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The embryonic development of the olfactory system in *Amphiprion melanopus* (Perciformes: Pomacentridae) related to the host imprinting hypothesis

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Development of the olfactory system in anemonefish embryos of the species *Amphiprion melanopus* was examined from day six post-fertilization, until hatching (day nine). An olfactory placode with receptor cells lining the epithelium and nerve axons from the placode into the olfactory bulb, was observed on newly hatched embryos. In addition, two different secondary bilateral receptor systems were found. These findings may firstly support the anemonefish host imprinting hypothesis, and secondly indicate that the ontogenetic timing of this imprinting mechanism occurs towards the end of the embryonic development.

INTRODUCTION

Olfactory signals and cues impinge on every aspect of fish ecology, from finding mates through to broad spatial distribution patterns (e.g. Hara, 1982, 1992). Sensitivity to chemical signals may be particularly useful to reef fish that live in the complex three-dimensional environment of a coral reef. Many coral reef fish display strong micro-habitat specificity, especially as juveniles (e.g. Fautin, 1986; McCormick & Makey, 1997; Both & Wellington, 1998). When the cost of a poor choice is death, there is strong selective force to develop sensitive means of detecting habitat that enhances survival. The use of chemical cues to detect a habitat seems particularly well developed in the damselfish (Pomacentridae) that form obligate associations with sea anemones, especially Indo–Pacific members of the family Stichodactylidae (Fautin & Allen, 1992). Evidence suggests that, during settlement, juvenile anemonefish detect and recognize their host anemones by means of olfactory cues released by these (e.g. Fricke, 1974; Murata et al., 1986; Miyagawa, 1989; Fautin, 1991; Arvedlund et al., 1999).

Recently, Arvedlund & Nielsen (1996) and Arvedlund et al. (1999) have demonstrated for two species of anemonefish (*Amphiprion ocellaris* and *A. melanopus*) that embryos imprint to their parental host anemone type and that this imprinting affects the choice by juveniles of a host at settlement. The mechanism underlying this imprinting phenomenon is not entirely clarified. However, one compulsory condition for this mechanism to function, is a requirement for the olfactory system and/or gustation and/or the 'common chemical sense' (Finger, 1988; Hara, 1992), to be fully functional, at least at hatching, in order for the fish larvae to imprint to the chemical cues from the parental anemone. There are four components required for a functional olfactory system: (1) receptor cells lining the olfactory epithelium; (2) axons from the olfactory receptor cells forming the olfactory nerve; (3) synaptic connections between the olfactory nerve fascicles and mitral cells in the olfactory bulb; and finally (4) connections between mitral cells and the telencephalon (Hara & Zielinski, 1989).

Fish detect chemical stimuli through at least two different channels of chemoreception, olfaction and gustation (Finger, 1988; Hara, 1992). The term olfaction denotes the system mediated by the bipolar receptors of the olfactory epithelium. The term gustation indicates the chemosensory system mediated by taste buds (Finger, 1988). A third chemosensory system, the 'common chemical sense' (Finger, 1988; Hara, 1992), might exist in form of solitary chemosensory cells scattered on the skin (Whitear, 1992). According to Hara & Zielinski (1989), and Marui & Caprio (1992), all available evidence indicates that olfaction is a major mediator of chemical signals and is involved in a diverse range of teleost behaviour, e.g. homing of salmonids to natal rivers.

The aim of the present study was to examine in detail the development of the olfactory system in anemonefish *A. melanopus* from the middle of the embryonic stage through to just after hatching, five days later.

MATERIALS AND METHODS

Sampling of embryos

Eggs of *Amphiprion melanopus* from breeding pairs held in captivity (Job et al., 1997; Arvedlund et al., 2000b) were collected from day six to day nine after spawning. On day nine the eggs were collected at 1700 hours, 3 h before hatching. Embryos were also collected immediately

after hatching. Each sample contained 10–15 eggs/newly-hatched embryos. Embryos were anaesthetized by placing them in a jar with marine water, in a freezer for 30 min.

Fixation of embryos for SEM, TEM and histology

To avoid histological artefacts associated with fixation, two fixatives were used on separate embryos for scanning electron microscopy (SEM) examination. Embryos were fixed in: (1) 70% ethanol, for the SEM observations of the embryonic development, and dissected from the egg-sack using chemically sharpened tungsten wire needles. The embryos were dehydrated to absolute ethanol. Specimens were then taken to a mixture of absolute ethanol and hexamethyldisilazane (HMDS) and then to 100% HMDS and allowed to air dry under cover for 12 h. Specimens were then sputter coated with gold-palladium, and examined in a JEOL 840 SEM. (2) The newly hatched embryos were fixed in 2.5% glutaraldehyde in marine water for 3 h at 4°C, postfixed for 1 h at 24°C, in 1% osmium tetroxide and marine water, and dehydrated in a graded series of ethanol. They were then critical point dried in a Petro CPD2, gold sputter coated and examined in a Philips X220 SEM.

For transmission electron microscopy (TEM) and examination using light microscopy, specimens were embedded in Spurr's resin (Spurr, 1968). The specimens were sectioned at 60 nm, and stained with uranyl acetate and lead citrate. Sections were examined in a Jeol 2000FX TEM, using 80 kV acceleration voltage.

For the examination using light microscopy, 1-μm sections were cut on an LKB ultramichrotome, and stained with 0.5% toluidine blue in 0.5% borax.

To determine whether the found structures of olfactory senses were indeed chemosensory, the criteria based on Hara (1982, 1992), Finger (1988), Laverack (1988), and Hara & Zielinski (1989), have been used for the olfactory placodes. For the solitary structures, the criteria of Whitear (1992) have been used.

RESULTS

As an overview of the whole embryo, the forepart of the embryo of *Amphiprion melanopus* is shown in Figure 1A (day seven post-fertilization), partly removed from its egg case.

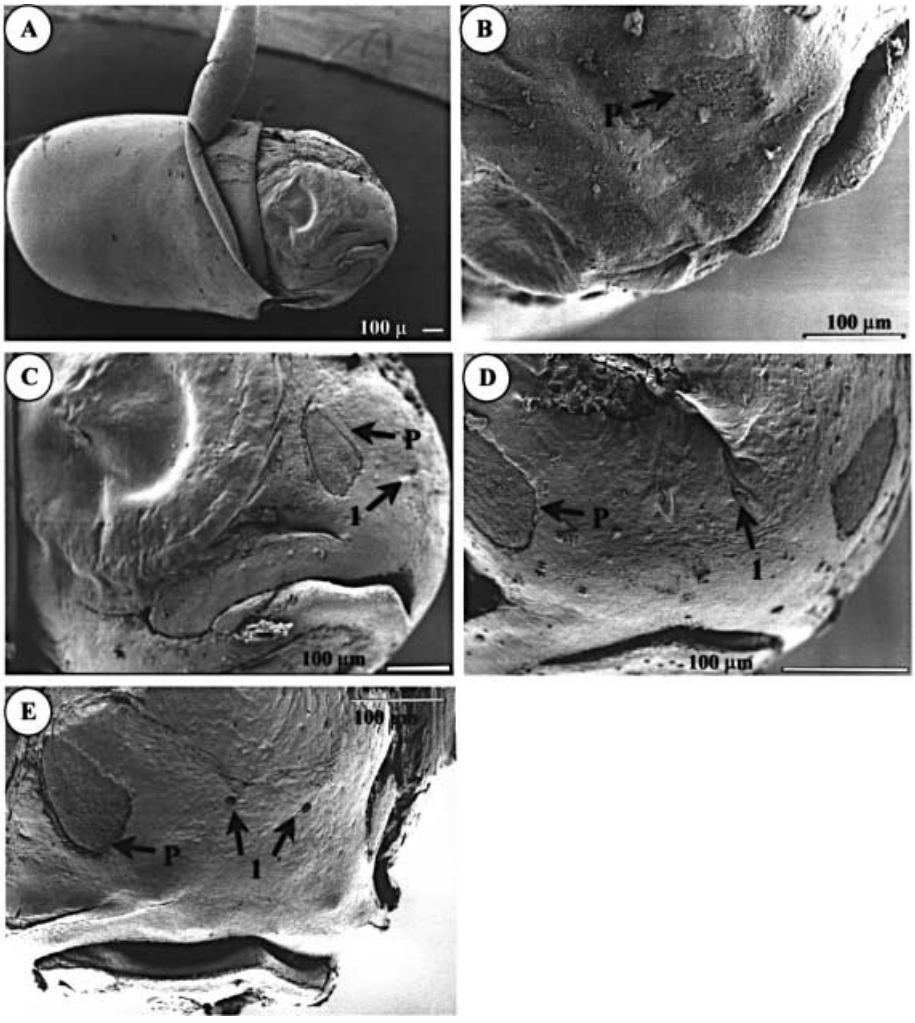

Figure 1. SEM images of embryonic development: (A) view of whole embryo in egg sack (day seven); (B) post-fertilized embryo day six: a preliminary olfactory placode is observed; (C) post-fertilized embryo day seven, a groovelike border is present, bordering the placode. Another chemoreceptor is also present (labelled as 1). (D) Post-fertilized embryo day seven, frontal close-up; (E) post-fertilized embryo day eight, frontal close-up. P, olfactory placode; 1, solitary chemosensory system.

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Day six

The olfactory placode was present as a low oval depression anterior to the eye (Figure 1B). The placode shows a slightly granular surface.

Day seven

The border of the olfactory placode was clearer and the placode had increased approximately 10% in size and attained an almost triangular appearance (Figure 1C). The granulation on the apical surface is clearly seen in Figure 1D.

Day eight

The olfactory apparatus was now deeper, like a depression, with a higher rim bordering the placodes. A bilateral different sensory receptor system (labelled '1') was present as a small protrusion anterior to the olfactory placode (Figure 1E).

Day nine, three hours before hatching

The pitlike structure of the olfactory placode had deepened further to a depression, with a 10–20 μm rim (Figure 2A,B). Ciliated cells had developed upon the granular surface of the placode (Figure 2B).

Day nine, immediately (i.e. less than 5 min) after hatching

The placode was now a deep invagination (Figures 2C,D) and the surface was covered with clusters of ciliated cells (Figure 2F). Two bilateral different sensory receptor systems (labelled '1' and '2', distinguished by the location) were present as a small protrusion anterior to the olfactory placode (Figure 2C). Figure 2E shows an overview of the head of the newly hatched anemonefish embryo. The two bilateral different sensory receptor systems could again be clearly seen as small protrusions anterior to the olfactory placode (Figure 2F).

Considerable variation was observed between the placodes on the three embryos immediately after hatching, examined by SEM. The placode on Figure 3A was fully covered with cilia, meanwhile the placode on Figure 3B was sparsely covered in a regularly pattern with tufts of cilia. This difference is not likely to occur because of artefacts, since the same method of fixation was used for all figures (3A–C). A third variant is shown on Figure 3C. Here the ciliated cells were dense, but a large area of the olfactory epithelium had no cilia.

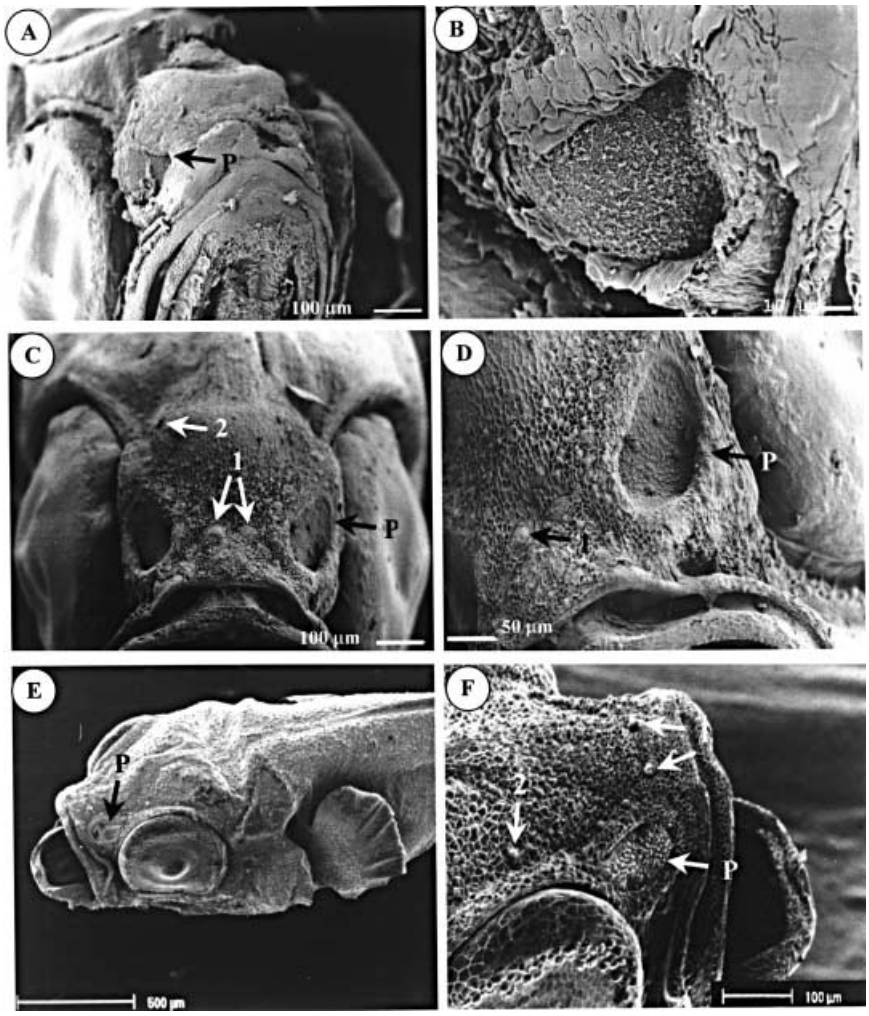

Figure 2. SEM images of embryonic development: (A) day nine, pre-hatching; (B) same, close-up; (C) day nine, post-hatching, frontal view; (D) same, right side close-up; (E) day nine, post-hatching, overview; (F) day nine, post-hatching, dorsal view. P, olfactory placode; 1, first solitary chemosensory system; 2, second solitary chemosensory system.

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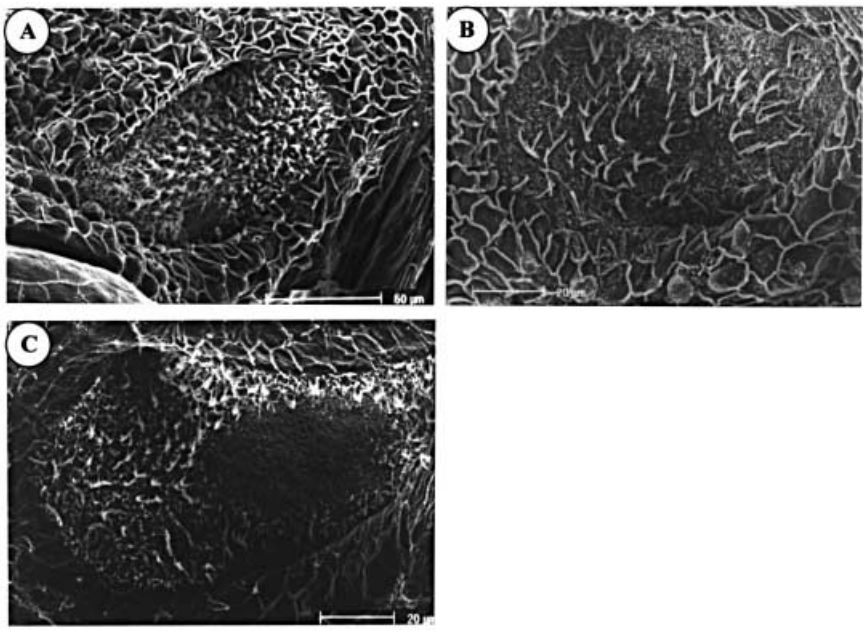

Figure 3. SEM images of day nine, post hatching, olfactory placode: specimen with full cilia coverage of the placode; (B) same, close-up; (C) specimen with incomplete ciliar coverage.

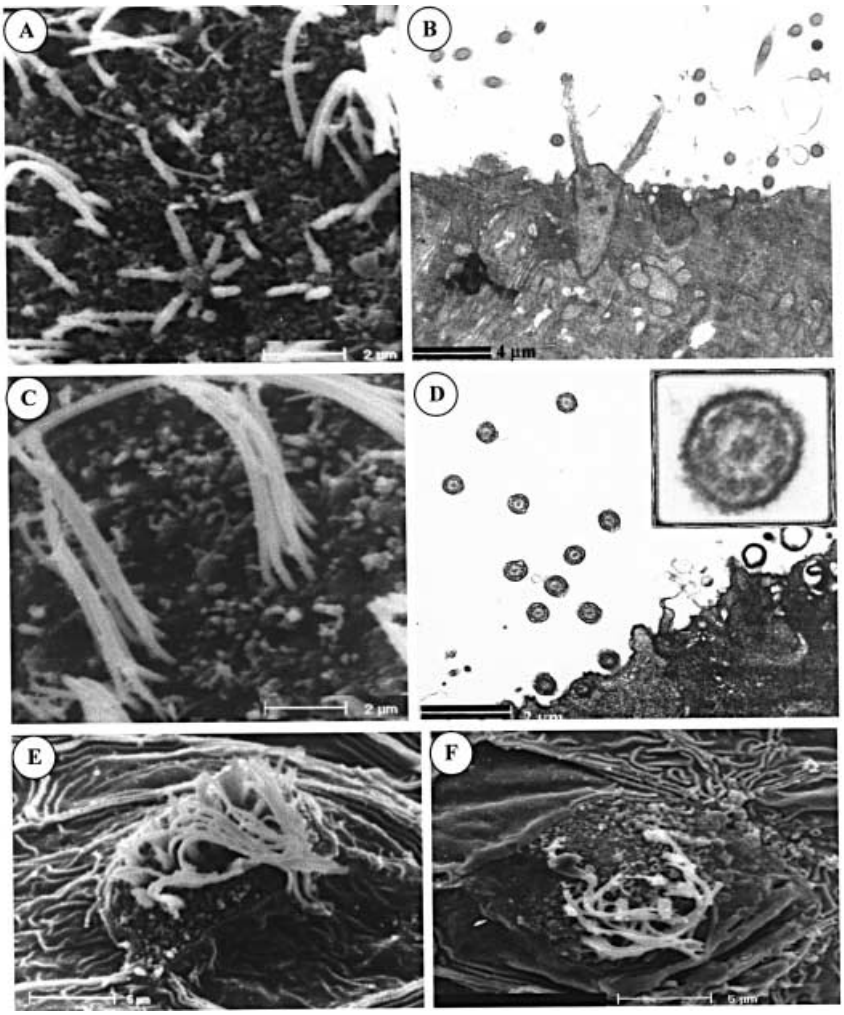

Figure 4. SEM and TEM of cilia structure, day nine, post-hatching: (A) SEM of cilia cluster around central granular; (B) TEM of same; (C) SEM of adhered cilia cluster; (D) TEM of same, right hand corner close-up; (E) cilia of solitary chemosensory cell one; (F) cilia of solitary chemosensory cell two.

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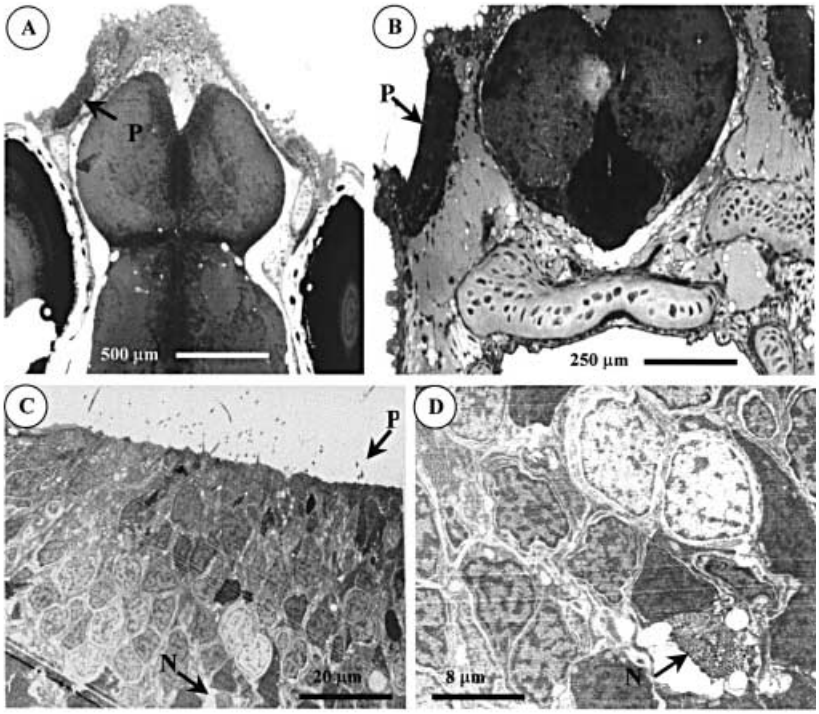

Figure 5. (A–B) Light microscopy (LM) of forebrain and olfactory placode. (C–D) TEM of olfactory placode, day nine post-hatching. P, placode; N, assemblage of olfactory nerves.

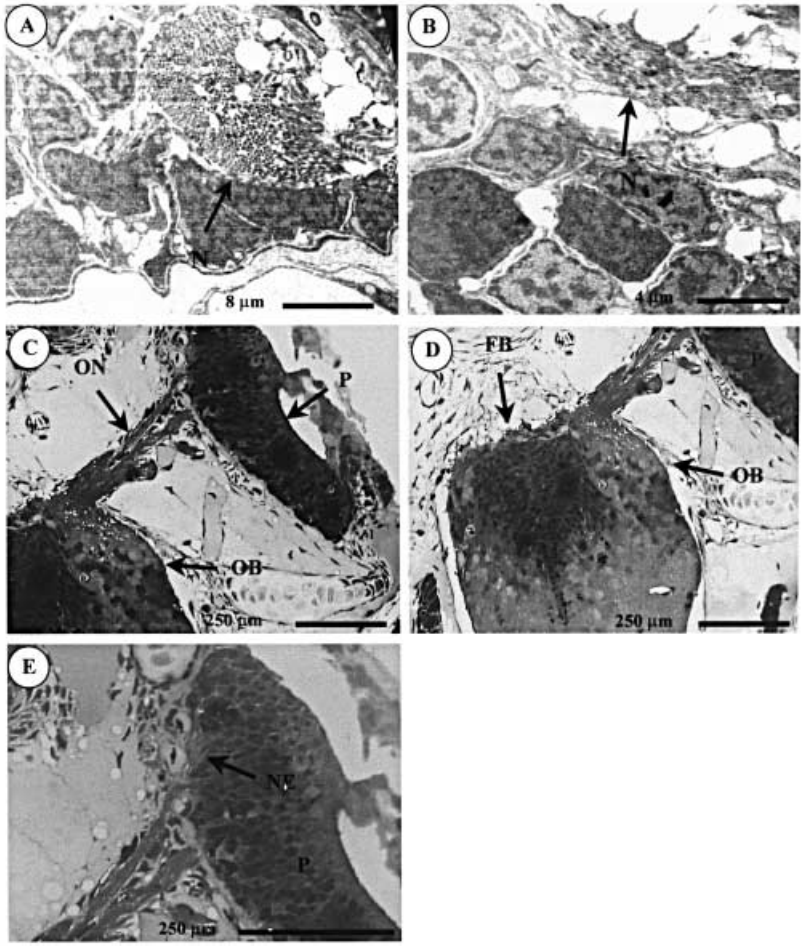

Figure 6. (A) TEM of olfactory placode; (B) assemblage of axons in placode; (C) overview of the nerve connection between the placode, the nerve connecting up in the olfactoric bulb next to the forebrain; (D) close-up of the olfactoric bulb and the forebrain; (E) close-up of the olfactoric nerve in the placode, nerve endings in the placode can be seen. N, assemblage of nerves; OB, olfactoric bulb; ON, olfactoric nerve; P, placode; FB, forebrain; NE, nerve endings.

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Two different types of ciliated cell clusters were observed on the olfactory epithelium. Figure 4A shows a group of seven, 1.5 μm cilia located on the periphery of a protruding cell. Figure 4B is a TEM of Figure 4A. Figure 4C shows a different group of ten 8-μm long cilia adhered to each other, however, the use of HMDS, which can cause ciliary structure to clump, was not applied here, instead fixation method no. 2 was applied. Figure 4D shows a TEM cross-section of Figure 4C. Inserted in Figure 4D, is an enlarged TEM cross section of one cilium. This enlargement demonstrates a distinctive 9+2 microtubuli structure was present. Two pairs of bilaterally symmetrical sensory systems were present with cilia (Figure 4E,F).

Figure 5A,B shows light microscopic trans-sections of the forepart of an embryo day nine, right after hatching. Figure 5A is an overview of where the placode is located (P). Figure 5B is a close up of Figure 5A: notice the surrounding cells of the placode (P), most likely glia cells. An assemblage of nerves was present in the olfactory placode (Figures 5C,D & 6A,B). A solid histologically identifiable olfactory nerve connection from the placode into the olfactory bulb was present (Figure 6C–E).

DISCUSSION

The morphological structures essential for a functional olfactory system are probably present at the time of hatching in *Amphiprion melanopus* embryos. Receptor cells are present in the olfactory epithelium, and axons from the olfactory receptor cells form the olfactory nerve, which leads into the olfactory bulb. We have not provided evidence for the presence of the olfactory tract and its connection into the telencephalon. However, it is highly unlikely that the other olfactory structures develop independently of the olfactory tract and its connection into telencephalon.

With regards to Figure 4A,B, showing a group of seven, 1.5 μm cilia located on the periphery of a protruding cell, this cell is clearly a chemoreceptor cell. Our findings are similar to a study of embryos of rainbow trout (*Oncorhynchus mykiss*) by Hara & Zielinski (1989) for embryos of *O. mykiss* before hatching. In the latter study, an almost identical figure to Figure 4A,B in this study, shows a group of cilia located on the periphery of a protruding cell, mentioned by the authors as a chemical receptor cell (Hara & Zielinski, 1989).

Werner & Lannoo (1994) described the development of the olfactory system of the white sucker, *Catostomus commersoni*, in relation to imprinting and homing. At hatching, the olfactory placode of the white sucker is present and beginning to differentiate. Fourteen days after hatching olfactory cilia are present, the olfactory tract is robust, and the telencephalon is beginning to differentiate. The developmental stage of the olfactory placode in *A. melanopus* at the time of hatching looks identical to the developmental stage of the white sucker 14 d after hatching. According to Werner & Lannoo (1994), the white sucker appears having the fundamental neural structures necessary for its imprinting mechanism at this stage.

The finding of two well-developed secondary bilateral symmetric chemoreceptor systems, between the placodes and above the eyes, supports Whitear's (1992) findings of solitary chemosensory cells in the sand goby *Pomatoschistus minutus*. Again, it suggests that the chemosensory system in newly hatched embryos of the anemonefish *A. melanopus* is well developed. However, the relative importance of this secondary receptor system, and whether the systems are involved with gustation or chemoreception, can only be speculated upon at this stage.

The olfactory developmental schedule described in this paper suggests that the sensitive period during which imprinting can occur may be between day six post-fertilization and day nine (day of hatching). A more precise time schedule of when imprinting occurs can only be speculated upon at this stage. However, the reader should notice that at day six, the placode is only present in a preliminary form, and the granular epithelium of the placode is also present in a preliminary state. No bilateral secondary receptor system is present until day seven. The granular epithelium of the placode, containing the ciliated receptor cells, is now more defined than on the placode of day six. Based on these findings, it therefore seems likely, that imprinting in *A. melanopus* can only happen from day seven post-fertilization.

Most of the changes associated with functionality of the olfactory system in the anemonefish *A. melanopus* occur just prior to hatching. This finding has several ramifications. Firstly, as a minimum one olfactory system is present at hatching, including at least one type of chemoreceptor cell. Presuming the egg case and the chorion membrane of the embryo are permeable for olfactory cues, our findings therefore support the hypothesis that anemonefish larvae of the species *A. melanopus* are capable of receiving chemical cues from their parental host anemone onto which they imprint. Thereby our findings also support the anemonefish host-imprinting hypothesis in general (Miyagawa, 1989; Fautin, 1991; Arvedlund & Nielsen, 1996; Arvedlund et al., 1999, 2000a). Secondly, our findings indicate the ontogenetic timing of this imprinting mechanism happens towards the end of the embryonic development for *A. melanopus*. Thirdly, our results point toward the second of the two hypotheses regarding mechanisms of how settling reef fish larvae recognize the reef. First hypothesis suggest that some reef fish larvae detect the reef by the use of sound, possibly created from the waves breaking on the fringing reefs (e.g. Leis et al., 1996). Second hypothesis suggest that some reef fish larvae use chemotaxis to detect their habitat during settlement (e.g. Fricke, 1974; Murata et al., 1986; Miyagawa, 1989, Arvedlund & Nielsen, 1996; Arvedlund et al., 1999, 2000a). We infer, that the findings in this study point to the hypothesis, that chemotaxis may play an important role in the larval settlement in some reef fish species.

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