CLOWNFISH CHEMICALLY RECOGNIZED THEIR SEA-ANEMONE HOST AT SETTLEMENT

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ABSTRACT. – One of the greatest challenges facing marine reef organisms with a pelagic larval stage that develops in offshore waters is how they relocate to patchily distributed reef environments in a vast oceanic matrix. In the present study, a series of aquarium experiments using a choice flume were conducted to test the abilities of Amphiprion ocellaris larvae (clownfish) to chemically recognize their host sea-anemone Heteractis magnifica. Moreover, to know if chemical abilities of fish change after settlement, the study was also conducted on juvenile stage. The results showed that both larvae and juveniles had a significant preference toward their host. Thus, our study showed that olfactory cues could play an important role in directing pelagic larval stage fishes to a suitable reef in which they can settle on.

INTRODUCTION

Most coral reef fish species have a stage-structured life history with a pelagic larval phase and a sedentary benthic stage (mostly juveniles and adults) (for review, see Leis & McCormick 2002). After the oceanic dispersal phase, fish larvae under metamorphosis return to a reef to find a suitable settlement habitat and continue their development into juvenile stage (Lecchini 2005). During this transition from the pelagic environment to a benthic environment, fish larvae are subjected to a strong selective pressure to choose a suitable reef habitat and can be removed by predation (e.g. Doherty et al. 2004, Lecchini et al. 2007). Thus, many fish species show a high selectivity concerning the habitats they choose at settlement (e.g. Nakamura et al. 2009a, b). This selection is based on specific substrates, presence of conspecifics, absence of predators and/or competitors for food and space (for review, see Doherty 2002). However, as it is unlikely that successful settlement is solely a matter of chance, fish larvae may use their swimming and sensory abilities (visual, chemical and/or acoustic cues) to detect suitable habitat on which conspecifics are already settled and to avoid predators or competitors (for review, see Leis et al. 2011). Some studies have thus demonstrated that chemical cues are mainly used by fish larvae to find a suitable reef habitat (e.g. Dixson et al. 2011, 2014, Lecchini et al. 2014a), to find conspecifics (Lecchini et al. 2005, Lecchini & Nakamura 2013), or to avoid predators (Dixson et al. 2012). Although these studies highlighted the differential ability of fish larvae to respond to olfactory stimuli from conspecifics, predator or habitat, it is still not known how fish larvae find their way back to a suitable settlement habitat.

The present study aimed to test if the clownfish Amphiprion ocellaris (Cuvier, 1830) at larval and juvenile stages could chemically recognize their suitable anemone Heteractis magnifica (Quoy and Gaimar, 1833). Clownfishes, which belong to the pomacentrids, live in symbiosis with sea anemones. Fish adults are sedentary and spend their entire life in the host that they choose at settlement stage. Some previous studies allowed to increase the scientific knowledge on the biology and ecology of clownfish at settlement: species-specific host anemone detection by the newly settled anemone fishes (e.g. Elliott et al. 1995), and proposed mechanisms for the innate or acquired ability for host or conspecific detection (e.g. Arvedlund & Nielsen 1996, Arvedlund et al. 1999, Dixson et al. 2014). For example, Arvedlund & Nielsen (1996) were the first ones to highlight the imprinting process in coral reef fish. Amphiprion ocellaris eggs were maintained with either the host anemone H. magnifica or without anemone. They showed that larvae from treated eggs settled in less than five minutes on the anemone whereas larvae from non-treated eggs remained indifferent to the anemone during 48 hours of experiment. Miyagawa-Kokshima et al. (2014) studied the embryonic and immediate post-hatching learning of chemical cues via the parents’ host (i.e. conspecific cues) in A. ocellaris through a host-exchange experiment with egg batches during hatching. They showed that innate recognition was found to exist at the time when juveniles first search for their conspecifics at settlement. Overall, these studies suggest that both processes, imprinting or innate capabilities, could occur at larval settlement of clownfishes to detect the presence of anemones or conspecifics. The present study will bring some new knowledge about the recognition of A. ocellaris larvae to chemical cues of their sea-anemone without imprinted the odor during the hatching.
MATERIALS AND METHODS

The clownfish *Amphiprion ocellaris* has been used in this study because it is easily kept in aquaria and larvae can be reared with high rates of success. A 8 year-breeding pair was reared in a 200 L tank filled with artificial sea-water at the aquarium of Canet-en-Roussillon (France). The pair laid egg clutches on a terracotta pot placed in their aquarium. Eight days after reproduction, larvae were transferred from the parental aquarium to a 40 L larval rearing aquarium filled with water taken from the parental aquarium to increase the rate of survival. Larvae were fed with rotifers at 15 individuals ml⁻¹ three times a day the first six days. *Artemia nauplii* were added at day 6 and the ratio *A. nauplii / rotifers* was increased until larvae were fed only with *A. nauplii* from day 11. In order to know if chemical abilities of fish could change after settlement, clownfish at juvenile stage was also tested. At day 20, juveniles (i.e. larvae have totally accomplished the metamorphosis process since they feature most of the adults' shape and pigmentary characteristics) were fed with pellet food mixed with astaxanthin. Thus, *A. ocellaris* tested at larval or juvenile stage did not have been in contact with an anemone during their rearing.

To test the ability of larvae (11 days post-hatching) and juveniles (24 dph) to chemically recognize their host *Heteractis magnifica*, a two-channel perspex choice flume of a similar design developed by Gerlach *et al.* (2007) was used. One individual was placed in the centre of the downstream end of the flume where it was freely able to explore the chamber and swim between the two adjacent flows on each side. Flow rate was maintained constant using variable area flow meters (Dwyer Instruments) at 200 ml min⁻¹. This low flow made possible by the small chamber size, which allowed the clownfish to swim without struggling against the current, ensuring movement was likely due to preference of location (Dixson *et al.* 2011). Preliminary dye tests were conducted to ensure a laminar flow on each side of the chamber without eddies or mixing and that each side was fully flushed of the previous water source after switching to another.

Experimental procedure was based on choice flume experiments of Atema *et al.* (2002). Each trial began with a fish placed within a piece of PVC pipe in the center of the downstream end of the chamber. The fish released could explore the chamber and acclimate to the water sources for a period of two minutes. Fishes which did not swim actively or explore both sides of the chamber during these two minutes were discarded from the trials (< 5 % of fish tested were discarded). After the acclimation period, the position of the fish, as being on one side of the chamber or the other (or in one water source or the other) was recorded every five seconds for another two minutes period (25 data points). Water sources entering the chamber from buckets feeding into the left and right sides were then switched, with one minute being allocated for the water sources to exchange and flush completely, in order to control for side preference in individuals. After switching water sources another two minutes acclimation period was given, followed by another two minutes observation period. Following testing fish were released back to the catch site and the chamber was rinsed thoroughly with freshwater.

Using this protocol, we tested the effect of the anemone on larvae and juveniles. Individuals were exposed to artificial seawater vs. anemone seawater. To create the anemone chemical cue, one *H. magnifica* was soaked in 20 L of artificial seawater for 4 hours before each trial. A total of 50 larvae and 23 juveniles were tested. The distribution of individuals was analyzed using the chi-square test (theoretical distribution = fish spent 50 % of their time in each compartment of choice flume).

RESULTS

In presence of artificial seawater vs. anemone seawater, fish larvae spent 54 % of their time in the compartment of choice flume filled with anemone seawater (Fig. 1). The chi-square test conducted on all the 50 individuals highlighted that this preference was significant (p-value < 0.01). The analysis at individual level confirmed this

![Fig 1. – Percentage of time spent by larvae and juveniles of *Amphiprion ocellaris* in the compartment filled with artificial seawater and in the compartment filled with anemone seawater.](image-url)
preference with 29 of the 50 A. ocellaris larvae tested (58%) showing an attraction for the anemone sea-water.

Amphiprion ocellaris at juvenile stage showed a similar significant attraction for the anemone seawater (i.e. 53% of their time in the compartment of choice flume filled with anemone seawater – chi-square test conducted on all juveniles: p-value = 0.03). Nevertheless, the analysis at individual level showed that only 52% of the juveniles spent more time in the compartment filled with anemone seawater (Fig. 1).

DISCUSSION

Perhaps one of the greatest challenges facing the majority of marine reef organisms with larval stages that potentially disperse and develop in offshore waters is how to relocate the relatively rare patches of coral reef habitat on which they settle and ultimately reside as adults (for review, see Leis et al. 2011). The answer must lie partly in the sensory modalities of fishes.

Our study showed a chemical attraction of both A. ocellaris larvae and juveniles for the anemone seawater (Fig. 1). Our protocol had, however, one weakness. We used a negative control (seawater with no anemone), but we did not use a positive control (seawater with an anemone of a different genus). Therefore, the observed difference could simply be the presence of chemical cues of any anemone species or marine-organism-soaked seawater, and could not be related to being a specific host sea-anemone of A. ocellaris. Nevertheless, several previous studies showed that clownfish recognized specifically the chemical cues of their host sea-anemone (e.g. Arvedlund et al. 1999, Dixson et al. 2014). Moreover, our result is in accordance with the rare studies done on the development of sensory organs of coral reef fish (e.g. Arvedlund et al. 1999, Lara 2008) which lead to the conclusion that the larvae at settlement had sensory modalities well developed and similar to those of juveniles. Therefore, our result confirms that clownfish larvae could recognize their settlement habitat using chemical cues and that clownfish at larval and juvenile stages had the same chemical abilities to detect an habitat. While the odors of various environmental components have been shown to attract or repel fish larvae (see references in Leis et al. 2011), determining the molecular basis of this chemotaxis is still unknown. Some rare studies have tried to identify the molecules. Murata et al. (1986) identified molecules produced by the sea anemone Heteractis crispa (amphikuemien and 4 analogs) and involved in specific research behavior of Amphiprion perideraion. Lecchini et al. (2005) isolated and (partially) identified a chemical conspecific cue used by Chromis viridis larvae. Identifying these specific compounds could reveal whether behavioral responses are dependent on the presence of a specific chemical component, or alternatively a range of habitat/conspecific/predator-specific chemicals will elicit similar responses (Lecchini et al. 2010, 2013). Therefore, the future studies should be focused on the identification of the nature of chemical cues responsible for larval attraction.

Our study highlights for one of the first time that A. ocellaris larvae could recognize their sea-anemone without imprinted the odor during hatching. Many studies conducted on fish settlement and their perception of information used naive larvae captured either with light traps or crest nets (e.g. Lecchini et al. 2005, Lecchini & Nakamura 2013). Some studies used also fish larvae reared in aquaria, especially for Amphiprion species (e.g. Dixson et al. 2011, 2014). Thus, fish larvae had no prior experience of settlement habitats (i.e. naive larvae). Therefore, how could these naive individuals recognize a conspecific (and not a heterospecific), a reef predator (and not any fish), or a specific host anemone (and not any anemone species)?

The scientists asked the question: is the recognition innate or imprinting at settlement (Dixson et al. 2014)? In our study, the low attraction of A. ocellaris larvae (only 54%) and juvenile (only 53%) could be explained by the rearing conditions. Amphiprion ocellaris tested at larval or juvenile stage did not have been in contact with an anemone during their rearing. Dixson et al. (2014) showed that clownfish larvae recognized innately their host, but this recognition was stronger when larvae were in contact with the anemone during their development. Our study supports thus the recent result of Dixson et al. (2014): Amphiprion larvae could recognize innately their host but need to imprint themselves during their development to increase the probability to find a suitable host.

To conclude, although our study showed that clownfish at larval and juvenile stage could chemically recognize their settlement habitat, some questions remain still unsolved about “how fish locate the relatively rare patches of coral reef habitat on which they settle”. For example, imprinting process can happen in oviparous species with demersal eggs, which represent 40% of coral reef fish including clownfishes. However, for the other species called egg-laying fish, the eggs are carried away from parental environment by currents, therefore any imprinting can occur. In that case, how can we explain the chemical preference for conspecifics or specific habitats of egg-laying fish? This is a fruitful research avenue for further studies: do oviparous species with demersal eggs recognize more their settlement habitat than egg-laying fish species? Moreover, only the olfactory cues was tested in our study, but other stimuli could have contributed the process of host recognition (visual, sound cues – see Holles et al. 2013, Lecchini et al. 2014b). Thus, the main priority of future studies on sensory abilities of fish larvae at settlement should be to conduct in situ experiment in order to test the chemical, acoustic and visual cues of conspecifics, habitat or predator in a real environment in which several other cues are present.
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