The Journal of Experimental Biology 202, 3485–3495 (1999)
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JEB2377

IN VIVO SEQUENTIAL CHANGES IN CHLORIDE CELL MORPHOLOGY IN THE YOLK-SAC MEMBRANE OF MOZAMBIQUE TILAPIA (*OREOCHROMIS MOSSAMBICUS*) EMBRYOS AND LARVAE DURING SEAWATER ADAPTATION

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Accepted 8 October; published on WWW 29 November 1999

Summary

Changes in chloride cell morphology were examined in the yolk-sac membrane of Mozambique tilapia (*Oreochromis mossambicus*) embryos and larvae transferred from fresh water to sea water. By labelling chloride cells with DASPEI, a fluorescent probe specific for mitochondria, we observed *in vivo* sequential changes in individual chloride cells by confocal laser scanning microscopy. In embryos transferred from fresh water to sea water 3 days after fertilization, 75 % of chloride cells survived for 96 h, and cells showed a remarkable increase in size. In contrast, the cell size did not change in embryos and larvae kept in fresh water. The same rate of chloride cell turnover was observed in both fresh water and sea water. Using differential interference contrast (DIC) optics and whole-mount immunocytochemistry with anti-Na^+/K^+-ATPase, we classified chloride cells into three developmental stages: a single chloride cell without an apical pit, a single chloride cell with an apical pit, and a multicellular complex of chloride and accessory cells with an apical pit. DIC and immunofluorescence microscopy revealed that single chloride cells enlarged and were frequently indented by newly differentiated accessory cells to form multicellular complexes during seawater adaptation. These results indicate that freshwater-type single chloride cells are transformed into seawater-type multicellular complexes during seawater adaptation, suggesting plasticity in the ion-transporting functions of chloride cells in the yolk-sac membrane of tilapia embryos and larvae.

Key words: chloride cell, yolk-sac membrane, DASPEI, Na^+/K^+-ATPase, confocal laser scanning microscopy, tilapia, *Oreochromis mossambicus*, adaptation.

Introduction

In adult teleosts, chloride cells in the gill epithelium are the major site of ionic regulation. Chloride cells are characterized by numerous mitochondria and an extensive tubular system, in which the ion-transporting enzyme Na^+/K^+-ATPase is located (Karnaky et al., 1976; Philpott, 1980; McCormick, 1995). These cells are involved in the secretion of excess ions from the body fluid in sea water, and possibly in ion uptake in fresh water (Foskett and Scheffey, 1982; Zadunaisky, 1984; Marshall, 1995; Perry, 1997). In embryos and larvae of several teleost species, chloride cells have been detected in the epithelia covering the yolk and body, and these extrabranchial chloride cells are considered to be the site of ionic regulation during the early developmental stages in the absence of functional gills (Shelbourne, 1957; Lasker and Threadgold, 1968; Hwang and Hirano, 1985; Alderdice, 1988; Hwang, 1989, 1990; Ayson et al., 1994; Kaneko et al., 1995; Tytler and Ireland, 1995; Wales and Tytler, 1996; Sasai et al., 1998b; Hiroi et al., 1998a; Watanabe et al., 1999).

Mozambique tilapia (*Oreochromis mossambicus*) can mature and breed in both fresh water and sea water, and the embryos can survive direct transfer from fresh water to sea water and *vice versa*. Ayson et al. (1994) reported that tilapia embryos and larvae adapted to both fresh water and sea water possess numerous chloride cells in the yolk-sac membrane, and that chloride cells in seawater embryos and larvae are larger than those in freshwater embryos. Moreover, chloride cells in the yolk-sac membrane become larger in response to transfer from fresh water to sea water, whereas they become smaller when the fish are transferred from sea water to fresh water. According to Shiraishi et al. (1997), multicellular complexes, consisting of well-developed chloride cells and accessory cells, are formed in the yolk-sac membrane of seawater tilapia embryos and larvae. These morphological observations suggest the occurrence of freshwater-type and seawater-type chloride cells in the yolk-sac membrane of tilapia embryos and larvae, as is the case with gill chloride

cells in adult fish (Pisam et al., 1987, 1993; Uchida et al., 1996; Sasai et al., 1998a; Hirai et al., 1999). The marked euryhalinity of tilapia embryos and larvae seems to be attributable to these chloride cells, which may function as the sites of ion uptake or ion secretion in fresh water and sea water, respectively. However, the mechanism for altering their structure and function when exposed to different osmotic environments has not yet been documented.

From both physiological and morphological points of view, it is of great interest to determine whether chloride cells are replaced by newly differentiated cells of a different function after transfer from fresh water to sea water or from sea water to fresh water, or whether the same chloride cells function in both fresh water and sea water. The most effective way to answer this question would be to examine sequential changes in individual chloride cells during adaptation to a different salinity. However, conventional methods do not allow us to follow time course changes in the same chloride cells in the gills of adults: separated gill filaments are not a suitable material in which to examine the time course of changes in chloride cells, because of their complex, three-dimensional structures; primary cultures of the gill epithelium may also be unsuitable, since dispersed chloride cells do not survive under *in vitro* conditions (Pärt and Bergström, 1995; Avella and Ehrenfeld, 1997; Wood and Pärt, 1997). In contrast, the yolk-sac membrane of embryos and larvae is a suitable model in which to examine the time course of changes in chloride cells *in vivo* because of its simple flat structure.

In the present study, we followed individual chloride cells in the yolk-sac membrane of tilapia embryo and larvae transferred from fresh water to sea water. An intact fish was immersed in a medium containing a mitochondrial fluorescent probe, which has been used to identify chloride cells, and morphological changes in individual chloride cells were followed continuously under a confocal laser scanning microscope. Meanwhile, chloride cells in the fixed yolk-sac membrane were examined by immunocytochemistry using an antiserum specific for Na^+/K^+-ATPase α-subunit, a key enzyme for ion transport in chloride cells.

Materials and methods

Fish

Mature tilapia (*Oreochromis mossambicus*) were maintained in tanks containing recirculating fresh water (Na^+, 0.74 mmol l^{-1}; Ca^{2+}, 0.54 mmol l^{-1}; Mg^{2+}, 0.26 mmol l^{-1}) at 25 °C. Fertilized eggs were obtained from the mouth of brooding females 3 days after fertilization. The chorion of embryos was then removed using sharp-pointed forceps under a dissecting microscope, since the chorion is not permeable to fluorescent dye and dissolves at hatching to cause water fouling. In the following experiments, water temperature was maintained at 25 °C. Tilapia embryos typically hatch after 5 days of incubation at 25 °C, and yolk absorption is completed by 10 days after hatching.

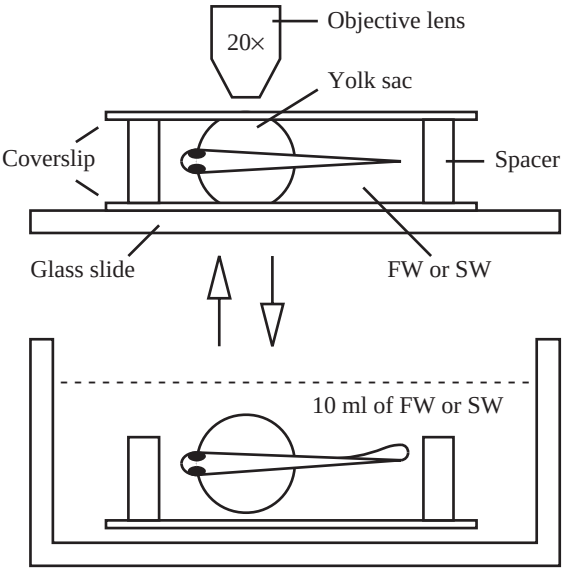


Fig. 1. Schematic representation of the incubation chamber for *in vivo* sequential observation of chloride cells in the yolk-sac membrane. An individual embryo previously incubated in DASPEI solution was placed in the chamber and covered with a coverslip. The flattened surface at the top of the yolk sac was examined using a confocal laser scanning microscope. The coverslip above the fish was carefully removed as soon as the observation was finished, and the chamber including the fish was immersed in 10 ml of fresh water (FW) or sea water (SW). Confocal images of the same area were obtained repeatedly at 6 h intervals for 96 h.

Vital staining and sequential observation of individual chloride cells

Dechorionated embryos (3 days after fertilization or 2 days before hatching) were incubated in fresh water containing 250 μmol l^{-1} 2-(4-dimethylaminostyryl)-1-ethylpyridinium iodide (DASPEI; Sigma, St Louis, MO, USA) for 6 h prior to initial observation. DASPEI is a mitochondrial vital probe which has been used to identify chloride cells (Bereiter-Hahn, 1976; Karnaky et al., 1984). An individual embryo was then briefly rinsed in DASPEI-free fresh water, and placed in a chamber filled with fresh water using a large-mouthed pipette (Fig. 1). The chamber consisted of a coverslip (24 mm×24 mm) and a spacer slightly thinner than the thickness of the yolk sac. The chamber was then placed on a glass slide and covered with another coverslip, so that the fish was sandwiched between two coverslips. The flattened surface at the top of the yolk sac was examined using a Zeiss 310 confocal laser scanning microscope (Carl Zeiss, Oberkohen, Germany). It is difficult to observe DASPEI-positive chloride cells in the yolk-sac membrane using a conventional fluorescence microscope, because of the strong autofluorescence of the yolk. In contrast, laser scanning microscopy enables us to observe the sectional image of the yolk-sac membrane, which is not affected by the autofluorescence of the yolk. The 488 nm line of an argon ion laser was used as the excitation wavelength, and the emission was recorded at 515–565 nm. An area of the yolk-sac

membrane where chloride cells were densely distributed was selected, and a confocal image of 0.41 mm^2 was obtained with a 20×/0.50 objective lens (width of optical section, 12.1 μm).

After the initial observation, the coverslip on the top was carefully removed, and the chamber including the embryo was immersed in 10 ml of artificial sea water (Na^+, 409 mmol l^{-1}; Ca^{2+}, 10 mmol l^{-1}; Mg^{2+}, 47 mmol l^{-1}; Jamarin Laboratory, Osaka, Japan) containing 250 μmol l^{-1} DASPEI. At 2, 6 and 12 h after transfer to sea water containing DASPEI, confocal images were obtained from the area including most of the chloride cells identified in the initial observation. The pattern of distribution of melanophores in the yolk sac helped in identifying the area used. Confocal images of the same area were obtained repeatedly at 6 h intervals until 96 h after transfer to sea water. The incubation medium (DASPEI-containing sea water) was replaced with DASPEI-free sea water at 6 h intervals: the fish was incubated in DASPEI-free sea water for 12–18, 24–30, 36–42, 48–54, 60–66, 72–78 and 84–90 h, and in sea water containing DASPEI for 18–24, 30–36, 42–48, 54–60, 66–72, 78–84 and 90–96 h after seawater transfer. As controls, other embryos were kept in fresh water and examined using the same procedure. The experiment was conducted with two broods produced by different parents. Sequential images were obtained from three individuals kept in fresh water (one and two individuals from two different broods) and from four individuals transferred from fresh water to sea water (two individuals from each of two broods) throughout the experimental period of 96 h.

The successive confocal images obtained from both freshwater and seawater embryos and larvae at 0, 24, 48, 72 and 96 h after transfer were used for morphometric analyses of chloride cells. The cross-sectional area of DASPEI-positive chloride cells was measured on an Apple Macintosh computer using the public domain NIH Image program (available on the Internet at http://rsb.info.nih.gov/nih-image/).

DIC and immunofluorescence observations

Another brood of dechorionated embryos 3 days after fertilization was separated into two groups: half the embryos were transferred directly to artificial sea water, and the other half were maintained in fresh water. The ambient water was renewed once a day. The freshwater embryos 3 days after fertilization (2 days before hatching) and the larvae of both groups 48 and 96 h after the transfer (corresponding to 0 and 2 days after hatching) were anaesthetized with 2-phenoxyethanol and fixed in 4% paraformaldehyde in 0.1 mol l^{-1} phosphate buffer (pH 7.4) for 50 min at 4 °C. The yolk sac was then incised, the yolk was carefully scraped out, and the connective tissue and capillaries under the basal membrane were removed using sharp-pointed forceps. The embryos and larvae were further fixed overnight at 4 °C and preserved in 70% ethanol.

Whole-mount immunocytochemistry was carried out following the method of Ohtani et al. (1989) with some modifications. After treatment with 0.1% sodium cyanoborohydride in 0.01 mol l^{-1} phosphate-buffered saline (PBS, pH 7.2) for 1 h, the samples were incubated overnight at 4 °C with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-Na^+/K^+-ATPase diluted 1:500 with PBS containing 0.05% Triton X-100, 10% normal goat serum, 0.1% bovine serum albumin, 0.02% keyhole limpet haemocyanin and 0.01% sodium azide. After rinsing in PBS for 1 h, the yolk-sac membrane was removed from the body trunk, mounted on a slide in PBS and examined by laser scanning microscopy. The excitation and emission wavelengths were the same as those used for DASPEI observations.

Both confocal fluorescence and differential interference contrast (DIC) images were taken using a laser scanning microscope equipped with a 63×/1.4 oil-immersion objective lens (width of optical section, 0.75 μm). The total area of the yolk-sac membrane was approximately 3 mm^2, and ten images corresponding to 0.42 mm^2 were obtained from each sample. DIC images were superimposed on fluorescence images using Photoshop 4.0 (Adobe, San Jose, CA, USA), and the cross-sectional area and density of immunopositive chloride cells were measured using the NIH Image program. Two or more immunopositive chloride cells which shared one apical pit and formed a multicellular complex were regarded as one unit; immunopositive chloride cells that touched but possessed their own apical pits were measured separately.

Antibody

A polyclonal antibody was raised in a rabbit against a synthetic peptide corresponding to part of the highly conserved region of the Na^+/K^+-ATPase α-subunit (Ura et al., 1996). The amino acid sequence of the synthetic peptide was Cys-Val-Thr-Gly-Val-Glu-Glu-Gly-Arg-Leu-Ile-Phe-Asp-Asn-Leu-Lys-Lys. The antibody was further purified by affinity chromatography and conjugated to FITC. The specificity of the antibody was confirmed by western blotting (Uchida et al., 1999).

Statistical analyses

Significant differences in the cross-sectional area of DASPEI-positive and Na^+/K^+-ATPase-immunopositive chloride cells between freshwater and seawater groups at each sampling time were examined using the two-sample *t*-test, as the homogeneity of variance was established. Significant differences in the frequencies of DASPEI-positive chloride cells existing at 0 h and cells appearing after that between freshwater and seawater groups at each sampling time were tested using the χ^2-test for independence. Significant differences in the density of Na^+/K^+-ATPase-immunopositive chloride cells between freshwater and seawater groups at each sampling time were tested using the Mann–Whitney test.

Results

Vital staining and sequential observation of individual chloride cells

Confocal images of representative individuals kept in fresh water and transferred to sea water are shown in Fig. 2. The

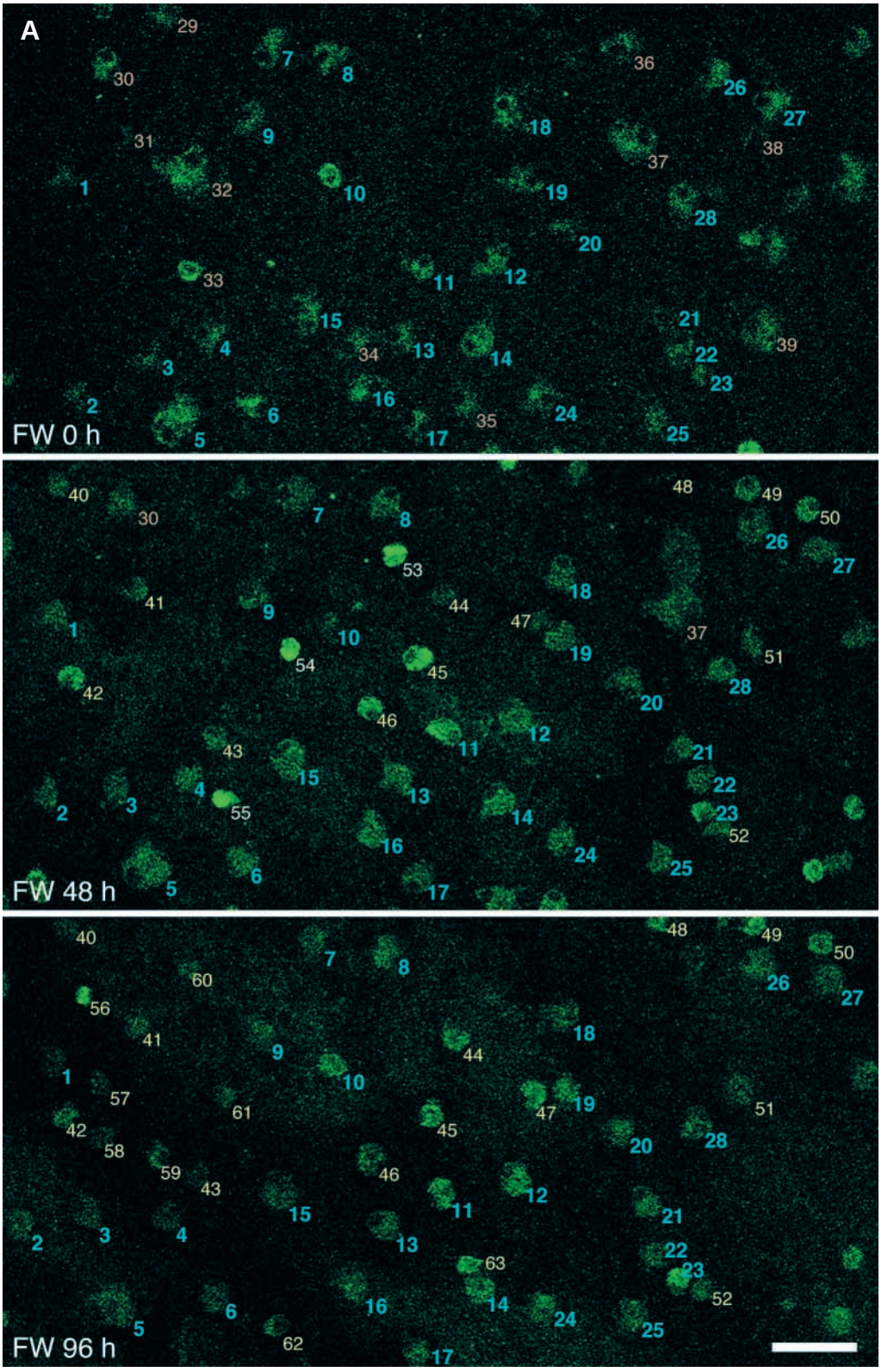
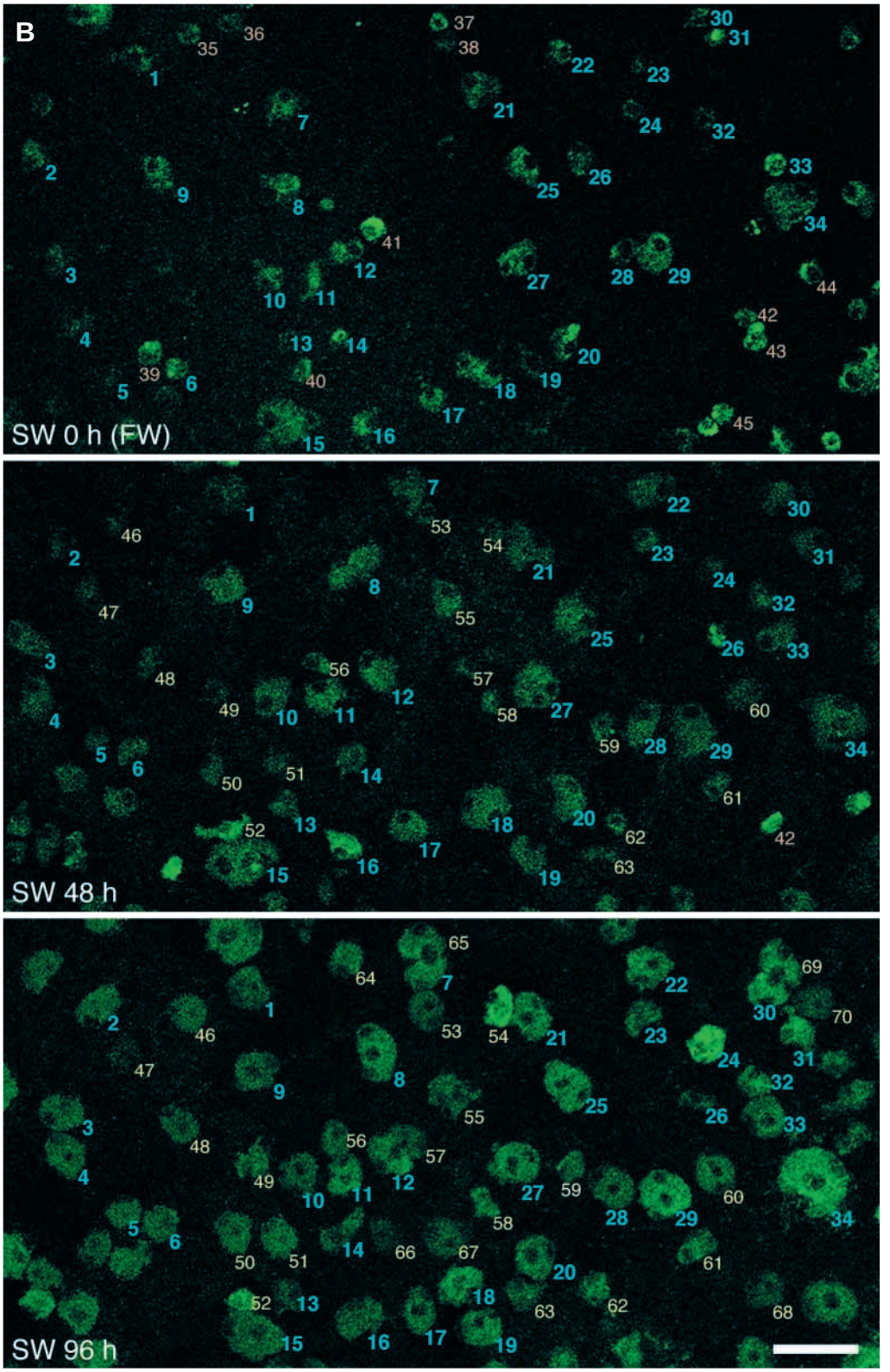


Fig. 2. Sequential confocal images of DASPEI-stained chloride cells in the yolk-sac membrane of a fish kept in fresh water (A) (FW 0 h, FW 48 h, FW 96 h) and of a fish transferred from fresh water to sea water (B) (SW 0 h, SW 48 h, SW 96 h) at 0 h, 48 h and 96 h. Chloride cells detectable throughout the experiment are labelled with blue numbers. Cells existing at 0 h and disappearing thereafter are numbered in red, those newly appearing but disappearing are numbered in pink, and those newly appearing and surviving until 96 h are numbered in yellow. Scale bar, 50 μm.

patterns of distribution of chloride cells were largely maintained during the experiment, unless the yolk-sac membrane was injured externally. Consequently, individual chloride cells were readily identified by sequential observation at 6 h intervals.

Time course changes in the area of each chloride cell identified in Fig. 2 are illustrated in Fig. 3A,B. The area of chloride cells did not change greatly in fresh water, while most chloride cells tended to increase in area after transfer to sea water. This is further confirmed by changes in the mean area



of chloride cells obtained from three freshwater and four seawater individuals: the mean cell area was 200–240 μm^2 in fresh water; the area increased steadily and reached 420 μm^2 at 96 h after transfer to sea water (white circles in Fig. 3C,D). Significant differences in cell area between freshwater and seawater individuals were observed at 48, 72 and 96 h (two-sample *t*-test, $P<0.05$ at 48 h, $P<0.01$ at 72 h and 96 h). On the basis of their patterns of appearance, chloride cells that survived until 96 h were classified into five subgroups: cells pre-existing at 0 h, and those appearing at 24 h, 48 h, 72 h and

96 h. Fig. 3C,D also shows changes in the mean area of these subgroups obtained from three freshwater and four seawater individuals. In freshwater individuals, the area of pre-existing cells remained constant throughout the experiment. The area of cells appearing at 24 h, 48 h and 72 h also remained constant, although these cells tended to be smaller than the pre-existing cells at the time of their appearance. In contrast, the pre-existing cells showed a remarkable increase in area after seawater transfer. Although the area of cells appearing after seawater transfer was comparable with that of pre-existing cells in fresh water at the beginning of the experiment, it increased thereafter.

In the freshwater individual shown in Fig. 2, the number of pre-existing chloride cells decreased gradually, and 72 % of the cells remained at 96 h, while new cells amounting to 64 % of the initial cell number appeared over 96 h. In the individual transferred to sea water, 76 % of the initial cells remained at 96 h, and new cells equivalent to 51 % of the initial cell number appeared. Changes in the number of chloride cells obtained from three freshwater and four seawater individuals are shown in Fig. 3E,F. There was no significant difference in the frequencies of pre-existing cells and newly appearing cells between freshwater and seawater individuals at any of the sampling times (χ^2-test for independence).

DIC and immunofluorescence observations

DIC observation revealed three distinct cell types in the yolk-sac membrane: pavement, chloride and putative undifferentiated cells. Pavement cells with ridge structures and apical openings of chloride cells were clearly visible on the outer surface of the yolk-sac membrane (Fig. 4A,D,G), and underlying chloride cells and putative undifferentiated cells were detectable at a deeper plane (Fig. 4B,E,H). Both chloride and undifferentiated cells possessed a nucleus of a smooth appearance which contained two or more nucleoli. The cytoplasm of chloride cells was readily distinguishable by its rough appearance, while that of undifferentiated cells was indistinct. Undifferentiated cells were abundantly distributed all over the yolk-sac membrane, frequently attached to chloride cells. Na^+/K^+-ATPase immunoreactivity was detected only in the cytoplasm of chloride cells (Fig. 4C,F,I).

The combination of DIC and immunofluorescence images allowed us to classify immunopositive chloride cells into three subtypes: a single chloride cell without an apical pit; a single chloride cell with an apical pit open; and a multicellular complex of chloride and accessory cells with an apical pit (Fig. 4). Although the single chloride cell without a pit was small, the cytoplasm showed strong immunoreactivity to Na^+/K^+-ATPase; an immunonegative nucleus was located in the centre

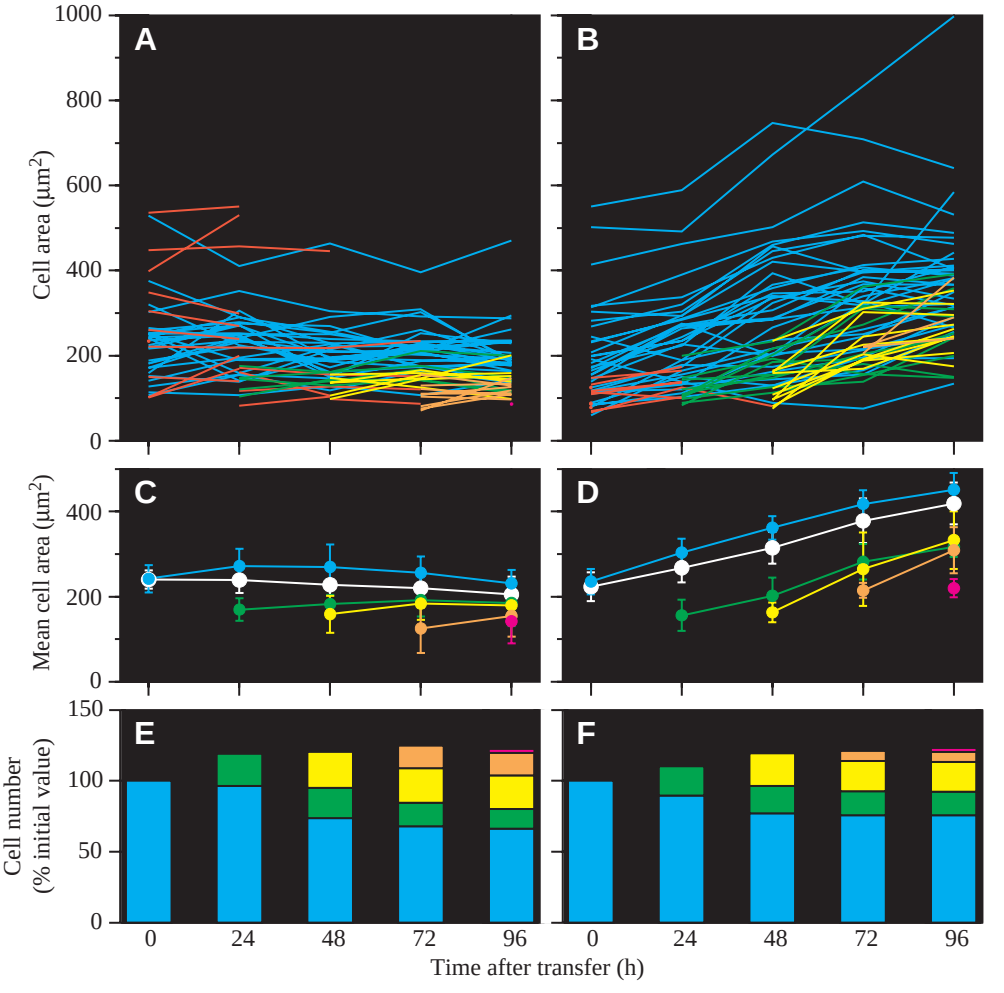


Fig. 3. (A,B) Time course changes in the cross-sectional area of individual chloride cells in the yolk-sac membrane of the freshwater (A) and seawater (B) individuals shown in Fig. 2. Chloride cells detectable throughout the experiment are plotted in blue. Cells appearing at 24 h, 48 h, 72 h and surviving until 96 h are shown in green, yellow and orange, respectively, while those appearing at 96 h are shown in magenta. Cells that disappeared are shown in red. (C,D) Time course changes in the mean area of chloride cells obtained from three freshwater (C) and four seawater (D) fish. The mean area of all chloride cells is plotted in white, and those of five subgroups (cells pre-existing at 0 h, and those appearing at 24 h, 48 h, 72 h and 96 h) are shown in blue, green, yellow, orange and magenta, respectively. Vertical bars represent standard deviations. (E,F) Time course changes in the number of chloride cells in freshwater (E) and seawater (F) fish. Data obtained from three freshwater and four seawater fish were combined for each observation time. The cell numbers for the five subgroups are shown in the same colours as in C and D.

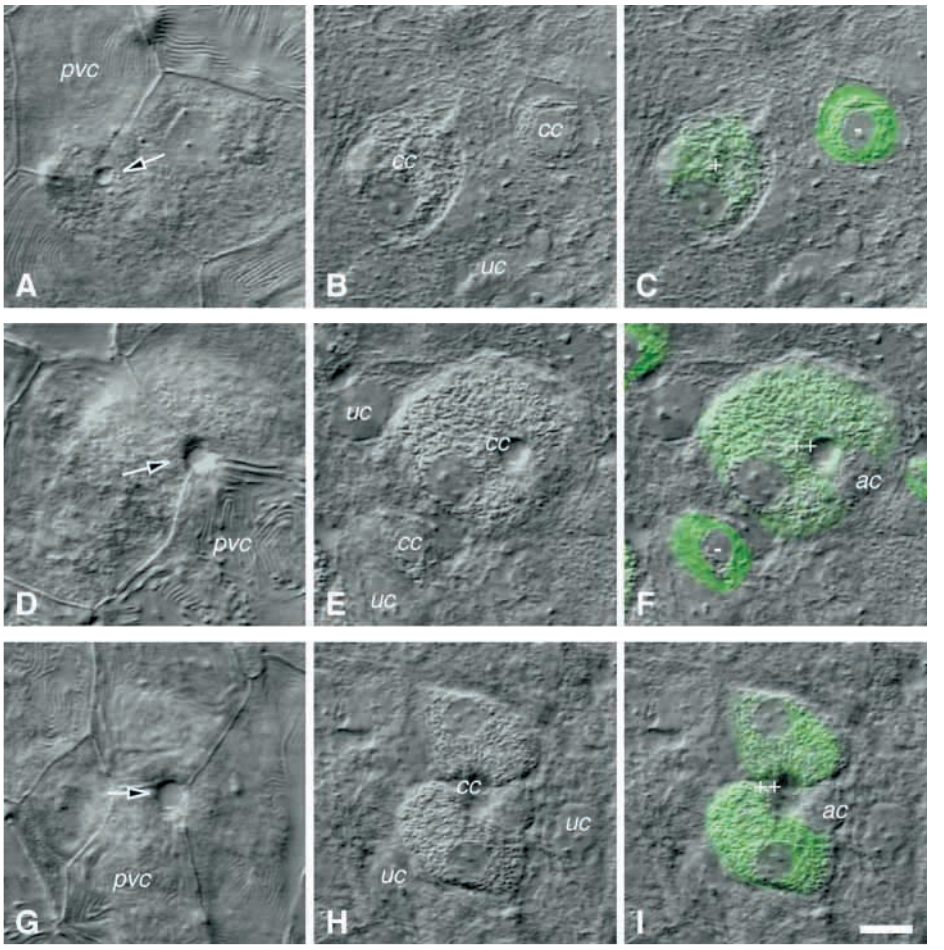


Fig. 4. Differential interference contrast (DIC) and immunofluorescence images of chloride cells in the fixed yolk-sac membrane. (A–C) Freshwater fish at 96 h; (D–I) Seawater fish at 96 h. The ridge structures and boundaries of pavement cells (*pvc*) and the apical pits of chloride cells (arrows) are observable on the surface (A,D,G), while the underlying chloride cells (*cc*) and undifferentiated cells (*uc*) are in focus at a deeper plane of the same tissues (B,E,H). These DIC images are combined with immunofluorescence images (C,F,I). Na^+/K^+-ATPase-immunopositive chloride cells (stained green) are classified into three subtypes: a single chloride cell without an apical pit (–); a single chloride cell with an apical pit open (+); and a multicellular complex of chloride cells and accessory cells (*ac*) with an apical pit (++). Scale bar, 10 μm.

(Fig. 4C,F). The single chloride cell with a pit possessed more cytoplasm, most of which was immunopositive; the pit was located in the centre and the nucleus peripherally (Fig. 4C). The multicellular complex usually consisted of an immunopositive main chloride cell and one or more accessory cells (Fig. 4F), and occasionally consisted of two or more immunopositive main chloride cells of similar size, also accompanied by accessory cells (Fig. 4I). All multicellular complexes possessed an apical pit, and accessory cells were located close to the pit. The cytoplasm of accessory cells was immunonegative or only slightly immunopositive for Na^+/K^+-ATPase.

Size/frequency distributions of immunopositive chloride cells in freshwater and seawater individuals at 0, 48 and 96 h are shown in Fig. 5. In fresh water, chloride cells were consistently small, the mean area being 180–220 μm^2. Following transfer to sea water, chloride cells became larger, and the mean area reached 310 μm^2 at 96 h. Significant differences in cell area between freshwater and seawater groups were observed at 48 and 96 h (two-sample *t*-test, $P<0.01$). Although there was no significant difference in the density (cells mm^{-2}) of chloride cells between freshwater and seawater individuals at 48 h and 96 h, the density of multicellular complexes in seawater groups was significantly

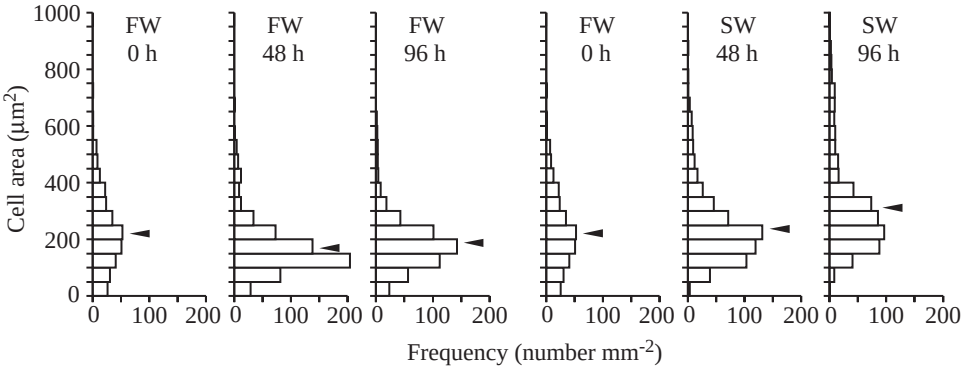


Fig. 5. Changes in the size/frequency distributions of Na^+/K^+-ATPase-immunopositive chloride cells in the fixed yolk-sac membrane. Data obtained from five individuals were combined for each group. Arrowheads indicate the mean cell area.

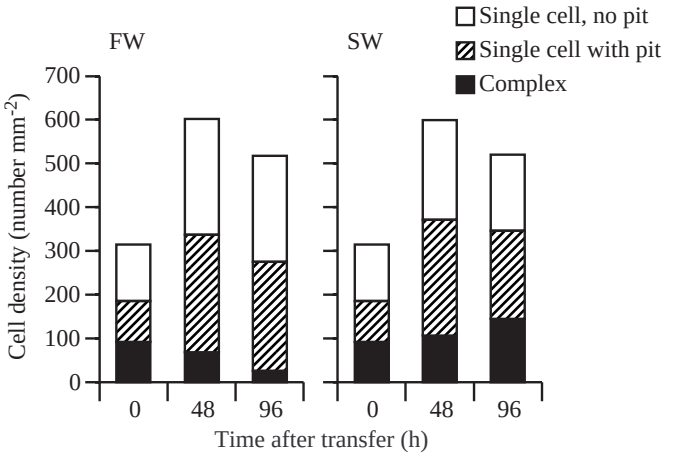


Fig. 6. Changes in the density of three types of Na^+/K^+-ATPase-immunopositive chloride cells in the fixed yolk-sac membrane. Chloride cells are classified into a single cell without a pit, a single cell with a pit and a multicellular complex of chloride and accessory cells with a pit. Data obtained from five individuals were combined for each group. FW, freshwater fish; SW, seawater fish.

greater than that in freshwater groups (Fig. 6, Mann–Whitney test, $P<0.05$ at 48 h and $P<0.01$ at 96 h).

Discussion

The enlargement of chloride cells after transfer from fresh water to sea water has been reported not only in the yolk-sac membrane of tilapia embryos and larvae, but also in the gills of various teleost species (Pisam and Rambourg, 1991; Ayson et al., 1994). However, it has not been demonstrated whether chloride cells existing in fresh water vanish, to be replaced with newly differentiated chloride cells after seawater transfer, or whether chloride cells survive after seawater transfer and increase their size. In the present study, we have demonstrated for the first time sequential changes in individual chloride cell morphology by observing DASPEI-stained chloride cells in the yolk-sac membrane of live tilapia embryos and larvae transferred from fresh water to sea water. This *in vivo* sequential observation confirmed that 75 % of the initial chloride cells remained 96 h after seawater transfer and that individual chloride cells increased their size markedly. Although 25 % of the initial cells disappeared and new cells amounting to 50 % of the initial cell number appeared after seawater transfer (providing direct evidence for cell turnover), the same rate of turnover was also observed in fish kept in fresh water. Chloride cell size did not change greatly in fish kept in fresh water, in contrast with those kept in sea water. From these results, we can conclude that the freshwater-type small chloride cells possess the ability to survive after direct transfer from fresh water to sea water and to transform to the seawater-type large chloride cells. Although the function of chloride cells was not addressed in the present study, chloride cells are generally considered to be involved in ion secretion in sea water and in ion uptake in fresh water. Therefore, these results also imply that chloride cells possess the plasticity to alter their ion-transporting function.

In addition to the *in vivo* sequential observation, we examined chloride cells in the fixed yolk-sac membrane by whole-mount immunocytochemistry with anti-Na^+/K^+-ATPase. The size of immunopositive chloride cells was somewhat smaller than that of DASPEI-positive chloride cells *in vivo*, probably because of the shrinkage of the fixed sample. Since the time course of changes in the mean size and number of DASPEI-positive chloride cells *in vivo* accorded well with those of immunopositive chloride cells, the treatment of living fish with DASPEI is considered to have little influence on the nature of chloride cells.

Li et al. (1995) and van der Heijden et al. (1997) labelled chloride cells in the gills of tilapia with both DASPMI and concanavalin-A/fluorescein (the former is a mitochondrial vital probe similar to DASPEI, and the latter stains glycoproteins in the apical pit of chloride cells) and clearly identified different developmental stages of chloride cells: mature chloride cells that are in contact with the water *via* the apical pit, and immature chloride cells that are not. We did not utilize this co-labelling method in our *in vivo* sequential observations in order to examine the living fish with minimum disturbance. However, the combination of DIC and immunofluorescence images of chloride cells in the fixed yolk-sac membrane provided information about the developmental processes of chloride cells. We identified three subtypes of chloride cell: a single chloride cell without an apical pit, a single chloride cell with an apical pit, and a multicellular complex of chloride and accessory cells with an apical pit.

The single chloride cells without pits are probably immature cells, since they are not exposed to the external environment and are smaller in size. However, their strong cytoplasmic Na^+/K^+-ATPase immunoreactivity indicates that the tubular system has already developed, because Na^+/K^+-ATPase is located in this system. These immature chloride cells may originate from undifferentiated cells with a nucleus similar to that of chloride cells, and then enlarge their cytoplasm and come into contact with the ambient water by forming apical pits. The single chloride cells with an apical pit seem to be mature chloride cells ready to function as ion-transporting cells.

The multicellular complex generally consisted of one main chloride cell and one (rarely two) accessory cells. Occasionally, two or more chloride cells of similar size shared one pit, and this type of complex was also associated with one or two accessory cells. Observations of the fixed yolk-sac membrane indicate that accessory cells develop from undifferentiated cells attaching to the main chloride cells and gradually intruding into the main chloride cells. Accessory cells are thought to be young stages of chloride cells (Sardet et al., 1979; Hootman and Philpott, 1980; Wendelaar Bonga and van der Meij, 1989) or a cell type specific to seawater-adapted fish (Laurent and Dunel, 1980; Chretien and Pisam, 1986). If accessory cells are young chloride cells, they would develop further and replace the main chloride cells when the

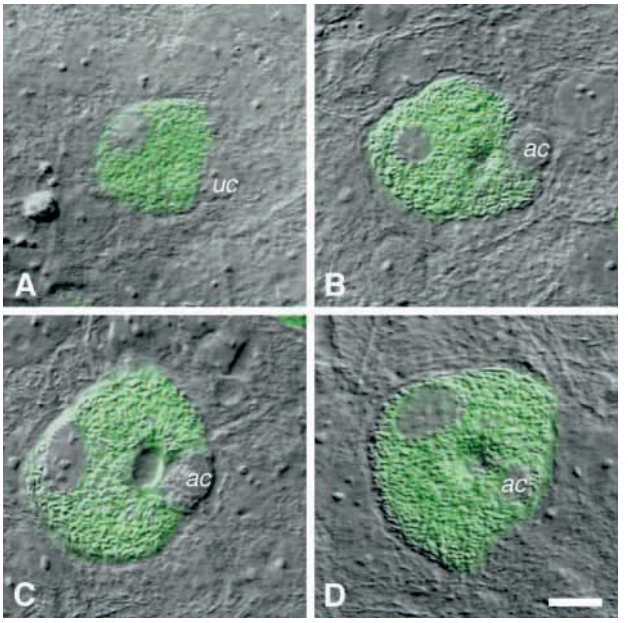


Fig. 7. A series of differential interference contrast (DIC) and immunofluorescence images of multicellular complexes of chloride and accessory cells arranged in the order of presumed developmental stages. (A) Seawater fish at 48 h; (B–D) seawater fish at 96 h. (A) At first, an undifferentiated cell (*uc*) is attached to a single, small chloride cell (stained green). (B,C) The chloride cell becomes larger, indented by an accessory cell (*ac*) originating from the undifferentiated cell. (D) The accessory cell is finally enveloped by the chloride cell, so that the boundary between these cells becomes indistinct. Scale bar, 10 μm.

main chloride cells degenerate. Although the replacement of main chloride cells with accessory cells is not indicated here, this may be partly because the experimental period of 96 h was too short for any cell turnover within the complex to be observed.

Although all three subtypes of chloride cell were found in both freshwater and seawater fish throughout the experiment, the number of multicellular complexes varied greatly in freshwater and seawater fish. The multicellular complexes increased in number after seawater transfer, whereas they decreased in number in fresh water. Considering these results and those obtained from the *in vivo* sequential observations, we presume that both the enlargement of main chloride cells and the formation of complexes occur simultaneously during seawater adaptation, as shown in Fig. 7. In their transmission electron microscopic observation of the yolk-sac membrane of seawater-adapted tilapia larvae, Shiraishi et al. (1997) have shown that main chloride cells and adjacent accessory cells interdigitate with each other to form multiple junctions. The multicellular complex is considered to be advantageous for the secretion of excess Na^+ from the body fluid, since secretion of Na^+ may occur down its electrochemical gradient *via* a paracellular pathway between the main chloride cells and accessory cells (Silva et al., 1977; Marshall, 1995; McCormick, 1995). Therefore, the multicellular complexes found in the yolk-sac membrane of tilapia embryos and larvae in sea water are probably the site of secretion of excess ions from the body fluid. In addition, it should be noted that a considerable number of multicellular complexes important for seawater adaptation were present in freshwater embryos. The number of complexes decreased as the fish developed in fresh water. Seawater adaptability in freshwater tilapia is high before hatching: freshwater embryos readily survive direct transfer to sea water; however, seawater adaptability decreases after hatching, although freshwater larvae are able to adapt to sea water when transferred gradually (K. Shiraishi and T. Kaneko, unpublished data). The decrease in seawater adaptability during development may be related to decrease in the number of multicellular complexes. The strong euryhalinity of tilapia embryos may be attributed to the occurrence of seawater-type multicellular complexes as well as freshwater-type single chloride cells in the yolk-sac membrane.

An increase in chloride cell size and density has been reported in the gills of adult teleosts transferred from fresh water to sea water (Pisam and Rambourg, 1991). In the present study, however, the density of chloride cells in the yolk-sac membrane did not differ between freshwater and seawater fish. This result is consistent with previous studies of the yolk-sac membrane of the same species (Ayson et al., 1994; Shiraishi et al., 1997). However, the density of chloride cells in seawater fish may have been underestimated because of the formation of multicellular complexes during seawater adaptation in the present and previous studies; multicellular complexes may have been counted as one cell. If the precise number of cells in the complex had been determined, the chloride cell number would have been greater in seawater than in freshwater fish.

Chretien and Pisam (1986) reported that cell differentiation and renewal in the gill epithelium of 50 % seawater-adapted guppy are three times faster than in freshwater-adapted fish. According to Uchida and Kaneko (1996), chloride cell turnover in the gills of chum salmon fry is approximately three times faster in seawater than in freshwater fish. In the present study, however, there was no significant difference in the turnover rate of chloride cells between freshwater and seawater fish. The longevity of chloride cells after seawater transfer may be a characteristic of those in the yolk-sac membrane, or the response of chloride cells to different osmotic conditions could vary among species.

Although the role of Na^+ and Cl^- excretion in chloride cells of seawater fish is well established (Foskett and Scheffey, 1982), their role in the uptake of Na^+ and Cl^- in freshwater fish is still controversial. Recent studies have suggested that pavement cells, rather than chloride cells, are the site of Na^+ uptake (Goss et al., 1992; Morgan et al., 1994; Potts, 1994; Kültz and Somero, 1995). We have also demonstrated that immunoreactivity to vacuolar-type H^+-ATPase, which generates a favourable electrochemical gradient for passive electrodiffusion of Na^+ through a Na^+ channel (Avella and Bornancin, 1989; Lin and Randall, 1995; Sullivan et al., 1995, 1996), is not detectable in chloride cells but is detectable in pavement cells in the yolk-sac membrane of freshwater tilapia

larvae (Hiroi et al., 1998b). Therefore, pavement cells, but not chloride cells, seem to be the site of Na^+ uptake in tilapia yolk-sac membrane. Recently, Avella and Ehrenfeld (1997) suggested that pavement cells are also involved in Cl^- secretion in sea water. Freshwater-type, single chloride cells in the yolk-sac membrane of tilapia embryos and larvae are probably the site of Ca^{2+} uptake, as suggested in the gills of adult fish (Flik et al., 1995).

Chloride cells in the yolk-sac membrane are not only important for osmotic and ionic regulation in the early life stages of teleosts without functional gills, but also provide excellent models for future research on chloride cell function generally because of their structural simplicity.

We wish to express our gratitude to Professor Howard A. Bern, University of California, Berkeley, for critical reading of the manuscript. We also thank Dr Takeo Kurihara, Seikai National Fisheries Research Institute, for his advice on statistical analyses. This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Japan. J.H. was supported by Research Fellowships of the Japan Society for the Promotion of Science for Young Scientists.

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