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Authors: Otsuka, Machiko, and Nagai, Shin-ichi

Source: Zoological Science, 14(3) : 475-481

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.14.475

ZOOLOGICAL SCIENCE **14**: 475–481 (1997)

Neuromast Formation in the Prehatching Embryos of the Cod Fish, *Gadus macrocephalus* Tilesius

Machiko Otsuka and Shin-ichi Nagai

Department of Biology, Dokkyo University School of Medicine, Mibu-machi, Tochigi 321-02, Japan

ABSTRACT—To clarify when the free neuromasts of fish become functional, the neuromast formation in the embryonic development of the cod fish *Gadus macrocephalus* Tilesius was investigated. The initial appearance of placodes and an increase in the number and distribution of free neuromasts were followed using light microscopy and scanning electron microscopy. Subsequently, the appearance of afferent and efferent nerve endings of the free neuromasts was investigated by transmission electron microscopy. Sections of embryos 72 hr before hatching revealed a pair of placodes on the head and two pairs on the trunk. The placodes increased in number, and some of them differentiated into free neuromasts during the embryonic stage. There were three pairs of free neuromasts on the head and four pairs on the trunk in newly hatched larvae. The afferent nerve endings were recognized first in the free neuromasts on the trunk at about 36 hr before hatching, and the efferent nerve endings were recognized on the trunk at about 24 hr before hatching. On the head, afferent nerve endings were seen at about 12 hr before hatching; the efferent nerve endings were not yet seen in newly hatched larvae.

INTRODUCTION

Free neuromasts are known as a type of fish lateral line organ that are formed on the head and body during the early developmental stages. They first appear as placodes and differentiated into free neuromasts. Some of them are incorporated into canals at the last larval stage.

There are many reports concerning the development, morphological changes, distribution and sensory cell polarity of the free neuromasts during the larval stage (Blaxter, 1984a,b; Blaxter *et al.*, 1983; Cahn and Shaw, 1962; Harvey *et al.*, 1992; Iwai, 1963b, 1967, 1972; Kawamura and Ishida, 1985; Kawamura *et al.*, 1983; Metcalfe *et al.*, 1985; Mukai and Kobayashi, 1995; Mukai *et al.*, 1992; Sato, 1952). Some species of fish embryos are known to have some free neuromasts and cupulae on the head and body (Iwai, 1963a, 1964, 1965; Mukai and Kobayashi, 1993). However, few reports have described the initial formation of placodes and their subsequent differentiation as the embryo grows (Iwai, 1963a; Sato, 1952). To investigate the functions of free neuromasts, some investigators have used electrophysiology methods (Katsuki and Yoshino, 1950; Katsuki *et al.*, 1950, 1951; Kawamura and Yamashita, 1983; Suga, 1967). Histologically, it is generally believed that neuromasts showing two types of nerve endings, afferent and efferent, are functional (Iwai, 1972). Metcalfe *et al.* (1985) reported on the nerve endings of the neuromasts in five-day-old zebrafish larvae. However, there are neither electrophysiological nor histological studies on the functions of free neuromasts in fish embryos.

This paper describes the results of an investigation into the morphology and function of free neuromasts in the developing cod embryo of *Gadus macrocephalus* Tilesius using light microscopy, scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

MATERIALS AND METHODS

Artificially fertilized *Gadus macrocephalus* Tilesius eggs were obtained from the Japan Cultivation Fisheries Association of Noto Station, Ishikawa Prefecture. They were transferred to the laboratory aquarium where the water was kept at 9°C.

Embryos were fixed every 6-12 hr from 7 days after fertilization without being anesthetized in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M phosphate buffer solution (pH. 7.4) for 1.5 hr at room temperature. They were rinsed with the buffer solution for more than 12 hr at 4°C. The egg membrane was carefully removed by forceps in the buffer solution under a binocular and post-fixed in 1% osmic acid for 2 hr at 4°C. For SEM observation, specimens were dehydrated in graded ethanols, dried at the critical point with liquid CO_2, sputtered with gold and examined with a HITACHI S-600 microscope. For the TEM examination, specimens were dehydrated in graded acetones and embedded in an epoxy resin (Epon 812). A JEOL 200 CX microscope was used for observation. Semithin sections (about 1 μm) of the epoxyembedded specimens were also observed under a light microscope after staining with toluidine blue.

Embryonic stages are designated by hours before hatching (-72 hr, -48 hr etc.). The placodes and neuromasts are numbered in order of appearance, on the head (I, II, etc.) and on the trunk and tail (i, ii, etc.).

* Corresponding author: Tel. +81-282-87-2118; FAX. +81-282-86-6214.

RESULTS

Embryonic development and formation of free neuromasts

The incubation period was about 240 hr, and the isolation of a tail from the yolk sac was recognized around 92 hr after fertilization. Table 1 shows the development of the free neuromast, and Figs. 1 and 2 show the distribution of the free neuromasts formed during the embryonic stage. In the embryos 72 hr before hatching (-72 hr), the protuberance of the embryonic body from the yolk sac is slight and the end of the tail reached to an eye by curling of the body. Lightcolored melanophores were distributed all over the body. The nasal cavities were seen, the lenses of the eyes were already developed, and pectoral fins were seen as bud-like processes. Sections showed that a pair of narrow cracks (gill holes) are open to the exterior below the auditory vesicles. At this stage there were a pair of placodes on the head, placode I, about 50 μm wide and 23 μm high, located upper-posterior to the eyes (Fig. 3A). On the trunk, there were two pairs of placodes: placode i, about 50 μm wide and 22 μm high, posterior to the pectoral fin and placode ii, about 38 μm wide and 20 μm high, at the middle of the trunk. These placodes showed cupula-holes. The diameter of the cupula-hole of placode I was 3 μm, of placode i, 7.5 μm, and of placode ii, 2.5 μm.

At about -48 hr, the number of placodes increased (Table 1). On the head, in addition to placode I, there were another four pairs of placodes: placode II, between the nasal cavities; placode III, upward of the eyes; placode IV and placode V, anterior and posterior to the abdominal side of the eyes (Fig. 2). There were one or two more placodes, placode iii and placode iv, on either side of the trunk (Fig. 1).

At about -40 hr, placode i was differentiated into a free neuromast (Table 1) whose apical surface was visible by SEM and was about 7.5 μm in width, with a few kinocilia and stereocilia.

At about -36 hr, the tail curved over the trunk and head and its tip reached the pectoral fin which protruded semicircularly (Fig. 4A). The arrowhead of Fig. 4C shows a gill hole cracking inside the body, and its external appearance by SEM is shown by the arrowhead in Fig. 4A.

Twenty-four hr before hatching, the melanophores were distributed on the head and aggregated on the dorsal side of

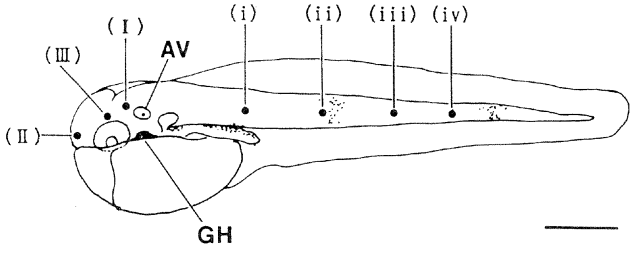

Fig. 1. Distribution of free neuromasts (black spots) on the body in a newly hatched larva of the cod fish *Gadus macrocephalus*. AV, auditory vesicle; GH, gill hole. Scale bar = 0.5 mm.

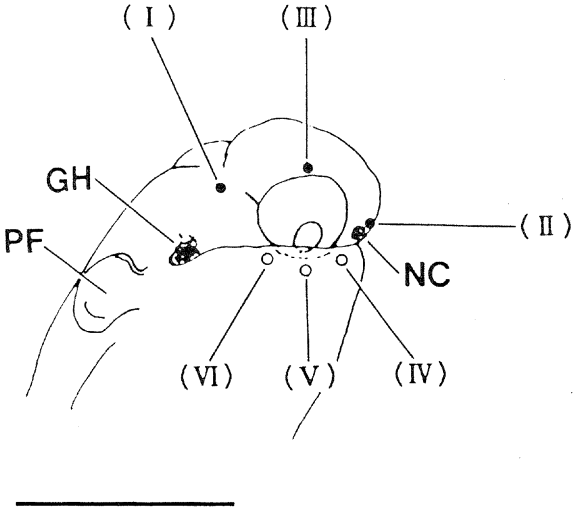

Fig. 2. Distribution of free neuromasts and placodes on the head of a newly hatched larva of the cod fish. Black spots show free neuromasts, and open spots show placodes. NC, nasal cavity; GH, gill hole; PF, pectoral fin. Scale bar = 0.5 mm.

Table 1. Free neuromast formation in *Gadus macrocephalus* embryos

	Organs											
Age (hr)	I	II	III	IV	V	VI	i	ii	iii	iv	v	vi
−72	PL, CH						PL, CH	PL, CH				
−48		PL, CH	PL	PL	PL				PL	PL		
−40			CH				NM					
−36							aNE		CH			
−24	NM, aNE	NM		CH	CH		eNE	NM		CH	PL, CH	
−12						PL, CH		aNE, eNE	NM			PL, CH
newly hatched larva	NM, aNE	NM	NM	PL, CH	PL, CH	PL, CH	NM*	NM*	NM	NM	PL, CH	PL, CH

Developmental stages of neuromasts are shown in five steps. PL, placode; CH, cupula-hole; NM, neuromast; aNE, afferent nerve ending; eNE, efferent nerve ending.
NM*, functional neuromast.

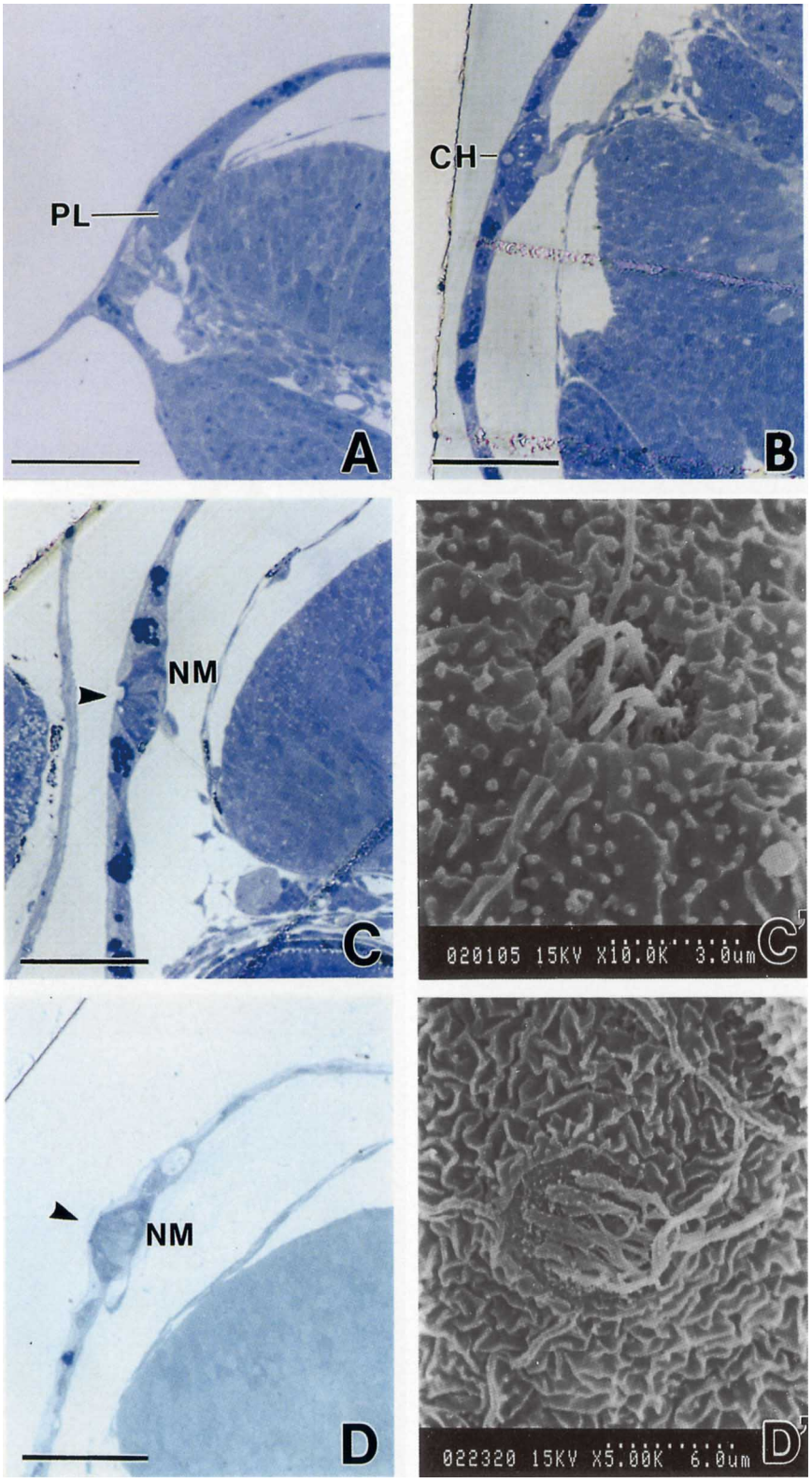

Fig. 3. Morphological changes of free neuromast I, from placodes to neuromasts of the cod fish. **A**, **B**, **C**, **D**, photomicrographs; **C'**, **D'**, scanning electron micrographs; **A**, -72 hr; **B**, -40 hr; **C**, **C'**, -24 hr; **D**, **D'**, newly hatched larva. Higher magnifications of apical surface of neuromasts in **C** and **D** (arrowheads) are shown in **C'** and **D'**. PL, placode; CH, cupula-hole; NM, neuromast. Scale bars in **A**, **B**, **C**, **D** = 50 μm.

the digestive duct and two places of the tail. At this stage, placodes I and II on the head and placode ii on the trunk differentiated into free neuromasts (Table 1). The apical surface of neuromast I was caved in and was about 8 μm in width with five-to-six kinocilia about 4.7 μm long (Fig. 3 C,C'). The diameter of the apical surface of neuromast II was 7.5 μm, and there were few cilia; the apical surface of neuromast i was about 10 μm wide, and nine-to-ten kinocilia about 3.6 μm long were seen.

Twelve hr before hatching, placode VI with a cupula-hole was seen behind the placode V on the head (Fig. 2). On the trunk, placode iii became visible by SEM.

Just after hatching, the body length of the larva was about 3.8 mm. On the head, only the top of the snout was isolated from the yolk sac (Fig. 4B). There were still only one pair of gill holes, but gills were observed in the sections cut at the level of the holes (Fig. 4B,D). An anus was formed. The pectoral fins became disc-like shapes (Fig. 4B). Newly hatched larvae had three pairs of free neuromasts and three pairs of placodes on the head (Fig. 2) and four free neuromasts and two placodes on either side of the trunk. Figure 3D shows a free neuromast projected from the epidermis with a flat apical surface. The cupula-holes had disappeared by this stage. The apical surface of a free neuromast detected by SEM is shown in Fig. 3 D'. Free neuromast i exhibited the largest apical surface, 12.5 μm in width.

The mouth was recognized as a thin groove in larvae about one day old. In about larvae two days old, with a body length of 4.5 mm, the movement of the mouth, twitching, could be seen. After hatching, the pigmentation of the eyes developed, but heavy black pigmentation was seen two days after hatching. The cupulae could be seen clealy in one-day-old larvae for the first time; it was about 35 μm long, present on free neuromast i.

TEM observation and formation of the nerve endings

At about -36 hr, a nerve bundle about 2.7 μm in diameter was in contact with a free neuromast. In free neuromast i, an

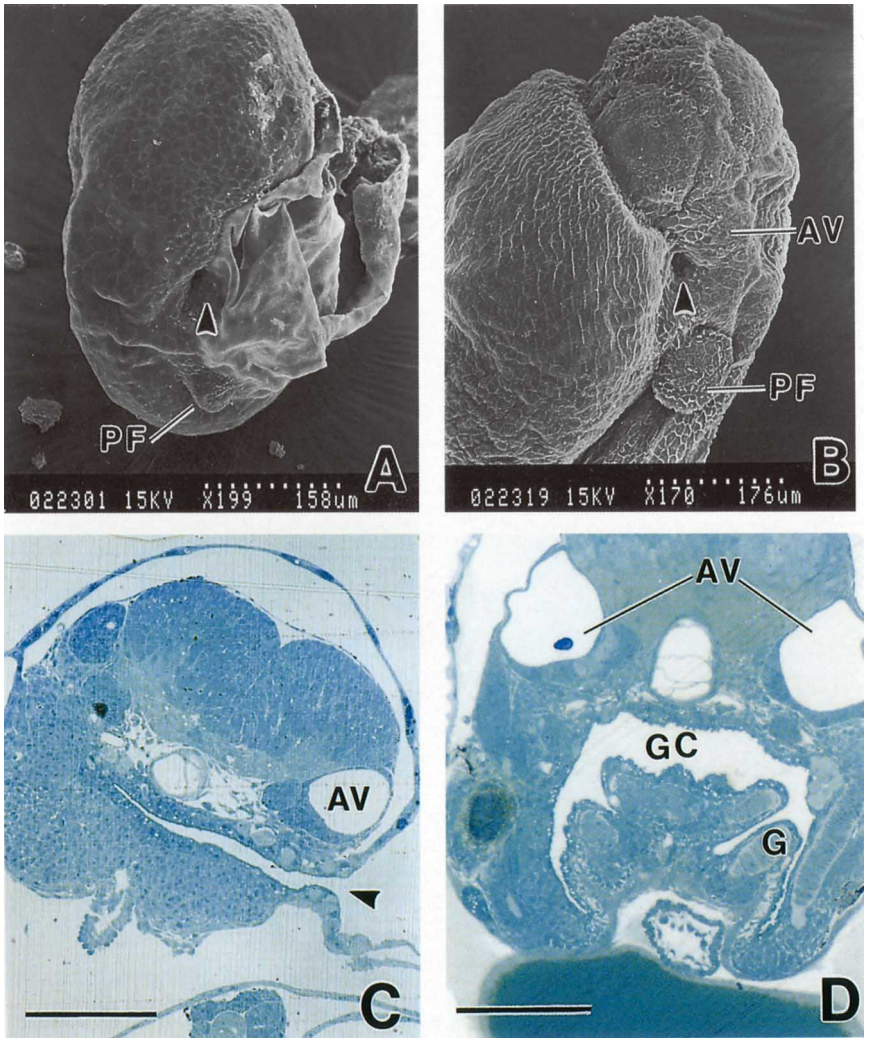

Fig. 4. Scanning electron micrographs and photomicrographs of embryo and newly hatched larva of the cod fish. **A**, **C**, -36 hr embryo; **B**, **D**, newly hatched larva; **C**, **D**, transverse sections at about the position of gill holes (arrowheads). PF, pectoral fin; AV, auditory vesicle; G, gill; GC, gill cavity. Scale bars in **C**, **D** = 100 μm.

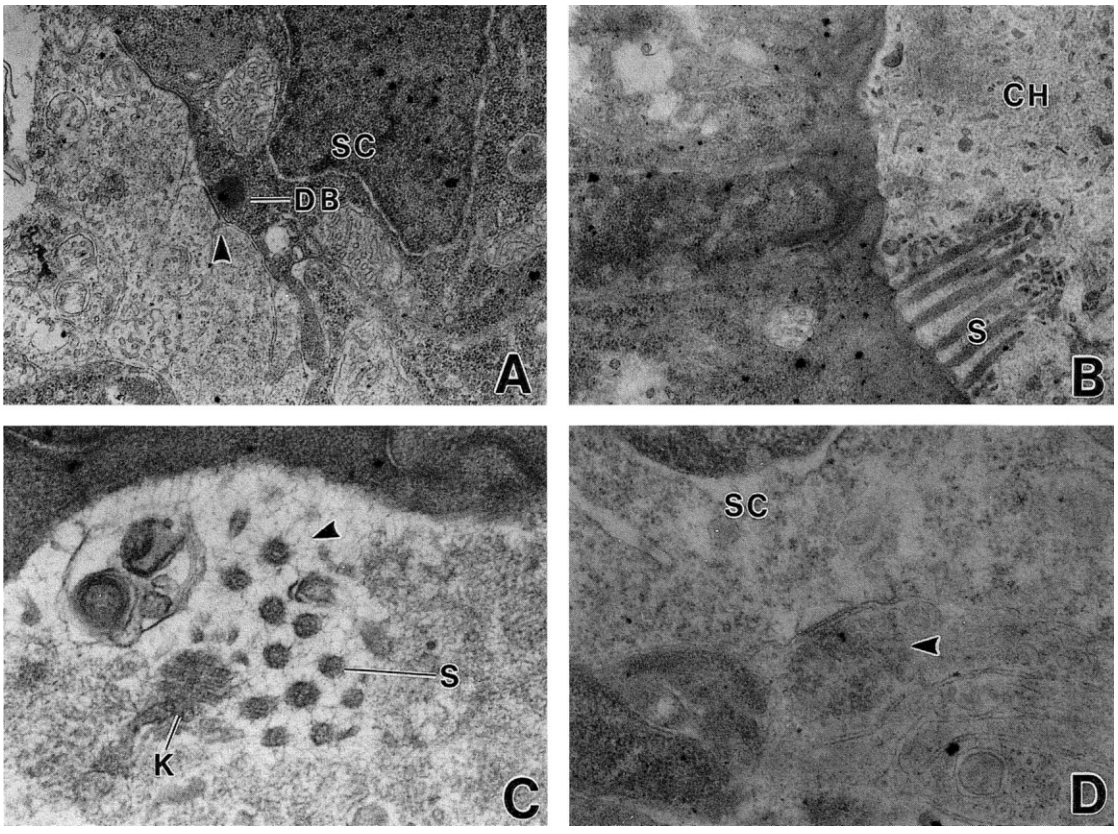

Fig. 5. Electron micrographs of placodes of the cod fish. **A**, **B**, **C**, -36 hr; **D**, -24 hr. (**A**) Afferent nerve ending (arrowhead) formed in free neuromast i. × 20,000. (**B**) Cupula-hole of free neuromast I, and indeterminate formed substances. × 20,000. (**C**) Cupula-hole of free neuromast ii. There is a fibrous network (arrowhead) between stereocilia. × 50,000. (**D**) Efferent nerve ending of neuromast i. A terminal of the neuron contains synaptic vesicles (arrowhead). × 30,000. SC, sensory cell; DB, dense body; CH, cupula-hole; S, stereocilia; K, kinocilia.

afferent nerve ending was formed between the nerve cell and the sensory cell. A dense body about 250 μm in diameter was seen in the base of the sensory cell (Fig. 5A). The apical surface of neuromast I was caved in (cupula-hole) and filled with indeterminate formed substances (Fig. 5B). One kinocilium and at least 29 streocilia composed a group of cilia, and the kinocilium was located in the periphery of this group. There were at least five groups of cilia in one neuromast, and they were located in opposite directions from each other. There was a fine fibrous network (Flock, 1965) between the stereocilia (Fig. 5C).

At about -24 hr, in neuromast I, at least five kinocilia were recognized and an afferent nerve ending was also seen. An efferent nerve ending was observed in free neuromast i; there was an accessory double membrane at the sensory cell side, and the nerve cell adjacent to this double membrane contained synaptic vesicles (Fig. 5D).

At about -12 hr, afferent and efferent nerve endings were recognized in neuromast ii. The periphery of the nucleus of the sensory cells often shows deep recesses. The nucleus of the supporting cells arranged at the basement of the neuromast, and there were well-developed endoplasmic reticulum around the nucleus. In neuromast i, one kinocilium and at least 32 stereocilia comprised a group of cilia.

DISCUSSION

We observed that in *Gadus macrocephalus*, the placodes increased in number, and some of them differentiated into free neuromasts before hatching. The number of free neuromasts of newly hatched larvae depends on the species (Iwai, 1972; Mukai and Kobayashi, 1995). *Gadus morhua* L. possess two pairs of free neuromasts on the head and five on either side of the trunk (Blaxter, 1984b), but *G. macrocephalus* has three pairs on the head and four on either side of the trunk. According to Blaxter (1984b), the proliferation of neuromast organs in *G. morhua* is slow; 40 days after hatching, the larvae still only have nine pairs on the trunk. Blaxter suggested that this is due to the more shortened body form of this cod larvae.

Three pairs of placodes with cupula-holes ware recognized in the -72 hr *G. macrocephalus* embryos. As shown in Table 1, placode i, which is located posterior to the pectoral fins on the trunk (Fig. 1) was the placode that was formed first and also differentiated into a free neuromast first. The time required for the differentiation from placode to free neuromast varied. Placode i took about 30 hr to differentiate into a free neuromast. Placode I, on the head, took about 48 hr to differentiate into a free neuromast. Neuromasts IV and V were

formed on the abdominal side of the eyes and became visible by SEM after the yolk was absorbed. Neuromasts IV was seen in 1-day-old larvae. On the trunk, the proliferation of free neuromasts seemed to occur in sequence from anterior to posterior in proportion to the body growth.

Placodes are made in the epidermis, and there is a cupula-hole at the center of the placodes (Fig. 3 A,B). The cupula-hole is a hollow made as a result of the radially arranged cells of the placode (Iwai, 1963a) and filled with indeterminate formed substances (Fig. 5B). When the apical surface was open to the exterior, it was caved in and became visible by SEM (Fig. 3C,C'). By hatching, free neuromasts projected from the epidermis and their apical surface became flat (Fig. 3D,D') and the cupula-holes disappeared. According to Iwai (1967), cupula-holes are seen for a short period before the cupula appears. The apical surface of free neuromasts of the two-day-old larvae is rather hemispherically projected.

In killifish medaka, *Oryzias latipes*, Sato (1952) described that in some cases one placode is differentiated into two or three neuromasts during the embryonic growth. We did not observe such a phenomenon in *G. macrocephalus* embryos.

There is general agreement that the formation of two kinds of nerve endings of the sensory organs indicates that the organs are functional (Iwai, 1967,1972). The neuromasts in oneday-old medaka larvae (Iwai, 1964), and in five-day-old zebrafish larvae (Metcalfe *et al.*, 1985) are suggested to be functional. Iwai (1964) also reported that these two types of nerve endings appear after the cupula formation is accomplished. Therefore, in *G. macrocephalus*, neuromast i seems to be functional at least at -24 hr, and neuromast ii is functional at about -12 hr. However, the cupulae were not observed until the larva was 24 hr old. They might have been lost due to handling, because they are easily broken. On the head, in contrast, efferent nerve endings were not seen during the embryonic stage. They seem to be formed by the first feeding stage of the larva (Blaxter and Fuiman, 1989). In *G. macrocephalus* embryos, the free neuromasts found on the trunk are functional earlier than those on the head.

The developmental stage at which free neuromasts would first be functional seems to differ among species. It probably depends on habitat and habits, especially just after hatching. The ayu *Plecoglossus altivelis* and cyprinid fish *Gnathopogon elongatus caerulescens* have well developed free neuromasts with cupula when they hatch. The former needs such well-developed neuromasts because just after hatching, they swim actively and downward into the sea. In the latter, which usually feed on animal plankton, it is thought that well-developed neuromasts are essential to detect the fine water flow produced by their prey. In contrast, another cyprinid fish, *Zacco platypus*, is hatched with undeveloped free neuromasts or eyes. They stay in the spawing bed at the bottom of the river about four days after hatching until these organs develop further. (Kawamura and Ishida, 1985; Kawamura *et al.*, 1983).

In *G.macrocephalus*, we observed that during the embryonic development, there were a pair of ectodermal invaginations below the auditory vesicles, as indicated by the arrowheads in Fig. 4A and B. Sections cut at this level showed that these invaginations eventually meet the gill cavity (Fig. 4C,D). We coined the term "gill hole" to refer to them, since there is no previous report of these holes. We recognized the same invagination in other kinds of fish embryos (unpublished data). We suspect that they are involved with respiration, and are one of the important aspects of morphogenesis taking place in the fish embryo.

ACKNOWLEDGMENTS

We are grateful to Prof. M. Tanaka of the Kyoto University and Mr. K.Yoseda of the Japan Cultivation Fisheries Association of Noto Station for kindly supplying the fertilized eggs.

REFERENCE

Blaxter JHS (1984a) Cupular growth in herring neuromasts. J Mar Biol Ass UK 64: 935–938

Blaxter JHS (1984b) Neuromasts and cupular growth of cod larvae. Flødevigen Rapportserie 1: 183–188

Blaxter JHS, Fuiman LA (1989) Function of the free neuromasts of marine teleost larvae. In "The Mechanosensory Lateral Line: Neurobiology and Evolution" Ed by S Coombs, P Görner, H Münz, Springer-Verlag, New York, pp 481–499

Blaxter JHS, Gray JAB, Best ACG (1983) Structure and development of the free neuromasts and lateral line system of the herring. J Mar Biol Ass UK 63: 247–260

Cahn PH, Shaw E (1962) The first demonstration of lateral line cupulae in the mugiliformes. Copeia 1: 109–114

Flock Å (1965) Electron microscopic and electrophysiological studies on the lateral line canal organ. Acta Oto-laryngol Suppl 199: 1–90

Harvey R, Blaxter JHS, Hoyt RD (1992) Development of superficial and lateral line neuromasts in larvae and juveniles of place (*Pleuronectes platessa*) and sole (*Solea solea*). J Mar Biol Ass UK 72: 651–668

Iwai T (1963a) Development of lateral-line cupulae in the gobioid fish, *Tridentiger trigonocephalus* (Gill). Bull Misaki Marine Biol Inst Kyoto Univ 4: 1–20

Iwai T (1963b) Sensory cupulae found in newly hatche larvae of *blennius yatabei* Jordan et Snyder. Bull Japan Soc Sci Fish 29: 503–506

Iwai T (1964) Development of cupulae in free neuromasts of the Japanese medaka, *Oryzias latipes* (Temminck et Schlegel). Bull Mar Biol Ins Kyoto Univ 5: 31–37

Iwai T (1965) Notes on the cupulae of free neuromast in larvae of the goldfish. Copeia 1965: 379

Iwai T (1967) Structure and development of lateral line cupulae in teleost larvae. In "Lateral Line Detectors" Ed by P Cahn, Indiana Univ Press, Bloomington, pp 27–44

Iwai T (1972) On the free neuromasts of some teleost larvae. Japan J Ichthyol 19: 307–311 (in Japanese with English abstract)

Katsuki Y, Yoshino S (1950) The electro-physiological and histological study of the lateral-line nerve system of fish. Japan J Ichthyol 1: 127–134 (in Japanese with English resume)

Katsuki Y, Yoshino S, Chen J (1950) Action current of the single lateral-line nerve fiber of fish. I. On the spontaneous discharge. Jap J Physiol 1: 87–99

Katsuki Y, Yoshino S, Chen J (1951) Action current of the single lateral-line nerve fiber of fish. II. On the discharge due to stimulation. Jap J Physiol 1: 179–194

Kawamura G, Ishida K (1985) Changes in sense organ morphology and behavior with growth of the flounder *paralichthys olivaceus*.

Bull Jap Soc Sci Fish 51: 155–165
Kawamura G, Mukai Y, Ohta H (1983) Morphology of the eye and free neuromast of larval ayu and pale chub and its relation to behavior in water flow. Bull Jap Soc Sci Fish 49: 1527–1532 (in Japanese with English abstract)
Kawamura T, Yamashita S (1983) Chemical sensitivity of lateral line organs in the Goby, *Gobius giurinus*. Comp Biochem Physiol 74A: 252–258
Metcalfe E, Kimmel CB, Schabtach E (1985) Anatomy of the posterior lateral line system in young larvae of the zebrafish. J Com Neu 233: 377–389
Mukai Y, Kobayashi H (1993) Extremely long cupulae of embryonic neuromasts in cyprinid fish. Copeia 1993: 1157–1159
Mukai Y, Kobayashi H (1995) Development of free neuromasts with special reference to sensory polarity in larvae of the willow shiner, *Gnathopogon elongatus caerulescens* (Teleostei, Cyprinidae). Zool Sci 12: 125–131
Mukai Y, Kobayashi H, Yoshikawa H (1992) Development of free and canal neuromasts and their directions of maximum sensitivity in the larvae of ayu, *Plecoglossus altivelis*. Japan J Ichthyol 38: 411–417 (in Japanese with English abstract)
Sato M (1952) Medaka no sokusenki no hassei. Kagaku 22: 544–545 (in Japanese)
Suga N (1967) Electrosensitivity of canal and free neuromast organs in a gymnotid electric fish. J Comp Neur 131: 453–458

(Received November 8, 1996 / Accepted February 24, 1997)