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The effect of dietary sodium chloride on some osmoregulatory parameters of the teleost, *Oreochromis niloticus*, after transfer from freshwater to seawater

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Abstract

The aim of this work was to determine the effects of supplemental dietary sodium chloride on salt water acclimation of tilapia *Oreochromis niloticus*. Fish were fed a basal diet supplemented with NaCl (8%) during three weeks in fresh water (FW) and then transferred to salt water (SW) at 15 and 20‰. Changes in plasma osmolality, chloride ion concentration (Cl^-), plasma level of cortisol and gill Na^+, K^+-ATPase activity were measured at 6, 12, 24, 48, 72 and 168 h after transfer to 15‰SW, while the higher strength SW group (20‰) was only monitored up to 24 h. Morphological changes in the gill mitochondria-rich (MR) cells were examined in relation to environmental salinity. The changes associated with dietary NaCl were sporadic and of small magnitude. The plasma osmolality and Cl^- increased immediately after transfer up to 12–24 h, but fish fed dietary salt (S) showed lower values than the control group (C). The S group showed higher plasma levels of cortisol than the control, which maintained its initial levels during the experiment. Gill Na^+, K^+-ATPase activity of the S group began to increase in the first hours after transfer, reaching maximum at 12 h and returned to basal level at 24 h, while the control group maintained basal levels. The differences between gill Na^+, K^+-ATPase activity of S and C fish were significant ($p < 0.05$) at 12 h. Transmission electron microscopy (TEM) revealed that MR cells in SW show more mitochondria and a more developed tubular system arising from the basolateral membrane. The MR cells of both groups frequently formed a multicellular complex in SW, consisting of a main MR and one or more accessory cells. Such complexes are rarely observed in FW. Some MR cells of fish fed supplemented dietary salt displayed convex apical membrane in FW.

Introduction

Tilapia of the genus *Oreochromis* is a suitable biological model for studying the mechanism of osmoregulation in teleost fish, because they can survive direct transfer from freshwater (FW) to salt water (SW). In tilapia, the transition from FW to SW is associated with a temporary elevation in plasma osmolality and sodium (Na^+) and chloride (Cl^-) ion concentrations (Assem and Hanke 1979; Hwang et al. 1989), accompanied by a transient rise in plasma cortisol and growth hormone (GH) levels (Assem and Hanke 1979; Yada et al. 1994); plasma prolactin levels ($tPRL_{177}$ and $tPRL_{188}$) decrease (Morgan et al. 1997). Morphological changes in gill mitochondria-rich (MR) cells (Hwang 1987) and an increase in gill Na^+, K^+-ATPase activity have also been observed in SW-adapted tilapia (Hwang et al. 1989; Morgan et al. 1997). Some studies have examined the effects of different factors on SW acclimation (Jürss et al. 1984; Vijayan et al. 1996).

Pre-acclimation has a crucial physiological significance for some euryhaline teleosts during SW adaptation. Hwang et al. (1989) reported that tilapia *O.*

mossambicus when transferred to 30‰ SW with a pre-acclimation to lower salinity (20‰) for 24 h, exhibited a more rapid increase in gill Na^+, K^+-ATPase activity and less dehydration than fish transferred directly to 20 or 30‰ SW. Similar data concerning plasma parameters have been reported in chum salmon (*Oncorhynchus keta*) and ayu (*Plecoglossus altivelis*) (Iwata et al. 1982; Hasegawa et al. 1983). The acclimation with a diet supplemented with NaCl is an other acclimation method utilized for adapting rainbow trout and other salmonids to SW (Zaugg et al. 1983; Salman and Eddy 1987; Arzel et al. 1993). This is not an universal finding among teleosts and the effect of this method on the osmoregulation mechanism in tilapia is unknown. However, it is reasonable to expect that dietary salt could have a practical effect on the activation of the physiological mechanisms involved on salt secretion. One hypothesis is that dietary salt supplementation improves the ability of tilapia to adapt to direct increases in water salinity.

MR cells (so called chloride cells) are specialized for ionic regulation in gill epithelia (Hwang and Hirano 1985; Goss et al. 1993; Zadunaisky 1996) and they are the site of ion extrusion in SW-adapted fish (Foskett and Scheffey 1982; McCormick 1995). MR cells are characterized by the presence of numerous mitochondria and an extensive tubular systems continuous with the basolateral membrane (Pisam and Rambourg 1991). It has been suggested that MR cells are multifunctional and do more than just excrete chloride in SW-adapted fish (Foskett and Scheffey 1982). Indeed it is now well established that MR cells play a crucial role in NaCl uptake, Ca^{2+} uptake and acid-base balance in FW-adapted teleosts (Laurent and Perry 1991). Some studies have reported morphological alterations in MR cells in response to environmental salinity (Yoshikawa et al. 1993; Kultz et al. 1995; Lee et al. 1996; Shiraishi et al. 1997). Lee et al. (1996) suggested the existence of more than one type of MR cells in euryhaline species adapted to either SW or FW. These authors have also reported some morphological changes of different types of MR cells that occurred within 24 h after tilapias were transferred to various hypotonic milieus. However, the different types of MR cells remain to be determined.

The main objective of this study was to obtain detailed information on development of osmoregulatory mechanisms and to examine alterations in the short term in tilapia *O. niloticus* transferred directly from FW to SW (15 and 20‰). Plasma cortisol levels, gill Na^+, K^+-ATPase activity and plasma osmolality and Cl^- concentration were the main parameters investigated. The morphological structure of MR cells was examined by transmission electron microscopy (TEM).

Materials and methods

Fish and diet formulation

Adult male tilapia (*Oreochromis niloticus*) with a 30–40 g body weight were obtained from a laboratory stock at the University of Tras-os-Montes and Alto Douro. These fish originated from a population at the Fish Laboratory of INRA, Rennes. The fish used for this experiment had been treated with 17-α-methyltestosterone (MT, Sigma Co.) to produce all-male offspring, following the procedure described by Guerrero (1985). The treatment ended two months before the start of this experiment. Although sexual hormones can affect MR cell number and morphology (Madsen and Korsegaard 1989; Coimbra et al. 1993), this effect disappears within a few weeks of the treatment, because the lifecycle of MR cells is only a few days (Laurent et al. 1994). Pre-treatment is therefore unlikely to have any effect on this experiment.

Using data obtained in a previous experiment (Fontaínhas-Fernandes et al. 2000), a practical diet was formulated by adding supplemental salts (8% of NaCl) to a basal diet. The diet supplemented with NaCl was given for 3 weeks before the direct transfer from FW to SW; the control group (C) were fed the basal diet without salt supplementation. The fish were fed to visual satiation, by hand, twice a day at about 10 a.m. and 5 p.m., during the acclimation period. Fish were fasted for 24 h before sampling.

Experimental design

Two acclimation experiments were conducted in an experimental system with 60 l tanks and a water flow rate of 0.5 l min^{-1}: Experiment 1 - Two groups of fish (S and C) reared in FW were transferred directly to 15‰ SW; fish were sampled at 6, 12, 24, 48, 72 and 168 h after transfer. Experiment 2 - Two groups of fish (S and C) reared in FW were transferred directly to 20‰ SW; fish were sampled only at 6, 12 and 24 h after transfer. Before the transfer to SW eight fish were sampled. The two experiments were conducted under the same conditions. Water salinity was maintained at the desired level by mixing synthetic sea salts. Water temperature was kept similar in each

treatment tank (25 ± 1 °C) and supplemental aeration was provided to maintain dissolved oxygen near saturation. Some other water quality parameters such as ammonia, nitrite, nitrate and suspended solids were maintained at acceptable levels by mechanical and biological filtration. A diurnal light: dark cycle of 12L: 12D was provided by fluorescent lighting controlled with a timer.

Sampling and analytical procedures

The fish were anaesthetized by immersion in an ethylene glycol monophenyl ether bath (0.4 ml l^{-1}) and stunned by a blow to the head; blood was drawn from the caudal vessels into a heparinized syringe and centrifuged at 3000× *g* for 10 min. Plasma was immediately stored at −20 ° C for later analysis. After blood collection, the fish were decapitated and gill filaments were dissected from the second gill arch on the left side of the fish, placed in ice-cold sucrose buffer (0.25 M, pH 7.4) to remove blood, quickly frozen in liquid nitrogen and then stored at −70 ° C until the measurement of Na^+, K^+-ATPase was made.

Plasma Cl^- was measured by electrometric titration using a chloride meter (Jenway, model PCLM3). Plasma osmolality (mOsm Kg^{-1}) was measured with a micro-osmometer (model 3 MO plus, Advanced Instruments, Lda) and each sample was examined at least three times. The plasma cortisol level was determined using a commercial radioimmunoassay kit cortisol (^{125} I) (Coat-a-Count, Diagnostic Products Corporation), according to Iwama et al. (1989). The intra–assay coefficient was 5.8% and inter-assay variation was avoided by measuring all samples in the same assay. Cross-reactivity with cortisone, the other major corticosteroid in teleostean fish, was 7.0%. Gill Na^+, K^+-ATPase activity, expressed as mmoles phosphate (Pi) mg prot.$^{-1}$.h^{-1}, was determined according to the method of Epstein et al. (1967), as modified by Lasserre et al. (1978). The amount of inorganic phosphate (Pi) liberated from ATP was determined according to the method of Fiske and Subbarow (1925) and the concentration of the total protein was determined by the method of Lowry et al. (1951), using crystalline bovine albumin as standard.

Morphological methods

To examine the MR cells in tilapia, a partial filament dissection method, similar to that described by Laurent and Dunel (1980), was performed. A minimum 6 fish were sampled for each examination. For light microscopy (LM) and transmission electron microscopy (TEM), small pieces of gill filaments were fixed at 4 °C in a 3% glutaraldehyde and 0.1 M sodium cacodylate mix in 0.2 M phosphate buffer (pH 7.2), according to the method of Russell-Pinto et al. (1996). They were rinsed for 2 h with 3 changes of 0.1 M phosphate buffer (pH 7.2) at 4 °C and then post-fixed with 1% osmium tetroxide in 0.2 M phosphate buffer (pH 7.2) for 1 h. Gill filaments were rinsed in buffer as above, and dehydrated in increasing concentrations of ethanol from 50% to total, after which they were filtrated and embedded in parafin. Sections were cut with glass or diamond knives in an ultramicrotome (LKB, Bromma). Semithin sections (1 μm) for LM observations were mounted on glass slides, stained with methylene blue, and observed with a Nikkon light microscope under oil immersion. Ultrathin gray sections were mounted on formvar-coated copper grids, double stained in uranyl acetate and lead citrate and examined with a JEOL JEM 100CXII electron microscope at 60 kV. Interlamellar chloride cell density was measured by counting the number of MR cells in a 470 μm diameter field (40 × objective) and dividing by the number of lamellae. At least 12 fish per group were measured.

Statistical analysis

Data are presented as means ± standard error (SE). Two-way analysis of variance (ANOVA) was used to test for treatment and time effects. Significant differences between treatment means were identified using Student-Newman-Keuls multiple comparison test ($p < 0.05$).

Results

Osmolality and Cl^-

Following direct transfer from FW to 15‰ SW, plasma osmolality and Cl^- concentration increased and reached a peak after 12 h (Figures 1 and 2). These parameters began to decline at 24 h, and reached the basal level (FW) at 168 h. At all sampling periods, S fish showed lower values of plasma osmolality and Cl^- concentration than the C group; however, the differences were not statistically significant ($p > 0.05$). At 12 and 24 h after the transference to 20‰ SW the Cl^- concentration of the S group was significantly lower than C ($p < 0.05$). At 24 h, the same relationship occurs to plasma osmolality (Figure 1).

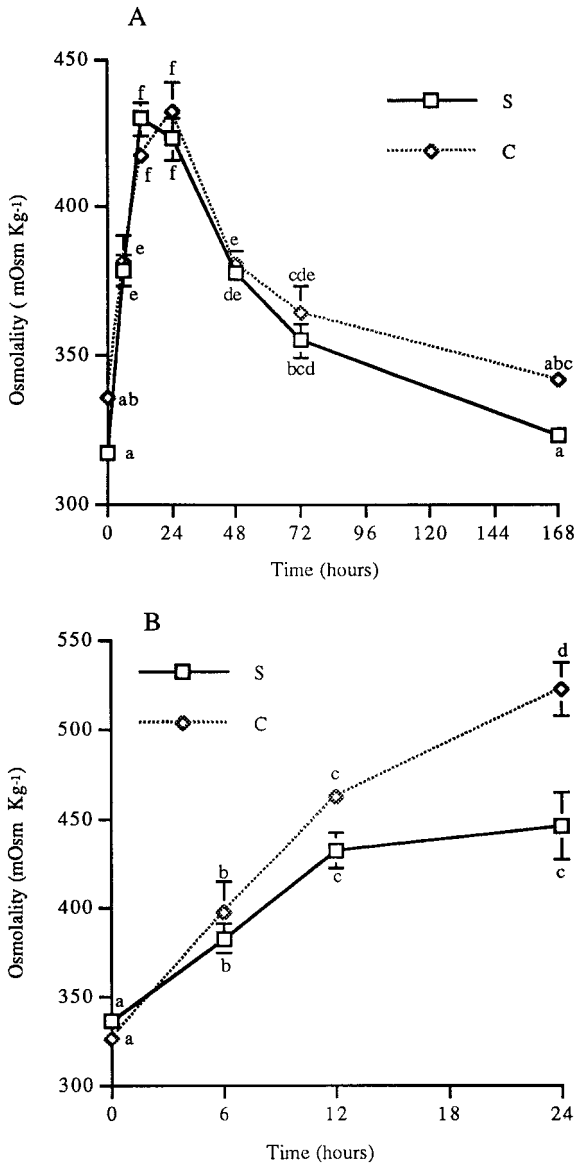

Figure 1. Osmolality of tilapia *O. niloticus* after transfer from FW to 15‰ SW (A) and 20‰ SW (B). Data are presented as means ± SE (n = 6–8). Inset shows significant treatments effects; significant interaction at each period is shown by letters next to symbols, and means with different letters are significantly different ($p < 0.05$, two-way ANOVA).

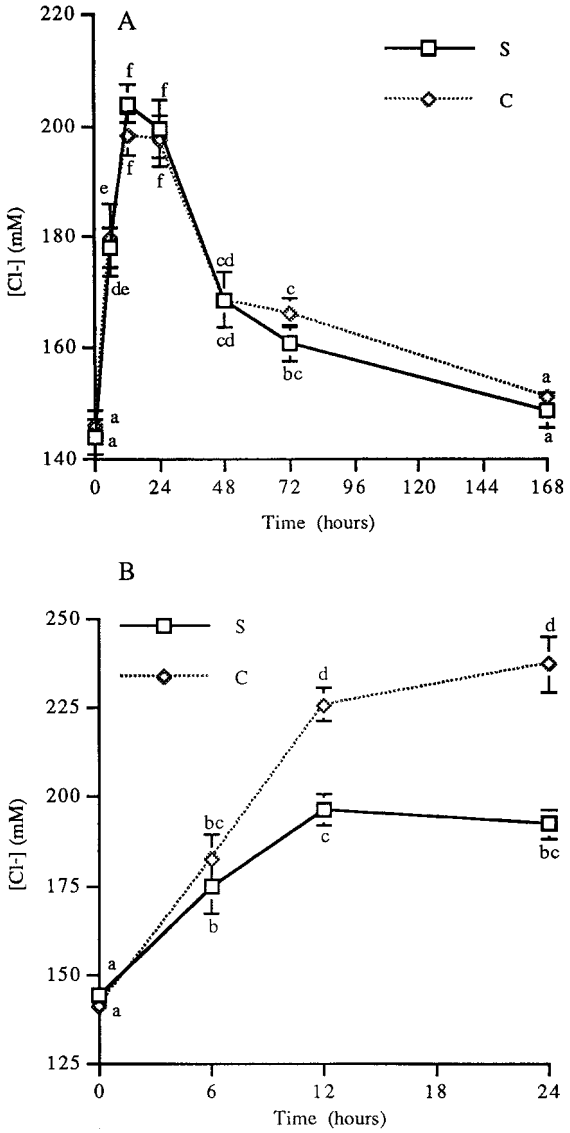

Figure 2. Cl^- of tilapia *O. niloticus* after transfer from FW to 15‰ SW (A) and 20‰ SW (B). Data are presented as means ± SE (n = 6–8). Inset shows significant treatments effects; significant interaction at each period is shown by letters next to symbols, and means with different letters are significantly different ($p < 0.05$, two-way ANOVA).

Cortisol

Following transfer from FW to 15‰ SW both groups exhibited a small decrease in plasma cortisol levels, but this began to increase at 48 h. At all sampling periods, the S group showed similar values of cortisol to those obtained with the control group (Figure 3). S fish transferred to 20‰ SW showed plasma cortisol levels higher than the C group, and the differences were statistically significant ($p < 0.05$) at 6 and 24 h.

Gill Na^+, K^+-ATPase activity

Gill Na^+, K^+-ATPase activity of S fish and the control group increased following the transference from FW to 15‰ SW (Figure 4). At 168 h, gill Na^+, K^+-ATPase activity of both groups was significantly

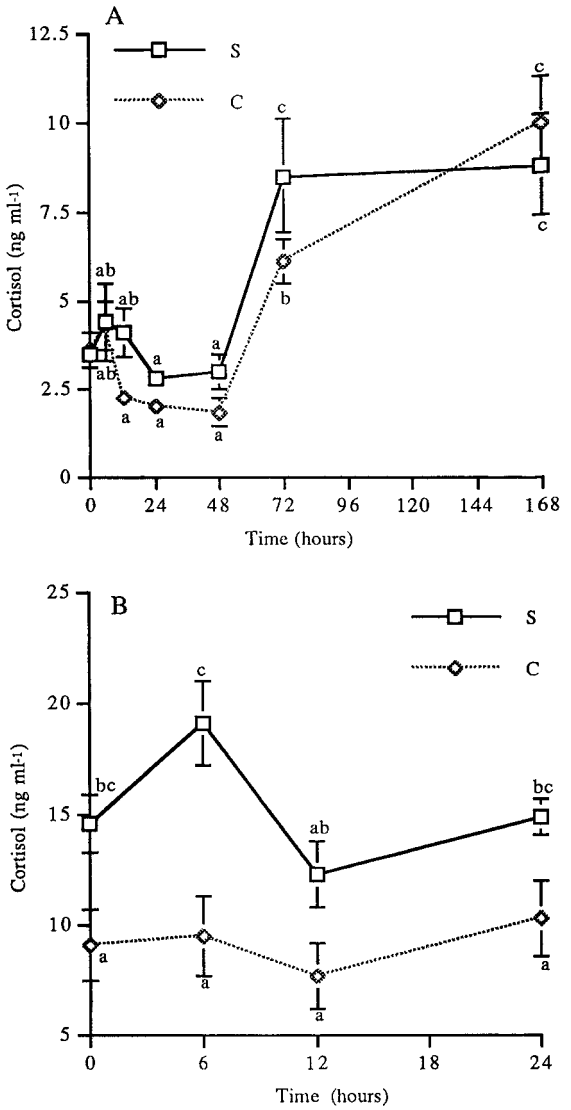

Figure 3. Plasma cortisol of tilapia *O. niloticus* after transfer from FW to 15‰ SW (A) and 20‰ SW (B). Data are presented as means ± SE (n = 6). Inset shows significant treatments effects; significant interaction at each period is shown by letters next to symbols, and means with different letters are significantly different ($p < 0.05$, two-way ANOVA).

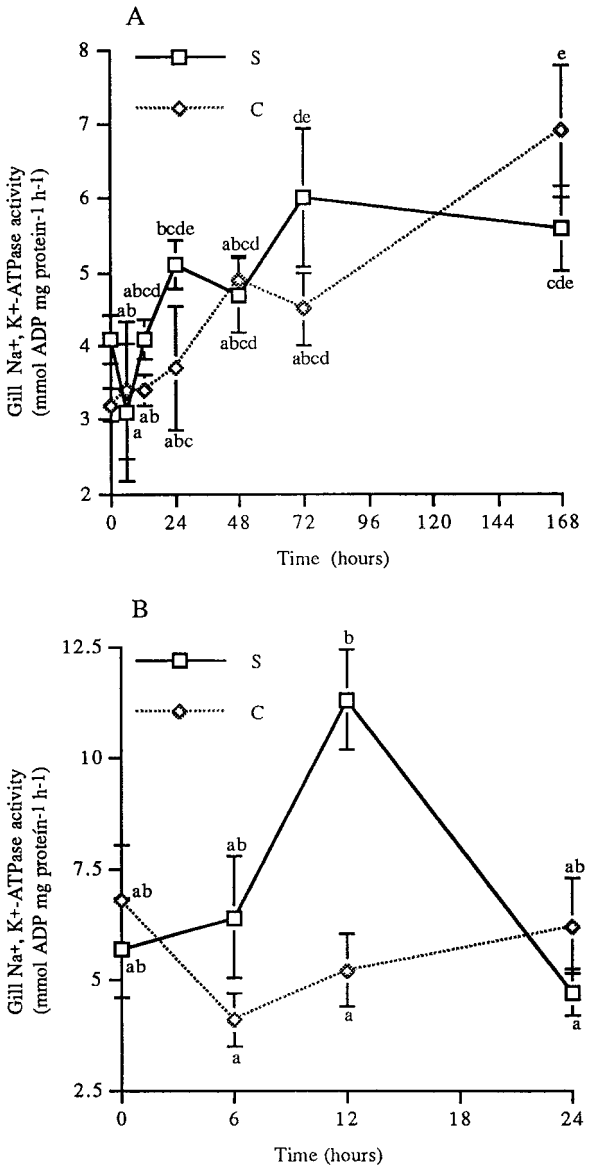

Figure 4. Gill Na^+, K^+-ATPase acitvity of tilapia *O. niloticus* after transfer from FW to 15‰ SW (A) and 20‰ SW (B). Data are presented as means ± SE (n = 6). Inset shows significant treatments effects; significant interaction at each period is shown by letters next to symbols, and means with different letters are significantly different ($p < 0.05$, two-way ANOVA).

higher than FW ($p < 0.05$). When tilapia were transferred to 20‰ SW, S fish showed an increase of gill Na^+, K^+-ATPase activity, while the C group maintained basal levels. At 12 h, the differences between S and C fish were statistically significant ($p < 0.05$).

MR cells

Table 1 summarizes the measurements of the MR cell number in the interlamellar primary epithelium after the transference from FW to 20‰ SW. No statistically significant differences ($p > 0.05$) in the number of MR cells were observed during the sampling period. Figure 5 shows representative TEM's micrographs illustrating the MR cell's ultrastructure in the control group in FW and at 168 h after transfer (A, B) and in the dietary salt group in FW and at 168 h (D, E).

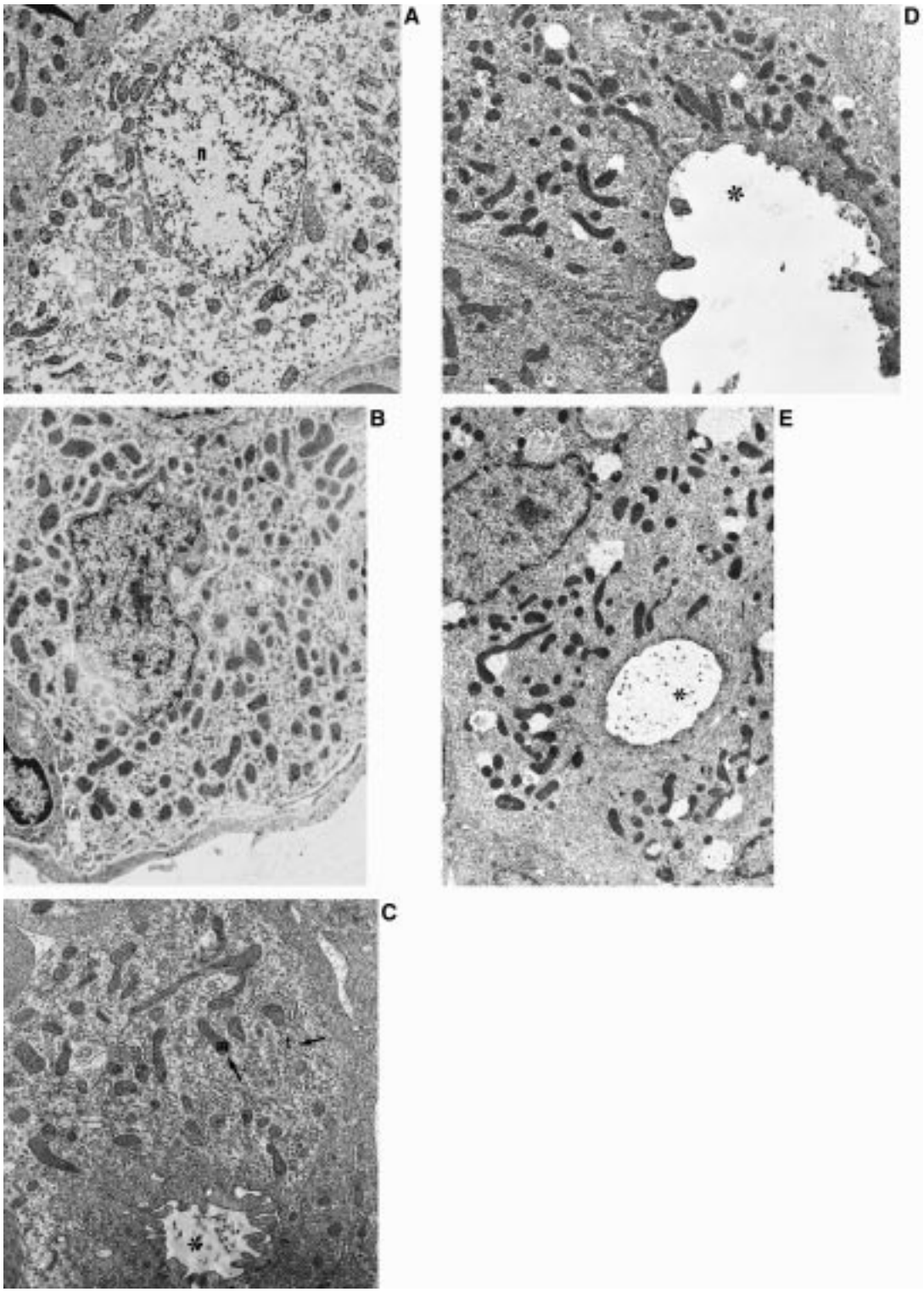

Figure 5. Transmission electron micrographs illustrating the MR cell ultrastructure of untreated tilapia in FW and 168 h after transfer (a, b), and fed dietary salt in FW (c) and 168 h after transfer (d, e) (× 12 000). Its cytoplasm contains numerous mitochondria (m), a tubular system (t) and a basal ovoid nucleus (n). The formation of apical crypt (*) appeared more frequently in SW.

Table 1. Number of gill MR cells in tilapia *O. niloticus* after direct transfer from FW to 20‰ SW.

Treatment	Hours after transfer	Chloride cell density (number of MR per interlamellar area)
CS	0	3.3 ± 0.29^a
	6	3.4 ± 0.37^a
	24	2.9 ± 0.22^a
SS	0	3.0 ± 0.33^a
	6	4.0 ± 0.66^a
	24	2.8 ± 0.46^a

*Means with same letters are not significantly different ($p < 0.05$); (n = 12).

The MR cells contained numerous mitochondria, a well-developed tubular system arising from the basolateral membrane and a large ovoid nucleus. The MR cells were in direct contact with the external medium via their apical membrane on the mucosal side, and with the basement membrane on the serosal side. After the transference to SW, the number and shape of MR apical structures increased or changed. Most of the MR cells displayed a convex apical membrane causing them to protrude into the water (Figure 5D). Fish treated with dietary salt showed, in FW, some MR cells forming an apical crypt (Figure 5C) that was not seen in the control group reared in FW (Figure 5A). However, at 168 h after transference to 15‰ SW mitochondria were more numerous and the tubular system formed a denser network of anastomed tubules than in FW (Figure 5D, E). At 168 h it was possible to observe frequent multicellular complexes in both groups consisting of one main cell and one or more accessory cells, which were attached to each side of the main cell (Figure 5D).

Discussion

The present study shows an increase of plasma osmolality and Cl^- concentration immediately after the transference of *O.niloticus* from FW to SW. At 12 h after transition to 15‰ SW, the decrease of plasma osmolality and Cl^- levels of S fish had began, while the decrease of the control group occurred later (24 h). S group showed plasma osmolality and Cl^- values significantly lower ($p < 0.05$) than the control group at 12–24 h in 20‰ SW. These results are consistent with other studies in rainbow trout *Oncorhynchus mykiss* (Hegab and Hanke 1986), chum salmon *Oncorhynchus keta* (Iwata et al. 1982) and ayu *Plecoglossus altivelis* (Hasegawa et al. 1983).

Previous studies have shown that the acclimation of tilapia *O. mossambicus* to SW involves two different physiological periods, i.e, the crisis period and the stabilization period (Hwang et al. 1989). Immediately post-transfer to SW, the critical problem faced by teleosts is dehydration, caused by osmotic removal of water in gill and gut epithelial (Bath and Eddy 1979). This crisis period occurs in rainbow trout (*O. mykiss*) at 8 to 12 h after transition to SW (Bath and Eddy 1979; Leray et al. 1981), while in tilapia *O. mossambicus* it appears to be within 6 to 12 h (Hwang et al. 1989). The subsequent 12–24 h mark the beginning of the stabilization period, where the plasma osmolality and Cl^- concentration start to decrease. Assem and Hanke (1979) also reported a strong increase of the osmolality and Na^+ concentration after the transference to SW in *O. mykiss*, peaking before 24 h, followed by a decline to basal level. Present results reveal that the control group shows a more rapid increase in plasma osmolality and Cl^- concentration than fish fed dietary salt, especially when tilapia are transferred to 20‰ SW.

There are some studies showing the ease of SW-adaptation by dietary salt. Zaugg et al. (1983) suggested an increase of gill Na^+, K^+-ATPase activity in chinook salmon fed salt supplemented diets. Other experiments in salmonids also found this positive correlation (Salman and Eddy 1987; Arzel et al. 1993). In this study, gill Na^+, K^+-ATPase activity shows an increase at 24 h in 15‰ SW. Following transfer from FW to 20‰ SW, the initial increase of gill Na^+, K^+-ATPase activity is higher in S than in the control group. At 12 h gill Na^+, K^+-ATPase activity of S group in 20‰ SW was two fold higher than in FW, and the differences are significant ($p < 0.05$) in relation to the control group. The timing of the decrease in plasma Cl^- and osmolality and the increase in gill Na^+, K^+-ATPase activity observed in this study are consistent with the known role of this enzyme in salt secretion to maintain ionic balance in SW fish (Zadunaisky 1984). Avella et al. (1993) also analyzed this parameter to test the SW tolerance of *O. niloticus* and *O. aureus*. They observed that transfer from FW to 20‰ SW significantly enhanced the Na^+, K^+-ATPase activity, 1.6 times in *O. niloticus* and 3 times in *O. aureus*. Similar results were observed in the present study in 20‰ SW.

The present results are in accordance with those obtained by Luke et al. (1994) who found that in

the European eel (*Anguilla anguilla*), changes in the activity and expression of Na^+, K^+-ATPase were associated with three phases: 1) immediately upon transfer, gill Na^+, K^+-ATPase increased by a factor of three within 6 hours compared to activities found in FW groups; 2) the initial peak of Na^+, K^+-ATPase was followed some 3 days after transfer by a gradual and more prolonged increase in Na^+, K^+-ATPase activity, leading to levels at 21 days which matched the 6-h peak; 3) finally, in the adapted fish held in SW for 6 months, the increased levels of enzyme activity were not sustained, and at this time, levels had returned to near those found in the FW group.

In teleost fish, cDNA from at least two Na^+, K^+-ATPase α subunits isoforms (α_1 and α_3) and different β subunits like isoforms (designed β_1, β_3, β_{179}, β_{185}, β_{185b}, β_{233}) were identified (Cutler et al. 1995, 1996, 1997; Lee et al. 1998). In the above study, Luke et al. (1994), measuring the levels of α_1 and β_1 subunits mRNA production, observed that during the first peak of Na^+, K^+-ATPase activity, immediately after transfer to SW, there was no increase in α_1 or β_1 subunit mRNA production, supporting the view that the higher regulation of enzyme activity is probably associated with either the recruitment of the pre-formed sequestered units to the plasma membrane, or may be the result of a direct activation of the enzyme by phosphorylation/desphosphorylation mechanisms. The second and more gradual enhancement of Na^+, K^+-ATPase activity that follows, paralleled an increase in the abundance of α_1 and β_1 subunit mRNAs up to maximum after 3 weeks of adaptation. A further study by Cutler et al. (1995a, b) shows that higher levels of α_1 and β_1 mRNAs were detected 3 weeks after the transference in fish adapted to higher salinities (20‰). Results obtained in the present study concerning the gill Na^+, K^+-ATPase activity after transfer to SW suggest that, as in the study of eels (Luke et al. 1994), the initial enhancement of enzyme activity after SW transfer is associated with an activation of pre-existing molecules of Na^+, K^+-ATPase, or the recruitment of pre-formed sequestered units to the plasma membrane.

The number of MR cells and the observation of morphological changes are usually used to study SW adaptation. The results of the present study showed that the acclimation with dietary NaCl has no effect on the number of MR cells, neither in FW nor after transfer to SW (Table 1). Cioni et al. (1991) also observed that following adaptation to SW, the number of MR cells did not change, but the surface area of their section profile increased significantly in *O. niloticus* and *O. mossambicus*. Wendelaar-Bonga and van der Meij (1989) found that MR cells are more frequently observed at 3–5 days after the transfer of tilapia *O. mossambicus* from FW to SW. They usually occurred in groups of two or more cells, i.e., as accessory, mature and degenerated MR cells. The accessory cells increase in size and number after transition.

Although some variation occurs among species, MR cells in FW look different from those in SW (Laurent and Perry 1991). In FW the MR cells are generally smaller and less columnar, have a less pronounced tubular system, and often do not have apical and serosal contacts. In SW-adapted tilapia MR cells showed morphological changes similar to those previously described by Hwang (1987) and Cioni et al. (1991). Most of the MR cells exhibited a deep apical pit, in which a multicellular complex opens (Figure 5D). The elongated mitochondria were more numerous, and frequently located in close association with the tubular system. Apoptotic cells were usually found at 168 h in both groups (Figure 5B). At this time many MR cells showed different degrees of apoptotis, while MR cells in necrosis were rarely observed. The presence of an apical crypt has been generally reported as a distinctive feature of MR cells of marine teleosts or as a structural change that these cells display when euryhaline species are transferred from FW to SW (Laurent 1984). However, apical pits of the MR cells have already been observed in the gills of FW-adapted tilapias (Wendelaar Bonga and van der Meij 1989; Maina 1990).

Tilapia fed dietary salt for 3 weeks showed some morphological features in FW similar to those previously described in SW-adapted tilapia. Most of the MR cells exhibited a deep apical crypt and a more developed tubular system than in the control group (Figure 5A, B). There was also a higher gill Na^+, K^+-ATPase activity and higher plasma levels of cortisol than in the control group. In addition the morphological features of MR cells of both tested groups are similar at 168 h. Further structural changes of both groups at 168 h include the organization of most MR cells in multicellular complexes (Figure 5D), and the development of apical interdigitations and leaky junctions. These changes are similar to those described generally in the MR cells of marine and SW-adapted teleost species (Hwang 1987; Cioni et al. 1991).

Moreover, Wendelaar Bonga and van der Meij (1989) demonstrated that the MR cells are pleomorphic. Lee et al. (1996) also observed that reversible

changes of MR cells occurred within 24 h after the tilapia were transferred to various hypotonic milieus. The phenotypic changes of MR cells in rainbow trout occurred as early as 12 h, while the cell division increased significantly 48 h after the transference to ion-poor water (Laurent et al. 1994).

In conclusion, some changes of MR cells found in the present study may represent the response of gill cells to SW adaptation and indicate the close association of structure with function. The interaction between the acclimation method tested and the MR cells morphology needs to be studied in more detail to improve knowledge in this area.

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