leis, 1991; Shulman and Bermingham, 1995; Kingsford *et al.*, 2002; Lecchini *et al.*, 2005 a,b). It is the dispersal process that determines patterns of connectivity among benthic populations and helps drive the dynamics of local populations (Sale, 2004; Cowen and Spoonaugle, 2009). Reef fish can potentially disperse large distances during their pelagic larval phase, but at the conclusion of this period are faced with the challenge of locating appropriate settlement habitats. The mechanisms that fish larvae use to navigate through the pelagic environment, eventually locating the suitable benthic settlement sites remain unclear (Montgomery *et al.*, 2001;

Leis and McCormick, 2002; Kingsford *et al.*, 2002; Leis, 2007). However determining the processes responsible for complex dispersal patterns by reef fish larvae, and how these processes might change during the larval phase, is vital for effective management and conservation of their populations (Leis and McCormick, 2002).

Coral reef fish larvae rarely achieve the dispersal distance predicted when models include only physical factors (Swearer *et al.*, 2002; Taylor and Hellberg, 2003; Jones *et al.*, 2005; Almany *et al.*, 2007; Gerlach *et al.*, 2007; Leis *et al.*, 2007). Settlement-stage larvae have displayed phenomenal swimming capabilities (Fisher *et al.*, 2005; Hogan *et al.*, 2007), endurance (Stobutzki and Bellwood, 1997; Leis *et al.*, 2007), orientation skills (Leis and Carson-Ewart, 2003; Irisson *et al.*, 2009), and acute sensory abilities (Kingsford *et al.*, 2002). Field data also suggests that larvae are able to migrate vertically in the water column to exploit different current speeds, another mechanism for controlling their dispersal patterns (Paris and Cowen, 2004). Larval behaviour has also been used to explain natal retention in locations where a greater portion of recruits settle back to natal reefs than would be expected if larvae were passively drifting in ocean currents (Jones *et al.*, 2005; Almany *et al.*, 2007). Most of the research conducted on behaviour attributes of larval reef fish has focused on the end of the pelagic phase, when larvae are competent to settle. A full understanding of marine connectivity will not be achieved until the ontogenetic development of larval abilities throughout the entire larval period is considered. To date, very little information has been collected through the entire larval period (Leis *et al.*, 2007).

Although it is likely that a suite of sensory cues are used for orientation during the larval stage, the most likely senses used to influence of dispersals of marine fish larvae

are auditory, visual and olfactory (Leis, 2007). The use of olfactory cues in settlement site selection by coral reef fish is well documented (Atema *et al.*, 2002; Kingsford *et al.*, 2002; Leis and McComrick, 2002; Wright *et al.*, 2005). Late stage larval fish respond positively to olfactory cues produced by reef substrate, preferred habitats and conspecifics (Sweatman, 1988; Booth, 1992; Elliott *et al.*, 1995; Lecchini *et al.*, 2005 a,b). Reef fish have a well-developed olfactory system (Arvedlund *et al.*, 1999; Lara, 2008) that is likely to be used for locating appropriate settlement habitat, demonstrated by the fact that reef fish often settle at night during the new moon when light leaves are at their lowest and visual cues may be less useful (Sweatman, 1985). Reef fish larvae already possess a well-developed olfactory bulb at hatching, indicating that olfaction may be important through the pelagic larval phase (Arvedlund *et al.*, 1999). However, the chemical cues preferred at hatching might be very different from those at settlement. For example, larvae of reef-associated species adapted for pelagic dispersal might avoid olfactory cues of reefs during the early larval phase by becoming attracted to them near the end of the larval phase when they become competent to settle.

The influence that physical stimuli have on the ontogenetic changes associated with settlement behaviour has been identified as an important factor in settlement preferences of invertebrate larvae. Recently, the behavioural ontogenetic shift in vertical migration was shown to effect recruitment models for the Caribbean spiny lobster (*Panulinus argus*) (Butler *et al.*, 2011). When ontogenetic vertical movement patterns of *P. argus* larvae were incorporated into biophysical models, a greater portion of larvae were predicted to settle much closer to the natal reefs than expected to occur solely on hydrodynamic patterns. Chemical cues have also been shown to stimulate settlement in

many marine invertebrates such as abalone (Morse and Morse, 1984; Barlow, 1990), echinoderms (Pearce and Scheibling, 1990; Johnson *et al.*, 1991), barnacles (Maki *et al.*, 1990; Wright and Boxshall, 1999), polychaetes (Toonen and Pawlik, 1996; Walters and Wethey, 1996) and oysters (Fitt *et al.*, 1990; Titar *et al.*, 1992; Turner *et al.*, 1994). Research indicates that the chemical cues produced by specific bacteria and substrate can facilitate settlement and elicit enhanced metamorphosis in some invertebrate species (Cole and Knight-Jones, 1939; Anderson, 1996; Zhao *et al.*, 2003). These results emphasize the impacts of physical stimuli on larval behaviour in shaping the dispersal kernels and connectivity among populations of marine invertebrates, highlighting the importance of full comprehension of larval behavioural capabilities. Understanding the role cues and the important physical stimuli play through the developmental process of marine fish larvae is of great interest in the marine environment even through they do not undergo the same developmental metamorphosis as invertebrate larvae during the transition from a pelagic to a benthic lifestyle.

The objective of this study was to investigate the ontogenetic development of olfactory cue use in two species of coral reef fish (*A. percula* and *A. melanopus*) throughout their larval phases. This study was conduced at James Cook University's Aquarium Facilities during November 2008-February 2009. Reef-associated olfactory cues were used to determine: (1) whether newly hatched larvae could respond to appropriate olfactory stimuli and (2) whether olfactory preferences that may affect dispersal and settlement sites selection changed throughout the larval phase. Specifically, both species of larvae were tested throughout their larval phase for behavioural preferences of chemical cues produced by their host anemone species, a common coral

Porites cylindrica, and for *A. percula*, the chemical cues from a range of terrestrial plants that appear to be important in locating reefs associated with vegetated islands (Dixson *et al.*, 2008).

4.3 MATERIALS AND METHODS

4.3.1 Study species

Anemonefish are an ideal group for testing olfactory preferences. They are characterized by the symbiotic relationship formed with sea anemones. The degree of specificity in host anemone selection implies an ability to travel long distances to ensure that larvae are able to find not only a suitable reef, but also one that supports their specific anemone host. Anemonefish have already been shown to use olfactory cues to locate their anemone habitat (Elliott *et al.*, 1995; Arvedlund and Neilsen, 1996; Arvedlund *et al.*, 1999). The anemonefishes, *A. percula* and *A. melanopus*, were used in this study because they are amendable to laboratory breeding and offspring can be reared with a high rate of success compared with other coral reef fish. In some regions, such as Papua New Guinea, *A. percula* are often found near vegetated offshore islands (Dixson *et al.*, 2008). The chemical cues associated with the fallen terrestrial leaf litter have been shown to be an innate settlement site cue for this species. Both *A. melanopus* and its most commonly associated host anemones do not display any island association.

4.3.2 Breeding and larval rearing techniques

The clownfish, A. percula and A. melanopus, were reared in a 700,000 litre recirculating seawater system at James Cook University's experimental marine aquarium facility. Larvae tested were offspring of breeding pairs of each species collected from the Great Barrier, Australia and kept at the experiment facility for one (*A. melanopus*) to three years (*A. percula*). Pairs were maintained in separate 70 l aquariums. Breeding pairs laid egg 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 aquariums had no water exchange during the day and were 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 (*Brachionus* sp.) at five individuals ml^{-1} each morning for the first three days. *Artemia* nauplii were added at one individual ml^{-1} each morning beginning at day three. The ration of *Artemia* nauplii to rotifers was increased each day until larvae were fed only five individuals of *Artemia* nauplii ml^{-1} from day eight. Larvae were reared until they were competent to settle at 11-days post-hatching.

4.3.3 Olfactory testing

The response of larvae to olfactory cues 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 stimuli. Larvae were released at the downstream end where they were free to move in the chamber. Using the protocols outlined in Gerlach *et al.* (2007), a constant gravity-driven flow of 100 ml min^{-1} per channel was maintained through all trials with flow meters. The small flume chamber is designed to hold a volume of 52 ml per channel, allowing a low flow rate, which ensures that the larvae are not struggling to maintain their desired location within the flume and therefore only olfactory preferences are being tested. Dye tests were conducted to determine that a full flushing occurred when water sources were switched and to ensure laminar flow with no eddies or areas of turbulence. 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 two minutes. Fish that did not swim actively during the acclimation period were discarded (<1% of fish from both species were discarded). Following the acclimation period, the position of the fish in the chamber was recorded at five-second intervals for a two minutes testing period. The chamber was divided into left and right sides by visually extending the line of the central partition to the end of the chamber, allowing the fish's position in the chamber to be recorded on one side o the other. This was followed by a one minute rest period, during which the water sources were switched, providing a control for potential side preferences. Following the switch of water sources, the entire test, including the acclimation period, was repeated. Each fish was used only once.

Early-stage larvae displayed a preference for reflected light on the sides of the chamber; therefore, trials conducted on larvae at one, three and five days post-hatching were conducted in low light conditions. At day seven post-hatching, larval responses were insensitive to light conditions therefore overhead lights were used during trials. With the design of the two-channel flume chamber, it is impossible to distinguish between an avoidance of the odour sources being tested or an attraction to untreated seawater. Due to the ambiguity, for this study only, larval preferences are discussed.

4.3.4 Odour cues

It is well documented that anemonefish species are attracted to the chemical cues of their host anemones (Elliott *et al.*, 1995; Arvedlund and Neilsen, 1996; Arvedlund *et al.*, 1999). Therefore, the preference of both anemonefish species for the chemical cues of a preferred host anemone (*Stichodactyla gigantea* for *A. percula* and *Herteractis magnifica* for *A. melanopus*) was tested. Settlement-stage larvae of both species were also expected to prefer the chemical cues from a live coral, as it would indicate the presence of a reef. Furthermore, a preference for the chemical cues from coral has been documented among other coral reef species (Lecchini *et al.*, 2005b; Ben-Tzvi *et al.*, 2010; McCormick *et al.*, 2010). Therefore, the preference for chemical cues of a common branching coral, *Porites cylindrica*, was tested in both anemonefish species. *P. cylindrica* was chosen because it is a widely distributed coral that is common in lagoonal and back-reef habitats where *A. percula* and *A. melanopus* are commonly found. It is also easily kept healthy in captivity. Finally, previous research has revealed that

settlement-stage *A. percula* use olfactory cues produced by terrestrial tropical plant leaves to identify reefs surrounding vegetated islands (Dixson *et al.*, 2008), and that they can discriminate between the odours from a range of different plant species (Munday *et al.*, 2009). Therefore, *A. percula* were tested for the ontogenetic development of terrestrial plant cue use.

Artificial seawater (Red Sea Brand) assumed to contain no biological cues was used to create olfactory testing water. Due to the constraints of laboratory experiments, the same coral branches and anemones were used to create all treatments. Three hundred and twenty-three grams of healthy *P. cylindrica* branches, collected from a mid-shelf reef on the Great Barrier Reef near Orpheus Island, were used to create the olfactory cues for coral. One *S. gigantea* anemone, weighting 341 g was used in all trials to create *A. percula's* anemone chemical cue. One *H. magnifica* anemone, weighting 357 g was used in all trials to create A. melanopus' anemone chemical cue. Soaking each item in 10 litre of seawater for 2 hours created the chemical cue. As the natural range of concentrations of specific chemical cues in the environment is unknown, this protocol was designed simply to test for a reaction to the odour at an arbitrary concentration. All olfactory sources were removed from the seawater before the treated water was used in the choice chamber. Specific olfactory tests for both *A. percula* and *A. melanopus* included (1) untreated seawater compared with untreated seawater, this was used as a blank control; (2) seawater treated with the chemical cue produced by the associated anemone species compared with untreated seawater; and (3) seawater treated with the chemical cue produced by branches of *P. cylindrica* compared with untreated seawater.

Water containing leaf litter chemical cues were created by soaking 20 g of fresh broken leaves (approximately 1 cm^2 pieces) for two hours in 10 litre of artificial seawater. Specifically, larval *A. percula* were given the choice of water streams in the flume chamber containing olfactory cues from: (1) seawater treated with leaves from the common tropical plant *Xanthostemon chrysanthus* (Myrtaceae) against untreated seawater; settlement-stage larvae have previously shown a preference for this cue (Munday *et al.*, 2009). (2) Seawater treated with leaves from the swamp-plant *Melaleuca nervosa* against untreated seawater; settlement-stage larvae have previously shown a repulsion of this cue (Munday *et al.*, 2009). (3) Lastly, water treated with the chemical cue from a common grass species, *Panicum maximum*, was tested against untreated seawater; settlement-stage larvae previously showed no preference for or against this cue (Munday *et al.*, 2009).

4.3.5 Statistical Analysis

All trials were conducted on larvae from a minimum of three parental groups. For each trial, 30 individual larvae (1 from each of three different parental groups) were randomly selected from the rearing tub and tested in the choice flume on every second day post hatching, starting the morning following hatching (n=30 per trial). All larvae were only tested once. There was no statistical difference in olfactory preferences between larvae from the three parental groups in any o the trials conducted (Kolmogrov-Smirnov test, $p>0.10$); therefore, parental groups were pooled for each larval age within an olfactory cue. Each fish was scored as exhibiting a preference for a cue if it spent

more than 50% of this time in the stream of water containing the cue. Log-linear models were then used to test the relationship between age post hatching and preferences of avoidance of each olfactory cue (Quinn and Koeogh, 2002). Starting with the saturated model, model terms were removed until the removal of a term resulted in a significant increase in deviance from one model to the next. Because we were only interested in testing the effects of cue type and age on the preference exhibited by the larvae for each cue, we only removed terms that contained "preferences" in the model selection process.

4.4 RESULTS

Age had no effect on the response of larvae to the blank trial consisting of untreated seawater compared with untreated seawater, with both species of larvae spending equal time in either side of the chamber. This trial strongly supports the conclusion that larvae recognize and respond to the olfactory cues presented to them within the flume chamber throughout their larval stage beginning at hatching.

4.4.1 *Amphiprion percula*

Amphiprion percula exhibited strong discrimination and responses to olfactory cues throughout the larval stage (Fig. 4.1a). The model that best described the data contained the term cue*age + age*preference (χ^2 = 0.000, df = 6, p = 1.000, Table 1), indicating that preferences for a particular cue were dependent on larval age, but were

independent of the olfactory cue presented or an interaction between preference, cue and age. Initially, newly hatched larvae preferred blank seawater over the anemone cue, with newly hatched larvae spending only 10% of their time in the anemone olfactory cue water choice (Fig. 4.1a). As larvae developed, the attraction to the anemone odour increased, with larvae preferring this same odour 92% of the time at 7-day post-hatching. Preferences established by day seven were retained to the end of the larval stage at day 11 (Fig. 4.1a). The same ontogenetic trend was shown in the preference for olfactory cues produced by the coral, *P. cylindrica*.

The saturated model, containing the term cue*age*preference, best described the relationship between reference, age and the different terrestrial cues presented to the *A. percula* larvae (Table 4.2). This indicates that preference of larvae for a cue was dependent on age, the olfactory cue tested and the interaction between the two. As for the chemical cues from the anemone species and the coral, *A. percula* larvae displayed and increased attraction to the olfactory cue produced by the tropical plant leaf litter (Fig. 4.1a), initially displaying only a 16% attraction to the olfactory cue as newly hatched larvae that increased to a 92% attraction at settlement stage. A positive correlation between the larval sage and the affinity for the olfactory cue produced by the terrestrial grass species was also shown. Newly hatched larvae initially preferred blank seawater over the grass odour, while settlement-stage larvae showed no apparent preference for or against the same cue, spending only 50% of their time on the associated side of the chamber (Fig. 4.1a). Larvae age had no effect on the olfactory response to the chemical cue produced by the swamp-plant *Melaleuca nervosa*. All larvae preferred blank seawater compared with this cue regardless of age, spending a maximum of 15% of their

time in the cue at day five post hatching. Settlement-stage larvae completely avoided the water treated with *M. nervosa* (Fig. 4.1a).

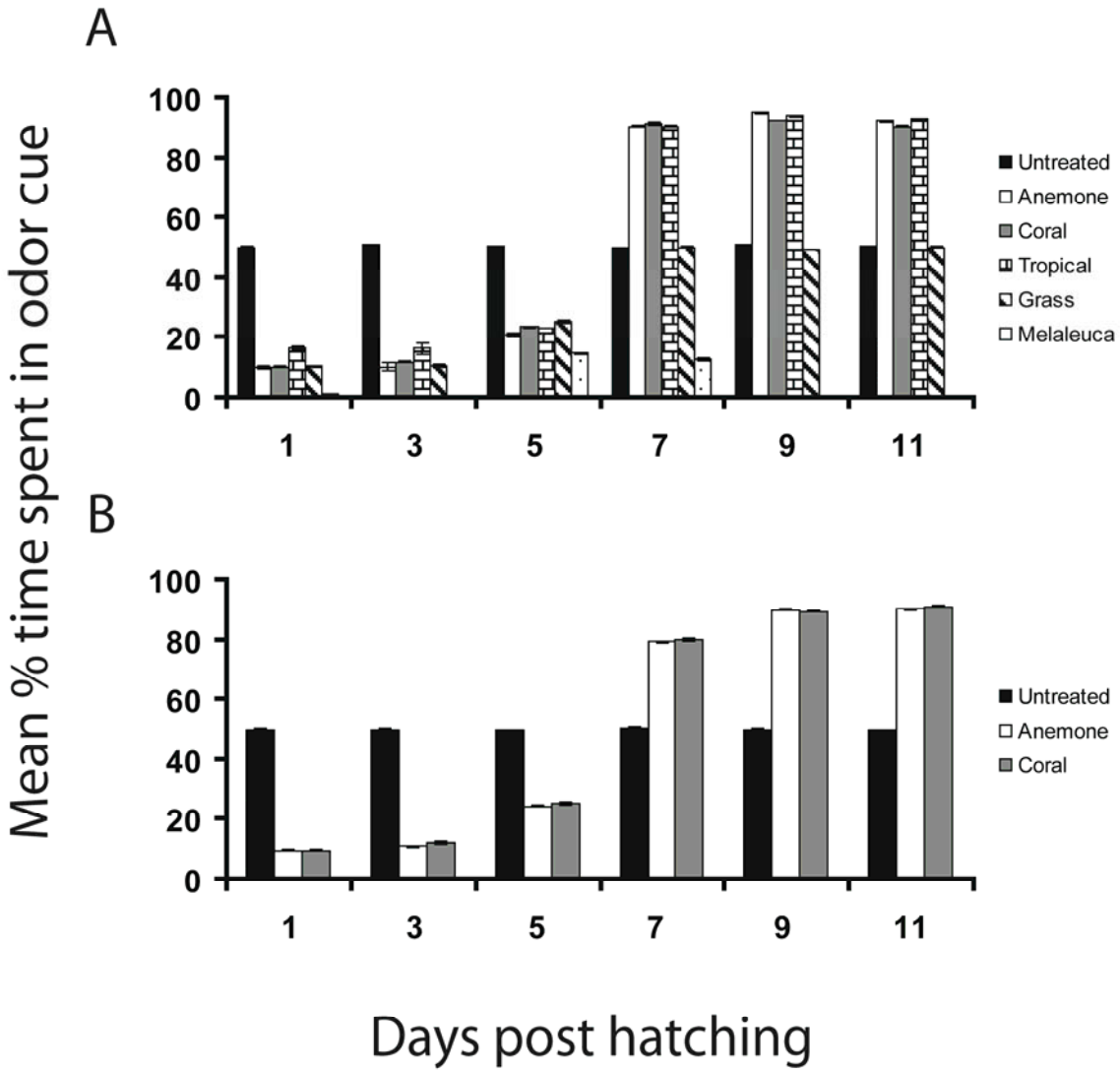

Figure 4.1 Behaviour of (a) *Amphiprion percula* and (b) *Amphiprion melanopus* in the presence of reef-associated settlement cues (±SE)

Table 4.1 Log-linear analysis of relationships between larval age, olfactory cue presented (associated anemone ad coral *Porites cylindrica*) and preference or avoidance of each cue by larval *Amphiprion percula*

Model	Likelyhood ratio χ^2	df	Differences between models	df
(1) C*A*P	0.0	0		
(2) C*A + C*P + A*P	0.0 NS	5	1 and 2, 0.0 NS	5
(3) C*A + A*P	0.0 NS	6	2 and 3, 0.0 NS	1
(4) C*A + C*P	454.27***	10	2 and 4, 454.27***	5
(5) C*A + P	454.27***	11	3 and 5, 454.27***	5

Results are identical for *A. melanopus*
C = Olfactory cue, A = Larval age, P = Olfactory preference
*P<0.05, **P<0.01, ***P<0.001, NS not significant (P>0.05)

4.4.2 *Amphiprion melanopus*

Amphiprion melanopus exhibited a very similar trend in the ontogenetic development of olfactory preferences to *A. percula* (Fig. 4.1b). For both the anemone odour and the coral olfactory cue, the best-fit model only included the effect of larval age on the cue preference (cue*age + age*preference (χ^2 = 0.000, df = 6, p = 1.000, Table 1). At first, larvae preferred blank seawater compared with the anemone or coral chemical cues, with newly hatched larvae spending only 9% of their time in the water containing these cues. Preference for both of these cues increased to approximately 80% at seven day post-hatching and increased to 90% at the end of the larval stage (Fig. 4.1b).

Table 4.2 Log-linear analysis of relationships between larval age, olfactory cues presented (tropical plants, grass, *Melaeuca nervosa*), and preference or avoidance of each cue by larval *Amphiprion percula*

Model	Likelyhood ratio χ^2	df	Differences between models	df
(1) C*A*P	0.0	0		
(2) C*A + C*P + A*P	20.85 NS	10	1 and 2, 0.0 NS	10
(3) C*A + A*P	233.34 NS	12	2 and 3, 0.0 NS	2
(4) C*A + C*P	328.86***	15	2 and 4, 454.27***	5
(5) C*A + P	468.54***	17	3 and 5, 454.27***	5

C = Olfactory cue, A = Larval age, P = Olfactory preference
*P<0.05, **P<0.01, ***P<0.001, NS not significant (P>0.05)

4.5 DISCUSSION

The importance of olfactory cues in the settlement process has been shown during the latter portion of the larval phase (Leis and McCormick, 2002; Kingsford *et al.*, 2002; Atema *et al.*, 2002; Wright *et al.*, 2005; Gerlach *et al*., 2007); however, this is the first study demonstration that the behaviour response to olfactory odours begins at hatching and changes during the larval period. Importantly, we found that both species of anemonefish exhibited strong preferences for untreated seawater when tested against reef-associated olfactory cues during the first few days after settlement. This may be an adaptation to reduce the exposure of larvae to the high densities of planktivorous fishes found on coral reefs (Almany and Webster, 2006). It would also predispose larvae to disperse away from their natal reefs. In the middle of the larval phase, an increase in preference for all olfactory cues is shown; however, cues are not significantly preferred over untreated seawater until seven days post-hatching. Towards the end of the larval phase, the larvae display a strong preference for the olfactory cues indicating a reef

(anemone odour, coral odour and for *A. percula,* terrestrial tropical plant odour). Larvae also exhibited the ability to discriminate between olfactory cues indicating a reef (anemone odour, coral odour, and for *A. percula,* terrestrial tropical plant odour). Larvae also exhibited the ability to discriminate between olfactory cues by displaying a strong preference for untreated seawater when tested against *M. nervosa*, and an indifference for the olfactory cues produced by the species of grass. These results highlight the early use of olfactory cues by larval coral reef fish and the change in preference that occurs during the larval stage. Although the conclusions that can be drawn from this study show larvae are able to use olfactory cues for navigation in the field are limited, due to the extreme difficulty in conducting such experiments with pre-settlement-stage larvae in the open ocean. The importance of ontogenetic shifts in olfactory preferences are likely to be important in influencing dispersal and settlement patterns.

Larval coral reef fish may be predisposed for dispersal, with innate olfactory preferences drawing newly hatched larvae away from reefs and near-shore environments, and into the pelagic environment. Although early-stage larvae may not possess the swimming ability to overcome physical oceanic characteristics, such as currents, vertical movement in the water column may allow larvae to obtain their desired portion. Importantly, even if newly hatched fishes are retained in near-shore waters, these data suggest that they will be unlikely to settle due to the innate avoidance of the typical settlement habitat cues.

There was an increased affiliation for settlement cues seven days post-hatching that strengthens as larvae move closer to settlement stage. Interestingly, at five days post-hatching, larvae spent an average of 15% of the testing time in the water stream

treated with *M. nervosa*; while this water stream was completely avoided by all fish during late state larval trials (nine and 11-days post-hatching). This may indicate that larvae are not as selective in their olfactory preferences at day five of development. The turning point for both species, where settlement olfactory cues were preferred, occurs approximately halfway through the pelagic larval duration (PDL). These results coincide with the ontogenetic development of the swimming ability. Fisher *et al*. (2000) tested the ontogenetic development of *A. melanopus* and found that this species develops its sustained swimming ability at five days post-hatching, and unpublished data discussed in the same study revealed a similar swimming trend with *A. percula* larvae. Additional species, with a longer PLD will need to be tested to determine whether olfactory changes occurs at a similar halfway point. Both *A. percula* and *A. melanopus* were strongly attracted to the benthic habitat cues for a relatively short time towards the end of their larval phase. However, with initially strong critical swimming speeds useful in covering short distances for short periods of time, abilities important in terms of potential for larvae to move between locations on a small scale, such as vertical migration and access to different current regimes, could grant larvae greater control over their initial position in the water column (Fisher *et al.*, 2000). It is possible that a species with a much longer PLD may exhibit a wider window for the attraction to settlement cues with increased variability in olfactory preferences and settlement timing.

High levels of self-recruitment on isolated island reefs have raised questions about how larvae locate settlement sites, and the influence larval behaviour may have on the dispersal process (Jones *et al.*, 1999; Almany *et al.*, 2007). For example, Almany *et al.* (2007) have shown that after a pelagic period of 10-12 days, up to 60% of the juvenile

A. percula settling at one off-shore island in Papua New Guinea were offspring of the same island's resident adults. This same result was found in *Chaetodon vagabundus*, which has an average pelagic duration of 38 days. It is possible that the some of the offshore islands species do not encounter olfactory cues of other reef habitats before the ontogenetic shift in cue preference occurs and larvae become attracted to cues from their natal reefs. As the dispersion of olfactory cues is current dependent, locating settlement habitat would require upstream orientation and movement once a favourable cue has been identified (Leis *et al.,* 2011).

A better understanding of sensory cues, the behavioural response of larvae (Kingsford *et al.,* 2002), and physical studies integrating behaviour (Armsworth, 2000; Paris and Cowen, 2004; Largier, 2004; Paris, 2007) has raised awareness about the role behaviour plays in dispersal and recruitment (Metaxas, 2001; Leis *et al.,* 2007). Flexibility of behaviour in response to hydrological conditions (Cowen and Sponaugle, 2009), and other sensory stimuli such as sound (Leis *et al.,* 2003), gives larvae an unexpected opportunity in controlling their movements. Accurate dispersal models require the influence of larval behaviour in settlement site selection to be incorporated throughout the entire larval stage rather than only the latter half. This study indicates that larval behaviour early in the pelagic stage along with changes in sensory preferences during ontogenetic development could play an important role in determining patterns of dispersal and connectivity.

CHAPTER 5: **Experimental evaluation of imprinting as a mechanisms for habitat selection in a coral reef fish**[†]

[†] Dixson DL, Munday PL, Planes S, Pratchett M, & Thorrold SR (2011) Experiential evaluation of imprinting as a mechanisms for habitat selection in a coral reef fish. *Proceedings of the Royal Society of London B. In Preparation*

5.1 ABSTRACT

When faced with decisions about where to live, there is likely to be a strong selection for juveniles to choose habitats similar to where their parents successfully bred. As most marine animals have a pelagic larval stage, juveniles have only a brief embryonic period to acquire information from their place of birth. For fishes, it has been shown that developing embryos can imprint on chemical cues from their natal habitat. However, to show that imprinting is ecologically important, it must be shown that both juveniles can distinguish among potential imprinted cues, and also respond positively towards cues from natal environments. Here we show that juveniles of a coral reef anemonefish (*Amphiprion percula*) are capable of imprinting on and responding to a variety of olfactory cues, including their host anemone species. However, field studies using parentage analysis to relate settled juveniles to their natal habitat, showed that juvenile settlement was independent of the parent's host anemone. While imprinting does not

appear to explain anemone choice by settling larvae, settlement choices are likely moderated by the presence of specific conspecifics and other critical settlement cues. We hypothesize that the ability of embryos to imprint on a diversity of chemical cues may explain their ability to home to natal islands.

5.2 INTRODUCTION

It has been commonly observed among animals that juveniles have very similar habitat preferences to that of their parents (Davis and Stamps, 2004; Davis, 2008; Mabry and Stamps, 2008). Juveniles may maximize their chances of survival and successful reproduction by either remaining at or returning to their natal habitat, or dispersing to places with a similar habitat structure (Stamps, 2001; Stamps and Davis, 2006; Aubret and Shine, 2008). Parental habitat choice may be an innate response that evolves as long as juveniles selecting the same habitats as their parents consistently experience fitness advantages (Janz *et al.,* 2009; Grief and Siemers, 2010). However, the ability of juveniles to acquire or learn information about suitable habitats during early development can further maximize the chances of making the correct choice later in life (Stamps, 2001; Baker and Rao, 2004, Stamps *et al.,* 2005, 2009). While assessing habitat suitability is potentially advantageous, juveniles of many species are not fully developed for the brief period in which they are exposed to the same environment as their parents. It has been suggested that, in such species, juveniles may have gained mechanisms of imprinting through evolution that allow them to acquire, retain, and later use information

on their natal environment (Immeman, 1975). Imprinting is a behavioural response that occurs when individuals are exposed to a stimulus during a sensitive period early in life, and then respond to that stimulus later in development (Lorenz, 1937).

The phenomenon of imprinting is most widely recognized in relation to parental recognition in birds and mammals (Bateson, 1966; Bolhuis, 1991), with juveniles famously being imprinted on other species (Lorenz, 1937). The term ecological imprinting was proposed to encompass the role of imprinting in the establishment of food and habitat preferences or the identification of natal or home areas (Immelman, 1975). Among fishes, salmon are renowned for their ability to imprint using olfactory stimuli from natal streams (Cooper *et al.,* 1976; Dittman *et al.*, 1996; Yamamoto *et al.,* 2010). Most marine fishes have a pelagic larval phase, with juveniles spending only a very brief period early in life in close proximity to their parents. For imprinting to occur, juveniles must acquire information during the pre-dispersal embryonic stage. This was demonstrated by Arvedlund *et al*. (1999) who found that juvenile anemonefish *Amphiprion melanopus*, which had completed embryonic development near the host anemone *Entacmaea quadricolor* (as opposed to *Heteractis magnifica,* another species of anemone that this species also uses), used olfactory cues to recognize this anemone and later preferred it over other potential host anemones. This study provided the first evidence that coral reef fish embryos are capable of imprinting olfactory information and might potentially use this information to make habitat choices at settlement (Arvedlund and Nielsen, 1996).

To establish the ecological importance of imprinting, it must be established that both juveniles are capable of imprinting, and also that they use this acquired information

to make habitat choices in the natural environment. For fishes, laboratory rearing experiments provide a useful means of testing whether or not juveniles can imprint and make choice in response to imprinting (Arvedlund *et al.,* 1999; Gerlach *et al.,* 2008). For example, Gerlach *et al.,* (2008) found imprinting in zebrafish occurs within a 24-hour window six days post-fertilization 2 days after hatching occurs. However, for such choices to be considered ecologically important, they need to be verified in natural habitats. For marine fishes, the fate of offspring after a pelagic larval phase is usually unknown, making comparisons of early ecological stimuli and subsequent habitat choices impossible. However, it has recently been demonstrated that juveniles of many coral reef fishes can return to natal reefs (Jones *et al.,* 2005; Almany *et al.,* 2007; Planes *et al.,* 2009). For anemonefish, this means that the precise location of parents and settlement sites can be compared using parentage analysis (Jones *et al.*, 2005), making it possible to examine whether juveniles are more likely to settle on the same anemone species they were associated with at birth.

The overall aim of this study was to investigate the capacity for imprinting and the role it plays in settlement site selection in the coral reef anemonefish *Amphiprion percula*. This is an ideal species for testing the importance of imprinting as it commonly occupies at least two host anemone species (Fautin and Allen, 1992). Adults live in a life-long association with a host anemone and lay demersal eggs on the adjacent substratum, so that developing embryos have the potential to imprint on the host anemone species or other cues in the immediate environment. Recent studies show that a substantial number of juvenile *A. percula* return to natal islands (Almany *et al.,* 2007, Planes *et al.,* 2009), making it possible to determine whether juveniles are settling on the

same anemones species as their parents, and thus the same anemone species that the eggs and newly hatched larvae were exposed to. *A. percula* has been shown to be strongly associated with coral reefs surrounding vegetated islands, and larvae and juveniles exhibit strong innate responses to a range of waterborne chemical cues, including anemones, conspecifics and terrestrial vegetation (Dixson *et al.,* 2008). Imprinting on these signals may facilitate finding home islands and/or natal anemone species.

In the first part of this study, we carried out laboratory rearing experiments to test the ability of the anemonefish to imprint on a range of relevant and novel olfactory cues including the two main host anemone species (*Heteractis magnifica* and *Stichodactyla gigantea)*, and tropical leaf litter (which may signal proximity of an island) (Dixson *et al.,* 2008). We also tested the potential for imprinting on chemical cues derived from other plants that this species does not normally prefer (grasses) or actively avoids (the swamp tree *Melaleuca nervosa*) (Dixson *et al.,* 2008). Previous experiments using coral reef fishes have only focused on the host anemone/anemonefish relationship, testing host selection under constant laboratory conditions (Arvedlund *et al.,* 1999). Results from this study indicate that imprinting may be an important factor in host habitat selection; however, the role of imprinting was not directly tested. The aim of this study is to test the effect of imprinting directly on host selection in anemonefish. As the imprinting process should affect all chemical cues presented during the "sensitive period", it is important to test the full potential this mechanism would have on cues that are not innately preferred over both a short and long exposure time. Also, although imprinting has been studied under laboratory conditions, the role imprinting plays in habitat selection under natural conditions has not yet been determined. In the second part, we

conducted a field study in Kimbe Bay, Papua New Guinea, where we used genetic parentage analysis to determine whether settling juveniles are preferentially settling on their natal host anemone species. We hypothesized that juveniles would be capable of imprinting on olfactory cues regardless of their innate preferences, and that imprinting would increase the likelihood of juveniles settling on natal hosts.

5.3 MATERIALS AND METHODS

5.3.1 Laboratory induction and test of imprinting

Larvae were raised from a brood stock of 21 pairs of *Amphiprion percula* maintained in 70 litre tanks in a closed seawater system at the James Cook University's Marine Aquarium Facility, using methods described by Dixson *et al.*, (2008). Due to the requirement of parental care during the egg stage, and the facility used for breeding imprinting was only tested during the larval stage, with no manipulation of chemical cues during the egg stage. After hatching, larvae were split among six 70 litre rearing treatment tubs, where they were exposed to differential cues. One control treatment and five imprinting treatments made from the olfactory cues of: (1) the host anemone *Stichodactyla gigantea*; (2) the host anemone *Heteractis magnifica*; (3) a tropical rainforest plant *Xanthostemon chrysanthus*; (4) a swamp dwelling plant *Melaleuca nervosa* and; (5) a grass species *Panicum maximum*. All chemical cues used were chosen because they have previously been shown to elicit a behaviour response by larval

anemonefish at settlement stage (both as either a repellent and an attractant); early exposure to these cues may change the elicited behavioural response. Anemone olfactory cues were created by soaking anemones in a closed 70 litre system for two hours; 10 litre of water was then removed from the anemone-holding tank and slowly added to the larval tanks. Terrestrial leaf treatments were created by placing 10 grams of each leaf species held in nylon bags, into each larval rearing tank.

To determine if brief exposure early in development is sufficient for larval fish to imprint on chemical cues, or if prolonged exposure is required, clutches were either exposed to cues during just the first 24 hours after hatching, or for their entire larval stage. For clutches exposed to imprinting odours throughout the entire larval stage, nylon bags were replaced daily and fresh anemone odour cues were introduced to the larval rearing tank each morning. Artificial seawater was used throughout the experiment to ensure larvae were exposed to only intentional chemical signals.

5.3.2 Experimental design

Larvae were tested in a two-channel choice chamber (described below) at settlement stage (11 days post-hatching). All larvae were tested for their preference for each odour cue (*S. gigantea*, *H. magnifica*, tropical plant, *M. nervosa*, grass, control) against untreated seawater, regardless of their imprinting treatment, including control larvae that were not allowed to imprint (Fig. 5.1). In addition, we compared the preference of larvae for the two anemone species simultaneously in the flume. This enabled us to test if imprinting could cause larvae to exhibit different preferences

between the two anemones species, which might potentially cause them to make different decisions when faced with the odours of both anemone species in a normal reef setting. It was hypothesized that larvae imprinted on a specific chemical cue, regardless of the innate response, would display a heightened preference for that specific imprinted cue only. The response to all other cues tested would be unaffected by the imprinting process and remain consistent with the results of the response displayed by the control treated larvae. It was also hypothesized that exposure time would effect the strength of the preference, with larvae exposed to chemical cues for the entire larval phase displaying a greater preference for the imprinted cue than larvae exposed only during the first 24 hours.

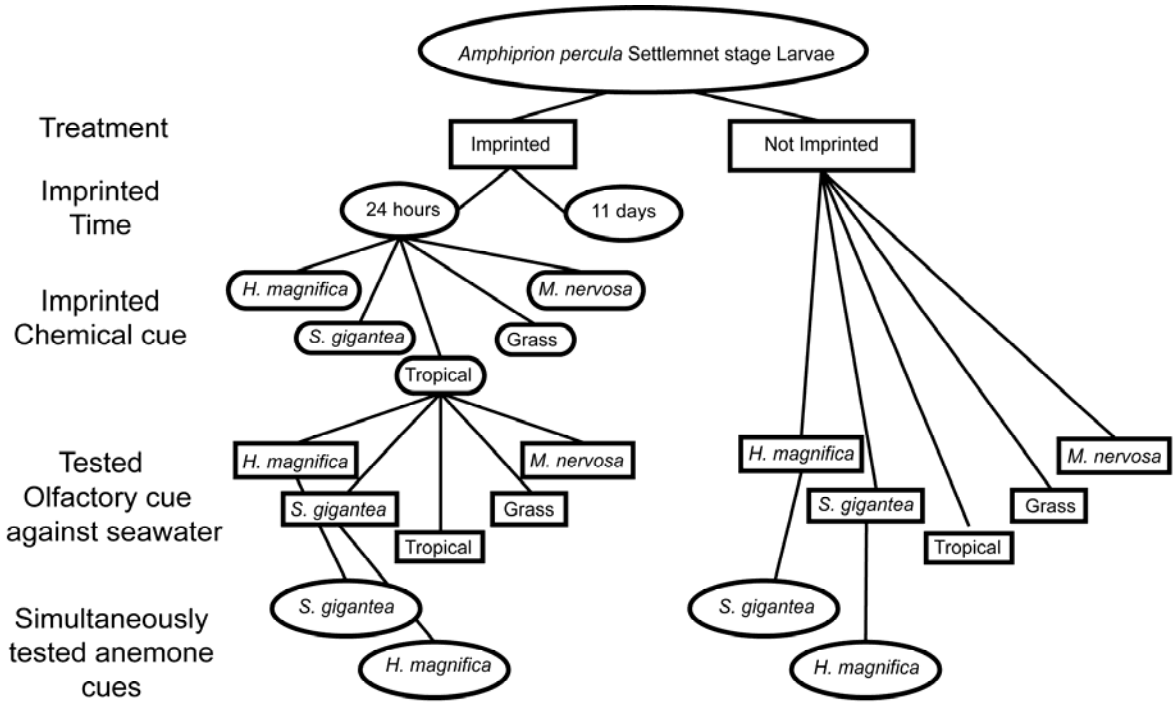

Figure 5.1 Experimental flow diagram of the imprinting process for *Amphiprion percula* larvae

5.3.3 Behavioural testing apparatus

A two-channel choice flume (13cm x 4cm) 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 flowing from two different sources. Water containing chemical cues for use in the flume was created by soaking 20 grams of leaves in ten litres of artificial seawater for two hours or soaking the anemone in 70 litre of artificial seawater for two hours. Leaves and anemones were removed prior to testing. Water was gravity-fed into the choice flume, which is partitioned along half of its length. Fish are released at the downstream end of the flume where they are free to move to either side or swim toward the preferred water source.

Using the protocols outlined in Gerlach *et al.* (2007), a constant gravity-driven flow of 100 ml/minute per channel was maintained throughout all trials. A two-minute acclimation period was followed by a two-minute testing period where the position of the fish, on either the right or left side of the chamber was recorded on five-second intervals. A one-minute rest period followed, were the water sources were exchanged from one side to the other. The test was then repeated, including the acclimation period, to ensure larvae were displaying a preference for the chemical cues in the water rather than one side of the chamber. Flow rates were measured using a flow meter and dye tests were conducted at each water change to ensure that the two channels exhibited parallel water flow, with no turbulence or eddies.

All trials were conducted on larvae from a minimum of three parental groups. For each parental group ten individual larvae were randomly selected from the rearing tub and tested in the choice flume. Each fish was tested only once. Using the Kolmogrov-Smirnov test, no statistical difference between the three parental groups was found; therefore parental groups were pooled for each analysis. Kolmogrov-Smirnov tests were also used to compare the proportion of time that individuals spent in the stream of water containing the olfactory cue when imprinting was absent, verses the proportion of time that individuals spent in the same olfactory cue when larvae were allowed to imprint. A separate Kolmogrov-Smirnov test was conduced for each olfactory cue. This statistical test was also used to compare the proportion of time that individuals spent in the olfactory cue when the exposure period for imprinting was 24-hours, verses the entire pelagic larval stage.

5.3.4 Field parentage study

The field study was carried out at Kimbe island (58°12.530 S, 150°822.801 E), a small, vegetated island 30 km offshore in Kimbe Bay, Papua New Guinea. The island supports a population of approximately 266 pairs of adult *A. percula* that live in association with two species of host anemones (*S gigantea* and *H. magnifica*). Female *A. percula* spawn benthic eggs that hatch after seven days of parental care. Larvae then spend an average of 11 days in the pelagic environment before settling into an anemone. At this island, approximately 50% of settled juveniles are offspring of adults from the same island (Almany *et al.*, 2007, Planes *et al.*, 2009).

All 266 anemones with adult fishes were located in April 2007, including 121 breeding pairs on *S. gigantea* and 145 pairs on *H. magnifica*. A total of 532 adults (the two largest fish in each colony) were captured using hand-nets and clove oil anaesthetic, measured using callipers underwater, and a small piece of fin tissue from the caudal fin collected underwater on site. The fin-clip was preserved in 85% ethanol in individual 2.0ml vials. Sampled fish were released back onto their host anemone.

Juvenile *A. percula* were collected using similar methods described above and measured using underwater callipers. When possible a small piece of tissue was taken *in situ* from the caudal fin and preserved in individual 2.0ml vials of 85% ethanol, the fish was then released back on to its host anemone. For some of the smallest recruits (<3 cm), the whole fish was taken and preserved. A total of 162 recently settled juveniles (<18mm SL) were captured and collected for genetic analysis.

DNA from all collected fish was extracted and polymorphic microsatellites were screened. Microsatellite screening was preformed by Matis-Prokaria (Iceland) using ABI capillary technology. Sixteen microsatellite DNA loci were screened, satisfying Hardy-Weinberg equilibrium assumptions (Planes *et al.*, 2009). Missing values accounted for 3.4% of the data distributed over all loci, but were concentrated in only a few individuals, which is likely due to the original quality of the DNA. The FAMOZ platform was used to assign juveniles back to adults (Gerber *et al.*, 2003; Planes *et al.*, 2009). Of the 162 new recruits, 104 were assigned to parents in the Kimbe Island population, including 91 that were independently assigned to a female and a male within a single anemone and 13 that were assigned to a single parent (male or female). Single parent assignments were likely due to missing adults during the collection process, turnover in the adult population

and blanks in the microsatellite scoring. No juvenile was ever assigned to parents residing in two different anemones.

A two by two chi-square test of independence was conducted to determine if the anemone species chosen at settlement in the field experiment was independent of the anemone species occupied by parents.

5.4 RESULTS

Table 5.1 Olfactory results showing mean per cent time larvae spent in one of 5 olfactory cues (SE) in control conditions compared to mean per cent time larvae spent in the olfactory cues (SE) when imprinting occurred after 24 hours of exposure or the entire larval stage (11 days).

Olfactory Cue	Mean % time spent in cue (control)	Mean % time spent in cue (imprinted 24 hrs)	Mean % time spent in cue (imprinted 11 days)
H. magnifica	85.79 (±0.42)	97.15 (±0.13)	98.65 (±0.15)
S. gigantea	86.77 (±0.18)	97.50 (±0.12)	97.99 (±0.19)
Tropical	92.92 (±0.52)	98.34 (±0.65)	99.51 (±0.10)
Grass	49.24 (±0.62)	86.46 (±1.15)	88.40 (±0.66)
Melaleuca nervosa	0 (±0.0)	27.64 (±0.87)	81.03 (±0.61)

5.4.1 Laboratory induction of imprinting

Settlement stage *A. percula* displayed a significant increase in attraction to all cues that they were exposed to for imprinting at the embryo stage (Fig 5.2, $p<0.01$ for tropical plant and $p<0.001$ for all other cues). The imprinting process only affected the response to the imprinted cue; there was no significant change in preference for any of the other chemical cues tested. Control larvae were innately attracted to cues from both

anemone species, spending approximately 86% of their time in the water stream containing these cues even though they had not previously been exposed to the olfactory cues from either anemone species (Table 1). For individuals exposed to *H. magnifica* at the embryo stage, this attraction increased to 97 and 98%, within 24 hours and the entire larval phase exposure to the cue, respectively (Table 1). There was no statistical difference in the preference expressed between the two exposure times when considering cues from host anemones. The same trend was found for individuals imprinted on *S. gigantea*, with attraction to the chemical cues of *S. gigantea*, increasing from 86% in non-imprinted larvae to 97.5 - 98% after imprinting for 24 hours or the entire larval stage, with no significant difference between the two exposure times (Table 1).

When imprinted anemonefish larvae were given the choice between odours of the two anemone species simultaneously, the anemone which larvae were imprinted on was always preferred over the non-imprinted host anemone ($p<0.001$ for both anemone species). Larvae imprinted on *H. magnifica* spent 68.42% (±0.47 SE) of their time in the *H. magnifica* odour cue and only 31.58% (±0.47 SE) in the *S. gigantea* olfactory cue after only 24 hours of imprinting. Anemone cue preferences for the imprinted chemical cue significantly increased, by 9.10% ±0.23 SE, when the imprinting process lasted through the entire larval period ($p<0.01$). Larvae imprinted on *S. gigantea* exhibited a preference for the olfactory cues produced by *S. gigantea* (64.99% ±0.67 SE) compared to the olfactory cues of *H. magnifica* (35.01% ±0.67 SE). As observed with the *H. magnifica* imprinted larvae, preferences for *S. gigantea* significantly increased, by 14.21% ±0.19 SE, for larvae imprinted on the *S. gigantea* for their entire larval phase compared with the 24 hour exposure, which was significant ($p<0.01$)

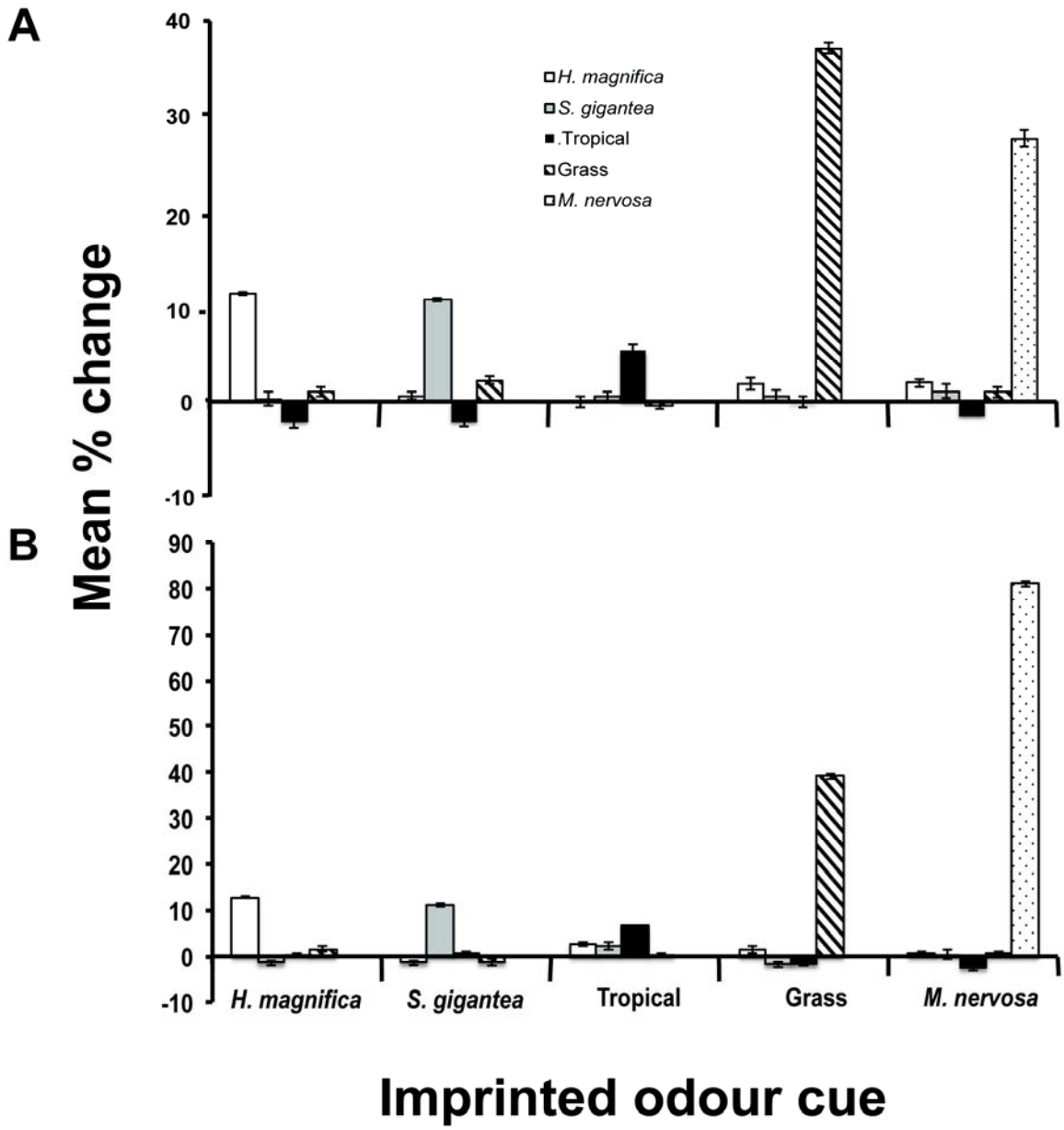

Figure 5.2 Mean per cent change in cue preference association by *Amphiprion percula* after (a) 24 hours or (b) 11 days of imprinting, when tested in a two-channel choice chamber (initial innate cue preference – cue preference after imprinting) on one of five different olfactory odour cues

For the plant odours, larvae displayed an innate preference for the chemical cues produced by the tropical plant, with control larvae spending 92% of their time in this odour cue. The strength of this preference increased significantly after 24 hours of exposure to tropical plant cue, with larvae spending 98% of their time in the water stream contained the tropical plant odour. However, as with the anemone olfactory cues, no significant change in preference was detected when the exposure period was increased to the entire larval period (Table 1). The larvae showed no preference for or against the chemical cues from the grass odour, with control larvae spending 49% of their time in the grass cue. The preference for grass significantly increased after 24 hour of exposure to the cues, with imprinted larvae spending 86% of their time in the grass cue (Table 1). Imprinting the larvae for the entire pelagic stage did not significantly increase the preference for the chemical cues produced by grass. Control larvae exhibited an innate avoidance to the olfactory cues produced by *M. nervosa*, with no larvae spending any time in the olfactory cues produced by this plant. After 24 hours of imprinting, the larvae increased their preference for this cue, spending 27% of their time in the odour, and increasing to 81% of their time in the *M. nervosa* odour cue when they had been exposed for their entire larval phase ($p<0.001$).

5.4.2 Field parentage study

We predicted a high correspondence between the anemone species chosen for settlement and the natal anemone species. Of the 59 recruits who were offspring from

adult pairs on *S. gigantea*, 34 individuals recruited back to *S. gigantea,* while 25 individuals settled on *H. magnifica*. For the 49 recruits that were offspring of adult pairs on *H. magnifica*, 25 recruits settled back onto the same species, while 24 individuals settled on *S. gigantea*. The observed distribution of settlers on the two anemone types was independent of their natal anemone species ($p>0.37$).

5.5 DISCUSSION

The laboratory studies show that larval anemonefish are able to imprint on a wide range of chemical cues within the first 24 hours of hatching, including olfactory cues produced by host anemones, potentially far-reaching habitat cues (such as the odour of tropical trees found on vegetated islands) and even chemical cues that are innately avoided (such as from the swamp tree *Melaleuca nervosa*). In all cases, when reared with the cue to induce-imprinting, settlement-stage juveniles displayed an increased attraction to or reduced repulsion from the cue than they do otherwise. Settlement-stage larvae could clearly display information retention for imprinted chemicals by responding to these odours once settlement stage was reached. Although laboratory trials highlight the ability to imprint in *A. percula*, specifically on host anemone species odour cues, the field experiment found no evidence that imprinting influences settlement site selection. The anemone chosen at settlement was independent of the anemone which the eggs and newly hatched larvae were exposed to prior to the pelagic larval stage. Hence, the potential for imprinting under controlled conditions and its significance under complex field conditions clearly differ. Settlement is a complex process wherein many important

factors will influence the settlement choice made. It is possible that the complex signals likely to be encountered in field settings, such as the influence of conspecifics and associated predation risk, may modify settlement choices, which were previously influenced by imprinting.

The ability of larvae to imprint has been speculated by numerous studies as a possible explanation for larval retention (Almany *et al.*, 2007, Planes *et al.*, 2009) and specific substrate choice (Arvedlund and Nielsen, 1996; Arvedlund *et al.*, 1999). Larvae displayed the strongest imprinting ability for the cues that innately showed no initial preference. For example, control larvae completely avoided *Melaleuca nervosa* odour, but spent 27% of their time in the cue after just 24 hours of exposure and over 80% of their time in the cue after imprinting for their entire larval phase. In contrast, there was relatively little increase in preference strength for the chemical cues when innate preferences already existed, such as with host anemones. Interestingly when the two anemone cues were presented simultaneously, a clear preference was shown for imprinted anemone species, with additional exposure time significantly increasing the preference for the imprinted chemical cues. As a result of the laboratory experiment it can be concluded that innate olfactory preferences can be modified as a result of imprinting.

Exposure time to the imprinted chemical cues had no significant effect on the preference strength for the habitats normally used by *A. percula* or the odours that were already innately preferred when tested against untreated seawater. Increasing the imprinting time period throughout the embryonic developmental stage only resulted in a significant increase in preference strength for the *Melaleuca nervosa* chemical cue and

the simultaneous comparison of the two host anemone species. These results indicate that a 24 hour exposure period reinforces an already innate preference through moderate exposure. This is consistent with previous studies wherein imprinting occurred when eggs were exposed to the chemical cues and newly hatched larvae were exposed to the anemone for only the first 30 minutes after hatching (Arvedlund *et al.*, 1999). Eggs in this study were not exposed to any of the chemical cues, indicating that it is possible for larvae to imprint post-hatching with no exposure during the egg stage.

One explanation for the conflicting laboratory and field data is that it is unlikely that a strong habitat specialist would avoid appropriate settlement habitat when encountered for the chance to settle on the imprinted habitat. Anemones are a limited resource for settling anemonefish (Fautin, 1993; Elliott and Mariscal, 2001; Hattori, 2005) and anemonefishes reside in a hierarchical social system, where dominant status and opportunity to breed is based on size (Fricke, 1979; Fautin, 1993; Buston, 2004 a,b). Consequently, settlement on saturated anemones often results in the eviction of new recruits (Buston, 2003a), or an increased mortality for low ranking settlers (Buston, 2003b). Due to the complex elements associated with the settlement process and because all host species offer breeding opportunities, it would be advantageous to settle on any suitable vacant host anemone when chances arise, rather than delay settlement in the hopes of locating an unsaturated imprinted host anemone species. Elliott and Mariscal (2001) observed the recruitment of nine species of anemonefish and found that recruitment occurred to anemones that were small with no residents or had comparatively fewer and smaller residents than the anemones, which did not receive new recruits. In *A. percula,* recruitment was found to be up to 75 times higher on anemones that had

residents removed as opposed to anemones not manipulated. A similar trend was found by Fautin, (1993), with a significantly increased recruitment rate to anemones that had residents removed. This suggests that locating a vacant settlement site on any host anemone species is likely the primary driver of settlement patterns. This is consistent with our genetic analysis, which indicated if imprinting is occurring in nature, it does not affect the distribution of juveniles among anemones.

Concentration levels of chemical cues found in the field vary through time and space, due to the influence of both tidal cycles and the distance from the source of the stimuli. As a result of this, the amount of chemical stimuli coral reef fish larvae are exposed to during their larval stage is unknown and would vary between locations and even individuals within an egg clutch. Therefore, another possible explanation for the disconnection between the laboratory and field result found in this study, is the uncertainty in concentration used for the imprinting process. Due to water mixing, the concentration of the imprinted chemical stimuli could be lower in the field than simulated in the laboratory experiment.

Although this study indicates that imprinting may not be as important in habitat selection as laboratory studies indicate, the potential importance of imprinting is highlighted by the capacity of larvae to strongly adjust preferences after imprinting has occurred, particularly on chemical cues for which innate preferences do not exist. It should also be considered that imprinting might occur on multiple chemical cues at once, possibly providing information that may be useful over different spatial scales during the settlement process. Imprinting may be more important in reef selection than micro-habitat selection, which would help to explain the high level of self-recruitment seen in

offshore island communities, including Kimbe Island, where the present study was conducted (Almany *et al.*, 2007, Jones *et al.*, 2005). Finding island locations may involve a range of chemical signals, which requires further testing. Previous experiments have shown larval fish capable of distinguishing between home reefs and other reefs within a ten km radius, through chemical cues alone (Gerlach *et al.*, 2007), and that reefs themselves possess unique chemical signatures which larvae are able to identify and respond to (Dixson *et al.*, 2008). It is possible that newly hatched larvae are able to imprint on these specific reef signature cues, which may aid in settlement site selection following the pelagic larval stage.

In conclusion, the anemonefish *A. percula* possesses the ability to imprint on a variety of chemical signals; however, chemical imprinting on specific settlement substrate is not used for settlement site selection. Instead, innate preferences appear to drive microhabitat selection. It is possible that anemonefish imprint on broad scale cues that may aid in returning to natal reefs, but this hypothesis requires further testing. The conflicting results between the laboratory experiment, which indicated strong imprinting potential, and the field research indicating imprinting does not influence anemone selection, highlights the complexity of cues that are likely to be encountered by settling larvae in the field setting and the significant amount of research required to elucidate complex decision processes.

CHAPTER 6: **Predation risk assessment by larval reef fishes during settlement site selection**[†]

[†] Dixson DL (2011) Predation risk assessment by larval reef fishes during settlement site selection. *Coral Reefs. In press. (*doi: 10.1007/s00338-011-0842-3)

6.1 ABSTRACT

Predation rates of marine species are often highest during the transition from the pelagic to the benthic life stage. Consequently, the ability to assess predation risk when selecting a settlement site can be critical to survival. In this study, pair-wise choice trials were used to determine if larvae of three species of anemonefish (*Amphiprion melanopus*, *A. percula* and *Premnas biaculeatus*) are able to: 1) assess the predation risk of potential anemone settlement sites through olfactory cues alone, and 2) alter their settlement choices depending on the options available (host or non-host anemone). When predation risk was assessed with host and non-host anemone species independently, all species of anemonefish significantly chose the odor associated with the low risk settlement option over the high-risk site. Most importantly, all species of anemonefish selected water with olfactory cues from their host anemone regardless of predation risk when paired against non-host anemone odor. These results demonstrate that larval reef fishes can use

olfactory cues for complex risk assessment during settlement site selection; however locating the correct habitat is the most important factor when selecting a settlement site.

6.2 INTRODUCTION

Early detection and avoidance of predators is vital for individual survival. Most marine organisms experience extreme predation risk during life history transitions such as at hatching or when returning to adult habitat following their pelagic larval phase (Caley et al. 1996; Almany and Webster 2004). On coral reefs, late stage larvae returning to reefs may experience a predation "gauntlet" as they navigate their way to suitable benthic settlement habitat (Hamner et al. 1988; Almany and Webster 2006). It would therefore be advantageous for larvae, at this important life-stage, to be capable of assessing the risk of predation at different settlement sites prior to making a potentially fatal choice.

Predator odor is one type of sensory cue aquatic species use to assess their risk of predation (Wisenden 2000; Kelley and Magurran 2003). Although a suite of sensory mechanisms is available for predator detection, olfaction plays a significant role in the initial detection of predators (Chivers et al. 2001). Settlement-stage fish larvae possess a highly developed visual system; however, for many species, settlement occurs at night around the new moon when visual detection of predators is less effective (Valles et al. 2009). Olfactory cues have been used in other important settlement-stage processes, such as, navigation to reefs (Gerlach et al. 2007), recognition of conspecifics (Sweatman

1988), and habitat selection (Elliott et al. 1995; Arvedlund et al. 1999; Lecchini et al. 2005b). While at least one reef fish can innately detect predators during the larval stage (Dixson et al. 2010), it is unknown whether this ability enables late-stage larvae to respond to multiple cues and minimize predation risk when choosing a settlement site.

The ability to assess predation risk and identify the most suitable settlement site is expected to be greatest in species that have a strong and lasting association with specific habitats (i.e., habitat specialists). Some settlement sites are likely to be in more favorable locations (Booth and Beretta 1994) or contain better quality habitat than others (Munday 2001). In many coral reef species, settlement site decisions are relatively permanent choices. For example, anemonefish are likely to spend their entire adult life in the anemone that they chose at settlement (Fricke and Fricke 1977; Buston 2004b). Therefore, the ability to assess settlement site suitability can have life-long implications for an individual's survival and reproductive success. Settlement sites can be a limited resource (Munday et al. 2001; Buston 2004b) and thus individuals may need to balance the benefits of searching for premium habitat against the risk of finding no settlement habitat. To date, studies on settlement-site selection in coral reef fish larvae have focused on either the olfactory cues from the settlement habitat (Arvedlund et al. 1999; Elliott et al. 1995; Lecchini et al. 2005b), or the ability to use olfactory cues for predator detection (Dixson et al. 2010). However, the combined effect these important olfactory cues may have in the critical evaluation of settlement habitat is unknown.

Twenty-eight anemonefish species exist in the Indo-Pacific region characterized by their symbiotic relationship with host sea-anemones; with each anemonefish species utilizing between one and ten sea-anemone species (Fautin and Allen 1992). This high

degree of habitat specialization makes anemonefish a model group for studying habitat selection. Due to their high degree of host-specificity, anemonefish larvae need to locate a reef, containing the sea-anemone species required for their survival.

In this study, a series of pair-wise choice trials were used to test whether larvae of three species of anemonefish (*Amphiprion melanopus*, *A. percula* and *Premnas biaculeatus*) are able to assess the predation risk of potential settlement sites through olfactory cues. Furthermore, this study assessed whether settlement choices are context dependent, based on the habitat options available. The first experiment determined the effect of predator and non-predator olfactory cues on settlement site preferences of anemonefish larvae for their host anemone. These trials tested the hypothesis, that larvae are capable of odor discrimination and will select the settlement option with the lowest risk of predation (i.e., host anemone with non-predator odor or host anemone odor alone). The second experiment examined whether predator and non-predator olfactory cues affect the settlement site preferences of larval anemonefish for a non-host anemone habitat or untreated seawater (which represents settlement avoidance). This tested the hypothesis that the larvae will maintain a preference for the low predation risk settlement option (i.e., non-predator odor), but will avoid the non-host anemone in favor of the untreated seawater, indicative of larvae delaying settlement to search elsewhere for suitable habitat. The third experiment determined if predator and non-predator olfactory cues affected settlement site preferences when either a host or non-host anemone was available. These trials tested the hypothesis that avoiding predation risk is more important than correct habitat selection and therefore larvae will continue to maintain a preference for the low predation risk option regardless of anemone species.

6.3 MATERIALS AND METHODS

6.3.1 Breeding protocols

Larvae were raised from breeding pairs of *A. percula*, *A. melanopus* and *P. biaculeatus* collected from the Great Barrier Reef and maintained in individual 70 l tanks in a closed 70,000 l seawater system. Breeding pairs laid egg clutches on the underside of a terracotta pot and on the night of hatching (6-8 days post-laying) egg clutches were transferred to a 70 l larval rearing aquarium. After hatching, larvae were reared in a semi-closed system, which 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-labitum* throughout the day and that any unconsumed food was removed each night. Larvae were fed rotifers (*Brachionus* sp.) at 5 individuals ml^{-1} each morning for the first three days. *Artemia* nauplii was added at 1 individual ml^{-1} each morning beginning at day three. The ratio of *Artemia* nauplii to rotifers 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 for *A. percula* and *A. melanopus*, and 14 days post hatching for *P. biaculeatus*.

6.3.2 Olfactory choice trials

A two channel choice flume developed by Gerlach et al. (2007) was used to assess the ability of larval anemonefish to discriminate between olfactory cues from different settlement sites. This apparatus was designed to conduct pair-wise choice

experiments, with fish able to freely choose between water flowing from two different sources. An individual larva was gently removed from the hatching aquarium and was released at the downstream end of the flume where it was free to move to either side or swim toward the preferred water source. Water flow was maintained at 100 ml min^{-1} per channel using flow meters. The small flume chamber is designed to hold a volume of 52 ml per channel, allowing a low flow rate, which ensures that the larvae are not struggling to maintain their desired location within the flume and therefore only olfactory preferences are being tested. Dye tests were conducted at each water change to ensure that the two channels exhibited parallel water flow. For each anemonefish species, a minima of 30 randomly selected larvae from three different breeding pairs were tested for each olfactory trial. Each individual larva was only tested once.

A total of nineteen different pair-wise water choice combinations were tested (Table 1). All water combinations were tested with the exception of the following. Host anemone olfactory cues were not tested against untreated seawater because it is well documented that anemonefish have an innate preference for the olfactory cues of their host anemone (Arvedlund et al. 1999; Elliott et al. 1995). Furthermore, Dixson et al. (2008) demonstrated this with the same testing method used in the present study. While trials were not run testing host anemone olfactory cues compared against non-host anemone odor with the addition of either predator or non-predator odor to both water choices, the more extreme trial of testing predator or non-predator olfactory cues combined with a host anemone against the odor of the non-host anemone species was tested. The clear preference for their host anemone in this trial by all three anemonefish species demonstrates that the anemone specie is a more important factor in habitat

selection than predation risk, suggesting that the omitted trial would not have provided additional information. Pair-wise tests were conducted with two predator/prey combination olfactory cues: (1) *Pseudochromis fuscus* (predator) compared to *Siganus corallinus* (non-predator); and (2) *Cephalopholis cyanostigma* (predator) compared to *Acanthurus pyroferus* (non-predator). Due to the constraints of laboratory experiments, only one individual from each species of predator, non-predator and anemone were used to create olfactory cues. Two combinations of predator and non-predator species were used due to limited number of individuals per species. Water containing chemical cues was created by soaking a predator, a non-predator, or an anemone in a closed system for two hours prior to testing. *Stichodactyla gigantea* is one of three host anemones used by *A. percula* in the wild. One *S. gigantea* anemone, weighting 341g was used in all trials to create *A. percula's* host anemone chemical cue. *Entacmaea quadricolor* is the exclusive anemone used by *P. biaculeatus* and one *E. quadricolor* (weighing 362g) was used in all trials to create *P. biaculeatus'* host anemone chemical cue. *Heteractis magnifica* is one of three host anemone species used by *A. melanopus* and one *H. magnifica* anemone (weighing 357g) was used in all trials to create *A. melanopus'* host anemone chemical cue. A single anemone was soaked in 10 l of seawater for two hours prior to the trials. Untreated seawater consisted of UV-sterilized filtered seawater and was tested to simulate a delay in settlement in search of more favorable habitat.

Cues for a high-risk settlement site were created by combining equal parts of water treated with the chemical cues of the anemone and water of a predator (*C. cyanostigma* or *P. fuscus*). Both predatory species were fed an artificial diet to ensure that any behavioral reaction observed by the larvae being tested was due to the predator

odor cues and not a result of chemical alarm cues. The predator diet consisted of NRD pellets (INVE) for *P. fuscus* and frozen fish dinners (Fish Fuel Co.) for *C. cyanostigma*. Cues for a low risk settlement site were created by combining equal parts of water containing chemical cues produced by the anemone with the olfactory cues of a non-predator herbivorous species (*S. corallinus* or *A. pyroferus*), which were fed *Caulerpa* spp..

6.3.3 Statistics

Kolmogorov-Smirnov tests were used to compare the proportion of time that individuals spent in the stream of water containing one of the olfactory cues versus the proportion of time that individuals spent on one side of the chamber when no cues were presented (i.e. results from the blank control, trial 1 in Table 1). Using the same statistical analysis, no statistically significant differences were detected between the three parental groups or the two predator/non-predator combinations used for each olfactory comparison ($p>0.10$ for all species); therefore, both parental groups and predator/non-predator combinations were pooled for each species.

Table 6.1 Pair-wise choice trials used to test settlement-site olfactory preference of anemonefish larvae, the cue listed in olfactory cue 1 is the predicted preference, the ratio of mean percent time spent in either cue per species per trial is also listed along with the p value generated from the Kolmogorov-Smirnov test comparing the results from trial 1 to the corresponding trial. Species differences are highlighted with (*)

Trial	Olfactory Cue 1	Olfactory Cue 2	*A. percula*	*P. biaculeatus*	*A. melanopus*
1	Untreated Seawater	Untreated Seawater	49:51 ±0.23	50:50 ±0.52	53:47 ±0.43
2	Non-predator + host anemone	Untreated Seawater	96:4 ±0.42 p<0.001	96:4 ±0.32 p<0.001	97:3 ±0.73 p<0.001
3	Untreated Seawater	Predator + host anemone	93:7 ±0.25 p<0.001	93:7 ±0.17 p<0.001	98:2 ±0.09 p<0.001
4	Host anemone	Non-predator + host anemone	53:47 ±0.03 p>0.05	53:47 ±0.05 p>0.05	55:45 ±0.19 p>0.05
5	Host anemone	Predator + host anemone	100:0 ±0.0 p<0.001	100:0 ±0.0 p<0.001	100:0 ±0.0 p<0.001
6	Non-predator + host anemone	Predator + host anemone	99:1 ±0.03 p<0.001	99:1 ±0.05 p<0.001	97:3 ±0.15 p<0.001
7*	Untreated Seawater	Non-host anemone	90:10 ±0.67 p<0.001	91:9 ±0.70 p<0.001	22:78 ±0.82 p<0.001
8*	Untreated Seawater	Non-predator + non-host anemone	91:9 ±0.42 p<0.001	94:6 ±0.34 p<0.001	23:77 ±0.77 p<0.001
9*	Untreated Seawater	Predator + non-host anemone	97:3 ±0.49 p<0.001	99:1 ±0.09 p<0.001	22:78 ±0.58 p<0.001
10	Non-host anemone	Non-predator + non-host anemone	52:48 ±0.14 p>0.05	51:49 ±0.21 p>0.05	48:52 ±0.12 p>0.05
11	Non-host anemone	Predator + non-host anemone	97:3 ±0.17 p<0.001	96:4 ±0.30 p<0.001	98:2 ±0.16 p<0.001
12	Non-predator + non-host anemone	Predator + non-host anemone	97:3 ±0.49 p<0.001	96:4 ±0.23 p<0.001	99:1 ±0.15 p<0.001
13	Host anemone	Non-host anemone	97:3 ±0.45 p<0.001	98:2 ±0.38 p<0.001	96:4 ±0.33 p<0.001

14	Non-host anemone	Predator + host anemone	1:99 ±0.11 p<0.001	0:100 ±0.0 p<0.001	3:97 ±0.23 p<0.001
15	Non-predator + host anemone	Non-host anemone	93:7 ±0.24 p<0.001	93:7 ±0.25 p<0.001	90:10 ±0.35 p<0.001
16	Host anemone	Predator + non-host anemone	100:0 ±0.0 p<0.001	100:0 ±0.0 p<0.001	100:0 ±0.0 p<0.001
17	Host anemone	Non-predator + non-host anemone	94:6 ±0.21 p<0.001	94:6 ±0.23 p<0.001	92:8 ±0.43 p<0.001
18	Non-predator + host anemone	Predator + non-host anemone	100:0 ±0.03 p<0.001	100:0 ±0.0 p<0.001	100:0 ±0.03 p<0.001
19	Non-predator + non-host anemone	Predator + host anemone	3:97 ±0.31 p<0.001	3:97 ±0.19 p<0.001	11:89 ±0.55 p<0.001

6.4 RESULTS

6.4.1 Experiment 1: Risk assessment on host anemone settlement preferences

When only the host anemones were used in trials, larvae of all three species were able to distinguish between the olfactory cues from the high and low risk settlement habitats, showing nearly identical trends in olfactory preferences (Table 1; Fig. 1). The presence of a predator significantly affected the habitat preference for all anemonefish species, larvae selected the water stream with the anemone cue 100% of the time opposed to the anemone cue combined with a predator odor (Fig. 1; $p<0.001$ for all species). When larvae were given the choice of a water stream containing the anemone odor combined with a non-predator odor compared to a water stream containing the anemone odor combined with a predator odor, the non-predator/anemone combination was chosen

100% of the time (Fig 1; $p<0.001$ for all species). When either the anemone/predator water combination or the non-predator/anemone combination was compared against untreated seawater larvae selected anemone odor >93% of the time, regardless of the presence of predator or non-predator odor. The presence of non-predator odor had no effect on habitat preference, with larvae spending approximately equal time in the water stream containing the non-predator/anemone water combination and the anemone cue alone (Fig. 1; $p>0.05$ for all species).

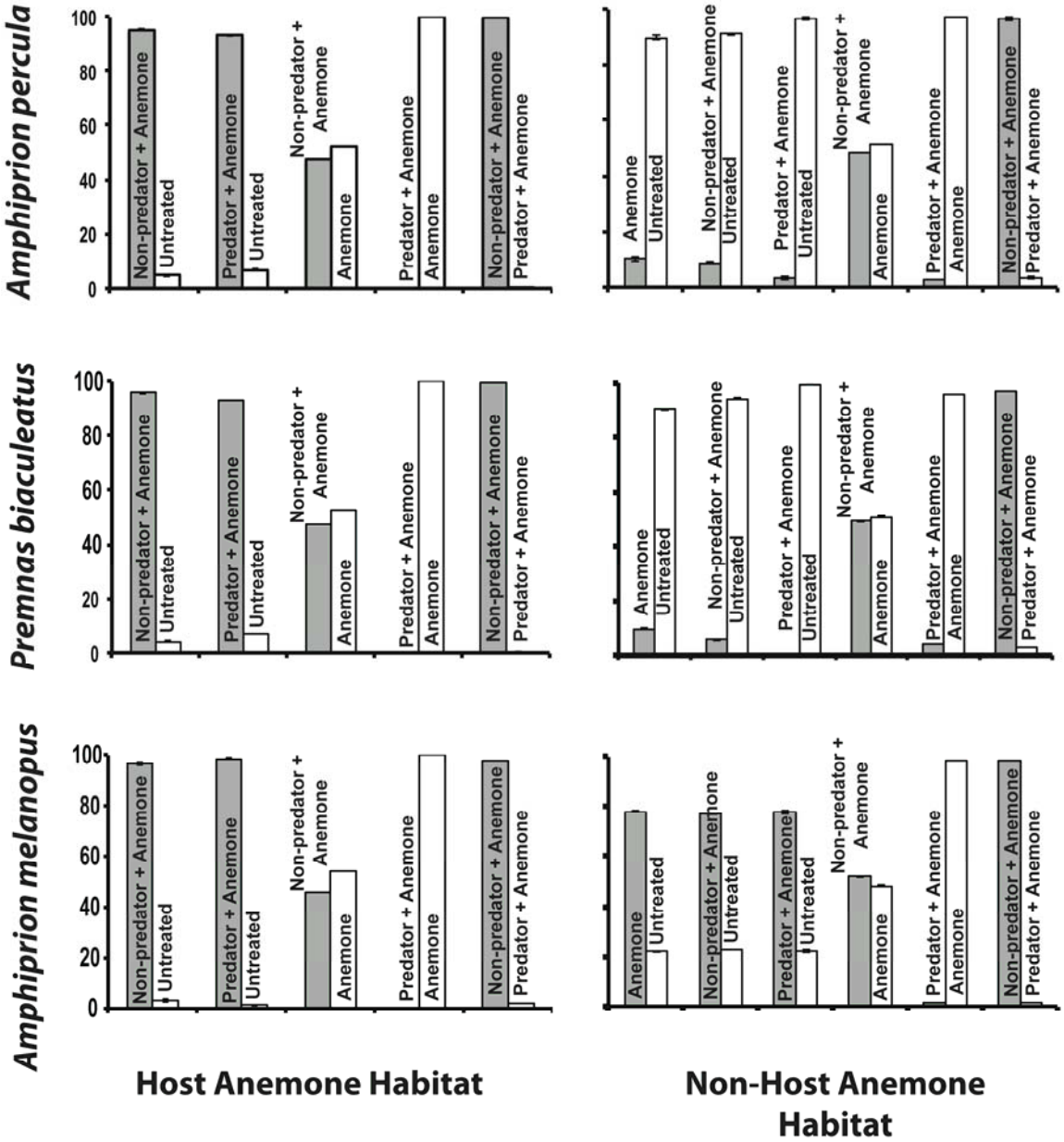

Figure 6.1 Mean per cent time (±SE) that larval *Amphiprion percula*, *A. melanopus* and *Premnas biaculeatus* spent on either side of the chamber, with cues simulating risk levels on their species specific host anemones or non-host anemones.

6.4.2 Experiment 2: Risk assessment on non-host anemone settlement preferences

Slight differences between species were found when risk assessment was tested with the olfactory cues produced from a non-host anemone species (Table 1; Fig. 1). Larvae of *A. percula* and *P. biaculeatus* chose untreated seawater over the predator/anemone water combination or the non-predator/anemone water combination. Larvae of both species spent <10% of their time in the treated water cue for either trial (Fig 1; $p<0.001$ for both species). *A. melanopus,* however, chose the predator/anemone water combination 78% of the time and the non-predator/anemone water combination 77% of the time over untreated water (Fig. 1; $p<0.001$). As seen in the host anemone trials, the non-predator/anemone water combination had no effect on the habitat preference when tested against anemone treated water alone (Fig. 1; $p>0.05$ for all species). Larvae of all species chose the anemone water treatment over the predator/anemone water combination, spending <5% of their time in this water cue (Fig. 1; $p<0.001$ for all species). Similar to the host anemone trials, larvae of all species chose the non-predator/anemone water combination over the predator/anemone water combination when non-host anemone olfactory cues were used (Fig. 1; $p<0.001$ for all species).

6.4.3 Experiment 3: Risk assessment comparing host anemone and non-host settlement preferences

The importance of the host anemone was shown in the choice experiments comparing risk options with host versus non-host anemones (Table 1; Fig. 2). Host

anemones were strongly preferred by all species of anemonefish regardless of the predation risk associated with this settlement option ($p<0.001$ for all species). Preferences were strongest in *P. biaculeatus*, with larvae choosing the host anemone habitat 100% of the time in three of the choice trials (predator/host anemone vs. non-host anemone; predator/non-host anemone vs. host anemone; and non-predator/host anemone vs. predator/non-host anemone).

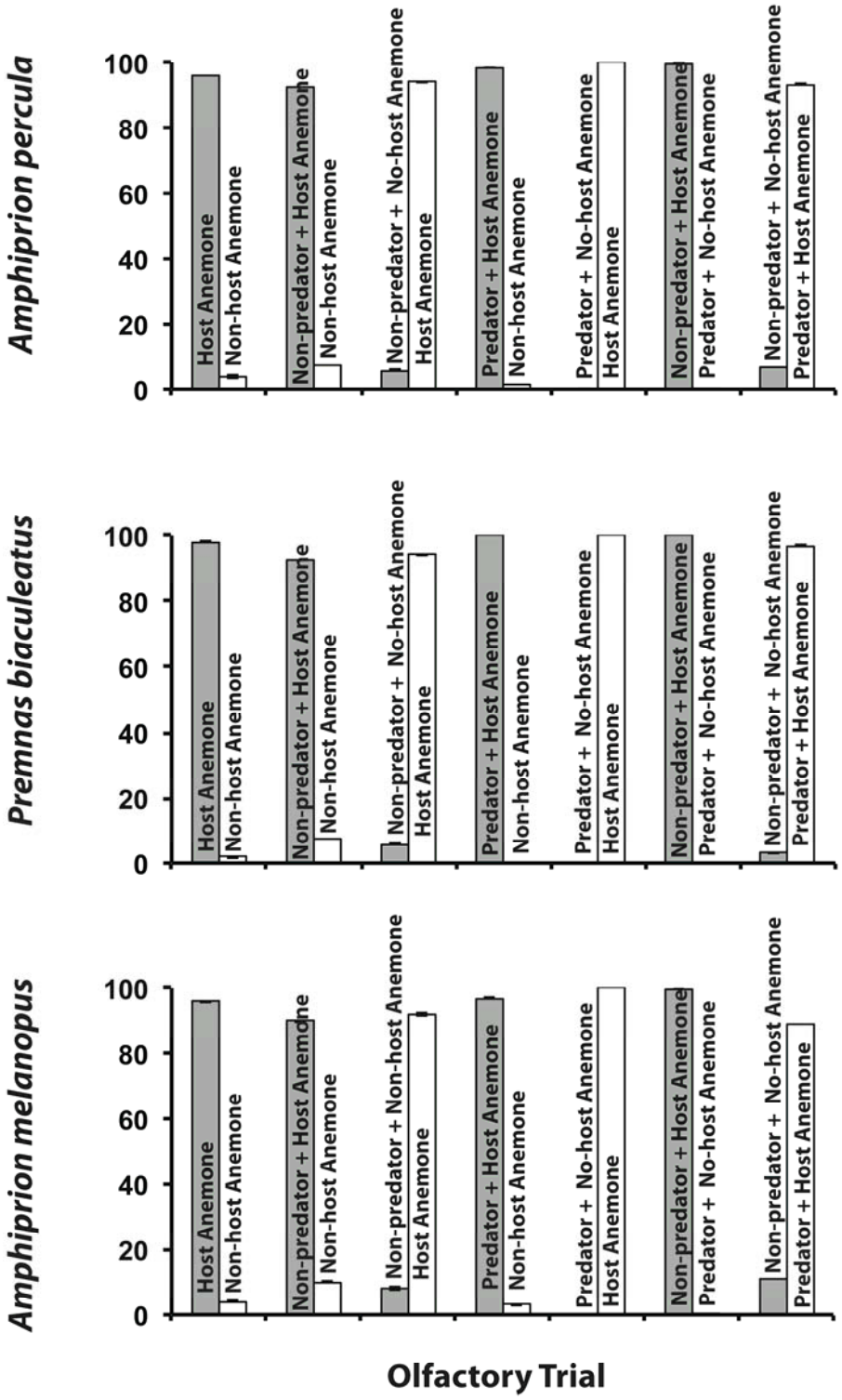

Figure 6.2 Mean percent time (±SE) that larvae spent on either side of the chamber, with olfactory cues simulating risk levels comparing host to non-host anemone species. (A) *A. percula* with its host *Stichodactyla gigantea* compared to non-host *Heteractis malu*. (B) *P. biaculeatus* with its host *Entacmaea quadricolor* compared to non-host *S. gigantea*. (C) *A. melanopus* with its host *E. quadricolor* compared to non-host *S. gigantea*.

6.5 DISCUSSION

This study demonstrates the capacity of larval anemonefish to make settlement site choices based on stimuli from multiple sources. It also highlights the importance of olfactory cues in assessing predation risk during habitat selection by reef fish larvae. The pair-wise choices indicate that correct settlement habitat is the first priority in settlement site selection, with all three species of anemonefish choosing the host anemone odor regardless of the level of predation risk.

Predator avoidance has a secondary influence on settlement site selection. In all trials involving the same settlement habitat (host anemone), all larvae were attracted to olfactory cues from low risk (predator-free) settlement habitat over cues indicating the presence of a predator. However, larvae chose the water stream containing cues from host settlement habitat associated with a predator when no other choice of settlement habitat was available. This indicates that settlement preferences are conditional and depend on the options available. Settlement-site decisions reflect the capacity of anemonefish to discriminate and preferentially associate with settlement-sites deemed to be of lower risk, but indicate settling into the correct habitat is favored over predator avoidance, with larvae choosing high-risk areas over continued habitat searching.

Although conclusions from this study are limited by the use laboratory based experimental methods, these results may help to explain the settlement patterns that have been observed in field-based experiments. Sweatman (1988) showed that not only is the damselfish *Dascyllus aruanus* attracted to the olfactory cues of conspecifics, but that other species use these same olfactory cues to avoid settlement on habitat that is already

occupied by *D. aruanus*. Sweatman's study shows fish use the olfactory cues produced by other fish species to avoid settlement sites in the same way that the anemonefish utilize the olfactory cues produced by a predator for risk assessment.

Both *A. percula* and *P. biaculeatus* highlight the importance of correct settlement-site selection with the rejection of the non-host anemone when tested against seawater. In contrast, *A. melanopus* significantly chose the non-host anemone in all trials. *A. melanopus* has been observed settling in soft corals and giant clams before moving to their host anemone (Arvedlund and Takemura 2005). *A. melanopus* may use settlement on a non-host anemone as a temporary strategy until a suitable habitat is found.

When ideal site selection is a crucial factor for individual success, informed decisions made at settlement can be of critical importance to fitness. Habitat choice can affect survival, growth and reproductive success of habitat specialists (e.g., Munday 2001). As habitat specialists, anemonefish are restricted to settling onto host anemone species. Although there are limited options for settlement, choosing a preferred habitat has lifelong implications. Through hierarchical choice decisions, larvae are able to adjust behavioral responses to different settlement situations, choosing the best situation available. Although generalist species have a wider range of settlement habitat options the same hierarchal decision-making skills might still be important in determining suitable settlement sites. Further research is required to determine whether olfactory cues in selecting settlement sites are just as important for habitat generalists.

It is well known that animals can adjust their behavior and habitat use based on the perceived distribution of predation risk (Lima and Dill 1990; Brown and Kotler 2004; Cresswell 2008). The lack of variation in risk assessment choices observed in the 3

anemonefish indicates the critical need for predator recognition in the settlement process of reef fishes. Given the high levels of predation during reef fish settlement (Almany and Webster 2006), there is an obvious adaptive advantage to having a strong ability to detect predators. The lack of variation in predator recognition among species and the high level of predation on all recruiting species indicate that innate predator recognition coupled with the ability for risk assessment may be widely used in reef fishes.

The ability of a larval fish to make complex risk assessments that influence habitat selection decisions is pivotal for an individual's survival. Anemonefish show clear preferences in habitat selection, indicating that settling on the correct habitat is more important than avoiding the risk of predation that may be associated with it. However, when host anemone species are not a limited resource, the low predation risk option was always chosen. This demonstrates the capability of anemonefish larvae to choose settlement-sites based on stimuli from multiple sources and highlights the importance of olfaction in a crucial life history transition stage in reef fishes.

CHAPTER 7: **Ocean acidification disrupts the innate ability of fish to detect predator olfactory cues** †

†Dixson DL, Munday PL, Jones GP (2010) Ocean acidification disrupts the innate ability of fish to detect predator olfactory cues. *Ecology Letters* 13: 68-75 (doi: 10.1111/j.1461-0248.2009.01400.x)

7.1 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 that 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 1000ppm 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.

7.2 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 a,b; Fabry *et al.,* 2008; Hall-Spencer *et al.,* 2008). Average ocean pH has already declined by 0.1 units since pre-industrial times due to the 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 centred 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 and Thornton, 2005; Bibby *et al.,* 2007) and declining growth of reef-building corals (Langdon and Atkinson, 2005; De'ath *et al.,* 2009) have been documented. Recently, the effects of acidification have been extended to other marine

organisms (Rosa and 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 and Dill, 1990). Consequently, many learned (Brown, 2003; Kelley and 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 habitats at the end of the pelagic larval phase (Caley *et al.,* 1996; Almany and Webster, 2004; 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 and 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 and 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*., 2006; Arvedlund *et al*., 1999; 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 would be impaired at the level of ocean acidification predicted to occur around 2100 on a business-as-usual scenario of CO_2 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 CO_2 (equivalent to 1000ppm atmospheric CO_2). This simulated ocean pH and CO_2 levels that could occur around 2100 due to present and future CO_2 emissions under the SRES A2 scenario (Meehl *et al.,* 2007). Predator-naïve 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 to the olfactory cues of predatory and non-predatory species.

7.3 MATERIAL AND METHODS

7.3.1 Study species and general protocol

Amphiprion percula were reared in a 70,000 litre 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 were kept at the experimental facility for three to five years. Pairs were maintained in separate 70 litre 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 (six to eight days post-laying) egg clutches were transferred from the parental aquarium to a 70 litre larval-rearing aquarium. Readiness to hatch was identified by the appearance of the embryos. After hatching, larvae were reared in a semi-closed system, in which 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 (*Brachionus* sp.) at five individuals m^{-1} each morning for the first three days. *Artemia* nauplii were added at one individual m^{-1} each morning beginning at day three. The ratio

of *Artemia* nauplii to rotifers was increased each day until larvae were fed only five individuals of *Artemia* nauplii $m^{-1}$from day eight. 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 predator species for olfactory trials. These are common and widely-distributed predatory fishes that are known to target newly-settled fish (Stewart and Jones, 2001; Beukers-Stewart and 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 litre aquariums that were isolated from the larvae. Predators were fed one portion of manufactured pre-packaged frozen fish food (Fish Dinner, Fish Fuel Co.) every second day. The non-predators were fed the green algae *Caulerpa lentillifera* every day. Chemical cues were collected from predators and non-predators by turning off water flow to their aquariums for two hours and then removing 20 litre of water.

The response of newlyhatched and settlement-stage larvae to the 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 versus untreated water (blank control); 2. *C. cyanostigma* (predator 1) versus untreated water; 3. *P. fuscus* (predator 2) versus untreated water; 4. *A. pyroferus* (non-predator 1) versus untreated water; 5. *S. corallinus* (non-predator 2) versus untreated water; 6. *C. cyanostigma* versus *A. pyroferus*

(predator 1 versus non-predator 1), and *P. fuscus* versus *S. corallinus* (predator 2 versus 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.

7.3.2 CO_2 manipulation

Clownfish were reared in either control seawater (pH= 8.15 ± 0.07) or CO_2-acidified seawater, from the day that the 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 Aquarientechnik, Germany) was attached to each aquarium to maintain pH at 7.8 by CO_2 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 CO_2. The solenoid injected a slow stream of CO_2 into a diffuser (Red Sea CO_2 Reaktor 500) 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 CO_2 was estimated to be 1050ppm (Munday *et al.,* 2009b), which is consistent with other studies that have used CO_2 to reduce seawater pH by 0.3-0.4 units (Havenhand *et al.,* 2008; Rosa and Seibel, 2008).

The pH of each aquarium was independently validated each day using a WP80 pH meter (TPS, Australia) calibrated daily with fresh pH buffers (Merk, Germany). CO_2 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 the mean ocean pH (The Royal Society 2005). Water temperature was maintained at 30^{o}C ± 0.6 (SD) using electric heaters. Oxygen levels were maintained above 90% saturation by the mixing action of the diffuser pump.

7.3.3 Olfactory choice tests

A two-channel choice flume (13cm x 4cm) developed by Gerlach *et al.,* (2007) was used to assess the ability of larval *A. percula* to discriminate between waters 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 toward their preferred water source. Using the protocols outlined in Gerlach *et al.,* (2007), a constant gravity-driven flow of 100 ml/min 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 hours 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 two minutes. Fish that did not swim actively during the acclimation period were discarded. Two per cent of fish tested were discarded from both the control and the 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 two minute period. This was followed by a one minute 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 water sources, the entire test including the acclimation period, was repeated. Each fish was used only once.

7.3.4 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 presented (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 versus the proportion of time that individuals spent in that stream of water when reared in acidified water. There was no statistical difference between the three parental groups used for each olfactory comparison, neither for larvae reared in control nor acidified water (Kolmogorov-Smirnov test $p>0.10$ for all comparisons). Therefore, parental groups were pooled for each analysis.

7.4 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 (with the mean per cent time on one side of the chamber 49.9 ±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 neither newly hatched nor 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 the control water innately recognized the chemical cues of predators and were are 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 versus untreated seawater (Fig.7.1a; $p<0.001$ for both comparisons). Newly hatched larvae also avoided water

containing olfactory cues from the two non-predator species when presented in combination with untreated seawater (Fig. 7.1a; $p<0.001$ 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. 7.1a; $p<0.001$ 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 versus untreated seawater remained in the untreated seawater 100% of the time (Fig. 7.1b; $p<0.001$ for both tests). In contrast, settlement-stage larvae displayed no preference or avoidance to 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. 7.1b; $p>0.1$ 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. 7.1b; $p<0.001$ for both tests), spending 100% of the time in the water stream containing olfactory cues from the non-predator.

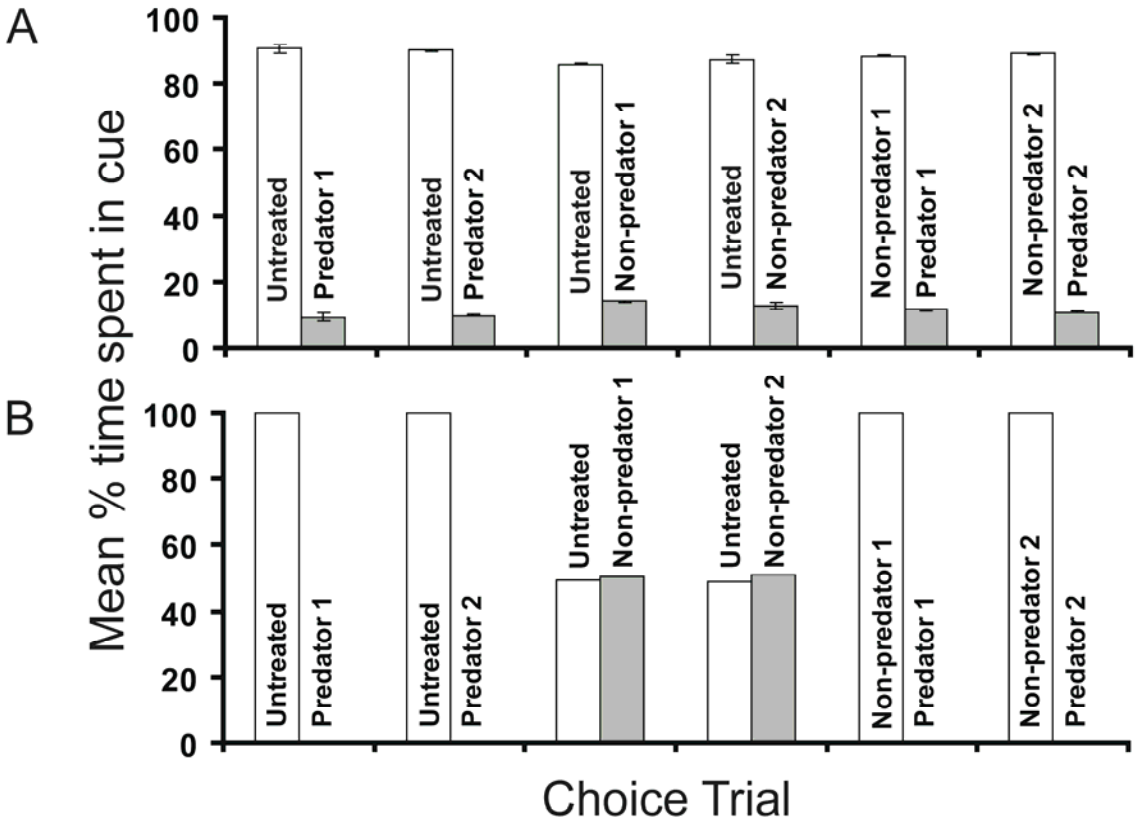

Figure 7.1 Response of larvae reared in control seawater to olfactory cue from predator and non-predator and non-predator species. (a) Mean per cent of time (± SE) that newly hatched *A. percula* larvae spent in either water stream when presented with different olfactory cues in a two-channel choice chamber. (b) Mean per cent time (± SE) that settlement-stage (day 11) *A. percula* larvae spent in water containing either predator or non-predator chemical cues. Predator 1 was *Cephalopholis cyanostigma*, predator 2 was *Pseudochromis fuscus*. Non-predator 1 was *Acanthurus pyroperus* and non-predator 2 was *Signaus corallines*

Exposure to acidified water affected the olfactory preferences of settlement-stage larvae, but not newly hatched larvae. Newly hatched *A. percula* larvae that were reared from the egg stage in CO_2-acidified water exhibited similar preferences to newly hatched larvae from control seawater for all comparisons (Fig. 7.1a, 2a; $p>0.1$). Newly hatched larvae from acidified water avoided water containing chemical cues from a predator when presented against untreated seawater (Fig. 7.2a; $p<0.001$). They also avoided chemical cues produced by non-predator species when presented against untreated seawater (Fig.

7.2a; $p<0.001$), 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 7.2a; $p<0.001$). This indicates that the olfactory preferences of larvae were not affected by exposure of the eggs to high CO_2 and low pH during embryogenesis.

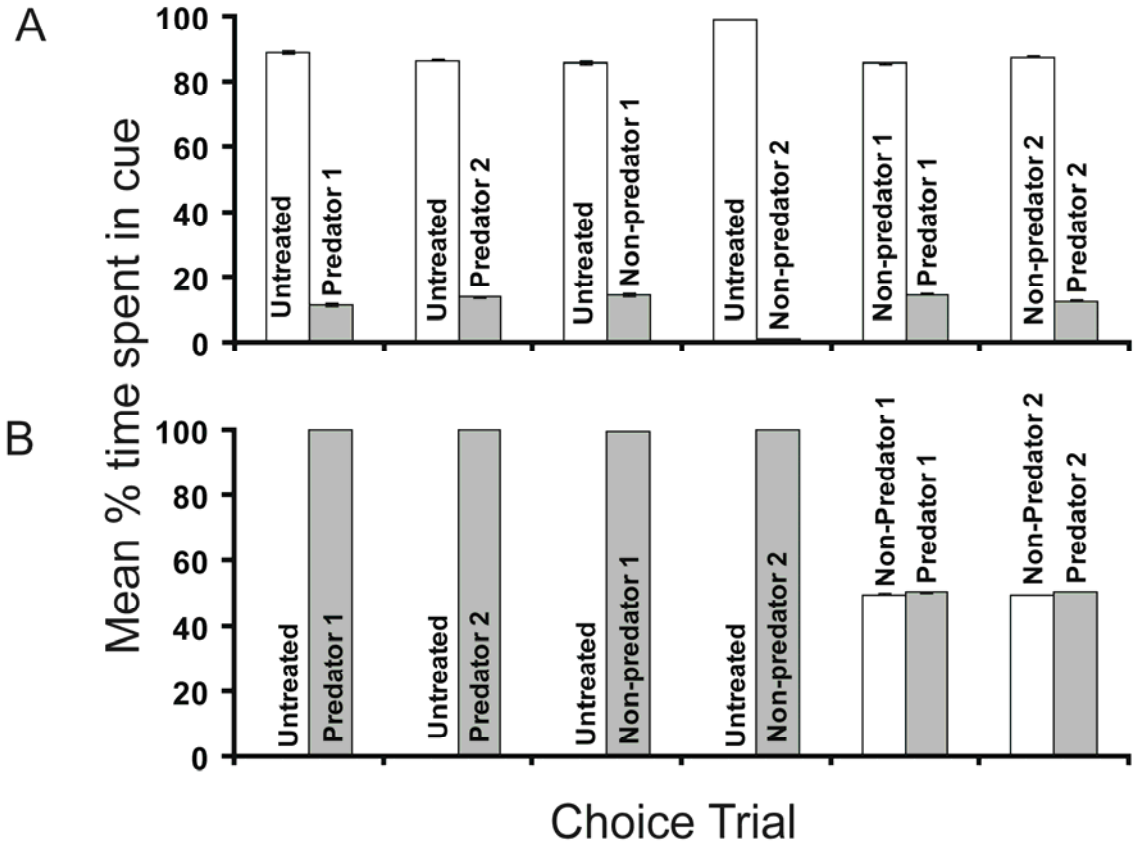

Figure 7.2 Response of larvae reared in CO_2-acidificed seawater (pH 7.8 and *c.* 1000 p.p.m., CO2) to olfactory cues from predator and non-predator species. (a) Mean per cent of time (± SE) that newly hatched *A. percula* from acidified seawater spent in either water stream when presented with different olfactory cues in a two-channel flow chamber. (b) Mean per cent of time (± SE) that settlement-stage (day 11) *A. percula* larvae from acidified seawater spent in water containing either predator or non-predator chemical cues. Predator 1 was *Cephalopholis cyanostimga*, predator 2 was *Pseudochromis fuscus*. Non-predator 1 was *Acanthurus pyroferus* and non-predator 2 was *Siganus corallines*.

In contrast, settlement-stage larvae reared in acidified water exhibited significantly different preferences to larvae reared in control seawater for all comparisons

(Fig. 7.2b; $p<0.001$ 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 CO_2 acidified water and presented with chemical cues from the same predator species as the control larvae spent 100% of their time in the stream of water containing the predator cue (Fig. 7.2b), whereas the control larvae completely avoided water with a predator cue. Larvae reared in acidified water also exhibited a strong preference for non-predators when presented with chemical cues from a non-predator versus untreated water (Fig. 7.2b). Finally, larvae reared in acidified water were not able to distinguish between the chemical cues of predators and non-predators, spending equal time on each side of the chamber when presented with predator and non-predator cues simultaneously (Fig. 7.2b; Kolmogorov-Smirnov test $p>0.1$), whereas control larvae were strongly attracted to the non-predator cue when presented simultaneously with the predator cue.

Changes in olfactory preferences were the only behavioural differences observed as a result of exposure to acidified water. There was no apparent difference in swimming behaviour in the test chamber between larvae reared in acidified water compared with control water.

7.5 DISCUSSION

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 approximately 1000ppm 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 (McCormick and Manassa, 2008; Brown, 2003), 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 away 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 habitats. Further experiments are now required to determine if the attraction to olfactory cues produced by a predator for fish reared in acidified water leads to increased larval mortality under natural conditions.

The potential for marine organisms to adapt to a 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 (~1000ppm CO2 and 7.8 pH). Whether the dramatic shift in behaviour exhibited by larvae reared in acidified water could be mitigated by adaption 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 by increasing CO_2 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 of larval behaviour adaptation 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 neurosensory 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 CO_2 and pH covaried in our experiments, as they would under naturally occurring ocean acidification, we were unable to separate the specific effects of CO_2 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 to CO_2 versus pH to this change and, 3) how variation in these environmental factors may alter the ontogenetic timing of changes in olfactory preferences.

In contrast to the results for settlement-stage larvae, the chemosensory ability of newly hatched larvae was not affected by the exposure of the eggs to CO_2 acidified water. It is likely that CO_2 levels within the egg are often higher than the surrounding water due to embryonic respiration. Additionally, clownfish deposit eggs that remain on the reef until hatching where developing embryos would be exposed to natural variation in pH and CO_2 close to the reef matrix. This is caused by the respiration and photosynthesis of reef organisms (Ohde and van Woesik, 1999; Kuffner *et al.,* 2008). The embryonic stage may be adapted to variation in CO_2 levels within the egg and this could explain why newly hatched larvae were unaffected by the CO_2 acidified water.

Differences in the olfactory preferences of newly hatched and settlement-stage larvae were also observed for fish reared in control water. Although innate predator

recognition was already fully-developed in newly hatched fish, these larvae also avoided water containing chemical cues from non-predators. The avoidance of odours from both predators and non-predators in favour of seawater with no additional chemical cues suggested that newly hatched larvae are genetically predisposed to disperse away from the reef and into the open ocean. Attraction of newly hatched larvae to ocean water would both reduce the risk of mortality from reef-based predators and promote dispersal between neighbouring populations. By the time the larvae are ready to settle to adult habitats, they no longer avoid the olfactory cues of non-predators, presumably because avoidance to non-predators at this stage conflicts with the need to locate adult habitat.

In species rich communities, such as coral reefs, it is important that individuals can distinguish potential predators from the many other species of similar size and appearance that they are likely to encounter. Predation risk is exceptionally high for reef fishes during (Doherty *et al.,* 2004) and immediately after settlement (Almany and Webster, 2006). Innate predator recognition provides prey with the ability to identify and locate threatening species without the requirement of prior experience. One potential cost associated with this mechanism of predator recognition is that an anti-predator response may be elicited to any predatory species, even though not all of them pose a threat to the particular prey species. However, experience and learning after settlement might fine-tune these responses so that juveniles are more capable of avoiding threats (McCormick and 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 the survival of larvae and newly-settled juveniles that are naïve of 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 odours does in fact 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.,* 2009a) 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 habitats in order 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.

CHAPTER 8: **GENERAL DISCUSSION**

The mechanism(s) that coral reef fish larvae use to locate reefs and select settlement habitat have yet to be fully understood. This thesis provides significant insights into the use of olfactory cues for settlement site selection on both a micro and macro scale, focusing on the species that require the highest precision in reef selection-habitat specialists. The six chapters of this thesis used a combination of field and laboratory techniques to better understand the complex decisions larval fish are required to make when settling on adult habitats. Although the use of olfactory cues in habitat selection has been speculated upon and documented on small scales, the importance of this sensory system to the ecology of reef fishes during their early life history is highlighted by the research presented here.

In Chapter 2, I demonstrated that coral reef fish exhibit innate preferences for olfactory cues that identify potential settlement sites. An unexpected discovery was that the species' associated with islands use unique terrestrial cues for island reef identification. The use of these cues may effectively increase the target area of island reefs for searching larvae because chemical cues such as leaf litter can be exported greater distances than other reef-based cues, such as coral odour (Chapter 2 and 3). Olfactory cue use begins at hatching with larvae able to detect and respond to different odours within their first day in the plankton. However, olfactory preferences change throughout the pelagic period, with fish showing an ontogenetic shift in cue preference, initially avoiding reef cues but developing a strong preference for reef cues approximately two thirds of the way through their pelagic larval stage (Chapter 4).

Olfactory imprinting has been shown to exist in a laboratory setting and to be important in anemonefish/anemone recognition. However, imprinting does not appear to influence habitat selection in nature, because the natal anemone species had no effect on the anemone species selected at the conclusion of the larval stage (Chapter 5). During settlement-site selection larvae are inundated with olfactory information, however, previous research on olfactory cue use focuses on just one cue at a time. In Chapter 6, I demonstrated that larvae are capable of making complex settlement decisions, weighing information on predation threat with correct habitat choice. Finally, in Chapter 7, I showed that rising carbon dioxide levels could affect the settlement process, because ocean acidification has detrimental effects on the olfactory system, rendering larvae unable to distinguish between different olfactory cues, including those of a predator and a non-predator. Together, these findings greatly improve our understanding of the role that olfactory cues play in habitat selection by coral reef fishes and the threats to this process posed by rapid climate change.

Knowledge about how marine fish larvae are able to navigate through the pelagic environment in search of a suitable adult settlement site is increasing. Current studies interested in gaining a better understanding of the sensory mechanisms utilized by larvae for settlement site location focus primarily on larvae's auditory, visual and chemosensory abilities. Previous studies have speculated on the distance from habitat in which these cues would be useful for settlement site detection. Auditory cues are considered to be the most useful cue at detecting settlement sites when larvae are the furthest away from potential reefs; however, auditory cues have also been shown to be useful in a closer proximity. Auditory cues are location-dependent, as many settlement habitats such as

reefs and mangrove forests provide a distinct soundscape that extends from the source independent of currents in all directions. This cue may be an important element in the initial recognition of settlement habitat. Visual cues are thought to be the last sense utilized in habitat assessment, as this cue is limited by water clarity and light levels. Due to the directional limitations of olfactory cues and the nature of chemical components, olfactory cues are thought to be utilized at a further distance than visual cues, but may not be as useful as auditory cues on a large scale. Research presented in this thesis has shown, however, that olfactory cues are important not only at greater distances than previously thought, but also that they are utilized throughout the larval stage. Additional information is necessary to determine the spatial scales at which different sensory cues provide information. As yet, we still do not know much about the ontogenetic development for both auditory and visual cue use and, in turn, the capacity of larvae to utilize sensory stimuli not only throughout their pelagic larval stage but also on different spatial scales. Increasing our understanding of larval capabilities for each cue individually is an important first step in comprehending the factors influencing larval decision-making during the pelagic stage. Another important area for further research is gaining a better understanding of the interactive effects sensory cues have on larval behaviour. As it is unlikely that larvae independently use only one sensory stimulus at a time, the combined effect of different sensory stimuli may result in a different behaviour than each stimulus independently might have caused. For example, larvae may react differently to an olfactory cue while receiving an auditory signal, than they would when receiving the same olfactory cue alone.

One of the most unexpected results from my research was the discovery that larval reef fish are attracted to the odour of tropical plant leaves. This is an important link between the marine and the freshwater environment, which requires additional investigation. It is clear from the research presented, that tropical plant leaves possess a chemical cue which is attractive to coral reef fish larvae, however this chemical cue is either not found in all terrestrial plants or is able to be masked by other more pungent odours. An important next step would be to identify the chemical cue present in the tropical plant leaves through biological assays. Once this has been done, the concentration threshold from which it is detectable can be determined for different coastal dwelling fish species. Identifying the chemical cue(s) responsible for the attraction would also allow other plant species to be profiled in order to seek its presence. This may be an important step for marine conservation initiatives through terrestrial management. More often than not, the marine and the terrestrial environment are looked at as two separate ecosystems, which are managed as separate entities. However, this research indicates that shoreline coastal development threatens both the terrestrial ecosystem in a very direct way and the marine ecosystem in an indirect way, by removing important olfactory cues that may be necessary for settling larvae to located suitable habitat. Data, not presented in this thesis, indicated that larvae respond negatively to the olfactory cues produced by plants commonly grown commercially in tropical habitats. Research now needs to be conducted to show if these agricultural plants have a negative impact on recruitment, and if so, whether planting the tropical plants used for reef identification by coral reef fish larvae as a shoreline olfactory buffer would help to minimize the effect coastal development has on the marine ecosystem.

Another novel finding presented in this thesis was that the effect ocean acidification has on the olfactory discrimination ability of coral reef fish larvae. As this is one of the first studies to look into the effect ocean acidification could have on behaviour, particularly on threat identification, future research is required to gain a better understanding of potential problems that may result. One of the most important areas to focus on determining the mechanisms, which are causing the inability to discriminate. As treated larvae are able to identify the chemical signals, it is likely that the problem is cognitive rather than an issue with the olfactory sense specifically. Since this paper has been published, a number of follow up studies have been conducted identifying ocean acidification as causing bolder behaviour and affecting other sensory systems such as auditory recognition, which then resulted in increased mortality. Gaining an understanding on how the process is being affected is vital in preparing for future climate change scenarios. Although early life history stages are inherently more vulnerable, additional research is necessary to determine the effect realistic future levels of increased carbon dioxide in the water is expected to have on adult organisms, especially in species which rely heavily on their sensory systems, such as sharks, eels and nocturnal species. Trough the study presented here investigated the role realistic future levels of ocean acidification would have on coral reef fish larvae, it was unable to account for the ability of reef fish to acclimate or adapt to the changing water conditions. This is another extremely important area to focus on.

A better understanding of reef fish settlement patterns and the stimuli that larvae use during the settlement process is important for marine conservation and management. Both fisheries' management and marine conservation require an understanding of larval

dispersal and patterns of population connectivity. Marine protected areas are developed with the expectation to fulfil both biodiversity conservation and fishery replenishment roles, and their effectiveness in both depends on the scale of connectivity. From a fisheries perspective, protected areas must be spaced accordingly in order to not only replenish themselves, but also to spill over into the non-protected area. Biodiversity conservation, on the other hand, requires genetic connectivity between different marine protected areas. As a result of the different emphasis required to fulfil both conservation and management needs, it is vital for the optimal marine protected network design to understand both the genetic and population spatial scales involved. Research into marine connectivity has yielded tremendous advances in determining the spatial scales of larval dispersal. Through new techniques, larvae can be mapped genetically, allowing researchers to know where larvae originated and where settlement occurred as well as the degree of genetic mixing that is occurring within populations. However, scientists still do not fully understand the mechanisms that larvae use to choose one site over another. The research in this thesis helps to address this knowledge gap by presenting important discoveries on the information obtained through olfaction during the settlement process. Complex behavioural choices are thought to be the primary driver in larval settlement habitat choice. The body of research presented here has added to the larval behavioural field, providing evidence that chemical cues emitted from settlement habitat influence larval choices at different spatial scales.

In conclusion, this study tested the importance of olfaction in the settlement process for coral reef fish larvae throughout their larval phase. To do this, I have used both field and laboratory techniques to assess the behavioural reactions that larvae

display to a variety of olfactory stimuli. This comprehensive study has demonstrated the significant role olfactory cues play in the settlement process, in predator recognition, and the potential problems that are likely to occur as a result of ocean acidification. Research presented in this thesis has added to the present understanding of the settlement process. Understanding connectivity in the marine environment requires a comprehension of the dispersal phase: where larvae are being transported to and which mechanisms are being used for settlement site identification. Without continued effort to understand the complex settlement process, our tools for protecting marine communities will continue to be limited.

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Biogeosciences

Effect of ocean acidification on otolith development in larvae of a tropical marine fish

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Abstract. Calcification in many invertebrate species is predicted to decline due to ocean acidification. The potential effects of elevated CO_2 and reduced carbonate saturation state on other species, such as fish, are less well understood. Fish otoliths (earbones) are composed of aragonite, and thus, might be susceptible to either the reduced availability of carbonate ions in seawater at low pH, or to changes in extracellular concentrations of bicarbonate and carbonate ions caused by acid-base regulation in fish exposed to high pCO_2. We reared larvae of the clownfish *Amphiprion percula* from hatching to settlement at three pH_{NBS} and pCO_2 levels (control: pH 8.15 and 404 µatm CO_2; intermediate: pH 7.8 and 1050 µatm CO_2; extreme: pH 7.6 and 1721 µatm CO_2) to test the possible effects of ocean acidification on otolith development. There was no effect of the intermediate treatment (pH 7.8 and 1050 µatm CO_2) on otolith size, shape, symmetry between left and right otoliths, or otolith elemental chemistry, compared with controls. However, in the more extreme treatment (pH 7.6 and 1721 µatm CO_2) otolith area and maximum length were larger than controls, although no other traits were significantly affected. Our results support the hypothesis that pH regulation in the otolith endolymph can lead to increased precipitation of $CaCO_3$ in otoliths of larval fish exposed to elevated CO_2, as proposed by an earlier study, however, our results also show that sensitivity varies considerably among species. Importantly, our results suggest that otolith development in clownfishes is robust to even the more pessimistic changes in ocean chemistry predicted to occur by 2100.

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1 Introduction

Absorption of additional carbon dioxide (CO_2) from the atmosphere is causing ocean pH to decline and is reducing the availability of carbonate ions required by many marine species to form calcium carbonate ($CaCO_3$) shells and skeletons (Feely et al., 2004; Orr et al., 2005). As the saturation states of aragonite and calcite decline, the calcification rate of corals, molluscs, echinoderms and other invertebrates that secrete these forms of $CaCO_3$ is predicted to decrease (Gattuso et al., 1998; Riebesell et al., 2000; Gazeau et al., 2007; Kleypas and Yates, 2009). If the current trajectory of global CO_2 emissions is maintained, atmospheric CO_2 concentrations could reach between 730–1020 ppm by the end of the century (Meehl et al., 2007; Raupach et al., 2007). This would cause ocean pH to decline 0.3–0.4 units compared to current-day levels (Caldeira and Wickett, 2005) and reduce the concentration of carbonate ions in the shallow ocean by nearly 50 % compared to the pre-industrial era. The consequences of such a reduction in carbonate ion concentration is likely to be serious for many calcifying species (Hoegh-Guldberg et al., 2007; Fabry et al., 2008; Doney et al., 2009; Hofmann et al., 2010). Possible impacts of elevated CO_2 and ocean acidification on non-calcifying species, such as fishes, is still poorly known, but could potentially include effects on a range of physiological (Pörtner and Farrell, 2008), developmental (Pankhurst and Munday, 2011) and behavioural processes (Munday et al., 2009, 2010). Early life history stages are likely to be most vulnerable because physiological homeostasis might not be fully developed and their small body size makes them more sensitive to environmental variation (Ishimatsu et al., 2008; Melzner et al., 2009).

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In general, marine fish appear to be relatively tolerant to mild increases in ambient CO_2, presumably because well-developed mechanisms for acid-base regulation allow them to compensate for cellular acidosis caused by exposure to elevated pCO_2 (Pörtner et al,. 2005; Ishimatsu et al., 2008; Melzner et al., 2009). A concern, however, is that fish otoliths (earbones) are composed of aragonite, and therefore, could be susceptible to the declining carbonate ion concentrations associated with ocean acidification. Just as calcification rates in corals decrease as the aragonite saturation state falls, it may become more difficult for fish to precipitate aragonite in their otoliths as the availability of carbonate ions in seawater declines. Alternatively, otolith growth may be affected by regulatory mechanisms used by fish to maintain their internal pH as ambient pCO_2 increases. Changes in extracellular concentrations of carbonate and bicarbonate caused by acid-base regulation in a high CO_2 environment could affect the precipitation of $CaCO_3$ in the otolith (Payan et al., 1997). Indeed, one recent study reported that otoliths were larger in larval fishes exposed to elevated CO_2, possibly because pH regulation caused carbonate concentrations to increase within the endolymph (Checkley et al., 2009). Maintenance of a steady pH within the endolymph, despite an influx of CO_2, would increase the abundance of bicarbonate and carbonate ions in the endolymph, both of which could potentially be used in aragonite precipitation. Finally, physiological stress can also affect otolith size, shape, and the symmetry between left and right otoliths (Gagliano and McCormick, 2004; Payan et al., 2004). Thus, even if acid-base regulation or declining aragonite saturation state do not directly affect otolith development, increased stress caused by elevated pCO_2 could potentially influence otolith shape and symmetry.

Changes to ocean chemistry associated with declining pH, or changes in extracellular dissolved inorganic carbon (DIC) concentration due to acid-base regulation, could also affect otolith chemistry. The chemical composition of otoliths responds to environmental variation, including concentrations of elements in ambient water, temperature, and salinity (Campana and Thorrold, 2001). In many instances ambient water chemistry is the primary control on the incorporation of elements in otoliths, as evidenced by high correlations between the ratio of these elements to Ca at the site of otolith deposition and their concentration in the external environment (e.g. Bath et al., 2000; Martin and Thorrold, 2005). Bicarbonate ion concentration and pH has been shown to influence Sr, but not Mg, incorporation in calcitic foraminifera (Dissard et al., 2010). However, the effect of changes in aqueous carbonate chemistry on fish otolith composition remains unknown.

Fish ears detect sound, body orientation and acceleration from the position of the otoliths in the inner ear and movement of the otoliths over sensory hair cells (Helfman et al., 1997; Popper and Lu 2000). Any substantial change to the size, shape, or symmetry of otoliths could have serious implications for individual performance and survival (Gagliano et al., 2008). To date, two studies have examined the potential effects of ocean acidification on otolith growth and development. Checkley et al. (2009) found that otolith size increased in larval seabass exposed to ∼1000 µatm CO_2 for eight days. In contrast, Munday et al. (2011) detected no effects of ∼850 µatm CO_2 on size, shape or symmetry of otoliths on juvenile spiny damselfish, a species without a larval phase. No studies have investigated the possible effects of ocean acidification on otolith chemistry. We reared larvae of a model species, the clownfish *Amphiprion percula*, through their entire larval phase at two ocean acidification scenarios to test if exposure to elevated pCO_2 and reduced carbonate ion concentrations affects otolith size, shape, symmetry (between left and right otoliths) and otolith chemistry compared with current-day controls. The experimental conditions are consistent with more extreme scenarios for the years 2100 (pH 7.8 and 1050 µatm CO_2) and 2200–2300 (pH 7.6, 1721 µatm CO_2) based on a business-as-usual trajectory of CO_2 emissions (Caldeira and Wickett, 2005; Meehl et al., 2007). This combination of treatments allowed us to test the susceptibility of otolith development in clownfish to changes in seawater chemistry and to investigate potential mechanisms involved.

2 Materials and methods

2.1 Larval rearing

Clownfish were reared in a 70 000 l recirculating seawater system at James Cook University's experimental marine aquarium facility. Four adult breeding pairs were kept in separate 70 l aquariums supplied with a continuous flow of filtered seawater at 18 l h^{-1}. Breeding pairs laid eggs on the underside of a terracotta pot placed in their aquarium. Pots were checked each morning for the presence of eggs. On discovering a new clutch of eggs, the parental aquarium was assigned to one of three pH_{NBS} levels (8.15 (control), 7.8, 7.6) and pH adjusted as described below. The breeding pairs produced new egg clutches on a 2–4 week cycle. The pH treatment assigned to each clutch was alternated so that each of the four parents were assigned to each pH treatment during the experiment. On the evening of hatching (6–8 days after laying) the egg clutch was removed from the parental aquarium and transferred to a 100 l larval rearing aquarium set to the same pH as the parental aquarium. Eggs hatched within several hours of darkness. Larvae were reared in a semi-closed system, where each aerated aquarium had no water flow during the day and was then slowly flushed with filtered seawater each night. This daily cycle ensured that larvae could feed ad-libitum throughout daylight hours and that any unconsumed food was removed each night. Larvae were fed rotifers (*Brachionus* sp.) at 5 individuals ml^{-1} each morning for the first three days. *Artemia naupli* were added

at 1 individual ml^{-1} each morning from day three. The ratio of *Artemia naupli* to rotifers was increased each day until larvae were only fed 5 *Artemia naupli* m^{-1} from 8 days post-hatching. A summer light cycle of 13 h light/11 h dark was simulated with fluorescent lights. Larvae were reared to the end of their larval phase (11-d post hatching), at which time behaviour consistent with competency to settle (attraction to the sides of the rearing aquarium) and the appearance of benthic colouration was observed.

Settlement stage larvae (11-d post hatching) were sacrificed with a lethal dose of clove oil anaesthetic (Munday and Wilson, 2007). Larvae from one breeding pair in the control group, and a different breeding pair in the pH 7.8 group, did not survive, therefore each treatment contained larvae from at least three of the four parental genotypes. A sample of 10–15 larvae from each clutch were stored in 75 % ethanol for otolith analysis. The remaining larvae were stored in formaldehyde for other analyses.

2.2 Seawater manipulation

Seawater pH in the parental aquariums and larval rearing tanks that had been allocated to the 7.8 or 7.6 treatments was adjusted by CO_2 injection. A separate pH-controller (Tunze Aquarientechnik, Germany) was attached to each aquarium to maintain pH at the desired level. 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 CO_2. The solenoid injected a slow stream of CO_2 into a diffuser (Red Sea Reactor 500) at the bottom of the aquarium whenever the pH of the aquarium seawater rose above the set point. A precision needle valve inserted before the solenoid was adjusted to ensure a slow, steady, delivery of CO_2 into the diffuser. Using this method is was possible to constantly maintain pH within $\pm$ 0.05 units of the desired level and there was no detectable gradient in seawater pH within the aquarium. A diffuser without CO_2 injection was placed in each aquarium and rearing tank assigned to control conditions. The pH_{NBS} of each aquarium was independently checked each day using a WP80 pH meter (TPS, Australia) calibrated with fresh pH buffers (Merk, Germany). CO_2 was only injected into aquariums to adjust pH when eggs or larvae were present. All water returned to a 60 000 l sump where it was degassed by stirring and using a 1000 l algal bio-remediation tank. Water temperature was maintained at 30 °C $\pm$ 0.6 (SD) using electric heaters. Oxygen saturation was checked regularly with an Oxi 340i oxygen probe (WTW, Germany) and was always above 90 %.

Total alkalinity (A_T) of seawater at each pH level was measured weekly by titration. Average pCO_2, bicarbonate and carbonate ion concentrations were then estimated from pH and A_T in the program CO2SYS, using the constants of Millero et al. (2006). All seawater chemistry parameters are shown in Table 1. Average pCO_2 for the 8.15 (control), 7.8 and 7.6 pH treatments was estimated to be 404, 1050 and 1721 µatm, respectively.

2.3 Otolith morphometrics and Fourier shape analysis

Larvae were removed from the preservative, blotted dry, weighed (nearest mg) and photographed in a lateral position under a stereomicroscope. Standard length (SL) to nearest 0.01 mm was estimated for each fish from the digital photograph using image analysis (Optimas 6.5, Media Cybernetics). Sagittal otoliths were then removed and stored dry in well-plates. The left and right otolith of each individual was photographed to produce a calibrated, grey-scale image. Morphometric measurements (otolith area, μm^2; maximum length, µm; maximum breadth, µm; rectangularity; circularity) and Fast Fourier descriptor were obtained from the images using Optimas. An automatic pixel gradient traced anticlockwise around the silhouette of the otolith, starting from a common landmark (distal edge of the rostrum). Fourier analysis reproduces the outline of a shape by considering it to be an aggregate of simple wave forms, describable by a series of sine and cosine curves (Younker and Ehrlich, 1977). Optimas provided a complex ($a + b_i$) Cartesian fast Fourier transformation of the x-y coordinates of 128 equidistant points around the otolith outline. The complex Fourier descriptors were converted ($\sqrt{a^2} + \sqrt{b^2}$) to an absolute value for each harmonic (Lestrel, 1997). The images of the right otoliths were flipped horizontally so that the 128 sampling points were measuring the same part of the otolith for left and right otoliths.

To remove any confounding effect of otolith image position or size from the data, the Fourier descriptors were standardized for differences in otolith position by setting the zeroth harmonic (H_0) to $0 + 0_i$, and for differences in otolith size by dividing all successive harmonics by the first harmonic (H_1). Greater than 97.0 % of the variance in otolith shape was accounted for by the first 19 standardized harmonics (H_2-H_{20}) in all instances (> 99.0 % in most instances). Consequently, we restricted our analysis to these standardized harmonics.

Morphometric measurements were calculated for 16 randomly selected individuals from each treatment and Fast Fourier coefficients were calculated for 19 randomly selected individuals from each treatment (Table 2). Samples at each treatment level contained 3–10 individuals from at least 3 different breeding pairs.

2.4 Otolith chemistry

Elemental chemistry of otoliths was quantified using laser ablation inductively coupled plasma mass spectrometry. Otolith chemistry was conducted on 5–7 randomly selected individuals from each treatment. One otolith chosen at random from each fish was mounted on a microscope slide with a cyanoacrylic glue, ground to the midplane using

Table 1. Average (± SD where estimated) seawater chemistry parameters over the duration of the experiment for the nominated pH treatments. Salinity (S), temperature (T) and total alkalinity (A_T) were measured directly. All other parameters were estimated in the program CO2SYS.

pH_{NBS}	S ppt	T °C	A_T µmol kg^{-1}	DIC µmol kg^{-1}	pCO_2 µatm	CO_2 µmol kg^{-1}	HCO_3^- µmol kg^{-1}	CO_3^{2-} µmol kg^{-1}	Ω Ar
8.15	33	30(0.6)	2043(131)	1768	404.1	10.24	1567.22	190.45	3.14
7.8	33	30(0.6)	2066(116)	1946	1050.8	26.64	1820.68	98.83	1.63
7.6	33	30(0.6)	2042(132)	1990	1721.4	43.63	1881.85	65.01	1.06

Table 2. Fish sample sizes and dimensions used in morphometric and Fourier analyses.

Treatment	Morphometrics			Fourier
	n	Fish standard length (mm)		n
		Mean (±SD)	Range	
pH 8.15	16	7.2 (±1.75)	5.2–10.6	19
pH 7.8	16	8.0 (±1.22)	6.0–9.7	19
pH 7.6	16	6.9 (±1.22)	5.6–9.0	19

3 µm Al_2O_3 lapping film, sonified in ultrapure water for 2 min and then dried under a laminar flow hood in a class 100 cleanroom. Cleaned otoliths were then remounted onto a petrographic slide (8 otoliths per slide) using double-sided tape and stored in plastic bags for transportation to the ICP-MS facility at the Woods Hole Oceanographic Institution. Sectioned otoliths were analyzed with a 193 nm excimer laser coupled with a Thermo Finnigan *Element2* high resolution ICP-MS. We sampled a 80 µm spot centered approximately 50 µm outside of the nucleus using a laser repetition rate of 5 Hz at 70 % power and a dwell time of 60 s. A He gas stream carried ablated material from the ablation chamber to the ICP-MS where it was mixed with an Ar sample gas and a wet aerosol (2 % HNO_3) supplied by a self-aspirating (20 µ min^{-1}) PFA nebulizer in the concentric region of the quartz dual inlet spray chamber. Initial testing found that isotopes from six elements (^{7}Li, ^{25}Mg, ^{43}Ca, ^{55}Mn, ^{88}Sr and ^{138}Ba) had count rates significantly higher than blank values and were free of isobaric interferences. Instrument blanks (2 % HNO_3) and a otolith certified reference material (Sturgeon et al. 2005), dissolved in 2 % HNO_3 and diluted to a final Ca concentration of 8 µg.g^{-1}, were run every eight samples and used to correct for blank values and instrument mass bias, respectively, following FitzGerald et al. (2004). Detection limits were calculated as 3 standard deviations of the blanks that were run periodically throughout the analyses ($n = 3$). These limits were 5 % of the average sample intensity for ^{7}Li, 3 % of ^{25}Mg, 0.01 % for ^{43}Ca, 8 % for ^{55}Mn, 0.004 % for ^{88}Sr, and 7 % for ^{138}Ba. We estimated external precision (relative standard deviation, RSD) of the analyses by running a powdered otolith lab standard, dissolved in 2 % HNO_3 and diluted to a Ca concentration of 8 µg.g^{-1}. Estimates of RSD ($n = 3$) were 2.2 % for Li:Ca, 1.7 % for Mg:Ca, 14 % for Mn:Ca, 0.5 % for Sr:Ca and 0.6 % for Ba:Ca.

2.5 Statistical analyses

2.5.1 Otolith size and shape

Fish size could potentially influence otolith size and shape, therefore, we first examined the relationship between SL and each of the otolith morphometric traits. Otolith circularity was the only trait that exhibited a significant relationship with SL and ANCOVA was used to determine if this morphometric trait differed among pH treatments, using fish standard length as the covariate. There was no relationship between SL and the other otolith traits. ANOVA was used to compare otolith breadth and rectangularity differed among pH treatments. The variance distributions for otolith area and maximum length were not homogeneous (Levene's test: $P < 0.05$) and were not improved by transformations. Therefore, non-parametric Kruskal-Wallis median tests were used to determine if these morphometric traits differed among pH treatments.

2.5.2 Size difference within otolith pairs

To investigate directional asymmetry of the otoliths (i.e. is the right or left otolith usually larger), signed differences in otolith morphometrics were obtained by subtracting the value for the left otolith from that of the right otolith (R-L) for otolith area, maximum length, and maximum breadth. This was not conducted for rectangularity and circularity because it is the absolute values of these parameters that have meaning. The frequency of positive versus negative scores among the pH treatments was then compared with a chi-square test of independence for each measure of otolith size.

To determine if pH treatment affected the magnitude of otolith asymmetry with respect to otolith area, maximum length, and maximum breadth, we used ANOVA to compare unsigned differences between left and right otoliths for these

traits. ANCOVA was not required as there was no relationship between SL and unsigned differences for any of these traits. Unsigned differences in area and maximum length were log transformed to meet assumptions of homogeneity of variances.

2.5.3 Fourier analysis of otolith asymmetry

To investigate differences in the degree of asymmetry among treatments, unsigned differences between right and left otoliths in the standardised harmonic amplitude were obtained for each harmonic number. The data were analysed in two ways. First, a Kruskal-Wallis test was performed on the unsigned differences in standardised harmonic amplitudes for each harmonic number (shape descriptors). Parametric tests were not used because transformation of data did not produce normality. Second, a one-way between-groups multivariate analysis of variance (MANOVA) was performed using the unsigned differences between right and left otoliths in harmonic numbers H_2 to H_{20} (shape descriptors) as dependent variables. The assumption of multivariate normality was checking by confirming normality for each of the dependent variables. The multivariate data were visualised using non-metric multidimensional scaling (MDS), which is presented with a stress coefficient that reflects how well the data have been reduced to two dimensions.

2.5.4 Otolith chemistry

A one-way MANOVA was used to compare multivariate elemental signatures among treatments. Assumptions of multivariate normality and homogenenity of covariance matrices were confirmed as above.

3 Results

3.1 Otolith morphometrics

3.1.1 Otolith size and shape

There was a significant difference among pH treatments in mean otolith area (left otoliths only) and otolith maximum length (left and right otoliths) (Table 3; Fig. 1). One-tailed t-tests (assuming unequal variance) revealed that mean area of otoliths in the 7.6 treatment was larger than controls for left otoliths ($P = 0.02$), but not right otoliths ($P = 0.11$) and that maximum length of otoliths in the 7.6 treatment was larger than controls for both left ($P = 0.01$) and right otoliths ($P = 0.03$). On average otoliths in the 7.6 treatment had a 15 % greater area and were 10 % longer than otoliths from control fish. There was also an apparent trend for otolith breadth to be larger in the 7.6 treatment (Fig. 1), however, this was not statistically significant (Table 3). In contrast to the 7.6 treatment, there were no apparent differences in average size or shape of otoliths in the 7.8 treatment compared with controls (Fig. 1).

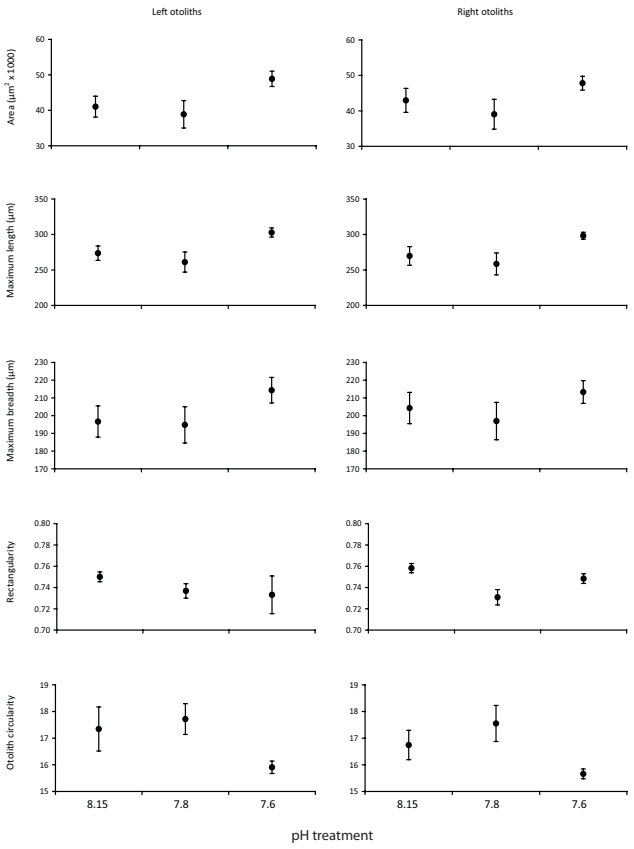

Fig. 1. Mean (± SE) otolith area, maximum length, maximum breadth, rectangularity and circularity for left and right otoliths of larval clownfish reared at pH_{NBS} 8.15, 7.8, and 7.6.

There was a statistically significant effect of pH treatment on otolith rectangularity for right otoliths only (Table 3), with a lower rectangularity value in the pH 7.8 treatment compared with controls ($P = 0.003$). However, the difference in the two values (0.76 and 0.73 for control and 7.8 respectively) represents a negligible difference in otolith shape, and there was no difference in shape between the pH 7.6 treatment and the controls. Therefore, this significant result does not appear to be causally related to the pH treatment. There was no significant effect of pH treatment on otolith circularity (Table 3), although there was a trend for decreased circularity for both left and right otoliths in the 7.6 treatment (Fig. 1).

Variation around the mean was lower in the pH 7.6 group for otolith area, maximum length and circularity (with one outlying value excluded) for both left and right otoliths (Fig. 1). This suggests that otoliths in this treatment were a more consistent size and shape than otoliths in the control and 7.8 groups.

Table 3. Results of Kruskal-Wallis median test (area, maximum length), ANOVA (breadth, rectangularity) and ANCOVA (circularity) for left and right otoliths of larval clownfish reared at three pH treatments (8.15, 7.8, and 7.6). Fish standard length (SL) is the covariate for ANCOVA.

Variable	Source	d.f.	χ^2 or F value	P
(a) Left otoliths				
Otolith area	pH treatment	2	$\chi^2 = 6.00$	**0.049**
Otolith maximum length	pH treatment	2	$\chi^2 = 9.50$	**0.009**
Otolith maximum breadth	pH treatment	2	1.488	0.237
Otolith rectangularity	pH treatment	2	0.62	0.54
Otolith circularity	pH treatment	2	1.280	0.288
	Fish SL	1	59.70	**0.000**
(b) Right otoliths				
Otolith area	pH treatment	2	$\chi^2 = 0.50$	0.778
Otolith maximum length	pH treatment	2	$\chi^2 = 6.00$	**0.049**
Otolith maximum breadth	pH treatment	2	0.878	0.422
Otolith rectangularity	pH treatment	2	6.31	**0.004**
Otolith circularity	pH treatment	2	1.32	0.277
	Fish SL	1	40.84	**0.000**

Mean SL (mm) of larvae was not significantly different among treatments (ANOVA: $F_{2,45}$ = 2.667, $P = 0.08$), however there was some variation in SL within treatments (Table 2). There was a highly significant effect of SL on otolith circularity for both left and right otoliths (Table 3). Regression analysis revealed a positive linear relationship between otolith circularity and fish standard length (left otoliths: y = 1.457x + 6.507; $r^2 = 0.517$; right otoliths: y = 1.060x + 8.880; $r^2 = 0.522$). Circularity is defined as otolith perimeter^2/otolith area. The minimum value of 4pi (12.75) is achieved only for a circular boundary, with values of ∼16 for a square boundary, and ∼20 for a triangular boundary. Circularity increased from ∼15 in the smallest fish (5–6 mm SL) to >20 in the largest fish (9–11 mm SL) indicating a shift from a square-shaped boundary towards a more triangular shape with increasing fish length.

3.1.2 Size difference within otolith pairs

There was no evidence of directional asymmetry, with either the left or the right otolith being the largest of a pair, and no difference in the distribution of positive and negative asymmetry among treatments for any of the otolith measurements (Chi-square $P > 0.15$ in all cases). Similarly, there was no significant difference among pH treatments in the magnitude of otolith asymmetry with respect to otolith area ($P = 0.24$), maximum length ($P = 0.79$), or maximum breadth ($P = 0.79$), although there was a tendency for the magnitude of difference between left and right otoliths be less in the 7.6 treatment compared to the controls and 7.8 treatment (Fig. 2). As observed above in the morphometric analysis, variation around the mean value was lower in the pH 7.6 group compared to the controls and pH 7.8 group.

3.1.3 Fourier analyses of otolith asymmetry

There were no significant differences among pH treatments in the Fourier descriptors of otolith shape, either when harmonic numbers were analysed individually (Kruskal-Wallis test $P > 0.05$ in all cases, except H_{13}) or in the multivariate analysis of all 19 harmonic numbers (MANOVA $F_{38,72}$ = 1.10, $P = 0.357$, Wilks' Lambda = 0.4). The similarity among treatments, based on the combination of all harmonic numbers, was clearly evident in the MDS plot (Fig. 3). For H_{13} there was a significant difference in median harmonic amplitude between the control and pH 7.8 treatment (Mann-Whitney U test $P = 0.02$), but no difference in any other pairwise comparison.

3.2 Otolith chemistry

Minor and trace element chemistry of larval clownfish otoliths appeared unaffected by the pH of ambient water in which they were reared. A one-way MANOVA found no significant differences in elemental signatures of otoliths among treatments (MANOVA $F_{10,22}$ = 1.07, $P = 0.42$, Wilks' Lambda = 0.45). Similarly, there were no consistent patterns in any of the individual elemental ratios as a function of pH (Fig. 4).

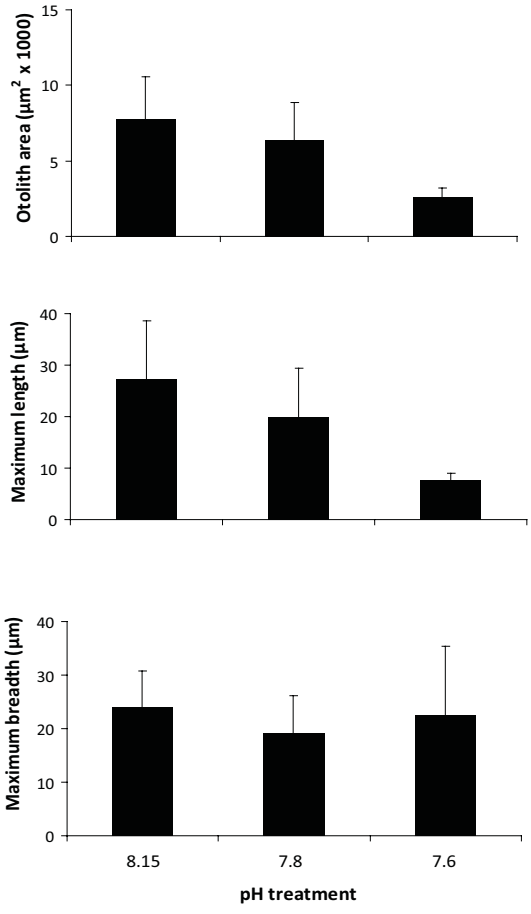

Fig. 2. Mean (± SE) unsigned differences in area, maximum length and maximum breadth between left and right otoliths of larval clownfish reared at pH_{NBS} 8.15, 7.8, and 7.6.

Fig. 3. MDS plot of Fourier descriptors of otolith shape for each individual sampled, grouped by pH treatment.

4 Discussion

We found that the size, shape, symmetry and elemental chemistry of otoliths in larval clownfish was unaffected by exposure to simulated levels of ocean acidification that could occur at the end of this century under a business-as-usual scenario of CO_2 emissions (pH 7.8 and 1050 µatm CO_2). However, in a more extreme treatment (pH 7.6 and 1721 µatm CO_2) otolith area and maximum length were larger than control otoliths. Two other recent studies have examined potential effects of ocean acidification on fish otoliths. Checkley et al. (2009) detected increased otolith size in larval seabass, *Atractoscion nobilis*, exposed to elevated CO_2 during the egg stage and up to 8 days post-hatching. Otoliths area was 7–9 % and 15–17 % larger than controls for fish reared at 993 and 2558 µatm CO_2, respectively. In contrast, Munday et al. (2011) did not detect any effect of elevated CO_2 on otolith size of juvenile spiny damselfish, *Acanthochromis polyacanthus*, reared for 3 weeks in treatments up to 841 µatm CO_2. The different results of these two studies could have been due to: (1) the higher pCO_2 levels used by Checkley et al. (2009), (2) the absence of a larval phase in the spiny damselfish, which could make them less susceptible to elevated CO_2, or (3) different durations of the two experiments. Our results presented here suggest that otolith size can indeed by affected by reduced pH and elevated pCO_2, as proposed by Checkley et al. (2009), however, sensitivity varies among species. Clownfish otoliths were not affected at ∼1000 µatm CO_2, whereas seabass otoliths were significantly larger at this concentration, but otoliths of both species were larger at higher pCO_2 concentrations. Thus, elevated pCO_2 appears to affect otolith development in both species, but larval clownfishes are more tolerant of elevated CO_2 than larval seabass. There is increasing evidence that ocean acidification affects different calcifying species in very different ways (Langer et al., 2009; Ries et al., 2009; Dupont et al., 2010; Kroeker et al., 2010) and these results indicate that the same types of variation might be observed among species without extensive carbonate skeletons.

The variable responses observed among the three species studied to date may be related to their life histories. The spiny damselfish, *Acanthochromis polyacanthus*, studied by Munday et al. (2010) has direct developing juveniles that remain on the reef after hatching. Juveniles shelter with their parents in small caves, where CO_2 levels are likely to become elevated due to respiration. Consequently, newly hatched *A. polyacanthus* may be adapted to periods of high ambient CO_2. The clownfish, *Amphiprion percula*, has pelagic larvae, but is a demersal spawner. Benthic eggs are likely to experience significant fluxes in ambient CO_2 due to consumption of CO_2 by photosynthesis during the day and release of CO_2 by respiration of reef organisms at night (Munday et al., 2008). Hatching from benthic eggs may precondition larval clownfishes to moderate increases in ambient CO_2. In contrast, the seabass *Atractoscion nobilis* is a pelagic spawner, so both eggs and larvae are likely adapted to the relatively

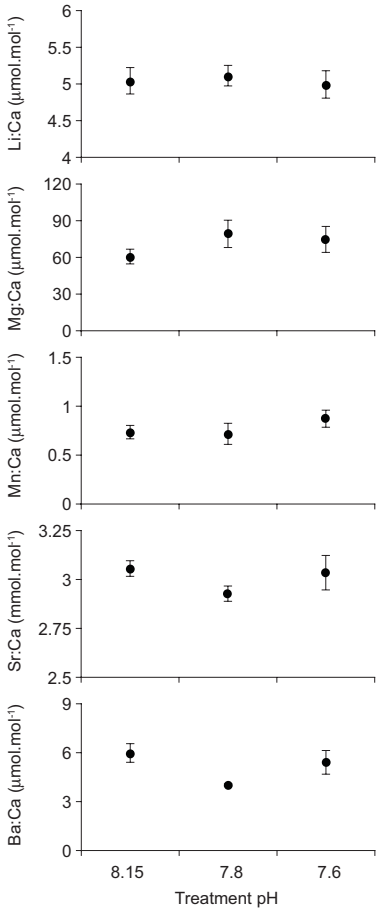

Fig. 4. Mean (± SE) Li:Ca, Mg:Ca, Mn:Ca, Sr:Ca and Ba:Ca ratios in otoliths of larval clownfish reared at pH_{NBS} 8.15 ($n = 7$), 7.8 ($n = 5$), and 7.6 ($n = 6$).

low and stable CO_2 environment characteristic of epipelagic ocean waters, and may be more susceptible to elevated CO_2. Further studies on a range of benthic and pelagic spawners are required to test this hypothesis.

If reduced seawater carbonate ion (CO_3^{2-}) concentrations affected the rate of otolith precipitation we would expect to see a decline in otolith size in acidified seawater. Instead there was a trend for otolith size to increase in these conditions. Fish actively regulate their acid-base balance through bicarbonate (HCO_3^-) accumulation and ion exchange across the gills (Claiborne et al., 2002; Evans et al., 2005; Brauner and Baker, 2009) and, thus, are able to compensate for intra- and extra-cellular acidosis caused by increased pCO_2. Changes to extracellular HCO_3^- and CO_3^{2-} concentrations caused by acid-base regulation at high pCO_2 could potentially influence the precipitation of $CaCO_3$ in structures such as otoliths (Payan et al., 1997). Checkley et al. (2009) proposed that larval fish controlled the concentration of H^+ and Ca^{2+} ions in the blood plasma and endolymph (the fluid surrounding an otolith), but not the neutral molecule CO_2. Consequently, maintenance of a constant pH in the endolymph despite elevated extracellular pCO_2 would lead to increased CO_3^{2-} concentrations in the endolymph due to diffusion of CO_2 from blood plasma across the endolyphatic membrane. Increased CO_3^{2-} concentrations in the endolymphatic fluid may, in turn, increase the rate of aragonite precipitation at the otolith surface. However, CO_2 concentrations are generally higher in endolymphic fluid than in blood plasma (Takagi, 2002), perhaps related to the removal of H^+ ions produced during disassociation of HCO_3^- to CO_3^{2-} at the time of aragonite precipitation (Payan et al., 1999). Under this scenario there would be net diffusion of CO_2 out of, not into, the endolymph. Alternatively, HCO_3^- accumulation as a result of acid-base compensation, either in the plasma or endolymph, may promote otolith accretion if HCO_3^- is the preferred dissolved inorganic carbon species for calcification (Allemand et al., 2007; Herfort et al., 2008). The precise mechanisms involved in acid-base regulation in larval marine fishes are not fully understood (Brauner, 2009), nor are the cellular mechanisms involved in calcification of many marine species (Hofmann et al., 2010). Nonetheless, acid-base regulation and biological control of endolymph pH are likely to be central to the changes in otolith growth observed in these two species of fish when exposed to high CO_2 (Romanek and Gauldie, 1996; Payan et al., 1997, 1998; Allemand et al. 2007).

Otoliths have an important role in fish hearing and body orientation. However, if the changes in otolith area and maximum length that we observed in the highest CO_2 treatment (pH 7.6, 1721 µatm CO_2) would be sufficient to affect these important functions is unknown. Otolith size can vary considerably among individual fish of the same somatic size, and otoliths of some species continue to grow even once somatic growth has ceased (Campana, 1990; Thorrold and Hare, 2002; Munday et al., 2004). Furthermore, we observed considerable variation in otolith morphometric traits among individuals in the control and less extreme CO_2 groups. Therefore, some variation in otolith size is commonplace and it is not clear that the relatively small change in otolith size observed here in the highest CO_2 treatment would be sufficient to cause substantive effects on larval fish.

A number of studies have shown that physiological stress can increase otolith asymmetry (Gagliano and McCormick, 2004; Payan et al., 2004); however, we found no evidence that ocean acidification is likely to decrease symmetry between left and right otoliths, even at relatively high CO_2 concentrations. In fact, there was a trend for smaller differences in otolith area and length between left and right otoliths in the highest CO_2 treatment, and for there to be less variation among individuals (i.e. lower sample variance). Increased calcification in the higher CO_2 treatment might have tended to ameliorate differences in otolith shape and size, and thus been responsible for the apparent increase in symmetry between otoliths, both within and among individuals. Variation in the size and shape of left and right otoliths may affect the ability of individuals to detect and localize sound (Popper

and Lu, 2000; Gagliano et al., 2008) and decreased otolith symmetry has been associated with higher mortality rates in larval reef fishes (Gagliano et al., 2008). Consequently, any trend toward increased symmetry, such as that observed here, is unlikely to have a negative impact on individual fitness.

Otolith circularity was the only trait that varied significantly with fish length. There was a linear relationship between SL and circularity, indicating a shift from a square-shaped boundary in smaller fish towards a more triangular shape in larger fish. Otolith deposition does not occur evenly around the otolith, instead, growth often occurs more rapidly on one axis than the other, leading to an otolith that is increasingly more elongate and triangular in shape (Gagliano and McCormick, 2004; Green et al., 2009). Otolith growth is often correlated with somatic growth and therefore larger, faster growing fish within treatments may be expected to have a more triangular otolith profile, as observed.

This is the first study to examine the potential effects of ocean acidification on otolith chemistry and we found no effects of altered seawater carbonate chemistry on the elemental chemistry of the otoliths. Elemental ratios of all five elements were within the range typically reported for aragonitic marine fish otoliths (Campana and Thorrold, 2001). Although sample sizes were relatively small (5–7 individuals per treatment) the low variance and consistency of results suggests that a larger sample sizes would not have changed our interpretation in any meaningful way. We also found no visual evidence of vaterite or calcite in the any of the treatment otoliths, which if present would have resulted in relatively low Sr:Ca values (Veizer, 1983; Tomas and Geffen, 2003). Taken together, our data suggest that the larval clownfish were capable of regulating endolymphic fluid chemistry even in waters with pH values significantly lower than open ocean values.

Our results demonstrate a number of important points. First, the tendency for otolith size to increase and become less variable in the higher CO_2 treatment is consistent with an effect on otolith development caused by the physiological process of pH regulation in fishes. Further studies are required to pinpoint the exact processes involved. Second, our results indicate that otolith development in larval clownfishes is robust to the levels of ocean chemistry change that may occur over the next 50–100 years, even if higher levels are likely to influence otolith size. Finally, we emphasize that there is likely to be considerable variation among species in their sensitivity to elevated CO_2 and reduced pH. Determining the traits that make some species more susceptible than others will aid in making predictions about the longer-term and ecosystem level effects of ocean acidification.

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