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RESEARCH ARTICLE

Effects of chronic exposure to 12‰ saltwater on the endocrine physiology of juvenile American alligator (*Alligator mississippiensis*)

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ABSTRACT

American alligator (*Alligator mississippiensis*) habitats are prone to saltwater intrusion following major storms, hurricanes or droughts. Anthropogenic impacts affecting hydrology of freshwater systems may exacerbate saltwater intrusion into freshwater habitats. The endocrine system of alligators is susceptible to changes in the environment but it is currently not known how the crocodilian physiological system responds to environmental stressors such as salinity. Juvenile alligators were exposed to 12‰ saltwater for 5 weeks to determine the effects of chronic exposure to saline environments. Following 5 weeks, plasma levels of hormones [e.g. progesterone, testosterone, estradiol, corticosterone, aldosterone (ALDO), angiotensin II (ANG II)] were quantified using liquid chromatography and tandem mass spectrometry. Compared with freshwater-kept subjects, saltwater-exposed alligators had significantly elevated plasma levels of corticosterone, 11-deoxycortisol, 17α-hydroxyprogesterone, testosterone, 17β-estradiol, estrone and estriol whereas pregnenolone and ANG II were significantly depressed and ALDO levels were unchanged (slightly depressed). On the one hand, saltwater exposure did not affect gene expression of renal mineralocorticoid and glucorticoid and angiotensin type 1 (AT-1) receptors or morphology of lingual glands. On the other hand, saltwater exposure significantly reduced plasma glucose concentrations whereas parameters diagnostic of perturbed liver function (aspartate aminotransferase and alanine aminotransferase) and kidney function (creatinine and creatine kinase) were significantly elevated. Except for plasma potassium levels (K^+), plasma ions Na^+ and Cl^- were significantly elevated in saltwater alligators. Overall, this study demonstrated significant endocrine and physiological effects in juvenile alligators chronically exposed to a saline environment. Results provide novel insights into the effects of a natural environmental stressor (salinity) on the renin–angiotensin–aldosterone system and steroidogenesis of alligators.

KEY WORDS: RAAS, Steroidogenesis, Plasma biochemistry, Angiotensin II, Aldosterone, Saltwater stress

INTRODUCTION

American alligators [*Alligator mississippiensis* (Daudin 1802)] mainly inhabit freshwater (FW) systems in the southeastern USA. Although no longer endangered, alligators are commonly affected by both anthropogenic and natural environmental stressors. Whereas anthropogenic stressors generally include pollutants from domestic, agricultural and industrial sources (Crain et al., 1997; Guillette, 2000; Guillette et al., 1994; Gunderson et al., 2016; Tellez and Merchant, 2015), they can also include activities that alter alligator habitat hydrology, such as construction of canals, dredging, logging and oil/gas exploration. These activities can reduce FW input (from rivers and streams), increasing the likelihood of droughts and risk of saltwater (SW) intrusion from nearby coastal areas (Day et al., 2000; Herbert et al., 2015). Less immediate anthropogenic impacts include gradually rising sea levels, which are expected to inundate alligator habitats along the Gulf of Mexico with SW (Emanuel, 2005; Hoyos et al., 2006). Environmental stressors include drought or storm surges following major storm and hurricane events. These further contribute to the salinization of alligator habitats, constituting a significant present and long-term threat along Gulf coast states.

Although larger alligators are known to forage in brackish water (0.5–30‰) and some have even been reported to forage in or near coastal areas (Elsey, 2005), alligators generally do not tolerate saline environments. This reduced salinity tolerance of alligators (Family Alligatoridae) compared with crocodiles (Family Crocodylidae), in which ocean-going species are known (e.g. saltwater crocodile, *Crocodylus porosus*), is due to osmoregulatory and physiological differences between the two families (Taplin, 1988). First, most crocodylids have extrarenal salt-secreting glands enabling them to excrete a concentrated solution of Na^+ and Cl^- in hyperosmotic conditions (Pidcock et al., 1997; Taplin, 1988; Taplin and Grigg, 1981; Taplin et al., 1982). These salt glands are located in mucus membranes on the surface of the tongue (Taplin and Grigg, 1981; Taplin et al., 1982). On the contrary, several studies have demonstrated an apparent absence of lingual salt glands in Alligatoridae (Grigg et al., 1998; Taplin, 1988; Taplin et al., 1982). Low secretory rates and secretions almost iso-osmotic with plasma in alligatorids suggest lingual glands are salivary rather than salt glands (Taplin, 1988; Taplin et al., 1982). Second, the cloaca in crocodiles, in addition to the lingual salt glands, is an active osmoregulatory organ as final urine composition in the bladder differs from ureteral urine. However, the cloaca in alligators does not seem to have a similar mechanism and they therefore lack the ability of post-renal osmoregulation (Pidcock et al., 1997).

The renin–angiotensin–aldosterone system (RAAS) is an endocrine system that regulates blood pressure and water–salt balance in all vertebrates (Morici, 1996; Nishimura, 2017; Silldorff and Stephens, 1992a,b). The main enzymes are renin and angiotensin-converting enzyme (ACE), which convert angiotensinogen to angiotensin I (ANG I) and ANG I to angiotensin II (ANG II), respectively. ANG II has several physiological effects in addition to stimulation of the

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mineralocorticoid steroid hormone aldosterone (ALDO). Some of these effects are an increase in blood pressure via Na^+ retention in kidneys (Fagyas et al., 2014; Singh et al., 2010). A functional RAAS has been demonstrated in American alligator as injections of fowl ANG I produced dose-dependent increases in arterial blood pressure (Silldorff and Stephens, 1992a,b). The increase in blood pressure was blocked by the ACE inhibitor, captopril, which demonstrates ACE involvement in ANG I to ANG II conversion. What is more, injection of ANG II caused significantly increased plasma ALDO levels in juvenile alligators (Morici, 1996). Injection of ANG I and ANG II into the spectacled caiman (*Caiman crocodilus*; Alligatoridae) similarly produced significant dose-dependent increases in mean arterial blood pressure (Butler, 2006).

ALDO is produced and secreted from the adrenal glands and is, along with ANG II, under regulatory control of plasma K^+ levels. ALDO exerts direct effects on kidney nephrons by enhancing retention of renal Na^+ in exchange for K^+ excretion and thereby restoration of blood volume and blood pressure (Bollag, 2014). Despite demonstrated presence of RAAS in alligators, studies on hormonal control of alligator osmoregulation, when exposed to saline environments, are few (Lauren, 1985; Morici, 1996). It is therefore not fully understood how long-term salinity exposure affects ANG II and ALDO levels in alligators. Information regarding the effects on young alligators is especially important as hatchlings and juvenile alligators are at higher risk of salinity stress due to smaller size and thinner integument. Furthermore, high abundance of hatchlings (~July–August) coinciding with the Gulf of Mexico hurricane season (June–December) and enhanced risk of storm surges and drought may impact recruitment to the adult population and have wide-ranging effects in a species that does not reach sexual maturity until 8–10 years of age (Lance et al., 2015; Wilkinson and Rhodes, 1997).

Whereas sex determination in alligators is predominantly temperature dependent (Rooney et al., 2004), sexual maturity and reproduction are mainly driven by elevated levels of sex steroid hormones, such as progesterone, 17β-estradiol and testosterone (Lance, 1989). Juvenile alligators show seasonal variations in plasma sex steroid hormones (Rooney et al., 2004). Thus, any changes in a young alligator's environment can potentially affect sex steroid hormone levels and impact reproductive ability. Previous studies demonstrated a negative correlation between plasma testosterone and 17β-estradiol levels and stress hormone corticosterone levels in sexually mature male and female alligators (Elsey et al., 1991; Lance and Elsey, 1986). Whether stressful conditions such as salinity exposure can significantly affect sex steroid hormone levels in sexually immature alligators is not known at present. However, sex steroid hormone production in alligators is susceptible to the presence of anthropogenic endocrine-disrupting compounds (EDCs) in the environment. For example, the decline of American alligator populations in Lake Apopka (FL, USA) was attributed to reproductive failure and reduced egg viability due to exposure to pesticides, such as DDT (dichlorodiphenyl trichloroethane) and its metabolite *p,p'*-DDE (dichlorodiphenyl dichloroethylene). The estrogenic potency of *p,p'*-DDE was postulated to be causal for the altered endocrine effects observed (Guillette et al., 1994, 1999). More recently, feminizing effects of corexit-enhanced water-accommodated fraction of crude oil were reported following *in vitro* exposure of gonads and gonad–adrenal–mesonephric organ complexes at male-producing temperatures during the embryonic thermo-sensitive period of alligator development (Williams et al., 2017).

Given that storm surges, drought and salinization of FW wetlands are continuous threats to alligator habitats, it is crucial to assess the effects of SW on juvenile alligator endocrine system and physiology. The goal of this study was to investigate the diagnostic effects of chronic (5 weeks) salinity stress (12‰) on the RAAS cascade, reproductive steroids and blood biochemistry in juvenile American alligators exposed to SW. To that end, juvenile (1–2 years old) alligators were exposed to 12‰ SW for 5 weeks and the effects on steroidogenesis, RAAS hormones (ANG II, ALDO), plasma biochemistry parameters, gene expression of RAAS hormone receptors and morphology of lingual gland were investigated. We hypothesized that plasma hormones of the RAAS would be elevated following chronic salt stress due to a reduction in blood volume caused by severe dehydration whereas levels of sex steroid hormones would be depressed due to high corticosterone levels.

MATERIALS AND METHODS

Animals and husbandry

Juvenile American alligators (*A. mississippiensis*, 1–2 years old) [mean body mass 639±40.3 g; 29.5±0.645 cm snout-to-vent length (SVL)] were generously donated by Rockefeller Wildlife Refuge (Grand Chenier, LA, USA) and transported back to Texas A&M University at Galveston (TAMUG), TX, USA. Alligators were housed in a constant temperature (26°C) and photoperiod (12 h:12 h light: dark cycle) controlled room. Animals were kept at a stocking density of 16 animals in 380 liter Rubbermaid stock tanks (Rubbermaidcommercial.com) containing 90 liters of FW and a basking plate. Tanks were fitted with a reptile 160 W UVB light heat lamp (Zoo Med Laboratories, Inc., San Luis Obispo, CA, USA) to maintain temperature at 26±1°C. Twenty-five percent of each tank was shaded to allow animals to shelter and thermoregulate. Animals were fed an average of 3% body mass per week of Mazuri® Reptile Diet (PMI Nutrition International, St Louis, MO, USA). Water changes were performed 24–30 h after each feeding (three times per week).

Experimental design

Juvenile alligators were exposed to either FW (control, N=8) or SW (12‰, N=8) for 5 weeks. The salinity concentration was based on previous studies (Lauren, 1985; Morici, 1996) in which juvenile alligators were exposed to salinity levels up to 20‰. However, in these studies, mortality was observed at levels higher than 14‰. These previous studies further assessed osmolality and reported 8‰ being iso-osmotic to alligator plasma, with 10‰ and above being hyper-osmotic (Lauren, 1985; Morici, 1996). To ensure salinity effects but to avoid mortalities, we therefore determined to use 12‰ as the highest salinity level. The FW group was maintained in dechlorinated city tap water at 0‰ for the duration of the exposure period. To ensure removal of chlorine, tap water was kept in stock tanks for 2 days before each water change. At the time of water change, chlorine was absent in stock tanks as determined by water quality test strips (LaMotte Inc., Chestertown, MD, USA). The treatment group was gradually exposed to increases in salinity over 8 days. Salinity was increased every 2 days from 0‰ to 4‰, 8‰ and finally maintained at 12‰ for the duration of the experimental period. SW was attained by mixing filtered and sterilized seawater with tap water in appropriate proportions. All seawater was obtained from the Gulf of Mexico off Galveston Island, TX, USA. During the course of the study, salinity levels in stock and exposure tanks [control (0‰) and SW (12‰)] were verified using a salinity meter (Oakton Instruments, Vernon Hills, IL, USA, WD-35604-00) twice daily.

Prior to the salinity trial, alligators in control and experimental tanks were fed a rate of 3% body mass weekly. Pellets were weighed

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and counted to determine the number of pellets corresponding to a weekly food intake of 3% body mass. During the trial, animals were offered food three times weekly and food intake was closely monitored by counting the number of pellets given to each tank. The SW group gradually refused feeding and, to avoid conflicting interactions between food deprivation and salinity effects, the SW group was fed first and the number of pellets eaten by the animals was counted. The FW group was then fed the same number of pellets.

After 5 weeks, 3 ml blood samples were collected from each alligator from the occipital sinus using a 23 gauge non-heparinized needle and a 3 ml syringe. Blood was placed in a non-heparinized microfuge tube and immediately centrifuged for 2 min at 10,000 ***g*** to separate the plasma. Plasma from each animal was then aliquoted into two lithium heparin tubes: one for hormone analysis, and one for plasma biochemistry analysis. Plasma samples were stored at −20°C until analysis. Following blood sampling, each alligator was then anesthetized by placing it in a plastic tote (~3.0 liters) with a cotton ball soaked with 1 ml of liquid isoflurane. When ventilation ceased, animals were checked for eye-blink and pedal reflexes and when absent, alligators were cranially pithed with a 14 gauge hypodermic needle. Each animal was then weighed and measured (snout-to-vent and total length) prior to being dissected. Liver, lungs, kidneys, heart and tail muscle were removed and placed in −80°C until further analysis. Small tissue samples were saved for mRNA expression (lungs and kidneys) and placed in RNAlater (Qiagen, Redwood City, CA, USA) and kept at −20°C before analysis. Although we randomized animals prior to the trial, upon dissection it was determined that all alligators (FW and SW) were male. All studies were in compliance with Texas A&M University's Animal Care Committee under AUP IACUC 2015-0347.

Blood plasma biochemistry

Determination of plasma biochemistry levels was performed by Texas A&M University's Veterinary Medical Diagnostic Laboratory (College Station, TX, USA). Plasma samples were analyzed on a Beckman Coulter AU480 analyzer (Beckman Coulter, Miami, FL, USA). Plasma levels of Na^+, K^+, Cl^-, uric acid, total protein, albumin, globulin, glucose, creatinine, bilirubin, creatine kinase, cholesterol, calcium, phosphorous, alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured.

Steroid hormone extraction from blood plasma

All chemicals and reagents used in sample preparation were purchased from Sigma-Aldrich (St Louis, MO, USA). Plasma samples were thawed on ice, and 500 µl aliquots were spiked with internal standards d_9-progesterone and d_3-estradiol. d_9-Progesterone was used as internal standard for progesterone, pregnenolone, 17α-hydroxyprogesterone, 17α-hydroxypregnenolone, androstenedione, testosterone, 5α-dihydrotestosterone, 11-deoxycortisol and corticosterone, whereas, d_3-estradiol was used as internal standard for 17β-estradiol, estrone and estriol. The plasma was suspended in 5 ml Milli-Q water and liquid:liquid extracted twice with 5 ml methyl tert-butyl ether (MTBE). Pooled MTBE layers were dried under nitrogen gas with the residue reconstituted in 50 µl of 30:70 methanol: Milli-Q water. Samples were transferred to small-volume inserts in 2 ml amber glass vials for liquid chromatography and tandem mass spectrometry (LC–MS/MS) analysis.

For estrogen analysis, a 25 µl sub-aliquot of MTBE-extracted steroids was derivatized with dansyl chloride prior to analysis (Li et al., 2005). The sub-aliquot was dried under nitrogen and reconstituted into 50 µl of 1 mg ml^{-1} dansyl chloride and 50 µl of 100 mmol l^{-1} sodium bicarbonate, and incubated at 60°C for 3 min. The resulting dansylated estrogens (Dns-estrogen) were suspended in 500 µl of Milli-Q water and liquid:liquid extracted twice with 500 µl of 1:1 hexane:ethyl acetate. Pooled solvent layers were dried under nitrogen with residue reconstituted in 30:70 methanol:Milli-Q water, placed into glass inserts and analyzed via LC–MS/MS.

LC–MS/MS analysis of steroid hormones

The LC–MS/MS system comprised an Agilent 1260 UHPLC system (Agilent, Santa Clara, CA, USA) with triple-quad 6420 mass detector. Steroid hormone chromatographic separations were enabled on an Agilent Poroshell EC-C18 column (3.0×50 mm, 5 µm particle size). The liquid mobile phases comprised Milli-Q water (A) and methanol (B), respectively, with each containing 5 mmol l^{-1} ammonium formate. The mobile phase gradient transitioned from 30% (B), increased linearly to 70% over 3 min and from 70% to 95% in 6 min. The gradient was subsequently decreased from 95% to 70% in 3 min and from 70% to 30% (initial condition) over 3 min with the flow rate maintained at 0.4 ml min^{-1} (and total run-time of 15 min).

Plasma hormones were detected in positive ion electrospray ionization (ESI+) mode with nitrogen as the desolvation gas heated to 350°C (gas flow of 12 l min^{-1}) and capillary voltage at 3.5 kV. Hormones were detected in multiple reaction monitoring mode with argon as the collision gas. The precursor>product ions monitored included: *m/z* (mass-to-charge ratio) 347.2→121.1 (corticosterone), *m/z* 347.2→97.2 (11-deoxycortisol), *m/z* 315.2→97.1 (progesterone), *m/z* 317.3→299.2 (pregnenolone), *m/z* 331.2→97.1 (17α-hydroxyprogesterone), *m/z* 333.2→315.2 (17α-hydroxypregnenolone), *m/z* 287.1→97.0 (androstenedione), *m/z* 291.2→255.1 (5α-dihydrotestosterone), *m/z* 289.2→97.0 (testosterone), *m/z* 324.3→100.2 (d_9-progesterone), *m/z* 504.2→171.1 (Dns-estrone), *m/z* 506.2→171.1 (Dns-estradiol), *m/z* 522.2→171.1 (Dns-estriol) and *m/z* 509.3→171.1 (Dns-d_3-estradiol). The mass transitions quantified for ANG II and ALDO were *m/z* 523.8→70.3 (ANG II) and *m/z* 361.2→343.2 (ALDO).

Reverse transcription qPCR (RT-qPCR)

Kidney RNA was analyzed for mineralocorticoid receptor (MR), glucocorticoid receptor (GR) and AT-1 gene expression using real-time qPCR. Forward and reverse primers were designed using Primer3 version 4.0.0 (Table S1), with the exception of GR primers, which were obtained from Gunderson et al. (Gunderson et al., 2006). Kidney tissue stored in RNAlater was extracted for total RNA using TRI Reagent (Sigma-Aldrich, catalog no. T9424). RNA concentration and purity based on a 260:280 (nucleic acid:protein) ratio was quantified using a Take3 plate and Cytation 5 imaging reader with Gen5 software (BioTek Instruments, Inc., Winooski, VT, USA). RNA was diluted to 40 ng $µl^{-1}$ and forward and reverse primers were each diluted to 10 mmol l^{-1} before performing RT-qPCR assays. Gene expression was quantified via SYBR Green detection using Rotor-Gene SYBR Green PCR kits (Qiagen, catalog no. 204074) and a Rotor-Gene Q PCR cycler (Qiagen). One-step RT-qPCR reactions were performed containing 12.5 µl SYBR Green, 0.25 µl RT mix, 4.75 µl RNase-free water, 2.5 µl each of 10 mmol l^{-1} forward and reverse primers, and 2.5 µl template RNA. RNA was reverse-transcribed at 55°C for 10 min followed by 95°C for 5 min. PCR reactions were performed over 40 cycles of denaturation (95°C for 5 s) and annealing/extension (60°C for 10 s). Total copies of cDNA per nanogram of template RNA were calculated as described by Tate et al. (2016).

Histology

Tongue samples from both treatment groups (N=8 animals, n=6 samples per animal) were harvested immediately post-euthanasia and fixed in 10% buffered formaldehyde. Samples were processed for paraffin histology using a Leica tissue processor (Leica Biosystems Inc., Buffalo Grove, IL, USA) under vacuum. Samples were moved through a dehydration series of alcohol, followed by xylene and paraffin. Treatment samples were embedded into cross-section and horizontal orientations equally. Subsequent tissue blocks were sectioned at 7 µm on a rotary microtome. Sections were mounted onto 1% gel subbed slides and stained with modified Masson's trichrome stain. Digital micrographs were collected using a Nikon E-400 (Nikon Instruments Inc., Melville, NY, USA) Eclipse light microscope fitted with a Spot Insight (Diagnostic Images, Sterling Heights, MI, USA) digital microscopy camera. Criteria for measurement of lingual glands in cross-section were: the greatest width of the gland was captured in the field of view as well as the pore from the surface of the tongue. Criteria for measurements of lingual glands in horizontal orientation were: the greatest width of the gland was captured in the field of view and a representation of the pore from the surface of the tongue could be visualized. Morphometrics were collected using Spot Imaging software (Diagnostic Images). Morphometrics collected on cross-sections were: greatest depth of gland, greatest width of gland, pore length, pore width and surface area of glandular region. Morphometrics collected on horizontal sections were: greatest width of glandular region, width 90 deg to greatest width of glandular region and surface area of glandular region.

Statistical analyses

Normality testing was performed using the Shapiro–Wilk test (P<0.05). Statistical significance was determined using parametric and non-parametric tests. Parametric analyses were conducted using two-tailed Student's t-tests. When normality was not met, non-parametric tests were performed. If assumptions for Mann–Whitney/Wilcox tests were not met, a permutation test was performed using 1000 permutations. P-values of <0.05 were considered statistically significant. Statistical analyses were performed using R version 3.3.1 (https://www.r-project.org) or GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA). All data shown are means±s.e.m.

Table 1. Body morphometrics and food intake in American alligators (*Alligator mississippiensis*) exposed to 12‰ saltwater for 5 weeks

	Freshwater	12‰ saltwater
Initial body mass (g)	497±37.5*	781±31.3
5 week body mass (g)	569±30.8	563±29.9
% Mass loss	−14.4±8.30*	27.9±3.90
SVL (cm)	30.3±0.663	28.8±1.09
Total length (cm)	62.4±1.34	61.7±1.28
K	2.04±0.0551	2.57±0.428
% Decrease in food intake	98.73	98.73

Values listed are means±s.e.m. All parameters shown except initial body mass were obtained at 5 weeks. SVL, snout–vent length; K, Fulton's condition factor estimated using SVL. Asterisks denote statistically significant differences between freshwater and saltwater groups (P≤0.05).

RESULTS

Food intake and body morphometrics

Body morphometrics and food intake rate are shown in Table 1. Prior to the trial, control and treatment tanks were fed the same amount of pellets. However, as the SW-exposed group gradually reduced food intake, we decided to feed the FW control group the same amount of pellets to avoid conflicting interactions between food deprivation and salinity effects. Food intake in the SW group decreased by 98% and the FW group's food intake was therefore equally reduced by 98% (Table 1). Despite the FW and SW groups eating the same amount of food, alligators exposed to 12‰ SW lost ∼28% of their body mass (wet body mass) after 5 weeks whereas the FW animals maintained mass. However, there were no significant differences between SVL and total length or in Fulton's condition factor between FW and SW alligators (Table 1).

Effects of salt stress on blood plasma biochemistry

Concentrations of plasma biochemistry parameters are listed in Table 2. Except for plasma potassium levels (K^+), which remained similar between FW- and SW-acclimated alligators, plasma ions

Table 2. Plasma biochemistry parameters in freshwater and 12‰ chronically (5 weeks) exposed juvenile American alligators (*Alligator mississippiensis*)

Blood chemistry parameter	Freshwater (means±s.e.m.)	Freshwater range	Saltwater (means±s.e.m.)	Saltwater range
Glucose (mg dl^{-1})	71.7±2.92*	62–83	60.9±3.31	43–70
Creatinine (mg dl^{-1})	0.84±0.08*	0.4–1.4	4.89±0.20	3.5–5.6
Bilirubin (mg dl^{-1})	0.1±0.00	0.1–0.1	0.12±0.01	0.1–0.2
ALP (U l^{-1})	17.4±0.73	14–22	16.6±0.91	11–22
Creatine kinase (U l^{-1})	717.7±74.2*	413–1205	9043.9±2509.6	364–25,650
AST (U l^{-1})	359.70±12.08*	277–425	518.70±42.94	381–807
ALT (U l^{-1})	33.20±1.12*	29–40	42.60±2.53	35–57
AST:ALTratio	10.87±0.38	9.08–11.73	12.22±0.63	7.86–19.63
Cholesterol (mg dl^{-1})	111.70±4.69*	91–144	245.90±8.11	211–281
Calcium (mg dl^{-1})	10.26±0.09*	9.6–10.5	11.59±0.12	10.8–11.9
Phosphorous (mg dl^{-1})	3.92±0.14*	3.2–4.8	5.32±0.23	3.9–6.3
Sodium (mmol l^{-1})	148.3±0.81*	147–152	202.00±1.42	194–208
Potassium (mmol l^{-1})	5.24±0.17	4.0–5.8	5.5±0.15	4.9–6.3
Na:K ratio	28.55±1.0*	26.7–36	36.95±1.01	30.8–38.5
Chloride (mmol l^{-1})	117.00±1.14*	103–122	173.60±2.99	161–189
Total protein (g dl^{-1})	4.01±0.04*	3.8–4.2	5.25±0.09	4.7–5.7
Albumin (g dl^{-1})	<1.5‡	–	1.73±0.03	1.6–1.9
Globulins (g dl^{-1})	<1.5‡	–	3.52±0.06	3.1–3.8
A:G ratio	–	–	0.5	0.5
Uric acid (mg dl^{-1})	<1.5‡	–	3.73±0.38	1.5–6

*Denotes significant difference (P<0.05) between freshwater and saltwater mean values. ‡Shows values below the detection limit. ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

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Na^+ and Cl^- were significantly elevated in SW alligators (Table 2). Furthermore, plasma uric acid, albumin and globulin levels were below the detection limit in FW alligators but were significantly higher in the 12‰ treatment group after 5 weeks. In addition, total protein levels were significantly elevated in SW alligators compared with FW alligators.

Chronic (5 weeks) exposure to SW significantly increased plasma creatine kinase, creatinine, AST, ALT and cholesterol levels (Table 2). Plasma minerals such as Ca^{2+} and phosphorous (P) were additionally significantly elevated after 5 weeks in 12‰ SW (Table 2). However, although the control and treatment groups ate the same amount of food, plasma glucose levels were significantly reduced in salinity exposed alligators (Table 2). In contrast, exposure to SW did not significantly affect plasma bilirubin or ALP levels.

Mineralocorticoid and glucocorticoid steroid hormones

Plasma levels of the biologically active RAAS hormone, ANG II, were significantly (P=0.01) lower in SW alligators after chronic salinity exposure. In fact, ANG II levels were almost 90% lower in SW alligators compared with FW alligators (Fig. 1). Plasma levels of the mineralocorticoid ALDO were not significantly affected by salinity exposure (P=0.21) although a minor depression in hormone levels was quantified in SW alligators compared with FW alligators (Fig. 1).

Effects of salt stress on steroidogenesis

The effects of salinity stress were assessed on four main classes of steroid hormones, namely progestogens (pregnenolone, progesterone, 17α-hydroxyprogesterone, 17α-hydroxypregnenolone), corticoids (corticosterone, 11-deoxycortisol), androgens (androstenedione, testosterone, 5α-dihydrotestosterone) and estrogens (17β-estradiol, estrone, estriol). Therefore, in total, 12 steroid hormones were quantified in plasma from alligators (Fig. 2). Pregnenolone, the precursor to progesterone or 17α-hydroxypregnenolone, was significantly decreased by 63% relative to FW control (Student's t-test, P=0.002) after 5 weeks of exposure to 12‰ SW (Fig. 3A). This decrease, however, did not affect productions of 'downstream' steroid hormones such as progesterone and 17α-hydroxypregnenolone, which were not significantly different from the FW group (Fig. 3A). In contrast, 17α-hydroxyprogesterone showed 4× higher levels in SW relative to FW (Permutation test, P=0.03) (Fig. 3A).

The elevated levels of 17α-hydroxyprogesterone are of interest as they also associate with elevated 11-deoxycortisol levels (transformation catalyzed by cyp21-hydroxylase) (Fig. 2, Fig. 4).

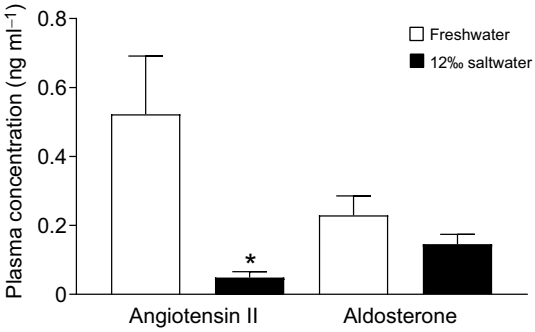

Fig. 1. Plasma levels of angiotensin II (ng ml^{-1}) and aldosterone (ng ml^{-1}) in juvenile American alligators exposed to freshwater or saltwater (12‰). Data shown are means±s.e.m. (N=8). Blood was sampled after 5 weeks in both groups. The asterisk denotes statistically significant differences (P≤0.05) between treatment groups.

Five weeks in 12‰ SW caused a significant increase in the glucocorticoid hormones, corticosterone and 11-deoxycortisol. Corticosterone was ∼7× higher in 12‰-exposed alligators whereas 11-deoxycortisol was ∼8× higher in SW compared with FW alligators (Fig. 4).

The cyp450 enzyme exhibiting 17,20-lyase activity (cyp17-lyase) catalyzes the conversion of 17α-hydroxyprogesterone to androstenedione (Figs 2 and 3B). Although 17α-hydroxyprogesterone levels were elevated in salinity-exposed, male juvenile alligators, there was no significant difference (P=0.23) in androstenedione levels between treatment groups (although androstenedione levels in salinity exposed animals were slightly lower) (Fig. 3B). In contrast, the conversion of androstenedione to testosterone [catalyzed by hydroxysteroid dehydrogenase (17β-HSD)] resulted in significantly (P=0.01) elevated levels of testosterone in salinity exposed juveniles. Testosterone levels were almost doubled in alligators chronically (5 weeks) exposed to 12‰ salinity (Fig. 3B). However, these high testosterone levels in 12‰ salinity exposed animals did not result in higher plasma 5α-dihydrotestosterone levels in SW alligators, suggesting no differences in 5α-reductase activity (Fig. 2).

The cyp450 enzyme aromatase (or cyp19a1a) converts androgens (androstenedione and testosterone) to estrogens (estrone and 17β-estradiol). In turn, estrone and 17β-estradiol can be converted to estriol via concerted activities of cyp450 (cyp3a5 and cyp1a1) and 17β-HSD enzymes (Fig. 2). Plasma levels of all three estrogens were significantly elevated in 12‰ SW-exposed alligators compared with FW controls (Fig. 3C).

Finally, ratios of various steroid hormones were used to assess predominant steroidogenic fluxes under control versus salinity stressed conditions. Specifically, the ratio of estrone to androstenedione was significantly (P=0.02) increased 9× in SW-exposed alligators relative to FW control (Table 3). However, there was no significant (P=0.22) difference in the ratio of 17β-estradiol to testosterone between FW- and SW-exposed alligators (Table 3).

Histology

Morphometric data of lingual glands in FW- and SW-exposed alligators are shown in Table 4 whereas micrographs of cross- and horizontal sections of FW and SW alligator tongues are shown in Fig. 5. Measurements from horizontal sections did not reveal any significant differences (P>0.05) in gland area or width (minimum and maximum) between FW- and SW-maintained alligators (Table 4). Furthermore, measurements from cross-sections of lingual glands also did not show any significant differences in pore and gland area, gland width, height or pore width between FW and SW alligators. However, the length of the pore from the gland to the surface of the tongue was significantly longer in SW-exposed alligators (P=0.001).

DISCUSSION

Food intake and body morphometrics

A significant loss of body mass (28%) had occurred after 5 weeks in 12‰ SW. Although the FW group ate the same amount of food as the SW group, they maintained mass and even gained a little after 5 weeks. Loss in body mass in the SW-exposed group is therefore attributed entirely to the effects of salinity treatment and is likely mainly due to osmotic water loss across the integument (Lauren, 1985) and/or mucus membranes, such as mouth, eyes and cloaca. Presence of severe dehydration was evident as total serum protein levels were significantly elevated in SW-exposed alligators. In mammals, higher total proteins levels are commonly indicative of significant dehydration due to a reduction in plasma volume and concentration of proteins (Burtis and Ashwood, 1999).

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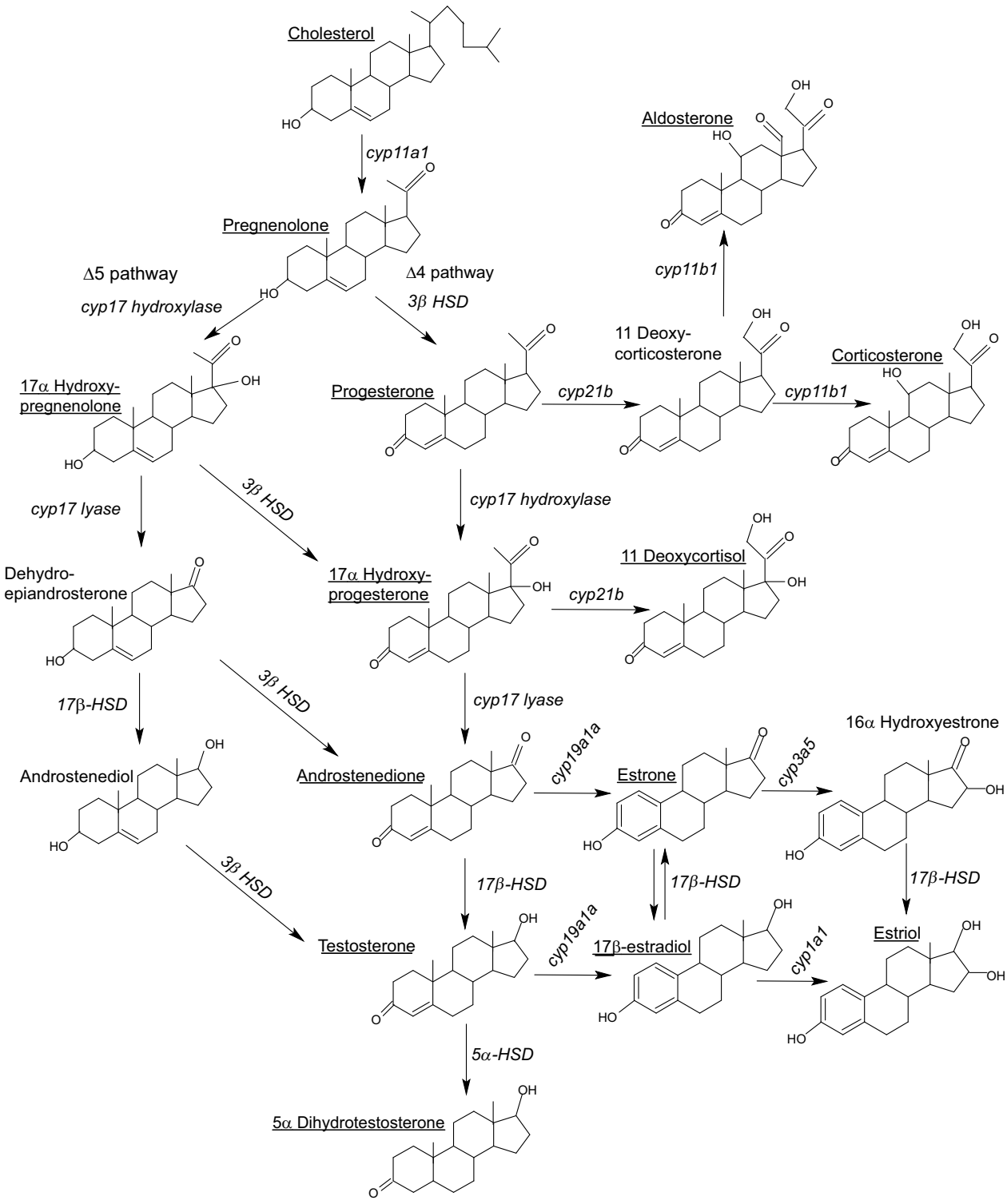

Fig. 2. Illustration of suggested steroidogenic pathway in American alligator. Arrows indicate catalyzed reactions with catalyzing enzymes listed in italics next to arrows. Underlined hormones were quantified in the current study.

Cessation of feeding in saline environments has previously been reported for juvenile alligators (Lauren, 1985; Morici, 1996) and FW turtles exposed to seawater for >2 weeks (Bower et al., 2016; Davenport and Ward, 1993). In FW turtles (*Chelodina expansa* and *Emydura macquarii*) exposed to 15‰ for 50 days, cessation of feeding was considered a behavioral response to reduce salt intake in order to further limit dehydration (Bower et al., 2016). It is likely that a similar behavioral response was displayed by alligators in the current study.

Blood plasma biochemistry parameters

SW exposure significantly elevated creatine kinase levels (Table 2). High creatine kinase levels can be responsible for elevated creatine to phosphocreatine conversion (high-energy phosphate depot for

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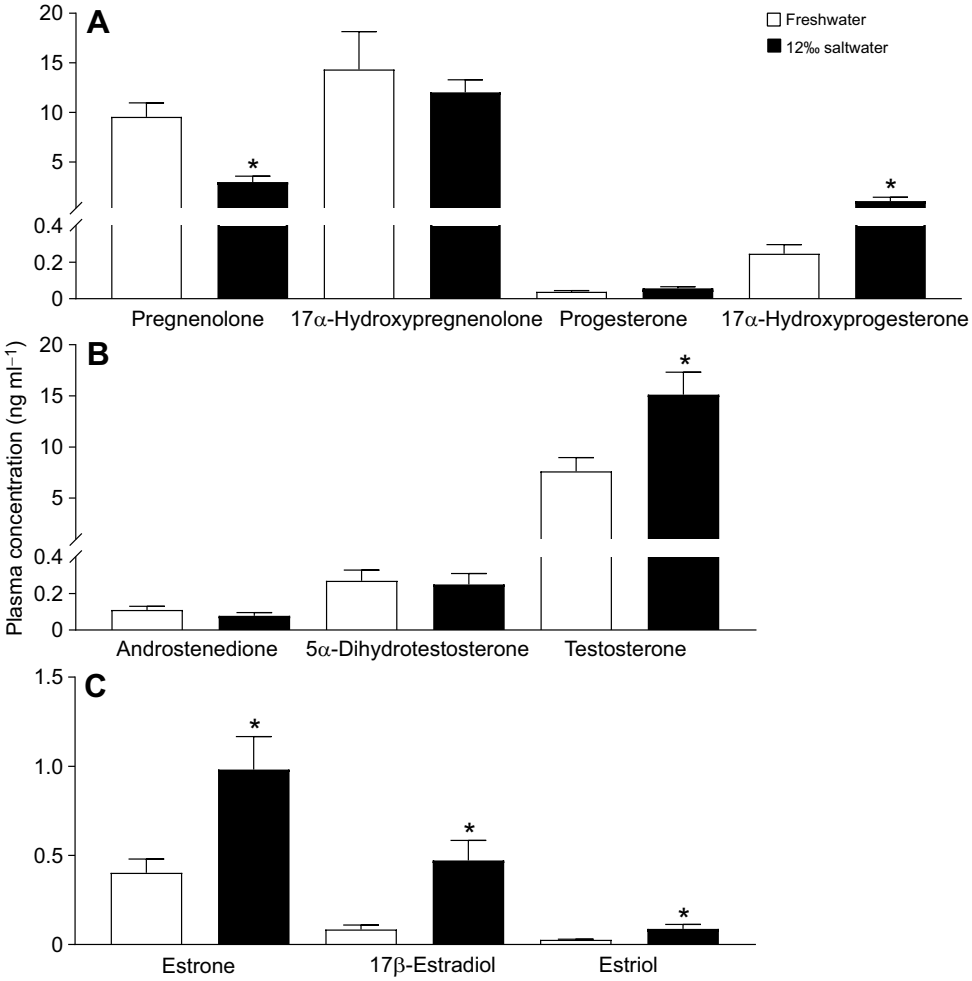

Fig. 3. Plasma sex steroidogenic hormone concentrations (ng ml^{-1}) in juvenile American alligators exposed to freshwater (0‰) or saltwater (12‰) for 5 weeks. Animals were sampled after 5 weeks. (A) Progestogens: pregnenolone, 17α-hydroxypregnenolone, progesterone and 17α-hydroxyprogesterone. (B) Androgens: androstenedione, 5α-dihydrotestosterone and testosterone. (C) Estrogens: estrone, 17β-estradiol and estriol. Data shown are means±s.e.m. (*N*=8). Asterisks denote significant differences ($P \leq 0.05$) between the freshwater and saltwater groups.

ATP recycling), and its subsequent breakdown to creatinine (Wallimann et al., 2011). Therefore, significantly high levels of creatine kinase and creatinine observed in the SW group attest to the elevation of creatine metabolism. In mammals, elevated serum creatine kinase activity is usually attributed to skeletal muscle disease or damage and has been used as a plasma biomarker of myocardial infarction and impaired kidney function (Burtis and Ashwood, 1999). Owing to the osmoregulatory capabilities of alligators (Braun, 1998; Taplin et al., 1982), the kidneys are likely to have been adversely affected by SW exposure in an attempt to compensate for the excess salt load.

AST is an important enzyme in amino acid metabolism (Burtis and Ashwood, 1999). The enzymes AST, ALP and ALT and bilirubin are commonly used in veterinary practices to determine potential liver damage. In particular, AST and ALT and their ratio (AST:ALT) become elevated when disease processes affect liver cell integrity, thus indicating liver damage (Burtis and Ashwood, 1999). Higher levels of AST and ALT as well as high AST:ALT ratio in SW alligators suggest SW compromises liver integrity and has adverse effects on hepatic function.

Dehydration in crocodilians can increase uric acid levels but has only in severe cases been reported to cause renal failure (Huchzermeyer, 2003). Because of the low solubility of uric acid, accumulation can trigger the formation and deposition of uric acid crystals in organs. Salinity acclimated alligators started to eliminate white precipitate (uric acid) after 1 week of exposure (P.C.F., personal observations). Uric acid crystals could thereafter be seen at

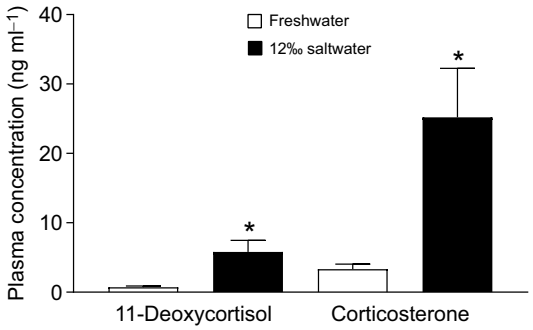

Fig. 4. Plasma 11-deoyxcortisol and corticosterone concentrations (ng ml^{-1}) in juvenile American alligators exposed to freshwater (0‰) or saltwater (12‰) for 5 weeks. Animals were sampled after 5 weeks. Data shown are means±s.e.m. (*N*=8). Asterisks denote significant differences ($P \leq 0.05$) between the freshwater and saltwater groups.

Table 3. Ratio of estrogens to androgens in freshwater- and saltwater-acclimated juvenile American alligators

	Freshwater	Saltwater
Estrone:androstenedione	3.12±0.18*	27.92±11.09
17β-Estradiol:testosterone	0.02±0.007	0.04±0.02

*Significant difference ($P<0.05$) between the freshwater and saltwater groups.

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Table 4. Morphometrics of lingual glands in American alligators exposed to either freshwater (0‰) or saltwater (12‰) for 5 weeks

	Freshwater	Saltwater	*P*-value
Horizontal sections (mm)‡			
Gland area	481.52±61.96	506.67±105.89	0.86
Maximum width	0.91±0.07	0.87±0.06	0.75
Minimum width	0.64±0.05	0.61±0.05	0.66
Pore width	0.08±0.01	0.10±0.02	0.24
Cross-sections (mm)§			
Gland area	211.12±45.12	238.34±19.27	0.61
Gland width	0.53±0.06	0.61±0.04	0.28
Gland height	0.43±0.06	0.52±0.03	0.25
Pore length	0.25±0.05*	0.55±0.05	0.001

*Significant difference (P<0.05) between the freshwater and saltwater groups. N=4 for FW, N=5 for SW.
‡Nine slides for horizontal sections. §Thirteen slides for cross-sections.

the bottom of tanks for the remainder of the exposure period (P.C.F., personal observations). These observations correlate well with the measured increase in uric acid plasma levels after 5 weeks in 12‰ (Table 2). Elimination of uric acid is a mechanism to conserve water while excreting nitrogenous waste and has previously been reported in salinity-exposed (5–20‰) juvenile alligators (Lauren, 1985). High corticosterone (CORT) levels could further have contributed to uric acid production as injection of cortisol into alligators resulted in increased uric acid synthesis and excretion (Coulson and Hernandez, 1959).

Finally, SW-exposed juvenile alligators had significantly raised plasma Na^+ (by ∼36%) and Cl^- levels (by ∼50%) after 5 weeks in 12‰. Correspondingly, Lauren (Lauren, 1985) recorded ∼30% increase in Na^+ at 15‰ while plasma Cl^- levels were elevated by almost 79% at 15‰ after 4 weeks. Comparably, chronic exposure (50 days) of two Australian FW turtles (*Chelodina expansa* and *Emydura macquarii*) to 15‰ increased Na^+ levels 42% and 50%, respectively, and Cl^- levels 65% and 78%, respectively (Bower et al., 2016). Reptile kidney nephrons do not possess a Loop of Henle (Braun, 1998; Willmer et al., 2009) and hence are unable to excrete hyperosmotic urine. Furthermore, although only a few studies have determined alligatorid integumental permeability to Na^+, there is evidence of Na^+ influx and efflux across the integument when alligators are in FW (Ellis and Evans, 1984; Taplin, 1988). Data further show influx of Na^+ across the integument when alligators are exposed to 35‰ for a few hours (Mazzotti and Dunson, 1984). Given the lack of functional salt glands in alligators, the higher plasma Na^+ in SW alligators was likely due to several factors such as continuous Na^+ influx across the integument and/or mucus membranes and inability of kidneys to excrete hyperosmotic urine. Although speculative, chloride ions potentially passively followed Na^+ influx causing the increased plasma Cl^- concentration. Further studies on the mechanisms of electrolyte transport across the integument are, however, needed. Interestingly, K^+ levels remained unchanged in our alligators and in salinity-exposed FW turtles (Bower et al., 2016), which suggests activation of ALDO, which stimulates increased renal excretion of K^+.

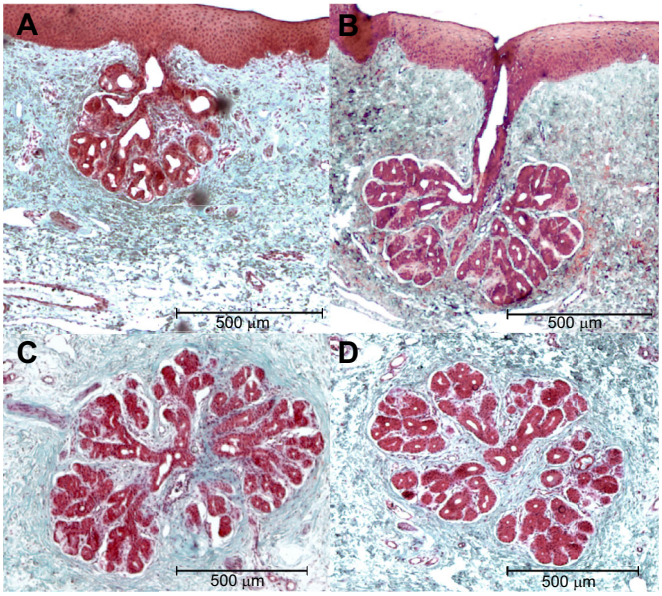

Fig. 5. Micrographs of alligator lingual glands. Left panels show pictures of tongues from freshwater-maintained juvenile alligators: (A) cross-section, (C) horizontal. Right panels show pictures of tongues from chronically (5 weeks) saltwater-exposed (12‰) juvenile alligators: (B) cross-section, (D) horizontal.

RAAS and lingual glands

To the best of our knowledge, this is the first study to determine ANG II levels in salinity-stressed alligators. Generally, very few studies have determined ANG II levels in reptiles but extensive studies exist on the effects of dehydration and hyperosmotic conditions in amphibians and fishes (Johnson et al., 2010; Tierney et al., 1995; Uchiyama et al., 2014). One objective of this study was to obtain true values of ANG II and ALDO in juvenile alligators following chronic exposure to SW. ANG II and ALDO levels are highly dependent on hemodynamic factors such as blood volume, blood pressure and hematocrit, all of which can be decreased by repeated blood sampling (Walsh et al., 1980). To avoid repeated blood sampling potentially masking any direct effects of salinity treatment on hormone levels, we therefore only assessed ANG II and ALDO after 5 weeks of exposure and compared values with those of a FW control group. Five weeks at 12‰ significantly decreased plasma ANG II levels in juvenile alligators, which rejects our research hypothesis of increased ANG II levels in severely dehydrated, salinity exposed alligators. ANG II has its own, independent from ALDO, Na^+ reabsorptive effects on the kidneys (Fournier et al., 2012). We therefore determined gene expression of the AT-1 receptor in the kidneys of FW and SW alligators to assess any genomic differences as a result of SW exposure. However, contrary to the reduction in plasma hormone levels, there was no significant difference in AT-1 expression (Fig. S1A). This finding suggests that gene expression changes may be transient as demonstrated for the glucose transporter gene in chronic hypoxic cod (Hall et al., 2009) or that effects on ANG II are non-genomic or post-translational. ANG II regulates blood pressure by increasing renal tubular Na^+ reabsorption in exchange for renal K^+ excretion. Sodium retention causes water to follow passively, leading to overall water retention and restoration of blood volume and hence blood pressure (Li and Zhuo, 2015). The significantly lower plasma ANG II levels in SW alligators therefore suggest strong negative feedback mechanisms acting at all or some major components of RAAS. Normally excess water loss will concentrate plasma Na^+ levels, and thereby stimulate ANG II secretion to restore plasma volume. However, exposure to SW seems to disrupt the normal function of RAAS by preventing Na^+ reabsorption in kidneys and resorption of water. Whether this was an active regulatory mechanism in an attempt to suppress Na^+ reuptake in an already Na^+-loaded animal is not known. As further studies are needed to determine these feedback mechanisms, we can only speculate as to

the site of inhibition. However, the enzymes renin and ACE both play significant roles in RAAS with renin being the rate-limiting enzyme. Renin catalyzes the conversion of angiotensinogen to ANG I and is stimulated by intrarenal baroreceptors that sense changes in renal arterial pressures at or near juxtaglomerular (JG) cells. Although non-mammalian vertebrate kidneys do not possess JG or have rudimentary JG, there are functional baroreceptor mechanisms in fish [e.g. toadfish (*Opsanus tau*) (Nishimura et al., 1979), reptiles (turtle, *Pseudemys scripta*) (Stephens and Creekmore, 1984) and birds (Nishimura, 2017)]. Indeed, in common slider turtles (*Pseudemys scripta*), barostatic mechanisms were responsible for renin release following hypotension caused by hemorrhage (Stephens and Creekmore, 1984). Reduction in blood volume (e.g. excess water loss) would have decreased renal arterial blood pressure and stimulate renin release. Therefore, it is likely that other negative feedback mechanisms exist. For instance, studies on rats show a negative correlation between renin and hepatic angiotensinogen production (Herrmann and Dzau, 1983). Continuous stimulation of renin could have suppressed angiotensinogen levels in the liver decreasing substrate availability for renin. However, the other key enzyme in RAAS is ACE, which converts the inactive ANG I to the biologically active ANG II (Wilson, 1984). It was only recently discovered in humans that serum albumin levels are endogenous ACE inhibitors as seen by negative correlation with plasma ACE levels (Fagyas et al., 2014). The significantly elevated albumin levels in SW alligators may therefore have contributed to an ACE inhibition and hence reduced ANG II levels.

Very few studies have investigated ALDO in salinity stressed or salt-loaded reptiles. For instance, ALDO levels were significantly depressed but CORT was elevated in salt-loaded sand goannas (*Varanus gouldii*, Gray) whereas dehydration resulted in elevated ALDO levels (Bradshaw and Rice, 1981). Juvenile Nile crocodiles (*Crocodylus niloticus*) kept in a hypertonic medium for 7 days exhibited a 33% increase in ALDO levels compared with FW-maintained animals (Balment and Loveridge, 1989). Furthermore, juvenile alligators exposed to 4‰ for 1 week doubled ALDO concentration whereas animals exposed to 8‰, 12‰ and 16‰ exhibited a minor increase in ALDO (Morici, 1996). Thus, these findings correspond well with results from the present study in which ALDO levels were not significantly different in SW alligators compared with FW alligators after 5 weeks. ALDO secretion in vertebrates is regulated by ANG II in addition to the adrenocorticotropic hormone (ACTH) and plasma K^+ (Nishimura, 2017). It has previously been demonstrated that ALDO and CORT secretions in alligators are stimulated by ACTH, which is released from the pituitary gland (Lance and Lauren, 1984; Morici, 1996). However, juvenile alligators implanted with CORT tablets had undetectable ALDO levels after 1 week of implants, showing a negative feedback of CORT on ALDO (Morici et al., 1997). The continuously high CORT levels at week 5 in our SW alligators did not seem to have significantly depressed ALDO levels although a reduction was evident between the FW and SW alligators. Indeed, we did not detect a significant correlation between CORT and ALDO in either FW or 12‰-exposed alligators at 5 weeks (Fig. S3B), suggesting long-term exposure to SW does not continuously result in a negative feedback of CORT on ALDO. On the contrary, data revealed a correlation between plasma K^+ and ALDO in SW alligators but not in FW alligators (Fig. S2F). Interestingly there was no correlation between ANG II and ALDO levels in either FW or SW alligators after 5 weeks (Fig. S3C). The lack of correlation between ANG II and ALDO in SW alligators therefore explains why ALDO levels in SW alligators did not mirror the significantly lower ANG II levels. Data from the present study therefore demonstrate ALDO secretion in salinity stressed alligators is tightly regulated by plasma K^+ levels and to a lesser extent ANG II or CORT.

ALDO plays a significant role in renal Na^+ retention and K^+ secretion in mammals and acts with high affinity on the MR (Kubzansky and Adler, 2010). The MR has recently been molecularly characterized in alligators (Oka et al., 2013), and we therefore determined gene expression of MR in the kidneys of FW and SW alligators. As MR equally binds the glucocorticoid CORT, we additionally determined gene expression of the GR, which selectively binds CORT (Funder, 2017). Consistent with plasma ALDO data, there were no significant differences in the number of RNA copies of kidney MR between acclimation groups (Fig. S1B). However, kidney GR gene expression data did not correspond with plasma CORT levels as no significant difference was quantified in GR gene expression between treatment groups (Fig. S1C). It is unknown why receptor gene expression data did not match plasma hormone levels. However, as described for AT-1 mRNA expression, gene expression of MR and GR were either transient or were not reflected post-translationally. In general, gene expression may not always be an accurate indicator of changes at the receptor/protein level and should therefore be used as suggestions rather than evidence for changes at the protein level.

This study shows that juvenile alligators do not appear to have osmoregulatory abilities to cope with even brackish (12‰) saline environments for extended periods of time. It has previously been argued that lingual glands in alligators would never become functional salt glands in saline environments (Taplin, 1988). To test this hypothesis, we studied histological sections of lingual glands in FW and SW alligators.

Chronic exposure to SW did not significantly change the overall morphology of lingual glands. The only parameter altered between FW and SW alligators was a longer length of the pore from the surface of the tongue to the glands (Fig. 5, Table 4). Alligatorids possess basic glands and, although these are more numerous, the glands are smaller and lack the complex lobulated structure and size seen in crocodylids (Taplin, 1982; Taplin and Grigg, 1981; Taplin et al., 1982). Taplin (1988) described mucous secretory droplets filling the cytoplasm, which corresponds well with observations in glands from FW alligators. The lingual glands in this study appeared filled with secretions as evidenced by wide open areas in the glands in cross- and horizontal sections (Fig. 5). Although there was no significant difference in glandular morphometrics (except pore length), histological sections of glands in SW alligators appeared to have fewer open areas (Fig. 5). The morphological changes in these glands from SW alligators were likely due to the severe dehydration. Whether that reduced salivary secretion rate is unknown, but data from this study support that lingual glands in juvenile alligators do not become functional salt glands in saline environments. Results contrast findings from SW-acclimated *C. porosus* where increases in secretory tubule size, increased mitochrondrial numbers and plasma membrane surface area were correlated with increased functional activity of lingual salt glands (Cramp et al., 2007, 2008).

Steroid hormones

To the best of our knowledge, this is the first steroidogenic pathway constructed for alligators based on multiple plasma steroid hormones. The pathway was created from previously suggested pathways in alligators (Guillette et al., 2007). However, with

increasingly sensitive analytical techniques, we are now able to detect and quantify multiple steroid hormones in small plasma samples, which enabled a more detailed pathway to be constructed.

The suggested pathway further enabled assessment of the effect of salinity on corticoid production. For instance, the concomitant elevation of 17α-hydroxyprogesterone and decrease (albeit non-significant) of 17α-hydroxypregnenolone suggests an increased demand for corticoid production, which may be indicative of a higher demand for the production of hormones capable of metabolic adaptation to stress. For example, high corticoid levels (cortisol, cortisone, corticosterone) can induce gluconeogenesis (via induction of phosphoenolpyruvate carboxykinase) or hydrolysis of triglycerides (via induction of lipoprotein lipase). The inductions of these enzymes can help maintain or even elevate glucose or fatty acid levels, fueling increased metabolic demands under stress (Mommsen et al., 1999; Morton, 2010). The significantly increased CORT levels but significantly lower plasma glucose levels in SW alligators could suggest that enhanced glucose production is fueling ATP-demanding processes (i.e. energy-demanding ion channels/transporters).

Following Hurricane Rita, which affected Louisiana, USA, in 2005, wild alligators showed a clear positive correlation between plasma CORT and osmolality (r^2=0.74) and plasma Na^+ (r^2=0.92) (Lance et al., 2010). Comparable to salinity-stressed wild alligators, data from the present study clearly show long-term exposure to SW results in chronic stress in juvenile alligators. Long-term elevated CORT levels have several negative physiological effects in alligators, such as reduced growth, significantly lower white blood cell count and a significantly higher heterophil:lymphocyte ratio (Morici et al., 1997). Thus, salinity exposed young alligators are at higher risk of infections and diseases due to a suppressed immune system and are further vulnerable to predators if growth rate is stunted.

Sex steroid hormones are key drivers for growth and reproduction in the American alligator (Guillette et al., 1997). While most studies have investigated the adverse effects of anthropogenic EDCs on endocrine or steroidogenic effects in alligators (Crain et al., 1997, 1998; Guillette et al., 1994; Vonier et al., 1996), to the best of our knowledge, this is the first study to demonstrate significant effects of an environmental stressor (SW) on juvenile alligator steroid hormones. We hypothesized that a natural stressor such as salinity would decrease sex steroid hormone levels due to high CORT levels but data revealed significant increases in androgens, progestogens and estrogens.

Cholesterol is the pre-cursor for sex steroid hormones and levels of cholesterol were doubled in SW alligators compared with their FW controls (Table 2). As food intake was significantly reduced, elevated cholesterol levels were not of immediate dietary origin. The high cholesterol levels suggest either enhanced β-oxidation of fatty acids to fuel acetyl-CoA availability for cholesterol biosynthesis (Berg et al., 2002) or mobilization of internal cholesteryl esters from lipid reserves (Hu et al., 2010). The significantly elevated cholesterol levels in SW-exposed alligators did not result in elevated levels of pregnenolone, which was significantly lower in SW-exposed alligators.

It is interesting to note that 17α-hydroxypregnenolone levels were almost two orders of magnitude higher than progesterone levels in both FW- and SW-exposed alligators (Figs 2 and 3). These data are highly suggestive of higher pregnenolone catalysis via the Δ5 versus Δ4 steroidogenic pathways (Fig. 2). Such differential metabolism of pregnenolone to 17α-hydroxypregnenolone (via cyp71-hydroxylase) or progesterone (via 3β-HSD) is expected to 'shunt' steroidogenesis through mainly androgen synthesis pathways (Δ5 shunt) versus progestogen synthesis pathways (Δ4 shunt) (Conley and Bird, 1997). The functional relevance of distributing steroidogenesis through alternative pathways is largely unexplored but has been shown to be differentially utilized during pubertal development and oocyte maturation in male and female catfish (*Clarias gariepinus*), respectively (Cavaco et al., 1997; Sreenivasulu and Senthilkumaran, 2009).

SW-exposed alligators also exhibited increased production of testosterone. Testosterone levels in alligators have previously been shown to negatively correlate with plasma CORT levels under stress conditions (capture/handling stress) in sexually mature alligators (Elsey et al., 1991; Lance and Elsey, 1986). However, the concomitant elevations of 17α-hydroxyprogesterone, 11-deoxycortisol, CORT and testosterone in SW-exposed alligators suggest differences between sexually mature and immature alligators with respect to the effect of CORT on testosterone and estradiol production. The increased testosterone levels under SW exposure may be responsible for driving elevated estrogen levels for 17β-estradiol, estrone and estriol. Conversion of androstenedione and testosterone to estrone and 17β-estradiol is catalyzed by the enzyme cyp19a1a (or aromatase) (Fig. 2). While there were no effects of SW exposure on androstenedione levels, testosterone levels were significantly elevated. Therefore, it appears that elevated estrogen levels are exclusively due to elevated testosterone levels.

Although all animals were male, the sex ratio was not affected by salinity as all animals were 1–2 years old at the time of experimentation. Circulating levels of estradiol in juvenile males have been reported in the range 0.01–0.06 ng ml^{-1} (Crain et al., 1997, 1998; Guillette et al., 1994, 1997, 1999; Milnes et al., 2002) and as low as 0.29–3.14 pg ml^{-1} (Lance et al., 2003). This range corresponds well with levels quantified in our FW male juvenile alligators in the present study (mean 0.08±0.07 ng ml^{-1}). Thus, although low, with increasingly sensitive analytical methods, we are able to detect low resting circulating levels of estradiol in male juvenile alligators. Determination of the estrogen:androgen ratio is informative of skewed sex hormone ratios in alligators. For instance, alligators exposed to various anthropogenic compounds exhibited altered estrogen:androgen (E_2:T) ratios (Crain et al., 1997; Guillette et al., 1994, 1995). Interestingly, the E_2:T ratio in juvenile alligators from the contaminated Lake Apopka was significantly higher in both male and female juveniles (Guillette et al., 1994). Comparably, the estrogen:androgen ratio for estrone:androstenedione increased 9× in SW alligators compared with FW animals whereas a non-significant increase in estradiol:testosterone was determined. Thus, increased salinity had a similar effects on the estrogen:androgen ratio as contaminant exposure. At this point, the exact role of estrogens in juvenile male alligators remains to be determined.

In summary, our data demonstrate novel and significant effects of a natural stressor (salinity) on the endocrine system in juvenile alligators. In addition, significantly elevated levels of most blood biochemistry parameters including those diagnostic of impaired hepatic and renal function suggest adverse effects of SW. This study has evoked several questions as to the underlying mechanisms attributing to the reported effects on plasma hormones and plasma biochemistry parameters. With changing climate and increased risk of coastal flooding it is imperative to further assess physiological changes in juvenile alligators.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.L.B., L.H.P.; Methodology: P.C.F., M.L.B., L.S., C.D.M., D.H., L.H.P.; Validation: P.C.F., D.H., L.H.P.; Formal analysis: P.C.F., L.S., C.D.M., D.H., L.H.P.; Investigation: P.C.F., L.H.P.; Resources: D.H., L.H.P.; Data curation: P.C.F., L.S., C.D.M.; Writing - original draft: P.C.F., L.H.P.; Writing - review & editing: P.C.F., M.L.B., C.D.M., D.H., L.H.P.; Visualization: M.L.B., L.H.P.; Supervision: D.H., L.H.P.; Project administration: P.C.F., D.H., L.H.P.; Funding acquisition: D.H., L.H.P.

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Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.181172.supplemental

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