Morphological and ion-transporting plasticity of branchial mitochondrion-rich cells in the euryhaline spotted green pufferfish, *Tetraodon nigroviridis*

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Running headline: Gill MR cells of euryhaline pufferfish

ABSTRACT

Morphological characteristics and chloride regulatory function of gill mitochondrion-rich (MR) cells of the pufferfish (*Tetraodon nigroviridis*) were investigated in this study because *T. nigroviridis* are widely used in the studies of fish osmoregulation. In scanning electron micrographs, the apical membrane structures of MR cells could be distinguished into three phenotypes including flat, intermediate-indentation, and concave-hole. The apical surfaces of most MR cells were flat with microvilli in fresh water (FW)-acclimated pufferfish, whereas most apical surfaces of MR cells were concave to form holes in brackish water (BW) and seawater (SW)-acclimated pufferfish. The observed changes in the proportion of the intermediate indentation type suggested that when transferred from FW to BW or SW, pufferfish MR cells were transformed from a flat type into a concave-hole type via different stages of the intermediate indentation type. Meanwhile, no significant difference in cell size and density were found in pufferfish acclimated to various salinities. To compare the ion-transporting functions of MR cells, both the chloride test and the double immunofluorescent staining of Na^+/K^+-ATPase (NKA) and chloride secreting channel, cystic fibrosis transmembrane conductance regulator (CFTR) were examined. The CFTR was presented in the apical membrane of NKA-immunoreactive cells in gills of SW-acclimated pufferfish but completely disappeared in FW-acclimated fish. Moreover, the chloride test directly demonstrated the chloride secretion function of the concave-hole MR cells. Taken

together, our findings suggested that different types of MR cells in pufferfish gills expressed various ion-transport proteins to conduct distinct functions necessary for salinity acclimation of this euryhaline species.

Key words: spotted green pufferfish, *Tetraodon nigroviridis*, mitochondrion-rich cells, cystic fibrosis transmembrane conductance regulator, Na^+/K^+-ATPase, salinity

INTRODUCTION

Teleosts have evolved to extend their habitats into varied environments. Euryhaline teleosts maintain the ionic concentrations and osmolality of body fluid at levels that are different from those of the external environments (Kaneko et al. 2008). In response to changes of environmental conditions, the ion-transporting epithelia play the roles of modulating ion fluxes. Although ionoregulation in euryhaline teleosts is mediated by a group of organs including the intestine and kidney, the gill is the major site for the balance of ion movement between gains and losses (Evans et al. 2005). Because gill epithelium mitochondrion-rich (MR) cells are the main sites of ion absorption and secretion in fresh water (FW) and seawater (SW) fish, respectively, they are important for adaptation of euryhaline teleosts to environments of various salinities. In SW MR cells, the apical chloride channel, cystic fibrosis transmembrane conductance regulator (CFTR), plays a crucial role in conducting chloride secretion (Hirose et al. 2003; Evans et al. 2005).

Some studies (Laurent and Perry 1990; Perry et al. 1992) reported that injection of exogenous cortisol or treatment using ion-deficient water (Avella et al. 1987; Perry and Laurent 1989; Greco et al. 1996; Tang et al. 2008) altered the morphology of apical surfaces of branchial MR cells. Other studies demonstrated the correlations between MR cells and Cl^- uptake in different species (Goss et al. 1992; Morgan et al. 1994; Morgan and Potts 1995; Chang et al. 2002, 2003). The apical structures of MR cells were closely related to the

ion-transporting activities (Kaneko et al. 2008). Scanning electron microscopy (SEM) was used to observe the structures of apical membranes and identify functional MR cells including FW and SW types in many species (reviewed in Hwang and Lee 2007; Kaneko et al. 2008). During SEM observation, the FW MR cells generally display flat or slightly invaginated surfaces like membrane patches with short cellular projections on them, while the SW type exhibited deeply invaginated surfaces with smaller orifices (Hwang and Lee 2007). In addition, Na^+/K^+-ATPase (NKA) was thought to be the marker of MR cells because it is a primary active transport pump providing the major driving force for ion-transporting functions in branchial MR cells (McCormick 1995; Marshall 2002; Hirose et al. 2003). Many studied euryhaline species altered the cell size, density, or NKA responses of their MR cells suggesting an adjustment of their ion-transporting functions when acclimated to environments of different salinities (Langdon and Thorpe 1985; Richman et al. 1987; McCormick 1995; Uchida et al. 1996, 1997, 2000; Heljden et al. 1997; Sasai et al. 1998; Katoh et al. 2001; Lee et al. 2003; Lee et al. 2006; Kaneko et al. 2008).

The spotted green pufferfish (*Tetraodon nigroviridis*) is an advanced tetraodontid teleost whose native range covers the rivers and estuaries of Southeast Asia (Rainboth 1996). Since our previous studies have demonstrated that *T. nigroviridis* can survive when transferred from FW to SW or vice versa, this species is an efficient osmoregulator (Lin et al. 2004; Lin and Lee 2005; Tang and Lee 2007a,b; Wang et al. 2008). The pufferfish is therefore a great

model organism used to study osmoregulatory mechanisms in several studies (Lin et al. 2004; Lin and Lee 2005; Tang and Lee 2007a, b, Bagherie-Lachidan et al. 2008, 2009; Wang et al. 2008). However, the morphological characterizations (i.e., apical membrane structure, cell size, and density) of branchial MR cells of this euryhaline pufferfish are not clear yet. The present set of experiments was designed to clarify the morphological features of MR cells and their implicated function in this euryhaline teleost.

MATERIALS and METHODS

Experimental animals and environments

The spotted green pufferfish, *Tetraodon nigroviridis*, were obtained from a local aquarium with the total length of 5.2±0.6 cm. After rearing in brackish water (BW; 15‰) for one month, the pufferfish were separated to rear in fresh water (FW), BW, or seawater (SW; 35‰) at 27 ± 1°C with a daily 12-hr photoperiod for two weeks before sampling. SW and BW used in this study were prepared from local tap water with proper amounts of synthetic sea salt (Instant Ocean, Aquarium Systems, Mentor, OH, USA). The water was continuously circulated through fabric-floss filters. The fish were fed with commercial arid shrimp daily.

Ultrastructure of apical membrane of mitochondrion-rich (MR) cells in gill filaments

After anesthetization with MS-222, fish were killed by spinal pithing, and their gills were excised. The first gill arch from each side was fixed at 4°C in the fixative consisting of 5% (w/v) glutaraldehyde and 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.3) for 12 hr. After rinsing with 0.1 M PB, specimens were postfixed with 1% (v/v) osmium tetroxide in 0.1 M PB for 1 hr. Subsequently, the specimens were serial dehydrated in ethanol, followed by critical-point drying using liquid CO_2 in a critical-point dryer (Hitachi HCP-2, Tokyo, Japan). The gills were mounted on an aluminum specimen plate and then coated with gold in an ion sputter (JFC-1600, JEOL, Tokyo, Japan). Then the samples were

examined with a scanning electron microscope (JSM-6700F, JEOL, Tokyo, Japan).

Antibodies

The primary antibodies used in this study included: (1) Na^+/K^+-ATPase (NKA) α5, a mouse monoclonal antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) raised against the α-subunit of avian NKA. The dilution was 1:200 for immunofluorescent staining; (2) anti-cystic fibrosis transmembrane conductance regulator (CFTR), a mouse monoclonal antibody to human CFTR (RandD Systems, MN, USA). This antibody was raised against a carboxy-terminal sequence of human CFTR and had been successfully applied on several fish species (Hiroi et al., 2008; Katoh and Kaneko, 2003; McCormick et al., 2003). The dilution was 1:100 for immunofluorescent staining; (3) NKA #11, a rabbit polyclonal antiserum kindly provided by Prof. P.P. Hwang (Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, Taiwan). This antiserum was raised against 565 amino acids corresponding to 392-939 amino acid sequence of α-subunit of the tilapia. This region shared high sequence identity with NKA α-subunit of vertebrates (Hwang et al., 1998). The secondary antibodies for immunoblotting were alkaline phosphatase conjugated goat anti-mouse IgG (Chemicon, Temecula, CA, USA). The dilution was 1:100 for immunofluorescent staining. For immunofluorescent staining, the secondary antibodies were Alexa-Fluor 546-conjugated goat anti-mouse IgG or Alexa-Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA). The dilution of both two secondary

antibodies was 1:200 for immunofluorescent staining.

Whole-mount double immunofluorescent staining

The procedure of whole-mount immunofluorescent staining was carried out as described by Tang and Lee (2007b). The gill filaments were removed from gill samples fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After washing in phosphate-buffered saline (PBS), the gill filaments were postfixed and permeated with 70% ethanol for 10 min at -20°C. The gill filaments were rinsed with phosphate buffered saline (PBS) and then incubated in 5% bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA). For the detection of MR cells in whole-mount preparations, the gill filaments were incubated at room temperature for 2 hr with the primary monoclonal antibody of NKA (α5). Following incubation, the gill filaments were washed several times with PBS and then labeled with Alexa Fluor 546-conjugated goat anti-mouse secondary antibody (Invitrogen) at room temperature for 2 hr. For the detection of chloride channel CFTR, the gill filaments were first stained with polyclonal antibody NKA#11 and labeled with Alexa Fluor 488-conjugated goat-anti-rabbit secondary antibody at room temperature for 2 hr. After the first staining, the gill filaments were washed several times with PBS to proceed the second staining. The gill filaments were subsequently incubated with anti-CFTR monoclonal antibody overnight at 4°C followed by labeling with Alexa Fluor 546-conjugated goat anti-mouse secondary antibody (Invitrogen) at room temperature for 2 hr. The samples were then washed with PBS,

mounted with a coverslip, and observed with a Leica TCS-NT confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany).

Quantitative analysis of MR cells

The MR cells stained by whole-mount immunofluorescent staining were measured on stored images by Leica TCS NT software. The cell size was determined as the greatest linear diameters of MR cells, and obtained from 10 cells per individual (n = 5), which were randomly selected from gill filaments. For determining the density of MR cells, an area corresponding to 100×100 μm^2 was randomly selected from the region of the afferent-vascular edge of gill filaments of each experimental fish (n = 5). The MR cells in the selected areas were counted, and the number of cells per square millimeter was reported.

Chloride test

To determine the chloride-secreting sites in gill epithelium, SW pufferfish were subjected to the chloride test modified from Kaneko and Shiraishi (2001). The fish were first immersed in deionized water (DW) three times (for 1 min each) to remove Cl^- on the gill surface, and immersed in 0.25% $AgNO_3$ in DW for 1 min. Newly secreted Cl^- reacted with Ag^+ to form photosensitive AgCl during the period of incubation in $AgNO_3$ solution. After a brief rinse with DW, the gills were removed and placed in a glass vial containing 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and exposed to strong light for 30 min because light exposure causes the reduction of AgCl to form Ag precipitates. To observe the

details of chloride-secreting activity of MR cells in gills of SW pufferfish, samples were examined using X-ray microanalysis. The gills which were subjected to the chloride test and fixed as described previously, were dehydrated in ethanol and dried using a Hitachi HCP-2 critical-point drier (Tokyo, Japan). The gills were mounted on an aluminum specimen stub, coated with gold in an ion sputter (JFC-1600, JEOL, Tokyo, Japan), and examined with a scanning electron microscope (JSM-6700F, JEOL, Tokyo, Japan) equipped with an energy-dispersive X-ray microanalyzer and detector (Oxford Inca Energy 350, Oxford Instruments, Oxfordshire, UK). The elemental profile of Ag was examined by detecting the X-ray characteristic of Ag at 2.644 keV (LI) and 2.984 keV (Lα1). For mapping the Ag profile, the X-ray signals were accumulated for 375 s.

Statistical analysis

Values were compared using a one-way analysis of variance (ANOVA) and post-hoc analyses were conducted using Tukey's pairwise method. Values were expressed as the means ± SEM. The significant difference was set as $P<0.05$.

RESULTS

The apical structure of gill mitochondrion-rich (MR) cells

The apical surface structure of MR cells distributed in the flat region of the afferent-vascular edge of gill filaments was observed by a scanning electron microscope (SEM) (Fig. 1). Three phenotypes of MR cells were found on the afferent side of gill filaments according to the apical surface structures, which were distinguished into the flat (Fig. 2A), different stages of intermediate indentation (Fig. 2 B, C, and D), and concave hole subtypes (Fig. 2E). Furthermore, the apical opening of most MR cells exhibited the flat structure in fresh water (FW)-acclimated pufferfish and the apical surface was about 2-3 μm in diameter (Fig. 3A). In brackish water (BW)- and seawater (SW)-acclimated fish, however, most MR cells formed the hole structure (Fig. 3B and 3C). The apical holes of MR cells were about 1-2 μm in diameter. In addition, the intermediate indentation type could be found in all salinity groups with different proportions.

Morphological characteristics of branchial MR cells

The cell size and density of MR cells were examined by immunostaining with Na^+/K^+-ATPase (NKA), the marker of MR cells, in whole-mount preparations of gill filaments from pufferfish acclimated to FW, BW, and SW (Fig. 4 A, B, and C). The cell size and density of branchial MR cells were similar in pufferfish of all salinity groups (Fig. 4 D and E). In FW-, BW-, as well as SW-acclimated pufferfish, two MR cells were sometimes

found to connect with each other to form complexes (arrowheads in Fig. 4 A, B, and C).

Immunolocalization of cystic fibrosis transmembrane conductance regulator (CFTR) in gill MR cells

Localization of branchial CFTR was determined by the whole-mount immunofluorescent staining and double-staining with NKA. The signal of CFTR immunoreaction completely disappeared in FW-acclimated individuals (Fig. 5 A, B, and C). In contrast, CFTR immunoreactions were detected in the apical membrane of NKA immunoreactive cells in gill filaments of SW-acclimated pufferfish (Fig. 5 D, E, and F).

Chloride-secreting activity

The chloride-secreting activity of branchial MR cells were examined because the channel for chloride secretion, CFTR, is expressed in the apical membrane of MR cells in SW-acclimated pufferfish (Fig. 5). The gill filaments of SW-acclimated pufferfish were subjected to the chloride test. These images showed the Ag distribution was confined evidently to the apical hole of the branchial MR cells and its adjacent area (Fig. 6). Because Ag was the reaction product of the chloride test, branchial MR cells in SW pufferfish definitely secreted Cl^- through their apical holes with CFTR localization (Fig. 5).

DISCUSSION

The gill filaments and lamellae are covered by the epithelium which not only provides a distinct boundary between the external environment and body fluids of fish but also plays a critical role in the physiological function of the fish gill (Evans et al. 2005). The mitochondrion-rich (MR) cell is one of the major cell types in gill epithelium and is considered to be one of the predominant sites of active physiological mechanisms (Wilson and Laurent 2002; Hirose et al. 2003; Evans et al. 2005; Marshall and Grosell 2006). Scanning electron microscopy (SEM) was used in the present study to observe MR cells, which were distributed mainly in the afferent-vascular edge of gill filaments in the spotted green pufferfish (*Tetraodon nigroviridis*), similar to previous findings in many other teleostean species (Wilson and Laurent 2002; Evans et al. 2005; Marshall and Grosell 2006). In addition, branchial MR cells were generally not found in the gill lamellae of *T. nigroviridis* acclimated to fresh water (FW), brackish water (BW; 15‰), and seawater (SW; 35‰) by both immunohistochemical (Lin et al. 2004) and SEM observations (data not shown). In the other species, such as milkfish (*Chanos chanos*) (Lin et al., 2003) and tilapia (*Oreochromis mossambicus*) (Tang et al., 2008), MR cells were scarcely found on the epithelium of the gill lamellae except under particular environmental conditions that are associated with the presence of lamellar MR cells (Evans et al. 2005; Hwang and Lee 2007). Thus, it will be intriguing to detect whether MR cells were present in gill lamellae when pufferfish were

acclimated to hypersaline water (45‰) in future studies and to compare the results to the other species (i.e., Japanese eel, *Anguilla japonica*, Sasai et al. 1998; sea bass, *Latealabrax japonicus*, Hirai et al. 1999; Atlantic salmon, *Salmo salar*, Hiroi et al. 2007; Tilapia, *O. mossambicus*, Tang et al. 2008) to clarify the roles of MR cells in pufferfish gill lamellae.

MR cells located in gill epithelium play the roles of ionoregulation because they have contact with the external environment (via the apical membrane) and blood (via the basolateral membrane) (Evans et al. 2005; Kaneko et al. 2008). Therefore, the apical membrane structure of MR cells in many species varies greatly with diverse environmental salinities, generally with an apical concave crypt in SW and convex surface equipped with numerous microvilli in FW (Marshall and Grosell 2006; Hwang and Lee 2007; Kaneko et al. 2008). From the scanning electron micrographs, three phenotypes (i.e., flat, intermediate indentation, and hole types) of the branchial MR cells could be identified in pufferfish depending on the structures of their apical surfaces. The flat type MR cells were found predominately in FW pufferfish while most MR cells in BW and SW pufferfish were the hole type (Fig. 3). In the time-course experiments of Mozambique tilapia that were transferred from hard FW (HFW) to 5‰ SW, wavy-convexed MR cells decreased in apical surface area at 3-hr post-transfer and formed deep-holes after 12-hr post-transfer (Lee et al. 1996). It might indicate that the transformation process of tilapia MR cells occurred efficiently. In addition, Katoh and Kaneko (2003) provided direct evidences to demonstrate that the

occurrence of intermediate types of MR cells in killifish was most frequently observed at 3-hr post-transfer from SW to FW, and the morphology of intermediate type MR cells were illustrated to show the larger apical surfaces and the shallower concave pits compared to those observed in the typical SW type MR cells. Combined with the results of the tilapia (Lee et al. 1996) and killifish (Katoh and Kaneko 2003) with the present study, we proposed that the transformation process of MR cells was from the flat structure (Fig. 2A) to form the concave hole structure (Fig. 2E) via the intermediate indentation structure (Fig. 2 B, C, and D) when pufferfish were transferred from FW to BW or SW. The present study not only distinguished different types of MR cells but also presumed the observed transformation process in the euryhaline pufferfish to provide more information about the apical opening morphology of branchial MR cells in euryhaline species.

Na^+/K^+-ATPase (NKA) expressed in the basolateral membrane of MR cells is a primary active enzyme to provide the driving force for the ion-transporting system of MR cells (McCormick, 1995). The antibody (α5) specific for this enzyme has been used for immunodetection in many studies of fish ionoregulation (Marshall et al. 2002; Lee et al. 2003; Tipsmark et al. 2004; Lin et al. 2006; Wang et al. 2008; Ivanis et al. 2008; Tang et al. 2008). In addition to SEM observation, this study also used whole-mount immunofluorescent staining of NKA to investigate the size and density of MR cells in gills of pufferfish. Whole-mount immunocytochemistry has been used to study MR cells in several teleosts

(Katoh et al. 2001; Katoh and Kaneko 2003; Lee et al. 2003; Lin et al. 2006; Tang and Lee 2007b; Hiroi et al. 2008; Inokuchi et al. 2008, 2009). The effect of environmental salinity on the cell size and density of branchial MR cells seems to vary among different euryhaline teleost species. Euryhaline Mozambique tilapia increased the cell size but decreased the density of gill MR cells with elevated environmental salinity (Heijden et al. 1997; Uchida et al. 2000). Moreover, the size of gill MR cells was larger in FW- than in SW-acclimated killifish, but the cell density was similar between FW- and SW-acclimated groups (Katoh et al. 2001). In Atlantic salmon, both the cell size and density of MR cells were found to increase after acclimation to SW or during smolting (Langdon and Thorpe 1985; Pelis et al. 2001). However, the cell size and number of MR cells on the primary filament decreased in postsmolts (Pelis et al. 2001). Interestingly, this study found there was no significant difference in the cell size and density of branchial MR cells in pufferfish acclimated to different salinities (Fig. 4). Meanwhile, the abundance of branchial NKA α-subunit protein was the lowest in BW-acclimated pufferfish compared to the FW- and SW-acclimated individuals (Lin et al. 2004). These findings suggested that modulating protein amounts of branchial NKA of the pufferfish acclimated to different environmental salinities were through modulating the protein amounts of NKA per cell rather than altering the size or density of MR cells, as reported in tilapia and killifish (Uchida et al. 2000; Katoh et al. 2001).

Cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel

which is expressed in apical membranes of MR cells and responsible for chloride secretion in teleosts acclimated to SW (reviewed in Hirose et al. 2003; Evans 2008; Kaneko et al. 2008). The localization and expression of ion transporters (i.e., NKA and CFTR) were used to classify the function of MR cells in euryhaline teleosts acclimated to different environmental salinities (Marshall et al. 2002; Katoh and Kaneko 2003; Hiroi et al. 2005a, b; Wilson et al., 2007a). In the present study, localization of branchial CFTR was determined by counter-staining with NKA. In SW-acclimated pufferfish, CFTR was expressed in the apical membranes of MR cells. In contrast, CFTR was undetectable in FW-acclimated pufferfish (Fig. 5). This finding was similar to previous studies in tilapia (Hiroi et al. 2005a, b) and killifish (Katoh and Kaneko 2003). Wilson et al. (2007b) also reported that the immunoreaction of CFTR in the apical membranes of branchial MR cells was positive in elvers while negative in the glass eel stage of the European eel (*Anguilla anguilla*). Furthermore, the SW-acclimated pufferfish were also examined by the chloride test to demonstrate the chloride secretion in SW MR cells. The method used in the chloride test had also been used in other euryhaline teleosts to detect the site of chloride secretion (Wong and Chan 1999; Kaneko and Shiraishi 2001). In this study, the presence of large amounts of silver precipitates was confined to the apical crypt of the hole- type MR cells of SW pufferfish (Fig. 6) with CFTR localized in apical membrane (Fig. 5). In FW pufferfish, however, a few silver precipitates were sprinkled on the gill filament rather than specifically located on the

apical surface (data not shown) and CFTR was immunonegative (Fig. 5). Taken together, our results provide direct evidence indicating different functions exhibited in SW- and FW-type MR cells in gills of the euryhaline pufferfish.

T. nigroviridis is a peripheral FW species that is often found in estuary and FW river (Helfman et al. 1997). Thus, the pufferfish were demonstrated an efficient osmoregulator in experimental conditions, as it could tolerate a direct transfer from FW to SW or vice versa (Lin et al. 2004; Lin and Lee 2005; Tang and Lee 2007a, b; Wang et al. 2008). Although previous studies investigated the iono- and osmoregulatory mechanisms in gills of *T. nigroviridis* (Lin et al. 2004; Tang and Lee 2007a, b; Wang et al. 2008; Bagherie-Lachidan et al. 2008, 2009), the morphological features of gill MR cells were not addressed in this species. The present study not only investigated the types of MR cells but also proposed the transformation process and further discussed the cell size and number of branchial MR cells in pufferfish acclimated to environments of various salinities. Taken together, changes of gill MR cell phenotypes, with positive or negative expression of apically located chloride secretion channel indicating altered functions of MR cells, are crucial for efficient responses to salinity challenge in pufferfish. We also concluded that the expressions of the key enzyme, NKA, as well as the other ion transport proteins, rather than modulation of the cell size and number of MR cells, were involved in regulating ion-transporting capacity of branchial MR cells of pufferfish acclimated to different salinities.

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REFERENCES

Avella M, A Masoni, M Bornancin, N Mayer-Gostan. 1987. Gill morphology and sodium influx in the rainbow trout (*Salmo gairdneri*) acclimated to artificial freshwater environments. J. Exp. Zool. **241**: 159-169.

Bagherie-Lachidan M, SI Wright, SP Kelly. 2008. Claudin-3 tight junction proteins in *Tetraodon nigroviridis*: cloning, tissue-specific expression, and a role in hydromineral balance. Am. J. Physiol. Regul. Integr. Comp. Physiol. **294**: R1638-1647.

Bagherie-Lachidan M, SI Wright, SP Kelly. 2009. Claudin-8 and -27 tight junction proteins in puffer fish Tetraodon nigroviridis acclimated to freshwater and seawater. J. Comp. Physiol. B. **179**: 419-431.

Chang IC, TH Lee, HC Wu, PP Hwang. 2002. Effects of environmental Cl^- levels on Cl^- uptake and mitochondria-rich cell morphology in gills of the stenohaline goldfish, *Carassius auratus*. Zool. Stud. **46**: 236-243.

Chang IC, YY Wei, FI Chou, PP Hwang. 2003. Stimulation of Cl^- uptake and morphological changes in gill mitochondria-rich cells in freshwater tilapia (*Oreochromis mossambicus*). Physiol. Biochem. Zool. **76**: 544-552.

Evans DH. 2008. Teleost fish osmoregulation: what have we learned since August Krogh, Homer Smith, and Ancel Keys. Am. J. Physiol. Regul. Integr. Comp. Physiol. **295**: R704-713.

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

Goss GG, SF Perry, CM Wood, P Laurent. 1992. Mechanisms of ion and acid-base regulation at the gills of freshwater fish. J. Exp. Zool. **263**: 143-159.

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

Heijden A, P Verbost, J Eygensteyn, J Li, S Bonga, G Flik. 1997. Mitochondria-rich cells in gills of tilapia (*Oreochromis mossambicus*) adapted to fresh water or sea water: quantification by confocal laser scanning microscopy. J. Exp. Biol. **200**: 55-64.

Helfman GS, BB Collette, DE Facey. 1997. The Diversity of Fishes. Oxford: Blackwell science.

Hirai N, M Tagawa, T Kaneko, T Seikai, M Tanaka. 1999. Distributional changes in branchial chloride cells during freshwater adaptation in Japanese sea bass *Lateolabrax*

japonicus. Zool. Sci. **16**: 43-49.

Hiroi J, SD McCormick. 2007. Variation in salinity tolerance, gill Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter and mitochondria-rich cell distribution in three salmonids *Salvelinus namaycush*, *Salvelinus fontinalis* and *Salmo salar*. J. Exp. Biol. **210**: 1015-1024.

Hiroi J, H Miyazaki, F Katoh, R Ohtani-Kaneko, T Kaneko. 2005a. Chloride turnover and ion-transporting activities of yolk-sac preparations (yolk balls) separated from Mozambique tilapia embryos and incubated in freshwater and seawater. J. Exp. Biol. **208**: 3851-3858.

Hiroi J, SD McCormick, R Ohtani-Kaneko, T Kaneko. 2005b. 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. **208**: 2023-2036.

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

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

Hwang PP, MJ Fang, JC Tsai, CJ Huang, ST Chen. 1998. Expression of mRNA and protein of Na^+-K^+-ATPase α subunit in gills of tilapia (*Oreochromis mossambicus*). Fish. Physiol. Biochem. **18**: 363-373

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

Inokuchi M, J Hiroi, S Watanabe, PP Hwang, T Kaneko. 2009. Morphological and functional classification of ion-absorbing mitochondria-rich cells in the gills of Mozambique tilapia. J. Exp. Biol. **212**: 1003-1010.

Inokuchi M, J Hiroi, S Watanabe, KM Lee, T Kaneko. 2008. 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 salinities. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. **151**: 151-158.

Ivanis G, AJ Esbaugh, SF Perry. 2008. Branchial expression and localization of SLC9A2 and SLC9A3 sodium/hydrogen exchangers and their possible role in acid-base

regulation in freshwater rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. **211**: 2467-2477.

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

Kaneko T, S Watanabe, KM Lee. 2008. Functional morphology of mitochondrion-rich cells in euryhaline and stenohaline teleosts. Aqua-BioSci. Monogr. **1**: 1-62.

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

Katoh F, T Kaneko. 2003. 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. **206**: 4113-4123.

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

Laurent P, SF Perry. 1990. Effects of cortisol on gill chloride cell morphology and ionic uptake in the freshwater trout, *Salmo gairdneri*. Cell. Tissue. Res. **259**: 429-442

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

Lee TH, SH Feng, CH Lin, YH Hwang, CL Huang, PP Hwang. 2003. Ambient salinity modulates the expression of sodium pumps in branchial mitochondria-rich cells of Mozambique tilapia, *Oreochromis mossambicus*. Zool. Sci. **20**: 29-36.

Lee TH, PP Hwang, HC Lin, FL Huang. 1996. Mitochondria-rich cells in the branchial epithelium of the teleost, *Oreochromis mossambicus*, acclimated to various hypotonic environments. Fish. Physiol. Biochem. **15**: 513-523.

Lin CH, TH Lee. 2005. Sodium or potassium ions activate different kinetics of gill Na, K-ATPase in three seawater- and freshwater-acclimated euryhaline teleosts. J. Exp. Zoolog. A. Comp. Exp. Biol. **303**: 57-65.

Lin CH, RS Tsai, TH Lee. 2004. Expression and distribution of Na, K-ATPase in gill and kidney of the spotted green pufferfish, *Tetraodon nigroviridis*, in response to salinity challenge. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. **138**: 287-295.

Lin LY, JL Horng, JG Kunkel, PP Hwang. 2006. Proton pump-rich cell secretes acid in skin of zebrafish larvae. Am. J. Physiol. Cell. Physiol. **290**: C371-378.

Lin YM, CN Chen, TH Lee. 2003. The expression of gill Na, K-ATPase in milkfish, *Chanos chanos*, acclimatized to seawater, brackish water and fresh water. Comp. Biochem. Physiol. A. **135**: 489-497.

Marshall WS, EM Lynch, RR Cozzi. 2002. Redistribution of immunofluorescence of CFTR anion channel and NKCC cotransporter in chloride cells during adaptation of the killifish *Fundulus heteroclitus* to sea water. J. Exp. Biol. **205**: 1265-1273.

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

Marshall WS, M Grosell. 2006. Ion transport, osmoregulation, and acid-base balance. In: Evans DH, Claiborne JB, editors. The Physiology of Fishes. Boca Raton, FL: CRC Press. p 179-214.

McCormick SD. 1995. Hormonal control of gill Na^+, K^+-ATPase and chloride cell function. In: Wood CM, Shuttleworth TJ, editors. Cellular and Molecular Approaches to Fish Ionic Regulation. New York: Academic Press.

McCormick SD, K Sundell, BT Bjornsson, CL Brown, J Hiroi. 2003. Influence of salinity on the localization of Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and CFTR anion channel in chloride cells of the Hawaiian goby (*Stenogobius hawaiiensis*). J. Exp. Biol. **206**: 4575-4583.

Morgan II, W Potts. 1995. The effects of thiocyanate on the intracellular ion concentrations of branchial epithelial cells of brown trout. J. Exp. Biol. **198**: 1229-1232.

Morgan II, W Potts, K Oates. 1994. Intracellular Ion Concentrations in Branchial Epithelial Cells of Brown Trout (*Salmo Trutta L.*) Determined by X-Ray Microanalysis. J. Exp. Biol. **194**: 139-151.

Pelis RM, J Zydlewski, SD McCormick. 2001. Gill $Na^+/K^+/2Cl^-$ cotransporter abundance and location in Atlantic salmon: effects of seawater and smolting. Am. J. Physiol. Regul. Integr. Comp. Physiol. **280**: R1844-1852.

Perry SF, GG Goss, P Laurent. 1992. The interrelationships between gill chloride cell morphology and ionic uptake in four freshwater teleosts. Can. J. Zool. **70**: 1775-1786.

Perry SF, P Laurent. 1989. Adaptational responses of rainbow trout to lowered external NaCl concentration: contribution of the branchial chloride cell. J. Exp. Biol. **147**:

147-168.

Rainboth WJ. 1996. Fishes of the Cambodian Mekong. FAO Species Identification Field Guide for Fishery Purposes Rome: FAO.

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

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

Tang CH, IC Chang, CH Chen, TH Lee, PP Hwang. 2008. Phenotypic changes in mitochondrion-rich cells and responses of Na^+/K^+-ATPase in gills of tilapia exposed to deionized water. Zool. Sci. **25**: 205-211.

Tang CH, TH Lee. 2007. The effect of environmental salinity on the protein expression of Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter, cystic fibrosis transmembrane conductance regulator, anion exchanger 1, and chloride channel 3 in gills of a euryhaline teleost, *Tetraodon nigroviridis*. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. **147**: 521-528.

Tang CH, TH Lee. 2007. The novel correlation of carbonic anhydrase II and anion exchanger 1 in gills of the spotted green pufferfish, *Tetraodon nigrovirids*. J. Exp. Zool. Part. A. Ecol. Genet. Physiol. **307**: 411-418.

Tipsmark CK, SS Madsen, RJ Borski. 2004. Effect of salinity on expression of branchial ion transporters in striped bass (*Morone saxatilis*). J. Exp. Zoolog. A. Comp. Exp. Biol. **301**: 979-991.

Uchida K, T Kaneko, H Miyazaki, S Hasegawa, T Hirano. 2000. 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. **17**: 149-160.

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

Uchida K, T Kaneko, K Yamauchi, T Hirano. 1996. Morphometrical analysis 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. **276**: 193-200.

Wang PJ, CH Lin, HH Hwang, TH Lee. 2008. Branchial FXYD protein expression in response to salinity change and its interaction with Na^+/K^+-ATPase of the euryhaline teleost Tetraodon nigroviridis. J. Exp. Biol. **211**: 3750-3758.

Wilson JM, P Laurent. 2002. Fish gill morphology: inside out. J. Exp. Zool. **293**: 192-213.

Wilson JM, A Leitão, AF Gonçalves, C Ferreira, P Reis-Santos, AV Fonseca, J Moreira da Silva, J Carlos Antunes, C Pereira-Wilson, J Coimbra. 2007. Modulation of branchial ion transport protein expression by salinity in glass eels (*Anguilla anguilla* L.). Mar. Biol. **151**: 1633-1645.

Wilson JM, P Reis-Santos, AV Fonseca, JC Antunes, P D.Bouça, J Coimbra. 2007. Seasonal changes in ionoregulatory variables of the glass eel *Anguilla anguilla* following estuarine entry: comparison with resident elvers. J. Fish. Biol. **70**: 1239-1253.

Wong CK, DK Chan. 1999. Isolation of viable cell types from the gill epithelium of Japanese eel *Anguilla japonica*. Am. J. Physiol. **276**: R363-372.

Figure legends

Fig. 1 The scanning electron micrograph of a gill filament of the euryhaline spotted green pufferfish (*Tetraodon nigroviridis*). The filament showed the afferent-vascular (AV) edge, efferent-vascular (EV) edge, and lamellae (L).

Fig. 2 The observed transformation of mitochondrion-rich (MR) cell phenotypes. From the scanning electron micrographs, apical structure phenotypes of MR cells were presumed to transform from the flat type (A) into the hole type (E), via the intermediate indentation types (i.e., I, II, and III) (B, C, and D). Scale bar, 2 μm. F, flat; I, intermediate indentation; H, hole.

Fig. 3 Scanning electron micrographs of the afferent-vascular area from gill filaments of pufferfish acclimated to fresh water (FW; A), brackish water (BW; B) and seawater (SW; C) for two weeks. The letters in the micrographs indicate subtypes of mitochondrion-rich (MR) cells with different apical structures. Most MRC of FW pufferfish were flat type (F), almost all MRC of SW fish were hole type (H), and both hole type and intermediate indentation type (I) were found in BW individuals. Scale bar, 10 μm.

Fig. 4 Confocal laser scanning micrographs of whole-mount preparations of gill filaments in pufferfish acclimated to fresh water (FW; A), brackish water (BW; B), and seawater (SW; C). Gill filaments were stained with anti-Na^+/K^+-ATPase (NKA) antibody (α5). Arrows indicate the multicellular complex of mitochondrion-rich (MR) cells consisting of two adjacent cells. No significant differences (n=5; mean ± SEM) were found in cell size (D) and density (E)

among all groups.

Fig. 5 Confocal laser scanning micrographs of whole-mount preparations of the gill filaments in fresh water (FW; A, B, and C)- and seawater (SW; D, E, and F)-acclimated pufferfish. Gill filaments were double stained with anti-Na^+/K^+-ATPase (NKA; green; A and D) (NKA #11) and anti-cystic fibrosis transmembrane conductance regulator (CFTR) (CFTR; red; B and E). The merged images (C and F) of double-stained gill filaments showed that CFTR was localized in the apical membrane of NKA immunoreactive cells in SW pufferfish (F), while the signal of CFTR expression completely disappeared in FW pufferfish (C).

Fig. 6 (A) The scanning electron micrograph showed the hole type of the mitochondrion-rich (MR) cell (MRC; arrow) in gill epithelium of seawater-acclimated pufferfish. (B) In the area of the MR cell apical crypt, a distribution image of Ag precipitates was detected by the X-ray characteristic of Ag at 2.644 and 2.986 keV. (C) The merged image of the MR cell apical crypt (A) and its X-ray signals (B) indicate that the presence of Cl^- was confined to the apical crypt of the MR cell (arrow) and its adjacent area. PVC, pavement cell. Scale bars, 1 μm.

Fig. 1

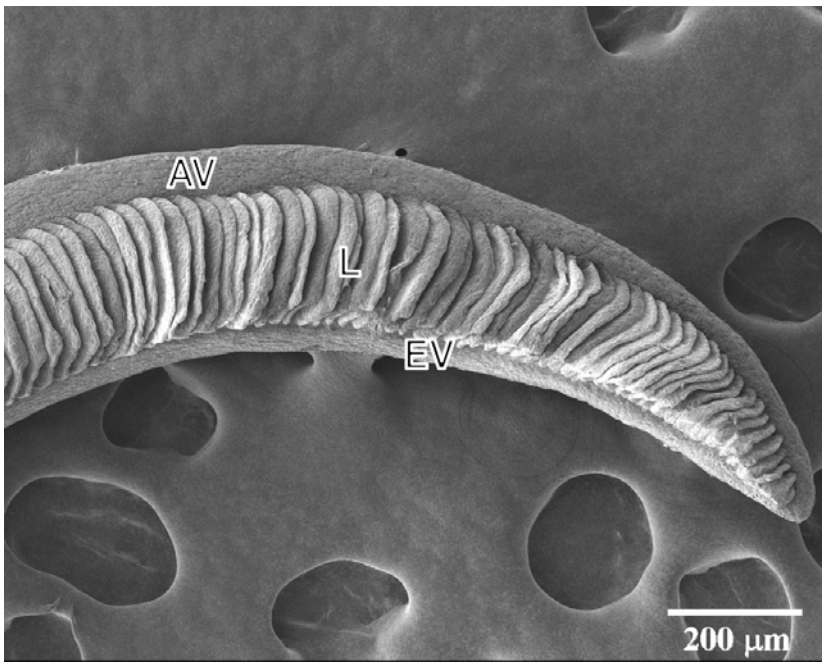
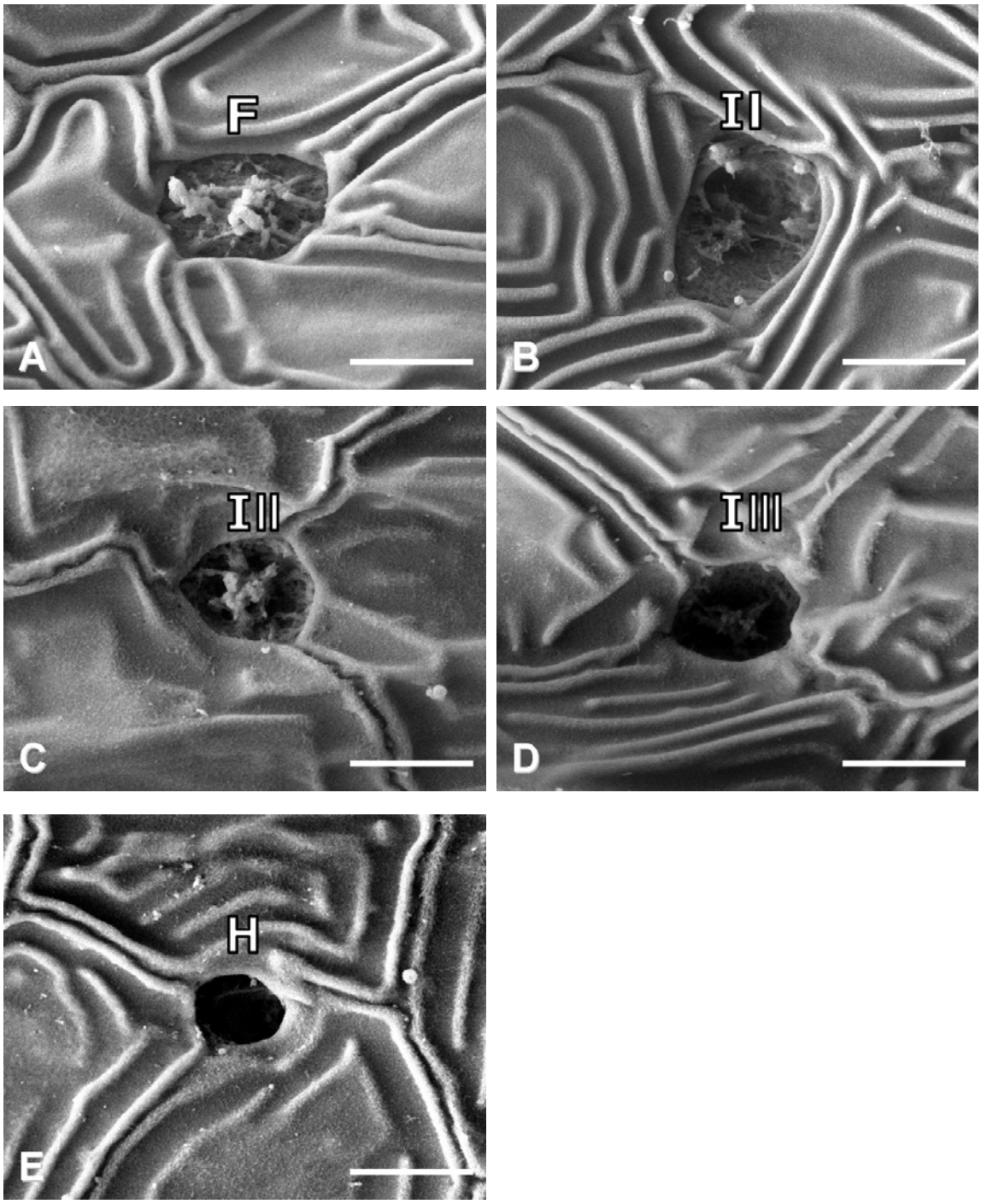


Fig. 2



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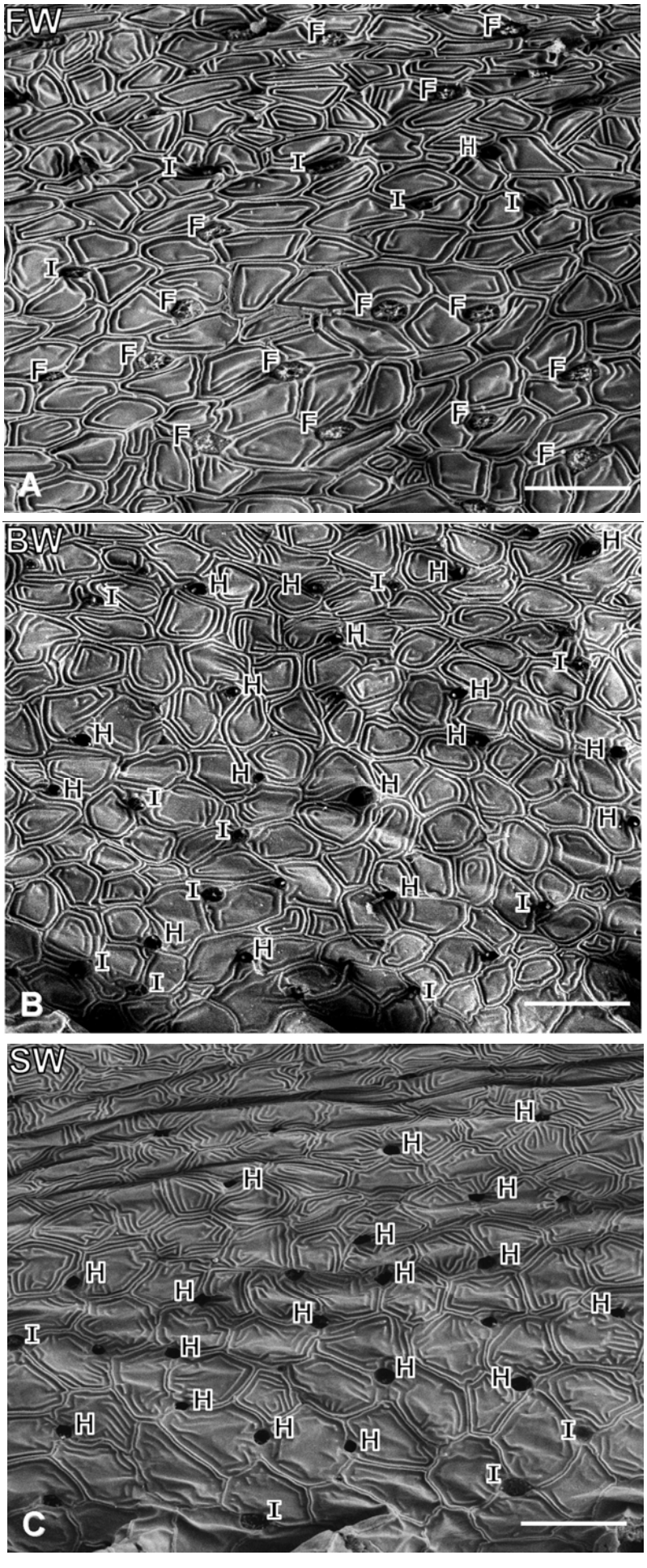

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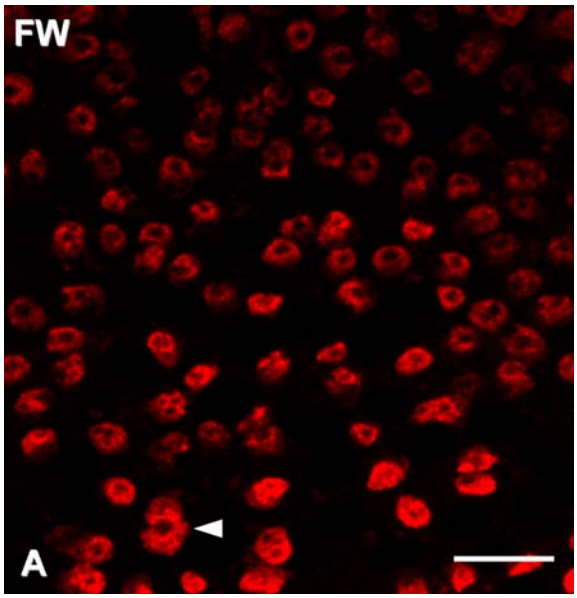
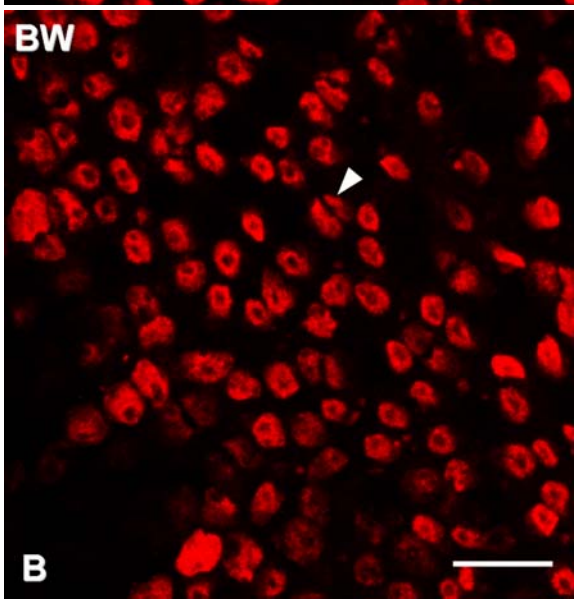
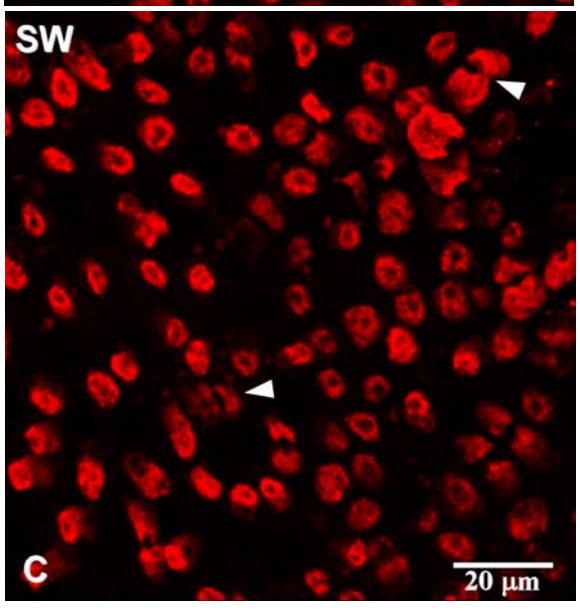
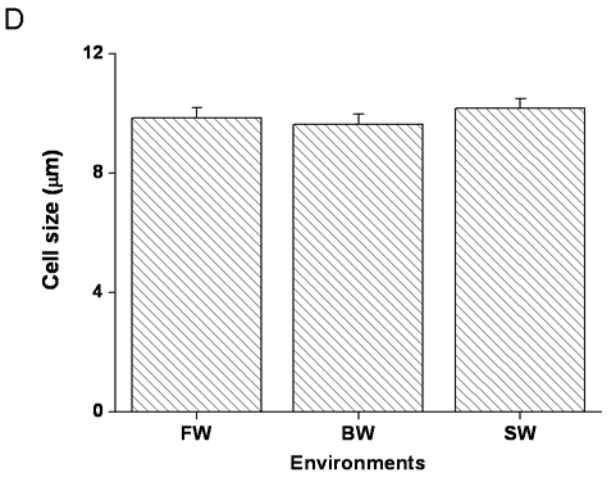

FW
A


BW
B


SW
C
20 µm


D
12
8
4
0
Cell size (µm)
FW
BW
SW
Environments

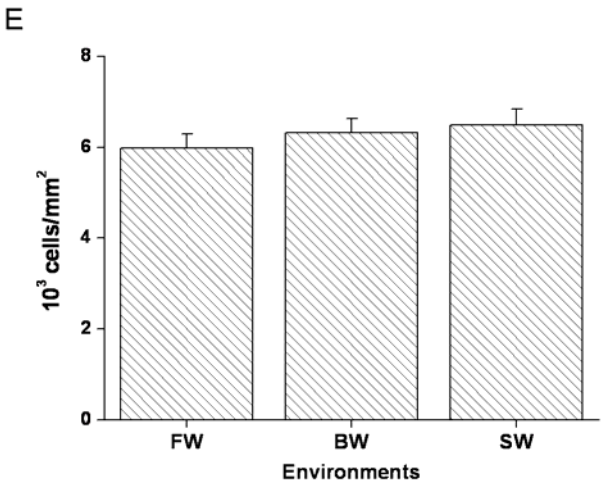

E
8
6
4
2
0
10³ cells/mm²
FW
BW
SW
Environments

Fig. 5

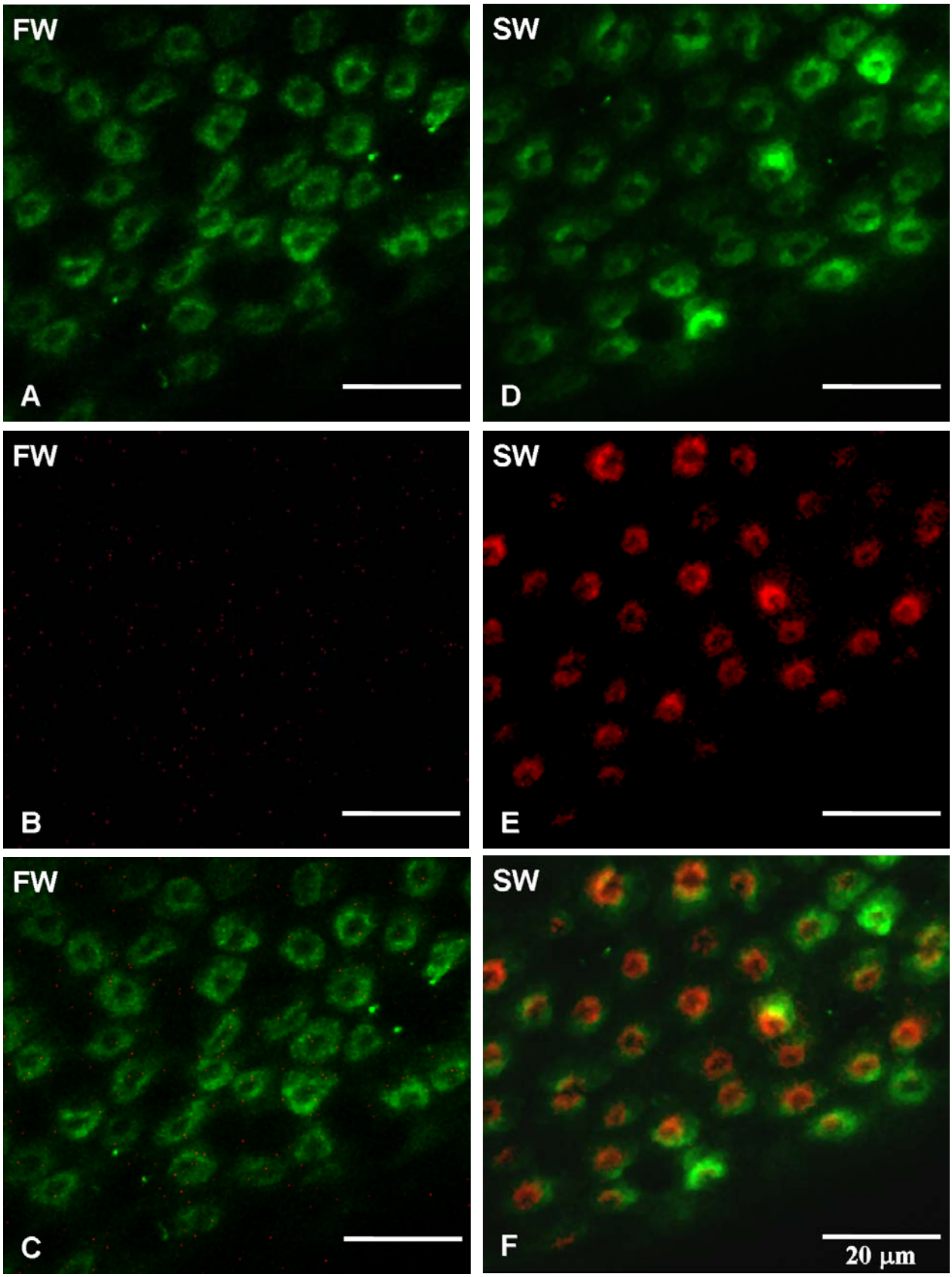


Fig. 6