PHYSIOLOGICAL AND BEHAVIORAL EFFECTS OF SALT STRESS ON JUVENILE AMERICAN ALLIGATORS (*Alligator mississippiensis*)

A Dissertation

by

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ABSTRACT

American alligators (*Alligator mississippiensis*) inhabit freshwater wetlands but are vulnerable to salt stress during storm surges and droughts. Anthropogenic alterations to freshwater system hydrology, runoff of coal mine spoils, and road de-icing using salt can further contribute to salinization of freshwater habitats. Juvenile alligators are especially vulnerable to salt stress due to their thinner integument and lower mobility and ability to avoid saltwater. Little is known about how crocodilian physiological systems respond to environmental stressors such as salinity.

To better understand the effects of saltwater on alligators, juvenile alligators were exposed to 12‰ saltwater for 5-week and 1-week periods and blood plasma biochemistry, components of the renin-angiotensin-aldosterone system, steroidogenesis, and behavior were assessed. Furthermore, to correlate findings in laboratory-based studies with conditions in the wild, juvenile male and female wild alligators in various salinities were opportunistically sampled each month excluding November, December, and January for one year at Rockefeller Wildlife Refuge in coastal Louisiana.

Compared to freshwater-kept alligators, 5-week exposure to 12‰ saltwater significantly elevated plasma corticosterone, 11-deoxycortisol, 17α-hydroxyprogesterone, testosterone, estrone, 17β-estradiol, and estriol. Conversely, alligators exposed to 12‰ saltwater for 1 week had significantly reduced estrone and 17β-estradiol, while corticosterone and 11-deoxycortisol were elevated and histology showed alterations in gonad tissues. Additionally, the progestogen 17α,20β-dihydroxypregnenone was

significantly, positively correlated with environmental salinities wild juvenile male alligators were found in. Angiotensin II was significantly reduced after 5- and 1-week exposure, which correlated with low renin and angiotensin-converting enzyme expression in kidney and lung tissues of alligators exposed for 1 week. Na^+ and Cl^- were significantly elevated after 5- and 1-week exposure, which corresponded well with Na^+ and Cl^- being strongly, positively correlated with environmental salinities wild juvenile male alligators were found in. Additionally, saltwater-exposed alligators largely ceased feeding within 1 week and spent significantly less time basking compared with pre-salinity observations.

This dissertation demonstrates behavioral changes and time-dependent, dynamic changes in physiology of juvenile alligator exposed to saltwater. This work further shows significant correlation of environmental salinity with electrolyte levels and a sex steroid in wild juvenile alligators, and to my knowledge represents the first measurement of 17α,20β-dihydroxypregnenone in alligators.

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NOMENCLATURE

3β-HSD	3β-Hydroxysteroid Dehydrogenase
5α-HSD	5α-Hydroxysteroid Dehydrogenase
11-DC	11-Deoxycortisol
17-OHP4	17α-hydroxyprogesterone
17-OHP5	17α-hydroxypregnenolone
17β-HSD	17β-Hydroxysteroid Dehydrogenase
A4	Androstenedione
ACE	Angiotensin-Converting Enzyme
ACTH	Adrenocorticotropic Hormone
ALDO	Aldosterone
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AMH	Anti-Müllerian Hormone
ANG I	Angiotensin I
ANG II	Angiotensin II
ANOVA	Analysis of Variance
AST	Aspartate Aminotransferase
AT-1	Angiotensin II Type 1 Receptor
AVP	Arginine Vasopressin
AVT	Arginine Vasotocin

°C	Degrees Celsius
CA	Catecholamine
Ca^{2+}	Calcium
CK	Creatine Kinase
Cl^-	Chloride
cm	Centimeter
CORT	Corticosterone
CYP	Cytochrome
cyp11b1	Steroid 11β-Hydroxylase
cyp19a1a	Aromatase
cyp21b	Steroid 21-Hydroxylase
CWAF	Corexit-Enhanced Water-Accommodated Fraction of Crude Oil
DAB	3’3-Diaminobenzidine
DDT	Dichlorodiphenyltrichloroethane
DHP	17α,20β-Dihydroxypregnenone
DHT	5α-Dihydrotestosterone
Dns	Dansyl
ESI+	Positive Electrospray Ionization
E_1	Estrone
E_2	17β-Estradiol
E_3	Estriol
FSH	Follicle-Stimulating Hormone

FW	Freshwater
× g	Times Gravity
g	Gram
GAM	Gonad-Adrenal Mesonephric
g h^{-1}	Grams per Hour
GnRH	Gonadotropin-Releasing Hormone
GR	Glucocorticoid Receptor
h	Hour
Hct	Hematocrit
HPG	Hypothalamic-Pituitary-Gonadal
HSI	Hepatic-Somatic Index
IPCC	International Panel on Climate Change
IR	Immunoreactive
JG	Juxtaglomerular
K	Fulton's Condition Factor
km	Kilometer
kV	Kilovolts
K^+	Potassium
L	Liter
LC-MS/MS	Liquid Chromatography and Tandem Mass Spectrometry
LH	Luteinizing Hormone
L min^{-1}	Liters per Minute

m	Meter
mg dl^{-1}	Milligrams per Deciliter
mg ml^{-1}	Milligrams per Milliliter
min	Minute
ml	Milliliter
ml 100 g^{-1} h^{-1}	Milliliters per 100 Grams per Hour
mm	Millimeter
mM	Millimolar
mmol L^{-1}	Millimoles per Liter
MR	Mineralocorticoid Receptor
MRM	Multiple Reaction Monitoring
MTBE	Methyl Tert-Butyl Ether
m/z	Mass-to-Charge Ratio
Na^{+}	Sodium
NaCl	Sodium Chloride
ng ml^{-1}	Nanograms per Milliliter
ng μl^{-1}	Nanograms per Microliter
P	Phosphorus
P4	Progesterone
P5	Pregnenolone
P450scc	Cholesterol Side-Chain Cleavage Enzyme
PBS	Phosphate Buffered Saline

PBT	Preferred Body Temperature
PCA	Principal Component Analysis
p,p'-DDE	Dichlorodiphenyl dichloroethylene
pRDA	Partial Redundancy Analysis
RAS	Renin-Angiotensin System
RAAS	Renin-Angiotensin-Aldosterone System
RT-qPCR	Real-Time Quantitative Polymerase Chain Reaction
s.e.m.	Standard Error of the Mean
SNS	Sympathetic Nervous System
SVL	Snout-Vent Length
SW	Saltwater
T	Testosterone
TAMUG	Texas A&M University at Galveston
UHPLC	Ultra-High Performance Liquid Chromatography
$U\ L^{-1}$	Units per Liter
UVB	Ultraviolet B
W	Watt
β-AR	β-Adrenergic Receptor
µl	Microliter
µm	Micrometer
µmol	Micromole
$\mu mol\ 100\ g^{-1}\ h^{-1}$	Micromoles per 100 Grams per Hour

CHAPTER I

INTRODUCTION

1.1. Salinization of Habitats Along the Gulf of Mexico

Gulf of Mexico coastal habitats are frequently exposed to external factors (e.g., natural or anthropogenic) that can negatively impact near-shore and coastal ecosystems. For instance, many habitats are increasingly being exposed to storm surges (saltwater inundation) (Jackson et al., 1995; Schriever et al., 2009) since hurricane frequency and intensity increases due to elevated sea surface temperature (Emanuel, 1987; Hoyos et al., 2006; O'Brien et al., 1992). Storm surges can cause displacement and widespread mortality of wildlife by introducing saltwater into freshwater habitats (Michener et al., 1997; Schriever et al., 2009). These saltwater intrusions can convert normally freshwater habitats into saline environments lasting up to one year and may be as high as 24‰ (Blood et al., 1991; Chabreck and Palmisano, 1973; Lance et al., 2010). Increased sea surface temperature also contributes to sea level rise, which can introduce saltwater into coastal habitats (Nicholls and Cazenave, 2010; Rahmstorf, 2007; Sheikh, 2005). Sea level has been projected to rise by up to 1.79 meters by 2100, with as much as 3.11×10^5 km^2 of coastal wetlands lost worldwide (Brown et al., 2016; Nicholls and Cazenave, 2010; Rahmstorf, 2007; Vermeer and Rahmstorf, 2009). Furthermore, rivers, lakes, and freshwater wetlands can be exposed to salinization due to anthropogenic perturbations that alter freshwater or underground water flow (Day et al., 2000; Herbert et al., 2015).

1.2. American Alligators as a Species Vulnerable to Salt Stress

American alligators [*Alligator mississippiensis* (Daudin, 1802)] inhabit a wide variety of aquatic habitats in the Gulf coast region (Fig. 1.1) (Joanen and McNease, 1987). Molecular phylogenetic analysis suggests separation or speciation between west (Louisiana, Texas) and east (Florida, Georgia) American alligator populations (Davis et al., 2002). Alligators are commonly found in freshwater or low-salinity habitats including marshes, swamps, rivers, ponds, and lakes, and they may occasionally venture into saline water for brief periods (Joanen and McNease, 1987). American alligators consume a wide variety of prey, with mammals, particularly nutria, being a major component of diet in the southeast United States. Their diet further varies with environmental salinity, comprising more arthropods and fish as salinity rises (McNease and Joanen, 1977). The diet of American alligators also varies ontogenetically; juveniles and subadults rely more on invertebrates than adults in Florida lakes (Delany, 1990; Delany and Abercrombie, 1986).

Age of sexual maturity in American alligators is dependent on body size, as they reach maturity between about 1.8 and 2.2 m. Therefore, in geographic areas with more warm "growing months" such as Louisiana, alligators reach sexual maturity at about 10 years compared with alligators in North Carolina reaching maturity in about 18 years. (Joanen and McNease, 1987; Lance, 1989). Alligators fast between October and March, during which they spend most of their time in holes dug under the banks of ponds. Alligators reemerge around late March or early April and subsequently resume feeding and begin courtship behaviors, coinciding with a seasonal increase in sex steroid hormones (testosterone, 17β-estradiol) in March and April (Rooney et al., 2004). Copulation occurs

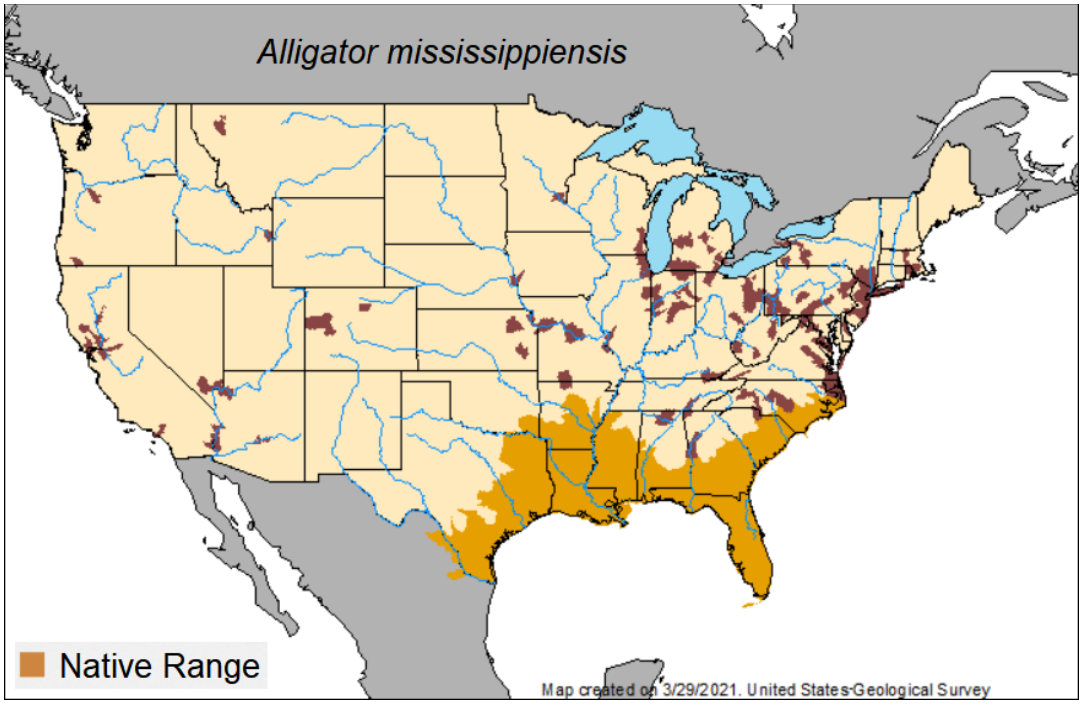

Figure 1.1. The range of *Alligator mississippiensis* (modified from https://nas.er.usgs.gov/).

between late May and early June, and females subsequently nest in June and July (Joanen, 1969; Lance, 1989). At Rockefeller Wildlife Refuge, LA, females nest in marsh water with an average salinity of about 3.8‰ (Joanen, 1969). Clutch sizes range from 2 to 58 with between 30.2% and 68.3% of nests being successful (Deitz and Hines, 1980; Joanen, 1969). Sex of young is determined by incubation temperature where eggs incubated below 30°C hatch as female, while eggs incubated above 34°C hatch as male (Ferguson, 1982). Females guard their nests throughout incubation (Fig. 1.2), and vocalizations from newly hatched young stimulate their mother to open the nest (Lance, 1989).

Figure 1.2. Nest guarded by female *Alligator mississippiensis* (Oberhofer).

1.3. American Alligators as a Keystone Species for Ecosystem Health

American alligators are a keystone species that control populations of lower trophic levels (Mazzotti et al., 2009; Mazzotti and Brandt, 1994). The activities of keystone species greatly influence community composition and stability (Paine, 1969). Furthermore, various other reptile species including mud snakes (*Farancia abacura*), Carolina anole (*Anolis carolinensis*), and various turtles are known to use alligator nests to incubate their eggs (Deitz and Jackson, 1979; Hall and Meier, 1993; Kushlan and Kushlan, 1980). Additionally, alligator-dug holes often hold water, providing refuge for aquatic species during droughts and serving as foraging sites for birds and mammals (Kushlan, 1974; Palmer and Mazzotti, 2004; Somma, 2016). Alligator-dug holes impact community structure by sustaining high species richness of plants and fishes relative to undisturbed marsh (Kushlan, 1974; Palmer and Mazzotti, 2004).

American alligators contribute significantly to the gulf coast economy by creating revenue through hunting, farming, and ecotourism. These activities are estimated to generate $80-90 million annually in Louisiana alone (Fisheries, 2015). Alligator hunting is regulated by many states and takes place in specified areas with limited allowances on harvest (Joanen and McNease, 1987). Alligator conservation requires careful management, making it important to have a comprehensive understanding of their life history and physiology, especially under changing environmental conditions such as climate change and increasingly frequent storm surges.

American alligators have historically been exploited since the 1800's for their hides. For instance, approximately 10,000 alligator skins were taken per year between

1922 and 1926, and skins of larger animals became scarce in the 1950's (Joanen and McNease, 1987). Because of depleted populations, Louisiana closed its season to hunting alligators in 1962 until implementing its first program to manage their sustainable harvest in 1972. In addition, the species was considered endangered in 1973 by the U.S. Fish and Wildlife Service. Due to successful management with strict quotas on numbers hunted, populations have successfully recovered (Joanen and McNease, 1987).

Today, alligators are faced with various anthropogenic and environmental stressors. In addition to hunting, alligators are threatened by aquatic habitat loss due to man-made land and water development (Mazzotti et al., 2009). Alligators can also be exposed to pollution of pesticides from agricultural areas or metals accumulating in aquatic systems (Burger et al., 2000; Guillette et al., 1995). Alligators cannot cope with high salinity for extended periods of time, making them vulnerable to saltwater inundation from storm surges, sea level rise, and alterations to freshwater flow. There is, however, very little information of the physiologically changes that occur in salt-stressed alligators. This information is imperative in order to assess whether alligators are able to live in saline environments.

1.4. Physiological Responses to Salt Loading

1.4.1. Hormonal Control of Osmoregulation in Reptiles

Various hormones of the hypothalamic-pituitary-adrenal axis as well as the renin-angiotensin-aldosterone system (RAAS) play important roles in maintaining salt and water balance in vertebrates. For instance, the RAAS hormone aldosterone in lizards can

promote salt retention in nasal salt glands even during salt stress, but aldosterone doesn't mediate salt absorption in colon and cloaca (Bradshaw and Rice, 1981; Shoemaker et al., 1972; Templeton et al., 1972). Furthermore, desert iguanas (*Dipsosaurus dorsalis*) when adrenalectomized to block secretion of aldosterone experienced increased Na^+ secretion via nasal glands (Templeton et al., 1972). Further, circulating aldosterone is decreased by salt loading in sand goannas (*Varanus gouldii*), but increased by dehydration (Rice, 1982). Aldosterone can also act on kidney tubules of salt-loaded water snakes (*Natrix cyclopion*) to increase Na^+ retention (LeBrie and Elizondo, 1969), however aldosterone had no apparent effect on kidney function of desert iguanas (Bradshaw et al., 1972).

In salt-loaded north African desert lizards (*Uromastix acanthinurus*), aldosterone was also associated with Na^+ retention in salt glands, however aldosterone didn't inhibit K^+ secretion (Bradshaw et al., 1984a). It is noteworthy that herbivorous north African desert lizards have high dietary potassium compared with sodium. Herbivorous desert iguanas also have high K^+ intake during their active season, and their nasal glands are stimulated preferentially by K^+ and Cl^- (Hazard, 2001). Furthermore, aldosterone itself is stimulated by increases in blood K^+ (Davis, 1974; Lumbers, 1999). Aldosterone administered in desert iguanas also increased urine K/Na ratios (Templeton et al., 1972), and increased total Na^+ content while reducing K^+ levels in muscle (Chan et al., 1970). Thus, aldosterone appears to preferentially promote secretion of K^+ in some reptiles and may play a role in maintaining K^+ balance in the body. On the other hand, while not statistically significant, plasma aldosterone was 33% greater in juvenile Nile crocodiles (*Crocodylus niloticus*) exposed to about 35‰ salinity compared with animals in

freshwater (Balment and Loveridge, 1989). However, another major component of RAAS promoting aldosterone, angiotensin II, is reduced by hypersaline NaCl injections in Japanese quail (*Coturnix japonica*) (Takei et al., 1985). Overall, the osmoregulatory role of aldosterone appears to vary among reptile groups.

The hypothalamic-pituitary-adrenal axis can also control osmoregulation in reptiles, often promoting Na^+ secretion. For example, NaCl injection in desert iguanas reduced kidney Na^+ reabsorption while dexamethasone administered to block adrenocorticotropic hormone (ACTH) increased Na^+ absorption in kidneys (Bradshaw et al., 1972). Salt loading in lizards also increases corticosterone levels (Bradshaw and Rice, 1981; Bradshaw et al., 1984b), and salt-loaded sand goannas furthermore exhibit antidiuresis which is blocked by hypothalamic lesions to inhibit ACTH (Bradshaw and Rice, 1981). Further, hypophysectomized desert iguanas had increased water content in plasma and muscle, while treatment with corticosterone prevented this change in water and electrolyte content (Chan et al., 1970). Conversely, adrenalectomized pekin ducks (*Anas platyrhynchos*) did not show change in secretion of NaCl or water conservation compared with sham-operated ducks, suggesting adrenocortical steroids do not primarily regulate salt/water balance in this species (Butler, 1987).

Finally, the antidiuretic hormone arginine vasotocin (AVT) released by the posterior pituitary also mediates reptile osmoregulatory function. AVT is elevated during salt loading and dehydration in sand goannas, and it is associated with antidiuresis resulting from reduced glomerular filtration rate and increased water permeability in kidney tubules (Bradshaw and Rice, 1981). Similarly, juvenile Nile crocodiles exposed to

35‰ saltwater tended to have elevated AVT, although not significantly due to high variability (Balment and Loveridge, 1989). Further, AVT has been implicated in playing a role in mediating salt gland function in green sea turtles (*Chelonia mydas*) by having a transient inhibitory effect during salt loading (Reina and Cooper, 2000).

Overall, the function of osmoregulatory hormones such as aldosterone and corticosterone vary among reptiles, while AVT appears to consistently play a role in salt and water retention. Variability in osmoregulatory function can be associated with life history and dietary intake of ions (Na^+, K^+). However, little is still known about the function of these hormones in alligators.

1.4.2. Cardiovascular Function

The RAAS plays a role in regulating cardiac function in mammals (De Mello and Danser, 2000; Lahera et al., 2006; Lee et al., 1980). For instance, angiotensin II stimulates the sympathetic nervous system (SNS) via the adrenal medulla and sympathetic ganglia (Reid, 1992; Saxena, 1992). The SNS releases neurotransmitters such as catecholamines (CAs) that affect target organs such as heart and blood vessels via β-adrenergic receptors (β-ARs). The RAAS and β-AR pathway is vital to regulating cardiac function in vertebrates, since CAs influence heart function (e.g., contractility) and resistance in blood vessels (e.g., blood pressure) (Harris and Van Petten, 1978; Privitera et al., 1969). Catecholamines elicit similar cardiovascular responses in all embryonic, juvenile, and adult vertebrates (Barry, 1950; Dawes et al., 1957; McCarty et al., 1960) and are associated with heart failure in mammals (Cohn et al., 1984; Engelhardt et al., 1999; Kiuchi et al.,

1993). Furthermore, high salt intake in vertebrates can increase aldosterone levels which are associated with cardiovascular dysfunction including fibrosis, inflammation, and eventually ventricular hypertrophy in mammals (Bollag, 2014; Lahera et al., 2006; Muiesan et al., 2008; Takeda et al., 2000). High levels of glucocorticoids such as corticosterone are further associated with muscle atrophy and dysfunction, as well as fibrosis and diastolic dysfunction in the left ventricle of vertebrates (Hattori et al., 2013). At present, it remains to be investigated whether reptile cardiac function is susceptible to salinity stress through these endocrine systems.

1.4.3. Impacts on Reptile Thermoregulation

Plasma osmolality and hydration status often influence thermoregulatory behavior in reptiles (Bradshaw et al., 2007; Ladyman et al., 2006). For instance, various lizards are known to reduce their preferred body temperature (PBT) during dehydration (Crowley, 1987), hypernatremia (Bradshaw et al., 2007; Dupré and Crawford Jr, 1985a), or hyperkalemia (Smits et al., 1986). While salt-loaded desert iguanas behaviorally reduced body temperature, dehydrating them to 80% initial bodyweight didn't impact thermoregulatory behavior and only slightly raised plasma osmolality (Dupré and Crawford Jr, 1985a). Further, desert lizards (*Sceloporus undulatus*) significantly reduced activity during dehydration (Crowley, 1987). It is noteworthy that hypothalamic lesions blocking AVT secretion prevented impacts on thermoregulation in agamid lizards (*Ctenophorus ornatus*), while subsequently administering AVT repaired the normal reduction in PBT (Bradshaw et al., 2007). Western tiger snakes (*Notechis scutatus*)

similarly lowered PBT when dehydrated (Ladyman and Bradshaw, 2003), and showed elevated circulating AVT while dehydrated or salt-loaded (Ladyman et al., 2006). Furthermore, tiger snakes injected with AVT select significantly lower body temperatures than control snakes despite having no difference in body mass, plasma Na^+, or plasma osmolality (Ladyman et al., 2006). Thus, AVT is strongly implicated in controlling thermoregulatory behavior in salt-loaded reptiles.

Osmotic challenge also affects panting and gaping behaviors in reptiles. Eastern bearded dragons (*Pogona barbatus*) experience elevated panting thresholds when dehydrated, while desert lizards such as Central bearded dragons (*Pogona vitticeps*) show a well-defined dose response of reduced gaping behavior when salt-loaded as well as reduced thermoregulatory shuttling (da Silveira Scarpellini et al., 2015). Further, salt-loading, dehydration, and reducing blood volume by exsanguination increased the temperature threshold to panting in desert iguanas (Dupré and Crawford, 1986; Dupré and Crawford Jr, 1985b). The responses to dehydration and salt-loading in reptiles fulfill a need to minimize evaporative water loss, consequently reducing activity (Bradshaw et al., 2007; Ladyman et al., 2006). This method of water conservation can potentially come at the cost of abandoning important routine activities like foraging or reproductive behavior as in the case of chuckwallas (*Sauromalus obesus*) observed during droughts (Nagy, 1972; Nagy, 1973). Less is currently known about the thermoregulatory response to osmotic stress in other reptile groups.

1.5. Alligator Physiology and its Vulnerability to Salt Stress

1.5.1 Effects of Salt Stress on Osmoregulation and the RAAS Endocrine System

Order Crocodilia contains the well-known families Crocodylidae (crocodiles) and Alligatoridae (alligators and caimans). Crocodiles are tolerant of high salinities, since they osmoregulate by excreting excess salt via buccal mucosa, the cloaca, and lingual salt glands. These lingual glands account for about 55% of sodium efflux in saltwater crocodiles (*Crocodylus porosus*) (Taplin, 1985). Unlike crocodiles, American alligators (referred to as "alligators" from this point on) are vulnerable to high salinity because their salt glands are underdeveloped and incapable of producing hypersaline solutions (Taplin et al., 1982). Thus, alligators are largely restricted to low salinity habitats and are susceptible to salt stress. Juveniles are especially at risk due to their smaller size and thinner skin, which allows rapid loss of bodily fluid, causing osmotic shock (Lance et al., 2010; Taplin, 1988). Furthermore, saltwater inundation brought on by storm surges have caused mass mortality in juvenile alligators (Laurén, 1985). Since frequency and intensity of storm surges are projected to increase, there is concern that mortalities of these animals will occur more frequently. Further, the hurricane season in the Southeastern United States occurs between June and November while the largest hurricanes tend to form in August and September (Curtis, 2008; Saunders and Lea, 2005). This hurricane season coincides with the hatching season of American alligator eggs during July and August (Joanen, 1969; Lance, 1989), leaving alligator hatchlings vulnerable to storm surges. Because alligators are important keystone predators, effects on alligator populations can have ecosystem-level impacts.

Adverse effects of salinity stress in juvenile alligators include elevation of stress hormone levels (corticosterone, catecholamines) and changes in electrolyte (Na^+/K^+) balance (Faulkner et al., 2018; Faulkner et al., 2019; Laurén, 1985; Morici, 1996). Changes in electrolyte balance as well as decreased renal blood flow rate stimulate RAAS, which includes biologically active hormones angiotensin II and aldosterone, both of which regulate blood pressure (Fig. 1.3) (De Mello and Danser, 2000; Lahera et al., 2006; Lee et al., 1980). Angiotensinogen secreted by the liver is converted to angiotensin I by renin from the kidneys, and subsequently to angiotensin II by angiotensin-converting enzyme from lung tissue. Finally, angiotensin II stimulates the adrenal cortex to synthesize aldosterone (Fig. 1.3).

Both angiotensin II and aldosterone stimulate kidney tubules to increase the active uptake of Na^+ ions from filtrate, allowing water in the collecting duct to passively follow the resulting osmotic gradient and become reabsorbed to increase blood volume (Ichikawa and Harris, 1991). Angiotensin II further acts to promote the release of arginine vasopressin (AVP) from the posterior pituitary of mammals (Ramsay et al., 1978). AVP stimulates cells of the kidney's collecting ducts to increase the number of aquaporins along collecting duct membranes, thus increasing their water permeability and the osmotic reabsorption of water. Arginine vasotocin (AVT) found in non-mammalian vertebrates is homologous to mammalian AVP and is similarly important in osmoregulation, but the role of angiotensin II in AVT secretion is not as well-understood (Balment and Loveridge, 1989; Gray and Erasmus, 1989; Konno et al., 2005). In addition to its effects on kidney function, angiotensin II stimulates the sympathetic nervous system (SNS) via the adrenal

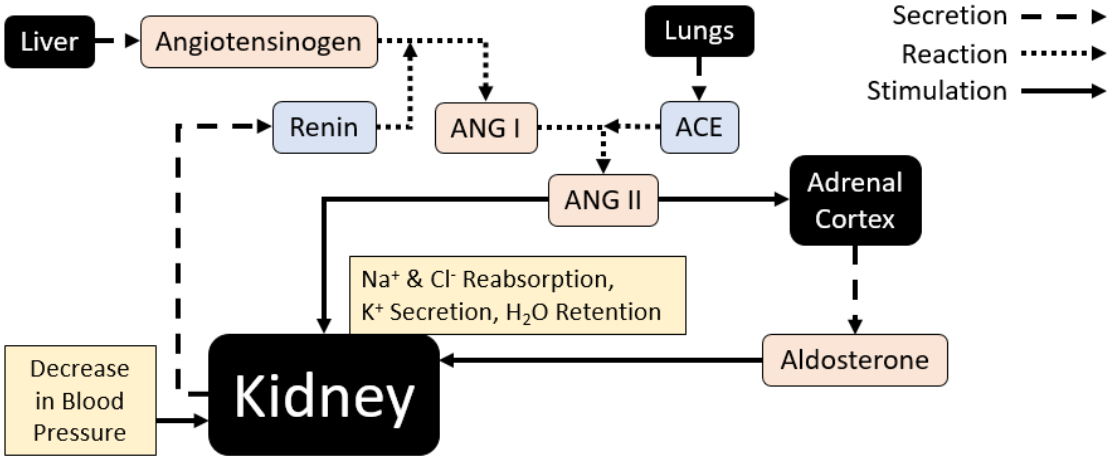

Figure 1.3. Components of the Renin-Angiotensin-Aldosterone System (RAAS) acting on osmoregulation. Abbreviated are angiotensin I (ANG I), angiotensin II (ANG II), and angiotensin-converting enzyme (ACE).

medulla and sympathetic ganglia (Reid, 1992; Saxena, 1992). The SNS subsequently stimulates the release of catecholamines including epinephrine and norepinephrine which promote vasoconstriction to increase blood pressure. Angiotensin II itself similarly has a direct pressor effect on arterioles (Nishimura, 2016). Collectively, RAAS is an important endocrine system which enables animals to respond to changes in the environment. Although we know alligators have a functional RAAS which is activated during salinity stress (Laurén, 1985; Morici, 1996), the detailed function of RAAS in salinity stressed alligators is still not fully understood. For instance, is RAAS equally activated during short- versus long-term saltwater exposure? And do all hormones of RAAS have similar functions as seen in other vertebrates?

1.5.2. The Role of Steroid Hormones in Regulating Alligator Endocrinology

Steroid hormones play crucial roles in sexual development, reproduction, metabolism, behavior, stress response, and physiological homeostasis (e.g., water/salt balance) (Ali et al., 2018; Bremer and Miller, 2014; Hughes, 2001; Lee et al., 2015; Mattsson and Olsson, 2007; Morton, 2010; Sinisi et al., 2003). Thus, sex steroids are commonly used as diagnostic biomarkers of physiological health (Ding et al., 2007; Naessen et al., 2010; Walther et al., 2016) and reproductive status (Orlando and Guillette, 2007; Tyler et al., 1998). Quantification of steroids can further aid in identifying the endocrine disrupting effects of anthropogenic (chemical contaminants) or environmental stressors (hypoxia, salinity) (Thibaut and Porte, 2004; Thomas et al., 2006). For example, endocrine-

disrupting pollutants are known to impact sex steroid levels and sexual development in juvenile alligators (Guillette et al., 1995; Guillette et al., 1994).

Steroid hormones are synthesized through steroidogenesis, which comprises a series of biochemical enzymatic pathways. The major steroidogenic pathways in vertebrates are the adrenal and gonadal pathways with the adrenal pathway located in the adrenal glands producing the glucocorticoids 11-deoxycortisol and corticosterone and the mineralocorticoid aldosterone. Glucocorticoids are involved in the stress response of the animal while aldosterone regulates salt/water balance as a component of RAAS. The gonadal pathway, located in the testes and ovaries, produce the sex steroid hormone classes androgens (androstenedione, testosterone, 5α-dihydrotestosterone), progestogens (pregnenolone, progesterone, 17α-hydroxyprogesterone, 17α-hydroxypregnenolone), and estrogens (17β-estradiol, estrone, estriol). These three hormone classes regulate sexual maturity, secondary sexual characteristics, and reproduction. Since steroid hormones are produced by a series of enzymatic catalyzed reactions, all steroid hormones, regardless of adrenal or gonadal origin, are derived from cholesterol and progestogens (Bremer and Miller, 2014; Payne and Hales, 2004). By measuring plasma steroid hormone levels, a comprehensive picture of the activity and interaction of endocrine pathways involved in stress response, salt/water balance, and reproduction can be obtained. While it is understood salinity stress in juvenile alligators elevates corticosterone levels (Faulkner et al., 2018; Faulkner et al., 2019; Laurén, 1985; Morici, 1996), and acute stress can suppress alligator sex steroid levels (Elsey et al., 1991; Lance and Elsey, 1986; Lance et al., 2004), it is unknown how salinity stress affects sex steroids in alligators.

1.5.3. Alligator Behavior

Because physiological function often influences behavioral changes (Blüm and Fiedler, 1965; Fitzsimons, 1998), effects of salinity stress on physiology likely further impact alligator behavior. For example, alligators exposed to saltwater greater than 10‰ for 4-5 weeks significantly reduced feeding (Faulkner et al., 2018; Laurén, 1985; Morici, 1996). However, it is unclear if the reduced feeding was caused by effects on foraging behavior, comprising an animal's ability to capture prey (Fitzpatrick, 1980; Remsen and Robinson, 1990).

Foraging and habitat use by alligators is driven by many factors including prey distribution and environmental salinity (Fujisaki et al., 2014; Rosenblatt and Heithaus, 2011; Rosenblatt et al., 2015). Wild alligator populations further exhibit individual niche specialization in which individuals show variable habitat use and foraging strategies independent of sex, size, and age (Nifong et al., 2015; Rosenblatt and Heithaus, 2011; Rosenblatt et al., 2013). Variation in foraging behavior and habitat use by ontogeny, sex, and individual niche specialization has important implications for community structure and function, as not all individuals within a population utilize the same resources equally (Bolnick et al., 2011; McCauley et al., 1996; Persson et al., 1998). Since alligator behavior is highly complex and variable, further clarifying the effects that environmental stressors such as salinity have on alligator behavior will aid our understanding of alligator habitat use.

1.6. Objectives

Although it is understood alligators are vulnerable to salt stress (Lance et al., 2010; Laurén, 1985; Taplin, 1988), we have a poor understanding of how salinity affects alligator physiology. Thus, this dissertation comprehensively examines how saltwater affects blood plasma biochemistry and electrolytes (Na^+, Cl^-, Ca^{2+}), RAAS-related hormones, and steroid hormones in juvenile alligator plasma. To address this knowledge gap, I have conducted key studies to assess the physiological effects of salinity on alligators:

I performed both chronic (5 weeks) and short-term (1 week) salinity exposure trials to determine time-dependent effects of salt stress on juvenile alligators. I was further interested in learning whether I could correlate findings in a laboratory setting with conditions in the wild. To this end, I worked with Dr. Ruth Elsey from Rockefeller Wildlife Refuge in LA who opportunistically blood sampled wild alligators found at different salinities. Alligators are emerging as an apt sentinel species for environment quality, stimulating considerable interest in characterizing their endocrinology and physiology (Brock et al., 2016; Crain et al., 1999; Lance and Bogart, 1992; McCoy et al., 2016; Tipton et al., 2017). This knowledge is important for effective management of wild alligators and perhaps other crocodilians. Successful management will support continuous revenue from hunting and ecotourism as well as ensuring ecosystem health. Finally, my research further investigates how juvenile alligator basking and feeding behaviors are impacted by salinity stress. Habitat use and foraging behavior in alligators is highly complex and variable (Nifong et al., 2015; Rosenblatt and Heithaus, 2011; Strickland et

al., 2020). Thus, behavioral observations allow for a more comprehensive understanding of how salinity stress affects juvenile alligators and their interactions with the environment. My Ph.D. dissertation research is thus based on the following four objectives and subsequent research hypotheses:

Objective 1. Determine effects of salinity stress on juvenile alligator physiology.

H_1: A high-salinity environment will elevate levels of plasma electrolytes, stress hormones, and RAAS hormones in juvenile *A. mississippiensis* due to severe dehydration, whereas sex steroid levels in plasma will decrease due to high corticosterone levels.

Objective 2. Determine effects of salinity stress on juvenile alligator behavior.

H_2: Juvenile *A. mississippiensis* exposed to high salinity will alter feeding behavior to maintain salt/water balance, and animals will spend more time basking to avoid saltwater.

Objective 3. Investigate time-dependent effects of salinity exposure on RAAS hormones in juvenile alligators.

H_3: Changes in RAAS hormone levels due to salinity in *A. mississippiensis* will be time-dependent, resulting in dynamic changes in RAAS after chronic vs. short-term exposure.

Objective 4. Determine effects of salinity stress on physiology of wild alligators.

H_4: Higher-salinity environments will correlate with greater plasma electrolyte and RAAS hormone levels in wild *A. mississippiensis* due to dehydration, while resulting elevations in plasma corticosterone will depress sex steroid levels.

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CHAPTER II

EFFECTS OF CHRONIC EXPOSURE TO 12‰ SALTWATER ON THE ENDOCRINE PHYSIOLOGY OF JUVENILE AMERICAN ALLIGATORS (*Alligator mississippiensis*)*

2.1. 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 effects of chronic exposure to saline environments. Following 5 weeks, plasma levels of hormones (e.g., progesterone, testosterone, estradiol, corticosterone, aldosterone, angiotensin II) were quantified using LC-MS/MS. Compared to freshwater kept subjects, saltwater exposed alligators had significantly elevated plasma levels of corticosterone, 11-deoxycortisol, 17α-hydroxyprogesterone, testosterone, 17β-estradiol, estrone and estriol while pregnenolone and angiotensin II were significantly depressed and aldosterone levels were unchanged

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(slightly depressed). However, saltwater exposure did not affect gene expression of renal mineralocorticoid and glucocorticoid (MR, GR) 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 (enzymes AST, ALT) and kidney function (creatinine, 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 renin-angiotensin-aldosterone system and steroidogenesis of alligators.

2.2. Introduction

American alligators [*Alligator mississippiensis* (Daudin, 1802)] mainly inhabit freshwater systems in the Southeastern USA. Although no longer endangered, alligators are commonly affected by both anthropogenic and natural environmental stressors. While 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 freshwater input (from rivers and streams), increasing the likelihood of droughts and risk of saltwater 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 saltwater (Emanuel, 2005; Hoyos et al., 2006). Environmental stressors include drought or storm surges following major storm and hurricane events. These further contribute to salinization of alligator habitats, constituting a significant present and long-term threat along Gulf coast areas.

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 isoosmotic 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; Silldorff and Stephens, 1992b). The main enzymes are renin and angiotensin-converting enzyme (ACE) which convert angiotensinogen to angiotensin I and angiotensin I to angiotensin II respectively. Angiotensin II has several physiological effects in addition to stimulation of the mineralocorticoid steroid hormone aldosterone. 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 system has been demonstrated in American alligator as injections of fowl angiotensin I produced dose-dependent increases in arterial blood pressure (Silldorff and Stephens, 1992a; Silldorff and Stephens, 1992b). The increase in blood pressure was blocked by the ACE inhibitor, captopril, which demonstrates ACE involvement in angiotensin I to angiotensin II conversion. What's more, injection of angiotensin II caused significantly increased plasma aldosterone levels in juvenile alligators (Morici, 1996). Injection of angiotensin I and angiotensin II into the spectacled caiman (*Caiman crocodilus*; Alligatoridae) similarly produced significant dose-dependent increases in mean arterial blood pressure (Butler, 2006).

Aldosterone is produced and secreted from the adrenal glands and is, along with angiotensin II, under regulatory control by plasma K^+ levels. Aldosterone 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 angiotensin II and aldosterone 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 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 before 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 is 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 hormone (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, decline of American alligator populations in Lake Apopka (FL, US) was attributed to the 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 and 1999). More recently, feminizing effects of Corexit-enhanced water-accommodated fraction of crude oil (CWAF) (Standard Oil, NJ) were reported following *in vitro* exposure of gonads and gonad-adrenal-mesonephric (GAM) 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 freshwater wetlands are continuous threats to alligator habitats, it is critical to assess effects of saltwater 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 saltwater. To that end, juvenile (1-2 years old) alligators were exposed to 12‰ saltwater for 5 weeks and effects on steroidogenesis, RAAS hormones (angiotensin II, aldosterone), plasma biochemistry parameters, gene expression of RAAS hormone receptors, and morphology of lingual gland were investigated. We hypothesized that plasma hormones of the RAAS system would be elevated following chronic salt stress due to a reduction in

blood volume caused by severe dehydration while levels of sex steroid hormones would be depressed due to high corticosterone levels.

2.3. Materials and Methods

2.3.1. Animals and Husbandry

Juvenile American alligators (*Alligator mississippiensis*, 1-2 years old) [average body mass 639 ± 40.3 g; 29.5 ± 0.645 cm snout-to-vent length (SVL)] were generously donated by Rockefeller Wildlife Refuge (Louisiana, USA) and transported back to Texas A&M University at Galveston (TAMUG). Alligators were housed in a constant temperature (26°C) and photoperiod (12:12 light:dark cycle) controlled room. Animals were kept at a stocking density of 15 animals in a 380 L Rubbermaid stock tanks (Rubbermaidcommercial.com) containing 90 L of freshwater and a basking plate. Tanks were fitted with a reptile 160 W UVB light heat lamp (Zoo Med Laboratories, Inc.) 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). Water changes were performed 24-30 hours after each feeding (three per week).

2.3.2. Experimental Design

Juvenile alligators were exposed to either freshwater (FW, control, N=8) or saltwater (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 18‰ and 20‰. However, in these studies mortality was observed at levels higher than 14‰. These previous studies further assessed osmolality and reported 8‰ being isoosmotic to alligator plasma with 10‰ and above being hyperosmotic (Lauren, 1985; Morici, 1996). To ensure salinity effects but to avoid mortalities, we therefore determined to use 12‰ as the highest salinity level. The freshwater 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 eight days. Salinity was increased every 2 days from 0‰ to 4‰, 8‰, and finally maintained at 12‰ for the duration of the experimental period. Saltwater was attained by mixing filtered and sterilized seawater with tap water in appropriate proportions. All seawater was obtained from Gulf of Mexico off Galveston Island, TX, USA. During the course of the study, salinity levels in stock and exposure tanks [control (0‰) and saltwater (12‰)] were verified with a salinity meter (Oakton Instruments, 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 and counted to determine number of pellets corresponding to a weekly food intake of 3% body mass. During the trial, animals were offered food 3 times weekly and food intake was closely monitored by counting number of pellets given to each tank. The saltwater group gradually refused feeding and to avoid conflicting interactions between food deprivation and salinity effects, the

saltwater group was fed first and number of pellets eaten by the animals was counted. The freshwater 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 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 2 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 L) 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) 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.

2.3.3. 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. 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 and are listed in Table 2.2.

2.3.4. Steroid Hormone Extraction from Blood Plasma

All chemicals and reagents used in sample preparation were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO). 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 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 30:70 methanol:Milli-Q water. Samples were transferred to small-volume inserts in 2 ml amber glass vials for 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 mM sodium bicarbonate, and incubated at 60°C for 3 minutes. The resulting

dansylated estrogens (Dns-estrogen) were suspended in 500 µl Milli-Q water and liquid:liquid extracted twice with 500 µl 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 liquid chromatography and tandem mass spectrometry (or LC-MS/MS).

2.3.5. LC-MS/MS Analysis of Steroid Hormones

The LC-MS/MS system comprised an Agilent 1260 UHPLC system 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 mM ammonium formate. The mobile phase gradient transitioned from 30% (B), increased linearly to 70% over 3 minutes and from 70% to 95% in 6 minutes. The gradient was subsequently decreased from 95% to 70% in 3 minutes and from 70% to 30% (initial condition) over 3 minutes with the flow rate maintained at 0.4 ml min^{-1} (and total run-time of 15 mins).

Plasma hormones were detected in positive ion electrospray ionization (ESI+) mode with nitrogen as desolvation gas heated to 350°C (gas flow of 12 L/min) and capillary voltage at 3.5 kV. Hormones were detected in multiple reaction monitoring (MRM) 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 (d9-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-d3-estradiol). The mass transitions quantified were *m/z* 523.8 → 70.3 (angiotensin II), *m/z* 361.2 → 343.2 (aldosterone), and *m/z* 324.3 → 100.2 (d9-progesterone).

2.3.6. Reverse Transcription qPCR (RT-qPCR)

Kidney RNA was analyzed for MR, GR, and AT-1 gene expression using real-time qPCR. Forward and reverse primers were designed using Primer3 version 4.0.0 (Table 2.1), 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 Co. LLC., MO, Cat.# T9424). RNA concentration and purity based on 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). RNA was diluted to 40 ng μl^{-1} and forward and reverse primers were each diluted to 10 mM before performing RT-qPCR assays. Gene expression was quantified via SYBR Green detection using Rotor-Gene SYBR Green PCR kits (Qiagen, CA, Cat.# 204074) and a Rotor-Gene Q PCR cycler (Qiagen, CA). 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 mM forward and reverse primers, and 2.5 µl template RNA. RNA was

Table 2.1. Primer sets for RT-qPCR of indicated genes and product length (base pairs).

Gene	Forward (5' to 3') Primer	Reverse (3' to 5') Primer	Product Length (bp)
AT-1	GAGTGACGCTGTTCGCAGTA	CCCAAACAGTCCTCTGCAAT	189
MR	GGCCATTGTTCCTCTCCCTT	AGAGCCCCGGTTTTCAATGT	297
GR	AAAAAACTGTCCCGCATGCC	CGTTGGACTGCTGAATTCCTTT	104

reverse-transcribed at 55°C for 10 minutes followed by 95°C for 5 minutes. PCR reactions were performed over 40 cycles of denaturation (95°C for 5 seconds) and annealing/extension (60°C for 10 seconds). Total copies of cDNA per nanogram of template RNA was calculated as described by Tate et al. (Tate et al., 2016).

2.3.7. 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 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 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 that 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, surface area of glandular region. Morphometrics collected on horizontal sections were: greatest width of glandular region, width 90° to greatest width of glandular region and surface area of glandular region.

2.3.8. Statistical analyses

Normality testing was performed using the Shapiro–Wilk's test ($p \leq 0.05$). Statistical significance was determined using parametric and nonparametric tests. Parametric analyses were conducted using two-tailed Student's *t*-tests. When normality wasn't met, nonparametric tests were performed. If assumptions for Mann-Whitney/Wilcox tests were not met, a permutation test was performed using 1,000 permutations. P-values ≤ 0.05 were considered statistically significant. Statistical analyses were performed using R version 3.3.1 or GraphPad Prism version 5.0. All data shown are mean ± s.e.m.

2.4. Results

2.4.1. Food Intake and Body Morphometrics

Body morphometrics and food intake rate are shown in Table 2.2. Prior to the trial, control and treatment tanks were fed the same number of pellets. However, as the saltwater exposed group gradually reduced food intake, we decided to feed the freshwater control group the same number of pellets to avoid conflicting interactions between food deprivation and salinity effects. Food intake in the saltwater group decreased by 98% and the freshwater group's food intake was therefore equally reduced by 98% (Table 2.2).

Despite the freshwater and saltwater groups eating the same amount of food, alligators exposed to 12‰ saltwater lost ~28% of their body mass (wet body mass) after 5 weeks while 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 2.2).

2.4.2. Effects of Salt Stress on Blood Plasma Biochemistry

Concentrations of plasma biochemistry parameters are listed in Table 2.3. Except for plasma potassium levels (K^+) which remained similar between FW- and SW-acclimated alligators, plasma ions Na^+ and Cl^- were significantly elevated in SW alligators (Table 2.3). Furthermore, plasma uric acid, albumin, and globulin levels were below 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 saltwater significantly increased plasma creatine kinase, creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and cholesterol levels (Table 2.3). Plasma minerals such as Ca^{2+} and phosphorous (P) were additionally significantly elevated after 5 weeks in 12‰ saltwater (Table 2.3). On the other hand, although the control and treatment groups ate the same amount of food, plasma glucose levels were significantly reduced in salinity exposed alligators (Table 2.3). In contrast, exposure to saltwater did not significantly affect plasma bilirubin or alkaline phosphatase (ALP) levels.

Table 2.2. Body morphometrics and food intake in American alligator (*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
% Weight Loss	-14.4 ± 8.30*	27.9 ± 3.90
S-V Length (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 mean ± s.e.m. All parameters shown except initial body mass were obtained at 5 weeks. K = Fulton's condition factor estimated using S-V length. Asterisks denote statistically significant differences between freshwater and saltwater groups ($p \leq 0.05$).

Table 2.3. Plasma biochemistry parameters in freshwater and 12‰ chronically (5 weeks) exposed juvenile American alligators (*Alligator mississippiensis*).

Blood Chemistry Parameter	Freshwater (mean ± s.e.m.)	Freshwater Range	Saltwater (mean ± 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	364-25650
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/ALT	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 ($p \leq 0.05$) between freshwater and saltwater mean values. †Shows values below detection limit. ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

2.4.3. RAAS Hormones

Plasma levels of the biologically active RAAS hormone, angiotensin II, were significantly (p=0.01) lower in SW alligators after chronic salinity exposure. In fact, angiotensin II levels were almost 90% lower in SW alligators compared with FW alligators (Fig. 2.1). Plasma levels of the mineralocorticoid aldosterone 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. 2.1).

2.4.4. 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 twelve steroid hormones were quantified in plasma from alligators. Pregnenolone, the precursor to progesterone or 17α-hydroxypregnenolone (Fig. 2.2), was significantly decreased by 63% relative to FW control (Student's *t*-test, p=0.002) after 5 weeks exposure to 12‰ SW (Fig. 2.3A). This decrease, however, did not affect productions of 'downstream' steroid hormones such as progesterone and 17α-hydroxypregnenolone, which were not significantly different from the freshwater group (Fig. 2.3A). In contrast, 17α-hydroxyprogesterone showed 4× higher levels in SW relative to FW (Permutation test, p=0.03) (Fig. 2.3A).

The elevated levels of 17α-hydroxyprogesterone are of interest as they also associate with elevated 11-deoxycortisol levels (transformation catalyzed by cyp21-hydroxylase) (Fig. 2.2, Fig. 2.4). Five weeks in 12‰ saltwater caused a significant increase in the glucocorticoid hormones, corticosterone and 11-deoxycortisol. Corticosterone was ~7× higher in 12‰ exposed alligators while 11-deoxycortisol was ~8× higher in SW compared with FW alligators (Fig. 2.4).

The cyp450 enzyme exhibiting 17,20-lyase activity (cyp17-lyase) catalyzes the conversion of 17α-hydroxyprogesterone to androstenedione (Fig. 2.2; Fig. 2.3B). 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. 2.3B). In contrast, the conversion of androstenedione to testosterone (catalyzed by 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. 2.3B). 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. 2.2).

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 hydroxysteroid dehydrogenase (17β-HSD) enzymes (Fig. 2.2).

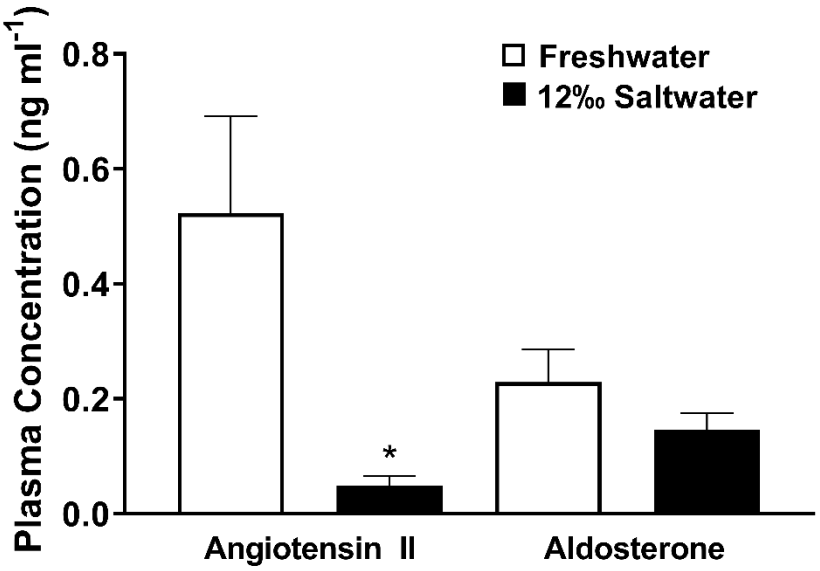

Figure 2.1. Plasma levels of angiotensin II (ng ml^{-1}) and aldosterone (ng ml^{-1}) in freshwater and salinity (12‰) exposed juvenile American alligators. Data shown are means ± s.e.m. (N= 8). Blood was sampled after 5 weeks exposure in both groups. Asterisk denotes statistically significant differences ($p \leq 0.05$) between treatment groups.

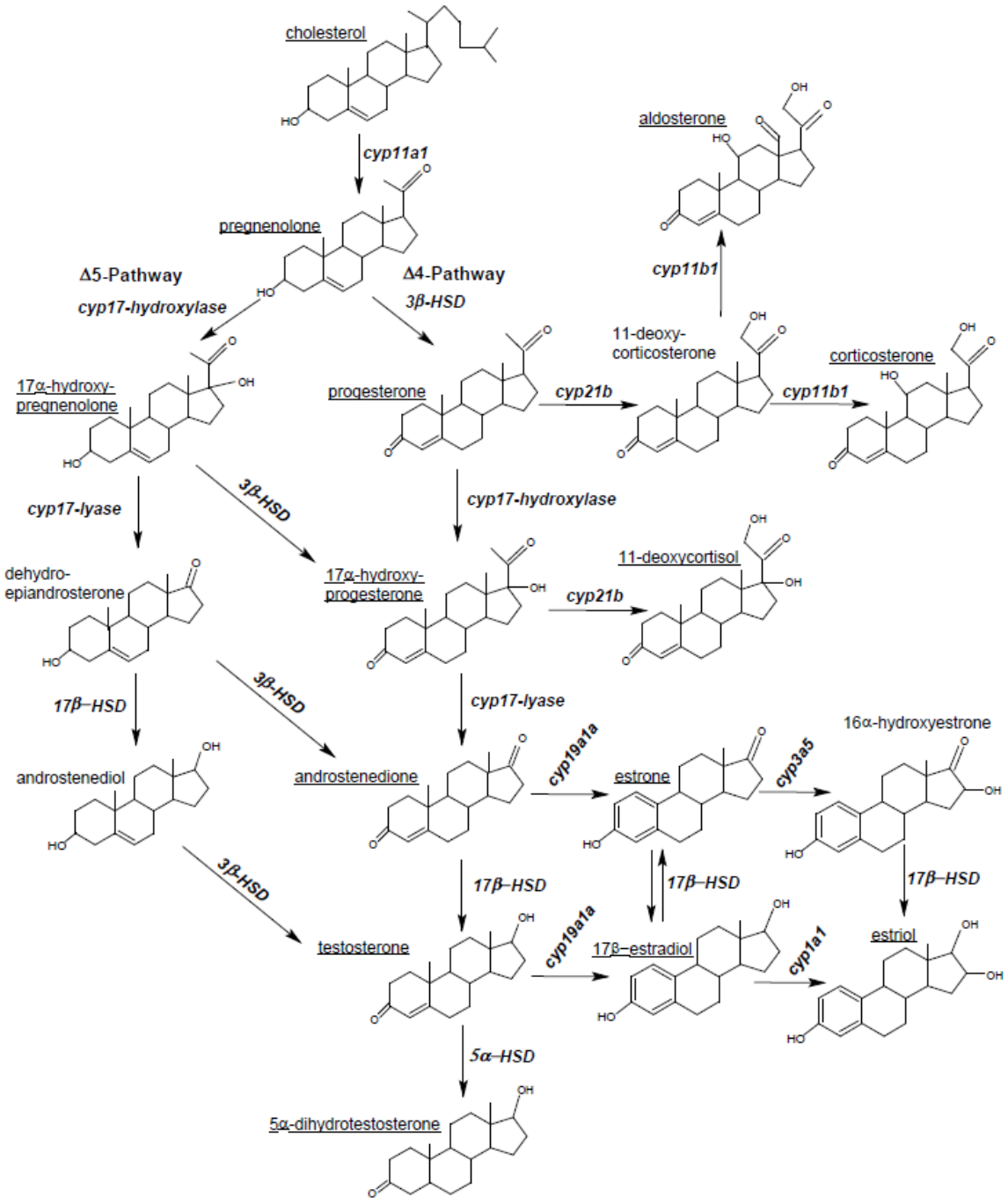

Figure 2.2. Illustration of suggested steroidogenic pathway in American alligator. Arrows indicate catalyzed reactions with catalyzing enzymes listed next to arrow. Underlined hormones were quantified in the current study.

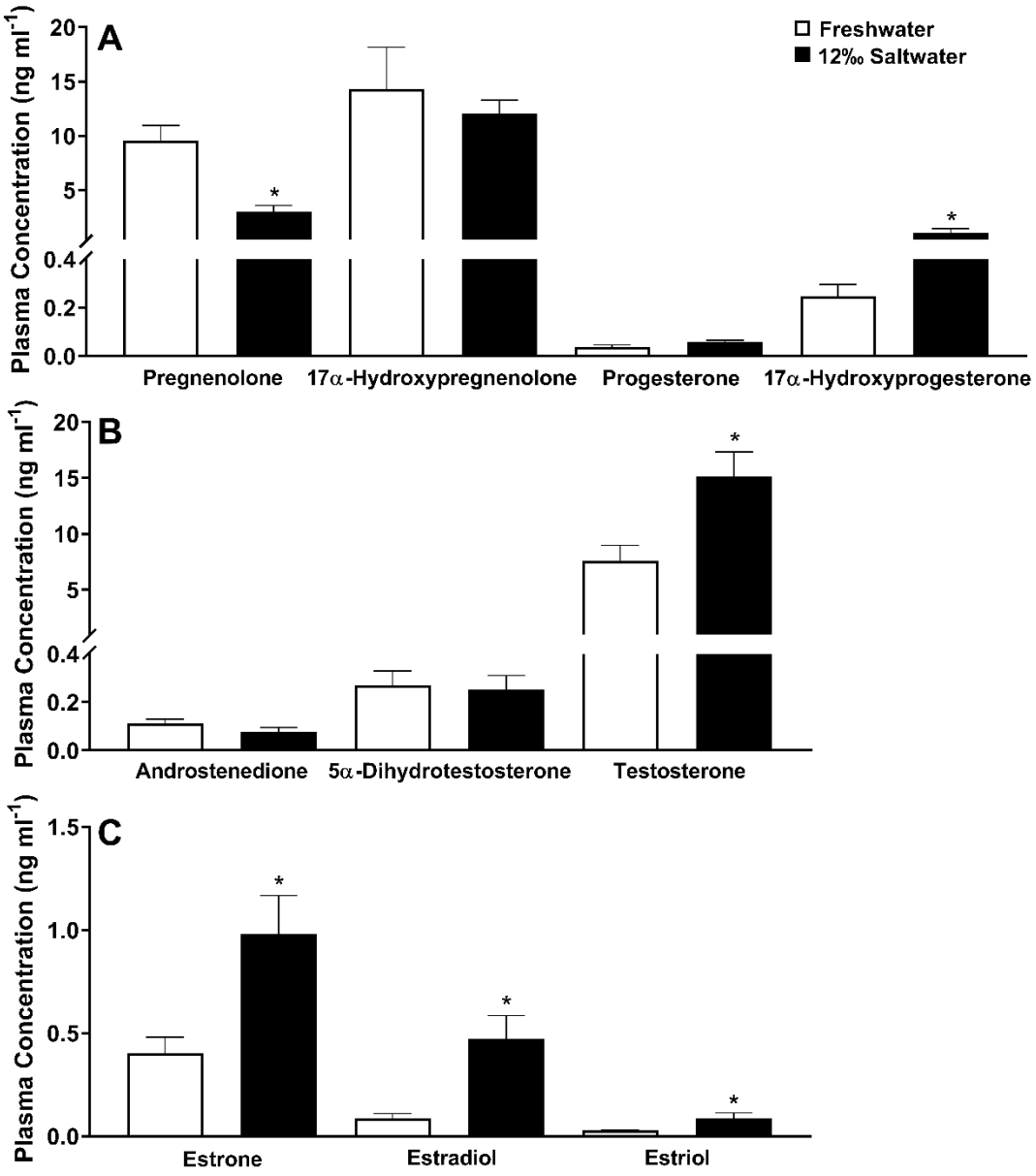

Figure 2.3. Plasma sex steroidogenic hormone concentrations (ng ml^{-1}) in juvenile American alligators exposed to freshwater (0‰) or saltwater (12‰) for 5 weeks. Animals were sampled after 5 weeks. A) Progestogens: pregnenolone, 17α-hydroxypregnenolone, progesterone and 17α-hydroxyprogesterone. B) Androgens: androstenedione, dihydrotestosterone and testosterone. C) Estrogens: estrone, 17β-estradiol and estriol. Data shown are average (N=8) ± s.e.m. *Denotes significant difference ($p \leq 0.05$) between freshwater and saltwater group.

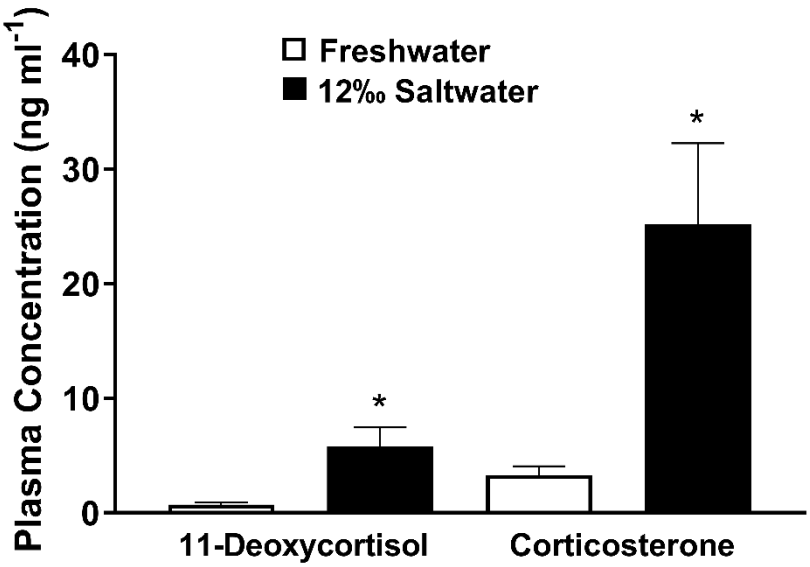

Figure 2.4. Plasma 11-deoyxcortisol and corticosterone concentrations (ng ml^{-1}) in juvenile American alligators exposed to freshwater (0‰) or saltwater (12‰) for 5 weeks. Animals were sampled after 5 weeks. Data shown are average (N=8) ± s.e.m. *Denotes significant difference ($p \leq 0.05$) between freshwater and saltwater group.

Plasma levels of all three estrogens were significantly elevated in 12‰ saltwater exposed alligators compared with freshwater controls (Fig. 2.3C).

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 2.4). However, there was no significant ($p=0.22$) difference in the ratio of 17β-estradiol to testosterone between FW- and SW-exposed alligators (Table 2.4).

2.4.5. Histology

Morphometric data of lingual glands in FW- and SW-exposed alligators are shown in Table 2.5 while micrographs of cross- and horizontal sections of FW and SW alligator tongues are shown in Figure 2.5. Measurements from horizontal sections did not reveal any significant differences ($p>0.05$) in gland area or width (min and max) between freshwater and saltwater maintained alligators (Table 2.5). Furthermore, measurements from cross sections of lingual glands did also not show any significant differences in pore and gland area, gland width, height or pore width between freshwater and saltwater alligators. However, the length of the pore from the gland to surface of tongue was significantly longer in saltwater exposed alligators ($p=0.001$).

Table 2.4. 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
Estradiol/Testosterone	0.02 ± 0.007	0.04 ± 0.02

*Denotes significant difference ($p \leq 0.05$) between FW and SW groups.

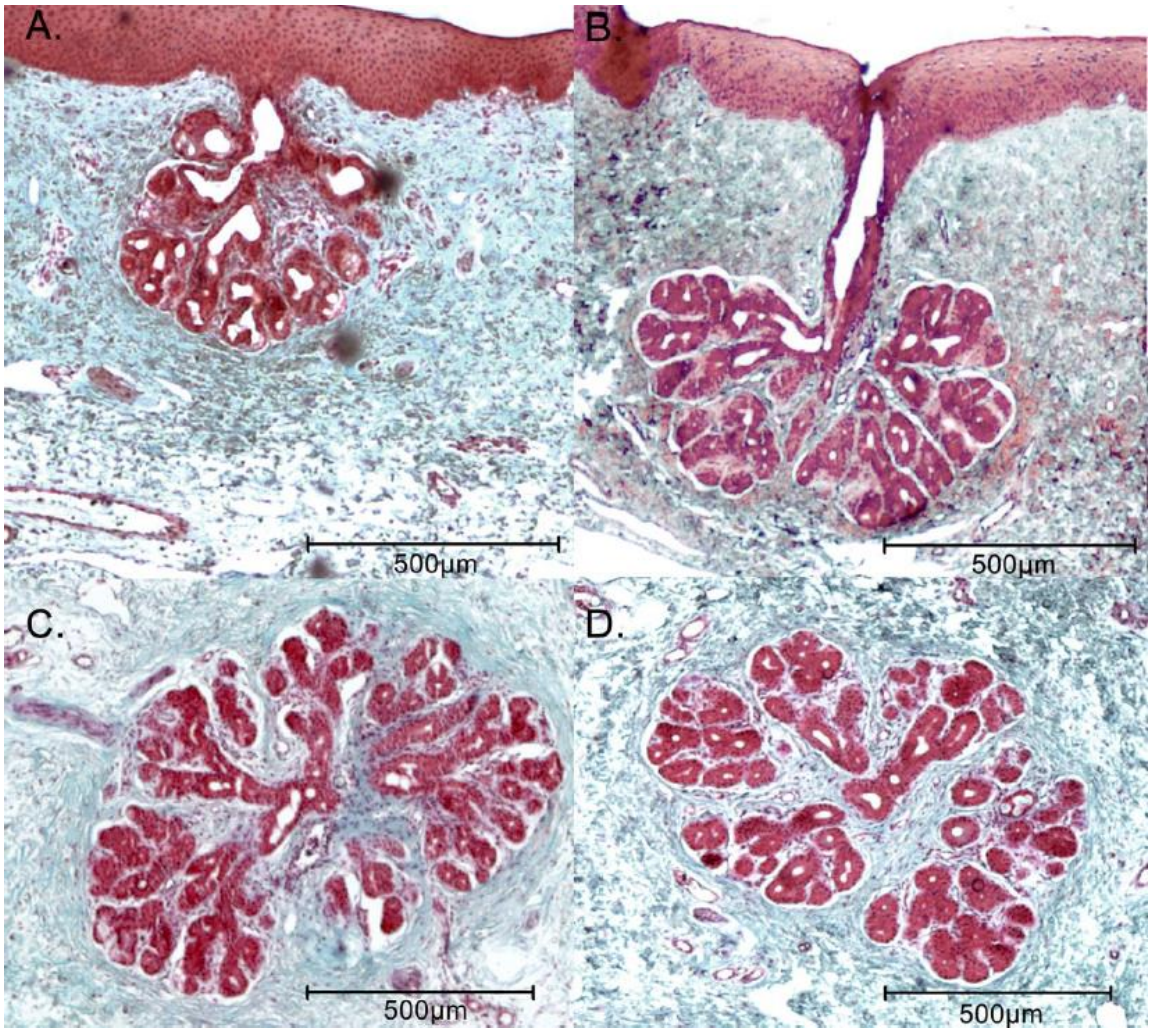

Figure 2.5. Micrographs of alligator lingual glands. Left panel shows pictures of tongues from freshwater-maintained juvenile alligators: (A) cross-section, (C) horizontal. Right panel shows pictures of tongues from chronically (5 weeks) saltwater-exposed (12‰) juvenile alligators: (B) cross-section, (D) horizontal.

Table 2.5. Morphometrics of lingual glands in American alligator 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
Max width	0.91 ± 0.07	0.87 ± 0.06	0.75
Min 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

*Denotes significant difference ($p \leq 0.05$) between FW and SW groups. N=4 for FW, N=5 for SW. †9 slides for horizontal sections and ‡13 slides for cross-section.

2.5. Discussion

2.5.1. 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 saltwater 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 saltwater 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).

Cessation of feeding in saline environments has previously been reported for juvenile alligators (Lauren, 1985; Morici, 1996) and freshwater turtles exposed to seawater for >2 weeks (Bower et al., 2016; Davenport and Ward, 1993). In freshwater 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.

2.5.2. Blood Plasma Biochemistry Parameters

SW exposure significantly elevated creatine kinase levels (Table 2.2). High creatine kinase levels can be responsible for elevated creatine to phosphocreatine

conversion (high-energy phosphate depot for ATP recycling), and its subsequent breakdown to creatinine (Wallimann et al., 2011). Therefore, significantly high levels of creatine kinase and creatinine observed in the saltwater 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). Due to the osmoregulatory capabilities of alligators (Braun, 1998; Taplin et al., 1982), the kidneys are likely to have been adversely affected by saltwater 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 saltwater compromises liver integrity and has adverse effects on hepatic function.

Dehydration in crocodilians can increase uric acid levels but have only been reported to cause renal failure in severe cases (Huchzermeyer, 2003). Because of the low solubility of uric acid, accumulation can trigger the formation and deposition of uric acid crystals in organs. SW-acclimated alligators started to eliminate white precipitate (uric acid) after 1 week of exposure (Faulkner, personal observations). Uric acid crystals could thereafter be seen at the bottom of tanks for the remainder of the exposure period

(Faulkner, personal observations). These observations correlate well with the measured increase in uric acid plasma levels after 5 weeks in 12‰ (Table 2.2). 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 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 alligator 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 freshwater 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 few studies have determined alligatorid integumental permeability to Na^+, there is evidence of Na^+ influx and efflux across the integument when alligators are in freshwater (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 freshwater turtles (Bower et al., 2016) which suggests activation of aldosterone which stimulates increased renal excretion of K^+.

2.5.3. RAAS and Lingual Glands

To the best of our knowledge this is the first study to determine angiotensin II levels in salinity stressed alligators. Generally, very few studies have determined angiotensin II levels in reptiles, but extensive studies exist on 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 angiotensin II and aldosterone in juvenile alligators following chronic exposure to saltwater. angiotensin II and aldosterone 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, 1980). To avoid blood sampling potentially masking any direct effects of salinity treatment on hormone levels, we therefore only assessed angiotensin II and aldosterone after 5 weeks exposure and compared values with a freshwater control group. Five weeks at 12‰ significantly decreased plasma angiotensin II levels in juvenile alligators which rejects our research hypothesis of increased angiotensin II levels in severely dehydrated salinity exposed alligators. angiotensin II has

its own, independent from aldosterone, Na^+ reabsorptive effects on the kidneys (Fournier et al., 2012). We therefore determined gene expression of the AT-1 receptor in kidneys of FW and SW alligators to assess any genomic differences as a result of saltwater exposure. However, contrary to the reduction in plasma hormone levels there was no significant difference in AT-1 expression (Fig. 2.6A). 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 angiotensin II are non-genomic or post-translational. angiotensin II regulates blood pressure by increasing renal tubular Na^+, Cl^- 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 angiotensin 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 angiotensin II secretion to restore plasma volume. However, exposure to saltwater 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 conversion of angiotensinogen to angiotensin I and is stimulated by intrarenal baroreceptors that sense changes in renal arterial pressures at or near

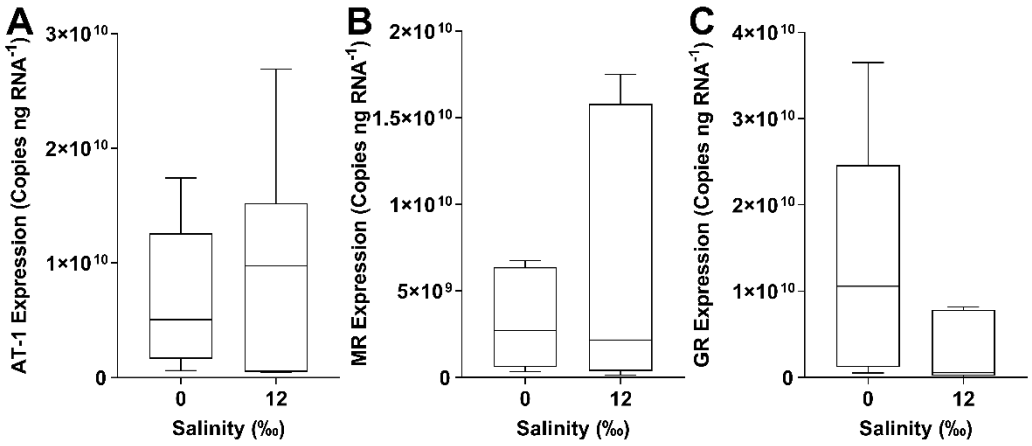

Fig. 2.6. Box plots of gene expression of angiotensin II receptor (AT-1) (A), mineralocorticoid receptor (MR) (B) and glucocorticoid receptor (GR) (C) in American alligator kidneys. Animals were exposed to freshwater (0‰) and saltwater (12‰) for 5 weeks. Expression is shown as copies of RNA per ng RNA (N=8 for each group).

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 angiotensin I to the biologically active angiotensin 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 angiotensin II levels.

Very few studies have investigated aldosterone in salinity stressed or salt loaded reptiles. For instance, aldosterone levels were significantly depressed but corticosterone was elevated in salt-loaded sand goannas (*Varanus gouldii*, Gray) while dehydration resulted in elevated aldosterone levels (Bradshaw and Rice, 1981). Juvenile Nile crocodiles (*Crocodylus niloticus*) kept in hypertonic medium for 7 days exhibited a 33%

increase in aldosterone levels compared with freshwater-maintained animals (Balment and Loveridge, 1989). Furthermore, juvenile alligators exposed to 4‰ for one week doubled aldosterone concentration while animals exposed to 8‰, 12‰, and 16‰ exhibited a minor increase in aldosterone (Morici, 1996). Thus, these findings correspond well with results from the present study in which aldosterone levels were not significantly different in SW alligators compared with FW alligators after 5 weeks. aldosterone secretion in vertebrates is regulated by angiotensin II in addition to the adrenocorticotropic hormone (ACTH) and plasma K^+ (Nishimura, 2017). It has previously been demonstrated that aldosterone and corticosterone 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 corticosterone tablets had undetectable aldosterone levels after 1 week of implants showing a negative feedback of corticosterone on aldosterone (Morici et al., 1997). The continuously high corticosterone levels at week 5 in our SW alligators did not seem to have significantly depressed aldosterone levels although a reduction was evident between the FW and SW alligators. Indeed, we did not detect a significant correlation between corticosterone and aldosterone in either freshwater or 12‰ exposed alligators at 5 weeks (Fig. 2.7B) suggesting long-term exposure to saltwater does not continuously result in a negative feedback of corticosterone on aldosterone. On the contrary, data revealed a correlation between plasma K^+ and aldosterone in SW alligators but not in FW alligators (Fig. 2.8F). Interestingly there was no correlation between angiotensin II and aldosterone levels in either FW or SW alligators after 5 weeks (Fig. 2.7C). The lack of correlation between angiotensin II and aldosterone in SW alligators therefore explains

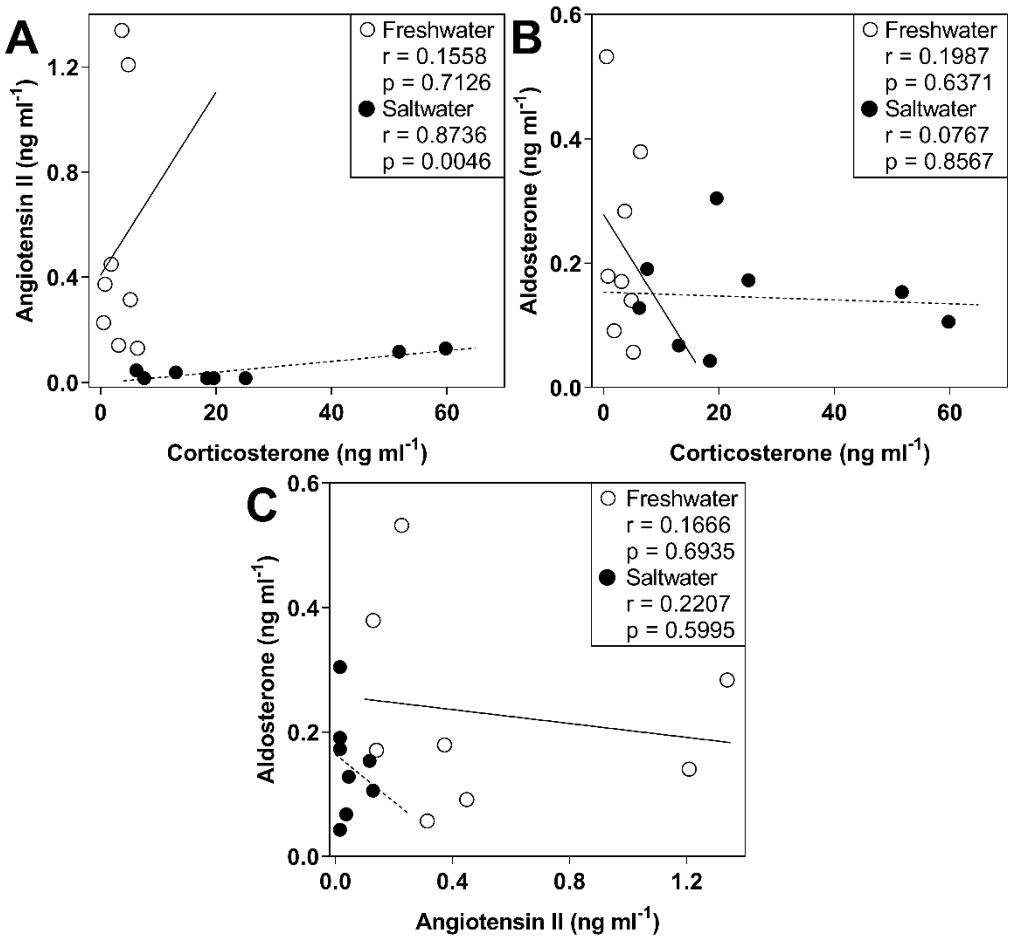

Fig. 2.7. Correlation between plasma hormones in American alligators exposed to either freshwater (0‰) or saltwater (12‰). A) correlation between plasma angiotensin II and corticosterone, B) correlation between plasma aldosterone and corticosterone and C) correlation between plasma aldosterone and angiotensin II. r = correlation coefficient, p = significance of correlation between plasma hormone and electrolyte within each group.

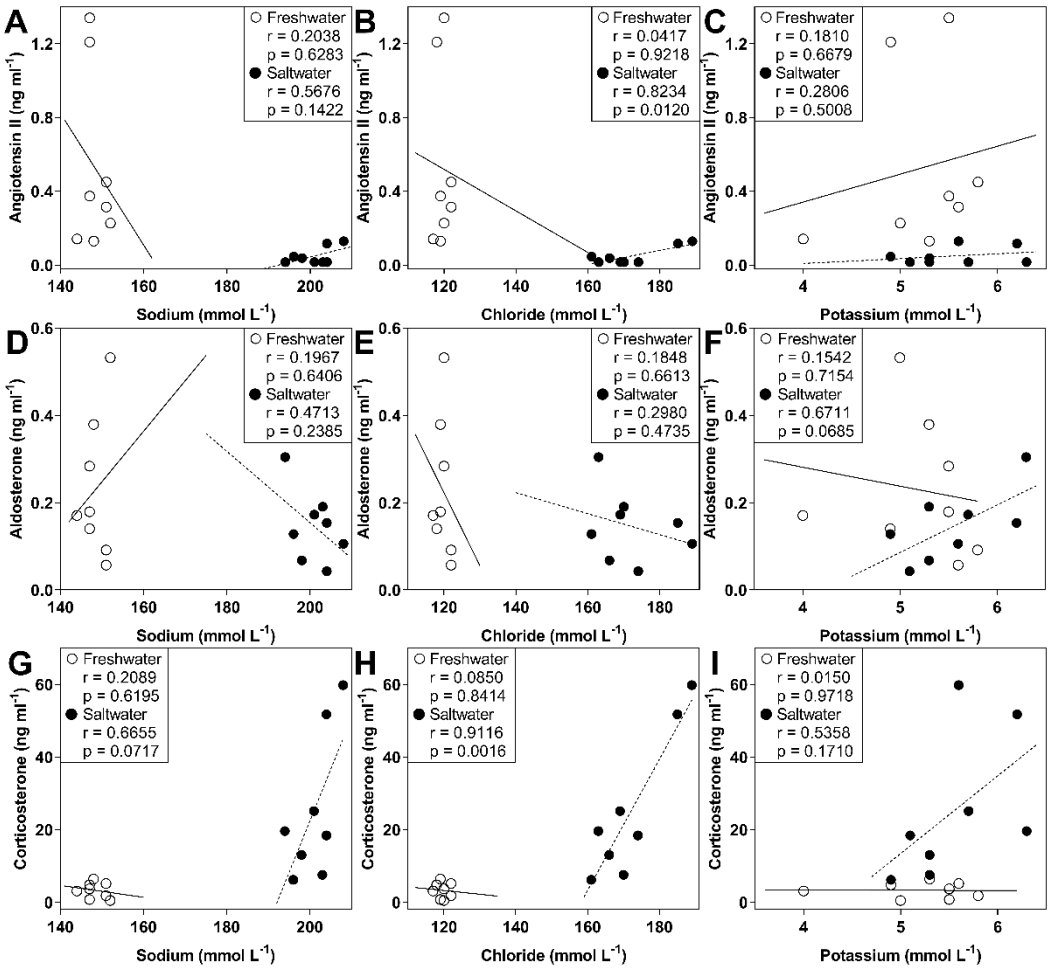

Fig. 2.8. Correlation between plasma hormones and plasma Na^+ (left panel), Cl^- (middle panel) and K^+ (right panel) in American alligators exposed to either freshwater (0‰) or saltwater (12‰). A, B, C denote correlation between angiotensin II and plasma electrolytes. **D, E, F** denote correlation between aldosterone and plasma electrolytes. **G, H, I** denote correlation between plasma corticosterone and plasma electrolytes. r = correlation coefficient, p = significance of correlation between plasma hormone and electrolyte within each group.

why aldosterone in SW alligator levels did not mirror the significantly lower angiotensin II levels. Data from the present study therefore demonstrate aldosterone secretion in salinity stressed alligators is tightly regulated by plasma K^+ levels and to a lesser extent angiotensin II or corticosterone.

aldosterone plays a significant role in renal Na^+ retention and K^+ secretion in mammals and acts with high affinity on the mineralocorticoid receptor (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 kidneys of FW and SW alligators. As MR equally binds the glucocorticoid corticosterone, we additionally determined gene expression of the glucocorticoid receptor (GR) which selectively binds corticosterone (Funder, 2017). Consistent with plasma aldosterone data, there were no significant differences in the number of RNA copies of kidney MR between acclimation groups (Fig. 2.6B). However, kidney GR gene expression data did not correspond with plasma corticosterone levels as no significant difference was quantified in GR gene expression between treatment groups (Fig. 2.6C). 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 accurate indicators 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 saltwater did not significantly change 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. 2.5, Table 2.5). 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, 1983; Taplin and Grigg, 1981; Taplin et al., 1982). Taplin et al. (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. 2.5). 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. 2.5). 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 saltwater acclimated *C. porosus* where increases in secretory tubule size, increased mitochondrial numbers and plasma membrane surface area were correlated with increased functional activity of lingual salt glands (Cramp et al., 2007; Cramp et al., 2008).

2.5.4. 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 productions of hormones capable of metabolic adaptation to stress. For example, high corticoid levels (cortisol, cortisone, corticosterone) can induce gluconeogenesis (via induction of phosphoenolpyruvate carboxykinase, PEPCK) or hydrolysis of triglycerides (via induction of lipoprotein lipase, LPL). 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 corticosterone 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 corticosterone 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 saltwater result in chronic stress in juvenile alligators. Long-term elevated corticosterone 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 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 adverse effects of anthropogenic endocrine disrupting compounds on endocrine or steroidogenic effects in alligators (Crain et al., 1997; Crain et al., 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 (saltwater) on juvenile alligator steroid hormones. We hypothesized that a natural stressor such as salinity would decrease sex steroid hormone levels due to high corticosterone levels but data revealed significant increases in androgens, progestogens, and estrogens.

Cholesterol is the precursor for sex steroid hormones and levels of cholesterol were doubled in SW alligators compared with their FW controls (Table 2.2). As food intake was significantly reduced, elevated cholesterol levels were not of immediate dietary origin. The high cholesterol levels suggest either enhanced beta-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 saltwater exposed alligators did not result in elevated levels of pregnenolone, which was significantly lower in saltwater 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 (Fig. 2.2, 2.3). These data are highly suggestive of higher pregnenolone catalysis via the Δ5 versus Δ4 steroidogenic pathways (Fig. 2.2). 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 corticosterone 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, corticosterone, and testosterone in saltwater exposed alligators suggest differences between sexually mature and immature alligators with respect to the effect of corticosterone on testosterone and estradiol production. The increased testosterone levels under saltwater 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. 2.2). While there were no effects of saltwater 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 of 0.01-0.06 ng ml^{-1} (Crain et al., 1997; Crain et al., 1998; Guillette et al., 1994; Guillette et al., 1997; Guillette et al., 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 freshwater male juvenile alligators in the present study (average 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 to 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., 1995; Guillette et al., 1994). Interestingly, 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 while a non-significant increase in estradiol:testosterone was determined. Thus, increased

salinity had a similar effect on 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 saltwater. 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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CHAPTER III

EFFECTS OF SALTWATER ON JUVENILE AMERICAN ALLIGATOR (*Alligator mississippiensis*) BASKING AND FORAGING BEHAVIOR

3.1. Abstract

American alligators (*Alligator mississippiensis*) are native to freshwater habitats in the Southeastern United States which are susceptible to salinity increases due to droughts, storm surges, sea level rise, and anthropogenic perturbations of freshwater flow. Juvenile alligators are especially vulnerable to salt stress due to their thinner integument and lower mobility and ability to avoid saltwater. Previous studies have reported high physiological stress in alligators exposed to saltwater. To better understand the effects of saltwater on alligator behavior, juvenile alligators were exposed to 12‰ saltwater for 5 weeks while resting and feeding behaviors were recorded to be compared with observations prior to exposure. Behaviors were recorded via instantaneous sampling, in which sampling intervals of 1 minute occurred throughout a 20-minute observation period, and scores were generated as a proxy for time spent on each behavior. Saltwater-exposed alligators largely ceased feeding within the first week. In addition, alligators exposed to saltwater spent significantly less time basking compared with pre-salinity observations, likely as a strategy to reduce flux of ions and water across integument. However, bite force was unaffected by saltwater exposure. Overall, the present study showed significant impacts of saltwater exposure on foraging and basking behavior in juvenile alligators.

3.2. Introduction

American alligators [*Alligator mississippiensis* (Daudin, 1802)] are native to the Southeastern United States and inhabit mainly freshwater areas. Although large individuals are known to forage in brackish water and some have even been reported foraging in estuarine and marine saltwater areas (Elsey, 2005), alligators prefer low saline environments or need frequent access to freshwater if foraging in saline environments. However, many freshwater alligator habitats frequently experience salinity increases. These increases can stem from months with low rainfall which enhances the concentration of salts and solutes as freshwater evaporates (Lance et al., 2010). Furthermore, storm surges following large storms or hurricanes can push seawater into coastal freshwater areas causing short- or long-lasting saline environments (Jackson et al., 1995; Michener et al., 1997; Schriever et al., 2009). However, gradual changes in climate results in subtle but persistent salinity changes that are not likely reversible. For instance, the International Panel on Climate Change (IPCC) predicts that by the end of the 21st century sea levels will rise by 28-48 cm above levels at the beginning of the century causing saline environments in coastal freshwater habitats.

Sea level rise can also introduce saline water into inland groundwater fed wetlands resulting in salinization of further inland areas (Wood and Harrington, 2015). Indeed, recent predications estimated that considerable areas of wetlands will be lost from the Mississippi river delta by 2050 (Barras et al., 2008; Barras et al., 2003). Finally, increased sea surface temperatures in the tropics (e.g., 1°C over the past century) is correlated with increased hurricane intensity as shown by an increase in category 4 and 5 hurricanes

(DeLaune and White, 2012; Emanuel, 2005; Hoyos et al., 2006) with ensuing increased frequency in storm surges. The hurricane season in the Southeastern United States ranges from June-November with most large hurricanes forming in the months of August and September (Curtis, 2008; Saunders and Lea, 2005). This overlaps with reproductive season of American alligators since eggs hatch in the months of July and August (Joanen, 1969; Lance, 1989) so emergence of hatchlings coincides with peak hurricane season.

In contrast to crocodiles, alligators do not possess lingual salt glands and are therefore unable to regulate electrolytes (Na^+, Cl^-, K^+) in hyperosmotic conditions (Faulkner et al., 2018; Faulkner et al., 2019; Laurén, 1985; Pidcock et al., 1997; Taplin, 1988). While lack of salt glands prevents excretion of excessive Na^+ and Cl^- ions, the permeable integument fails to prevent diffusive loss of body water to external saline environments. Elevated salinity levels therefore result in dehydration and increased plasma electrolyte levels (Faulkner et al., 2018; Faulkner et al., 2019; Laurén, 1985; Pidcock et al., 1997; Taplin, 1988). While adult alligators are affected by saline environments, juvenile alligator integument is thinner than adults and they are therefore at a higher risk of dehydration and influx of ions. Furthermore, juvenile alligators are less mobile and their inability to leave affected areas pose an additional risk of high salinity effects. Laboratory-based studies demonstrated that high salinity in juvenile alligators results in extreme stress as measured by elevated stress hormones corticosterone and 11-deoxycortisol (Faulkner et al., 2018; Faulkner et al., 2019; Laurén, 1985), dehydration, and significantly increased plasma electrolytes and osmolality (Faulkner et al., 2018; Faulkner et al., 2019; Lance et al., 2010; Lance et al., 2000; Laurén, 1985; Morici et al.,

1997). Since physiological function commonly influences behavior (Blüm and Fiedler, 1965; Fitzsimons, 1998), it is likely that physiological changes associated with salinity stress can affect certain behaviors in alligators. For example, chronic (4-5 weeks) exposure of juvenile alligators to brackish water (>10‰) significantly reduced feeding (Faulkner et al., 2018; Laurén, 1985; Morici, 1996), resulting in decreases in growth and body mass (Faulkner et al., 2018; Laurén, 1985). However, it is unknown whether the reduction in feeding was brought about by changes in foraging behavior which encompasses the ability to capture and ingest prey (Fitzpatrick, 1980; Remsen and Robinson, 1990). Furthermore, alligators rely on behavioral thermoregulation to adjust body temperature, such as basking on river banks or logs to elevate body temperature which is commonly seen in post-absorptive animals to increase digestive processes (Diefenbach, 1975; Lang, 1979) and on colder days (Brisbin Jr et al., 1982; Lang, 1987). Time spent in the water versus basking may be indicative of changes in alligator physiological function.

Because alligators are considered a keystone species, salinization of freshwater wetlands can have significant negative ecosystem level effects (Mazzotti et al., 2009; Mazzotti and Brandt, 1994). Since salinization events are unpredictable and environmental observations are often confounded by many factors, the current study used a laboratory-based investigation to assess juvenile alligator feeding and basking behavior when animals were chronically (5 weeks) exposed to saltwater (12‰). The behavioral study was performed using video recordings to avoid influence of observer presence on behavioral responses. This study was part of a larger physiologically based study (Faulkner et al., 2018; Chapter 2) and where applicable references to observed behaviors will be related to

physiological results. Based on saltwater's effects on food intake in freshwater reptiles (alligators and turtles) (Bower et al., 2016; Faulkner et al., 2018; Laurén, 1985; Morici, 1996), we hypothesized that feeding behavior would be significantly altered in saltwater exposure animals, and that animals would spend more time basking to avoid saltwater.

3.3. Materials and Methods

3.3.1. Animals and Husbandry

Juvenile American alligators (1-2 years old; average body mass 639 ± 40.3 g; snout-vent length 29.5 ± 0.645 cm) were generously donated by Rockefeller Wildlife Refuge (Louisiana, USA) and transported to Texas A&M University at Galveston (TPWD SPR-0416-097). Alligators were housed in a controlled photoperiod (12:12 light:dark cycle) and a constant temperature (26°C) using a 160 W UVB light heat lamp (Zoo Med Laboratories, Inc.). Animals were kept at a stocking density of 16 animals in a 380L Rubbermaid stock tanks (Rubbermaidcommercial.com) containing 90L of freshwater and a 35.5cm×35.5cm basking plate. Twenty five percent of each tank was shaded to allow animals to shelter and thermoregulate. Animals were fed on average 3% body mass per week of Mazuri® Reptile Diet (PMI Nutrition International), and complete water changes were performed 24-30 hours after each feeding (three per week). Animals were maintained in freshwater cultures for approximately 6 months prior to experimentation.

3.3.2. Experimental Design

Juvenile alligators were exposed to saltwater (SW, 12‰, N=16) for 5 weeks. The salinity concentration was based on previous studies in which juvenile alligators were exposed to salinity levels up to 18‰ and 20‰ (Laurén, 1985; Morici, 1996). However, these studies observed mortality at levels higher than 14‰. These studies further assessed plasma osmolality and reported 8‰ being isoosmotic to alligator plasma with 10‰ and above being hyperosmotic (Laurén, 1985; Morici, 1996). To ensure salinity effects while avoiding mortalities, we used 12‰ as the highest salinity level. The freshwater group was kept in dechlorinated city tap water at 0‰ for the duration of the exposure period. Chlorine was removed by keeping tap water in stock tanks for 2 days before each water change. Water quality test strips (LaMotte Inc., Chestertown, MD, USA) were subsequently used to confirm chlorine was absent in stock tanks at the time of water change. The saltwater treatment group was gradually exposed to increasing salinity levels over eight days in which salinity was increased every 2 days from 0‰ to 4‰, 8‰, and finally 12‰ for the duration of the experiment. Saltwater was attained by mixing freshwater with filtered and sterilized seawater from the Gulf of Mexico off Galveston Island, TX, USA to achieve 12‰. Throughout the present study, salinity levels in stock and exposure tanks [control (0‰) and saltwater (12‰)] were measured with a salinity meter (Oakton Instruments, WD-35604-00) twice daily. Prior to the trial, alligators were fed an average of 3% body mass weekly by offering food 3 times per week. Food intake of alligators was closely monitored by counting pellets given in each tank. Since saltwater-exposed alligators gradually refused food, to avoid interactions between food deprivation and saltwater

exposure, saltwater-exposed alligators were fed first while the number of pellets eaten was recorded. Alligators in freshwater were subsequently fed the same number of pellets.

3.3.3. Behavioral Observations

Two main categories of alligator behaviors were determined as "resting" and "feeding," and preliminary observations were conducted to construct ethograms of the most common "resting" and "feeding" behaviors. Resting and feeding behaviors were further defined within each category and approximately 10 different behaviors were recorded (see ethogram in Table 3.1). "Resting" behaviors included behaviors only observed when animals were left undisturbed and post-absorptive, and were recorded on non-feeding days (Tuesdays, Thursdays, Saturdays). The feeding behavior category included behaviors that only occurred during the feeding event. To avoid changes in behavioral responses due to observer presence, resting observations were always conducted using video recordings using a Canon EOS Rebel T2i (18.0 megapixel) camera positioned on a tripod. This allowed for observations of all alligators in the tank at one time. Each video segment was filmed in 20 min intervals 2-3 times a day on days the animals were not fed. Feeding events were equally filmed but always in presence of investigator. However, alligators used in the present study were in culture for at least 6 months prior to experimentation and were thus used to investigator presence during feeding. Feeding behaviors were equally filmed in 20 min segments but only on feeding days (Monday, Wednesday, and Friday). All video recordings were performed 2 weeks prior to treatment (in freshwater) and during the course of the 5-week saltwater exposure study. Thus, observations ranged a total of 7

Table 3.1. The ethogram used to assess the basking and water behaviors of juvenile American alligators prior to and during 5 weeks of 12‰ saltwater exposure.

Activity	Definition
Fighting-Biting	Biting either faces, toes, or tails
Death Roll	Grab each other by the mouths and roll
Floating-Grabbing	Floating stationary in water while grabbing each other by the mouths
Submerged-Grabbing	Completely submerged and stationary while grabbing each other by the mouths
Swimming	Moving in the water
Diving	Complete submersion
Foraging-air	On the basking plate, waiting to grasp food from air
Foraging-water	Looking for food in the water
Foraging-plate	Moves a side of its face back and forth looking for food on the basking plate
Foraging-half	Head and front feet on plate, body in the water. Waiting for food pellets to drop on plate.
Water-Open Mouth	Stationary in the water with mouth open
Floating-Head	Only the head is above water, body is submerged
Plate Dominance	Scaring others off the plate
Feeding-water	Actively grabbing food from water
Feeding-plate	Actively grabbing food from plate
Climbing-tank	Climbing up tank wall trying to get food
Climbing-plate	Climbing on/off basking plate
Vocalization	Hissing at other animals or chirping
Whipping tail	Moving tail side to side in water
Eyes closed	Stationary in water with eyes closed

weeks. Following the trial, recordings of resting behaviors were randomly selected from different days with a minimum of 6 observations per week. Feeding observations averaged 3 per week prior to the salinity trial. However, although animals were offered food 3 times per week during saltwater exposure, no feeding activity was observed during some of these feeding events. Feeding observations therefore averaged 2 per week for 5-week salinity exposure.

3.3.4. Data Collection and Statistical Analyses

Behaviors were sampled using focal sampling which encompassed observing the entire group of 16 saltwater-exposed alligators. Behaviors were recorded using instantaneous sampling recording methods in which each sampling interval was 1 min providing 20 sample points per observation period of 20 min. Score was calculated as number of times the behavior was observed divided by total number of sample intervals (X/20). As score based on instantaneous sampling often (if sample intervals are short, e.g., a few minutes) is similar to proportion of time the group spent on a behavior, we use score as a proxy for time spent on each behavior in all graphs. Most of the data did not meet criteria for normal Gaussian distribution, and hence non-parametric tests followed by post-hoc tests were performed on all data. Score and number of animals performing a behavior during pre-salinity observation and during 5 weeks of saltwater exposure were compared using Kruskal-Wallis followed by Dunn's post-hoc tests. Significance was assumed at $p \leq 0.05$ for all data points. All data shown are mean ± s.e.m.

3.3.5. Multivariate Analysis

Frequency of behavior occurrence across all individuals over the sampling period was used to explore behavioral patterns before and after the salinity trial. All resting and feeding behaviors were included in an unconstrained correspondence analysis (CA) that used chi-square distances to infer relationships among stages of the salinity trial and fourteen different resting and feeding behaviors. The angle and magnitude of each vector is representative of its correlation (angle) and strength of relationship (magnitude) with a given time or behavior. Results of the CA were ordinated using the first two axes, which comprised 94.23% of the total variation.

3.4. Results

3.4.1. Multivariate Analysis

Most of the variation in this dataset (73.48%) is distributed along the CA1 axis, followed by the CA2 axis (20.75%). The pre-salinity time period separated out from all other stages during the salinity trial on the far-left of the ordination (Fig. 3.1). Weeks 1 and 2 were both opposite Weeks 3-5 along the CA2 axis. The 'fighting,' 'death roll,' 'feeding-plate,' 'foraging-plate,' 'submerged-grabbing,' and 'floating-grabbing' behaviors occurred with greatest frequency during the pre-salinity period compared to all others during the salinity trial. Additionally, these behaviors are all tightly correlated given their high level of overlap with each other. The 'floating body,' 'swimming,' 'diving,' and 'foraging-water' behaviors fall approximately along the y-axis, which is indicative of no major differences in frequency between pre-salinity compared to salinity periods. Only

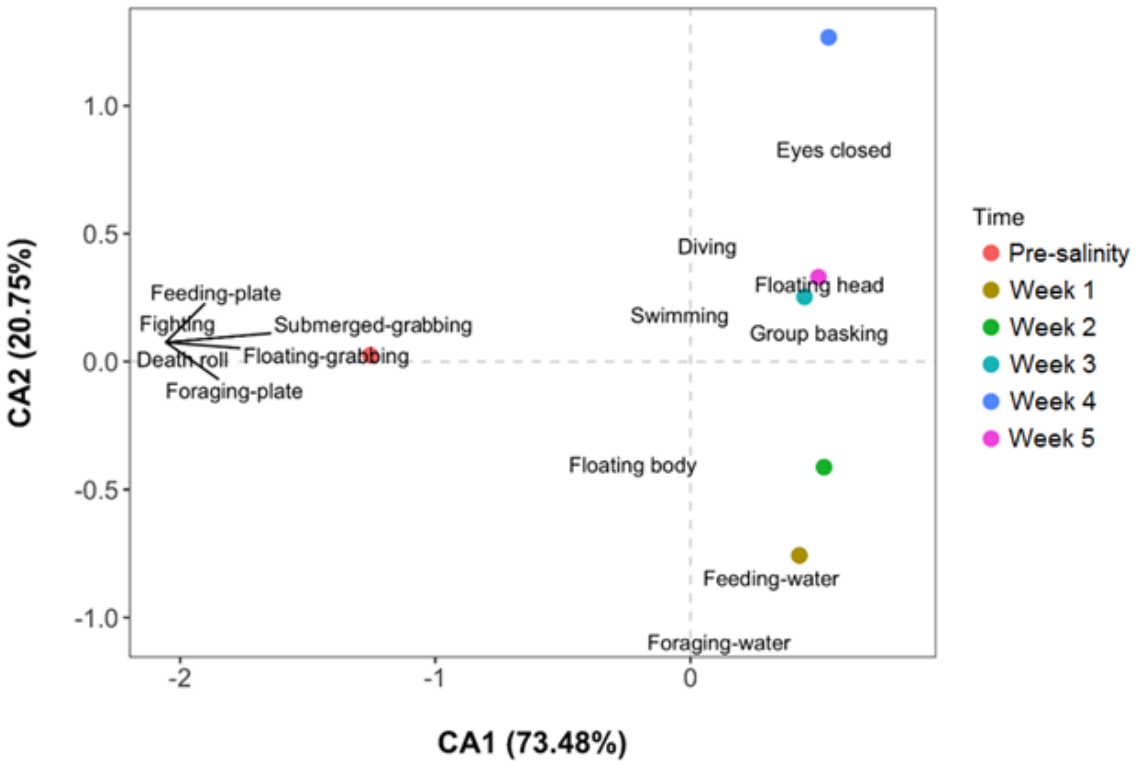

Figure 3.1. PCA plot of alligator foraging and feeding behaviors pre-salinity (freshwater) and during 5-week exposure to 12‰ saltwater. The CA1 axis shows a separation between pre-salinity observations and observations during the trial, while variability across time points during salinity exposure were distributed across the CA2 axis.

‘feeding-water’ showed a positive relationship with weeks 1 and 2 of the salinity trial, while ‘group basking,’ ‘floating head,’ and ‘eyes closed’ behaviors increased in frequency in Weeks 3-5.

3.4.2. Resting/Post-Absorptive

We observed several different behaviors when alligators were post-absorptive and undisturbed, and at any one time many animals were seen floating in the water. The most frequently displayed behavior in the water (Table 3.1) which was displayed by most animals (Fig. 3.2) we called “floating-head” in which the animal’s head was visible but the rest of the body was under water. We also observed a “floating-body” behavior where animals would float at the surface with their entire body visible. However, fewer animals floated at the surface compared with the “floating-head” behavior. True diving behaviors were also observed which involved the entire animal’s body being submerged. In some cases, diving involved swimming under water for a short period of time but most often it included the animal submerging and re-appearing in the same area. Compared with floating with only the head visible, few animals (~5%) were observed to perform actual diving (score of ~0.4) in freshwater, while more animals engaged in swimming at the surface (~30%). When alligators were in freshwater the next most frequently displayed behavior was basking (score of 0.77) (Fig. 3.3), although animals took turns basking and on average ~10-15% of animals were basking at a time.

Exposure to saltwater had significant effects on alligator resting behaviors. For example, we saw gradual and significant reductions in basking behavior as fewer animals

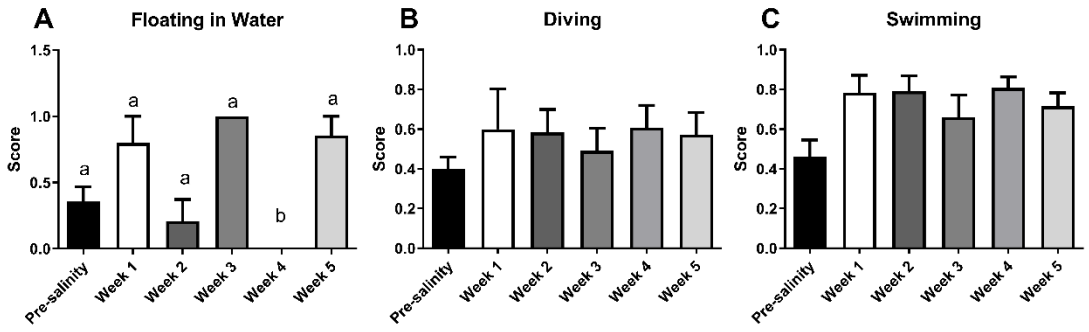

Figure 3.2. **Instantaneous sampling score of animals displaying behaviors related to resting/post-absorptive water activities pre-salinity (freshwater) and during 5-week exposure to 12‰ saltwater.** Data shown are average ± s.e.m. N=16. Dissimilar letters indicate significant ($p \leq 0.05$) between treatments.

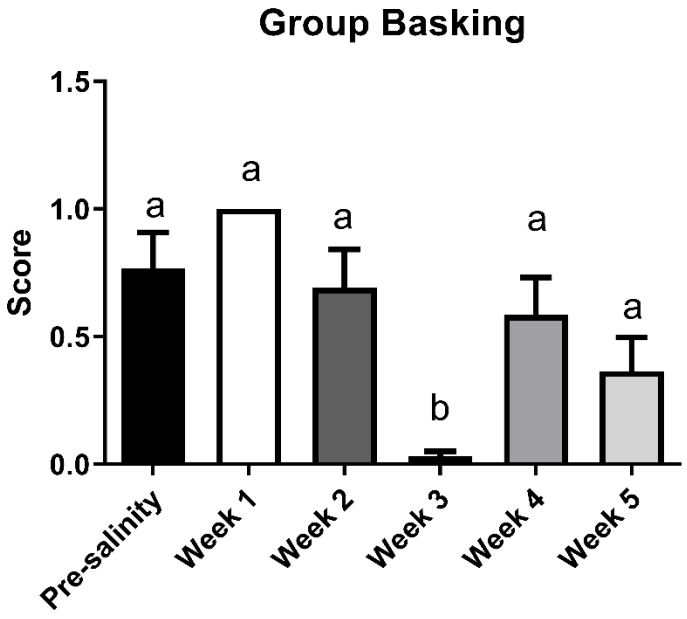

Figure 3.3. Instantaneous sampling score of alligator displaying group basking (more than 1 animal on basking plate) pre-salinity (freshwater) and during 5-week exposure to 12‰ saltwater. Data shown are average ± s.e.m. N=16. Dissimilar letters indicate significant ($p \leq 0.05$) between treatments.

basked when in saltwater. This reduction was evident already during the first week of saltwater exposure and continued until the end of the study. The change in basking behavior coincided with the changes observed in water behaviors. For instance, when alligators were exposed to saltwater, increases (score and number of animals) in swimming, diving, and "floating-body" behaviors were observed. However, the time spent floating in the water with only head visible was not affected by saltwater. In fact, most animals (up to 80%) exhibited this behavior after 2 weeks in saltwater.

One significant and unexpected behavior seen in saltwater alligators was the presence of eye closure when in saltwater. This was a behavior that was not observed when animals were in freshwater but gradually become more prevalent over the course of saltwater exposure. By week 3, alligators spent significantly more time in the water with eyes closed compared with freshwater (Fig. 3.4). At the end of the study, 20% of animals displayed eye closure when in the water.

3.4.3. Feeding

When juvenile alligators were maintained in freshwater, feeding behaviors included grabbing food pellets by either floating at the surface or from a submerged position. Alligators equally fed (score of ~0.06) from a floating and submerged position even though slightly more animals were observed to feed while floating (Fig. 3.5). Alligators also had the opportunity to feed either in the water (pellets dropped in the water) or on the basking plate (pellets dropped on the basking plate). Alligators equally engaged

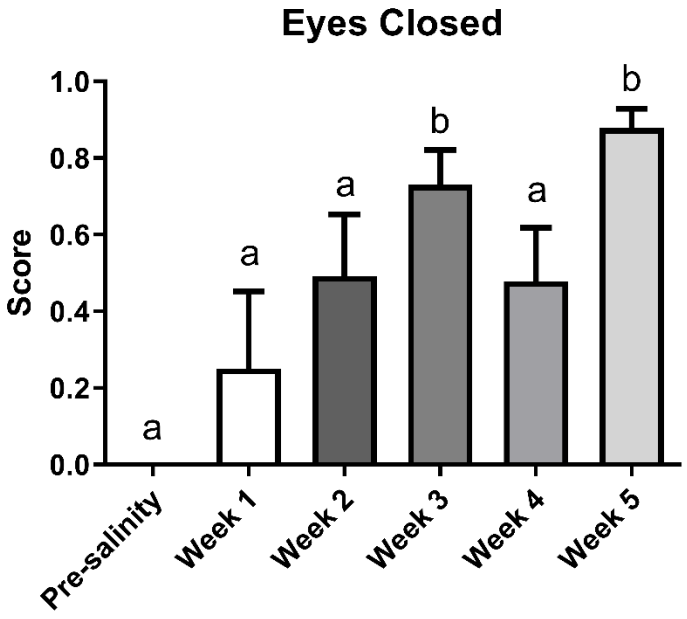

Figure 3.4. Instantaneous sampling score of alligators closing their eyes while in freshwater (pre-salinity) and 12‰ saltwater for 1-5 weeks. Data shown are average ± s.e.m. N=16. Dissimilar letters indicate significant ($p \leq 0.05$) between treatments.

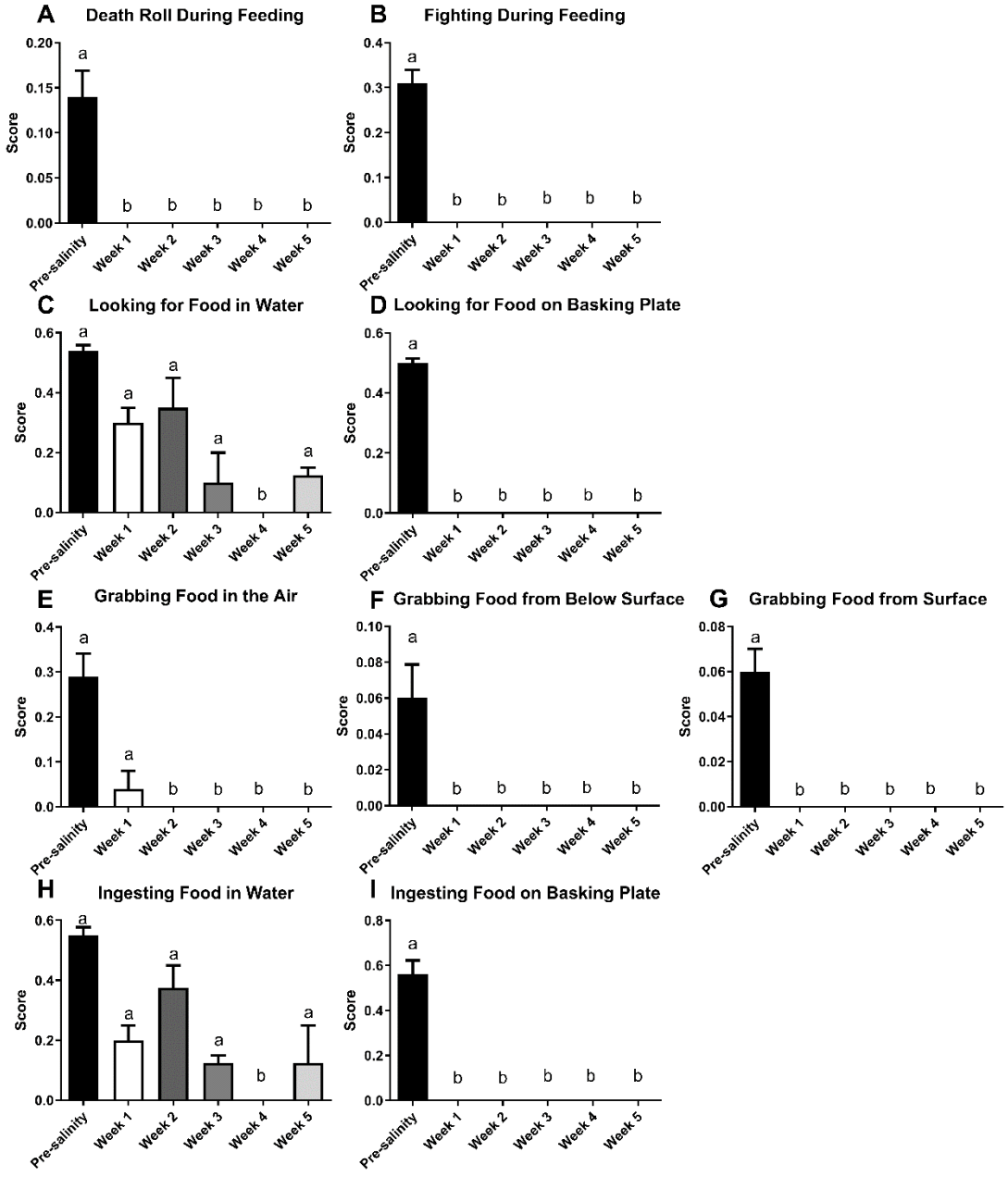

Figure 3.5. Instantaneous sampling score of alligators displaying foraging and feeding behaviors while in freshwater (pre-salinity) and 12‰ saltwater for 1-5 weeks. Data shown are average ± s.e.m. N=16. Dissimilar letters indicate significant ($p \leq 0.05$) between treatments.

in foraging (score of ~0.05) in the water and the plate in freshwater even though a larger number of animals fed from the water.

Exposure to 12‰ saltwater had an immediate and significant ($p \leq 0.05$) effect on feeding behaviors. For instance, a complete cessation of grabbing food pellets from a floating or submerged position was observed. The cessation of grabbing pellets was not only immediate but lasted throughout the duration of salinity exposure. Furthermore, alligators completely ceased to search for pellets on the basking plate and only foraged in the water.

Some aggression was observed during feeding in freshwater as evidenced by death rolling another individual by grappling that animal and both rolling over multiple times during feeding event. Direct fighting included biting another animal most commonly on the snout or head region. Although only few (~12-20%) animals displayed aggressive behaviors towards other alligators, aggressive behaviors completely ceased during the first week of saltwater exposure and were not seen during the entire exposure period.

Finally, to assess morphological effects of saltwater on feeding and feeding ability, we determined bite force in animals maintained in freshwater throughout the study and in alligators maintained in freshwater (pre-salinity) and later exposed to 12‰ for 5 weeks (Fig. 3.6). Although bite force was not significantly affected by saltwater, a small depression in bite force was observed between pre-salinity and 0‰ alligators and after 5-week exposure to 12‰.

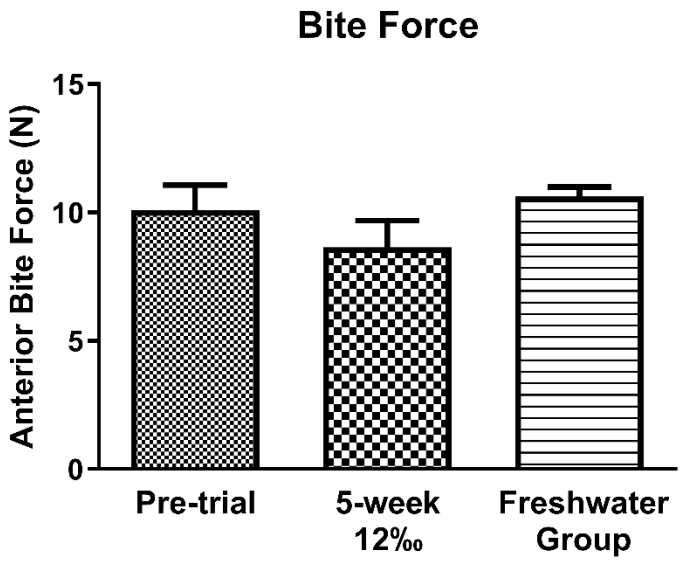

Figure 3.6. Anterior bite force in Newton (N) in alligators pre-salinity (freshwater) and during 5-week exposure to 12‰ saltwater. Data shown are average ± s.e.m. N=10.

3.5. Discussion

This study investigated how juvenile alligators behaviorally responded to exposure to 12‰ saltwater. Significant behavioral changes were observed in all animals both at the onset and during the course of saltwater exposure. Further, behavioral changes occurred in both alligator feeding and resting/basking behavior.

3.5.1. Basking Behavior

When crocodiles experience saline environments, they exhibit distinct behavioral strategies to alleviate an osmotically challenging environment. One strategy is to move to more favorable (less saline) environments or if movement is restricted to seek a more favorable microhabitat which can include burrowing or even aestivation (Taplin, 1988). One of the most significant findings from the present study pertains to basking behaviors. Alligators spent significantly more time basking when in freshwater than when in saltwater, and the percentage of animals basking decreased significantly in the saline environment (~6%, 1 week) compared with pre-salinity (~20%). Crocodiles and alligators use behavioral thermoregulation to adjust body temperature to different physiological needs (Grigg and Seebacher, 2001; Lang, 1987). For example, feeding is followed by digestion which in ectotherms necessitates the elevation of body temperature to accommodate digestive processes and therefore involves finding areas with higher temperatures (Blouin-Demers and Weatherhead, 2001; Gatten Jr, 1974; Slip and Shine, 1988). Saltwater exposure depressed food intake in alligators which may explain the reduction in number of animals basking alleviating the need to elevate body temperature.

Indeed, a correlation with the reduced feeding rate is further plausible as recently fed alligators select for higher temperature environments (Lang, 1979) which decreases gastric residence time (Diefenbach, 1975). On the other hand, fasted alligators select for lower temperatures (Lang, 1979). However, saltwater exposure significantly affected physiology and endocrinology of alligators as seen by elevated levels of plasma Na^+, K^+, and Cl^- and significant reduction in body mass (Faulkner et al., 2018). It is therefore very likely that the reduction in basking was closely tied to changes in the animals' physiology. For instance, when animals basked during freshwater exposure, each animal could frequently spend up to 1-2 or more hours on the basking plate (Petersen, personal observations) and would be completely dry at the end of the basking period. As saltwater exposure caused significant dehydration (Faulkner et al., 2018; Laurén, 1985), limiting basking may have been a behavioral response to avoid further dehydration. Concomitant with these observations, an increased number of animals were seen in saltwater compared with freshwater. For example, at the end of the study (by week 5) most animals were seen in the water with just the head above water and the body below. There was further an increase in animals floating with their entire body at the water's surface, while more animals were seen diving and swimming in salt- versus freshwater. In addition, thermoregulating to reduce body temperature can play a role in responding to salinity stress. Alligators exhibit a lower body temperature in water than while out of water (Brisbin Jr et al., 1982; Diefenbach, 1975). Because lower metabolism may reduce flux of ions and water (Beaupre, 1996; Raven and Smith, 1978), animals in the present study

spending more time in the water (water temp = 24.14 ± 0.46°C) than basking (air temp = 26.00 ± 0.79°C) may aid in alleviating dehydration.

The increased presence of alligators in saltwater seems juxtaposed to physiological data showing that alligators are unable to prevent Na^+ influx in saltwater. For example, in unfed alligators ranging 310-586 g (similar to animals used in the present study), Na^+ influx averaged ~10.8 μmol 100 g^{-1} h^{-1} when alligators were exposed to 35‰ for up to 4 hours while water efflux was 0.25 ml 100 g^{-1} h^{-1} (Mazzotti and Dunson, 1984). Freshwater also has significant effects on electrolyte balance as net Na^+ and K^+ loss occurs in solutions up to 1 mmol L^{-1} Na^+ and 0.4 mmol L^{-1} K^+ and in freshwater (Ellis and Evans, 1984; Taplin et al., 1982). In addition, hatchlings (0.03-0.07 g) in freshwater exhibited a whole-body Na^+ efflux of 3.9 μmol 100 g^{-1} h^{-1} (Ellis and Evans, 1984). Thus, as alligator integument is not impermeable to fluxes of electrolytes it seems contradictory that juvenile alligators would spend more time in saltwater which would increase risk of dehydration and influx of ions (Na^+, Cl^-, K^+). The lack of access to freshwater, burrowing, or aestivation is likely to have resulted in behaviors that alleviate negative physiological effects of the enhanced time in saltwater. One novel behavior never previously observed in freshwater was the presence of closing eyelids when sitting immobile in saltwater. Alligators have an upper and lower eyelid in addition to a third nictitating membrane that is transparent and enables the animal to see under water (Rehorek et al., 2005). It is unknown whether alligators used the nictitating membrane to cover the eye as the outer eyelid was closed in water. Alligators in saltwater began showing signs of closing the outer eyelid as early as the first week of exposure (Fig. 3.5). The occurrence of this

behavior significantly increased with time and peaked during week 5. It is important to note, however, that not all alligators in saltwater displayed this behavior, but about 20% of animals in saltwater had their eyes closed. Closing the outer eyelid is usually a protective measure to protect the eye during prey capture (Kerfoot et al., 2016). However, mucus membranes in eyes, nostrils, mouth, or vent provide a thinner barrier through which ions can be exchanged between the body and environment (Taplin, 1985). Thus, closing of the eyelid can be interpreted as a protective measure to prevent influx of ions (Na^+, K^+, Cl^-) and likely efflux of water when alligators were in saltwater. The observed behavioral responses thus correspond well with demonstrated physiological responses described previously (Faulkner et al., 2018).

Collectively, data reveal an overall significant increase in behaviors related to the water when alligators are confined to saline environments. Novel behaviors related to alleviation of dehydration and ion influx were observed. Observations further showed that saltwater exposure, in particular when alligators have limited access to freshwater, has a significant effect on alligator's thermoregulatory behavior as evidenced by reduced basking which could affect important physiological functions such as digestion and ion flux.

3.5.2. Feeding

When maintained in freshwater, alligators equally fed on food pellets from either a floating position in the water, from a submerged position or climbed the basking plate and took pellets from the plate. These observations correspond well with a previous study

on captive juvenile alligators which demonstrated that juveniles fed equally well above compared with underwater (Kerfoot et al., 2016). However, studies on wild adult alligators in which feeding behaviors were recorded using an attached crittercam revealed that position in the water column significantly affected success rate of prey capture. For example, alligators were twice as successful at capturing prey from a submerged position compared to attempts made at the water surface (Nifong et al., 2015). As conditions between captive-raised and wild alligators are different (i.e., variability in food abundance in the wild), feeding attempts would differ between captive and wild individuals. However, juvenile alligators in the present study displayed similar feeding behaviors (from surface and submerged position) as seen in adult wild alligators which suggests behaviors displayed in the culture tanks comprise innate feeding behaviors.

Behavioral strategies of crocodilians when exposed to elevated saline environments include selective drinking and feeding (Taplin, 1988). In laboratory settings, including the present study, juvenile alligators refused feeding already during the first week of salinity exposure a trend which was continuous through-out the course of salinity exposure (Faulkner et al., 2018; Laurén, 1985). The reduction in feeding is, however, attributed to osmotic water loss across the integument rather than starvation (Faulkner et al., 2018; Laurén, 1985) since the control group (0‰) eating similar amounts of food as the saltwater (12‰) group maintained and even gained a small amount of weight in 5 weeks (Faulkner et al., 2018). Due to physiological differences between Alligatorids and Crocodylids, osmotic strategies significantly differ between the two crocodilian families (Taplin, 1988; Taplin et al., 1982). For example, American crocodiles (*Crocodylus acutus*)

maintained in 10‰ continued feeding and even rapidly gained weight, and *Crocodylus acutus* exposed to 18‰ still gained weight albeit at a slower rate (Dunson, 1982). In two freshwater turtles (*Chelodina expansa* and *Emydura macquarii*) exposed to 15‰ saltwater 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). Whether similar behavioral responses occurred in juvenile alligators is possible. However, contrary to freshwater turtles, juvenile alligators lose significant amounts of body mass in saline environments (Faulkner et al., 2018; Laurén, 1985) and therefore are not able to tolerate prolonged exposure to even low (12‰) saline environments. Furthermore, as many animals depend on metabolic water from food items (Willmer et al., 2009) the reduced feeding could have exacerbated water loss and attributed to significant dehydration.

As expected, significant changes in feeding behaviors (grabbing, foraging, active feeding) were concomitant with the reduced food intake observed at the onset of saltwater exposure. For instance, aggressive behaviors during feeding were completely abolished during the first week of salinity exposure and remained absent throughout the study period. Foraging for pellets and actively feeding from air or water were significantly reduced while feeding from the basking plate was completely absent starting from the first week of saltwater exposure. After Week 2 alligators no longer attempted to catch pellets in the air and only resided to forage for and eat pellets in the water for the remaining exposure period. However, the number of animals observed foraging and actively feeding dropped significantly to less than 5% after only one week of exposure. As grabbing pellets from either water's surface or submerged position completely ceased during the first week of

exposure, alligators were observed feeding by slowly moving towards the pellets and gradually moving the head to ingest them. Prior to the study we had hypothesized whether saltwater would interfere with normal muscle function due to the high influx of electrolytes Na^+, Cl^-, and K^+. Muscle function of the jaw muscles are particularly important in alligators as the jaws are used to catch, subdue and ingest prey (Reilly et al., 2001). Thus, any negative effects of ion perturbations on muscle function may have disrupted jaw movement and function. Although data did not reveal any significant effects of saltwater on bite force, a small depression in bite force was observed compared with the same alligators prior to exposure and compared with alligators maintained in freshwater (Fig. 3.6).

It is currently unknown whether the reduction in feeding is brought about by changes in foraging behavior or whether changes in foraging behavior affected food intake. However, this study shows that saltwater affects feeding behavior which may ultimately affect ability to catch, subdue, and ingest prey which could result in starvation when alligators are exposed to even low saline environments.

Overall, behavioral frequency exhibited during the pre-salinity trial period is very different from all weeks of the trial. With the exception of feeding underwater, the pre-salinity trial period had greater frequencies of all other feeding and agonistic behaviors, whereas resting behaviors tended to occur at greater frequencies during the salinity trial. The increased frequency of resting behaviors under salinity stress may be one of the mechanisms alligators use to cope during periods of salinity stress. The reduction of active behaviors could also potentially occur in response to other sources of physiological stress.

In summary, alligators exposed to saltwater exhibited behaviors related to the osmotic physiological challenges experienced by the animals. To avoid further dehydration, animals reduced basking behavior and remained inactive in the water most often with eyelids closed to reduce ion and water fluxes. Feeding behaviors were also reduced which could have further affected basking behavior. The reduced feeding and previously reported reduction in weight due to dehydration indicates that alligators cannot tolerate prolonged exposure to even low (12‰) saline environments. Future studies should investigate behaviors of alligators during night time where most wild alligator spend the time in the water. How saltwater affects diurnal behaviors in captivity versus wild alligators is an avenue worth pursuing. Together, this study provided novel information of alligator behavior in low saline environments. Facilitating knowledge of how future storm surges or droughts affect alligator physiology and behavior is pertinent in understanding population effects and the impacts of salinization in freshwater near coastal Gulf of Mexico ecosystems.

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CHAPTER IV

SHORT-TERM EXPOSURE TO 12‰ SALTWATER HAS SIGNIFICANT EFFECTS ON THE ENDOCRINE PHYSIOLOGY OF JUVENILE AMERICAN ALLIGATORS (*Alligator mississippiensis*)*

4.1. Abstract

American alligators (*Alligator mississippiensis*) mainly inhabit freshwater habitats but can be exposed to a wide range of salinities during storm surges, droughts, or from alterations in freshwater flows. Although some salinization events last weeks, others only last a few days. This study assessed changes in the endocrine function of the renin-angiotensin-aldosterone system (RAAS) and steroid hormone production (steroidogenesis) in juvenile alligators exposed to saltwater (12‰) for 7 days. We quantified plasma levels of angiotensin II and the corticosteroids (aldosterone, corticosterone, and 11-deoxycortisol). Various progestogens, androgens, and estrogens were further assessed. The protein expression for the RAAS enzymes, renin and angiotensin converting enzyme (ACE), was quantified immunohistochemically in kidney and lung tissue, respectively, and histology was performed on kidney, lung and gonad tissues. Finally, blood biochemistry parameters such as electrolyte levels and diagnostic indicators for dehydration, renal, and hepatic function were measured. Corticosterone,

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11-deoxycortisol, Na^+, Cl^-, total protein, albumin, uric acid, and cholesterol levels were all significantly elevated in alligators exposed to saltwater compared with alligators in freshwater. The levels of 17β-estradiol and estrone were significantly lowered while histology showed alterations in gonad tissue in the saltwater exposed group. In contrast, while there were no effects of exposure on aldosterone levels, angiotensin II was significantly reduced in saltwater exposed alligators. These results correlated with significantly decreased expressions for both renin and ACE in kidney and lung tissue. Overall, this study showed that short-term exposure of alligators to 12‰ saltwater has significant endocrine effects on juvenile alligators.

4.2. Introduction

American alligators [*Alligator mississippiensis* (Daudin, 1802)] are native to southeastern United States and although larger alligators are known to forage in saltwater and some have even been reported foraging off-shore (Elsey, 2005), alligators do not tolerate saline environments for prolonged periods of time without access to freshwater (Faulkner et al., 2018; Lauren, 1985; Pidcock et al., 1997). Nevertheless, many alligator habitats are prone to frequent saltwater intrusions from storm surges which push seawater into normally freshwater areas (Emanuel, 2005; Hoyos et al., 2006). Rivers, lakes and freshwater wetlands are further frequently exposed to salinization due to anthropogenic perturbations that cause changes in freshwater or underground water flow (Day et al., 2000; Herbert et al., 2015). Furthermore, gradually rising sea levels can introduce saline water into inland groundwater fed wetlands resulting in salinization of inland areas not

directly affected by seawater intrusion (Wood and Harrington, 2015). Finally, predicted impacts of salinization are higher for freshwater than saltwater marshes in the Gulf of Mexico coastal areas (Twilley et al., 2016).

Steroid hormones play crucial roles in coordinating sex determination and differentiation, metabolism, behavior, and responses to stress (Ali et al., 2018; Hughes, 2001; Lee et al., 2015; Mattsson and Olsson, 2007; Morton, 2010; Sinisi et al., 2003). Steroid hormones are produced by the metabolic process of steroidogenesis, which converts cholesterol to the major bioactive steroid hormones such as glucocorticoids, mineralocorticoids, androgens, estrogens, and progestogens (Bremer and Miller, 2014; Payne and Hales, 2004). The major classes of steroid hormones produced (and quantified in this study) include progestogens (pregnenolone, progesterone, 17α-hydroxyprogesterone, 17α-hydroxypregnenolone), androgens (androstenedione, testosterone, 5α-dihydrotestosterone), estrogens (17β-estradiol, estrone, estriol), and corticosteroids (aldosterone, 11-deoxycortisol, corticosterone).

While glucocorticoid hormones (such cortisol) are mainly involved with stress responses (Exton, 1979), mineralocorticoid hormones (such as aldosterone) regulate salt/water balance through the endocrine system known as the renin-angiotensin-aldosterone system (RAAS) (Norris and Carr, 2013). The other three hormone classes (progestogens, androgens, estrogens) regulate sexual maturity, secondary sexual characteristics, and reproduction/reproductive cycling. Steroidogenesis occurs in several endocrine glands but the hormones produced are gland specific as enzyme activities are regulated differently depending on endocrine gland (Bremer and Miller, 2014). However,

by measuring plasma steroid hormone levels a comprehensive picture of endocrine pathways involved in the stress response, salt/water balance and reproduction can be obtained.

The renin-angiotensin-aldosterone system (RAAS) is an important endocrine system of vertebrates that is involved with maintaining homeostasis of water/salt balance, and regulating physiological responses under dehydration or high salt loads (Nishimura, 2017). A drop in blood volume and/or sodium concentration stimulates the release of the enzyme renin from the kidneys. Once released, renin catalyzes the conversion of the hepatic peptide angiotensinogen to angiotensin I. In turn, angiotensin I is converted by the angiotensin converting enzyme (ACE) (mainly located in the endothelial cells of lung capillaries) to angiotensin II. The peptide hormone angiotensin II stimulates the adrenal cortex to synthesize and release the mineralocorticoid steroid hormone aldosterone which exerts direct effects on kidney nephrons by enhancing retention of renal Na^+ in exchange for K^+ excretion which results in restoration of blood volume and pressure via the passive reuptake of water (Bollag, 2014; Norris and Carr, 2013).

In a recent study, we demonstrated that long-term (5 weeks) exposure of juvenile alligators to low salinity (12‰) caused significant decreases in plasma angiotensin II, while aldosterone levels were unchanged. In contrast, plasma electrolyte (Na^+, Cl^-) levels were significantly elevated and animals showed clear signs (body mass loss) of dehydration (Faulkner et al., 2018). It is currently not known how RAAS in saltwater exposed alligators is regulated, and where in the RAAS cascade the inhibition of angiotensin II occurs. As described above, renin and angiotensin converting enzyme

(ACE) are two key enzymes of RAAS (Nishimura, 2017). Granulated renin producing cells are located in the juxtaglomerular (JG) area along the afferent arteriole in the glomeruli in the kidney nephrons (Nishimura, 2017; Sokabe and Ogawa, 1974). The juxtaglomerular area further has a tubular component located near the distal tubules (macula densa) in mammals (Nishimura, 2017). At present, there is no evidence that a tubular component of JG exists in non-mammalian vertebrates (Bailey and Nishimura, 1984; Nishimura, 2017). The apparent lack of the macula densa in reptiles, such as alligators, is interesting as this area senses Na^+/Cl^- levels in tubular fluid, and exerts a negative feedback on renin synthesis when levels are high. The absence of this system in alligators therefore suggests different regulatory mechanisms of RAAS compared with mammals. Whether regulation involves only renin, or the other RAAS enzyme ACE, is not understood. ACE is found in most endothelial cells in the vasculature and is particularly highly expressed in lung tissue (Fournier et al., 2012). In mammals, regulation of ACE varies with tissue type but infusion of angiotensin II or pulmonary hypertension reduces serum ACE activity in rats (Jederlinic et al., 1988; Schunkert et al., 1993). Therefore, it is of interest to understand when during 12‰ saltwater exposure angiotensin II suppression occurs, and whether aldosterone levels are affected initially.

Interestingly, chronic (5 weeks) exposure to an environmental stressor, such as salinity, significantly elevated 17α-hydroxyprogesterone, testosterone, 17β-estradiol, estrone, and estriol levels in juvenile alligators exposed to 12‰ saltwater (Faulkner et al., 2018). These findings suggest potential reproductive consequences from long-term salinity exposure but further studies are needed to determine short-term effects and to

assess changes in gonad morphology which could indicate important effects on alligator steroidogenesis.

As duration of salinization of freshwater wetlands vary (Ramsey et al., 2011) and alligators can be equally exposed to short-term (few days) salinization events, it is imperative to further investigate endocrine pathways in alligators exposed to saline environments. Considering the significant endocrine effects reported in juvenile alligators (Faulkner et al., 2018) after 5 weeks exposure to 12‰ saltwater, it is expected that time-dependent responses exist. Therefore, alligators may be able to tolerate short-term saltwater exposure if physiological responses differ from long-term exposure.

The goal of this study was therefore to assess how saltwater (0.5-30‰) affects key endocrine pathways (RAAS and steroidogenesis) in alligators, and to explore changes in plasma biochemistry (diagnostic biomarkers for dehydration, electrolytes, hepatic and renal function). To that end, juvenile alligators were exposed to 12‰ saltwater for 7 days and effects on angiotensin II, and the steroid hormones comprising: androgens (androstenedione, testosterone, 5α-dihydrotestosterone), progestogens (pregnenolone, progesterone, 17α-hydroxyprogesterone, 17α-hydroxypregnenolone), estrogens (17β-estradiol, estrone, estriol), and corticosteroids (aldosterone, corticosterone, 11-deoxycortisol), were determined. In addition, histology was performed on lung, kidneys, and gonad tissues to examine morphological changes in key RAAS and steroidogenic organs, and immunohistochemistry was used to determine renin and ACE protein expressions in kidneys and lungs, respectively. Based on findings from our 5-week chronic saltwater study (Faulkner et al., 2018), and that saltwater exposed animals displayed

behavioral changes (e.g., more time spent in water compared with basking) (Petersen unpublished), we hypothesized that 7 days exposure to 12‰ saltwater would equally result in significant endocrine effects.

4.3. Materials and Methods

4.3.1. Animals and Husbandry

Juvenile American alligators (2-3 years old; average body mass: 1,243.1 ± 41.7 g; snout-to-vent length: 38.3 ± 0.4 cm) were generously donated by Rockefeller Wildlife Refuge (Grand Chenier, LA, USA), and transported back to Texas A&M University at Galveston (TAMUG). Alligators were housed in a constant temperature (26°C) and photoperiod (12:12 light:dark cycle) controlled room to ensure water and air temperature were maintained at 26 ± 1°C (tested periodically). Experimental animals were kept at a stocking density of 8 animals in 380 L Rubbermaid stock tanks (Rubbermaidcommercial.com) containing 90 L of fresh water and a basking plate. Preliminary data on plasma stress hormones levels (data not shown) showed no effect of this stocking density on stress levels. A 160 W UVB light heat lamp (Zoo Med Laboratories, Inc., San Luis Obispo, CA, USA) was placed over the basking plate to allow animals to bask. Prior to and during the trial animals were fed an average of 3% body mass per week of Mazuri® Reptile Diet (PMI Nutrition International, St Louis, MO, USA) and to avoid build-up of waste products, water was changed in each tank approximately 24-30 hours after each feeding (three water changes per week).

4.3.2. Experimental Design

Prior to this study, juvenile alligators had been at TAMUG's culture facility for over 1 year at the above stocking density. At the initiation of the study, one group was exposed to freshwater (FW, control, N=8) and another to saltwater (SW, 12‰, N=8) for 1 week (7 days). The selection of a salinity concentration of 12‰ was based on a previous study by Faulkner et al. (2018), and was chosen to allow comparison between short-term (1 week) and long-term (5 weeks) saltwater exposure on alligator endocrine systems. This level of salinity is further considered hyper-osmotic (isoosmotic ~10‰) to alligator plasma (Lauren, 1985; Morici, 1996), and represents an environmentally relevant level as ongoing environmental monitoring studies performed by our lab show most wild juvenile alligators to be found in salinities ranging from 0.4-13‰, with few (5 out of 54) captured in salinities as high as 22‰ (see Chapter V).

While all alligators were still in fresh water, blood was sampled (pre-trial) (see details below) to determine within-group effects. The SW group was then gradually exposed to increases in salinity over eight days. Salinity was increased every 2 days from 0‰ to 4‰, 8‰, and 12‰. On the 8^{th} day, the salinity level of 12‰ was maintained for 7 days, after which another blood sample was taken (post-trial) to allow comparison within and between groups. The freshwater group was maintained in dechlorinated city tap water 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). Saltwater was attained by mixing filtered and treated seawater

with tap water in appropriate proportions. Water was monitored for water quality parameters (e.g., nitrogenous compounds, pH etc.) once a week using LaMotte Inc. water quality test strips. All seawater was obtained from Gulf of Mexico off Galveston Island, TX, USA. During the entire trial, salinity levels in stock and exposure tanks [freshwater (0‰) and saltwater (12‰)] were verified with a salinity meter (WD-35604-00, Oakton Instruments, Vernon Hills, IL, USA) twice daily. There were no fluctuations in either freshwater 0‰ or saltwater (12‰) during the trial.

4.3.3. Blood and Tissue Sampling

A 3 ml blood sample was collected from each alligator from the occipital sinus using a 23-gauge non-heparinized needle and 3 ml syringe. Blood was quickly placed in a non-heparinized microfuge tube and immediately centrifuged for 2 min at 10,000 × g to separate the plasma. Non-heparinized needles, syringes, and tubes were used as plasma biochemistry analyzes (see below) required the absence of heparin in collection syringes/tubes (recommended by Texas A&M University's Veterinary Medical Diagnostic Laboratory). Blood clotting was not an issue. Plasma from each animal was then aliquoted into 2 lithium heparin tubes; one for hormone analysis and one for plasma biochemistry analysis. Plasma samples were stored at -20°C until analysis (max storage duration < 1 month). Following post-trial blood sampling, each alligator was then anesthetized by placing it in a plastic tote (~3.0 L) 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. Condition factor (K) was determined by $K=W/L^3$ where W = weight; L = snout-vent length (SVL). Liver, lungs, kidneys, heart, and tail muscle were removed and placed in -80°C until further analysis. The animals were randomized prior to the trial, upon dissection it was determined that the freshwater and saltwater groups were comprised of both females and males. All studies were in compliance with Texas A&M University's Animal Care Committee under AUP IACUC 2015-0347.

4.3.4. Blood Plasma Biochemistry Analysis

Determination of plasma biochemistry levels was performed by Texas A&M University's Veterinary Medical Diagnostic Laboratory (College Station, TX, USA). Samples were analyzed on a Beckman Coulter AU480 analyzer and were quantified for 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) (Table 4.2).

4.3.5. Steroid Hormone Extraction from Blood Plasma

All chemicals and reagents used in sample preparation were purchased from Sigma-Aldrich Co. LLC. (St. Louis, MO, USA). Plasma samples were thawed on ice, and 500 µl aliquots spiked with internal standards d9-progesterone and d3-estradiol. A stable isotope dilution method was used for the quantification of all analytes (Pitt, 2009). This

method used the response ratio of an analyte relative to a stable isotope internal standard, in conjunction with the standard curve slope equation, to calculate analyte concentrations. A representative analyte (as a stable isotope) was chosen as the internal standard in any given chromatographic method. Therefore, d9-progesterone was used as internal standard for progesterone, pregnenolone, 17α-hydroxyprogesterone, 17α-hydroxypregnenolone, androstenedione, testosterone, 5α-dihydrotestosterone, 11-deoxycortisol, corticosterone, angiotensin II, and aldosterone. Whereas, d3-17β-estradiol was internal standard for 17β-estradiol, estrone, and estriol. The plasma was suspended in 5 ml Milli-Q water and liquid:liquid (L:L) extracted twice using methyl tert-butyl ether (MTBE). The L:L extraction involved mixing (by vortexing) the plasma with 5 ml MTBE for 10 seconds to allow the partitioning of analytes from the aqueous phase (i.e., plasma) to the solvent (MTBE) phase. Mixtures were then centrifuged for 5 minutes at 2,000 × g to let the plasma and MTBE to separate into distinct layers. Pooled MTBE layers were dried under nitrogen gas with the residue reconstituted in 50 µl 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 mM sodium bicarbonate, and incubated at 60°C for 3 minutes. The resulting dansylated estrogens (Dns-estrogen) were suspended in 500 µl Milli-Q water and L:L extracted twice with 500 µl 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.

4.3.6. LC-MS/MS Analysis of Steroid Hormones

The LC-MS/MS system consisted of an Agilent 1260 UHPLC system 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 mM ammonium formate. The mobile phase gradient transitioned from 30% (B), increased linearly to 70% over 3 minutes and from 70% to 95% in 6 minutes. The gradient was subsequently decreased from 95% to 70% in 3 minutes and from 70% to 30% (initial condition) over 3 minutes with the flow rate maintained at 0.4 ml min^{-1} (and total run-time of 15 mins).

Plasma hormones were detected in positive ion electrospray ionization (ESI+) mode with nitrogen as 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 (MRM) mode with argon as 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 (d9-

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), *m/z* 509.3 → 171.1 (Dns-d3-estradiol), *m/z* 523.8 → 70.3 (angiotensin II), and *m/z* 361.2 → 343.2 (aldosterone).

4.3.7. Histological Procedures

Lung, kidney, and gonad tissues were fixed in 4% paraformaldehyde solution, dehydrated in a series of increasing concentrations of ethanol (50-100%), embedded in paraffin, and sectioned at 7 μm thickness on a rotary microtome (Leica, Buffalo Grove, IL). Sections were then deparaffinized in xylene, rehydrated through decreasing concentrations of ethanol (100-50%), stained with hematoxylene and eosin solutions (Sigma-Aldrich, St. Louis, MO, USA), and mounted with Cytoseal mounting media (Fisher Scientific, Hampton, NH, USA). Histological pictures were captured by a Cool-SNAP camera (Photometrics, Tucson, AZ, USA) using a light microscope (Nikon Eclipse E600, Nikon, Japan). For gonad histology, each cell type was identified by its morphology as described by (Guillette et al., 1994). Cells were counted with a tally counter and analyzed for each treatment group.

4.3.8. Immunohistochemistry

The immunoreactive (IR) signals of ACE and renin protein expressions in lung and kidney tissues, respectively, were detected by 3’3-diaminobenzidine (DAB) substrate (Vector Laboratories, Burlingame, CA, USA) according to Moore et al., (Moore et al., 2009), and Rahman and Thomas (Rahman and Thomas, 2018). Briefly, the paraffin-

embedded lung and kidney tissues were sectioned at 7 μm, deparaffinized in xylene and rehydrated in a decreasing series of ethanol. Sections were then rinsed with phosphate buffered saline (1× PBS, pH 7.4; Fisher Scientific), and treated with retrieval solution (10 mM citric acid, pH 6) for 10 min, rinsed in 1× PBS, and incubated in blocking solution (1% bovine serum albumin containing PBS) at room temperature for 1 h. After blocking, sections were rinsed in 1× PBS and incubated with mouse monoclonal anti-ACE (Sigma-Aldrich) and anti-renin (Novus Biologicals, Littleton, CO, USA) primary antibodies (dilution 1:100) overnight at 4°C. These monoclonal antibodies were generated against conserved amino acid sequences, specific to the C-terminal, in other vertebrate ACE and renin genes which are distinctly identical (~60%) to the corresponding region of amino acid sequences (C-terminal) in alligator ACE and renin genes. These antibodies have been validated previously in other vertebrates (Danilov et al., 2014; Flores-Monroy et al., 2014; Hu et al., 2014). The negative control slides were incubated with PBS instead of ACE or renin primary antibodies in lung or kidney tissues respectively. The specificity of the immunohistochemical staining reaction with renin antibody in the alligator kidney was also confirmed by blocking with recombinant protein (Novus Biologicals) corresponding to the amino acid sequence of vertebrate renin. After incubation, slides were rinsed in 1× PBS and incubated with anti-mouse secondary antibody (Cell Signaling, Danvers, MA, USA) at room temperature for 2 h. The IR signals were detected using DAB substrate in dark condition, and the photographs were captured using Nikon Eclipse microscope. The IR intensity of ACE and renin protein expressions were estimated using ImageJ software according to Schneider et al. (Schneider et al., 2012).

4.3.9. Statistical Analyses

Normality testing was performed using the Shapiro–Wilk's test ($p \leq 0.05$). Statistical significance was determined using parametric and nonparametric tests. Parametric analyses were conducted using one-way ANOVAs with Tukey post-hoc tests, or where appropriate, Student's *t*-tests. When normality was not met, nonparametric testing was performed using Mann-Whitney U tests. All end-points (blood biochemistry parameters and steroid hormones) were analyzed for differences between sexes (male vs female within each group and saltwater-exposed female (or males) versus freshwater-exposed female (or male) alligators between groups). Only estrogens showed significant sex differences between groups (females only) while blood biochemistry parameters (Na^+, Cl^-, uric acid) were significantly different between groups for each sex. However, as results for individual sexes were reflected in the whole group assessments, we chose to combine sexes and analyze differences based on whole group effects and within group effects (pre- vs post-trial) rather than focus on sex differences. All data presented shown in the text, figures and tables are group mean ± standard error of the mean (s.e.m.), and p values ≤ 0.05 were considered statistically significant. Statistical analyses were performed using GraphPad Prism version 5.0.

4.4. Results

4.4.1. Food Intake and Body Morphometrics

Body morphometrics are shown in Table 4.1. The similar food intake in the SW and FW groups resulted in negligible changes in body mass (wet body mass), snout-vent-

length (SVL), and total length within and between groups (Table 4.1). Condition factor (K) was significantly increased within FW and SW groups post-trial compared with pre-trial (Table 4.1). Finally, liver wet weight was recorded upon dissection and hepatic/somatic index (HSI) was significantly ($p=0.003$) lower in SW compared with FW alligators (Table 4.1).

4.4.2. The Endocrine Effects of 7-Days Saltwater Exposure on Blood Plasma Biochemistry

Concentrations of plasma biochemistry parameters in FW and SW alligators are shown in Table 4.2. Plasma potassium levels (K^+) remained similar pre- and post-trial within FW and SW groups, while no significant differences on either sampling day were detected between FW- and SW-acclimated alligators. Plasma Na^+ and Cl^- levels were further similar between FW and SW alligators prior to the trial and did not significantly change in FW alligators, however, both Na^+ and Cl^- were significantly elevated at the end of the 7-day saltwater exposure compared with pre-trial and pre- and post-trial levels in FW alligators (Table 4.2). Plasma total protein, albumin, globulin levels and A/G ratio were similar between FW and SW alligators pre-trial but both total protein and albumin levels were significantly higher post-trial compared with pre-trial values in SW alligators. Uric acid levels were below detection limit (1.5 mg dl^{-1}) in FW and SW alligators before the trial and did not change in FW alligators, however, levels were significantly higher in the SW group after 7 days at 12‰ compared with pre-trial values. On the other hand, parameters diagnostic of renal and hepatic function, creatine kinase, creatinine, aspartate

aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and bilirubin levels (Table 4.2) were not significantly affected by 7-days exposure to 12‰ saltwater. Plasma minerals such as phosphorous (P) were significantly elevated in the SW group after 7 days in 12‰ saltwater (Table 4.2). Cholesterol levels were similar in FW and SW alligators pre-trial, and did not change in the FW group, but these levels were significantly elevated in the SW group compared to pre-trial values (Table 4.2).

4.4.3. The Endocrine Effects of 7-Days Saltwater Exposure on Steroid Hormones and the Renin-Angiotensin-Aldosterone System

The levels of both glucocorticoids, 11-deoxycortisol and corticosterone (CORT), were significantly elevated after 7 days in 12‰ saltwater compared with pre-trial values (Fig. 4.1A,B). Interestingly, this finding is contrary to FW alligators where both hormones were significantly reduced post- compared with pre-trial. Thus, corticosterone and 11-deoxycortisol levels in SW alligators were significantly higher in the SW group compared with the FW group post-trial.

The analysis of progestogens showed pregnenolone, which is a precursor to progesterone and 17α-hydroxypregnenolone, to be significantly reduced post-trial compared with pre-trial values in SW alligators ($p=0.05$) (Fig. 4.2A). However, pregnenolone levels were not significantly different as compared with the FW group (pre- and post-trial). Interestingly, the 'downstream' productions of steroid hormones such as progesterone, 17α-hydroxyprogesterone, and 17α-hydroxypregnenolone were not significantly affected by 1 week exposure to 12‰ saltwater (Fig. 4.2,B,C,D).

The androgen levels for androstenedione and testosterone showed no statistically significant differences within or between groups (Fig. 4.2E,F). In contrast, 5α-dihydrotestosterone levels were significantly increased in SW alligators pre- versus post-trial while no changes were observed between FW and SW alligators or within the FW group (Fig. 4.2G).

Both androstenedione and testosterone are catalyzed by the cytochrome P450 (cyp450) enzyme, aromatase (cyp19a1a), to the estrogens: estrone and 17β-estradiol respectively (Payne and Hales, 2004). Of the steroid hormones quantified in this study, the estrogens showed most pronounced effects within, and between groups. For instance, plasma levels of estrone, 17β-estradiol, and estriol were all significantly decreased post-trial compared with pre-trial values within both groups (Fig. 4.2H,I,J). However, saltwater had a significantly stronger effect on estrone and 17β-estradiol as post-trial levels in the SW group were significantly lower compared with the FW group (Fig. 4.2H,I). It is noteworthy to mention that both FW and SW groups were comprised of males and females, and thus we decided to assess effects of sex differences between the quantified steroid hormones, although sample sizes were low (SW group: n=5 females; n=3 males. FW group: n=3 females; n=5 males). The only hormone class in which sex had a significant effect on steroidogenic hormones (data not shown) was the estrogens, where post-trial estrone and 17β-estradiol levels were significantly decreased in SW females compared with FW females. However, since these results were similar to the overall group differences, we decided to study within and between group effects by combining sexes.

Table 4.1. Body morphometrics in American alligators (*Alligator mississippiensis*) exposed to freshwater or 12‰ saltwater for 1 week.

Morphometrics	Freshwater Pre-trial	Freshwater Post-trial	Saltwater Pre-trial	Saltwater Post-trial
Body Mass (g)	1,228.8 ± 61.7	1,233.3 ± 62.6	1,257.5 ± 59.9	1,204.3 ± 59.1
% Weight Loss	-	-0.37	-	4.23
S-V Length (cm)	37.7 ± 0.6	37.0 ± 0.8	38.9 ± 0.5	36.8 ± 0.7
Total Length (cm)	77.6 ± 1.3	79.0 ± 1.5	78.6 ± 1.2	79.5 ± 1.2
Condition factor (K)	2.29 ± 0.07^{a}	2.44 ± 0.08^{b}	2.13 ± 0.05^{a}	2.41 ± 0.09^{b}
Hepatic/Somatic Index	-	1.33 ± 0.05^{a}	-	1.10 ± 0.04^{b}

Values listed are mean ± s.e.m. K = estimated using S-V length. Dissimilar letters denote significant differences within and between groups ($p \leq 0.05$). Results from both sexes were combined because they were not significantly different.

Table 4.2. Plasma biochemistry parameters in juvenile American alligators (*Alligator mississippiensis*) exposed to freshwater or 12‰ saltwater for 1 week.

Blood Chemistry Parameter	Freshwater Pre-trial	Freshwater Post-trial	Saltwater Pre-trial	Saltwater Post-trial
Glucose (mg dl^{-1})	91.50 ± 3.66	86.13 ± 3.06	90.38 ± 2.22	89.25 ± 1.69
Creatinine (mg dl^{-1})	< 0.2†	< 0.2†	< 0.2†	0.26 ± 0.02
Creatine Kinase (U L^{-1})	774 ± 122.3	921.9 ± 443.4	948.1 ± 378.1	424.3 ± 133.6
Bilirubin (mg dl^{-1})	0.10	0.10	0.10	0.10
ALP (U L^{-1})	11.75 ± 0.59	12.75 ± 0.70	12.88 ± 0.71	13.38 ± 1.52
AST (U L^{-1})	386.1 ± 18.51	402.6 ± 24.63	330.9 ± 29.83	338.9 ± 34.17
ALT (U L^{-1})	50.38 ± 4.52	50.25 ± 4.26	39.13 ± 2.39	40.63 ± 3.45
AST/ALT	7.88 ± 0.42	8.12 ± 0.25	8.33 ± 0.49	8.25 ± 0.51
Cholesterol (mg dl^{-1})	78.50^{a} ± 6.36	78.38^{a} ± 5.20	76.25^{a} ± 5.90	101.0^{b} ± 8.30
Calcium (mg dl^{-1})	10.69 ± 0.18	10.61 ± 0.16	10.20 ± 0.11	10.84 ± 0.09
Phosphorous (mg dl^{-1})	3.70^{a} ± 0.08	3.51^{a} ± 0.14	3.62^{a} ± 0.06	4.83^{b} ± 0.10
Sodium (mmol L^{-1})	150.9^{a} ± 0.58	148.8^{a} ± 0.98	149.5^{a} ± 0.78	182.0^{b} ± 1.04
Potassium (mmol L^{-1})	5.06 ± 0.13	4.58 ± 0.12	4.71 ± 0.15	4.98 ± 0.16
Na/K ratio	29.93^{a} ± 0.73	32.65^{a} ± 0.82	31.94^{a} ± 1.03	36.81^{a} ± 1.09
Chloride (mmol L^{-1})	114.0^{a} ± 2.2	112.9^{a} ± 2.71	117.80^{a} ± 0.25	146.50^{b} ± 1.21
Total Protein (g dl^{-1})	5.14 ± 0.17^{a}	5.05 ± 0.15^{a}	4.6 ± 0.12^{b}	5.23 ± 0.13^{a}
Albumin (g dl^{-1})	1.59 ± 0.05^{a}	1.56 ± 0.04^{a}	1.50 ± 0.0^{b}	1.59 ± 0.02^{a}
Globulins (g dl^{-1})	3.56 ± 0.12	3.60 ± 0.11	5.67 ± 2.22	3.73 ± 0.08
A/G ratio	0.45 ± 0.02	0.42 ± 0.02	0.45 ± 0.05	0.41 ± 0.01
Uric Acid (mg dl^{-1})	$< 1.5†^{a}$	$< 1.5†^{a}$	$< 1.5†^{a}$	9.34 ± 0.61^{b}

†Shows values below detection limit. Dissimilar letters denote significant differences within and between groups ($p \leq 0.05$). Data shown are mean ± s.e.m. Results from both sexes were combined because they were not significantly different.

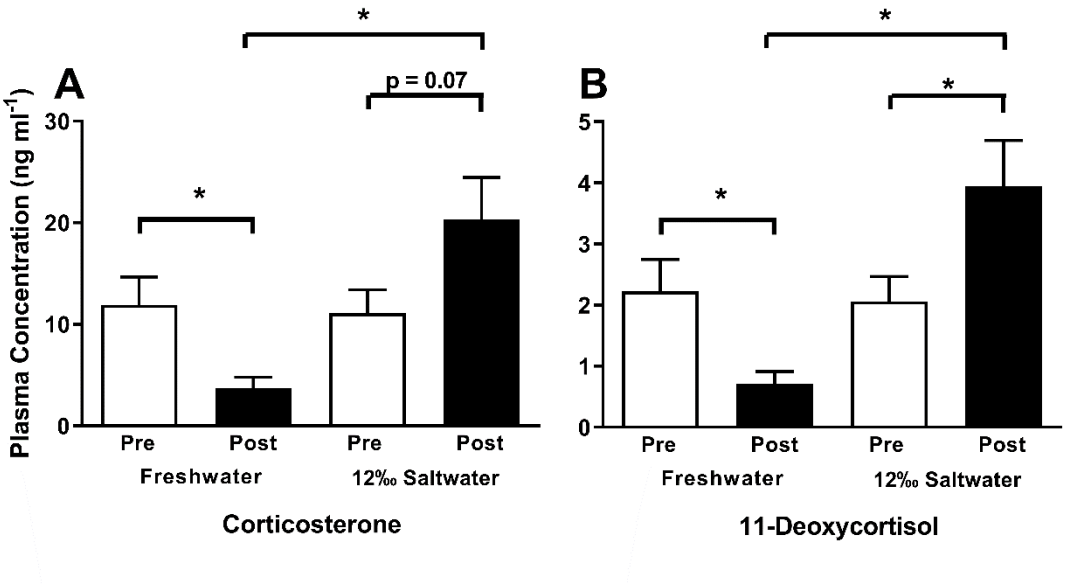

Figure 4.1. Plasma levels of corticosterone (A) and 11-deoxycortisol (B) in juvenile American alligators exposed to freshwater and saltwater (12‰) for 1 week. Animals were blood sampled pre-trial (all animals in freshwater) and post-trial (after 7 days). Data shown are mean ± s.e.m. (N=8). *Asterisk denotes significant differences ($p \leq 0.05$, one-way ANOVA) between and within freshwater and saltwater groups as shown. Results from both sexes were combined because they were not significantly different.

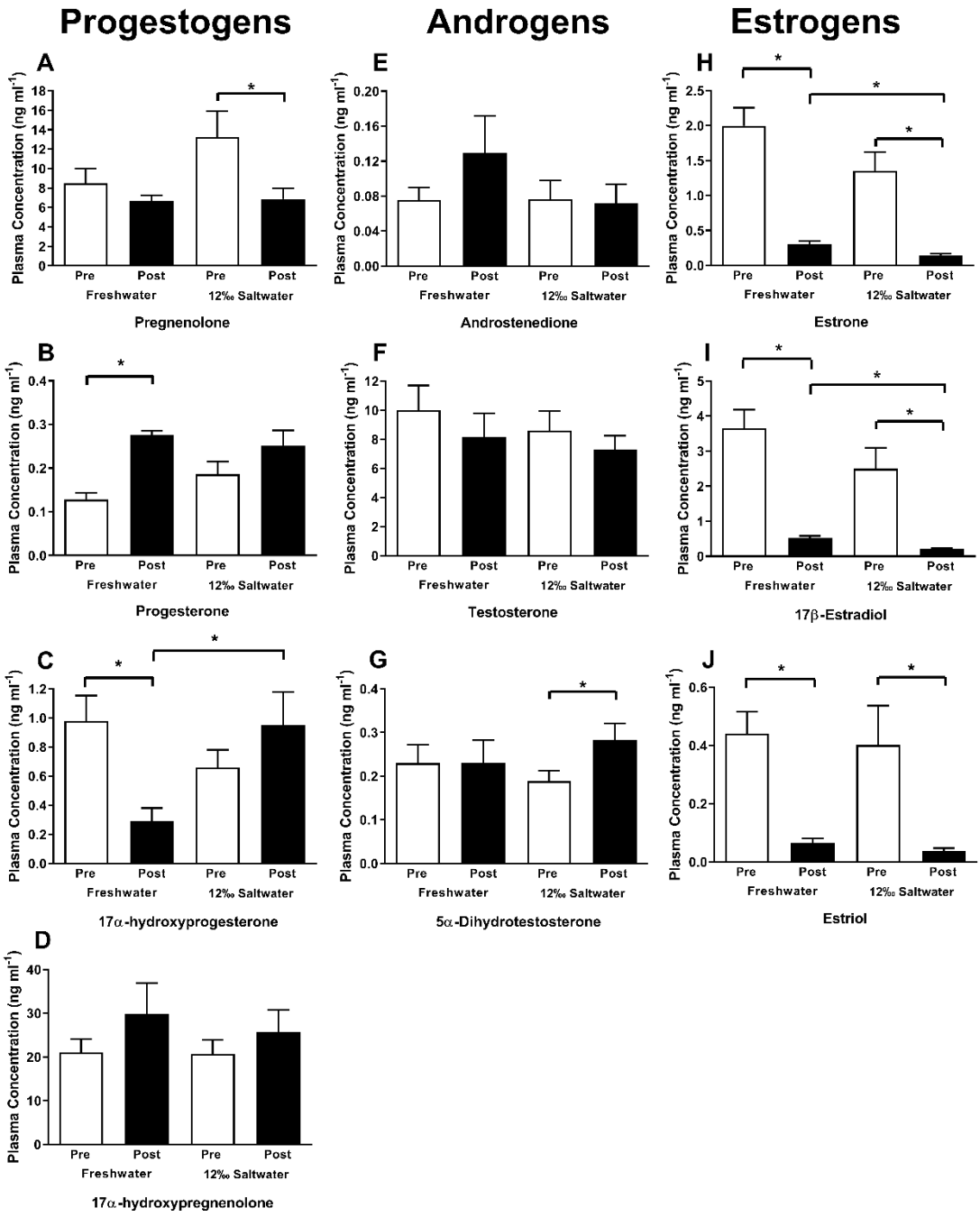

Figure 4.2. Plasma steroidogenic hormone concentrations (ng ml^{-1}) in juvenile American alligators exposed to freshwater (0‰) or saltwater (12‰) for 1 week. Animals were blood sampled pre-trial (all animals in freshwater) and post-trial (after 7 days). Data shown are mean ± s.e.m. (N=8). *Asterisk denotes significant differences ($p \leq 0.05$, one-way ANOVA) between and within freshwater and saltwater groups. Results from both sexes were combined because they were not significantly different.

The ratios of estrogens (estrone and 17β-estradiol) to androgens (androstenedione and testosterone) can also be used to determine the relative catalytic capability of aromatase. Such assessment serves as an additional biomarker of endocrine physiology (Guillette et al., 1994). When the two groups were compared (combined females and males), no differences in estrone to androstenedione (E_1/A) ratios was found between groups either pre- or post-trial. The E_1/A ratios were, however, significantly ($p=0.001$) reduced in FW alligators post-trial compared with pre-trial values, and while there was a decrease in E_1/A ratios between days in SW alligators, it was not significant ($p=0.06$) due to larger variation in the data (Table 4.3). Ratios of 17β-estradiol to testosterone (E_2/T) were significantly lower post-trial compared with pre-trial values within FW ($p=0.008$) and SW groups ($p=0.02$) (Table 4.3). Even though not significant at the $p\leq0.05$ level ($p=0.07$), E_2/T ratio in SW alligators was ~3× lower compared with FW alligators post-trial (Table 4.3). Accounting for sex differences, SW females had a significantly lower E_1/A ratio compared with SW males post-trial, but no differences were detected pre-trial. Furthermore, SW female E_2/T ratio was significantly lower compared with FW females post-trial.

Finally, the plasma levels of the biologically active RAAS hormone, angiotensin II was significantly ($p=0.0004$) lower in SW alligators post-trial compared with pre-trial values, and compared with post-trial FW alligators (Fig. 4.3A). In contrast, levels of the mineralocorticoid steroid hormone, aldosterone, was not affected by either time or saltwater treatment (Fig. 4.3B).

4.4.4. Effects of Salt Stress on Lung, Kidney, and Gonad Histology

Histological examination of lung tissue showed morphological changes in SW alligators. It appeared that alveolar-capillary membranes were decreased while alveolar spaces were more numerous in SW alligators (Fig. 4.4A,B). Histological analysis of kidney tissue did not show marked differences in kidney tubules between SW and FW alligators (Fig. 4.5A,B).

Immunohistochemical analysis showed significantly lower expression of ACE in lung tissue in SW alligators compared with FW alligators (Fig. 4.4C,D,E). No immunoreactive signals indicating ACE protein expression were detected in the negative control sections of lung tissue (Fig. 4.6).

Immunohistochemical analysis of renin expression in kidney tissue showed a significant decrease in renin expression in SW alligators compared with FW alligators (Fig. 4.5C,D,E). No immunoreactive signals indicating renin protein expression were detected in the negative control sections of kidney tissue (Fig. 4.7).

Representative micrographs of gonad histology are shown in Figure 6A-D. Freshwater female gonads showed presence of oocytes, while seminiferous tubules with Sertoli cells and surrounding Leydig cells were evident in FW male gonad tissue. Gonad histology in both male and female SW alligators showed marked differences with the FW group. In females, there was evidence of atresia as oocytes were no longer clearly visible and more open spaces without presence of cells or oocytes were seen (Fig. 4.8A,B). In males, clear seminiferous tubules were no longer evident, and Sertoli and germ cells were significantly lower ($p<0.001$) in SW males compared with FW males (Fig. 4.8C-F).

Table 4.3. Ratio of estrogens to androgens in juvenile American alligators (*Alligator mississippiensis*) exposed to freshwater and 12‰ saltwater for 1 week.

Sex and group	Estrone/ Androstenedione Pre-trial	Estrone/ Androstenedione Post-trial	17β-Estradiol/ Testosterone Pre-trial	17β-Estradiol/ Testosterone Post-trial
FW-combined	31.64 ± 6.34†	3.48 ± 0.86	0.49 ± 0.12†	0.08 ± 0.02
SW-combined	27.99 ± 10.71	3.86 ± 0.94	0.35 ± 0.11†	0.03 ± 0.01
FW-female	19.9 ± 2.36	3.46 ± 1.64	0.22 ± 0.08	0.10 ± 0.03^{+}
FW-male	38.67 ± 8.83	3.48 ± 0.99	0.64 ± 0.14	0.07 ± 0.04
SW-female	36.43 ± 16.45	2.72 ± 0.76*	0.41 ± 0.16	0.03 ± 0.005
SW-male	13.92 ± 3.18	6.72 ± 0.92	0.25 ± 0.09	0.05 ± 0.02

†Denotes significant difference ($p \leq 0.05$) between days within each group. *Denotes significant difference between males and females within each group. $^{+}$Denotes significant difference between same sex between groups. SW group: n=5 females; n=3 males. FW group: n=3 females; n=5 males. Data shown are mean ± s.e.m.

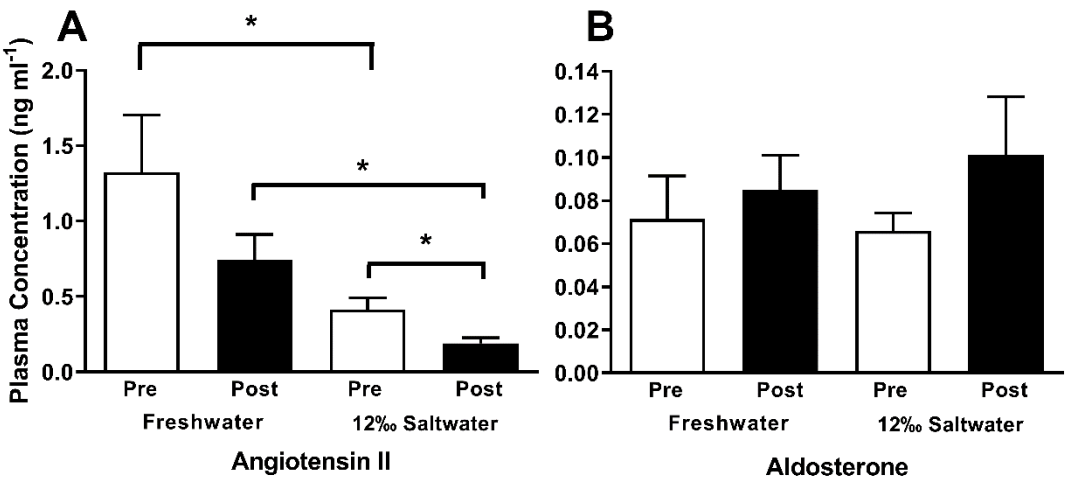

Figure 4.3. **Plasma levels of angiotensin II (ng ml^{-1}) (A) and aldosterone (ng ml^{-1}) (B) in juvenile American alligators exposed to freshwater and saltwater (12‰) for 1 week**. Animals were blood sampled pre-trial (all animals in freshwater) and post-trial (after 7 days). Data shown are mean ± s.e.m. (N=8). *Asterisk denotes significant differences ($p \leq 0.05$, one-way ANOVA) between and within freshwater and saltwater groups. Results from both sexes were combined because they were not significantly different.

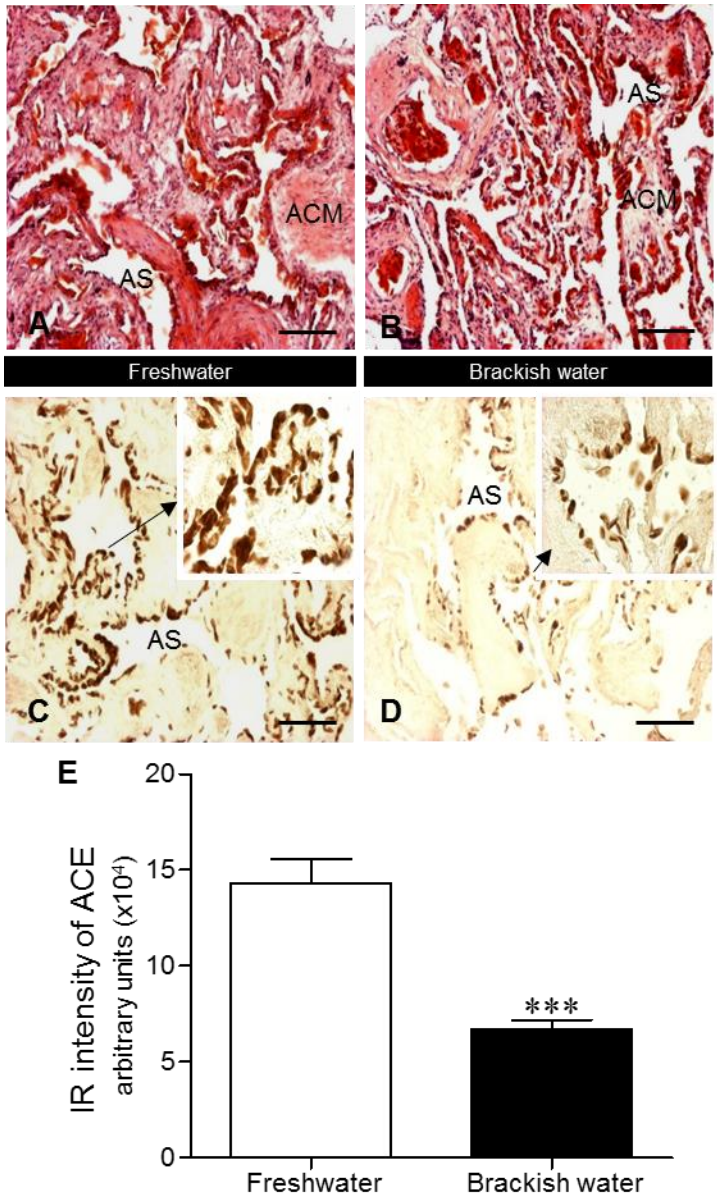

Figure 4.4. **Morphology and protein expression of ACE in lung tissue in American alligator exposed to freshwater and saltwater (12‰) for 1 week.** Histology (A,B), immunohistological expression (C,D) and levels (E) of ACE in lung tissue. (C,D) Arrow-heads indicate higher magnification of ACE expressions in lung tissue. Scale bar = 100 μm. Each value (E) represents the mean ± s.e.m. (N = 60-70 for ACE immunostaining intensity). AS, Alveolar space; ACM: Alveolar-capillary membrane. *Asterisks indicate significant difference ($p < 0.001$, Student's *t*-test).

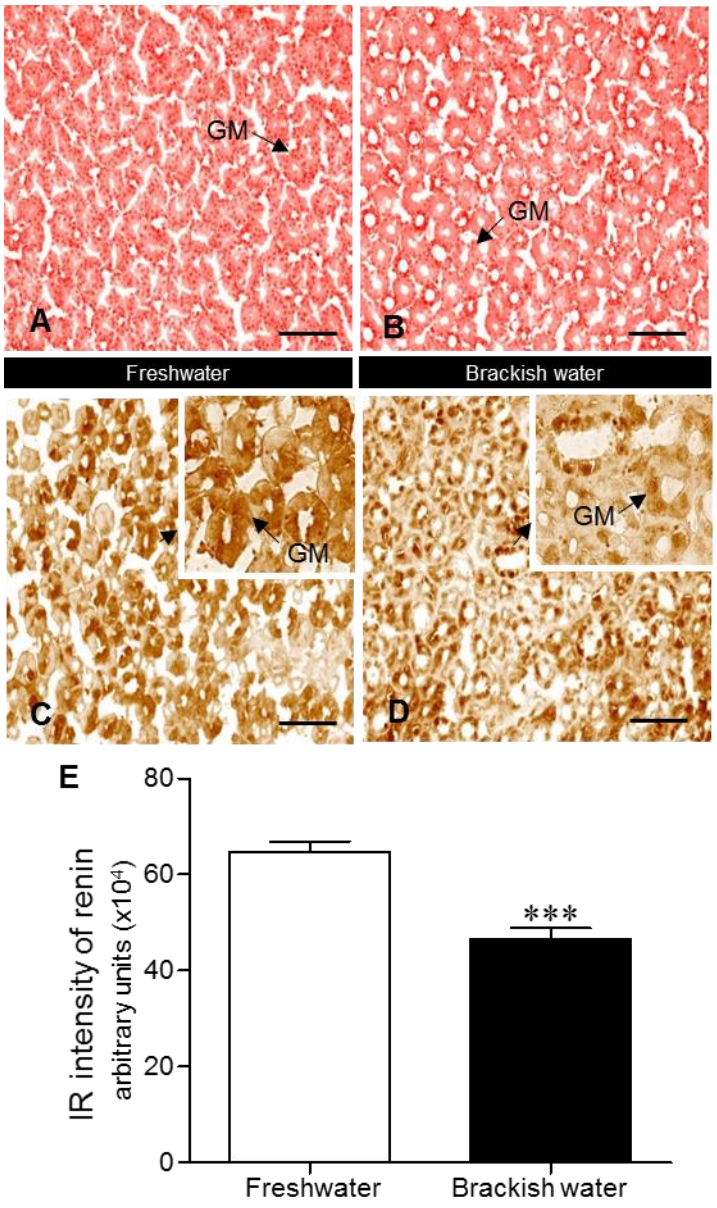

Figure 4.5. Morphology and protein expression of renin in kidney tissue in American alligator exposed to freshwater and saltwater (12‰) for 1 week. Histology (A,B), immunohistological expression (C,D) and levels (E) of renin in kidney tissue. (C,D) Arrow-heads indicate higher magnification of renin expressions in kidney tissue. Scale bar = 100 μm. Each value (E) represents the mean ± s.e.m. (N = 60-65 for renin immunostaining intensity). GM, glomerulus. *Asterisks indicate significant difference ($p < 0.001$, Student's *t*-test).

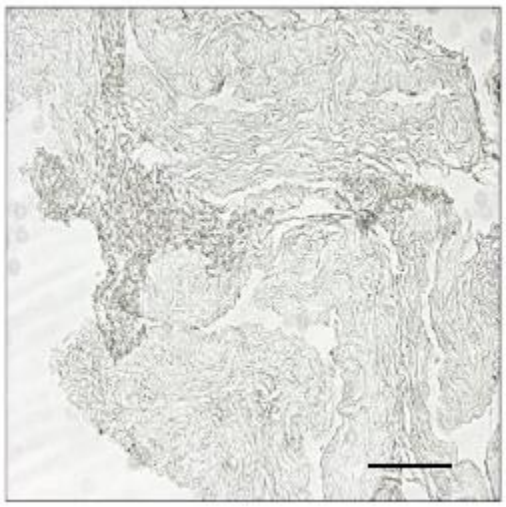

Figure 4.6. Negative control of ACE expression in lung tissue of American alligator. The negative control slide was incubated with phosphate-buffered saline instead of ACE primary antibody. No immunohistochemical signal was detected in the negative control of lung tissue section. Scale bar = 100 μm.

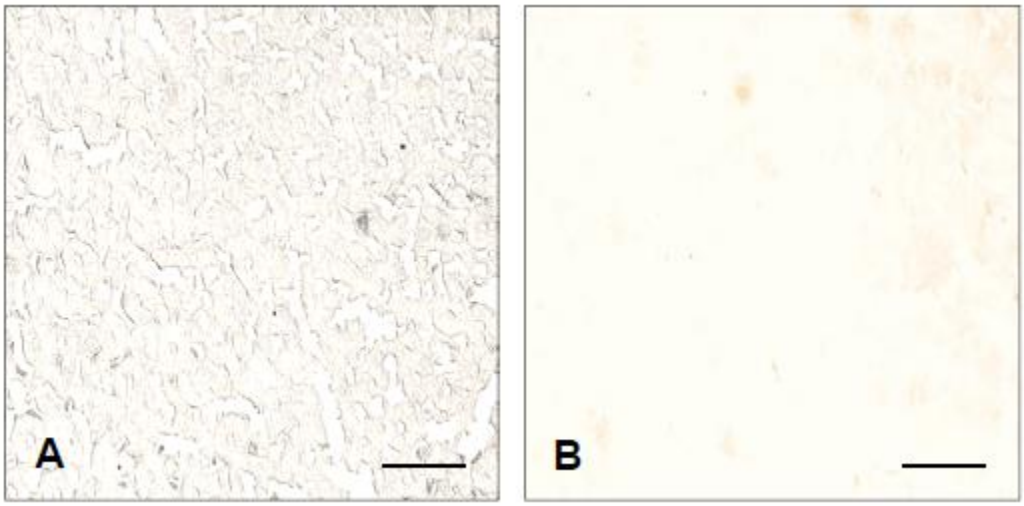

Figure 4.7. Negative controls of renin expression in kidney tissue of American alligator. (A) The negative control slide incubated with phosphate-buffered saline instead of renin primary antibody. (B) The specificity of the immunohistochemical staining reaction with renin antibody in the alligator kidney tissue section was confirmed by blocking with recombinant protein corresponding to the amino acid sequence of vertebrate renin (B). No immunohistochemical signals were detected in the negative controls of kidney tissue sections (A,B). Scale bar = 100 μm.

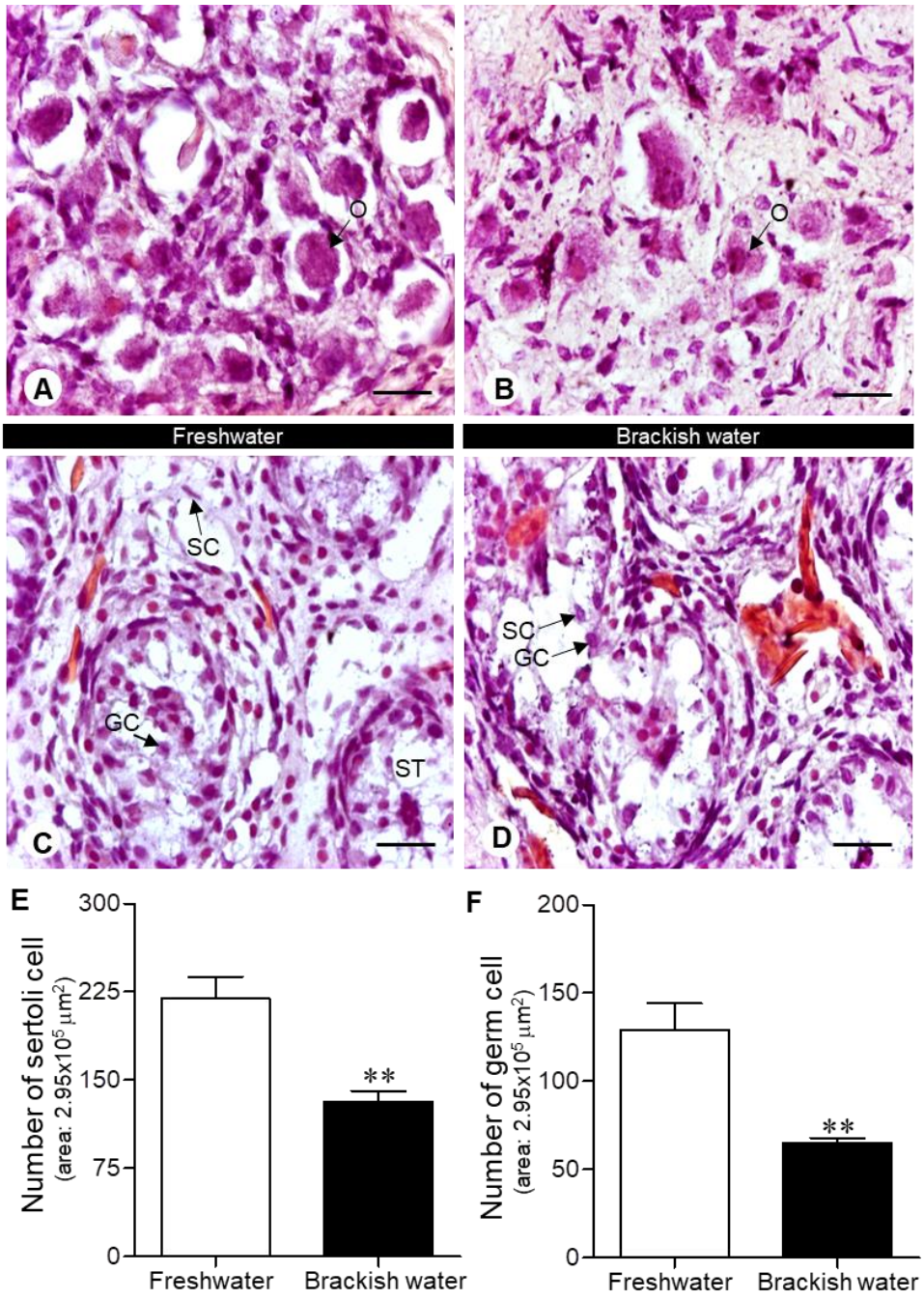

Figure 4.8. Histology of gonadal tissue in American alligator exposed to freshwater and saltwater (12‰) for 1 week. Histology of ovarian (A,C) and testicular (B,D) tissues in alligator. Note: histological observation reveals a large number of oocytes (O) present in freshwater alligator (A,C). Testes from saltwater exposed males show poorly organized seminiferous tubules (ST) and fewer germ (G) cells (B,D). *Asterisks indicate significant difference ($p < 0.001$, Student's *t*-test). N=3-5. SC, Sertoli cell; GC, germ cell. Scale bar = 20 µm.

4.5. Discussion

4.5.1. Food Intake and Body Morphometrics

Cessation of feeding is commonly observed in juvenile alligators (Faulkner et al., 2018; Lauren, 1985; Morici, 1996), and freshwater turtles (Bower et al., 2016; Davenport and Ward, 1993) exposed to saline environments. For example, when alligators were exposed to 12‰ saltwater for 5 weeks, cessation of feeding occurred after 1 week and animals had lost ~28% of body mass by week 5 (Faulkner et al., 2018). During short-term 12‰ exposure, all alligators (fresh- and saltwater) continued feeding and, as expected, no loss of body mass was measured after 7 days.

4.5.2. Blood Plasma Biochemistry

The significantly elevated total plasma protein and albumin levels post-trial in SW alligators suggest that 7 days in 12‰ saltwater is sufficient to initiate dehydration in juvenile alligators. When animals lose body fluids total plasma protein~~s~~ (and albumin and globulin) levels become more concentrated (Burtis and Ashwood, 1999) and although other factors can contribute to elevated plasma protein levels, dehydration was a significant factor in elevated plasma proteins in 5-week salinity exposed juvenile alligators (Faulkner et al., 2018).

In mammals, elevated serum creatine kinase and creatinine levels have been used as plasma biomarkers of impaired kidney function (Burtis and Ashwood, 1999). Contrary to long-term (5 week) 12‰ saltwater exposure, in which significant increases in both creatine kinase and creatinine levels were detected (Faulkner et al., 2018), short-term

exposure to 12‰ did not significantly affect creatine metabolism. Elevated aspartate aminotransferase (AST) levels are good indicators of hepatic disease, renal disease, and muscle damage in reptiles (Eatwell et al., 2014), and while AST was elevated in alligators after 5 weeks at 12‰ (Faulkner et al., 2018), 1-week exposure did not result in significant changes in AST. These results indicate that saltwater effects on hepatic and/or kidney function are not diagnostic after 1 week.

Alligators mainly excrete nitrogenous end-products as a mix of ammonia and uric acid (Coulson and Hernandez, 1959; Singer, 2003). Plasma uric acid levels were significantly elevated after only 7 days in 12‰ saltwater, and while urine composition was not measured, we observed copious white precipitate elimination from the cloaca during dissection. Thus, even short-term exposure to saltwater causes physiological changes towards increased production of uric acid in juvenile alligators.

Pre-trial potassium (K^+) levels in FW and SW alligators were comparable to previously published values from wild juvenile and adult alligators [K^+ 4.9 ± 0.11 mmol L^{-1} (Hamilton et al., 2016), 3.8 mmol L^{-1} (Divers and Mader, 2006)]. Interestingly, K^+ levels did not change in SW alligators after 1 week, which was similarly demonstrated after 5 weeks in 12‰ saltwater (Faulkner et al., 2018). Plasma K^+ levels are regulated by aldosterone, whose synthesis and secretion from the adrenal gland is controlled by plasma angiotensin II, adrenocorticotropic hormone (ACTH) and high plasma K^+ levels (Nishimura, 2017). For instance, high plasma K^+ levels can also stimulate aldosterone secretion and as aldosterone enhances renal K^+ excretion and Na^+ absorption, K^+ levels will stabilize again upon aldosterone release. Continuous stable K^+ levels can therefore

indicate elevated aldosterone levels. However, in SW alligator plasma aldosterone levels were not elevated post-trial despite similar K^+ levels pre- versus post-trial. These data therefore suggest that saltwater does not affect K^+ levels or that K^+ levels are regulated by another hormone.

Pre-trial plasma Na^+ levels in FW and SW alligators (Table 4.2) corresponded well with values obtained in wild juvenile [140.3 ± 0.69 mmol L^{-1} (Hamilton et al., 2016)] and adult alligators (146 mmol L^{-1} (Divers and Mader, 2006)). In addition, plasma chloride levels (Table 4.2) were similarly comparable to previously published values from wild juvenile and adult alligators [Cl^- 110 mmol L^{-1} (Divers and Mader, 2006)]. Seven days exposure to 12‰ saltwater caused significant elevation of plasma Na^+ and Cl^- levels compared with pre-trial values and with FW alligators (pre- and post-trial). The rapid elevation of plasma Na^+ and Cl^- levels in 7 day 12‰ saltwater alligators were likely due to integumental influx of ions from the saline environment in combination with some water loss (seen by significantly elevated plasma protein levels). The integument constitutes a significant route for ion and water exchange in juvenile alligators. For instance, integumental whole body Na^+ efflux averaged 3.9 µmol 100 g^{-1} h^{-1} in hatchling alligators (body mass 0.03-0.07 g) when maintained in freshwater (Ellis and Evans, 1984). When briefly (4 hours) exposed to 35‰ saltwater integumental Na^+ influx into juvenile alligators (230-586 g) was 10.8 µmol 100 g^{-1} h^{-1} while water efflux was 0.25 ml 100 g^{-1} h^{-1} (Mazzotti and Dunson, 1984). During the 1-week and 5-week trials (Faulkner et al., 2018) we closely observed alligator behavior (e.g., water and basking activities) and found that SW alligators spent more time in the water than on the basking plate (see Chapter III).

Whether this was a behavioral response to prevent dehydration on the basking plate is unknown. The observed physiological changes therefore correspond well with behavioral observations and demonstrate that short-term exposure to saline environments is sufficient to significantly elevate plasma Na^+ levels and cause water loss. Although loss of body mass was not as drastic as seen after 5 weeks in 12‰ water, some dehydration was evident as discussed above. Of interest were the Na^+ levels which after only 1 week in 12‰ saltwater (~182 mmol L^{-1}) were only slightly lower compared with levels measured after 5 weeks (~202 mmol L^{-1}) (Faulkner et al., 2018). Reptile kidneys do not possess Loop of Henle (Willmer et al., 2009), and hence are unable to excrete hyperosmotic urine which suggests that a continuous influx of ions cannot eliminated sufficiently in the alligator which lacks salt glands.

4.5.3. Steroidogenic Hormones and the Renin-Angiotensin-Aldosterone System

Seven days in 12‰ saltwater raised corticosterone levels from 11 ng ml^{-1} to 20 ng ml^{-1}, which corresponds with corticosterone levels measured after 5 weeks in 12‰ saltwater (~25 ng ml^{-1}) (Faulkner et al., 2018). Furthermore, 11-deoxycortisol levels were significantly elevated in SW alligators after 1 week (~4 ng ml^{-1}) and corresponded with 11-deoxycortisol levels after 5 weeks (~6 ng ml^{-1}). Thus, despite significantly reduced plasma pregnenolone levels in SW alligators, data show that this precursor did not affect adrenal production of stress hormones in SW alligators. Stress hormone levels are known to have significant physiological effects (e.g., stunted growth, depressed immune system,

elevated glucose production) (Morici et al., 1997), and thus short-term saltwater exposure can have negative effects on alligator physiology.

The precursor to all steroid hormones is cholesterol which is mainly produced in the liver. Plasma cholesterol levels were significantly elevated in SW alligators while the hepatic-somatic index (HSI) was significantly lower. The significance of the correlation between HSI and plasma cholesterol levels is interesting, yet not fully understood, but it is noteworthy that plasma cholesterol levels were similarly elevated after 5 weeks in 12‰ saltwater (Faulkner et al., 2018). Some plasma cholesterol is shuttled to the inner mitochondrial membrane (as mediated by the steroidogenic acute regulatory (StAR) protein), and is converted to the progestogen pregnenolone (catalyzed by the cytochrome P450 side chain cleavage (P450scc) enzyme) (Stocco and Clark, 1996). Despite the significantly elevated plasma cholesterol levels after 7 days, there were significantly depressed levels of pregnenolone within the SW group. This decrease could suggest that saltwater exposure causes a decrease in the activity of P450scc, or that the high plasma cholesterol is used in other metabolic pathways besides steroidogenesis. Despite a decrease in pregnenolone levels in SW alligators, there was no difference in levels between FW and SW alligators and these results therefore differ from findings after 5 weeks where pregnenolone levels were significantly lower in SW compared with FW alligators (Faulkner et al., 2018). It is likely that these time-dependent differences relate to changes in the steroidogenic enzyme activity of P450scc. On the contrary, 17α-hydroxyprogesterone levels were significantly higher in SW compared with FW alligators after both 1 and 5 weeks (Faulkner et al., 2018), indicating elevated activities of

hydroxysteroid dehydrogenase and cyp450 enzymes (such as 3β-HSD and cyp17-hydroxylase) within 7 days of saltwater exposure.

Five weeks exposure to 12‰ saltwater has been shown to cause significantly elevated testosterone levels in juvenile American alligators (Faulkner et al., 2018). In this study, the unchanged androgen levels under short-term saltwater exposure are interesting as levels of 17β-estradiol and estrone were significantly lower post-trial in the SW compared with FW group. In contrast, all three estrogen levels were significantly elevated after long-term saltwater exposure (Faulkner et al., 2018). Estrogen to androgen ratios [e.g., estrone/androstenedione (E_1/A); 17β-estradiol/testosterone (E_2/T)] can be used as an indicator of the steroidogenic environment (Guillette et al., 1994). For instance, E_2/T ratio in male and female juvenile alligators from a contaminated lake (from agricultural waste, sewage, and pesticide spill) were significantly higher compared with alligators from a reference lake (Guillette et al., 1995; Guillette et al., 1994). We have previously demonstrated significantly higher E_1/A ratio in juvenile alligators following 5 weeks exposure to 12‰ saltwater (Faulkner et al., 2018). In the current study, E_1/A and E_2/T decreased in FW and SW alligators, but none of these changes were significant. A significantly lower E_2/T ratio, however, was evident post-trial in SW females compared with FW females (Table 4.3). Collectively, plasma steroid hormone analysis along with observed changes in gonad histology from male and female alligators showed marked changes between treatment groups. The implications of these changes need to be determined further to assess potential long-term reproductive effects.

Finally, angiotensin II levels in FW alligators post-trial (0.8 ng ml^{-1}) were similar to levels quantified in FW alligators (~0.5 ng ml^{-1}) maintained under similar culture conditions for 5 weeks (Faulkner et al., 2018). On the contrary, one week in 12‰ saltwater was sufficient to significantly decrease plasma angiotensin II levels by ~75% compared with FW alligators. The decrease in angiotensin II levels in the present study, therefore, suggests an early depression of RAAS which is sustained long-term as shown the by 90% reduction in angiotensin II after 5 weeks (Faulkner et al., 2018). It is likely that this suppression of angiotensin II was due to depression of the RAAS enzymes renin and ACE and we therefore quantified protein expression in kidneys and lungs.

Immunohistochemical analysis demonstrated a significant reduction in renin expression in alligator kidneys after 1 week in 12‰ saltwater, which suggests inhibition of synthesis. In mammals, an increase in plasma Na^+ levels often correspond with elevated blood pressure both of which inhibit renin release from the kidneys (Hayakawa et al., 2015; Lopez and Gomez, 2010; Nishimura, 2017). Renin synthesis and release is further inhibited by elevated plasma angiotensin II levels, and an increase in tubular fluid Na^+/Cl^- concentration (Gomez and Sequeira-Lopez, 2018; Nishimura, 2017). However, as reptiles seem to lack the macula densa in kidney tubules (Nishimura, 2017), high plasma Na^+/Cl^- levels and potentially elevated blood pressure could explain the reduced expression of renin in SW alligator kidney tissue. However, while renin synthesis and activity are likely to be key to the reduced angiotensin II levels, ACE is the enzyme that plays an important physiological role as it converts the inactive angiotensin I to the biologically active angiotensin II (Wilson, 1984). The reduced expression of ACE in SW alligator lung tissue

is noteworthy, but it is currently unknown whether negative feedback mechanisms acted on both renin and ACE or whether initial suppression of renin caused a reduction of the entire RAAS cascade. Finally, studies in mammals show that a continuous salt load provides a negative feedback on systemic RAAS hormones (angiotensin II, aldosterone) while it additionally induces release of angiotensin II and aldosterone in tissues such as the heart (Ouvrard-Pascaud et al., 2005; Lahera et al., 2006, Bollag, 2014). Although it is beyond the scope of this study to examine cardiovascular effects of saltwater exposure in alligators, this would be an interesting avenue to further investigate.

Aldosterone levels in FW and SW alligators pre-trial ranged between 0.07-0.08 ng ml^{-1} (current study). However, aldosterone levels in freshwater maintained captive alligators were 0.022 ± 0.002 ng ml^{-1} (Morici et al., 1997) while levels in captive breeding and non-breeding alligators ranged 0.01-0.025 ng ml^{-1} (Balment and Loveridge, 1989). Aldosterone levels in wild alligators, however, were higher ranging 0.0252-0.209 ng ml^{-1} (Lance et al., 2001). Thus, aldosterone levels seem to vary considerably between studies making comparisons difficult. In the current study, aldosterone levels only rose slightly to 0.1 ng ml^{-1} post-trial. Unaffected aldosterone levels in salinity exposed alligators were further demonstrated after 5 weeks in 12‰ saltwater (averaged 0.15 ng ml^{-1}) (Faulkner et al., 2018). Results therefore demonstrate only moderate changes in this mineralocorticoid in salinity stressed alligators. These findings correspond well with previous studies on salinity exposed alligators which likewise showed only minor increases in plasma aldosterone concentration. For instance, 1 week exposure to 8‰, 12‰, or 16‰ saltwater had no effect on plasma aldosterone concentrations. Furthermore, 5 weeks at 8‰ and 12‰

equally had no effect and although aldosterone levels were elevated in the 16‰ saltwater group this was attributed to an artefact of weekly blood sampling which lowered blood volume (Morici, 1996). Thus, it is likely that aldosterone does not have an osmoregulatory function in alligators in saline environments. Indeed, a recent review states that ALDO's role in renal Na^+ and K^+ transport in non-mammalian vertebrates has not been established (Nishimura, 2017). In contrast, juvenile Nile crocodiles (*Crocodylus niloticus*) kept in hypertonic medium (Na^+ 660 mM, K^+ 0.5 mM, Cl^- 612 mM ~35‰) for 7 days exhibited a 33% increase in aldosterone levels compared with freshwater maintained animals (Balment and Loveridge, 1989). Although data remain extremely limited, differences in aldosterone responses in saltwater exposed crocodilians suggest differences between crocodilian families (Crocodylidae versus Alligatoridae) with regards to the role of aldosterone as an osmoregulatory hormone. Indeed, studies have shown that there are marked osmoregulatory differences (e.g., cloaca being an active secondary osmoregulatory organ in crocodiles in addition to presence of salt gland) between alligatorids and crocodylids when exposed to saltwater (Pidcock et al., 1997) which may involve differences in the role of aldosterone. Finally, it is noteworthy to mention that the high glucocorticoid levels (corticosterone) could have had mineralocorticoid actions. Although this suggestion is entirely speculative, the lack of elevated glucose levels in SW alligators indicates other actions of corticosterone besides a traditional stress hormone effect. Collectively, the functional role played by aldosterone and corticosterone in osmoregulation in the two crocodilian families remains to be fully determined.

4.5.4. Conclusions

Our data demonstrated novel and significant effects of short-term saltwater exposure on the endocrine physiology (RAAS and steroidogenesis) of juvenile alligators. This study complements a previous chronic study on salt stress in alligators. Common for both studies was the reduced angiotensin II and elevated plasma Na^+ and Cl^- levels. Clear differences were also shown in steroidogenic hormone levels, demonstrating that saltwater exposure can cause endocrine effects. Furthermore, blood biochemistry parameters demonstrated that even short-term saltwater exposure has significant effects on juvenile alligator water and salt balance (e.g., dehydration and high electrolyte levels) and further confirms the low tolerance of alligators to saline environments.

This study evokes further questions as to the physiological changes in alligators exposed to saline environments. Future studies should delineate the roles of arginine vasotocin (AVT), adrenocorticotropic hormone (ACTH) and aldosterone in reptilian osmoregulation. Further analysis should examine the time to recovery following salinity exposure. Such information will assist in understanding whether alligators can physiologically acclimatize to brackish areas. This knowledge will be pertinent in understanding how future storms and storm surges affect alligator populations inhabiting vulnerable Gulf of Mexico ecosystems.

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CHAPTER V

CORRELATIONS BETWEEN ENVIRONMENTAL SALINITY LEVELS, BLOOD BIOCHEMSITRY PARAMETERS, AND STEROID HORMONES IN WILD JUVENILE AMERICAN ALLIGATORS (*Alligator mississippiensis*)

5.1. Abstract

American alligators (*Alligator mississippiensis*) inhabit freshwater wetlands that are vulnerable to salinization caused by anthropogenic alterations to freshwater flow, in addition to storm surges, sea level rise, and droughts. Salinization of coastal freshwater habitats is a growing concern in a changing climate due to increased frequency and intensity of storm surges and drought conditions. This study opportunistically sampled juvenile male and female wild alligators in various salinities each month excluding November, December, and January for one year at Rockefeller Wildlife Refuge in coastal Louisiana. Blood plasma biochemistry parameters including electrolyte levels were subsequently measured. In addition, levels of various renin-angiotensin-aldosterone system hormones, glucocorticoids, androgens, estrogens, and progestogens were analyzed using liquid chromatography and tandem mass spectrometry. Only males were sampled in hyperosmotic environments (>10‰) during dry conditions in late summer 2018. In juvenile males, plasma Na^+, Cl^-, and the progestogen 17α,20β-dihydroxypregnenone were significantly and positively correlated with environmental salinity. However, variation in glucocorticoids, androgens, and estrogens were not associated with hypersaline water while sex steroids showed significant seasonal variation. This study demonstrated

significant correlation of environmental salinity with electrolyte levels and a sex steroid in wild juvenile alligators, and to our knowledge represents the first measurement of 17α,20β-dihydroxypregnenone in alligators.

5.2. Introduction

American alligators [*Alligator mississippiensis* (Daudin, 1802)] do not tolerate saline environments for prolonged periods of time without access to freshwater (Faulkner et al., 2018; Faulkner et al., 2019; Laurén, 1985; Pidcock et al., 1997). However, many freshwater alligator habitats are prone to frequent saltwater intrusion from storm surges (Emanuel, 2005; Hoyos et al., 2006). Rivers, lakes, and freshwater wetlands are frequently exposed to salinization due to anthropogenic perturbations that cause changes in freshwater or underground water flow (Day et al., 2000; Herbert et al., 2015). Furthermore, gradually rising sea levels may introduce saline water into inland groundwater fed wetlands resulting in salinization of inland areas not directly affected by seawater intrusion (Wood and Harrington, 2015).

Juvenile alligators are especially at risk of salinization effects due to their smaller size and thinner skin, allowing more rapid loss of water (Lance et al., 2010; Taplin, 1988). Since American alligators are a keystone species that control populations at lower trophic levels, salinization events can have disruptive ecosystem-level impacts (Mazzotti et al., 2009; Mazzotti and Brandt, 1994). Salinization is a growing concern for coastal freshwater habitats due to the effects of climate change. For instance, projected increases in sea surface temperature are associated with growing intensity and frequency of hurricanes

with ensuing storm surges (Hoyos et al., 2006; Michener et al., 1997; O'Brien et al., 1992). Furthermore, frequency of drought conditions is projected to increase due to rising greenhouse gas emissions (Li et al., 2009; Strzepek et al., 2010), contributing to the concentration of saltwater in wetlands and exacerbating salt inundation as seen in southwest Louisiana following Hurricane Rita (Lance et al., 2010).

Alligators use various osmoregulatory mechanisms to cope with changing salinity levels. For instance, they can modify the composition of urine in the kidney to excrete higher levels of sodium and chloride (Laurén, 1985; Pidcock et al., 1997), although they are unable to excrete urine hyperosmotic to body fluids due to their kidney nephrons lacking loops of Henle (Braun, 1998). In addition, alligators are known to behaviorally osmoregulate by selecting lower-salinity habitats (Dunson and Mazzotti, 1989; Mazzotti and Dunson, 1989; Taplin, 1988). Although few studies have investigated the physiological effects of saltwater on alligators, there is emerging evidence that saltwater has significant impacts on alligator endocrine physiology (Faulkner et al., 2018; Faulkner et al., 2019; Laurén, 1985; Morici, 1996). For instance, juvenile alligators ceased feeding and experienced weight loss and showed elevated plasma corticosterone and electrolytes (Na^+, Cl^-) when exposed to salinities between 10 and 20‰ for 4 weeks (Laurén, 1985) and 5 weeks (Morici, 1996). Further, mortality was observed in juvenile alligators exposed to 15‰ and 20‰ saltwater after 3 weeks (Laurén, 1985) while 4 mortalities occurred out of 10 animals exposed to 16‰ for 5 weeks (Morici, 1996). In addition, exposure of juvenile alligators to 12‰ saltwater for 5 weeks significantly elevated circulating levels of electrolytes (Na^+, Cl^-), lowered angiotensin II [a renin-angiotensin-aldosterone system

(RAAS) hormone], and elevated important steroid hormones involved in stress response (glucocorticoids) and reproduction and growth (progestogens, androgens, estrogens) (Faulkner et al., 2018). Long-term exposure to brackish saltwater levels (12‰) therefore caused important alterations in reproductive ability while depressing the endocrine system responsible for water/salt regulation. Short-term (1 week) exposure to 12‰ saltwater similarly elevated Na^+ and Cl^- and glucocorticoid levels in juvenile alligators while estrogen levels decreased and androgen levels were largely unaffected (Faulkner et al., 2019). Collectively, these studies demonstrated that saltwater exposure can cause time-dependent changes in sex steroids important for growth and reproduction.

Steroid hormones are vital in controlling various physiological processes such as growth, development, reproduction, and response to stress (Ali et al., 2018; Hughes, 2001; Lee et al., 2015; Mattsson and Olsson, 2007; Morton, 2010; Sinisi et al., 2003). Thus, steroids are commonly used as biomarkers to assess the physiological impacts of environmental or anthropogenic stressors (Guillette et al., 1994). It is well-documented that wild alligators are susceptible to endocrine disruption via exposure to contaminants such as the estrogenic pesticides dicofol, DDT, and various metabolites such as p,p'-DDE (Guillette et al., 1995; Guillette et al., 1994; Guillette et al., 1999). For instance, male juvenile alligators in the contaminated Lake Apopka had significantly greater 17β-estradiol and lower testosterone than in an uncontaminated lake, while juvenile females in the contaminated lake showed significantly elevated 17β-estradiol (Guillette et al., 1995; Guillette et al., 1994; Guillette et al., 1999). While we have significant knowledge on anthropogenic compounds, little information exists as to the effects of a natural stressor

(e.g., salinization) on alligator endocrine physiology. Understanding the impacts of environmental stressors, such as salinity, on alligators is crucial to the successful conservation and management of wild alligator populations in a changing climate.

While we have some information on how salinity affects alligator physiology from laboratory studies there is currently limited information (Lance et al., 2010) regarding the effect of salinity on the physiology of juvenile alligators found in the wild. Thus, the objective of this study was to determine whether wild juvenile alligator endocrine physiology is affected by exposure to varying salinity levels. Our working hypothesis was that endocrine effects observed in laboratory-kept juvenile alligators exposed to 12‰ salinity (changes in blood biochemistry, stress hormones, sex steroids) would be reflected in wild juvenile alligators found in hyperosmotic environments.

Wild juvenile alligators were opportunistically caught and blood sampled at Rockefeller Wildlife Refuge in Grand Chenier, LA each month over the course of one year (July 2018-July 2019). Environmental salinity levels were measured at the site of capture. Hormones involved in the RAAS (angiotensin II, aldosterone), stress response (glucocorticoids: 11-deoxycortisol, corticosterone), and reproduction and growth [progestogens (pregnenolone, 17α-hydroxypregnenolone, progesterone, 17α-hydroxyprogesterone, 17α,20β-dihydroxypregnenone), androgens (androstenedione, 5α-dihydrotestosterone, testosterone), and estrogens (estrone, 17β-estradiol, estriol)] were determined. Partial redundancy analysis was performed to determine correlation between salinity and hormones as well as various blood plasma biochemistry parameters (e.g., Na^+, Cl^-). This study presents new knowledge by monitoring monthly changes in a wide range

of hormones and blood biochemistry parameters in male and female juvenile American alligators.

5.3. Materials and Methods

5.3.1. Wild Alligator Sampling and Blood Plasma Collection

Fifty-four blood samples were opportunistically collected from juvenile alligators (<1.8 m total length) (Joanen and McNease, 1980) by the Louisiana Department of Wildlife and Fisheries during the day between July 2018 and July 2019 at Rockefeller Wildlife Refuge (Grand Chenier, LA) (Fig. 5.1). Alligators were not sampled in November, December, and January when cool temperatures during winter months limit their activity. Varying numbers of alligators were sampled per month and per sex due to the opportunistic sampling method. After alligators were located, animals were restrained and blood was quickly drawn from the post-occipital spinal venous sinus and placed in 10 ml lithium heparin vacutainers, centrifuged at 1,318 × g for 10 min, then transferred to microfuge tubes for storage at -20°C until chemical analysis. Morphometrics [total length (66-178 cm), and sex (male N = 33, female N = 21)] were recorded from each animal in addition to salinity level (0.4-22.2‰) in the vicinity of each animal's capture site.

5.3.2. Blood Biochemistry Analysis

Blood plasma biochemistry levels were measured by Texas A&M University's Veterinary Medical Diagnostic Laboratory (College Station, TX, USA). A Beckman Coulter AU480 analyzer was used to measure Na^+, K^+, Cl^-, Ca^{2+}, phosphorus, uric acid,

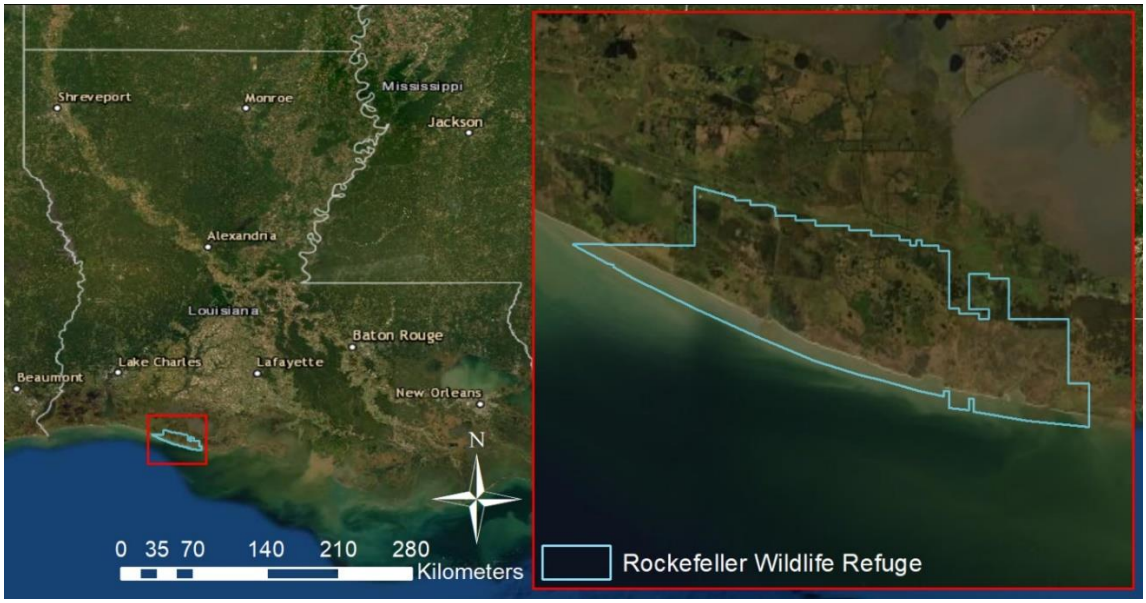

Figure 5.1. Rockefeller Wildlife Refuge in Grand Chenier, Louisiana (U.S.A.) where wild American alligators (*Alligator mississippiensis*) were sampled from various locations.

total protein, albumin, globulin, bilirubin, glucose, cholesterol, creatinine, creatine kinase, alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT).

5.3.3. Hormone Extraction from Blood Plasma

Hormone extractions were performed as previously described (Faulkner et al., 2018; Faulkner et al., 2019), with the following modifications. Plasma samples were thawed on ice, and 500 µl aliquots of plasma were spiked with surrogate standards d9-progesterone and d3-17β-estradiol. d9-Progesterone was used as the surrogate standard to quantify progesterone, 17α-hydroxyprogesterone, pregnenolone, 17α-hydroxypregnenolone, 17α,20β-dihydroxypregnenone, angiotensin II, aldosterone, 11-deoxycortisol, corticosterone, androstenedione, 5α-dihydrotestosterone, and testosterone, while d3-17β-estradiol was used as the surrogate standard to quantify estrone, 17β-estradiol, and estriol. The plasma was suspended in 5 ml Milli-Q water and liquid:liquid extracted twice using methyl tert-butyl ether (MTBE). Liquid:liquid extraction involved vortexing to mix the plasma with 5 ml MTBE for 10 seconds to allow for partitioning of analytes from the aqueous phase (plasma) to the solvent phase (MTBE). The mixture was subsequently centrifuged for 5 min at 2000 × g to separate the plasma and MTBE into distinct layers. Pooled MTBE layers were gently dried under nitrogen gas, and the residue was 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 analysis via liquid chromatography and tandem mass spectrometry (LC-MS/MS).

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

5.3.4. LC-MS/MS Analysis of Steroid Hormones

Chemical analyses were as described previously (Faulkner et al., 2018; Faulkner et al., 2019), with the following modifications. The LC-MS/MS system comprised an Agilent 1260 UHPLC system and a triple-quad 6420 mass detector. Chromatography separation of steroid hormones was achieved with an Agilent Poroshell EC-C18 column (3.0×50 mm, 5 µm particle size). The liquid mobile phase consisted of Milli-Q water (A) and methanol (B), each containing 5 mM ammonium formate. The mobile phase gradient transitioned from 30% (B) linearly to 70% B over 3 min and from 70% to 95% in 6 min. The gradient subsequently decreased from 95% to 70% over 3 min and from 70% to 30% (initial condition) in 3 min with the flow rate maintained at 0.4 ml min^{-1} (total run-time of 15 min).

Plasma hormones were ionized in positive ion electrospray ionization (ESI+) mode using nitrogen as the desolvation gas heated to 350°C (gas flow of 12 L min^{-1}) and a

capillary voltage of 3.5 kV. Hormones were detected in multiple reaction monitoring (MRM) mode with argon as the collision gas. The precursor→product ions monitored included: *m/z* (mass-to-charge ratio) 523.8→70.3 (angiotensin II), *m/z* 361.2→343.2 (aldosterone), *m/z* 347.2→97.2 (11-deoxycortisol), *m/z* 347.2→121.1 (corticosterone), *m/z* 317.3→299.2 (pregnenolone), *m/z* 333.2→315.2 (17α-hydroxypregnenolone), *m/z* 315.2→97.1 (progesterone), *m/z* 331.2→97.1 (17α-hydroxyprogesterone), m/z 333.2→97.1 (17α,20β-dihydroxypregnenone), *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 (d9-progesterone); and dansyl chloride (Dns) derived estrogens: *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-d3-estradiol).

5.3.5. Statistical Analyses

Normality testing was performed using Shapiro-Wilk's test ($p \leq 0.05$) and data were $\log_{10}$ transformed which ensured homogeneity prior to correlation analysis. Partial redundancy analysis (pRDA) was performed to determine correlations between physiological parameters and environmental salinity using the vegan package in R. A permutation test using 999 permutations was used to test the significance of relationships between physiological parameters and environmental salinity. Spearman's rank-order correlation analysis was used to further test the correlation between environmental salinity and all blood biochemistry parameters and hormones. One-way ANOVAs were used to test for variation in individual hormone levels and blood biochemistry parameters in each

sex by month. ANOVAs were followed by post-hoc Scheffe's tests, which was selected as a conservative test to reduce the chances of type I error while performing a large number of analyses. Differences in physiological parameters between male and female alligators within each month were determined by Student's *t*-tests for parametric data and Wilcoxon tests for nonparametric data. If Wilcoxon test assumptions were not met, permutation tests using 1000 permutations were performed instead. All statistical analyses were performed using R version 3.6.3. All data shown in figures, tables, and text are mean ± s.e.m. Statistical significance was assumed at $p \leq 0.05$.

5.4. Results

5.4.1. Alligator Size and Environmental Salinity Levels

Total length of wild male and female juvenile alligators did not vary significantly by month of sampling with one exception. Average total length of males was significantly higher than females during August 2018 (*t*-test, $p<0.001$) (Fig. 5.2). Similarly, no significant variance in environmental salinity at capture sites of males and female was detected between months. However, some males were found in significantly higher environmental salinity than females during August 2018 (permutation test, $p=0.022$), corresponding with the (not significant) trend of the highest average environmental salinity occurring in August 2018 (Fig. 5.2). In total, 7 of the 33 juvenile males were found in hyperosmotic salinities of 10‰ or greater (Laurén, 1985; Morici, 1996) during the present study. These comprised 5 males found in water between 16.2 and 22.2‰ during August 2018, one male in 10.9‰ in September 2018, and one male in 12.4‰ during

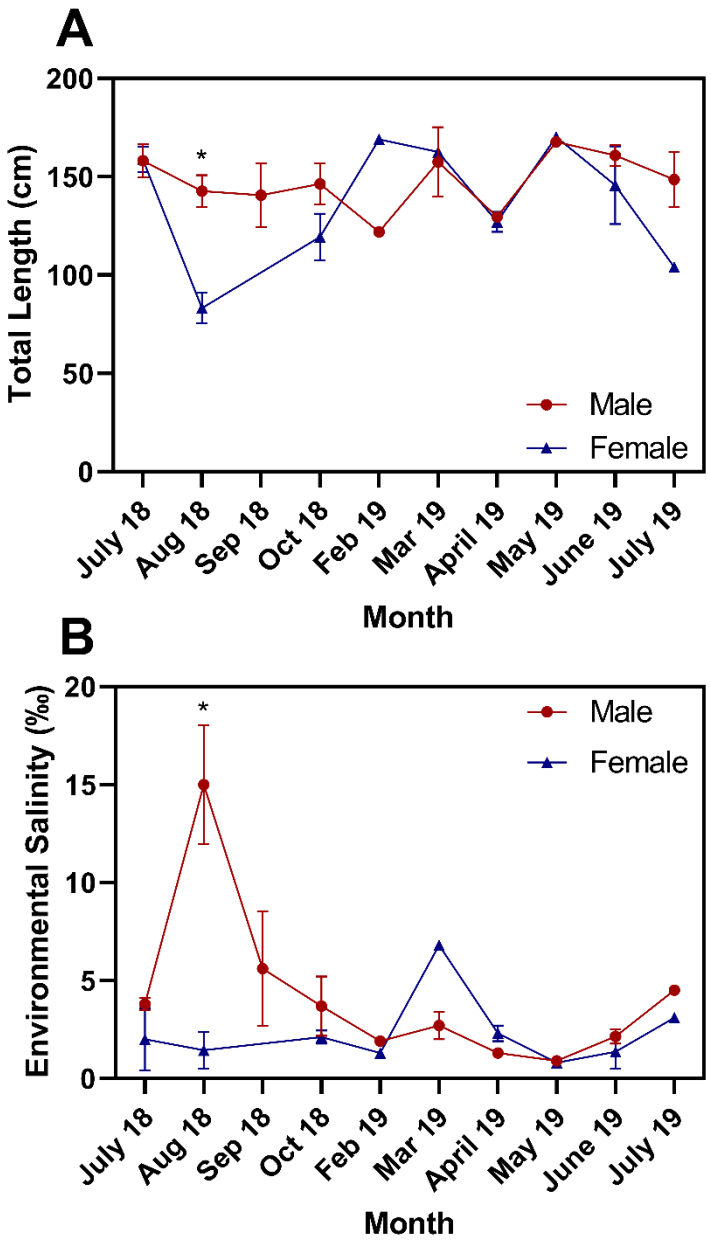

Figure 5.2. Monthly variation in total length (A) of wild juvenile American alligators (*Alligator mississippiensis*) and environmental salinity in which animals were located (B). *Significant differences between males and females during the indicated month. Error bars indicate s.e.m. of 2-8 animals.

October 2018. On the other hand, none of the 21 juvenile females were found in hyperosmotic environments.

5.4.2. Multivariate Analysis of Blood Chemistry and Hormones

The pRDA found significant correlation between environmental salinity and blood chemistry parameters ($p=0.003$), but a low adjusted r^2 of 0.031 indicates a low proportion of variance in blood chemistry levels overall is explained by environmental salinity. Blood plasma Na^+ and Cl^- appear positively correlated with environmental salinity, albeit not very strongly, while 17α-hydroxyprogesterone, 11-deoxycortisol, and corticosterone are more weakly, but positively correlated with salinity (Fig. 5.3). On the other hand, 17α-hydroxypregnenolone, androstenedione, 5α-dihydrotestosterone, 17β-estradiol, and estriol appear negatively, but weakly correlated with environmental salinity. Other parameters are closer to 90-degree angles compared with salinity with respect to the origin, signifying weaker correlation.

5.4.3. Correlations of Blood Biochemistry and Hormones with Environmental Salinity

Spearman rank correlations showed significant, positive correlations between environmental salinity and both plasma Na^+ ($p=5.26e-9$) and Cl^- ($p=4.26e-4$) in juvenile male alligators, while plasma K^+ was also positively, albeit weakly ($p=0.035$) correlated with environmental salinity (Fig. 5.4). In addition, environmental salinity of males was also positively, but weakly correlated with plasma 11-deoxycortisol ($p=0.045$) and corticosterone ($p=0.046$) (Fig. 5.5), while showing a stronger positive correlation with

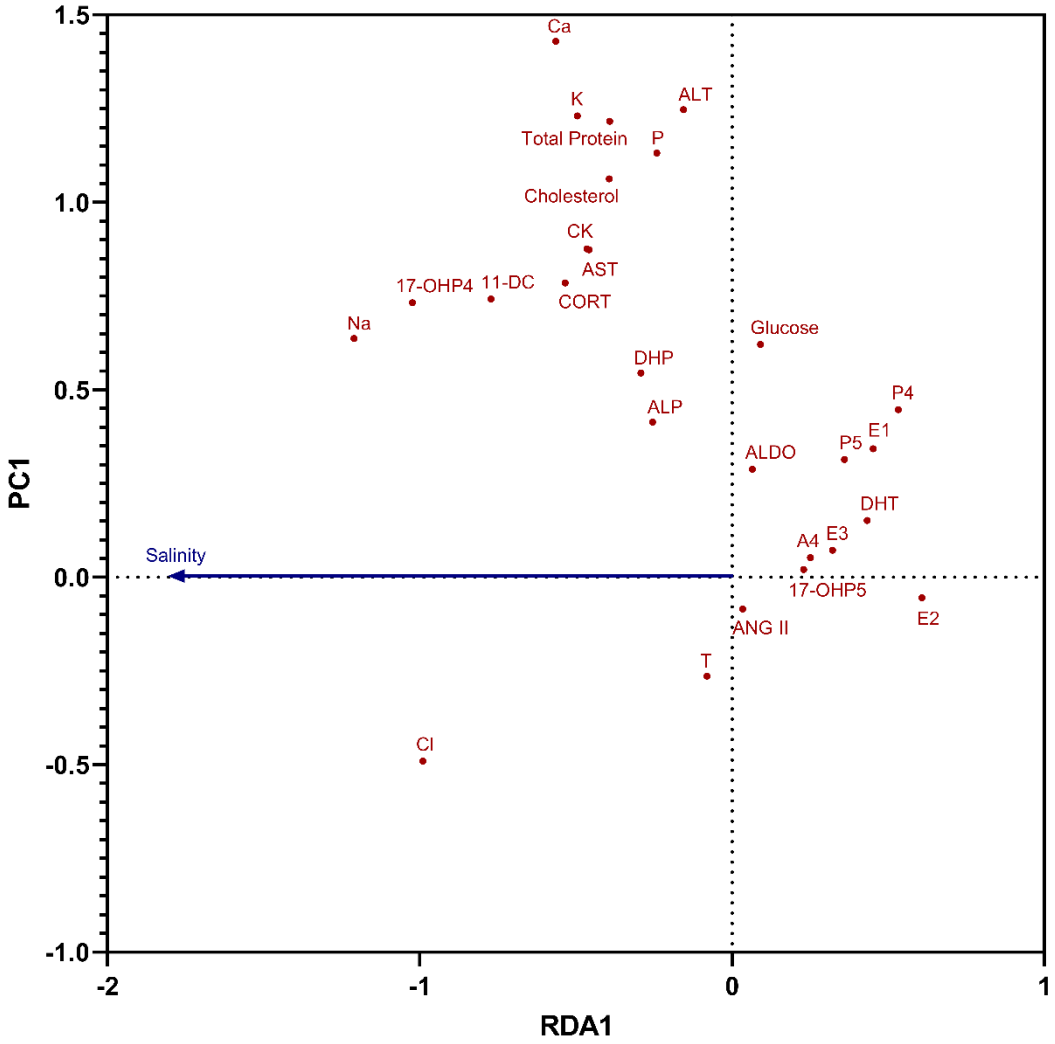

Figure 5.3. Partial redundancy analysis using environmental salinity in which juvenile American alligators (*Alligator mississippiensis*) were found as a predictor and holding total length, sex, and month constant. Abbreviated are angiotensin II (ANG II), aldosterone (ALDO), 11-deoxycortisol (11-DC), corticosterone (CORT), pregnenolone (P5), 17α-hydroxypregnenolone (17-OHP5), progesterone (P4), 17α-hydroxyprogesterone (17-OHP4), 17α,20β-dihydroxypregnenone (DHP), androstenedione (A4), 5α-dihydrotestosterone (DHT), testosterone (T), estrone (E1), 17β-estradiol (E2), estriol (E3), sodium (Na), chloride (Cl), potassium (K), calcium (Ca), phosphorus (P), alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT).

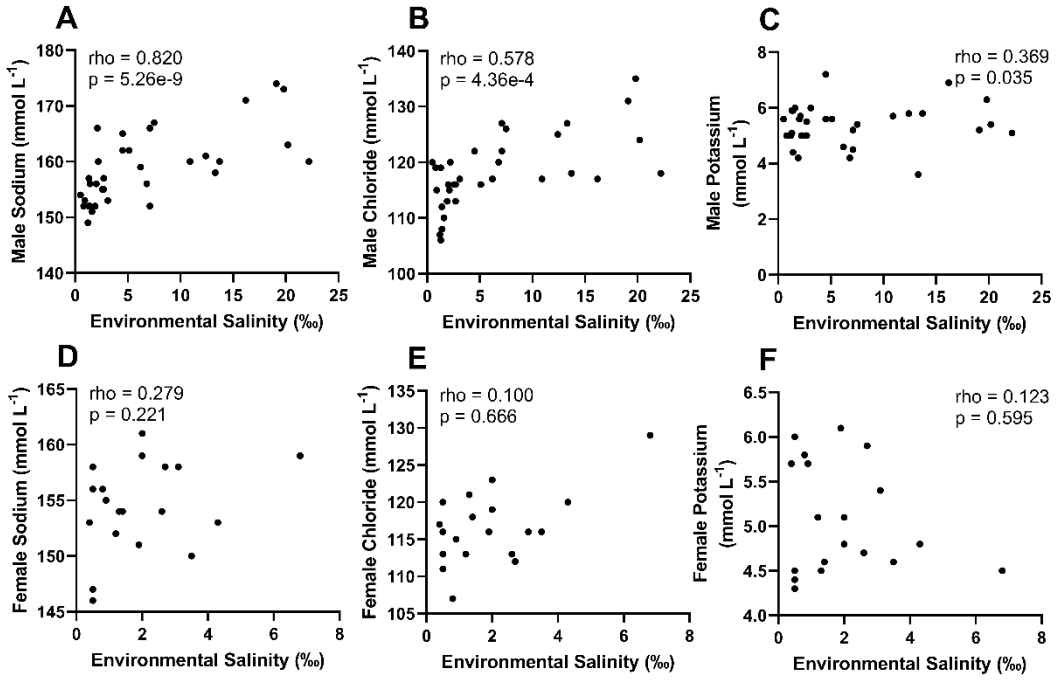

Figure 5.4. Spearman rank correlation between environmental salinity and plasma sodium (A,D), chloride (B,E), and potassium (C,F) in juvenile male and female American alligators (*Alligator mississippiensis*). rho = Spearman's rho, p = significance of correlation.

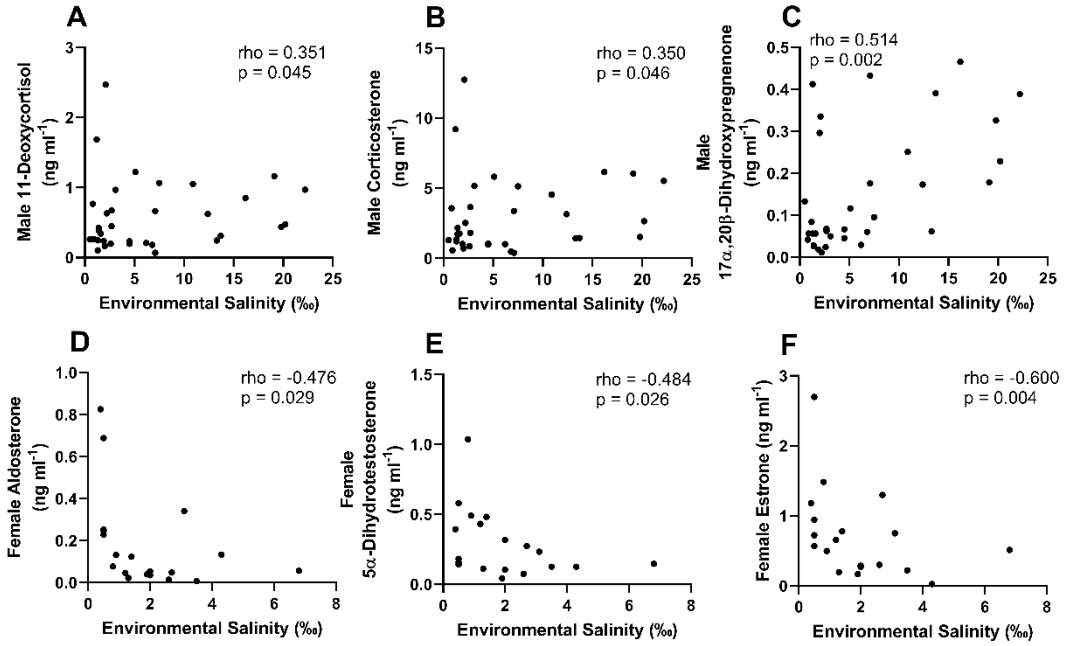

Figure 5.5. Spearman rank correlation between environmental salinity and plasma hormone levels found to be significant ($p < 0.05$) in juvenile male (A,B,C) and juvenile female (D,E,F) American alligators (*Alligator mississippiensis*). rho = Spearman's rho, p = significance of correlation.

17α,20β-dihydroxypregnenone (p=0.002) (Fig. 5.5). Although female alligator ion concentrations in plasma were not correlated with environmental salinity, environmental salinity was significantly negatively correlated with plasma aldosterone (p=0.045), 5α-dihydrotestosterone (p=0.046), and estrone (p=0.002) levels in females (Fig. 5.5).

5.4.4. Differences in Blood Biochemistry Parameters Between Months and Sex

Plasma ion concentrations (Na^+, K^+, Cl^-, Ca^{2+}, P) did not vary significantly by month in male or female juvenile alligators. However, males had significantly greater blood Na^+ (*t*-test, p=0.003), Ca^{2+} (*t*-test, p=0.014), and p (*t*-test, p=0.033) levels compared with females during August 2018 (Table 5.1) when salinities were higher due to lower rainfall. Among plasma biochemistry parameters, ALP was the only parameter to vary significantly by month as male ALP in October 2018 was significantly lower than in both July 2018 (p=0.036) and June 2019 (p=0.017) (Table 5.2). Male juvenile alligators had significantly greater creatine kinase levels in plasma compared with females in August 2018 (*t*-test, p=0.040). During the same month of August, females had significantly lower blood plasma ALT (*t*-test, p=0.050) and significantly greater AST:ALT ratios than in males (permutation test, p=0.042) (Table 5.2). Plasma albumin, globulin, creatinine, and uric acid levels were below the detection limit in most animals.

5.4.5. Differences in Hormone Levels Between Months and Sex

Plasma hormone levels varied significantly during certain months, such as corticosterone levels in male alligators which were significantly greater in July 2018 than

Table 5.1. Plasma ion concentrations in wild juvenile American alligators (*Alligator mississippiensis*).

Ion	Sex	July 2018	Aug 2018	Sep 2018	Oct 2018	Feb 2019	Mar 2019	April 2019	May 2019	June 2019	July 2019
Sodium (mmol l^{-1})	Male	158.50± 0.50	166.86± 3.29	158.00± 3.06	156.63± 1.93	152.00	159.50± 3.50	157.00	153.00	154.33± 1.41	163.50± 1.50
	Female	155.50± 2.50	150.50± 2.40*	-	155.00± 1.71	154.00	159.00	154.50± 3.50	156.00	157.33± 0.67	153.00
Potassium (mmol l^{-1})	Male	5.50± 1.00	5.61± 0.27	5.43± 0.22	5.16± 0.17	4.20	5.85± 0.25	5.90	5.00	5.32± 0.19	6.40± 0.80
	Female	5.90± 0.20	4.80± 0.32	-	4.82± 0.09	4.50	4.50	6.00± 0.10	5.80	5.30± 0.44	6.00
Na:K ratio	Male	29.80± 5.30	30.03± 1.16	29.13± 0.67	30.53± 0.90	35.30	27.30± 0.60	26.60	30.60	29.22± 1.06	25.90± 3.00
	Female	26.35± 0.45	31.68± 1.50	-	32.23± 0.55	34.20	35.30	25.80± 1.00	26.90	30.10± 2.46	25.50
Chloride (mmol l^{-1})	Male	115± 5.00	123.86± 2.59	117.33± 0.88	116.38± 2.60	113.00	116.00± 0.00	106.00	115.00	115.67± 1.48	122.00± 0.00
	Female	112.50± 4.50	117.50± 1.85	-	117.00± 1.57	121.00	129.00	114.00± 2.00	107.00	115.67± 2.60	117.00
Calcium (mg dl^{-1})	Male	10.45± 0.65	11.24± 0.39	10.17± 0.34	10.51± 0.41	11.00	11.95± 0.75	12.30	9.90	11.12± 0.25	11.35± 0.55
	Female	11.10± 0.80	9.78± 0.28*	-	10.62± 0.37	10.00	9.20	10.65± 0.35	10.90	11.50± 0.26	10.80
Phosphorus (mg dl^{-1})	Male	5.55± 1.25	5.87± 0.65	4.83± 0.32	3.98± 0.29	3.40	6.00± 0.90	4.40	4.70	6.40± 0.39	5.40± 1.40
	Female	3.80± 0.20	3.80± 0.50*	-	4.18± 0.19	3.00	3.50	5.65± 0.95	5.90	6.27± 0.57	4.40

*Significant difference between males and females during the indicated month ($p < 0.05$). Values shown are averages ± s.e.m. of 2-8 animals. S.e.m. is not shown in cases including only one animal.

Table 5.2. Plasma biochemistry parameters in wild juvenile American alligators (*Alligator mississippiensis*).

Blood Chemistry Parameter	Sex	July 2018	Aug 2018	Sep 2018	Oct 2018	Feb 2019	Mar 2019	April 2019	May 2019	June 2019	July 2019
Total Protein (mg dl^{-1})	Male	4.20± 1.20	4.39± 0.37	4.17± 0.32	4.26± 0.28	5.70	5.00± 0.50	5.30	4.00	4.22± 0.30	4.20± 0.60
	Female	4.95± 0.15	3.63± 0.23	-	4.68± 0.39	4.00	3.00	5.15± 0.15	5.60	4.40± 0.21	4.70
Bilirubin (mg dl^{-1})	Male	0.10± 0.00	0.10± 0.00	0.10± 0.00	0.10± 0.00	0.10	0.10± 0.00	0.10	0.10	0.10± 0.00	0.10± 0.00
	Female	0.10± 0.00	0.10± 0.00	-	0.10± 0.00	0.10	0.10	0.10± 0.00	0.10	0.10± 0.00	0.10
Albumin (mg dl^{-1})	Male	<1.50†	1.54± 0.04	<1.50†	1.56± 0.04	<1.50†	1.65± 0.15	<1.50†	<1.50†	<1.50†	<1.50†
	Female	1.55± 0.05	1.53± 0.03	-	1.60± 0.05	<1.50†	<1.50†	1.55± 0.05	1.70	<1.50†	<1.50†
Globulins (mg dl^{-1})	Male	3.90 (<1.50†)	3.50± 0.30	<1.50†	3.30± 0.12	<1.50†	3.70 (<1.50†)	3.80	<1.50†	4.00 (<1.50†)	<1.50†
	Female	3.20 (<1.50†)	2.70 (<1.50†)	-	3.28± 0.48	<1.50†	1.50	3.70 (<1.50†)	3.90	<1.50†	<1.50†
A:G ratio	Male	0.40	0.47± 0.03	-	0.50± 0.00	-	0.50	0.40	-	0.40	-
	Female	0.50	0.60	-	0.55± 0.06	-	-	0.40	0.40	-	-
Creatinine (mg dl^{-1})	Male	<0.20†	0.37± 0.09	<0.20†	0.23± 0.02	<0.20†	<0.20†	<0.20†	<0.20†	<0.20†	0.25± 0.05
	Female	0.30± 0.00	<0.20†	-	0.27± 0.04	<0.20†	<0.20†	<0.20†	<0.20†	<0.20†	0.30
Creatine kinase (U l^{-1})	Male	792.00± 300.00	687.29± 205.27	362.67± 49.24	321.00± 44.58	381.00	786.50± 56.50	677.00	411.00	945.50± 274.56	551.00± 130.00
	Female	227.50± 52.5	149.75± 6.97*	-	691.17± 297.96	1984.00	1264.00	693.50± 82.50	670.00	525.00± 43.10	1212.00
ALP (U l^{-1})	Male	22.50± 0.50^{a}	14.57± $2.29^{a,b}$	11.00± $0.58^{a,b}$	7.75± 0.70^{b}	$5.00^{a,b}$	18.50± $0.50^{a,b}$	$22.00^{a,b}$	$14.00^{a,b}$	17.67± 2.33^{a}	18.00± $0.00^{a,b}$
	Female	13.00± 3.00	11.25± 1.93	-	7.83± 0.79	7.00	29.00	19.00± 2.00	19.00	17.00± 3.79	22.00
AST (U l^{-1})	Male	167.00± 45.00	185.14± 39.58	152.67± 25.83	162.00± 9.52	155.00	226.00± 52.00	188.00	156.00	187.00± 15.11	183.50± 31.50
	Female	163.50± 15.50	188.00± 7.36	-	222.33± 35.38	136.00	129.00	218.50± 7.50	215.00	178.67± 4.91	240.00
ALT (U l^{-1})	Male	31.00± 2.00	36.00± 6.40	35.33± 5.04	40.75± 4.51	29.00	42.50± 6.50	60.00	35.00	54.33± 6.86	42.50± 8.50
	Female	39.50± 0.50	18.75± 4.17*	-	45.67± 5.10	38.00	22.00	48.00± 17.00	56.00	43.00± 3.06	74.00
AST:ALT ratio	Male	5.32± 1.11	5.97± 1.37	4.31± 0.31	4.20± 0.35	5.34	5.25± 0.42	3.13	4.46	3.55± 0.27	4.35± 0.13
	Female	4.15± 0.45	11.31± 1.92*	-	4.98± 0.66	3.58	5.86	5.15± 1.67	3.84	4.18± 0.18	3.24
Glucose (mg dl^{-1})	Male	89.00± 4.00	96.43± 7.56	108.33± 2.85	73.13± 4.06	49.00	88.00± 3.00	88.00	82.00	95.67± 3.63	80.00± 13.00
	Female	71.50± 3.50	96.75± 2.39	-	77.67± 4.48	60.00	53.00	95.00± 12.00	93.00	106.67± 12.71	78.00
Cholesterol (mg dl^{-1})	Male	55.50± 25.50	75.14± 10.57	75.33± 7.86	61.75± 7.14	65.00	87.00± 5.00	78.00	37.00	73.50± 5.02	89.00± 35.00
	Female	64.00± 11.00	77.25± 14.05	-	73.50± 12.39	27.00	44.00	73.00± 30.00	61.00	93.33± 22.58	54.00
Uric acid (mg dl^{-1})	Male	1.70± 0.20	2.46± 0.55	1.70± 0.20	1.65± 0.11	1.70	<1.50†	1.80	<1.50†	<1.50†	<1.50†
	Female	1.60± 0.10	<1.50†	-	1.60± 0.10	<1.50†	<1.50†	1.55± 0.05	<1.50†	<1.50†	<1.50†

Dissimilar letters show significant differences between months within the given sex. *Significant difference between males and females during the indicated month ($p < 0.05$). †Values below detection limit. Values shown are averages ± s.e.m. of 2-8 animals. S.e.m. is not shown in cases including only one animal. Outliers excluded from analyses are shown in parentheses.

in March 2019 ($p=0.042$) due to higher salinities in summer compared to winter. Male pregnenolone levels in September 2018 were also significantly higher than in June 2019 ($p=0.039$), and 17α-hydroxypregnenolone in females was greater in August 2018 compared with females in October 2018 ($p=0.032$). Male progesterone levels were also significantly lower in October 2018 than in both July 2018 ($p=0.048$) and June 2019 ($p=0.021$). Furthermore, testosterone levels in juvenile male alligators were significantly greater in March 2019 compared with July 2018 ($p=0.016$) and August 2018 ($p=0.050$). Differences in plasma concentrations of two hormones were also detected between sexes, including angiotensin II which was significantly greater in juvenile male alligators than in females during October 2018 (Wilcoxon test, $p=0.038$), while in August 2018 females had greater plasma 17α,20β-dihydroxypregnenone than males (*t*-test, $p<0.001$) (Table 5.3).

Table 5.3. Plasma hormone levels in wild juvenile American alligators (*Alligator mississippiensis*).

Hormone	Sex	July 2018	Aug 2018	Sep 2018	Oct 2018	Feb 2019	Mar 2019	April 2019	May 2019	June 2019	July 2019
Angiotensin II (ng ml^{-1})	Male	1.15	0.26± 0.03	1.21± 0.65	0.45± 0.29	0.38	0.43± 0.14	0.06	1.05	0.19± 0.07	0.05± 0.01
	Female	1.12± 0.19	0.66± 0.53	-	0.21± 0.16*	0.16	0.16	0.13± 0.02	2.26	0.11± 0.04	0.02
Aldosterone (ng ml^{-1})	Male	0.16± 0.07	0.28± 0.08	0.17± 0.06	0.11± 0.04	0.10	0.12± 0.08	0.02	0.39	0.25± 0.07	0.18± 0.09
	Female	0.46± 0.37	0.35± 0.12	-	0.05± 0.02	0.02	0.06	0.04± 0.00	0.08	0.27± 0.03	0.10
11-Deoxycortisol (ng ml^{-1})	Male	1.91± 0.40	0.95± 0.32	1.01± 0.13	0.70± 0.16	0.24	0.23± 0.07	0.25	0.26	0.48± 0.08	0.22± 0.02
	Female	0.56± 0.16	0.40± 0.14	-	1.08± 0.20	0.16	0.33	0.3± 0.18	0.33	1.1± 0.23	0.97
Corticosterone (ng ml^{-1})	Male	10.40± 1.71^{a}	5.34± 1.66^{a,b}	4.64± 0.65^{a,b}	3.49± 0.92^{a,b}	1.02^{a,b}	0.56± 0.14^{b}	1.21^{a,b}	0.56^{a,b}	2.42± 0.48^{a,b}	1.01± 0.04^{a,b}
	Female	3.29± 1.02	1.64± 0.54	-	5.15± 1.04	0.12	0.17	1.59± 1.03	1.17	4.99± 1.14	5.15
Pregnenolone (ng ml^{-1})	Male	17.97± 3.62^{a,b}	28.56± 9.79^{a,b}	42.42± 3.17^{a}	20.78± 4.86^{a,b}	9.92^{a,b}	5.18± 0.30^{a,b}	3.13^{a,b}	2.73^{a,b}	4.77± 1.21^{b}	7.33± 1.45^{a,b}
	Female	15.6± 4.88	21.78± 6.41	-	37.28± 16.33	8.25	3.83	3.18± 0.88	26.61	7.37± 3.20	1.35
17α-Hydroxypregnenolone (ng ml^{-1})	Male	425.03± 67.30	342.28± 48.74	318.87± 119.43	141.87± 17.25	122.02	147.44± 15.13	157.41	382.25	217.25± 33.41	251.08± 36.72
	Female	255.93± 68.64^{a,b}	466.18± 71.93^{a}	-	107.72± 26.21^{b}	118.06^{a,b}	152.95^{a,b}	94.93± 7.09^{a,b}	321.09^{a,b}	274.63± 87.88^{a,b}	183.53^{a,b}
Progesterone (ng ml^{-1})	Male	0.79± 0.03^{a}	0.35± 0.08^{a,b}	0.28± 0.02^{a,b}	0.17± 0.02^{b}	0.10^{a,b}	0.30± 0.08^{a,b}	0.09^{a,b}	1.21^{a,b}	0.58± 0.09^{a}	0.17± 0.05^{a,b}
	Female	0.24± 0.04	0.18± 0.08	-	0.20± 0.10	0.15	0.18	0.10± 0.05	0.82	0.76± 0.22	1.22
17α-Hydroxyprogesterone (ng ml^{-1})	Male	0.41	0.62± 0.27	1.06± 0.29	0.60± 0.30	0.10	0.08± 0.04	0.12	0.11	0.30± 0.07	0.15± 0.02
	Female	0.63± 0.34	0.28± 0.09	-	0.76± 0.15	0.03	0.07	0.13± 0.10	0.28	0.46± 0.19	0.74
17α,20β-Dihydroxypregnenone (ng ml^{-1})	Male	0.30± 0.02	0.36± 0.04	0.14± 0.06	0.11± 0.05	0.02	0.18± 0.12	0.06	0.06	0.21± 0.13	0.06± 0.01
	Female	0.18± 0.05	0.08± 0.01*	-	0.06± 0.01	0.09	0.04	0.02± 0.01	0.05	0.12± 0.02	0.05
Androstenedione (ng ml^{-1})	Male	0.10± 0.06	0.15± 0.09	0.25± 0.23	0.11± 0.07	0.02	1.13± 0.52	0.04	0.13	1.14± 0.49	0.24± 0.16
	Female	0.07± 0.02	0.03± 0.02	-	0.05± 0.02	0.02	0.03	0.05± 0.04	0.46	0.20± 0.04	0.03
5α-Dihydrotestosterone (ng ml^{-1})	Male	0.43± 0.30	0.70± 0.37	0.46± 0.18	0.55± 0.20	0.06	2.52± 0.69	0.10	0.21	0.72± 0.17	0.25± 0.04
	Female	0.26± 0.14	0.32± 0.11	-	0.26± 0.07	0.11	0.15	0.16± 0.12	1.04	0.19± 0.02*	0.05
Testosterone (ng ml^{-1})	Male	1.00± 0.09	5.70± 1.62	10.55± 7.40	8.06± 2.70	1.56	-	0.86	7.30	25.74± 5.98	8.39± 4.29
	Female	9.15± 4.24	16.72± 9.12	-	10.12± 2.05	3.34	6.64	4.10± 2.44	28.38	8.63± 0.75*	2.03
Estrone (ng ml^{-1})	Male	0.32± 0.14	1.31± 0.32	0.24± 0.07	0.65± 0.23	0.45	0.56± 0.09	1.72	0.99	0.72± 0.06	0.51± 0.04
	Female	0.59± 0.07	0.41± 0.08	-	1.10± 0.28	0.45	0.46	1.19± 0.53	0.99	0.82± 0.07	0.65
17β-Estradiol (ng ml^{-1})	Male	1.65± 0.10	1.48± 0.04	2.31± 0.08	1.41± 0.35	1.68	1.51± 0.11	0.38	1.54	1.11± 0.10	0.71± 0.09
	Female	1.64± 0.04	1.73± 0.31	-	0.42± 0.30	1.93	2.01	1.00± 0.81	2.42	1.06± 0.15	0.57
Estriol (ng ml^{-1})	Male	0.14± 0.03	0.82± 0.21	0.81± 0.18	2.28± 0.64	0.24	0.18± 0.00	0.55	0.33	1.12± 0.36	1.85± 0.84
	Female	0.36	1.44± 0.40	-	1.57± 0.39	0.06	0.07	0.19± 0.08	0.20	1.57± 0.44	1.99

Dissimilar letters show significant differences between months for the given sex. *Significant difference between males and females during the indicated month ($p < 0.05$). †Values below detection limit. Values shown are averages ± s.e.m. of 2-8 animals. S.e.m. is not shown in cases where data was only obtained from one animal.

5.5. Discussion

5.5.1. Alligator Sex and Environmental Salinity

During this study, opportunistically sampled animals were most commonly found in environmental salinities considered hypoosmotic to alligator bodily fluids. This assumption is based on previous studies which have estimated an environmental salinity level of 8‰ to be hypoosmotic to alligator body fluids with environmental salinities of 10‰ or greater being considered hyperosmotic (Laurén, 1985; Morici, 1996). Correlating the opportunistic sampling of male and female alligators throughout the year of 2018-2019 (July-July), no clear trend between salinity levels at capture and sex was apparent. It is noteworthy, however, that more juvenile males were captured in hyperosmotic environments in August 2018 while female alligators were not sampled from any hyperosmotic areas in this study. The reason why we captured smaller males in these hyperosmotic areas may be due to territoriality of alligators. For instance, large male alligators are very territorial and will defend their territory against other males (Garrick, 1975; Garrick and Lang, 1977). This behavior often results in smaller and sexually immature males being pushed out of favorable areas (Garrick and Lang, 1977) by the territory holder and into less favorable areas (e.g., higher salinity environments). However, there is evidence that juvenile alligator populations in coastal Louisiana are significantly male-biased with 58% males of 3,000 juveniles collected over 6 years (Lance et al., 2000), which can further explain the greater number of males in the present study. While sex differences in tolerance to saltwater could be a contributing factor, there were no significant differences in plasma biochemistry and hormone levels between males and

females exposed to 12‰ saltwater for 1 week (Faulkner et al., 2019) and it is therefore more likely that the observed trend was due to behavioral than physiological effects.

A review of climate data (https://www.ncdc.noaa.gov/cdo-web/) from Rockefeller Wildlife Refuge during the sampling period of July 2018-July 2019 showed that during August 2018, there was lower rainfall during August 2018 (13.1 cm) than average during the sampling period (15.9 cm). In addition, August 2018 had high average temperature (28.4°C) compared with other months during the sampling period (21.5°C). Drought conditions brought on by low rainfall can be exacerbated by high evaporation rates due to high temperature, which correlates well with the highest salinity levels recorded during that month compared with other months in 2018-2019. It is possible that during dry conditions as seen in August 2018, fewer freshwater habitats may be available, or are occupied by large males as discussed above. Therefore, it is likely that young male alligators utilize less favorable, high-salinity environments in the process of avoiding larger males.

Alligator habitat use is highly complex and is driven by a number of factors including prey distribution and environmental salinity (Fujisaki et al., 2014; Rosenblatt and Heithaus, 2011; Rosenblatt et al., 2015), in addition to social interactions (Garrick and Lang, 1977). Individual niche specialization can also play a role, i.e., individuals within a population can show variable habitat use and foraging strategies independent of sex, size, and age (Nifong et al., 2015; Rosenblatt and Heithaus, 2011; Rosenblatt et al., 2013). While there is evidence that larger alligators favor freshwater habitats (Chabreck, 1965; Rosenblatt and Heithaus, 2011), adult alligators are more able to endure salt stress to

forage in higher-salinity areas. For example, adult alligators were observed to be more likely than juveniles to utilize high-salinity habitats in the Shark River Estuary in Florida (Fujisaki et al., 2016), and large alligators can be seen making offshore excursions into full strength seawater (Elsey, 2005). On the other hand, previous data also demonstrate that sub-adult males tend to utilize higher-salinity habitats in the Shark River Estuary compared with larger adult males which were mainly found in low salinity areas (Rosenblatt and Heithaus, 2011). This use of high-salinity habitats by smaller alligators can be explained by the seeking of prey-rich areas at the cost of salt stress, as well as by smaller alligators evading territories of larger males (Nifong et al., 2015; Rosenblatt and Heithaus, 2011). These studies further highlight the complexity of alligator behavior and habitat use. For example, high variability in adult alligator movements driven by modified environmental salinity has been observed following Hurricane Irma (Strickland et al., 2020). Responses after the hurricane included alligators moving into river channels or travelling downstream, both likely due to changes in prey distribution, while others showed no change in habitat use. Since alligator behavior can be highly variable and complex, further studies are warranted to better understand the driving factors behind the use of saline habitats by young alligators (Strickland et al., 2020).

5.5.2. Correlation of Blood Plasma Biochemistry with Salinity

Average plasma Na^+ levels in juvenile wild alligators (157.26 ± 0.86 mmol l^{-1}) corresponded well with values obtained in laboratory-kept juvenile alligators [148.3 ± 0.81 mmol l^{-1} (Faulkner et al., 2018), 150.9 ± 0.58 mmol l^{-1} (Faulkner et al., 2019)], and

wild juvenile [140.3 ± 0.69 mmol l^{-1} (Hamilton et al., 2016)] and adult alligators [146 mmol l^{-1} (Divers and Mader, 2005)]. In addition, plasma Cl^- (117.22 ± 0.80 mmol l^{-1}) and K^+ (5.33 ± 0.09 mmol l^{-1}) levels were similarly comparable to previously published values from juvenile laboratory-kept alligators and wild juvenile and adult alligators [Cl^- 117.00 ± 1.14 mmol l^{-1} (Faulkner et al., 2018), 114.0 ± 2.20 mmol l^{-1} (Faulkner et al., 2019), 110 mmol l^{-1} (Divers and Mader, 2005), K^+ 5.24 ± 0.17 mmol l^{-1} (Faulkner et al., 2018), 5.06 ± 0.13 mmol l^{-1} (Faulkner et al., 2019), 4.9 ± 0.11 mmol l^{-1} (Hamilton et al., 2016), 3.8 mmol l^{-1} (Divers and Mader, 2005)].

Wild juvenile male alligators sampled from a range of environmental salinities showed a significant positive correlation between salinity and plasma Na^+ and Cl^- levels. Like most animals, alligators are unable to prevent Na^+ influx when in saline environments. For example, unfed alligators ranging in size from 310-586 g showed an average Na^+ influx of ~10.8 μmol 100 g^{-1} h^{-1} when exposed to 35‰ for up to 4 hours while water efflux was 0.25 ml 100 g^{-1} h^{-1} (Mazzotti and Dunson, 1984). Freshwater also has significant effects on electrolyte balance, as net Na^+ and K^+ loss occurs in solutions up to 1 mmol L^{-1} Na^+ and 0.4 mmol L^{-1} K^+ in freshwater (Ellis and Evans, 1984; Taplin et al., 1982). Additionally, hatchlings (0.03-0.07 g) exhibit a whole-body Na^+ efflux of 3.9 μmol 100 g^{-1} h^{-1} in freshwater (Ellis and Evans, 1984). Thus, as alligator integument is not impermeable to fluxes of electrolytes, the positive and significant correlations in wild alligators are therefore likely due to passive influx of ions through the integument and mucous membranes, while water loss also contributed to elevated ion levels as seen in laboratory kept alligators (Faulkner et al., 2019).

There were no significant trends in any other blood biochemistry parameters (Table 5.1, 5.2, Fig. 5.3) dependent on environmental salinity. However, while uric acid was not detected in most samples, uric acid was more consistently above the detection limit and tended to be higher in August 2018 males (not significant), which were found in higher saline environments. Although this trend is not significant, it does correlate well with findings from laboratory studies where uric acid significantly increased in juvenile alligators exposed for 1 and 5 weeks to 12‰ saltwater (Faulkner et al., 2018; Faulkner et al., 2019).

5.5.3. Correlation of RAAS and Steroid Hormones with Salinity

While plasma K^+ in wild juvenile males significantly and positively correlated with environmental salinity, the low variability in male plasma K^+ across salinity levels would suggest activation of aldosterone to promote K^+ excretion. However, aldosterone in males was not correlated with salinity, which corresponds with a lack of increased aldosterone in laboratory-kept juvenile alligators exposed to 12‰ saltwater (Faulkner et al., 2018; Faulkner et al., 2019). As suggested previously (Faulkner et al., 2018; Faulkner et al., 2019), the mechanisms controlling K^+ excretion and the role of aldosterone in osmoregulation in saltwater-exposed alligators are not fully understood and require further investigation. This observation is further supported by data from female alligators which albeit were not found in hyperosmotic salinity, but data analysis revealed aldosterone to be strongly and negatively correlated with environmental salinity in females. The observation that aldosterone is higher in hypoosmotic environments further demonstrates

aldosterone does not regulate water reabsorption in alligators and supports the previous argument for a yet unknown but non-osmoregulatory role of aldosterone in alligators. Similar to aldosterone, the other RAAS hormone angiotensin II was not affected by hyperosmotic salinity levels in the present study. This is in contrast to laboratory studies on 1- and 5-week saltwater-exposed juvenile alligators where angiotensin II was significantly decreased after 5 weeks in 12‰ and reduced after 1 week in 12‰ (Faulkner et al., 2018; Faulkner et al., 2019). Therefore, the present study provides further evidence that RAAS is not active in juvenile alligators exposed to hyperosmotic salinities.

We detected a significant, weakly positive correlation between environmental salinity and the glucocorticoids corticosterone and 11-deoxycortisol in wild juvenile male alligators. However, glucocorticoid levels of animals found in hyperosmotic water (<6 ng ml^{-1} corticosterone, <2 ng ml^{-1} 11-deoxycortisol) did not exceed those previously seen in unstressed alligators [11.91 ± 2.74 ng ml^{-1} corticosterone and 2.22 ± 0.52 ng ml^{-1} 11-deoxycortisol (Faulkner et al., 2019), 5.5 ± 0.8 ng ml^{-1} corticosterone (Laurén, 1985)]. Thus, the present study does not show elevated corticosterone or 11-deoxycortisol resulting from salinity exposure. These findings are likely due to confounding factors in the wild, such as variable or less time spent in hyperosmotic environments. While corticosterone is an indicator of stress in reptiles (Cockrem, 2013), corticosterone has been implicated to play an osmoregulatory role in lizard species such as sand goannas (*Varanus gouldii*) (Bradshaw and Rice, 1981) and desert iguanas (*Dipsosaurus dorsalis*) (Bradshaw, 1972; Bradshaw et al., 1972) by promoting Na^+ excretion in kidneys. Since salt stress has been well documented to be associated with elevated corticosterone in wild and captive

alligators (Faulkner et al., 2018; Faulkner et al., 2019; Lance et al., 2010; Laurén, 1985), it is likely that glucocorticoids play an osmoregulatory role in crocodilians. As previously speculated, it is possible corticosterone may have mineralocorticoid effects since elevated glucocorticoid levels were not seen alongside increased plasma glucose, suggesting a nontraditional effect of corticosterone (Faulkner et al., 2019).

Androgen (androstenedione, 5α-dihydrotestosterone, testosterone) and estrogen (estrone, 17β-estradiol, estriol) levels in wild alligators were not correlated with hyperosmotic environmental salinity in males. This result is likely due to less time of exposure in the wild. Previous lab studies showed time-dependent dynamic changes in sex hormones, in which 5-week saltwater exposure significantly increased testosterone and estrogen levels (Faulkner et al., 2018), whereas 1-week exposure significantly decreased estrone and 17β-estradiol levels (Faulkner et al., 2019). Conversely, in females we found significant negative correlations between environmental salinity (0.4-6.8‰) and 5α-dihydrotestosterone and estrone. However, these significant correlations are likely an artifact of seasonal variation in sex hormones since higher values occurred during the months of April, May, July, August, and October. These months have been associated with increased female testosterone and 17β-estradiol levels (see discussion of seasonal hormone cycling in section 5.5.4) (Guillette et al., 1997; Hamlin et al., 2014; Rooney et al., 2004), it is more likely these higher levels are explained by seasonal cycling than by hypoosmotic environments. The fact that sex steroid levels were not explained by differences in salinity in the present study could be due to the many confounding factors

found in the wild compared with laboratory-based studies, small sample sizes, and unknown duration of exposure to environmental salinity.

The progestogen, 17α,20β-dihydroxypregnenone (DHP) levels were positively and significantly correlated with salinity in males. Most of our knowledge of DHP in ectotherms are from studies of fish where it acts as an oocyte maturation inducing hormone in female teleosts (Nagahama and Adachi, 1985; Thomas et al., 2007) and in maturation of sperm and induction of sperm motility in males (DeFraipont and Sorensen, 1993; Miura et al., 1992). To the best of our knowledge, this study presents the first measurement of DHP in alligators, and in archosaurs DHP has only been previously detected in domestic chickens (*Gallus gallus domesticus*) (Marrone et al., 1985). It is currently unknown what role DHP plays in juvenile alligators exposed to salinity. It is interesting to note, however, that DHP is involved in reproduction in fish, so therefore could potentially play a similar role in egg-laying reptiles. As there is evidence from laboratory studies that salinity levels affect reproduction (changes in gonad histology in males and females) and affects key sex steroid hormone levels, the significant correlation between DHP and salinity in males provides further evidence of potential reproductive disruptions in alligators exposed to salinity.

5.5.4. Blood Biochemistry Parameter and Hormone Variation Across Sex and Month

Several steroid hormones varied by month in wild male juvenile alligators, including an increase in testosterone (not significant) during May and peaking in June, which corresponded with similar trends of increased androstenedione and 5α-

dihydrotestosterone. Increased testosterone around March and April is followed by the onset of mating season which normally starts in April (Joanen and McNease, 1980; Lance, 1989). Indeed, previous studies have demonstrated cycling of testosterone in wild adult and juvenile alligators during the months of March, April, and May (Hamlin et al., 2011; Lance, 2003; Lance et al., 2015; Rooney et al., 2004). However, the present study only detected increased male androgens in May and June. Testosterone peaks in June correspond well with increased testosterone previously reported in 90-119 cm males (Lance et al., 2015) and in other juvenile males (Rooney et al., 2004). However, the peak testosterone levels presented in this study (25.74 ng ml^{-1}) are closer to those of adult males less than 135 cm snout-vent length (~25 ng ml^{-1}) (Hamlin et al., 2011) than to wild juvenile alligators between 56-172 cm (Rooney et al., 2004) and 59-180 cm total length (Lance et al., 2015) (below 8 ng ml^{-1}). The variable levels of sex hormones seen in reptiles can be due to various environmental factors. For instance, seasonal variation in lizard sex hormones can be influenced by temperature (Bourne et al., 1986; Pearson et al., 1976) or diet (Lovern and Adams, 2008; Ruiz et al., 2010). Further study is required to understand the factors controlling the seasonal fluctuation in juvenile alligators across size classes and whether salinity levels can impact this seasonal variation in sex steroid hormone levels.

Although only one female alligator was opportunistically sampled during May, the higher (not significant) 17β-estradiol in this sample compared with females sampled during other months corresponds well with the higher level of its substrate testosterone, as well as androstenedione and 5α-dihydrotestosterone. Seasonal variation in 17β-estradiol has previously been reported in other alligator populations where adult (Guillette

et al., 1997; Hamlin et al., 2014) and juvenile (Rooney et al., 2004) female alligators in Florida exhibited increases in 17β-estradiol during the months of March, April, and May. Smaller, subsequent increases in sex steroid hormones can also occur, such as 17β-estradiol in August, September, and October (Guillette et al., 1997; Hamlin et al., 2014; Rooney et al., 2004) or in testosterone during August (Rooney et al., 2004). Furthermore, estrone levels in juvenile females, in the current study, also tended to increase in April, similarly coinciding with mating season. On the other hand, estriol levels remained low during April and May, but tended to be higher during June, correlating with alligator nesting season. While not significant, progesterone and 17α-hydroxyprogesterone tended to increase in juvenile females during May and June, similar to trends seen in progesterone levels of adult females sampled in Florida lakes (Guillette et al., 1997). However, adult female alligators sampled on a barrier island have shown more stable progesterone levels across seasons (Hamlin et al., 2014), illustrating differences between some alligator populations. Overall, sex steroid levels in immature alligators appear to reflect similar seasonal variation as seen in adults. Furthermore, the fluctuations in sex steroids reported in the present study occur independently from varying environmental salinity since hyperosmotic samples were only obtained in August, September, and October 2018. Further work is needed however to understand the role these fluctuations play in the maturation of juvenile alligators, and if changes in these hormone levels resulting from environmental changes (e.g., salt inundation) impact the long-term fitness of juvenile alligators.

5.5.5. Conclusions

The present study demonstrates significant correlations between hyperosmotic saltwater exposure and blood plasma electrolyte concentrations (Na^+, Cl^-) in wild juvenile alligators, similar to changes seen in laboratory-kept juvenile alligators exposed to 12‰ saltwater. In addition, higher environmental salinity was associated with elevated levels of the progestogen DHP in juvenile male alligators. Glucocorticoid, androgen, and estrogen levels were not clearly impacted in hyperosmotic salinities, likely due to confounding factors present in the wild. Further, the RAAS hormones angiotensin II and aldosterone were not associated with high salinity levels, and aldosterone was in fact significantly correlated with hypoosmotic environments in juvenile female alligators.

These findings evoke questions regarding the physiological changes in alligators exposed to hyperosmotic environments. Further research is needed to understand what role DHP plays in alligators. Future studies should also investigate if saltwater exposure during developmental stages of juvenile alligators impacts long-term fitness, and to observe whether salt inundation affects wild male alligators differently than females due to territorial social interactions and differential levels of sex steroid hormones. This information will be crucial in understanding how wild alligator populations in vulnerable Gulf of Mexico ecosystems respond to natural stressors such as salinity in a changing climate.

5.6. Acknowledgments

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CHAPTER VI

CONCLUSIONS

6.1. Summary

In this dissertation, I performed laboratory-based experiments to investigate how chronic (5 weeks) and short-term (1 week) exposure to saltwater (12‰) affected the physiology of juvenile American alligators [*Alligator mississippiensis* (Daudin, 1802)]. In addition, I investigated the effects of 5-week 12‰ saltwater exposure on juvenile alligator basking and foraging behavior. Finally, blood biochemistry and hormones in wild juvenile alligators were measured to compare findings in the lab with animals opportunistically sampled in the wild. In the first experiment (Chapter II), I investigated how 5-week exposure to 12‰ saltwater impacted the renin-angiotensin-aldosterone system (RAAS), steroidogenesis, blood plasma biochemistry, and lingual gland morphology in juvenile alligators. Saltwater-exposed alligators showed significantly higher Na^+ and Cl^- levels in blood plasma, while K^+ was unaffected. Further, 5 weeks of 12‰ saltwater exposure significantly lowered plasma concentrations of the RAAS hormone angiotensin II, while aldosterone levels decreased but were not significantly changed. As neither RAAS hormone was elevated during salt stress, this suggests that the RAAS was depressed or not activated in salt-stressed alligators. Conversely, the glucocorticoid stress hormones corticosterone and 11-deoxycortisol were significantly elevated. However, gene expression of angiotensin II, aldosterone, and glucocorticoid receptors were unaffected by salt stress, indicating physiological changes were post-translational. Additionally,

significantly elevated plasma 17α-hydroxyprogesterone and reduced 17α-hydroxypregnenolone (not significant) can be associated with higher demand for corticoid production. Furthermore, the androgen testosterone and the estrogens (estrone, 17β-estradiol, estriol) were each significantly elevated in saltwater-exposed alligators, associated with significantly greater estrone:androstenedione ratios. Saltwater-exposed alligators also experienced significant weight loss due to dehydration, as indicated by higher total plasma protein concentration. Finally, lingual gland morphology was largely unchanged except for significantly increased pore length in salt-stress alligators, likely due to severe dehydration.

In addition to physiological changes, I investigated how exposure to 12‰ saltwater for 5 weeks influenced juvenile alligator behavior. In Chapter III, feeding and resting behaviors were observed in saltwater-exposed juvenile alligators, and bite force was measured before and after 5 weeks. Saltwater exposure significantly reduced feeding within the first week, and cessation of feeding continued throughout the duration of exposure. Aggressive behaviors also ceased entirely in saltwater-exposed alligators. Although a small reduction in bite force was seen after saltwater exposure, bite force was not significantly affected, indicating the cessation of feeding was due to behavioral changes rather than morphological changes influencing feeding ability. Saltwater exposure also impacted resting behaviors, as alligator spent significantly less time basking over the course of exposure and spent more time in saltwater. Interestingly, saltwater-exposed alligators also rested with eyelids closed significantly more frequently during exposure, likely to reduce water loss across mucus membranes.

The initial 5-week exposure study in Chapter II raised a number of questions, such as how time of saltwater exposure influenced changes in alligator physiology. For instance, is the RAAS in alligators activated briefly before being subject to negative feedback? Furthermore, are the enzymatic components of the RAAS, renin and angiotensin-converting enzyme (ACE), affected by salt stress? In Chapter IV, I studied time-dependent dynamic changes in alligator physiology during short-term (1 week) 12‰ saltwater exposure. After 1 week of saltwater exposure, I investigated juvenile alligator blood biochemistry parameters, RAAS and steroid hormones, gonad histology, and expression of renin and ACE in kidney and lung tissues respectively. As seen after 5-week exposure, Na^+ and Cl^- were significantly elevated after 1 week, albeit to a lesser degree. Also similar to the previous 5-week exposure, angiotensin II levels in plasma were significantly reduced after 1 week of saltwater exposure while aldosterone was not significantly affected. These results were associated with significantly reduced renin and ACE expression, providing further evidence that the RAAS is indeed not active or is depressed in salt-stressed alligators. The glucocorticoids corticosterone and 11-deoxycortisol were also significantly elevated after 1-week saltwater exposure, although these increases were less pronounced compared with the 5-week exposure. Contrasting the findings after the 5-week saltwater exposure, testosterone was not significantly affected after 1 week. However, 5α-dihydrotestosterone was significantly elevated in salt-stressed juvenile alligators. Furthermore, estrone, 17β-estradiol, and estriol were significantly decreased after 1 week, contrasting the increased estrogens seen after 5 weeks. Finally, gonad histology revealed atresia in female gonads after 1-week saltwater

exposure as oocytes were no longer clearly visible. Furthermore, Sertoli and germ cells in male testes were significantly less abundant in salt-stress juvenile alligators. Overall, juvenile alligators exposed to 12‰ saltwater for 1 week showed similar effects on the RAAS while renin and ACE expression was also decreased by saltwater exposure. However, the 1-week study demonstrated time-dependent effects of sex hormones important for growth and sexual development.

As described in Chapter V, I measured changes in juvenile wild alligator physiology across varying environmental salinities to compare the effects of salinity in wild alligators with those observed in previous laboratory-based studies. To this end, blood plasma samples of juvenile wild alligators in coastal Louisiana were opportunistically obtained over the course of 1 year (July 2018-July 2019). Correlations between environmental salinity and various blood biochemistry parameters, RAAS, and steroid hormones were subsequently analyzed, and seasonal variation of hormones was assessed. Likely due to chance, only males were sampled in hyperosmotic salinities (>10‰) during late summer 2018. Na^+ and Cl^- levels were strongly and positively correlated with salinity in males, corresponding well with findings from previous laboratory-based studies. Angiotensin II was not correlated with salinity, although aldosterone in juvenile female alligators was negatively correlated with environmental salinity levels, further suggesting the RAAS is not activated by increased salinity. To my knowledge, this study represents the first measurement of the progestogen 17α,20β-dihydroxypregnenone (DHP) in alligators. Interestingly, DHP was significantly positively correlated with environmental salinity in juvenile males. Finally, this study demonstrated

seasonal variation of sex hormones in juvenile wild alligators. For instance, testosterone in juvenile males increased during May and peaked in June (not significant), corresponding with similar trends of increased androstenedione and 5α-dihydrotestosterone around the onset of adult mating season. Juvenile females experienced fluctuations in sex hormones as well, as 17β-estradiol tended to increase during May (not significant), also coinciding with adult mating season. However, estriol levels remained low during April and May, but increased during June, correlating with nesting season. Overall, sex steroid levels in immature alligators largely reflect similar season variation as seen in adult alligators.

6.2. Perspectives and Future Research

The above research highlights the challenges posed to alligators by high salinity levels, and this dissertation demonstrates various ways salt stress impacts juvenile alligator physiology and behavior. However, this work also highlights various knowledge gaps in how juvenile alligators respond to salt stress, and that further study is necessary to elucidate the complex mechanisms by which salt stress drives physiological and behavioral changes. First, components of the RAAS were not activated by salinity stress as hypothesized, and angiotensin II levels were in fact reduced in salt-stressed alligators. These findings evoke questions as to the role of the RAAS in alligators, as well as which endocrine mechanisms regulate salt/water balance in salt-loaded alligators. In addition, how are other physiological systems in alligators affected by salt stress? For instance, how does the local, tissue-specific cardiovascular RAAS respond to salt-loading? Furthermore,

what drives alligators' behavioral response to salt stress? Finally, since salt stress had significant impacts on sex hormone levels in immature alligators, does salt-stress affect their sexual development or long-term fitness?

In Chapters II and IV, endocrine mechanisms by which reptiles regulate salt/water balance were discussed. Indeed, osmoregulatory mechanisms appear diverse among reptiles. For instance, angiotensin II was reduced by salt-loading in Japanese quails (*Coturnix japonica*) (Takei et al., 1985) as seen in alligators in Chapters II and IV. However, in contrast, Nile crocodiles (*Crocodylus niloticus*) experienced elevated aldosterone when exposed to hyperosmotic water (Balment and Loveridge, 1989). Further, it is important to note the osmoregulatory function of corticosterone in reptiles. For example, salt-loaded sand goannas (*Varanus gouldii*) experienced elevated corticosterone and reduced aldosterone levels (Bradshaw and Rice, 1981), which corresponds well with the elevated corticosterone seen in salt-loaded alligators. Further, corticosterone has been implicated to influence osmoregulation in reptile kidneys, as blocking adrenocorticotropic hormone (ACTH) with dexamethasone is associated with increased Na^+ reabsorption in kidneys of desert iguanas (*Dipsosaurus dorsalis*) (Bradshaw et al., 1972). Further, salt-loaded *V. gouldii* experienced antidiuresis which was inhibited by blockage of ACTH by hypothalamic lesions (Bradshaw and Rice, 1981). Blocking ACTH via hypophysectomy in desert iguanas also increased plasma and muscle water content, while corticosterone treatment prevented these effects (Chan et al., 1970). Although speculative, corticosterone may have mineralocorticoid action in reptiles. This is supported by the lack of elevated plasma glucose in salt-loaded alligators (Faulkner et al., 2018; Faulkner et al., 2019),

suggesting corticosterone may have actions besides traditional stress hormone effects such as mobilization of glycogen stores in the body. Furthermore, injections of the glucocorticoid cortisol in alligators increased uric acid production, a mechanism to conserve water during elimination of nitrogenous wastes (CoulsonHernandez1959). Thus, it is possible corticosterone may promote the conservations of water in this way. Further study is warranted to elucidate the endocrine control of osmoregulation in alligators.

While saltwater exposure reduced components of the RAAS in alligator plasma, less is known about the impacts of salt-loading on other physiological systems. For instance, the function of the tissue-specific, cardiac renin-angiotensin system (RAS) which produces angiotensin II independent of the systemic RAAS previously discussed is an important concern in salt-stressed animals. Activation of the intracardiac RAS is associated with fibrosis and ventricular hypertrophy during healing after myocardial infarction (Baker et al., 1992; Dostal and Baker, 1999; Sun, 2010), although these effects also occur in noninfarcted myocardium which is associated with cardiac dysfunction (Sun, 2010). Mammalian studies show continuous salt-loading induces negative feedback on systemic RAAS hormones (angiotensin II, aldosterone) similar to alligators, while promoting release of angiotensin II in the intracardiac RAS (Bollag, 2014; Lahera et al., 2006; Ouvrard-Pascaud et al., 2005). The effects of saltwater exposure on the intracardiac RAAS in alligators and cardiac health requires further study.

It was interesting to observe in Chapter III that saltwater-exposed juvenile alligators preferred to spend more time in saltwater than basking. It is worth noting that thermoregulation can play a role in responding to salinity stress. The alligator cultures

maintained for this work consistently had lower water temperature than air temperature, and alligators indeed tend to show lower body temperatures in water compared with out of water (Brisbin Jr et al., 1982; Diefenbach, 1975). Selecting lower body temperatures reduces metabolism, which can reduce ion and water flux (Beaupre, 1996; Raven and Smith, 1978). Thus, it is likely that juvenile alligators selected lower body temperatures by spending more time in water, reducing ion and water flux and alleviating dehydration. Similarly, the tendency for salt-stressed juvenile alligators to rest with eyelids closed likely aided in reducing ion and water flux across mucus membranes.

Sex hormone levels are important in regulating growth and reproduction in alligators (Guillette et al., 1997; Guillette et al., 1994), and the impacts of pollutants on alligator reproductive physiology has been well-documented (Crain et al., 1998; Crain et al., 1997; Guillette et al., 1994; Vonier et al., 1996). For example, the estrogenic pesticides dicofol, DDT, and various metabolites such as p,p'-DDE significantly impact juvenile alligator endocrine physiology (Guillette et al., 1995; Guillette et al., 1994; Guillette et al., 1999). Compared with an uncontaminated lake, male juvenile alligators in the contaminated Lake Apopka had significantly greater 17β-estradiol and lower testosterone, which was associated with poorly organized testes and reduced phallus size (Guillette et al., 1995; Guillette et al., 1994; Guillette et al., 1999). Further, juvenile female alligators in Lake Apopka had significantly greater 17β-estradiol (Guillette et al., 1995; Guillette et al., 1994). Additionally, endocrine disruption in Lake Apopka alligators is associated with reduced offspring viability and reproductive success (Guillette et al., 1994; Guillette et al., 1999). While the effects of pollutants on reproductive endocrinology in alligators have

been thoroughly investigated, there is relatively little information about the impacts of salt stress on alligator reproductive development and function. The data presented in this dissertation raise concerns as to the long-term reproductive health of alligators exposed to salt inundation. Thus, future studies should investigate the potential impacts on long-term fitness by saltwater as an endocrine disruptor.

6.3. Implications of Current Research

The research in my dissertation provides important insight into the physiological and behavioral response of alligators exposed to high salinity. While alligators are unable to cope with excess salinity for extended periods of time, juvenile alligators are the most vulnerable due to their smaller size and thinner integument allowing for more rapid water loss to hyperosmotic environments (Lance et al., 2010; Taplin, 1988). Furthermore, the largest hurricanes of the Southeastern United States hurricane season occur around August and September (Curtis, 2008; Saunders and Lea, 2005), coinciding with alligator hatching season in July and August (Joanen, 1969; Lance, 1989), leaving hatchlings vulnerable to storm surges. Sea surface temperature is also projected to increase, which is associated with greater frequency and intensity of hurricanes and ensuing storm surges (Hoyos et al., 2006; Michener et al., 1997; O'Brien et al., 1992). Additionally, rising greenhouse gas emissions are associated with projected increases in drought frequency (Li et al., 2009; Strzepek et al., 2010). As these events induce and exacerbate salinization of freshwater habitats, it is important to consider the challenges posed to alligators by salt stress in a changing climate. Other factors which further contribute to salinization of freshwater

ecosystems include anthropogenic alterations to freshwater flow (Herbert et al., 2015), runoff of coal mine spoils (Li et al., 2014), and the use of salt to de-ice roads (Szeligowski et al., 2020).

In addition to salt stress, alligators face a number of other growing challenges including habitat loss (Mazzotti et al., 2009) and metal and organic pollutants (Burger et al., 2000; Guillette et al., 1995). As alligators play important roles in ecosystems by controlling populations of lower trophic levels, changes in alligator populations may have ecosystem-level impacts (Mazzotti et al., 2009; Mazzotti and Brandt, 1994). Careful and ongoing management is required to ensure continued conservation of wild alligator populations and ecosystem health, and further studies are necessary to better understand how saltwater impacts alligator populations in the wild in a changing climate.

6.4. References

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