Aqua-BioSci. Monogr. (ABSM), Vol. 1, No. 1, pp. 1–62 (2008)

www.terrapub.co.jp/onlinemonographs/absm/

Functional Morphology of Mitochondrion-Rich Cells in Euryhaline and Stenohaline Teleosts

Toyoji Kaneko,* Soichi Watanabe and Kyung Mi Lee

Department of Aquatic Bioscience
Graduate School of Agricultural and Life Sciences
The University of Tokyo
Bunkyo, Tokyo 113-8657, Japan

Abstract

In teleosts, mitochondrion-rich (MR) cells, or chloride cells, are importantly involved in freshwater and seawater adaptation and acid-base regulation. In this paper, we reviewed recent advances in functional morphology of MR cells in relation to environmental adaptation in euryhaline and stenohaline teleost species with different life histories and adaptability to osmotic and ionic environments. MR cells already existed as early as during the embryonic stages before the formation of the gills. The principal site for MR-cell distribution shifts from the yolk-sac membrane and body skin during the embryonic stages to the gills in larval and later developmental stages, although the timing of the shift differs in different species. In euryhaline teleosts, MR cells alter their morphology and ion-transporting functions to meet unexpected environmental salinity changes, whereas alteration of MR-cell functions takes place as a preadaptive response for the forthcoming upstream and downstream migrations in diadromous teleosts. In stenohaline teleosts inhabiting either freshwater or seawater, on the other hand, MR cells do not possess the functional plasticity to switch the direction of ion transport, but merely adjust the degree of unidirectional ion transport. Thus, euryhalinity or stenohalinity of teleosts is primarily determined by their ability of functional alteration and plasticity of MR cells.

Received on April 15, 2008
Accepted on June 18, 2008
Published online on October 10, 2008

Keywords
- mitochondrion-rich cell
- chloride cell
- euryhalinity
- stenohalinity
- diadromous migration
- Mozambique tilapia
- killifish
- chum salmon
- Japanese eel
- fugu
- Japanese dace
- ion transport

1. Introduction

Among vertebrate species, teleost fishes have evolved to extend their habitats into diverse aquatic environments, such as rivers, lakes and oceans. It is an essential prerequisite for vertebrate species, including teleosts, to maintain various chemical and physical conditions of the body fluid within certain physiological ranges, in order to ensure normal activities of the cells that constitute the body. Among the important factors prescribing the characteristics of the body fluid is osmotic pressure, or osmolality, which is determined by the total concentration of the solutes, mostly inorganic electrolytes present in the body fluid. Since Na^+ and Cl^- are the major electrolytes

*Corresponding author at:
Department of Aquatic Bioscience
Graduate School of Agricultural and Life Sciences
The University of Tokyo
1-1-1 Yayoi, Bunkyo, Tokyo 113-8657, Japan
Phone/Fax: +81-3-5841-5286
e-mail: kaneko31@marine.fs.a.u-tokyo.ac.jp

in the body fluid, the regulation of both Na^+ and Cl^- is critical for osmoregulation. Another important factor is regulation of water balance, since water serves as a solvent for osmolytes. Vertebrates apparently regulate their body fluid osmolality by controlling ion concentration and water content.

Teleost species that inhabit aquatic environments face osmotic problems which differ from those in terrestrial vertebrates. The aquatic animals of teleosts possess the gill, which is permeable to both ions and water to a large extent, as their respiratory organ. Ion and water movements occur according to concentration and osmotic gradients, respectively, between the body fluid and external aquatic environments. This is not the case with terrestrial vertebrates in which gas exchanges take place in the air through the lung. To maintain ion and water balances in a wide variety of environmental salinity, teleost fish have developed superior osmoregulatory mechanisms.

As in most other vertebrate species, teleosts maintain the osmolality of the body fluid at constant levels of approximately one-third of seawater (SW) osmolality. Osmoregulation in adult teleosts is largely the result of integrated ion and water transport activities of the gills, kidney and intestine (Bentley 2002; Evans *et al*. 2005; Marshall and Grosell 2006). Freshwater (FW) teleosts face water load and salt loss through their permeable body surfaces, the most of which is occupied by the gill epithelia. To meet these problems, they discharge excess water by producing dilute urine in the kidney, and absorb ions through the gill epithelia. On the other hand, marine teleosts must deal with water loss and salt load. The loss of water is compensated for by drinking seawater and absorbing water in the intestine, while the excess ions are actively excreted from the gills and kidney. Thus, in adult fish the gills, kidney and intestine are important osmoregulatory organs, creating ionic and osmotic gradients between the body fluid and external environments. Among ion-transporting epithelial cells in those osmoregulatory organs, mitochondrion-rich (MR) cells, or chloride cells, present in gill epithelia are the major site of ion secretion and absorption, being important in SW and FW adaptation, respectively. Moreover, MR cells are also importantly involved in acid-base regulation and Ca^{2+} uptake, thus playing crucial roles in adaptation to various osmotic and ionic aquatic environments.

During early life stages, however, those osmoregulatory organs in adult fish are not yet developed or not fully functional, and gill MR cells are lacking. Nevertheless, teleost embryos are also able to maintain the osmotic balance of the body fluid (Guggino 1980; Alderdice 1988; Kaneko *et al*. 1995), suggesting the presence of osmoregulatory mechanisms that are different from those in adult fish. During the early developmental stages of several teleosts, extrabranchial MR cells have been found in the epithelia covering the body and yolk sac (for reviews, see Kaneko *et al*. 2002; Kaneko and Hiroi 2008). These findings appear to show the possible involvement of extrabranchial MR cells in osmoregulation during early life stages when functional gills and gill MR cells are lacking.

For the past dozen years or so, we have investigated physiological roles and functional differentiation of MR cells in teleosts during early developmental and adult stages. In this review, we summarize recent advances in functional morphology of MR cells in relation to environmental adaptation in various teleost species, including euryhaline and stenohaline teleosts, and consider future perspectives of MR-cell research.

2. Mitochondrion-rich (MR) cells

Keys and Willmer (1932) are the first to describe special secretory cells for Cl^- in the gills of the eel *Anguilla unlgaris*, and called them "chloride cells". Since then, the term "chloride cell" has long been used for those cells specializing in active ion transport in the gill epithelia and other tissues in fish, although it has been proven that the ion-transporting function of chloride cells is not restricted to Cl^- secretion, but involves bidirectional transport of various ions. It would be thus more appropriate to refer to chloride cells as mitochondrion-rich (MR) cells, which are widely used for ion-transporting cells, or ionocytes, in general. In this review, we adopt "MR cells" in place of "chloride cells" throughout the text.

2-1. General characteristics of MR cells

The MR cells are literally characterized by the presence of numerous mitochondria in the cytoplasm, which allows us to detect MR cells with mitochondrion-specific fluorescent probes, such as 2-(4-dimethylaminostyryl)-1-ethylpyridinium iodide (DASPEI), Rhodamine 123 and MitoTrackers, under a fluorescence or confocal laser scanning microscope. The cells are also characterized by an extensive tubular system developing in the cytoplasm. The tubular system is continuous with the basolateral membrane, resulting in a large surface area for the placement of ion-transporting proteins. Among them, Na^+/K^+-ATPase is a key enzyme in ion-transporting functions of MR cells, creating ionic and electronic gradients and providing the major driving force for ion secretion and absorption in MR cells (McCormick 1995). An antiserum specific for this enzyme serves as a specific marker for their immunocytochemical detection (Ura *et al*. 1996; Uchida *et al*. 1996). Since Na^+/K^+-ATPase is located in the basolateral membrane and continuous tubular system, the widespread distribution of the tubular system in the cytoplasm results in labeling over the cell with the nucleus remaining unstained. In addition to immunocytochemical detection of Na^+/K^+-ATPase, overall activity of MR cells can be assessed by biochemical measurement of Na^+/K^+-ATPase activity.

The fine structures of MR cells can be clearly observed by means of transmission electron microcopy (TEM); the abundance of mitochondria and the tubular system in the cytoplasm are greatly affected by cellular activities. In highly activated MR cells, mitochondria become well developed to produce much energy for active ion transport. In MR cells of fish adapted to SW, the tubular system expands its distribution over the cytoplasm, except for the sub-apical region, to enlarge the surface area, through which Na^+ and Cl^- are transported from the serosal side into the cell. Another morphological characteristic of MR cells in SW-adapted fish is the formation of multicellular complexes together with adjacent accessory cells. Such a structure of MR cells is considered to be advantageous to Na^+ extrusion (Silva *et al*. 1977; Zadunaisky 1984; Towle 1990).

Scanning electron microscopic (SEM) observations also provide useful information on fine structures of the apical membrane of MR cells which are suggestive of the osmoregulatory status of teleosts. Functional MR cells are in direct contact with external environments through their apical membrane which acts as the primary site of ion transport between MR cells and external environments. The apical membrane is located at the boundary of pavement cells in the gill and other epithelia. The apical structure varies greatly according to environmental conditions, and is closely related to the ion-transporting activities in MR cells. In SW-adapted fish, the apical membrane typically forms a crypt, through which Na^+ and Cl^- are secreted. In FW-adapted fish, the apical membrane of MR cells serves as ion-entry sites, and is often enlarged when ion uptake is in high demand, for example, in fish exposed to extremely low-ion water.

2-2. Molecular mechanisms of ion-transporting functions of MR cells

Since Foskett and Scheffey (1982) demonstrated that MR cells are the site of active Cl^- secretion in SW-adapted tilapia, it has become well accepted that MR cells are responsible for salt secretion in SW-adapted fish. A currently accepted model for NaCl secretion by MR cells consists of the cooperative action of three major ion transporters: Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter (NKCC), and cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel (Marshall 2002; Hirose *et al*. 2003; Evans *et al*. 2005). The basolaterally-located Na^+/K^+-ATPase creates low intracellular Na^+ and a highly negative charge within the cell. The Na^+ gradient is used to transport Na^+, K^+ and $2Cl^-$ into the cell through basolateral NKCC. Then, Cl^- leaves the cells down on an electrical gradient through an apical Cl^- channel which is homologous to human CFTR; Na^+ is transported back outside the cells via Na^+/K^+-ATPase, and then secreted by the paracellular pathway between MR and accessory cells.

On the other hand, mechanisms underlying ion uptake in MR cells of FW fish are still unclear and controversial. Several different models have been proposed for ion absorption in MR cells. However, the difficulties lie in the facts that different species may possess different mechanisms, and that one species may have more than one type of ion-absorbing MR cells

(Marshall 2002; Hirose *et al*. 2003; Perry *et al*. 2003; Evans *et al*. 2005; Hwang and Lee 2007). Further details on this issue will be discussed later.

3. Euryhalinity and stenohalinity of teleosts

Euryhalinity and stenohalinity of teleosts are primarily defined by their ability and inability, respectively, to maintain blood osmolality within narrow physiological ranges under different salinity conditions. Figure 1 shows a schematic representation of adaptable ranges of environmental salinity (expressed as dilution of SW in %) and blood osmolality in some euryhaline and stenohaline teleosts. Euryhaline species, such as Mozambique tilapia *Oreochromis mossambicus*, killifish *Fundulus heteroclitus* and Japanese eel *Anguilla japonica*, can be adapted to a wide range of salinity from FW to SW with blood osmolality maintained within narrow ranges from 300 to 330 mOsm/kg H_2O (mOsm). In goldfish *Carassius auratus*, a stenohaline FW teleost, blood osmolality in FW is typically 260–270 mOsm, being slightly lower than that in euryhaline species. Although goldfish can tolerate up to 40% SW, blood osmolality becomes higher with increasing environmental salinity and exceeds 400 mOsm in 40% SW. Goldfish cannot survive in higher salinities with blood osmolality over the physiological range. In a SW-dwelling stenohaline teleost of fugu *Takifugu rubripes*, blood osmolality in SW is maintained at 360–380 mOsm, slightly higher than in euryhaline species. The lower limit of salinity tolerance of fugu lies between 5 and 10% SW; fugu is not adaptable to FW, in which blood osmolality decreases below a tolerable level. It is of great interest that goldfish and fugu can co-exist at environmental salinities between 10 and 30% SW.

The following sections deal with functional and morphological aspects of MR cells in relation to environmental adaptation in several teleosts with different life histories and adaptability to osmotic and ionic environments. Those teleost species that we have investigated include: 1) Mozambique tilapia, a euryhaline fish with FW preference; 2) killifish, a euryhaline fish with SW preference; 3) chum salmon *Oncorhynchus keta*, an anadromous salmonid; 4) Japanese eel, a catadromous eel; 5) fugu, a stenohaline marine teleost; and 6) Japanese dace *Tribolodon hakonensis*, an acid-tolerant teleost.

4. Mozambique tilapia

Tilapia, the genus *Oreochromis*, are widely distributed in the wild and cultivated in fish farms owing to their hardy nature, rapid growth rates, and tolerance of varied environmental salinity (Pullin 1991). Among those tilapia species, Mozambique tilapia *O. mossambicus* is one of the most suitable fish species for studies on osmoregulation, because this euryhaline tilapia is adaptable not only to a wide range of salinity from FW to SW but also to extremely low-ion water and even to concentrated SW (Dange 1985; Suresh and Lin 1992; Kültz *et al*. 1995; Vonck

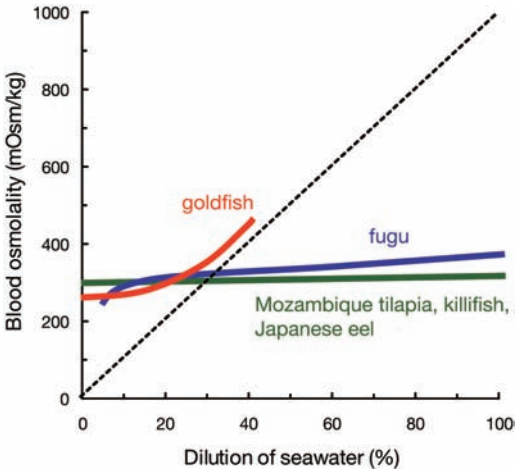

Fig. 1. Schematic representation of adaptable ranges of environmental salinity (expressed as dilution of seawater in %) and blood osmolality in euryhaline (Mozambique tilapia, killifish, Japanese eel) and stenohaline (goldfish, fugu) teleosts.

et al. 1998; Uchida *et al*. 2000; Inokuchi *et al*. 2008). Furthermore, they can breed either in FW or in SW, and embryos develop normally in respective media. The tilapia embryos are also able to survive direct transfer from FW to SW and *vice versa*. Their strong euryhalinity during both early life and adult stages intrigued us to explore the mechanisms of osmoregulation and salinity tolerance.

4-1. MR cells in the yolk-sac membrane of tilapia embryos and larvae

Tilapia typically hatch after five days' incubation at 25°C. Ayson *et al*. (1994) examined the development and changes in the activity of MR cells in the yolk-sac membrane of tilapia embryos and larvae that spawned and hatched in either FW or SW. According to their observations, DASPEI-positive MR cells were detected in the yolk-sac membrane in both FW and SW embryos, starting from two days after fertilization, or three days before hatching (day –3). The density of MR cells in both FW and SW increased towards hatching and, thereafter, decreased as the yolk was gradually absorbed. Using newly hatched tilapia larvae, Ayson *et al*. (1994) also investigated the effects of transfer between FW and SW on MR-cell morphology. When newly hatched larvae in FW were transferred directly to SW, the size of MR cells increased significantly, whereas cell size did not change or decreased slightly in those larvae retained in FW (Fig. 2A). Conversely, the cell size decreased when newly hatched larvae in SW were transferred to FW, whereas cell size was maintained in those larvae retained in SW (Fig. 2B). These findings indicate that MR cells are consistently larger in fish in SW compared with those in FW, suggesting that MR cells play a significant role in SW environments in tilapia embryos and larvae.

The larger size of MR cells of tilapia embryos and larvae in SW than in FW has also been confirmed by Shiraishi *et al*. (1997) who detected MR cells by means of whole-mount immunocytochemistry with anti-Na^+/K^+-ATPase in tilapia embryos and larvae reared in FW and those transferred to SW on day –1. They observed morphological differences in MR cells in the yolk-sac membrane between FW and SW tilapia embryos and larvae by SEM. In embryos and larvae maintained in FW, the average size of the apical openings did not vary greatly from day –1 to day 5, ranging between 1.8 and 3.1 μm^2. Small openings of less than 1 μm^2 were observed most frequently, whereas a small proportion of MR cells possessed relatively large apical openings

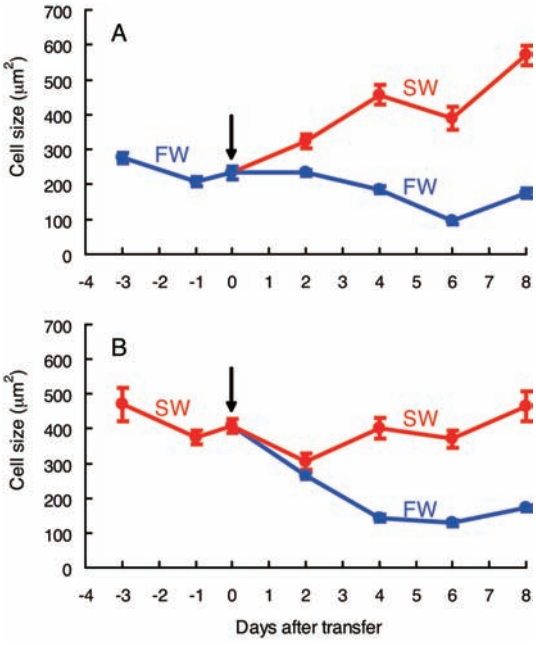

Fig. 2. Changes in MR-cell size after direct transfer of newly hatched tilapia larvae from freshwater (FW) to seawater (SW) (**A**) and from SW to FW (**B**) at the time of hatching (arrows). The values are the mean ± S.E.M. Modified from *J. Exp. Zool*., **270**, 1994, 129–135, with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

exceeding 10 μm^2. Following transfer of the embryos to SW, the apical opening increased in size significantly, the average size being approximately 5 μm^2 on days 1 to 5.

4-2. FW- and SW-type MR cells in tilapia embryos and larvae

In addition to the differences in cell and apical sizes between FW- and SW-adapted embryos and larvae, TEM observations revealed more distinct differences in their structure. Shiraishi *et al*. (1997) compared the ultrastructure of MR cells in the yolk-sac membrane between tilapia larvae in FW and those transferred to SW. In FW fish, the apical membrane of MR cells formed a shallow pit, possessing villous cytoplasmic projections (Fig. 3A). In contrast, the apical membrane in SW fish was invaginated deeply to form an enlarged apical pit (Fig. 3C). In the larvae transferred to SW, mitochondria were more numerous and the tubular system formed a denser network of anastomosed tubules than in those of FW larvae (Fig. 3B, D). Furthermore, two or three cells frequently congregated and shared a common apical pit in SW, forming a multicellular complex. Such a multicellular complex was observed only rarely in FW larvae. The complex consisted typically of one main MR cell and one or two accessory cells, which were attached on the shoulder of the main cell (Fig. 3C, E). Accessory cells were also equipped with many mitochondria and a tubular system. They interdigitated with the main cell and extended cytoplasmic processes to its apex. Cytoplasmic processes of the main and accessory cells were arranged alternately on a horizontal section cut through an apical pit (Fig. 3F). From these observations, MR cells in the yolk-sac membrane can be classified into two distinct types: a single FW type and a multicellular SW type.

4-3. Functions of multicellular complexes of SW-type MR cells

The SW-type MR cells are considered to secrete excess Na^+ and Cl^- in hyperosmotic environments. In view of the possible significance of multicellular complexes of MR cells in the yolk-sac membrane, we attempted to localize Cl^--secreting sites in the yolk-sac membrane of SW-adapted tilapia larvae using the chloride test (Kaneko and Shiraishi 2001). This technique is based on the chemical reaction that Cl^- reacts with Ag^+ to form photosensitive AgCl, which is reduced to Ag deposition after exposure to sunlight. Under a microscope equipped with a Nomarski differential interference contrast device, MR cells in the yolk-sac membrane were readily identified as large, round cells when focused on the subsurface plane. The dark deposition, representing the presence of Cl^-, was detected in the apical pits of multicellular SW-type MR cells, whereas deposition was not associated with FW-type cells (Fig. 4). These findings indicated that Cl^- is secreted from the apical pit of SW-type MR cells.

To further confirm the Cl^--secreting activity of MR cells in SW larvae, the SW samples subjected to the chloride test were examined using X-ray microanalysis (Kaneko and Shiraishi 2001). The elemental profile of Ag showed that the Ag distribution was confined to the apical pit of the MR cell and its adjacent area (Fig. 5). This indicates that Ag is the reaction product of the chloride test, and that multicellular SW-type MR cells definitely secrete Cl^- through the apical pit in SW tilapia larvae. These findings also provide direct evidence for the ion-transporting function of MR cells in the yolk-sac membrane of fish embryos and larvae in which gill MR cells are not fully functional.

On the other hand, although ion-absorbing mechanisms are less understood, it has been shown that increased Cl^- uptake is closely associated with enlarged apical surfaces of MR cells (Lin and Hwang 2001). When ambient Cl^- levels were extremely reduced, MR cells enlarged their apical area, probably to upregulate their Cl^- uptake capacity; conversely, when ambient Cl^- levels were increased, MR cells were inactivated by constriction of their apical openings and became totally covered by adjacent pavement cells. More details about ion-absorbing mechanisms in MR cells are discussed later.

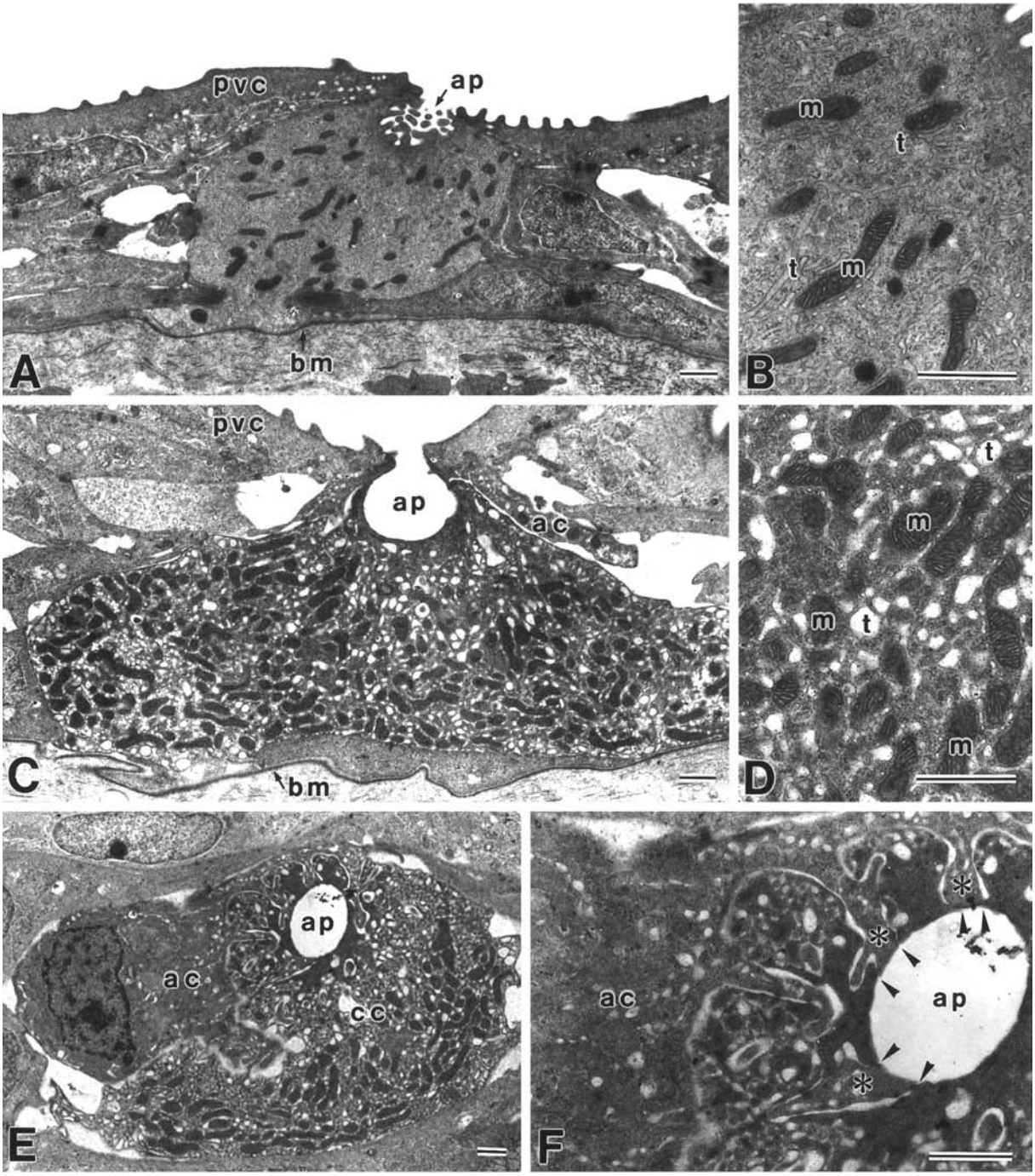

Fig. 3. A, C. Transmission electron micrographs of MR cells in the yolk-sac membrane of tilapia larvae (day 3) maintained in freshwater (FW: **A**) and those transferred from FW to seawater (SW) 1 day before hatching (**C**). ac, accessory cell; ap, apical pit; pvc, pavement cell; bm, basement membrane. **B, D.** Magnified views of MR cells in FW (**B**) and SW (**D**). Note the numerous mitochondria (m) and well-developed tubular system (t) of MR cells in SW compared with those in FW. **E.** Horizontal section cut through an apical pit of the multicellular complex of a MR cell in SW. The MR-cell complex consists of a main cell (cc) and an accessory cell (ac), sharing a common apical pit (ap). **F.** Magnified view of the apical region of the MR-cell complex. The accessory cell interdigitates with the main MR cell and extends cytoplasmic processes (asterisks) toward the apical pit. Note the multiple shallow junctions (arrowheads) between the main and accessory cells. Bars: 1 μm. Reprinted from *Cell Tissue Res*., **288**, 1997, 583–590, Development of multicellular complexes of chloride cells in the yolk-sac membrane of tilapia (*Oreochromis mossambicus*) embryos and larvae in seawater, Shiraishi *et al*., Fig. 4. With kind permission of Springer Science+Business Media.

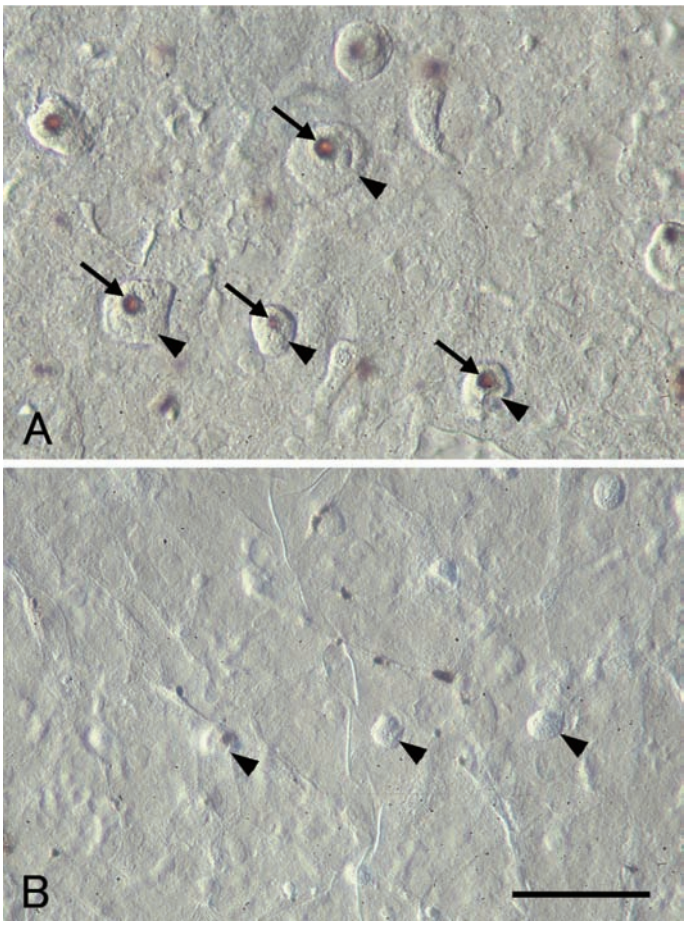

Fig. 4. Yolk-sac membranes of seawater (SW)-adapted (**A**) and freshwater (FW)-adapted (**B**) tilapia larvae subjected to the chloride test. MR cells (arrowheads) appear as large, round cells when observed with a microscope equipped with a Nomarski's differential interference contrast device. The brown deposition (arrows) is observed in the apical pit of MR cells in SW-adapted larvae, but not in FW-adapted fish. Bar: 50 μm. Reprinted with permission from Kaneko and Shiraishi, Evidence for chloride secretion from chloride cells in the yolk-sac membrane of Mozambique tilapia larvae adapted to seawater, *Fish. Sci*. 2001, **67**, 541–543, Wiley-Blackwell.

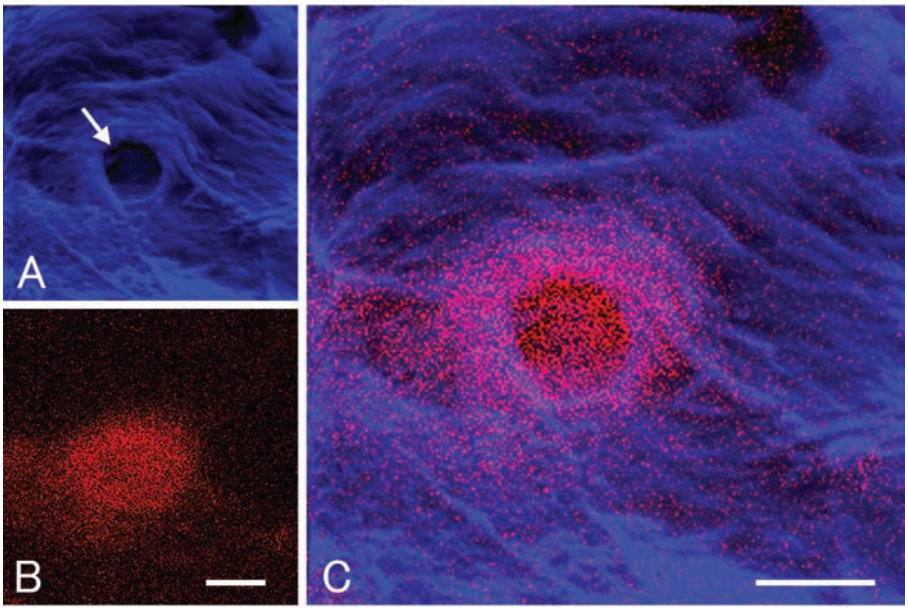

Fig. 5. A. Scanning electron microscopic image of the yolk-sac membrane of a seawater-adapted tilapia larva (blue). **B.** Distribution image of Ag detected by X-ray microanalysis (red). **C.** Overlapped image of A and B. X-ray signals, representing the presence of Cl^-, are confined to the apical pit (arrow) of the MR cell and its adjacent area. Bars: 5 μm. Reprinted with permission from Kaneko and Shiraishi, Evidence for chloride secretion from chloride cells in the yolk-sac membrane of Mozambique tilapia larvae adapted to seawater, *Fish. Sci*. 2001, **67**, 541–543, Wiley-Blackwell.

4-4. Functional differentiation of MR cells in the yolk-sac membrane

From both physiological and morphological points of view, it is of great interest to determine whether MR cells are replaced by newly differentiated cells of a different function after transfer from FW to SW or whether the same MR cells function in both FW and SW. The most effective way to answer this question would be to examine sequential changes in individual MR cells during adaptation to a different salinity. However, conventional methods do not allow us to follow time-course changes in the same MR cells in the gills of adults; separated gill filaments are not suitable material for examining the time-course changes in MR cells because of their complex 3-D structures.

Using tilapia embryos, Hiroi *et al*. (1999) succeeded, for the first time, in following *in vitro* sequential changes in MR cell morphology. Dechorionated embryos were immersed in a medium containing DASPEI, a mitochondrial fluorescent probe, and morphological changes in individual MR cells were continuously examined under a confocal laser scanning microscope. Because the distribution patterns of MR cells were largely maintained during the experimental period of 96 h, each individual MR cell was readily identified by the sequential observation (Fig. 6). The area of MR cells did not change greatly in FW, whereas most MR cells tended to increase in area after SW transfer to form multicellular complexes. Their observations at a higher magnification on the fixed yolk-sac membrane have clearly showed that accessory cells develop from undifferentiated cells attaching to the main MR cells and gradually intrude into the main MR cells (Fig. 7). These findings demonstrate that FW-type single MR cells are transformed into SW-type multicellular complexes during SW adaptation, suggesting plasticity in ion-transporting functions of MR cells in the yolk-sac membrane of tilapia embryos and larvae. This sequential observation technique also provides direct evidence of cell turnover. In FW individuals, the number of pre-existing MR cells decreased gradually and 72% of the cells remained at 96 h, while new cells amounting to 64% of the initial cell number appeared during 96 h. In those individuals transferred to SW, 76% of the initial cells remained, and new cells equal to 51% of the initial cell number appeared. These findings indicate that MR cells are continuously replaced with newly-differentiated cells under either a constant or changeable osmotic condition.

4-5. Functional classification of MR cells in the yolk-sac membrane

In addition to the crude classification of MR cells into FW and SW types, Hiroi *et al*. (2005a) have proposed more detailed functional classification of MR cells on the basis of distribution patterns of ion-transporting proteins involved in ion secretion and absorption, including Na^+/K^+-ATPase, NKCC, and CFTR Cl^- channel. Based on the distribution patterns of those ion transporters determined by triple-color whole-mount immunofluorescence staining, MR cells in tilapia embryos have been classified into four distinct types: type I, showing only basolateral Na^+/K^+-ATPase staining; type II, basolateral Na^+/K^+-ATPase and apical NKCC; type III, basolateral Na^+/K^+-ATPase and basolateral NKCC; and type IV, basolateral Na^+/K^+-ATPase, basolateral NKCC and apical CFTR. More recently, some other molecular components have been identified as presumably being involved in ion transport of MR cells in tilapia: NKCC1a, a distinct isoform of NKCC; Na^+/Cl^- cotransporter (NCC, Hiroi *et al*. 2008); and Na^+/H^+ exchanger 3 (NHE3, Watanabe *et al*. 2008). Introducing those additional ion transporters, Hiroi *et al*. (2008) have successfully upgraded the "four-type model" of MR cells. An amazing simultaneous quintuple-color immunofluorescence staining for Na^+/K^+-ATPase, NKCC1a, NCC, NHE3 and CFTR Cl^- channel has clearly shown differential expression patterns of those proteins in the distinct four types of MR cells (Fig. 8).

Among the four cell types, type-I MR cells showed only basolateral Na^+/K^+-ATPase and lacked other ion-transporting proteins examined. This type appeared constantly independent of environmental salinity changes. Because of their relatively small size, possessing only Na^+/K^+-ATPase immunoreactivity, and relatively small apical opening, type-I MR cells are considered to be immature MR cells, being able to develop into other MR-cell types.

Type-II MR cells were defined by apical NCC and basolateral Na^+/K^+-ATPase, and showed FW-specific changes: they were absent in SW, appeared following transfer from SW to FW, and

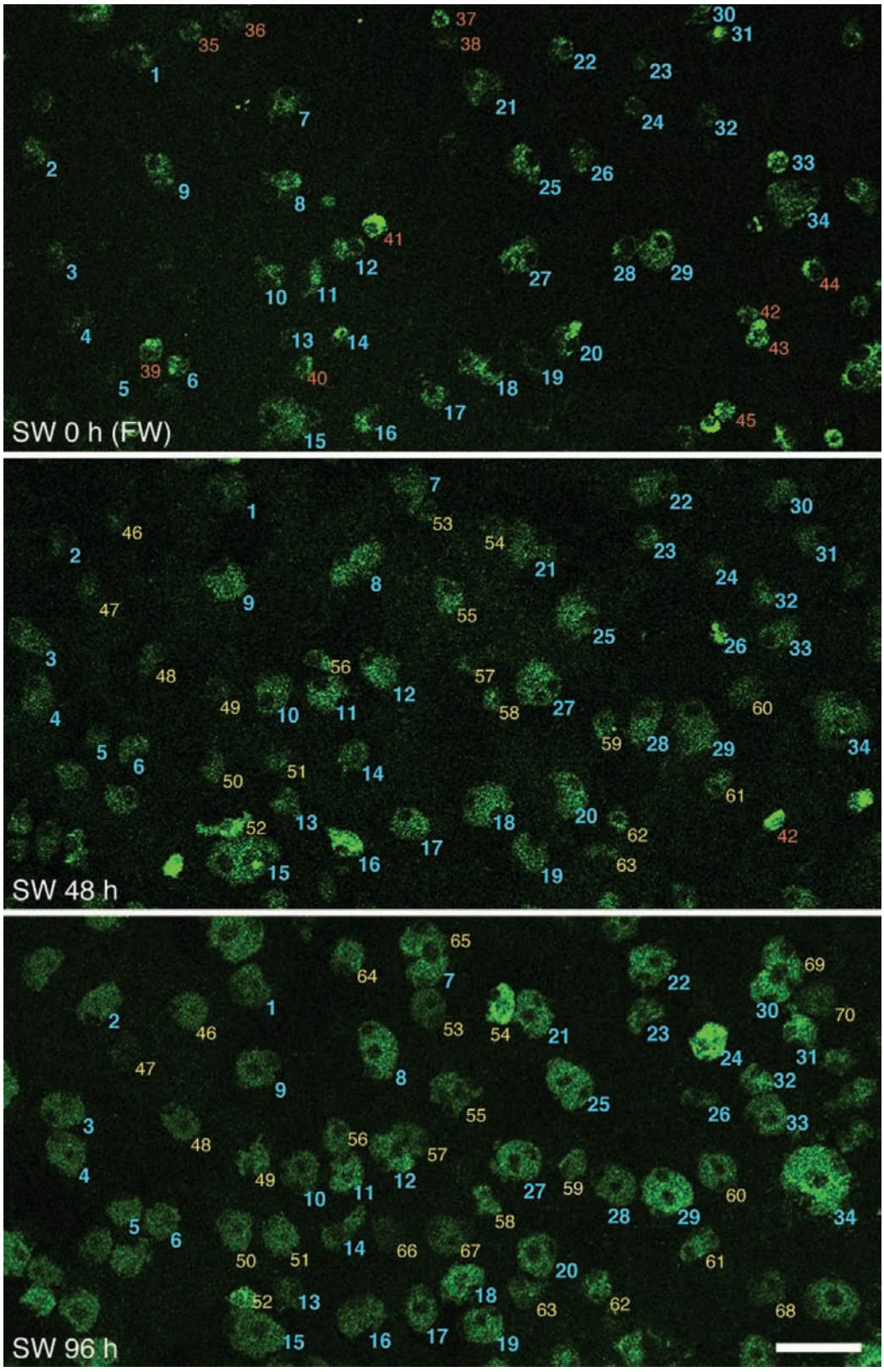

Fig. 6. Sequential confocal images of DASPEI-stained MR cells in the yolk-sac membrane of a tilapia transferred from freshwater (FW) to seawater (SW) at 0, 48 and 96 h. Bar: 50 μm. Reprinted from *J. Exp. Biol.*, **202**, Hiroi *et al.*, *In vitro* sequential changes in chloride cell morphology in the yolk-sac membrane of Mozambique tilapia (*Oreochromis mossambicus*) embryos and larvae during seawater adaptation, 3485–3495, 1999, The Company of Biologists Limited.

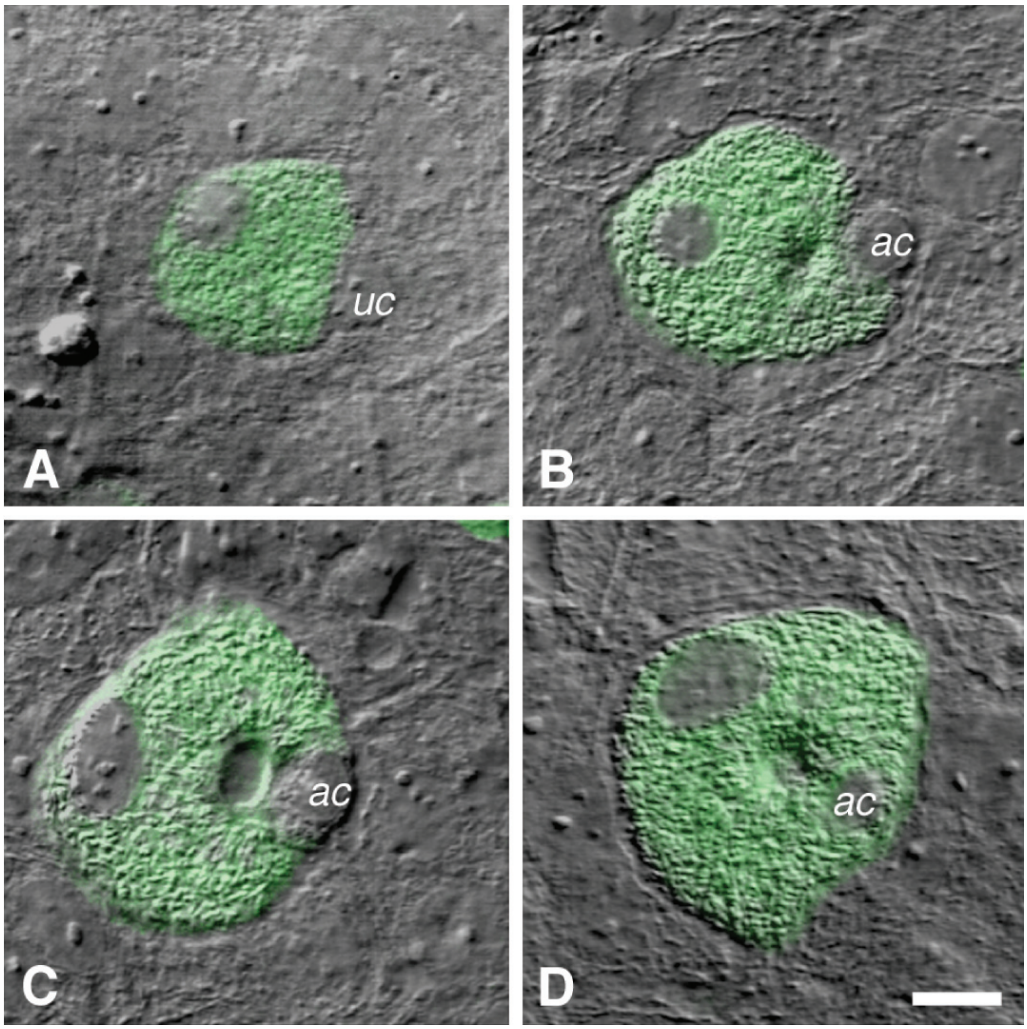

Fig. 7. A series of differential interference contrast (DIC) and immunofluorescence images of multicellular complexes of MR and accessory cells arranged in the order of presumed developmental stages. **A.** At first, an undifferentiated cell (*uc*) is attached to a single, small MR cell (stained green). **B, C.** The MR cell becomes larger, indented by an accessory cell (*ac*) originating from the undifferentiated cell. **D.** The accessory cell is finally enveloped by the MR cell so that the boundary between those cells becomes indistinct. Bar: 10 μm. Reprinted from *J. Exp. Biol.*, **202**, Hiroi *et al.*, *In vitro* sequential changes in chloride cell morphology in the yolk-sac membrane of Mozambique tilapia (*Oreochromis mossambicus*) embryos and larvae during seawater adaptation, 3485–3495, 1999, The Company of Biologists Limited.

decreased following transfer from FW to SW. The NCC immunoreactivity was restricted to the apical membrane of type-II MR cells and not observable in other cell types. Therefore, it seems likely that type-II MR cells are the FW-type ion-absorbing cells, and that the apically-located NCC protein would cotransport Na^+ and Cl^- from the external environment into the cell.

Meanwhile, types-III and IV cells are closely associated with each other, both possessing basolateral Na^+/K^+-ATPase and basolateral NKCC1a. Type-III MR cells are another type of ion-absorbing cells, being characterized by apical NHE3 in addition to basolateral Na^+/K^+-ATPase and basolateral NKCC1a, and showed FW-specific changes: they rarely appeared in SW, rapidly increased in number following transfer from SW to FW, and disappeared rapidly following transfer from FW to SW. In spite of the presence of basolateral NKCC1a, considered to be related to ion secretion, type-III MR cells are likely responsible for Na^+ uptake in exchange for H^+ secretion through apical NHE3 in FW. In contrast, type-IV MR cells were defined by apical

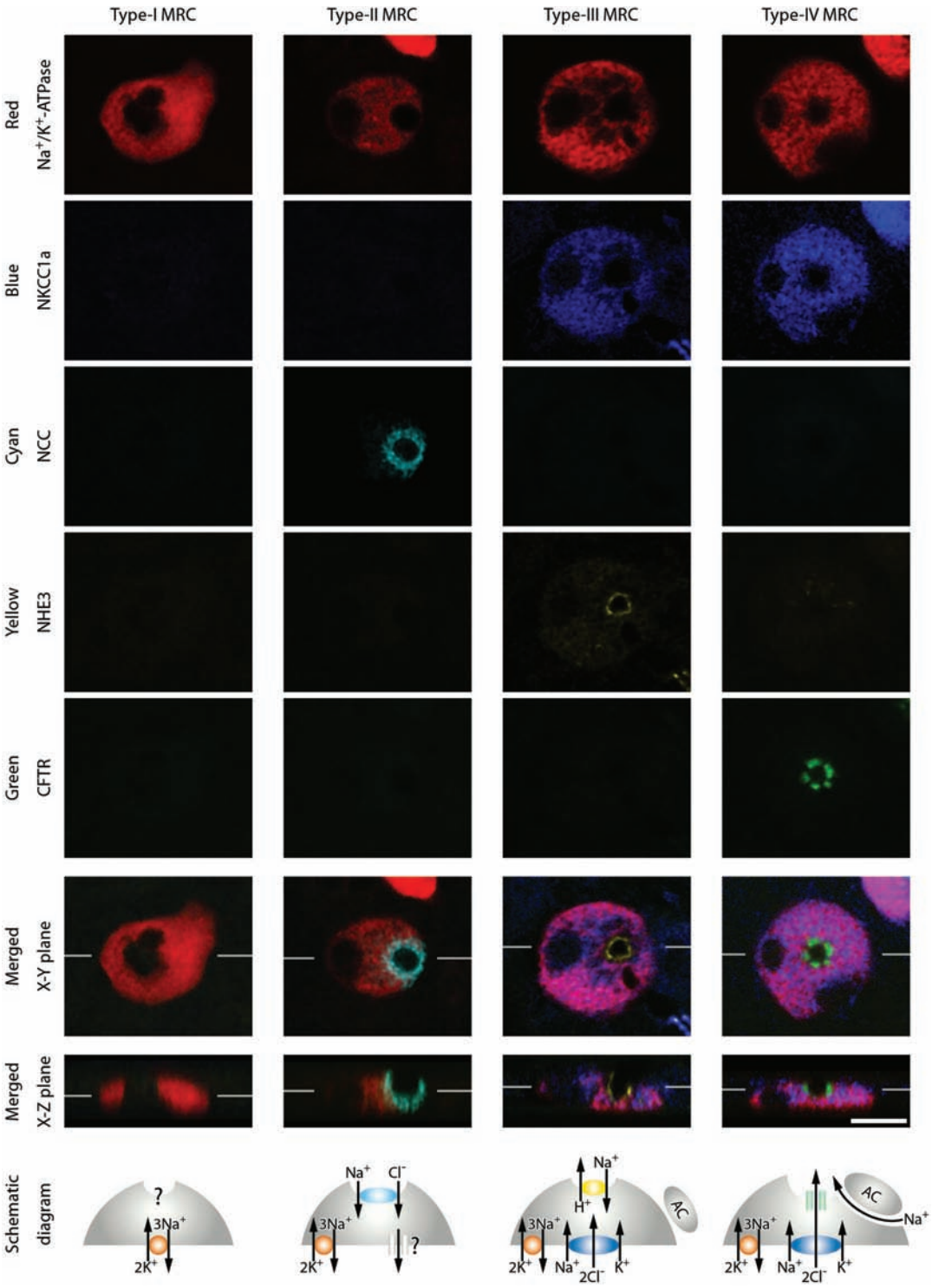

Fig. 8. Classification of MR cells in the yolk-sac membrane of Mozambique tilapia embryos into four types, by means of quintuple-color immunofluorescence staining: type-I (A), type-II (B), type-III (C), and type-IV (D). The signals for Na^+/K^+-ATPase (red), NKCC1a (blue), NCC (cyan), NHE3 (yellow) and CFTR (green) are shown as separate channels, and the five channels are merged in Merged *X–Y* and *X–Z* planes. Merged *X–Z* plane, the *X–Z* optical section cut transversely at the horizontal lines indicated in Merged *X–Y* plane. Merged *X–Y* plane, the *X–Y* optical section cut at the lines indicated in Merged *X–Z* plane. *n*, Nucleus. Bar: 10 μm. Schematic diagrams of each of the four cell type are presented in the bottom row, showing the apical or basolateral localization patterns of Na^+/K^+-ATPase (red), NKCC1a (blue), NCC (cyan), NHE3 (yellow) and CFTR (green). Reprinted from *J. Exp. Biol.*, **211**, Hiroi *et al.*, Evidence for an apical Na–Cl cotransporter involved in ion uptake in a teleost fish, 2584–2599, 2008, The Company of Biologists Limited.

CFTR, basolateral Na^+/K^+-ATPase and basolateral NKCC1a, and this distribution pattern was completely consistent with the current accepted model for ion secretion by MR cells in SW. This cell type was purely SW specific: they were not observable in FW, rapidly appeared following transfer from FW to SW, and rapidly disappeared following transfer from SW to FW. From these facts it can be deduced that type-IV MR cells are the SW-type, ion-secretory cells, and that NKCC1a localized at the basolateral membrane probably cotransports Na^+, K^+ and Cl^- from the internal environment into the cell.

The rapid appearance or disappearance of type-III MR cells was largely completed within 24 h following SW-to-FW or FW-to-SW transfer, and was obviously inverse to that of type-IV MR cells. Such an inverse relationship between types-III and IV MR cells suggests that types-III and IV MR cells would transform into each other; in other words, types-III and IV MR cells have the same cell origin, and are only counted as type-III or IV according to the presence of either apical NHE3 or CFTR. As mentioned above, we have demonstrated that most MR cells are able to survive following transfer from FW to SW, by observing *in vivo* sequential changes in individual MR cells. The proposed model of functional classification of MR cells can account not only for ion uptake in FW and ion secretion in SW, but also for plasticity in the altering ion-transporting functions of MR cells in tilapia.

4-6. "Yolk ball" incubation system

Although many studies have focused in the past on the effects of environmental salinity and hormones, their direct effects on MR-cell differentiation are not yet clear. This may be partly due to the lack of suitable *in vitro* experimental models. Under these circumstances, Shiraishi *et al*. (2001) have established a unique *in vitro* experimental model named "yolk balls", in which the yolk sac was separated from a tilapia embryonic body and subjected to *in vitro* incubation (Figs. 9A–D). The yolk-ball preparation consists of the yolk and the covering yolk-sac membrane, which contains a rich population of MR cells. After appropriate cutting, the incision on the yolk ball was healed during incubation in a balanced salt solution for 3 h, so that the yolk-sac membrane completely enclosed the yolk (Figs. 9C–F).

The yolk-ball incubation system has great advantages over conventional *in vitro* experimental models for MR cells. Primary cultures of isolated cells from gill epithelia have been utilized in physiological studies. However, dispersed MR cells do not survive under *in vitro* conditions. Another problem of primary cultures is the loss of the cellular polarity, since the location of respective ion-transporting proteins in the plasma membrane is critical for ion-transporting mechanisms of MR cells. Although the opercular and yolk-sac membrane preparations maintain the cellular polarity, MR cells in those preparations can neither survive long nor differentiate under *in vitro* conditions. Moreover, the yolk balls prepared in balanced salt solution are able to survive transfer to FW and SW. The Cl^- turnover rate measured by whole-body influx of $^{36}Cl^-$ was about 60 times higher in yolk balls in SW than in FW, while there was no essential difference between them in Cl^- content (Hiroi *et al*. 2005b). This is exactly what happens in intact embryos (Miyazaki *et al*. 1998), thus indicating that the yolk-sac membrane of the yolk balls maintain the ion-transporting properties of the intact membrane.

Shiraishi *et al*. (2001) examined whether or not functional differentiation of MR cells in the yolk-sac membrane occurred in the yolk balls without embryonic control. The yolk balls prepared from FW embryos were incubated either in FW or in SW up to 48 h to elucidate the morphological alteration of MR cells in response to the environmental salinity. The MR cells in the yolk balls incubated in SW became larger than those in FW, and formed multicellular complexes, characteristic of SW-type MR cells (Figs. 10C–F). In those transferred to FW, on the other hand, the cells remained small and rarely formed a complex. Such responses of MR cells are identical to those observed in intact embryos (Figs. 10A, B). More precisely, types I, II and III MR cells were identified in FW yolk balls (Hiroi *et al*. 2005b). It is likely that FW yolk balls preserve the MR-cell population of the intact embryos in FW. The yolk balls incubated in SW, on the other hand, are characterized by the predominant occurrence of type-IV cells, which can account for active ion secretion in SW yolk balls. These findings indicate that the MR cells in

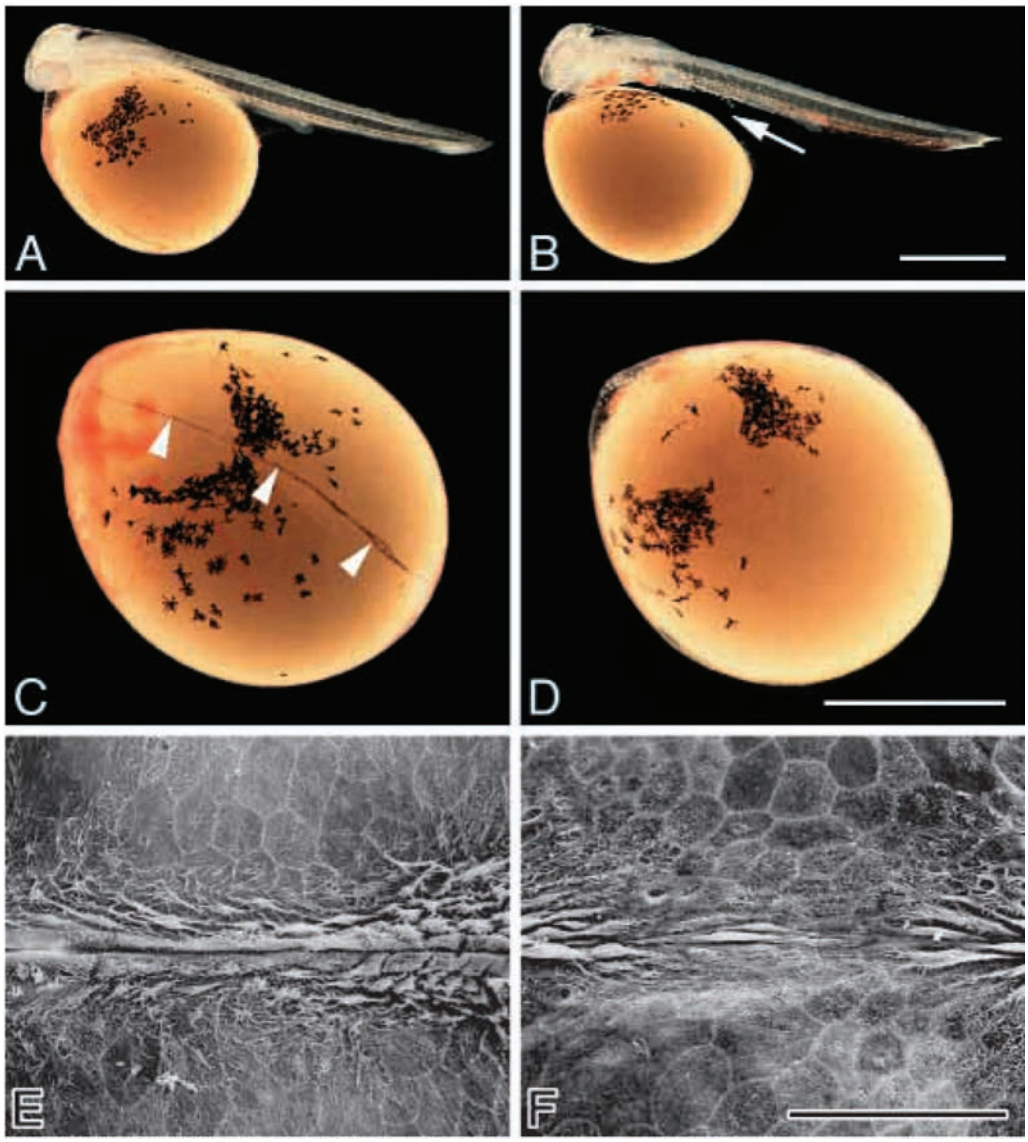

Fig. 9. A, B. An intact tilapia embryo two days before hatching (**A**), and an embryo whose yolk sac has been almost severed from the embryonic body (**B**). An arrow indicates the incision. **C–F.** Incised wounds on the yolk-ball preparations at 0 h (**C, E**) and 3 h (**D, F**) after surgery stained with trypan blue (**C, D**) and those observed by scanning electron microscopy (**E, F**). The wound (arrowheads) had almost healed after incubation for 3 h in BSS. Note the symmetrical distribution of melanophores on the dorsal side of the yolk-sac membrane. Bars: **A–D**, 1 mm; **E, F**, 100 μm. Reprinted from *J. Exp. Biol.*, **204**, Shiraishi *et al.*, *In vitro* effects of environmental salinity and cortisol on chloride cell differentiation in embryos of Mozambique tilapia, *Oreochromis mossambicus*, measured using a newly developed "yolk-ball" incubation system, 1883–1888, 2001, The Company of Biologists Limited.

the yolk-sac membrane are equipped with an autonomous mechanism of functional differentiation, independent of embryonic endocrine and nerve systems, although it is generally believed that MR cell activities in adult fish are under hormonal control.

4-7. Salinity tolerance of adult tilapia

In addition to excellent euryhalinity which has already been developed during the embryonic and larval stages, adult Mozambique tilapia are adaptable to an extremely wide range of

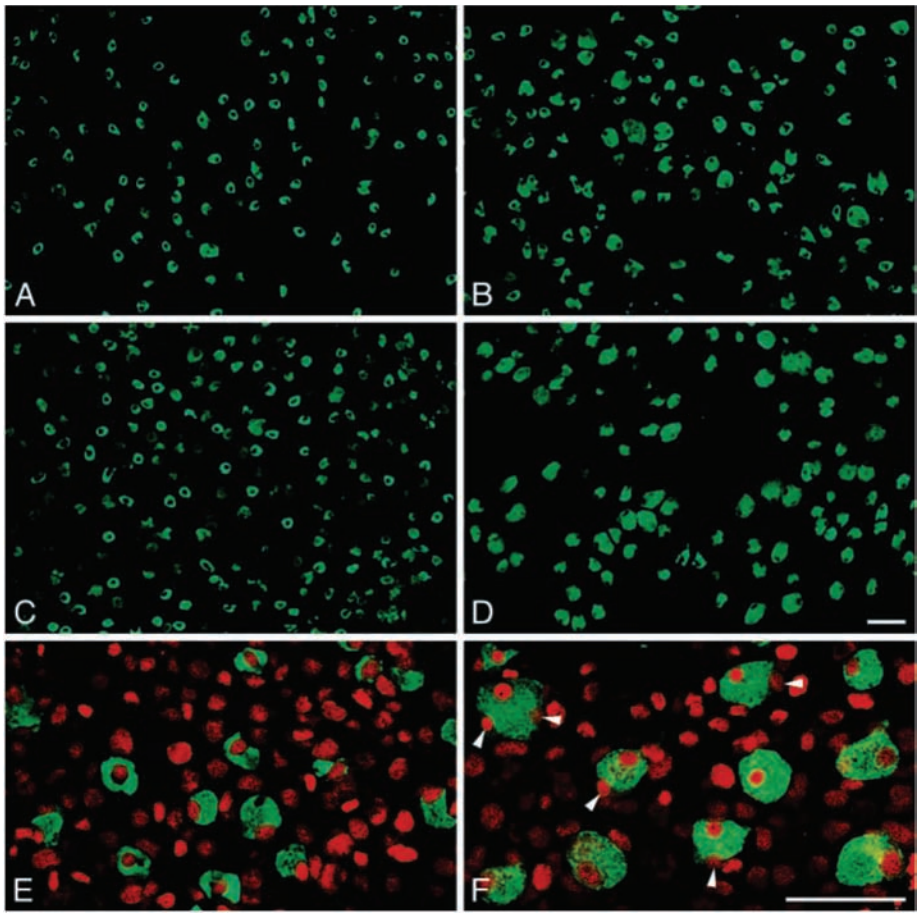

Fig. 10. A–D. MR cells in the yolk-sac membrane of intact tilapia embryos (**A, B**) and yolk-ball preparations (**C, D**) incubated in freshwater (FW: **A, C**) and seawater (SW: **B, D**) for 48 h. MR cells were detected by whole-mount immunocytochemistry with FITC-labeled anti-Na^+/K^+-ATPase. **E, F.** MR cells in the yolk-sac membrane of yolk-ball preparations incubated in FW (**E**) and SW (**F**) for 48 h, double-stained with FITC-labeled anti-Na^+/K^+-ATPase and propidium iodide. MR cells in SW form multicellular complexes with accessory cells (arrowheads), as demonstrated by the presence of more than one propidium-iodide-labeled nucleus. Bars: 50 μm. Reprinted from *J. Exp. Biol.*, **204**, Shiraishi *et al.*, *In vitro* effects of environmental salinity and cortisol on chloride cell differentiation in embryos of Mozambique tilapia, *Oreochromis mossambicus*, measured using a newly developed "yolk-ball" incubation system, 1883–1888, 2001, The Company of Biologists Limited.

salinity. Uchida *et al*. (2000) have shown that adult tilapia can be well adapted to up to 180% concentrated SW with blood osmolality maintained within a physiologically normal range. Freshwater tilapia were acclimated to 100 and 180% SW in a stepwise fashion, and osmoregulatory parameters and MR-cell morphology were compared among FW-, SW- and 180% SW-acclimated tilapia. Although the plasma osmolality of those fish became slightly higher with increasing environmental salinity, the levels stayed within a relatively narrow range of 300–350 mOsm, indicating their successful adaptation to those salinity environments. Gill Na^+/K^+-ATPase activity was increased in accordance with elevated environmental salinities. The activity was twice higher in SW fish and five times higher in 180% SW fish than that in FW fish, suggestive of more enhanced activity of gill MR cells in fish exposed to higher salinity. In the gills, Na^+/K^+-ATPase-immunoreactive MR cells were detected on the afferent vascular half of the filament epithelia: mainly at the flat region of the afferent edge where lamellae were absent, and at the base of lamellae and the interlamellar region to a lesser extent (Fig. 11). The size of immunoreactive MR cells was twice larger in SW and 4 times larger in 180% SW than in FW. Confocal

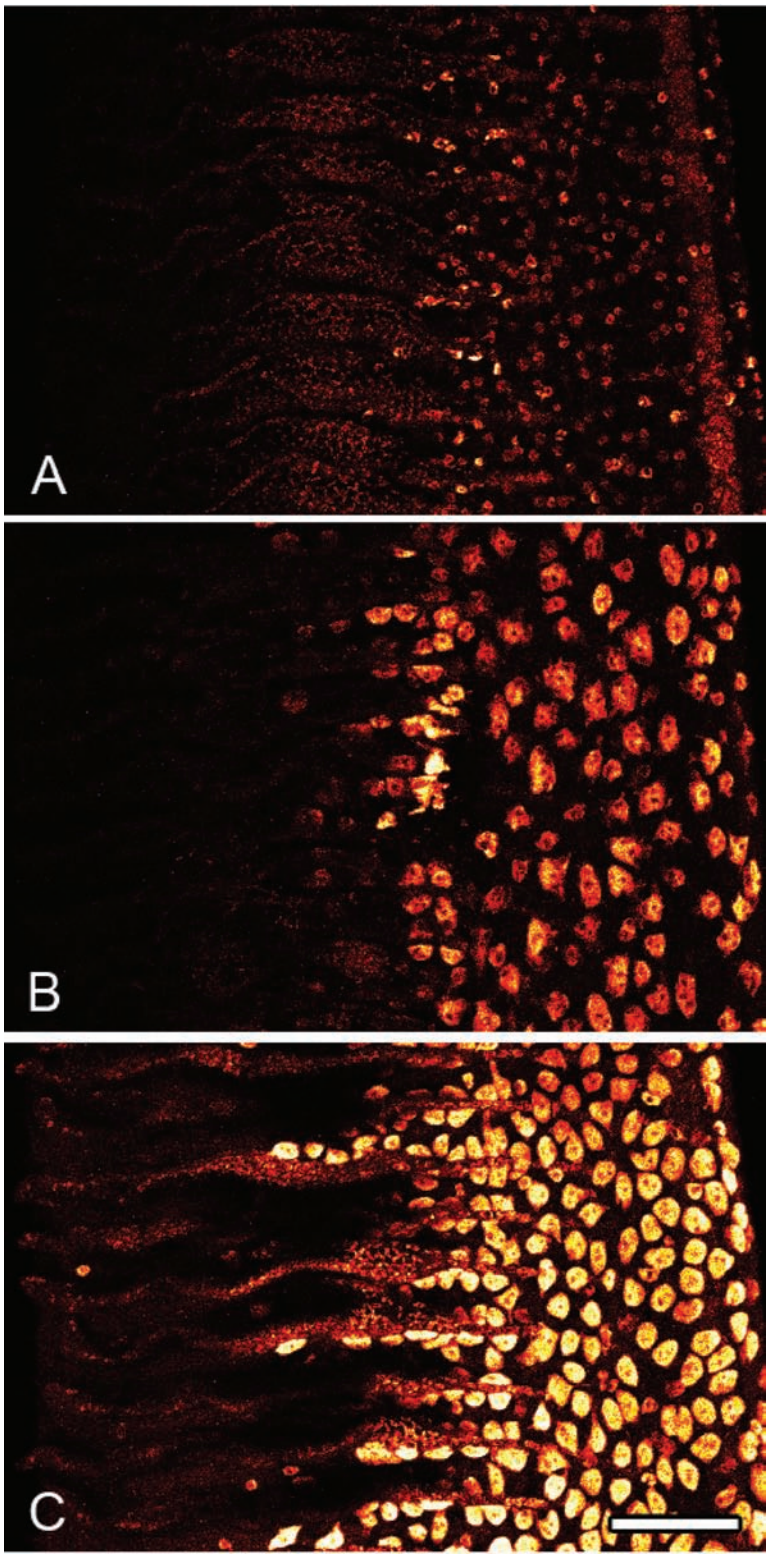

Fig. 11. Confocal laser scanning micrographs of the whole-mount preparations of gill filaments stained with anti-Na^+/K^+-ATPase in tilapia adapted to freshwater (FW: **A**), seawater (SW: **B**) and concentrated SW (180% SW: **C**). Note that immunoreactive MR cells are observed mainly on the afferent side of the filament and well developed with increasing salinity. Bar: 100 μm. Reprinted with permission from *Zool. Sci.*, **17**, Uchida *et al.*, Excellent salinity tolerance of Mozambique tilapia (*Oreochromis mossambicus*): elevated chloride cell activity in the branchial and opercular epithelia of the fish adapted to concentrated seawater, 149–160, 2000, The Zoological Society of Japan.

laser scanning microscopic and TEM observations revealed the frequent occurrence of MR-cell complexes under hypertonic conditions. A deeply invaginated apical crypt and well-developed tubular network were observed in MR cells of SW and 180% SW fish, indicating their enhanced salt-secreting activity. The excellent salinity tolerance of tilapia can be attributed, at least in part, to their ability to develop MR cells in response to increased environmental salinity.

Adult tilapia can also survive extremely low-ion conditions. Inokuchi *et al*. (2008) explored molecular and morphological alteration in gill MR cells in tilapia acclimated to deionized FW (DFW), FW, 1/3-diluted SW (1/3 SW) and SW. Tilapia could be well adapted to DFW, where Na^+ and Cl^- concentrations were less than 0.05 mM, with blood osmolality maintained at about 290 mOsm, whereas osmolalities in fish acclimated to FW, 1/3 SW and SW ranged from 310 to 330 mOsm. It should be noted that gill Na^+/K^+-ATPase activity was increased in fish acclimated to DFW, being equivalent to that in SW-acclimated fish and significantly higher than that of 1/3 SW-acclimated fish. According to SEM observations, the apical membrane of MR cells appeared as a flat or slightly projecting disk in hypotonic waters (DFW and FW), being larger in DFW than in FW; the apical membrane formed a pit structure in isotonic (1/3 SW) and hypertonic (SW) waters. The enlargement of the apical membrane of MR cells and increased gill Na^+/K^+-ATPase activity in DFW may reflect enhanced ion-absorbing activities of MR cells in the ion-deficient environment. Furthermore, in view of the proposed molecular mechanisms of ion transport in tilapia yolk-sac MR cells (Hiroi *et al*. 2008), Inokuchi *et al*. (2008) investigated mRNA expressions of NHE3, NCC and NKCC1a in the gills of tilapia acclimated to DFW, FW, 1/3 SW and SW. Expression levels of NHE3 and NCC increased with decreasing environmental salinity, whereas NKCC1a expression was upregulated by increasing salinity. Immunofluorescence staining showed that the MR-cell population of DFW- and FW-acclimated tilapia consisted mostly of MR cells with apical NHE3 and those with apical NCC; MR cells with basolateral NKCC1a dominated in SW-acclimated fish. There results indicate that apical-NHE3/NCC and basolateral-NKCC1a MR cells are responsible for ion uptake and secretion, respectively, in the gills of adult tilapia, as is the case with MR cells in the yolk-sac membrane. In fish acclimated to 1/3 SW, both ion-absorbing and secreting MR cells existed in the gills, suggesting that fish in the near-isotonic water are equipped with mechanisms of both hyper- and hypo-osmoregulation to prepare for environmental salinity changes.

4-8. Possible osmoreception by MR cells

The functional differentiation of MR cells independent of embryonic endocrine and nerve systems (Shiraishi *et al*. 2001) implies the existence of a mechanism that MR cells detect environmental salinity changes. It is probable that, in response to transfer from FW to SW, FW-type MR cells can sense salinity changes in a direct or indirect manner, and this triggers transformation into SW-type cells. One possible way is to detect a fluctuation in the external salinity via the apical membrane of MR cells facing the external medium. It is more likely, however, that increased environmental salinity results in a slight increase in internal osmolality, which could be detected by the basolateral membrane of MR cells. In fact, changes in osmolality on the basolateral side have been shown to affect the rate of Cl^- secretion by opercular epithelia with a rich population of MR cells in killifish (Zadunaisky *et al*. 1995; Marshall *et al*. 2000).

Meanwhile, we have recently identified aquaporin 3 (AQP3), a water-channel molecule, from the tilapia gills, and demonstrated that AQP3 is intensively located in the basolateral membrane of MR cells (Fig. 12) (Watanabe *et al*. 2005). Based on this finding, it is hypothesized that basolateral AQP3 is involved in osmoreception by MR cells. It is expected that the AQP3-rich basolateral membrane is more permeable to water than the other membranes. This is supported by the finding that AQP3-expressed Xenopus oocytes exhibited higher water permeability than the control oocytes, resulting in oocyte swelling in the hypoosmotic medium (Watanabe *et al*. 2005). Since the surface area of the basolateral membrane is enlarged because of intensive infoldings of the tubular system, the ratio of the surface area to the cell volume is supposed to be much higher in MR cells than in any other cell type in the gills or yolk-sac membrane. Such structural characteristics may further enhance the possible osmosensitivity of MR cells. Transfer of fish from SW to FW, for example, may induce a slight decrease in blood osmolality, which

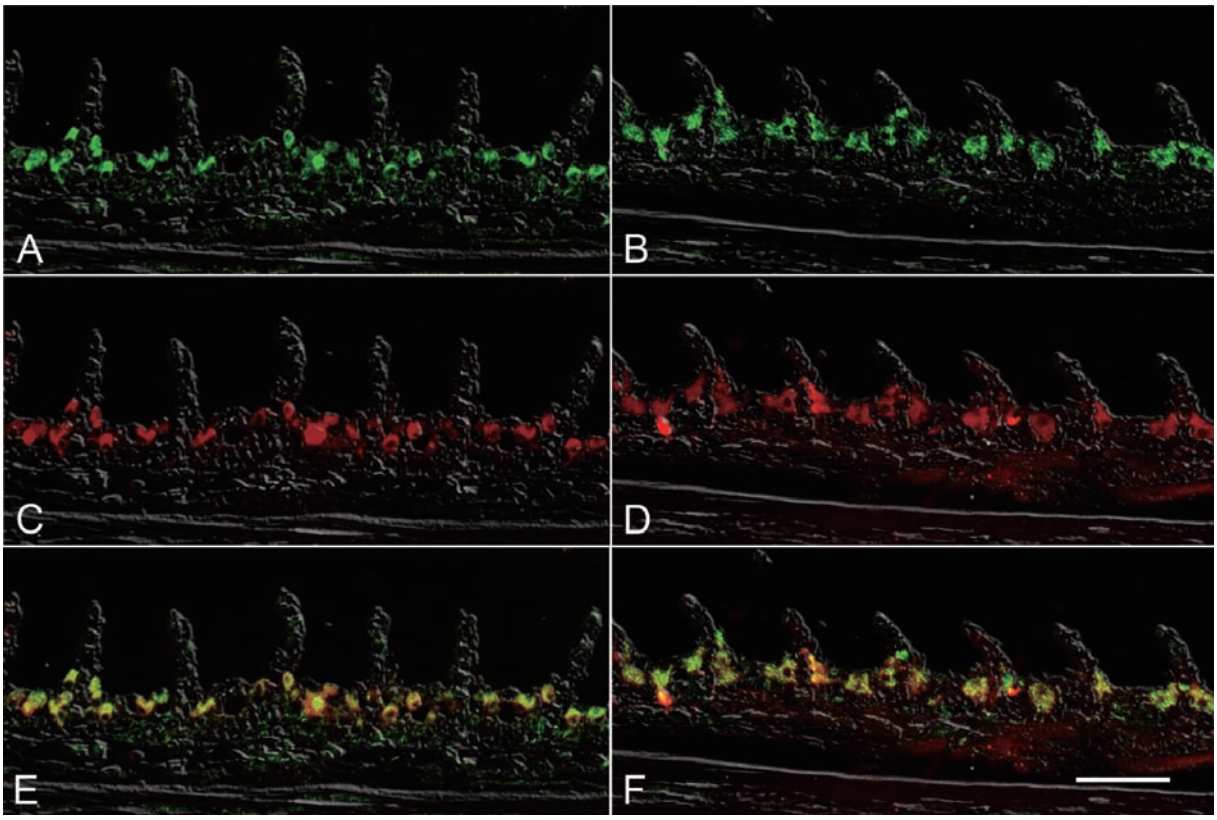

Fig. 12. Double immunofluorescence microscopy of gills in tilapia adapted to freshwater (FW: **A, C, E**) and seawater (SW: **B, D, F**), stained with anti-tilapia AQP3 (**A, B**) and anti-Na^+/K^+-ATPase as a marker of MR cells (**C, D**). E and F are merged images of A, C and B, D, respectively. AQP3-immunoreactive cells are observed in both FW and SW gills (**A, B**), and immunoreactions for AQP3 and Na^+/K^+-ATPase coincided completely with each other (**E, F**). Bar: 50 μm. Reprinted from *J. Exp. Biol.*, **208**, Watanabe *et al.*, Aquaporin-3 expressed in the basolateral membrane of gill chloride cells in Mozambique tilapia *Oreochromis mossambicus* adapted to freshwater and seawater, 2673–2682, 2005, The Company of Biologists Limited.

creates an osmotic gradient between the intracellular fluid and blood. Subsequently, water moves into MR cells through basolateral AQP3 according to the osmotic gradient, resulting in cell swelling. Conversely, transfer from FW to SW may leads to shrinkage of MR cells. In mammalian species, AQP4 in the brain has been suggested to be involved in osmoreception for vasopressin regulation (Nielsen *et al.* 1997; Wells 1998). Although more detailed information is required for clarifying the osmoreception in fish gills, AQP3 expressed in MR cells may be one of the strong candidates for effectors in branchial osmosensing processes.

5. Killifish

Killifish *Fundulus heteroclitus* is a euryhaline estuarine teleost which can be adapted to both FW and SW (Griffith 1974; Hardy 1978). In spite of their excellent euryhalinity, killifish has preference for SW rather than FW. Judging from the kidney anatomy, killifish falls into a marine teleost; the nephron in the killifish kidney lacks the distal tubules responsible for reabsorption of Na^+ and Cl^-, as is generally characteristic of marine stenohaline teleosts (Edwards 1933). This is in sharp contrast with euryhaline teleosts with FW preference, such as Mozambique tilapia which possesses distal tubules. While most studies on gill MR cells have been carried out with euryhaline teleosts with FW preference, killifish serves as an excellent model to investigate MR cell functions, particularly those in FW.

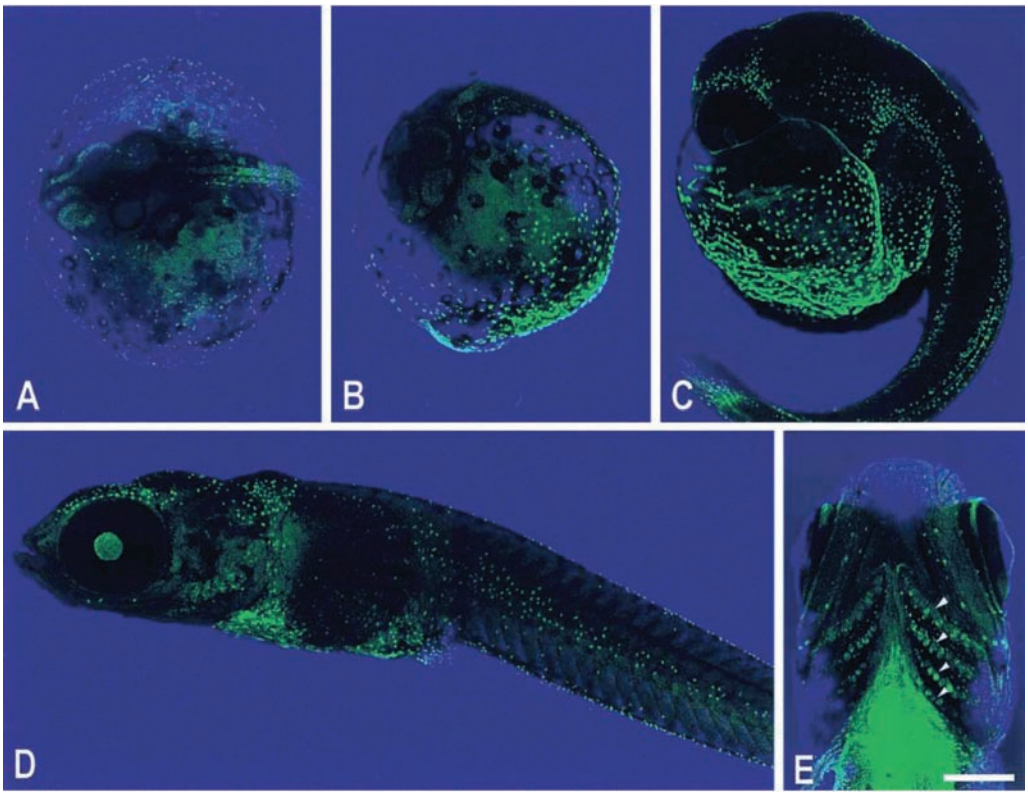

Fig. 13. MR cells in the yolk-sac membrane and body skin of killifish embryos and larvae at 2 (**A**), 4 (**B**), 8 (**C**) and 15 days (**D, E**) after fertilization. MR cells were detected by whole-mount immunocytochemistry with FITC-labeled anti-Na^+/K^+-ATPase, and observed by conforcal laser scanning microscopy. The ventral view of a larva (**E**) shows the arrays of MR cells (arrowheads) in the developing gills. Bar: 500 μm. Reprinted with permission from *Zool. Sci.*, **17**, Katoh *et al.*, Shift of chloride cell distribution during early life stages in seawater-adapted killifish, *Fundulus heteroclitus*, 11–18, 2000, The Zoological Society of Japan.

5-1. Transitional processes of MR-cell distribution during early life stages

In SW-adapted killifish, the shift of MR cell distribution was investigated during early life stages by Katoh *et al*. (2000), in which MR cells were detected by immunocytochemistry with an antiserum specific for Na^+/K^+-ATPase in whole-mount preparations and paraffin sections of the embryos and larvae.

At an incubation temperature of 20°C, killifish eggs typically hatched 11 days after fertilization, and the day of fertilization was designated as day 0. By day 2, fertilized eggs complete gastrulation, and the yolk-sac membrane completely encloses the yolk sac (Hardy 1978). In embryos on day 2, small MR cells were sparsely distributed over the yolk-sac membrane (Fig. 13A). On days 4 to 10, a rich population of MR cells was detectable in the yolk-sac membrane (Figs. 13B, C). However, yolk-sac MR cells disappeared as the yolk absorption was completed on day 15 (Figs. 13D, E). In addition to the yolk-sac membrane, MR cells also appeared in the body skin on day 6 and later developmental stages (Figs. 13C, D). MR cells in the body skin were rather small on day 6, increased to the maximal size on day 10, and decreased in size thereafter. Immunoreactive MR cells in the yolk-sac membrane and body skin during early developmental stages of SW-adapted killifish often appeared to be closely associated with each other to form cellular complexes, as evidenced by the presence of more than one immunonegative nucleus. The formation of such cellular complexes is characteristic of SW-type MR cells responsible for secretion of Na^+ and Cl^-.

The gills were not detectable in embryos on days 2 and 4. Four pairs of gill arches first appeared on day 6, although gill filaments and lamellae had not yet developed. Gill filaments started developing on day 10, followed by the development of gill lamellae on day 15 (Fig. 14). Eosinophilic MR cells of spherical shape were first observed in the gill filaments on day 10, when gill lamellae were not yet formed. However, since these cells lacked apical pits and were only faintly immunoreactive to anti-Na^+/K^+-ATPase, those cells may not be functional yet (Figs. 14A, B). Eosinophilic and intensely immunoreactive MR cells equipped with apical pits became evident in the gill filaments of larvae on days 15 to 25 (Figs. 14C–F). During these developmental stages, a rich population of immunoreactive MR cells was also observed in the opercular membrane with which the operculum was lined. Similar to those in the yolk-sac membrane and body skin, MR cells in the gills and opercular membrane of SW-adapted killifish often formed multicellular complexes which shared a common apical pit.

Four pairs of gills were well developed in adult fish. Each gill consisted of a gill arch, filaments and lamellae. Two rows of gill filaments radiated posteroventrally from the arch. The gill lamellae extended laterally from both sides of the filament. A large number of immunoreactive MR cells were detected in the gill filaments. These cells were round or columnar in shape and mostly distributed along a flat region of the trailing edge (afferent vascular side) and between lamellae in the filament. No or few MR cells were detectable in the gill lamellae. In addition to the gills, a large number of MR cells were also present in the opercular membrane, as is the case with the larvae.

All these observations clearly show transitional processes of the MR-cell distribution during early life stages; MR cells first appear in the yolk-sac membrane in the early embryonic stage, followed by their appearance in the body skin in the late embryonic stages. The principal site for the MR cell distribution then shifts to the gills and opercular membrane in larval and later developmental stages.

5-2. Distinct FW- and SW-type MR cells

In most euryhaline and migratory species, such as Mozambique tilapia, Japanese eel and salmonids, MR cells become larger and denser when fish are transferred from FW to SW (Langdon *et al*. 1985; Richman *et al*. 1987; Uchida *et al*. 1996, 2000; Sasai *et al*. 1998a), and this is often accompanied by enhanced gill Na^+/K^+-ATPase activity. Thus, its activity has generally been considered to serve as a reliable index of SW adaptability in those fishes (McCormick 1995). However, this is not the case in killifish; gill Na^+/K^+-ATPase activity as well as oxygen consumption does not differ between SW- and FW-adapted killifish (Marshall *et al*. 1999; Katoh *et al*. 2001), suggesting that MR cells are equally active in the two environments but exert different ion-transporting functions.

Katoh *et al*. (2001) described occurrence of distinct SW- and FW-type MR cells in the gills and opercular membrane in killifish. In the gills of SW-adapted fish, the MR cells are located mostly in the afferent side of the gill filament (Fig. 15A), as occurs in Mozambique tilapia (Uchida *et al*. 2000). In FW-adapted fish, their distribution extends toward the efferent side and, consequently, more cells are observed between lamellae in the filament (Fig. 15B). Interestingly, MR cells are larger in FW- than in SW-adapted fish in both gills and opercular membrane, which is in accordance with observations by Marshall *et al*. (1997). In addition, TEM observations revealed striking differences in the ultrastructure of MR cells between SW- and FW-adapted fish (Fig. 16). In SW-adapted fish, the apical membrane of MR cells was invaginated to form a pit. As is seen in embryos and larvae, MR cells interdigitate with neighboring accessory cells in SW-adapted fish, forming multicellular complexes. In FW-adapted fish, on the other hand, the enlarged apical membrane is flat or even shows projections. These morphological observations clearly indicate two distinct FW and SW types of MR cells in killifish.

5-3. Functional alteration and replacement of MR cells

Although the structural difference is evident between FW- and SW-type MR cells in killifish, it is not clear whether one cell type is replaced by another, or whether one cell type changes its

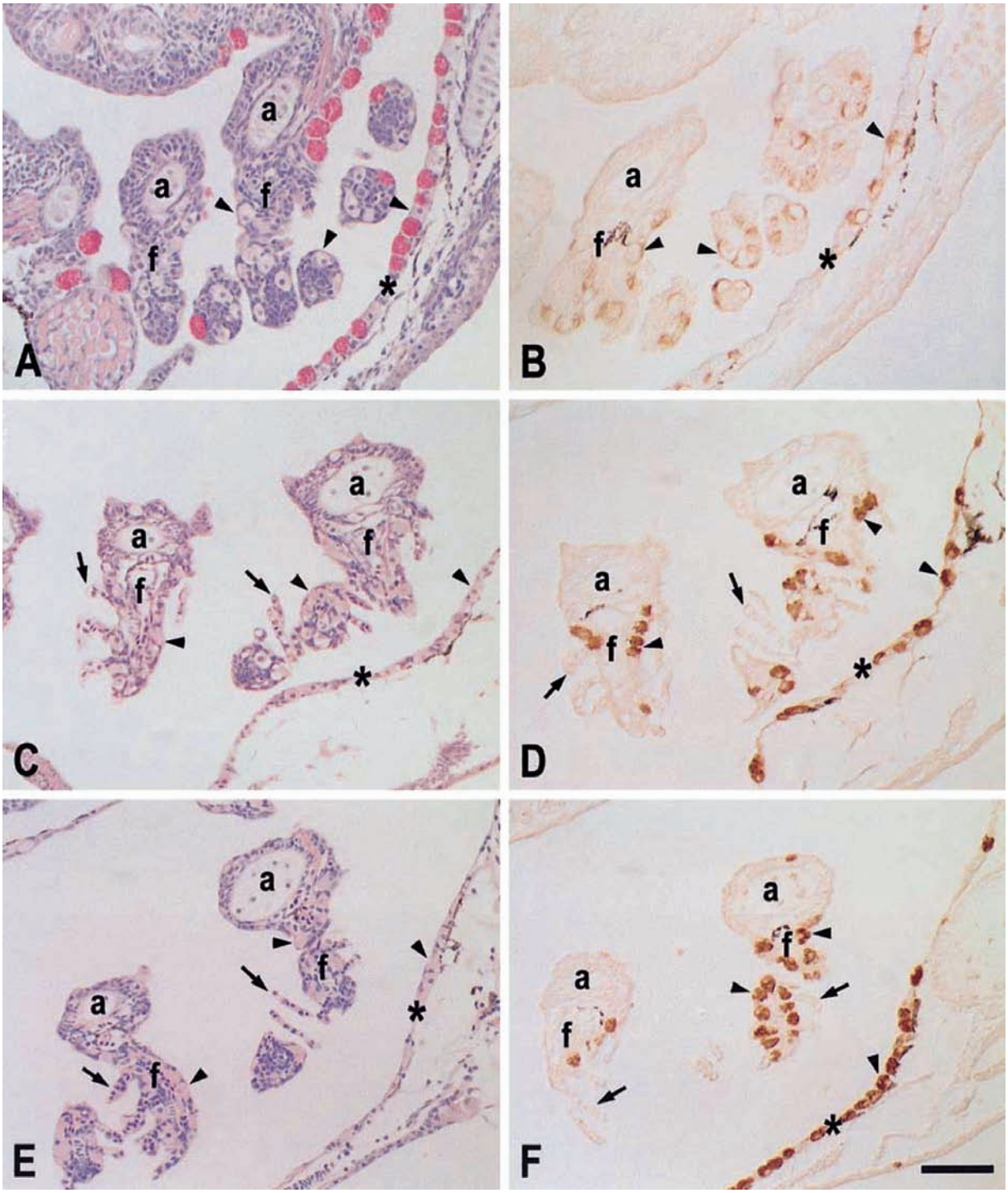

Fig. 14. Cross sections of killifish embryos and larvae at 10 (**A, B**), 15 (**C, D**) and 25 (**E, F**) days after fertilization, showing the development of the gills. **A, C, E.** Hematoxylin and eosin staining. **B, D, F.** Immunocytochemical detection of MR cells with anti-Na^+/K^+-ATPase. MR cells (arrowheads) are observed in the gill filaments (f) and the opercular membrane (asterisks), but not in the gill lamellae (arrows). a, Gill arch. Bar: 50 μm. Reprinted with permission from *Zool. Sci.*, **17**, Katoh *et al.*, Shift of chloride cell distribution during early life stages in seawater-adapted killifish, *Fundulus heteroclitus*, 11–18, 2000, The Zoological Society of Japan.

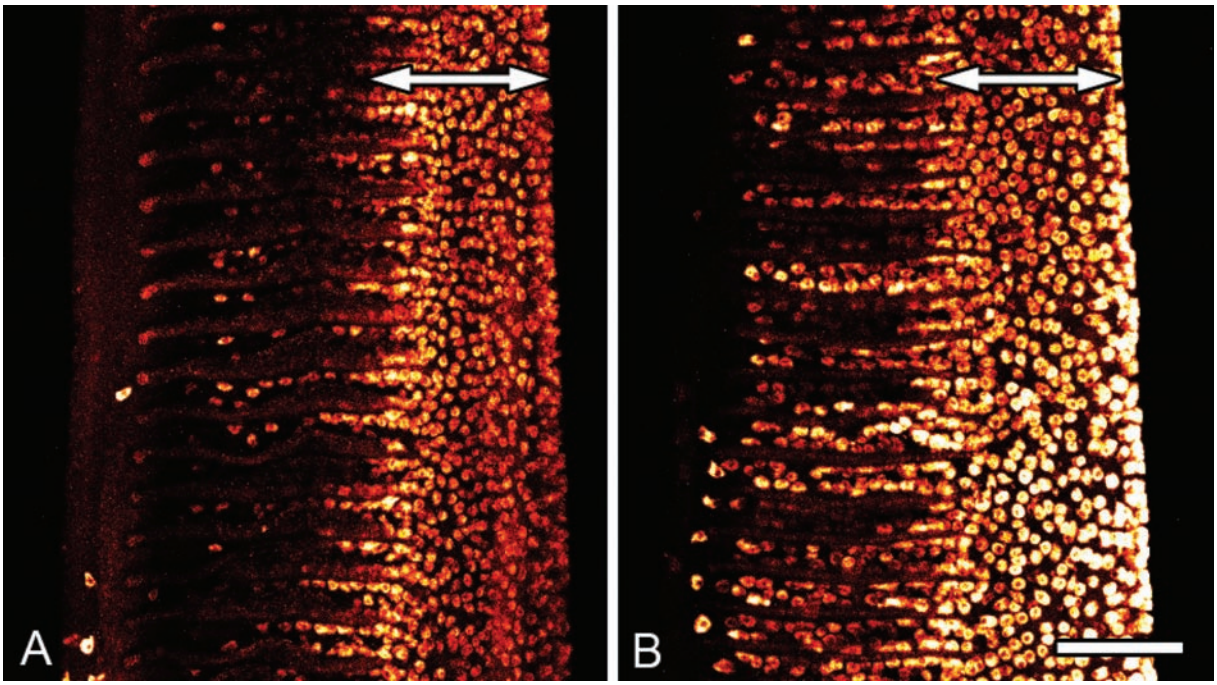

Fig. 15. Confocal laser scanning micrographs of whole-mount preparations of gill filaments in killifish adapted to seawater (SW: **A**) and freshwater (FW: **B**). The gill filaments were stained with FITC-labeled anti-Na^+/K^+-ATPase. The arrows indicate the flat region of the gill filament. Bar: 100 μm. Reprinted with permission from *Can. J. Zool.*, **79**, Katoh *et al.*, Distinct seawater and freshwater types of chloride cells in killifish, *Fundulus heteroclitus*, 822–829, 2001, NRC Research Press.

function and morphology into another type, following transfer between FW and SW. To answer this question, Katoh and Kaneko (2003) examined short- and long-term responses to direct transfer from SW to FW in gill MR cells of killifish.

In a short-term response within 24 h after transfer, it has been shown that SW-type MR cells are transformed into FW-type cells (Fig. 17). The SEM and TEM observations revealed the occurrence of the intermediate type, which was most frequently observed at 3 h after transfer from SW to FW (Figs. 17C, D). The intermediate-type cells were accompanied by accessory cells, which is characteristic of the SW type. However, the apical openings were larger and the pits were shallower than those observed in the typical SW type, and more like those in the FW type. Poorly developed microvilli are observed on the apical membrane through the enlarged opening, which is not evident in the SW type. Considering that the intermediate-type cells appear within 3 h after transfer to FW, it is most probable that SW-type cells are transformed into the intermediate type. Since typical FW-type cells are frequently observed at 12 h (Figs. 17E, F), the intermediate-type cells appear to be further transformed into the FW type.

This transformation process was accompanied by the disappearance of neighboring accessory cells and CFTR Cl^- channel located in the apical membrane (Fig. 18). In killifish, the CFTR has been identified as the apically-located Cl^- channel (Marshall *et al*. 1995; Singer *et al*. 1998), and thus the existence of CFTR in the apical membrane could provide evidence for functional SW-type MR cells. Immunoreactive CFTR was evident in the apical membrane of SW-type cells (Figs. 18A, B); however, it disappeared by 24 h after FW transfer as SW-type cells are transformed into the FW type (Figs. 18G, H). These observations indicate that the SW type is both morphologically and functionally transformed into the FW type as a short-term response to FW transfer, suggesting a functional plasticity of MR cells.

MR-cell replacement was also examined in killifish as a long-term adaptation to FW transfer (Katoh and Kaneko 2003). For this purpose, we have established a "time differential double fluorescent staining (TDS)" technique, in which *in vivo* labeling of MR cells was performed on

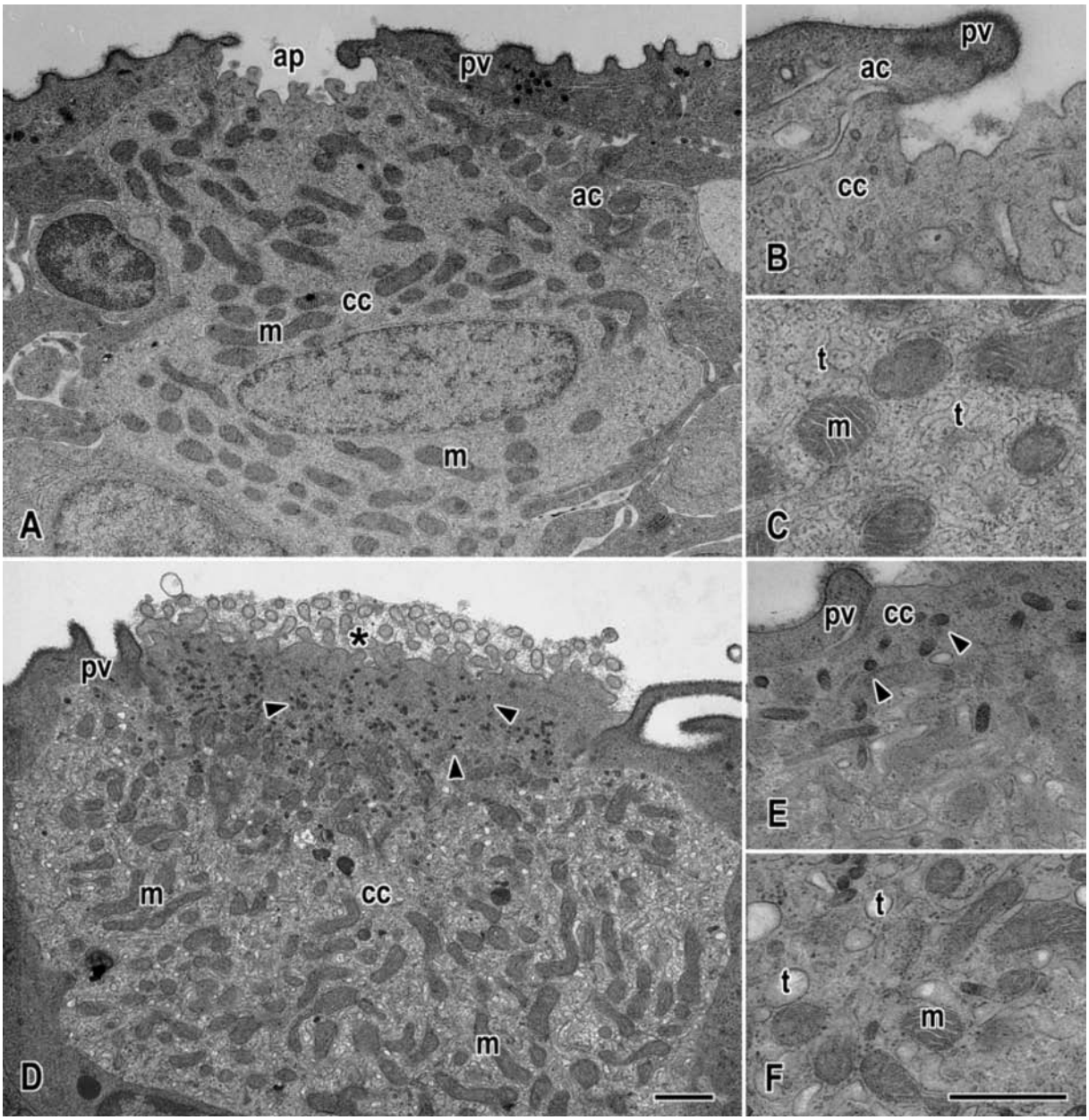

Fig. 16. Transmission electron micrographs of MR cells (cc) in gill filaments of killifish adapted to seawater (SW: **A–C**) and freshwater (FW: **D–F**). In SW-adapted fish, the apical membrane of MR cells forms an apical pit (ap). In FW-adapted fish, on the other hand, the apical membrane is flat or shows projections, being equipped with numerous microvilli (asterisk). **B** and **E** are magnified views of the apical region of MR cells. In SW-adapted fish, the MR cells interdigitate with accessory cells (ac) and the junctions are shallow and leaky (**B**). In FW-adapted fish, the junctions between pavement cells (pv) and MR cells are deep and tight. Subapical vesicles (arrowheads) are characteristic of FW-adapted fish (**E**). **C** and **F** are magnified views of the cytoplasm of MR cells in SW- and FW-adapted fish. Note the numerous mitochondria (m) in both SW- and FW-adapted fish, and a better developed tubular system (t) in SW- than in FW-adapted fish. Bars: **A, D,** 1 μm; **B, C, E, F,** 0.5 μm. Reprinted with permission from *Can. J. Zool.*, **79**, Katoh *et al.*, Distinct seawater and freshwater types of chloride cells in killifish, *Fundulus heteroclitus*, 822–829, 2001, NRC Research Press.

two separate days, using two distinguishable mitochondria-specific fluorescent probes: Rhodamine 123 and MitoTracker Red (Fig. 19). Compared with conventional methods using ^{3}H-thymidine or BrdU, the newly developed TDS technique provides a more convenient and precise method for assessing MR cell turnover. The fish acclimated to either FW or SW were first immersed in Rhodamine 123 for 3 h to label MR cells. Half of SW-acclimated fish were then transferred to FW. Three days after transfer, the fish were immersed in MitoTracker Red for 3 h for the second labeling. Consequently, MR cells which are newly differentiated during the last three days appear as Rhodamine 123-negative, MitoTracker Red-positive cells. During the experimental period of three days, although the total number of MR cells did not change, the ratio of newly differentiated MR cells to the total cells was markedly higher in the FW-transferred group (14.7%) than those in FW- and SW-maintained groups (1.8% and 1.2%, respectively).

These results indicate that the turnover rate of MR cells is relatively low under constant osmotic environments, but is accelerated after transfer from SW to FW. Under constant osmotic conditions, a small proportion of MR cells are continuously replaced with the same type of cells. In contrast, the enhanced MR cell turnover after transfer seems to be the result of replacement of pre-existing SW-type MR cells with newly differentiated FW-type cells. Thus, the replacement of MR cells after transfer not only sustains the MR cell population but also contributes to the alteration in ion-transporting functions of the gills. This may serve as a long-term adaptive response to a different osmotic environment, together with a short-term response of morphological and functional transformation of pre-existing MR cells.

5-4. Ion-absorbing mechanisms of MR cells

To confirm the involvement of MR cells in ion absorption in hypoosmotic environments in killifish, the MR cell morphology was compared in fish acclimated to defined FWs with different NaCl concentrations together with 0.5 mM $CaCl_2$: low (0.1 mM)-, mid (1 mM)- and high (10 mM)-NaCl FWs (Katoh *et al*. 2003). As a result, MR cells were larger in low- and mid-NaCl FWs than in high-NaCl FW (Figs. 20A–F). According to SEM and TEM observations, the apical membranes of well-developed MR cells in low- and mid-NaCl FWs are flat or slightly projecting, and equipped with microvilli to expand the surface area exposed to external environments. These features are characteristic of typical FW-type cells. On the other hand, in high-NaCl FW, the apical membrane was invaginated to form a pit, and MR cells often formed multicellular complexes with accessory cells. These cells were more like SW-type cells, although the NaCl concentration in high-NaCl FW (10 mM) was much lower than that in the plasma (about 150 mM). These observations provide morphological evidence that FW-type MR cells with the enlarged apical surface area are involved in active ion uptake in killifish.

Then, in order to clarify the molecular mechanism of ion uptake in MR cells of FW killifish, Katoh *et al*. (2003) explored a possible involvement of vacuolar-type proton ATPase (H^+-ATPase) in Na^+ absorption. In a proposed model, H^+-ATPase in the apical membrane and Na^+/K^+-ATPase in the basolateral membrane create the driving force permitting passive electrodiffusion of Na^+ through the apically-located Na^+ channel (Avella *et al*. 1989). H^+-ATPase, one type of ATP-dependent proton pumps, is responsible for the acidification of intracellular compartments of eukaryotic cells (Forgac 1999) and also energizes animal plasma membranes (Harvey and Wieczorek 1997). H^+-ATPase is composed of a catalytic V1 domain responsible for ATP hydrolysis and an integral V0 domain that forms a channel for H^+ to cross the plasma or vacuolar membranes (Forgac 1999). Most studies on H^+-ATPase in fish have been performed in the context of acid-base regulation (Lin *et al*. 1994; Sullivan *et al*. 1996; Perry *et al*. 2000). Although the gills have been identified as the site of H^+-ATPase activity, the cellular localization of H^+-ATPase in the gills is still controversial. Immunocytochemical studies have shown that H^+-ATPase is distributed in both MR and pavement cells in rainbow trout *Oncorhynchus mykiss* (Lin *et al*. 1994; Wilson *et al*. 2000a) and in pavement cells but not in MR cells in tilapia (Hiroi *et al*. 1998b; Wilson *et al*. 2000a). In view of such controversy, we have cloned a cDNA encoding the A-subunit of H^+-ATPase from killifish gills (Katoh *et al*. 2003). The deduced amino acid sequence shows high identity with H^+-ATPase A-subunits from other vertebrate species. Light

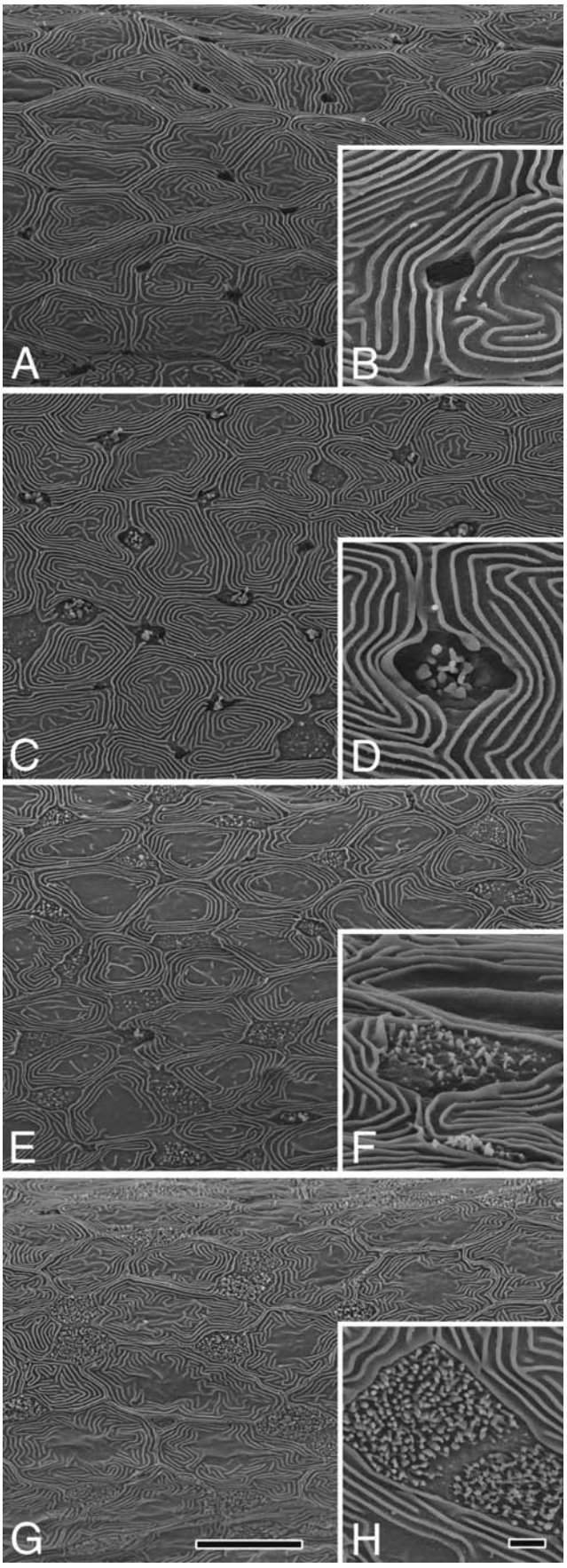

Fig. 17. Scanning electron micrographs of gill filaments in killifish at 0 h (**A, B**), 3 h (**C, D**), 12 h (**E, F**) and 30 days (**G, H**) after transfer from seawater to freshwater. **B, D, F, H.** Enlarged views of the apical region of MR cells. Bars: **A, C, E, G,** 10 μm; **B, D, F, H,** 1 μm. Reprinted from *J. Exp. Biol.*, **206**, Katoh and Kaneko, Short-term transformation and long-term replacement of branchial chloride cells in killifish transferred from seawater to freshwater, revealed by morphofunctional observations and a newly established "time-differential double fluorescent staining" technique, 4113–4123, 2003, The Company of Biologists Limited.

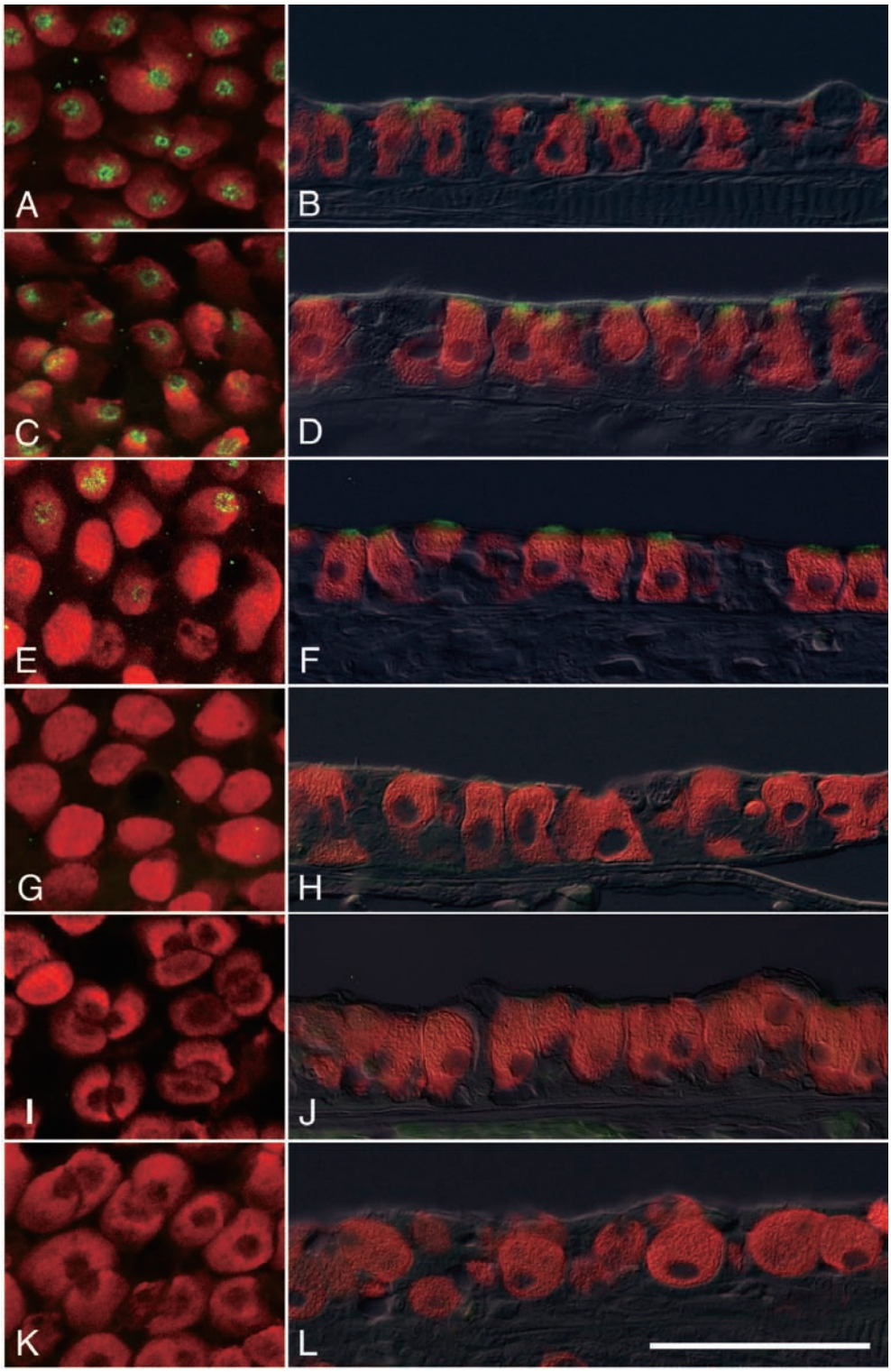

Fig. 18. Confocal laser scanning micrographs of whole-mount preparations of the gill filaments (**A, C, E, G, I, K**) and fluorescence microscope images of gill cryosections (**B, D, F, H, J, L**) in killifish at 0 h (**A, B**), 3 h (**C, D**), 12 h (**E, F**), 1 day (**G, H**), 7 days (**I, J**) and 30 days (**K, L**) after transfer from seawater to freshwater. Gill filaments were double stained with anti-cystic fibrosis transmembrane conductance regulator (CFTR, green) and anti-Na^+/K^+-ATPase (red). Bar: 50 μm. Reprinted from *J. Exp. Biol.*, **206**, Katoh and Kaneko, Short-term transformation and long-term replacement of branchial chloride cells in killifish transferred from seawater to freshwater, revealed by morphofunctional observations and a newly established "time-differential double fluorescent staining" technique, 4113–4123, 2003, The Company of Biologists Limited.

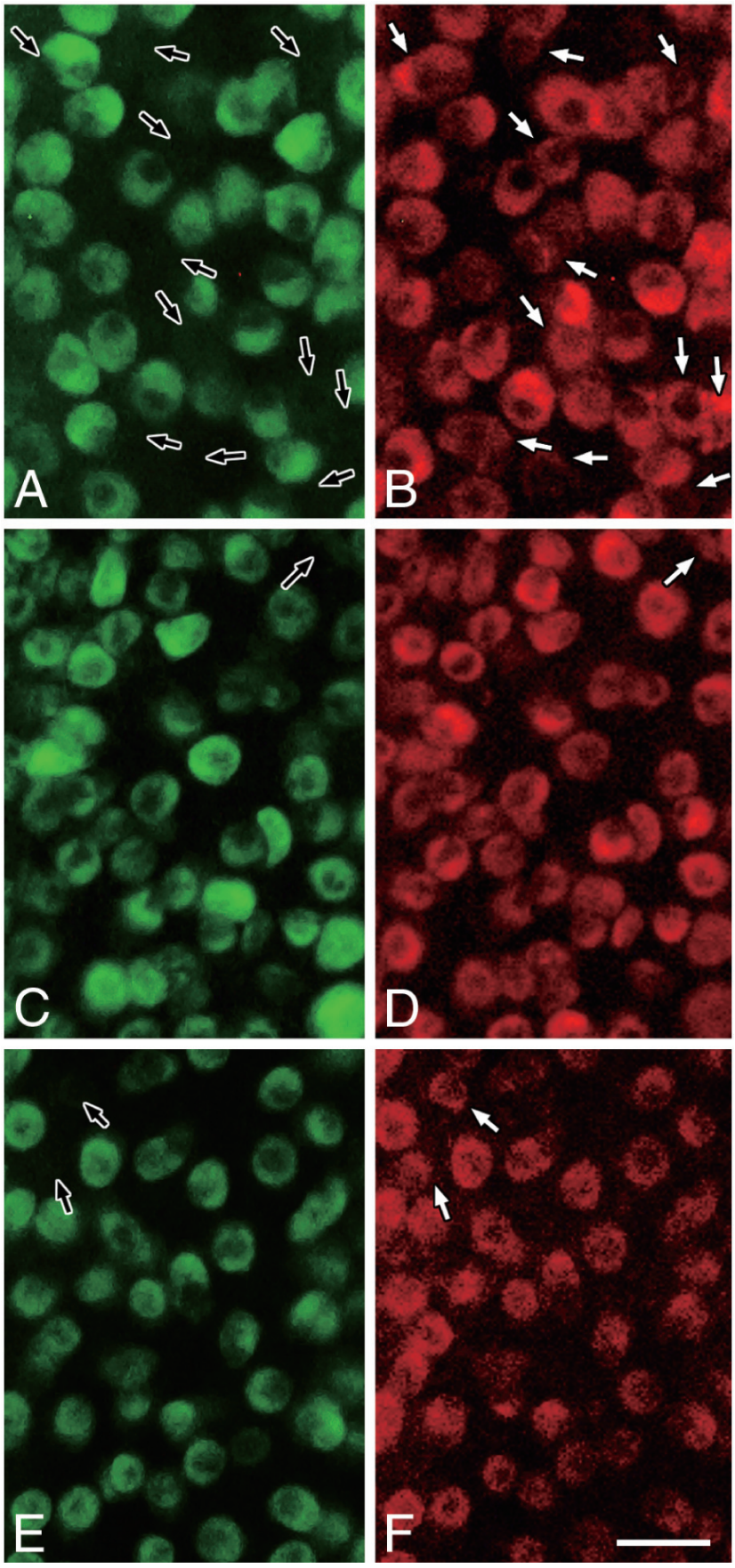

Fig. 19. Time-differential double fluorescent staining of branchial MR cells in killifish transferred from seawater (SW) to freshwater (FW) (**A, B**) and in those maintained in FW (**C, D**) or in SW (**E, F**). Gill MR cells were double-labeled *in vivo* with Rhodamine 123 (**A, C, E**) just before transfer (day 0) and MitoTracker (**B, D, F**) on day 3. Cells positive for both Rhodamine 123 and MitoTracker were pre-existing. Arrows indicate newly differentiated MR cells, which are Rhodamine 123-negative (black arrows)/MitoTracker positive (white arrows). Bar: 20 μm. Reprinted from *J. Exp. Biol.*, **206**, Katoh and Kaneko, Short-term transformation and long-term replacement of branchial chloride cells in killifish transferred from seawater to freshwater, revealed by morphofunctional observations and a newly established "time-differential double fluorescent staining" technique, 4113–4123, 2003, The Company of Biologists Limited.

microscopic immunocytochemistry, using a homologous antibody, has revealed H^+-ATPase-immunoreactivity in Na^+/K^+-ATPase immunoreactive MR cells of killifish adapted to low (0.1 mM)-NaCl FW, whereas the immunoreactivity is much weaker in higher (1 and 10 mM) NaCl FWs (Figs. 20G–I). These results indicate that activation of MR cells in the ion-poor environment is accompanied by enhanced expression of H^+-ATPase.

Furthermore, the immunoelectron microscopy revealed H^+-ATPase to be located in the basolateral membrane of MR cells (Katoh *et al*. 2003), which disagrees with previous observa-

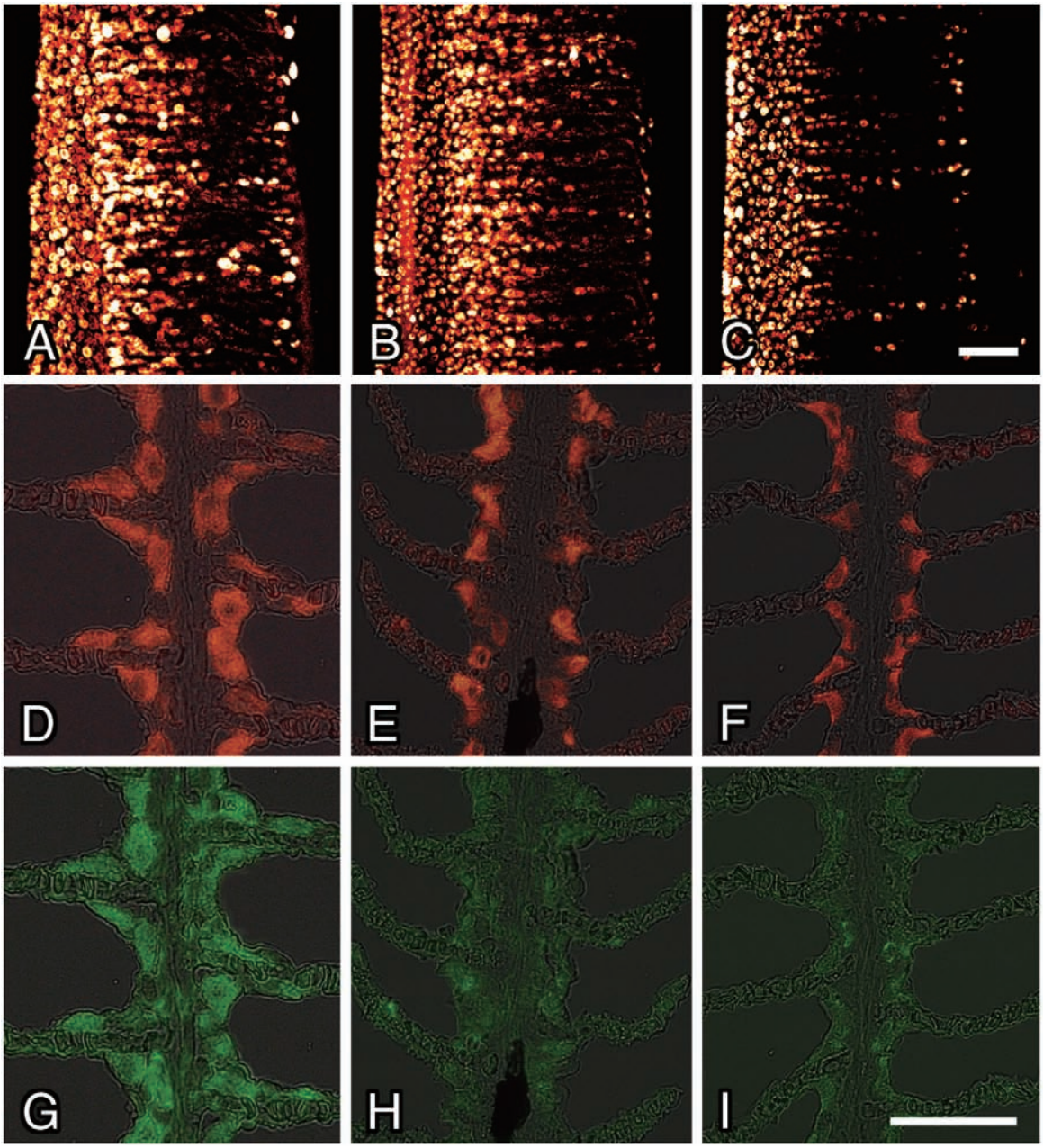

Fig. 20. MR cells in gill filaments of killifish acclimated to defined freshwaters with low- (**A, D, G**), mid- (**B, E, H**) and high- (**C, F, I**) NaCl. **A–C**. Whole-mount preparations stained with anti-Na^+/K^+-ATPase. **D–I**. Double immunofluorescence staining of Na^+/K^+-ATPase (**D–F**) and V-ATPase (**G–I**). Bars: **A–C,** 100 μm; **D–I,** 50 μm. Reprinted from *J. Exp. Biol*., **206**, Katoh *et al*., Vacuolar-type proton pump in the basolateral membrane energizes ion uptake in branchial mitochondria-rich cells of killifish *Fundulus heteroclitus*, adapted to a low ion environment, 793–803, 2003, The Company of Biologists Limited.

tions in teleost gills. In gill epithelia of rainbow trout, H^+-ATPase is located in the apical membranes of both MR and pavement cells (Wilson *et al*. 2000a; Perry and Fryer 1997). H^+-ATPase immunoreactivity has also been detected in the apical membrane of lamellar MR cells in mudskipper (*Periophthalmodon schlosseri*) (Wilson *et al*. 2000b) and in pavement cells in Mozambique tilapia (Hiroi *et al*. 1998b). In the gills of euryhaline stingray *Dasyatis sabina*, however, H^+-ATPase immunoreactivity was detected in the cytoplasm of gill epithelial cells (Piermarini and Evans 2001), presumably in vesicles or the basolateral membrane, which is in accordance with the observation in killifish. These conflicting results may indicate possible diversity in the distribution and function of H^+-ATPase in the gill epithelia among different species.

Based on the observation that Na^+/K^+-ATPase and H^+-ATPase are co-localized in the basolateral membrane in gill MR cells, we propose a model for Na^+ uptake through FW-type MR cells in killifish. When basolaterally located Na^+/K^+-ATPase and H^+-ATPase transport Na^+ and H^+, respectively, from MR cells to the serosal side, the MR cells would be negatively charged. According to the electrical gradient established by Na^+/K^+-ATPase and H^+-ATPase, Na^+ is absorbed via apically located Na^+ channels. In killifish, H^+-ATPase may facilitate the creation of a steeper electrical gradient in collaboration with Na^+/K^+-ATPase for absorption of Na^+ from low Na^+ environments. It is highly possible that the Na^+ channel is located in the apical membrane of MR cells, although the localization of Na^+ channels has not been addressed in killifish.

6. Chum salmon

Most anadromous salmonids such as sockeye (*Oncorhynchus nerka*), coho (*O. kisutch*), chinook (*O. tshawytscha*), masu (*O. masou*), Atlantic salmon (*Salmo salar*) and steelhead trout (*O. mykiss*) migrate to the sea after parr-smolt transformation, which involves morphological and physiological changes for preadaptation to SW entry. In these species, gill Na^+/K^+-ATPase activity increases during smoltification, in correlation with increased salinity tolerance and also with the increase in number and size of branchial MR cells (Langdon and Thorpe 1985; Richman *et al*. 1987; McCormick 1995). In contrast with these salmonids, chum (*O. keta*) and pink (*O. gorbuscha*) salmon go downstream to the sea in early life stages, acquiring hypoosmoregulatory ability during the alevin stages (Weisbart 1968; Iwata *et al*. 1982; Hasegawa *et al*. 1987).

6.1. Hypoosmoregulatory ability of chum salmon embryo

At a late embryonic stage, chum salmon show salinity resistance and osmoregulatory ability to some extent. Kashiwagi and Sato (1969) observed 25% hatching success when chum salmon eggs were transferred to SW during the eyed stage. In our experiment, eyed-stage eggs survived direct transfer from FW to SW, the survival rate for two days being more than 95%. To evaluate the osmoregulatory ability of eyed-stage embryos of chum salmon, Kaneko *et al*. (1995) examined changes in osmolality of the perivitelline fluid (PVF) and blood following transfer from FW to 50 and 100% SW. At the beginning of the experiment, osmolality of the PVF was 32 mOsm. In the FW control group, osmolality of the PVF remained relatively low during the experiment for two days. In contrast, the osmolality of the PVF increased rapidly after transfer to 50 and 100% SW and reached the levels of the corresponding environments at 3 h after transfer. The high levels of osmolality were maintained afterward in both groups. This indicates that the egg shell is permeable to ions and water in agreement with previous reports (Lönning and Davenport 1980; Davenport *et al*. 1981).

Since ions and water seem to exchange freely across the egg shell, the embryo can be considered to be exposed to the environmental medium. Then, changes in blood osmolality were investigated in chum salmon embryos transferred to 50 and 100% SW (Fig. 21). The blood osmolality was maintained at a constant level of 360 to 370 mOsm in FW during the experiment, but increased markedly to 440 mOsm in 50% SW and to 462 mOsm in 100% SW 3 h after transfer. Afterwards, the osmolality decreased gradually to 383 mOsm in 50% SW and 407 mOsm in 100% SW at 168 h, but both levels were still higher than that of the controls maintained in FW. These observations clearly indicate that eyed-stage embryos of chum salmon have already acquired a hypoosmoregulatory mechanism.

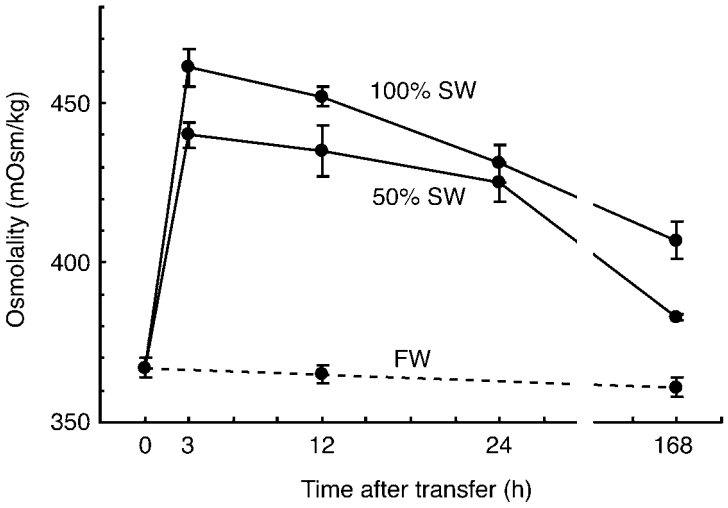

Fig. 21. Changes in blood osmolality following transfer from freshwater (FW) to 50% and 100% seawater (SW). Data are expressed as the mean ± S.E.M. (n = 6). Reprinted from *Mar. Biol.*, **122**, 1995, 165–170, Hypoosmoregulatory ability of eyed-stage embryos of chum salmon, Kaneko *et al.*, Fig. 3. With kind permission of Springer Science+Business Media.

In a hyperosmotic environment, fish require a mechanism that extrudes excess salts to maintain ion balance. In adult fish, MR cells in the gills are responsible for salt extrusion in SW. In chum salmon embryos, the gills do not seem to be functional neither as a respiratory epithelium nor as a site of salt secretion. Our histological observations reveal that gill secondary lamellae, considered to be a functional unit in the gill, are not yet developed in the eyed-stage embryos, although the gill arches were recognizable. Thus, the gills are not the primary site for ion exchange, even if the epithelium of the poorly developed gills may contain some MR cells. Instead, as is the case with Mozambique tilapia and other teleost species, a rich population of MR cells is present in the yolk-sac membrane. To investigate the involvement of yolk-sac MR cells during SW adaptation, morphological changes in those cells were examined following transfer to hypertonic environments (Kaneko *et al*. 1995). The sectional area of the MR cells was significantly increased, by about 40%, two days after transfer to 50 and 100% SW, whereas such increase was not observed in the embryos maintained in FW. SEM observations also showed that apical openings of MR cells were frequently observed among the pavement cells in the yolk-sac membrane of the embryos transferred to hyperosmotic environments (50 and 100% SW). The apical openings were only occasionally found in the embryos maintained in FW, being covered completely with the pavement cells. The activation of MR cells in SW, as evidenced by the increased cell size and frequent appearance of apical openings, indicates MR cells in the yolk-sac membrane are the primary site for salt extrusion in SW in place of gill MR cells during the late embryonic stage.

6-2. Seawater adaptability in chum salmon fry

When chum salmon grow to be fry in FW, they develop strong SW adaptability prior to the forthcoming downstream migration to the sea. Uchida *et al*. (1996) examined functional modification of gill MR cells during SW adaptation of chum salmon fry. The gill Na^+/K^+-ATPase activity in chum salmon fry (0.6–3 g) was increased prior to SW entry, being twice as high as that in late alevin stages (0.3–0.4 g).

In chum salmon, Na^+/K^+-ATPase-immunoreactive MR cells were detected on both filament and lamellar epithelia in the gills (Fig. 22). MR cells on the filament were usually round or columnar and located at the base of the lamella. In contrast, the cells on the lamella were flat and appeared as protrusions on the lamella. The two distinct types of MR cells showed different morphological and morphometrical changes following SW transfer (Fig. 22). The number of MR cells in the filament was not affected by SW transfer, whereas lamellar MR cells were significantly decreased. The size of filament MR cells was 1.8-fold larger on 21 days after SW transfer than that of FW fish, whereas there was no change in the size of lamellar MR cells

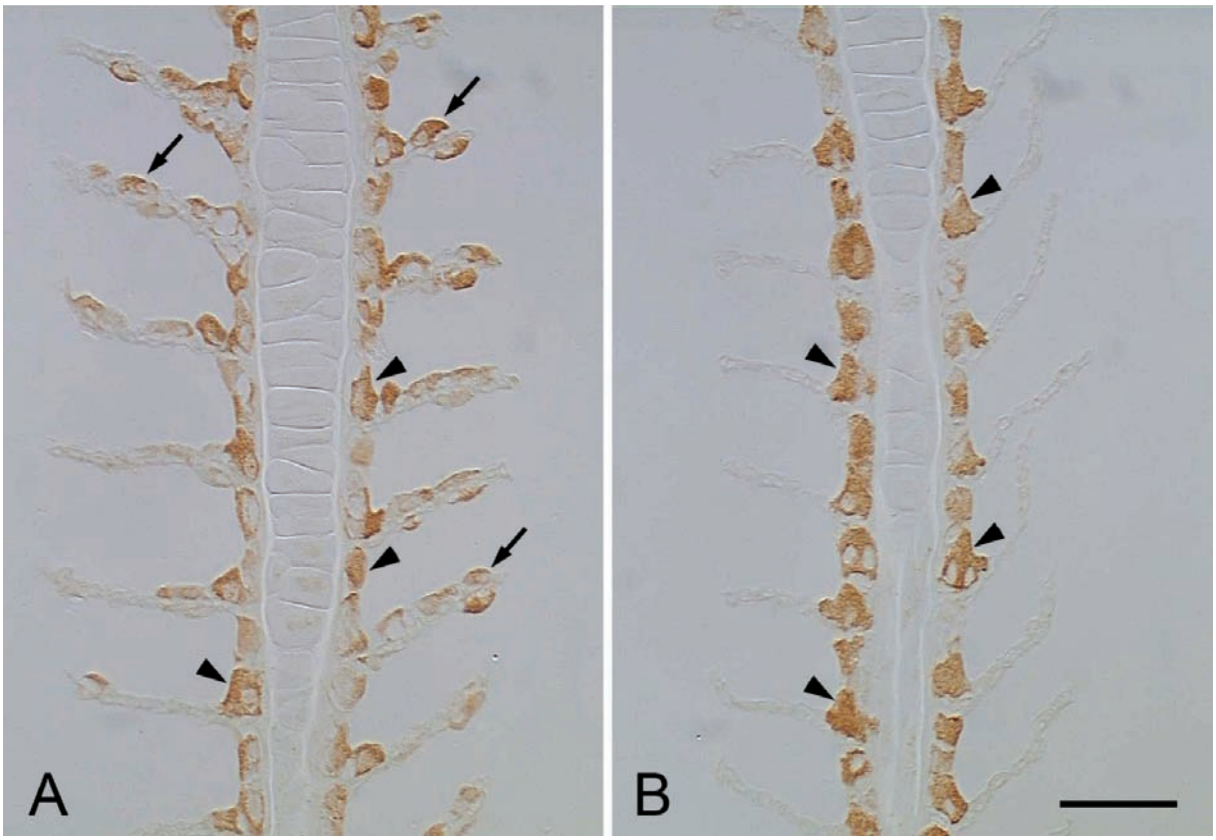

Fig. 22. Sagittal sections of the gills stained with anti-Na^+/K^+-ATPase in chum salmon fry. A. Freshwater (FW). Both filament (arrowheads) and lamellar (arrows) MR cells are present in FW. B. Seawater (SW). In SW-adapted fish, filament MR cells (arrowheads) are well developed, but lamellar MR cells are absent. Bar: 50 μm.

throughout the experiment. The intensity of immunoreaction gradually increased after transfer to SW in filament MR cells. According to TEM observations, MR cells were moderately developed in both filaments and lamellae of FW fish. In the SW-adapted fish, well-developed MR cells were detected only in the filament. Degenerating MR cells were occasionally found on the lamellar epithelia in SW-transferred fish, whereas such cells were not evident in FW. These results suggest that MR cells in the lamella are the site of ion uptake in FW chum salmon fry, and that the well-developed MR cells in the filament are responsible for salt secretion in SW. The occurrence of a rich population of filament MR cells, concomitant with enhanced gill Na^+/K^+-ATPase activity, in FW fry may imply preadaptation to SW entry.

6-3. MR-cell turnover in the gills of chum salmon fry

As mentioned above, filament MR cells became activated following transfer of chum salmon fry to SW. To examine whether the activation of MR cells was accompanied by enhanced turnover of those cells, Uchida and Kaneko (1996) explored the differentiation and renewal of gill MR cells in FW- and SW-adapted chum salmon fry and in those during SW adaptation, using 5-bromo-2′-deoxyuridine (BrdU) incorporated into nuclei during DNA synthesis as a marker of newly-differentiated cells.

Chum salmon fry in FW were injected intraperitoneally with BrdU at 100 μg/g body weight in saline. After the injection, the fry were either maintained in FW (FW group) or transferred directly to SW (SW-transferred group). Another group of intact fry were transferred directly to SW at the same time. After acclimation to SW for two weeks, they were injected with BrdU in

the same manner and kept in SW (SW group). In all groups, gills were sampled on days 1, 2, 3, 4, 5 and 7 after BrdU injection. To determine the degree of differentiation of gill MR cells, MR cells and BrdU-incorporated nuclei were detected immunocytochemically with antibodies specific for Na^+/K^+-ATPase and BrdU, respectively. The numbers of filament MR cells was constant in FW, SW and SW-transferred groups. In contrast, lamellar MR cells were fewer in the SW group than in the FW group, and decreased gradually in SW-transferred group. In all groups, newly-differentiated cells with a BrdU-immunoreactive nucleus were mainly detected in the vicinity to the central venous sinus in the filament on day 1. Thereafter, BrdU-labeled cells spread out, especially in the basement of the lamellar epithelia. Observations on adjacent sections stained with anti-Na^+/K^+-ATPase and anti-BrdU revealed that a large proportion of BrdU-labeled cells corresponded to Na^+/K^+-ATPase-immunoreactive MR cells. MR cells with a BrdU-immunoreactive nucleus were 48–56% of total BrdU-labeled cells in the entire gills on day 7. Since MR cells comprise only a small population (less than 5%) of gill epithelial cells, the turnover of MR cells appears much greater than that of the other cell types in the gills. Considering that the total numbers of filament MR cells were constant, MR cells in the filament are continuously replaced; newly differentiated cells are recruited and the equivalent number of cells degenerated.

In the filament, BrdU-labeled MR cells increased during the experimental period. The percentage of BrdU-labeled MR cells to total MR cells in the filament in the FW group increased gradually and reached the maximal value of about 30% on day 5. In the SW group, the percentage of BrdU-labeled MR cells was three times greater than that of the FW group on day 1, and reached a plateau level of about 30% on day 4. In SW-transferred group, the percentage rapidly increased and attained a maximal level on day 1. The occurrence of BrdU-labeled MR cells during the first 24 h was 8, 21 and 28% of the total MR cells in the filament in FW, SW and SW-transferred groups, respectively. These results indicate that the activated MR cells following transfer from FW to SW seem to be differentiated rapidly, probably within a few days after SW entry. The enhanced turnover of filament MR cells in response to SW transfer adds support to the notion that filament MR cells are responsible for salt secretion in SW.

In contrast, lamellar MR cells labeled with BrdU were rarely observed in the three groups; MR-cell differentiation in the gill lamellae seems less active than in the gill filaments. This is in accordance with the result obtained by Laurent *et al*. (1994) that no cell proliferation was seen in the lamellar epithelium of rainbow trout within 48 h after transfer to ion-poor water. In Japanese sea bass, Hirai *et al*. (1999) suggested that lamellar MR cells originated from the filaments and migrated to the lamellae during FW adaptation. In chum salmon, lamellar MR cells are considered to be derived from undifferentiated cells in the filament epithelia.

6-4. Loss of hypoosmoregulatory ability in mature chum salmon

Maturing chum salmon return to their natal streams after spending 3 to 5 years in the ocean. At northern Honshu Island, Japan, they usually spawn close to the sea, sometimes less than 1 km from the river mouth. Interestingly, the fully matured fish, with ovulation or spermiation completed, lose their hypoosmoregulatory ability in SW, although they adapt well to FW (Morisawa *et al*. 1979; Hirano *et al*. 1990). The maladaptation to SW seems to be due primarily to an increased plasma osmolality (Hirano *et al*. 1990). In response to increased plasma osmolality, secretion of cortisol and growth hormone, which are the central SW-adapting hormones, was stimulated. On the other hand, plasma prolactin, a FW-adapting hormone in fish, remained low in SW fish, indicating that hypersecretion of prolactin is not the cause of maladaptation to SW (Hirano *et al*. 1985, 1990; Bolton *et al*. 1986; Ogasawara *et al*. 1996).

Uchida *et al*. (1997) investigated functional modification of MR cells during spawning migration of mature chum salmon. Mature female chum salmon, weighing 3 to 5 kg, were caught by a set-net in Otsuchi Bay, a small semiclosed bay on the Pacific coast of the northern Honshu Island, Japan, in December 1995. The returning chum salmon spend about a week in the bay in late November and early December, and complete gonadal maturation while in the bay before ascending the river (Ogasawara *et al*. 1996). Fish were transported to the Otsuchi Marine Research Center of the Ocean Research Institute, University of Tokyo. The fish were placed in SW

tanks on arrival, and kept for 3 h to allow them to recover from the transporting stress. The fish were then separated into two groups: one maintained in SW and the other transferred to FW.

No mortality was seen in the mature salmon transferred to FW during the experimental period of five days. On the other hand, four out of seven mature fish kept in SW died after day 3, and the other fish survived until day 5. The initial level of plasma osmolality of the mature chum salmon caught in the bay was about 370 mOsm. Plasma osmolality of the SW-maintained fish that died after three days increased markedly on day 3, whereas that of the remaining three fish was maintained at the initial levels and tended to increase on day 5 (Fig. 23). In contrast, plasma osmolality of the FW-transferred fish decreased to about 300 mOsm on day 1, but no further decrease was observed until day 5 (Fig. 23). Different mortality seen in the SW-maintained fish suggests heterogeneity of the fish collected in Otsuchi Bay. The fish surviving for three days might have stayed longer in the Bay, whereas those for five days might have arrived later. However, the late arriving fish also lose hypoosmoregulatory ability within a few days, concomitant with a marked increase in plasma osmolality.

Such mortality of mature chum salmon is closely related to gill MR cells. When gills in immature chum salmon migrating in the Pacific Ocean were examined, Na^+/K^+-ATPase-immunoreactive MR cells were present mainly on the filament (Fig. 24A). Round- or columnar-shaped MR cells were also present in contact with the base of the lamellar epithelium, but typical lamellar cells were absent. In the mature fish caught in the bay, MR cells were located in both filament and lamellar epithelia (Fig. 24B). Lamellar MR cells in the bay fish were generally flat and located individually. They often appeared as protrusions on the lamella. In fish maintained in SW, the intensity of the immunoreaction in the filament MR cells decreased markedly on day 5, whereas flat-shape MR cells were still present in the lamellae (Fig. 24C). In the fish transferred to FW, filament MR cells were weakly stained, whereas lamellar were similar to those in the fish kept in SW (Fig. 24D).

Quantitative analysis clearly indicated that the number of filament MR cells in the bay fish (day 0) was significantly less than that in ocean fish. The number of filament MR cells in bay fish maintained in SW decreased significantly on days 3 and 5. Filament MR cells in FW-transferred fish also decreased significantly on day 5. In contrast with filament MR cells, lamellar

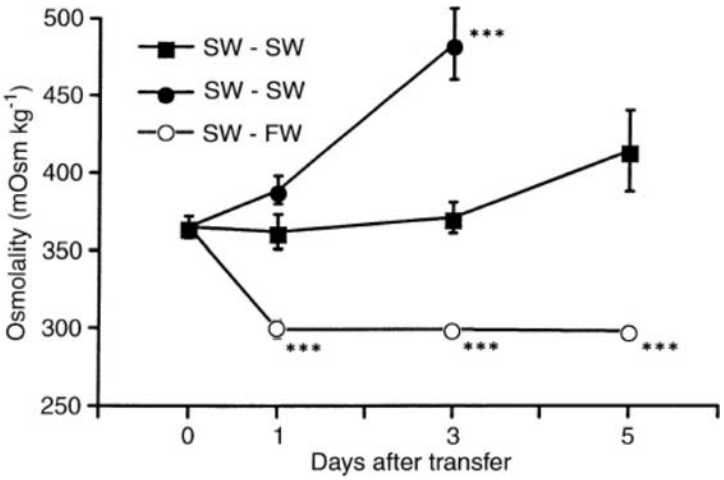

Fig. 23. Changes in plasma osmolality after transfer of mature chum salmon from seawater (SW) to freshwater (FW) or those maintained in SW (SW-SW). Data are expressed as means ± S.E.M. The numbers of fish used are as follows: SW fish caught in the bay (day 0), $n = 7$; SW fish that survived until day 3 but died by day 5 (●), $n = 4$; SW fish survived by day 5 (■), $n = 3$; and FW fish (○), $n = 5$. ***$p < 0.001$, Significantly different from the initial level. Reprinted from *Mar. Biol.*, **129**, 1997, 247–253, Reduced hypoosmoregulatory ability and alteration of gill chloride cell distribution in mature chum salmon (*Oncorhynchus keta*) migrating upstream for spawning, Uchida *et al.*, Fig. 1. With kind permission of Springer Science+Business Media.

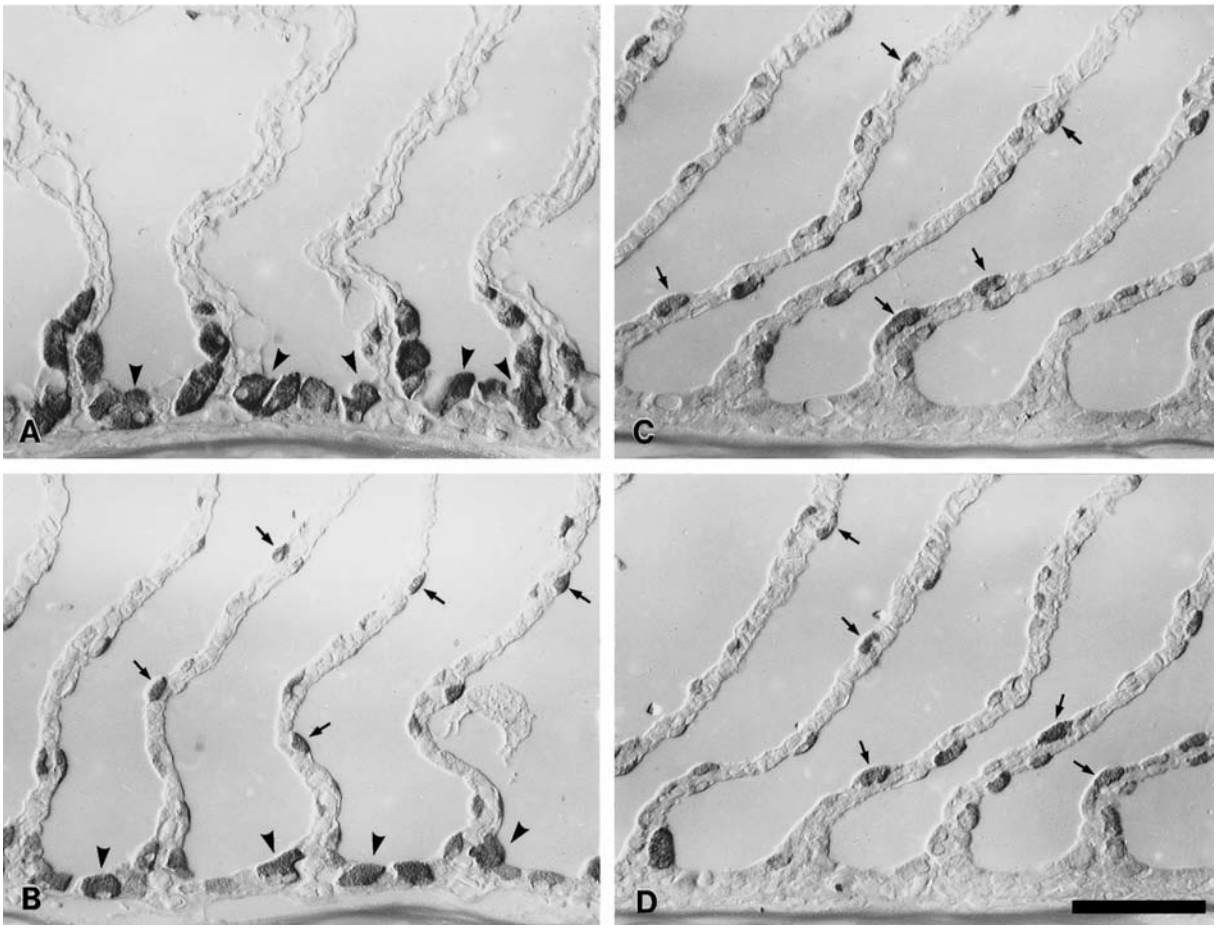

Fig. 24. Sagittal sections of the gills stained with anti-Na+/K+-ATPase in an immature fish caught in the ocean (**A**), a mature fish caught in Otsuchi Bay (**B**, day 0), a fish kept in seawater (**C**, day 5), and a fish kept in freshwater (**D**, day 5). Well-developed filament MR cells (arrowheads) are present in ocean fish, whereas flat-shape lamellar MR cells (arrows) are present in mature fish caught in the bay. Bar: 50 μm. Reprinted from *Mar. Biol*., **129**, 1997, 247–253, Reduced hypoosmoregulatory ability and alteration of gill chloride cell distribution in mature chum salmon (*Oncorhynchus keta*) migrating upstream for spawning, Uchida *et al*., Fig. 4. With kind permission of Springer Science+Business Media.

MR cells in ocean fish were much less numerous than in mature fish. There was no change in lamellar MR cells between the fish transferred to FW and those kept in SW until day 5. Considering that filament and lamellar MR cells may be the sites of ion secretion and absorption, respectively, the loss of hypoosmoregulatory ability in mature chum salmon may be attributed, at least in part, to a decreasing filament MR cell number. In view of the strong hyperosmoregulatory ability of mature chum salmon in FW, the appearance of lamellar MR cells may be a response preparatory to the forthcoming upstream migration and may perhaps be one of the causes of their maladaptation to SW.

These findings suggest the involvement of filament and lamellar MR cells in hypoosmoregulation in SW and hyperosmoregulation in FW, respectively, as is the case with chum salmon fry as described above (Uchida *et al*. 1996). Thus, hyperosmoregulatory ability may predominate over hypoosmoregulatory ability in fully matured salmon. Such a shift of osmoregulatory mechanisms in chum salmon may motivate upstream migration to rivers for spawning.

7. Japanese eel

In teleost fishes, a number of species perform spectacular migrations between SW and FW environments. Whereas chum salmon is an anadromous teleost, Japanese eel, *Anguilla japonica*, is one of those species that perform extensive catadromous migrations. The spawning area of Japanese eel is located in the North Equatorial Current west of Mariana Islands in the western North Pacific, where many small leaf-like larvae, termed leptocephali, were collected during summer (Tsukamoto 1992, 2006). Leptocephali drift westward over several months and grow to about 60 mm in length, before they metamorphose into glass eel and move to coastal areas of East Asia. After entering rivers, the eel spend sedentary life in FW for 5–12 years, and then go downstream to the sea for spawning. Therefore, the life cycle of Japanese eel is closely associated with migration between FW and SW environments; the eel are equipped with mechanisms for adaptation to both FW and SW, which are adjusted to forthcoming environments during their upstream and downstream migrations.

7-1. Epidermal MR cells in embryos and larvae

In Japanese eel, although the occurrence of extrabranchial MR cells had been predicted in the body surfaces in their early life stages, samples had not been readily available for the detection of MR cells because of their scarcity. Even though the precious samples caught from the natural habitat are available, their body surfaces would be seriously damaged by netting so that MR cells could hardly be observed. However, recent advances in artificial maturation techniques for the eel have enabled us to use eel embryos and larvae for MR cell research. This is mostly owing to the remarkable advances in the field of fish reproductive endocrinology for the past two decades. Using the eel embryos and larvae obtained from maturation-induced eel, we succeeded in detecting MR cells in the body surface of Japanese eel embryos and larvae, in which the gills were not yet developed (Sasai *et al*. 1998b).

To obtain Japanese eel embryos and larvae, females and males were artificially induced to mature by repeated injections of salmon pituitary extracts and human chorionic gonadotropin, respectively. The fertilized eggs were incubated in SW at 23°C, and typically hatched after 36-h incubation. As shown in Fig. 25, a large number of Na^+/K^+-ATPase-immunoreactive MR cells were detected in the yolk-sac membrane, which occupied a large proportion of the body surface at this developmental stage. However, very few MR cells were observed in the epithelium covering the developing embryo. Two or three MR cells often appeared to be closely associated with each other to form a cellular complex, as evidenced by the presence of more than one immunonegative nucleus, suggesting their ion-secreting function.

The occurrence and distribution of MR cells were further examined in eel larvae on 0, 2, 4 and 6 days after hatching (Kaneko *et al*. 2003). On the day of hatching, large MR cells were distributed extensively in the epithelium of the anterior half of the body, which included the yolk-sac membrane (Fig. 26). As the fish grew, the density of MR cells decreased and the cell size tended to become small, suggesting a decline in the cellular activity. Since the survival rate decreased remarkably after hatching, the mortality of the eel larvae obtained from maturation-induced fish could be attributed to the observed inactivation of MR cells present in the body skin. In the larvae that survived until day 6, however, MR cells in the body surface were increased in number, arranging along the muscle segments. As is the case in embryos, MR cells in the larvae often formed multicellular complexes in the epithelium covering the body surface.

In the late embryonic stages, MR cells were detected in the yolk-sac membrane, but rarely observed in other parts of the body surface. In the larval stages, MR cells appeared in the integument of the developing body, indicating a shift of the functional site of salt secretion from the yolk-sac membrane to the integument of the body surface during the embryonic and larval stages. It is most probable that MR cells in the integument of the body surface function as major osmo- and iono-regulatory sites until the development of gills and gill MR cells at a late leptocephalus stages.

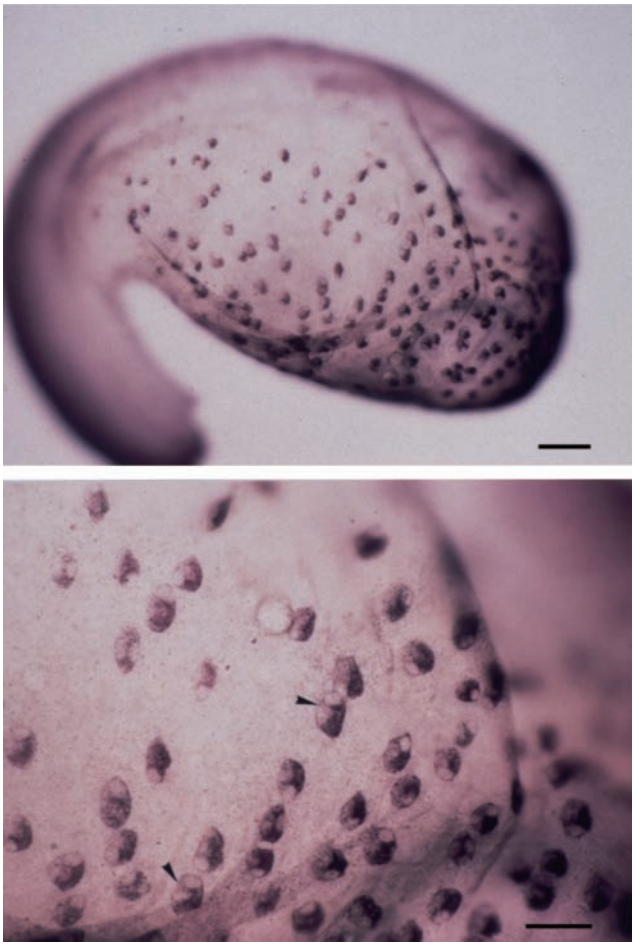

Fig. 25. Extrabranchial MR cells in a Japanese eel embryo at 35 h after fertilization, detected by whole-mount immunocytochemistry with anti-Na^+/K^+-ATPase. **B.** magnified view of the yolk-sac region. Reprinted from *Ichthyol. Res.*, **45**, 1998, 95–98, Extrabranchial chloride cells in early life stages of the Japanese eel, *Anguilla japonica*. Sasai, *et al.*, Fig. 1. With kind permission of Springer Science+Business Media.

7-2. Ontogenic changes in MR cells during leptocephalus and glass eel stages

Ecological studies using otolith analysis have revealed that Japanese eel have an extremely long larval period and undergo a drastic metamorphosis from planktonic leptocephali to benthic glass eel (Otake *et al*. 1994; Arai *et al*. 1997; Kawakami *et al*. 1999). However, little information had been available on the osmoregulatory mechanisms of anguillid eels during their unique larval stage. Recently, Sasai *et al*. (2007) explored the developmental changes in gill MR cells during the leptocephalus and glass eel stages, using specimens collected in their natural habitats (Fig. 27). By means of immunocytochemistry with the antiserum specific for Na^+/K^+-ATPase, MR cells in the gill epithelium were first recognized in a leptocephalus at a length of 32.2 mm, in which the buds of the gill filaments were barely differentiated (Figs. 27E, F). These immunoreactive cells had weak staining intensity and mottled appearance, which may indicate their low cellular activity. Therefore, gill MR cells may not yet be functional at this developmental stage. Gill MR cells that were uniformly and intensively immunoreactive to anti-Na^+/K^+-ATPase were observed in 54.8 and 58.3 mm leptocephali, in which the gill filaments had started developing (Figs. 27G, H). In contrast, gill lamellae, which enlarge the surface area, were never seen even in the late leptocephalus stage. In the glass eel that had just metamorphosed, gill lamellae were already present on the extended gill filaments. However, the lamellae were still poorly developed, and the surface area of the gills was still smaller than that in the adult form of the gills (Fig. 27I). MR cells were extensively distributed on the filaments, but were absent from the lamellae (Fig. 27J). From otolith studies, the estimated duration of the leptocephalus stage extends as long as 80–150 days in Japanese eel (Otake *et al*. 1994; Arai *et al*. 1997; Kawakami *et al*. 1999). It should be noted that Japanese eel do not develop fully functional gills as a respiratory

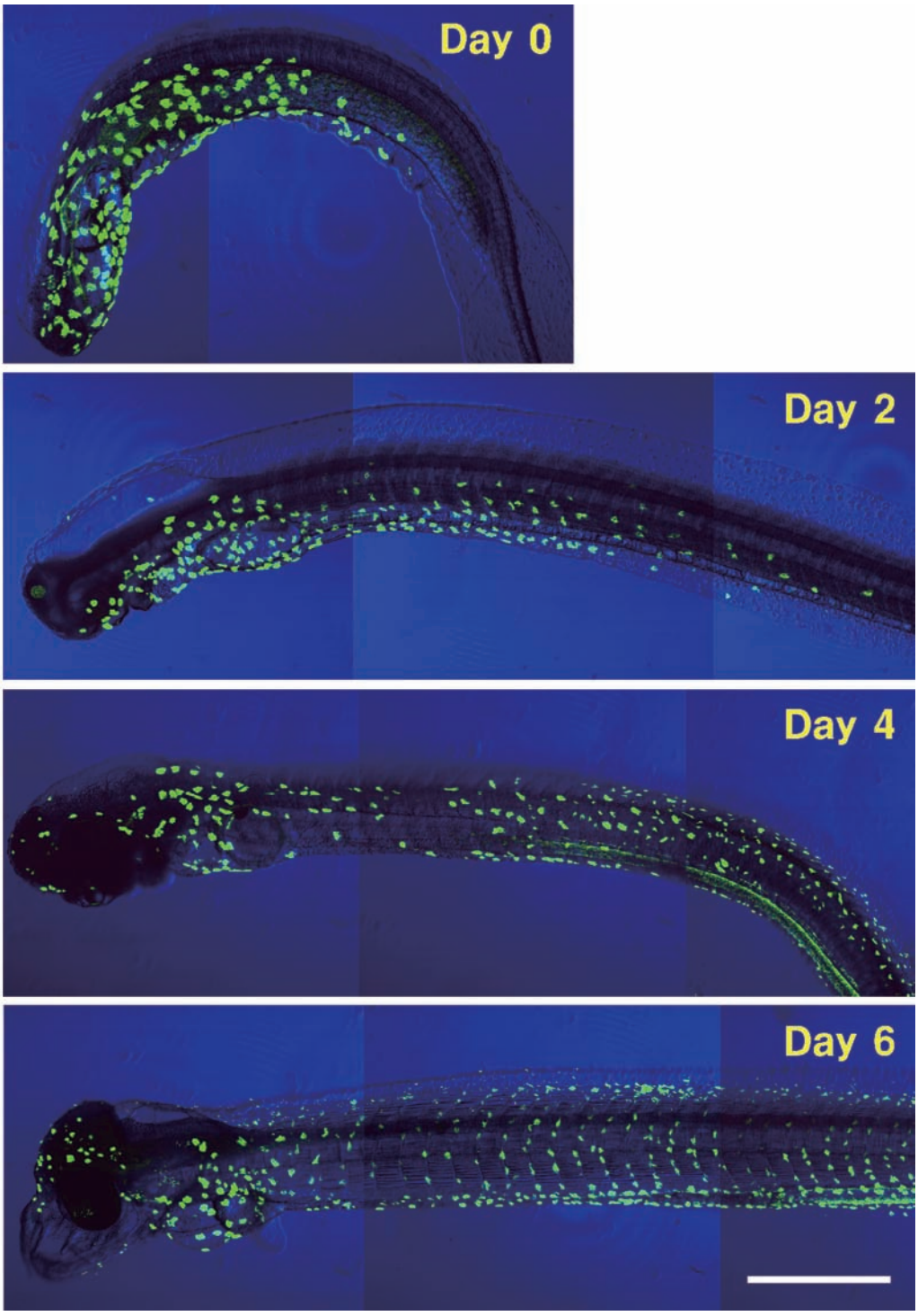

Fig. 26. Extrabranchial MR cells in Japanese eel larvae on 0, 2, 4 and 6 days after hatching, detected by whole-mount immunocytochemistry with anti-Na^+/K^+-ATPase labeled with FITC and observed with a confocal laser scanning microscope. Scale: 500 μm.

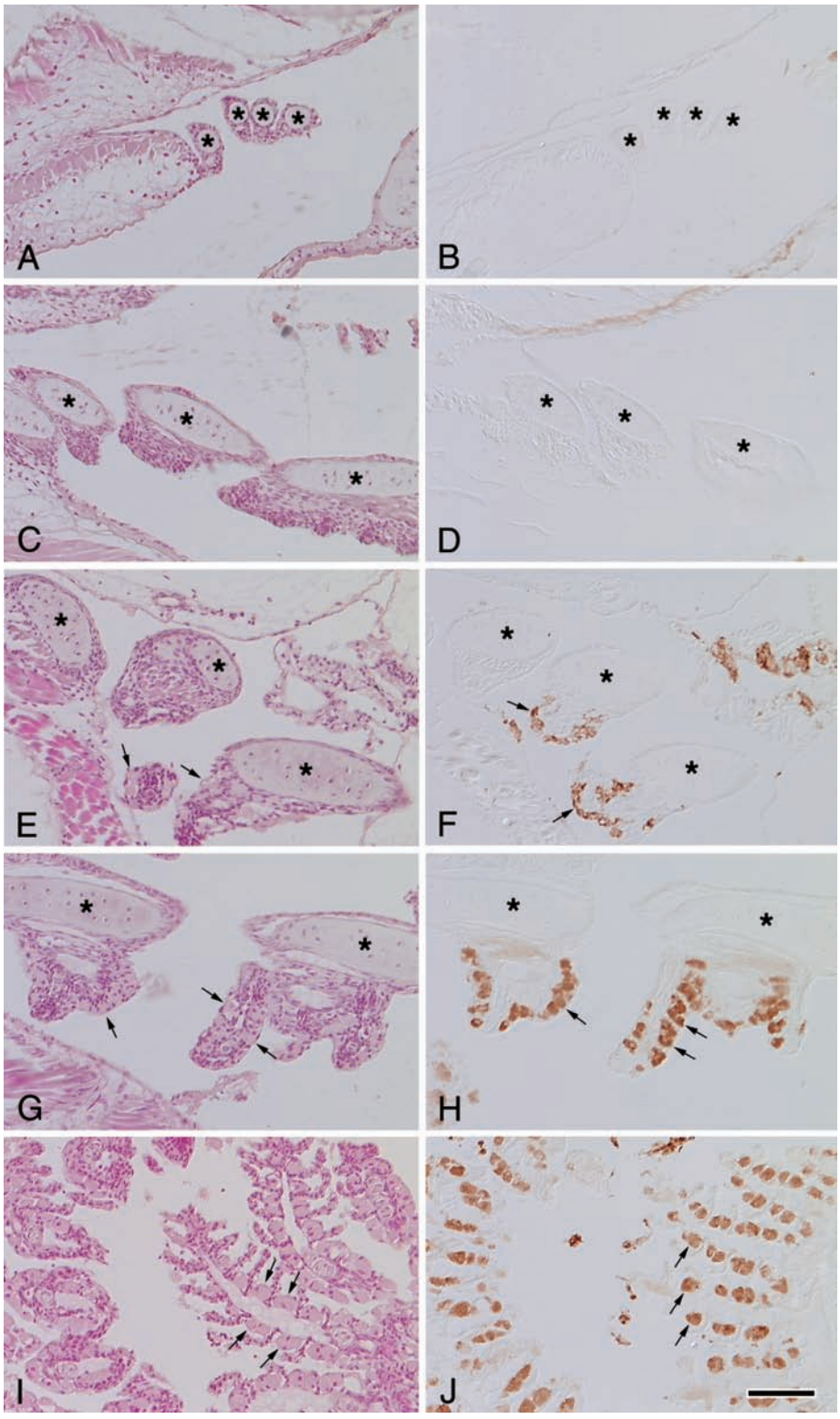

Fig. 27. Sagittal sections of leptocephali of Japanese eel at 17.0 (**A, B**), 22.0 (**C, D**), 32.2 (**E, F**) and 54.8 (**G, H**) mm, and glass eel just after metamorphosis (**I, J**), showing the development of the gills. Panels **A, C, E, G,** and **I** show tissues stained with hematoxylin and eosin. Panels **B, D, F, H,** and **J** show tissues immunocytochemically stained with anti-Na^+/K^+-ATPase for detection of MR cells. Arrows and asterisks indicate the location of MR cells and gill arches, respectively. Bar: 50 μm. Reprinted from *Mar. Biol.*, **150**, 2007, 487–496, Ontogenic change of gill chloride cells in leptocephalus and glass eel stages of the Japanese eel, *Anguilla japonica*, Sasai *et al.*, Fig. 3. With kind permission of Springer Science+Business Media.

organ, in spite of the occurrence of MR cells, during the long-lasting larval stages. As suggested in Japanese flounder by Hiroi *et al*. (1998a), the primary function of the gills during the larval stages may be ion regulation rather than gas exchanges.

The leptocephalus is generally thought to have little or no ability to maintain a stable osmotic gradient between their body fluids and the external environments (Hulet *et al*. 1972). Using live, actively swimming leptocephali of the anguilliforms, Hulet and Robins (1989) reported that blood osmolality in younger individuals was nearly equal to the value of the ambient SW, whereas prior to the onset of metamorphosis, the value decreased progressively with length. The osmolality of the intravascular fluid of the leptocephali of a congrid eel, *Rhynchoconger flavus*, decreased from 1030 to 535 mOsm as the leptocephali grew from 50 to 117 mm in total length. Nevertheless, our preliminary experiment showed that tissue osmolality of pre-leptocephali and leptocephali of Japanese eel stayed within a range between 370 and 500 mOsm, which is apparently higher than that observed in adult fish, but much lower than SW osmolality (Lee and Kaneko, unpublished data). Considering the development of gill MR cells and their occurrence in the integument, it is most probable that leptocephali develop osmoregulatory ability to some extent during the extremely long larval stages.

7-3. MR cells in glass eel acclimated to FW

Sasai *et al*. (2007) have also examined changes in MR cells in glass eel transferred from SW to FW. In glass eel collected at the coast of Lake Hamana, Shizuoka Prefecture, the gill filaments were well developed and the lamellae extended from gill filaments (Fig. 28A). MR cells were extensively distributed in the gills. These cells were cuboidal in shape, occupying a large proportion of the gill filament epithelia. Although typical MR cells were absent in the

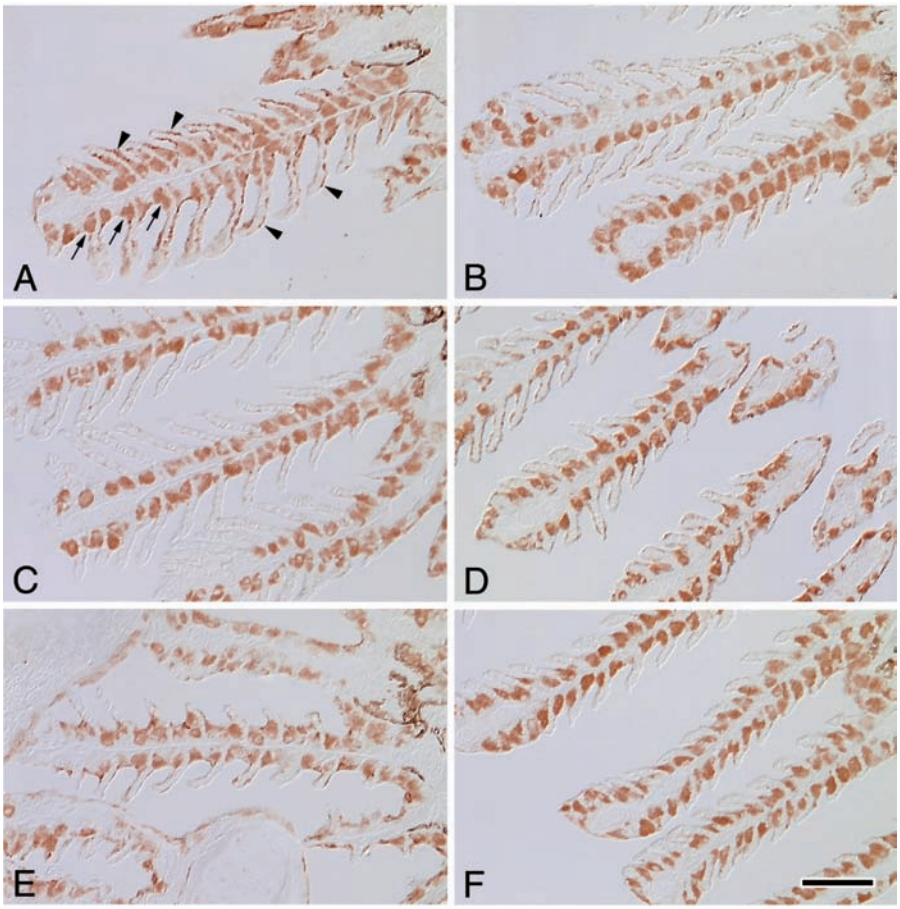

Fig. 28. Sagittal sections of the gills stained with anti-Na^+/K^+-ATPase in glass eel of Japanese eel at 0 (**A**), 1 (**B**), 3 (**C**), 7 (**D**), and 14 (**E**) days after transfer from seawater (SW) to freshwater (FW), and those maintained in SW for 14 days (**F**). Arrows and arrowheads indicate MR cells in the filaments and immunoreaction in the lamellae, respectively. Bar: 50 μm. Reprinted from *Mar. Biol*., **150**, 2007, 487–496, Ontogenic change of gill chloride cells in leptocephalus and glass eel stages of the Japanese eel, *Anguilla japonica*, Sasai *et al*., Fig. 4. With kind permission of Springer Science+ Business Media.

lamellae, distinct Na^+/K^+-ATPase immunoreaction was often observed along the surface of gill lamellae. Following transfer of those glass eel to FW, the size of filament MR cells decreased in a time-dependent manner to be about 60% of the initial size on day 14 (Figs. 28A–E). The filament MR cells in the control fish maintained in SW for 14 days also significantly decreased to 70% in size (Fig. 28F). In contrast, there was no difference in the number of filament MR cells throughout the experiment. The immunoreaction on the lamellar epithelium, which were frequently observed in SW fish at the beginning of the experiment, was also present in all FW-transferred fish (Figs. 28A–E), whereas it disappeared in the control fish maintained in SW for 14 days (Fig. 28F).

Filament MR cells in the gills of glass eel seem to be important in SW adaptation, presumably acting as salt secretion sites in hyperosmotic environments. This result is broadly consistent with that obtained in yellow and silver eel (Sasai *et al*. 1998a), as described below. Unexpectedly, MR cells in the gill filament decreased in size in fish maintained in SW for 14 days. Supposing that filament MR cells are SW-type cells responsible for salt secretion in hyperosmotic environments, the progressive decrease in size could be considered as a preadaptive response for upstream migration to FW, reducing the hypoosmoregulatory ability to some extent. However, they do not lose SW adaptability completely since no mortality was seen in SW-maintained glass eel, but slightly reduced hypoosmoregulatory ability could motivate upstream migration to rivers. Tsukamoto *et al*. (1998) reported that some Japanese eel caught in the East China Sea had spent their entire lives in SW, and had not been residing in FW during their juvenile growth phase. Considering the occurrence of such "sea eel", some glass eel may go upstream into rivers; others may stay in estuaries or coastal waters.

On the basis of their location and response to SW or FW transfer, two different types of MR cells have been identified in the gill filament and lamellar epithelia of several euryhaline species, such as the adult form of Japanese eel (Sasai *et al*. 1998a), chum salmon (Uchida *et al*. 1996), and Japanese sea bass (Hirai *et al*. 1999). Filament and lamellar MR cells were thought to be important in SW and FW adaptation, respectively. As mentioned above, SW-type filament MR cells were consistently observed in the gills of glass eel. However, typical MR cells were rarely observed in the lamellar epithelium of the glass eel in SW and those transferred to FW. Instead, some Na^+/K^+-ATPase-immunoreaction was observed on the gill lamellae. It is possible that pavement cells are responsible for ion uptake in place of lamellar MR cells in glass eel. In some teleost species in FW, pavement cells are considered to be the site of Na^+ uptake in exchange for H^+ secretion through apically-located H^+-ATPase (Goss *et al*. 1992; Lin *et al*. 1994; Morgan *et al*. 1994; Sullivan *et al*. 1995; Hiroi *et al*. 1998b).

7-4. Gill MR cells in eel cultured in FW and those acclimated to SW

After spending a sedentary life in FW, Japanese eel undergo metamorphosis from yellow to silver eel, which is referred to as silvering. During silvering, the belly changes from yellow to silvery white, whereas the dorsal part of the body turns brown to black. Many important morphological and physiological changes are associated with these color modifications. A series of metamorphic events includes preadaptation to marine migration; SW adaptability is highly enhanced during silvering in advance of SW entry (Fontaine 1975). Among various metamorphic events, functional modification of the gills seems to be closely related to enhancement of SW adaptability. Although eel go downstream to the sea after metamorphosis to silver eel in nature, those cultured in FW, corresponding to yellow eel, also show excellent SW adaptability.

Sasai *et al*. (1998a) examined osmoregulatory ability and general morphology of gill MR cells in FW-cultured eel and those acclimated to SW for two weeks. Plasma Na^+ levels were maintained within a narrow range (145–163 mM) in both FW and SW eel, but were slightly higher in SW fish than in FW fish. Plasma osmolality was also slightly but significantly higher in SW fish. However, both plasma Na^+ and osmolality stayed within physiological ranges, indicating that the SW eel prepared by transfer of FW eel to SW were well adapted to the SW environment. Histological observations revealed that Na^+/K^+-ATPase-immunoreactive MR cells were extensively distributed in the gill epithelia (Fig. 29). According to their location and shape, MR cells were classified into two types, similar to those in chum salmon described above. One

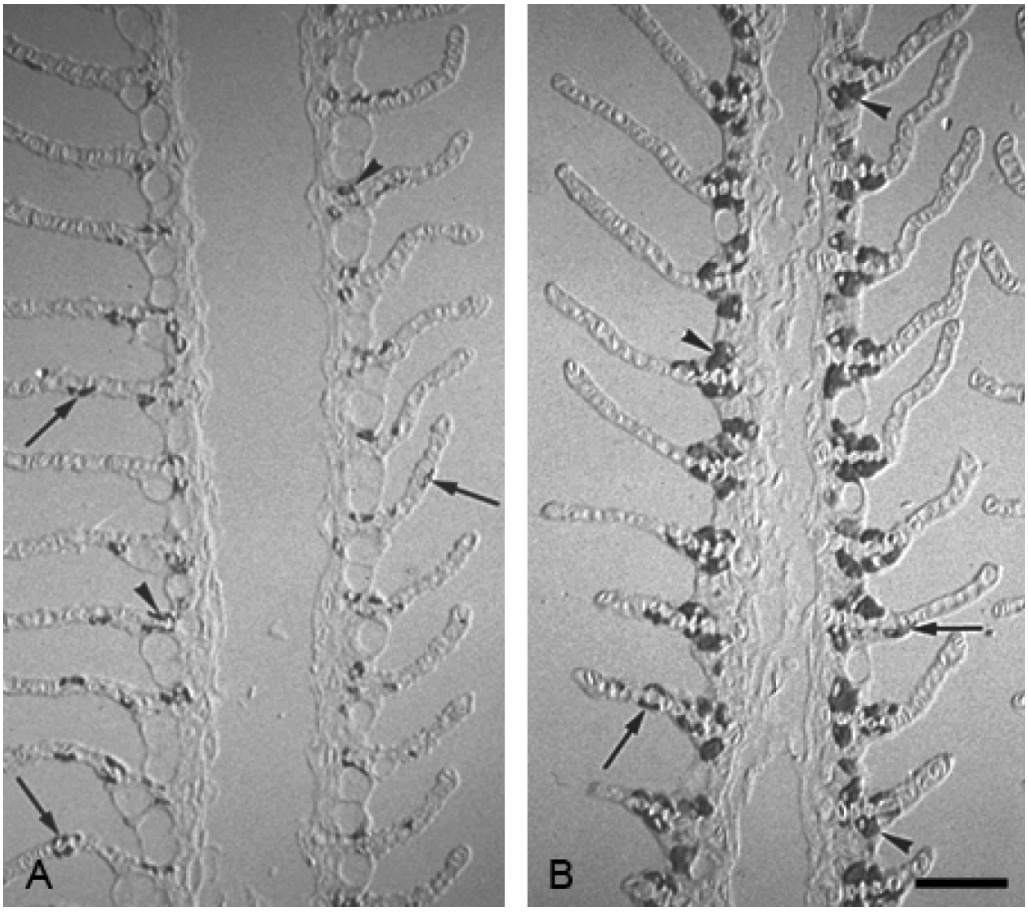

Fig. 29. Sagittal sections of the gills stained with anti-Na^+/K^+-ATPase in cultured Japanese eels acclimated to freshwater (**A**) and seawater (**B**). Immunoreactive MR cells are classified into filament (arrowheads) and lamellar (arrows) MR cells, based on their location and shape. Scale: 50 μm. Reprinted with permission from *Can. J. Zool.*, **76**, Sasai *et al.*, Morphological alteration in two types of gill chloride cells in Japanese eels (*Anguilla japonica*) during catadromous migration, 1480–1487, 1998, NRC Research Press.

cell type was round or columnar in shape and was located at the base of lamellae in the gill filament (filament MR cells). The other cell type was flat and observed in the gill lamella (lamellar MR cells). Although the two distinct types of gill MR cells were detectable in both FW and SW eel, there were apparent differences in their number and size between FW and SW fish. Filament MR cells in SW eel were more abundant and larger, and stained more intensely than in FW fish (Fig. 29). This finding indicates the activation of filament MR cells in SW, again suggesting that this cell type is responsible for secretion of excess salt in SW. In contrast with filament MR cells, no apparent difference was observed in lamellar MR cells between FW and SW eel (Fig. 29). As mentioned above, lamellar MR cells are generally considered as FW-type cells responsible for ion uptake in hypotonic environments. Under natural conditions, maturing eel go downstream to the sea for spawning. Since the cultured eel used were too young to mature, those fish might not be fully ready for downstream migration in spite of their potential capability for SW adaptation. Activation of filament MR cells could be interpreted as an adaptive response to unexpected exposure to SW, whereas the presence of lamellar MR cells in the SW-transferred, cultured eel may imply retention of FW adaptability.

7-5. MR cells in yellow and silver eel

Based on the results obtained in the cultured eel, Sasai *et al*. (1998a) further investigated the morphological modification of gill MR cells in eel caught in wild stocks with special reference to the difference between yellow and silver eel. For this study, four different groups of Japanese eel captured in natural habitats were used. Color of the integument is the most commonly used criterion of maturity; immature eel are often referred to as yellow eel and maturing eel as silver eel from their body color. Yellow eel in FW weighing 100–192 g (small yellow eel) were caught in the Tone River, Chiba Prefecture. Another group of yellow eel (large yellow eel), weighing 443–536 g, were captured in Lake Inba, Chiba Prefecture. Silver eel in FW (FW silver eel; mean body mass, 576 g) were caught by net-fishing in the Tone River. Silver eel were also caught in brackish water (BW) at the mouth of the Chikugo River, Saga Prefecture (BW silver eel; the mean body weight, 300 g). Coloration of small yellow eel caught in the Tone River was typically yellow, whereas that of large yellow fish in Lake Inba was gray rather than yellow. The belly of silver eel in both FW and BW was whitey silver, and apparently differed from that of yellow eel. These eel possessed small but recognizable gonads, except for small yellow eel. GSI values in silver eel were significantly higher than in yellow eel, being more than 1%.

In small and large yellow eel, MR cells were detected in both filament and lamellar epithelia of the gills. There was no significant difference in the filament MR cell number between small and large yellow eel, whereas lamellar MR cells were fewer in large yellow eel than in small yellow eel. Lamellar MR cells in large yellow eel were slightly smaller than those in small yellow eel, whereas there was no difference in filament MR cell size between them. Filament MR cells were the dominant cell type in FW and BW silver eel. Lamellar MR cells observed frequently in yellow eel were decreased markedly in both silver eel groups. Filament MR cells in silver eel were significantly larger than those in yellow eel. Moreover, the cell size was larger in BW silver eel than in FW eel.

The development of filament MR cells in silver eel is essentially in accordance with the results obtained in cultured eel, suggesting the importance of filament MR cells in SW adaptation. In contrast, lamellar MR cells were more abundantly observed in yellow eel than in silver eel, although the cell size was no different. This implies a significant role of lamellar MR cells in FW adaptation. However, this finding disagrees with that obtained from cultured eel, in which there was no apparent difference in lamellar MR cells between FW- and SW-acclimated eel. The discrepancy may be related to differences in age and gonadal development between natural and cultured eel used in this study. As the eel grow in FW environments and start gonadal development under natural conditions, they may be developing hypoosmoregulatory ability as preadaptation for downstream migration to the sea, and reducing hyperosmoregulatory ability to some extent at the same time. On the other hand, transfer of the cultured eel to SW may not affect hyperosmoregulatory ability.

Toward the downstream migration of the eel to the sea, the ratio of filament MR cells was increasing, which resulted from increase in filament MR cells and concomitant decrease in lamellar MR cells (Fig. 30). This could be interpreted as a preadaptive response to forthcoming SW entry. Furthermore, our preliminary study showed that the gill epithelium of a silver eel caught in the East China Sea lacked lamellar MR cells, although only one specimen was available (Fig. 30).

8. Fugu

Fugu *Takifugu rubripes* is a SW-dwelling pufferfish. This marine teleost is one of a few vertebrate model organisms that have been used for genomic studies because of their small genome size (Venkatesh 2000; Aparicio 2002). The compact genome of fugu has been almost completely sequenced. Therefore, this marine teleost could serve as an excellent experimental model to investigate physiological and molecular mechanisms of osmoregulation in marine teleosts.

Whereas the mechanisms of ionic and osmotic regulation have been well investigated in euryhaline teleosts, which are adaptable to both FW and SW environments, less attention has

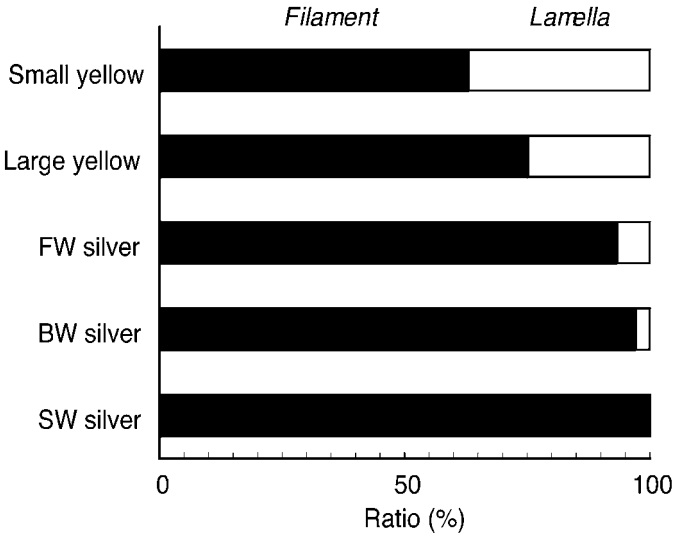

Fig. 30. Ratios of gill filament and lamella MR cells in five different stages during the downstream migration of Japanese eel.

been paid to those in stenohaline species. The mechanisms of FW and SW adaptation in stenohaline teleosts are considered to be essentially the same as in euryhaline species. Although marine teleosts in general appear to have a limited ability for FW adaptation, some marine species have been shown to possess some low-salinity tolerance (Wu and Woo 1983; Mancera *et al*. 1993; Woo and Chung 1995; Kelly *et al*. 1999; Kelly and Woo 1999). This suggests that those marine teleosts might exert hyperosmoregulatory ability, to some extent, to maintain blood osmolality within certain physiological ranges.

8-1. Low-salinity tolerance of fugu

To examine the adaptability of fugu to low-salinity environments, juvenile fish weighing 3.8–5.7 g were transferred from full-strength (100%) SW to FW and 25, 50, 75 and 100% SW, and maintained for three days (Lee *et al*. 2005). Fugu could not survive in pure FW, but they were adaptable to 25% SW or higher salinities, judging from their survival rates and blood osmolality. Within the salinity range from 25 to 100% SW, blood osmolality became slightly lower with decreasing environmental salinity. However, osmolality levels seemed to be maintained within physiological levels. Time-course changes indicated that blood osmolality decreased transiently at 12 h, which were restored thereafter, in fish transferred to 25 and 50% SW. This observation is similar to findings in another marine teleost, gilthead sea bream *Sparus aurata* exposed to brackish water (7‰ salinity) (Mancera *et al*. 1993). Such profiles indicate that fugu as well as gilthead sea bream possess the ability to deal with decreased blood osmolality. In contrast, transfer to FW caused a rapid decrease in blood osmolality below 300 mOsm, which was not restored; reduced osmolality less than 300 mOsm may indicate forthcoming death.

Based on those results, we explored the lower limit of salinity that fugu could tolerate. To examine the adaptability of fugu to 25% SW or lower salinity environments, fish were transferred from 100% SW to FW and 1, 5, 10, 15 and 25% SW, and maintained for three days. Whereas all fish survived in 5–25% SW groups, some fish died in FW and 1% SW. Although 30% of fish survived in FW and 60% of fish survived in 1% SW for three days after direct transfer from 100% SW, physical activity was extremely low in the surviving fish and their blood osmolality was markedly decreased, compared with those in higher salinities (Fig. 31). Therefore, the surviving fish in FW and 1% SW seem to be approaching death, even though they were still alive three days after transfer. Despite no death in 5% SW, fish in this group were less active and blood osmolality was decreased to approximately 300 mOsm. Therefore, the lower limit of salinity tolerance of fugu is estimated to lie between 5 and 10% SW. In stenohaline teleosts, there seems to be a narrow salinity range that divides successful osmoregulation from

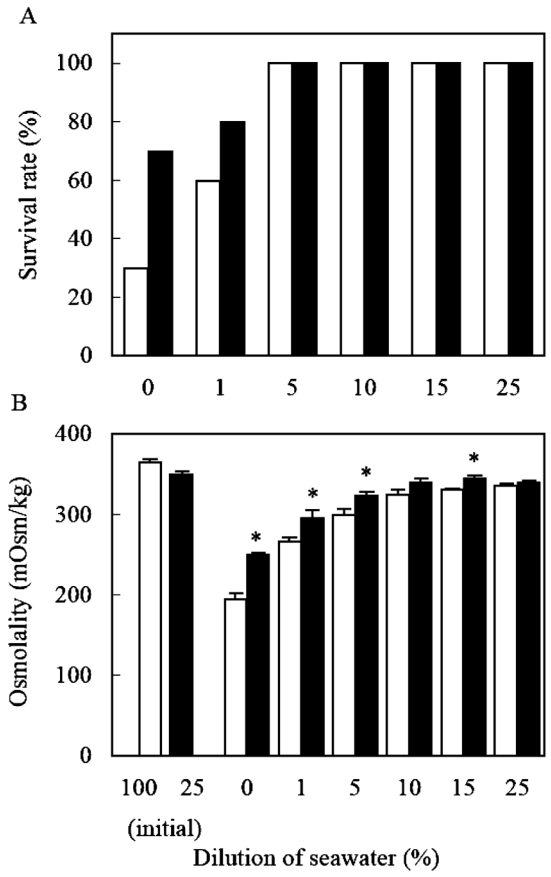

Fig. 31. Survival rates (**A**) and blood osmolality (**B**) in fugu transferred to freshwater (FW) and 1, 5, 10, 15 and 25% seawater (SW), directly from SW (open columns) and after preacclimation in 25% SW for seven days (filled columns). Asterisks indicate significant differences ($p < 0.05$) in blood osmolality, compared with the values in fish transferred directly from 100% SW. Reprinted with permission from Lee *et al*., Low-salinity tolerance of a marine pufferfish, fugu *Takifugu rubripes*, *Fish. Sci*., 2005, **71**, 1324–1331, Wiley-Blackwell.

osmoregulatory failure; mortality appears to occur once the environmental salinity reaches a certain critical level.

To further examine whether or not preacclimation to 25% SW improved their adaptability to lower salinities, fish were first transferred from 100 to 25% SW, and acclimated for seven days with feeding. Then, the fish were randomly divided into six groups, and transferred to FW and 1, 5, 10, 15 and 25% SW. Although survival rates and blood osmolality were slightly improved by preacclimation in 25% SW, improvement of the low-salinity tolerance was very limited; blood osmolality decreased markedly in salinities of less than 10% SW, as was seen in the direct transfer (Fig. 31). Therefore, maladaptation to low salinities might not be attributed to stress caused by direct transfer, but to lack of potential low salinity adaptability.

8-2. Gill MR cells in fugu

In order to examine the involvement of gill MR cells in low-salinity tolerance of fugu, MR-cell morphology and gill Na^+/K^+-ATPase activity were examined in those juvenile fugu transferred to low-salinity environments. No distinct difference in MR-cell distribution and morphology was observed in fish three days after transfer to low salinities. Moreover, neither the MR-cell size nor Na^+/K^+-ATPase activity showed significant differences among experimental groups (Lee *et al*. 2005). To confirm this notion, time-course changes in MR-cell morphology were examined at 1, 3 and 7 days in fugu (75–170 g) transferred from 100 to 25% SW (Lee *et al*. 2006b). In accordance with the previous result, there was no significant change in average size of Na^+/K^+-ATPase-immunoreactive MR cells in fish transferred to 25% SW. However, SEM observations revealed a distinct difference in their apical structures (Fig. 32A). The apical openings of MR cells formed an apical pit in fish acclimated to SW, appearing as a hole among pavement cells. Following transfer to 25% SW, the size of the opening became smaller and the

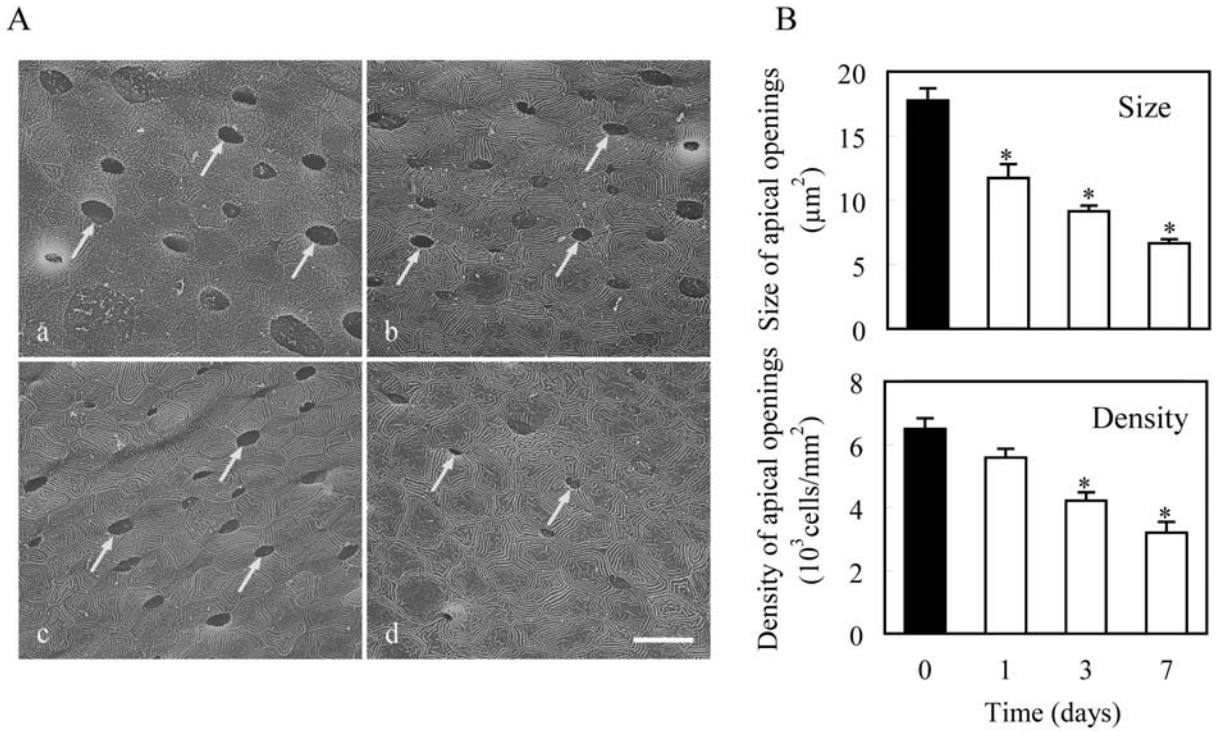

Fig. 32. A. Scanning electron micrographs of gill filaments in fugu before (**a**), and 1 day (**b**), 3 days (**c**) and 7 days (**d**) after transfer from 100% to 25% seawater (SW). Arrows indicate the apical openings of MR cells. Bar: 10 μm. **B.** Changes in size and density of the apical openings of gill MR cells in fugu after transfer from 100 to 25% SW. Asterisks indicate significant differences ($p < 0.05$), compared with the initial levels. Values are means ± S.E.M. ($n = 4$). Reprinted from *Gen. Comp. Endocrinol.*, **149**, Lee *et al.*, Prolactin gene expression and gill chloride cell activity in fugu *Takifugu rubripes* exposed to a hypoosmotic environment, 285–293, 2006, with permission from Elsevier.

mean area of the opening was reduced from 18 μm² on day 0 down to 7 μm² on day 7 (Fig. 32B). At the same time, the density of the apical openings of MR cells decreased steadily to about 50% on day 7 (Fig. 32C). These findings suggest that fugu may lack the ability to switch MR cell functions from ion secretion to ion absorption, but that MR cells developed in SW may reduce their ion-secreting activity by closing their apical openings as an adaptive response to exposure to hypoosmotic media. Similarly, mudskipper *Periophthalmus modestus* tolerates hypotonicity by closing the apical pit of existing SW-type MR cells on exposure to FW (Sakamoto *et al.* 2000).

Although fugu is less likely to develop FW-type MR cells in response to exposure to hypoosmotic media, they are supposed to exert hyperosmoregulation in 10–25% SW. Fugu were fully adaptable to 25% SW, the osmolality of which was approximately 260 mOsm. Because blood osmolality of fugu adapted to 25% SW was approximately 350 mOsm, 25% SW is evidently hypoosmotic to the blood. It has been shown that fugu can be adapted down to 10% SW, maintaining their blood osmolality above the critical level of 300 mOsm. These findings indicate that fugu exerts, to some extent, hyperosmoregulatory ability which allows them to survive in low-salinity environments. Among the osmoregulatory organs other than the gills, the kidney is not able to absorb ions from external environments, but merely retains them by reabsorption in the convoluted tubules. Most probably, the intestine is responsible for ion absorption to compensate for osmotic ion loss in fugu exposed to hypoosmotic environments. Marine teleosts ingest a large amount of water and water absorption is considered to take place in the intestine following active ion absorption (Loretz 1995; Aoki *et al.* 2003; Veillette and Young 2005). Therefore, the intestine of marine teleosts is essentially equipped with a mechanism of ion uptake, and fugu can use this mechanism to absorb ions from ingested water and diet. Limited ability of the

intestine for ion uptake might explain their maladaptation to and death in 5% SW or lower salinities.

8-3. Functional significance of prolactin in a marine teleost of fugu

Prolactin (PRL) is a polypeptide hormone, which is synthesized in the adenohypophysis and secreted into blood circulation throughout vertebrate species except for cyclostomes. A wide variety of physiological actions have been shown in different classes of vertebrates for this hormone, including milk production, water and electrolyte balances, development and growth, metabolism, reproduction, and immunoregulation (Bole-Feysot *et al*. 1998). These diverse functions of PRL seem to be closely associated with the water-to-land transition during the evolution of vertebrates. Pickford and Phillips (1959) originally showed that ovine PRL was effective in teleosts in promoting survival in FW of hypophysectomized killifish. Since then, many investigations have shown that PRL plays an important role in adaptation to FW in diadromous and euryhaline teleosts (Hirano 1986). During FW adaptation, PRL is known to reduce Na^+ efflux and water permeability (Dharmamba and Maetz 1972, 1976; Wendelaar Bonga and Van der Meij 1981).

Assuming that the major function of PRL in teleosts lies in FW adaptation, it should be of great interest to investigate the function of PRL in marine species in order to expand our knowledge of functional diversity of PRL. Lee *et al*. (2006a) have cloned a cDNA encoding PRL from the fugu pituitary and examined expression levels of PRL mRNA in the pituitary. The fugu PRL cDNA consisted of 966 bp, containing 54 bp of a 5′-untranslated region (UTR), 639 bp of an open reading frame (ORF) and 270 bp of a 3′-UTR except for a poly(A) tail. The ORF encoded a protein of 213 amino acid residues, composed of a putative signal peptide of 24 amino acid residues and a mature protein of 189 amino acid residues. Using specific primers for fugu PRL, a time-course change in PRL mRNA levels was investigated in fugu transferred to from 100 to 25% SW. The levels of PRL mRNA expression in the pituitary were significantly increased in response to 25% SW transfer; the expression levels were increased about five times by day 3, and the increased levels were maintained on day 7 (Fig. 33). In contrast with the profile of PRL expression, gene expression levels of growth hormone (GH) measured simultaneously showed a reverse response. GH mRNA levels were decreased to about 50% on days 3 and 7 following transfer to 25% SW (Fig. 33). These results suggest that PRL functions in fugu as a hyperosmoregulatory hormone and GH as a hypoosmoregulatory one, which is on the same line of FW- and SW-adapting functions of PRL and GH, respectively, in euryhaline teleosts (Yada *et al*. 1994; Sakamoto *et al*. 1997; Shepherd *et al*. 1997).

Considering that PRL initiates its actions through binding to a specific cell surface PRL receptor, Lee *et al*. (2006a) have also cloned a cDNA encoding fugu PRL receptor, and examined

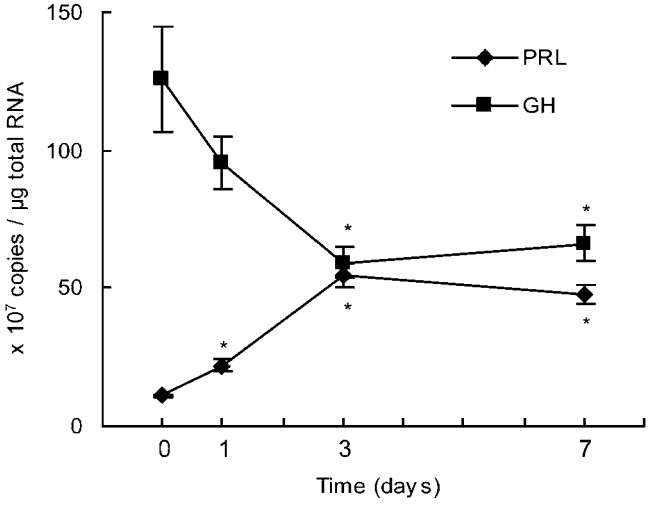

Fig. 33. Time-course changes in prolactin (PRL) and growth hormone (GH) mRNA levels in the pituitary of fugu transferred from 100 to 25% seawater. Asterisks indicate significant differences ($p < 0.05$), compared with the initial levels. Values are means ± S.E.M. ($n = 10$). Reprinted from *Gen. Comp. Endocrinol*., **149**, Lee *et al*., Prolactin gene expression and gill chloride cell activity in fugu *Takifugu rubripes* exposed to a hypoosmotic environment, 285–293, 2006, with permission from Elsevier.

tissue and cellular distribution of PRL receptor mRNA. Such attempts enable the identification of target tissues and cells of PRL and provide important information on PRL functions in a stenohaline SW teleost of fugu. The fugu PRL receptor cDNA consisted of 2198 bp, containing an ORF encoding 510 amino acid residues, similar to the long form of the mammalian PRL receptor. The 5′-UTR comprised 184 nucleotides, and the 3′-UTR consisted of 481 nucleotides except for a poly(A) tail. The deduced amino acid sequence of the fugu PRL receptor was composed of a signal peptide, an extracellular domain, a single transmembrane domain, and an intracellular domain. The fugu PRL receptor conserved some features characteristic of vertebrate PRL receptors. The extracellular domain contained two pairs of cysteines and a WSXWS motif. Highly conserved box 1 and box 2 regions were found within the intracellular domain of PRL receptor.

The RT-PCR analysis demonstrated that PRL receptor was intensively expressed in the gills, kidney, intestine and ovary. It should be noted that those PRL receptor-expressing organs, except for the ovary, are closely associated with osmoregulation, suggesting the involvement of PRL and PRL receptor in regulation of hydromineral balance in those organs. In accordance with those results, *in situ* hybridization for the PRL receptor mRNA showed that hybridization signals were detected in osmoregulatory epithelia in those organs; MR cells in the gills, proximal convoluted tubule cells in the kidney and mucosal epithelial cells in the intestine (Fig. 34). It seems reasonable that PRL receptor transcripts were detected in MR cells, since PRL has been shown to have marked effects on the morphology, distribution and number of MR cells in euryhaline species (for a review, see McCormick 1995). Similarly, PRL receptors have been localized to gill MR cells in tilapia (Weng *et al*. 1997) and sea bream (Santos *et al*. 2001). These findings also provide evidence that gill MR cells are the target cells for PRL, expressing its specific receptor.

Earlier studies have suggested that PRL acts on the kidney to increase Na^+ reabsorption and water excretion (Clarke and Bern 1980). However, the mechanisms by which PRL exerts these effects are still poorly understood. Considering that PRL exerts its actions towards increasing blood osmolality, it is highly possible in fugu that PRL decreases secretion of divalent ions from the proximal tubules into primitive urine, which may be mediated by the PRL receptor. Another important function of PRL in the kidney is to enhance water secretion by increasing the glomerular filtration rate; it has been observed that the glomerular size as well as urine output increases following PRL treatment (Braun and Dantzler 1987). It is less likely, however, that PRL directly regulates the glomerulus filtration rate in fugu because the PRL receptor was not expressed in the glomerulus.

Occurrence of PRL receptor transcripts in the mucosal epithelia of the intestine also suggests osmoregulatory actions of PRL in fugu. In SW-adapted fish, as described above, water absorption in the intestine takes place according to an osmotic gradient created by active absorption of monovalent ions. The ion absorption in the intestine is considered to be driven by Na^+/K^+-ATPase, which is seemingly regulated by PRL. Collie and Hirano (1987) reported that PRL decreased intestinal NaCl absorption as well as the subsequent water absorption by reducing Na^+/K^+-ATPase activity. This is also the case with brown trout (Seidelin and Madsen 1999).

Although PRL is apparently a multifunctional hormone, one of its major functions in fugu seems to lie in the regulation of ion and water balances. Fugu spend their whole life in a marine environment without experiencing a severe hypoosmotic challenge. Nevertheless, fugu has been shown to possess low-salinity tolerance to some extent, although the fish cannot survive in FW. The higher PRL-gene expression in the pituitary in 25% SW than in 100% SW implies a possible hyperosmoregulatory action of PRL in this marine teleost. To maintain blood osmolality within narrow physiological ranges, teleosts are supposed to be equipped with mechanisms of both hyper- and hypoosmotic regulation. While hypoosmoregulation seems to predominate in marine teleosts, antagonistic hyperosmoregulation should also be important to correct overshooting decrease in blood osmolality. Most probably, PRL in marine stenohaline teleosts may act on osmoregulatory organs through the PRL receptor and reduce their functions favorable to SW adaptation for the precise adjustment of osmolality, but not develop FW adaptability. Thus, it would be more appropriate to refer to PRL as a hyperosmotic or anti-hypoosmotic hormone rather than a FW-adapting hormone.

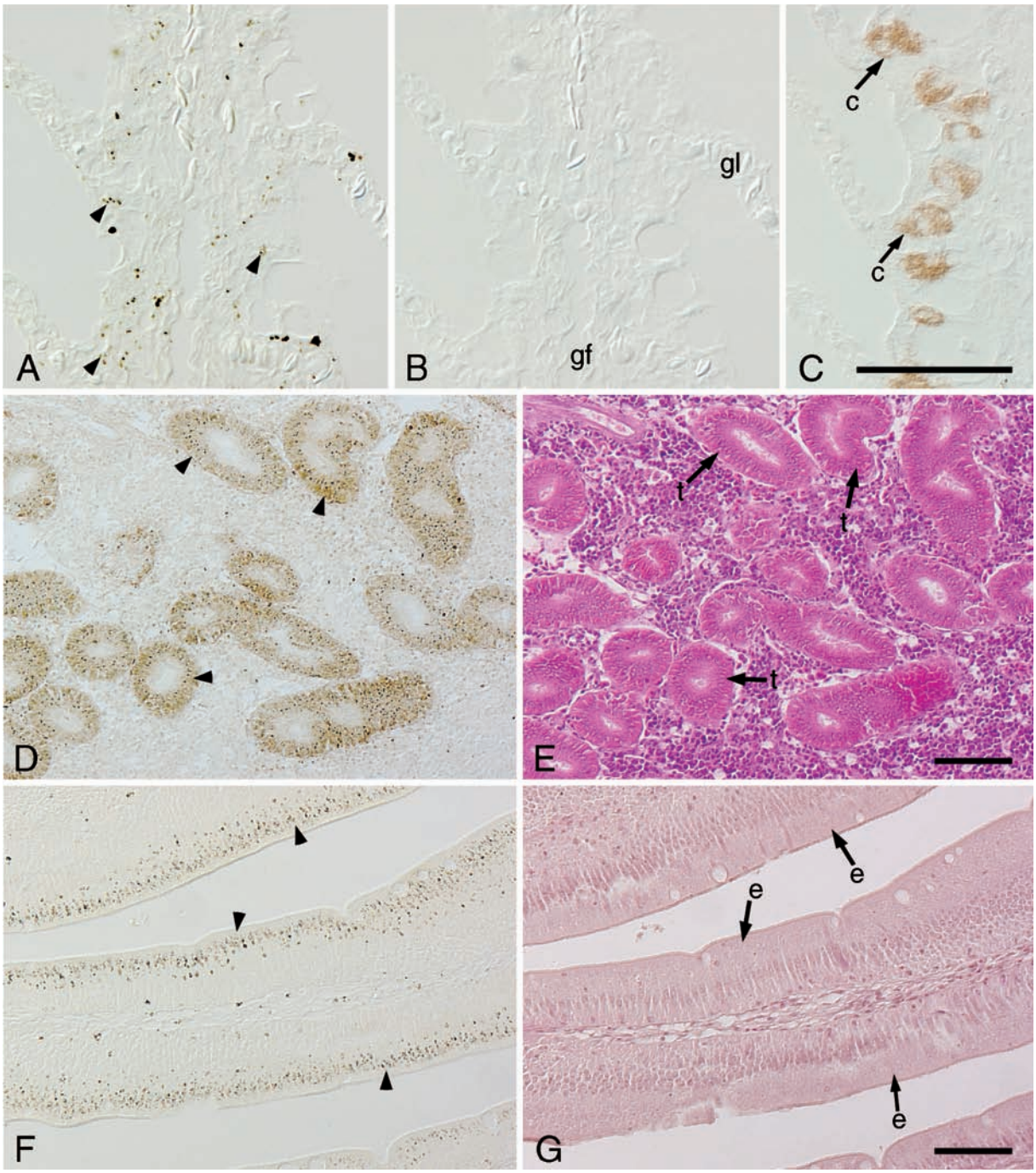

Fig. 34. Adjacent sections of the gill (**A**, **B**), kidney (**D**, **E**), and intestine (**F, G**) of fugu. Sections (**A, D, F**) were subjected to *in situ* hybridization with a digoxigenin-labeled PRL receptor oligonucleotide probe, and their counterparts were stained immunocytochemically with anti-Na^+/K^+-ATPase (**C**) or with hemotoxylin and eosin (**E, G**). An adjacent section (**B**) of the gill (**A**) was subjected to hybridization with the unlabeled probe as a negative control. Hybridization signals (arrowheads) for PRL receptor mRNA are detected in Na^+/K^+-ATPase-immunoreactive MR cells (c) in the gill (**A, C**), the proximal convoluted tubules (t) in the kidney (**D, E**), and mucosal epithelial cells (e) in the intestine (**F, G**). gf, gill filament; gl, gill lamella. Bars: 50 μm. Reprinted from *Gen. Comp. Endocrinol.*, **146**, Lee *et al.*, Prolactin and prolactin receptor expressions in a marine teleost, pufferfish *Takifugu rubripes*, 318–328, 2006, with permission from Elsevier.

8-4. Comparison of growth in fugu reared in 25 and 100% SW

In order to investigate long-term effects of a low-salinity environment on growth, juvenile fugu (16–30 g) were reared in a 2 m^3 tank either with recirculating 25% or with 100% SW (n = 30 each) for an extended period of eight weeks. During this period, the fish were fed on commercial pellets of suitable sizes at 3% body weight per day. Body weight and standard length were measured weekly. Under the conditions that fish were given a defined amount of food, they grew similarly in both media; there was no difference in body weight and standard length at any weekly sampling point (Fig. 35). In fish sampled at the end of the experiment, MR-cell morphology and gill Na^+/K^+-ATPase activity were compared between 25 and 100% SW. Although no significant difference was seen in the average size of Na^+/K^+-ATPase-immunoreactive MR cells in the gills between fish reared in 100% SW and those in 25% SW, gill Na^+/K^+-ATPase activity was significantly lower in 25% SW than in 100% SW. According to SEM observations, the size of the apical openings of MR cells was smaller and the density was lower in 25% SW than in 100% SW, in agreement with results in the short-term experiment described above.

One may expect that fugu in 100% SW may grow faster than those in 25% SW, because 100% SW should be a more natural and thus preferable condition than 25% SW that they may not experience during their lifetime. On the other hand, one may also consider that fish require less energy for osmoregulation in 25% SW than in 100% SW, since the osmotic gradient between the blood and external environment is less in fish reared in 25% SW than those in 100% SW. The lower gill Na^+/K^+-ATPase activity in 25% SW than in 100% SW supports the notion that 25% SW is a less energy-consuming condition in terms of gill ion-transporting activities.

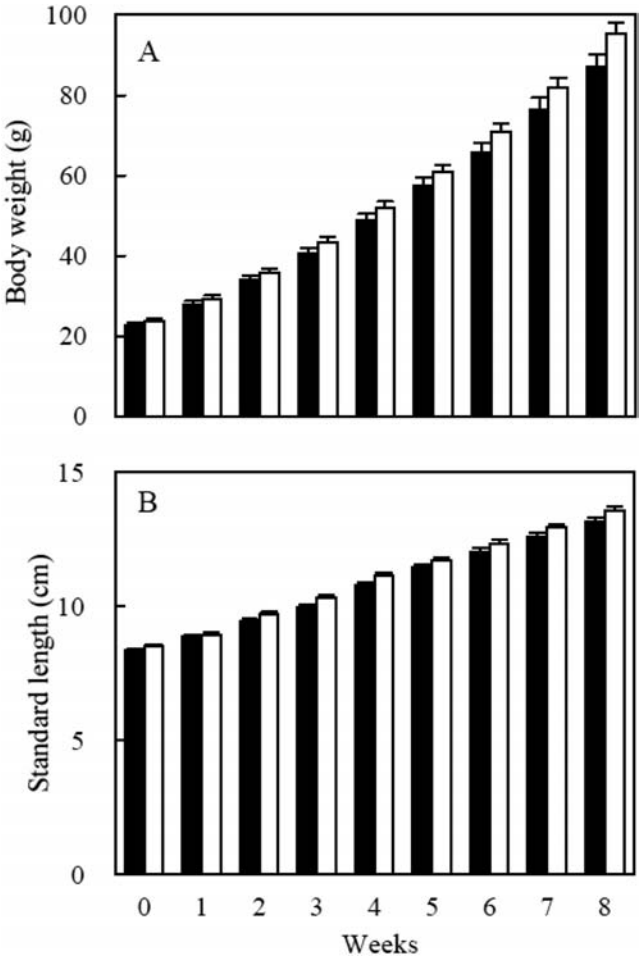

Fig. 35. Time-course changes in body weight (**A**) and standard length (**B**) of fugu reared in 100% (filled columns) and 25% seawater (open columns) for eight weeks. There was no significant difference in body weight and standard length between the two groups at any point examined. Values are means ± S.E.M. (n = 30). Reprinted from *Gen. Comp. Endocrinol.*, **149**, Lee *et al.*, Prolactin gene expression and gill chloride cell activity in fugu *Takifugu rubripes* exposed to a hypoosmotic environment, 285–293, 2006, with permission from Elsevier.

9. Japanese dace

In addition to osmoregulatory roles, MR cells are involved in acid-base regulation. Among teleosts that are generally intolerant of acid environments, Japanese dace *Tribolodon hakonensis* is the only teleost species inhabiting an acid lake, Lake Osorezan (Mashiko 1940). Lake Osorezan is located in Shimokita Peninsula, lying in the northernmost district of Honshu, the mainland of Japan. Strong acidity of the lake water (pH 3.4–3.8) is derived from hot springs containing sulfuric acid (Mashiko 1940; Satake *et al*. 1995). Thus, the dace in Lake Osorezan is expected to possess excellent acid tolerant mechanisms. Exposure of teleost fish to acid environments has been reported to alter gill MR-cell number, distribution and morphology (Jagoe and Haines 1990; Leino and McCormick 1984; Leino *et al*. 1987; Wendelaar Bonga *et al*. 1990), implying the involvement of MR cells in adaptation to acidic environments. Mashiko *et al*. (1973) have described well-developed complexes of MR cells in the gills of the dace in Lake Osorezan; MR cells are arranged in a radial fashion, forming a gland-like structure. Occurrence of these MR-cell complexes may be related to the strong acid tolerance of the dace. Considering the strong acid-tolerant ability, the dace would be a suitable model to search for mechanisms of adaptation to acid environments in teleosts.

9-1. Acid tolerance of Osorezan dace

To assess the acid tolerance and involvement of branchial MR cells in acid-base regulation, we examined physiological and morphological changes in response to environmental acidification in Osorezan dace (Kaneko *et al*. 1999). During the spawning period of the dace in Lake Osorezan, extending from mid-May to early August, mature fish migrate from the acid lake up to neutral streams. The fish were caught by means of netting in one of the neutral streams in early August 1994. The fish caught at the end of the spawning season were considered to be acclimated well to neutral-stream water, presumably having spent several weeks in the stream. They were stocked in fish cages immersed in the stream water. The dace were stocked in the stream for two days and directly transferred to acid lake water. The dace were sampled at 0 (before transfer), 1, 3, 6, 12, 24, 48 and 72 h after transfer. After measurement of the body weight, blood was drawn into a syringe from the caudal vessels and the gills were fixed for histological observations. Blood samples were subjected to pH determination and measurement of ion concentrations.

The dace survived direct transfer from neutral stream water to acid lake water; no fish died during the 72 h after transfer. Plasma Na^+ levels were significantly decreased at 1 h after transfer and then increased at 6 h. The increased levels, still significantly lower than those before transfer, were maintained until 72 h (Fig. 36A). Plasma Cl^- also showed a transient decrease at 1 h. However, the decrease in Cl^- levels was less evident than that in Na^+. The decreased Cl^- was restored to the initial levels by 3 h, followed by a significant increase at 72 h (Fig. 36B). Following acid exposure, blood pH was decreased significantly by 0.13 unit at 6 h after transfer, and partially restored by 24 h (Fig. 36C). Exposure to acid environments has been reported to cause ion losses in fish, and plasma ion levels have proved to be a fairly reliable indicator of sublethal acid stress (Goss *et al*. 1995; Leivestad and Muniz 1976). The observed decreases in plasma ion levels may be caused by increased membrane permeability in acid environments (Goss *et al*. 1995; McDonald *et al*. 1991; Wood 1989). Although plasma ion levels and blood pH were greatly disturbed just after exposure to the acid water, those physiological parameters tended to be restored thereafter, indicating that excellent acid tolerance of Osorezan dace. Such excellent abilities of Osorezan dace to prevent blood acidosis and ion losses are likely to be unique to Osorezan dace, because one-third of control dace, a different strain from Ueda, Nagano Prefecture, died within 24 h after transfer to acid lake water with a sharp decrease in plasma Na^+ levels (Hirata *et al*. 2003).

At 72 h after transfer to acid water, well-developed MR cells were arranged in a radial fashion, forming a follicular or gland-like structure (Fig. 37A). The follicular arrangement of MR cells was observed more abundantly on the afferent vascular side than on the efferent. These structures are mostly located in the interlamellar regions and in the afferent edge of the gill

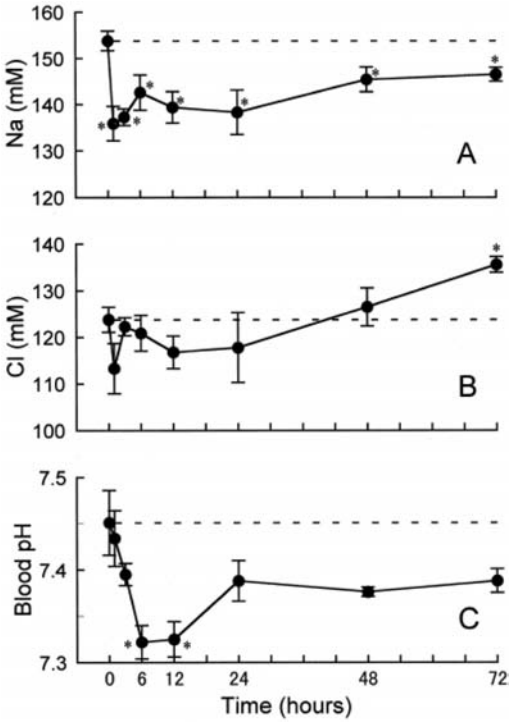

Fig. 36. Changes in plasma concentrations of Na^+ (**A**) and Cl^- (**B**) and blood pH (**C**) following transfer of Osorezan dace from neutral stream water (pH 6.8–7.2) to acid lake water (pH 3.6–3.7). Data are expressed as the mean ± S.E.M. ($n = 7–11$). Asterisks indicate significant difference ($p < 0.05$) from the initial values. Reprinted with permission from *Zool. Sci.*, **16**, Kaneko *et al.*, Acid tolerance of Japanese dace (a Cyprinid teleost) in Lake Osorezan, a remarkable acid lake, 871–877, 1999, The Zoological Society of Japan.

filament. A large follicular structure often consists of more than 10 MR cells on a histological section with an expanded lumen in the center. MR cells are linked to each other by tight junctions (Fig. 37B), and no cytoplasmic interdigitation is formed between MR cells. The tubular system and mitochondria in each cell are moderately developed in the cytoplasm and a round nucleus is usually located in the basal side. The cells are equipped with an apical pit, which faces a common lumen leading to the external environment. In the fish adapted to neutral stream water, the follicular arrangement of MR cells was not developed; MR cells are sparsely located in the gill filament (Fig. 37C). Moreover, such a follicular arrangement of MR cells is considered to be characteristic of Osorezan dace, since it was not observed in another strain of dace exposed to the acid environment. The formation of follicular structures of MR cells results in a great increase in the total apical surface area of MR cells without reducing the gill respiratory surface. A great increase in MR-cell population without forming follicles would considerably reduce the respiratory surface of pavement cells (Greco *et al*. 1996). Thus, the follicular formation may increase the ion-transporting activity of MR cells without affecting gas exchange efficiency. Well-developed MR cells with increased apical surface area in Osorezan dace suggest their active roles in H^+ secretion and/or HCO_3^- retention to correct blood acidosis.

9-2. Molecular mechanisms of acid adaptation

To clarify the molecular mechanism underlying the acid tolerance, Hirata *et al*. (2003) attempted to identify those proteins whose expressions were markedly increased in acid-adapted Osorezan dace, and performed subtraction cloning. Among several clones isolated from a cDNA library enriched in acid-inducible messages, there was a clone encoding Na^+/K^+-ATPase that generates the driving force for ion transport, and surprisingly its message level was markedly increased in the gill, but not in the kidney, of acid-adapted Osorezan dace. These observations, together with the life history and acid tolerance of the animal, suggested that MR cells in the gill of Osorezan dace play an important role in maintaining the acid-base balance in an acidic environment by excreting H^+ and transporting HCO_3^- into the blood for neutralization of plasma and that these functions are supported by the driving force generated by the Na^+/K^+-ATPase. Hirata *et al*. (2003) further hypothesized that carbonic anhydrase (CA), which catalyzes the reversible

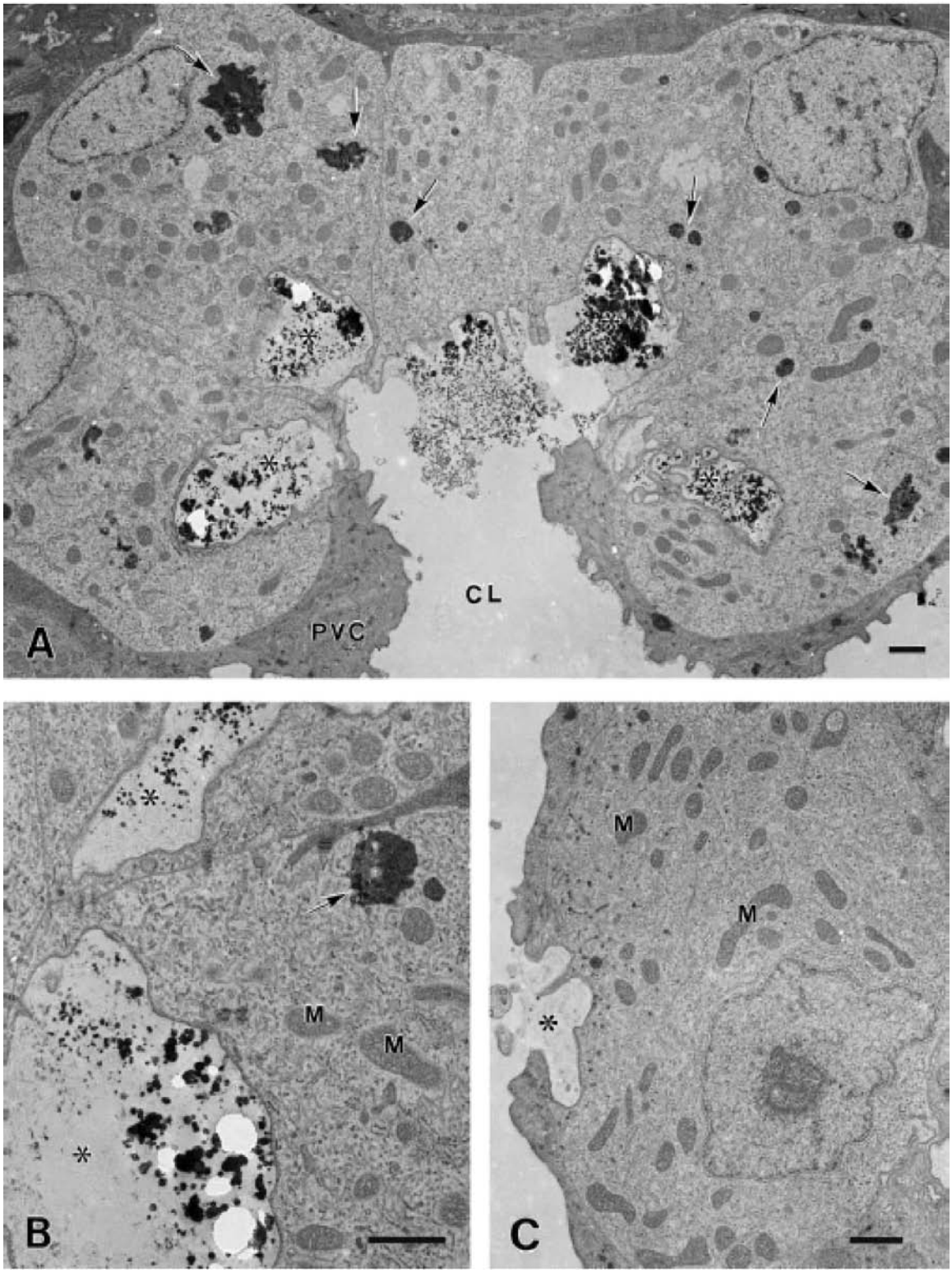

Fig. 37. Transmission electron micrographs of follicular arrangement of MR cells in Osorezan dace transferred to acid lake water (**A, B**), and an individual MR cell in those acclimated to neutral stream water (**C**). Follicular MR cells are equipped with an apical pit (asterisk), which faces a common lumen (CL) leading to the external environment. Arrows indicate electron-dense bodies in the cytoplasm of follicular MR cells. M, mitochondria; PVC, pavement cell. Bars: 1 μm. Reprinted with permission from *Zool. Sci.*, **16**, Kaneko *et al.*, Acid tolerance of Japanese dace (a Cyprinid teleost) in Lake Osorezan, a remarkable acid lake, 871–877, 1999, The Zoological Society of Japan.

hydration of CO_2 and contributes to control of pH and ion transport in the stomach, pancreas and kidney in mammals (Sciortino and Romero 1999), is also required for these processes in Osorezan dace. Hirata *et al*. (2003) therefore cloned the cDNAs for CA and candidate H^+ and HCO_3^- transporters from this fish species and determined the localization and regulation of expression of the corresponding genes. It has been shown that CA-II, Na^+/HCO_3^- cotransporter (NBC1), and NHE3 are indeed highly expressed, with the expected polarities, in MR cells of the acid-adapted Osorezan dace, compared with the low levels of expression apparent in fish acclimated to neutral water. An additional attempt to identify genes that are specifically expressed in the dace gill under acidic conditions by screening a subtracted cDNA library suggested that the gene for a water channel AQP3 might be one such candidate. Hirata *et al*. (2003) determined the localization and regulation of expression of AQP3 and revealed AQP3 immunoreaction to be located in MR cells in a pattern similar to that of Na^+/K^+-ATPase.

Based on these findings, Hirata *et al*. (2003) proposed a molecular mechanism of MR cells, by which Osorezan dace secrete acid (H^+) out of and retain base (HCO_3^-) in the body for adaptation to acidic environments (Fig. 38). In this model, NHE3 in the apical membrane excretes H^+ in exchange for Na^+, whereas the electrogenic NBC1 in the basolateral membrane provides HCO_3^- for neutralization of plasma using the driving force generated by Na^+/K^+-ATPase and CA-II. Meanwhile, AQP3 transports water into the cell across the basolateral membrane, probably to provide a substrate for CA-II. In this model, apically-located NHE3 plays a crucial role not only for excretion of H^+ but also for absorption of Na^+, both of which are favorable to acid adaptation. Figure 39 shows a time-course change in NHE3 protein expression in gill MR cells, detected by immunocytochemistry with a specific antiserum, in Osorezan dace transferred from neutral water to acid water (pH 4.0). In fish acclimated to neutral water, faint NHE3 immunoreaction was observed in the cytoplasm of MR cells located sparsely in the gill filament. Following transfer to acid water, MR cells increased the number and formed follicular structures, which were observed as early as one day after transfer. Concomitant with the structural changes in MR cells, intense NHE3 signals became evident in the apical region of those MR cells. Five days after transfer to acid water, follicular structures of MR-cell were enlarged and apical regions facing the common lumen showed strong NHE3 immunoreaction, whereas the cytoplasm of MR cells showed no or only faint immunoreaction. The enhanced NHE3 protein expression in response to acid exposure is in accordance with upregulation of NHE3 mRNA in acid-adapted dace. Recently, involvement of NHE3 in acid-base regulation has also been reported in tilapia (Watanabe *et al*. 2008) and zebrafish *Danio rerio* (Yan *et al*. 2007).

Through a series of studies on acid-tolerant Osorezan dace, it has become increasingly clear that MR cells play an important role in maintaining acid-base balance. It is highly possible that a similar molecular mechanism could exist in other teleost species for acid adaptation or acid-base regulation, but this ability may be greatly exaggerated in Osorezan dace.

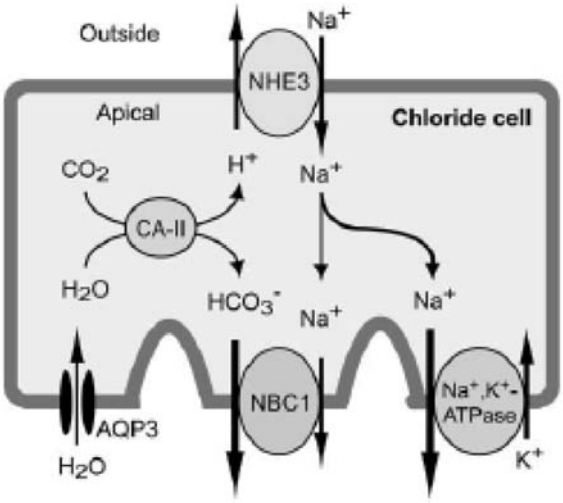

Fig. 38. A model for H^+ secretion and HCO_3^- retention in MR cells of Osorezan dace exposed to acid water. NHE3 in the apical membrane excretes H^+ in exchange for Na^+, whereas the electrogenic NBC1 in the basolateral membrane provides HCO_3^- for neutralization of plasma using the driving force generated by Na^+/K^+-ATPase and CA-II. Figure 11A from Taku Hirata, Toyoji Kaneko, Toshihiro Ono *et al*., Mechanism of acid adaptation of a fish living in a pH 3.5 lake, *Am. J. Physiol. Regul. Integr. Comp. Physiol.,* **284**, R1199–R1212, 2003; used with permission of The American Physiological Society.

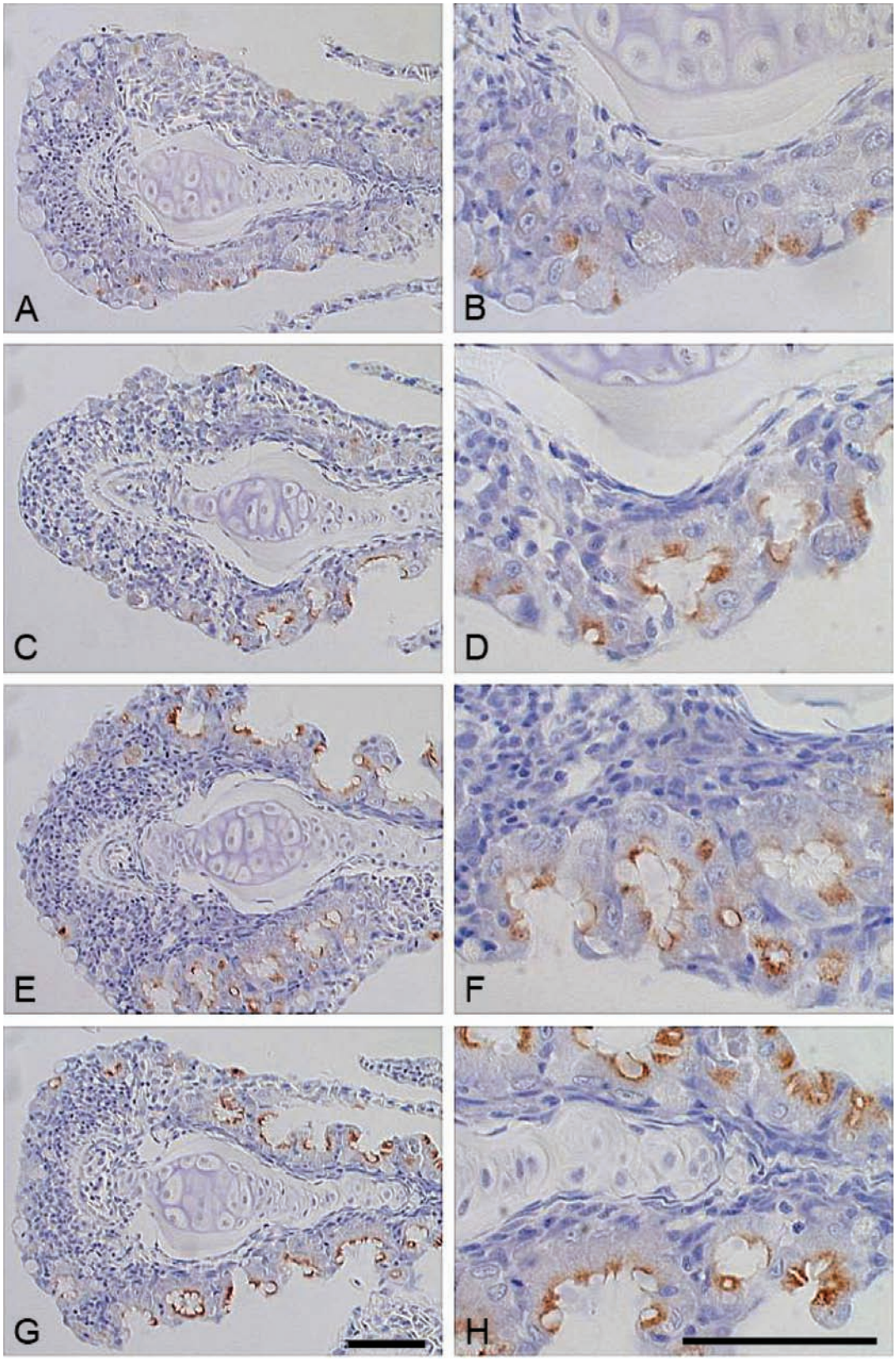

Fig. 39. Immunocytochemical detection of NHE3 in MR cells of Osorezan dace on 0 (**A, B**), 1 (**C, D**), 2 (**E, F**) and 5 (**G, H**) days after transfer from neutral water to acid water (pH 4.0). B, D, F and H are magnified views of A, C, E and G, respectively. Bars: 50 μm.

10. Conclusions and future perspectives

As clearly shown in Mozambique tilapia, killifish, chum salmon and Japanese eel, MR cells already exist as early as during the embryonic stages before the formation of the gills. MR cells are extensively distributed in the yolk-sac membrane and body surface until the gills become fully functional as a respiratory and osmoregulatory organ after hatching. It can be generalized that the principal site for MR-cell distribution shifts from the yolk-sac membrane and body skin during embryonic stages to the gills in larval and later developmental stages, although the timing of the shift differs in different species. Those embryonic MR cells apparently play a central role in osmotic and ionic regulation during early developmental stages in place of gill MR cells.

The capacity of functional alteration and plasticity of MR cells is one of critical differences that determine euryhalinity and stenohalinity in teleosts. It has been demonstrated in euryhaline teleosts that MR cells alter their morphology and ion-transporting functions to meet abrupt environmental osmotic changes. Using a sequential observation technique, Hiroi *et al*. (1999) have revealed that single FW-type MR cells in the yolk-sac membrane of Mozambique tilapia are transformed into SW-type multicellular complexes during SW adaptation. In killifish, Katoh and Kaneko (2003) have demonstrated that SW-type MR cells are transformed into FW-type cells in response to transfer from SW to FW. In euryhaline teleosts such as Mozambique tilapia and killifish, the functional alteration of MR cells is interpreted as an acute adaptive response to a new environment and allows them to deal with unexpected salinity changes. Although diadromous teleosts, such as anadromous chum salmon and catadromous Japanese eel, are also adaptable to a wide range of salinity, they are distinct from typical euryhaline teleosts in the sense that MR-cell functions may alter in advance to meet forthcoming new environments during their diadromous migration. In stenohaline teleosts inhabiting either FW or SW, on the other hand, ion-transporting functions of MR cells do not possess the plasticity to switch the direction of ion transport, but merely adjust the degree of unidirectional ion transport. For example, a marine teleost of fugu can tolerate hypotonic environments to some extent by closing the apical openings of gill MR cells to reduce or stop their ion-secreting activity.

In contrast with the well-defined mechanism of ion-secreting MR cells, molecular mechanisms of ion uptake in MR cells are still controversial in both early developmental and adult stages. In hypoosmotic environments, teleost fish absorb Na^+ and Cl^- through MR cells to compensate for diffusional ion loss, whereas involvement of pavement cells in Na^+ and Cl^- uptake cannot be ruled out (Hiroi *et al*. 1998b). The current proposed models for apical uptake of Na^+ in MR cells include 1) epithelial Na^+ channel (ENaC) electronically coupled to apically-located H^+-ATPase, 2) electroneutral Na^+/H^+ exchange by NHE, and 3) cotransport of Na^+ and Cl^- through NCC (Marshall 2002; Perry *et al*. 2003; Hirose *et al*. 2003; Evans *et al*. 2005; Hwang and Lee 2007; Hiroi *et al*. 2008). In our studies, mRNA expression of H^+-ATPase was not associated with reduced environmental salinity in Mozambique tilapia (Inokuchi *et al*. 2008), whereas H^+-ATPase was shown to be located in the basolateral membrane to energize Na^+ absorption in killifish (Katoh *et al*. 2003) . In tilapia, both NHE3 and NCC are considered to be importantly involved in ion absorption of MR cells. Moreover, NHE3 in the apical membrane excretes H^+ in exchange for Na^+ in Osorezan dace exposed to acid water (Hirata *et al*. 2003), indicating a deep involvement of NHE3 in acid-base regulation. The inconsistency in ion-absorbing mechanisms of MR cells in different teleosts may be attributed, at least in part, to species specificity.

Whereas genetic information is very limited in euryhaline species that have been widely used as experimental models, zebrafish has been recognized as a powerful model fish to elucidate ion uptake mechanisms. Using a non-invasive electrophysiological technique, Hwang and his coworkers have succeeded in demonstrating H^+ secretion in the skin of zebrafish larvae (Lin *et al*. 2006). Strong signals of H^+ secretion are detected at the yolk-sac membrane and pericardial surface, which contained a rich population of MR cells. Surprisingly, H^+ secretion is attributed to a novel H^+-secreting cells, which are H^+-ATPase rich and distinct from the conventional MR cells (Na^+/K^+-ATPase-rich cells). Knockdown of the gene product of H^+-ATPase subunit A resulted in several abnormalities including suppression of acid secretion, indicating the critical role of H^+-ATPase-rich cells in acid secretion and ion balance (Horng *et al*. 2007). Using a Na^+-specific fluorescent reagent (Sodium Green), Esaki *et al*. (2007) provided direct evidence

that Na^+ absorption takes place in H^+-ATPase-rich cells on the body skin of zebrafish larvae. Further studies with zebrafish will surely provide new insights into the current models of ion transport, although models proposed from zebrafish studies could not necessarily apply to teleosts in general.

Acknowledgments

We thank Prof. Tetsuya Hirano and Prof. Katsumi Aida for discussions and their encouragement. We also extend our thanks to the following colleagues for their great contributions to the original research: Prof. Tsuyoshi Ogasawara, Ms. Sanae Hasegawa, Dr. Susumu Hyodo, Dr. Felix G. Ayson, Dr. Katsuhisa Uchida, Dr. Junya Hiroi, Dr. Kiyono Shiraishi, Dr. Seiji Sasai, Dr. Fumi Katoh, and Ms. Mayu Inokuchi.

References

Alderdice DF. Osmotic and ionic regulation in teleost eggs and larvae. *In*: Hoar WS, Randall DJ (eds). *Fish Physiology*, Vol. XIA. Academic Press, San Diego. 1988; pp. 163–251.

Aoki M, Kaneko T, Katoh F, Hasegawa S, Tsutsui N, Aida K. Intestinal water absorption through aquaporin 1 expressed in the apical membrane of mucosal epithelial cells in seawater-adapted Japanese eel. *J. Exp. Biol*. 2003; **206**: 3495–3505.

Aparicio S, Chapman J, Stupka E, Putnam N, Chia J, Dehal P, Christoffels A, Rash S, Hoon S, Smit A, Gelpke MDS, Roach J, Oh T, Ho IY, Wong M, Detter C, Verhoef F, Predki P, Tay A, Lucas S, Richardson P, Smith SF, Clark MS, Edwards YJK, Doggett N, Zharkikh A, Tavtigian SV, Pruss D, Barnstead M, Evans C, Baden H, Powell J, Glusman G, Rowen L, Hood L, Tan YH, Elgar G, Hawkins T, Venkatesh B, Rokhsar D, Brenner S. Whole-genome shotgun assembly and analysis of the genome of *Fugu rubripes*. *Science* 2002; **297**: 1301–1310.

Arai T, Otake T, Tsukamoto K. Drastic changes in otolith microstructure and microchemistry accompanying the onset of metamorphosis in the Japanese eel *Anguilla japonica*. *Mar. Ecol. Prog. Ser.* 1997; **161**: 17–22.

Avella M, Bornancin M. A new analysis of ammonia and sodium transport through the gills of the freshwater rainbow trout (*Salmo gairdneri*). *J. Exp. Biol*. 1989; **142**: 155–175.

Ayson FG, Kaneko T, Hasegawa S, Hirano T. Development of mitochondrion-rich cells in the yolk-sac membrane of embryos and larvae of tilapia, *Oreochromis mossambicus*, in fresh water and seawater. *J. Exp. Zool*. 1994; **270**: 129–135.

Bentley PJ. *Endocrines and Osmoregulation: A Comparative Account in Vertebrates*. Zoophsyiology 39. Springer-Verlag, Berlin. 2002.

Bole-Feysot C, Goffin V, Edery M, Binart N, Kelly PA. Prolactin (PRL) and its receptor: actions, signal transduction pathways and phenotypes observed in PRL receptor knockout mice. *Endocr. Rev.* 1998; **19**: 225–268.

Bolton JP, Takahashi A, Kawauchi H, Kubota J, Hirano T. Development and validation of a salmon growth hormone radioimmunoassay. *Gen. Comp. Endocrinol*. 1986; **62**: 230–238.

Braun EJ, Dantzler WH. Mechanisms of hormone actions on renal function. *In*: Pang PKT, Schreibman MP (eds). *Vertebrate Endocrinology: Fundamentals and Biomedical Implications*, Vol. 2. Academic Press, San Diego. 1987; pp. 189–210.

Clarke WC, Bern HA. Comparative endocrinology of prolactin. *In*: Li CH (ed). *Hormonal Proteins and Peptides*, Vol. VIII. Academic Press, New York. 1980; pp. 105–197.

Collie NL, Hirano T. Mechanisms of hormone actions on intestinal transport. *In*: Pang PKT, Schreibman MP (eds). *Vertebrate Endocrinology: Fundamentals and Biomedical Implications*, Vol. 2. Academic Press, San Diego. 1987; pp. 239–270.

Dange AD. Branchial Na^+, K^+-ATPase activity during osmotic adjustments in two freshwater euryhaline teleosts, tilapia (*Sarotherodon mossambicus*) and orange chromid (*Etroplus maculatus*). *Mar. Biol*. 1985; **87**: 101–107.

Davenport J, Lönning S, Kjorsvik E. Osmotic and structural changes during early development of eggs and larvae of the cod, *Gadus morhua* L. *J. Fish. Biol*. 1981; **19**: 317–331.

Dharmamba M, Maetz J. Effects of hypophysectomy and prolactin on the sodium balance of *Tilapia mossambica* in fresh water. *Gen. Comp. Endocrinol*. 1972; **19**: 175–183.

Dharmamba M, Maetz J. Branchial sodium exchange in sea water-adapted *Tilapia mossambica*: effects of prolactin and hypophysectomy. *J. Endocrinol*. 1976; **70**: 293–299.

Edwards JG. The renal unit in the kidney of vertebrates. *Am. J. Anat*. 1933; **53**: 55–87.

Esaki M, Hoshijima K, Kobayashi S, Fukuda H, Kawakami K, Hirose S. Visualization in zebrafish larvae of Na^+ uptake in mitochondria-rich cells whose differentiation is dependent on foxi3a. *Am. J. Physiol. Regul. Integr. Comp. Physiol*. 2007; **292**: R470–R480.

Evans DH, Piermarini PM, Choe KP. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* 2005; **85**: 97–177.

Fontaine M. Physiological mechanisms in the migration of marine and amphihaline fish. *Advances in Marine Biology*. Elsevier. 1975; **13**: pp. 241–355.

Forgac M. Structure and properties of the vacuolar (H^+)-ATPases. *J. Biol. Chem*. 1999; **274**: 12951–12954.

Foskett KJ, Scheffey C. The chloride cell: definitive identification as the salt secreting cell in teleosts. *Science* 1982; **215**: 164–166.

Goss CG, Laurent P, Perry SF. Evidence for a morphological component in acid-base regulation during environmental hypercapnia in the brown bullhead (*Ictalurus nebulosus*). *Cell Tissue Res*. 1992; **268**: 539–552.

Goss G, Perry S, Laurent P. Ultrastructural and morphometric studies on ion and acid-base transport processes in freshwater fish. *In*: Wood CM, Shuttleworth TJ (eds). *Cellular and Molecular Approaches to Fish Ionic Regulation*. Academic Press, San Diego. 1995; pp. 257–284.

Greco AM, Fenwick JC, Perry, SF. The effects of soft-water acclimation on gill structure in the rainbow trout *Oncorhynchus mykiss*. *Cell Tissue Res*. 1996; **285**: 75–82.

Griffith RW. Environment and salinity tolerance in the genus *Fundulus*. *Copeia* 1974; **1974**: 319–331.

Guggino WB. Salt balance in embryos of *Fundulus heteroclitus* and *F. bermudae* adapted to seawater. *Am. J. Physiol*. 1980; **238**: R42–R49.

Hardy DJ Jr. *Development of Fishes of Mid-Atlantic Bight*, Vol. 2. Government Printing Office, Washington DC. 1978.

Harvey WR, Wieczorek H. Animal plasma membrane energization by chemiosmotic H^+ V-ATPase. *J. Exp. Biol*. 1997; **200**: 203–216.

Hasegawa S, Hirano T, Ogasawara T, Iwata M, Akiyama T, Arai S. Osmoregulatory ability of chum salmon, *Oncorhynchus keta*, reared in fresh water for prolonged periods. *Fish. Physiol. Biochem*. 1987; **4**: 101–110.

Hirai N, Tagawa M, Kaneko T, Seikai T, Tanaka M. Distributional changes in branchial chloride cells during freshwater adaptation in Japanese sea bass *Lateolabrax japonicus*. *Zool. Sci*. 1999; **16**: 43–49.

Hirano T. The spectrum of prolactin action in teleosts. *In*: Ralpha CH (ed). *Comparative Endocrinology: Developments and Directions*. Liss, New York. 1986; pp. 53–74.

Hirano T, Prunet P, Kawauchi H, Takahashi A, Ogasawara T, Kubota J, Nishioka RS, Bern HA, Takada K, Ishii S. Development and validation of a salmon prolactin radioimmunoassay. *Gen. Comp. Endocrinol*. 1985; **59**: 266–276.

Hirano T, Ogasawara T, Hasegawa S, Iwata M, Nagahama Y. Changes in plasma hormone levels during loss of hypoosmoregulatory capacity in mature chum salmon (*Oncorhynchus keta*) kept in seawater. *Gen. Comp. Endocrinol*. 1990; **78**: 254–262.

Hirata T, Kaneko T, Ono T, Nakazato T, Furukawa N, Hasegawa S, Wakabayashi S, Shigekawa M, Chang MH, Romero MF, Hirose H. Mechanism of acid adaptation of a fish living in a pH 3.5 lake. *Am. J. Physiol. Regul. Integr. Comp. Physiol*. 2003; **284**: R1199–R1212.

Hiroi J, Kaneko T, Seikai T, Tanaka M. Developmental sequence of chloride cells in the body skin and gills of Japanese flounder (*Paralichthys olivaceus*) larvae. *Zool. Sci*. 1998a; **15**: 455–460.

Hiroi J, Kaneko T, Uchida K, Hasegawa S, Tanaka M. Immunolocalization of vacuolar-type H^+-ATPase in the yolk-sac membrane of tilapia (*Oreochromis mossambicus*) larvae. *Zool. Sci*. 1998b; **15**: 447–453.

Hiroi J, Kaneko T, Tanaka M. *In vitro* sequential changes in chloride cell morphology in the yolk-sac membrane of Mozambique tilapia (*Oreochromis mossambicus*) embryos and larvae during seawater adaptation. *J. Exp. Biol*. 1999; **202**: 3485–3495.

Hiroi J, McCormick SD, Ohtani-Kaneko R, Kaneko T. Functional classification of mitochondrion-rich cells in euryhaline Mozambique tilapia (*Oreochromis mossambicus*) embryos, by means of triple immunofluorescence staining for Na^+/K^+-ATPase, Na^+/K^+2Cl^- cotransporter and CFTR anion channel. *J. Exp. Biol*. 2005a; **208**: 2023–2036.

Hiroi J, Miyazaki H, Katoh F, Ohtani-Kaneko R, Kaneko T. Chloride turnover and ion-transporting activities of 'yolk balls', yolk-sac preparations separated from Mozambique tilapia embryos, incubated in freshwater and seawater. *J. Exp. Biol*. 2005b; **208**: 3851–3858.

Hiroi J, Yasumasu S, McCormick SD, Hwang PP, Kaneko T. Evidence for an apical Na–Cl cotransporter involved in ion uptake in a teleost fish. *J. Exp. Biol*. 2008; **211**: 2584–2599.

Hirose S, Kaneko T, Naito N, Takei Y. Molecular biology of major components of chloride cells. *Comp. Biochem. Physiol. B* 2003; **136**: 593–620.

Horng JL, Lin LY, Huang CJ, Katoh F, Kaneko T, Hwang PP. Knockdown of V-ATPase subunit A (*atp6v1a*) impairs acid secretion and ion balance in zebrafish (*Danio rerio*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2007; **292**: R2068–R2076.

Hulet WH, Robins CR. The evolutionary significance of the leptocephalus larva. *In*: Böhike EB (ed). *Fishes of the Western North Atlantic*, Mem. Seans. Fdn. Mar. Res. 1, New Haven. 1989; pp. 669–677.

Hulet WH, Fischer J, Rietberg BJ. Electrolyte composition of anguilliform leptocephali from the straits of Florida. *Bull. Mar. Sci.* 1972; **22**: 432–448.

Hwang PP, Lee TH. New insights into fish ion regulation and mitochondrion-rich cells. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 2007; **148**: 479–497.

Inokuchi M, Hiroi J, Watanabe S, Lee KM, Kaneko T. Gene expression and morphological localization of NHE3, NCC and NKCC1a in branchial mitochondria-rich cells of Mozambique tilapia (*Oreochromis mossambicus*) acclimated to a wide range of salinity. *Comp. Biochem. Physiol. A* 2008; **151**: 151–158.

Iwata M, Hirano T, Hasegawa S. Behavior and plasma sodium regulation of chum salmon fry during transition into seawater. *Aquaculture* 1982; **28**: 133–142.

Jagoe CH, Haines TA. Morphometric effects of low pH and limed water on the gills of Atlantic salmon (*Salmo salar*). *Can. J. Fish. Aquat. Sci.* 1990; **47**: 2451–2460.

Kaneko T, Hiroi J. Osmo- and ionoregulation. *In*: Finn RN, Kapoor BG (eds). *Fish Larval Physiology*. Science Publishers, Enfield. 2008; pp. 163–183.

Kaneko T, Shiraishi K. Evidence for chloride secretion from chloride cells in the yolk-sac membrane of Mozambique tilapia larvae adapted to seawater. *Fish. Sci.* 2001; **67**: 541–543.

Kaneko T, Hasegawa S, Takagi Y, Tagawa M, Hirano T. Hypoosmoregulatory ability of eyed-stage embryos of chum salmon. *Mar. Biol.* 1995; **122**: 165–170.

Kaneko T, Hasegawa S, Uchida K, Ogasawara T, Oyagi A, Hirano T. Acid tolerance of Japanese dace (a cyprinid teleost) in Lake Osorezan, a remarkable acid lake. *Zool. Sci.* 1999; **16**: 871–877.

Kaneko T, Shiraishi K, Katoh F, Hasegawa S, Hiroi J. Chloride cells in early life stages of fish and their functional differentiation. *Fish. Sci.* 2002; **68**: 1–9.

Kaneko T, Hasegawa S, Sasai S. Chloride cells in Japanese eel (*Anguilla japonica*) during their early life stages and downstream migration. *In*: Aida K, Tsukamoto K, Yamauchi K (eds). *Eel Biology*. Springer, Tokyo. 2003; pp. 457–468.

Kashiwagi M, Sato R. Studies on the osmoregulation of the chum salmon, *Oncorhynchus keta* (Walbaum). I. the tolerance of eyed period eggs, alevins and fry of the chum salmon to sea water. *Tohoku. J. Agric. Res.* 1969; **20**: 41–47.

Katoh F, Kaneko T. Short-term transformation and long-term replacement of branchial chloride cells in killifish transferred from seawater to freshwater, revealed by morphofunctional observations and a newly established "time-differential double fluorescent staining" technique. *J. Exp. Biol.* 2003; **206**: 4113–4123.

Katoh F, Shimizu A, Uchida K, Kaneko T. Shift of chloride cell distribution during early life stages in seawater-adapted killifish, *Fundulus heteroclitus*. *Zool. Sci.* 2000; **17**: 11–18.

Katoh F, Hasegawa S, Kita J, Takagi Y, Kaneko T. Distinct seawater and freshwater types of chloride cells in killifish, *Fundulus heteroclitus*. *Can. J. Zool.* 2001; **79**: 822–829.

Katoh F, Hyodo S, Kaneko T. Vacuolar-type proton pump in the basolateral membrane energizes ion uptake in branchial mitochondria-rich cells of killifish *Fundulus heteroclitus*, adapted to a low ion environment. *J. Exp. Biol.* 2003; **206**: 793–803.

Kawakami Y, Mochioka N, Nakazono A. Immigration patterns of glass-eels *Anguilla japonica* entering river in northern Kyushu, Japan. *Bull. Mar. Sci.* 1999; **4**: 315–327.

Kelly SP, Woo NYS. Cellular and biochemical characterization of hyposmotic adaptation in a marine teleost, *Sparus sarba*. *Zool. Sci.* 1999; **16**: 505–514.

Kelly SP, Chow IN, Woo NYS. Haloplasticity of black seabream (*Mylio macrocephalus*): hypersaline to freshwater acclimation. *J. Exp. Zool.* 1999; **283**: 226–241.

Keys AB, Willmer EN. 'Chloride secreting cells' in the gills of fishes, with special reference to the common eel. *J. Physiol.* 1932; **76**: 368–378.

Kültz D, Jurss K, Jonas L. Cellular and epithelial adjustments to altered salinity in the gill and opercular epithelium of a cichlid fish (*Oreochromis mossambicus*). *Cell Tissue Res.* 1995; **279**: 65–73.

Langdon JS, Thorpe JE. The ontogeny of smoltification: developmental patterns of gill Na^+, K^+-ATPase, SDH, and chloride cells in juvenile Atlantic salmon *Salmo salar* L. *Aquaculture* 1985; **45**: 83–95.

Laurent P, Dunel-Erb S, Chevalier C, Lignon J. Gill epithelial cells kinetics in freshwater teleost, *Onchorhynchus mykiss* during adaptation to ion-poor water and hormonal treatments. *Fish Physiol. Biochem.* 1994; **13**: 353–370.

Lee KM, Kaneko T, Aida K. Low-salinity tolerance of a marine pufferfish, fugu *Takifugu rubripes*. *Fish. Sci*. 2005; **71**: 1324–1331.

Lee KM, Kaneko T, Aida K. Prolactin and prolactin receptor expressions in a marine teleost, pufferfish *Takifugu rubripes*. *Gen. Comp. Endocrinol*. 2006a; **146**: 318–328.

Lee KM, Kaneko T, Katoh F, Aida K. Prolactin gene expression and gill chloride cell activity in fugu *Takifugu rubripes* exposed to a hypoosmotic environment. *Gen. Comp. Endocrinol*. 2006b; **149**: 285–293.

Leino RL, McCormick JH. Morphological and morphometrical changes in chloride cells of the gills of *Pimephales promelas* after chronic exposure to acid water. *Cell Tissue Res*. 1984; **236**: 121–128.

Leino RL, McCormick JH, Jensen KM. Changes in gill histology of fathead minnows and yellow perch transferred to soft water or acidified soft water with particular reference to chloride cells. *Cell Tissue Res*. 1987; **250**: 389–399.

Leivestad H, Muniz IP. Fish kill at low pH in a Norwegian river. *Nature* 1976; **259**: 391–392.

Lin H, Pfeiffer DC, Vogl AW, Pan J, Randall DJ. Immunolocalization of H^+-ATPase in the gill epithelia of rainbow trout. *J. Exp. Biol*. 1994; **195**: 169–183.

Lin LY, Hwang PP. Modification of morphology and function of integument mitochondria-rich cells in tilapia larvae (*Oreochromis mossambicus*) acclimated to ambient chloride levels. *Physiol. Biochem. Zool*. 2001; **74**: 469–476.

Lin LY, Horng JL, Kunkel JG, Hwang PP. Proton pump-rich cell secretes acid in skin of zebrafish larvae. *Am. J. Physiol. Cell Physiol*. 2006; **290**: C371–C378.

Lönning S, Davenport J. The swelling egg of the long rough dab, *Hippoglossoides platessoides limandoides* (Bloch). *J. Fish. Biol*. 1980; **17**: 359–378.

Loretz CA. Electrophysiology of ion transport in teleost intestinal cells. *In*: Wood CM, Shuttleworth TJ (eds). *Cellular and Molecular Approaches to Fish Ionic Regulation*. Academic Press, San Diego. 1995; pp. 25–56.

Mancera JM, Perez-Figares JM, Fernandez-Llebrez P. Osmoregulatory responses to abrupt salinity changes in the euryhaline gilthead sea bream (*Sparus aurata* L.). *Comp. Biochem. Physiol*. 1993; **106A**: 245–250.

Marshall WS. Na^+, Cl^-, Ca^{2+} and Zn^{2+} transport by fish gills: retrospective review and prospective synthesis. *J. Exp. Zool*. 2002; **293**: 264–283.

Marshall WS, Grosell M. Ion transport, osmoregulation, and acid-base balance. *In*: Evans DH, Claiborne JB (eds). *The Physiology of Fishes*. CRC Press, Boca Raton. 2006; pp. 177–230.

Marshall WS, Bryson SE, Midelfart A, Hamilton WF. Low conductance anion channel activated by cAMP in teleost Cl^-secreting cells. *Am. J. Physiol*. 1995; **268**: R963–R969.

Marshall WS, Bryson SE, Darling P, Whitten C, Patrick M, Wilkie M, Wood CM, Buckland-Nicks J. NaCl transport and ultrastructure of opercular epithelium from a freshwater-adapted euryhaline teleost, *Fundulus heteroclitus*. *J. Exp. Zool*. 1997; **277**: 23–37.

Marshall WS, Emberley TR, Singer TD, Bryson SE, McCormick SD. Time course of salinity adaptation in a strongly euryhaline estuarine teleost, *Fundulus heteroclitus*: a multivariable approach. *J. Exp. Biol*. 1999; **202**: 1535–1544.

Marshall WS, Bryson SE, Luby T. Control of epithelial Cl^- secretion by basolateral osmolality in the euryhaline teleost *Fundulus heteroclitus*. *J. Exp. Biol*. 2000; **203**: 1897–1905.

Mashiko K. Limnological study of Lake Osoresan-ko, a remarkable acidotrophic lake in Japan. *Sci. Rep. Tohoku Imp. Univ. 4th Ser., Biology* 1940; **15**: 331–356.

Mashiko K, Jozuka K, Asakura K. Different type of chloride cells in the gills of *Tribolodon hakonensis* from Lake Osoresanko. *Ann. Rep. Noto Mar. Lab*. 1973; **13**: 33–37.

McCormick SD. Hormonal control of gill Na^+, K^+-ATPase and chloride cell function. *In*: Wood CM, Shuttleworth TJ (eds). *Cellular and Molecular Approaches to Fish Ionic Regulation*. Academic Press, San Diego. 1995; pp. 285–315.

McDonald DG, Freda J, Cavdek V, Gonzalez R, Zia S. Interspecific differences in gill morphology of freshwater fish in relation to tolerance of low-pH environments. *Physiol. Zool*. 1991; **64**: 124–144.

Miyazaki H, Kaneko T, Hasegawa S, Hirano T. Developmental changes in drinking rate and ion and water permeability during early life stages of euryhaline tilapia, *Oreochromis mossambicus*, reared in fresh water and seawater. *Fish Physiol. Biochem*. 1998; **18**: 277–284.

Morgan IJ, Potts WTW, Oates K. Intracellular ion concentrations in branchial epithelial cells of brown trout (*Salmo trutta* L.) determined by X-ray microanalysis. *J. Exp. Biol*. 1994; **194**: 139–151.

Morisawa M, Hirano T, Suzuki K. Changes in blood and seminal plasma composition of the mature salmon (*Oncorhynchus keta*) during freshwater adaptation. *Comp. Biochem. Physiol. A* 1979; **64**: 325–329.

Nielsen S, Nagelhus EA, Amiry-Moghaddam M, Bourque C, Agre P, Ottersen OP. Specialized membrane

domains for water transport in glial cells: high-resolution immunogold cytochemistry of aquaporin-4 in rat brain. *J. Neurosci*. 1997; **17**: 171–180.

Ogasawara T, Sakamoto T, Hirano T. Prolactin kinetics during freshwater adaptation of mature chum salmon, *Oncorhynchus keta*. *Zool. Sci*. 1996; **13**: 443–447.

Otake T, Ishii T, Nakahara M, Nakamura R. Drastic changes in otolith strontium/calcium ratios in leptocephali and glass eels of Japanese eel *Anguilla japonica*. *Mar. Ecol. Prog. Ser.* 1994; **112**: 189–193.

Perry SF, Fryer JN. Proton pumps in the fish gill and kidney. *Fish Physiol. Biochem*. 1997; **17**: 363–369.

Perry SF, Matthew LB, Johnson DA. Cloning and molecular characterization of the trout (*Oncorhynchus mykiss*) vacuolar H^+-ATPase B subunit. *J. Exp. Biol*. 2000; **203**: 459–470.

Perry SF, Shahsavarani A, Georgalis T, Bayaa M, Furimsky M, Thomas SL. Channels, pumps, and exchangers in the gill and kidney of freshwater fishes: their role in ionic and acid-base regulation. *J. Exp. Zool. A* 2003; **300**: 53–62.

Pickford GE, Phillips JG. Prolactin as a factor in promoting survival of hypophysectomized killifish in fresh water. *Science* 1959; **130**: 454–455.

Piermarini PM, Evans DH. Immunochemical analysis of the vacuolar proton-ATPase B-subunit in the gills of a euryhaline stingray (*Dasyatis sabina*): effects of salinity and relation to Na^+/K^+-ATPase. *J. Exp. Biol*. 2001; **204**: 3251–3259.

Pullin RV. Cichlids in aquaculture. *In*: Keenleyside MA (ed). *Cichlid Fishes: Behaviour, Ecology and Evolution*. Chapman and Hall, London. 1991; pp. 280–299.

Richman NH, Tai de Dias S, Nishioka RS, Prunet P, Bern HA. Osmoregulatory and endocrine relationships with chloride cell morphology and density during smoltification in coho salmon (*Oncorhynchus kisutch*). *Aquaculture* 1987; **60**: 265–285.

Sakamoto T, Shepherd BS, Maden SS, Nishioka RS, Siharath K, Richman NH, Bern HA, Grau EG. Osmoregulatory actions of growth hormone and prolactin in an advanced teleost. *Gen. Comp. Endocrinol*. 1997; **106**: 95–101.

Sakamoto T, Yokota S, Ando M. Rapid morphological oscillation of mitochondria-rich cell in estuarine mudskipper following salinity changes. *J. Exp. Zool*. 2000; **286**: 666–669.

Santos CRA, Ingleton PM, Cavaco JE, Kelly PA, Edery M, Power DM. Cloning, characterization, and tissue distribution of prolactin receptor in the sea bream (*Sparus aurata*). *Gen. Comp. Endocrinol*. 2001; **121**: 32–47.

Sasai S, Kaneko T, Hasegawa S, Tsukamoto K. Morphological alteration in two types of gill chloride cells in Japanese eels (*Anguilla japonica*) during catadromous migration. *Can. J. Zool*. 1998a; **76**: 1480–1487.

Sasai S, Kaneko T, Tsukamoto K. Extrabranchial chloride cells in early life stages of the Japanese eel, *Anguilla japonica*. *Ichthyol. Res*. 1998b; **45**: 95–98.

Sasai S, Katoh F, Kaneko T, Tsukamoto K. Ontogenic change of gill chloride cells in leptocephalus and glass eel stages of the Japanese eel, *Anguilla japonica*. *Mar. Biol*. 2007; **150**: 487–496.

Satake K, Oyagi A, Iwao Y. Natural acidification of lakes and rivers in Japan: The ecosystem of Lake Usoriko (pH 3.4–3.8). *Water Air Soil Pollution* 1995; **85**: 511–516.

Sciortino CM, Romero MF. Cation and voltage dependence of rat kidney electrogenic Na^+-HCO_3^- cotransporter, rkNBC, expressed in oocytes. *Am. J. Physiol. Renal Physiol*. 1999; **277**: F611–F623.

Seidelin M, Madsen SS. Endocrine control of Na^+, K^+-ATPase and chloride cell development in brown trout (*Salmo trutta*): interaction of insulin-like growth factor-I with prolactin and growth hormone. *J. Endocrinol*. 1999; **162**: 127–135.

Shepherd BS, Ron B, Burch A, Sparks R, Richman NH, Shimoda SK, Stetson MH, Lim C, Grau EG. Effects of salinity, dietary level of protein and 17*a*-methyltestosterone on growth hormone (GH) and prolactin (tPRL177 and tPRL188) levels in the tilapia, *Oreochromis mossambicus*. *Fish Physiol. Biochem*. 1997; **17**: 279–288.

Shiraishi K, Kaneko T, Hasegawa S, Hirano T. Development of multicellular complexes of chloride cells in the yolk-sac membrane of tilapia (*Oreochromis mossambicus*) embryos and larvae in seawater. *Cell Tissue Res*. 1997; **288**: 583–590.

Shiraishi K, Hiroi J. Kaneko T, Matsuda M, Hirano T, Mori T. *In vitro* effects of environmental salinity and cortisol on chloride cell differentiation in embryos of Mozambique tilapia, *Oreochromis mossambicus*, measured using a newly developed "yolk-ball" incubation system. *J. Exp. Biol*. 2001; **204**: 1883–1888.

Silva P, Solomon R, Spokes K, Epstein, F. Ouabain inhibition of gill Na-K-ATPase: relationship to active chloride transport. *J. Exp. Zool*. 1977; **199**: 419–426.

Singer TD, Tucker SJ, Marshall WS, Higgins CF. A divergent CFTR homologue: highly regulated salt transport in the euryhaline teleost *F. heteroclitus*. *Am. J. Physiol*. 1998; **274**: C715–C723.

Sullivan GV, Fryer JN, Perry SF. Immunolocalization of proton pumps (H^+-ATPase) in pavement cells of rainbow trout gill. *J. Exp. Biol.* 1995; **198**: 2619–2629.

Sullivan GV, Fryer JN, Perry SF. Localization of mRNA for the proton pump (H^+-ATPase) and Cl^-/HCO_3^- exchanger in the rainbow trout gill. *Can. J. Zool.* 1996; **74**: 2095–2103.

Suresh AV, Lin CK. Tilapia culture in saline waters: a review. *Aquaculture* 1992; **106**: 201–226.

Towle DW. Sodium transport systems in gills. *In*: Kinne RKH (ed). *Comparative Aspects of Sodium Cotransport Systems*. Karger, Basel. 1990; pp. 241–263.

Tsukamoto K. Discovery of the spawning area for Japanese eel. *Nature* 1992; **356**: 789–791.

Tsukamoto K. Spawning of eels near a seamount. *Nature* 2006; **439**: 929.

Tsukamoto K, Nakai I, Tesch FW. Do all freshwater eels migrate? *Nature* 1998; **396**: 635–636.

Uchida K, Kaneko T. Enhanced chloride cell turnover in the gills of chum salmon fry in seawater. *Zool. Sci.* 1996; **13**, 655–660.

Uchida K, Kaneko T, Miyazaki H, Hasegawa S, Hirano T. Excellent salinity tolerance of Mozambique tilapia (*Oreochromis mossambicus*): elevated chloride cell activity in the branchial and opercular epithelia of the fish adapted to concentrated seawater. *Zool. Sci.* 2000; **17**: 149–160.

Uchida K, Kaneko T, Yamaguchi A, Ogasawara T, Hirano T. Reduced hypoosmoregulatory ability and alteration of gill chloride cell distribution in mature chum salmon (*Oncorhynchus keta*) migrating upstream for spawning. *Mar. Biol.* 1997; **129**: 247–253.

Uchida K, Kaneko T, Yamauchi K, Hirano T. Morphometrical analysis of chloride cell activity in the gill filaments and lamellae and changes in Na^+, K^+-ATPase activity during seawater adaptation in chum salmon fry. *J. Exp. Zool.* 1996; **276**: 193–200.

Ura K, Soyano K, Omoto N, Adachi S, Yamauchi K. Localization of Na^+, K^+-ATPase in tissues of rabbit and teleosts using an antiserum directed against a partial sequence of the α-subunit. *Zool. Sci.* 1996; **13**: 219–227.

Veillette PA, Young G. Tissue culture of sockeye salmon intestine: functional response of Na^+-K^+-ATPase to cortisol. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2005; **288**: R1598–R1605.

Venkatesh B, Gilligan P, Brenner S. Fugu: a compact vertebrate reference genome. *FEBS Lett.* 2000; **476**: 3–7.

Vonck APMA, Wendelaar Bonga SE, Flik G. Sodium and calcium balance in Mozambique tilapia, *Oreochromis mossambicus*, raised at different salinities. *Comp. Biochem. Physiol. A* 1998; **119**: 441–449.

Watanabe S, Kaneko T, Aida K. Aquaporin-3 expressed in the basolateral membrane of gill chloride cells in Mozambique tilapia *Oreochromis mossambicus* adapted to freshwater and seawater. *J. Exp. Biol.* 2005; **208**: 2673–2682.

Watanabe S, Niida M, Maruyama T, Kaneko T. Na^+/H^+ exchanger isoform 3 expressed in apical membrane of gill mitochondrion-rich cells in Mozambique tilapia *Oreochromis mossambicus*. *Fish. Sci.* 2008; **74**: 813–821.

Weisbart M. Osmotic and ionic regulation in embryos, alevins, and fry of the five species of Pacific salmon. *Can. J. Zool.* 1968; **46**: 385–397.

Wells T. Vesicular osmometers, vasopressin secretion and aquaporin-4: A new mechanism for osmoreception? *Mol. Cell. Endocrinol.* 1998; **136**: 103–107.

Wendelaar Bonga SE, Van der Meij JCA. Effect of ambient osmolarity and calcium on porlactin cell activity and osmotic water permeability of the gills in the teleost *Sarotherodon mossambicus*. *Gen. Comp. Endocrinol.* 1981; **43**: 432–442.

Wendelaar Bonga SE, Flik G, Balm PHM, Van der Meij JCA. The ultrastructure of chloride cells in the gills of the teleost *Oreochromis mossambicus* during exposure to acidified water. *Cell Tissue Res.* 1990; **259**: 575–585.

Weng CF, Lee TH, Hwang PP. Immune localization of prolactin receptor in the mitochondria-rich cells of the euryhaline teleost (*Oreochromis mossambicus*) gill. *FEBS Lett.* 1997; **405**: 91–94.

Wilson JM, Laurent P, Tufts B, Benos DJ, Donowitz M, Vogl AW, Randall DJ. NaCl uptake by the branchial epithelium in freshwater teleost fish: an immunological approach to ion-transport protein localization. *J. Exp. Biol.* 2000a; **203**: 2279–2296.

Wilson JM, Randall DJ, Donowitz M, Vogl AW, Ip AKY. Immunolocalization of ion-transport proteins to branchial epithelium mitochondria-rich cells in the mudskipper (*Periophthalmodon schlosseri*). *J. Exp. Biol.* 2000b; **203**: 2297–2310.

Woo NYS, Chung KC. Tolerance of *Pomacanthus imperator* to hypoosmotic salinities: changes in body composition and hepatic enzyme activities. *J. Fish Biol.* 1995; **47**: 70–81.

Wood CM. The physiological problems of fish in acid waters. *In*: Morris R, Taylor EW, Brown DJA, Brown JA (eds). *Acid Toxicity and Aquatic Animals*. Cambridge University Press, Cambridge. 1989; pp. 125–152.

Wu RSS, Woo NYS. Tolerance of hypo-osmotic salinities in thirteen species of adult marine fish: implications for estuarine fish culture. *Aquaculture* 1983; **32**: 175–181.

Yada T, Hirano T, Grau EG. Changes in plasma levels of the two prolactins and growth hormone during adaptation to different salinities in the euryhaline tilapia, *Oreochromis mossambicus*. *Gen. Comp. Endocrinol*. 1994; **93**: 214–223.

Yan JJ, Chou MY, Kaneko T, Hwang PP. Gene expression of Na^+/H^+ exchanger in zebrafish H^+-ATPase-rich cells during acclimation to low-Na^+ and acidic environments. *Am. J. Physiol. Cell Physiol*. 2007; **293**: C1814–C1823.

Zadunaisky JA. The chloride cell. In Hoar WS, Randall DJ (eds). *Fish Physiology*, Vol XB. Academic Press, San Diego. 1984; pp. 130–176.

Zadunaisky JA, Cardona S, Au L, Roberts DM, Fisher E, Lowenstein B, Cragoe EJ Jr, Spring KR. Chloride transport activity by plasma osmolarity during rapid adaptation to high salinity of *Fundulus heteroclitus*. *J. Membr. Biol*. 1995: **143**: 207–217.

Abbreviations

AQP:	aquaporin
BrdU:	5-bromo-2′-deoxyuridine
CA:	carbonic anhydrase
cDNA:	complementary DNA
CFTR:	cystic fibrosis transmembrane conductance regulator
DASPEI:	2-(4-dimethylaminostyryl)-1-ethylpyridinium iodide
DFW:	deionized FW
FW:	freshwater
GH:	growth hormone
H^+-ATPase:	vacuolar-type proton ATPase
mOsm:	mOsm/kg H_2O
MR cell:	mitochondrion-rich cell
mRNA:	messenger RNA
NBC:	Na^+/HCO_3^- cotransporter
NCC:	Na^+/Cl^- cotransporter
NKCC:	$Na^+/K^+/2Cl^-$ cotransporter
ORF:	open reading frame
PRL:	prolactin
PVF:	perivitelline fluid
RT-PCR:	reverse transcription-polymerase chain reaction
SEM:	scanning electron microscopy
SW:	seawater
TDS:	time differential double fluorescent staining
TEM:	transmission electron microcopy
UTR:	untranslated region