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REGULAR ARTICLE

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Appearance of cuboidal cells in relation to salinity in gills of *Fundulus heteroclitus*, a species exhibiting branchial Na^+ but not Cl^- uptake in freshwater

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Abstract *Fundulus heteroclitus* (killifish) is a model organism for ionoregulatory studies, particularly because of its opercular epithelium, although the gills are the major sites of ion exchange. Whereas Na^+ and Cl^- are excreted through the gills in seawater (SW), the killifish is unusual in taking up only Na^+ and not Cl^- at the gills in freshwater (FW). We describe morphological changes in the branchial epithelium following transfer from an acclimation medium of 10% SW to 100% SW or FW. In 10% SW, mitochondria-rich cells resemble typical seawater chloride cells (SWCCs) with accessory cells. After transfer to 100% SW, no change occurs in pavement cell (PVC) morphology or mitotic rate (measured by bromo-deoxyuridine technique), although the density of SWCC apertures increases several fold because of the uncovering of buried SWCCs by PVCs, in accord with increased rates of Na^+ and Cl^- efflux. After transfer to FW, PVC morphology remains unchanged, but SWCCs and accessory cells are quickly covered by PVCs, with many undergoing apoptosis or necrosis. The mitotic rate doubles by 10–14 h but typical freshwater chloride cells (FWCCs) do not appear. Instead, a wedge-shaped cell type that is moderately rich in apically oriented mitochondria, with a large ovoid nucleus, thin cytoplasmic layer, paucity of vesicular-tubular network, and variably villous surface rapidly (by 3 h) and progressively appears in the filament epithelium, by both uncovering and mitosis. This cell type is similar to that recently identified as the site of Na^+ uptake in the FW trout gill. We propose the new term "cuboidal cell" for this cell, based on its morphology, to avoid confusion with traditional terminology (of PVC). We hypothesize that the cuboidal cells are the sites of active Na^+ uptake in FW *F. heteroclitus* and suggest that the lack of Cl^- uptake is attributable to the absence of typical FWCCs previously described in teleosts.

Keywords Cuboidal cells · Chloride cells · Pavement cells · Gills · Osmoregulation · Killifish, *Fundulus heteroclitus* (Teleosti)

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Introduction

The common killifish or mummichog, *Fundulus heteroclitus*, has served as a useful model of euryhalinity (see Karnaky 1986; Wood and Marshall 1994; Zadunaisky 1996; Marshall et al. 1999; Marshall 2003) but appears to employ different ionoregulatory strategies from most other euryhaline teleosts. Perhaps most importantly, when living in freshwater (FW), it takes up Na^+ but not Cl^- at the gills (Wood and Marshall 1994; Patrick et al. 1997a; Patrick and Wood 1999; Wood and Laurent 2003). In the FW killifish, the bulk of Cl^- acquisition comes presumably from the diet, although active Cl^- uptake does seem to occur to a limited extent at the opercular epithelium (Marshall et al. 1997; Burgess et al. 1998).

In general, mitochondria-rich cells (MRCs) present in the gill epithelia of FW and seawater (SW) fish are widely considered as being the site of active Cl^- transport in both FW (Cl^- influx) and SW (Cl^- outflux), in accord with their traditional name "chloride cells". However, their involvement in Na^+ transport, at least in FW fish, is more controversial, with some authors suggesting that the whole filament population of pavement cells (PVCs) either contributes to this process or is involved exclusively in this process (Goss et al. 1992a,b, 1994, 1995; Laurent et al. 1994b; Laurent and Perry 1995; Sullivan et al. 1996; Perry 1997; Claiborne 1998; Wilson et al. 2000; Marshall 2002). Goss and colleagues in particular have championed the

view that a particular sub-population of PVCs are the actual sites of active Na^+ uptake coupled to H^+ excretion in FW (Goss et al. 2001; Galvez et al. 2002; Reid et al. 2003; G. G. Goss, J. Brumbach, G. S. Hawkings, K. M. Gilmour, F. Galvez, in preparation). In the rainbow trout (*Oncorhynchus mykiss*), they have termed these cells MR-PVCs, based on ultrastructural features (irregularly shaped nuclei, dense peripheral chromatin, absence of vesicular-tubular network) that group them with traditional PVCs despite their relative abundance of mitochondria, in contrast to the classic MR-chloride cells (with regularly shaped nuclei, dense vesiculo-tubular network, and abundant mitochondria). They have also termed the MR-PVCs as "PNA-negative MRCs" based on the finding that they do not bind peanut lectin agglutinin (PNA) even though they separate in a density gradient with other MRCs that do bind PNA. The latter PNA-positive MRCs are hypothesized to be the classic MR-chloride cells responsible for active Cl^- uptake and coupled HCO_3^- excretion in FW. They appear to be the same cells as those termed freshwater chloride cells (FWCC) by other workers (cf. Laurent and Perry 1990).

The killifish offers a powerful model system for evaluating these proposals in a euryhaline species. Since *Fundulus heteroclitus* excretes Na^+ and Cl^- at the gills in SW and intermediate salinities, yet takes up only Cl^- but not Na^+ in FW (Wood and Marshall 1994; Patrick et al. 1997a; Patrick and Wood 1999; Wood and Laurent 2003; Scott et al. 2004), we have hypothesized that the MR-PVCs might appear upon FW adaptation, whereas the MR-chloride cells would not be present in the FW-adapted gill. In the present study, we have tested this hypothesis and have performed a detailed ultrastructural analysis of changes in gill epithelial morphology when killifish acclimated to 10% SW are transferred to either 100% SW or FW. Changes in gill morphology have been evaluated by transmission (TEM) and scanning electron microscopy (SEM) and changes in the density of cell populations by the immunodetection of cell proliferation via the bromodeoxyuridine (BrdU) technique (Laurent et al. 1994a). Cell death (apoptosis) has been investigated by conventional methods with a commercially available anti-caspases antibody against the cleavage product of cytokeratin 18. Parallel measurements of unidirectional Na^+ and Cl^- fluxes at the gills by using radioisotopes (Wood and Laurent 2003) have provided functional data to aid interpretation. Overall, our results support the hypothesis stemming from the ideas of Goss and colleagues; we propose a new term (cuboidal cell) based on cell morphology to overcome the confusion associated with terms such as MR-PVC and PNA-negative MRC, which may confound traditional ideas concerning MRC and PVC function in the FW gill.

Materials and methods

Three successive studies have been carried out, which have allowed us to develop a functional interpretation of the structures in relation to ionic fluxes measurements as reported by Wood and Laurent (2003).

In 1978 in Pennsylvania University, Philadelphia, we sampled the first gill arch from six fish (approximately 5 g, 5 cm long) supplied by a pet store from a long-term SW-adapted stock. We experimented on three SW-adapted fish and three fish 1 week after acute transfer to FW. Samples were fixed in 5% glutaraldehyde and 4% sucrose for 1 h (Laurent and Hebibi 1989).

In 1996, killifish (approximately 4.7 g, 3.8 cm long) were collected in estuaries close to Antigonish, Nova Scotia and air-shipped to McMaster University, Hamilton, where they were adapted to 10% SW (~47 mM Na^+, ~57 mM Cl^-, 5.5 Mg^{2+}, 1.7 mM Ca^{2+}) for several months. Fish were fed once daily to satiation with a mix of 50% commercial flakes (Wardley Total Tropical Gourmet Flake Blend, Hartz Mountain) and 50% frozen brine shrimp; feeding was stopped 24 h before BrdU injection (see below). Care was taken to keep the starvation period constant to avoid any differences in mineral loading from the diet. Fish for TEM and SEM analysis only were transferred to FW (dechlorinated Hamilton tapwater: ~0.6 mM Na^+, ~0.7 mM Cl^-, ~0.4 mM Mg^{2+}, ~1.1 mM Ca^{2+}) or 100% SW (~500 mM Na^+, ~550 Cl^-, ~54 mM Mg^{2+}, ~11 mM Ca^{2+}) and killed at periods of up to 7 days after transfer (minimum of three fish per sample time). Additionally, we experimented on five batches of five fish each to examine the rate of cell division in the gills: one batch was sampled in 10% SW, and other batches were sampled after 14 h and 50 h in FW and after 14 h and 50 h in 100% SW. In each case, the fish were injected via the caudal vessel with a dose of 50 mg/kg BrdU (Sigma) in physiological saline (10 ml/kg) 4 h prior to being killed. Therefore, the 14-h sample captured the cell divisions occurring from 10 to 14 h, whereas the 50-h sample captured the cell divisions occurring from 46 to 50 h after transfer. We sampled gill arches, operculum, and intestine for TEM, SEM, and BrdU immunohistochemistry and processed them by the methods described by Laurent et al. (1994a,b), including the methods employed for counting BrdU-labeled cells.

In 2003 in McMaster University, Hamilton, five batches of six fish each (approximately 4.9 g, 4.5 cm long) from the same source as that of the 1996 fish were adapted to 10% SW (composition as above) for several months and then transferred into dechlorinated Hamilton tapwater (composition as above) for various durations. The feeding regime and 24-h feeding suspension were identical to those used in the 1996 study. In each case, the fish were injected with a dose of 50 mg/kg BrdU (Sigma) in 10 ml/kg physiological saline 4 h prior to being killed. Case 1 consisted of fish maintained in 10% SW (i.e., 0 h in FW) and BrdU-injected 4 h prior to sacrifice. Case 2 comprised fish kept for 3 h in FW and BrdU-injected 4 h prior to sacrifice. Case 3 fish were kept for 8 h in FW and BrdU-injected 4 h prior to sacrifice. In case 4, fish were maintained for 12 h in FW and were BrdU-injected 4 h prior to sacrifice. Case 5 fish remained for 3 days in FW and were BrdU-injected 4 h prior to sacrifice. Therefore, cases 1–3 provided a contin-

uous record of cell divisions over the first 12 h after transfer to FW.

Samples collected comprised gill filaments of the second branchial arch, pieces of the middle segment of intestine, and the opercular inner lining carefully dissected from the cartilage. Tissue from all fish was fixed in paraformaldehyde and paraffin-embedded for light immunohistology. In addition, samples from two of the six fish were glutaraldehyde-fixed and embedded in LR White for TEM analysis, or glutaraldehyde-fixed and incubated in 70% ethanol for SEM, or paraformaldehyde-fixed and LR White-embedded for the immunogold technique according to Laurent et al. (1994a,b). Cell proliferation was quantified by the BrdU method according to Laurent et al. (1994a,b). Apoptotic cells were determined by using an anti-caspases antibody against the cleavage product of cytokeratin 18 (Cytodeath Roche) according to the method rcommended by the supplier. All morphometric measurements, including sampling strategies, measurements of surface areas, and counting of apertures, were performed with methods identical to those described in detail by Laurent and Hebibi (1989) and Goss et al. (1994). The results of the 1996 and 2003 studies on *Fundulus* gut and operculum in relation to environmental salinity are not reported here and constitute separate reports. However, the opercular samples were screened for the possible presence of the cuboidal cells described here in the gill epithelium.

Results

We examined the cellular morphology of the filament epithelium from the gills of killifish exposed to various salinities and for various times (Figs. 1, 2, 3, 4, 5, 6, 7, 8, 9, 10) by both TEM and SEM.

Gill epithelial cell components under control conditions (10% SW)

The structural organization of the filament epithelium of *Fundulus* when adapted to 10% SW is illustrated in Fig. 1. The most obvious components are the large MRCs, also known as seawater chloride cells (SWCCs). In spite of the relatively low NaCl content of their adaptation medium, SWCCs are still present in fish adapted to 10% SW, with identical characteristics as those in full strength SW. However, in contrast to fish adapted to 100% SW, not all SWCCs in killifish adapted to 10% SW are in contact with the external milieu through a pit but are sometimes hidden, together with their adjacent accessory cells, within the epithelium (Fig. 2). These cells are presumably not functional because they are not in contact with the external medium.

Under SEM, most SWCCs in killifish adapted to either 10% or 100% SW could be clearly distinguished and easily counted because of their pit openings on the recessed apical membrane. Each pit observed with the SEM corresponded to a single chloride cell accompanied by its accessory cell.

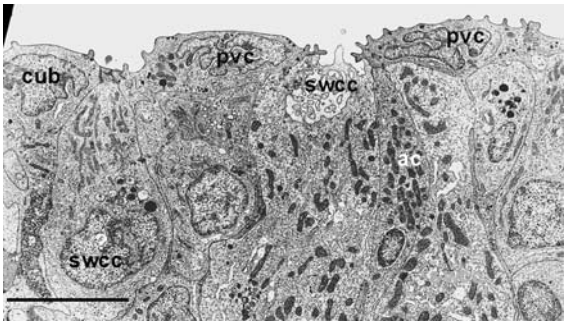

Fig. 1 Representative transmission electron micrograph of the filament epithelium in a killifish adapted to 10% SW. A typical seawater chloride cell (SWCC; *swcc*) is flanked by an accessory cell (*ac*) and pavement cells (PVCs; *pvc*). A second SWCC (*left*) is not open to the environment. Compare the shapes of the cuboidal cell (*Cub*) and the PVCs: the nucleus is notched in the cuboidal cell but elongated and lobate in PVCs. Contrary to FW-adapted *Fundulus*, the apical surface of the cuboidal cell has few microvilli. *Bar* 10 μm

SWCCs were only present on the trailing edge of the filament epithelium, their typical position in most marine fish (Laurent and Dunel 1980) and a position presumably facilitating ion efflux. Additional morphologic criteria indicative of the SW type included: (1) invagination of the basolateral membrane forming an intracellular network of tubules; (2) a rich population of mitochondria in close relationship with the tubules; (3) an accessory cell sending cytoplasmic processes to constitute an intricate system of leaky junctions with the apical surface of the main cell. In *Fundulus* adapted to 10% SW, in those transferred to 100% SW (i.e., the 1996 McMaster University fish), and in those long-term adapted to 100% SW (the 1978 Pennsylvania University fish), SWCCs complied with these criteria (Fig. 1). Interestingly, the morphology of gill SWCCs appeared virtually identical to that of opercular SWCCs. These characters are worth specifying because they differ from those of the FWCCs that occur in other species, and that are known to be different in function (see Discussion).

Apart from the mucus cells that were not the subject of our present study, PVCs accounted for almost all the cell components of the gill epithelia, whereas MRCs counted for no more than 1% of the total. The morphology of PVCs

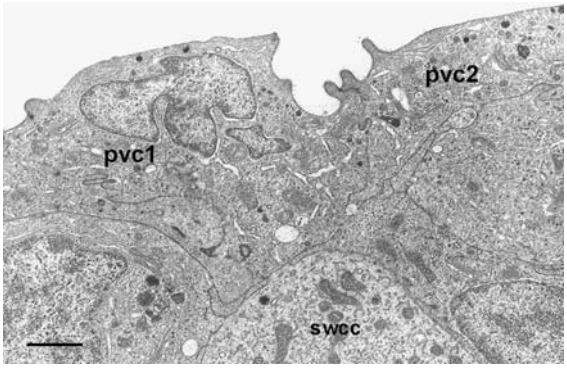

Fig. 2 Transmission electron micrograph of characteristic PVCs (*pvc1*, *pvc2*) on the filament epithelium in a killifish adapted to 10% SW. Note presence of a hidden SWCC (*swcc*); the other cells have not been identified. *Bar* 1 μm

differed according to their location. Those on the lamellar epithelium averaged about 100 μm^2 in surface area but were highly variable in thickness (less than 0.3 μm in some places and up to 3 μm in others) and displayed long and irregular spiny-shaped processes on the external surface and an extremely protruding nucleus. The cytoplasm was poor in mitochondria but contained numerous vesicles and Golgi. Underneath, a 1-μm-thick stroma of connective tissue separated the most external lining from a basement lamina supporting blood capillaries (for more details, see Laurent 1984).

The PVCs constituting the lining of the filament were completely different, displaying an apical surface differing from that typical of most other SW species. In *Fundulus*, the apical surface was unvaryingly ornamented (resembling a fingerprint) with a few concentric ridges on its periphery and various short ridges in the center of a polygonal cell face measuring 10–30 μm in its largest dimension, with a total apical surface area comparable with that of lamellar PVCs. Unlike lamellar PVCs, the internal organization of PVCs in the filament was complex (Fig. 1). The centrally located nucleus exhibited several lobes, and the Golgi system was well developed. Several types of numerous dense or clear vesicles and a rich endoplasmic reticulum filled the cytoplasm. Notably, PVCs on the filament epithelium contained a small population of mitochondria contrasting with the density of other cell organelles (Figs. 1, 2). Long intermembrane junctions (tight) attached PVCs to each other.

Effect of transfer from 10% SW into 100% SW

When *Fundulus* was transferred into full SW, the density of SWCC pit apertures increased several fold by 12 h, presumably by the uncovering of previously hidden SWCCs, and thereafter the morphology was stable and identical to that of individuals that had been long-term adapted to 100% SW. The pits were widely opened, and the apical surfaces of the SWCCs were readily observed. Assuming that the number of open pits reflected the number of active SWCCs, then the functioning rate of this cell population was increased by at least three-fold in 100% SW in comparison with 10% SW. Indeed, our radioisotopic flux data showed that sodium and chloride turnover rates increased by 4.5-fold (Wood and Laurent 2003). Interestingly, BrdU analysis indicated no significant change in mitotic events (see below), and so, the increase in the "open" SWCCs upon transfer to 100% SW appeared to occur entirely because of their exposure by the PVCs. At this time, no organizational changes were observed in PVCs on either the lamellar or filament epithelia.

Effect of transfer from 10% SW into FW

Following transfer into true FW, the most evident change was the rapid disappearance of the SWCC pit openings after only 3 h. This speedy covering process appeared to be attributable to the coordinated movements of the neighboring PVCs (Fig. 3). TEM allowed us to find many aspects of necrosis (Fig. 4) or apoptosis (Fig. 8) in the SWCCs, but other "hidden" SWCCs remained in good morphological condition (Fig. 9). However, their loss of external contact undoubtedly impaired their function. Apical pits of SWCCs were only occasionally seen to persist at 12 h or longer after transfer to FW. Furthermore, typical FWCCs were not observed in our studies on *Fundulus*, as outlined below.

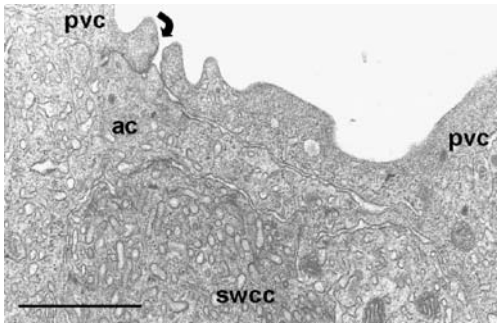

Fig. 3 TEM of filamental epithelium 12 h after transfer from 10% SW into FW: note that a SWCC (*swcc*) and its accessory cell (*ac*) are covered by two PVCs (*pvc*) and that the apical aperture is closing (*arrow*). *Bar* 1 μm

The standard PVC morphology in *Fundulus*, on both the filament and lamellar epithelia, remained stable during and after transfer from 10% SW to FW. However, at this time, we noted the prominent appearance in the filament epithelium of a cell type designated here as the "cuboidal cell" (similar to that earlier identified by Goss and coworkers as an MR-PVC or PNA-negative MRC; see Introduction and Discussion).

Cuboidal cells

Cuboidal cells were found intermingled with the usual PVCs but were easily identifiable by their relatively

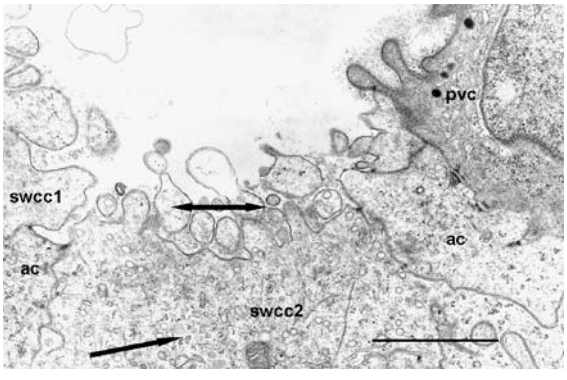

Fig. 4 Necrosis (*arrows*) occurs in the filament epithelium after 48 h in FW, perhaps attributable to osmotic shock in two SWCCs (*swcc1*, *swcc2*) and their adjacent accessory cells (*ac*) not protected by a PVC (*pvc*) covering. These structures may eventually be exfoliated. *Bar* 1 μm

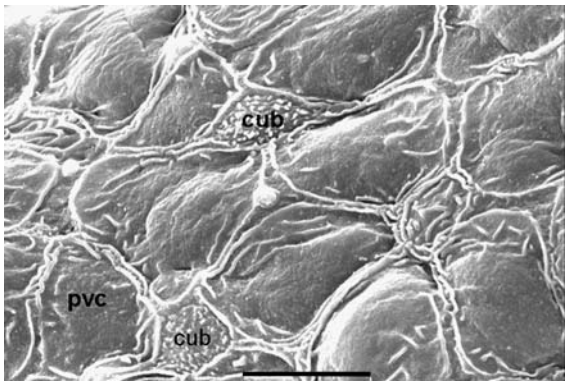

Fig. 5 Representative SEM of filament epithelium 3 h after transfer to FW. Note the apical surfaces of two cuboidal cells (*cub*) emerging between PVCs (*pvc*). *Bar* 5 μm

abundant mitochondria and unusual shape in FW-adapted killifish. This cell type (5–6 μm in length, width, and depth) generally took the form of a massive wedge between the PVCs of the filament lining. The apical surfaces were triangular, square, or rounded, and were either smooth or villous in surface relief (Figs. 5, 6, 7). By TEM, cuboidal cells were approximately cubical or wedge-shaped and displayed a large round or ovoid nucleus, which was notched rather than elongated and lobate as in PVCs. The nucleus was surrounded by a thin envelope of cytoplasm. There was no vesicular-tubular network. Mitochondria, in variable density, were mainly located beneath the apical surface (Fig. 10a–c). Our present investigation could not exclude the possibility that some cuboidal cells were present in the SW-adapted *Fundulus* gill epithelium as hidden dormant cells. However, in animals adapted to 10% SW, we had clear evidence of their presence (Fig. 10a). In addition, we often noticed cells having the same aspect as the cuboidal cells among the several layers of the filament epithelium (e.g., Figs. 2, 3). We interpreted these cells as dormant cuboidal cells.

We observed significant changes in the cuboidal cell population with time after transfer into FW. By 3 h after transfer (the shortest delay that we used), cuboidal cells were present in a proportion of about 10% relative to the

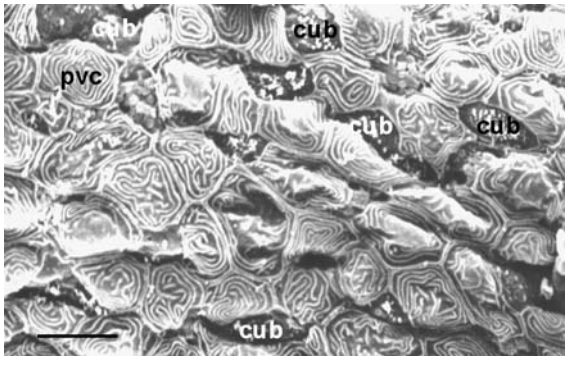

Fig. 6 Representative SEM of filament epithelium 12 h after transfer to FW. Among PVCs (*pvc*), there are more cuboidal cells (*cub*) with larger apical surface areas. Compare with Fig. 5 (3 h). *Bar* 10 μm

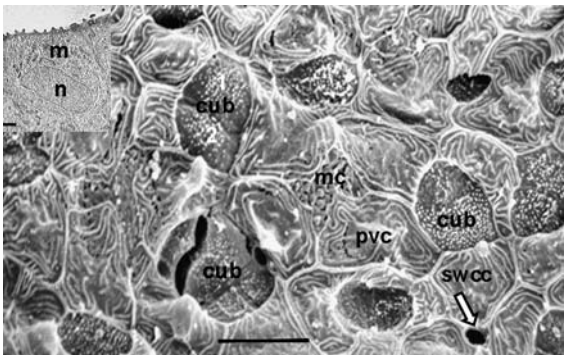

Fig. 7 SEM of filamental epithelium 24 h after transfer to FW, showing clusters of several cuboidal cells (*cub*). Note varied density and distribution of microvilli on their apical surfaces; compare their larger surface areas with those shown in Figs. 5, 6. An apical aperture (*arrow*) of a SWCC (*swcc*) is still visible; a mucus cell (mc) and a PVC (pvc) are also present. *Bar* 10 μm. *Inset* Transmission electron micrograph of transverse section of a clusters of cuboidal cells; only the middle cell is shown in full. Note the number and location of mitochondria (*m*) and the nucleus (*n*). *Bar* 2 μm

total population of PVCs (Figs. 1, 5). Individual cuboidal cell surface area was not as large as that of their neighboring PVCs. After 12 h in FW, cuboidal cells were found singly or sometimes in clusters of two or three (Fig. 7). Furthermore, the apical surface of individual cells was multiplied by a factor of approximately four between 3 h (Fig. 5) and 12 h (Figs. 6, 7) in FW. Their moderately villous aspect contrasted with the fingerprint morphology of PVCs. Between 48 h and 7 days, the morphologic aspect did not change significantly, but we obtained too few data at longer time-points to indicate the final FW-adapted aspect of cuboidal cells. On the other hand, we observed apoptosis of cuboidal cells 12 h after transfer into 100% SW (Fig. 10c).

In contrast with the well-known FWCCs of salmonids (Laurent and Perry 1990), the surface aspect of the cuboidal cells by SEM was generally disorganized. Furthermore, TEM examination demonstrated that *Fundulus* cuboidal cells differed fundamentally from the well-known

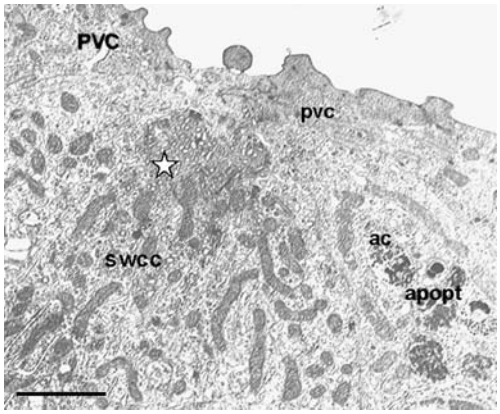

Fig. 8 Transmission electron micrograph of an apoptotic SWCC (*swcc*) and its accessory cell (*ac*) in the filament epithelium, 48 h after transfer to FW. The tubular network within the cytoplasm is disorganized (*star*) and the mitochondria are disrupted. Note the apoptotic bodies (*apopt*) in the accessory cell. *Bar* 2 μm

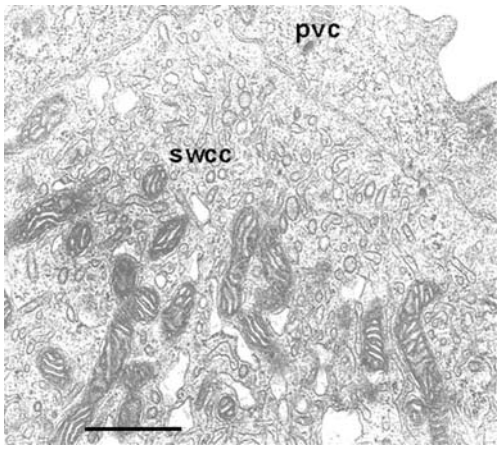

Fig. 9 "Hidden" SWCC (*swcc*) still intact after 5 days in FW. Covered and protected from the external environment by a PVC (*pvc*), this cell might be able to resume its function when the fish returns to SW. *Bar* 2 μm

salmonid FWCCs (cf. the internal anatomy of a *Fundulus* cuboidal cell in Fig. 10a with that of the trout FWCC in Fig. 10d). From their internal organization, cuboidal cells could not be considered as traditional MRCs, because they exhibited no tubular network, a variable density of apical villi, and large variations in mitochondria density, all of which were less than those in typical FWCCs of other species. However, mitochondrial density and typical cuboidal cell size appeared to increase with time after exposure to FW (Fig. 10a,b).

Proliferation versus apoptosis

We conducted two studies to determine proliferation versus apoptosis: (1) by injecting BrdU into *Fundulus* adapted to 10% SW before transfer and at different times after transfer to FW and (2) by immunohistochemistry for caspases.

We noticed that the rate of cell proliferation on the filament epithelium increased abruptly two-fold at 10–14 h after transfer to FW and stabilized thereafter to reach the control values by 46–50 h (Fig. 11). In contrast, no significant change was observed in the rate of cell proliferation at either of these times after transfer to

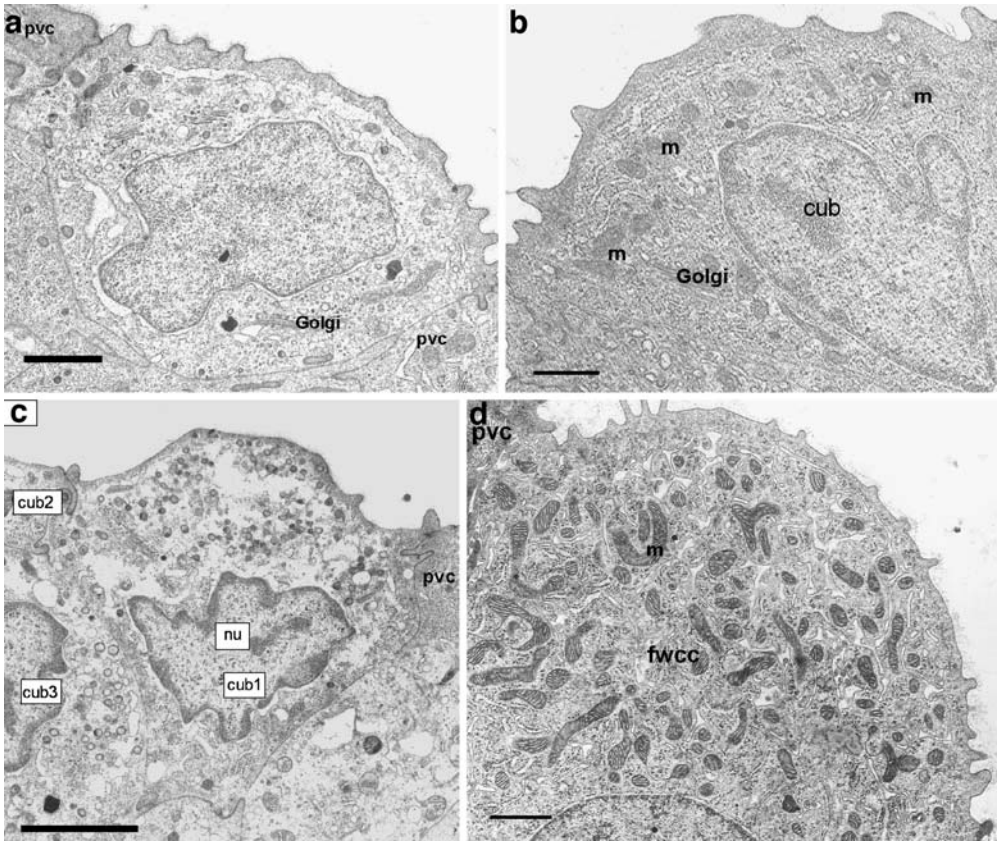

Fig. 10 **a** A representative cuboidal cell, bordered by PVCs (*pvc*). Filament epithelium of fish adapted to 10% SW. Note the abundant Golgi, small population of mitochondria, and nucleus to cytoplasm ratio close to 1. The nucleus is notched but not elongated as in PVCs (see Fig. 1). *Bar* 1 μm. **b** Representative micrograph showing that, after 5 days in FW, typical cuboidal cells (*cub*) are larger; more mitochondria (*m*) are located beneath a convex apical surface. These features are characteristic of cuboidal cells in FW (cf. **a**). *Bar* 1 μm. **c** Example of a cluster of three cuboidal cells (*cub1–3*), 12 h after transfer to 100% SW. Note the smooth apical surface, absence of mitochondria, picnotic nuclei (*nu*), and process of internal membranes vesiculization. *Right* An apparently normal PVC (*pvc*). *Bar* 1 μm. **d** Micrograph of a typical trout freshwater chloride cell (FWCC; *fwcc*) adjacent to a PVC (*pvc*). This cell type has not been found in our study of *Fundulus* but becomes abundant in salmonids transferred into ion-poor water (Laurent et al. 1985). *Bar* 1 μm

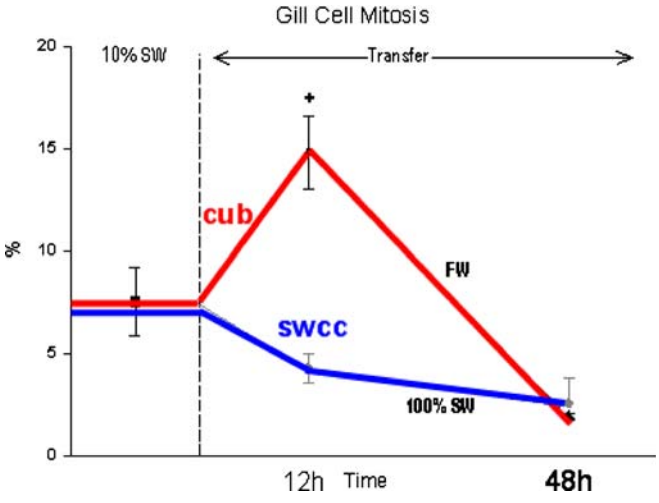

Fig. 11 Changes in the frequency of mitotic events (BrdU-labeled cells expressed as a percentage of total number of cells examined) in the filamental epithelia in killifish at various times after transfer to FW from their adaptation medium (10% SW). At each time point, the BrdU incubation period was 4 h (*cub* cuboidal cells, *swcc* SWCCs). Means ± 1 SEM (n=5 at each time point). $^{+}P<0.05$

100% SW. In a second BrdU series, we found that the mitosis observed under control conditions (10% SW) concerned a few oblong nuclei presumably belonging to PVCs (Fig. 12a). Starting at the 3-h sample point after transfer to FW and continuing through to 12 h, we noted the presence of large rounded nuclei labeled by BrdU inside the filament epithelium (Fig. 12b). However, by 48 h, their incidence declined. Presumably such nuclei belonged to a new cell population, distinct from the PVCs (cf. the size and shape of cell nuclei in Fig. 12b versus a). We hypothesized that these nuclei belonged to new cuboidal cells.

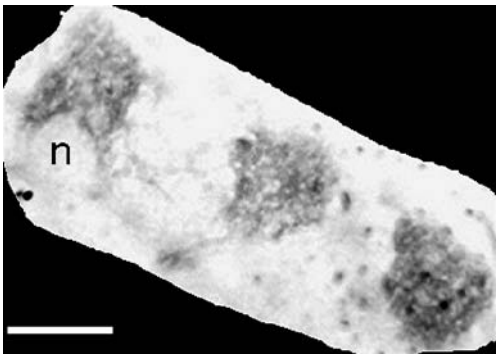

Fig. 13 Immunostaining of caspases (mediator of apoptosis). Filament epithelium 3 h after transfer from 10% SW into FW. The caspase anti-body reveals intense cytosolic labeling in three cells, contrasting with the non-reactive nuclei (*n*). The morphology of the stained cells suggests that they are apoptotic SWCCs. *Bar* 10 μm

Immunohistochemistry was used to confirm that after transfer to FW, SWCCs were affected by apoptosis (Fig. 13) as earlier shown with TEM (Fig. 8). A commercial antibody against caspases confirmed that within 3 h of transfer to FW, a significant proportion of these cells were immunostained. The morphology of the stained cells (Fig. 13) strongly suggested that these were SWCCs affected by apoptosis as shown by TEM observations. Interestingly, whereas immunolabeled cells were numerous at 3 h after transfer to FW, they became scarce by 8 h and remained scarce through 3 days post-transfer. These observations confirmed that apoptosis was a transitory process following transfer into FW.

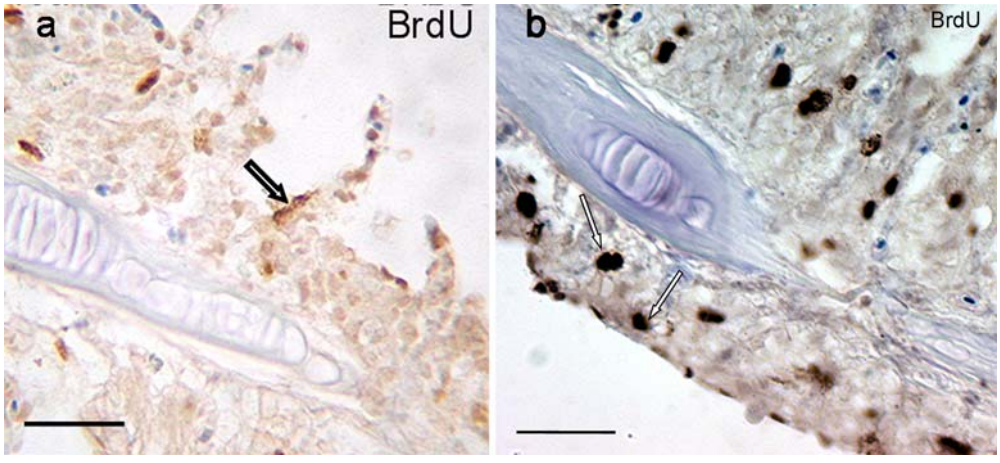

Fig. 12 **a** Mitoses labeled by BrdU observed in filament epithelium of *Fundulus* adapted to 10% SW. Under these conditions, the rate of cell renewal was slow (see Fig. 11). Note that mitosis only concerns elongated nuclei characteristic of PVCs (*arrow*). *Bar* 100 μm. **b** Representative mitotic labeling over a 4-h period after BrdU injection in the filament epithelium of a *Fundulus* originally adapted to 10% SW. In this case, the 4-h period included the first 3 h after transfer to FW, a period when the frequency of mitosis starts to increase (see Fig. 11). Note the change in labeling in comparison with **a**. Mitotic labeling mainly concerns large round nuclei located within the filament epithelium, characteristic of cuboidal cells (*arrows*). *Bar* 100 μm

Discussion

Na^+ and Cl^- excretion when *Fundulus* is transferred into 100% SW

In both 10% and 100% SW, *Fundulus* gill filaments and opercular epithelium contain an abundance of typical SWCCs, opening to the external milieu via apical pits, and bordered by adjacent accessory cells. Similar SWCCs are present in all steno- and euryhaline marine species (Laurent and Dunel 1980) and are considered to be the site of NaCl cotransport, resulting in transcellular Cl^- excretion and paracellular Na^+ excretion via the tight junctions with their associated accessory cells (Silva et al. 1977; Wood and Marshall 1994; for a review, see Marshall 2002). Their persistence in *F. heteroclitus* adapted to 10% SW is in accord with many previous reports indicating that SW morphology is maintained down to a low salinity (1% SW) in this species, at least in the MRCs of the opercular epithelium, which have been studied more extensively than in those of the gills (Copeland 1950; Lacy 1983; Philpott and Copeland 1963; Karnaky 1986). The present study has demonstrated that, whereas SWCCs are still present in the gill filaments in 10% SW (Fig. 1), some of them are hidden inside the epithelium, probably dormant (inactive; Figs. 2, 9). Presumably, when *Fundulus* are returned again to full SW, the SWCCs are quickly re-activated, by withdrawal of the overlying PVCs. Indeed, in the present study, we have observed a several-fold increase in the number of SWCC apertures by 12 h, without any evidence of increased mitosis (Fig. 11). Unidirectional flux measurements with radioisotopes under identical conditions have demonstrated a 4.5-fold increase in unidirectional Na^+ and Cl^- fluxes by 12 h after transfer from 10% SW to 100% SW, and that these elevated fluxes persist unchanged for at least 7 days (Wood and Laurent 2003). Presumably, this represents activation of the classic co-transport system for NaCl excretion. Whole-body Na^+ and Cl^- levels, although disturbed at 12 h, are restored by 3 days (Wood and Laurent 2003). In nature, this pattern of rapid covering and uncovering of the SWCCs probably occurs daily as killifish move back and forth between dilute SW and 100% SW on each tidal cycle (Marshall 2003).

Na^+ uptake when *Fundulus* is transferred into FW

A key point to emphasize in the interpretation of our results is that after *Fundulus* are transferred from 10% SW to FW, unidirectional radioisotopic flux measurements have demonstrated that, within 12 h post-transfer, Na^+ is vigorously and actively taken up at about 20%–30% of the influx rate in 10% SW, but virtually no uptake of Cl^- has been measured at the gills in FW (Wood and Laurent 2003; Scott et al. 2004). The active uptake of Na^+ increases moderately from 12 h to 48 h and is soon sufficient to counter the diffusive efflux of Na^+. Whole-body Cl^- falls to a much greater extent than whole-body Na^+, but both recover by 7 days in fed killifish. This active Na^+ uptake follows a Michaelis-Menten saturable relationship (Patrick et al. 1997a; Wood and Laurent 2003). The flux pattern is stable, at least up to day 7. This agrees with earlier work showing the same strong Na^+ uptake but negligible Cl^- uptake in killifish adapted to FW for up to a month (Wood and Marshall 1994; Patrick et al. 1997a; Patrick and Wood 1999).

Our morphological observations suggest that, when *Fundulus* are transferred into FW, SWCCs become non-functional and are therefore unlikely to represent the sites of active Na^+ uptake. Some undergo apoptosis (Fig. 13), resulting in their elimination (Fig. 8), whereas others appear to be eliminated by necrosis and exfoliation, perhaps following osmotic shock (Fig. 4). Still others are conserved, isolated from the FW, being masked by neighboring PVCs and confined within the innermost layer of the gill epithelium in which they appear to remain dormant (Figs. 3, 9). Deprived of external contact, they keep their structural integrity but are probably inactivated until the next return of the fish to SW. This mechanism seems to be employed even in 10% SW (e.g., Fig. 2), presumably in order to adjust the machinery to need. A similar mechanism of MRC occlusion has been shown to exist in other species of fish when they adapt to changing environmental conditions (Goss et al. 1992a,b; Perry and Laurent 1993; Laurent et al. 1994a; Daborn et al. 2001).

Instead, we propose that the cells that we have here described as "cuboidal cells", and that appear to be comparable to the MR-PVCs or PNA-negative MRCs described by Goss and colleagues (Goss et al. 2001; Galvez et al. 2002; Reid et al. 2003; G. G. Goss, J. Brumbach, G. S. Hawkings, K. M. Gilmour, F. Galvez, in preparation) in *O. mykiss* (see below) are the sites of active Na^+ uptake in FW-adapted *F. heteroclitus*. We further propose that there is no Cl^- uptake from FW because there are no FWCCs in the gills of these animals.

If this concept is correct, then a rapid proliferation of cuboidal cells is involved in the adaptation of *Fundulus* to FW after transfer from 10% SW, in accord with the data of Fig. 11, and in contrast to the situation after transfer to 100% SW. Certainly, increased mitosis of cuboidal cells (the round labeled nuclei do not belong to PVC) is seen within the first 3 h after transfer (Fig. 12b) and continues through 12 h but declines by 48 h. At the same time, the following occurs: the uncovering of apparently dormant cuboidal cells, a three-fold to four-fold increase in their mean apical surface area, and an increase in their mean size and mitochondrial density (Figs. 2, 3, 5, 6, 7, 12a,b). Radioisotopic flux measurements over this period (Wood and Laurent 2003) have demonstrated that, by 12 h, unidirectional Na^+ influx reaches about 70% of the final FW level and stabilizes at the latter by 48 h. In future studies, measurements of unidirectional Na^+ fluxes within the initial 10–12 h period of adaptation to FW will be of interest.

Our present observations sharply contrast with those of Katoh et al. (2001, 2003) and Katoh and Kaneko (2003) who have reported the presence of FWCCs in the gills of FW-adapted *Fundulus*. These cells appear by the rapid

transformation and later replacement of SWCCs after transfer into FW, an observation that we are unable to confirm. Indeed, we have been unable to find any FWCCs in the gill of *Fundulus* after 1 week of transfer into FW, but rather a loss of some of the SWCCs by apoptosis (Figs. 8, 13) and necrosis (Fig. 4) and a paving over of the remainder by the PVCs (Figs. 3, 9). At the same time, cuboidal cells appear and increase their apical exposure (Figs. 1, 6, 7, 10), associated with an increase in mitosis (Figs. 11, 12b).

The reasons for the differences between our results and those of Katoh et al. (2001, 2003) and Katoh and Kaneko (2003) are unclear. Many of the pictures shown in these papers resemble traditional FWCCs, but these workers have not measured Na^+ or Cl^- uptake rates in their fish. Differences in experimental protocols and specific water chemistries may be involved: Katoh and Kaneko (2003) transfer their killifish directly from 100% SW to FW, whereas we transfer our killifish from 10% SW to FW. A possible associated explanation is the resulting differences in time courses between the studies. In the present study, we have tracked gill morphology for only up to 7 days after transfer to FW. If FWCCs differentiate much later after the less severe transfer in our fish, we suggest that their functions could be related to Ca^{2+} uptake (Perry and Wood 1985; Patrick et al. 1997b). Another important possibility might involve differences in killifish races. We have used wild-caught animals from the north-east coast of North America ("northern race"), whereas Katoh et al. (2001, 2003) and Katoh and Kaneko (2003) have employed an inbred laboratory population of the Arasaki strain (Shimizu 1997). Important differences have recently been shown for FW adaptation parameters in wild-caught North American *F. heteroclitus* between "northern" and "southern" races (Scott et al. 2004). These differences are especially evident in the relative abilities to regulate plasma Cl^- and diffusive Cl^- efflux after transfer, although in both races, active branchial Cl^- influx is negligible. Scott et al. (2004) have also reported differences in FWCC abundance between the two races but have used only surface morphology by SEM to distinguish FWCCs from SWCCs; they may have mistaken cuboidal cells for FWCCs. Certainly some cells identified as FWCCs or "intermediate cells" in their report appear to be cuboidal cells by our criteria. Furthermore, the inset in Fig. 4e in the paper of Katoh and Kaneko (2003) suggests to us the apical surface of a well-developed cuboidal cell, so morphological criteria may also be a source of some of the differences.

In most fish living in FW, both Na^+ and Cl^- are actively taken up from FW to compensate diffusive efflux. In early work, evidence was presented for the electroneutral exchange of both Na^+ [$Na^+/H^+(NH_4^+)$] and Cl^- [Cl^-/HCO_3^-], in some way coupled to Na^+,K^+ ATPase activity in FWCCs (Krogh 1939; Maetz and Garcia-Romeu 1964; Garcia-Romeu and Maetz 1964). These systems were later averred impossible for thermodynamic reasons. Avella and Bornancin (1989) have proposed instead that Na^+ uptake across the apical membrane occurs via selective Na^+ channels energized by a vacuolar H^+-ATPase. The identity and location of the Na^+ channel has still not been established in FW fish gills, but immunolocalization studies suggest that vacuolar H^+-ATPase is localized in the apical membranes of both PVCs and FWCCs in trout, but preferentially in the apical membranes of PVCs in FW-adapted tilapia (Wilson et al. 2000; for a review, see also Perry et al. 2003). However, an apical Na^+/H^+ exchanger has also been detected in tilapia. An alternative ion uptake scheme consisting of an amiloride-sensitive Na^+/H^+ exchanger (an apical non-selective NHE protein) in PVCs and an apical Cl^- uptake mediated by an electroneutral Cl^-/HCO_3^- anion exchange protein in FWCCs (i.e. the Maetz and Garcia-Romeu model, but in different cell types) has therefore been proposed for FW-adapted tilapia. Curiously, Katoh et al. (2003) have reported the presence of a basolaterally located V-ATPase in apparent FWCCs in the gills of *Fundulus* and suggest that it may power Na^+ and/or Cl^- uptake. In the FW-adapted stingray, Piermarini and Evans (2001) have argued that a similar V-ATPase localization serves to power apical Cl^- uptake by an apical Cl^-/HCO_3^- exchanger. However, since there is essentially no Cl^- uptake by the gills of *Fundulus* (at least in the first few weeks after transfer; Patrick et al. 1997a,b; Patrick and Wood 1999; Wood and Laurent 2003; Scott et al. 2004), this explanation cannot apply in the case of the present fish.

Interestingly, in the population of *Fundulus* studied here, Scott et al. (2005) have recently shown that the gills (which take up Na^+ but not Cl^-) have a fundamentally different pattern of mRNA expression from the opercular epithelium (which actively takes up small amounts of Cl^- but not Na^+; Marshall et al. 1997; Burgess et al. 1998) after transfer to FW. Although the aim of the present paper has not been to report on the opercular epithelium, we have sought but not found cuboidal cells in this tissue, irrespective of the

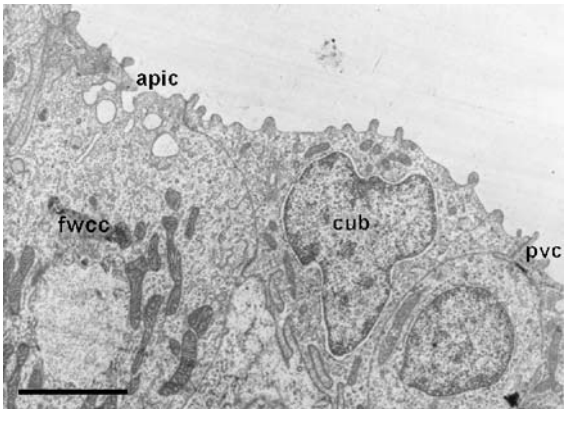

Fig. 14 Catfish gill filament epithelium. This micrograph provides a significant example of the concomitant presence, side by side, of a typical FWCC (*fwcc*, not observed in *Fundulus*) and a cuboidal-like cell (*cub*) in a FW teleost. Their simultaneous presence establishes that they are distinct entities. In this FW catfish, parallel influxes of Cl^- and Na^+ occur contrary to *Fundulus* in which no Cl^- influx exists. A specific population of cells has been considered as a site of Na^+ uptake (see Fig. 6b in Goss et al. 1994). This population co-habits with FWCC in most teleosts but not in *Fundulus*. However, comparison with Fig. 10a of the present paper suggests great similarity with the cuboidal cells of FW-adapted *Fundulus*. *Bar* 10 μm

environmental conditions. We have however noted the presence of numerous typical SWCCs in this epithelium in *Fundulus* adapted to 10% SW and full SW. In contrast, after 12 h in full FW, many buried SWCCs (presumably not functioning), but no FWCCs or cuboidal cells can be observed on the opercular epithelium, as far as we are presently aware.

Based on these expression data, Scott et al. (2005) have proposed that gill Na^+ uptake occurs via apical Na^+/H^+ exchange (NHE-2) fueled with H^+ ions by intracellular carbonic anhydrase (CA2) in our FW-adapted *Fundulus*. Branchial V-ATPase, whose expression does not change after transfer to FW, plays no direct role in active Na^+ uptake in this model. Recent protein expression studies with specific antibodies for various NHE isoforms also support the involvement of NHE-2 as a key branchial Na^+/H^+ exchanger in FW-adapted *Fundulus* (Edwards et al. 2005). In future studies, positive immunodetection of the Na^+/H^+ exchanger in the apical membrane might help to support our theory that cuboidal cells are specific sites of Na^+ uptake in FW-adapted *Fundulus*.

Are cuboidal cells a type of PVC?

Throughout our many studies on gill morphology, we have had extensive opportunity to observe and compare PVCs among many different species and have found strong inter-specific differences. In marine fish, the apical ornamentation of PVC resembles fingerprints, consisting of concentric circles (Laurent and Dunel-Erb 1980). This is the case in *Fundulus* in which the standard PVC apical surface does not change after transfer into FW. On the contrary, in tilapia, bullhead catfish, trout, and eel, changes in the apical ornamentation of PVCs are uniformly observed in the whole filament epithelium after transfer to FW (Laurent and Perry 1995; Goss et al. 1995). However, we believe that whether the cell identified here as a cuboidal cell is really a type of modified PVC or a unique cell type remains an open question; for this reason, we have given it this new name, based solely on its morphology and without the confounding connotations of grouping it with PVCs or MRCs, because its morphology is so dramatically different from that of standard PVCs. The most important of these morphological differences from the traditional PVC are: (1) the cubic shape of the cell; (2) the round, often notched shape of the nucleus; (3) the location of the mitochondria within the sub-apical portion of the cell; (4) the infolded baso-lateral cell membrane; (5) the numerous fine macro-villi on the apical cell membrane, with increased density in FW-adapted individuals.

We must emphasize that many studies have argued for the presence of at least two and sometimes three different types of MRC in the gills of FW fish; Wilson and Laurent (2002) provide a detailed review. From this, it is clear that classification criteria vary greatly between studies; indeed, a functional analysis has been performed only in rainbow trout (*O. mykiss*). Based on the finding that the gills of *F. heteroclitus* take up only Na^+ and not Cl^- from FW, we believe that the killifish cuboidal cell is probably analogous or homologous to the MR-PVC or PNA-negative MRC of the FW-adapted trout gill, which Goss and colleagues have identified as the primary site of Na^+ uptake (Goss et al. 2001; Galvez et al. 2002; Reid et al. 2003; G. G. Goss, J. Brumbach, G. S. Hawkings, K. M. Gilmour, F. Galvez, in preparation). In classifying this cell as a modified PVC, Goss et al. (2001) and Galvez et al. (2002) have used morphological criteria of similarity to PVCs but have examined only dispersed rounded-up cells, rather than cells in situ. The characteristic cubic shape of the cuboidal cell or flat shape of the PVC would thus not have been seen, and so the diagnosis is problematic. Nevertheless, these workers have developed a powerful technique, based on differential gradient centrifugation, PNA binding, and magnetic bead separation, to separate these PNA-negative MRCs from PNA-positive MRCs (probably traditional FWCCs), allowing the demonstration of preferential Na^+ uptake in the former (Reid et al. 2003; G. G. Goss, J. Brumbach, G. S. Hawkings, K. M. Gilmour, F. Galvez, in preparation). It would be of great interest to apply these techniques to the *Fundulus* gill.

Cuboidal cells might be present in a number of other teleost species but may have been previously overlooked. The similarity of the cuboidal cells observed in *Fundulus* gill in FW to cells previously considered to be a particularly active type of PVC in the gills of brown bullhead catfish after cortisol injection is exciting (Goss et al. 1994) in light of recent findings concerning the involvement of cortisol in adaptation to FW and branchial epithelial cell proliferation in *F. heteroclitus* (G. Scott, personal communication). A representation (Fig. 14) taken from the study of Goss et al. (1994) demonstrates that the putative cuboidal cell and the FWCC might co-exist in the gills of this species. Furthermore, we have also found cuboidal-like cells in the gills of the FW-adapted eel, a teleost that, like the killifish, takes up Na^+ but not Cl^- from FW (Kirsch and Mayer-Gostan 1972; Hyde and Perry 1987; Grosell et al. 2000). More investigations are thus needed to clarify the respective roles of traditional PVCs, FWCCs, and cuboidal cells in a range of FW fish species.

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