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Development of Free Neuromasts with Special Reference to Sensory Polarity in Larvae of the Willow Shiner, *Gnathopogon elongatus caerulescens* (Teleostei, Cyprinidae)

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ABSTRACT—To find how larval fish sense mechanical stimuli *via* their free neuromasts, we examined morphological changes in free neuromasts in the larval willow shiner, *Gnathopogon elongatus caerulescens*. Free neuromasts were found on the body surface of newly hatched larvae and their number increased on both the head and trunk with larval growth. The apical surface of free neuromasts changed in outline from a circle to a lozenge shape as the number of sensory cells increased in the prelarval stage, and then the cupulae of the free neuromasts changed from a stick-like to a blade-like shape. Seven-day-old larvae were at the postlarval stage and had many free neuromasts that were nearly mature. All free neuromasts contained sensory cells of opposing polarity. The orientation of the maximum sensitivity of free neuromasts, decided from the polarity of the sensory cells, coincided with the minor axis of the lozenge-shaped outline of the apical surface of the free neuromasts, and was in the same axis as the direction in which the blade-like cupulae bent. The change to a blade-like shape would cause a stimulus parallel to the minor axis to be perceived as being stronger than the same stimulus from other directions. The polarity of trunk neuromasts was usually oriented along the antero-posterior axis of the fish body, but a few had dorso-ventral orientation. On the head, free neuromasts were oriented on lines tangential to concentric circles around the eye.

INTRODUCTION

Free neuromasts, which are mechanosensory organs in larval fish, are important for the perception of physical stimuli from predators [3] or planktonic prey [11, 14]. A free neuromast consists of sensory cells surrounded by supporting cells and a cupula protruding from the body surface [1, 9, 10]. Displacement of the cupula by physical stimuli is transmitted to the sensory hairs of the sensory cells, exciting them [1, 5]. The apical surface (sensory epithelium) of free neuromasts is the part that connects the cupula and sensory hairs, so this surface seems to be important for an understanding of free-neuromast functions. Many studies have been done of the distribution, development, and sensory-cell polarity of free neuromasts [6, 8, 13, 15, 16, 20, 22, 25, 26], but we know of no studies of the relationship of the morphological changes of cupulae and such changes in the apical surface of free neuromasts.

The willow shiner, *Gnathopogon elongatus caerulescens*, inhabits calm lakes and feeds on plankton [27]. The larvae of the willow shiner have large cupulae and feed on naupli of *Artemia salina* in complete darkness by using information received by their free neuromasts. The cupular size is proportion to the ability of larval willow shiners to detect and capture *Artemia* [24]. Moreover, the cupulae of willow shiners in prelarval to postlarval stages change from a stick-like to a blade-like shape [19]. The development of free-neuromast functions must be related to the changes in cupular shape during larval growth. Here, we examined the free

neuromasts of larval willow shiners to identify the relationship among the morphological changes of the cupula, such changes in the apical surface, and the orientation of maximum sensitivity of free neuromasts.

MATERIALS AND METHODS

Eggs fertilized by artificial insemination of the willow shiner, *Gnathopogon elongatus caerulescens*, were obtained from Shiga Prefectural Fisheries Experimental Station. The larvae were fed an artificial diet and the water temperature of the aquarium was kept at 16–20°C.

For observations by scanning electron microscopy (SEM), the larvae in the aquarium were transferred to Karnovsky's fixative by a pipet without being anesthetized and were preserved in the same solution, as in the method of Jones and Janssen [14]. Specimens were dehydrated in a graded series of ethanol concentrations, dried at the critical point with liquid CO₂, sputter-coated with gold, and examined under a Jeol JSM T-200 microscope.

A total of 49 larvae, from newly hatched to 35 days old which was the intermediate postlarval stage, were examined by SEM. The number of trunk neuromasts was shown as the mean value of the measurements obtained from several specimens. The number of head neuromasts was the sum of neuromasts observed from dorsal, lateral, and ventral views of different larvae on specimen mounts. The morphological changes in the apical surface of free neuromasts were examined in specimens from which the cupulae were removed by immersion in 30 ppm NaOH for 10–20 min before fixation. The major and minor axes of the outline of the apical surface were measured, and the number of sensory cells was counted in ten free neuromasts. The neuromasts were selected as the first ten in order from front to rear on the trunk, all with sensitivity in the antero-posterior orientation.

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RESULTS

Distribution of free neuromasts

Figure 1 shows typical changes in the distribution of free neuromasts with larval growth. The number of free neuromasts increased during larval growth; in particular, many neuromasts appeared on the ventral side of the head. Table 1 shows the number of free neuromasts at each stage. In newly hatched larvae, there was a mean of 51 free neuromasts on one side of the body. On the day of hatching the larvae were already sensitive to minute displacements of water in the aquarium.

At seven days after hatching, the larvae had absorbed all of the yolk-sac, thus entering the postlarval stage. A mean

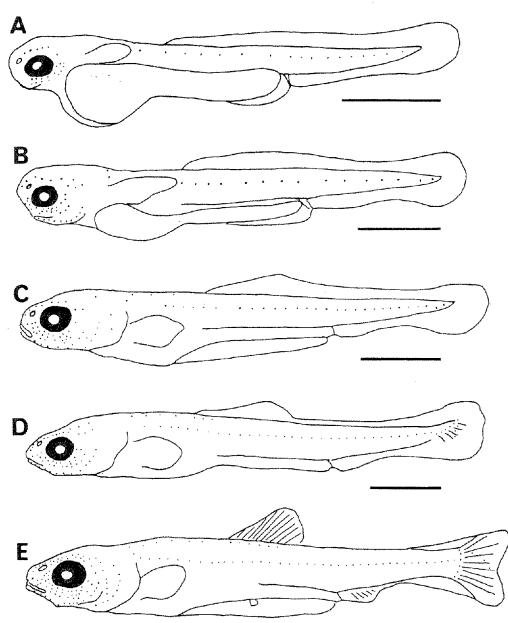


FIG. 1. Distribution of free neuromasts (black spots) at different stages of larval growth of the willow shiner, *Gnathopogon elongatus caerulescens*. A, newly hatched larva; B, one-day-old larva; C, seven-day-old larva; D, 15-day-old larva; E, 35-day-old larva. Scale bars, 1 mm.

of 94 neuromasts was found on one side of the body. The distribution of the head neuromasts was almost symmetrical (Fig. 2A). These neuromasts were found around the eyes, between the nostrils, on the opercular and occipital regions, and on the lower jaw. On the trunk, free neuromasts were arrayed in the middle of the flank (Fig. 2B). The trunk neuromasts were arranged asymmetrically on the right and left sides of living larva inspected from above by phase-contrast microscopy. Neuromasts were found on the dorsum of the trunk and on the caudal fin (Fig. 2C). At 35 days, there was one neuromast per segment along the middle of the flank.

Development of free neuromasts

The apical surface of free neuromasts expanded during larval growth. The major axis of the outline of the apical surface increased gradually, as well; it was 10.2 ± 1.2 (mean \pm SD) μm in hatching larvae and almost three times that in 35-day-old larvae. The minor axis increased less (Table 2). The mean number of sensory cells increased from eight in hatching larvae to 35 at 35 days (Table 2). The outline of the apical surface changed from round to lozenge-shaped (Fig. 3A-D). The changes in shape are expressed as the degree of narrowness of the outline (Table 2). The ratio of the major axis to the minor axis increased during the prelarval stage, but it was almost unchanged in the postlarval stage up to day 35, the end of the observation period.

Each sensory cell of the neuromasts had one kinocilium and 30-40 stereocilia (Fig. 3E, F). In seven-day-old larvae, the kinocilium was about $8 \mu\text{m}$, and the longest stereocilia were about $2 \mu\text{m}$. The appearance of each sensory cell did not change much during the 35 days of observations.

Sensory cell polarity

The orientation of the kinocilium in relation to the stereocilia governs the polarity of the sensory cell, since the sensory cell is excited only when the hair bundle is displaced in the direction from the stereocilia towards the kinocilium [7, 18]. The polarities of sensory cells are the same as the orientation of maximum sensitivity of the free neuromast [2].

TABLE 1. Number (mean \pm SD) of free neuromasts on one side of larval willow shiners

Age, days	*1n	Trunk	Dorsum	*2Dorso-ventral type	*3Head
0	5	18 \pm 2	[†] 0 - 2	(0)	32
1	4	17 \pm 2	2 \pm 0	[†] (0 - 1)	48
3	5	20 \pm 2	3 \pm 0	[†] (0 - 2)	54
7	5	27 \pm 2	4 \pm 0	(4 \pm 0)	63
15	5	32 \pm 3	7 \pm 1	(4 \pm 1)	71
35	5	43 \pm 5	12 \pm 3	(7 \pm 1)	115

*1 n; the number of specimens of which the trunk was inspected.

*2 The number of trunk neuromasts of the dorso-ventral type. Numerals parenthesized are included in the number of trunk neuromasts.

*3 The number of head neuromasts was the sum of the number of the neuromasts from dorsal, lateral, and ventral views. For details, see Results section.

[†] The numbers are the minimum and maximum.

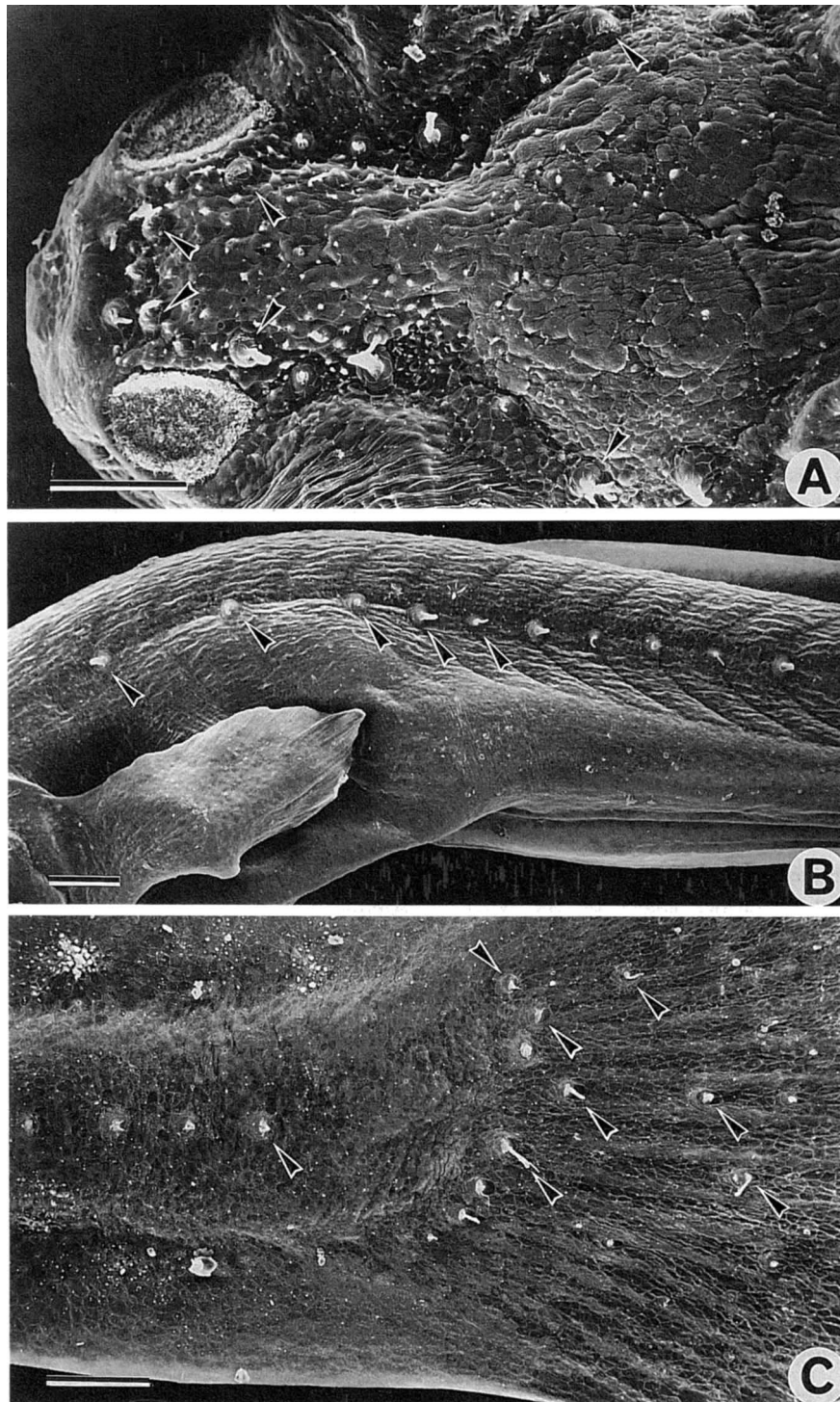


FIG. 2. Scanning electron micrographs of free neuromasts of the willow shiner. A, dorsal view of the head of a seven-day-old larva; B, trunk of a seven-day-old larva; C, caudal fin of a 15-day-old larva. Arrowheads indicate free neuromasts. Scale bars, 100 μ m.

Every free neuromast examined contained a mixture of sensory cells of opposing polarity in approximately equal numbers. The polarities of sensory cells coincided with the minor axis of the outline of the apical surface of each neuromast (Fig. 3E, F) and were in agreement with the direction in which the blade-like cupula bent [19].

Figure 4A shows the orientation of maximum sensitivity of free neuromasts on the trunk. Two types of neuromasts with regard to orientation were found there. One type had an antero-posterior orientation, parallel to the fish body axis. All neuromasts in Figure 3 had antero-posterior orientations on the trunk. The major axis of the outline of the apical

TABLE 2. Morphological changes (mean \pm SD) of the apical surface of ten free neuromasts with an antero-posterior orientation

Age, days	* ¹ Major axis (μ m)	* ² Minor axis (μ m)	Ratio (major/minor)	* ³ Number of sensory cells
0	10.2 \pm 1.2	7.0 \pm 1.1	1.48 \pm 0.18	8 \pm 3
1	11.7 \pm 1.0	6.7 \pm 0.8	1.76 \pm 0.19	16 \pm 1
3	17.7 \pm 1.7	9.0 \pm 1.0	1.97 \pm 0.19	21 \pm 3
7	16.3 \pm 3.1	8.0 \pm 1.8	2.06 \pm 0.28	23 \pm 8
15	19.9 \pm 5.9	9.2 \pm 2.3	2.17 \pm 0.22	24 \pm 11
35	26.1 \pm 9.7	12.3 \pm 3.0	2.07 \pm 0.40	35 \pm 12

*¹ Length of the outline of the apical surface longitudinally.

*² Width of the outline of the apical surface latitudinally.

*³ The number of sensory cells per free neuromast (mean \pm SD).

surface was perpendicular to the fish body axis, and the minor axis was parallel to this axis. The other type had a dorso-ventral orientation. In these dorso-ventral neuromasts, the major axis of the outline of the apical surface was parallel to the body axis. On the trunk, all neuromasts of newly hatched larvae had an antero-posterior orientation. Dorso-ventral neuromasts on the trunk were first found on one-day-old larvae, and seven-day-old larvae had a mean of four neuromasts of this type. The number of dorso-ventral neuromasts increased slowly until day 35 (Table 1). Neuromasts with an antero-posterior orientation were more common than those with the other orientation on the trunk during the pre- and postlarval stages.

Figure 4B-D shows the orientation of maximum sensitivity of free neuromasts on the head. The minor axis of these neuromasts was oriented in various angles on the head surface. The orientation was more variable on the head than on the trunk. The orientation of head neuromasts formed lines tangential to concentric circles around the eye. On the lower jaw, the orientation was approximately symmetrical on the right and left side, and the orientations were various, although many neuromasts had a left-right component.

On the caudal fin, all neuromasts had an antero-posterior orientation. On the dorsum of the trunk, the orientation of neuromasts from the occipital area to the start of the dorsal fin was perpendicular to the fish axis, but the ones at the base of the dorsal fin had an antero-posterior orientation.

DISCUSSION

As a rule, the first neuromasts to develop in a fish embryo later become presumptive canal neuromasts [25]. Other types of free neuromasts are secondary or third neuromasts, which appear later than presumptive canal neuromasts and remain on the body surface or become pit organs [6, 17, 29]. The number of presumptive canal neuromasts is almost identical with the number of scales finally bearing canal structures [26]. The number of free neuromasts on the trunk of willow shiners 35 days old (Table 1) was almost the same as that of scales of the lateral line in adult fish as mentioned by Nakamura [28]. Therefore, the free neuromasts on the

trunk that we measured in various ways must be presumptive canal neuromasts.

The major axis of the outline of the apical surface of the neuromasts and the number of sensory cells per neuromast continued to increase until 35 days, but the ratio of the major axis to the minor axis of the outline had already become constant at the beginning of the postlarval stage (Table 2). The changes in the ratio reflect the development of the cupular shape, with fully developed cupulae with a blade-like shape appearing in many free neuromasts at the beginning of the postlarval stage [19]. Stick-like cupulae can probably bend in various directions, but blade-like cupulae bend only in the direction of the minor axis of the apical surface. Cupular shape changed with larval growth, so the free neuromasts seem to change in their sensitivity to directional stimuli.

Seven-day-old larvae could eat *Artemia* in complete darkness, by sensing vibrations produced by their prey swimming [24]. This ability requires free neuromasts, which seemed therefore to be almost completely developed at the start of the postlarval stage. At that stage, feeding begins, and the chance of encountering predators increases, as well. The timing of the appearance of free neuromasts in ontogenetic development during the prelarval stage appears to be an adaptation for survival.

The orientation of maximum sensitivity of free neuromasts, decided from the polarity of sensory cells, coincided with the minor axis of the apical surface outline (Fig. 3E, F). This relationship was different from that in the free neuromasts of adult *Xenopus laevis* [5] or in canal neuromasts of adult sprats, *Sprattus sprattus* [4] or adult mottled sculpins, *Cottus bairdi* [13], in which the orientation of sensory cell polarities parallels the major axis. The same pattern as with willow shiners was found in free neuromasts with a lozenge-shaped outline of the apical surface of herring larvae, *Clupea harengus* [2], a species of *Eigenmannia* larvae [30], ayu, *Plecoglossus altivelis* [21], pale chub, *Zacco platypus*, Japanese dace, *Tribolodon hakonensis* [23], and crucian carp, *Carassius auratus grandoculis* (unpublished data). Moreover, in some of these species [21, 23], the orientation was in parallel with the direction in which the blade-like cupulae bent, as in the willow shiner. Therefore, the morphology of

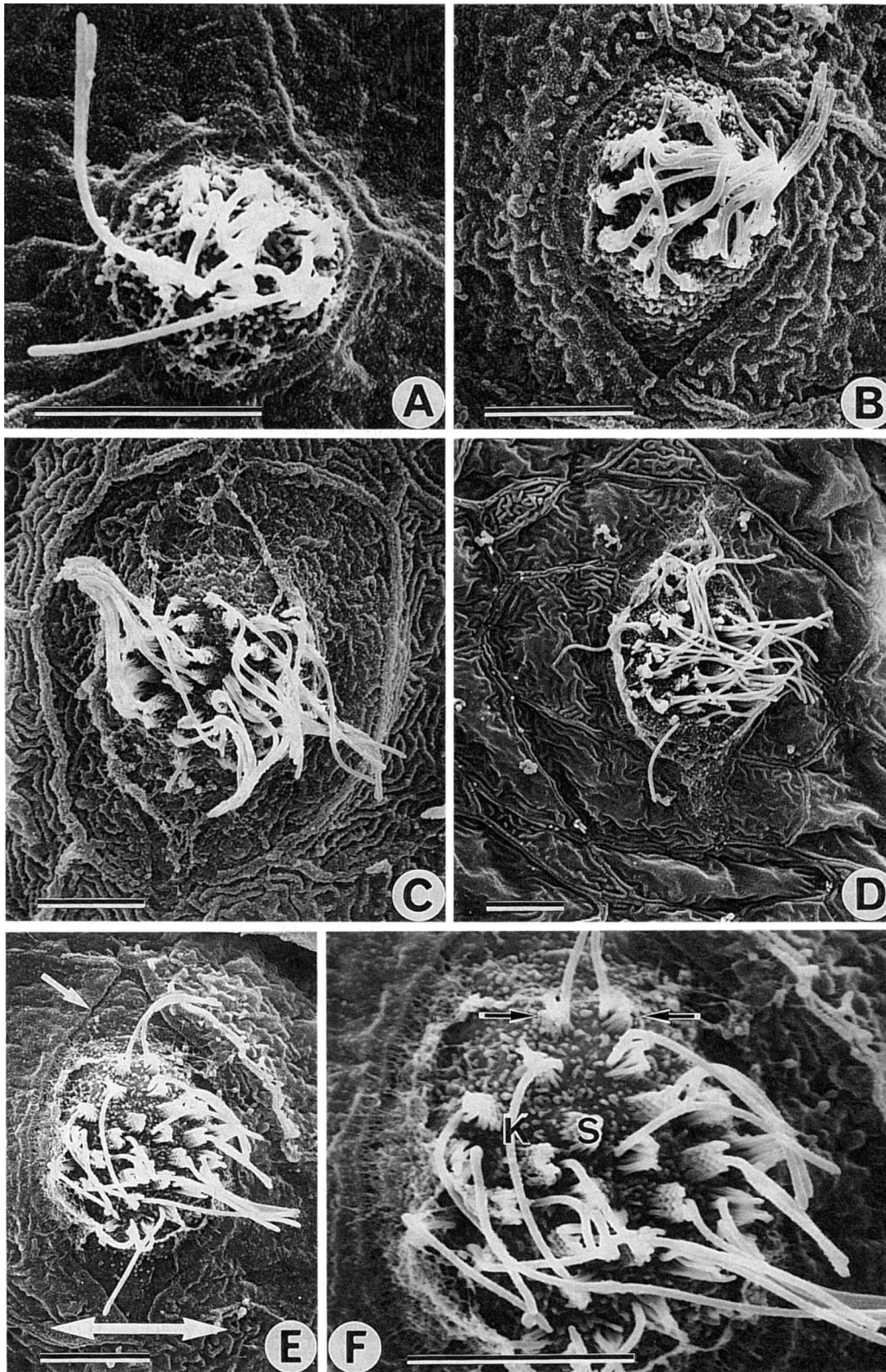


FIG. 3. Scanning electron micrographs showing development of free neuromasts on the trunk of the willow shiner. A, neuromast of a newly hatched larva; B, neuromast of a one-day-old larva; C, neuromast of a seven-day-old larva; D, neuromast of a 35-day-old larva; E and F, neuromast of a three-day-old larva. E, Arrow indicates the outline of the apical surface of the free neuromast. Double-headed arrow indicates the orientation of maximum sensitivity of the free neuromast in terms of the sensory cell polarity of F. F, higher magnification of E; Arrows indicate sensory cell polarity; K, kinocilium; S, stereocilia. The directions of right and left in all micrographs are in parallel with the fish body axis, and the fish heads are to the left side. Scale bars, 5 μ m.

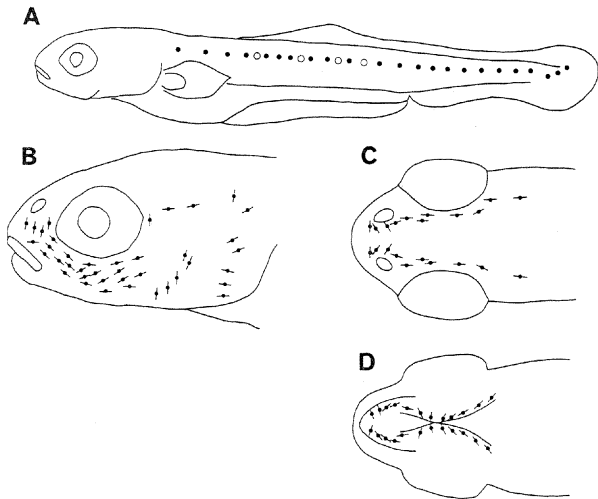


FIG. 4. Typical arrangement of neuromasts and their orientation in terms of sensory cell polarity in seven-day-old larvae of the willow shiner. A, free neuromasts on the trunk; solid circles, antero-posterior orientation; open circles, dorso-ventral orientation. B-D, free neuromasts on the head; B, lateral view; C, dorsal view; D, ventral view. Bars through the dots indicate the neuromast orientation in terms of sensory cell polarity.

the free neuromasts in willow shiner larvae seemed to be typical of that in cyprinid larvae.

The antero-posterior neuromasts on the trunk would become canal neuromasts in adult willow shiners and receive mechanical stimuli along with the canal axis parallel to the fish body axis. The various orientations of free neuromasts on the head are in accord with the complicated arrangement of head canal organs in the adults. The second type of free neuromasts with dorso-ventral polarity on the trunk has been found on juvenile cichlids; they are orthogonal to presumptive canal neuromasts with antero-posterior polarity [31]. However, the dorso-ventral type of free neuromasts on the trunk is present in an early stage of fish ontogeny, in the larvae of herring [2], anchovy, *Engraulis japonica* [12], ayu [21], pale chub, Japanese dace [23], crucian carp (unpublished data), and the willow shiner (Table 1). This dorso-ventral type might not be the second type of free neuromasts, because it is formed early, in the prelarval or early postlarval stage. Therefore, there are two possibilities; one is that this dorso-ventral type remains on the body surface and another is that this type changes to canal neuromasts.

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