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Relationships of Salinity Tolerance to Immunolocalization of Na^+, K^+ -ATPase in the Gill Epithelium during Seawater and Freshwater Adaptation of the Guppy, *Poecilia reticulata*

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ABSTRACT—The relationships of salinity tolerance to immunolocalization of Na^+, K^+ -ATPase in the gill epithelium were examined during seawater and freshwater adaptation of the guppy. In fresh water, immunoreactivity for Na^+, K^+ -ATPase appeared in two types of chloride cells, which are located on the primary lamellae of the gills. Immunoreactivity was strong in the chloride cells located at the base of the secondary lamellae and weak in the chloride cells located at the interlamellar region. During seawater adaptation, the strongly-immunoreactive chloride-cells increased in number and size while the weakly-immunoreactive chloride-cells decreased in number with an increase in salinity tolerance. In the fish of the seawater-adapted strain, on the other hand, most of the chloride cells were located at the base of the secondary lamellae and showed strong immunoreactivity. During freshwater adaptation, the strongly-immunoreactive chloride-cells decreased in number and size while the weakly-immunoreactive chloride-cells increased in number with a decrease in salinity tolerance. A positive correlation was observed between the salinity tolerance and the occupying area of the strongly-immunoreactive chloride-cells while a negative correlation was observed between the salinity tolerance and the occupying area of the weakly-immunoreactive chloride-cells during the seawater and freshwater adaptation. These results directly suggested that not only the occupying area of chloride cells but also the expression of Na^+, K^+ -ATPase protein in the cells is important with respect to the osmoregulatory function in the gills and hypoosmoregulatory ability at the individual level.

INTRODUCTION

One of the most important functions in the gills of teleosts is ion transport for the maintenance of the constancy of their internal ionic environment in hyperosmotic or hypoosmotic environments. Branchial epithelia are heterogeneous with respect to cell type and function. Chloride cells are identified as external sites of chloride ion extrusion in seawater (Foskett and Scheffey, 1982) and are thought to act as sites for ion uptake in a hypoosmotic environment (Laurent and Dunel, 1980; Perry and Wood, 1985; Avella *et al.*, 1987; Perry and Laurent, 1989; Marshall *et al.*, 1997). Several studies have reported the existence of two distinct types of chloride cells in the gills of euryhaline teleosts (Doyle and Gorecki, 1961; Straus, 1963; Shirai and Utida, 1970; Pisam *et al.*, 1987, 1988, 1990; Richman *et al.*, 1987; Franklin and Davison, 1989; Pisam and Rambourg, 1991). Using an acid fuchsin stain, Shirai and Utida (1970) studied the gill epithelium of the Japanese eel and identified two types of chloride cells. They reported that the one cell type increased in number while the other cell type underwent a progressive degeneration and disappeared when

eels were transferred to seawater. Some investigators have also reported that two types of chloride cells were observed in the gill epithelium of salmonids (Richman *et al.*, 1987; Pisam *et al.*, 1988; Franklin and Davison, 1989) and that the constitutions of the two types of chloride cells changed during smoltification with an increase in sodium-potassium adenosinetriphosphatase (Na^+, K^+ -ATPase) activity in the gills (Richman *et al.*, 1987; Pisam *et al.*, 1988). The changes in the constitutions of the two types of chloride cells during seawater adaptation and smoltification are strongly suggestive of the changes in the osmoregulatory function of the gills.

Na^+, K^+ -ATPase is one of the most important enzymes in ion transport in the gills. The enzyme is composed of two different protein subunits, α and β , both of which exist in several distinct isoforms (Shull *et al.*, 1986). The α -subunit is the catalytic part of the Na^+/K^+ exchange and shows a highly conserved amino acid sequence in diverse vertebrate and invertebrate species (Shull *et al.*, 1985; Kawakami *et al.*, 1985; Takeyasu *et al.*, 1988; Lebovitz *et al.*, 1989). It has been revealed that the enzyme is mainly located in the basolateral membrane of chloride cells using tritiated ouabain autoradiography (Karnaky *et al.*, 1976a) and ultracytochemical localization of K^+ -NPPase (Hootman and Philpott, 1979). Immunohistochemical studies also showed that immunoreactivity for

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Na^+, K^+ -ATPase appeared in chloride cells in the gills (Ura *et al.*, 1996; Witters *et al.*, 1996). Uchida *et al.* (1996) reported that the immunoreactivity for the Na^+, K^+ -ATPase α -subunit in chloride cells and also gill Na^+, K^+ -ATPase activity increased when chum salmon were transferred from fresh water to seawater. Thus, immunolocalization of Na^+, K^+ -ATPase will provide useful information about the changes in the osmoregulatory function in the gills during seawater or freshwater adaptation.

The guppy is a useful fish in which to study the osmoregulatory function because both freshwater strains and a seawater-adapted strain were established (Shikano and Fujio, 1997). For the further understanding of the osmoregulatory function of the gills in euryhaline teleosts, the present study examines the relationships between salinity tolerance and immunolocalization of Na^+, K^+ -ATPase in the gill epithelium during seawater and freshwater adaptation in the guppy.

MATERIALS AND METHODS

Animals

Two guppy strains, F22 and F22-SW, were used in the present study. The F22 strain originated from the offspring yielded by one gravid female of the F strain and was maintained in fresh water (Shikano and Fujio, 1994). The F22-SW strain was made from the F22 strain by means reported in a previous paper (Shikano and Fujio, 1997) and maintained in 35 ppt artificial seawater (Aquasalz, Nissei, Japan). Each strain was maintained as a closed colony in a 60 l aquarium with a density of 200–300 individuals at a temperature of $23 \pm 2^\circ\text{C}$ with lighting for 10 hr per day. The fish were fed twice daily with dry ground carp pellets and dried *Daphnia* as a supplementary diet.

Seawater and freshwater adaptation

Guppies of the freshwater strain were exposed to 15 ppt dilute artificial seawater for 5 days and subsequently transferred to 35 ppt artificial seawater. On the other hand, guppies in the seawater-adapted strain were transferred directly to fresh water. To examine the changes in salinity tolerance and immunolocalization of Na^+, K^+ -ATPase in the gills, fish were sampled on days 0, 1, 2, 3, 5, 6, 7, 8, 10, 15, 20 and 25 during the seawater adaptation and on days 0, 1, 3, 5, 10, 20 and 30 during the freshwater adaptation.

Salinity tolerance

Mature guppies (more than 60 days old) were transferred to 20 ppt, 25 ppt, 30 ppt, 35 ppt, 40 ppt, 45 ppt, 50 ppt, 55 ppt or 60 ppt seawater during the seawater adaptation and the freshwater adaptation. Survival rates were examined 24 hr after the transfer. LD_{50} was calculated from 3 to 4 factors of survival rate data, which showed better regression.

Immunohistochemistry

Gill arches of the fish whose standard body length was 25.0 ± 1.0 mm were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 8 hr at 4°C . The tissue samples were dehydrated in ethanol and embedded in paraffin. Serial sections ($5 \mu\text{m}$) were cut parallel to the long axis of the primary lamellae at a right angle to the secondary lamellae or at the base of the secondary lamellae. The sections were mounted on polylysine-coated glass slides. The sections were deparaffinized in xylene, hydrated in ethanol and washed with phosphate-buffered saline (PBS). For immunocytochemical staining, the avidin-biotinylated alkaline phosphatase method (Vectastain ABC-AP kit, Vector Laboratories, Inc., Burlingame, CA, USA) was used ac-

cording to the manufacturer's recommendation. To reduce nonspecific staining, the sections were treated with 2% normal goat serum in PBS for 30 min at room temperature. The sections were incubated with a primary antibody at a 1:2000 dilution overnight at 4°C . The primary antibody was a rabbit polyclonal raised against a synthetic peptide corresponding to part of the highly conserved region of the Na^+, K^+ -ATPase α -subunit (Ura *et al.*, 1996). The sites of the immunoreactions were visualized by incubating the sections successively with a solution of biotinylated anti-rabbit IgG for 30 min, ABC-AP reagent for 60 min and alkaline phosphatase substrate solution for 4 min. Non-immunostained sections were also stained with hematoxylin-eosin. The immunostained sections and the hematoxylin-eosin stained sections were observed with a light microscope equipped with a Nomarski differential interference contrast device. No staining was detected when normal rabbit serum was used instead of the primary antibody.

The occupying area of the immunoreactive cells was determined using an image analysis system (NIH Image). The occupying area of the immunoreactive cells was measured in sections cut parallel to the long axis of the primary lamellae at the base of the secondary lamellae and expressed as a ratio of the total occupying area of the immunoreactive cells to the surface area of the gill epithelium on which the cells were situated.

Statistics

Data of the occupying area of the immunoreactive cells in each experimental group are shown as the mean \pm S.E.M. of five individuals. Statistical comparisons were performed using the Student's *t*-test.

RESULTS

Seawater adaptation

Figure 1 shows changes in the LD_{50} following transfer of the freshwater fish to seawater. Although LD_{50} was 24.8 ppt in fresh water, LD_{50} increased to 39.0 ppt during the first 5 days in 15 ppt dilute seawater and then increased to 52.5 ppt up to 20 days after subsequent transfer to 35 ppt seawater.

Hematoxylin-eosin stainings of the gill sections of freshwater fish are shown in Fig. 2-A and B. Large eosinophilic cells were observed on the primary lamellae (Fig. 2-A). The surface of the primary lamellae was observed at the base of

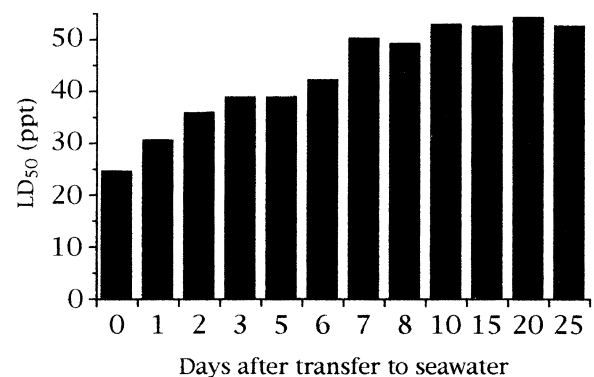


Fig. 1. Changes in LD_{50} following transfer of freshwater fish to seawater. The fish were transferred to 15 ppt dilute seawater on day 0 and subsequently transferred to 35 ppt seawater on day 5. LD_{50} was calculated from the data of survival rates 24 hr after transfer to 20, 25, 30, 35, 40, 45, 50, 55 or 60 ppt seawater.

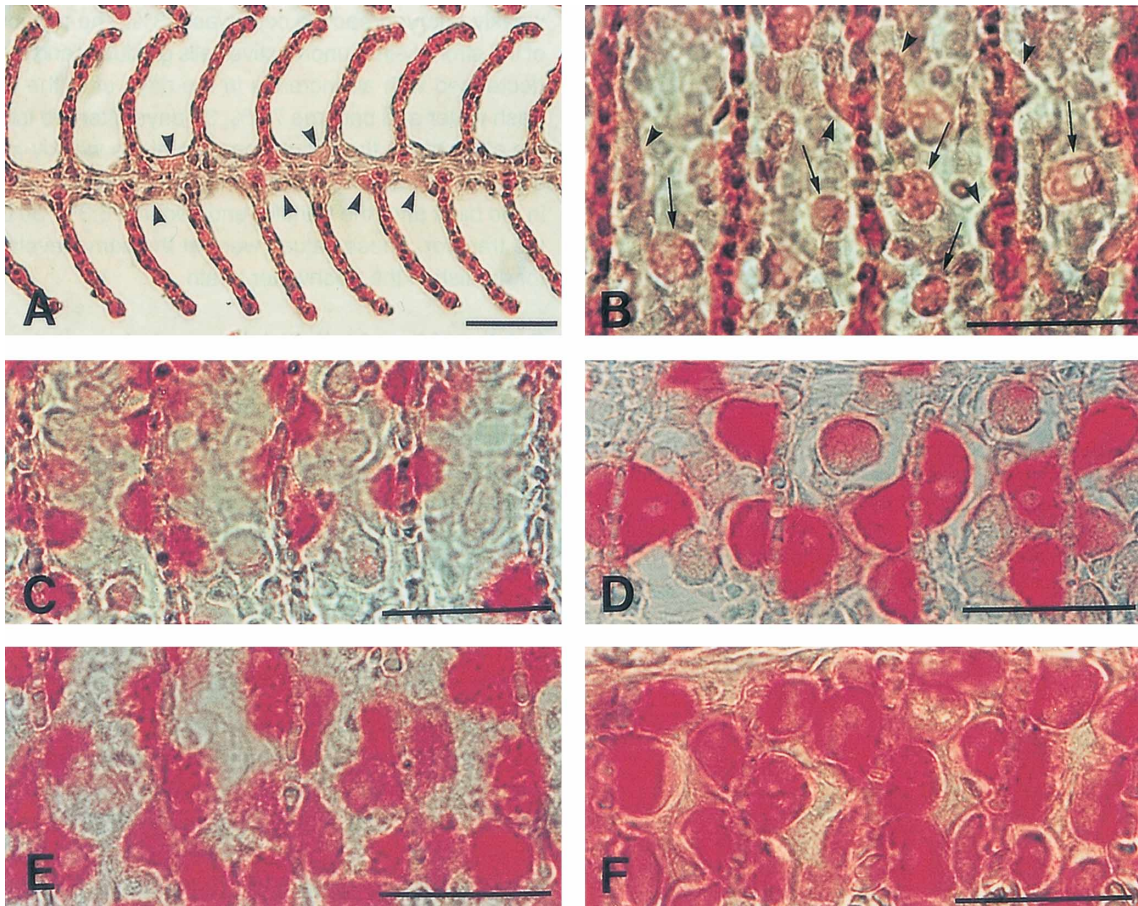


Fig. 2. Gill sections of the guppy in fresh water (**A**, **B** and **C**) and during seawater adaptation (**D**, **E** and **F**). (**A**) Sagittal section stained with hematoxylin-eosin in freshwater fish. Large eosinophilic cells were observed on the primary lamella (arrowheads). (**B**) Level surface section stained with hematoxylin-eosin in freshwater fish. The bases of the secondary lamellae were observed as vertical stripes. Large eosinophilic cells were observed at the base of the secondary lamellae (arrowheads) and at the interlamellar region (arrows). (**C**) Level surface section stained with the antibody to Na⁺,K⁺-ATPase in freshwater fish. Strong immunoreactivity was observed in the large eosinophilic cells located at the base of the secondary lamellae while little immunoreactivity was observed in the large eosinophilic cells located at the interlamellar region. (**D**) Level surface section stained with the antibody to Na⁺,K⁺-ATPase in fish transferred to 15 ppt dilute seawater for 5 days. (**E**) Level surface section stained with the antibody to Na⁺,K⁺-ATPase in fish transferred to 35 ppt seawater for 5 days. (**F**) Level surface section stained with the antibody to Na⁺,K⁺-ATPase in fish transferred to 35 ppt seawater for 15 days. The strongly-immunoreactive cells increased in number and size while the weakly-immunoreactive cells decreased in number during the seawater adaption (**C-F**). Scale bar, 30 μ m.

the secondary lamellae (Fig. 2-B). Large eosinophilic cells were observed at the base of the secondary lamellae and at the interlamellar region. Immunohistochemical stainings showed that strong immunoreactivity for Na⁺,K⁺-ATPase was observed in the large eosinophilic cells located at the base of the secondary lamellae while weak immunoreactivity was observed in the large eosinophilic cells located at the interlamellar region (Fig. 2-C).

When the fish were exposed to 15 ppt dilute seawater for 5 days and subsequently transferred to 35 ppt seawater, the strongly-immunoreactive cells increased in number and size while the weakly-immunoreactive cells decreased in number and gradually failed, as shown in Fig. 2-D, E and F. Most of the strongly-immunoreactive cells were observed at the base of the secondary lamellae while most of the weakly-immunoreactive cells were observed at the interlamellar region during the seawater adaptation. Figure 3 shows changes in

the occupying area of the strongly- and the weakly-immunoreactive cells following transfer to seawater. In fresh water, the occupying area of the strongly-immunoreactive cells was 7.3% and that of the weakly-immunoreactive cells was 6.4%. The occupying area of the strongly-immunoreactive cells gradually and significantly increased with an increase in the days after the transfer to seawater and became 35.5%, 25 days after the transfer. On the other hand, the occupying area of the weakly-immunoreactive cells gradually and significantly decreased with an increase in the days after the transfer and became 0.5%, 25 days after the transfer.

Freshwater adaptation

Figure 4 shows changes in the LD₅₀ after direct transfer of the fish of the seawater-adapted strain to fresh water. Although the LD₅₀ was 51.9 ppt in the seawater-adapted fish, the LD₅₀ gradually decreased with an increase in the days

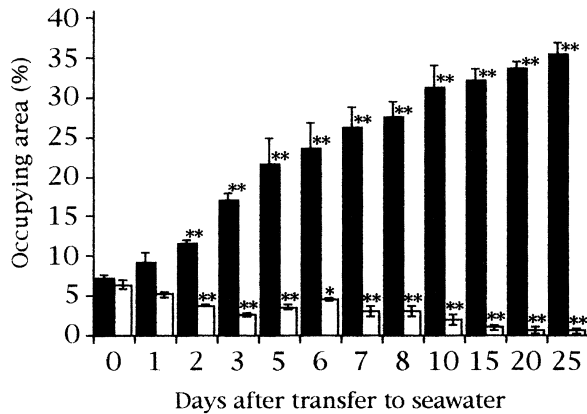


Fig. 3. Changes in the occupying area of strongly- and weakly-immunoreactive cells following transfer of freshwater fish to seawater. The fish were transferred to 15 ppt dilute seawater on day 0 and subsequently transferred to 35 ppt seawater on day 5. Significantly different from the initial control fish with * $P < 0.05$ and ** $P < 0.01$. □, Strongly-immunoreactive cells; ■, weakly-immunoreactive cells. Data are expressed as the mean \pm S.E.M. with $n = 5$.

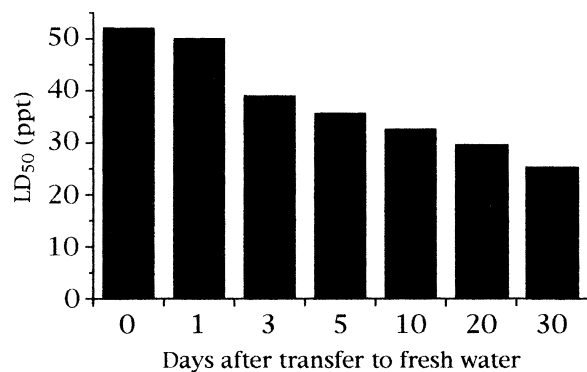


Fig. 4. Changes in LD₅₀ following transfer of seawater-adapted fish to fresh water. LD₅₀ was calculated from the data of survival rates 24 hr after transfer to 20, 25, 30, 35, 40, 45, 50, 55 or 60 ppt seawater.

after the transfer to fresh water and became 25.3 ppt, 30 days after the transfer. This value was the same level as that for the fish of the freshwater strain.

Hematoxylin-eosin staining of the gill section of the fish in the seawater-adapted strain is shown in Fig. 5-A. Large spherical eosinophilic cells were observed on the primary lamellae at the base of the secondary lamellae and showed strong immunoreactivity for Na⁺,K⁺-ATPase (Fig. 5-B, C).

When the fish were transferred to fresh water, the strongly-immunoreactive cells decreased in number and size while the weakly-immunoreactive cells increased in number as shown in Fig. 5-D, E and F. Most of the strongly-immunoreactive cells were observed at the base of the secondary lamellae while most of the weakly-immunoreactive cells were observed in the interlamellar region during the freshwater adaptation. Figure 6 shows changes in the occupying area of the strongly- and the weakly-immunoreactive cells following transfer to fresh water. In the seawater-adapted fish, the occupying area of the strongly-immunoreactive cells was 38.7% while that of the

weakly-immunoreactive cells was 0.3%. The occupying area of the strongly-immunoreactive cells gradually and significantly decreased with an increase in the days after the transfer to fresh water and became 7.5%, 30 days after the transfer. On the other hand, the occupying area of the weakly-immunoreactive cells gradually and significantly increased with increase in the days after the transfer and became 6.3%, 30 days after the transfer. These values were at the same levels as those for the fish in the freshwater strain.

Relationships of salinity tolerance to immunolocalization

Figure 7 shows the relationships of LD₅₀ to the occupying areas of the strongly-immunoreactive cells and the weakly-immunoreactive cells during the seawater and freshwater adaptations. An intimate positive correlation was observed between the LD₅₀ and the occupying area of the strongly-immunoreactive cells while an intimate negative correlation was observed between the LD₅₀ and the occupying area of the weakly-immunoreactive cells.

DISCUSSION

Immunohistochemical localization of Na⁺,K⁺-ATPase in the gill epithelium has been performed in several fish species (Ura *et al.*, 1996; Witters *et al.*, 1996; Uchida *et al.*, 1996). Witters *et al.* (1996) reported that Na⁺,K⁺-ATPase-positive cells correspond to the cells labelled with the chloride-cell-specific staining of DASPMI (dimethylaminostyrylmethylpyridiniumiodine) in the rainbow trout. Ura *et al.* (1996) also reported that immunoreactivity appeared in the large spherical eosinophilic cells in the gill epithelium of the masu salmon, and that the cells correspond to chloride cells. Autoradiographic and ultracytochemical studies have demonstrated that Na⁺, K⁺-ATPase in the gills is mainly located on the chloride cells (Karnaky *et al.*, 1976a; Hootman and Philpott, 1979). In the present study, immunoreactivity appeared in large eosinophilic cells located on the primary lamellae, which correspond to chloride cells as shown by Ura *et al.* (1996). However, immunoreactivity significantly differed among the cells in the freshwater guppy. Immunoreactivity was strong in the large eosinophilic cells located at the base of the secondary lamellae and weak in the large eosinophilic cells located at the interlamellar region. Pisam *et al.* (1987) reported the existence of two types of chloride cells on the primary lamellae of the freshwater guppy on the basis of location, shape and ultrastructural features. They described that one cell type, the α -chloride cell, is an elongated form of the cell located at the base of the secondary lamellae while another cell type, the β -chloride cell, is an ovoid cell form located in the interlamellar region of the primary epithelium. On the basis of their location and shape, the strongly- and the weakly-immunoreactive cells exactly correspond to the α - and the β -chloride cells, respectively.

When freshwater guppies were transferred to seawater, their LD₅₀ gradually increased from 24.8 ppt to 52.5 ppt. This indicates a significant increase of salinity tolerance. During the seawater adaptation, the strongly-immunoreactive cells

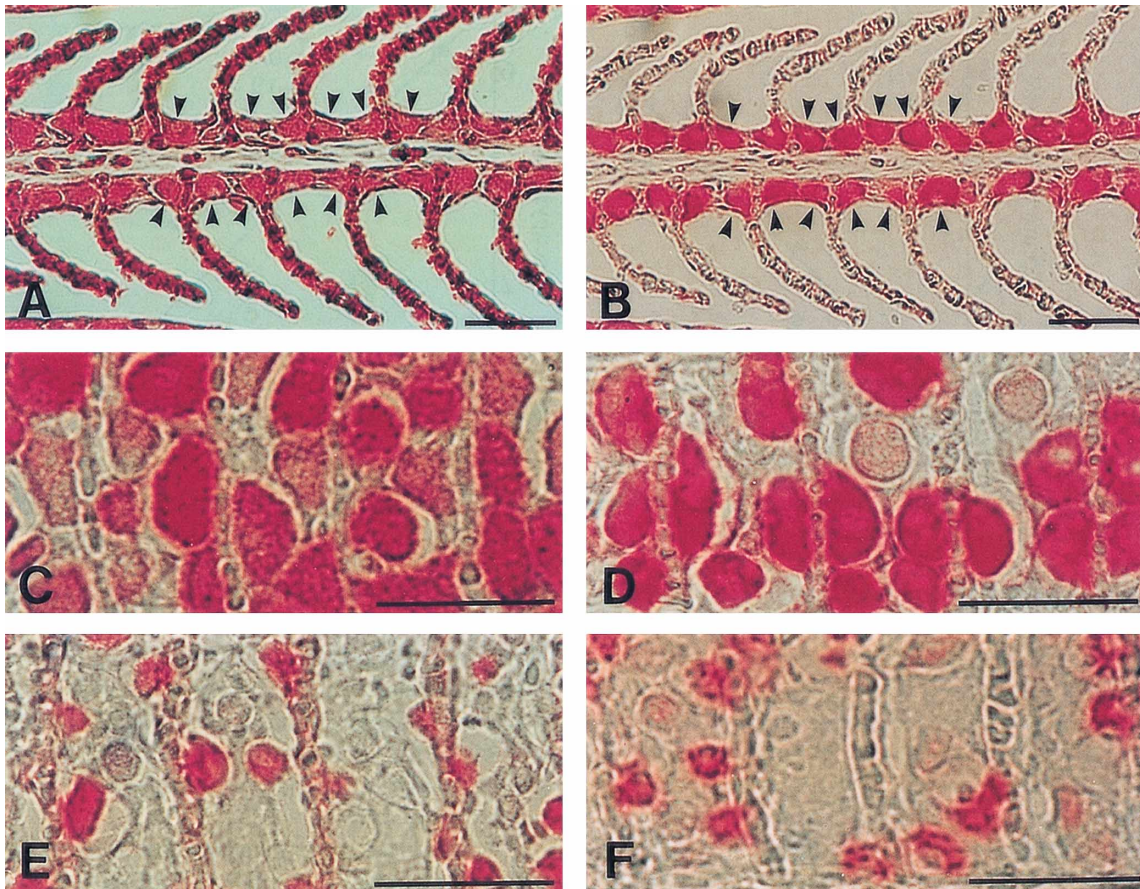


Fig. 5. Gill sections of the guppy in the seawater-adapted strain (A, B and C) and during freshwater adaptation (D, E and F). (A) Sagittal section stained with hematoxylin-eosin in fish of the seawater-adapted strain. Large eosinophilic cells were observed on the primary lamella (arrowheads). (B) Sagittal section stained with the antibody to Na⁺,K⁺-ATPase in fish of the seawater-adapted strain. Strong immunoreactivity was observed in the large spherical eosinophilic cells (arrowheads). (C) Level surface section stained with the antibody to Na⁺,K⁺-ATPase in fish of the seawater-adapted strain. The bases of the secondary lamellae were observed as vertical stripes. Strongly-immunoreactive cells were observed at the base of the secondary lamellae. (D) Level surface section stained with the antibody to Na⁺,K⁺-ATPase in fish transferred to fresh water for 3 days. (E) Level surface section stained with the antibody to Na⁺,K⁺-ATPase in fish transferred to fresh water for 5 days. (F) Level surface section stained with the antibody to Na⁺,K⁺-ATPase in fish transferred to fresh water for 20 days. The strongly-immunoreactive cells decreased in number and size while the weakly-immunoreactive cells increased in number during the freshwater adaption (C-F). Scale bar, 30 μ m.

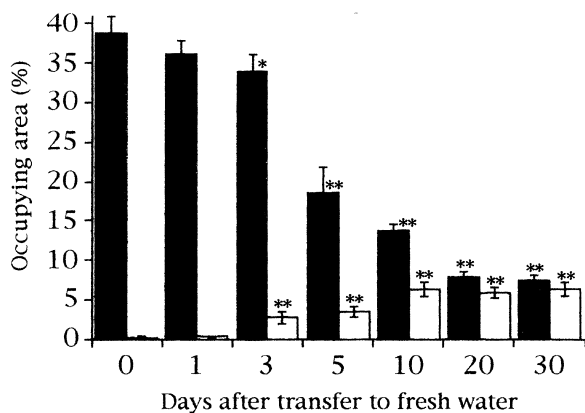


Fig. 6. Changes in the occupying area of strongly- and weakly-immunoreactive cells following transfer of seawater-adapted fish to fresh water. Significantly different from the initial control fish with * $P < 0.05$ and ** $P < 0.01$. ■, Strongly-immunoreactive cells; □, weakly-immunoreactive cells. Data are expressed as the mean \pm S.E.M. with $n = 5$.

increased in number and size and their occupying area gradually extended from 7.3% to 35.5%, while the weakly-immunoreactive cells decreased in number and their occupying area gradually reduced from 6.4% to 0.5%. Pisam *et al.* (1987) reported that the α -chloride cells increased in size while the β -chloride cells underwent a progressive degeneration and disappeared when guppies were adapted from fresh water to seawater. The changes in the strongly- and the weakly-immunoreactive cells during the seawater adaptation conformed with those of the α - and the β -chloride cells. Many investigators have reported that the transfer of freshwater fish to seawater caused increases in the size and/or number of chloride cells (Shirai and Utida, 1970; Thomson and Sargent, 1977; Foskett *et al.*, 1981; Uchida *et al.*, 1996) and Na⁺,K⁺-ATPase activity in the gills (Utida *et al.*, 1971; Karnaky *et al.*, 1976b; Thomson and Sargent, 1977; Epstein *et al.*, 1980). Uchida *et al.* (1996) reported that immunoreactivity for Na⁺,K⁺-ATPase in chloride cells on primary lamellae and also gill Na⁺,K⁺-ATPase activity significantly increased after the transfer of

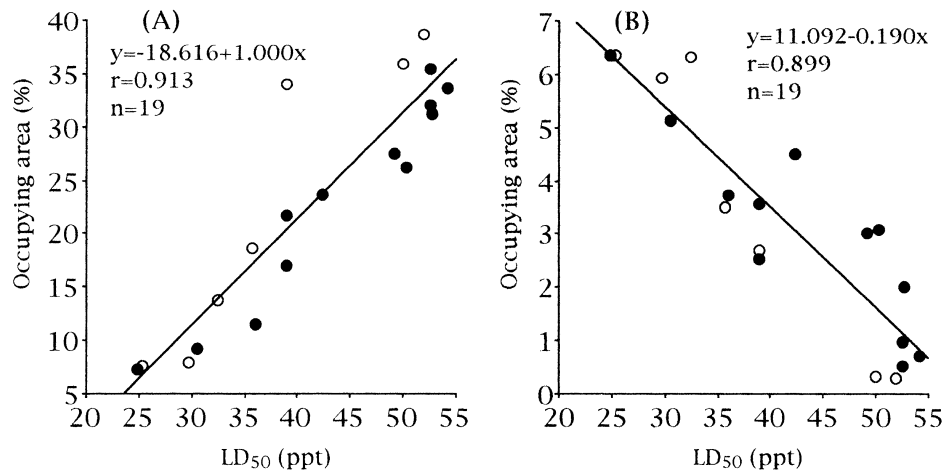


Fig. 7. Relationships of LD₅₀ to the occupying area of strongly-immunoreactive cells (A) and weakly-immunoreactive cells (B). ●, Data from the seawater adaptation; ○, from the freshwater adaptation.

freshwater chum salmon to seawater. Therefore, not only the number and size of the chloride cells but also the expression of Na⁺,K⁺-ATPase protein in the chloride cells may be important for seawater adaptation.

In the fish of the seawater-adapted strain, most of the large spherical eosinophilic cells on the primary lamellae showed strong immunoreactivity for Na⁺,K⁺-ATPase, similar to the fish that adapted from fresh water to seawater. Foskett and Scheffey (1982) provided direct evidence that chloride cells are the sites of salt secretion in seawater. Considering the existence of the strong immunoreactivity for Na⁺,K⁺-ATPase in most chloride cells, chloride cells may need a high expression of Na⁺,K⁺-ATPase protein for salt secretion in seawater.

When seawater-adapted guppies were transferred to fresh water, the strongly-immunoreactive cells decreased in number and size and their occupying area gradually reduced from 38.7% to 7.5% while the weakly-immunoreactive cells increased in number and their occupying area gradually extended from 0.3% to 6.3%. Shirai and Utida (1970) reported that both weakly-acidophilic chloride cells and strongly-acidophilic chloride cells exist in the gills of the Japanese eel. Although only strongly-acidophilic chloride cells were observed in seawater-adapted eels, the number of the cells decreased following transfer to fresh water. The decrease in the number of the strongly-immunoreactive cells in the present study is similar to that of the strongly-acidophilic chloride cells reported by Shirai and Utida (1970). The present results revealed that freshwater adaptation of the guppy is accompanied by significant reductions in the strongly-immunoreactive cells and salinity tolerance.

An intimate positive correlation was observed between salinity tolerance and the occupying area of the strongly-immunoreactive cells during the seawater and freshwater adaptation. This directly indicates that the occupying area of the strongly-immunoreactive cells in the gill epithelium reflects salinity tolerance at the individual level. Because Na⁺,K⁺-ATPase in the chloride cells plays a crucial role in ion trans-

port (Marshall, 1995; McCormick, 1995), not only the occupying area of the chloride cells but also the expression of Na⁺,K⁺-ATPase protein in the cells may be important with respect to the osmoregulatory function in the gills and hypoosmoregulatory ability at the individual level. This notion was supported by an intimate negative correlation between salinity tolerance and the occupying area of the weakly-immunoreactive cells.

The changes in the constitutions of two types of chloride cells in the gill epithelium were also observed in some salmonids during smoltification (Richman *et al.*, 1987; Pisam *et al.*, 1988). It was reported that parr-smolt transformation is accompanied by improvement in hypoosmoregulatory ability (Koch and Evans, 1959) and elevation of Na⁺,K⁺-ATPase activity in the gills (Zaugg and McLain, 1970, 1972; Boeuf and Prunet, 1985; Richman *et al.*, 1987; Pisam *et al.*, 1988). Although the existence of significant differences in immunoreactivity for Na⁺,K⁺-ATPase in the two types of chloride cells in salmonids was not previously reported, it is possible that the changes in the constitutions of the two types of chloride cells are important for the improvement of the hypoosmoregulatory ability during smoltification.

The present study directly suggests that not only the occupying area of chloride cells in the gill epithelium but also the expression of the Na⁺,K⁺-ATPase protein in the cells is important with respect to the osmoregulatory function in the gills and hypoosmoregulatory ability at the individual level. Because of the existence of significant differences in the expression of the Na⁺,K⁺-ATPase protein between the two types of chloride cells, the guppy is a good fish for further studies of the osmoregulatory function in the two types of chloride cells.

ACKNOWLEDGMENTS

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