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Physiological, biochemical and morphological indicators of osmoregulatory stress in 'California' Mozambique tilapia (*Oreochromis mossambicus* × *O. urolepis hornorum*) exposed to hypersaline water

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Summary

The salinity tolerance of the 'California' Mozambique tilapia (*Oreochromis mossambicus* × *O. urolepis hornorum*), a current inhabitant of the hypersaline Salton Sea in California, USA, was investigated to identify osmoregulatory stress indicators for possible use in developing a model of salinity tolerance. Seawater-acclimated (35 g l^{-1}) tilapia hybrids were exposed to salinities from 35–95 g l^{-1}, using gradual and direct transfer protocols, and physiological (plasma osmolality, [Na^+], [Cl^-], oxygen consumption, drinking rate, hematocrit, mean cell hemoglobin concentration, and muscle water content), biochemical (Na^+, K^+-ATPase) and morphological (number of mature, accessory, immature and apoptotic chloride cells) indicators of osmoregulatory stress were measured. Tilapia tolerated salinities ranging from 35 g l^{-1} to 65 g l^{-1} with little or no change in osmoregulatory status; however, in fish exposed to 75–95 g l^{-1} salinity, plasma osmolality, [Na^+], [Cl^-], Na^+, K^+-ATPase, and the number of apoptotic chloride cells, all showed increases. The increase in apoptotic chloride cells at salinities greater than 55 g l^{-1}, prior to changes in physiological and biochemical parameters, indicates that it may be the most sensitive indicator of osmoregulatory stress. Oxygen consumption decreased with salinity, indicating a reduction in activity level at high salinity. Finally, 'California' Mozambique tilapia have a salinity tolerance similar to that of pure Mozambique tilapia; however, cellular necrosis at 95 g l^{-1} indicates they may be unable to withstand extreme salinities for extended periods of time.

Key words: 'California' Mozambique tilapia, *Oreochromis mossambicus* × *O. urolepis hornorum*, osmoregulatory stress, Salton Sea, chloride cell, salinity challenge.

Introduction

Saline lakes exist on every continent world wide, and comprise a total volume of approximately 104 000 km^3 (Williams, 1996). The Salton Sea is a 980 km^2, highly saline lake that formed in 1905–1906 when Colorado River water flooded the Imperial Valley of south-eastern California. This inland sea has a high evaporation rate and lacks outflow; consequently, salinity has continually risen since its formation. The current salinity is approximately 43 g l^{-1}, but is continually increasing by 0.3 g l^{-1} year (Watts et al., 2001). Because reproductive failures and high larval mortality among introduced marine fish species have been partially attributed to high salinity (Costa-Pierce and Riedel, 2000), the continual salinity increase has generated concern due to the threat it places on the current fishery. In spite of this challenge, one species that has successfully inhabited the sea is a Mozambique–Wami tilapia hybrid (*Oreochromis mossambicus* × *O. urolepis hornorum*), which has been referred to as the 'California' Mozambique tilapia (Costa-Pierce and Doyle, 1997). Introduction of this tilapia was reported to have occurred in 1964–1965, when hybrids from local aquacultural farms escaped to drainage ditches leading into the sea (Costa-Pierce and Doyle, 1997). Although Mozambique tilapia are typically freshwater and estuarine, they have been observed to tolerate very saline water during experimental short-term direct transfers (Kultz et al., 1992; Kultz and Onken, 1993; Stickney, 1986); very little is known regarding the salinity tolerance of the Wami tilapia, but they have been reported to be able to grow and reproduce at salinities ranging up to 39 g l^{-1} (Talbot and Newell, 1957). This study will be the first to address the saline tolerance of the California hybrid, which is a hypersaline lake inhabitant.

Teleosts exposed to hyperosmotic environments experience osmotic water loss coupled with diffusive ion gains. To offset the loss of water, an increase drinking rate becomes necessary (Brocksen and Cole, 1972; Foskett et al., 1981; Hwang et al., 1989; Cioni et al., 1991); in the intestine water is absorbed

osmotically, following active absorption of salts across the epithelium (Wilson et al., 1996). In order to offset the increased ion load, and ultimately survive in saline waters, fish must actively excrete Na^+ and $Cl^–$. Gills are the primary organ involved in osmoregulation in teleost fishes. Ion-transporting chloride cells, located on the branchial filamental epithelium, are rich in Na^+, K^+-ATPase, which drives excretion mechanisms. Chloride cells are responsible for ion excretion in both juvenile and adult seawater-acclimated fish (Foskett et al., 1981; Laurent and Dunel, 1980; Marshall and Bryson, 1998; Perry, 1997). Chloride cells in seawater-acclimated fish are generally rich in mitochondria, bear an extensive basolateral tubular reticulum, and have direct contact with ambient water through their apical membrane and with blood through the basolateral cellular membranes (Laurent, 1984; van der Heijden et al., 1997; Wendelaaar Bonga and van der Meij, 1989). Four stages of the chloride cell life cycle have been identified, and include (1) large columnar mature cells, which have an ultrastructure that is typical of actively functioning cells, (2) slim crescent-shaped accessory cells, which are less abundant in mitochondria and have a poorly developed tubular reticulum, (3) tear-shaped immature cells, structurally intermediate between mature and accessory cells and (4) small and round degenerating, or apoptotic cells, which have a highly condensed cytoplasm, multi-lobular and heterochromatic nucleus, and dilated mitochondria and tubular reticulum (Wendelaar Bonga and van der Meij, 1989). Salinity tolerance in fishes is dependent upon the appropriate physiological, biochemical and morphological adjustments to a given salinity.

The salinity tolerance of pure Mozambique tilapia *Oreochromis mossambicus* has been previously investigated; however, studies have generally focused on seawater acclimation (35 g $l^{–1}$) or hypersaline (>35 g $l^{–1}$) acclimation following large increases in salinity (i.e. direct transfers). This study reports on the ability of a hybrid of this euryhaline species to gradually acclimate to salinities exceeding that of seawater (up to 95 g $l^{–1}$), and incorporates physiological (plasma osmolality, [Na^+], [$Cl^–$], oxygen consumption, drinking rate, hematocrit, mean cell hemoglobin concentration, and muscle water content), biochemical (Na^+, K^+-ATPase) and morphological (the number of mature, accessory, immature and apoptotic chloride cells) indicators of osmoregulatory stress. The ultimate goal of this study was to identify the most appropriate parameters that can be used to model salinity tolerance for fish species that inhabit hypersaline lakes, using a species that is currently known to inhabit such an environment, and to compare the salinity tolerance of this hybrid with that of pure Mozambique tilapia.

Materials and methods

Fish

Pacific Aquafarms in Niland, California, USA donated 300 juvenile tilapia hybrids (*Oreochromis mossambicus* L. × *O. urolepis hornorum* L.), which were transported to San Diego State University, where all experiments were conducted. Tilapia, averaging 36.05±0.414 g, were fed commercial trout food daily, except within 24 h prior to sampling. Approximately 30 seawater-acclimated fish were held in each of ten 60 liter glass aquaria maintained at 23–25°C; aquaria were fitted with charcoal filters and air stones. Hybrids were acclimated stepwise to 35 g $l^{–1}$ over three transfers (0–10 g $l^{–1}$, 10–20 g $l^{–1}$ and 20–35 g $l^{–1}$), with 4 days allowed for acclimation at each stage. Tilapia were then allowed to acclimate for 2 weeks at full strength seawater (35 g $l^{–1}$). Ammo-lock (Aquarium Pharmaceuticals Inc., Chalfont, PA, USA) was added to detoxify ammonia, and three-quarters of the water volume was changed every 2.5 days. Seawater was prepared using Instant Ocean synthetic sea salt in dechlorinated tapwater, and salinity was measured using a light refractometer.

Series I: 28 day acclimation to seawater (35 g $l^{–1}$ salinity)

To determine the time course for seawater-acclimation in this hybrid, two tanks of fish were kept at 35 g $l^{–1}$ salinity for 28 days, and seven fish were removed and sampled at 0, 24 h (1 day), 120 h (5 days), 336 h (14 days), 504 h (21 days) and 660 h (28 days) following transfer for measurement of plasma osmolality, [Na^+], and [$Cl^–$], muscle water content, Hematocrit (Hct) and mean cell hemoglobin concentration (MCHC). Fish sampled at 14 days were also sampled for Na^+, K^+-ATPase activity, and for gill morphological parameters.

Tilapia were anaesthetized using 0.7 g $l^{–1}$ benzocaine, which was first dissolved in 3 ml of ethanol. Fish were patted dry and weighed, and the caudal peduncle was severed. Blood was collected into heparinized microhematocrit capillary tubes for measurement of Hct and total hemoglobin. Approximately 1.5 g of the left dorsal epaxial muscle was removed to determine muscle water content. Muscle tissue was placed into pre-weighed, plastic scintillation vials and weighed prior to, and following drying to constant mass for 72–96 h at 70°C.

The second and third right gill arches and approximately 1.5 cm of duodenal intestine were removed, frozen on dry ice and stored at –80°C for later analysis of Na^+, K^+-ATPase activity. The second and third left gill arches were removed from the left side of five fish at the 120 h sampling time, and immediately fixed in cold Karnovsky fixative for at least 2 h. The middle third of each gill arch was cut off by a razor blade, and gill arches about 1 mm long, bearing up to 20 filaments in both anterior and posterior rows were used for scanning electron microscopy (SEM), while individual filaments were cut for transmission electron microscopy (TEM) and light microscopy (LM) studies. All specimens were rinsed three times for 10 min each in the phosphate-buffered saline (PBS), and post-fixed in 1% osmium tetroxide for 1 h.

Series II: Direct transfer from seawater to 60 g $l^{–1}$ and 85 g $l^{–1}$ salinity

Thirty seawater-acclimated tilapia were directly transferred to salinities of 60 and 85 g $l^{–1}$, *via* a three-quarter tank water change. Seven fish from each tank were sampled at 0, 3, 24,

and 120 h following transfer. Fish from all sampling times were sampled for measurement of plasma osmolality, [Na^+], [Cl^-], muscle water content, Hct and MCHC, while those samples at 120 h were also analysed for Na^+, K^+-ATPase activity; sampling was conducted as described in Series I.

Series III: Gradual salinity increase from seawater to 95 g l^{-1} salinity

Seawater-acclimated tilapia in the remaining tanks were gradually exposed to increased salinity. Salinity in all tanks was increased 10 g l^{-1} every 5 days, *via* a three-quarter water change, up to 95 g l^{-1}; this yielded experimental salinities of 45, 55, 65, 75, 85 and 95 g l^{-1}. At each salinity, seven fish were sampled at 0, 3, 24 and 120 h. Fish from all sampling times were sampled for measurement of plasma osmolality, [Na^+], [Cl^-], muscle water content, Hct and MCHC, while those samples at 120 h were also analysed for Na^+, K^+-ATPase activity and morphology; sampling was conducted as described Series I.

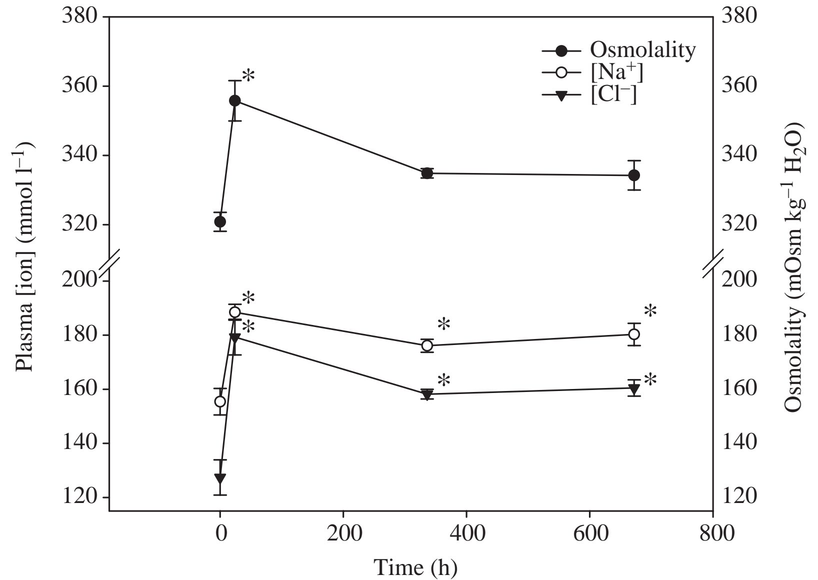

Fig. 1. The effect of transfer from 25 g l^{-1} to 35 g l^{-1} seawater on plasma osmolality, [Na^+] and [Cl^-] in tilapia hybrids from 0 to 28 days following transfer. *Significant difference relative to 0 h (P<0.001, N=7).

Series IV: Oxygen consumption and drinking rate

Oxygen consumption rate ($\dot{M}_{O_2}$) and drinking rate were measured in fish acclimated to 35, 55, 75 and 95 g l^{-1}. $\dot{M}_{O_2}$ was measured according to the methods of Gonzalez and McDonald (1994). Following measurements at 35 g l^{-1}, salinity was increased 10 g l^{-1} every 4 days until the next target salinity was reached. Fish were left at the target salinity for 1 week before measurements were made, after which salinity was increased again as previously described. When measuring $\dot{M}_{O_2}$, six fish were weighed and transferred to individual 140 ml chambers that were connected to a 60 l recirculating system filled with water of the appropriate salinity. Flow rate into individual chambers was approximately 100 ml min^{-1}, and fish were allowed to recover for 24 h before measurements were taken. To initiate measurements, water samples were withdrawn from each chamber while water was still flowing. Flow was then immediately stopped and the chambers were sealed. After 15 min, a second water sample was taken and flow was restored. The initial and final water samples were analyzed for P_{O_2} with a Cameron (Guelph, ON, Canada) O_2 meter. Using the initial and final P_{O_2}, and chamber volume, as well as fish mass, time, and the oxygen absorption coefficient for the target salinity, moles of O_2 consumed per unit time were calculated. Two sets of six fish were measured on consecutive days.

Drinking rate was measured using a modified method of Wilson et al. (1996). Fish were placed in individual 250 ml chambers connected to a recirculating system filled with 50 l of the appropriate salinity at least 24 h prior to the beginning of the measurement period. 5 μCi (185 kBq) of titrated PEG was added to each chamber and 5 ml water samples were taken after 4 and 8 h. After 8 h fish were killed using MS-222, rinsed, weighed and ligated gut removed. The whole guts were put in 4 ml 8% perchloric acid, homogenized for 1 min, and centrifuged. The supernatant and water samples were assayed for radioactivity. Drinking rate was calculated from the average activity per ml H_2O over the 8 h and the total number of counts taken up by the gut in that period.

Analyses

Blood parameters

Heparinized microhematocrit capillary tubes were centrifuged at 11 500 revs min^{-1} for 3 min in a Damon IEC MB (Needham Heights, MA, USA) microhematocrit centrifuge. Hct was recorded in duplicate or triplicate, depending on available blood volume, and plasma was expelled into Eppendorf tubes and frozen at –80°C for analyses of plasma osmolality, [Na^+] and [Cl^-]. Whole blood total hemoglobin concentration ([Hb]) was measured using a Sigma total hemoglobin assay kit, with absorbance measured using a spectrophotometer (Beckman DU640) at 540 nm; MCHC was calculated as [Hb]/(Hct/100). Plasma osmolality was measured using an osmometer (Wescor 5500V.P.), and expressed as mOsm kg^{-1} H_2O. Plasma [Cl^-] was measured using the calorimetric mercuric thiocyanate method (Zall et al., 1956), and plasma [Na^+] was measured using an atomic absorption spectrophotometer model 3100A; (Perkin Elmer, Wellesey, MA, USA). Plasma [Na^+] and [Cl^-] are expressed as mmol l^{-1} plasma.

Na^+, K^+-ATPase activity

Gill tissue taken from fish exposed to salinity for 120 h were homogenized in 1 ml of SEID buffer (250 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA·Na_2, 50 mmol l^{-1} imidazole, pH 7.3). Na^+,

K^+-ATPase activity at 120 h was determined using the method of Gibbs and Somero (1990) and expressed as μmol l^{-1} ADP h^{-1} mg^{-1} total protein; protein was determined by the Biuret method (Gibbs and Somero, 1990).

Scanning electron microscopy.

Fixed specimens were dehydrated in a graded ethanol series, concluding at 100%, critical-point-dried with liquid CO_2, mounted on stubs, sputter-coated with gold–palladium, and examined using a scanning electron microscope (Hitachi S 2700; Tokyo, Japan) at the accelerating voltage of 10 kV. Areas on the trailing (afferent) surface of filament behind the secondary lamellae and without interlamellar regions were randomly chosen. Photographs at 2000× magnification of this area (2400 μm^2) from ten different filaments per fish and three fish per group were used for quantification of apical pits and general examination of the superficial structure of filament epithelium.

Light microscopy and transmission electron microscopy.

Fixed specimens were dehydrated in a graded ethanol/acetone series with the final change in absolute acetone before infiltration and embedding in Epon epoxy resin. Longitudinal semithin (1 μm) and ultrathin (60–70 nm) sections, made parallel to the long axis of filaments, were cut using a microtome (LKB; Uppsala, Sweden). Semithin sections were mounted on glass slides, stained with 0.5% Methylene Blue, and examined on a Diastar microscope. Ultrathin sections were mounted on the copper grids, double stained with 1% uranyl acetate followed by 0.5% lead citrate, and examined in an electron microscope (Philips 410; Eindhoven, The Netherlands) at 80 kV. The ultrastructure of chloride cells was studied in the interlamellar areas of the filament epithelium. The percentage of mature, accessory, immature, apoptotic and necrotic chloride cells was determined, following the method described by Wendelaaar

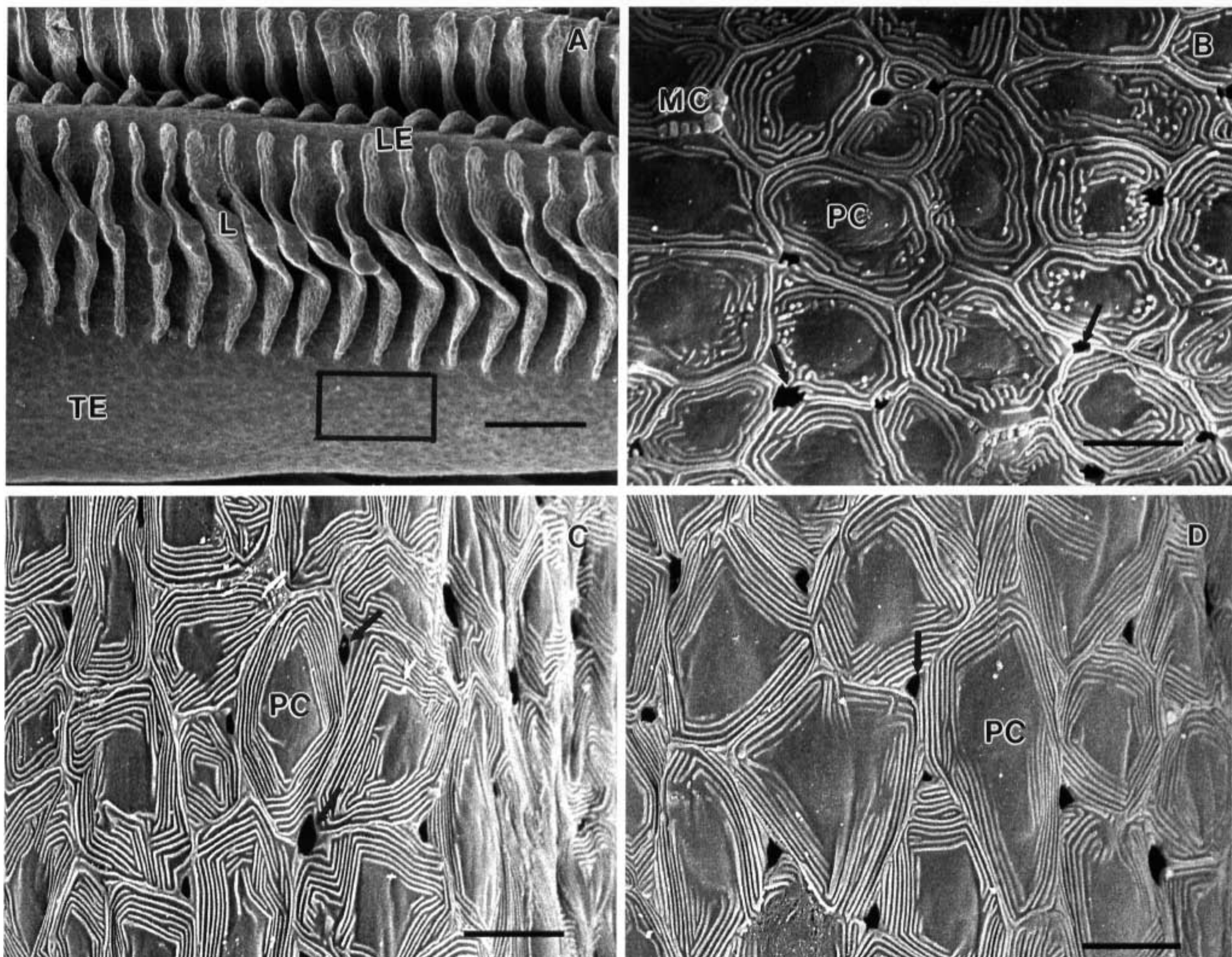

Fig. 2. SEM showing the surface of gill filaments of tilapia adapted to 35–55 g l^{-1} salinity for 14 days. (A) Middle of gill filament from 35 g l^{-1}-exposed fish; the rectangle marks the area examined on the trailing edge. L, secondary lamellae; LE, leading edge; TE, trailing edge. (B) Filamental epithelial surface from 35 g l^{-1}-exposed fish; arrows indicate the chloride cell apical pits. MC, mucus cells; PC, pavement cell. (C) Filamental epithelial surface in 45 g l^{-1}-exposed fish. (D) Filamental epithelial surface in 55 g l^{-1}-exposed fish. Scale bars: 100 μm (A), 10 μm (B–D).

Bonga and van der Meij (1989), on 20 randomly selected interlamellar areas, in three filaments per fish, and for four fish per group.

Statistics

The effects of salinity on Hct, MCHC, plasma osmolality, [Na$^+$], [Cl$^-$], muscle water content and morphological data were analyzed using one-way analysis of variance (ANOVA) followed by a *post-hoc* Dunnet's test. $\dot{M}_{O_2}$ and drinking rate were analyzed by one-way ANOVA followed by a *post-hoc* Tukey test. ANOVAs were performed using JMP (SAS Institute 2000). A value of $P<0.05$ was taken for significance in all statistical tests.

Results

Series I: 28 day acclimation to seawater (35 g l^{-1} salinity)

Plasma osmolality, [Na$^+$] and [Cl$^-$]

No mortality was observed in tilapia hybrids during acclimation to seawater. Plasma osmolality, [Na$^+$] and [Cl$^-$]

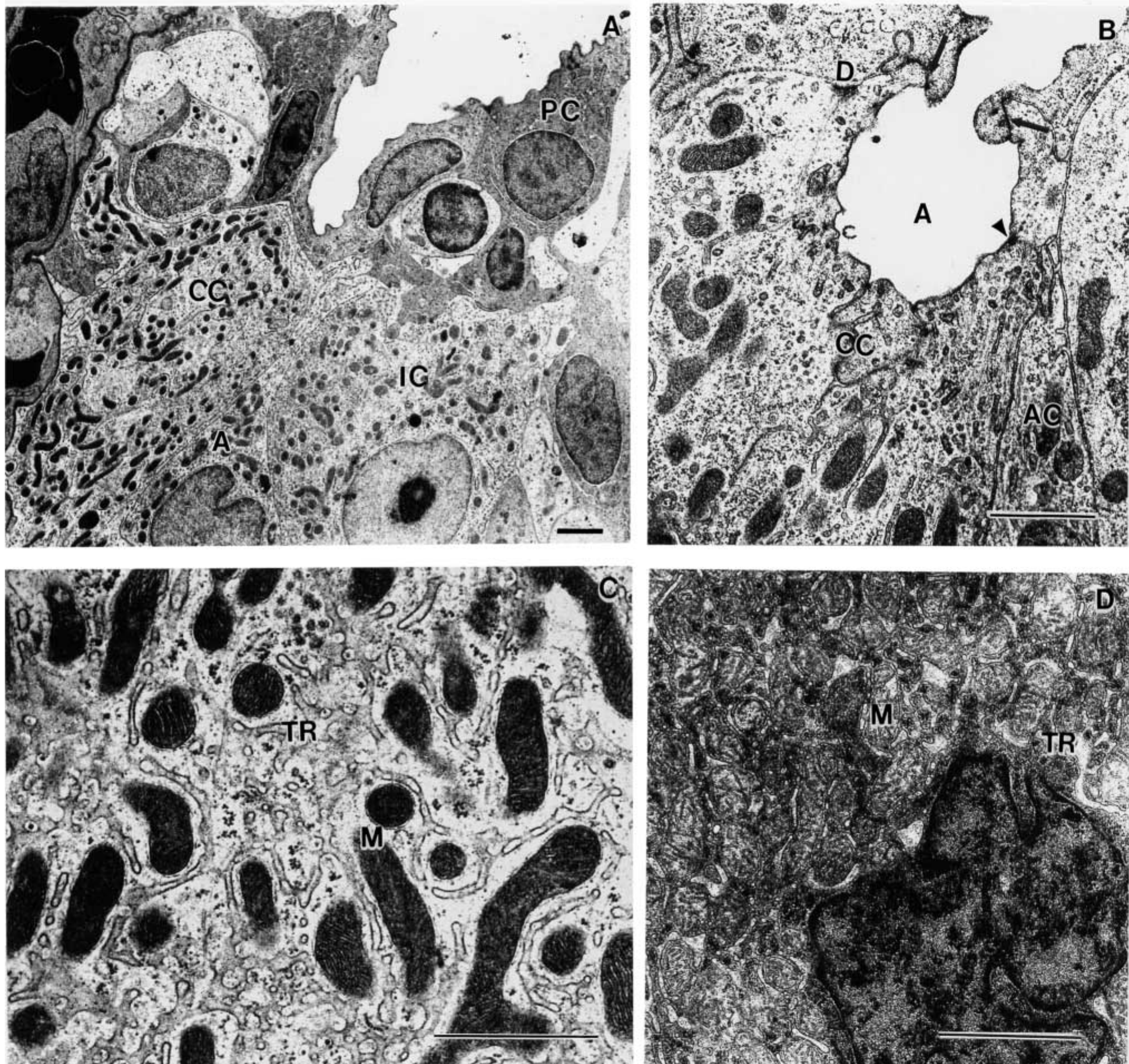

Fig. 3. TEM showing the chloride cell ultrastructure from the filamental epithelium of tilapia exposed to 35 g l^{-1} salinity for 14 days. (A) Interlamellar epithelium. AC, accessory cell; CC, mature chloride cell; IC, immature chloride cell; A, apical pit. (B) Multicellular complex formed by mature and accessory chloride cells; arrowhead indicates short apical tight junctions between CC and AC. Arrows indicate long tight junctions between CC and PC. A, apical pit; D, desmosome. (C) Perinuclear cytoplasm of a mature chloride cell. M, mitochondria; TR, tubular reticulum. (D) Apoptotic cell with nuclear and cytoplasm condensation and distention of the tubular reticulum. Scale bars: 1 μm.

Table 1. *Cellular parameters measured under scanning and transmission electron microscopy*

	Salinity (g l^{-1})						
Number per selected area	35	45	55	65	75	85	95
Apical pits (1)	8.4±1.9	9.2±2.7	8.8±2.1	17.0±3.4*	16.9±4.6*	13.6±2.4*	13.1±3.5*
Mature chloride cells (2)	36.7±4.1	35.1±4.0	36.2±3.3	37.2±3.5	20.5±4.5*	14.3±2.6*	11.0±2.9*
Accessory chloride cells (2)	50.3±4.4	50.2±5.0	53.8±5.6	89.9±5.9*	107.4±8.6*	127.3±6.9*	136.8±5.2*
Immature chloride cells (2)	11.8±3.1	11.5±2.2	12.4±2.5	13.8±5.2	22.5±4.6*	27.5±2.5*	33.8±3.6*
Apoptotic chloride cells (2)	6.7±1.2	7.3±1.4	7.4±1.5	17.6±3.8*	29.8±4.0*	49.2±6.5*	52.5±3.0*

(1) Number of apical pits on the rectangle area of filamental epithelium surface (2400 μm^2).
(2) Number of different subtypes of chloride cells in 20 interlamellar regions.
Values are means ± S.E.M. *P<0.001.

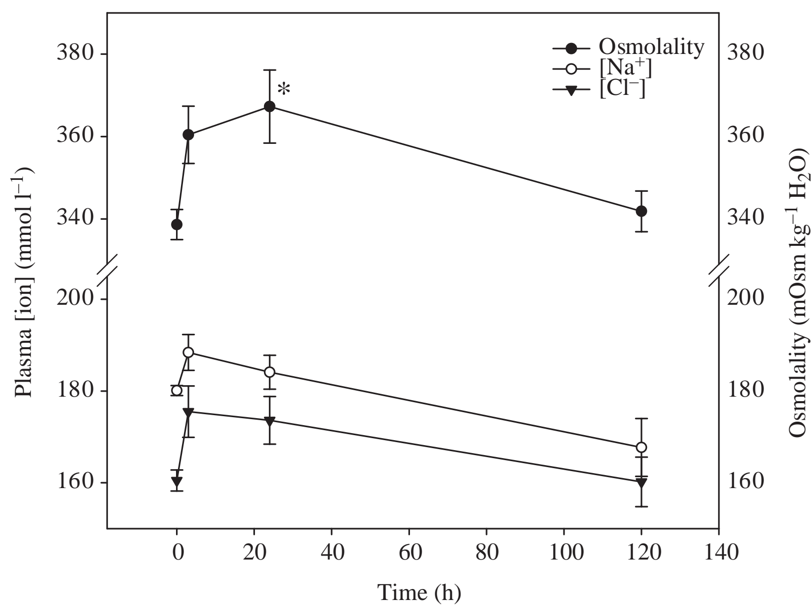

Fig. 4. The effect of transfer from 35 g l^{-1} to 65 g l^{-1} salinity on plasma osmolality, [Na$^+$] and [Cl$^-$] in tilapia hybrids from 0 to 120 h following transfer. *Significant difference relative to 0 h value (P=0.01, N=7).

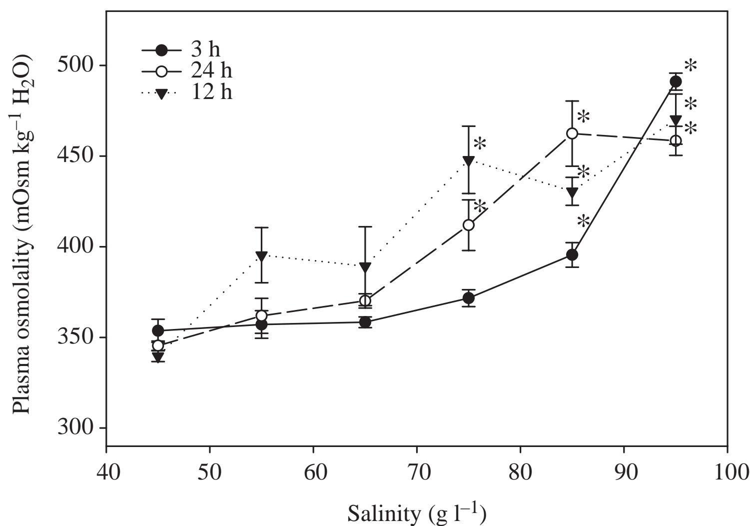

Fig. 5. The effect of salinity on plasma osmolality in tilapia hybrids following graded transfers to 45, 35, 55, 65, 75, 85 or 95 g l^{-1} salinity. Different lines represent sampling times of 3, 24 or 120 h following transfer (see key). *Significant difference relative to 45 g l^{-1} value (P<0.001, N=7).

were significantly elevated 24 h following transfer from 20 to 35 g l^{-1} salinity; however, after 24 h plasma osmolality decreased to a consistent level, not significantly different from pre-transfer values (Fig. 1). Plasma [Na$^+$] and [Cl$^-$] remained at elevated, but consistent levels throughout the remainder of the experiment. There were no changes in muscle water content at any time throughout the 28 day exposure (pooled mean±S.E.M.=80.59±0.12%), and hematological parameters such as Hct and MCHC remained unaffected as well [pooled means ± S.E.M. = 30.42±0.29% and 3.44±0.031 [Hb]/(Hct/100), respectively].

Morphology

Following 2 weeks of exposure to 35 g l^{-1}, pavement cells, a predominant component of the outer layer of filamental epithelium, displayed both long and short microridges organized into a concentric pattern with a flat central area. Numerous deep apical pits of multicellular chloride complexes and few pores of mucous cells were observed on the surface of filamental epithelium (Fig. 2B). Chloride complexes combined mature, immature and accessory cells that were linked by short apical tight junctions and followed desmosomes (Fig. 3A,B). No interdigitations between apexes of chloride cells within the multicellular complexes were found. Aging chloride cells degrading by apoptosis displayed a reduced size, highly condensed cytoplasm, a markedly indented nucleus with condensed chromatin, and

numerous enlarged mitochondria surrounded by distended tubules of the network (Fig. 3D). Numbers of mature, accessory, immature, and apoptotic cells are presented in Table 1.

Series II: Direct transfer from seawater to 60 g l^{-1} and 85 g l^{-1} salinity

Direct transfer from 35 g l^{-1} to 85 g l^{-1} salinity resulted in 100% mortality. In contrast, all fish survived direct transfer from 35 g l^{-1} to 60 g l^{-1}, but significant effects on sub-lethal indicators of osmoregulatory stress were observed. Plasma osmolality, $[Na^+]$ and $[Cl^-]$ increased only qualitatively 3 h post-transfer, while significant increases over pre-transfer values were observed 24 h after, but only in osmolality (Fig. 4). Plasma osmolality, $[Na^+]$ and $[Cl^-]$ all returned to near pre-transfer values by 120 h. Na^+, K^+-ATPase activity significantly increased from 2.46±0.48 to 8.87±1.62 μmol h^{-1} μg^{-1} protein between 0 and 120 h, respectively, which represents a near 260% increase in activity following transfer from 35 to 60 g l^{-1} salinity.

Series III: Gradual salinity increase from seawater to 95 g l^{-1} salinity

Plasma osmolality, $[Na^+]$ and $[Cl^-]$

With the exception of minor mortality observed at 85 g l^{-1} salinity, tilapia hybrids survived a 10 g l^{-1} salinity increase every 5 days up to a salinity of 95 g l^{-1}. Plasma osmolality values were not significantly different, relative to those at 45 g l^{-1}, in fish exposed to 55–65 g l^{-1} salinity, and were consistent with values obtained from 35 g l^{-1} fish sampled at 14 and 28 days. However, in fish exposed to 75 g l^{-1} salinity, osmolality was significantly increased at 24 and 120 h following transfer. At the 3 h sampling time, plasma osmolality was significantly increased relative to 45 g l^{-1} in fish exposed to salinities of 85 and 95 g l^{-1} (Fig. 5). Plasma $[Na^+]$ and $[Cl^-]$ followed similar trends, and were significantly elevated at 85 and 95 g l^{-1}, with the exception of $[Cl^-]$ at 3 h, which was only significantly increased at 95 g l^{-1} salinity (Fig. 6).

Na^+, K^+-ATPase activity

Branchial Na^+, K^+-ATPase activity remained relatively constant in gills exposed to 35–65 g l^{-1} salinity, but was significantly increased at 75 and 85 g l^{-1} (Fig. 7). Gut Na^+, K^+-ATPase activity averaged 5.30±0.41 mmol ADP h^{-1} mg^{-1} protein over the range of salinities, and did not vary significantly (data not presented).

Morphology

The superficial pattern of pavement cells was largely unchanged from that of a typical seawater-acclimated epithelium in fish exposed to 45–65 g l^{-1} salinity (Figs 2, 8A), although the first appearance of interdigitated junctions between mature and accessory chloride cells was in fish exposed to 55 g l^{-1} salinity (Fig. 9). However, at 75–95 g l^{-1} salinity the surface of pavement cells bore fewer marginal

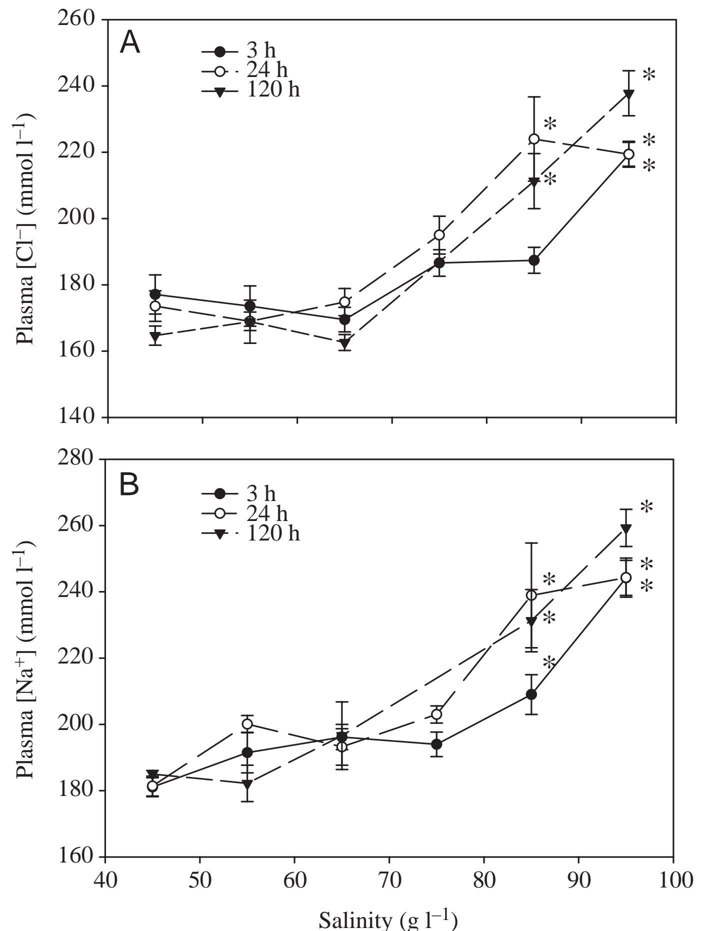

Fig. 6. The effect of salinity on (A) Plasma $[Cl^-]$ and (B) plasma $[Na^+]$ in tilapia hybrids following graded transfers to 45, 35, 55, 65, 75, 85 or 95 g l^{-1} salinity. Different lines represent sampling times of 3, 24 or 120 h following transfer (see key). *Significant difference relative to 45 g l^{-1} value (P<0.001, N=7).

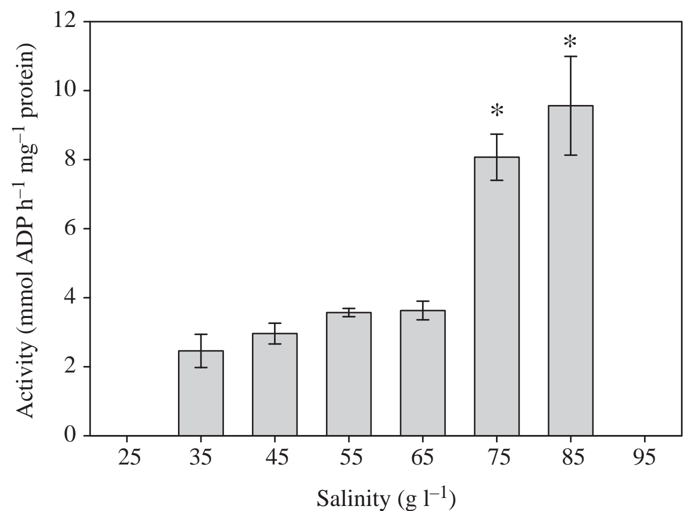

Fig. 7. Branchial Na^+, K^+-ATPase activity measured in tilapia hybrids acclimated to 35, 45, 55, 65 or 85 g l^{-1} salinity for 120 h. *Significant difference relative to 35 g l^{-1} value (P<0.001, N=7).

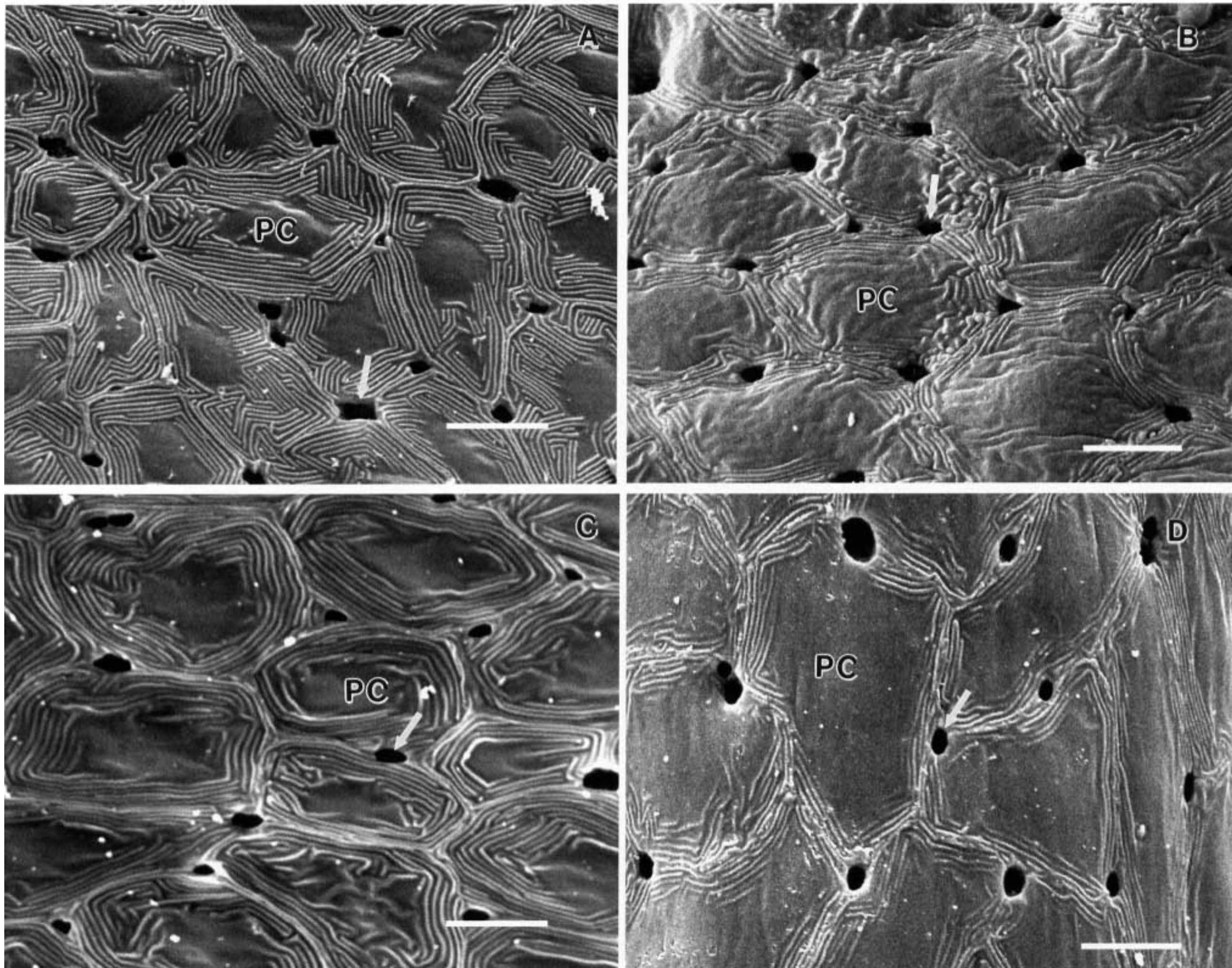

Fig. 8. SEM showing the surface of filamental epithelium in tilapia exposed to (A) 65 g l^{-1}, (B) 75 g l^{-1}, (C) 85 g l^{-1} and (D) 95 g l^{-1} salinity for 120 h. Scale bars: 10 μm. PC, pavement cell.

microridges, and cells had an expanded flat central area (Fig. 8B–D). The number of apical pits per 2400 μm^2 remained relatively consistent in the epithelia of tilapia exposed to 35–55 g l^{-1}, but then was significantly increased from 65–95 g l^{-1} salinity (Table 1). No mucous cells were observed in the filamental epithelium of fish exposed to 75 g l^{-1} salinity or greater. The first appearance of interdigitated mature and accessory chloride cells was noted in fish exposed to 55 g l^{-1} salinity (Fig. 9A).

The proportion of cells within the four stages of the chloride cell life cycle also changed with salinity. The number of cells in mature, accessory, immature or apoptotic stages quantified within 20 interlamellar regions, remained relatively consistent in fish exposed to 35–55 g l^{-1} salinity (Table 1). At 65–95 g l^{-1} salinity, the number of accessory and apoptotic chloride cells significantly increased, and at 75 g l^{-1} the number of immature cells became significantly increased, while the number of mature cells was reduced. Changes in cellular parameters are summarized in Table 1, and TEMs representing different stages of chloride cells exposed to various salinities are presented in Figs 10–12. Finally, several cells from epithelia of fish exposed to 95 g l^{-1} salinity exhibited features that are considered signs of cellular necrosis, including electron-transparent vacuolated cytoplasm, swollen mitochondria possessing fragmented cristae, an irregularly organized tubular reticulum, and a nucleus with a low electron density (Fig. 12F–G).

Series IV: Oxygen consumption rate and drinking rate

Oxygen consumption rate ($\dot{M}_{O_2}$) decreased with salinity. In fish acclimated to 95 g l^{-1} salinity, $\dot{M}_{O_2}$ decreased 40.5% relative to fish acclimated to seawater. Relative decreases in $\dot{M}_{O_2}$ at 55 and 75 g l^{-1} salinity were 17.6 and 29.5%, respectively (Fig. 13). Drinking rate remained constant at 35 and 55 g l^{-1} salinity, but was significantly increased at 75 g l^{-1} salinity (Fig. 14). Fish acclimated to 95 g l^{-1} for 2 weeks did not show an increased drinking rate, but these fish were in poor condition and means are based on N=3 due to mortality.

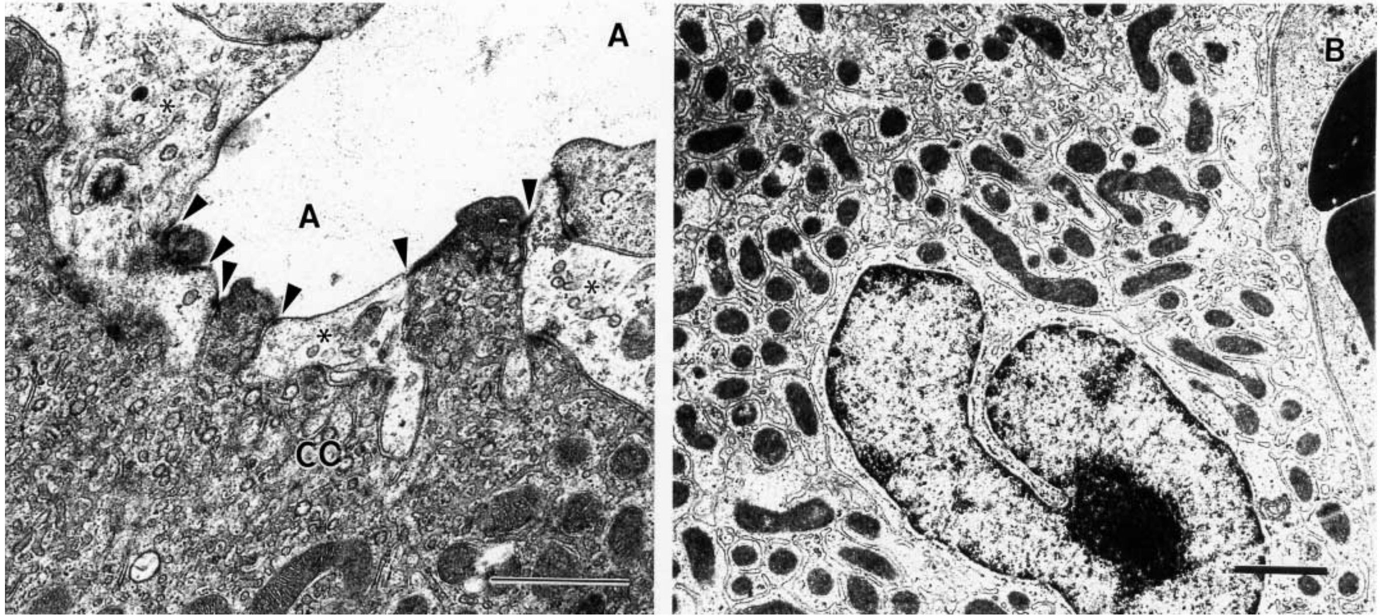

Fig. 9. TEM showing chloride cell ultrastructure in the filament epithelium of tilapia exposed to 45 g l^{-1} and 55 g l^{-1} salinity for 120 h. (A) Apical pit (A), formed by interdigitated apex of mature chloride cells (CC) and cytoplasmic projections of accessory cells (AC; asterisks), in fish exposed to 55 g l^{-1} salinity; arrowheads, short junctions between CCs and ACs. (B) Mature chloride cell with a deeply indented nucleus. Scale bars: 1 μm.

Discussion

'California' Mozambique tilapia are very saline tolerant, surviving up to 5 days in salinities as high as 95 g l^{-1}, which is similar to the salinity tolerance of pure Mozambique tilapia (Kultz and Onken, 1993; Stickney, 1986). When directly transferred from seawater, hybrid tilapia cannot survive in 85 g l^{-1} salinity, but they do survive transfer to 60 g l^{-1} salinity, while experiencing some osmoregulatory disturbances; however, plasma osmolality, [Na^+] and [Cl^-] were restored within 120 h.

When tilapia hybrids are gradually transferred to increased salinity, with 120 h between transfers, they survive exposures to salinity as great as 65 g l^{-1} before showing any signs of osmoregulatory stress. Tilapia no longer maintained a steady state with respect to osmoregulation above 65 g l^{-1} salinity. Changes in gill morphology were most sensitive, and first appeared after 120 h of exposure to 55 g l^{-1} salinity, indicating that they may be good indicators of osmoregulatory stress in this species. More specifically, it was concluded that the number of apoptotic chloride cells would best serve as an indicator of stress, due to their sizable increase immediately prior to other signs of osmoregulatory failure such as elevated plasma osmolality, [Na^+], [Cl^-], and branchial Na^+, K^+-ATPase.

Series I: Seawater acclimation of 'California' Mozambique tilapia

Physiological, biochemical and morphological data indicate that 2 weeks is sufficient for tilapia hybrids to become acclimated to seawater. Transfer from 20 to 35 g l^{-1} salinity resulted in increases plasma osmolality, [Na^+] and [Cl^-] 24 h following transfer (Fig. 1), but plasma osmolality decreased to a relatively constant level, near 340 mOsm kg^{-1}, when measured at 2 and 4 weeks. Although plasma [Na^+] and [Cl^-] remained significantly elevated over the 4 week period, concentrations consistently remained near 180 and 160 mmol l^{-1}, respectively, from 2 to 4 weeks. Hwang et al. (1989) found that seawater-acclimated Mozambique tilapia had a plasma osmolality and [Cl^-] of 343.8±17 mOsm kg^{-1} and 156±8.8 mmol l^{-1}, respectively, and Uchida et al. (2000) found seawater-acclimated tilapia had an osmolality of approximately 330 mOsm kg^{-1}. Furthermore, the general morphology and ultrastructure of the branchial epithelium of tilapia hybrids acclimated to 35 g l^{-1} salinity were similar to those previously observed in other seawater-acclimated species of tilapia (Cioni et al., 1991; Hwang, 1987; van der Heijden et al., 1997; Wendelaaar Bonga and van der Meij, 1989).

Series II: Mortality and sub-lethal effects of direct transfer to increased salinity

Survival was 100% following direct transfer from 35 g l^{-1} to 60 g l^{-1} salinity; however, significant effects on plasma osmolality, [Cl^-] (Fig. 4) and Na^+, K^+-ATPase activity were observed. Plasma [Na^+] and [Cl^-] followed a similar pattern to plasma osmolality, which was significantly elevated at 24 h following transfer but returned to near pre-transfer values by 120 h (Fig. 4). The return of plasma osmolality to pre-transfer levels, in spite of a greatly increased osmotic gradient, is probably due to the nearly 260% increase in Na^+, K^+-ATPase activity that was observed 120 h following transfer. Kultz et al. (1992) also observed large increases in enzyme activity

when transferring Mozambique tilapia from 10 g l^{-1} to 45 g l^{-1}, and 60 g l^{-1} salinity.

III: Sub-lethal effects of graded transfer to increased salinity

The increase in salinity from 35 g l^{-1} to 65 g l^{-1} represents a more than twofold increase in the osmotic gradient between the water and the blood, yet no significant increases in plasma osmolality, [Na^+] or [Cl^-] (Figs 5, 6) were observed, nor was there an increase in Na^+, K^+-ATPase activity (Fig. 7). Kultz and Onken (1993) observed a dramatic reduction in chloride cell–accessory cell leaky junction conductance over the range of salinities from 35 g l^{-1} to 60 g l^{-1}, and suggested that Mozambique tilapia reduce their whole body permeability in hypersaline water up to 60 g l^{-1}, which could offset the cost of osmoregulation. This hypothesis is consistent with the data obtained in the current study. The formation of interdigitation junctions in fish exposed to 55 g l^{-1} salinity may indicate that the fish are approaching the limit for effective osmoregulation *via* epithelial permeability reduction, and that a more traditional mechanism of salt excretion, such as an increase in Na^+, K^+-ATPase activity or drinking rate, is needed (Laurent, 1984). A similar phenomenon was described in Mozambique tilapia after 5 days of acclimation to full-strength seawater (Wendelaar Bonga and van der Meij, 1989).

Another possible explanation for the lack of change in plasma osmolality, [Na^+] and [Cl^-] could be that the measured level of Na^+, K^+-ATPase activity is sufficient to deal with environmental salinities up to 65 g l^{-1}. Furthermore, in spite of the lack of change in gut Na^+, K^+-ATPase, the role of the gut in water absorption should not be ruled out; drinking rate was

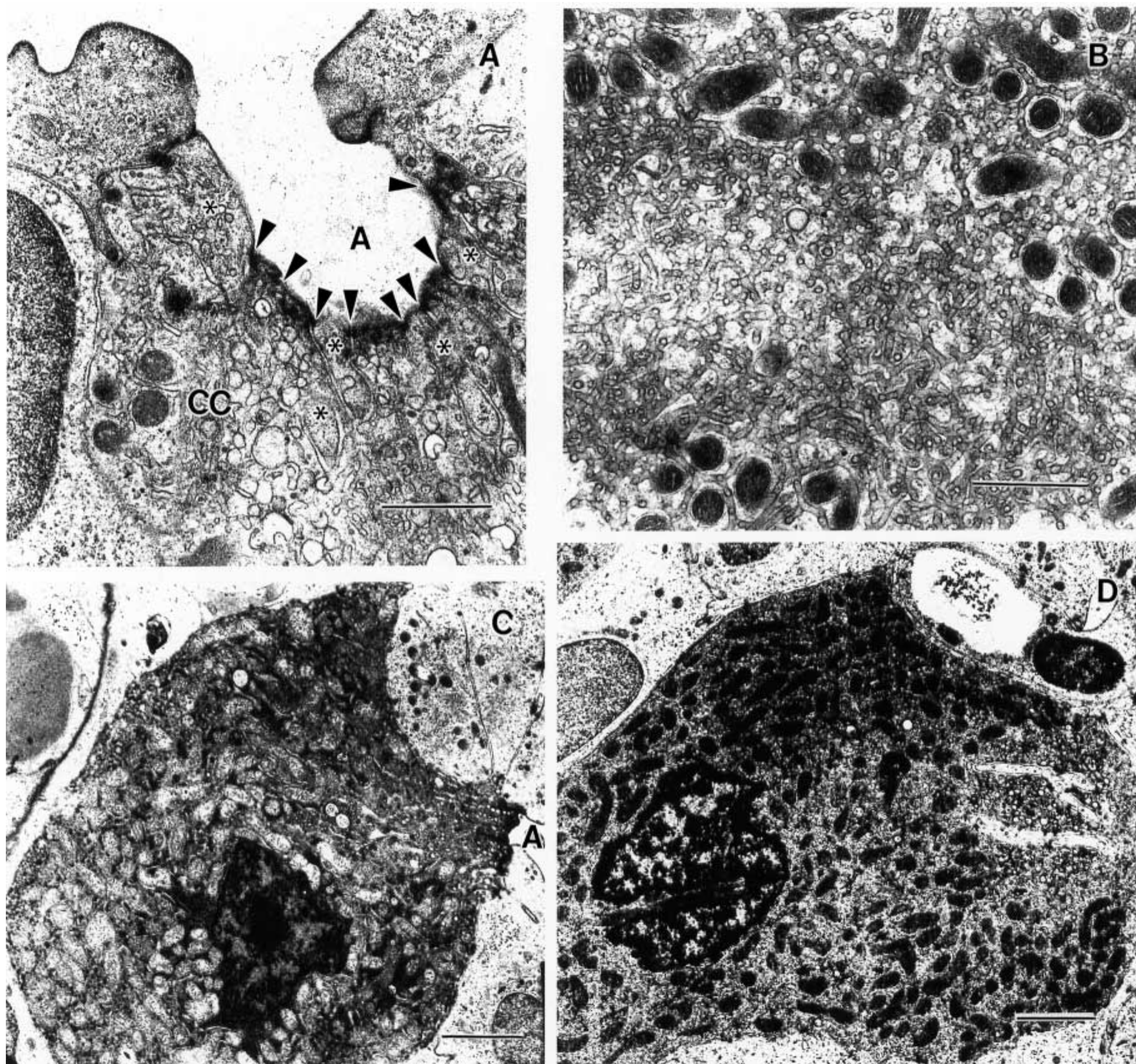

Fig. 10. TEM showing the chloride cell ultrastructure from the filamental epithelium of tilapia exposed to 65 g l^{-1} salinity for 120 h. (A) Deep apical pit (A) of a multicellular complex formed by interdigitated chloride cells and accessory cells (ACs); arrowheads, short tight junctions; asterisks, projections of ACs. (B) Perinuclear cytoplasm of a mature chloride cell, showing the extensively developed tubular reticulum. (C) Chloride cell at the early stage of apoptosis. (D) Apoptotic chloride cell with a cleft nucleus and highly condensed cytoplasm. Scale bars: 1 μm.

unchanged between 35 and 55, but increases in water absorption rate still may have occurred.

At exposure to salinities greater than 65 g l^{-1}, there are significant changes in physiological, biochemical and morphological indicators of osmoregulatory stress, which are probably indicative of osmoregulatory failure at the highest salinities. Plasma osmolality became significantly elevated, relative to seawater, 3 h following transfer to 85 g l^{-1} and 95 g l^{-1} salinity and at 24 and 120 h following transfer to 75–95 g l^{-1} salinity; with plasma [Na^+] and [Cl^-] following similar trends (Figs 5, 6). These increases in osmolality and ion levels coincide with a dramatic increase in Na^+, K^+-ATPase

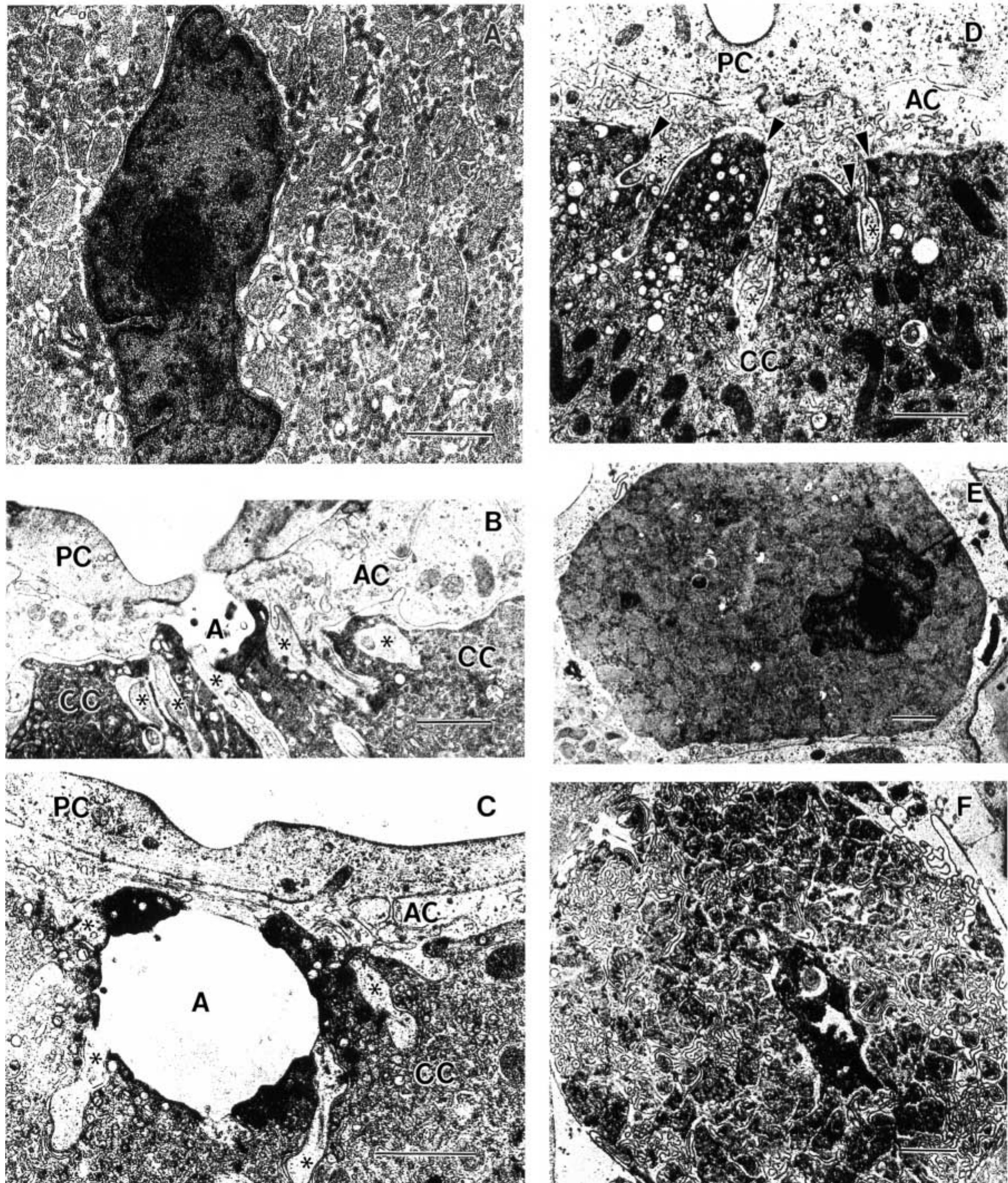

Fig. 11. TEM showing the chloride cell ultrastructure from the filamental epithelium of tilapia exposed to 75 g l^{-1} salinity for 120 h. (A) Nucleus and perinuclear cytoplasm of an apoptotic chloride cell (CC). (B) Apical pit of multicellular complex that is partially covered by pavement cells (PC) projections; asterisks, cytoplasmic projections of accessory cells (AC). (C) Apical pit (A) that is completely separated from the ambient water by layer of PCs. (D) A multicellular complex without an apical pit; arrowheads indicate desmosomes joining CCs and ACs. (E) Chloride cell degrading by apoptosis. (E) Chloride cell at the final stage of apoptosis. Scale bars: 1 μm.

activity at 75 g l^{-1} and 85 g l^{-1} (Fig. 7), which is a good indication of an osmoregulatory challenge.

Exposure to salinities greater than 65 g l^{-1} increased the turnover rate of branchial chloride cells. Changes in

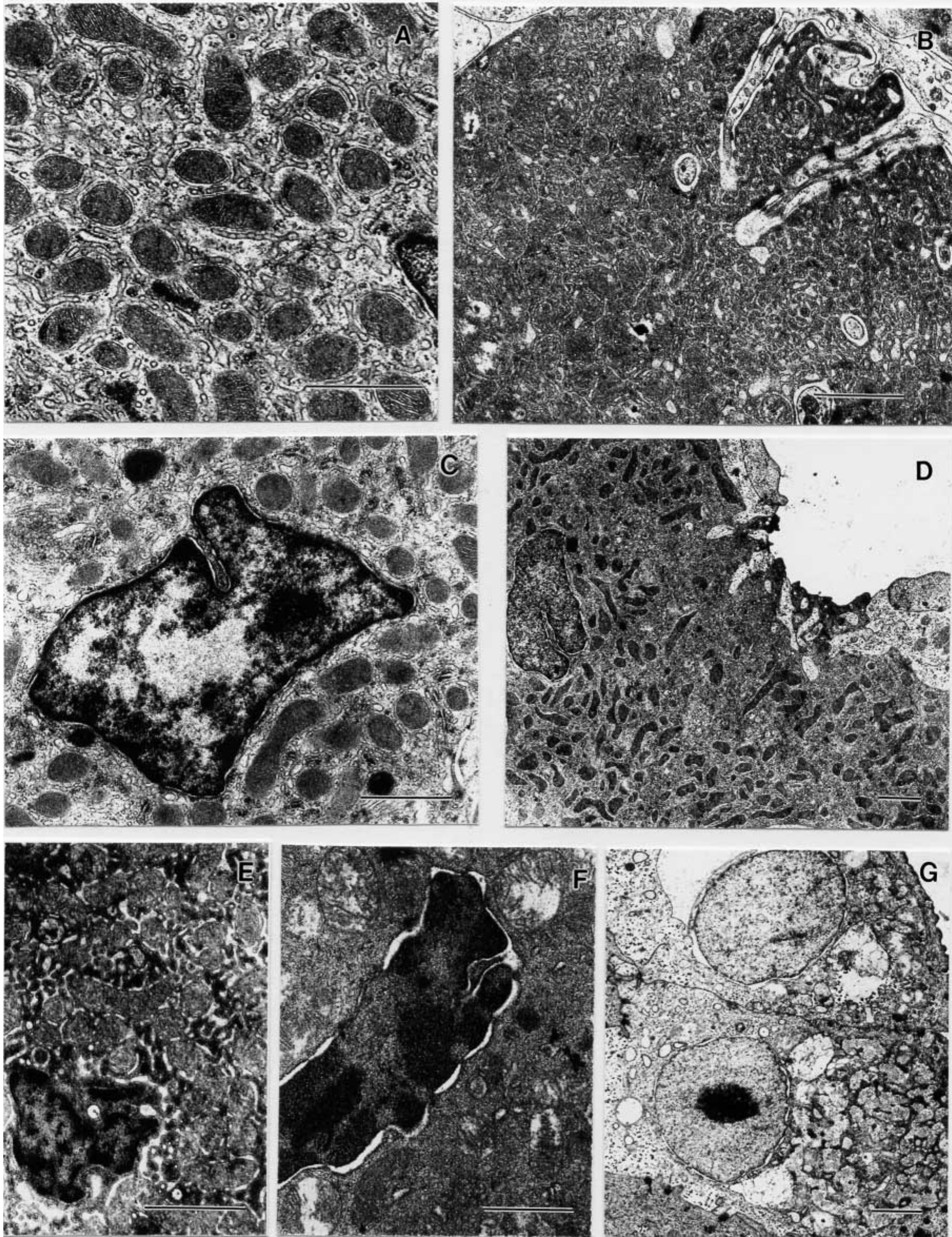

Fig. 12. TEM showing the chloride cell ultrastructure of the filamental epithelium from tilapia exposed to 85 g l^{-1} (A,B) and 95 g l^{-1} (C–G) salinity for 120 h. (A) Perinuclear cytoplasm of a mature chloride cell. (B) Chloride cell at the early stage of degradation by apoptosis. (C) Nucleus and perinuclear cytoplasm of a mature chloride cell. (D) Multicellular complex with a shallow apical pit, formed by a mature cell at the early stage of apoptosis and accessory cell projections. (E) Apoptotic chloride cell. (F) Degenerated chloride cells with condensed cytoplasm, pycnotic nuclei, and distended and ruptured mitochondria. (G) Chloride cells degrading by necrosis. Scale bars: 1 μm.

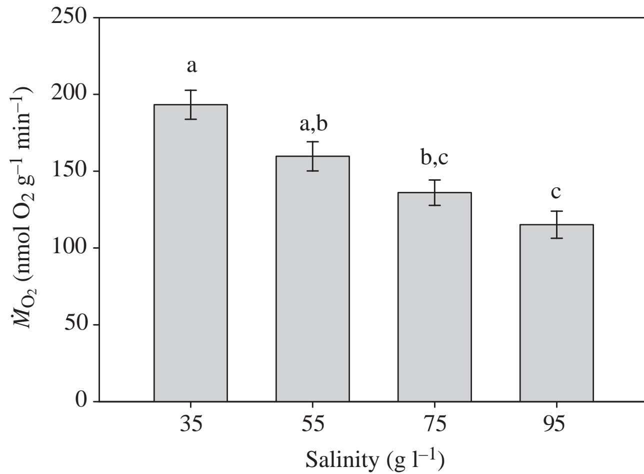

Fig. 13. Rate of oxygen consumption ($\dot{M}_{O_2}$) measured in tilapia hybrids acclimated for 2 weeks to 35, 55, 75 or 95 g l^{-1} salinity. Letters indicate significant differences (P<0.001, N=6).

physiological and biochemical parameters in fish exposed to 65–95 g l^{-1} were associated with wide-scale morphological alterations along the branchial epithelium. In 65 g l^{-1}-exposed fish, the number of accessory cells and apoptotic chloride cells increased nearly twofold and threefold, respectively, but the number of mature cells was similar to that in fish exposed to 35–55 g l^{-1} salinity. Exposure to 75–95 g l^{-1} salinity resulted in dramatic changes in the ratio between different subtypes of chloride cells. Mature chloride cells constituted only 11% of the total number of cells in 75 g l^{-1}-exposed fish, and only 5% in 95 g l^{-1}-exposed fish; in contrast, mature cells constituted 32–36% of all chloride cells in fish exposed to 35–55 g l^{-1} salinity (Table 1). Similar results observed in freshwater-acclimated tilapia, exposed to a number of stressful factors (e.g. high salinity, low pH or cadmium), were attributed to an increased turnover of chloride cells due to rapid aging (van der Heijden et al., 1997; Wendelaaar Bonga and van der Meij, 1989).

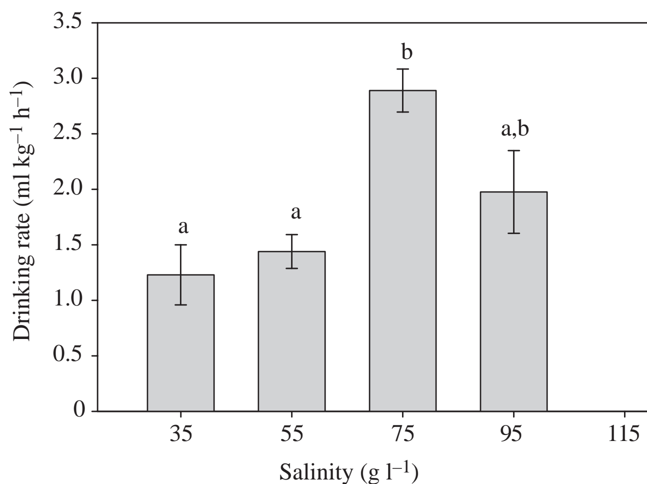

Fig. 14. Drinking rate measured in tilapia hybrids acclimated for 2 weeks to 35, 55, 75 or 95 g l^{-1} salinity. Letters indicate significant differences (P<0.001, N=6).

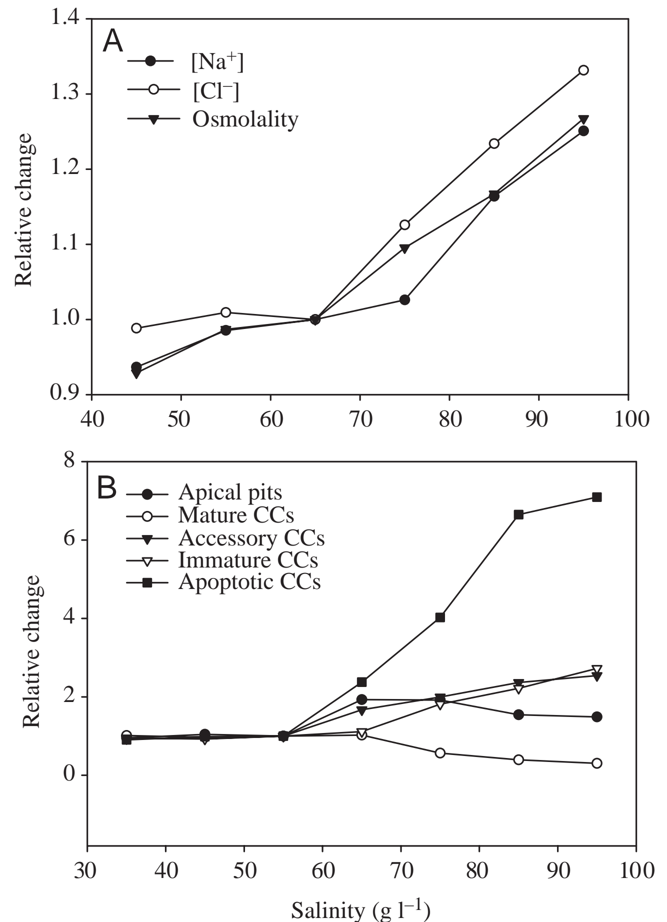

Fig. 15. Relative changes in (A) plasma parameters, with values at 65 g l^{-1} set to 1.0, and (B) cellular parameters, with values at 55 g l^{-1} set to 1.0.

TEM photos of the tilapia epithelium exposed to 75 g l^{-1} in the current study reveal partial and completely covered apical pits (Fig. 11B,C), which probably occurred at other high salinities as well. It is unclear to what end tilapia in hypersaline water are using a low-salinity acclimation strategy, but it may represent an attempt to seal off chloride cell leaky junctions to prevent further gain of salts or loss of water to the external environment. A more detailed investigation into this phenomenon will certainly prove interesting.

Fish exposed to salinities above 65 g l^{-1} face a further osmoregulatory challenge, as the branchial epithelium may have a drastically reduced capacity for ion transport. It has been previously shown that the ion-transporting capacity of the epithelium is dependent on the number of mature cells more so than on total number (Kultz et al., 1992). Functional activity of accessory and immature cells should be lower relative to mature cells, due to their poorly developed tubular reticulum and a limited number of mitochondria. Furthermore, a very low ion-transporting capacity, if any, may be expected from degenerating cells, and chloride cells occluded by pavement

cells may be considered functionally silent. Significant increases in plasma osmolality, [Na^+] and [Cl^-] are observed in concert with a significant decrease in the number of mature chloride cells. Interestingly, Na^+, K^+-ATPase activity was highest at 75 g l^{-1} and 85 g l^{-1} salinity (Fig. 7); the source of the increase in enzyme activity in a largely degenerating epithelium is another point of interest for further study. The significance of the increased turnover rate of chloride cells with respect to survival of the animal is also unclear, but it is unlikely that fish could survive long exposure periods at salinities above 55–65 g l^{-1}, in particular at 95 g l^{-1} when signs of cellular necrosis in the epithelium become apparent.

Series IV: Oxygen consumption rate and drinking rate

Oxygen consumption rate ($\dot{M}_{O_2}$) decreased with salinity, by as much as 40.5% from 35 g l^{-1} to 95 g l^{-1} (Fig. 13). Although the majority of previous work on how salinity affects $\dot{M}_{O_2}$ has focused on changes during acclimation from freshwater to seawater (Farmer and Beamish, 1969; Morgan et al., 1997), Swanson (1998) observed a reduction in $\dot{M}_{O_2}$ at 55 g l^{-1} salinity relative to 35 g l^{-1} in milkfish *Chanos chanos*, and attributed it to a reduction in activity level, allowing for greater use of metabolic energy for osmoregulation. An alternative hypothesis was proposed by Haney and Nordlie (1997), when sheepshead minnow *Cyprinodon variegatus* $\dot{M}_{O_2}$ decreased nearly 33% over a range of salinities from 40 g l^{-1} to 100 g l^{-1}; this decrease is relatively comparable to that observed in the current study. Haney and Nordlie (1997) suggested that the drop in $\dot{M}_{O_2}$ was related to a change in the permeability of the branchial epithelium, as described by Kultz and Onken (1993), as the reduction in branchial permeability would result in less oxygen diffusion into the animal. These hypotheses are not mutually exclusive, and it is probable that both play a role in tilapia hybrid osmoregulation.

Drinking rate was not significantly changed at salinities below 75 g l^{-1} (Fig. 14). The lack of an increase over this salinity range indicates that the tilapia are not losing excess water to the environment in spite of the increase from 35 g l^{-1} to 55 g l^{-1}, and the large increase in osmotic gradient between the blood and the water. This is consistent with the hypotheses formed in Series III that suggest a preventative strategy of osmoregulation in hypersaline water up to a salinity of 65 g l^{-1} *via* a reduction in branchial permeability.

Conclusions

The 'California' Mozambique tilapia has a similar salinity tolerance to pure Mozambique tilapia (Costa-Pierce and Riedel, 2000; Hwang et al., 1989; Kultz and Onken, 1993; Stickney, 1986; Uchida et al., 2000; van der Heijden et al., 1997).

Morphology, in particular the number of apoptotic chloride cells in the epithelium, appears to be the most sensitive and dramatic indicator of osmoregulatory stress. Gill morphology remained relatively unchanged until fish were exposed to a salinity of 55 g l^{-1}, but plasma parameters tended not to change until fish were exposed to salinities greater than 65 g l^{-1}. To better visualize these changes, morphological parameters were plotted on a scale of relative change, with values obtained at 55 g l^{-1} set to 1.0, and the same with plasma parameters obtained at 65 g l^{-1} that were also set to 1.0 (Fig. 15). This interpretation provides a good template for potential salinity tolerance modeling for this species when exposed to graded salinity increases. Whether or not the use of gill morphology as an osmoregulatory stress indicator is effective over longer durations of exposure to hypersalinity remains to be seen.

The final goal of this study was to gain insight into the salinity tolerance of 'California' Mozambique tilapia, which has ecological relevance considering the continual increase in salinity of the Salton Sea; it may be concluded that with all other variables under strict control, these hybrids can tolerate salinities up to 65 g l^{-1} and show little to no change in physiological parameters, with the possible exception of the decreased activity level that was indicated by a reduced $\dot{M}_{O_2}$ values. Furthermore, these tilapia hybrids seem to employ a strategy involving a reduction in permeability in hypersaline conditions, which has been previously described by Kultz and Onken (1993). Above 65 g l^{-1} salinity, changes in physiological, biochemical and morphological indicators of osmoregulatory stress become apparent, indicating a limit to salinity tolerance *via* permeability reduction. To further add to this model, the effects of other abiotic factors such as temperature or hypoxia, which have been observed to fluctuate in saline lakes such as the Salton Sea, need to be included; temperature in particular has been shown to greatly reduce salinity tolerance in this tilapia hybrid (Sardella et al., 2003), and reduce osmoregulatory ability (Al Amoudi et al., 1996).

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