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Citation	北海道大學水産學部研究彙報, 37(2), 95-100
Issue Date	1986-05
Doc URL	https://hdl.handle.net/2115/23910
Type	departmental bulletin paper
File Information	37(2)_P95-100.pdf



**Appearance and disappearance of chloride cells throughout
the embryonic and postembryonic development of the
goby, *Chaenogobius urotaenia***

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Abstract

The chloride cells in the embryos and larvae of *Chaenogobius urotaenia* were observed by using a light and transmission electron microscope.

Although no cells were found which could be identified as chloride cell in the embryo before retinal pigmentation, the eosinophilic cells appeared in the epithelium enveloping the region of the ventral aorta on the sixth or seventh day after retinal pigmentation. These eosinophilic cells were identified with chloride cells by an electron microscope. These cells decreased in number and eventually disappeared as chloride cells occurred in increasing numbers in the gills. These facts suggest that the cells in the epithelium of the region along the ventral aorta may be the site for osmoregulation in larvae which have no gill filament.

Keys and Willmer (1932) first described an eosinophilic cell located along the gill filament of an eel and suggested that the cell might be responsible for the transport of monovalent ions. They named it "chloride secretory cell". Since then, many studies have been carried out to clarify this hypothesis (Copeland, 1948; Parry, 1966).

Recently, electron microscopic studies have demonstrated that the chloride cell of euryhaline teleosts undergoes cytological changes during the course of adaptation from sea water to fresh water and the reverse course. When the euryhaline fishes are kept in sea water, the chloride cells increase not only in number, but also in size and in numbers of mitochondria. In addition, an apical crypt appears in the apex of the cell (Kessel and Beams, 1962; Ernst van Lennep, 1968; Utida and Shirai, 1970; Dunel-erb and Laurent, 1980).

Although there are many studies regarding osmoregulation in adult fishes, the study as for newly hatched larvae are comparatively few in number. The gills of newly hatched larvae are too undeveloped to play a role in osmoregulation. Threadgold *et al.* (1968) found cells morphologically similar to the chloride cell of teleost gills in the skin of larval sardine. Iwai (1969) noted peculiar cells resembling the chloride cell in the fine structure of the skin of larvae of the puffer, *Fugu niphobles*, and regarded these as cells playing a role in osmoregulation. Robert *et al.* (1973) observed a chloride cell in the skin of a newly hatched plaice *Pleuronectes platessa* L.. According to Yamashita (1978), chloride cells were observed in the skin of the larvae of red seabream *Pagrus major* for 15 days after hatching, but thereafter these cells gradually disappeared. The stage of the larvae stated above is the transitional stage, from yolk feeding to external feeding, and the organ systems

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develop rapidly during this stage. The observations stated above show that the larval skin of sea water teleosts may play a role in osmoregulation and the decrease in number of chloride cells in the skin may be the result of the development of osmoregulatory organs such as gills. Sea water teleosts are exposed to insignificant changes in the salinity of external media throughout their lives. However, larvae of fish, which spawn in fresh water are brought by currents into the sea just after hatching and are exposed to significant changes in the salinity of the media. Although there are many studies for osmoregulation of the larvae of sea water teleosts, studies on euryhaline fishes are few.

In the goby, *Chaenogobius urotaenia* (*Chaenogobius annularis*), which spawns in a river, chloride cells appear along the gill filament with the development of the secondary lamellae from the fourteenth to the sixteenth day after hatching (Hamada, 1968). Larvae of this species are swept into the sea just after hatching, therefore there must be an organ taking the place of the undeveloped gill to play a part in osmoregulation.

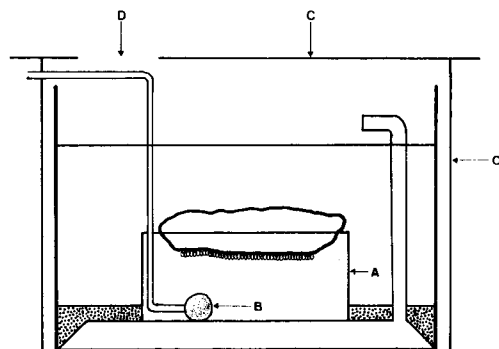
The purpose of this study is to clarify the site for osmoregulation in larvae of *Chaenogobius urotaenia* which have no gill filament. The term chloride cell will be used here for convenience without any implication of an active transport of chloride.

Materials and methods

The animals and eggs used in this study are middle type *Chaenogobius urotaenia*. (Nakanishi, 1968). These specimens were collected in the Hekiriji River running through the suburbs of Hakodate, Hokkaido. The eggs were collected with the male still taking care of the eggs attached to the underside of a stone in order to identify the type of parent of the eggs.

The embryos at the morula stage or earlier were carried with the stone to the laboratory and incubated in the through shown in Text-fig. 1. The water temperature was kept at $13 \pm 1^\circ\text{C}$ and dead embryos were removed once a day. Newly hatched larvae were transferred to 50‰ sea water and fed with *Brachionus plicatilis*.

For cytological or histological observations, embryos were fixed in Bouin's fluid every 12 hours after the beginning of pigmentation in the retina. Larvae were fixed once a day after hatching. The gills of the embryo which were removed from the freshwater-adapted or seawater-adapted fish were fixed in Bouin's solution. These pieces were embedded in Tissue-prep (Fisher Scientific Co.), and sectioned at 5-7



Text-fig. 1 Schematic diagram of an aquarium used to incubate the eggs of *C. urotaenia*. A: small aquarium to support eggs attached to the underside of a stone. B: air stone. C: A shield to shut off the light. D: window.

μm . The sections were stained with Delafield's hematoxylin and eosin. For electron microscope observations, the embryos were immersed for 2 hours in 2.5% glutaraldehyde, buffered to pH 7.4 with 0.1 M cacodylate buffer and postfixed for 1 hour in 1% osmium tetroxide. After dehydration in a graded series of ethanol at room temperature, the tissues were embedded in Epon-812. Blocks were sectioned with "Porter-Blum MT-1" ultramicrotome, stained with uranyl acetate and lead citrate at room temperature, and examined with a Hitachi H-300 transmission electron microscope.

Results

Because the eggs used in this study were collected in the river as stated previously, the time of fertilization was impossible to confirm. The beginning of pigmentation in the retina was used, for convenience, as the criterion in the description of developmental stages. No cells which could be identified with a chloride cell were found in embryos before pigmentation.

Light microscopic observations

(1) The first day after pigmentation in the retina

The embryo measured 3.5 mm in length. The fundamental structure of the eye has already formed. Both of the third and the fourth branchial pouches perforated the sides of the pharynx to form the gill clefts. The cell considered to be a chloride cell was not observed, but large eosinophilic cells were found over the epithelia of the yolk sac and the embryonic body.

(2) The second and third day

The embryo measured 3.9 mm in length. Four pairs of branchial arches were established, and the chondrification began in each arch. (Fig. 1) Large eosinophilic cells appeared in the epithelium of the chin and the adjacent regions. These cells were similar to the cells found in the yolk sac and the epithelium of the embryonic body as stated above.

(3) The fourth and fifth day

The embryo measured 4.5 mm in length. Meckel's cartilage, the hyomandibular, the ceratohyal, the basihyal and the branchials have formed and the median continuation of the ventral aorta formed the external carotid. By this time large eosinophilic cells appeared in the epithelia of the underside of the lower jaw and of the region along the ventral aorta (Figs. 2, 3). These same cells were also found in the epithelium at the base of the pectoral fin. (Fig. 4)

(4) The sixth and seventh day

The embryo measured 4.8-5.0 mm in length. Meckel's cartilage was well developed. The hyoid arch and part of the mandibular began to form the operculum enclosing four pairs of branchial arches. Each of the branchial arches was supplied by an unbranched aortic arch, and blunt, rounded gill filament primordia have formed on the external surface of branchial arches. The posterior intermandibularis muscles have developed considerably. The large eosinophilic cells seen at the previous stage in the epithelium of the underside of the lower jaw gradually disappeared as the gill developed. (Figs. 5, 6). In contrast, the eosinophilic cells appeared in the epithelium enveloping the region of the ventral aorta. These

eosinophilic cells were identified as chloride cells using an electron microscope.

(5) Hatching

Newly hatched larvae measured 5.3 mm in length. There were many chloride cells in the epithelium of the region along the ventral aorta. These cells were eosinophilic, round or oval in shape, and $17\ \mu\text{m}$ in diameter. The nucleus was usually located in the basolateral part of the cell. The plasmalemma of the free surface of cells sometimes assumed the form of a crypt-like invagination. (Figs. 7, 8) The pseudobranchia was already visible. (Fig. 9) At this stage, the afferent and efferent branchial arteries were not yet differentiated, but chloride cells appeared in the primordium of the gill filament this time. (Fig. 10)

(6) Four to eight days after hatching

The larvae, which were kept in 50% seawater after hatching measured 6.0 mm in length. The afferent and the efferent branchial arteries have already differentiated. In the pseudobranchia, a gill ray was visible. The ray was composed of a column of chondroblasts arranged in a row. The chloride cells were still observable in the epithelium enveloping the region along the course of the ventral aorta.

(7) Nine to fifteen days after hatching

The larvae which were kept in 50% sea water after hatching measured 7-8 mm in length. The secondary lamellae began to develop at about 11 days after hatching. The first appearance of secondary lamellae was the formation of small cell masses across the filament core (Fig. 11) About 15 days after hatching, the pillar cells formed columns to support respiratory epithelia. Many chloride cells appeared in the gill epithelium, especially on the same side as the afferent filament artery (Fig. 12), and were also distributed in the epithelia enveloping the region along the course of the ventral aorta, the operculum, the base of pectoral fin and the region of the forehead.

(8) Thirty or more days after hatching

The young measured about 14 mm in length. The gill possessed the same fundamental structure as that of the adult. The chloride cells increased in number in the gill filament. It is likely that the gill has already played a functional role in respiration and osmoregulation. The chloride cells decreased rapidly in number in the skin of the region along the ventral aorta and eventually disappeared.

Electron microscopic observations

Chloride cells densely located in the skin of the region along the ventral aorta were easily distinguishable from other cells based on the cytoplasm containing numerous mitochondria and agranular endoplasmic reticula (Fig. 13). The cells extended from the basement membrane to the free surface of epithelium. The apices of the cells were covered with pavement cells and were tightly connected to each other by desmosomes. The apex of chloride cells exposed to the external medium was occasionally invaginated and formed an apical crypt. The cytoplasmic zone immediately adjacent to the crypt plasmalemma was characterized by the presence of dense electron materials. The nucleus was spherical in shape and $5-6\ \mu\text{m}$ in diameter, or oval and $7-8\ \mu\text{m}$ in length and $5-6\ \mu\text{m}$ in width, and was usually located in the basolateral part of the cell. Mitochondria occasionally contained vacuoles, and are oval with an average length of $4-5\ \mu\text{m}$. The cristae were irregular

in their grouping and tortuous in appearance (Fig. 14).

Discussion

It has generally been accepted that the chloride cells in the gills of teleosts play important roles in maintaining a constant internal osmotic pressure independent of their hyper osmotic environment, *i.e.* in marine fishes, these cells control osmotic balance by the secretion of salt against the gradient of the external environment. The gills of newly hatched larvae, however, have no gill filament and chloride cells were not identified in the gills.

Shelbourne (1957) suggested the epidermis as a site for regulation of salt in plaice larvae. However, Holliday and Blaxter (1960) pointed out that the technique to demonstrate this is not completely specific. Thereafter, Threadgold and Lasker (1967) observed a mode of mitochondriogenesis in certain integumentary cells in the skin of the larval sardine, *Sardinops caerulea*. They believed that these cells are the site of special osmoregulatory functions. Again, the chloride cell of the larval sardine skin and physiological evidence for the osmoregulatory role of these cells were described in detail by them in 1968. According to them, characteristics of these cells are a branching system of smooth-walled tubules, numerous mitochondria and a localized area of the outer plasma membrane exposed to the environment.

According to Robert (1973), the chloride cells observed in the skin of the larvae of plaice, *Pleuronectes platessa* L. disappear as the dermis develops. Yamashita (1978) observed that the chloride cells in the skin of the larvae of red seabream, *Pagrus major*, decrease in number in the transition stage from yolk-feeding to external-feeding.

The middle type of *Chaenogobius urotaenia* used in this study are euryhaline, and the larvae are swept from a river down into the sea after hatching. According to Hamada (1968), in *Chaenogobius urotaenia*, chloride cells occur in the gill epithelium on the same side as the afferent artery with the development of the secondary lamellae after about 15 days from hatching. Therefore, the site for osmoregulation in the larvae swept down into the sea is not the gills, but another organ or tissue.

As stated above, chloride cells are found in the epithelium of the region along the ventral aorta in newly hatched larvae. These cells seem to play an important role as a site for osmoregulation in the larvae. Through this study, it has been observed that newly hatched larvae kept in freshwater were not able to live beyond a week. Although the cause of death was not clear, it is likely that the larvae of the middle type of *Chaenogobius urotaenia* were unable to maintain a constant, internal, osmotic pressure in freshwater. The chloride cells located in the epithelium of the region along the ventral aorta decreased in number and eventually disappeared, as the chloride cells occurred and in increasing numbers in the gills. This fact may be an indication that the cells located in the epithelium enveloping the region along the ventral aorta are the site of osmoregulation in the larvae, and the chloride cells function in place of the gills for osmoregulation as the larvae grow.

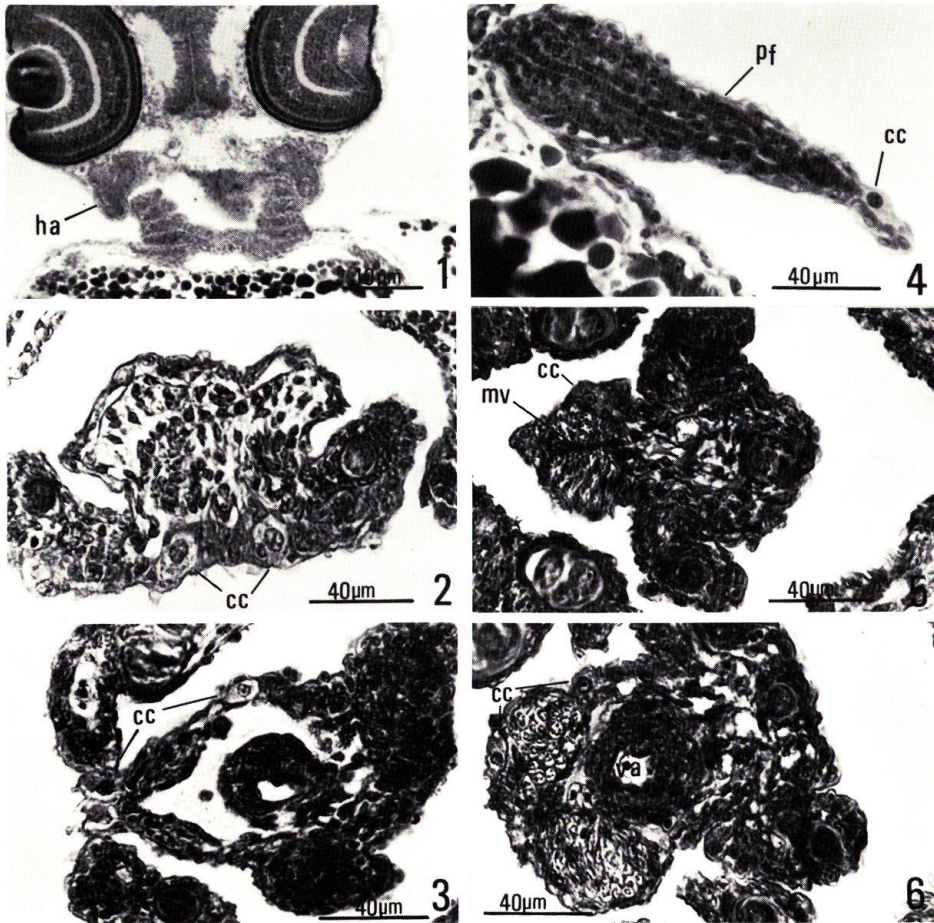
Acknowledgements

The authors wish to thank Drs. F. Yamazaki and A. Goto, Faculty of Fisheries,

Hokkaido Univ., and H. Onozato, Nanae Fish Culture Experimental Station, Hokkaido Univ., for their valuable comments, and are grateful to the students at the Laboratory of Embryology and Genetics, Faculty of Fisheries, Hokkaido Univ., for their help in the present study.

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Explanation of Plates

PLATE I

- Fig. 1. Frontal section through branchial arches. Hyoid arch (ha) not covering branchial arches.
Fig. 2. Transverse section through lower jaw. Appearance of chloride cell (cc) in the epithelium.
Fig. 3. Transverse section through ventral aorta. Appearance of chloride cells in the epithelium near the ventral aorta.
Fig. 4. Frontal section through pectoral fin (pf). Appearance of chloride cells in the epithelium on the tip of pectoral fin.
Fig. 5. Transverse section through muscle carinatus ventralis (mv).
Fig. 6. Transverse section basibranchial and ventral aorta. Note many chloride cells in the epithelium surrounding ventral aorta. (va).

PLATE II

- Figs. 7, 8. Transverse section through basibranchial. Note many chloride cells with an apical crypt (cac) in the epithelium converging ventral aorta.
- Fig. 9. Transverse section through opercular gill (ob). Note acidophil cell (ac).
- Fig. 10. Transverse section through branchial arch. Note chloride cells in epithelia of gill and about afferent branchial artery (af).
- Fig. 11. Section through gill filament. Formation of afferent filament artery (av), efferent filament artery (ev) and gill ray (cg).
- Fig. 12. Section through gill filament. Note chloride cells in the epithelium surrounding afferent filament artery. Blood cell (bc), pilaster cell (pc).

PLATE III

- Fig. 13. Chloride cells in the epithelium about ventral aorta. 4 days hatching. pvc : pavement cell, n : nucleus.
- Fig. 14. Apical region of chloride cell. Note formation of an apical crypt. m : mitochondria.

