Morphological variation and distribution of free neuromasts during half-smooth tongue sole *Cynoglossus semilaevis* ontogeny*

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Abstract   This study was conducted to clarify the distribution and morphology of free neuromasts during the development of half-smooth tongue sole (*Cynoglossus semilaevis*) using scanning electron microscopy. During development, (1) the apical surface of free neuromasts changed in shape from a circle to a four-point star; (2) the external structure changed from being level with the epidermis to papilla-like above the level of the epidermis; (3) the neuromast cupula changed from cylindrical to blade-shaped; (4) the free neuromasts went from occurring singly to being in clusters of a few single organs; (5) the arrangement changed from a linear array to no discernable pattern; (6) there was a significant increase in the number of free neuromasts after metamorphosis. In adult *C. semilaevis*, free neuromasts were only observed on the abocular side of the head. Thus, there were more free neuromasts located on the abocular side of the head with a higher concentration around the anterior nostril and mouth, which may have a mechanical sensory function to help locate food as an adaptation to a benthic mode of life.

Keyword: morphology; structure; cupula; metamorphosis; scanning electron microscopy (SEM)

1 INTRODUCTION

The lateral line system in teleost fish is sensitive to water movements, including currents and acoustic displacement (Harris and van Bergeijk, 1962). It generally comprises a canal system in which the sensory organs, the neuromasts, are enclosed in canals in a regular linear series along the head and trunk, and also a system of free neuromasts that are distributed in either rows or fields of varying density, again on the head and trunk (Blaxter, 1987).

The neuromast, the functional unit of lateral line system, consists of a cupula, a shield-shaped mantle, and a sensory plate containing hundreds to thousands of hair cells (Iwai, 1965; Blaxter, 1987; Münz, 1989; Northcutt, 1989). Although a free neuromast and a canal neuromast exhibit the same basic organization, they differ from one another in a number of ways, including morphology of hair cell arrangement, the type of hair cell, and innervation pattern (Münz, 1989; Song and Northcutt, 1991a, b). The free neuromasts appear to be more sensitive to low frequency vibration than the canal system (Münz, 1985; Kroese and Van Netten, 1989), acting as a type of mechanoreceptor, which play an

important role in detecting planktonic prey and physical stimuli from predators (Mukai, 2006).

Half-smooth tongue sole, *Cynoglossus semilaevis* (Günther, 1873), is one of the main cultured flatfish in China. The adult fish is large with asymmetrical jaws and non-toothed gill rakers typical of benthic-feeding flatfish, while the bothid has symmetric jaws and toothed gill rakers typical of midwater-feeding flatfish (Ma et al., 2006). Previous studies have found that the half-smooth tongue sole attack prey predominantly using information from the lateral line system on the head (Ma et al., 2007a, b). Therefore, the lateral line system may play an important role in feeding. The half-smooth tongue sole lateral line system comprises both the canal and free neuromast systems. The canal system is similar to that of other teleosts, however, the free neuromast system differs from that of previous reports (Ma et al., 2007a). In adult fish, there are two distinct differences: one is that the free neuromasts only occur on the abocular side of the head in high density, the other is that of the morphology of a free neuromast is like a nipple located on the skin and they occur in clusters (Ma et al., 2007a). Flatfish feeding habits closely reflect their morphological and sensory specialization (Livingston, 1987). Therefore, knowledge on the development and distribution of the free neuromast system in half-smooth tongue sole can enhance information on free neuromasts in teleost fish and provide a better understanding of their role in feeding.

2 MATERIAL AND METHOD

2.1 Rearing conditions

Fish were purchased from the Mingbo Aquaculture Company (Yantai, Shandong, China). Rearing water was filtered, UV-sterilized, and maintained at 23±0.3°C. Light intensity was 10–200 lx during the day, and <1 lx at night, with a photoperiod of 12 h L: 12 h D. Feeding with rotifers was initiated 3 days post hatching (dph) with *Artemia nauplii* at 20 dph. Commercial aquaculture diets were used from 40 dph. All experiments were done according to the policies of the University of Maryland and Animal Care and Use Committee.

2.2 Selection of *C. semilaevis*

The half-smooth tongue sole life cycle is divided into five important ontogenetic stages, including the prelarval (1–3 dph), postlarval (3–20 dph), juvenile (20–60 dph), young (60–80 dph), and adult stages.

During its life cycle, the metamorphosis period is generally from 25 to 30 dph. Free neuromasts were examined at 1 dph (body length: 2.50±0.52 mm), 10 dph (7.20±0.325 mm), 15 dph (11.50±0.582 mm), 40 dph (29.80±3.650 mm), 50 dph (49.65±3.389 mm), 60 dph (62.05±3.890 mm), 80 dph (76.25±5.125 mm), and in adults (1.5 years, 330.02±1.420 mm). The eight stages covered the five important ontogenetic stages in the half-smooth tongue sole. Thirty were taken to obtain accurate data for each stage analyzed.
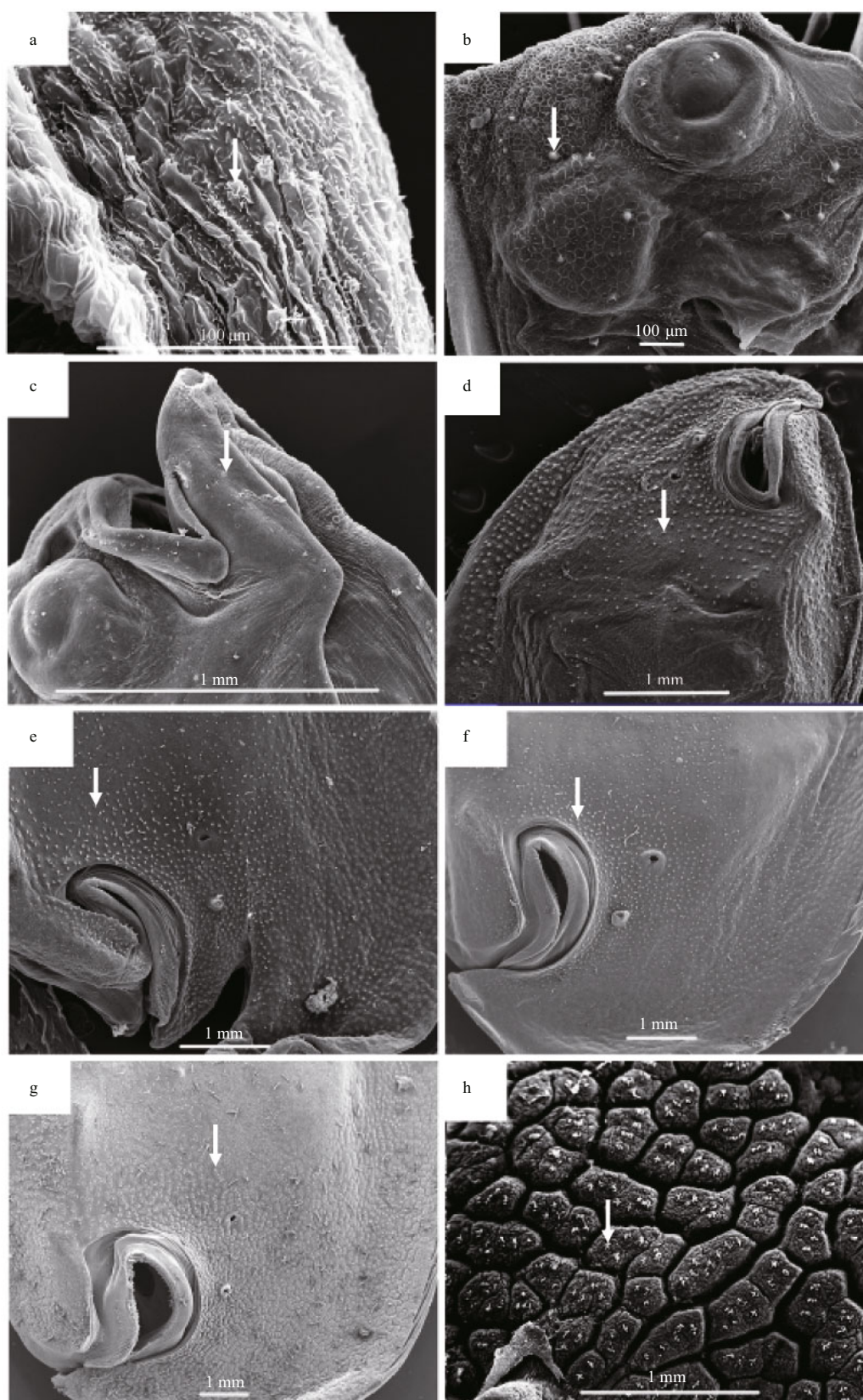
2.3 Experimental procedure

The fish were anaesthetized with a few drops of 25 g/L benzocaine in methanol. Specimens were immediately double-fixed in 5% glutaraldehyde and 1% osmic acid, serially dehydrated with ethanol, and passed through isoamyl acetate (banana oil) (amyl acetate, n-butyl acetate). They were then desiccated with a HCP-2 critical point desiccators (Hitachi, Japan), gold-coated with an Eiko-IB-3 ionic sprayer (Tokyo, Japan), and examined under a KYKY-2800B scanning electron microscope (Beijing, China).

3 RESULT

3.1 Free neuromast distribution in relation to ontogenetic stage

There were no neuromasts visible in 1-dph eleuthero embryos (Fig.1a). Approximately 20 were detected in 10-dph larvae, primarily located on the head, particularly around the eyes and olfactory pits. Neuromasts were also visible on the operculum and cranium (Fig.1b). In 15-dph larvae, neuromasts were chiefly located along the hyomandibular line and the lower jaw (Fig.1c). However, far more neuromasts were observed in 40-dph juveniles than the 15-dph larvae. They were distributed on the entire abocular side of the head. Neuromasts were single and arranged in a regular linear series (Fig.1d). In 50-dph juveniles, the neuromasts were also single, but increased in density. In contrast to the linear array arrangement in the 40-dph juvenile stage, they did not form a discernible pattern (Fig.1e). The neuromasts still occurred singly in the 60-dph young fish stage, in particularly high concentration around the anterior nostril (Fig.1f). In 80-dph young fish, the neuromasts were grouped and clusters of 3–5 neuromasts on the abocular side were separated by rugae in the epidermis. The neuromasts no longer exhibited a linear pattern. The highest concentration of neuromasts was observed around the anterior nostril and mouth, while the lowest

Fig.1 Changes in the distribution of free neuromasts during ontogenetic stages on the abocular side of the head in *C. semilaevis*

a. the skin near the right eye in a 1-dph eleuthero embryo; scale=100 μm; b. 10-dph larva; scale=100 μm; c. 15-dph larva; scale=1 mm; d. 40-dph juvenile; scale=1 mm; e. 50-dph juvenile; scale=1 mm; f. 60-dph juvenile; scale=1 mm; g. 80-dph juvenile; scale=1 mm; h. adult fish; scale=1 mm. White arrows indicate free neuromasts.

was observed along the dorsal edge of the fish (Fig.1g). In adult fish, the distribution of neuromasts, which were only present on the abocular side of the head, was the same as that in the 80-dph young fish. They extended from the tip of the head to just inside the scaly region and sometimes to the posterior of the operculum, but a higher concentration of neuromasts occurred around the anterior nostril and the mouth (Fig.1h).

3.2 Free neuromast morphology in relation to ontogenetic stage

The 1-dph eleuthero embryo had partially folded rugae (Fig.2a). In 10-dph larvae, the external neuromast shape was circular on the skin surface, the round sensory plate of the neuromasts' apical surface contained many cilia (Fig.1b). In 15-dph larvae, the neuromast began to rise up to the surface of the skin (Fig.2c), forming a bump that we called papilla in 40-dph juveniles (Fig.2d). The apical surface at the top of each neuromast expanded and began to take on a narrow rhombus shape in the 15-dph larvae (Fig.2c). After metamorphosis, in 40-dph juveniles, a narrow rhombus shape was apparent across the top of each papilla (Fig.2d). The outline of the apical surface of the papilla appeared as a four-pointed area in the 50-dph juveniles, the sensory plate increased in size as the outline of the apical surface changed from round to rectangular (Fig.2e). Rugae appeared in the epidermis around the papilla (Fig.2e). The outline of the apical surface and the external papilla shape in the 60- and 80-dph juveniles were similar to that of the adult fish. Rugae were more defined around the papillae (Fig.2f, g). In adult fish, the papillae occurred in clusters surrounded by rugae, with a few single and one four-lobed mantle creating a four-point opening over each papilla; the sensory plate consisted of hundreds of hair cells with cilia (Fig.2h).
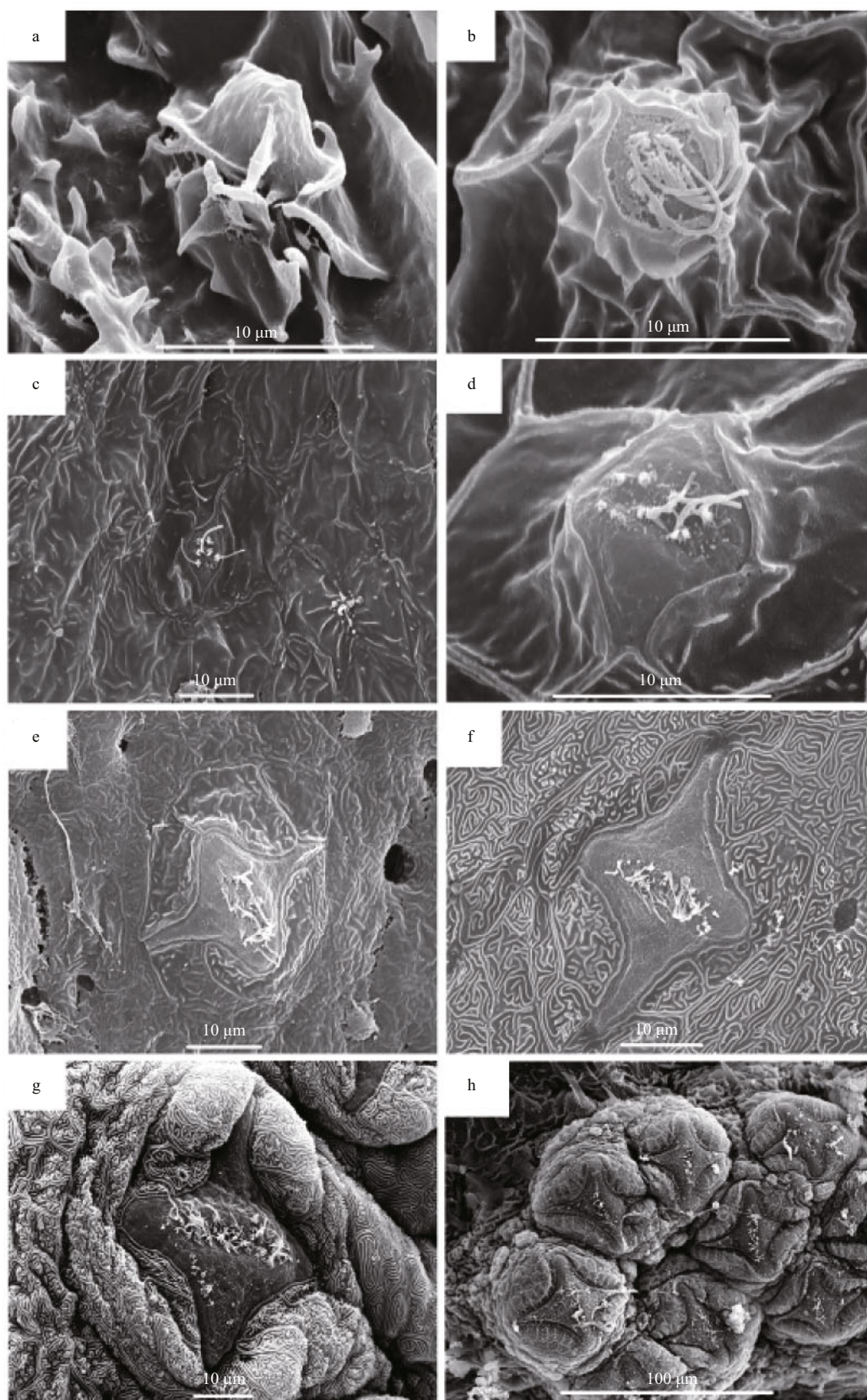
The cupula was blade-shaped protruding from the apical surface of the neuromast. In 10-dph larvae, the cupula was cylindrical (Fig.3a). After metamorphosis (40-dph juveniles), the cupula progressed from cylindrical to blade-shaped (Fig.3b). In 50-dph juveniles, the cupula was larger and flatter than in the 40-dph juveniles (Fig.3c). In adult fish, the cupula was approximately 50 μm in width, wider than in the juvenile fish (Fig.3d).

4 DISCUSSION

In this study, a few free neuromasts were observed in 1-dph half-smooth tongue sole fry, similar to other flatfish fry. Kawamura and Ishida (1995) described

Japanese flounder, *Paralichthys olivaceus* (Temminck and Schlegel, 1846), as having one neuromast on each side of the head on hatching. In plaice, *Pleuronectes platessa* (Linnaeus, 1758), there are three neuromasts on each side of the head on hatching (Neave, 1986). In 10-dph half-smooth tongue sole larvae, the free neuromasts were mostly found around the eyes and olfactory pits, similar to the distribution reported for plaice and sole, *Solea solea* (Linnaeus, 1758) (Harvey et al., 1992). In previous studies, 3-dph larvae began to ingest rotifers, but the retinomotor responses do not appear until 15 dph (Ma et al., 2007b). At that stage the canal neuromasts had not formed; presumably, the free neuromasts played an important role in detecting stimuli to help feeding in larvae. Bluefin tuna, *Thunnus orientalis* (Temminck and Schlegel, 1844), larvae can avoid glass pipets upon hatching because of the presence of free neuromasts (Kawamura et al., 2003). After metamorphosis in half-smooth tongue sole, the bilaterally symmetrical larva changes to a metamorphosed fish that lies on one side; the feeding behavior also changes because the bilaterally symmetrical larva fish are pelagic, feeding on plankton using their visual sense. The metamorphosed fish inhabit sandy or muddy sea beds, feeding at night using the lateral line system on the head to detect prey (Ma et al., 2007a, b). Therefore, at the 40-dph stage, more free neuromasts located on the abocular side of the head may play a mechanical sensory role to help locate prey as an adaptation to a benthic mode of life.
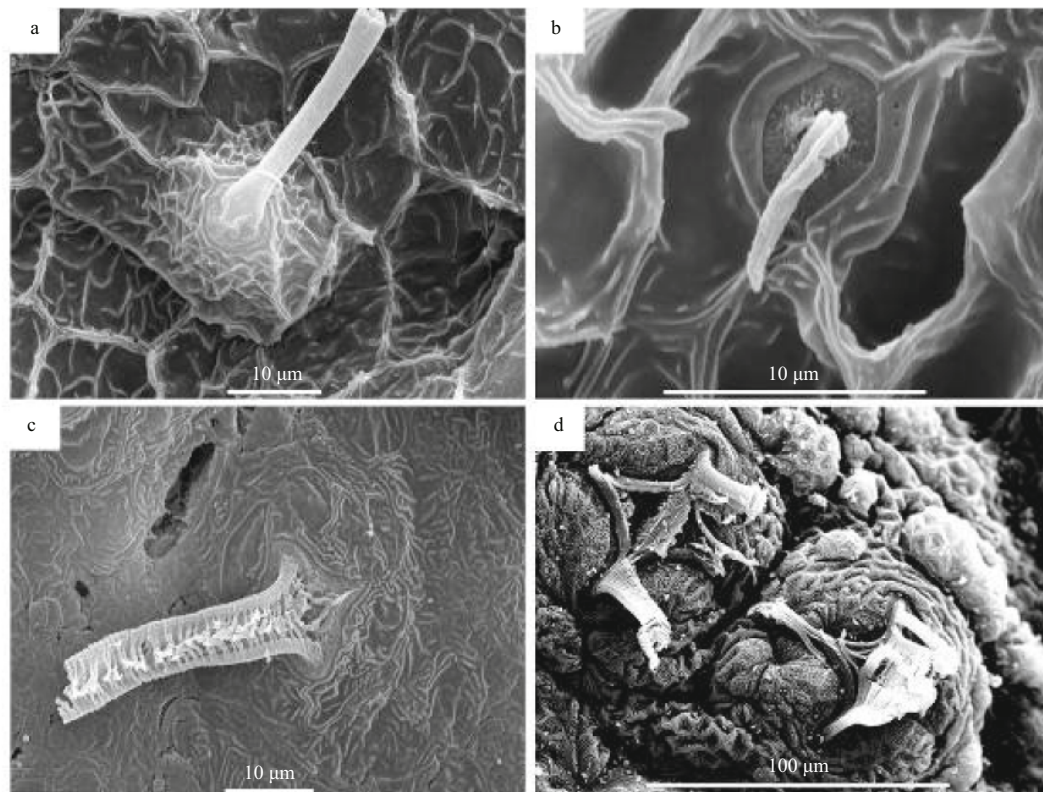
This is the first report on the distribution of free neuromasts in adult half-smooth tongue sole. In contrast to reports on other flatfish, we only observed free neuromasts on the abocular side of the head, arranged irregularly in adult fish. In Senegalese sole, *Solea senegalensis* (Kaup, 1858), free neuromasts arranged in a linear form were found mainly on the abocular side of the fish (Appelbaum and Schemmel, 1983). In New Zealand sole, *Peltorhamphus novaezeelandiae* (Günther, 1862), three longitudinal rows of free neuromasts are present on the body, one on the ocular side and two on the abocular side in addition to the lateral line canals (Roper, 1981). In plaice and sole, free neuromasts also appear on both the ocular and abocular sides (Harvey et al., 1992). *S. solea* have more free neuromasts on the abocular side than plaice (Harvey et al., 1992). We also found a higher concentration around the anterior nostril in adult half-smooth tongue sole. Sole are most active at night and probably use a combination of olfaction and mechanoreception to detect prey using the lateral line system (De Groot,

Fig.2 Morphological variation of free neuromasts during ontogenetic stages in *C. semilaevis*

a. 1-dph eleuthero embryo; scale=10 μm; b. 10-dph larva; scale=10 μm; c. 15-dph larva; scale=10 μm; d. 40-dph juvenile; scale=10 μm; e. 50-dph juvenile; scale=10 μm; f. 60-dph juvenile; scale=10 μm; g. 80-dph juvenile; scale=10 μm; h. adult fish; scale=100 μm.

Fig.3 Changes in free neuromast cupula morphology during ontogenetic stages in *C. semilaevis*

a. 10-dph larva; scale=10 μm; b. 40-dph juvenile; scale=10 μm; c. 50-dph juvenile; scale=10 μm; d. adult fish; scale=100 μm.

1971). In contrast, plaice depends mainly on vision for feeding and do not appear to depend on the lateral line system (Harvey et al., 1992). Thus, the number of free neuromasts on the abocular side in sole and half-smooth tongue sole may be indicative of their role in feeding. Large numbers of free neuromasts on the abocular side of the head in half-smooth tongue sole, arranged randomly and in groups, suggested that abrasion of neuromasts is not a serious problem, and the sensitivity of the abocular side in all directions may be enhanced for prey detection.

The free neuromast morphology observed in adult half-smooth tongue sole has been rarely reported in other flatfish. In *P. novaezealandiae*, the free neuromast is protected by two lappets, with the largest and best-developed on the abocular side of the head (Roper, 1981). In Senegalese sole, free neuromasts on the head are protected by bud-like epidermal structures, which may be up to 3 mm long (Appelbaum and Schemmel, 1983). In sole, a papilla-like structure is located next to the free neuromast and does not constitute a part of the organ (Harvey et al., 1992). However, the structure of the free neuromast in half-smooth tongue sole differed in that it was papilla-like and formed groups separated at the base by epidermal

rugae. Curiously, the structure of the free neuromast in half-smooth tongue sole was similar to that of the deep-sea fish *Phrynichthys wedli* (Pietschmann, 1926), in which shield-shaped neuromasts with elongated flanges overlay each papilla (Marshall, 1996). In our study, the papilla was a component of the free neuromast. The papilla on the top of the epidermal rugae may protect the apical surface from damage (Roper, 1981; Appelbaum and Schemmel, 1983; Harvey et al., 1992). We found extensive rugae around the free neuromast, which may protect the free neuromast from damage, as do lappets, which are epidermal bud-like structures (Appelbaum and Schemmel, 1983; Harvey et al., 1992). However, our study provides no evidence for this and the function of the rugae remains unknown.

The apical surface of free neuromasts changed from circular to a four-pointed star, and the cupula changed from cylindrical to blade-shaped during the same stage of development. The sensory cell cilia on the apical surface are excited as a result of displacement of the cupula by physical stimuli (Dijkgraaf, 1963; Blaxter, 1987). The polarity of sensory cells coincides with the minor axis of the neuromast apical surface (Mukai and Kobayashi, 1995). Thus, the cylindrical cupula can

probably bend in any direction, while blade-like cupula only bend in the direction of the apical surface minor axis (Mukai et al., 1992). Therefore, in adult half-smooth tongue sole, the four-pointed star-shaped apical surface and the blade-like cupula seem to be crucial to free-neuromast function and may impart specific polarity and directional sensitivity to the neuromast. It is likely that the orientation of free neuromasts in adult half-smooth tongue sole follows a definite pattern that can provide accurate information on the movements of potential prey.

5 CONCLUSION

Fish feeding behavior is associated with the interaction of organs receptive to visual, mechanical, chemical, and electromagnetic stimuli (Faucher et al., 2006). To compensate for loss of vision, the lateral line system plays an important role in feeding (Ma et al., 2007a, b). In our study, the free neuromasts mainly performed a mechanosensory role in feeding by sensing the water environment in all directions. Our results suggest that the unusual morphology and irregular distribution of the free neuromasts may enhance sensitivity to detect stimuli and help feeding. The function of free neuromasts in half-smooth tongue sole should be further investigated through behavioral and genetic studies to confirm this.

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