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Functional and morphological plasticity of crocodile (*Crocodylus porosus*) salt glands

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SUMMARY

The estuarine crocodile, *Crocodylus porosus*, inhabits both freshwater and hypersaline waterways and maintains ionic homeostasis by excreting excess sodium and chloride ions *via* lingual salt glands. In the present study, we sought to investigate the phenotypic plasticity, both morphological and functional, in the lingual salt glands of the estuarine crocodile associated with chronic exposure to freshwater (FW) and saltwater (SW) environments. Examination of haematological parameters indicated that there were no long-term disruptions to ionic homeostasis with prolonged exposure to SW. Maximal secretory rates from the salt glands of SW-acclimated animals (100.8±14.7 μmol 100 $g^{-0.7}$ body mass h^{-1}) were almost three times greater than those of FW-acclimated animals (31.6±6.2 μmol 100 $g^{-0.7}$ body mass h^{-1}). There were no differences in the mass-specific metabolic rate of salt gland tissue slices from FW- and SW-acclimated animals (558.9±49.6 and 527.3±142.8 μl O_2 g^{-1} h^{-1}, respectively). Stimulation of the tissue slices from SW-acclimated animals by methacholine resulted in a 33% increase in oxygen consumption rate. There was no significant increase in the metabolic rate of tissues from FW-acclimated animals in response to methacholine. Morphologically, the secretory cells from the salt glands of SW-acclimated animals were larger than those of FW-acclimated animals. In addition, there were significantly more mitochondria per unit volume in secretory tissue from SW-acclimated animals. The results from this study demonstrate that the salt glands of *C. porosus* are phenotypically plastic, both morphologically and functionally and acclimate to changes in environmental salinity.

Key words: osmoregulation, reptile, tissue respirometry, stereology, transmission electron microscopy.

INTRODUCTION

The estuarine crocodile, *Crocodylus porosus*, displays broad euryhaline capabilities, with animals observed in waters ranging in salinity from 0‰ to over 60‰ (Grigg et al., 1986). Data from field surveys demonstrate that animals can maintain plasma osmolarity within a narrow range (298–309 mOsm) along such salinity gradients (Grigg, 1981). In order to successfully osmoregulate in saline environs, *C. porosus* utilise extra-renal salt-secreting glands (Taplin and Grigg, 1981), much like marine reptiles and pelagic sea birds. Marine birds have paired orbital salt glands, which secrete excess salt (Fänge et al., 1958; Schmidt-Nielsen et al., 1958), whereas in marine turtles, lachrymal salt glands are responsible for salt secretion (Schmidt-Nielsen and Fänge, 1958). Sea snakes, however, have sublingual salt glands, which open into the oral cavity (Dunson and Taub, 1967). In crocodiles, the salt-excreting glands are located on the tongue immediately below the lingual epithelium and are responsible for the excretion of sodium and chloride ions (Taplin and Grigg, 1981; Taplin et al., 1982).

Lingual salt glands, unique to crocodilians, were first identified and described in *C. porosus* by Taplin and Grigg (Taplin and Grigg, 1981). The salt glands appear as 20–40 distinct pores on the posterior half of the lingual surface. Classified as compound, branched, tubular structures, each gland is composed of 10–14 distinct lobes. Lobes consist of a number of short, blind-ending secretory tubules which join together to form multiple interlobular ducts. Secretions exit the gland *via* these ducts. Numerous arterioles and venules punctuate the connective tissue around and between gland lobes, and a dense network of capillaries can be found in the connective tissues both surrounding and between the secretory tubules (Franklin and Grigg, 1993). Nerve fibres are also present in large numbers in the connective tissues between and throughout the salt gland lobes and tubules (Cramp et al., 2007; Franklin et al., 2005). Methacholine chloride, an acetylcholine agonist, stimulates lingual salt gland secretion in *C. porosus* (Taplin and Grigg, 1981) suggesting that active secretion may be under the control of the cholinergic nervous system (Franklin et al., 2005). In marine birds and turtles, salt loading by intravenous or intraperitoneal infusion of hypertonic salt solutions also stimulates secretions from the salt glands (e.g. Fänge et al., 1958; Schmidt-Nielsen and Fänge, 1958; Schmidt-Nielsen et al., 1958). Salt loading of restrained crocodiles does not stimulate secretion from the salt glands; an increase in the rate of secretion from the salt glands following salt loading only occurs in those that are freely moving and unstressed, implying a significant involvement of the adrenergic nervous system in regulating (suppressing) crocodilian salt gland secretion (Franklin et al., 2005; Taylor et al., 1995).

Avian salt glands show remarkable levels of phenotypic plasticity – both morphological and physiological – as a consequence of drinking saline water (for a review, see Shuttleworth and Hildebrandt, 1999). In birds that have never been exposed to saline conditions, salt glands secrete little; initial exposure to an osmotic stressor initiates a cascade of morphological and physiological responses, which results in a marked increase in salt gland secretion rate. The most striking change in avian salt glands upon initial exposure to salt water is the increase in gland size (Ellis et al., 1963) which is brought about by changes in secretory cell volume, cell

number and blood flow to and within the gland (Ballantyne and Wood, 1969; Hanwell et al., 1971; Hanwell and Peaker, 1975; Hossler, 1982; Knight and Peaker, 1979). These changes in morphology accompany increases in mitochondrial and glycolytic enzymes (McFarland et al., 1965; Spannhof and Jurss, 1967; Stainer et al., 1970) and increases in the abundance and activity of Na^+,K^+-ATPase (e.g. Ernst et al., 1967; Ernst and Mills, 1977; Fletcher et al., 1967; Stewart et al., 1976). In addition, most of these adaptive responses appear to be reversible during de-adaptation (e.g. Hossler et al., 1978; Mcarthur and Gorman, 1978).

Given the wide range of salinities across which the estuarine crocodile *Crocodylus porosus* can successfully osmoregulate in the wild, we speculate that crocodile salt glands should demonstrate a broad functional flexibility. Moreover, this functional flexibility should also be manifested in the cellular morphology of the glands. In previous studies, it has been shown that the gross morphology of the salt glands changes significantly with prolonged acclimation to saline environments (Cramp et al., 2007; Franklin and Grigg, 1993). Although we know that *C. porosus* transferred from fresh to salt water are immediately capable of maintaining the plasma ion homeostasis (Taplin, 1985), suggesting that salt gland function can be upregulated rapidly, little is known about long-term acclimatory changes in the structure and function of the glands following prolonged exposure to hypersaline environs.

In the present study, we have examined whole animal osmoregulatory capacities, maximal secretory rates, and tissue metabolic rate in *C. porosus* salt glands following prolonged acclimation to both fresh and hypersaline environments. Additionally, we performed a detailed morphological study of salt gland secretory cells from acclimated animals to determine if morphological changes to cells occur with prolonged saltwater acclimation which may correlate with functional changes in the tissue.

MATERIALS AND METHODS

Animal collection and maintenance

Estuarine crocodiles *Crocodylus porosus* Schneider 1801 were obtained from Fleays Wildlife Park (Queensland, Australia), as eggs. The eggs were incubated in a Clayson (Brendale, Queensland, Australia) IM 1000 incubator at 30°C in plastic containers covered in moist vermiculite. Upon hatching, all animals were transferred to 1000 l Nally bins containing 20 l of heated (30±2°C) freshwater. A basking area was supplied with a standard 40 W globe, turned on daily from 09:00 h until 16:00 h. The crocodiles were fed *ad libitum* on a mixture of live crickets and dead baby mice. Water changes were carried out twice weekly. Once all animals were feeding well (~2 weeks), animals were assigned to salinity treatment groups.

Hatchling crocodiles were randomly assigned to one of two treatment groups: freshwater (FW) or saltwater (SW) acclimation. SW-acclimated animals were placed into 70% seawater made using commercially available artificial sea salt (Aquasonic, Wauchape, New South Wales, Australia). Animals in both treatments were fed and had their water changed twice weekly. Animals remained in these treatments for 6 months.

Maximal secretory rate

Animals (N=6 per treatment group) were fasted for 1 week prior to experimentation. Animals were removed from holding tanks, weighed and restrained on a foam-lined board and their mouths propped open with a cork and secured with fabric tape. The tongue surface was rinsed clean with water and then blotted dry. Animals then received 2 mg kg^{-1} methacholine chloride in sterile saline *via* intraperitoneal injection and were left undisturbed for 10 min. Whatman filter papers pieces were cut to the size and shape of the animal's tongue. Secretions from the salt gland pores were collected by placing the filter papers over the tongue and pores and allowing the secretion to be absorbed into the filter paper. Filter paper pieces were large enough that they did not become completely saturated with secretion during the sampling period and that ensured secretions were continually absorbed by capillary action into the paper. Filter paper pieces were left on the tongue for 10 min. At the end of 10 min, the filter paper was removed and placed into a glass vial containing 1 ml of ultrapure water (Millipore, 18.2 MΩ cm). The process of secretion collection was repeated three more times. Vials containing salt secretions were allowed to sit overnight for the salt to diffuse into the surrounding fluid. Na^+ concentrations were measured with an EEL flame photometer (Corning Glassworks, Corning, NY, USA), taking into account the additional Na^+ present in the filter paper prior to the addition of the secretions. Na^+ secretion rate data were scaled to body mass and are presented as μmol 100 $g^{-0.7}$ body mass h^{-1}. The highest rate of secretion achieved over the 40 min period was deemed the maximal secretory rate. Mass and maximal secretory rates were compared with Student's t-tests using the statistical program SigmaStat™. Data are presented as means ± s.e.m.

Blood collection and processing

A 1 ml blood sample was taken from the cervical sinus of six crocodiles from each treatment group into individual heparinised syringes. Form these, duplicate 50 μl samples were taken and placed in haematocrit tubes and centrifuged for 2 min at 13 000 ***g***. The remaining blood sample was centrifuged at 2500 ***g*** for 3 min and the plasma collected. Osmotic pressure was measured by vapour pressure osmometry, and Na^+, K^+ and Cl^- concentrations were determined using an Olympus AU400 automated analyser (Olympus America Inc., Melville, NY, USA) using the ion-selective electrode (ISE) method. All haematological parameters were compared with Student's t-tests or where data failed tests of normality or equal variance, non-parametric Mann–Whitney rank sum tests were performed. All analyses were performed with the statistical program SigmaStat™. Data are presented as means ± s.e.m.

Tissue respirometry

In vitro salt gland oxygen consumption rate was measured using a Strathkelvin Instruments Mitocell S200 micro respirometry system comprising a 782 oxygen meter, 1302 oxygen electrode MT200 respirometer (Strathkelvin Instruments, Motherwell, Scotland). Crocodiles (N=5 per treatment) were killed by overdose of anaesthetic (sodium thiopentone, Jurox, Rutherford, New South Wales, Australia). The tongue was excised whole and placed into ice-cold crocodile ringer (containing in mmol l^{-1}, NaCl 116, KCl 4, $CaCl_2$ 2, $MgCl_2$ 2, glucose 15, sodium pyruvate 5, Hepes-Na 10). The salt glands were dissected free of the underlying connective tissue and overlying epithelia. Small (<0.5 mm thick↔1 mm^2) slices of salt gland tissue were placed into beakers containing ringer at room temperature bubbled continuously with air and allowed to incubate for 15 min. One piece was then placed into a MT200 microrespirometer containing a magnetic stirrer bar and 0.5 ml of air-saturated crocodile ringer at 25°C. The chamber was sealed and the rate of oxygen consumption by the tissue in the chamber was recorded instantaneously until the partial pressure of oxygen in the chamber had dropped by 20% (approximately 30 min, depending on the treatment group). At the end of this period the chamber was

opened and the ringer was replaced with fresh oxygenated ringer containing 0.1 mmol l^{-1} methacholine chloride. The chamber was once again sealed and the rate of oxygen consumption measured until the partial pressure of oxygen in the chamber had dropped by 20%. Prior to the commencement of experimentation, trials were conducted to ensure that oxygen consumption rates did not drop substantially over the course of the experiment. The data (not shown) indicated that, apart from an immediate drop in the metabolic rate of the tissue following excision (first 15 min), the metabolic rate of the tissue was subsequently stable for at least 4 h. Care was taken to ensure that all experimental measures were completed within this 4 h window. At the completion of the experiment, tissue pieces were blotted dry and weighed. Prior to the commencement of, and at the end of each trial, the rate of background oxygen consumption in the chamber (from contaminants and the electrode) in the absence of any tissue was recorded and this mean rate subtracted from the tissue rates. In order to minimize bacterial contamination of the chamber, chambers were cleaned prior to trials with 70% ethanol and the chamber ringer was filtered through 0.2 μm Millipore syringe filters. Rates of oxygen consumption were standardized to tissue wet mass and are reported as $\mu l\ O_2\ g^{-1}\ h^{-1}$. Data were compared statistically using a two-way repeated-measures AVOVA. *Post-hoc* comparisons were made with Holm–Sidak tests. All statistical comparisons were made using the statistical program SigmaStat™. Data are presented as means ± s.e.m. and treatments were considered as significantly different if $P<0.05$.

Transmission electron microscopy

Crocodiles (N=4 per treatment) were euthanased and salt glands dissected out as described previously. Small pieces (*circa* 2 mm^2) containing at least one visible pore were dissected out from the surface layer and were placed into cold 2% glutaraldehyde (ProSciTech, Thuringowa, Australia) in 0.1 mol l^{-1} phosphate buffer pH 7.4 for 24 h. Following glutaraldehyde fixation, tissues were washed in phosphate buffer and then post-fixed in 1% osmium tetroxide (ProSciTech, Thuringowa, Australia) in phosphate buffer. Tissue pieces were then dehydrated through an ascending ethanol gradient and infiltrated with Spurrs medium (ProSciTech). Tissues were blocked and allowed to polymerise at 60°C for 48 h. Semi-thin sections were cut, stained with 1% Toluidine Blue in 0.1 mol l^{-1} phosphate buffer and viewed under a microscope to determine if the appropriate position in the tissue piece had been reached (i.e. there were secretory cells present). Once the appropriate position within the tissue block was confirmed, ultrathin sections (60 nm) were cut with a diamond knife and collected onto 2 mm copper grids. All sectioning was performed with a Leica Ultracut UC6 Ultramicrotome. Sections were stained with uranyl acetate and lead citrate and viewed at 80 kV in a Jeol 1011 transmission electron microscope. Images were captured with a MegaView III digital camera using the iTEM™ software package.

Stereological analysis

Between 50 and 70 images of different secretory cell were collected by transmission electron microscopy (TEM) from four animals per treatment group. Mitochondrial volume density, plasma membrane surface area, intercellular space volume, and mitochondrial cross-sectional area were measured using the morphometrics software package, SigmaScanPro™.

Volume density of mitochondria within secretory tissue ($V_{V\,mit}$)

The volume density of mitochondria within glandular secretory tissue ($V_{V\,mit}$) was determined by systematic point counting of mitochondria in ultrathin sections. Using image analysis software, a coherent test lattice (30×24 μm; with lines spaced 2 μm apart) was superimposed over digital transmission electron micrographs of glandular tissue. The number of points of intersection (i.e. where test lines cross one another) over mitochondria was then counted. To calculate $V_{V\,mit}$, this number was divided by the total number of points of intersection over secretory tissue.

Volume density of intercellular space within secretory tissue ($V_{V\,ics}$)

The volume density of intercellular space within glandular secretory tissue ($V_{V\,ics}$) was determined by systematic point counting using the same coherent test lattice and digital TEMs of glandular tissue as used to determine $V_{V\,mit}$. To determine intercellular space, the number of points of intersection over areas of intercellular space was counted and divided by the total number overlaying secretory tissue.

Surface density of basolateral cell membrane within secretory tissue ($S_{D\,cm}$)

The surface density of basolateral cell membrane within secretory tissue ($S_{D\,cm}$) was determined using a coherent test lattice (30×24 μm; with lines spaced 4 μm apart) superimposed over the same TEMs used to determine $V_{V\,mit}$ and $V_{V\,ics}$. To determine $S_{D\,cm}$, the number of times test lines intersected the basolateral membrane of secretory cells was counted in micrographs from each animal. To calculate $S_{D\,cm}$, this number was doubled and then divided by the total length of line overlaying secretory tissue (L_C). L_C was calculated using the equation: $L_C=L_T\times V_{V\,st}$, where L_T is the total length of test line applied and $V_{V\,st}$ is the volume density of secretory tissue (st) in micrographs.

Cross-sectional area of mitochondria (μm^2)

Differences in $V_{V\,mit}$ between treatment groups could reflect a difference in the size and/or density of mitochondria. To investigate differences in $V_{V\,mit}$, we compared mitochondrion cross-sectional area between treatment groups. We did this by measuring the cross-sectional area of 200 mitochondria from each animal in both treatment groups. Cross-sectional area was measured directly from digital TEMs of glandular tissue using the image analysis program (SigmaPlot™ 7.0).

Cell density

$S_{D\,cm}$ will depend not only on the extent of elaboration of the basolateral cell membrane, but also the number of cells per unit volume of secretory tissue. To investigate the effects of saltwater and freshwater acclimation on basolateral cell membrane area more thoroughly, we compared the number of nucleated cells, per unit volume density of secretory tissue ($V_{V\,st}$), in micrographs from each animal. We did this by counting the number of nucleated cells in TEM micrographs and dividing this by the volume density ($V_{V\,st}$) of glandular secretory tissue. All morphological parameters were compared with Student's t-tests using the statistical program SigmaStat™. Data are presented as means ± s.e.m.

Chemicals

All reagents were sourced from Sigma Aldrich (Sydney, Australia) unless otherwise specified.

RESULTS

Maximal secretory rate

There were no differences in mean animal mass between treatment groups (P=0.5193, t=0.6679, d.f.=10l; FW-acclimated range

110–375 g; SW-acclimated range 130–234 g). Maximal Na^+ secretion rates from the lingual salt glands of *C. porosus* were highly variable (FW-acclimated range: 12–53; SW-acclimated range: 33–137 μmol 100 $g^{-0.7}$ body mass h^{-1}). There was a marked increase in the mean maximal salt gland secretory rate of the SW-acclimated animals when compared with their freshwater counterparts (*t*-test, P=0.002, t=–4.053, d.f.=10; Fig. 1A). In SW-acclimated animals, maximal secretion rates were attained 15 min post methacholine injection and declined steadily over the subsequent 40 min period. In FW-acclimated animals, secretion rates appeared to have attained their maximal level within 15 min, but remained constant over the subsequent collection period (Fig. 1B).

Osmoregulation

The haematocrit of blood from FW-acclimated animals was significantly lower than that of SW-acclimated animals (*t*-test, P=0.042; Fig. 2A). Plasma osmolarity was also significantly lower in FW-acclimated animals relative to SW-acclimated ones (*t*-test, P<0.05; Fig. 2A). Both plasma Na^+ and K^+ concentrations were significant higher in SW-acclimated individuals (Na^+-SW= 173±2.9, Na^+-FW=147±9.5 mmol l^{-1}; K^+-SW=5.9±0.18, K^+-FW=4.8±0.35 mmol l^{-1}) but Cl^- concentrations were not different between the treatment groups (Mann–Whitney rank sum test, P=0.065; Fig. 2B).

Tissue oxygen consumption

There was no statistically significant difference in the control (pre-drug) metabolic rates of tissue slices from FW- and SW-acclimated treatment groups (two-way RM ANOVA P=0.842, t=0.205; Fig. 3). Addition of methacholine chloride to tissue slices from FW-acclimated animals had no statistically significant effect on oxygen consumption (P=0.065, t=2.137) whereas in SW-acclimated animals, methacholine chloride addition resulted in a 33% increase in oxygen consumption (P=0.02, t=4.426).

Ultrastructure of principal secretory cells

The secretory tubules of *C. porosus* consist of a single layer of simple cuboido-columnar epithelial cells. The epithelium is composed primarily of just one cell type, the principal secretory cell, although this cell type appeared to have several morphological states. At the blind end of the secretory tubules, the principal cells were typically conical in cross section. They were, on average approximately 15 μm long and 7 μm wide at their widest point, tapering to approximately 3 μm at the apical tip of the cell (Fig. 4A). Morphologically, the principal cells were characterised by a greatly amplified basolateral membrane (BLM). Lateral, microvilli-like projections of the BLM intermeshed with those of adjacent cells (Fig. 4B). By contrast, the apical membrane surface area was relatively reduced. A few, small microvilli projected from the apical surface of the secretory cells into the secretory tubule lumen (Fig. 4C). Adjacent principal cells were linked at their apexes by zona occludens and zona adherens junctions and along their basolateral membranes by zona adherens junctions. Principal cells contained a large, basally located nucleus composed primarily of euchromatin, with small regions of heterochromatin at the nuclear periphery. The cells generally contained a large number of mitochondria, which were evenly dispersed throughout the cell. Numerous secretory vesicles were readily visible at the apical tip and, to a lesser degree, along the basolateral membrane of the principal cells. Large amounts of smooth endoplasmic reticulum

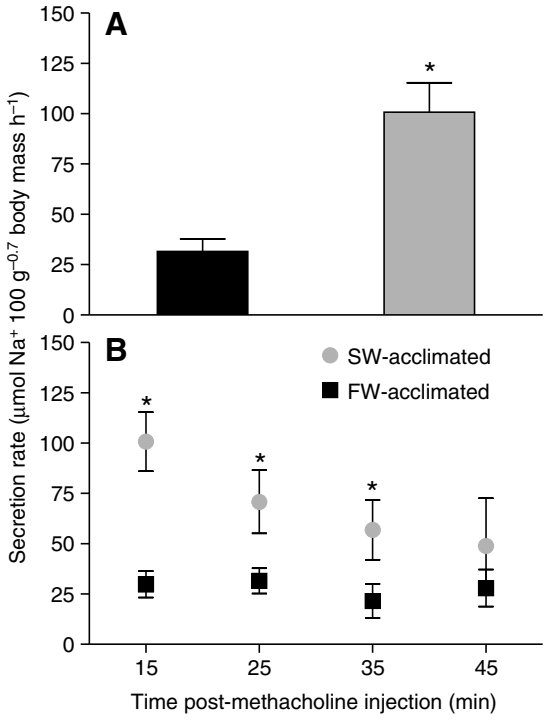

Fig. 1. (A) Maximal Na^+ secretion rate from lingual salt glands of restrained *C. porosus* juveniles acclimated to either freshwater (black bars) or saltwater (grey bars) conditions. (B) Na^+ secretion rates from lingual salt glands over 40 min post-stimulation by methacholine chloride (2 mg kg^{-1}). Values are means ± s.e.m.; N=6 per treatment. Asterisks indicate a significant difference between FW- and SW-acclimated treatment groups at the level of P<0.05.

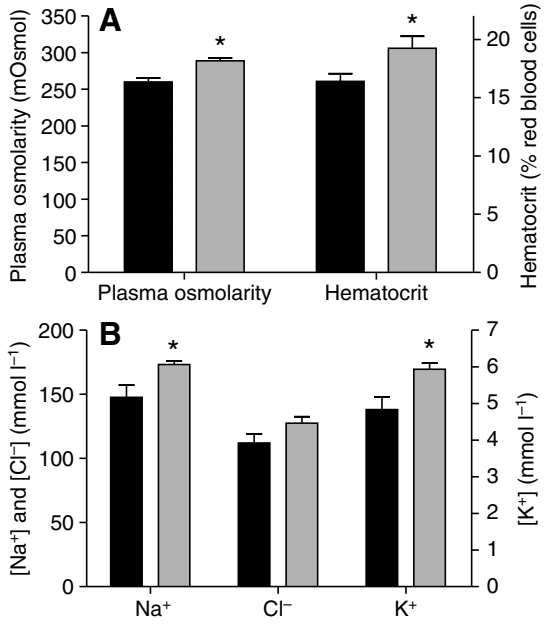

Fig. 2. Blood chemistry of freshwater (black bars) or saltwater (grey bars)-acclimated *C. porosus* juveniles. (A) Haematocrit (% red blood cells) of whole blood, and plasma osmolarity; (B) plasma electrolytes, Na^+, Cl^- and K^+. Values are means ± s.e.m.; N=6 per treatment. Asterisks indicate a significant difference between FW- and SW-acclimated treatment groups at the level of P<0.05.

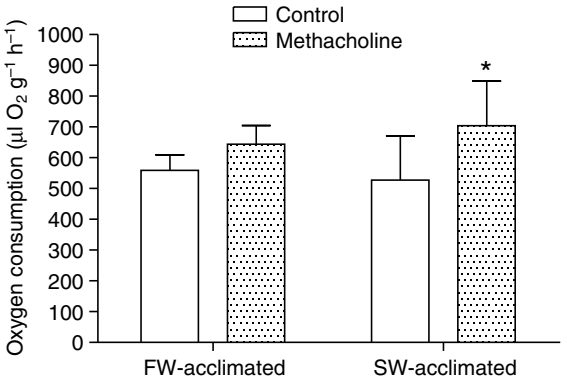

Fig. 3. Respiration of crocodile salt gland slices from freshwater- (black bars) or saltwater- (grey bars) acclimated *C. porosus* and the effect of 0.1 mmol l^{-1} methacholine chloride on salt gland tissue oxygen consumption rate. Values are means ± s.e.m.; *N*=5 per treatment. Asterisks indicate a significant difference between control and methacholine treatments at the level of $P<0.05$.

(ER) could be seen throughout the cell. Rough ER was less evident in principal cells. In cross section most cells could be seen to contain at least one Golgi complex, generally located close to the nucleus. Many secretory cells also contained small electron-opaque, non-membrane bound vesicles. Principal cells rested upon a well-developed basement membrane and were in close proximity to capillaries (Fig. 4D).

Morphological plasticity of principal secretory cells

There was no significant effect of saltwater acclimation on either the surface density of the basolateral cell membrane within secretory tissue ($S_{D\,cm}$; Table 1; *t*-test, $P=0.714$, d.f.=7) or the volume of intercellular space between secretory cells ($V_{V\,ics}$; Table 1; *t*-test, $P=0.546$, d.f.=7). There was a significant effect of saltwater acclimation on the volume density of mitochondria within the principal secretory cells; there was an increase of almost 23% in mitochondrial volume per μm^3 in SW-acclimated animals relative to FW-acclimated animals (Table 1; *t*-test, $P=0.024$, d.f.=7).

There were no differences in mitochondrial cross-sectional areas between FW- and SW-acclimated animals (*t*-test, $P=0.55$, d.f.=7), suggesting that the increase in mitochondrial volume density is most probably due to an increase in mitochondrial numbers, rather than mitochondrial size.

There was a significant difference in the numbers of cell nuclei per μm^2 per micrograph, with SW-acclimated animals having significantly fewer nuclei than FW-acclimated animals (Table 1; *t*-test, $P=0.046$, d.f.=7), suggesting that cells from SW-acclimated animals were larger than those of FW-acclimated animals.

DISCUSSION

We examined indices of salt gland function both *in vivo* and *in vitro* and the ultrastructual morphology of the salt secreting tissue of the estuarine crocodile, *Crocodylus porosus* to determine its long-term adaptive capacity and phenotypic plasticity following chronic exposure to hypo- and hyperosmotic environmental conditions. As previously established, salt glands play a pivotal role in the maintenance of osmotic homeostasis in *C. porosus* living

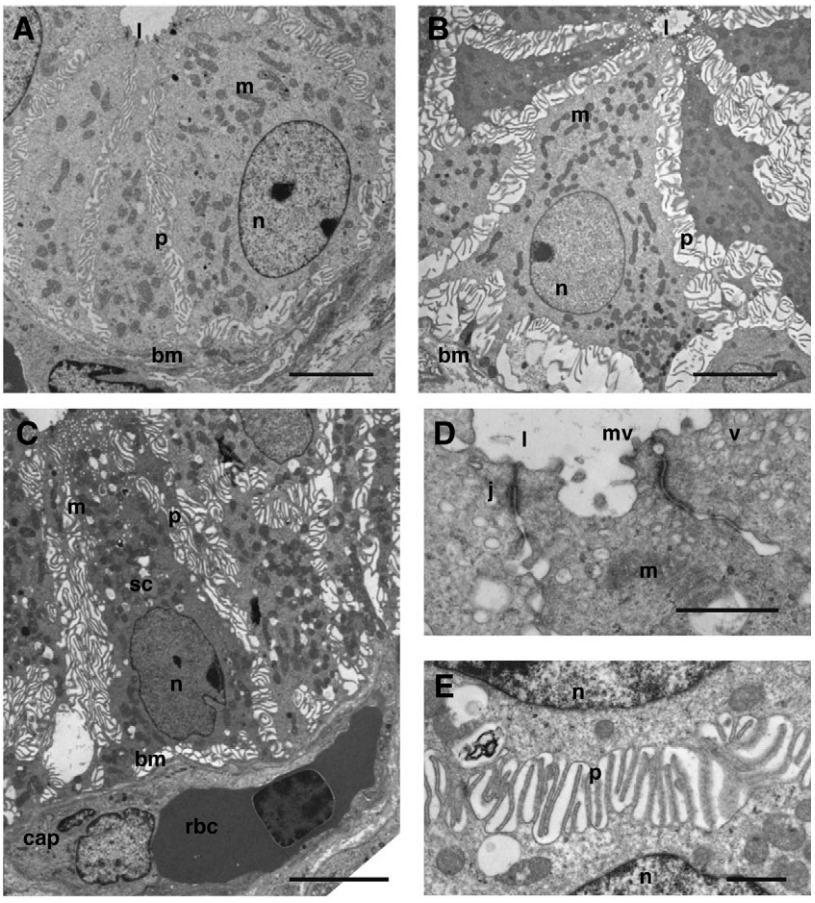

Fig. 4. Transmission electron micrographs of principal secretory cells from the salt gland of (A) FW-acclimated and (B) SW-acclimated *C. porosus.* Transverse sections through a whole cell showing ultrastructural arrangement of organelles typical of the principal secretory cells. Scale bars, 5 μm. (C) Transmission electron micrographs of principal secretory cells from the salt gland of a FW-acclimated *C. porosus* showing close apposition between secretory cells and capillaries. Scale bar, 5 μm. (D) High-resolution transverse section through the apical region of a principle secretory cell showing junctional complex and short, irregularly shaped microvilli protruding into the secretory tubule lumen. Scale bar, 1 μm. (E) High-resolution transverse section though the lateral margins of two adjoining cells demonstrating the interlocking arrangement of the long microvilli (plici) of the basolateral membrane. Scale bar, 1 μm. l, secretory tubule lumen; p, plicae; bm, basement membrane; n, nucleus; m, mitochondria; j, junctional complex; mv, apical microvilli; v, small, clear, apical vesicles; rbc, red blood cell; bm, basement membrane; cap, capillary; sc, secretory cell.

Table 1. The effects of chronic exposure to environmental salinity on the ultrastructure of the secretory cells in the lingual salt glands of juvenile freshwater- and saltwater-acclimated *C. porosus*

Ultrastructual measurement	FW-acclimated group	SW-acclimated group	*P* value
Volume density of mitochondria ($\mu m^3\ \mu m^{-3}$)	0.0736±0.00543	0.0908±0.00193	0.024*
Volume density of intercellular space ($\mu m^3\ \mu m^{-3}$)	0.0544±0.00573	0.0611±0.009	0.546
Surface density of plasma membrane ($\mu m^2\ \mu m^{-3}$)	1.0580±0.0809	1.1260±0.1570	0.714
Number of cell nuclei per μm^2	0.0054±0.0007	0.00356±0.00024	0.046*

Values are means ± s.e.m. (*N*=4).*Significant difference between treatments (*P*<0.05).

in hyperosmotic conditions. However, little was known about long-term effects of prolonged exposure to hyperosmotic environments on the maintenance of osmotic homeostasis. All haematological values measured in this study for SW-acclimated animals were within the ranges reported for wild-caught *C. porosus* living in water bodies of varying salinities (Grigg, 1981; Wells et al., 1991), suggesting that SW-acclimated crocodiles were able to successfully maintain osmotic homeostasis despite extended exposure to hyperosmotic conditions. Moreover, extended exposure to hypersaline conditions appeared to have little long-term impact on the animal's growth, suggesting that the additional energy required in maintaining homeostasis was either relatively minimal or was compensated for by increased food intake (animals were fed *ad libitum*, so we cannot readily distinguish between these possibilities).

Surprisingly, blood haematocrit, plasma osmolarity and plasma Na^+ and K^+ concentrations were significantly lower in FW-acclimated crocodiles when compared with their SW-acclimated counterparts. In previous studies of plasma Na^+ concentrations and water flux in *C. porosus*, Taplin (Taplin, 1985) demonstrated that FW-acclimated *C. porosus* actively drank freshwater when available, but when living in hyperosmotic environments, animals avoided drinking saltwater and obtain all necessary water though ingested food and metabolic water (Taplin, 1985). The active consumption of freshwater by *C. porosus*, when available, is thought to be related to the method of excretion of nitrogenous waste utilised while in freshwater [i.e. predominantly ammonia in freshwater as opposed to insoluble urates when in saltwater (Grigg, 1981)] and the associated high requirement for water. The ingestion of such an electrolyte-depleted medium, whilst important for waste excretion, may secondarily be responsible for the relatively reduced plasma osmolarity and haematocrit, and lower electrolyte levels in FW-acclimated animals observed in this study. Moreover, the integument of the cephalic region (including the buccal cavity, but excluding the salt glands) is also highly permeable to Na^+ and is a major secondary route for Na^+ efflux (and possibly other ions) in *C. porosus* (Taplin, 1985). Hence, in freshwater, maintaining a lower plasma osmolarity, relative to SW-acclimated animals, may aid in reducing the rate of Na^+ efflux from the buccal integument into the surrounding medium.

To investigate salt gland functional capacity *in vivo*, methacholine chloride, an acetylcholine (ACh) agonist, was used to stimulate maximal salt gland secretory rates in restrained *C. porosus*. Previously, Taplin reported that short term exposure (~160 h) to saline environments in *C. porosus* hatchlings reared in freshwater was not long enough to induce differences in maximal salt gland secretion rates relative to animals maintained continuously in freshwater (Taplin, 1985). However, in this study we found that chronic exposure to hypersaline environments resulted in adaptive changes to the salt gland, which enabled a significantly elevated rate of Na^+ secretion. Maximal mean lingual gland secretory rates were more than three times higher in SW-acclimated *C. porosus* relative to FW-acclimated animals. In FW-acclimated *C. porosus*, methacholine stimulation appeared to have relatively little effect on secretion rate. These results possibly suggest that prolonged saltwater acclimation results in changes to the cholinergic pathways responsible for salt secretion.

Although acetylcholine can act on numerous aspects of the tissue to increase secretion rate, we believe that there is at least an underlying direct increase in the response of the secretory tissue to exogenous methacholine, which may increase the secretory capacity of the tissue. In SW-acclimated *C. porosus* salt gland tissue slices, application of methacholine had a marked effect on the *in vitro* metabolic rate of the tissue, raising it by over 30%. A comparable metabolic response to methacholine application, *in vitro*, has been observed in both avian and turtle salt gland slices (Shuttleworth and Thompson, 1987). In contrast to tissue slices from SW-acclimated crocodiles, those from FW-acclimated animals showed relatively little response to methacholine. This result suggests that tissue slices from SW-acclimated crocodiles are more responsive to ACh than those of FW-acclimated crocodiles.

It was recently demonstrated that crocodile salt glands are richly innervated by cholinergic neurons, suggesting a large role for ACh in the regulation of salt gland secretion (Franklin et al., 2005). Similarly, avian salt-secreting tissues also appear to be under the control of the cholinergic system (e.g. Ash et al., 1969; Schmidt-Nielsen and Fänge, 1958). Moreover, avian salt gland secretory cells also possess large numbers of muscarinic ACh (mACh) receptors, which when activated, initiate a multitude of secretion-related processes within the cell (e.g. Borut and Schmidt-Nielsen, 1963; Hootman and Ernst, 1981; Hootman and Ernst, 1982; Stewart et al., 1979; van Rossum and Ernst, 1978). In addition, saltwater acclimation results in a 3-fold increase in the number of mACh receptors on duck salt gland secretory cells (Hootman and Ernst, 1981), suggesting that ACh plays a large, multifaceted role in regulating the secretory process. We suspect that, as in avian models, ACh is likely to act directly on the secretory cells in the salt glands of *C. porosus* to regulate the secretory activity of the tissue. Moreover, saltwater acclimation is likely to result in an increase in ACh receptor numbers in *C. porosus* secretory cells, although this, and the other potential sites of action by ACh, remains to be empirically determined.

Increases in the maximal secretory capacity of SW-acclimated crocodiles were reflected by changes in secretory tissue morphology. Studies on birds and turtles have demonstrated that salt-secreting tissues are morphologically plastic and are capable of responding rapidly to changes in plasma osmolarity (e.g. Abel and Ellis, 1966; Benson and Phillips, 1964; Ernst and Ellis, 1969; Reina, 2000; Schmidt-Nielsen and Kim, 1964). Changes include increases in blood flow to the gland (Hanwell et al., 1970) and the rapid increase in salt gland size by both cellular hypertrophy and hyperplasia (Abel and Ellis, 1966; Benson and Phillips, 1964; Reina, 2000; Schmidt-

Nielsen and Kim, 1964). In addition, changes in secretory cell ultrastructure, particularly marked increases in plasma membrane surface area and increases in the number of mitochondria (Abel and Ellis, 1966; Bokenes and Mercer, 1998) accompany increases in gland size. In *C. porosus* we found no increase in the surface density of plasma membrane per unit area in SW-acclimated animals, but did observe an increase in the volume density of mitochondria. As mitochondrial cross-sectional areas were not different across treatments, it suggests that changes in secretory cell mitochondrial volume per unit area arise from an increase in mitochondrial numbers (density per unit volume) in SW-acclimated crocodiles. Mitochondrial density is an important proxy for estimates of the metabolic activity of a cell. Hence cells involved in energetically expensive processes such as secretion and absorption often have large numbers of mitochondria. Salt-secreting cells generally have relatively large numbers of mitochondria. Indeed, actively secreting avian salt gland cells have approximately twice the volume of mitochondria compared with their inactive counterparts (Bokenes and Mercer, 1998). Moreover, the metabolic rate of secretory cells from SW-acclimated ducks is almost double that of naive (FW-acclimated) animals (Hootman and Ernst, 1980).

In the present study, we also examined the rate of oxygen consumption by isolated crocodile salt gland tissue and found there was no difference in the mass-specific metabolic rate of tissues from FW- and SW-acclimated crocodiles. There are fewer cells per unit mass in SW-acclimated salt gland relative to FW-acclimated ones (Cramp et al., 2007) and when oxygen consumption rates are expressed in terms of the number of cells per unit mass, the metabolic rate of SW-acclimated crocodile salt gland tissue would be higher than that of FW-acclimated cells. Similar values are seen in duck salt gland models (Hootman and Ernst, 1980). Given that there are almost 23% more mitochondria per unit volume in SW-acclimated crocodiles, and again, since these secretory cells are so much larger than those of FW-acclimated crocodiles, this figure is also probably an underestimate of the total cellular volume of mitochondria.

Following prolonged saltwater acclimation, we observed no increase in the density of plasma membrane per unit volume of salt gland tissue relative to that from FW-acclimated crocodiles. As detailed above, this result is likely to be an underestimate of the actual value since there are fewer secretory cells per unit volume of SW-acclimated salt gland (Cramp et al., 2007). We suspect that when the surface area measurements are expressed relative to total cells size, there would be an increase in plasma membrane surface area of salt gland following acclimation to hypersaline environments. However, the extent of the change in plasma membrane area from SW-acclimated crocodile cells is significantly less than is observed in duck salt gland cells following osmotic challenge, in which there can be a fivefold increase in plasma membrane surface area (Merchant et al., 1985). These differences in plasma membrane response to salt loading may simply reflect species differences between crocodiles and ducks, but may reflect greater underlying differences in their abilities to respond to osmotic challenges over long periods of time. Further work is required to determine whether the ultrastructural changes we observed in chronically SW-acclimated salt glands are different to those that occur following acute osmotic challenge.

Relative to the salt glands of birds, the functional and morphological effects of saltwater acclimation in *C. porosus* were comparatively small. Although it is possible that these differences reflect different time courses over which these responses occurred, it is also possible that differing relative salt loads may also play a role. *C. porosus* can successfully obtain all necessary water requirements from ingested food and metabolic water production alone, without the need to maintain water balance by drinking seawater (Taplin, 1984; Taplin, 1985). Indeed, several species of marine reptiles (e.g. sea snake *Pelarmis platurus*, estuarine turtles *Callagur borneoensis* and *Malaclemys terrapin* and *Crocodylus acutus*) are thought to also maintain water balance without the need to drink seawater (Dunson, 1982; Dunson and Moll, 1980; Dunson and Robinson, 1976; Evans and Ellis, 1977; Robinson and Dunson, 1976). Taplin (Taplin, 1985) provided data on the relative salt loads of saline water food items of *C. porosus* in the Liverpool/Tomkinson River System, northern Australia, which showed that the Na^+ content of these items ranged from ~55 to ~215 mmol Na^+ kg^{-1} body mass (the mean Na^+ content of food items fed to crocodiles in the present study was 145 mmol kg^{-1} body mass). Hence when living in seawater, a crocodile's salt load would be equivalent to that of the food it consumes plus any incidental water ingested (i.e. during swallowing). By contrast, marine birds maintain hydration by consuming seawater and excreting the excess salt through their salt glands. With an average [Na^+] of around 450 mmol l^{-1}, the ingestion of seawater would contribute to a much greater relative salt load than that experienced by crocodiles. Hence, differences in acclimatory responses to seawater exposure between marine birds and elasmobranchs and crocodiles may reflect different relative salt loads. We would hypothesise that increases in dietary salt loads may stimulate a great phenotypic response in the salt glands of *C. porosus*.

Crocodylus porosus has a remarkable ability to successfully inhabit severely hypersaline water bodies (>60‰) (Grigg et al., 1986). Animals maintain their osmotic balance by employing extra-renal lingual salt glands to concentrate and remove excess salt accumulated as a consequence of living in marine habitats. Data from the current and previous studies has shown that the salt glands of *C. porosus* can respond rapidly to changes in environmental salinity/plasma osmolarity (Cramp et al., 2007; Franklin and Grigg, 1993). The salt glands of *C. porosus* respond to increasing environmental salinity in much the same way as those of other marine reptiles and birds, in that they become larger, have larger secretory cells, larger blood vessels (Franklin and Grigg, 1993) and show some evidence of neural plasticity upon hypertrophying (Cramp et al., 2007). We have shown that a change in the ultrastructure of salt gland secretory cells (i.e. increased mitochondrial numbers and increase plasma membrane surface area) correlates with an increased functional activity of the glands. Thus, the salt glands of crocodiles exposed to hypersaline conditions have a greater secretory rate and higher tissue metabolic rate than those of freshwater-acclimated animals.

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REFERENCES

Abel, J. H., Jr and Ellis, R. A. (1966). Histochemical and electron microscopic observations on the salt secreting lacrymal glands of marine turtles. *Am. J. Anat.* **118**, 337-357.

Ash, R. W., Pearce, J. W. and Silver, A. (1969). An investigation of the nerve supply to the salt gland of the duck. *Q. J. Exp. Physiol. Cogn. Med. Sci.* **54**, 281-295.

Ballantyne, B. and Wood, W. G. (1969). Mass and function of avian nasal gland. *Cytobios* **4**, 337-345.

Benson, G. K. and Phillips, J. G. (1964). Observations on the histological structure of the supraorbital (nasal) glands from saline-fed and freshwater-fed domestic ducks (*Anas platyrhynchus*). *J. Anat.* **98**, 571-578.

Bokenes, L. and Mercer, J. B. (1998). A morphometric study of the salt gland in freshwater- and saltwater-adapted eider ducks (*Somateria mollissima*). *J. Exp. Zool.* **280**, 395-402.

Borut, A. and Schmidt-Nielsen, K. (1963). Respiration of avian salt-secreting gland in tissue slice experiments. *Am. J. Physiol.* **204**, 573-581.
Cramp, R. L., Hudson, N. J., Holmberg, A., Holmgren, S. and Franklin, C. E. (2007). The effects of saltwater acclimation on neurotransmitters in the lingual salt glands of the estuarine crocodile, *Crocodylus porosus*. *Regul. Pept.* **140**, 55-64.
Dunson, W. A. (1982). Osmoregulation of crocodiles: salinity as a possible limiting factor to *Crocodylus acutus* in Florida Bay. *Copeia* **2**, 374-385.
Dunson, W. A. and Moll, E. O. (1980). Osmoregulation in seawater of hatchling emydid turtles *Callagur borneoensis* from a Malaysian sea beach. *J. Herpetol.* **14**, 31-36.
Dunson, W. A. and Robinson, G. D. (1976). Sea snake skin-permeable to water but not to sodium. *J. Comp. Physiol.* **108**, 303-311.
Dunson, W. A. and Taub, A. M. (1967). Extrarenal salt excretion in sea snakes (Laticauda). *Am. J. Physiol.* **213**, 975-982.
Ellis, R. A., Delellis, R. A., Goertemiller, C. C. and Kablotsky, Y. H. (1963). Effect of a salt water regimen on development of salt glands of domestic ducklings. *Dev. Biol.* **8**, 286-308.
Ernst, S. A. and Ellis, R. A. (1969). The development of surface specialization in the secretory epithelium of the avian salt gland in response to osmotic stress. *J. Cell Biol.* **40**, 305-321.
Ernst, S. A. and Mills, J. W. (1977). Basolateral plasma membrane localiztion of ouabain-sensitive sodium transport sites in the secretory epithelium of the avian salt gland. *J. Cell Biol.* **75**, 74-94.
Ernst, S. A., Goertemiller, C. C., Jr and Ellis, R. A. (1967). The effect of salt regimens on the development of (Na^+K^+)-dependent ATPase activity during the growth of salt glands of ducklings. *Biochim. Biophys. Acta* **135**, 682-692.
Evans, D. H. and Ellis, T. M. (1977). Sodium balance in hatchling American crocodile, *Crocodylus acutus*. *Comp. Biochem. Physiol.* **58A**, 159-162.
Fänge, R., Schmidt-Nielsen, K. and Robinson, M. (1958). Control of secretion from the avian salt gland. *Am. J. Physiol.* **195**, 321-326.
Fletcher, G. L., Stainer, I. M. and Holmes, W. N. (1967). Sequential changes in the adenosinetriphosphatase activity and the electrolyte excretory capacity of the nasal glands of the duck (*Anas platyrhynchos*) during the period of adaptation to hypertonic saline. *J. Exp. Biol.* **47**, 375-391.
Franklin, C. E. and Grigg, G. C. (1993). Increased vascularity of the lingual salt glands of the estuarine crocodile, *Crocodylus porosus*, kept in hyperosmotic salinity. *J. Morphol.* **218**, 143-151.
Franklin, C. E., Taylor, G. and Cramp, R. L. (2005). Cholinergic and adrenergic innervation of lingual salt glands of the estuarine crocodile, *Crocodylus porosus*. *Aust. J. Zool.* **53**, 345-351.
Grigg, G. C. (1981). Plasma homeostasis and cloacal urine composition in *Crocodylus porosus* caught along a salinity gradient. *J. Comp. Physiol.* **144**, 261-270.
Grigg, G. C., Taplin, L. E., Green, B. and Harlow, P. (1986). Sodium and water fluxes in free living *Crocodylus porosus* in marine and brackish conditions. *Physiol. Zool.* **59**, 240-253.
Hanwell, A. and Peaker, M. (1975). Control of adaptive hypertrophy in salt glands of geese and ducks. *J. Physiol. Lond.* **248**, 193-205.
Hanwell, A., Linzell, J. L. and Peaker, M. (1970). Avian salt-gland blood flow and the extraction of ions from the plasma. *J. Physiol. Lond.* **207**, 83P-84P.
Hanwell, A., Linzell, J. L. and Peaker, M. (1971). Salt-gland secretion and blood flow in goose. *J. Physiol. Lond.* **213**, 373-387.
Hootman, S. R. and Ernst, S. A. (1980). Dissociation of avian salt gland: separation procedures and characterization of dissociated cells. *Am. J. Physiol.* **238**, C184-C195.
Hootman, S. R. and Ernst, S. A. (1981). Characterization of muscarinic acetylcholine receptors in the avian salt gland. *J. Cell Biol.* **91**, 781-789.
Hootman, S. R. and Ernst, S. A. (1982). [^{3}H]QNB binding to muscarinic receptors in intact avian salt gland cells. *Am. J. Physiol.* **243**, C254-C261.
Hossler, F. E. (1982). On the mechanism of plasma membrane turnover in the salt gland of ducklings. Implications from DNA content, rates of DNA synthesis, and sites of DNA synthesis during the osmotic stressing and destressing cycle. *Cell Tissue Res.* **226**, 531-540.
Hossler, F. E., Sarras, M. P. and Allen, E. R. (1978). Ultrastructural, cytochemical and biochemical observations during turnover of plasma-membrane in duck salt-gland. *Cell Tissue Res.* **188**, 299-315.
Knight, C. H. and Peaker, M. (1979). Adaptive hyperplasia and compensatory growth in the salt glands of ducks and geese. *J. Physiol. Lond.* **294**, 145-151.
Mcarthur, P. D. and Gorman, M. L. (1978). Salt-gland of incubating eider duck *Somateria mollissima* – effects of natural salt deprivation. *J. Zool.* **184**, 83-90.
McFarland, L. Z., Martin, K. D. and Freedland, R. A. (1965). The activity of selected soluble enzymes in the avian nasal salt gland. *J. Cell. Comp. Physiol.* **65**, 237-242.
Merchant, J. L., Papermaster, D. S. and Barrnett, R. J. (1985). Correlation of Na^+,K^+-ATPase content and plasma membrane surface area in adapted and de-adapted salt glands of ducklings. *J. Cell Sci.* **78**, 233-246.
Reina, R. (2000). Salt gland blood flow in the hatchling green turtle, *Chelonia mydas*. *J. Comp. Physiol. B* **170**, 573-580.
Robinson, G. D. and Dunson, W. A. (1976). Water and sodium balance in estuarine diamondback terrapin (*Malaclemys*). *J. Comp. Physiol.* **105**, 129-152.
Schmidt-Nielsen, K. and Fänge, R. (1958). Salt glands in marine reptiles. *Nature* **182**, 783-785.
Schmidt-Nielsen, K. and Kim, Y. (1964). The effect of salt intake on the size and function of the salt gland of ducks. *Auk* **81**, 160-172.
Schmidt-Nielsen, K., Jorgensen, C. B. and Osaki, H. (1958). Extrarenal salt excretion in birds. *Am. J. Physiol.* **193**, 101-107.
Shuttleworth, T. J. and Hildebrandt, J. P. (1999). Vertebrate salt glands: short- and long-term regulation of function. *J. Exp. Zool.* **283**, 689-701.
Shuttleworth, T. J. and Thompson, J. L. (1987). Secretory activity in salt glands of birds and turtles: stimulation via cyclic AMP. *Am. J. Physiol.* **252**, R428-R432.
Spannhof, L. and Jurss, K. (1967). Studies on the genesis of some enzymes in the salt glands of young common gulls. *Acta Biol. Med. Ger.* **19**, 137-144.
Stainer, I. M., Ensor, D. M., Phillips, J. G. and Holmes, W. N. (1970). Changes in glycolytic enzyme activity in duck (*Anas platyrhynchos*) nasal gland during period of adaptation to salt water. *Comp. Biochem. Physiol.* **37**, 257-260.
Stewart, D. J., Semple, E. W., Swart, G. T. and Sen, A. K. (1976). Induction of the catalytic protein of (Na^++K^+)-ATPase in the salt gland of the duck. *Biochim. Biophys. Acta Biomembranes* **419**, 150-163.
Stewart, D. J., Sax, J., Funk, R. and Sen, A. K. (1979). Possible role of cyclic GMP in stimulus-secretion coupling by salt-gland of the duck. *Am. J. Physiol.* **237**, C200-C204.
Taplin, L. E. (1984). Drinking of fresh water but not seawater by the estuarine crocodile (*Crocodylus porosus*). *Comp. Biochem. Physiol. A* **77**, 763-767.
Taplin, L. E. (1985). Sodium and water budgets of the fasted estuarine crocodile, *Crocodylus porosus* in sea water. *J. Comp. Physiol. B* **155**, 501-513.
Taplin, L. E. and Grigg, G. C. (1981). Salt glands in the tongue of the estuarine crocodile *Crocodylus porosus*. *Science* **212**, 1045-1047.
Taplin, L. E., Grigg, G. C., Harlow, P., Ellis, T. M. and Dunson, W. A. (1982). Lingual salt glands in *Crocodylus acutus* and *Crocodylus johnstoni* and their absence from *Alligator mississipiensis* and *Caiman crocodilus*. *J. Comp. Physiol.* **149**, 43-47.
Taylor, G. C., Franklin, C. E. and Grigg, G. C. (1995). Salt loading stimulates secretion by the lingual salt glands in unrestrained *Crocodylus porosus*. *J. Exp. Zool.* **272**, 490-495.
van Rossum, G. D. and Ernst, S. A. (1978). Effects of ethacrynic acid on ion transport and energy metabolism in slices of avian salt gland and of mammalian liver and kidney cortex. *J. Membr. Biol.* **43**, 251-275.
Wells, R. M. G., Beard, L. A. and Grigg, G. C. (1991). Blood viscosity and hematocrit in the estuarine crocodile, *Crocodylus porosus*. *Comp. Biochem. Physiol. A* **99**, 411-414.