# BRANCHIAL CHLORIDE CELLS IN LARVAE AND JUVENILES OF FRESHWATER TILAPIA *OREOCHROMIS MOSSAMBICUS*

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## Summary

Branchial chloride cells in the developing larvae and juveniles of freshwater tilapia, *Oreochromis mossambicus*, were identified and the membrane Na$^+$/K$^+$-ATPase was localized *in situ* through binding of the fluorescent dye anthroylouabain. After co-labelling of the cells with the fluorescent probes DASPMI and Con-A-FITC, the mitochondria and apical crypt in the same chloride cells were visualized using confocal laser scanning microscopy. The high density of apical crypts indicated that many chloride cells were functional. The density of branchial chloride cells in larvae 10 days after hatching was approximately 6000 mm$^{-2}$. An extremely high Na$^+$/K$^+$-ATPase specific activity of approximately 1500 $\mu$mol P$_i$ h$^{-1}$ mg$^{-1}$ was measured in the gills 10 days after hatching. With the development of secondary lamellae and hence an increase in the amount of branchial epithelial protein, a concomitant decrease in the specific activity of the enzyme in the gill tissues was observed. Total Na$^+$/K$^+$-ATPase activity increased markedly in the early life stages. Our data indicate that in larval stages of fish the gills form a functional ionoregulatory organ before they start functioning as a gas-exchange organ.

Key words: chloride cells, larvae, juveniles, freshwater tilapia, *Oreochromis mossambicus*, fish gills, Na$^+$/K$^+$-ATPase, mitochondria, Concanavalin-A, confocal laser scanning microscopy.

## Introduction

In the gills of adult fish, two morphologically distinct epithelia are found: the multilayered filament epithelium, which is largely involved in ion exchange, and the bilayered lamellar epithelium involved in gas exchange between the blood and the ambient water (Laurent, 1984). For a variety of species, including the tilapia studied in this paper, it has been demonstrated that the chloride cells (or ionocytes) of adult fish are mainly located in the filament epithelium (Wendelaar Bonga *et al*. 1990). These cells are the site of the active Ca$^{2+}$ transport that underlies transepithelial Ca$^{2+}$ uptake, in both freshwater and seawater fish (Marshall *et al*. 1992; McCormick *et al*. 1992; Verbost *et al*. 1994). Although their role in NaCl excretion in seawater species is clearly established (Foskett and Scheffey, 1982), their role in NaCl handling in freshwater fish is currently under debate: some conclude that these cells are involved in the uptake of both Na$^+$ and Cl$^-$ (e.g. Laurent *et al*. 1985; Laurent and Perry, 1990), others believe that the chloride cells are the site of Cl$^-$ uptake alone and that uptake of Na$^+$ occurs through respiratory cells (Goss *et al*. 1992a,b; Laurent *et al*. 1994; Morgan *et al*. 1994).

In general, a functional chloride cell is structurally characterized by the presence of many mitochondria, an abundant tubular system, where ion-transporting enzymes such as Na$^+$/K$^+$-ATPase, Ca$^{2+}$-ATPase and a Ca$^{2+}$/Na$^+$ exchanger are located, and an apical crypt facing the water (Hootman and Philpott, 1979; Flik *et al*. 1985; Wendelaar Bonga *et al*. 1990; Flik and Verbost, 1993).

The embryos and larvae of teleosts are able to maintain a constant hydromineral level in their body fluids, even though their gills and kidneys appear poorly developed (Alderdice, 1988). During the early developmental stages of teleosts, extrabranchial chloride cells located in the skin have been proposed as the site of ionoregulation. Indeed, Shelbourne (1957) suggested that these integumental chloride cells play an ion-transporting role in larvae of the plaice *Pleuronectes platessa*. Later, several studies focused on extrabranchial chloride cells in a number of embryonic and larval stages of teleosts (Alderdice, 1988; Hwang, 1989, 1990; Ayson *et al*. 1994). Hwang *et al*. (1994) reported that the first chloride cells to appear in the skin of freshwater tilapia embryos were found 48 h after fertilization. Some chloride cells with apical crypts facing the water were observed in the skin of newly hatched larve (96 h post-fertilization). They suggested that, up to 10

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days after fertilization, these integumental chloride cells are the main site of active ion transport in freshwater tilapia. However, little is known about the development of branchial chloride cells in fish larvae and when they become operational.

We have applied morphological, histochemical and biochemical methods to identify functional chloride cells in the gills of larval and juvenile freshwater tilapia. Three criteria were advanced to identify mature chloride cells (i.e. cells in contact with the water). First, through their abundance of mitochondria, chloride cells were visualized with a mitochondrion-specific, fluorescent vital stain. Second, through their high $Na^+/K^+$-ATPase content, the same cells were visualized with the fluorescent dye anthroylouabain (which binds to an extracellular binding site on the enzyme). Third, using fluorescein-conjugated Concanavalin-A, which appears to bind specifically to glycoproteins in the apical crypt of chloride cells, the presence of apical crypts was demonstrated. We used live tissue for our morphological studies. The specific and total $Na^+/K^+$-ATPase activities of gills were measured biochemically.

## Materials and methods
### Animals

Batches of eggs close to hatching were collected from adult female tilapia *Oreochromis mossambicus* (Peters). At 27 °C, hatching occurs approximately 4 days after fertilization (Lanzing, 1976; Hwang *et al.* 1994). Groups of at least six larvae and juveniles 3, 10 and 24 days after hatching, ranging in mass from 4 to 150 mg, were used. Masses refer to the animals after their yolk sac had been removed. A comparison was made with 180-day-old fish (adult). Animals were kept in Nijmegen tap water containing (in $mmol\,l^{-1}$): $Na^+$, 5.0; $K^+$, 0.06; $Ca^{2+}$, 0.8; $Mg^{2+}$, 0.2; $Cl^-$, 4.2; $SO_4^{2-}$, 0.5; pH 7.6; the water temperature was kept constant at 27 °C. Lights were on for 12 h per day. Fish were killed by spinal transection. For biochemical studies, gill arches were excised under a binocular microscope and kept in a Ringer's solution containing (in $mmol\,l^{-1}$) NaCl, 120; KCl, 5.0; $CaCl_2$, 1.25; $MgCl_2$, 1.0; Hepes, 10.0; glucose, 10.0; pH 7.4 (adjusted with Tris) at 4 °C until further use.

### Vital staining and density measurement of chloride cells

When 'baby' fish were studied, they were allowed to swim in vital stain dissolved in the water. The fish were first placed in Concanavalin-A–fluorescein conjugate (Con-A-FITC; $1\,mg\,ml^{-1}$) for 45 min. This probe binds to the carbohydrate groups of glycoproteins (Blithe *et al.* 1982; Angelides *et al.* 1988) that are present in the apical crypts of the chloride cells (Pisam, 1981). The procedure was completed by co-staining with the specific mitochondrial probe 2-(4-dimethylaminostyryl)-1-methylpyridinum iodide ($5\,\mu mol\,l^{-1}$; DASPMI, Molecular Probes, Eugene, USA) for 5 min. The fish were then killed by spinal transection and the gill arches excised, rinsed three times in Ringer's solution and placed in

$20\,\mu l$ of Ringer's solution in a rounded silicone chamber on a glass microscope coverslip. Using this method, we prevented the Con-A-FITC from entering the tissue through the cut edges (DASPMI, in contrast, is very lipophilic and will penetrate the tissue easily from the water). The successful staining of the apical crypts of chloride cells by this *in vivo* labelling technique suggests that the gills of the very young fish are being ventilated. When adult specimens were examined, individual filaments of the first and second gill arches were cut and stained using Ringer's solution to which Con-A-FITC and DASPMI had been added (at the same concentrations as above).

A confocal laser scanning microscope (CSLM, MRC-600, Bio-Rad) was employed to identify the chloride cells. Imaging conditions were as follows: we used the 488 nm line of the argon ion laser as the excitation wavelength and a filter block that allowed the concurrent collection of the emission wavelengths of Con-A-FITC (530 nm) and DASPMI (609 nm). All images were recorded and processed with an IBM-compatible computer. The objective lenses used were a 10× and a 40× oil-immersion type. The thickness of optical sections was $0.5\,\mu m$.

In gills of juvenile fish, chloride cells were equally distributed throughout the (single layer) branchial epithelium. Optical sections were made in a plane parallel to the surface of the filament. For chloride cell counting, at least four optical sections of different filaments from each fish were randomly photographed. In gills of adult fish (where secondary lamellae have developed, the chloride cells occur mainly on the trailing edge of the filament and where the epithelium has become multilayered), optical sections were made through the superficial cell layer of the epithelium.

All DASPMI-stained cells in the surface layer of the filaments were counted. From each recorded image ($0.5\,mm^2$), three areas of $2500\,\mu m^2$ were analysed and the density of stained cells was expressed per $mm^2$ of section area. In total, three filaments from each fish and at least four different fish per group were analysed in this way. For comparison of chloride cell densities during development, we refer to the total filament surface in juvenile stages and to the trailing edge area of the filament in adult fish. Sites were chosen at a point one-third of the way along the filament.

### Localization of $Na^+/K^+$-ATPase in chloride cells

Freshly excised first and second gill arches were incubated in $5\,\mu mol\,l^{-1}$ (final concentration) anthroylouabain (Sigma) in low-$K^+$ tilapia Ringer's solution (LKTR) for 60 min at room temperature in the dark. The LKTR solution contained (in $mmol\,l^{-1}$): NaCl, 140; $NaHCO_3$, 10; $MgSO_4$, 1; $CaCl_2$, 2; $NaH_2PO_4$, 1; KCl, 0.1; Hepes, 10; glucose, 5, adjusted to pH 7.4 with Tris. As a control, in some samples, a high concentration ($1\,mmol\,l^{-1}$) of non-fluorescent ouabain (Sigma) was added to the incubation medium to confirm the specific binding of anthroylouabain to membrane $Na^+/K^+$-ATPase (McCormick, 1990). After labelling with the fluorescent dye, the tissues were rinsed for 2 min in LKTR and placed on a

microscope coverslip as described above. Anthroylouabain has an excitation wavelength of 365–368 nm and an emission wavelength of 480 nm (Fortes, 1977). For these observations, a Zeiss epifluorescence microscope was used at a magnification of 250×.

*Determination of Na$^+$/K$^+$-ATPase activity*

The Na$^+$/K$^+$-ATPase activity in whole gill tissue homogenate was determined as described by Flik *et al.* (1983). Freshly dissected gills of each larval, juvenile or adult fish were placed in 100 $\mu$l of membrane isolation medium containing (in
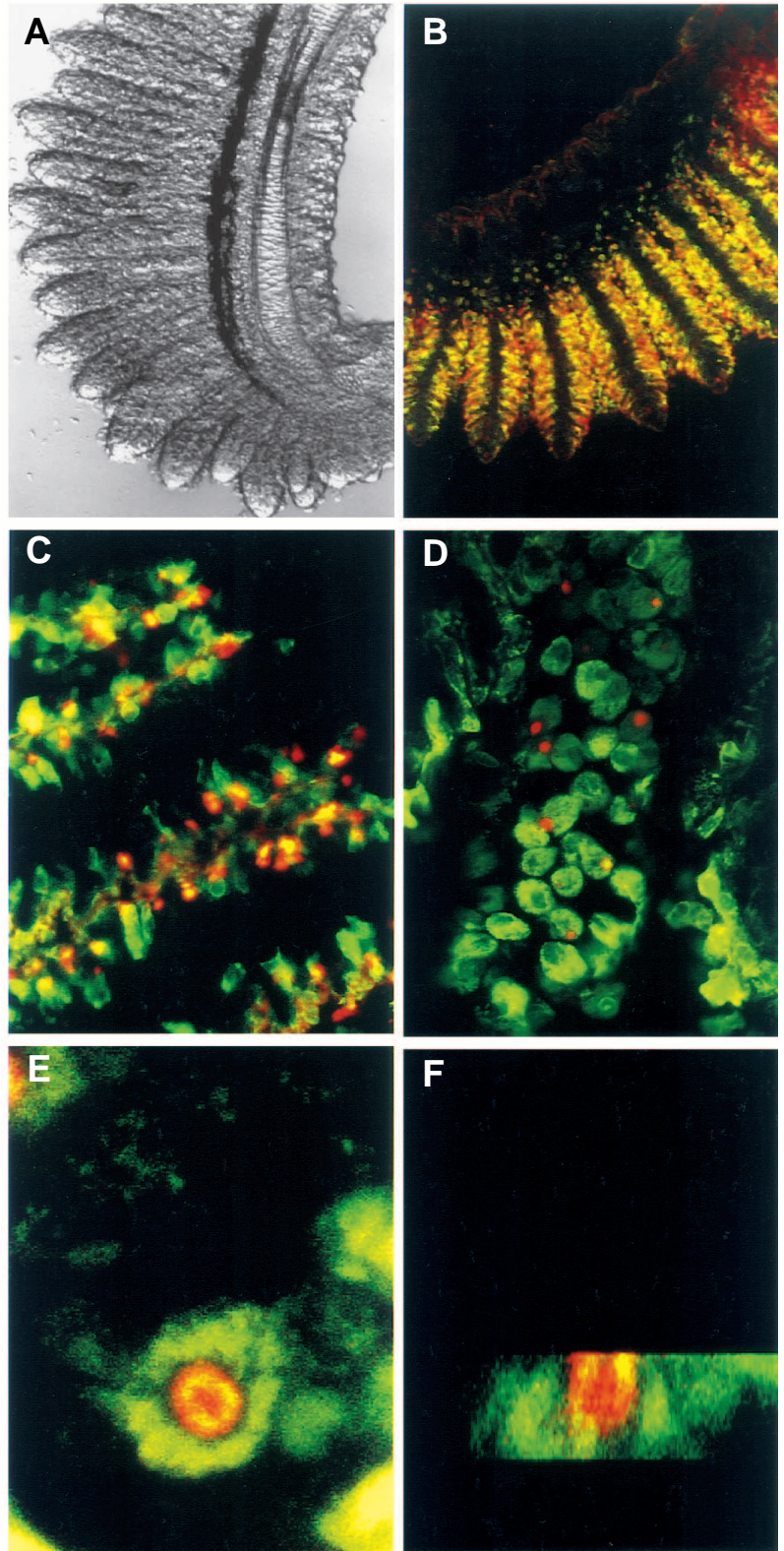


Fig. 1. Confocal laser scanning micrographs of the gill filaments of a 3-day-old freshwater tilapia after *in vivo* co-labelling with Con-A-FITC (red) and DASPMI (green). (A) Second gill arch, transmission light microscopy; the central cartilage can be seen in the gill arch; filament tips point to the left of the picture; magnificaion 100×. (B) Confocal cross section (section thickness 0.2 $\mu$m); magnification 100×. Note that the DASPMI-positive cells form an essentially confluent sheet. The apical crypts (red spots) are found only at the interface with the water. The central part of the filament is almost free of dye. Filament tips point towards the bottom of the picture. (C) Confocal cross section (section thickness 0.2 $\mu$m) near the gill arch; apical crypts, indicated by Con-A-FITC staining, face the water; filament tips point to the bottom left of the picture; magnification 400×. (D) Superficial optical section (thickness 0.2 $\mu$m) through the filament tip; rounded DASPMI-positive cells with a nuclear shadow are seen; some of the cells in this section shown their apical crypt (red spots); magnification 480× (including a zoom factor of 1.2×). The filament tip points towards the bottom of the picture. (E) A single DASPMI-positive cell co-labelled with Con-A-FITC. This picture is composed of 31 superimposed and subsequent optical sections (0.5 $\mu$m each) collected in the *z*-direction. Note the absence of DASPMI staining adjacent to the Con-A-FITC-labelled space, in accordance with the absence of mitochondria in this region shown by electron microscopy (see Fig. 2). Magnification 900× (including a zoom factor of 1.5×). (F) Reconstructed cross section (90° tilted) of the cell shown in E; the cross section was made perpendicular to the plane of view shown in E, from left to right. This side view shows the depth of the apical crypt. Magnification 900× (including a zoon factor of 1.5×).

mmol l$^{-1}$): sucrose, 250; NaCl, 15; Na$_2$EDTA, 0.5; Hepes, 5, adjusted to pH 7.4 with imidazole and 2 $\mu$l ml$^{-1}$ aprotinin (Sigma, 1.9 mg ml$^{-1}$ protein, 3.6 trypsin inhibitor units per mg protein), and 1 mmol l$^{-1}$ dithiothreitol (DTT) was added immediately before use. The tissue was homogenized by sonication for 10 s. To remove cellular debris and cartilage rods, the homogenate was centrifuged for 5 min at 4000 $g$, and the supernatant analysed for Na$^+$/K$^+$-ATPase activity. Optimal Na$^+$/K$^+$-ATPase activity was obtained by adding saponin (0.2 mg mg$^{-1}$ protein) to permeabilize the membranes (Verbost *et al*. 1994). Protein was determined using a commercial Coomassie Blue kit (Bio-Rad), using bovine serum albumin as a reference.

### Electron microscopy

Gill filaments from juvenile tilapia were dissected and fixed for electron microscopy as described by Wendelaar Bonga and Van der Meij (1989).

## Results

### General observations

Three days after hatching, larvae had not yet absorbed their yolk sac. Without the yolk sac, they weighed about 4 mg and had a body length of approximately 7 mm. On each side of the head, four newly developed gill arches could be distinguished microscopically. The first and most exterior arch was slightly bigger than the second one; the third and fourth arches were smaller still. Gill filaments were already present, in particular on the first two arches. Under a phase-contrast microscope, numerous large, spherical or ovoid granular cells with a centrally located nucleus, which resembled chloride cells, could be observed in the epithelium of filaments (Wendelaar Bonga *et al*. 1990; McCormick, 1990). Lamellae had just started to develop on day 3. In 10-day-old larvae, the yolk sac was essentially absorbed and lamellae had begun to develop on both sides of all filaments. In 24-day-old larvae, the yolk sac had completely disappeared and adult-like lamellae were observed.

### Chloride cells

The numerous large, ovoid cells with prominent nuclei seen on the newly developed gill filaments in 3-day-old tilapia using phase-contrast microscopy were identified as chloride cells by DASPMI staining using the CLSM; apical crypts were demonstrated by co-labelling the cells with Con-A-FITC (Fig. 1). The filamental epithelium consisted of a single layer of DASPMI-positive cells. Essentially all the cells had apical crypts as indicated by Con-A-FITC staining. The presence of apical crypts was confirmed by electron microscopical observations (Fig. 2). Fig. 3 presents the density of chloride cells (mm$^{-2}$): 4630±489 in 3-day-old fish, 6280±271 in 10-day-old fish and 6600±219 in 24-day-old fish. In adult fish, the density of chloride cells in the trailing edge area was 6519±710 mm$^{-2}$.

### Localization and activity of Na$^+$/K$^+$-ATPase

Exposure of isolated larval gills to 5 $\mu$mol l$^{-1}$ anthroylouabain in low-K$^+$ Ringer's solution resulted in the appearance of many fluorescent cells (Fig. 4). These cells appeared to be identical to the DASPMI-stained cells on the basis of their size, morphology and location. A shadow caused
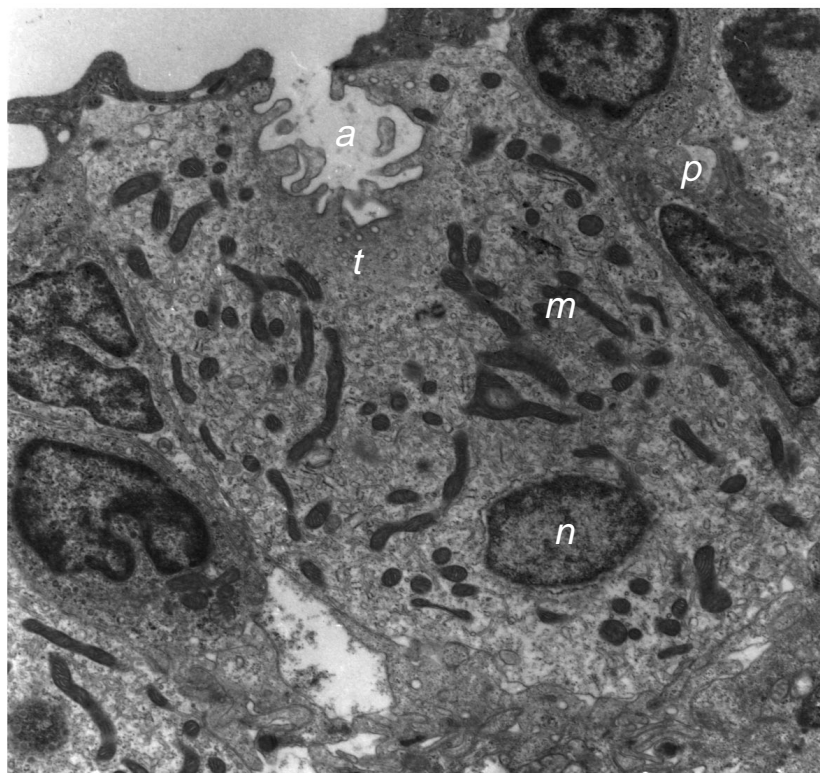


Fig. 2. An electron micrograph of a chloride cell from a 3-day-old freshwater tilapia. *t*, tubular system; *m*, mitochondria; *a*, apical crypt; *n*, nucleus; *p*, pavement cell. Magnification 8000×.
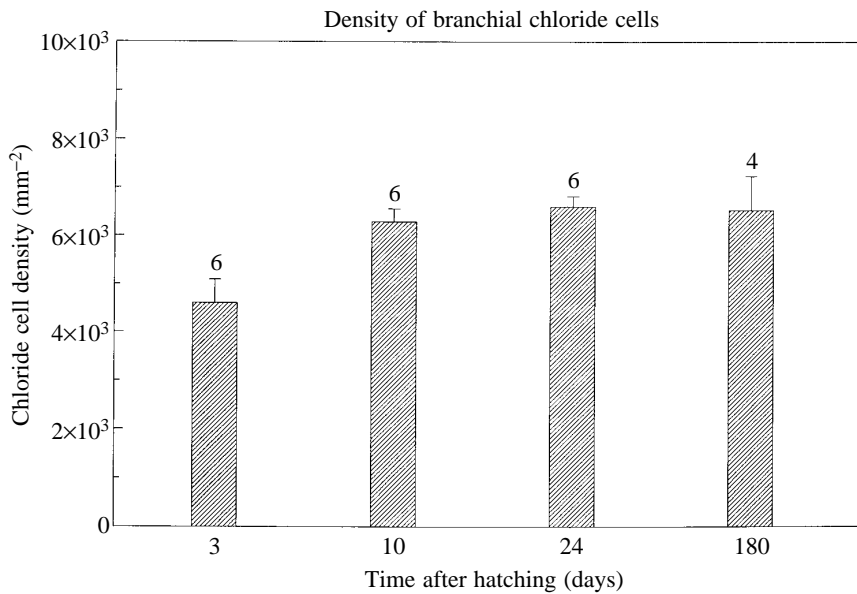
Density of branchial chloride cells

Fig. 3. Densities of chloride cells ($mm^{-2}$) in gills of freshwater tilapia of different ages. The densities in the juvenile fish refer to filamental epithelium; in the adult fish, they refer to the 'trailing edge' half of the filament epithelium. Values shown are means + S.D. Number of observations (*N*) are indicated above the columns.

by the nucleus could often be seen. Simultaneous exposure of the gills to $5\,\mu mol\,l^{-1}$ anthroylouabain and $1\,mmol\,l^{-1}$ ouabain completely abolished the fluorescent signals, demonstrating that branchial cells at this early stage of development contain functional ouabain-binding sites. This was confirmed by the high $Na^+/K^+$-ATPase specific activity in the gill homogenate (Table 1). A $Na^+/K^+$-ATPase specific activity of approximately $1500\,\mu mol\,P_i\,h^{-1}\,mg^{-1}$ in whole-tissue homogenate was measured in 10-day-old larvae. This was more than 370 times higher than the specific activity found in adults ($4.04\pm0.63\,\mu mol\,P_i\,h^{-1}\,mg^{-1}$). After 10 days, the branchial $Na^+/K^+$-ATPase specific activity gradually decreased, while the total activity of gill $Na^+/K^+$-ATPase increased (Table 1).

## Discussion

In this paper, we present evidence that numerous functional chloride cells are present in the gills of freshwater tilapia (yolk-sac) larvae, as early as 3 days after hatching. We have based this conclusion on four observations. First, in addition to the numerous mitochondria indicated by DASPMI-staining, most chloride cells possess an apical crypt in contact with the water, as shown by electron microscopy and by *in vivo* Con-A-FITC staining. Second, the chloride cells possess a high density of ouabain-binding sites, as shown by their ability to bind fluorescent anthroylouabain. Third, an extremely high $Na^+/K^+$-ATPase specific activity was found in the gill tissue of these larvae. Fourth, electron micrographs of the chloride cells confirm the presence of numerous mitochondria and an extensive tubular membrane system where transport ATPases and carriers have been shown to reside (Flik *et al.* 1985; Flik and Verbost, 1993).

Indeed, functional chloride cells in teleosts are structurally characterized by many small mitochondria and anastomosing tubules, where ion-transporting enzymes such as $Na^+/K^+$-ATPase, transport $Ca^{2+}$-ATPase and a $Na^+/Ca^{2+}$ exchanger are

located (Hootman and Philpott, 1979; Flik *et al.* 1985; Wendelaar Bonga *et al.* 1990; Kültz and Jürss, 1993; Verbost *et al.* 1994). The tubules have a lumen that is continuous with the extracellular space through orifices in the basolateral membrane (Sardet *et al.* 1979; Laurent and Dunel, 1980; Wendelaar Bonga *et al.* 1990), and the cell apical plasma membrane surface forms a crypt (or pit) facing the water. The latter contains glycoproteins and from its structure is considered to be an ion gradient region, a typical feature of functional chloride cells (Alderdice, 1988). To confirm the presence of intact functional chloride cells, a combination of three specific vital fluorescent probes (DASPMI, labelling mitochondria, Con-A-FITC labelling apical crypts, and anthroylouabain for indicating $Na^+/K^+$-ATPase) was applied successfully in this study. Since chloride cells are the *only* cells in gill epithelia containing an abundance of mitochondria,
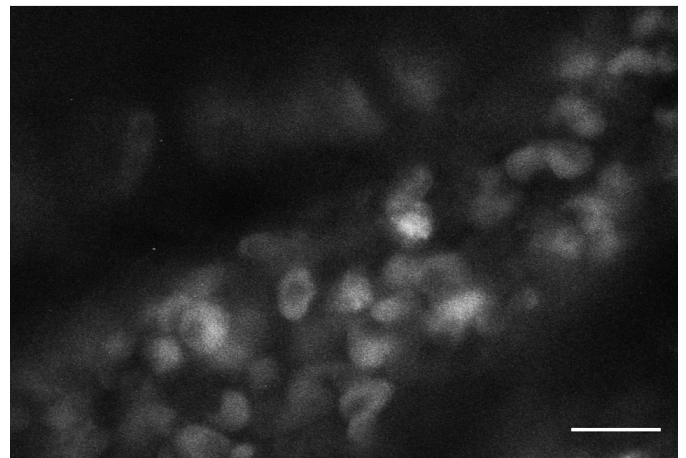


Fig. 4. Anthroylouabain staining of gill filaments of 3-day-old freshwater tilapia. After staining for 1 h with $5\,\mu mol\,l^{-1}$ anthroylouabain solution, filaments were examined with a fluorescence microscope. Scale bar, $25\,\mu m$.

Table 1. *Total and specific Na⁺/K⁺-ATPase activity in gills of larval, juvenile and adult freshwater tilapia*

| Time after hatching (days) | $V_{total}$ (N=6) | $V_{spec}$ (N=6) |
|---|---|---|
| 10 | 57±17 | 1558±366 |
| 24 | 233±42 | 914±192 |
| 180 (adult) | 371±58 | 4±0.6 |

$V_{total}$, the total Na⁺/K⁺-ATPase activity in $\mu$mol $P_i$ h⁻¹ ($V_{spec} \times$ mg protein).

$V_{spec}$, the specific Na⁺/K⁺-ATPase activity in $\mu$mol $P_i$ h⁻¹ mg⁻¹.

Data are means ± S.D. for six fish.

these cells may provide the energy required for active transepithelial ion transport (Na⁺, Cl⁻, Ca²⁺) against the sizeable electrochemical gradients known to exist between the blood and the external medium in freshwater fish (for a review, see Evans, 1982). Therefore, the specific vital dyes for mitochondria, DASPEI and DASPMI, are suitable for identifying and quantifying the mitochondria-rich chloride cells in epithelia of teleosts (Bereiter-Hahn, 1976; Foskett and Scheffey, 1982; Karnaky *et al.* 1984; McCormick, 1990; Marshall *et al.* 1992). The co-labelling with DASPMI and Con-A-FITC enabled us to identify the mature (functional) chloride cells in living tissues because Concanavalin-A-positive glycoproteins are concentrated in the apical pits of chloride cells (Zadunaisky, 1984).

A high level of Na⁺/K⁺-ATPase activity in branchial epithelia of fish is indicative of ion transport, and this enzyme has been localized almost exclusively in the chloride cells (Hootman and Philpott, 1979; Wendelaar Bonga *et al.* 1990). Karnaky (1986) estimated that teleost gill chloride cells have a higher concentration of Na⁺/K⁺-ATPase (up to $1.5 \times 10^8$ molecules per cell) than respiratory cells. Using [³H]ouabain autoradiography (Karnaky *et al.* 1976) and cytochemistry (Hootman and Philpott, 1979), Na⁺/K⁺-ATPase was localized in the extensive tubular system of chloride cells, which extends throughout the cell to the apical membrane. McCormick (1990) demonstrated, by co-labelling the operculum of seawater tilapia with DASPMI and anthroylouabain, that most of the Na⁺/K⁺-ATPase was located in the chloride cells; however, the procedure was not successful for freshwater tilapia in the study. Our results, in contrast, clearly demonstrate that the branchial chloride cells of larval and juvenile freshwater tilapia contain high concentrations of Na⁺/K⁺-ATPase, as shown by anthroylouabain binding in the intact gills as well as by biochemical measurement of enzymatic activity.

Many morphological, ultrastructural, physiological and biochemical studies on chloride cells in adult teleosts have been carried out (reviewed by Philpott, 1980; Foskett *et al.* 1983; Wendelaar Bonga *et al.* 1990). However, most of the investigations on this cell type in embryos and larvae of freshwater or seawater fish focused on the extrabranchial chloride cells, in particular on those in the yolk-sac epithelium and the body skin (for a review, see Alderdice, 1988; Ayson

*et al.* 1994). Hwang *et al.* (1994) suggested that extrabranchial chloride cells participate in ion transport and osmoregulation during the early developmental stages of tilapia, in particular *before* their gills become functional. Indeed, extrabranchial chloride cells begin to disappear during the post-yolk-sac stage when the gills start to develop. However, the exact timing of chloride cell development in fish gill is a matter of debate (Alderdice, 1988). We have found that 3 days after hatching, before the lamellae have formed, the epithelium of the gill filaments is almost entirely occupied by chloride cells (about 4000 cells mm⁻²). Also, in 10-day-old larvae, where the lamellae are still poorly developed, the density of chloride cells in the filaments reaches approximately 6000 cells mm⁻². This density remains essentially constant up to the adult stage, at least when we only consider the parts of the epithelium where chloride cells occur. Our values for chloride cell densities in adult fish are of the same order of magnitude as those reported for adult gill filaments of eel, trout and catfish (Perry *et al.* 1992; Laurent *et al.* 1994). Clearly, the gills in tilapia at all stages studied here participate in active ion transport. Lanzing (1976) reported that the gills of 8-day-old tilapia fry were sufficiently developed to assume a respiratory function, as shown by the observation that Indian ink particles placed in front of the mouth were swept inside and soon reappeared behind the opercular slit. We observed that it is approximately at this time that secondary lamellae start to develop. The presence of numerous mature chloride cells in the gill filaments of tilapia 3 days after hatching suggests that larvae at a very early stage may absorb ions from the ambient water via their gills. Hwang *et al.* (1994) reported that 10-day-old tilapia were able to accumulate sodium, potassium and calcium to levels about 8, 2 and 60 times, respectively, above those of the embryo. The authors attributed an important role in this accumulation of minerals to chloride cells near the pectoral fins. However, our data favour the conclusion that chloride cells in the gills are numerically by far the most important cell type in gills of young fish. From the numbers given by Ayson and coworkers (1994), we calculate a maximum number of 1500 chloride cells in the yolk sac membrane (total surface 3 mm², chloride cell density around 500 mm⁻²). Assuming that the gill filaments in these early life stages are more or less cylindrical, have an average length (*l*) of 250 $\mu$m and a diameter of 70 $\mu$m, we calculate a surface area ($2\pi rl$, where *r* is radius) of 0.055 mm² for an average, single filament. At a density of 4630 filament chloride cells per mm², we calculate that each filament contains about 255 chloride cells at 3 days after hatching. With an average of 20 filaments on the first and second arches, it follows that the branchial apparatus contains at least 20 400 ($4 \times 20 \times 255$) chloride cells (and there are more cells in the other arches). Clearly, the chloride cell population in the gills outnumbers that in the skin and the yolk sac and these cells may play a major role in active ion transport during the early developmental stages.

This conclusion is further supported by the presence of numerous ouabain-binding sites and significant Na⁺/K⁺-ATPase activity *in vitro*. The Na⁺/K⁺-ATPase specific activity

in gills of 10-day-old tilapia is almost 400 times higher than that of an adult tilapia. In 24-day-old tilapia, more lamellae had developed, while the density of branchial chloride cells in the filaments did not change. This resulted in the observed decrease of Na$^+$/K$^+$-ATPase specific activity an increase of total Na$^+$/K$^+$-ATPase activity of the gills.

In summary, our results demonstrate the presence of mature branchial chloride cells in very young freshwater tilapia and that these cells are equipped to participate in ion transport, even before the gills have adult morphological characteristics.

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