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RESEARCH ARTICLE

Salt-water acclimation of the estuarine crocodile *Crocodylus porosus* involves enhanced ion transport properties of the urodaeum and rectum

Martin Grosell[1,‡], Rachael M. Heuer[1], N. C. Wu[2], Rebecca L. Cramp[2], Yadong Wang[1], Edward M. Mager[1,*], Ross G. Dwyer[2] and Craig E. Franklin[2]

ABSTRACT

Estuarine crocodiles, *Crocodylus porosus*, inhabit freshwater, estuarine and marine environments. Despite being known to undertake extensive movements throughout and between hypo-osmotic and hyperosmotic environments, little is known about the role of the cloaca in coping with changes in salinity. We report here that, in addition to the well-documented functional plasticity of the lingual salt glands, the middle of the three cloacal segments (i.e. the urodaeum) responds to increased ambient salinity to enhance solute-coupled water absorption. This post-renal modification of urine serves to conserve water when exposed to hyperosmotic environments and, in conjunction with lingual salt gland secretions, enables *C. porosus* to maintain salt and water balance and thereby thrive in hyperosmotic environments. Isolated epithelia from the urodaeum of 70% seawater-acclimated *C. porosus* had a strongly enhanced short-circuit current (an indicator of active ion transport) compared with freshwater-acclimated crocodiles. This enhanced active ion absorption was driven by increased Na^+/K^+-ATPase activity, and possibly enhanced proton pump activity, and was facilitated by the apical epithelial Na^+ channel (ENaC) and/or the apical Na^+/H^+ exchanger (NHE2), both of which are expressed in the urodaeum. NHE3 was expressed at very low levels in the urodaeum and probably does not contribute to solute-coupled water absorption in this cloacal segment. As *C. porosus* does not appear to drink water of salinities above 18 ppt, observations of elevated short-circuit current in the rectum as well as a trend for increased NHE2 expression in the oesophagus, the anterior intestine and the rectum suggest that dietary salt intake may stimulate salt and possibly water absorption by the gastrointestinal tract of *C. porosus* living in hyperosmotic environments.

KEY WORDS: Osmoregulation, Salt and water balance, Crocodylians, NKA, ENaC, NHE2, NHE3, Phenotypic plasticity, Salt glands, Cloaca, Spatial ecology, Telemetry

INTRODUCTION

The estuarine crocodile, *Crocodylus porosus* Schneider 1801, can be found inhabiting freshwater, estuarine, marine and hypersaline environments, and is capable of maintaining ionic and osmotic homeostasis across this broad range of salinities (Grigg, 1981; Taplin, 1984a,b). The success of *C. porosus* in maintaining homeostasis across a wide salinity range is due in part to the plasticity of osmoregulatory organs, including lingual salt glands, the cloaca and kidneys (Franklin and Grigg, 1993; Cramp et al., 2010; Kuchel and Franklin, 1998, 2000). In *C. porosus*, the salt glands are particularly plastic both morphologically and functionally, showing increased vascularization, increased mitochondrial density and increased Na^+/K^+-ATPase α-subunit gene expression and protein abundance, which all probably facilitate an ∼3-fold increase in salt secretion rates in seawater-acclimated compared with freshwater-acclimated animals (Cramp et al., 2010; Cramp et al., 2008; Franklin and Grigg, 1993). In addition to the roles of the lingual salt gland, post-renal modification of urine probably plays an important role for osmoregulation in hyperosmotic environments. Crocodyloidea appear to also have distinct cloacal function during osmotic challenge when compared with Alligatoridae, examined in *Alligator mississippiensis* (Pidcock et al., 1997). The cloaca of *A. mississippiensis* has little influence on the composition of excreted urine while *C. porosus* displays extensive post-renal modification of urine in the cloaca (Pidcock et al., 1997). The cloaca of *C. porosus* consists of three chambers: the coprodaeum, the urodaeum and the proctodaeum (Kuchel and Franklin, 2000). The coprodaeum, the most anterior of the chambers, is small compared with the other chambers, with an epithelial lining rich in mucus cells, while the most distal chamber, the proctodaeum, houses the genitalia. The urinary papilla opens into the urodaeum, the largest of the three chambers, which stores urine prior to release (Kuchel and Franklin, 2000). The three chambers are separated from each other and from the rectum by tight muscular sphincters preventing retrograde movement of urine into the coprodaeum and the rectum, leaving the urodaeum the primary site for post-renal urine modification (Kuchel and Franklin, 2000). The urodaeum is capable of reabsorption of Na^+ and Cl^- and presumably water in *C. porosus* held in hypersaline water (Kuchel and Franklin, 1998). The reclaiming of water by the urodaeum, initially lost via renal excretion, is of significance for osmoregulation as both water loss and salt gain are challenging to *C. porosus* in hypersaline waters (Taplin, 1985). In parallel to the plasticity of the lingual salt glands, the urodaeum also appears to show plasticity according to ambient salinity. While an increase in surface area of the mucosal surface of the urodaeum in seawater-compared with freshwater-acclimated *C. porosus* has been suggested (Kuchel and Franklin, 2000), the functional responses of the urodaeum to ambient salinity remain unknown. Field-based studies, where *C. porosus* were captured, blood sampled and the salinity of the water recorded, have provided excellent insight into the osmoregulatory capabilities of this species (Grigg, 1981;

[1]Rosenstiel School of Marine and Atmospheric Science, University of Miami, Miami, FL 33149, USA. [2]School of Biological Sciences, The University of Queensland, Brisbane, QLD 4072, Australia.
*Present address: University of North Texas, Denton, TX 76203, USA.

‡Author for correspondence (mgrosell@rsmas.miami.edu)

M.G., 0000-0001-6117-8601; N.C.W., 0000-0002-7130-1279

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Taplin, 1985). These, however, are single, point in time, measurements and provide no information as to how long the individuals had been at that particular salinity at the point of capture. Both spatial and temporal changes in environmental salinity are important when considering the physiological plasticity of the osmoregulatory organs of *C. porosus*. In recent years, telemetry (Franklin et al., 2009) has revealed large movements of *C. porosus* (Read et al., 2007; Campbell et al., 2010, 2013) but these movements have to date not been matched with changes in environmental salinity that they would encounter.

The first objective of this study was to examine how wild *C. porosus* move in relation to natural variation in environmental salinity. Next, to test the hypothesis that functional adjustments of transport properties in the mucosal epithelium of the urodaeum occur to facilitate water retention and allow for prolonged occupancy in marine environments, we conducted a series of laboratory experiments. Specifically, predictions of enhanced Na^+ absorption by isolated epithelia from the urodaeum were tested using Ussing chambers. These expectations were met and followed by pharmacological characterization of putative Na^+ transport pathways in the urodaeum as well as cloning of putative Na^+ transporters. Finally, the third objective was to test the hypothesis of enhanced expression of genes coding for Na^+ transporters involved in solute-coupled water absorption by the urodaeum of seawater-acclimated *C. porosus*.

MATERIALS AND METHODS

Tracking *C. porosus* movements

To investigate movement and habitat use of crocodiles in relation to environmental salinity, we conducted a field-based study whereby electronic tracking devices were deployed on four wild adult male *C. porosus* to track their behaviour remotely. Field study was conducted on the Wenlock River in Cape York, Australia (Fig. 1), a tropical tidal river that flows in a westerly direction before draining into the eastern Gulf of Carpentaria. Crocodiles were captured in freshwater/brackish extents of the Wenlock River, using large aluminium traps baited with wild pig *Sus scrofa*. Upon capture, crocodiles were measured and fitted with an Argos-linked GPS tag (TGM-4310 Telonics, Mesa, AZ, USA), which was attached externally on the animals' nuchal shield and secured using 200 kg nylon-coated stainless wire leader and aluminium crimps. Geographical positioning was acquired via satellite twice daily (06:00 h and 20:00 h) and relayed via satellite to Argos processing centres where the data were available for download. All procedures were carried out with approval from The University of Queensland Animal Ethics Committee (SBS/215/14/AUST ZOO/ARC) and a Queensland Environment Protection Agency Permit (WISP13189313).

Environmental salinity

Salinity measurements throughout estuarine and freshwater components of our study area were obtained between November 2015

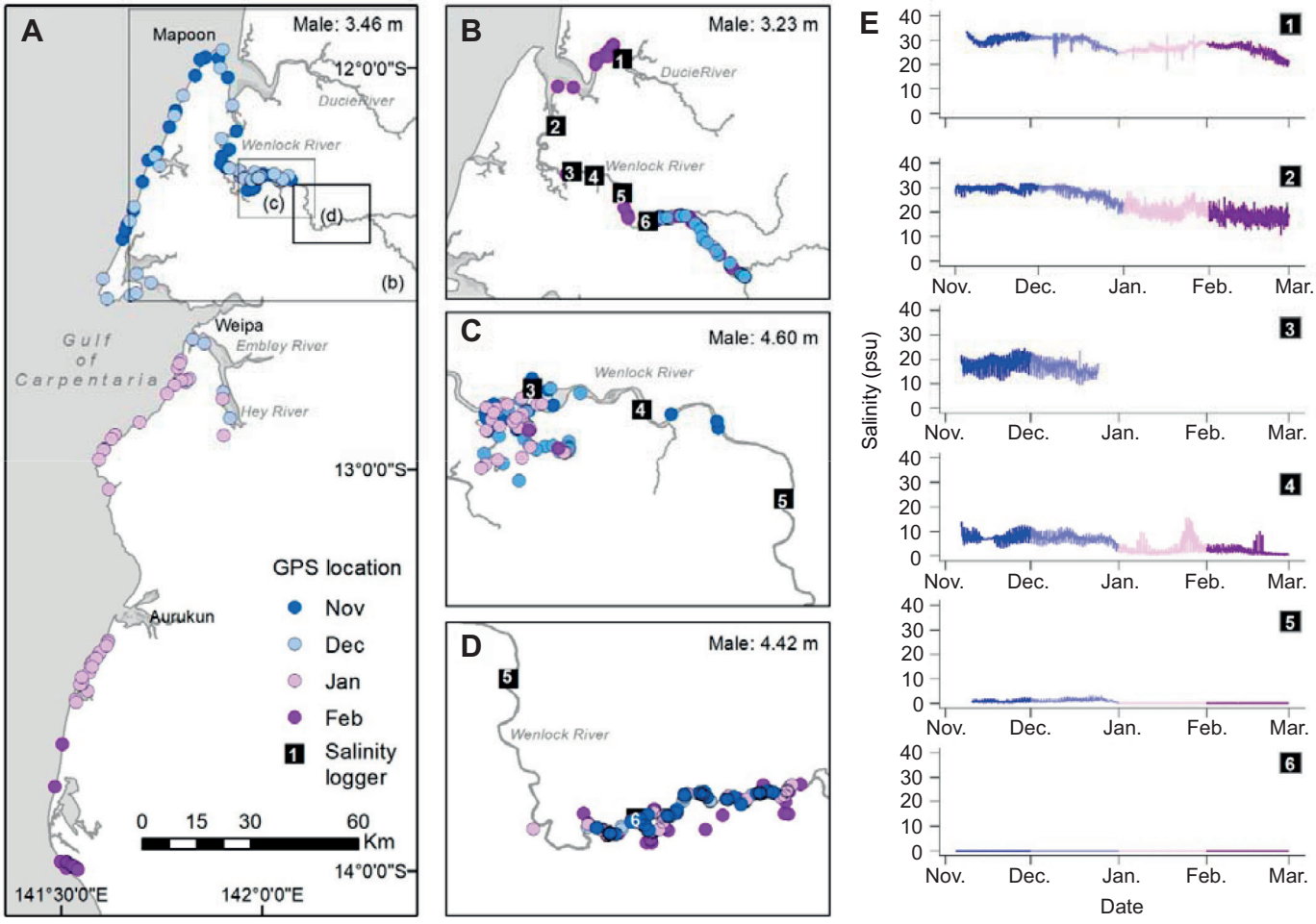

Fig. 1. *Crocodylus porosus* movements and salinity in the study area. Movements of four satellite-tagged free-ranging estuarine crocodiles in Cape York, Australia (A–D) in relation to local environmental salinity at six discrete locations (E).

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and March 2016 using six environmental loggers (3× *In-Situ* Aqua TROLL 200; 2× SeaBird SBE37; 1×Solinst Levelogger Edge; 1× Schlumberger Water Services CTD-Diver). These devices were fitted to moorings positioned 33 km upstream from Cullen Point within the Ducie River, and 28, 60, 69, 80 and 101 km upstream within the Wenlock River (Fig. 1B). Distances are provided as adopted middle thread distances (AMTD) from Cullen Point, Mapoon (11.957°S 141.909°E). Salinities are expressed as mean hourly salinity weight of solute per thousand parts of solution (psu).

Maintenance of animals in the lab

All experiments were carried out in compliance and accordance with The University of Queensland's Animal Ethics policies (SBS/314/14). *Crocodylus porosus* were obtained as eggs from Fleay's Wildlife Park and incubated at 31°C in a temperature-controlled cabinet. Upon hatching, they were transferred to enclosures and fed a mixture of chopped ox heart, chicken necks and beef mince. The hatchling crocodiles had access to a temperature-controlled pool (25°C) and a basking area heated with an infrared lamp. Juvenile *C. porosus* (240–1396 g) were randomly assigned to two experimental treatments, freshwater (FW) treatment (n=6) made with tap water and 70% seawater (SW) treatment (n=6). SW was made with tap water mixed with Sunray swimming pool salt (Cheetham Salt Limited, Melbourne, VIC, Australia) and animals were acclimated for 6 weeks prior to experimentation. During the acclimation period, crocodiles were fed 3–4 times a week with a mixture of minced beef supplemented with reptile-specific Repti-vite (multi-vitamin, mineral and amino acid) and Repti-cal (calcium and vitamin D3) powders (Aristopet Pty Ltd, Eagle Farm, QLD, Australia). For the SW treatments, food was moistened with saltwater (70%), while the food for FW treatments was moistened with tap water. Enclosures were checked daily and cleaned out once a week. Salinity of the SW treatment tanks was recorded using a refractometer and adjusted to 24.5±0.5 ppt. Animals were fasted for 7 days before experimentation.

Sampling of intact animals

A 1 ml blood sample was taken from lab-reared *C. porosus* using a heparinized (ammonium heparinate) syringe fitted with a 23 g needle; subsamples of blood were collected into heparinized capillary tubes in duplicate for haematocrit measurements and plasma was obtained after centrifugation and frozen for subsequent analyses. Animals were then killed with an overdose of thiopentone sodium (Troy Laboratories Pty Ltd, Glendenning, NSW, Australia) injected via the cervical sinus. The kidney, cloaca, oesophagus, anterior intestine and rectum were subsequently obtained by dissection and subsamples were immediately frozen in liquid nitrogen for subsequent RNA extraction and enzyme analyses (see below). During dissections, cloacal fluids were obtained when present and frozen for later analyses. A sample of tail white muscle was also obtained by dissection for duplicate determination of tissue water content. Segments of epithelia from the rectum and the urodaeum were placed in saline (125 mmol l^{-1} NaCl, 20 mmol l^{-1} $NaHCO_3$, 5 mmol l^{-1} Hepes sodium salt, 5 mmol l^{-1} Hepes free acid, 4 mmol l^{-1} KCl, 3 mmol l^{-1} $CaCl_2$ and 1.5 mmol l^{-1} $MgCl_2$, gassed with 5% CO_2 in O_2, pH 7.4) for subsequent Ussing chamber experiments to determine epithelial electrophysiology parameters.

Electrophysiological parameters of epithelia from the rectum and urodaeum

Isolated segments of epithelia from the rectum and urodaeum of crocodiles acclimated to FW and SW were mounted in tissue holders (Physiological Instruments: P2405 0.4 cm^2 or P2413 0.71 cm^2 depending on the size of tissue available) and subsequently placed in Ussing chambers (Physiological Instruments, P2400) after which each half chamber was rinsed 3 times and filled with 2 ml of physiological saline. Each half chamber was fitted with one current (Ag) and one voltage (Ag/AgCl) electrode for recordings of short circuit current (I_{sc}) and transepithelial potential (TEP), respectively. Electrodes (mucosal ground) were connected to an amplifier (Physiological Instruments, VCC600) interfaced with a computer using Acknowledge software (BIOPAC Systems, Goleta, CA, USA). Salines were continuously mixed by airlift gassing (5% CO_2 in O_2). Following mounting, tissues were allowed to stabilize under voltage-clamp conditions until constant and stable I_{sc} readings were obtained. During these and subsequent I_{sc} measurements, tissues were subjected to 3 s voltage pulses of 1 mV every 60 s to allow calculation of epithelial conductance (G). Once stable readings were obtained, conditions were changed to current clamp, allowing recordings of TEP, after which I_{sc} recordings were re-established. I_{sc} measurements were negative, reflecting Na^+ absorption, but are presented here as positive numbers.

Following recording of baseline conditions, pharmacological manipulations were initiated to target the clear absorptive I_{sc} seen especially in tissues from SW-acclimated crocodiles. Bumetanide, an inhibitor of Na^+:K^+:2Cl^- co-transporters, was first applied to the mucosal saline. It was dissolved in ethanol (3%), added to heated saline at 10^{-3} mol l^{-1} and allowed to cool to room temperature prior to addition to mucosal saline at a final concentration of 10^{-4} mol l^{-1} in 0.3% ethanol. Following a 10 min measurement period, amiloride, an inhibitor of Na^+ channels and Na^+/H^+ exchangers, was added to the luminal saline in increasing concentrations ranging from 5×10^{-7} to 10^{-4} mol l^{-1} at 5 min intervals, which was sufficient to yield stable I_{sc} readings. Amiloride was dissolved in ethanol (3%), added to heated saline at 10^{-3} mol l^{-1} and allowed to cool to room temperature prior to addition to luminal saline at the indicate concentrations.

Na^+/K^+-ATPase and proton pump enzymatic activity assays

Tissues were trimmed of as much connective tissue as possible, weighed and homogenized in SEID buffer [150 mmol l^{-1} sucrose, 10 mmol l^{-1} EDTA, 50 mmol l^{-1} imidazole and 0.01% sodium deoxycholate, with protease inhibitor cocktail (Sigma-Aldrich P2714) pH 7.3] for 60 s. The homogenized sample was then centrifuged at 6000 ***g*** for 2 min to pellet insoluble material. A 10 µl sample of homogenate supernatant (20 µl for urodaeum) was added to each of four wells (six if using 100 nmol l^{-1} bafilomycin) of a cold 96-well plate and 200 µl of assay mixture containing 2.8 mmol l^{-1} phosphoenolpyruvate, 0.7 mmol l^{-1} ATP, 0.22 mmol l^{-1} NADH, 4 U ml^{-1} lactate dehydrogenase, 5 U ml^{-1} pyruvate kinase, 50 mmol l^{-1} imidazole, 47 mmol l^{-1} NaCl, 2.6 mmol l^{-1} $MgCl_2$ and 10.5 mmol l^{-1} KCl, pH 7.3, was added to the first two wells; 200 µl of the assay mixture containing 1 mmol l^{-1} ouabain was added to the remaining two wells. The absorbance at 340 nm was measured at 30°C every 15 s for 5 min in a multiwell plate reader (DTX 880 Multimode Detector, Beckman Coulter). An ADP standard curve (from 0 to 100 nmol) was generated to convert absorbance values to ADP concentration. Protein concentration of the homogenates was determined using the Bradford procedure (Sigma-Aldrich). Pierce Bovine Serum Albumin Standard, 2 mg ml^{-1} (Thermo Fisher Scientific, Scoresby, VIC, Australia) as used as standard: 0–1.8 mg ml^{-1}, diluted in tissue homogenization buffer.

RNA extraction and cDNA synthesis

Total RNA was isolated as per the manufacturer's protocol from tissue samples homogenized in RNA STAT-60 solution

(Tel-Test, Friendsworth, TX, USA) using a tissue disperser (IKA, Wilmington, NC, USA). From each isolate, 10 µg of total RNA was treated with DNase I to remove any traces of genomic DNA (Turbo DNA-*free* Kit; Thermo Fisher Scientific). Following DNase I treatment, the integrity of each RNA was confirmed by gel electrophoresis. cDNA was synthesized from 1 µg DNase I-treated total RNA using the SuperScript IV First-Strand Synthesis System (Invitrogen, Waltham, MA, USA) with random hexamers. Following the final step of RNase H treatment, all reactions were diluted tenfold in TE buffer.

cDNA cloning of target transcripts

Segments of each target transcript were cloned by PCR using primers designed from CLUSTAL alignments of various reptile and avian sequences for each respective transcript. A mixture of equivalent volumes from each tissue cDNA was prepared as a template for cloning by aliquoting from random replicate samples for each tissue. Products were amplified using the Taq PCR Core Kit (Qiagen, Germantown, MD, USA), cloned using the TOPO-TA Cloning Kit (Thermo Fisher Scientific) and sequenced. Full-length sequences were subsequently obtained for the epithelial Na^+ channel (*ENaC*) and elongation factor 1α (*EF1α*) gene by rapid amplification of cDNA ends (RACE) in both 5′ and 3′ directions. Gene-specific primers for RACE were designed from the sequenced PCR products. RACE reactions were performed using the SMARTer RACE 5′/3′ kit (Takara Bio USA, Mountain View, CA, USA) with 1 µg DNase I-treated tongue total RNA following the manufacturer's protocol. Touchdown PCR cycling conditions were as follows: 5 cycles of 94°C for 30 s, 70°C for 30 s and 72°C for 2 min followed by 5 and 25 cycles as previously except at 68°C and 66°C annealing temperatures, respectively. Products were gel purified and initial cloning attempts of RACE products made using the In-Fusion approach (Takara Bio USA). These attempts were unsuccessful and therefore products were cloned using the TOPO-TA cloning approach as described above. All primers used in this study were purchased from Integrated DNA Technologies (Coralville, IA, USA) and are provided in Table 1.

Quantitative PCR (qPCR)

All qPCR experiments were carried out using a Stratagene Mx4000 multiplex qPCR system with Power SYBR Green Master Mix (Thermo Fisher Scientific) following the manufacturer's protocol. Briefly, 25 µl reactions were performed using 2 µl of diluted cDNA as template and 400 nmol l^{-1} of each primer. All qPCR primers were designed from the coding regions of each target gene (Table 1). Cycling was as follows: 95°C for 5 min followed by 40 cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 30 s. Amplicon identity was confirmed by sequencing and a single, corresponding melting peak was verified following all amplifications. Duplicate reactions of 5–6 biological replicates were performed for each tissue and treatment. Each plate contained no-template controls and a calibrator, both run in triplicate. Relative mRNA expression levels were determined using the Real-time PCR Miner software (Zhao and Fernald, 2005). Expression data for all genes examined were normalized to that of *EF1α* and expressed relative to the treatment and tissue with the lowest expression level.

Analytical techniques

For determination of muscle water content, fresh muscle tissue was weighed, and placed on aluminium foil in an oven at 70°C until constant dry mass was obtained. The pH of the cloacal fluids was measured using a micro pH electrode (Accumet 13-620-96; Thermo Fisher Scientific) coupled to a Hach H160 pH meter. Cations were analysed using flame atomic absorption spectrometry with an air/acetylene flame (Varian 220, Palo Alto, CA, USA) while anions were analysed by anion chromatography (Dionex 120). Total ammonia in cloacal fluids was measured using a commercial kit (EnzyChrom™ Ammonia/Ammonium Assay Kit, BioAsssay Systems, Hayward, CA, USA). Samples were diluted 1:100 prior to analysis.

Statistical analyses

Data are expressed as means±s.e.m. throughout. Results from FW- and 70% SW-acclimated crocodiles were compared using two-tailed Student's *t*-tests unless data failed a normality test, in which

Table 1. Primers used for cloning and qPCR

Primer	Purpose	Sequence (5′→3′)	Product size (bp)
ENaC-F1	Initial cloning	ATGAAGACRGCCTTCTGGTC	1619
ENaC-R1	Initial cloning	ACAGCYTCRACAGTGAAGC	
NHE-2-F1	Initial cloning	TCTATCACAAGTTGCCCTCG	731
NHE-2-R1	Initial cloning	CATGGTCATGGCACAAGCTG	
NHE-3-F1	Initial cloning	CAGACCACATTGCCTCCTTC	771
NHE-3-R1	Initial cloning	CAGCTGAAATTCCCAGGAAC	
EF1α-F1	Initial cloning	GGAGAARACCCAYATCAACATCG	1351
EF1α-R1	Initial cloning	CTTTGTGACCTTGCCAGCTCC	
ENaC-5p	5′ RACE	GATTACGCCAAGCTTCATGTCCATCAGTGTCTGGTGGGTGATCCG	1148
ENaC-3p	3′ RACE	GATTACGCCAAGCTTCGTCGTCATCACCTGTATCCTGTCCTTCCG	750
EF1α-5p	5′ RACE	GATTACGCCAAGCTTCAATTGTGATACCACGCTCACGTTCAGCC	371
EF1α-3p	3′ RACE	GATTACGCCAAGCTTGATGCTGCCATTGTTGATATGATCCCAGGC	562
ENaC-F2	qPCR	GAGCAGAACGACTTCATTCC	113
ENaC-R2	qPCR	CCTGGACGTACATTGAAGCC	
NHE-2-F2	qPCR	CCCACCTGCAATGAAGAGTG	134
NHE-2-R2	qPCR	TGCCCACTACAGCATACAAC	
NHE-3-F2	qPCR	TTGTACGCTGTCATTGGAAC	124
NHE-3-R2	qPCR	TGCCAAACAGGAGAAAGTCC	
EF1α-F2	qPCR	CTACAACCCAGACACTGTG	148
EF1α-R2	qPCR	AGCTTCAAGCAGGGTAGTGC	

Note that all RACE primers include 15 nucleotides at the 5′ end designed to facilitate In-Fusion (Takara Bio USA) cloning and that sizes for RACE products include 45 bp corresponding to the Long Universal Primer sequence (Takara Bio USA).

Table 2. Haematocrit, plasma ion concentrations, osmolality and white muscle water content of freshwater (FW)- and seawater (SW)-acclimated crocodiles

Parameter	FW	SW	Normality	*P*-value
Haematocrit (%)	23.2±1.8	24.5±2.5	Y	0.513
Plasma Na^+ (mmol l^{-1})	157.5±3.8	171.2±10.1	N	0.329
Plasma K^+ (mmol l^{-1})	5.2±0.2	5.3±0.2	Y	0.638
Plasma Ca^{2+} (mmol l^{-1})	2.9±0.2	3.2±0.1*	Y	0.006
Plasma Mg^{2+} (mmol l^{-1})	1.2±0.1	1.5±0.26	N	0.052
Plasma Cl^- (mmol l^{-1})	117.5±5.0	143.3±12.9*	N	0.030
Plasma SO_4^{2-} (mmol l^{-1})	3.1±0.3	2.9±0.3	Y	0.729
Osmolality (mosm kg^{-1})	305±18.6	341±26.5	N	0.329
White muscle water content (%)	75.4±0.8	77.0±2.2	Y	0.485

Data are means±s.e.m. of *n*=5–6 animals. A single blood sample from a FW-acclimated animal was lost as a result of coagulation.
*Significant difference from corresponding FW value.

case a Mann–Whitney rank sum test was performed. Means were considered significantly different at $P<0.05$.

RESULTS

Wild *C. porosus* movements in relation to environmental salinity

Of the four wild *C. porosus* fitted with Argos-linked GPS tags, one individual exited the river system and moved into the eastern Gulf of Carpentaria, where it spent extended periods of time in full-strength SW (Fig. 1A). This 3.46 m male crocodile spent a total of 120 days in coastal areas interrupted by three brief (5–27 days) forays in river systems while it travelled approximately 400 km from its capture site between November and March. The other three tagged crocodiles remained within the Wenlock River catchment, with one 3.23 m male (Fig. 1B) travelling over 400 km between 1 November 2015 and 1 March 2016, making extensive downstream and upstream movements where it regularly traversed hypersaline (~30 psu) and hyposaline (<0.1 psu) environments. The two largest crocodiles (a 4.60 m male and a 4.42 m male) remained in confined stretches of the Wenlock River during the 4 month tracking period (Fig. 1C,D); one remained in brackish waters where salinity ranged between 5 and 23 psu (Fig. 1C) whereas the other remained in FW throughout (Fig. 1D).

Integrative experimental responses

Neither haematocrit nor muscle water content differed between FW- and SW-acclimated *C. porosus*. Similarly, most measured plasma ions did not show significant differences between FW- and 70% SW-acclimated animals, although plasma Cl^- and Ca^{2+} were significantly elevated in 70% SW-acclimated compared with FW-acclimated crocodiles (Table 2). A similar trend was observed for Na^+ and in combination with Cl^- probably accounted for the trend towards elevated plasma osmolality in SW-acclimated individuals. Fluids obtained from the cloaca showed elevated osmotic pressure and ion concentrations, with exception of ammonia, K^+ and Mg^{2+}, in 70% SW-acclimated compared with FW-acclimated crocodiles (Table 3).

Epithelial transport properties and biochemical measurements

Measurements of resting electrophysiological parameters of epithelial tissue from the rectum revealed no differences between SW- and FW-acclimated animals (Fig. 2) although TEP tended to be lower and the absorptive I_{sc} tended to be higher in SW-acclimated than in FW-acclimated crocodiles (Fig. 2A,B). Indeed, later measurements found significantly elevated I_{sc} in rectal epithelia from 70% SW-acclimated animals versus FW-acclimated controls (Table 4, Fig. 3A). The properties of epithelia from the rectum and the urodaeum were similar in FW-acclimated crocodiles with the exception that the urodaeum displayed lower conductance and thus higher resistance than the rectum (Fig. 2C). However, in contrast to the rectum, the urodaeum from *C. porosus* showed strong responses to 70% SW acclimation with an approximately 3- and 4-fold increase in TEP and absorptive I_{sc}, respectively, over values observed in FW-acclimated animals (Figs 2A,B and 4A).

In an attempt to identify the apical Na^+ transporter(s) responsible for the absorptive I_{sc} in both the rectum and the urodaeum, and the greatly enhanced I_{sc} in the urodaeum of 70% SW-acclimated crocodiles, pharmacological agents were applied to target first co-transporters and subsequently epithelial Na^+ channels and Na^+/H^+ exchangers (NHEs). When applied to the mucosal saline, bumetanide, an inhibitor of Na^+:Cl^- and Na^+:K^+:Cl^- co-transporters, had no effect on I_{sc} or conductance in epithelia from either the rectum or urodaeum from FW- or SW-acclimated crocodiles (Table 4). In contrast, amiloride, an inhibitor of Na^+

Table 3. Chemistry and osmolality of cloacal fluids obtained from FW- and SW-acclimated crocodiles

Parameter	FW	SW	Normality	*P*-value
pH	6.94±0.7 (3)	5.34±0.5 (2)	Y	0.216
Ammonia (mmol l^{-1})	112.9±12.7 (3)	137.5±3.8 (4)	Y	0.114
Na^+ (mmol l^{-1})	10.4±2.4 (3)	49.9±13.1 (3)*	Y	0.041
K^+ (mmol l^{-1})	16.8±8.9 (3)	16.4±8.5 (3)	Y	0.975
Ca^{2+} (mmol l^{-1})	0.09±0.02 (3)	0.24±0.04 (3)*	Y	0.031
Mg^{2+} (mmol l^{-1})	0.73±0.07 (3)	0.92±0.38 (3)	Y	0.829
Cl^- (mmol l^{-1})	7.1±1.2 (3)	50.4±6.6 (3)*	Y	0.003
SO_4^{2-} (mmol l^{-1})	45.0±5.5 (3)	61.1±1.8 (3)*	Y	0.050
PO_4^{2-} (mmol l^{-1})	32.3±2.5 (3)	67.7±12.1 (3)*	Y	0.045
Osmolality (mosm kg^{-1})	254±21.7 (3)	396±14.7 (3) *	Y	0.002

Data are means±s.e.m. of *n*=3 animals. Not all individuals had fluids in the cloacal segments.

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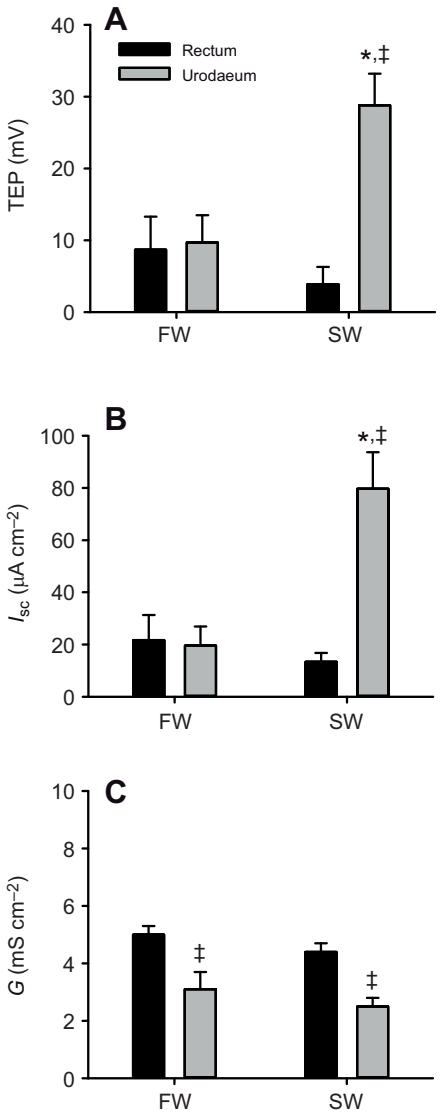

Fig. 2. Epithelial transport properties in isolated segments of the rectum and urodaeum of freshwater (FW)- and seawater (SW)-acclimated estuarine crocodiles. (A) Transepithelial potential (TEP). (B) Short-circuit current (I_{sc}), shown as positive values. (C) Conductance (G). Means±s.e.m. for n=5 FW-acclimated and n=5 SW-acclimated animals. *Significant difference from FW-acclimated animals; ‡significant difference between rectum and urodaeum (Student's t-test, P<0.05).

channels and NHEs, added to the mucosal saline in concentrations ranging from 0.5 to 100 μmol l^{-1} reduced I_{sc} in a concentration-dependent manner in epithelia from the urodaeum of both 70% SW- and FW-acclimated animals (Fig. 4A). The concentration required to inhibit I_{sc} to 50% (IC_{50}) was >1 μmol l^{-1} for the urodaeum of both 70% SW- and FW-acclimated animals (Fig. 4A), demonstrating high efficacy. The conductance of the urodaeum was not affected by amiloride (Fig. 3B). The I_{sc} observed in rectal epithelia from 70% SW-acclimated crocodiles also displayed high sensitivity to amiloride. The I_{sc} of the rectum from SW-acclimated animals appeared to be fully inhibited by the second amiloride concentration tested (1 μmol l^{-1}), demonstrating a low effective concentration with an IC_{50} <1 μmol l^{-1} although an IC_{50} could not be determined accurately because of low resolution of the dose–response curve. The conductance of the rectal epithelia, similar to that of the urodaeum, was not altered by amiloride.

Table 4. Short circuit current (I_{sc}) and conductance (G) of isolated segments of the rectum and urodaeum from FW- and SW-acclimated estuarine crocodiles under control symmetrical conditions and after addition of bumetanide to the mucosal saline

	FW		SW	
	Control	Bumetanide	Control	Bumetanide
Urodaeum				
I_{sc} (μA cm^{-2})	16.8±7.0	15.9±7.0	88.9±11.3*	85.6±11.9
G (mS cm^{-2})	2.8±0.3	2.9±0.3	2.3±0.6	2.2±0.3
Rectum				
I_{sc} (μA cm^{-2})	5.3±1.0	7.2±1.4	20.4±5.9*	19.8±4.5
G (mS cm^{-2})	4.3±0.5	4.2±0.4	3.9±0.2	4.0±0.2

The Na^+:K^+:Cl^- co-transporter inhibitor bumentanide was added at a concentration of 10^{-4} mol l^{-1}. Data are means±s.e.m. of n=5 animals.
*Significant difference from corresponding FW control.

In light of the pronounced increase in absorptive I_{sc} in epithelia from the urodaeum of SW-acclimated crocodiles, activity of the two main ATPase drivers for ion transport, the V-type proton pump and the Na^+/K^+-ATPase (NKA), was measured in this tissue. NKA activity was increased approximately 4- to 5-fold in urodaeum from SW-acclimated crocodiles versus FW-acclimated animals (t-test, P<0.002) (Fig. 5). A similar trend was observed for the proton pump, although this trend escaped statistical significance (t-test, P<0.126) (Fig. 5).

Gene cloning and expression

Full-length sequence (3004 base pairs, bp) of the gene encoding the epithelial Na^+ channel ENaC alpha subunit (GenBank accession number: KU351662.1) was cloned and showed 95% and 97% similarity with ENaC genes of *Alligator mississippiensis* (XM_019476484.1) and *Gavialis gangeticus* (XM_019527391.1), respectively. For the NHE2 gene, a 731 bp segment was cloned and sequenced (GenBank accession number: MG642818), and showed 98% similarity with the NHE2 gene of *A. mississippiensis* (XM_019485034.1), *G. gangeticus* (XM_019525318.1) and *Alligator sinensis* (XM_025213187.1). Finally, a 771 bp segment of the NHE3 gene was cloned (GenBank accession number: MG642819) and showed 97% similarity with that of *A. mississippiensis* (XM_006259235.2), *G. gangeticus* (XM_019509144.1) and *A. sinensis* (XM_006018996.2). All mRNA expression was normalized to that of *EF1α* (GenBank accession number: KU351663.1) and is reported relative to the lowest observed expression of the gene in question. As expected, the ENaC alpha subunit gene was highly expressed in the urodaeum as well as in the kidney and rectum of FW-acclimated crocodiles (Fig. 6A). However, in contrast to expectations, expression was downregulated in the urodaeum of 70% SW-acclimated compared with FW-acclimated animals. NHE3 gene expression showed no difference between FW- and 70% SW-acclimated animals (Fig. 6B). In contrast to NHE3, the NHE2 gene showed expression in all examined tissues, with a statistically significant increase in expression in the urodaeum of 70% SW- compared with FW-acclimated crocodiles (Fig. 6C). A similar trend was observed in all other examined tissues but none reached the level of statistical significance.

DISCUSSION

The present study confirms previous reports of extensive movements by wild *C. porosus* (Read et al., 2007) and furthermore demonstrates that these movements may traverse salinity gradients, necessitating switching between hyperosmoregulatory and hypo-osmoregulatory strategies. Further, juvenile *C. porosus* in captivity successfully

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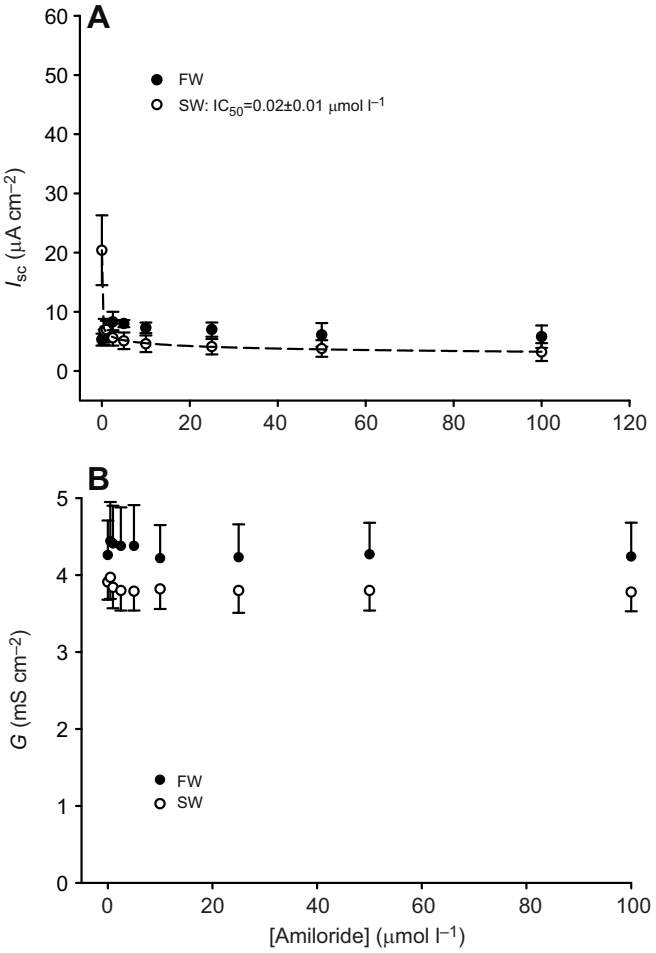

Fig. 3. Apical Na^+ transporters in isolated segments of the rectum of FW- and SW-acclimated estuarine crocodiles. (A) I_{sc} and (B) conductance (G) in the presence of the Na^+ channel inhibitor amiloride. Means±s.e.m. for n=3–4 FW-acclimated and n=4–5 SW-acclimated animals. The I_{sc} and conductance of the rectum from FW-acclimated crocodiles were not impacted by amiloride whereas those from the rectum of SW-acclimated crocodiles were significantly reduced at all amiloride concentrations (ANOVA, and *post hoc* *t*-tests, P<0.05). The concentration of amiloride required to inhibit the I_{sc} by 50% (IC_{50}) is indicated (means±s.e.m.).

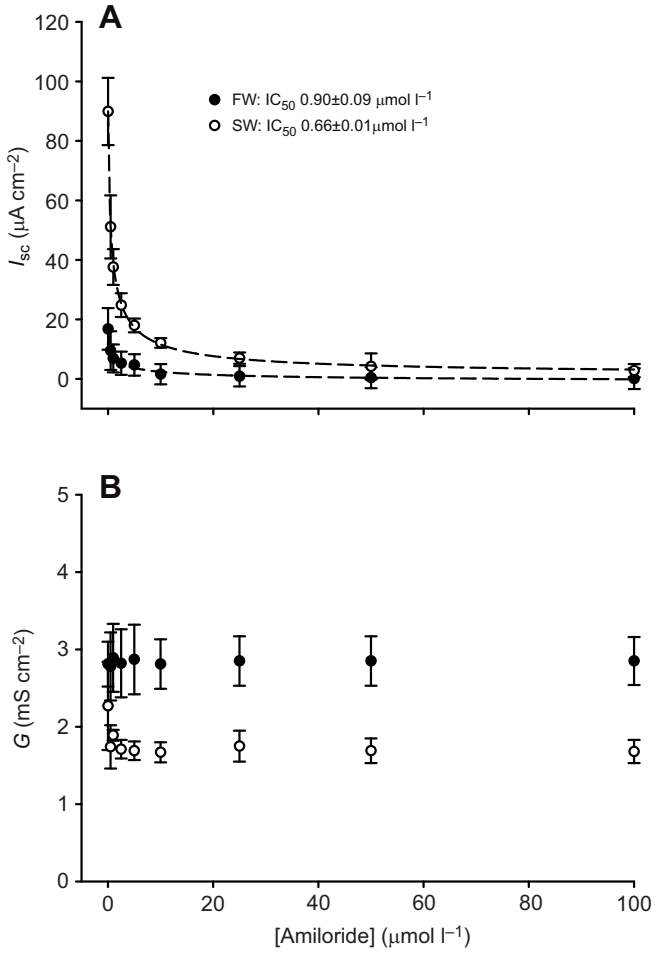

Fig. 4. Apical Na^+ transporters in isolated segments of the urodaeum of FW- and SW-acclimated estuarine crocodiles. (A) I_{sc} and (B) conductance (G) in the presence of the Na^+ channel inhibitor amiloride. Means±s.e.m. from n=4 FW-acclimated and n=5 SW-acclimated animals. The I_{sc} of the urodaeum from FW- and SW-acclimated crocodiles at all amiloride concentrations was significantly different from that of controls (ANOVA, and *post hoc* *t*-tests, P<0.05) while the conductance of the urodaeum from SW- but not FW-acclimated crocodiles showed significant depression at all tested amiloride concentrations.

acclimate to FW as well as hypersaline water and maintain near-constant osmoregulatory balance. We demonstrate that, in addition to the well-documented plasticity of the lingual salt glands (Cramp et al., 2010; Cramp et al., 2008; Franklin and Grigg, 1993), the mucosal epithelium of the urodaeum is also plastic and that both organs change to accommodate salt and water balance in hyperosmotic environments. Marked stimulation of an absorptive I_{sc} by the urodaeum supports previous suggestions of enhanced solute-coupled water absorption (Kuchel and Franklin, 1998) in this cloacal segment of SW-exposed *C. porosus*. In the urodaeum of SW-acclimated *C. porosus*, elevated NKA activity as well as stimulated gene expression of NHE2 provide a tentative pathway for Na^+ absorption across this epithelium.

Laboratory-hatched and -raised *C. porosus* in the present study maintained salt and water balance in 70% SW, although there were slight indications of challenged homeostasis (elevated plasma Cl^- and a tendency for elevated Na^+ and osmotic pressure). Elevated plasma Cl^- and a trend for elevated osmotic pressure in juvenile *C. porosus* held in 70% SW has been reported previously (Kuchel and Franklin, 1998), but is in contrast to reports of perfect salt balance in juvenile *C. porosus* collected from the wild at salinities as high as 64 ppt (Grigg, 1981; Taplin, 1984b). Although the captive *C. porosus* in the present study successfully hypo-osmoregulate, with respect to their environment, they perform less well than wild individuals. It is not possible to determine what caused this difference between captive and wild *C. porosus* but feeding rates may explain the differences. *Crocodylus porosus* do not drink in waters of >18 ppt (Taplin, 1984a) and in fact do not survive in SW without feeding (Taplin, 1985), suggesting that they obtain water from their diet to replace excretory losses. Indeed, Na^+ concentrations of lingual salt gland secretions are barely above those of SW (Grigg and Shine, 2015), meaning that very little water would be gained from SW ingestion as most of the imbibed water would be eliminated with salt secretion. Although the captive animals used in the present study fed and grew in both tested salinities, they may not have fed at rates comparable to those of wild individuals and at rates sufficient to maintain perfect salt and water balance. Further, the 7 day fasting period prior to sampling may have contributed to the less than perfect osmoregulatory balance. Nevertheless, the animals used in the present study appeared healthy

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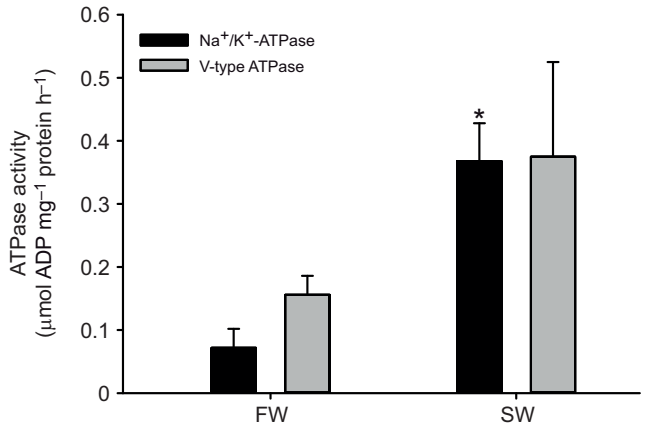

Fig. 5. Activity of Na^+/K^+-ATPase and V-type ATPase in isolated urodaeum epithelia from FW- and SW-acclimated estuarine crocodiles. Means±s.e.m. from *n*=5 FW-acclimated and *n*=6 SW-acclimated animals. *Significant difference from FW-acclimated animals (Student's *t*-test, $P<0.05$).

and clearly responded in adaptive manners to elevated salinity as discussed further below.

The cloacal urine sampled in the present study showed elevated osmotic pressure in 70% SW- compared with FW-acclimated animals, which is in agreement with previous reports from both wild and captive *C. porosus* (Grigg, 1981; Kuchel and Franklin, 1998), although the increase in osmotic pressure was higher in the present study. Similarly, although much lower than plasma levels, Na^+ and Cl^- levels in cloacal urine from *C. porosus* were higher than in these previous studies, again suggesting that the animals in the present study may have performed less well in hypersaline water, possibly as a consequence of less than adequate food ingestion and/or fasting prior to experimentation. However, it should be noted that the concentrations are lower than values reported for ureteral urine (Kuchel and Franklin, 1998), pointing to cloacal Na^+ and Cl^- absorption. Further, solute-coupled water absorption may not necessarily render Na^+ (and/or Cl^-) less concentrated in the lumen as salts and water are moving in parallel. Indeed, isotonic fluid absorption, which would not deplete luminal Na^+ and Cl^- concentrations, is common for leaky epithelia, like that of the *C. porosus* urodaeum (Larsen, 2000; Larsen et al., 2007; Nedergaard et al., 1999). Elevated concentrations of Ca^{2+}, SO_4^{2-} and PO_4^{2-}, and to a lesser extent ammonium, in the cloacal urine from 70% SW-acclimated *C. porosus* in the present study may be due to water absorption rendering these ions more concentrated. Overall, concentrations of divalent cations in the cloacal urine, even in the 70% SW-acclimated animals, are very low compared with, for example, what is found in bladder urine of marine fish (Larsen et al., 2014). These low concentrations of at least the divalent cations are probably attributable to complexation on urate salts (Grigg, 1981). Although low sample size prevents the detection of a statistically significant difference, it is noteworthy that the pH is rather low in cloacal urine of 70% SW-acclimated animals. Acidification of the cloacal lumen in *C. porosus* held in hypersaline water may be explained, at least in part, by the Na^+ absorption mechanism(s) discussed below and would facilitate precipitation of uric acids which have a p*K* of 5.4. Copious amounts of precipitated uric acid were observed in the 70% SW-acclimated *C. porosus* but it was absent in FW-acclimated animals.

Isolated epithelia from the urodaeum of 70% SW-acclimated *C. porosus* displayed a strongly stimulated ion absorption (I_{sc}) when compared with that of animals held in FW. Activity of NKA in the

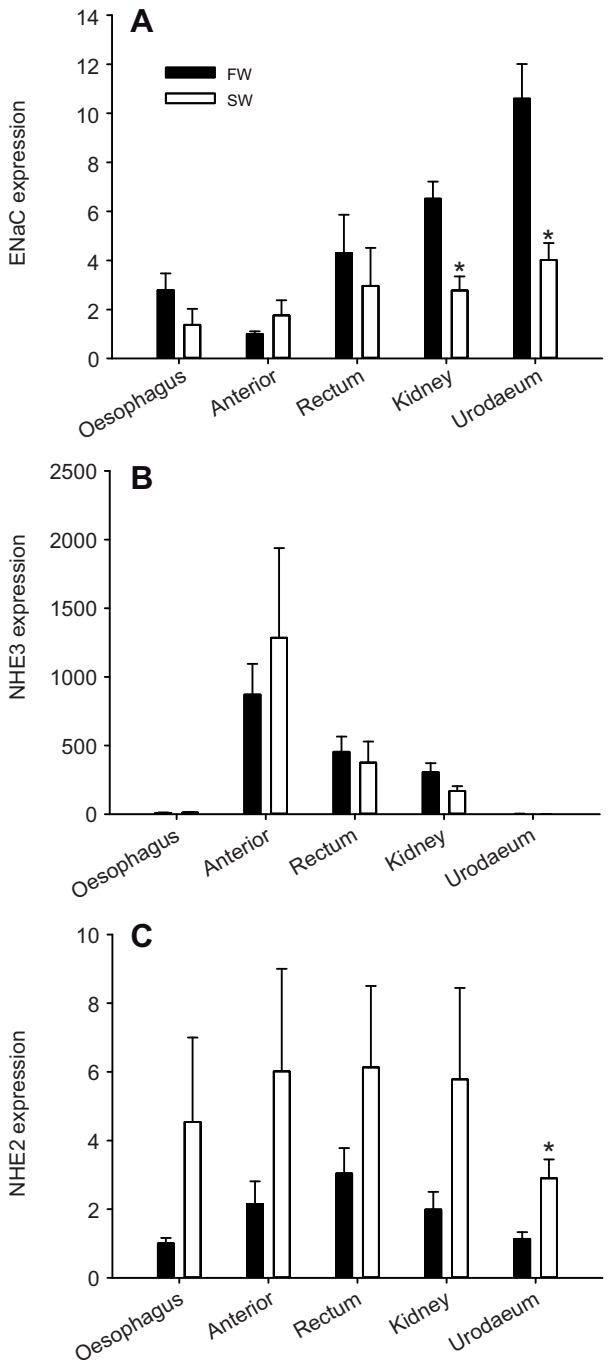

Fig. 6. Gene expression of the epithelial Na^+ channel alpha subunit and Na^+:H^+ exchanger in tissues from FW- and SW-acclimated crocodiles. (A) Epithelial Na^+ channel (ENaC) and Na^+:H^+ exchanger (B) NHE3 and (C) NHE2 gene expression levels were normalized to that of elongation factor 1α (*n*=5 or 6, means±s.e.m.). *Significant difference from FW-acclimated animals (Student's *t*-test, $P<0.05$).

epithelium from the urodaeum was stimulated in parallel, suggesting that this enzyme provides the driving force for Na^+ absorption. The apical contribution to the stimulated absorptive I_{sc} was hypothesized to be via Na^+:K^+:$2Cl^-$ co-transport, ENaC, NHE2 or NHE3, and a pharmacological approach was employed to identify the most likely transport pathway. The possible involvement of the co-transport pathway was ruled out by the lack of effect of luminal application of bumetanide. Subsequent

experiments titrating the absorptive I_{sc} by increasing concentrations of luminal amiloride revealed high sensitivity to this blocker (IC_{50} <1 µmol l^{-1}). Amiloride is a known inhibitor of several Na^+ transporters, of which Na^+ channels, like ENaC, typically display the highest sensitivity, characterized by IC_{50} values in the 1 µmol l^{-1} range (Kleyman and Cragoe, 1988). Thus, the high sensitivity to amiloride supports the involvement of ENaC in the stimulated I_{sc} in the urodaeum of *C. porosus* acclimated to 70% SW. The tendency of elevated proton pump activity in the same tissue may further support the possible role of ENaC in Na^+ absorption as this ATPase has been shown to drive Na^+ uptake via ENaC or/and other Na^+ channels in fish, presumably by hyperpolarizing the apical membrane (Bury and Wood, 1999; Grosell and Wood, 2002). However, perhaps in contrast to the pharmacological observations, mRNA expression of the ENaC alpha subunit was reduced, rather than stimulated, in the urodaeum of *C. porosus* acclimated to 70% SW. Similarly, NHE3 mRNA expression was not increased by acclimation to 70% SW. Expression of NHE2, in contrast, was stimulated >2-fold by acclimation to 70% SW, possibly suggesting that NHE2 could account for the observed increase in I_{sc}. Although NHEs in general are less sensitive to amiloride than Na^+ channels, sensitivity has been shown to vary substantially among species and according to test conditions (Kleyman and Cragoe, 1988), and IC_{50} values in the low micromolar range for NHE inhibition by amiloride have been reported (Kleyman and Cragoe, 1988). Additional pharmacological experiments using amiloride analogues such as EIPA (inhibits preferentially NHEs) and phenamil (inhibits primarily Na^+ channels) would be useful to differentiate between the two putative transport pathways. Further, quantification of NHE and ENaC protein in the apical membrane of the urodaeum mucosa would be useful as it cannot be ruled out that mRNA expression in the urodaeum does not reflect the levels of mature and active transport protein in this tissue. Although it is impossible to conclude which of these apical transport pathways are at play in the urodaeum based on the present data, it is worth noting that cloacal urine contains very high levels of ammonia. NHEs as well as the proton pump have been implicated in ammonia excretion through NHE-Rh protein or proton pump-Rh protein metabolons, respectively, effectively exchanging Na^+ for NH_4^+ (Wright and Wood, 2009; Wu et al., 2010). Furthermore, both putative Na^+ transport pathways may explain the tendency for acidification of cloacal urine in animals acclimated to 70% SW.

Interestingly, the rectum of *C. porosus* acclimated to 70% SW displayed elevated I_{sc} with amiloride sensitivity similar to that of the urodaeum and also a lack of bumetanide sensitivity. As two sphincters guard against movement of urine from the urodaeum to the rectum (Kuchel and Franklin, 2000) and as *C. porosus* do not deliberately drink in 70% SW (Taplin, 1984a), this stimulated I_{sc} may be a response to salt ingestion associated with feeding. Because the food was moistened with water from the holding tanks prior to being offered to the animals, 70% SW-acclimated animals obtained a greater dietary salt load. This practice was applied to simulate involuntary water ingestion associated with feeding in the wild. Regardless, increased dietary salt load may explain the stimulated I_{sc} in the rectum of the 70% SW-acclimated *C. porosus* and, although not a direct osmoregulatory response, may in fact have contributed to water absorption in the rectum and thus overall salt and water balance. The higher dietary salt load in 70% SW-acclimated animals may also explain the trend for increased NHE2 expression in the oesophagus, the anterior intestine and the rectum. Although dietary salt intake seems a likely explanation for the observations of increased I_{sc} in the rectum as well as enhanced NHE2 expression in several gastrointestinal segments, studies with varied dietary salt intake are required to test this suggestion.

Overall, this study identifies part of an important osmoregulatory pathway that facilitates the utilization of saline habitats by *C. porosus*. Although all examined Crocodyloidea possess lingual salt glands, nothing is known about whether salts glands and the urodaeum in species other than *C. porosus* respond to elevated salinity to facilitate osmoregulation in hyperosmotic environments. Further studies of the role on the gastrointestinal tract as well as the urodaeum in salt and water balance are clearly warranted.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: M.G., C.E.F.; Methodology: M.G., R.M.H., N.C.W., R.L.C., Y.W., E.M.M., R.G.D., C.E.F.; Formal analysis: M.G., Y.W., C.E.F.; Investigation: M.G., R.M.H., N.C.W., R.L.C., Y.W., E.M.M., R.G.D., C.E.F.; Resources: M.G.; Data curation: M.G.; Writing - original draft: M.G.; Writing - review & editing: R.M.H., N.C.W., R.L.C., Y.W., E.M.M., R.G.D., C.E.F.; Visualization: R.G.D.; Supervision: M.G., C.E.F.; Project administration: C.E.F.; Funding acquisition: C.E.F.

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