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EPITHELIUM
GILL
SILURUS GLANIS
ULTRASTRUCTURE
WELS CATFISH

ABSTRACT. – Gill epithelium of fish provides a dynamic ion transporting mechanism that consists of mitochondria rich cells (MRC), pavement cells (PC) and mucous cells (MC). In freshwater, MRC are the ones implicated in active uptake of Cl^- induced by a Na^+/K^+-ATPase pump located in the basolateral part of the cell plasma membrane. Wels catfish *Silurus glanis* was held in closed experimental recirculation systems. Two groups were maintained for 51 days at a constant temperature of 26°C and two different salinities: freshwater (0.3 ‰) and brackish water (11 ‰) respectively. At the end of the experiment, gills were sampled for routine histology, TEM, SEM and immunocytochemistry in order to describe ultrastructural changes as well as localizing ATPase α-subunit activity induced by the environmental change. *S. glanis* gill structure shows typically the presence of MRC located in interlamellar epithelium both in freshwater and brackish environment, similar to the distribution observed in other freshwater-adapted species. However, in brackish water, dark MR cells go through a degeneration and necrosis process while small and compact immature cells with dark cytoplasm resembling β MR cells are frequently observed tightly enveloped by α MR cells. Evaluating overall appearance and functionality of *S. glanis* gill epithelium, we conclude that even though it shares many characteristics with other euryhaline fish, it also shows a peculiar process of differentiation and maturation of β MR cells enclosed by the cytoplasm of pale MR cells.

INTRODUCTION

Teleosts maintain ion concentration and osmolality of their body fluids at a level different from the external environment, generally at about one-third of seawater osmolality both in fresh and seawater (Evans 1993). Osmoregulation in adult teleosts is largely the result of integrated ion and water transport activity of the gills, kidney and intestine, which creates an ionic and osmotic gradient between the body fluids and the external environment (Kaneko *et al*. 2002). Both NaCl secretion and absorption are evolving processes that can be modified into changed hypo- or hyperosmolality conditions enabled by different expression of transport proteins. This implies that cells involved in this transport – *i. e*., mitochondria rich cells (MRC) – can also evolve and modify their morphology and number at best for present environment conditions. This is documented in fish switching from freshwater to brackish or marine environment (Katoh & Kaneko 2003) in conditions of heavy metal pollution (Zia & McDonald 1994, Haaparanta *et al*. 1997, Fernandes *et al*. 1998, Pawert *et al*. 1998) or elevated suspended solids (Au *et al*. 2004). Since transport protein expression is regulated by cortisol secretion, it is also presumed that stress condition has a distinct effect on the variability and number of MRC (Eckert *et al*. 2001, Sakamoto *et al*. 2001).

Gill epithelium of freshwater fish provides a dynamic ion transporting mechanism that can regulate blood pH by manipulating the relative rates of Cl^- and Na^+ uptake (Perry *et al*. 1981). The scheme that consists of mitochondria rich cells (MRC), previously classified as chloride cells (CC), pavement cells (PC), and mucous cells (MC), is generally adopted. Mitochondria rich cells are the ones implicated in active uptake of Cl^- induced by a Na^+/K^+-ATPase pump located in the basolateral part of the cell plasma membrane. Their second activity is Na^+ uptake coupled with H^+ extrusion induced by a V type H^+ ATPase located in the apical part of the plasmalemma. Based on MRC ultrastructural differences, earlier studies pointed to the existence of two main types of cells: α and β MRC (Pisam *et al*. 1987). However, a third type named accessory cell and mainly noticed in marine fish, was described later (Pisam *et al*. 1989).

Pavement cells constitute the major part of gill epithelium, showing a large difference in ultrastructure from MRC, suggesting a milieu poor in mitochondria and vesiculotubular web. Nevertheless, H^+ ATPase activity was demonstrated in a small part of PC, supporting their role in osmoregulation (Galvez *et al*. 2002). Mucous cells have an excretory role carrying numerous vesicles aimed at washing off pathogens, toxic materials or particles of detritus. For instance, in case of heavy metal exposition, the first defense mechanism of gills is mucous secretion (McDonald & Wood 1993).

Since osmoregulation uses a high proportion of the available energy, ranging from 20 to > 50 % of the total energy expenditure dependent on the environmental salinity (Toepfer & Barton 1992), water quality of reared fish can have a great influence on the overall fish performance. As part of the study on sustainability of freshwater channel catfish (*Silurus glanis*) commercial growth in brackish water, an investigation was carried out in order to clarify the morphological and physiological basis of the adaptability of *Silurus* sp. to an increase in water salinity in order to establish the optimal condition for its intensive rearing.

MATERIALS AND METHODS

Fish and experimental systems: Wels catfish *Silurus glanis* was held in closed experimental systems. Two groups were maintained at two different salinities: freshwater (0.3 ‰) and brackish water (11 ‰) at the IFREMER Palavas Station, France. Both groups (each N = 100) were held for 51 days at a constant temperature of 26°C, were fed with a catfish commercial diet (eco 15-n°8), and the resulting average individual weight was 1.38-2.78 kg. No mortalities occurred during the experimental period. Schematic representation, technical details, fish husbandry and water quality sampling in the recirculation system were as described earlier (Deviller *et al*. 2004). Briefly, total ammonia nitrogen (TAN) concentration was measured using the method described by Solorzano (1969). Nitrites (NO_2) were measured by the molecular absorption method described by Bendschneider & Robinson (1952). Nitrates (NO_3) were measured by the same method, after nitrite reduction on a cadmium column (Wood *et al*. 1967). The sum of these three components is equal to the dissolved inorganic nitrogen (DIN). Determination of phosphates (PO^{3-}_4) was carried out using the spectrometric method (AFNOR NF T90-023) described by Murphy & Riley (1962). Nutrient levels in the recirculation system are shown in Table I.

Fish were killed by a sharp blow on the forehead and samples of gills were immediately dissected and fixed for routine histology and electron microscopy. As far as routine histology is concerned, gill tissue was fixed in a modified Davidson fixative (stock solution: filtered sea water 120 ml, ethanol 96 % 120 ml, formaldehyde 36-40 % 80 ml, glycerin 40 ml; working solution: stock solution: glacial acetic acid = 9:1), embedded in Histowax, cut at 6 μm and stained with hematoxylin-eosin method.

Table I. – Water quality analysis in freshwater (FW) and brackish water (BW) recirculation systems.

	FW (±SD)	BW (±SD)
T (°C)	26	26
salinity (‰)	0.3	11
pH	9.37	8.49
N-NH_4 (mg/ L)	0.07 ± 0.0027	0.05 ± 0.0042
N-NO_3 (mg/ L)	0.36 ± 0.08	0.4 ± 0.05
N-NO_2	0.04 ± 0.0011	0.038 ± 0.0009
N-DIN	0.35 ± 0.11	0.43 ± 0.08
P-PO_4	0.03 ± 0.002	0.03 ± 0.00

Transmission electron microscopy: Small fragments of gill filaments were collected and fixed in 3.5 % paraformaldehyde and 3 % glutaraldehyde in 0.1 M PBS (phosphate buffer solution) (pH 7.2-7.4). Tissue was postfixed in 1 % osmiumtetroxide for 1 hour, then dehydrated in an ascending series of acetone and embedded in Durcupan resin (Sheehan & Hrapchak 1980). Semi-thin sections of gill epithelium were stained with methylene blue and examined under a light microscope (Olympus BX41). The ultrathin sections (0.05 μm) were made from the chosen area of interest. In order to enhance osmiumtetroxide resolution, sections were further contrasted over night at room temperature in 2 % uranyl acetate and lead citrate (Reynolds 1963). The electron microscope FEI Morgagni 268D was used for examination of the ultrathin sections.

Scanning electron microscopy: The specimens were fixed in 3 % glutaraldehyde and in a 0.15 M phosphate buffer solution (PBS) for 24 hours. Then the tissues were washed in PBS and postfixed in 1 % osmiumtetroxide for 1 hour. After washing in PBS, the tissues were dehydrated twice in 70 % ethanol for 15 minutes, and twice in 96 % ethanol for 15 minutes. Furthermore, four changes of absolute ethanol were used: 15 minutes, 30 minutes, over night and a change before drying during 15 minutes. The specimens were then dried in a critical-point drier, mounted on specimen holders, coated in a sputter-coater and examined at the scanning electron microscope.

Immunocytochemistry: Paraffin-embedded gill sections were stained for immunocytochemistry in order to assess the activity of Na^+/K^+ ATPase α-subunit at two different salinities. After deparaffinisation and rehydration in decreasing alcohol concentrations, slides were stored in PBS for half an hour, passed in BSA 1 % and gelatin 0.1 %, and in PBS for 5 min. After another PBS washing, sections were encircled with a "Dakopen" paraffin pen. Unlabelled monoclonal antibody (diluted to 10 μg/ ml) raised against the α5-subunit of the chicken Na^+/K^+ ATPase (mouse anti-chicken IgG α5) was added on the sections (Developmental Studies Hybridoma bank, University of Iowa). Control sections were subjected to the same conditions, but PBS was used instead of monoclonal antibody. Slides were incubated in a dark humid chamber for 1h at 37°C. After rinsing, all sections were incubated with the florescein (FITC)-conjugated goat anti-mouse IgG. The slides were washed, mounted with anti-bleaching mounting medium and rapidly examined using a Leica DM4000 B microscope with 450 and 490 nm filters. Accompanying digital camera and LAS (Leica Application Suite) software were used to record and process the pictures. Since basic LAS software did not include the fluorescence quantification necessary for setting a borderline of immunoreactive intensity, fluorescence intensity of the background tissue was subtracted from positive-staining MRC using image analysis macros pro-

vided with MetaMorph (Universal Imaging Corp, Downingtown, PA) software. The correction of the background was set automatically at 70 units.

Pictures were loaded in Olympus DP-Soft 3.1 software to measure MRC diameter and abundance (average ± SD). Diameter of MRC (N = 30, sectioned through the nucleus, along the length of a primary filament containing three secondary lamellae, measured at three different areas) from both salinities was first measured by selecting a magnification and then adding a measurement by an arbitrary distance (Mylonas *et al*. 2009). Abundance of MRC (N = 30 at three different sites, from three different primary lamellae) was first calculated by choosing area/ perimeter manually, and then cell touch-counting in the given area. The same procedure was followed to measure the size and abundance of mitochondria in different salinities (N = 30 from three randomly selected cells at three different sites and from three different primary lamellae).

In order to analyze differences in MRC and mitochondria size and number between two salinities at a minimum significance level of $P < 0.05$, data were subjected to Student's t-test, using STATISTICA 6 software.

RESULTS

Scanning electron microscopy

Lamellar organization of the branchial filament of the freshwater *Silurus glanis* has been described by scanning electron microscopy (Fig. 1a), showing lamellar and filament epithelium, with short apical microridges (see Wilson & Laurent 2002). Observed at higher magnifications, interlamellar space situated between the branchial lamellae (Fig. 1b), consists of microridged pavement cells (PC) interspaced with mitochondria rich cells (MRC) (Fig. 1c). MRC are spread throughout the base of the branchial lamellae, in the interlamellar regions and in the lamellar epithelium. Pavement cells contain abundant microridges on their surface, while the surface of the mitochondria rich cells has microvillous appearance (Fig. 1d). On the leading edge (afferent board), the MC are predominant among PC while the trailing edge (efferent board) is more abundant in MRC. In brackish-adapted fish, lamellar organization is congruent with that observed in freshwater fish, except for the increased number of interlamellar MRC differentiating in two cell types based on the size and abundance of microvilli: a cell type with small and abundant microvilli, and a cell type with larger but less abundant microvillosities (Fig. 1e). Occasionally, several mucous cells (MC) were observed (Fig. 1f).

Immunocytochemical localization of the MRC

Control sections without primary antibody showed no immunolabeling (figure not shown). The abundance of MRC spaced out in the interlamellar epithelium was detected by FITC-conjugated antibody labeling. Freshwater wels catfish had in the interlamellar space smaller (28.5 μm ± 3.11) and less numerous MRC (N = 8) (Fig. 2a-c), compared to brackish water fish, which showed larger (36 μm ± 4.16) and more abundant MRC (N = 16) (Fig. 2d-f). The difference in size ($p = 0.00562$) and abundance ($p = 0.0247$) was statistically significant at a level of $P < 0.05$ after 51 days.

Transmission electron microscopy

Interlamellar spaces in both experimental groups consist of two different types of MRCs: low (pale) electron dense cytoplasm MRCs and high (dark) electron dense cytoplasm MRCs. The pavement cells are inserted among MRCs (Fig. 3a). In freshwater fish, pale MRC (or α-type) have a pale cytoplasm containing numerous mitochondria (1.5 μm x 0.6 μm), opening with a narrow apical crypt on the surface, thus forming the area of enzymatic activity (Fig. 3b and 3d). Dark MRC (or β-type), in the same experimental group, has a thick electron dense cytoplasm with a large nucleus, and poorly visible mitochondria (2.5 μm x 1.25 μm), compared to α-type MRC (Fig. 3c). Desmosomal attachments are interconnecting MRCs and adjacent pavement cells (Fig. 3e).

In the gill filament epithelium of fish exposed to brackish water, two or more MRC frequently appear together sharing a single apical crypt and forming a multicellular complex of chloride cells that have shallower junctions between MRCs. These MRCs are variable in their ultrastructure, particularly in the electron density of cytoplasm. Namely, α-type MRCs have significantly more mitochondria ($p = 0.00834$; diameter 1.8 x 0.9 μm) compared to freshwater cells, and an abundant vesiculotubular system scattered throughout cytoplasm (Fig. 3f), containing large membranous compartments of Golgi apparatus (Fig. 4a). In this experimental group, the apical crypt (wideness 8.4 μm) of the α-type MRCs has a wider surface compared to the same cell type in freshwater-reared fish (Fig. 4b). The marked feature in brackish water fish is a characteristic envelope of β-type MRCs (10.5 x 6.25 μm) by large and distended α-type MRCs (Fig. 4d and 5e). In such cases, when both cells are localized at the very tissue surface, vacuolisation of β-type MRCs cytoplasm and fragmentation of its plasma membrane are noticed. Tubular system in dark MRC is enlarged, with smudged edges that suggest degeneration signs (Fig. 5d), whereas in pale MRC this system is compact with sharp and narrow canaliculi (Fig. 5e). Sometimes in deeper tissue layers, two β-type MRCs are observed (9.5 μm x 5 μm) embedded in the α-type MRC (Fig. 4e). Pavement and mucous cells are abundant and adjacent to MRCs (Fig. 4c and 4f). MRC surface is covered by numerous microvilli (Fig. 5a), while long and slender interdigitations connect neighbouring MRCs and adjacent small accessory cells in the interlamellar spaces (Fig. 5b and

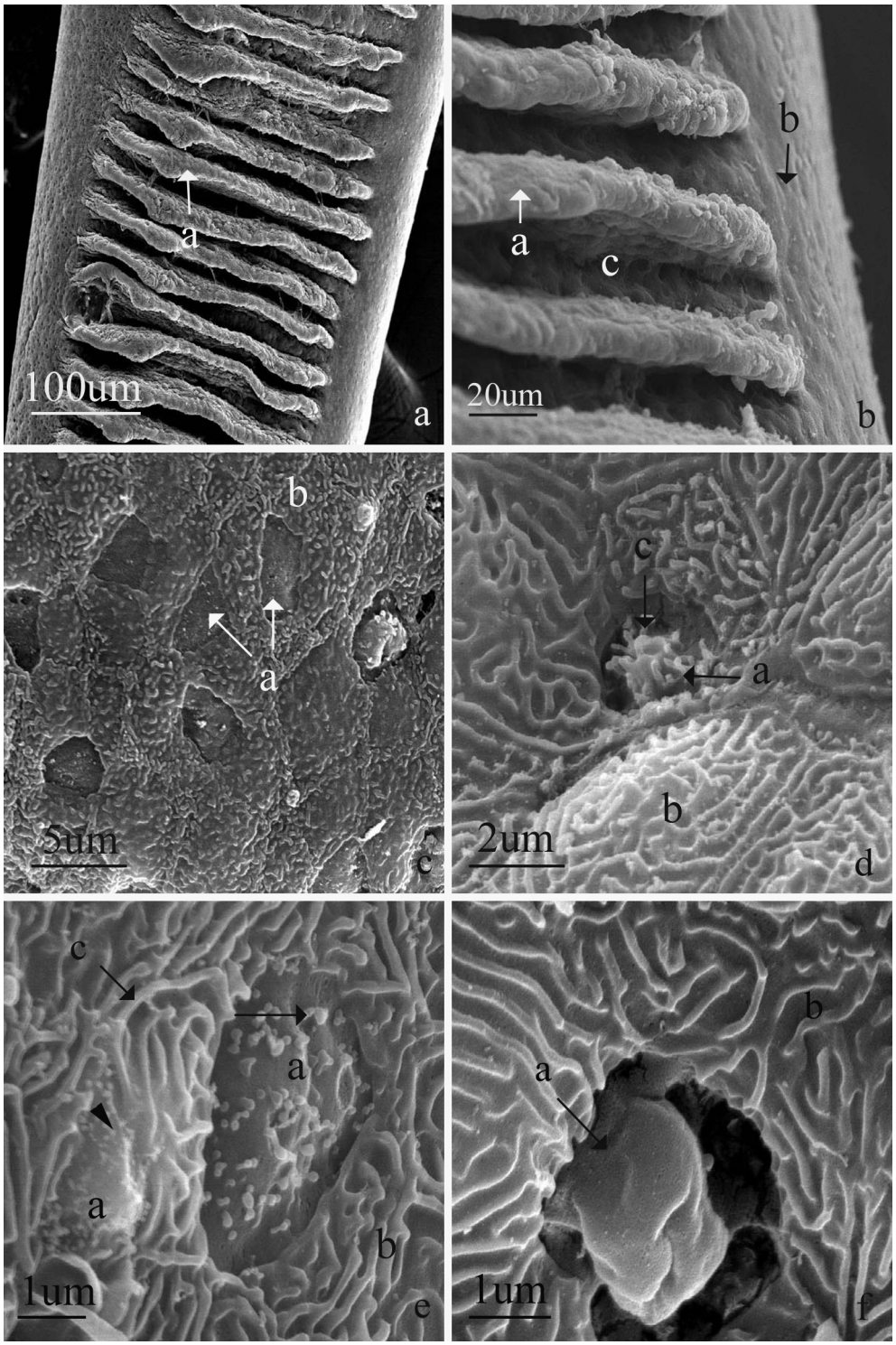

Fig. 1. – Scanning electron microscopy (SEM) of the branchial filament in freshwater *S. glanis*. a) Low magnification of the branchial filament: a, branchial lamellae. b) Higher magnification of the branchial filament: a, lamellar epithelium, b, filament epithelium, c, interlamellar space. c) Interlamellar space containing pavement cells and mitochondria rich cells: a, mitochondria rich cells, b, pavement cell. d) Mitochondria rich cell surrounded by pavement cells: a, mitochondria rich cell, b, pavement cell, c, microvilli on the surface of the mitochondria rich cell. e) Mitochondria rich cells (a) in brackish adapted fish. Note two types of MRC; with small and abundant microvilli (arrowhead), and with large and less abundant microvilli (arrow): b, pavement cell, c, microridges on the surface of the pavement cells. f) Mucous cell (a) inserted between pavement cells (b) in brackish adapted fish.

5c). Accessory cells are half the size of MRC with condensed and electron dense cytoplasm, and abundant in mitochondria.

Mucous cells are large, rounded (16.25 x 11.25 μm), with an apical opening, the nucleus compressed basally and mucin granules concentrated in the cytoplasm. While

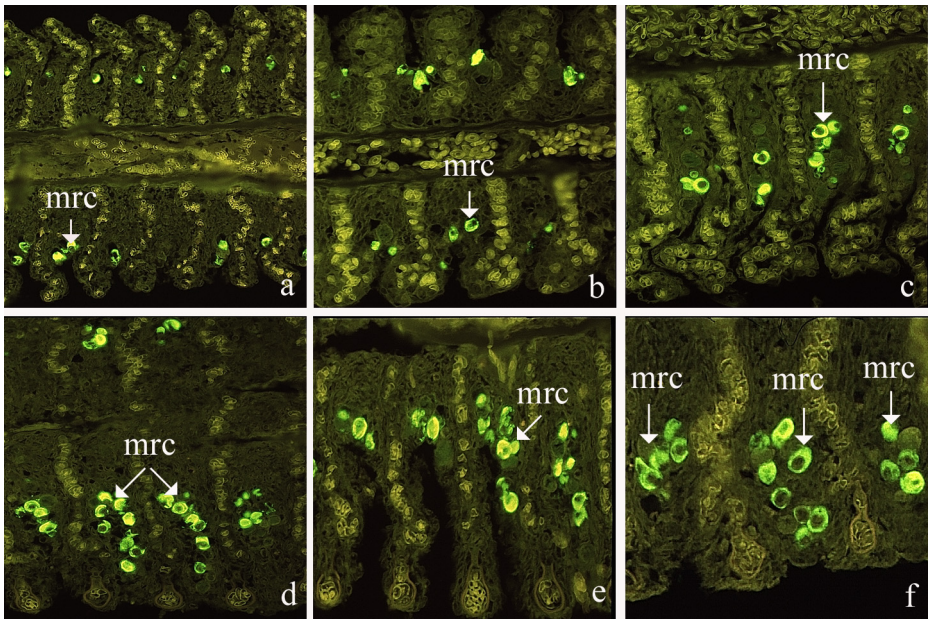

Fig. 2. – Immunocytochemical localization of the mitochondria rich cells (arrows) on the branchial epithelium of *Silurus glanis* in freshwater (a-c) and brackish water (d-f).

in freshwater fish these cells are more spherical or sac-like, and their mucin granules are electron dense on the periphery and lighter in the centre (Fig. 5f), in brackish water fish mucous cells are more goblet-like with significantly electron-lucent granules (Fig. 5g).

DISCUSSION

This study describes for the first time structural modifications and quantitative changes of MR cells in relation with different salinities that occur in *S. glanis* gills, as evidenced by TEM, SEM and immunocytochemistry. *S. glanis* gill structure shows a typical presence of mitochondria rich cells located in the interlamellar epithelium both in freshwater (FW) and brackish (BW) environment, similar to the distribution observed in FW-adapted chum salmon (*Oncorhynchus keta*) (Uchida *et al*. 1996), Japanese seabass (*Lateolabrax japonicus*) (Hirai *et al*. 1999), European seabass (*Dicentrarchus labrax*) (Varsamos *et al*. 2002) or milkfish (*Chanos chanos*) (Lin *et al*. 2006). Interlamellar space accommodates ventilatory flow of water, enabling oxygen uptake through a thin layer of lamellar epithelium (Wilson & Laurent 2002). Because of MRC proliferation and increase in size after the transfer to brackish water, diffusion distance increases impeding adequate gas exchange (Sollid & Nilsson 2006) but the process allows the necessary salt-secreting function regulating the ion and acido-base balance in brackish water as demonstrated in this study, as well as in saline and hypersaline environments (Mylonas *et al*. 2009, Ouatar *et al*. 2009).

Usually, in order to accommodate abrupt environmental ionic changes, channel catfish gill epithelia follow the same pattern as described in the group of fish like salmon, eel, tilapia that respond to a hyperosmotic environment by an increase in NAK (Na^+/K^+ ATPase pump) correlated with an increase in the number of MR cells (Hwang & Lee 2007). While lamellar MRC were observed in FW, filament MRC proliferated in BW, resulting in the rise of two cell types: α or pale MRC, and β or dark MRC type. The latter were enveloped by α MRC when located in the filament, whereas in the gill lamellae they were undergoing degenerative processes. This pleads in favor of a significant difference in functionality of filament and lamellar MRC; the former being activated following transfer from freshwater to seawater as a site for salt secretion, and the latter observed in freshwater enabling ion uptake, and disappearing after seawater transfer (Uchida *et al*. 1996). Moreover, Hirai *et al*. (1999) evidenced that lamellar MRC after the transfer of the Japanese sea bass from seawater to freshwater, originated from the gill filaments, which – given the localization of small dark MRC enveloped by pale MRCs – suggests the same process as in the catfish adapted to BW. Transformations of MR cell subtypes (α and β types) were similar to those described in previous studies: pale MR cells increase in volume and develop a distinct rich vesiculotubular system with larger and numerous crested mitochondria. Both dark β and α pale MR cells gain more space in the apical crypt, opening on the surface with numerous relatively short evaginations of their apical membrane. However, in FW, pavement cells cover most of their apical part, enabling only a minimal aperture providing a contact with the external media. Similar shift in the size of apical crypts was noticed in the euryhaline tilapia (*Oreochromis mossambica*) in hyposmotic media (Van der Hejiden *et al*. 1999).

Whereas pale α MR cells generate their activity in SW,

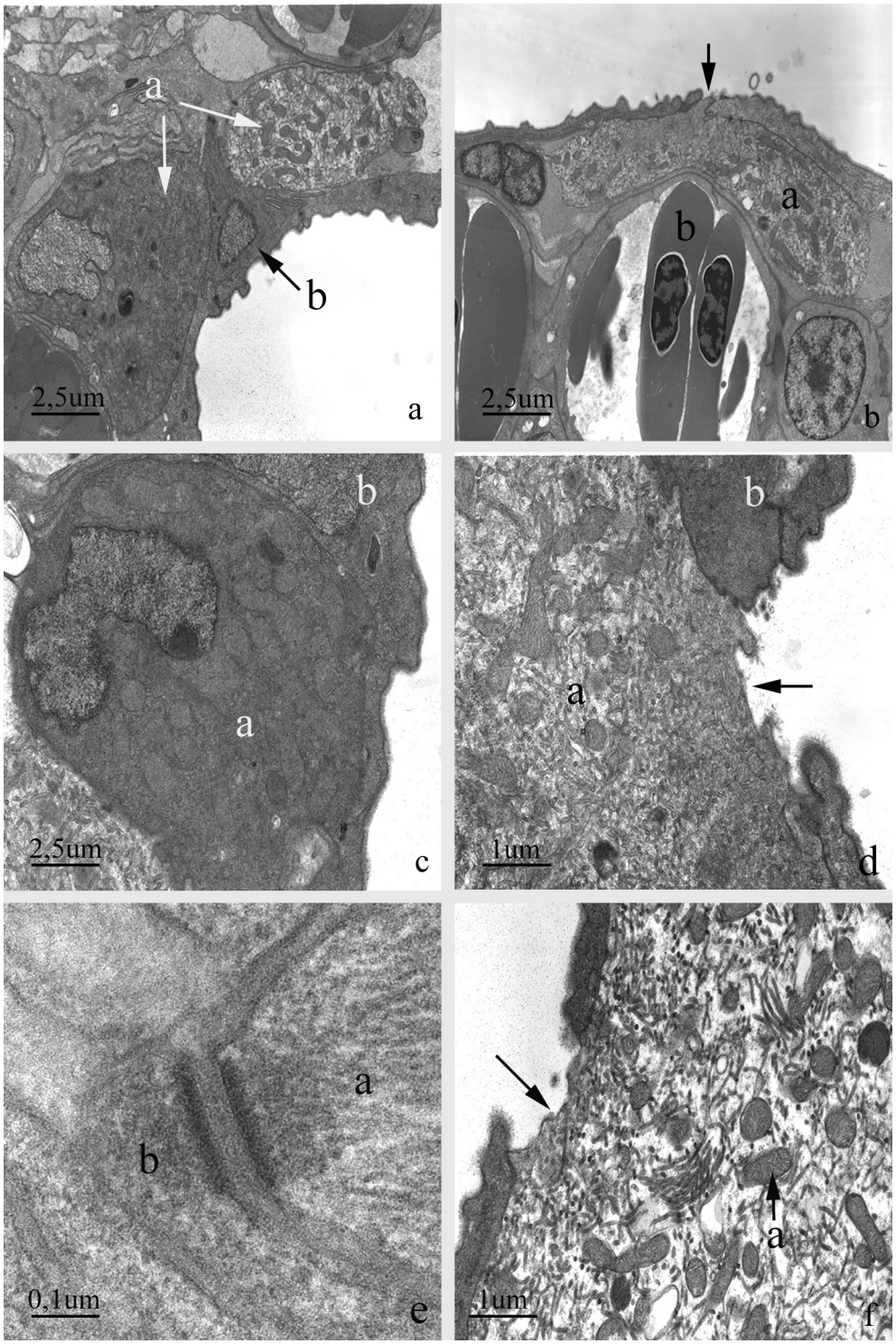

Fig. 3. – Transmission electron microscopy (TEM) of the branchial filament in fresh and brackish water *S. glanis*. a) Two mitochondria rich cells (a) with different density of cytoplasm, and pavement cell (b) inserted between them. b) A section through branchial epithelium: a, mitochondria rich cell type α (with pale cytoplasm) and its apical crypt (↑), b, erythrocytes inside the capillaries. c) A section through branchial epithelium: a, mitochondria rich cell type β (with dark cytoplasm), b, pavement cell. d) α type mitochondria rich cell (a) and its apical crypt (↑) surrounded by pavement cells (b). e) A desmosome between mitochondria rich cell (a) and pavement cell (b). f) α type mitochondria rich cell with abundance of mitochondria (a) and its apical crypt (↑).

it was accepted that dark β MR cells slightly change their size, start irreversible degeneration and decrease in number (Pisam *et al*. 1987). This was also documented in BW-adapted channel catfish, where vacuolization in the basal part of cytoplasm, and a degradation of the vesiculotubular system and mitochondria was observed. However, Hiroi *et al*. (1999), then Lin and Hwang (2004), demonstrated that actually both types of MR cells survive

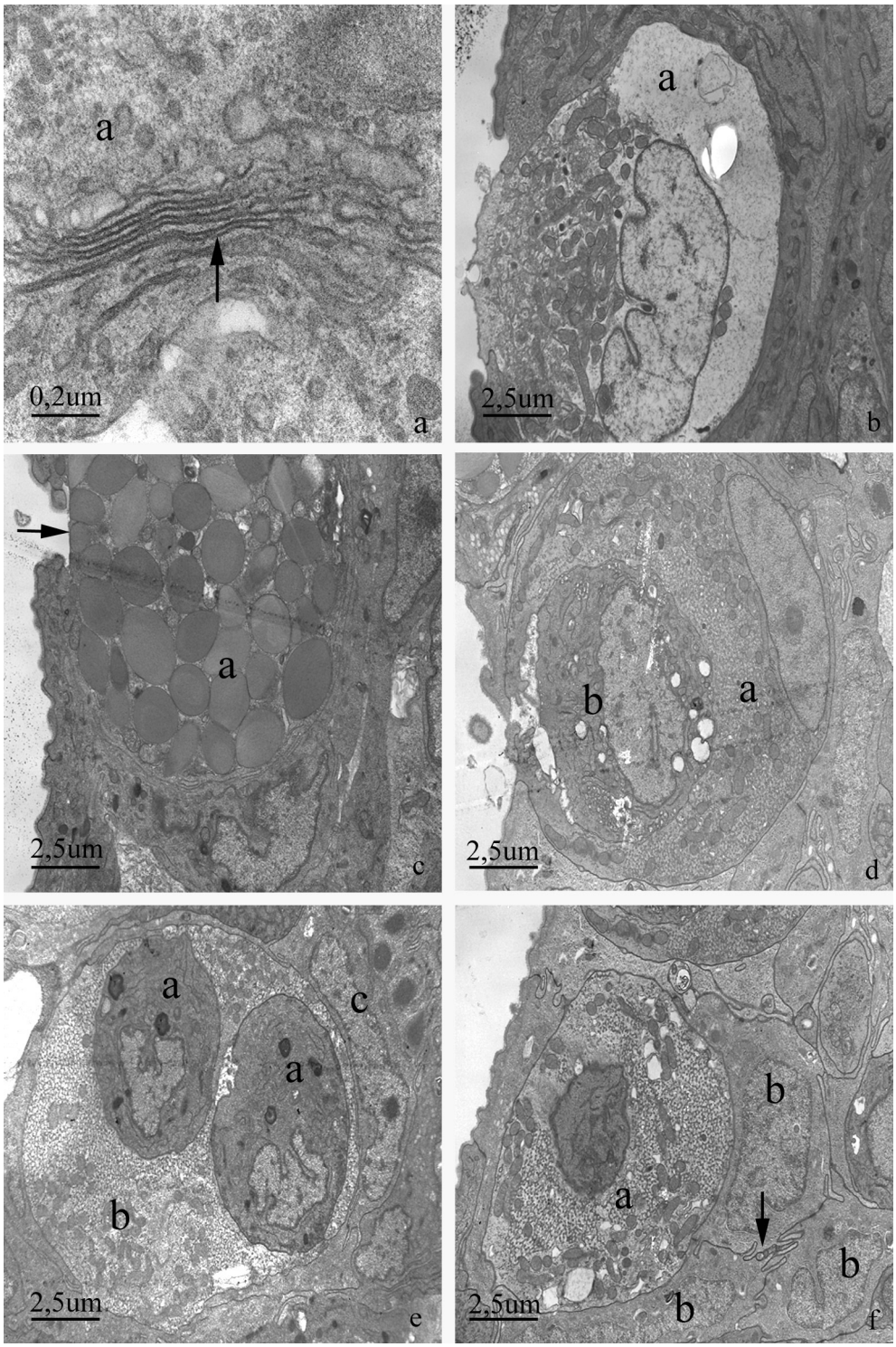

Fig. 4. – Transmission electron microscopy (TEM) of the branchial filament in fresh and brackish water *S. glanis*. a) Golgi apparatus (↑) in mitochondria rich cell (a). b) α type mitochondria rich cell (a) with its apical crypt. c) A mucous cell (a) with its apical crypt (↑). d) α type (a) enveloping β type (b) mitochondria rich cell. e) Two β type cells (a) inside α type (b) mitochondria rich cell surrounded by pavement cell (c). f) α type mitochondria rich cell (a) surrounded by pavement cell (b). Note interdigitations between adjacent cells (↑).

FW-SW transfer and are able to be renewed from undifferentiated cells, following the same turnover rate (10 % of new recruitment). Wong & Chan (1999) found convincing evidence about the MR cell origin from undifferentiated cells named stem cells. They represent a small population of nonchloride cells entering the DNA synthesis phase that ultimately differentiate into SW chloride cells. In *Silurus* sp., even though dark MR cells went through a degeneration and necrosis process, small and compact immature cells with dark cytoplasm resembling

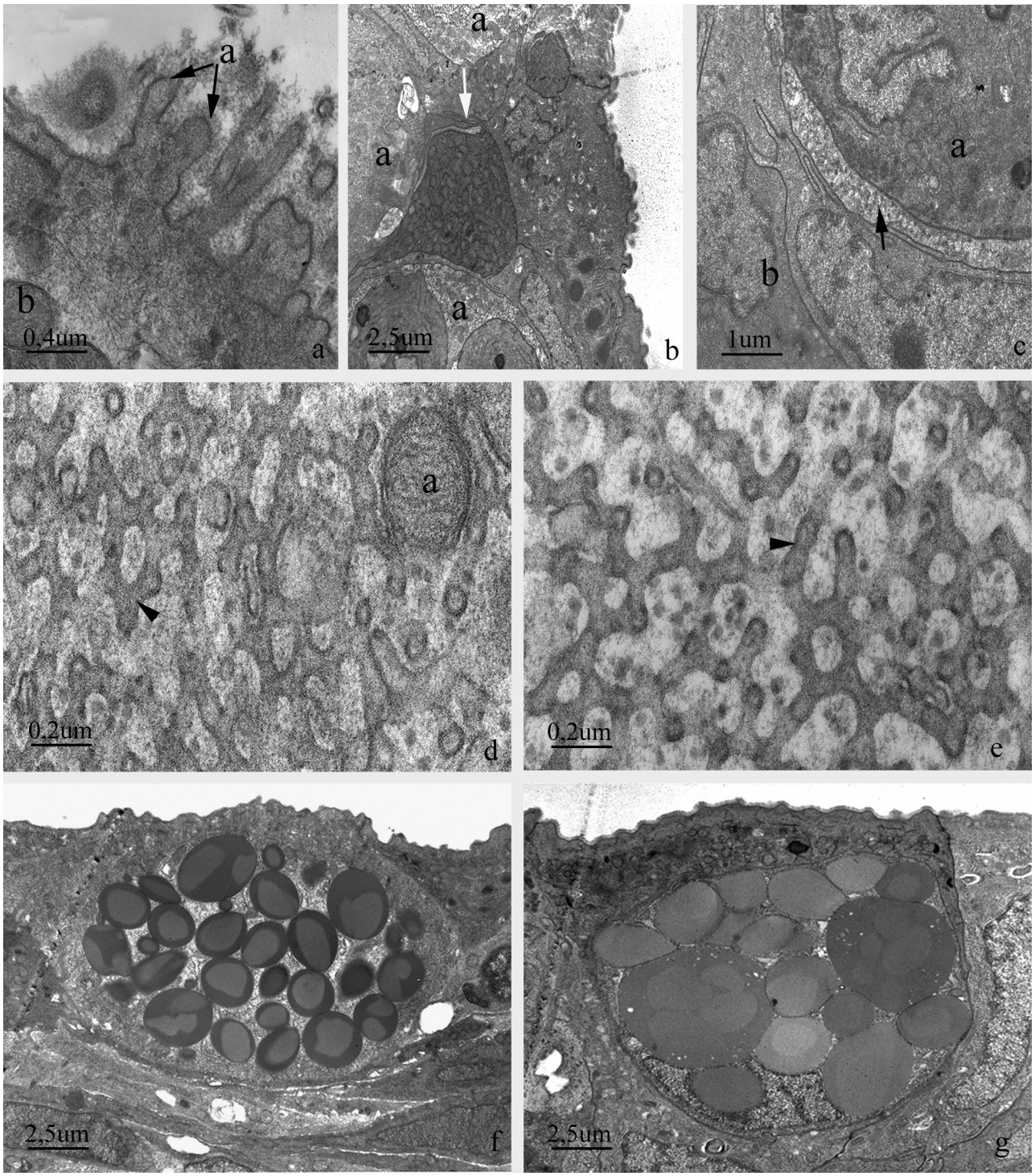

Fig. 5. – Transmission electron microscopy (TEM) of the branchial filament in fresh and brackish water *S. glanis*. a) Surface of the mitochondria rich cell: a, microvilli, b, mitochondria. b) Interdigitations (↑) between several mitochondria rich cells (a) and adjacent accessory cells. c) Mitochondria rich cell (a) with dense cytoplasm (β type) surrounded by a pale cytoplasm of an α type mitochondria rich cell (↑) and pavement cell (b). d) Tubular system in dark MRC: canaliculi (arrowhead), a, mitochondria. e) Tubular system in pale MRC: canaliculi (arrowhead). f) Mucous cell in branchial epithelium of fresh water *S. glanis*. g) Mucous cell in branchial epithelium of brackish water *S. glanis*.

β MR cells, were frequently observed enveloped by α MR cells. This is in accordance with the recruitment theory (Hiroi *et al*. 1999, Lin & Hwang 2004). However, the conspicuous location of these immature cells tightly enclosed by another MR cell subtype is documented for the first time. At this point it is hard to postulate whether or why this situation is only characteristic to *S. glanis*, since as far as we know no such description was previ-

ously given. It is worth noting that even when β cell necrosis is part of the process, the cell can be observed enveloped by intact and active α MR cells.

Another fraction of cells present in both FW and SW are small accessory cells entwined with larger MR cells that have a leaky paracellular single-stranded pathway for cation (Na^+) expulsion (Sardet *et al*. 1979). In *S. glanis,* they were present only in BW fish, with cytoplasmatic interdigitation that dissembled in leaky junction, suggesting the creation of additional pathways that may increase branchial permeability as an adaptive response of channel catfish to the progressive salinity change. Architectural difference was also noticed in salinity challenged *S. glanis* mucus cells and the morphology of their granule compared to fish reared in FW. Their increase in number along with an increase in salinity is correlated with the function of polyanionic mucosubstances secreted by these cells, which attract cations and maintain an ion concentration gradient that provides a microenvironment less exposed to drastic salinity shifts (Fernandes & Perna-Martins 2002). Mylonas *et al*. (2009) observed that mucous cells of the shi drum (*Umbrina cirrosa*) exposed to higher salinity contained acid mucins and are neutral at low salinity, suggesting the change in the mucin production is a salinity-adaptive process.

Evaluating overall appearance and functionality of *S. glanis* gill epithelium, we conclude that even though it shares many characteristics with other euryhaline fish, it also shows a peculiar process of differentiation and maturation of β MR cells enveloped by pale MR cells.

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