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Evolutionary History and Adaptation to Salinity in American Alligators

John Konvalina
University of Central Florida

EVOLUTIONARY HISTORY AND ADAPTATION TO SALINITY IN AMERICAN ALLIGATORS (*ALLIGATOR MISSISSIPPIENSIS*)

by

JOHN DOUGLAS KONVALINA
B.S. University of Nebraska-Lincoln, 2013
M.S. Arkansas State University, 2016

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ABSTRACT

Stressful environments can commonly be found at the edge of a species range and may be a driver for adaption in suboptimal environments. Furthermore, the edge of a species' range can expand and contract over time, resulting in multiple independent invasions of the same stressful habitat. Elucidating population genetic structure and demographic history can aid in determining the which geologic factors impact range distributions and when climatic changes occurred driving genetic patterns observed in contemporary populations. Moreover, populations at the edge of the species range may adapt to the stressful environments that occur at the range edge and exhibit genetic traits divergent from populations in the core of the species range. In this dissertation, I first examined how a stressor (salinity) has impacted genetic structure and demographic history in a wide-ranging, large semi-aquatic species, the American alligator (*Alligator mississippiensis*; Chapter 2). I estimated the splitting of genetic clusters and matched them with geologic events of past sea level rise. Then, I tested if coastal populations respond differently to changes in salinity compared to alligators from inland populations (Chapter 3). To do this I randomly placed juvenile alligators from coastal and inland populations in one of three salinities (0, 10, or 20 ppt) for two weeks. I collected behavioral, physiological, and histological datasets and found a habitat by salinity interaction with coastal alligators exhibiting a pattern of increased plasticity relative to inland alligators. In Chapter 4, I hypothesized that coastal and inland alligators would exhibit differentially expressed genes in osmoregulatory organs in response to salt stress. My data supported this hypothesis, and I found that the most differentially expressed genes functioned in signal transduction, metabolic pathways, and secretion. In addition, I found that at high salinities, coastal alligators upregulated genes coding for solute carriers compared to inland alligators. Overall, my dissertation contributed to the study of

adaptive evolution by demonstrating that salinity has been a past and current stressor for American alligators. High salinity levels continue to limit the alligator's species range and lead to genetic differentiation among historically isolated regions. Yet, at the same time, I found evidence that coastal populations exhibit incipient adaptation to high salt environments. The patterns I found here are similar to other species that inhabit both freshwater and saltwater environments. As there appears to be evidence of convergent evolution for mechanisms to excrete salt in fully marine reptiles, my dissertation is starting to provide evidence for patterns of convergent evolution among reptiles that similarly use both freshwater and brackish water environments.

I dedicate this dissertation to my wife, Dierdre, who stood by my side during the most difficult moments of this journey.

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CHAPTER 1: INTRODUCTION

The presence of similar phenotypic features across taxa in response to the same environmental conditions is called adaptive evolution (MacColl, 2011). Adaptive evolution is driven by natural selection, which changes allele frequencies over time (Lewontin, 1974). Wright (1931) imagined that changing allele frequencies would create fitness landscapes, where decreases in fitness were represented by valleys while increases in fitness were represented by fitness peaks. While some evolutionary processes such as genetic drift can drive allele frequencies over time towards fitness valleys (Kimura, 1991; Moore, F. B. G., & Tonsor, 1994; Wade & Goodnight, 2008), other processes such as natural selection, drive allele frequencies towards a fitness peak, over time (Barton & Rouhani, 1987, 1993; Simpson, 1953; Wright, 1932). The rate at which allele frequencies move towards a fitness peak is determined by the strength of selection, with stronger selection resulting in a quicker allele frequency change (Whitlock, Phillips, Moore, & Tonsor, 1995).

Multiple studies have identified that stressors can cause strong selection (Jasnos, Tomala, Paczesniak, & Korona, 2008; Kondrashov, Houle, Kondrashov, & Houle, 1994; Parsons, 1987; Uyenoyama, 1993). Here, stress is defined as any environmental factor that decreases absolute fitness in a population (Hoffmann and Hercus, 2000; Agrawal and Whitlock, 2010). Stressful environments can commonly be found at the edge of a species range (Hoffmann and Parsons, 1997; Jones et al., 2012; Lesica and Allendorf 1995; Sexton et al., 2009) resulting in edge populations having much lower genetic diversity than populations in the species core range (Hardie & Hutchings, 2010). Edge populations adapt to stressful environments when selection acts upon either existing genetic variation or new mutations that happen to arise by chance in the

edge populations (Barrett & Schluter, 2008). The former is a more likely scenario as alleles that are present have already passed a selective filter by being selected for in past environments (Liti, Barton, & Louis, 2006; McGregor et al., 2007; Rieseberg et al., 2003). Differential survival results in fixation of certain beneficial alleles (Patwa & Wahl, 2008; Uecker & Hermisson, 2011). These alleles can be present in both regulatory regions of the genome (i.e. transcription factors; Stern, 2000; Wray, 2007; Carroll, 2008) or protein-coding regions (Hoekstra & Coyne, 2007).

The edge of a species' range can expand and contract over time (Fraser, 1999), resulting in multiple independent invasions of the same stressful habitat (Ashelby, Page, De Grave, Hughes, & Johnson, 2012; Yamanoue et al., 2011). Elucidating demographic history can aid in determining the chronological order and timing of these changes (Hoffman & Blouin, 2004). Demographic history models can also be used in conjunction with geologic data to explain how a species' range changed in response to past climatic fluctuations (Mahoney, 2004; Walker & Avise, 1998; Zamudio & Savage, 2003). Environmental and climatic factors, both past and present, determine the distributional range and limit of a species' range (Brown et al., 1996; Lomolino et al., 2010). Examples of barriers to range expansion are vast but commonly cited barriers include deserts (Mcrae, Beier, Dewald, Huynh, & Keim, 2005), rivers (Beneteau, Mandrak, & Heath, 2009), and oceans (Luiz et al., 2012). For saltwater organisms, freshwater can severely limit range expansion (Lee & Bell, 1999), yet some saltwater taxa such as copepods (Lee & Petersen, 2002b, 2002a) and fish (Jamniczky, Barry, & Rogers, 2015; Scott & Brix, 2013; Velotta et al., 2017) have evolved to survive in freshwater. Although less common, examples of a freshwater species adapting to saltwater include the Asian sea bass (Weakley,

Claiborne, Hyndman, & Edwards, 2012), diamondback terrapin (*Malaclemys terrapin*; Dunson and Mazzotti, 1989), and salt marsh snake (*Nerodia clarkia;* Dunson and Mazzotti, 1989).

One of the model organisms for studying adaptation to salinity changes has been the stickleback, a marine fish that has evolved freshwater populations (Hasan et al., 2017; Hiroi, Yasumasu, McCormick, Hwang, & Kaneko, 2008; McCormick, 2003; Tipsmark et al., 2002; Velotta et al., 2017; Whitehead, Zhang, Roach, & Galvez, 2013). Freshwater populations have higher survival rates in freshwater than ancestral anadromous populations (Divino et al., 2016). Additionally, gill morphology and gene expression shows that marine ecotypes have a more difficult time adjusting physiologically to the freshwater environment than the freshwater ecotype (Gibbons, McBryan, & Schulte, 2018). In freshwater, organisms are hyperosmotic to their environment so they must reabsorb ions and secrete water. Thus, genes involved in maintaining osmotic balance (Hasan et al., 2017; Whitehead et al., 2013), filtration maintenance (Hasan et al., 2017), ion-uptake (Velotta, Mccormick, & Schultz, 2015) and ion transport (Hasan et al., 2017) are upregulated while genes that maintain the sodium/potassium gradient (Hasan et al., 2017; Gibbons et al., 2017; Whitehead et al., 2013) are downregulated. In saltwater, ion-uptake genes are downregulated (Hasan et al., 2017) while genes maintaining the sodium/potassium gradient are upregulated (Hasan et al., 2017).

Most of the studies investigating adaptation to saltwater or freshwater have been on fully aquatic taxa (Divino et al., 2016; Hasan et al., 2017; Jones et al., 2012; Purcell, Hitch, Klerks, & Leberg, 2008; Velotta et al., 2017), with only a few including semi-aquatic species (Agha et al., 2018; Dunson & Mazzotti, 1989; Hong et al., 2019). Studies in both semi-aquatic and fully aquatic freshwater taxa have found changes in gene expression of genes related to glycolysis,

fatty acid metabolism and ATP production in response to a high salinity environment (Hong et al., 2019; Lavado, Aparicio-Fabre, & Schlenk, 2014; Tine et al., 2008). In contrast, semi-aquatic species differ from fully aquatic species in that they primarily use ureogenesis as a strategy to combat dehydration stress (Dantzler, W. H., & Schmidt-Nielsen, 1966; Hong, Zhang, Shu, Xie, & Shi, 2014). As the literature on adaptation to salinity by freshwater semi-aquatic species is greatly limited by the dearth of such studies, I aimed to fill in this knowledge gap with my dissertation by investigating how coastal populations of American alligators (*Alligator mississippiensis*) adapt to fluctuations in salinity and sea level.

American alligators (*Alligator mississippiensis*) are large, semi-aquatic reptiles native to the southeastern United States. A long-term mark recapture study of alligators in coastal South Carolina found evidence that they routinely live to 50 years old and possibly up to 70 years of age (Wilkinson, Rainwater, Woodward, Leone, & Carter, 2016). Ontogenetic shifts in diet are present with insects making up most (~70%) of the diet of hatchling alligators (<61 cm) while juveniles (61–122 cm) begin transitioning to a diet of fish (Delany, 1990). Within freshwater lakes, fish make up a majority of an adult alligators' (>180cm) diet (Rice, 2004). While *Alligator* sp. evolved as freshwater animals (Whiting & Hastings, 2015; Whiting, Steadman, & Krigbaum, 2016; Whiting, Steadman, & Vliet, 2016), they can be found in brackish waters and estuaries along the coast of the southeastern United States (Rosenblatt & Heithaus, 2011; Rosenblatt, Heithaus, Mazzotti, Cherkiss, & Jeffery, 2013). Although crocodiles have salt glands on their tongue that help them excrete salt and enabling them to stay in saltwater for prolonged periods of time, alligators lack salt glands and must return to freshwater frequently to avoid dehydration (Fujisaki et al., 2016; Taplin, Grigg, Harlow, Ellis, & Dunson, 1982). In addition to renal

osmoregulation, saltwater crocodiles (*Crocodylus porosus*) have cloacal modification of urine, a feature American alligators lack and which may be indicative of deep divergences in osmoregulatory physiology between the crocodylid and alligatorid lineages (Pidcock, Taplin, & Grigg, 1997).

Alligators are an ideal model organism to answer questions about adaptation to salinity because high aqueous salt levels create a stressful environment for alligators (P C Faulkner et al., 2018; Patricia C. Faulkner, Hala, Rahman, & Petersen, 2019) and limits their species range (Dunson & Mazzotti, 1989). For Chapter 2, I first characterized the current genetic structure of American alligators across their geographic range. Then, I used demographic modeling to identify how past changes in sea level would have create the current patterns of genetic structure. For Chapter 3, I tested if coastal populations respond differently to changes in salinity compared to alligators from inland populations. To do this, I placed coastal and inland alligators in three different salinities (0, 10, and 20 ppt) over two weeks and analyzed behavioral, physiological, and histological datasets. Next, in Chapter 4 I detailed the molecular response to changes in salinity in both freshwater and saltwater ecotypes. I looked at two key osmoregulatory organs, the kidney and the liver, and compared my results to what has been found in fully aquatic species. Finally, I summarized my findings in Chapter 5 and addressed how my dissertation contributes to the field of adaptive evolution.

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CHAPTER 2: HISTORIC AND CONTEMPORARY GENE FLOW IN AMERICAN ALLIGATORS (*ALLIGATOR MISSISSIPPIENSIS*)

Abstract

The cause of phylogeographic concordance is assumed to be an interaction between physical features of the landscape (i.e. mountains or rivers) and biogeographic events (i.e. glaciation, sea level rise). Here, we used thousands of genomic markers to evaluate the relationship between past geologic events and the demographic history of population clusters. We used the wide-ranging American alligator (*Alligator mississippiensis*) to test hypotheses about discontinuities in the southeastern United States. We found that American alligators are split into multiple genetic clusters and that the geographic regions between these clusters are associated with previously recognized barriers to gene flow in the southeastern United States. The oldest barrier was associated with the expansion and saltwater inundation of the Mississippi River 2.45 million years ago. The divergence of the other genetic clusters occurred more recently and did not coincide with interglacial periods of high sea level rise. Moreover, we found evidence that different processes impacted American alligators, such that genetic clusters portrayed historic barriers to gene flow that are overcome by contemporary movements. In conclusion, we found admixture across the typical barriers forming genetic clusters, suggesting that contemporary patterns of genetic structure for semi-aquatic taxa appear to be more similar to those of fishes (i.e. reflecting river drainages) than those of more terrestrial taxa.

Introduction

Understanding how past climatic conditions and biogeographic events influenced the current distribution of species is a fundamental aim of evolutionary biology (Knowles 2009). Tying together historic events and contemporary genetic structure has typically been investigated using phylogeography (Avise 2000; Knowles 2009). Phylogeography characterizes the geographic distribution of genotypes with the assumption that these patterns reflect organismal patterns of diversification (Avise 1998, 2000). When multiple species that live in the same geographic region exhibit similar genetic patterns – called phylogeographic concordance – it is assumed that common barriers to gene flow (i.e. mountains or rivers) or biogeographic events (i.e. glaciation, sea level rise; Walker and Avise, 1998; Pauly et al., 2007; Satler and Carstens, 2016) have created parallel impacts on the evolutionary history of these taxa. For example, the Iberian Peninsula served as a glacial refugia for flora and fauna in Europe during the Pleistocene, causing a high level of phylogeographic concordance among species in the region (Gómez and Lunt 2007). While phylogeographic comparisons help researchers identify common genetic patterns, this is not enough to identify the cause of these patterns.

Demographic models (Beerli and Felsenstein 2001; Excoffier and Foll 2011) are some of the first analyses that enabled researchers to evaluate the processes that influenced observable genetic patterns. Specifically, demographic models have been used to date divergence times between genetic clusters, allowing researchers to compare divergence times with the geologic record to see if estimated divergence dates concur with biogeographic events that could have led to the splitting of genetic clusters (Beaumont et al. 2010; Chan, Brown, and Yoder 2011; Nielsen and Beaumont 2009). Demographic models use coalescent theory to simulate complex

evolutionary scenarios (G. K. Chen, Marjoram, and Wall 2009; Ewing and Hermisson 2010). Coalescent simulation programs have risen in prominence because they generate data much faster than forward genetic simulators (Carvajal-Rodríguez 2008). Yet, standard simulation programs have problems dealing with large amount of data produced by massively parallel sequencing (Altshuler et al. 2010). For example, simcoal2 uses a slow generation-by-generation approach, while the newer version fastsimcoal2 uses a fast continuous-time coalescent framework capable of handling thousands of genetic markers (Excoffier and Foll 2011). These issues likely limited interpretation of early large-scale phylogeographic studies.

With the advent of massively parallel sequencing, the number of molecular markers used in phylogeographic studies has increased dramatically (Tucker, Marra, and Friedman 2009). Studies that use next generation sequencing more frequently identify discontinuities than studies that use microsatellites or mitochondrial DNA (Lyman and Edwards 2022). Here a discontinuity is defined as "a distinct geographic pattern in the distribution of alleles and relationships between them, often characterized by a sharp geographic boundary between genetic groups that corresponds to major geographic features" as per Lyman and Edwards (2022). For example, in the loblolly pine (*Pinus taeda*) weak genetic structure and no discontinuities were found with 18 microsatellites (Al-Rabab'Ah and Williams 2002), but when ~3,000 single nucleotide polymorphisms (SNPs) were used, the authors found three genetically distinct groups and identified the Mississippi River as a discontinuity (Eckert et al., 2010). The use of widely different numbers of molecular markers has led to a large variation in the dating of phylogeographic discontinuities (Campbell-Staton et al. 2012; Manthey et al. 2016). For example, a mitochondrial DNA study of the green anole (*Anolis carolinensis*) found the Florida Peninsula formed a discontinuity in the Miocene (6.8-12.6 mya; (Campbell-Staton et al., 2012).

A RADSeq study looking at the same discontinuity in the same species shifted the divergence date to the late Pliocene (2.78-6.64 mya; (Manthey et al., 2016). These trends highlight the need for the application of large genomic datasets to create fine-scale identification of genetic clusters and discontinuities. The combination of fine-scale identification of genetic clusters and coalescent demographic modeling provides the tools necessary to estimate divergence times more accurately providing better insight into the processes driving phylogeographic concordance.

An ideal geographic region in which to use demographic model simulations to better understand the biogeographic forces driving shared genetic patterns is a location that is both well-studied and contains multiple features and events impacting genealogical concordance across species. The southeastern United States meets both these criteria as it has been the subject of intense phylogeographic study for over three decades and has yielded multiple hypothesized discontinuities (Bermingham and Avise 1986; Lyman and Edwards 2022; Soltis et al. 2006; D. E. Walker and Avise 1998). The two major landscape features that have previously been attributed as drivers of differentiation for species distributed in the southeastern United States are the Mississippi River and the Appalachian Mountains/Apalachicola River (Lyman and Edwards 2022). During the Pleistocene (about 2.6 mya - 11.7 kya), the Laurentide Sheet covered North America and reached as far south as the 39°N latitude (Balco and Rovey 2010). While the southeastern United States remained unglaciated during this period, it served as a refuge for species moving south to avoid the glacier (Soltis et al. 2006). During Pleistocene interglacial periods, the sea levels rose, causing rivers to expand (Russell et al. 2009). This expansion of rivers is hypothesized to have caused the Mississippi and Apalachicola Rivers to drive the formation of genetic discontinuities by isolating species into three common glacial refugia in the

southeastern United States: 1) West of the Mississippi River, 2) east of the Mississippi River to the Apalachicola River, and 3) east of the Apalachicola River (Burbrink, Lawson, and Slowinski 2000). A recent review of 184 phylogeographic studies in the region found that 63% of papers identified a Mississippi River discontinuity and estimated that the divergence happened during the Pleistocene (Lyman and Edwards 2022). Additionally, the same review found that of the five studies that dated the divergence time for the Apalachicola River, two studies estimated a Pliocene divergence whereas the other three estimated a Pleistocene divergence (Lyman and Edwards 2022). Increasing the number of genetic markers, samples, and species will allow researchers to narrow the time range for the appearance of these discontinuities, possibly even to the point of identifying which interglacial period during the Pleistocene they occurred. The presence of one discontinuity but not the other in some species could mean that the Mississippi River and Apalachicola River discontinuities occurred during different interglacial periods of the Pleistocene.

A third driver of genetic discontinuity in the southeastern United States is the Florida peninsular discontinuity, with species population divergence occurring north/south of a line extending from the area around Jacksonville, FL to the Gulf of Mexico (i.e. the Gulf Trough; Morris et al. 2007). This discontinuity separated peninsular Florida from continental North America (Marsico et al. 2015). The Florida peninsula is the emergent portion of the Florida Platform and formed during the Miocene (23-5.3 mya; Scott, 2011). During the subsequent interglacial periods, only the central portion of the Florida peninsula was above sea level, isolating populations from continental North America (Brachert et al. 2014). Lyman and Edwards (2022) found 13 studies dating divergence times for the Florida peninsular discontinuity extending from the late-Miocene (6.64 mya) to the late Pleistocene (0.4 mya) with no epoch

favored. The wide range of divergence estimates could be because of the frequent fluctuations in sea level during the second half of the Cenozoic Era (Miller et al. 2020). Using single-locus data provides low-precision estimates of divergence dates (Edwards and Beerli 2000) MPS genotyping improves genetic resolution and thus the demographic models that use these data will provide more accurate divergence estimates (Nevado et al. 2018).

Here, we used thousands of SNPs identified using a ddRADSeq approach to evaluate parallels between dates associated with the geologic record and demographic model simulations. To accurately test hypotheses about drivers of discontinuities for species in the southeastern United States, we used the wide-ranging American alligator (*Alligator mississippiensis*) which spans multiple hypothesized regions of genetic differentiation in a variety of taxa throughout the southeastern United States. The American alligator is a semi-aquatic, highly mobile, and long-lived species that inhabits a large portion of the southeastern United States, from Texas and Oklahoma in the west to Florida and north to North Carolina in the east (Joanen 1974). The first study investigating American alligator genetic differentiation and diversity sequenced mitochondrial DNA (mtDNA) and found little genetic structure and even less diversity, suggesting a bottleneck or selective sweep in the mtDNA (Glenn et al. 2002). Subsequent genetic studies using microsatellites found an east-west split associated with the Mississippi River (Davis et al. 2001; Davis et al. 2002). However, the timing of this split is unknown. Furthermore, it is unknown whether the Apalachicola River or Florida Peninsula also serve as a driver of discontinuities. Specifically, we used *A. mississippiensis* to evaluate genetic diversity within populations to uncover discontinuities and estimate the divergence dates of genetic clusters on either side of these discontinuities. We sampled across the entire species range and used thousands of genetic markers per individual. We used these data to compare diversity patterns to

other crocodilian species to see if uncover evidence of reduced diversity in American alligators caused by population declines due to overharvesting during the 1970's (Joanen et al. 2021). We predicted that populations would exhibit depauperate patterns of genetic diversity as expected following recent range-wide demographic decline and previous findings in mtDNA for *A. mississippiensis* (Glenn et al. 2002). Second, we evaluated the role that known barriers to gene flow played in *A. mississipiensis* genetic structure. Furthermore, our genotype data allowed us to estimate divergence dates so that the formation of discontinuities could be more closely linked to specific events in the geologic record. We predicted that all discontinuities were formed during interglacial periods of the Pleistocene (Avise 2000; Lyman and Edwards 2022; Soltis et al. 2006). Additionally, based on previous work on other species that spans both the Mississippi and Apalachicola Rivers, (e.g. Burbrink, Lawson, and Slowinski 2000), we predicted that the Mississippi River was a driver of differentiation in alligators during the early Pleistocene and the Apalachicola River was a barrier to gene flow during the mid-Pleistocene. These data are compared to other studies to continue to build a better understanding how different processes can produce patterns of phylogeographic concordance.

Methods

Samples and data generation.

We collected muscle tissue from 282 *A. mississippiensis* individuals spanning their entire geographic range (Figure 1). We collected these tissue samples from alligator processors who subsampled from alligators harvested from annual hunting seasons or through collaborators in state Fish and Wildlife agencies. The locality information for each individual varied. For most individuals, we had exact geographic coordinates or the specific body of water in which they were captured. For the Texas samples we had the county in which they were harvested and for

the South Carolina samples we had the hunting zone in which they were harvested. Additionally, we sampled one individual from three closely related species (*Alligator sinensis*, *Paleosuchus trigonatus*, and *Paleosuchus palpebrosus*; total n = 285). We stored tissue samples in 100% ethanol and placed them in -20°C for long-term storage. We extracted genomic DNA using a Serapure bead protocol (Rohland and Reich 2012) and used a ddRAD protocol (Peterson et al. 2012) to create four reduced representation libraries (n = 24, 24, 96, 144, respectively). With an input amount of 500 ng of DNA per individual, we used *Sbf*I and *Sau3A*I to digest the genomic DNA at sequence-specific sites. Following P1 (barcoded adaptor) ligation, we pooled samples with similar concentration, based on Qubit analysis, into sub-libraries. After DNA quantification on a Tapestation 2200 (Agilent Inc), we selected fragments sized from 545 to 685 bp from each library using a PippinHT (Sage Science Inc). We used PCR amplification of each library to ligate another barcoded adaptor (P2) to each library. Finally, we sequenced each library on one lane of an Illumina HiSeq 2500 with 150-bp, paired-end reads (Genewiz Genomics, New Jersey).

ddRAD sequencing library construction and sequencing data processing.

We trimmed each sequencing read to ensure that the sequence started with the barcoded adaptor. Then we demultiplexed the raw data using Stacks v2.4.1 (Catchen et al. 2013). We mapped the resulting reads to the reference genome (NCBI accession number ASM28112v4; Green et al., 2014) using Bowtie2 v2.4.0 (Langmead and Salzberg 2012). We made genotype calls with gstacks (Catchen et al. 2013). Next, we ran Stacks' populations program to create input files for several downstream genetic analysis programs (i.e. VCF, genepop, STRUCTURE, F_{ST}, fasta, summary statistics). We included only the first SNP from each RAD locus to minimize linkage disequilibrium within a RAD locus. Then, we ran the output files through Bayescan 2.1 (Foll and Gaggiotti 2008) to identify outlier loci. We removed the outlier loci and

repeated the Stacks' populations program. We used PLINK v2.0 (Purcell et al. 2007) to remove loci with genotyping rate <60% and minor allele frequency (MAF) <0.02, and to remove individuals with missing rate >50%. We repeated the Stacks populations program with the filtered VCF file. The number of SNPs we used was variable based on the regions included in each of the downstream analyses and included with each analysis below.

Population identification and genetic diversity.

Because our samples were collected across their range and not always easily placed into *a priori* populations, we used an initial *ad hoc* approach for characterizing putative populations. We defined a population as a group of samples that were geographically close enough to one another (within 50 km) that could reasonably reproduce with each other. This estimate was based on two studies: one found that the maximum dispersal of an adult male alligator was 53 km (T. Joanen and McNease 1972); the second was an extensive mark-recapture study of juvenile and adult alligators that found that out of 16 individual alligators, 14 moved equal to or less than 50 km (Lance et al. 2011). Only two outlier individuals moved more than 50 km (Lance et al. 2011). Therefore, if we only had county data or hunting region data, we placed the geographic coordinates in the middle of the county/region. Likewise, if we had a body of water, we placed the geographic coordinates in the middle of the body of water. Using these criteria, we split the samples into 40 populations with an average number of 6.2 ± 2.9 individuals per population (mean ± SD; Table 1). We used these putative populations as input for running the populations program in Stacks to estimate expected heterozygosity within each of the 40 putative populations.

Gene flow.

We used these *ad hoc* populations to generate a pairwise F_{ST} matrix to evaluate genetic differentiation among putative populations. Moreover, we compared this F_{ST} matrix, along with a pairwise geographic distance matrix of Euclidian distances between populations, to test for a pattern of isolation by distance (IBD) via a Mantel test in *R* v4.1.2 with the *R* package "adegenet v2.1.2" (Jombart and Ahmed 2011).

Because isolation by distance does not consider geographic and topographic heterogeneity, we also used EEMS v0.0.0.9000 to visualize effective migration rates across the species' geographic range taking into account potential barriers to gene flow (Petkova, Novembre, and Stephens 2015). EEMS requires three input files: (1) a pairwise genetic dissimilarity matrix which is converted from a PLINK binary format file, (2) a list of the latitude and longitude of every sample in decimal degrees, and (3) a list of coordinates for vertices forming a polygon that encompasses all habitat (i.e. the total species range). The pairwise genetic dissimilarity matrix is defined as the average number of allelic differences between each pair of individuals across all genotyped loci (Petkova, Novembre, and Stephens 2015).We used a burnin of 10,000,000 iterations followed by 20,000,000 MCMC iterations thinned every 9,999 iterations. We ran a second independent run to confirm the results from the first run. Finally, we plotted the results using the R package "reemsplots2 v0.1.0" (Petkova, Novembre, and Stephens 2015).

Genetic clusters and phylogeography.

To test for historical signatures of genetic structure, we used two clustering programs. First, we used the R package "adegenet" v.2.1.2. to run a Discriminant Analysis of Principal Components (DAPC; Jombart et al., 2010) to identify discrete clusters. We could not use STRUCTURE to initially identify clusters because it does not perform well across ranges that

exhibit patterns of IBD (Perez et al. 2018), as observed here (see Results). Therefore, we used DAPC for a range-wide assessment of genetic clustering. Unlike STRUCTURE, DAPC is free of model-based assumptions, such as Hardy-Weinberg equilibrium, and aims to maximize variance between groups while minimizing variance within groups (Jombart, Devillard, and Balloux 2010). We transformed the data into principal components (PCs) to decrease the number of variables and reduce computing time. After choosing the minimum number of PCs necessary to describe the variation in the data, we selected the optimal number of discriminant functions (DFs) to describe the number of clusters.

To investigate the genetic patterns at the borders between the clusters identified by DAPC, we ran STRUCTURE v2.3 (Pritchard, Stephens, and Donnelly 2000) for samples that were found on either side of a DAPC identified boarder. We did this so that we could identify individuals of split ancestry indicating gene flow between the region-wide clusters. Additionally, we ran STRUCTURE on each cluster identified by DAPC to identify any sub-structuring. For both of these analyses, the geographic range was small enough that we did not expect IBD to be a problem as it would examining the entire range. We analyzed the resulting Structure output with the following parameters: min K =1, max K = 8, replications (independent runs) = 5, Burnin = 20,000, remaining steps kept =100,000. Next, we used Structure Harvester to evaluate the best K following the delta K criteria of Evanno et al. (2005). The number of SNPs used per STRUCTURE dataset is listed in Table 2 since each sub-structure run used a different subset of populations and hence a different number of SNPs per dataset.

Demographic history.

We used fastsimcoal v2.7, a coalescent simulator, to model different demographic scenarios and estimate the date for the split of genetic lineages identified above (Excoffier 2021).

Whereas standard simulation programs have problems dealing with large data sets such as those produced by massively parallel sequencing (Altshuler et al. 2010), fastsimcoal2 uses a fast continuous-time coalescent framework capable of handling thousands of genetic markers (Excoffier and Foll 2011). We ran demographic simulations on both east coast and range-wide datasets to estimate parameters associated with demographic history. For the east coast dataset, we tested the demographic history of the three eastern clusters identified by DAPC: Florida, Georgia, and the Carolinas (see Results). We predicted that we would see a pattern of northward migration as has been observed in other species (Mohn et al. 2021; Soltis et al. 2006). For the range-wide dataset, we tested the demographic history of the two remaining clusters identified by DAPC: West of the Mississippi River and east of the Mississippi River to west of the Apalachicola River, and the most ancestral of the eastern clusters as determined by the east coast simulation (see Results). We predicted the best model would show stepwise evolution from west of Mississippi River eastward, following the west to east migration that has been found in the fossil record (Whiting and Hastings 2015; Whiting, Steadman, and Krigbaum 2016; Whiting, Steadman, and Vliet 2016).

We used the mutation rate (7.9×10^{-9} substitutions site^{-1} generation^{-1}) and generation time (18 years) from Green et al. (2014). For our input file, we used a folded MAF site-frequency spectrum (SFS) because we did not know the ancestral state allele. To convert our VCF file into the pairwise observation files (.obs), we used an R script from (Liu et al., 2018). This gave us three pairwise two-dimensional SFS (1_0, 2_0, 2_1) files. We tested a total of 16 demographic models (Figure 2). For each of the 16 models, we ran 50 independent runs each with 40 expectation conditional maximum (ECM) cycles and 100,000 simulations per cycle. We selected the highest likelihood value among the 50 runs as the maximum likelihood estimate for that

model. Then, for each model, we performed 100 runs (with 1,000,000 simulations per cycle) of fastsimcoal2 using the parameter values from the best run. We compared the distribution of the likelihoods from these 100 runs among models. Then, we selected the model with the highest likelihood distribution. If the likelihood distribution of two models overlapped, indicating that they do not differ significantly, we ran AIC model selection. We selected the model with the lowest AIC for bootstrapping.

To calculate a confidence interval for the parameters estimated by the best model we used a block-bootstrapping (https://github.com/speciationgenomics/scripts; https://speciationgenomics.github.io/fastsimcoal2/) method which accounts for linkage between SNPs. We split the SNP genotype data into 100 files (i.e. blocks), each with an equal number of SNPs. We then randomly sampled these 100 blocks with replacement to create a new, bootstrapped VCF file. We repeated the block-bootstrapping procedure 50 times (Excoffier et al. 2021).

For each bootstrapped VCF file, we ran 100 iterations of fastsimcoal according to the best model selected above and selected the iteration with the highest likelihood. We used the parameters from these best runs (50 runs in total, one for each bootstrap replicate) to calculate the 95% confidence interval. Finally, we multiplied the number of generations by 18 years (generation time) to get the 95% confidence interval in years. This generation time comes from the publication for the reference genome (Green et al. 2014). After estimating the divergence dates for each dataset, we associated the date with a geologic event. We did this by looking for the highest amount of sea level rise estimated for the northern Gulf of Mexico over the past nine million years (Donoghue 2011) during the confidence interval of the divergence date.

Results

We obtained a total of 1,326,307 SNP loci across all 285 samples. The average number of mapped reads per sample with a Phred quality ≥30 was 11 million reads and each sample had an average coverage of 40x ± 28.6x (SD). We identified 53 outlier loci, which were removed from all datasets so as to not influence the theoretically neutral patterns of differentiation and diversity investigated by this study. We found that the 40 putative populations had an expected heterozygosity range of 0.040 – 0.25 and an average expected heterozygosity of 0.16 (see Table 1).

Gene flow.

Our maximum pairwise F_{ST} estimate was 0.45, between a central Arkansas population (population #7, Figure 1) and a coastal South Carolina population (population #38, Figure 1). Our minimum pairwise F_{ST} estimate was 0.039 between two populations located along the southern Mississippi River (populations #12 & 19, Figure 1). Overall, we found a range-wide pattern of isolation by distance, with 11.6% of the variance in genetic difference between populations explained by the geographic distance between populations ($P = 0.01$, $R^2 = 0.116$; Figure 3). Our EEMS run had a total of 241 individuals, 40 demes (i.e. populations) and 120,074 sites (i.e. SNPs). Our EEMS results (identical for both independent runs) showed above average gene flow within peninsular Florida, North and South Carolina, between the Apalachicola and Alabama Rivers, and along the Mississippi River (Figure 4). We found below average gene flow between the Red and Brazos Rivers, within eastern Mississippi and western Alabama, and directly east of the Apalachicola River (Figure 4).

Genetic clusters and phylogeography.

DAPC identified five genetic clusters (Figure 5). Cluster 1 included populations west of the Mississippi River. Clusters 2-4 comprised populations restricted to the states of Alabama,

Florida, and Georgia, respectively. Cluster 5 included all populations found in either North Carolina or South Carolina. The STRUCTURE plots of the edges between clusters (Figure 6) showed admixture within the states of Mississippi and Georgia. Finally, sub-structuring within Florida supported two different clusters, the panhandle and the peninsula.

Demographic history.

For the east coast dataset, our data supported a northward migration out of a southern glacial refugia. We found that the best model (Model R, Figure 7) showed Florida as the most ancestral population. We estimated that Florida split into an ancestral population about 729 thousand years ago (kya; 95% confidence interval: 641 – 818 kya) and this ancestral population subsequently split into Georgia and the Carolinas approximately 290 kya (95% CI: 238 – 343; Figure 8, Table 3).

For the range-wide dataset, our data supported our prediction of stepwise evolution from west to east across the landscape. Our best model (Model R, Figure 7) estimated that the west of the Mississippi River cluster split from an ancestral population 2.5 mya (95% confidence interval: 2.2 – 2.7). This ancestral population then split into Alabama and Florida about 1.5 years ago (95% confidence interval: 1.1 – 1.8; Figure 9, Table 4). The best model for both datasets (Model R) showed migration between all demes (i.e. Migration between West of Mississippi River-Alabama-Florida and migration between Florida-Georgia-Carolinas).

Discussion

Past studies investigating phylogeographic concordance in the southeastern United States (reviewed in Avise 2000, Soltis et al. 2006, Lyman and Edwards 2022) have identified three common barriers (i.e. the Mississippi River, the Apalachicola River, and peninsular Florida) that tend to cause discordant genetic clusters among species that span these barriers. We found that

American alligators are split into multiple genetic clusters and that the geographic areas between these clusters are associated with these previously established barriers to gene flow in the southeastern United States. Interestingly, these appear to have served as historic barriers such that contemporary alligators exhibit evidence of gene flow spanning these barriers. Moreover, demographic models show that genetic clusters diverged at different times, despite ubiquity of American alligator occurrence throughout the region. Finally, although the Mississippi River barrier corresponded to sea level rise and saltwater inundation, this does not seem to be the processes demarcating other genetic clusters of American alligators. Below, we discuss how various factors influenced genetic variation in American alligators to better understand phylogeographic concordance throughout the southeastern United States.

We found that the oldest divergence was associated with the Mississippi River discontinuity, a result corroborated by studies of mammals (e.g. Cullingham et al. 2008, Kierepka and Latch 2016), amphibians (reviewed in Zeisset and Beebee 2008), and reptiles (e.g. Burbrink, Lawson, and Slowinski 2000; Myers, McKelvy, and Burbrink 2020). Our demographic modeling of American alligators revealed that the 95% confidence interval for the divergence date of the west of Mississippi River cluster from the Alabama and Florida clusters was 2.2 – 2.7 mya, which corresponded with a period of 17-m sea level rise (Figure 10, Panel A; Donoghue, 2011). This amount of sea level rise greatly expanded the width of the Mississippi River and inundated it with seawater (Figure 10, Panel A). The large expanse of seawater may have been a barrier to gene flow for alligators and caused genetic divergence east and west of the Mississippi River as has been proposed in other species (Al-Rabab'Ah and Williams 2002; Barton and Wisely 2012; Near, Page, and Mayden 2001). Moreover, our estimated divergence date of 2.45 mya matches closely with what was found in ratsnakes (2.97 mya, Chen et al. 2017), but differs

greatly from estimates from diamond-backed watersnakes (1.4 mya; Brandley et al. 2010), chorus frogs (6.8 mya; Lemmon et al. 2007), and skinks (0.4 mya; Howes, Lindsay, and Lougheed 2006). The varying divergence estimates for the Mississippi River likely reflect the fact that the river has expanded multiple times during the many interglacial periods of the past 6.8 million years (Donoghue 2011).

The estimated divergence dates for the splitting of the other genetic clusters of American alligator are more recent and do not coincide with large levels of sea level rise (Figure 10, Panels B and D, Donoghue, 2011). For example, we estimated that the divergence date for the Apalachicola River was 1.4 mya, which falls within the range of other studies (late Pliocene (Milá et al. 2017) – late Pleistocene (Krysko et al. 2017). Other factors could be at play such as environmental heterogeneity and allopatric glacial refugia (Myers et al. 2019; Myers, McKelvy, and Burbrink 2020). Non-overlapping areas of core habitat (Barrow et al. 2017) can lead to allopatric glacial refugia (Walker et al. 2009; Waltari et al. 2007). Another possibility is that intraspecific groups are responding differently to climate change across time. This could lead to genetic differentiation, a phenomenon which has been found in plants (Maguire et al. 2018) as well as vertebrates (Pearman et al. 2010). Finally, vast differences in environmental conditions across a species' range could lead to the formation of separate genetic clusters (Myers et al. 2019; Myers, McKelvy, and Burbrink 2020).

Wetland habitat, in particular, seems to be an important factor contributing to gene flow (or lack thereof) in semi-aquatic species (e.g. Pearson et al. 2023). Previous studies have found that the density, growth rate, and body condition of alligators differ between coastal and inland wetlands (Saalfeld et al. 2008; Webb and Austin 2005). Alligator snapping turtle (*Macrochelys temminckii*) abundance was found to be positively correlated with greater surrounding wetland

area and river size and negatively correlated with terrain ruggedness (Pearson et al. 2023). Thus, the combination of wetland abundance, river width and terrain ruggedness may provide the explanation for the genetic patterns we found in American alligators.

While we see evidence of the Mississippi River being a historic barrier for American alligators, it seems that environmental heterogeneity and geographic distance between river drainages are the two main factors driving current genetic differentiation. Despite finding large-scale clustering that associated with the Mississippi river, the Apalachicola river, and the Florida peninsula, we found evidence of admixture for populations that spanned these 'leaky' barriers. Indeed, our EEMS results suggested that river drainages may be more important to contemporary gene flow than historic biogeographic barriers. River drainages appear to be the common factor influencing genetic structure in fully aquatic species occurring in the southeastern United States (e.g. fishes; Bermingham and Avise 1986; Soltis et al. 2006). As for other semi-aquatic species, a recent study on snapping turtles (*M. temminckii*), that occur in a largely overlapping range with American alligators, also found genetic divergence among river drainages (Apodaca et al. 2023). These data suggest that historic and contemporary features of the landscape likely provide a pattern of isolation-by-environment (Myers et al. 2019) driving patterns of genetic differentiation.

We hypothesized that American alligator populations would exhibit little within-population genetic diversity as had been found previously for mtDNA sequence data (Glenn et al. 2002). Glenn et al. (2002) attributed the reduced diversity to a population bottleneck during the Pleistocene (Glenn, Dessauer, and Braun 1998). The results here appear to reject our hypothesis, since the diversity we estimated is similar to other studies that investigated genome-wide SNP diversity in other crocodilians (Cao et al., 2020, Fukuda et al., 2022, Pacheco-sierra et

al. 2018, Nobrega, 2021; Table 5). Indeed, the lack of reduction of diversity estimated from nuclear markers does not support the hypothesis of a selective sweep for American alligators, as was found using mtDNA (Glenn et al. 2002). Finally, despite declines in larger sizes classes (Joanen et al. 1997) and being listed as a federally protected species in 1967 (Udall 1967) due to a presumption of overharvest, our data supported subsequent studies that stated that in some regions (e.g. Louisiana, Taylor and Neal 1984, Taylor et al. 1991) and refuges (Glenn et al. 2002) population sizes never reduced to levels that would show marked patterns of a genetic bottleneck. Furthermore, the patterns of genetic structure identified here reflect that of a natural species and do not seem to have been influenced by anthropogenic movement of alligators during the 1970s.

Overall, we found evidence that different evolutionary processes impacted American alligators, such that genetic clusters exhibited historic barriers to gene flow that are overcome by contemporary movements. The expansion and saltwater inundation of the Mississippi River during an interglacial period in the early Pleistocene led to the historic divergence of genetic clusters east and west of this river. The cause of the other divergences occurred more recently and are less clear with regard to the specific factors driving differentiation. However, environmental heterogeneity and geographic distance between river drainages possibly play a role. Currently, we find gene flow across the historic patterns of genetic clusters, highlighting the mobile nature of this species. Indeed, contemporary patterns of genetic structure for semi-aquatic taxa appear to be more similar to those of fishes (i.e. reflecting river drainages) than those of more terrestrial taxa.

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CHAPTER 3: COASTAL AMERICAN ALLIGATORS (*ALLIGATOR MISSISSIPPIENSIS*) EXHIBIT GREATER PHENOTYPIC PLASTICITY TO CHANGES IN SALINITY THAN INLAND ALLIGATORS

Abstract

Adaptation can occur quickly when selection is stronger than stochastic processes. Multiple studies have identified stressful environments as strong selective agents. For freshwater adapted organisms, saltwater presents a stressful environment. To adapt to the stress of saltwater exposure, vertebrates use the Renin-Angiotensin-Aldosterone System (RAAS), an endocrine system that regulates blood pressure and water–salt balance. Most vertebrate studies on adaptation to salinity have been on fish, with studies on semi-aquatic species far and few between. Coastal American alligators live in estuaries with widely fluctuating levels of salinity not found in inland locations. While alligators venture into high-salinity areas to forage for food, they must periodically return to low-salinity areas to effectively osmoregulate. Thus, this study system provides a good model to understand how semi-aquatic species adapt to changes in salinity compared to fully aquatic species. In this study, we used a common garden experiment to expose juvenile American alligators from coastal and inland populations to different salinities for two weeks to measure behavioral and physiological responses to salinity. Specifically, we recorded the position of the alligators (in water or on a stand) twice daily, blood plasma sodium level post-experiment, and histological responses in the liver and kidney (two key RAAS organs) post-experiment. Our primary research question was: Do alligators from coastal populations respond differently to changes in salinity compared to alligators from inland populations? We hypothesized that coastal alligators would exhibit a habitat by salinity interaction to fluctuating salinity levels, a result that has been found in other freshwater species with coastal/saltwater

populations. We found that all 12 characters measured exhibited habitat-by-salinity interactions and that coastal alligators exhibited a general pattern of increased plasticity relative to inland alligators. This study may highlight a unique circumstance in which the optimal foraging environment for this species actually increases physiological stress to the organism.

Introduction

A fundamental aim of evolutionary biology is to understand how organisms adapt to their environment. The adaptive process occurs when a heritable characteristic provides an advantage to an individual in a specific environment (West & Gardner, 2013). Adaptation occurs slowly when selection pressure is weak (Orr, 2000), migration is high (Savolainen et al., 2007), or population sizes are small (Aitken et al., 2008). However, adaptation occurs quickly under models of strong selection (Alemayehu et al., 2014) or large population sizes (Neher, 2013). Multiple studies have identified stressful environments, defined as "those that lead to a sharp reduction in fitness in populations" (Hoffmann & Hercus, 2000), as strong selective agents (Jasnos et al., 2008; Kondrashov et al., 1994; Parsons, 1987). For example, Kondrashov et al. (1994) compared the relative fitness of three genotypes of *Drosophila melanogaster* across 50 environments and found that stressful environments (i.e. dilution of food medium and crowding) exaggerated the fitness differences between lineages with and without natural selection.

Edges of a species range are often delimited by the occurrence of stressful environments further limiting the growth and expansion of populations (Sexton et al., 2009). Examples of barriers to range expansion are vast but commonly include deserts (Mcrae et al., 2005), rivers (Beneteau et al., 2009), and oceans (Luiz et al., 2012). For saltwater organisms, freshwater can severely limit range expansion (Lee & Bell, 1999), yet some saltwater taxa such as copepods (Lee & Petersen, 2002b, 2002a) and fish (Jamniczky et al., 2015; Scott & Brix, 2013; Velotta et al., 2017)

have adapted a means to survive in freshwater. For example, to increase survival in freshwater, alewives have altered their expression of gill ion exchange genes (Velotta et al., 2017). Although less common, examples of a freshwater species adapting to a saltwater barrier include the Asian sea bass (*Lates calcarifer*; Weakley et al., 2012), diamondback terrapin (*Malaclemys terrapin*; (Dunson and Mazzotti, 1989), American crocodile (*Crocodylus acutus*; Dunson & Mazzotti, 1989; Mazzotti & Dunson, 1989) and salt marsh snake (*Nerodia clarkia;* (Dunson and Mazzotti, 1989).

For vertebrates to evolve to changes in environmental salinity, we would expect an adaptive response in the Renin-Angiotensin-Aldosterone System (RAAS; (Velotta et al., 2015, 2017), an endocrine system that regulates blood pressure and water–salt balance (Morici, 1996; Nishimura, 2017; Silldorff and Stephens, 1992a,b). The main organs involved in the RAAS are the liver, kidney, adrenal gland, and lung. First, angiotensinogen is cleaved by renin into angiotensin I (ANG I). Next, angiotensin-converting enzyme (ACE) converts ANG I into ANG II, a physiologically active peptide that helps regulate volume and mineral balance of body fluids (Fraga-Silva & Pinheiro, 2008; RICE et al., 2004). ANG II causes an increase in sympathetic activity, arteriolar vasoconstriction, and prompts the adrenal gland to produce aldosterone (ALDO), which leads to renal tubular sodium reabsorption, potassium excretion, and water retention in an aim to restore blood volume and pressure (Bollag, 2014). Finally, ANG II also stimulates the pituitary gland to release antidiuretic hormone (ADH), which causes vasoconstriction and the kidney to reabsorb water.

Although freshwater and saltwater barriers are ideal study systems for investigating adaptive evolution, many of the studies exploring adaptation to saltwater or freshwater have been on fully aquatic taxa (Purcell et al., 2008; Jones et al., 2012; Divino et al., 2016; Hasan et al., 2017; Velotta et al., 2017), with only a few on semi-aquatic species (Agha et al., 2018; Bower et

al., 2016; Dunson & Mazzotti, 1989). Here, we aimed to fill this knowledge gap by investigating how coastal and inland populations of the American alligator (*Alligator mississippiensis*), a large semi-aquatic reptile, respond to salinity exposure. We defined a coastal population of alligators as one in which there was a high probability that individuals would be exposed to varying salinity levels within their lifetime (i.e. within an estuary). We defined an inland population of alligators as one in which there a low probability that individuals would be exposed to varying salinity levels within their lifetime (i.e. freshwater lakes).

Although American alligators use saltwater habitat for foraging grounds (Fujisaki et al., 2016; Rosenblatt et al., 2013), marine environments provide a boundary in which they cannot live fulltime (Jackson et al., 1996). American alligators must return to freshwater periodically because they require access to freshwater to drink (Nifong et al., 2015; Nifong & Silliman, 2017). Indeed, a four-year study on estuary habitat use found that the alligators spent a majority of their time (74%) in the mid-estuary zone (estuarine year-round; 18-30 ppt) and only 17% of their time in the downstream zone (estuarine/marine year-round; 30-40 ppt; (Rosenblatt et al., 2013). Interestingly, the alligators spent twice as much time downstream in the wet season compared to the dry season (Rosenblatt et al., 2013), likely reflecting the lower salinity level during the wet season (Romigh et al., 2006).

A functional RAAS exists in American alligators (Morici, 1996; Silldorff, E. P., & Stephens, 1992a, 1992b) and likely enables them to use marine habitats for foraging. One study exposed a group of juvenile alligators to 12 ppt saltwater for five weeks and compared hormone levels with a control group kept in freshwater (Faulkner et al., 2018). They found elevated plasma levels of seven hormones including the stress hormone corticosterone in the saltwater-exposed alligators. Compared to the freshwater alligators, saltwater-exposed alligators had significantly lower levels

of ANG II (Faulkner et al., 2018). A short-term exposure study by the same research group found that renin expression in the kidney of alligators held at 12 ppt for one week was significantly lower than that of alligators held in freshwater for the same time period (Faulkner et al., 2019). Taken together, these studies on alligator physiology (Faulkner et al., 2018, 2019; Morici, 1996) suggest that alligators modify the RAAS in response to changes in environmental salinity.

In this study, we used a common garden experimental design to measure behavioral and physiological responses of American alligators to changes in salinity. Our primary research question was: Do alligators from coastal populations respond differently to changes in salinity compared to alligators from inland populations? We hypothesized that coastal alligators would exhibit a habitat by salinity interaction to fluctuating salinity levels, following what has been found in other freshwater species with coastal/saltwater populations (Agha et al., 2018; Chun et al., 2019; Hasan et al., 2017; Plemenitaš et al., 2014; Steil et al., 2003).

Methods

Experimental setup

We collected wild juvenile alligators (<1.5 m) from two coastal sites and two inland sites (Figure 11; n = 21 per site) in June and July 2019. Using a refractometer, we measured the salinity level in the inland sites at 0.5 ppt and the salinity level at the coastal sites as 5 ppt. The inland-coastal population pairs were on either side of the Mississippi River, a porous barrier to gene flow for alligators (Chapter 2). To capture the alligators from three sites (i.e. coastal Mississippi, and both inland sites), we surveyed areas at night via boat. We captured the alligators using standard crocodylian capture techniques, including by hand and with pillstrom tongs (Shirley et al., 2016; Webb & Messel, 1977), and placed them in a 110-gallon polyethylene tub. For the coastal Louisiana site, we collected alligators from the Rockefeller Wildlife Refuge.

These alligators were wild collected as eggs from nests within the refuge, but were hatched and reared inside a captive facility on the refuge. The alligators were kept in freshwater, in a dark enclosure, and fed commercial alligator food. At the time we collected these alligators for our experiment they were around three years old. All individual alligators were transported in a van over a period of 12 hours to the Ara Drive Wildlife Facility at the University of Central Florida (UCF) for housing. All alligators were placed in freshwater holding tanks for acclimation while animal collection proceeded from other sites. As such, the alligators west of the Mississippi River were captured one month before the alligators east of the Mississippi River. Upon arrival to UCF, we recorded the following measurements for each individual: SVL, total length, sex, and weight. In addition, we attached a metal tag to the toe webbing which had a unique identification number. Our research protocol followed humane standards and was revised and approved by the UCF IACUC (Protocol #19-10W).

All animals were housed in two rooms: first, a holding room for captive housing acclimation, and then a separate experimental room (where salinity trials occurred). Both rooms were temperature controlled (27 °C) with a 12-hour light/dark cycle. For our common garden experiment, we placed the alligators in 1.23 m^2 black polyethylene tanks filled with a water height of 0.6 m. Each tank had a plastic stand (43.2 cm x 30.5 cm x 17.8 cm) in the middle of the tank for the alligators to exit the water at their discretion. We fed the alligators commercial Mazuri® crocodilian feed with daily and water changes occurred every other day. We placed four alligators in each tank, consistent with a low stocking density (Elsey et al., 1990). Alligators kept at low stocking density have significantly lower plasma corticosterone than alligators at high stocking density (Elsey et al., 1990).

To acclimate the alligators to the same captive conditions, we placed all alligators in freshwater for a minimum of two weeks and a maximum of six weeks. During this time, we physically interacted with the alligators on a daily basis to habituate them to the presence of humans. After the acclimation period, we randomly placed alligators in one of three salinities (0, 10, or 20 ppt) with each tank containing one alligator from each of the four capture locations (n = 4 per tank). By the time that the alligators were placed in the experimental salinities, we observed that they had reached behavioral plateaus (i.e. lack of hissing when humans entered the room) so that human presence should not influence data collection. The salinities used here were based on previous work that identified a salinity of 8 ppt as being iso-osmotic to alligator blood plasma and salinities of 10 ppt or greater as hyperosmotic to alligator blood plasma (Lauren, 1985; Morici, 1996). Additionally, the salinity choices were meant to mimic the salinity gradient found as juvenile alligators swim from freshwater marshes (0 ppt) to the upper estuary (10 ppt) and middle/lower estuary (20 ppt; ITO, 1959). We used Instant Ocean© sea salt to create the target salinities and we checked the salinity levels daily with a refractometer, adjusting as necessary to maintain water depth and salinity. We did not provide the alligators with a source of drinking water during this experimental period. At the end of two-week trial, we euthanized the alligators with a captive bolt and collected tissue samples from the liver and kidney within 15 minutes of euthanasia. Because the number of animals used (n = 84) exceeded the space available for experimental trials, we split the experiment into two separate blocks. The first block contained nine treatments (three replicates of each salinity; n=36 animals) and the second block contained 12 treatments (four replicates of each salinity; n=48 animals). The blocks were separated by a time period of two weeks and the animals not in the first experimental block were kept in the holding room until they were used in the second experimental block.

Behavior data collection

During the first block of the experiment, we haphazardly observed potential behavioral differences in time spent out of the water between treatments. Therefore, we developed a scheme to test for behavioral patterns during the second experimental block. Here, we standardized behavior observations to assess individual alligator location (e.g. in-water versus out-of-water). Twice daily, once in the morning (7:30 am – 12:00 pm) and once in the afternoon (1:00 pm – 5:00 pm), we recorded each alligator's position in each tank. The datasheet had a list of the tank numbers without tank salinity information. We excluded the first day (when they were first placed in the salinities) and the last day (when they were euthanized). As each tank had one alligator from each capture location, we painted the backs of each alligator with a different color of non-toxic nail polish for each source population. Our prediction was that the inland alligators would be on the stand significantly more than the coastal alligators at high salinities (10 ppt and 20 ppt) because they would be under greater physiological stress. This prediction is based on previous work that found that inland alligators naively drink saltwater whereas coastal alligators do not (Jackson et al., 1996), indicating coastal alligators may have behavioral adaptations to high salinities not found in inland populations.

Blood and histological sample collection

Immediately before placing each alligator into its assigned salinity tank, we used a 23-guage non-heparinized needle with a 3-mL syringe to collect 3 mL of blood from the post-occipital spinal venus sinus. The same procedure occurred at the end of the two-week treatment, directly before euthanasia. We placed the blood in three 1-mL lithium heparin tubes and immediately centrifuged at 10,000 g for two minutes to separate the plasma. We pipetted the resulting plasma into 2-mL screw-cap tubes and immediately placed in -20 °C for storage. Then, we sent the plasma samples to the University of Miami Veterinary Lab, who used ion-specific

electrode (ISE) technology to measure the amount of sodium ions present in the plasma samples. Once euthanized, we dissected each specimen and collected tissue samples (~200 mg) from two key RAAS organs, kidney and liver. We placed the tissue samples into 10% Neutral Buffered Formalin to preserve them for histological analyses.

Histology slide preparation and scoring

We prepared liver (n = 46) and kidney (n = 46) samples from across both experimental blocks for light microscopy (LM) (Table 6). First, we placed the tissue samples into vials of 2% glutaraldehyde (GTA) solution buffered with 0.1 M sodium cacodylate at a pH of 7.2 and allowed to fix for 2 h. For postfixation, we used 1% osmium tetroxide, buffered as above, for 2 h. Then, we fixed the plastic-embedded tissues in 2% GTA. We then dehydrated the tissues in a graded series of increasing ethanol solutions (50–100%), placed them in a 50%/50% acetone/plastic mixture for overnight infiltration, and finally embedded them in Mollenhauer's Epon-Araldite #2 (Dawes, 1988). For thick sectioning (~1 μm in thickness) and staining, we used glass knives on a type 4801A Ultrotome (LKB Instruments, Inc., Rockville, Maryland, USA) with Laddt multiple stain, respectively. Laddt multiple stain is a generic replacement for paragon epoxy tissue stain. For photomicroscopy, we utilized a Leica DMi8 fluorescent light microscope.

For analysis, we used five replicate slides per tissue type per specimen. For the livers, we measured: central vein diameter, bile duct diameter, hepatic artery diameter, and portal vein diameter. For the kidneys, we measured: renal corpuscle diameter, glomerulus diameter, distal tubule diameter, distal tubule epithelial height, proximal tubule diameter, and proximal tubule epithelial height. Each measurement was taken twice, once by each of two independent observers

who were blind to individual identity, location, or treatment of each sample, resulting in a total of 2,760 measurements for each structure.

Statistical analyses

We used three types of generalized linear mixed models with a Bayesian framework to assess differences in each of our response variables as a function of habitat (coastal versus inland) and tank salinity. First, we used a binomial distribution to analyze the binary behavioral data. Second, we used a Michaelis-Menten equation for the functional response of blood-sodium (Michaelis et al., 1913). This equation models the saturation rate for a given solute. Third, we used linear models with normal distributions and assessed models with and without interactions for the histology data (Table 7). If the Bayesian model includes an interaction effect it is equivalent to a graphical representation of a plot with different slopes (Liu et al., 2015; Ren et al., 2020). We included the random effects of tank, block, individual, recorder (histology datasets only) and in the case of the behavior data, event (date/time period when data was recorded). We found that tank and block were confounded with individual. Thus, of these three variables we only included individual as a random effect. We also included the following morphological data as fixed effects: sex, specimen, total length, SVL, and weight. We found that the three body size traits (total length, SVL, and body weight) were highly correlated. A previous study found that longer alligators are more resilient to salt stress than shorter alligators (Nifong et al., 2015). Therefore, we only included total length in the models as it was the most biologically relevant size variable. We used the widely applicable information criterion (WAIC; Gelman et al. 2014) to evaluate the relative information among models. We identified the most informative model of the chosen set using WAIC weight as an indicator. We completed all statistical analyses in R (Ihaka and Gentleman, 1996) and STAN (Carpenter et al., 2017).

Results

The same model was returned as the best model for each of the 12 analyses (i.e. behavior, plasma sodium, and the 10 histological structures). The best model was: Response ~ intercept + habitat + salinity_ten + salinity_twenty + (1|individual) + sex + state + length + habitat:salinity_ten + habitat:salinity_twenty (Table 7, Model 4). Salinity was treated as a categorical variable for 11 of the 12 analyses, but for plasma sodium levels, salinity was treated as a continuous variable. Below we provide the results for each response variable (Tables 8-10) and figures (Figures 12 – 22) of the posterior distribution curves. Each figure shows the posterior distributions for small (48 cm total length) Mississippi (MS) males only. This was done to visualize the trend between habitat (coastal vs inland) and tank salinity. This pattern of the trend is identical for other comparisons (i.e. other combinations of sex, size, and state (e.g. large, Louisiana, female), but only a single set combinations is shown for the straightforward visualization of the plot. Tables 7-9, show the actual coefficients for sex, total length, and state across all combinations can be found.

Behavior

Over the 14-day experiment, we collected 24 observations of alligator location in the tank. Overall, the average probability of an alligator being out of the water at 0, 10, and 20 ppt was 0.297, 0.193, and 0.336, respectively. For inland alligators the probabilities of being out of the water were 0.438, 0.224, and 0.323 for 0, 10, and 20 ppt, respectively. For coastal alligator the average probabilities of being out of the water were 0.156, 0.161, and 0.349 for 0, 10, and 20 ppt, respectively. The posterior distribution curves for inland alligators were similar at 0 and 20 ppt, but shifted to the left at 10 ppt. In contrast, the posterior distribution curves for coastal alligators were different for at each of the three salinities (Figure 12). Overall, with respect to

time spent out of water, we found that inland alligators qualitatively exhibited less plasticity than coastal alligators.

Blood Plasma Sodium

We collected 168 total blood plasma samples, 84 from day 1 of the treatment and 84 from day 14 of the treatment. The average plasma sodium levels at the end of the experiment across habitats for 0, 10, and 20 ppt were 137.3, 147.7, and 158.9 mmol/L, respectively. For inland alligators the average plasma sodium levels were 136.7, 146.1, and 157.0 mmol/L for 0, 10, and 20 ppt, respectively. Whereas coastal alligators exhibited post-experiment average plasma sodium levels of 137.9, 149.3, and 160.9 mmol/L for 0, 10 and 20 ppt, respectively. Both inland and coastal alligators had low plasma sodium levels at 0 ppt and increased levels at 10 ppt (Figure 13). At 20 ppt, coastal alligators increased their plasma sodium levels greater than inland alligators (Figure 13). Overall, with respect to plasma sodium levels, we found that inland alligators qualitatively exhibited less plasticity than coastal alligators.

Liver Histology

For some of the specimens, we were not able to get exactly 30 measurements per structure. Therefore, in total we had 2,462 measurements for each of the liver structures. For all four structures measured, we found that coastal alligators had a qualitatively more plastic response than inland alligators (Figures 14-17). We also found that the diameter of all four structures decreased at 20 ppt compared to 0 ppt for both coastal and inland alligators (Figures 14-17).

Across all treatments, we found an average central vein diameter of 70.1, 69.4, and 60.7 µm for 0, 10, and 20 ppt, respectively. For inland alligators, average central vein diameters were 68.5, 64.9, and 63.3 µm for 0, 10, and 20 ppt, respectively. For coastal alligators, average central vein diameters were 73.8, 72.5, and 57.4 µm for 0, 10, and 20 ppt, respectively. We found an average bile duct diameter across all samples of 16.9, 15.7, and 14.7 µm for 0, 10, and 20 ppt,

respectively. For inland alligators, average bile duct diameters were 18.8, 14.3, and 15.3 µm for 0, 10, and 20 ppt, respectively. For coastal alligators, average bile duct diameters were 14.7, 16.6, and 13.9 µm for 0, 10, and 20 ppt, respectively. For all samples, we found an average hepatic artery diameter of 24.7, 26.2, and 22.8 µm for 0, 10, and 20 ppt, respectively. For inland alligators, average hepatic artery diameters were 25.7, 24.5, and 24.3 µm for 0, 10, and 20 ppt, respectively. For coastal alligators, average hepatic artery diameters were 23.6, 27.3, and 21.0 µm for 0, 10, and 20 ppt, respectively. Finally, we found an average portal vein diameter across all samples of 89.0, 87.9, and 75.0 µm for 0, 10, and 20 ppt, respectively. For inland alligators, average portal vein diameters were 98.3, 94.0, and 80.3 µm for 0, 10, and 20 ppt, respectively. For coastal alligators, average portal vein diameters were 78.6, 83.8, and 68.6 µm for 0, 10, and 20 ppt, respectively.

Kidney Histology

As above, we were not able to get exactly 30 measurements per structure for all specimens. Therefore, we had 2,719 measurements for each of the kidney structures. For all six structures measured, we found that coastal alligators had a qualitatively more plastic response than inland alligators (Figures 18-23). We also found that the diameter of three structures (renal corpuscle diameter, glomeruli diameter, and distal tubule diameter) increased at 20 ppt compared to 0 ppt for both coastal and inland alligators (Figures 18-20). Proximal tubule diameter increased at 20 ppt compared to 0 ppt for coastal alligators only (Figure 22).

We found an average renal corpuscle diameter of 60.3, 63.8, and 64.1 µm for 0, 10, and 20 ppt, respectively. For inland alligators, average renal corpuscle diameters were 58.0, 61.8, and 62.5 µm for 0, 10, and 20 ppt, respectively. For coastal alligators, average renal corpuscle diameters were 63.0, 64.5, and 65.7 µm for 0, 10, and 20 ppt, respectively. The overall average

glomerulus diameters were 47.0, 49.7, and 50.3 µm for 0, 10, and 20 ppt, respectively. For inland alligators, average glomerulus diameters were 44.9, 46.4, and 49.2 µm for 0, 10, and 20 ppt, respectively. For coastal alligators, average glomerulus diameters were 49.5, 51.0, and 51.5 µm for 0, 10, and 20 ppt, respectively. We found an average distal tubule diameter of 34.3, 37.8, and 38.1 µm for 0, 10, and 20 ppt, respectively. For inland alligators, average distal tubule diameters were 34.6, 36.0, and 37.7 µm for 0, 10, and 20 ppt, respectively. For coastal alligators, average distal tubule diameters were 34.0, 38.4, and 38.5 µm for 0, 10, and 20 ppt, respectively. Average distal tubule epithelial height across all samples was 9.4, 10.1, and 10.0 µm for 0, 10, and 20 ppt, respectively. For inland alligators, average distal tubule epithelial heights were 9.6, 8.5, and 9.8 µm for 0, 10, and 20 ppt, respectively. For coastal alligators, average distal tubule epithelial heights were 9.2, 10.7, and 10.3 µm for 0, 10, and 20 ppt, respectively. Across all samples, we found an average proximal tubule diameter of 46.0, 49.3, and 46.7 µm for 0, 10, and 20 ppt, respectively. For inland alligators, average proximal tubule diameters were 45.0, 45.5, and 45.0 µm for 0, 10, and 20 ppt, respectively. For coastal alligators, average proximal tubule diameters were 47.1, 50.8, and 48.4 µm for 0, 10, and 20 ppt, respectively. Across all samples, average proximal tubule epithelial height of 16.8, 18.4, and 16.8 µm for 0, 10, and 20 ppt, respectively. For inland alligators, average proximal tubule epithelial heights were 16.5, 15.9, and 16.3 µm for 0, 10, and 20 ppt, respectively. For coastal alligators, average proximal tubule epithelial heights were 17.1, 19.3, and 17.2 µm for 0, 10, and 20 ppt, respectively.

Discussion

In this study, we used a common garden experimental design to measure behavioral and physiological responses of American alligators to changes in salinity. Our primary research question was: Do alligators from coastal populations respond differently to changes in salinity

compared to alligators from inland populations? We hypothesized that coastal alligators would exhibit a habitat by salinity interaction to fluctuating salinity levels. We found that for all twelve measured characters, the best model included a habitat by salinity interaction. This is evidence that coastal alligators have different phenotypic responses to salt stress than inland alligators**.** Specifically, we found that coastal alligators exhibited a more plastic phenotypic response than inland alligators. This provided evidence that the phenotypic changes exhibited by coastal alligators are indeed part of an adaptive response to changing salinity concentrations that are not observed by inland alligators.

For both coastal and inland alligators, plasma sodium levels increased as salinity increased providing evidence that salinity treatment did generate a physiological response within the alligators (Table 8, Figure 13). Indeed, our results are not unique as elevated plasma sodium levels have been found in two freshwater turtle species exposed to 15 ppt (Agha et al., 2018). Another study found that both saltwater and freshwater sticklebacks had increased plasma sodium levels at 34.5 ppt compared to a 10-ppt control salinity (Kusakabe et al., 2017). In addition to an effect of treatment, we also found that the response was different depending on the habitat of origin of our samples (i.e. coastal versus inland). Similarly, saltwater sticklebacks had slightly higher plasma sodium levels at the control salinity than the freshwater sticklebacks (Kusakabe et al., 2017). We found a similar result in that coastal alligators had slightly higher plasma sodium levels at the control salinity than the inland alligators. Taken together, these findings suggest that instead of increasing solute excretion to respond to salt stress, saltwater ecotypes may use physiological mechanisms to cope with high plasma sodium levels.

How do our histological data correspond to other studies making similar comparisons in other species? Alligators at 20 ppt had smaller central veins than alligators at 10 ppt, a result that

was also found in Nile tilapia kept at 15 ppt compared to 10 ppt (Dawood et al., 2022). Similarly, rabbitfish kept at 15 ppt had smaller central veins than those kept at 5 ppt (Abdel-aziz, 2017). The decrease in central vein diameter is likely a result of how much stress is put on the blood vessels at such high salinities. At high salinity, the sodium levels in the blood are likely leading to an increase in blood pressure and causing vasoconstriction (Pojoga et al., 2008). Smaller blood vessels would lead to a decrease in the amount of blood coming into the liver via the portal vein (Bosetti et al., 2016). To deal with the elevated plasma sodium levels caused by hyperosmotic environmental conditions, our results are consistent with coastal alligators making physiological modifications at a greater magnitude than inland alligators.

Sticklebacks are marine fish that have invaded freshwater habitats (Dennenmoser et al., 2017; Divino et al., 2016), making them an ideal study system to compare with American alligators to determine if our patterns are indicative of adaptation to salinity. In general, we found an increase in renal corpuscle diameter in high salinity compared to low salinity for both coastal and inland ecotypes – but coastal alligators exhibited larger renal corpuscles than inland alligators at high salinity. Wendelaar Bonga (1973) found a similar result when comparing seawater sticklebacks to their freshwater ecotype. A larger renal corpuscle increases filtration and reabsorption rate (Wendelaar Bonga, 1973) suggesting that coastal/seawater populations are adapted to high salinity environments in ways that inland/freshwater populations are not (Basir & Peyghan, 2020). Additionally, it has been found that stickleback from high-salinity populations had larger kidney tubule diameters at high salinity compared to a low-salinity population (Hasan et al., 2017). Interestingly, we also found that coastal alligator populations had larger proximal tubule diameters at low salinity (0 ppt) compared to inland populations. Indeed, (Hasan et al., 2017) concluded that osmoregulatory plasticity is energetically costly to maintain and that it is

only worth investing in the necessary energy in coastal areas where there is a large fluctuation in salinity levels. Similar to three-spined sticklebacks, coastal alligators seem to have evolved adaptation to local variation in salinity (Defaveri & Merilä, 2014).

However, not all our results are consistent with those that have been found in fish. In our study, we found that glomeruli diameter was higher in coastal populations than inland populations at all salinities. This contrasts with what was found when comparing freshwater and seawater stickleback ecotypes (Wendelaar Bonga, 1973), where seawater animals had smaller glomeruli than freshwater animals. Another study found a decrease in glomeruli diameter in sturgeon (*Huso huso*) kept at high salinity compared to a freshwater control (Krayushkina et al., 1996). Interestingly, the same study found that glomeruli diameter continued to decrease the longer the sturgeon were kept at high salinity (Krayushkina et al., 1996). While both alligators (our study) and sturgeon (Krayushkina et al., 1996) increased distal tubule diameter in response to high salinity, sturgeon decreased epithelial height in the tubule whereas alligators increased epithelial height. The differences stated here could be the result of evolutionary history. Alligators evolved as freshwater animals (Whiting et al., 2016) that later invaded saltwater habitats, whereas the two fish studies mentioned here involved marine species that later evolved freshwater populations (Krayushkina et al., 1996; Wendelaar Bonga, 1973). While both processes involved modifying the RAA system, some modifications may be unique to the direction of adaptation (Corl et al., 2018; Handelsman et al., 2013).

With regard to behavior, we found that both ecotypes were in water the most at 10 ppt. This result corroborates other studies that found that 8 ppt is iso-osmotic to alligator plasma (Lauren, 1985; Morici, 1996). Because 10 ppt is similar to iso-osmolality, being in the water at this salinity is likely least physiologically stressful than either of the other two treatments. At our

highest salinity level (20 ppt), both ecotypes were out of the water an equal amount of time, suggesting that both ecotypes were stressed. That high salinity is a stressful environment for alligators is corroborated by another study that found that mortalities occurred after three weeks when alligators were placed in 20 ppt saltwater (Lauren, 1985). A previous behavior study found that when placed in a tank with 30 ppt seawater, inland alligators naïvely drank the saltwater, whereas coastal alligators did not (Jackson et al., 1996). Behavioral differences as evidence of adaptation to different salinity levels has also been found in dice snakes (Brischoux et al., 2017)

Taken together, this information suggests that coastal alligators modify their behavior more than inland alligators in response to changes in environmental salinity. How would these behavioral modifications work in the wild? A four-year study on estuary habitat use found that the alligators spent a majority of their time (74%) in the mid-estuary zone (freshwater/estuarine year-round; 18-30 ppt) and only 17% of their time in the downstream zone (estuarine/marine year-round; 30-40 ppt; (Rosenblatt et al., 2013), and that they spent twice as much time downstream in the wet season compared to the dry season (i.e., when freshwater influx reduced saltwater ppt further downstream the estuary). This provides evidence that high salinity levels create a stressful environment for alligators (Rosenblatt et al., 2013), which our experimental results support.

In this study we found evidence from behavioral, physiological, and histological datasets to support the hypothesis that coastal alligators have increased phenotypic plasticity in response to changing salinity levels than inland alligators. A habitat by salinity interaction was found in all twelve responses that we measured. The higher plasticity in coastal alligators is likely due to the fact that spend more time in fluctuating salinities (Fujisaki et al., 2014; Nifong et al., 2015; Rosenblatt et al., 2013). Coastal alligators are known to invade marine/estuarine habitats to

consume marine/estuarine prey (Nifong et al., 2015). Thus, this study may highlight a unique circumstance in which the optimal foraging environment for this species actually increases physiological stress to the organism.

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CHAPTER 4: MECHANISMS OF ADAPTATION TO CHANGES IN SALINITY IN COASTAL AMERICAN ALLIGATOR POPULATIONS

Abstract

How organisms adapt to changes in the environment is an important field of study in evolutionary biology. Here, we investigated how a semi-aquatic species, American alligators (*Alliator mississippiensis*), alter their gene expression in response to changes in salinity. Specifically, we used a common garden experimental design to analyze the effect of salt stress on the complete transcriptome of the liver and kidney, two organs important in water-and-salt balance. We found an interactive effect between habitat and salinity in differentially expressed genes (DEGs) in each tissue. We found many more DEGs in the liver (n = 939) than in the kidney (n = 31), likely reflecting the highly conserved nature of kidney gene expression. Within the liver, the coastal alligators upregulated more genes than the inland alligators. We also found key changes in DEGs related to osmotic balance, metabolism, immunity genes: a phenomenon that has been found in adaptation to salinity studies in fish. Finally, our Gene Ontology groups for DEGs showed expression patterns in signal transduction, metabolism, and immunity: all of three of which have been found in DEGs from osmoregulatory organs in fish exposed to varying salinity levels. Overall, understanding how aquatic and semi-aquatic organisms adapt to changing salinity levels will provide insight into how climate change-driven seawater encroachment into freshwater habitats will affect the species that live in those habitats.

Introduction

Osmoregulation helps the body maintain its salt and water balance and is accomplished through the excretory system. When environmental salinity levels change, an organism's body must respond to the change to adjust hormone levels involved with water retention (Faulkner et al. 2018; Faulkner et al. 2019). Indeed, organisms do this type of adjustment of water retention on a daily basis to survive (Pidcock, Taplin, and Grigg 1997). However, some species live in environments with widely fluctuating salt levels and have evolved adapted responses to changes in salinity (Bower et al., 2016; Fang, Kemppainen, Momigliano, Feng, & Merilä, 2020; Scott & Brix, 2013). Many of these adaptive responses are in organs that are part of the renin-angiotensin-aldosterone system (RAAS), the endocrine system that regulates water and salt balance in vertebrates (Nishimura 2017). Previous studies have found morphological changes in the tissues of RAAS organs in response to varying salinity levels (Abdel-aziz, 2017; Dawood, Gewaily, & Sewilam, 2022; Wendelaar Bonga, 1973); Chapter 3). The next frontier in this area of research is to identify the molecular mechanisms occurring within cells in these tissues that allow them to adapt to varying salinity levels (Cao et al. 2021; Hasan et al. 2017). With the advent of massively parallel sequencing and bioinformatics, studies can now use an RNASeq approach to start to address these questions directly (Dong et al. 2022; Hasan et al. 2017; Liang et al. 2021; Lv et al. 2013).

One of the methods organisms use to maintain their osmotic balance is changing the regulation of saltwater ion transporters. Many fish use a Na-K-Cl cotransporter (NKCC) to transport sodium, potassium and chloride out of the cell and to the gills where the ions are secreted into the external environment (Hiroi et al. 2008; McCormick 2003; Christian Klbk Tipsmark et al. 2002; Velotta et al. 2017). NKCC has two isoforms; the first transports sodium, potassium and chloride from extracellular fluid (i.e. blood) into the lumen of exocrine glands. This isoform is upregulated when the fish is in saltwater (Velotta et al., 2017). The second isoform is expressed in kidney tubules and reabsorbs sodium, potassium and chloride from urine. This isoform is downregulated when the fish is in saltwater (Hasan et al. 2017). All cells maintain a sodium/potassium gradient across their cell membrane, which is crucial for maintaining cell

structure. For example, the membrane protein *ATP1A1* establishes and maintains the sodium/potassium gradient across the cell membrane and is upregulated in both gill and kidney tissues of sticklebacks in response to the stress of increased sodium ions in saltwater (McCairns and Bernatchez 2010).

Metabolism is also greatly affected by changing solute levels. One mechanism for how this works is that adenosine monophosphate (AMP)-activated protein kinase (AMPK), a cellular nutrient and energy sensor that helps to maintain energy homeostasis (Garcia and Shaw 2017; Hardie, Ross, and Hawley 2012), is activated when energy homeostasis is compromised (Hardie, Schaffer, and Brunet 2016). For example, red-eared slider turtles (*Trachemys scripta elegans*) had elevated liver AMPK mRNA levels at salinity levels of 5 and 15 ppt compared to freshwater (i.e. 0 ppt) (Hong, Li, et al. 2019). Lipid metabolism is also highly affected by the available amount of cellular energy (adenosine triphosphate – ATP) in mitochondria (Osellame, Blacker, & Duchen, 2012). Energetic changes in response to salinity can disrupt normal mitochondrial function and alter the composition of fatty acids (Liu et al. 2017). Furthermore, elevated salinity levels can lead to cells increasing the amount of fatty acids reabsorbed into their membrane (Lv et al. 2013).

In addition to osmoregulation and energy issues, large changes in salinity can elicit a general stress response. Stressors can be defined as any environmental condition causing stress to an organism (Muthukumar and Nachiappan 2010; Schulte 2014). Osmotic stress causes upregulation of many stress response genes in bacteria, as well as genes involved in creating cell walls (Burall et al., 2015). In bacteria, cell walls help to make cells less permeable to the flow of ions. Osmotic stress can also cause cells to crenate as water rushes out of the cell. When native freshwater sticklebacks are exposed to saltwater, they downregulate a gene (*MAP3K15*) that initiates cell death when the cell is under stress (Wang et al. 2014). In the swimming crab, *Portunus trituberculatus*, which naturally occurs in coastal waters across Asia, immunity-related genes in the gill were significantly upregulated in low-salinity waters (Lv et al. 2013). Taken together, these studies show organisms modifying their protein levels to adjust to environmental stress.

Locally adapted populations of the same species can show vast differences in gene expression when responding to the same salinity variations. For example, a high-salinity population of sticklebacks exhibited greater plasticity in kidney gene expression at varying salinity levels than a low-salinity population (Hasan et al. 2017). Another stickleback study compared gill transcriptomes of freshwater, anadromous, and marine populations in 11 ppt and 0.3 ppt water and found an interactive effect of ecotype and salinity, where marine ecotypes were more physiologically stressed in freshwater than the anadromous or freshwater ecotypes (Gibbons, McBryan, and Schulte 2018). Finally, another fish study showed that genes that regulate gill ion exchange had functionally diverged in freshwater versus anadromous populations of alewives (Velotta et al. 2017). Specifically, ion-uptake genes were expressed more in freshwater populations whereas ion secretion genes were expressed more in anadromous populations (Velotta et al. 2017).

Most gene expression studies on changes to salinity are in fish (Boutet, Long Ky, and Bonhomme 2006; Liang et al. 2021; Velotta et al. 2017; Wang et al. 2014). This ignores semi-aquatic organisms that live in environments with fluctuating salinities such as the diamondback terrapin (*Malaclemys terrapin*), salt marsh snake (*Nerodia clarkia*), and crocodilians such as the American crocodile (*Crocodylus acutus*) and American alligator (*Alligator mississippiensis*; Dunson and Mazzotti, 1989). Alligators can be found in brackish waters and estuaries along the coast of the southeastern United States (Rosenblatt & Heithaus, 2011; Rosenblatt, Heithaus, Mazzotti, Cherkiss, & Jeffery, 2013). Alligators inhabit these high-salinity areas to take advantage of the beneficial food resources found in more marine environments (Rosenblatt and Heithaus, 2011; Nifong and Silliman, 2013). Although crocodiles have salt glands on their tongue that help them excrete salt and enabling them to stay in saltwater for prolonged periods of time, alligators lack salt glands and must return to freshwater frequently to avoid dehydration (Fujisaki et al., 2016; Taplin, Grigg, Harlow, Ellis, & Dunson, 1982). The fact that coastal alligators must move back and forth between high and low-salinity areas makes them a perfect semi-aquatic organism to test for changes to expression patterns associated with changing salinity levels.

In this study we investigated how coastal American alligators (*Alliator mississippiensis*) alter gene expression in response to changes in salinity. Specifically, we used a common garden experimental design to house juvenile American alligators from coastal and inland sites in polyethylene tanks of different salinities and analyzed the effect of salt stress on the complete transcriptome of two RAAS organs, the liver and kidney. We had two aims for this experiment. First, to determine if there was an interactive effect of habitat and salinity in differentially expressed genes (DEGs) from the kidney and liver in alligators. Second, to determine if previously identified osmoregulatory genes were upregulated in response to high salinity in alligators. For the first aim, we predicted that we would find an interactive effect of habitat and salinity in DEGs from the kidney and liver as has been found in threespine sticklebacks (Wang et al. 2014). For the second aim, we predicted that coastal alligators would differentially express genes related to osmotic balance (i.e. solute carriers), metabolism and immunity at high salinities compared to inland alligators, similar to what has been found in other studies comparing high-salinity and low-salinity adapted ecotypes (Hasan et al. 2017; Hong, Jiang, et al. 2019; Lv et al. 2013; Velotta et al. 2017).

Methods

Experimental setup

The complete description of the common garden experimental setup can be found in the Methods section of Chapter 3. In brief, we collected wild juvenile alligators (<1.5m) from two coastal sites and two inland sites (n = 21 per site) in Summer 2019. The juvenile alligators were transported to the Ara Drive Wildlife Facility at the University of Central Florida (UCF) for housing. Upon arrival to UCF, the following measurements were recorded for each individual: SVL, total length, sex, weight. The alligators were placed in 1.23 m^2 black polyethylene tanks filled with a water height of 0.6 m. Four alligators were placed in each tank, consistent with what a previous study (Elsey et al. 1990) found to be considered low stocking density. The alligators were kept in a room with a 12 hour light/dark cycle and a constant

temperature closely around 26 °C. After a minimum two-week freshwater acclimation period, alligators were randomly placed in one of three salinities (0, 10, or 20 ppt). The salinity choices were meant to mimic the salinity gradient found as juvenile alligators swim from freshwater marshes (0 ppt) to the upper estuary (10 ppt) and middle/lower estuary (20 ppt; (ITO, 1959). At the end of two-week trial in the selected salinity, the alligators were euthanized. Because the number of animals used (n = 84) exceeded the space available for experimental trials, we split the experiment into two separate blocks. The first block contained 9 treatments (3 replicates of each salinity; n=36 animals) and the second block contained 12 treatments (4 replicates of each salinity; n=48 animals). Previous research (i.e. Chapter 3) found that salinity treatments impacted blood plasma sodium levels and physiological responses to the alligators. Here, small tissue samples from the left liver and left kidney were collected immediately after euthanization and placed in both liquid nitrogen and in RNA*later* (Qiagen, Redwood City, CA, USA) before being transferred to −80°C for storage to determine whether differences in expression patterns were impacted by habitat (coastal versus inland) and salinity treatment. Both tissues were preserved within 15 minutes of death by pithing and then decapitation.

Total RNA extraction and quantification

For the liver samples, we extracted total RNA from the RNA*later*-preserved samples because they yielded higher quality RNA than the liquid nitrogen-preserved samples. First, we took the sample out of the -80°C freezer and let the RNA*later*-preserved thaw to enable tissue access. Then, we placed the tissue in an impact-resistant tube with two metal 5/32” ball bearings (Wheels Manufacturing Inc) and placed the tube in a bead beater for one minute to homogenize the sample. For the kidney samples, we underwent the same process except we used the liquid nitrogen-preserved samples. Next, for both tissues, we followed a Trizol extraction protocol to extract total RNA which we then quantified using a Qubit RNA Broad-Range kit. We then used a Monarch Total RNA Miniprep Kit (NEB #T2010S) to remove any DNA contamination via a DNase I treatment. This DNA-free total RNA sample was then run on an Agilent 4150 TapeStation for quantification and QC analysis. For the kidney samples, all samples had

RIN scores ≥7. RIN scores for the liver were much lower (4-6), a result that has been found elsewhere (Nouvel et al. 2021). A previous study found that RIN scores for the liver were significantly lower than that of muscle and adipose tissue, suggesting the liver is more sensitive to RNA degradation than other tissue types (Nouvel et al. 2021). Thus, for the livers, we retained samples with RIN scores ≥5 and re-extracted samples with RIN scores <5. Once the sample had a RIN score >5, we moved on to library preparation for the samples.

RNASeq library preparation

For kidney samples, we used an input amount of 250 ng of DNase-free total RNA for Poly(A) mRNA isolation (NEB #E7490) and cDNA synthesis following the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (NEB #E7765L) protocol. We ligated adapters to the cDNA and cleaned the samples with beads. Next, we ran TapeStation on the adapted cDNA samples to make sure there were no residual adapters. Then we ran qPCR on the samples before pooling them. Finally, we ran TapeStation one last time on the pool for QC purposes. We sent the pool to be sequenced on one lane of a NovaSeq 6000 with 150-bp, paired-end reads (Genewiz Genomics, New Jersey). Liver RNA extracts were sent to Genewiz Genomics, who followed the same RNASeq protocol for library preparation and were similarly run on one lane of a NovaSeq 6000 with 150-bp, paired-end reads.

Bioinformatics

The resulting FASTQ files of both liver and kidney were checked for quality scores and number of reads. Reads with a Phred-scaled quality score of >30 and with a length of at least 100 base pairs were kept for further analyses. We used the software STAR v2.7.10b mapping (Dobin et al. 2013) to map reads to reference genome (NCBI accession number ASM28112v4; Green et al. 2014). First, we downloaded and unzipped the GTP annotation file for the reference genome. Next, we mapped the gzipped FASTQ files to the reference genome using STAR. One of the resulting output files was a file with gene counts for each sample, which we used for subsequent analyses. We then used the IRIS-EDA web interface (Monier et al. 2019) to analyze our data. After uploading our input files to the IRIS-EDA web interface,

we underwent quality control measures by evaluating boxplots and histograms of the read count distributions. This process helps to reduce the false-discovery rate (FDR). After the quality check, we used DESeq2 v1.41.5 (Love, Huber, and Anders 2014) to identify differentially expressed genes among habitat (coastal vs. inland) and tank salinity (0, 10, and 20 ppt). For both tissues, we used DESeq2 to run the following model: Number of transcripts ~ Habitat + Tank Salinity + Habitat:Tank Salinity with a *p*-value cutoff of 0.05 and a minimum fold change of 1. We only tested this model because we were interested in if there was or was not a habitat:salinity interaction as this would show that habitat has an effect on how the organism responds to environmental changes. Using the IRIS-EDA web interface (Monier et al. 2019), we ran a Principal Component Analysis (PCA) to create plot of the variance of DEGs and then we qualitatively identified clusters based on either salinities or habitat. Then, to visualize how many DEGs where shared amongst salinities, we created a Venn Diagram of DEGs across salinities for each tissue. Next, we used heatmapper to make a heatmap of DEGs across samples (Babicki et al. 2016) to determine whether expression patterns were tied to habitat or salinity. Next, using the National Center for Biotechnology Information (NCBI), we searched among the DEGs for RAAS specific genes (Takvam et al. 2021) to compare our results with those of other studies, amongst salinities and ecotype. Finally, we used the DAVID Functional Annotation tool from DAVID Bioinformatics Resources tool (Huang et al. 2007) to identify Gene Ontology (GO) groups for each habitat and salinity combination. This enables us to identify the functional groups of genes that were differentially expressed between coastal and inland alligators.

Results

The FASTQ files for one of our kidney samples was corrupted. Thus, our total sample size for liver was n = 84 and for kidney was n =83. Before mapping to the reference genome, we had an average of 23.2 million and 21.4 million reads per sample for the liver and kidney, respectively (Table 11). After mapping to the reference genome, we had an average of 8.63 million and 16.2 million reads per sample for the liver and kidney, respectively (Table 11). For both tissue types, our overarching model showed

that number of transcripts was indeed related to habitat, salinity, and an interaction between habitat and salinity.

Liver

General patterns of DEGs

Our PCA found one principal component explaining 27% of the variance and the other explaining 16% of the variance. We found general patterns of clustering related to habitat (Figure 24) but did not find any clustering related to salinity (Figure 25). Our general patterns of DEG identified that coastal populations exhibited more differentially expressed genes than inland populations at all salinities (Figure 26). The total number of liver DEGs between coastal and inland populations was 939. Among salinities, the number of liver DEGs between coastal and inland populations ranged from a maximum of 464 (0 ppt) to a minimum of 104 (20 ppt) and we found 69 DEGs shared between all three salinities (Figure 27). Our heatmap of liver DEGs found monophyletic clades of upregulated genes (Figure 28). However, these monophyletic clades were not clustered with respect to coastal vs. inland habitats (Figure 29).

RAAS specific genes

Solute carriers (monovalent and divalent ions)

Of the 939 DEGs found between habitats at the 3 different salinities, we identified 32 genes that serve as "Solute carriers". Specifically, we found 15 ion transporter DEGs at 0 ppt, 7 ion transporter DEGs at 10 ppt, and 10 ion transporter DEGs at 20 ppt. In general, ion transporter genes were upregulated in coastal habitats versus inland habitats (Figure 30).

Aquaporins

We found the following aquaporins in the liver transcriptome: AQP1, 3, 4, 8-11. At 0 ppt, coastal alligators upregulated AQP3 and AQP8 compared to inland alligators. The other aquaporins were not differentially expressed between the two habitats. At 10 ppt and 20 ppt, there was no difference in expression between habitats in any of the aquaporins (Figure 31).

Claudins

We found the following claudins in the liver transcriptome: CLDN1, 2, 5-7, 11, 12, 15, 16, 18-20, and 25. At 0 ppt, coastal alligators upregulated CLDN10 and 18, while inland alligators upregulated CLDN1 and 16. The other claudins were not differentially expressed between the two habitats. At 10 ppt, coastal alligators upregulated CLDN1, 6, and 15. The other claudins were not differentially expressed between the two habitats. At 20 ppt, coastal alligators upregulated CLDN10 and CLDN2. The other claudins were not differentially expressed between the two habitats (Figure 32).

Liver Gene Ontology Groups

Across all three treatments, the top three GO groups were identical with *helical transmembrane proteins* as the most abundant, followed by *signal* and then *metabolic pathways* (Figure 33). Of the top 10 GO groups per treatment, five GO groups were shared among all three salinities and one group was uniquely shared between 0 ppt and 10 ppt (Table 12). Fourteen total GO groups were found in only a single treatment (Table 12).

Kidney

General patterns of DEGs

Our PCA found one principal component explaining 24% of the variance and the other explaining 15% of the variance. We did not find any general patterns of clustering related to habitat (Figure 34) or salinity (Figure 35). For the kidney, coastal and inland populations exhibited few upregulated genes at 0 ppt (1 gene, inland) and at 10 ppt (1 gene, coastal). However, at 20 ppt we identified 29 total DEGs with more upregulated genes in the inland habitat than the coastal habitat (Figure 36). We found that there were 0 DEGs shared across salinities, hence the total number of kidney DEGs between coastal and inland populations was 31 (Figure 37). Our heatmap of kidney DEGs did not find any monophyletic clades of upregulated genes (Figure 38).

RAAS specific genes

We found two RAAS-specific DEGs: two solute carrier genes were upregulated in coastal alligators at 20 ppt.

Kidney Gene Ontology Groups

The top GO groups (Table 13) for coastal versus inland at 0 ppt were: signal, disulfide bond, and receptor binding. There were no DEGs at 10 ppt in the kidney. The top 10 GO groups for coastal vs. inland at 20 ppt were: *integral component of membrane*, *concanacalin A-like lectin/glucanase subgroup*, *mitchochondrial ATP transmembrane transport*, *adenine nucleotide translocated 1*, *ATP:ADP antiporter activity*, *DOMAIN:CMP/dCMP-type deaminase*, *CMP/dCMP daminase*, *zinc binding*, *cytidine deaminase-like*, *mitochondrial carrier protein*, and *PRY*.

Discussion

We used a common garden experiment to test for differentially expressed genes in American alligators from two habitats (coastal versus inland) across three salinities (0 ppt, 10 ppt, and 20 ppt). We first predicted that we would find an interactive effect of habitat and salinity on gene expression. Indeed, we did find evidence that coastal alligators differentially expressed genes in osmoregulatory organs in response to environmental salinity changes compared to inland alligators. Additionally, we found that DEG patterns exhibited increased upregulation in the coastal habitat for liver genes, whereas the opposite pattern was true for kidney genes. In support of our second prediction, that coastal alligators would differentially express genes related to osmotic balance, metabolism and immunity at high salinities compared to inland alligators, many of the DEGs identified here consisted of coastal alligators upregulating genes related to signal pathways, metabolism, and immunity. Additionally, we found many more DEGs in the liver than in the kidney. Below, we discuss how these results provide information on potential ecotype evolution in varying environments.

We found that coastal alligators differentially respond to salt-stress compared to inland alligators. Similar results have been found in fish. For example, when coastal marsh fish (*Gambusia affini*) from fresh, intermediate and brackish marshes were exposed to 25 ppt water,

the fish from brackish and intermediate marshes had increased salinity tolerance compared to their freshwater counterparts (Purcell et al. 2008). Descendants of these fish were reared for two generations in freshwater before being run through the same experiment. The authors found that the descendants of brackish fish had higher survival rates than the descendants of freshwater fish when placed in high salinity (Purcell et al. 2008). Similarly, other studies of fish show various isoforms of the Na-K-Cl cotransporter (NKCC), with some isoforms upregulated when the fish is in saltwater (Velotta et al., 2017) while other isoforms are downregulated when the fish is in saltwater (Hasan et al. 2017). Indeed, the membrane protein *ATP1A1* establishes and maintains the sodium/potassium gradient across the cell membrane and is has been shown to be upregulated in both gill and kidney tissues of sticklebacks in response to the stress of increased sodium ions in saltwater (McCairns and Bernatchez 2010). Furthermore, a study on the adaptation mechanisms of Hainan medaka (*Oryzias curvinotus*) to salinity found liver DEGs involved substance synthesis and transport, energy metabolism, signal transduction, and binding functions (Dong et al. 2022), similar to the GO groups we found in the livers of alligators. These data begin to shed light on how differences in gene expression provide the mechanisms used by organisms to respond to environmental changes in salinity. Similar to these studies, we found that American alligators differentially express genes involved in RAAS system for osmotic balance.

Solute carriers move ions across cell membranes and have been linked to adaptation to salinity in many species (Hong, Jiang, et al. 2019; Mohindra et al. 2019; Takvam et al. 2021). Across all three salinities, we found that all but one differentially expressed solute carrier (SLC) gene were upregulated in coastal alligators. This reflects what other studies have found, with more SLCs being identified as for adaptation to salinity than adaptation to freshwater (Mohindra et al. 2019). In fact, one of the genes upregulated in coastal alligators at 0 ppt, was identified as a marker for saltwater adaptation. Interestingly, the one gene that was upregulated in inland alligators at 0 ppt, SLC25A48, has not been implicated in adaptation to osmoregulation (Mohindra et al. 2019). Additionally, we found that SLC27A1 was

upregulated in coastal alligators at 10 ppt. A similar protein (SLC27A4) was upregulated in the liver of Japanese medaka at 30 ppt (Dong et al. 2022). Both proteins are transmembrane long-chain fatty acid transporters that help cells absorb fatty acids. By accumulating more fat, the cells increase in size, a result corroborated by histological analyses (Dong et al. 2022).

Aquaporins are a family of protein channels that reabsorb water and have been linked to the ability of organisms to adaptively osmoregulate (Watanabe, Kaneko, and Aida 2005). We found that coastal alligators upregulated AQP3 and AQP8 compared to inland alligators in 0 ppt. AQP3 was upregulated in the kidneys of saltwater salmon compared to their freshwater counterparts (Watanabe, Kaneko, and Aida 2005). Aquaporin-3 is found on the basolateral membranes of tubule cells and plays a role in water reabsorption (Watanabe, Kaneko, and Aida 2005). Similarly, AQP8 creates a transcellular pathway to reabsorb water so that it can re-enter the extracellular fluids (Mohindra et al. 2019). AQP-8b was found to be responsible for much of the water reabsorption in the intestines of seawater salmonids (Tipsmark et al. 2010). It seems that saltwater ecotypes are adapted to a hyperosmotic environment and so when they are placed in a freshwater hypoosmotic environment, they will upregulate aquaporins to increase water reabsorption.

Claudins are tight junction proteins that control the amount of space between epithelial cells (Mohindra et al. 2019). Changes in the amount of claudins can increase or decrease epithelia permeability in response to salinity changes (Rosenthal et al. 2010). Coastal alligators had higher expression levels of CLDN10 at 0 and 20 ppt. In the Japanese medaka (*Oryzias latipes*), kidney expression levels of CLDN10bs were higher in saltwater fish than freshwater fish (Bossus, Madsen, and Tipsmark 2015). This highlights that claudin10 may be playing a role in paracellular ion transport for saltwater ecotypes. Furthermore, the upregulation of CLDN2 in coastal alligators at 20 ppt, could be indicative of an effort to increase paracellular water transport. Researchers found that CLDN2 creates water channels that facilitate water transport in the epithelia of the renal proximal tubules (Rosenthal et al. 2010). The upregulation of CLDN15 at 10 ppt, could be indicative of a change in paracellular permeability as part of saltwater

acclimation as suggested by a study in Atlantic salmon (Tipsmark et al. 2010). Claudins 6, 10 and 15 were all upregulated in coastal alligators and all three have been linked to acclimation to seawater in fish (Mohindra et al., 2019; Bui & Kelly, 2014).

The GO groups for the liver correspond what has been found in the livers of fish that adapt to changes in salinity (Dong et al. 2022). For example, one of the most commonly represented GO groups across all three salinities was metabolic pathways. The liver plays an important role in lipid metabolism (Liu et al. 2017; Lv et al. 2013) and gluconeogenesis (Guo et al. 2018) and be these pathways can be disrupted by changes in salinity (Li et al. 2017). Specifically, elevated salinity levels can lead to cells increasing the amount of fatty acids reabsorbed into their membrane (Lv et al. 2013). Furthermore, we found the "secreted" GO term was in the top 10 in the liver at all salinities, which matches other research that found that coastal populations upregulated ion secretion genes compared to inland populations (Velotta et al. 2017). Specifically, they upregulated ATP1A1, SLC12A2, CFTR, KIR, KCNJ1, and KCNJ2 (Velotta et al. 2017), none of which were upregulated in our study. Similar to our study, signal transduction proteins were also found as DEGs from liver transcriptomes of marine medaka (*Oryzias melastigma*) placed in high salinities (45 ppt) compared to freshwater (Zhang et al. 2023). Another study of DEGs in of Japanese medaka kept at 0, 15, and 30 ppt salinities found that signal transduction mechanisms was the top GO term (Dong et al. 2022). The authors suggested that this may be evidence that fish change expression levels of signal transduction receptors on cell membranes to adjust cellular osmotic pressure in changing environmental salinities.

At 0 and 10 ppt, immunoglobulin-like domain was a top 10 GO term in the liver, but not at 20 ppt. Large variations in salinity have been shown to significantly affect immunity-related gene expression (Lv et al. 2013). For example, one study investigated liver transcriptomes in yellow croaker (*Larimichthys crocea*) kept at 12, 24, 36 ppt for 4 weeks and found that genes in complement and coagulation pathways changed expression levels with varying salinity (Zhang et al. 2022). Another study in Coho salmon (*Oncorhynchus kisutch*) found that four complement genes along with clusterin (clu) and beta polypeptide

(c8b) were downregulated in livers at high salinity compared to the control (Maryoung et al. 2015). The two studies mentioned here along with others (Evans and Somero 2008; Seear et al. 2010) provide evidence that high salinity causes suppression of the immune system. Any immune-related advantage in gene expression between the ecotypes at lower salinities disappears at 20 ppt, where both ecotypes are equally immunocompromised. Salt stress has been shown to have a big effect on the body's ability to fight off infections (Brand et al. 2001). Thus, the limited adaptation to saltwater that coastal alligators possess seems to be not enough to ward off these negative immunological phenomena.

Why did we find such striking different numbers of DEGs between kidney and liver? There are two explanations for this phenomenon: one technical and one biological. It is possible that we did not have enough samples or enough depth of coverage to identify the patterns of differentiation that do exist. Second, it is possible that kidney gene expression is highly conserved compared to liver gene expression. as has been found in other studies (Deanne and Woo, 2004; Deanne et al.). In sea bream exposed to hypoosmotic, isosmotic, and hyperosmotic salinities, there was no change in kidney expression of genes in the hsp70 family across salinities (Deanne and Woo, 2004). Given that we found many DEGs from liver with half the amount of mapped reads as the kidney (avg: 8 million vs. 16 million), we believe the biological explanation is more likely.

In summary, we found that the liver had many more DEGs between coastal and inland habitats than the kidney, likely reflecting the highly conserved nature of kidney gene expression (Deane, Luk, and Woo 2011; Deane and Woo 2004). Within the liver, the coastal alligators upregulated many more genes than the inland alligators. This adds to the evidence that coastal/saltwater organisms have greater phenotypic plasticity in response to changing salinity levels than their inland/freshwater counterparts (Bossus, Madsen, and Tipsmark 2015; Hong, Jiang, et al. 2019; Hong, Li, et al. 2019). We found key changes in RAAS-specific genes (i.e. solute carriers, aquaporins, and claudins), a phenomenon that has been found in adaptation to

salinity studies in fish (Bossus, Madsen, and Tipsmark 2015; Mohindra et al. 2019; Watanabe, Kaneko, and Aida 2005). Finally, our GO groups showed expression patterns in areas that we expected: signal transduction, metabolism, and immunity. All of three of these GO groups have been found in DEGs from osmoregulatory organs in fish exposed to varying salinity levels (Lv et al. 2013; X. Zhang et al. 2023). Overall, understanding how aquatic and semi-aquatic organisms adapt to changing salinity levels will provide insight into how climate change-driven seawater encroachment into freshwater habitats will affect the species that live in those habitats.

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CHAPTER 5: CONCLUSIONS

My dissertation found that salinity has played a major role in the evolutionary history of American alligators (*Alligator mississippiensis*) and contributed to local adaptation in coastal populations. In Chapter 2, I used ddRADSeq to generate thousands of genomic SNPs to uncover the population genetic structure, gene flow among populations, and demographic history within regions across the range of the American alligator. I found that patterns of gene flow and genetic structuring were associated with watersheds and elevational differences between populations. While major rivers (i.e. Mississippi, Alabama, Apalachicola) have been found to serve as barriers to gene flow for terrestrial taxa (Lyman & Edwards, 2022), for American alligators these watersheds instead house unique genetic clusters. Indeed, the terrestrial areas between these watersheds seem to form the barriers to gene flow for alligators. In addition, within a watershed, large differences in elevation are associated with low levels of gene flow. Past sea level rise has expanded rivers and inundated them with seawater (Brachert et al., 2014), a process which has been hypothesized to create genetic structuring in organisms on either side of the river (Lyman & Edwards, 2022). Demographic models enabled me to estimate the timing of the sundering point among genetic clusters and approximate the geologic events leading to cluster differentiation. I estimated that the 17m of sea level rise that occurred 2.45 mya expanded the Mississippi River to such a width that created a genetic split in alligators west and east of the river. Likewise, 4m of sea level rise 1.4 mya expanded the St Johns River to where populations on the east coast of Florida were genetically isolated from populations in the panhandle. Finally, around 300kya, when sea levels rose by 8m, mid-Atlantic coastal populations appear to have been wiped out, genetically isolating populations in North Carolina from those in Georgia. Overall, the

demographic history models show how salinity served as a barrier to gene flow for alligators and that changes in sea level contributed to genetic structuring throughout the species range.

In Chapter 3, I investigated whether coastal alligators respond differently to changes in salinity compared to alligators from inland populations. Here, I used a common garden experimental design and placed juvenile alligators from both coastal and inland populations into one of three salinity levels (0, 10, or 20 ppt) for two weeks and recorded behavioral, physiological, and histological data. I found that for all traits measured, there was a habitat by salinity interaction. This means that both coastal and inland alligators showed plasticity in response to increasing salinity levels, but the slope of the response differed between coastal and inland populations, indicating a habitat component to how populations responded to salt stress. Previous studies have investigated how freshwater organisms that encounter saltwater respond to such contact. Specifically, genetic adaptation to high salinity in freshwater organisms had been found in freshwater fish (Purcell, Hitch, Klerks, & Leberg, 2008), freshwater turtles (Bower et al., 2016; Hong et al., 2019) and snakes (Brischoux, Kornilev, & Lillywhite, 2017).

Predictions of physiological responses in alligators originated from studies investigating saltwater adaption in other species, such as a study where Dice snakes (*Natrix tessellata*), a freshwater natricine, were immersed in three salinities (0.1, 15, 34 ppt) and found that the snakes used specific physiological adaptations to endure the high salinity levels instead of simply reducing salt intake (Brischoux et al., 2017). This response is similar to what I found in American alligators, where coastal alligators had increased plasma sodium levels compared to inland alligators, indicating coastal alligators are adapting to high sodium levels as opposed to increasing the amount of sodium they excrete as a means to maintain a homeostatic plasma salinity concentration in line with inland alligators.

In Chapter 4, I used whole transcriptome data from the kidneys and livers of alligators reared in the treatments described in Chapter 3 to investigate the molecular mechanisms that coastal alligators use to adapt to a high salinity environment. First, I used model selection to investigate the biological factors that impacted differential gene expression in the liver and the kidney. For both tissues, I found that both habitat (inland versus coastal) and tank salinity (0 ppt, 10 ppt, 20 ppt) were factors driving differential gene expression in my common garden experiment. Second, I used DESeq2 to identify differentially expressed genes (DEGs) between coastal and inland alligators at each tank salinity and I organized these DEGs into Gene Ontology (GO) groups to better understand which biological processes were being differentially affected. I found that signal transduction proteins, metabolic pathways, and secretion proteins were top GO terms from DEGs between coastal and inland at all three salinity levels in the liver. This corroborates other studies which have identified high salinity levels as affecting signaling between cells (Dong et al., 2022), increasing lipid metabolism (Lv et al., 2013), and increasing ion secretion (Velotta et al., 2017). Additionally, immunity-related genes were top GO terms at 0 and 10 ppt. This result indicates that changes in salinity affect the immune system, a hypothesis supported by evidence in other species (Liu et al., 2017; Maryoung et al., 2015; Zhang et al., 2022).

By delving more deeply into the GO terms, I was able to identify specific RAAS proteins that were differentially expressed between coastal and inland populations at different salinities. For example, solute carriers were upregulated more in coastal alligators than inland alligators at every salinity level, a result that is in line with other studies identifying solute carriers as markers of adaptation to salinity (Dong et al., 2022; Mohindra et al., 2019). In freshwater (0 ppt), coastal alligators expressed more aquaporins, proteins that reabsorb water, than inland alligators.

Another study found that saltwater salmon also upregulated aquaporins compared to their freshwater counterpart (Watanabe, Kaneko, & Aida, 2005), indicating that saltwater ecotypes are adjusting to the hypoosmotic environment. The takeaway message for Chapter 4 is that both coastal and inland alligators are adapted to their native environments and when they are placed in the opposite environment, they modify their gene expression to deal with the drastic change in the number of ions in the environment.

Overall, my dissertation contributed to the study of adaptive evolution by demonstrating that salinity has been a past and current stressor for American alligators. High salinity levels limit the alligator's species range and lead to genetic differentiation. Yet, at the same time, I found evidence that coastal populations exhibit incipient adaptation to high salt environments. The patterns I found here are similar to other species that inhabit both freshwater and saltwater environments (Brischoux & Kornilev, 2014; Brischoux et al., 2017; Hong et al., 2019).
As there appears to be evidence of convergent evolution for mechanisms to excrete salt in fully marine reptiles (Dunson & Dunson, 1973; Dunson, Packer, & Dunson, 1971; Hudson & Lutz, 2017; Nicolson & Lutz, 1989), my dissertation is starting to provide evidence for patterns of convergent evolution among reptiles that similarly use both freshwater and brackish water environments (Brischoux & Kornilev, 2014; Brischoux et al., 2017; Hong et al., 2019).

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APPENDIX A: FIGURES

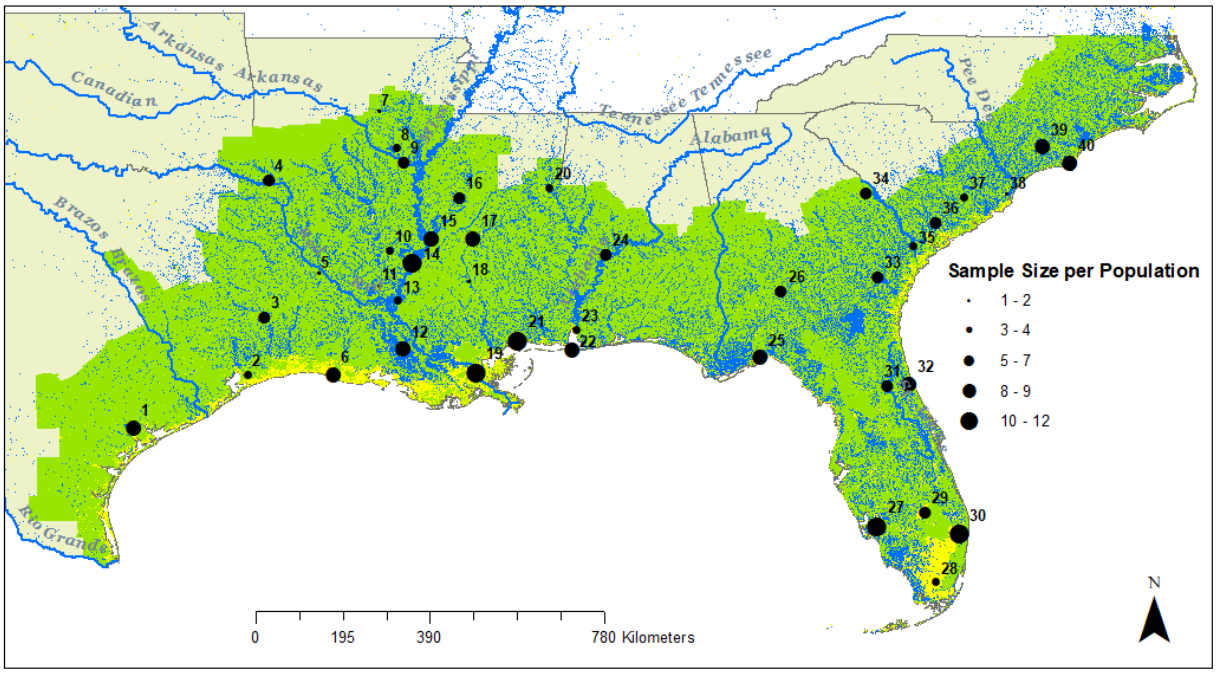

Figure 1. Map of woody wetlands (blue) and emergent herbaceous wetlands (yellow) throughout the range of the American alligator (green). Numbers indicate population ID (see Table 1 for more detail). The presence of wetlands seems to correspond to high levels of gene flow while the absence of wetlands seems to correspond to low levels of gene flow. Source layer: National Land Cover Database 2019.

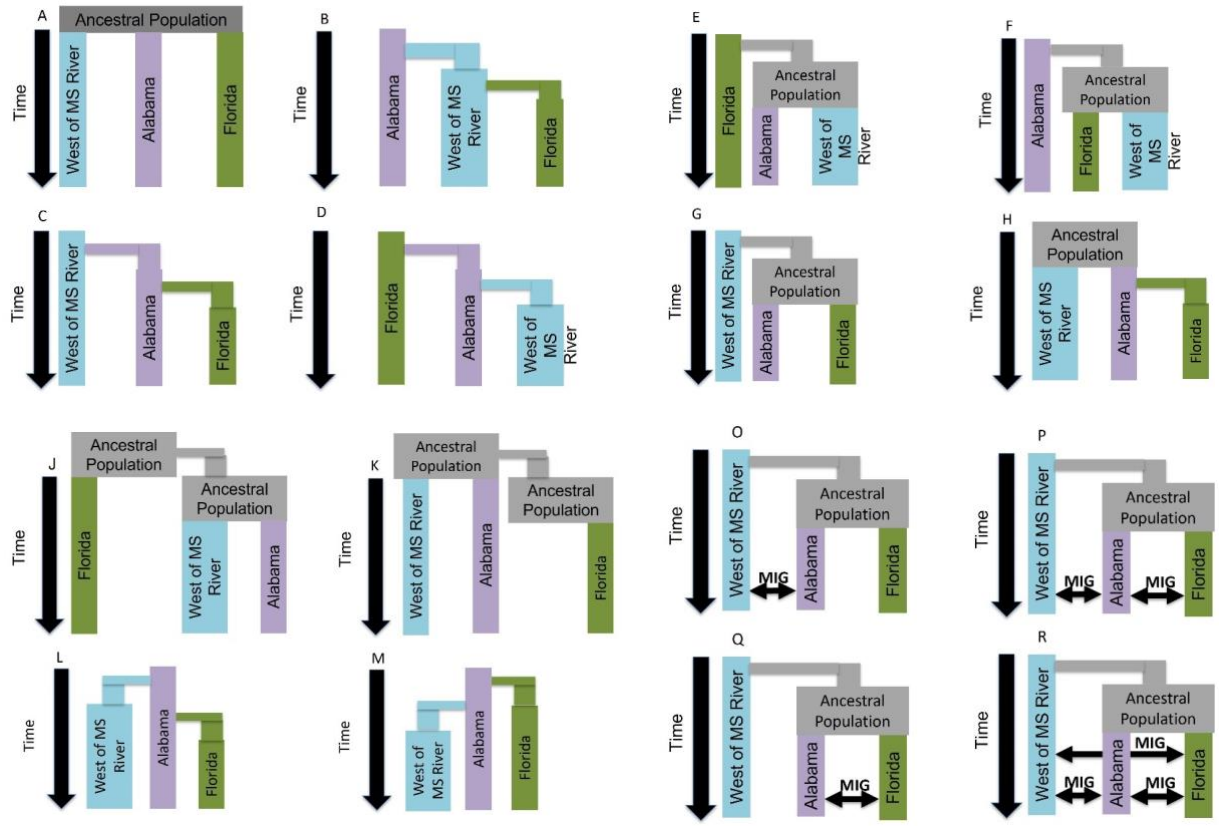

Figure 2. All Fastsimcoal2.7 models tested for the range-wide dataset. For the east coast dataset, the following substitutions were made: West of Mississippi River → Florida, Alabama → Georgia, Florida → Carolinas.

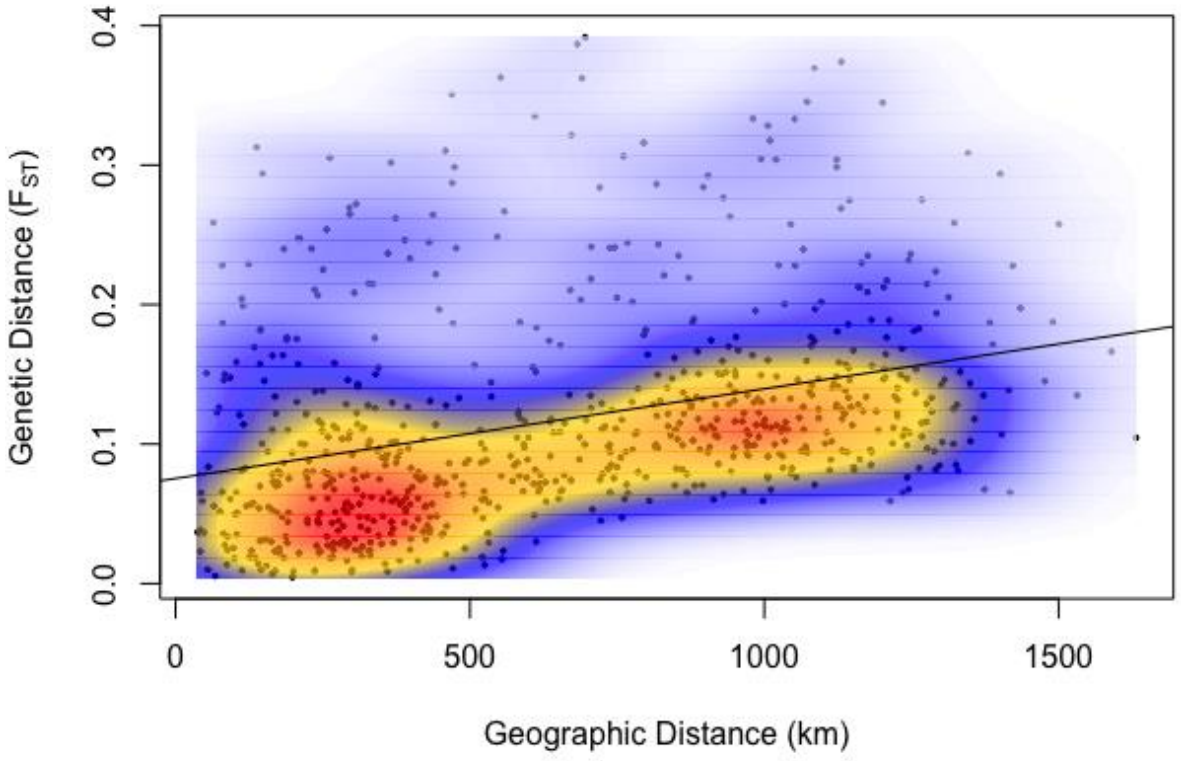

Figure 3. American alligator (Alligator mississippiensis) range-wide Isolation-by-Distance (IBD) plot. The continuous IBD model explains around 11.6% of the variance in genetic distance (R^2 = 0.1155, $p<0.01$).

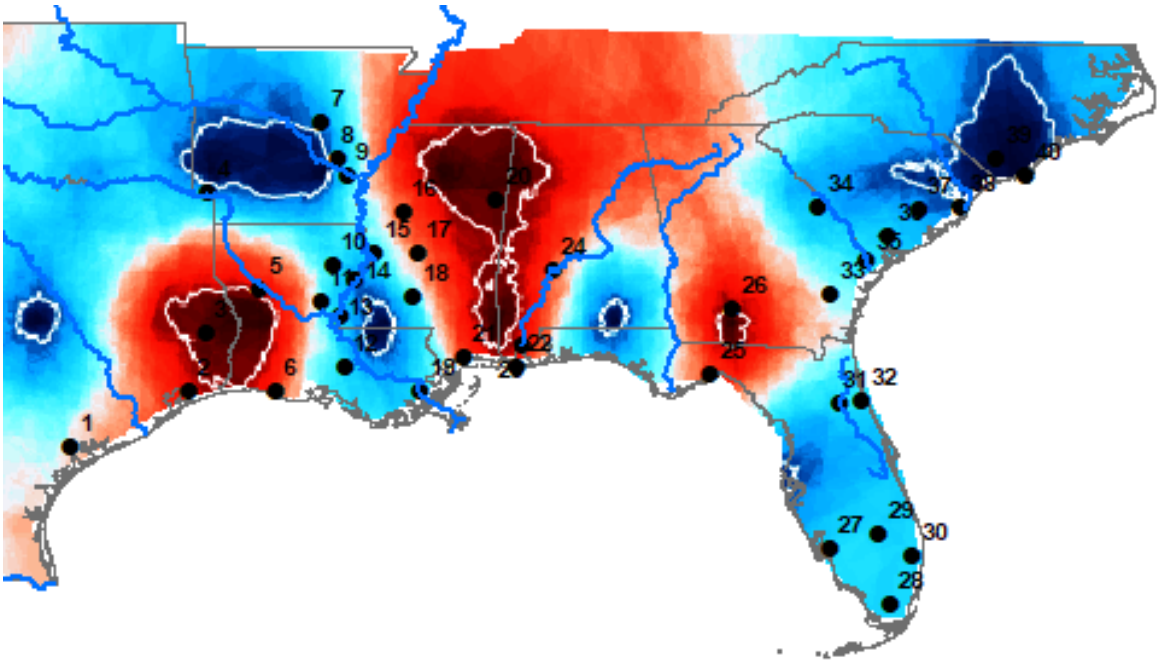

Figure 4. Estimated effective migration surface (EEMS) map. Blue = higher than average gene flow. Orange = lower than average gene flow. Black circles indicate populations and are numbered (see Table 1 for more detail). High gene flow is found between low elevation populations. Low gene flow is found between high elevation populations and low elevation populations.

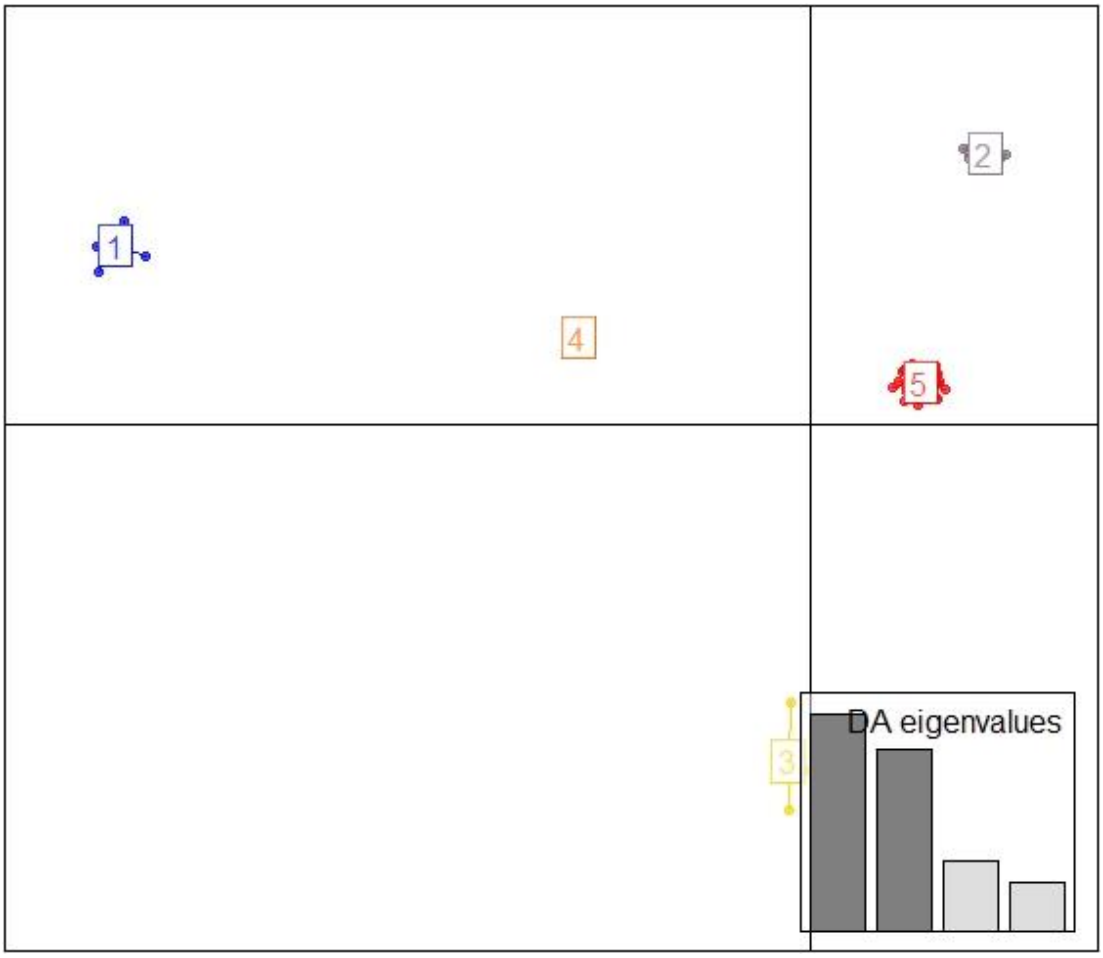

Figure 5.Discriminant Analysis of Principal Components (DAPC) plot showing that the range-wide samples grouped into five distinct genetic clusters: 1) West of Mississippi River, 2) East of the Mississippi River and West of the Apalachicola River, 3) Florida, 4) Georgia, and 5) North and South Carolina.

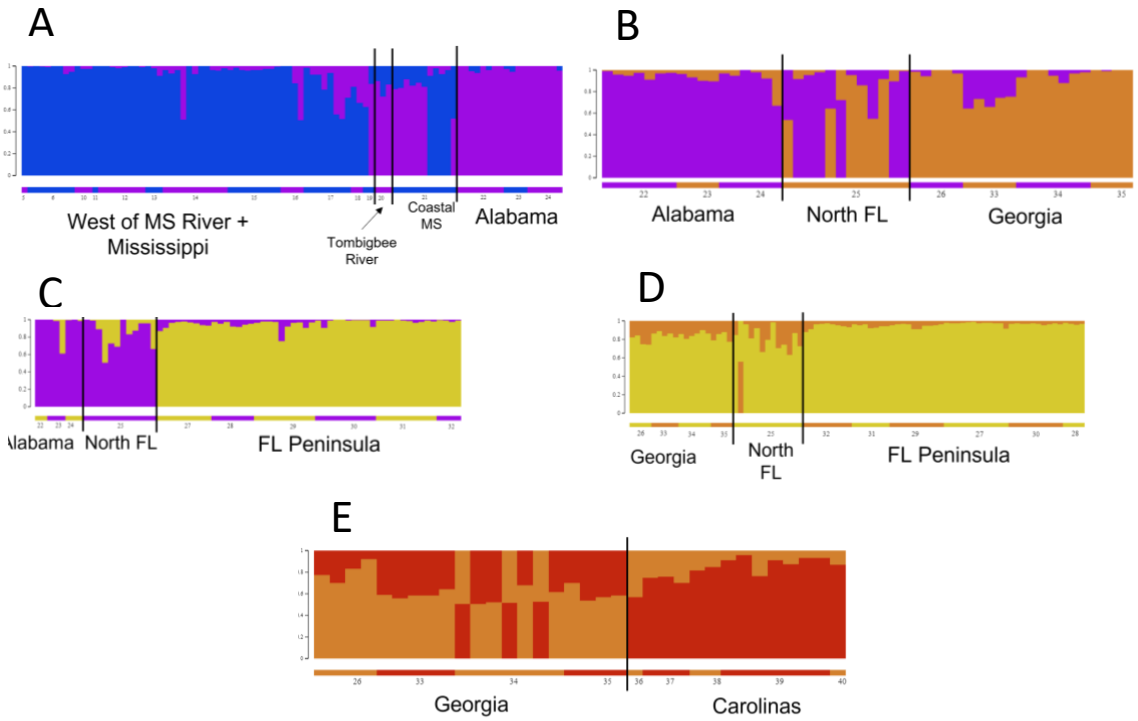

Figure 6. STRUCTURE plots of the borders between the clusters identified by DAPC (see Figure 4). A) STRUCTURE plot (K = 2) showing a break west and east of the Mississippi River watershed. Notice that the Tombigbee River groups with the Alabama cluster and that coastal Mississippi is admixed. B) STRUCTURE plot (K =2) showing that North Florida is an admixture of Alabama and Georgia clusters. C) STRUCTURE plot (K = 2) showing North Florida is more similar to Alabama than the Florida Peninsula. D) STRUCTURE plot (K = 1) showing genetic homogeneity when only Georgia, North Florida and the Florida Peninsula are included. E) STRUCTURE plot (K = 2) showing that Georgia has a large amount of admixture for the Carolinas cluster. FL = Florida, MS = Mississippi. Numbers indicate population number (see Table 1 for more detail).

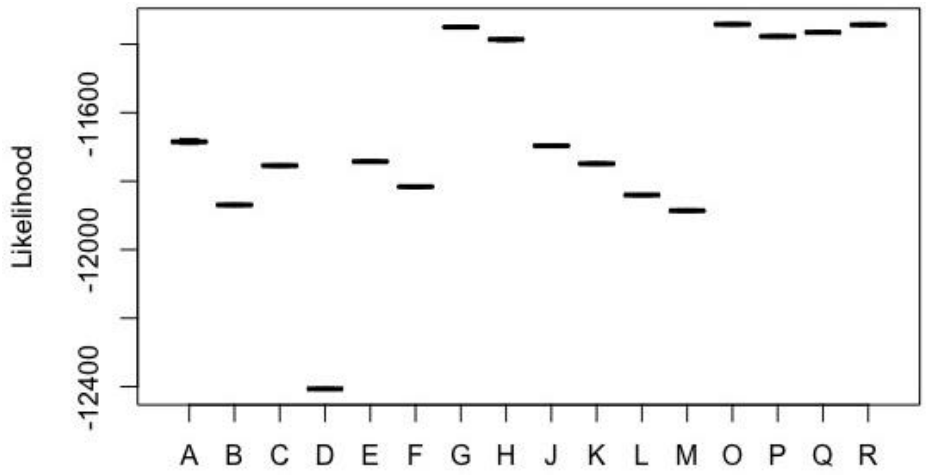

Figure 7. Likelihood distributions (Boxplot of 100 likelihoods) for models of the east coast dataset (Florida-Georgia-Carolinas). Models O and R were equally likely.

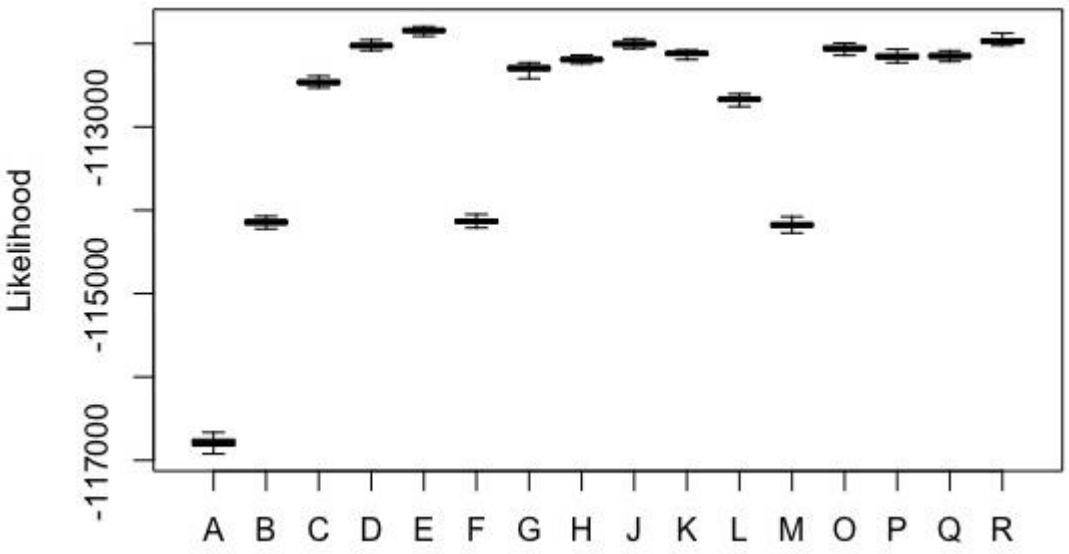

Figure 8. Likelihood distributions (Boxplot of 100 likelihoods) for models of the range-wide dataset (West-Alabama-Florida). Models E and R were equally likely.

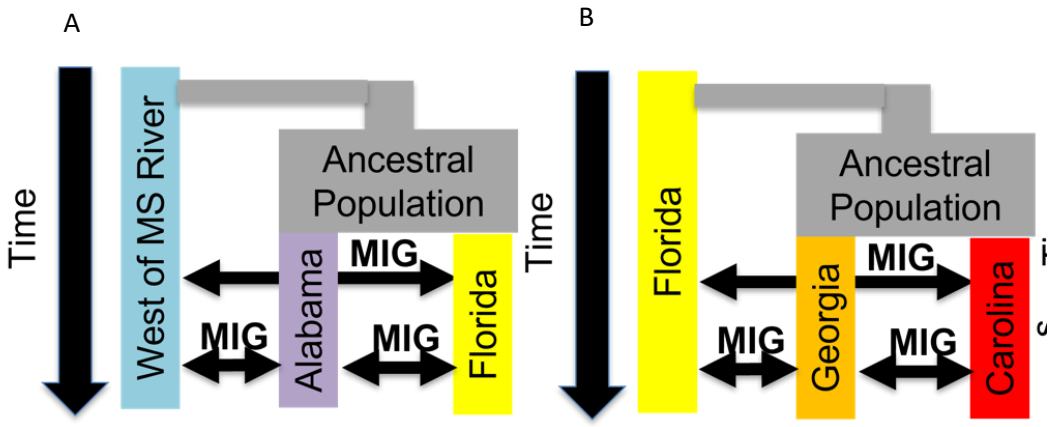

Figure 9. Best Fastsimcoal2.7 models for each of the east coast dataset and the range-wide dataset. A) West of Mississippi River, Alabama, and Florida. B) Florida, Georgia, and the Carolinas. C) North Florida and Peninsular Florida. MIG = current migration among demes.

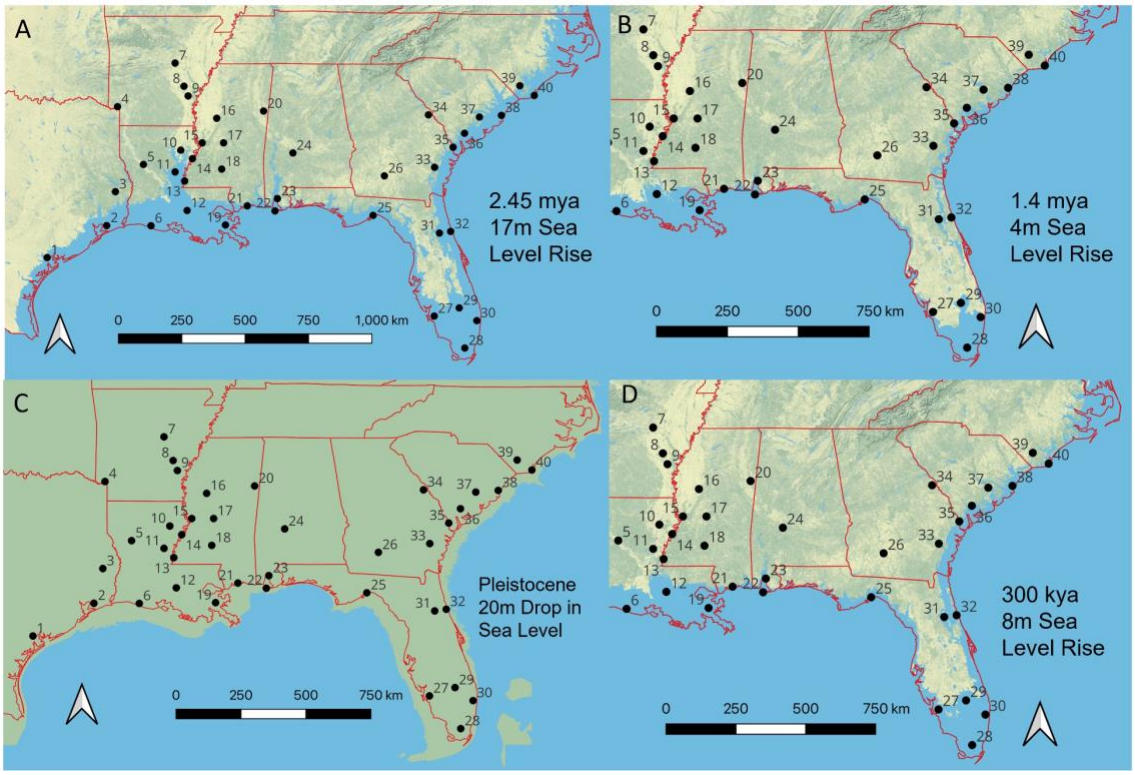

Figure 10. Illustration of sea level over time in the southeastern USA compared to contemporary sea level. A) The Mississippi River greatly expanded following a 17 m rise in sea level, leading to separate genetic clusters west and east of the Mississippi River watershed. B) Central Florida populations (#31 and #32) became isolated from the mainland following the flooding and saltwater inundation of the St. Johns River after 4 m of sea level rise. C) For much of the Pleistocene (2.58 mya – 11.5 kya) sea levels were 20 m below current levels. This would have increased connectivity among low elevation populations. D) Coastal Carolinas and coastal Georgia populations were non-existent when sea levels were 8m higher than today. This led to the geographic isolation and eventual genetic splitting of the above sea level Georgia (#26, #33, and #34) and Carolinas (#37 and #39) populations.

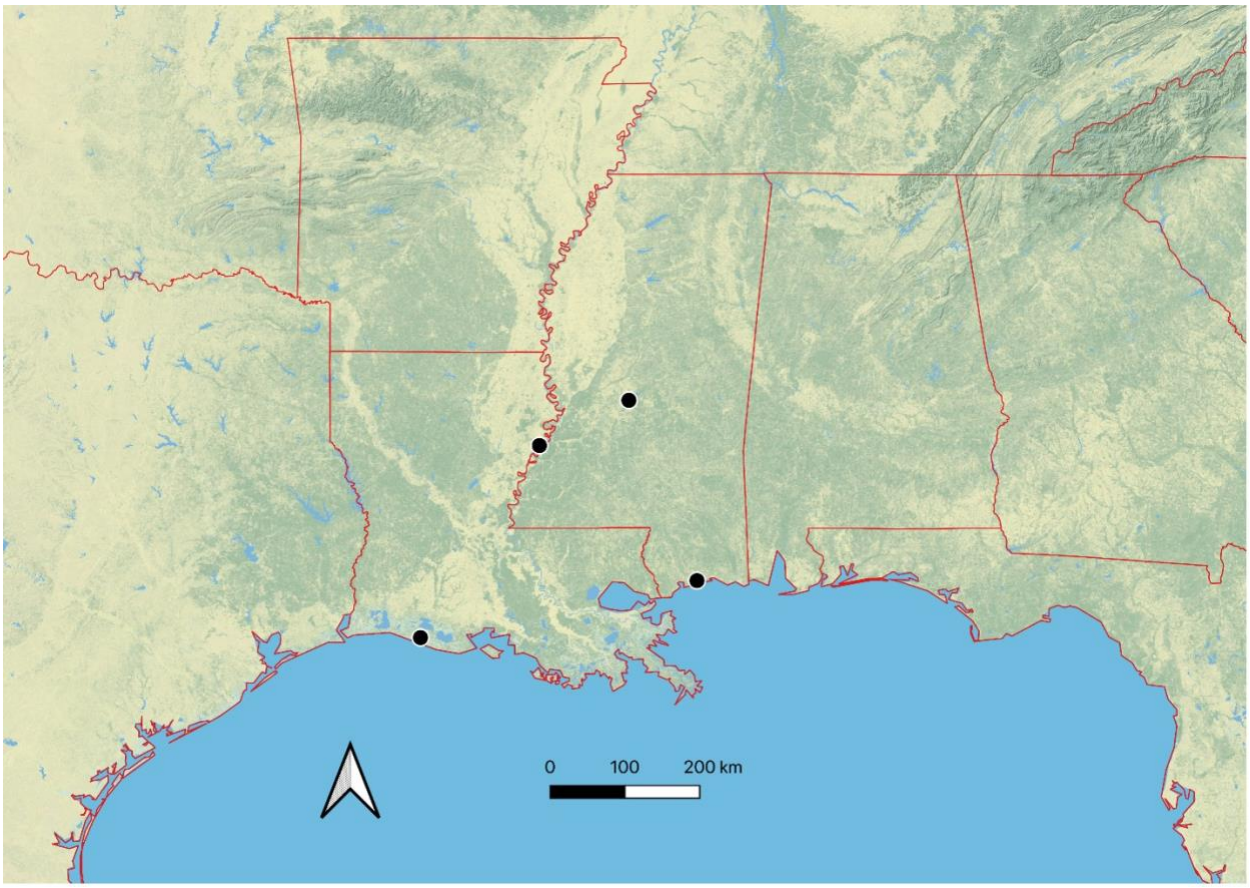

Figure 11. Map of sampled American alligator populations (2 coastal, 2 inland, total n = 4).

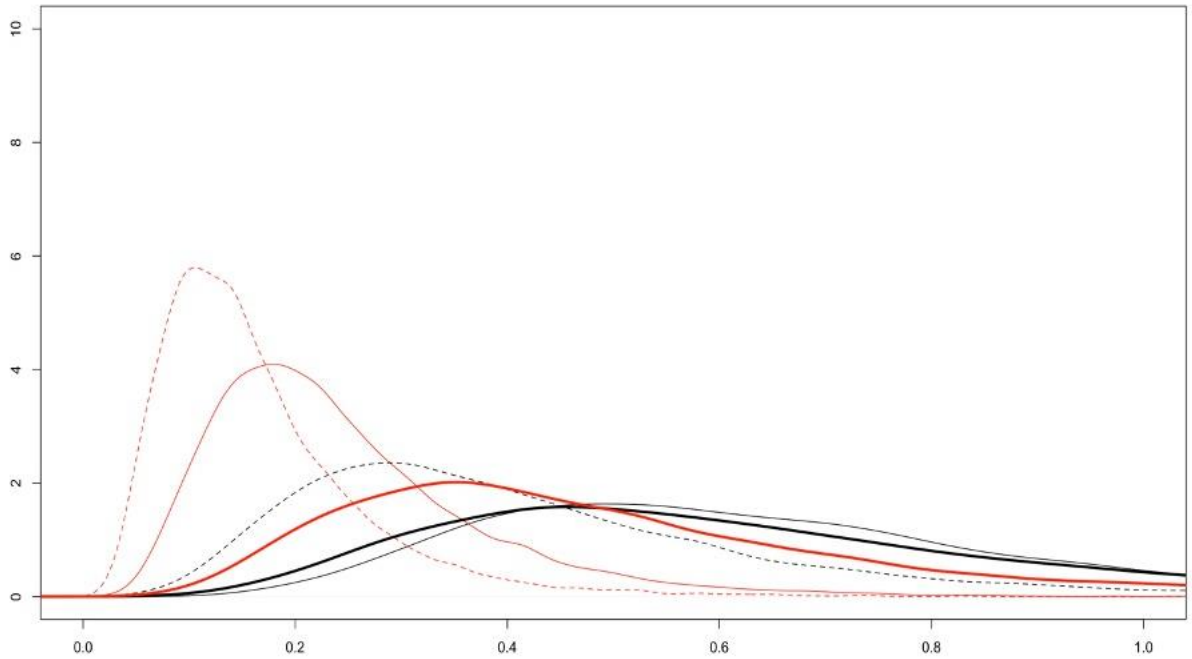

Figure 12. Bayesian posterior distributions of time spent on stand (i.e. out of the water) for small (total length = 48cm) Mississippi (MS) male American alligators. Black = inland animals. Red = coastal animals. Thin solid line = 0 ppt. Dotted line = 10 ppt. Thick solid line = 20 ppt. X-axis represents time on the stand: 0 = no time on the stand, 1 = always on the stand.

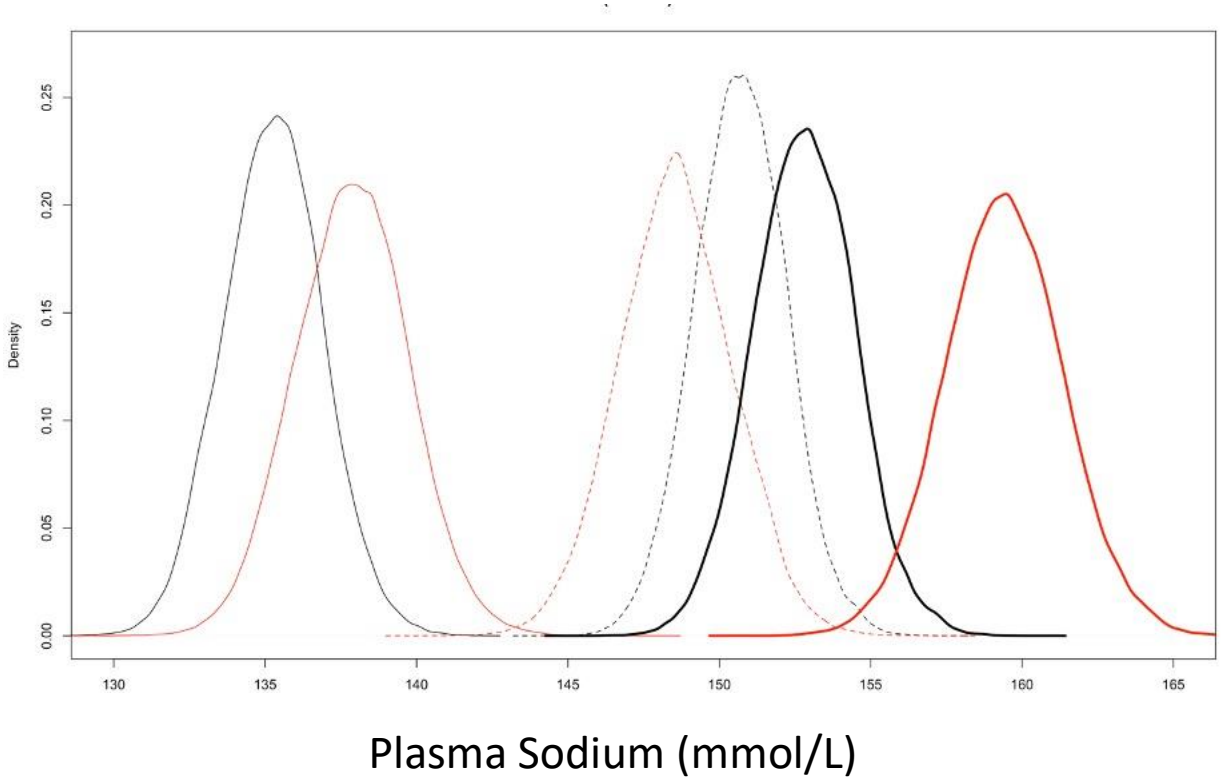

Figure 13. Bayesian posterior distributions of plasma sodium levels for small (total length = 48cm) Mississippi (MS) male American alligators. Black = inland animals. Red = coastal animals. Thin solid line = 0 ppt. Dotted line = 10 ppt. Thick solid line = 20 ppt. X-axis represents sodium levels (mmol/L).

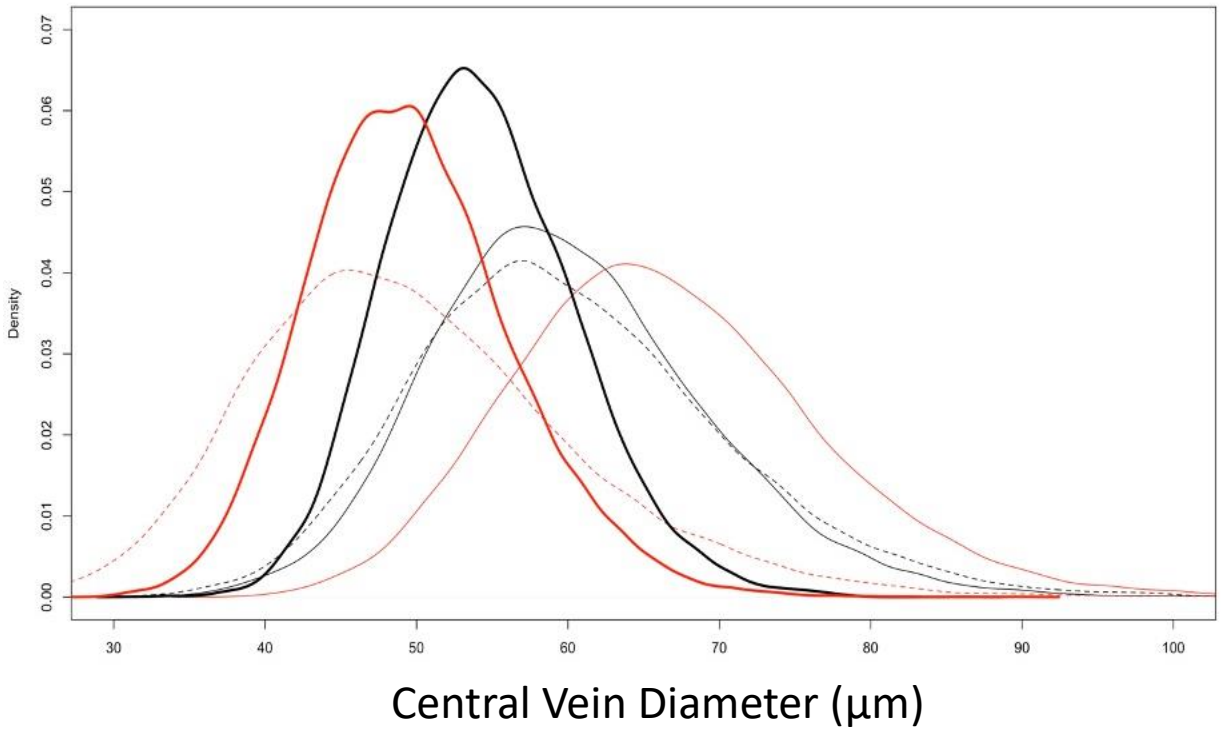

Figure 14. Bayesian posterior distributions of central vein diameter (liver) for small (total length = 48cm) Mississippi (MS) male American alligators. Black = inland animals. Red = coastal animals. Thin solid line = 0 ppt. Dotted line = 10 ppt. Thick solid line = 20 ppt. X-axis represents central vein diameter (µm).

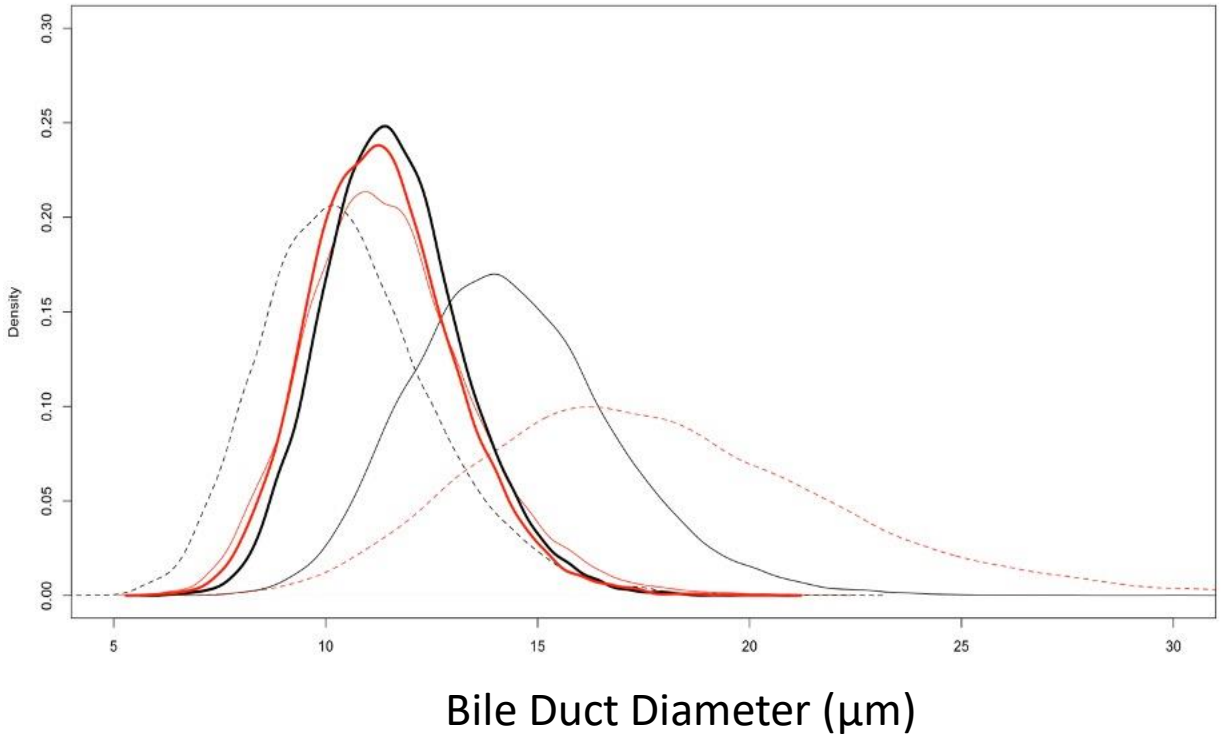

Figure 15. Bayesian posterior distributions of bile duct diameter (liver) for small (total length = 48cm) Mississippi (MS) male American alligators. Black = inland animals. Red = coastal animals. Thin solid line = 0 ppt. Dotted line = 10 ppt. Thick solid line = 20 ppt. X-axis represents bile duct diameter (µm).

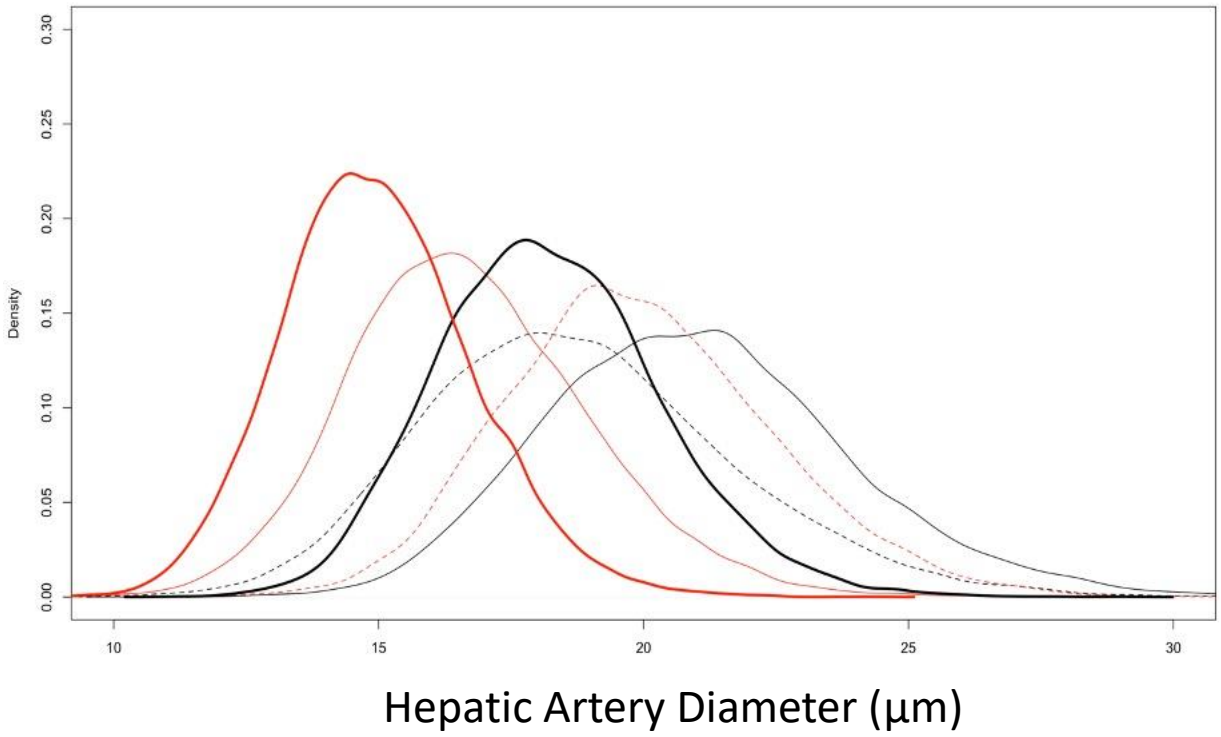

Figure 16. Bayesian posterior distributions of hepatic artery diameter (liver) for small (total length = 48cm) Mississippi (MS) male American alligators. Black = inland animals. Red = coastal animals. Thin solid line = 0 ppt. Dotted line = 10 ppt. Thick solid line = 20 ppt. X-axis represents hepatic artery diameter (µm).

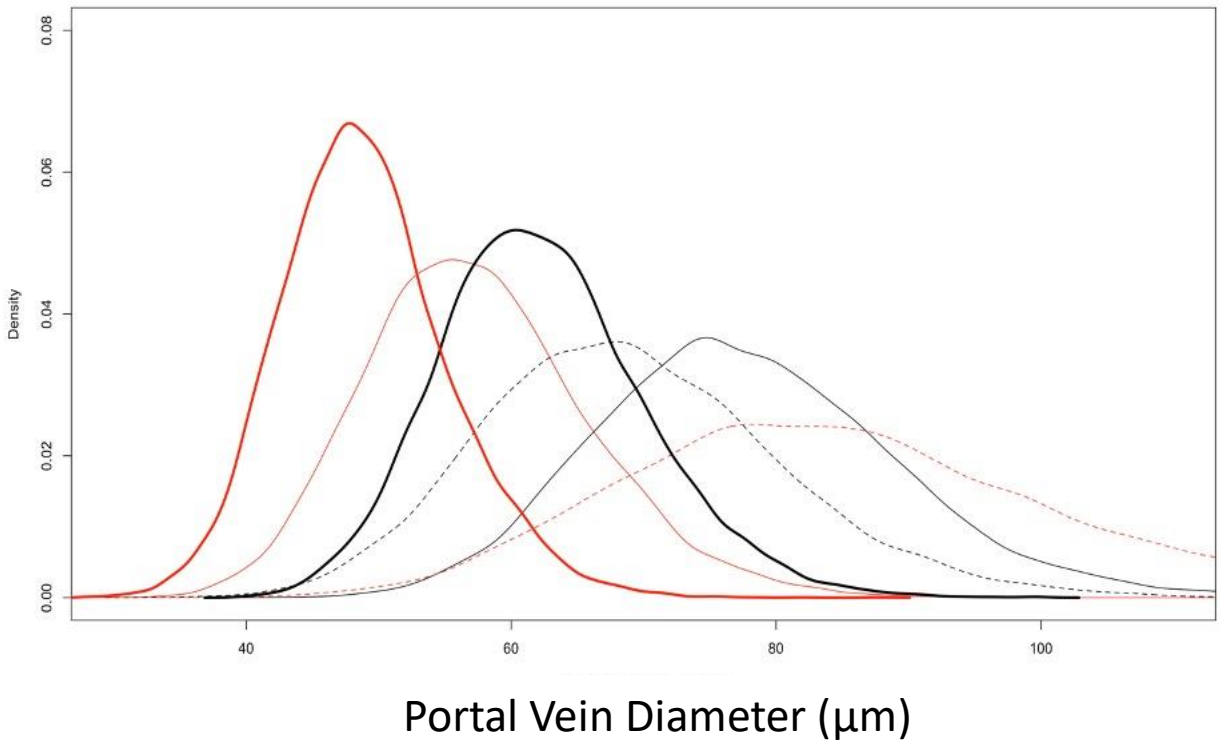

Figure 17. Bayesian posterior distributions of portal vein diameter (liver) for small (total length = 48cm) Mississippi (MS) male American alligators. Black = inland animals. Red = coastal animals. Thin solid line = 0 ppt. Dotted line = 10 ppt. Thick solid line = 20 ppt. X-axis represents portal vein diameter (µm).

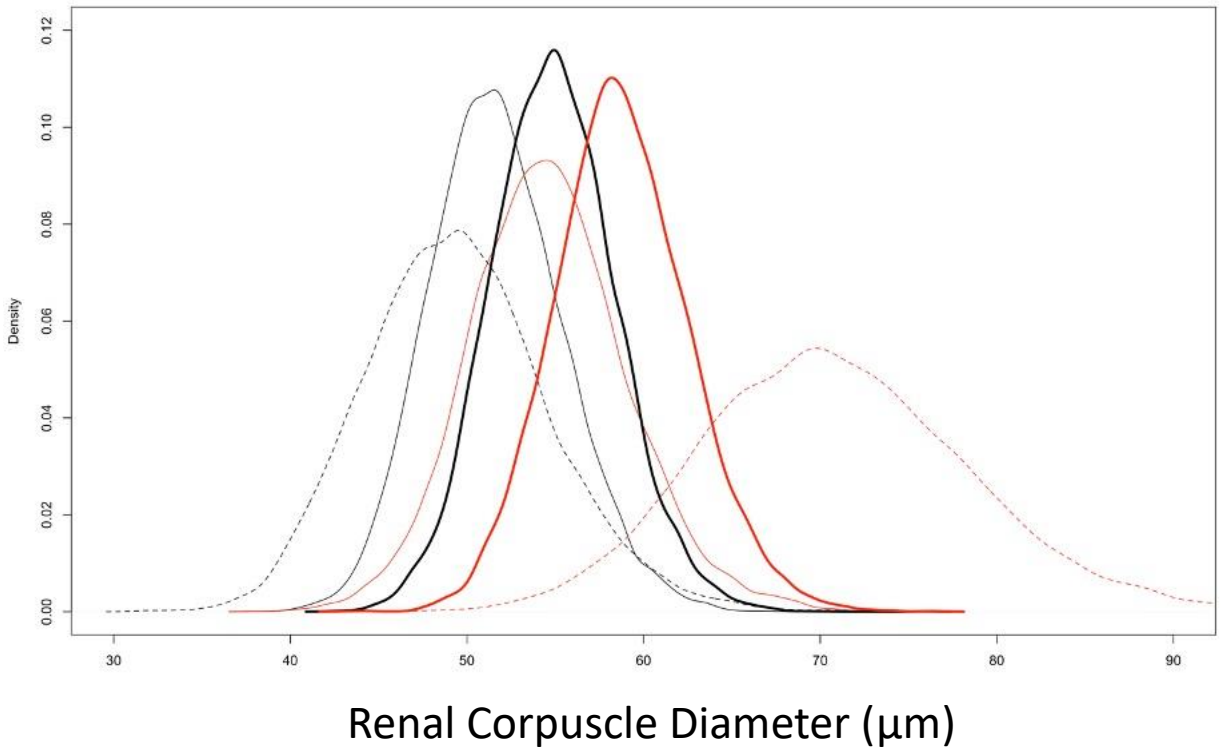

Figure 18. Bayesian posterior distributions of renal corpuscle diameter (kidney) for small (total length = 48cm) Mississippi (MS) male American alligators. Black = inland animals. Red = coastal animals. Thin solid line = 0 ppt. Dotted line = 10 ppt. Thick solid line = 20 ppt. X-axis represents renal corpuscle diameter (µm).

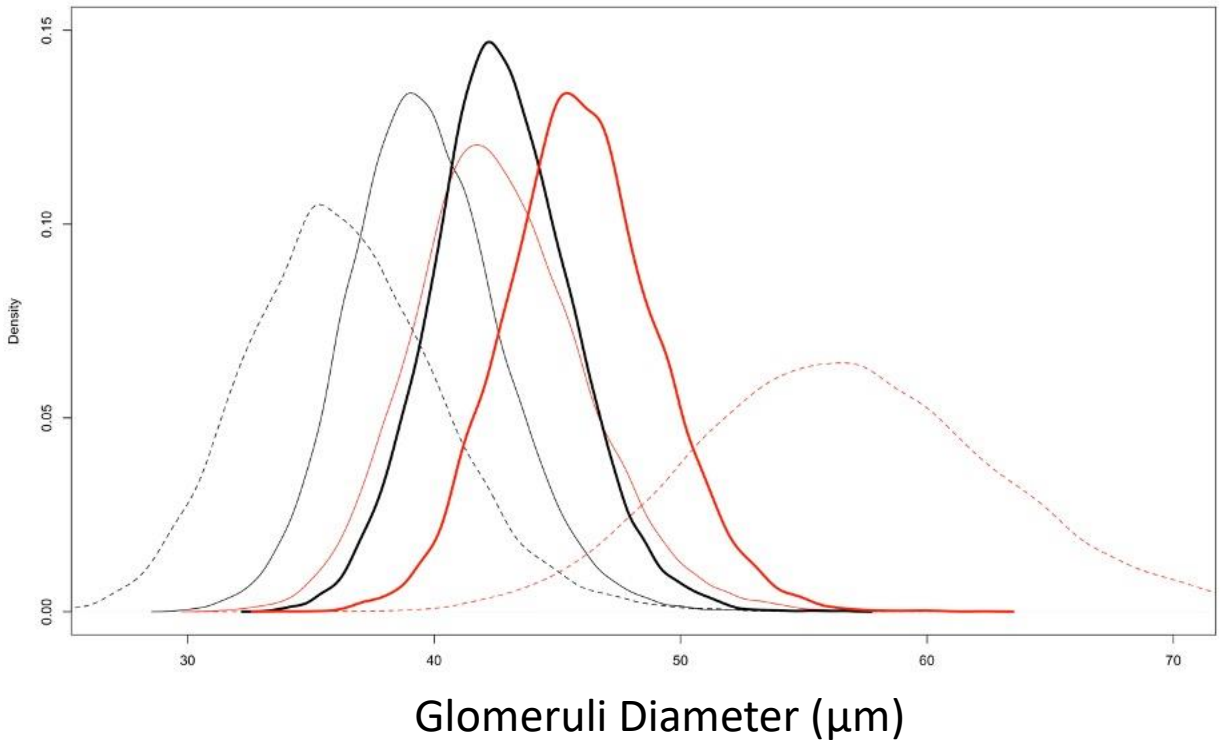

Figure 19. Bayesian posterior distributions of glomeruli diameter (kidney) for small (total length = 48cm) Mississippi (MS) male American alligators. Black = inland animals. Red = coastal animals. Thin solid line = 0 ppt. Dotted line = 10 ppt. Thick solid line = 20 ppt. X-axis represents glomeruli diameter (µm).

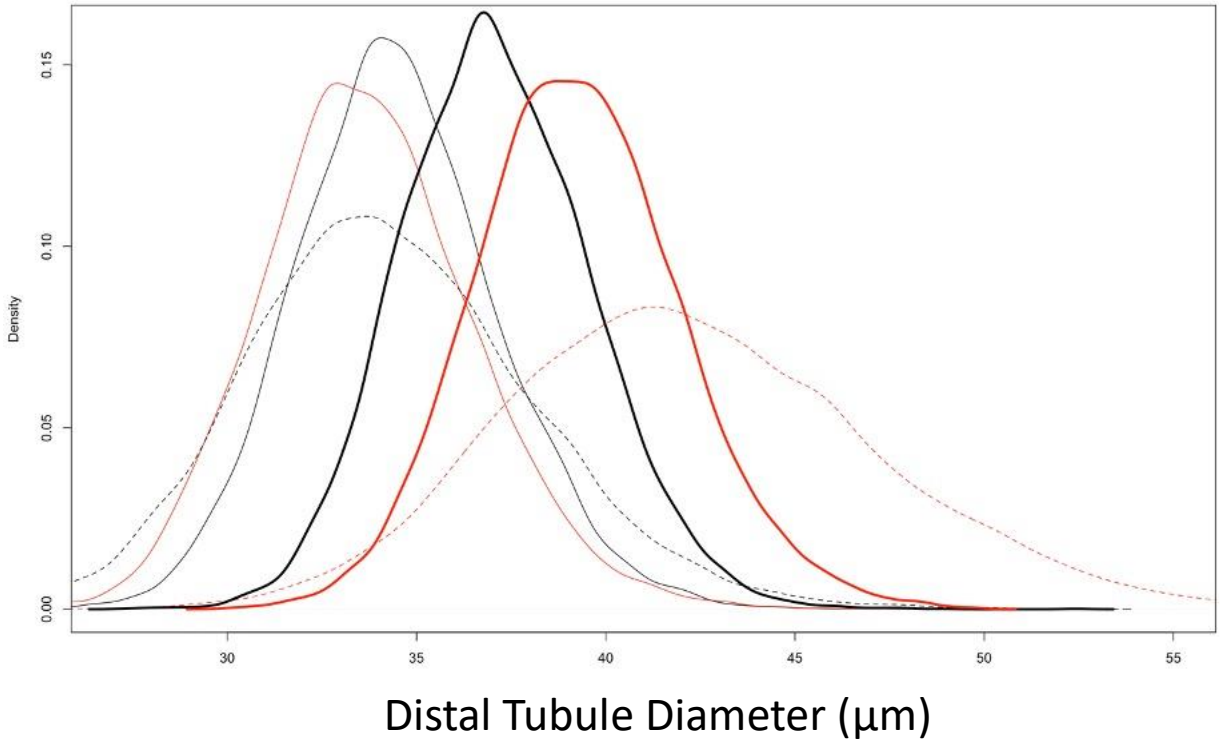

Figure 20. Bayesian posterior distributions of distal tubule diameter (kidney) for small (total length = 48cm) Mississippi (MS) male American alligators. Black = inland animals. Red = coastal animals. Thin solid line = 0 ppt. Dotted line = 10 ppt. Thick solid line = 20 ppt. X-axis represents distal tubule diameter (µm).

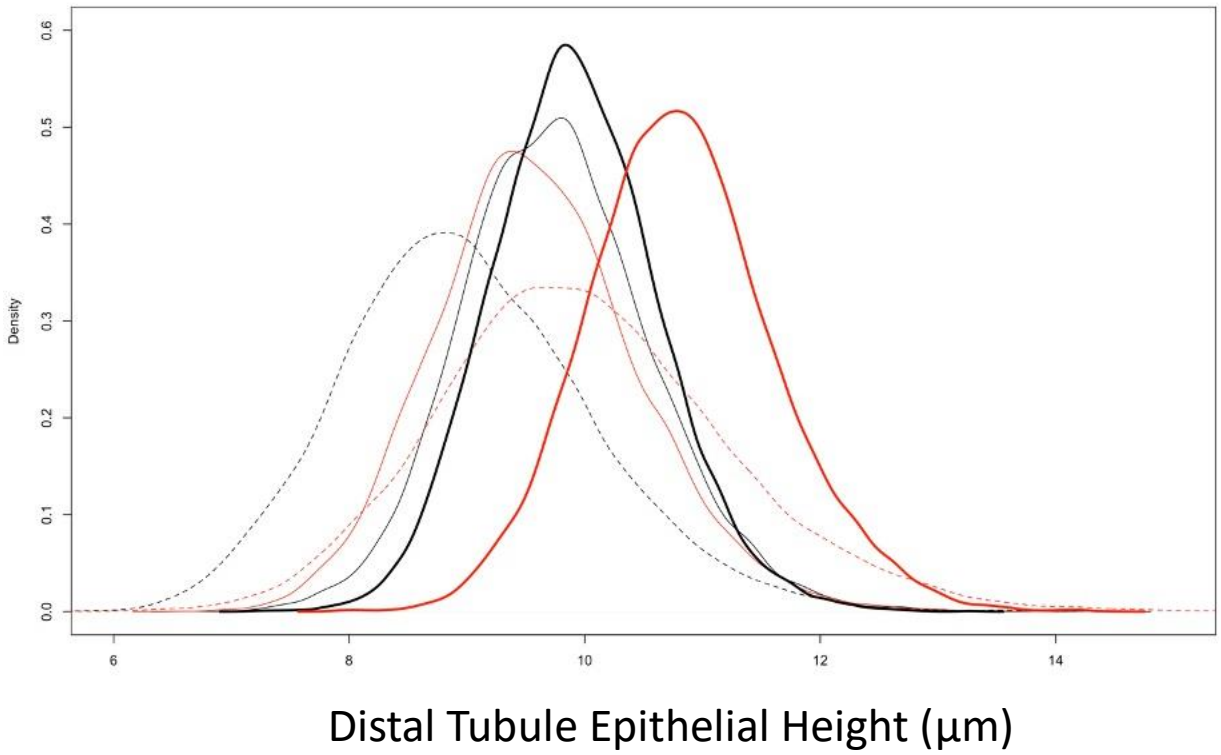

Figure 21. Bayesian posterior distributions of distal tubule epithelial height (kidney) for small (total length = 48cm) Mississippi (MS) male American alligators. Black = inland animals. Red = coastal animals. Thin solid line = 0 ppt. Dotted line = 10 ppt. Thick solid line = 20 ppt. X-axis represents distal tubule epithelial height (µm).

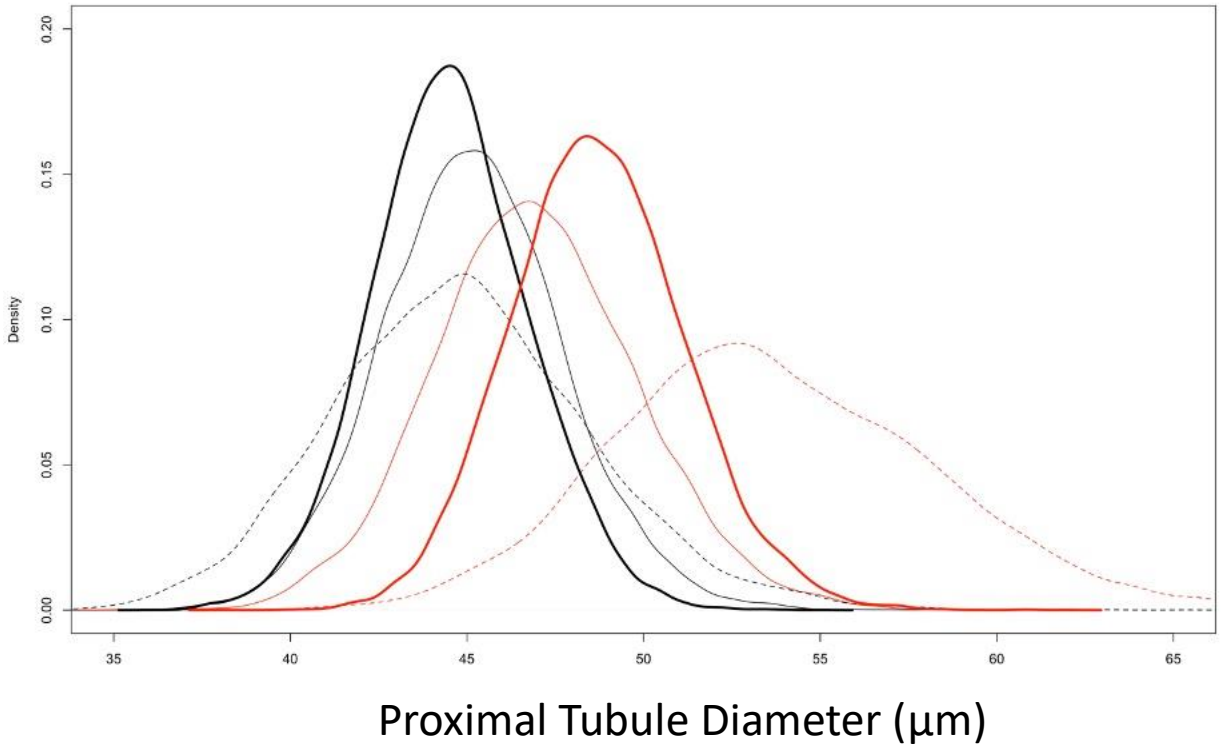

Figure 22. Bayesian posterior distributions of proximal tubule diameter (kidney) for small (total length = 48cm) Mississippi (MS) male American alligators. Black = inland animals. Red = coastal animals. Thin solid line = 0 ppt. Dotted line = 10 ppt. Thick solid line = 20 ppt. X-axis represents proximal tubule diameter (µm).

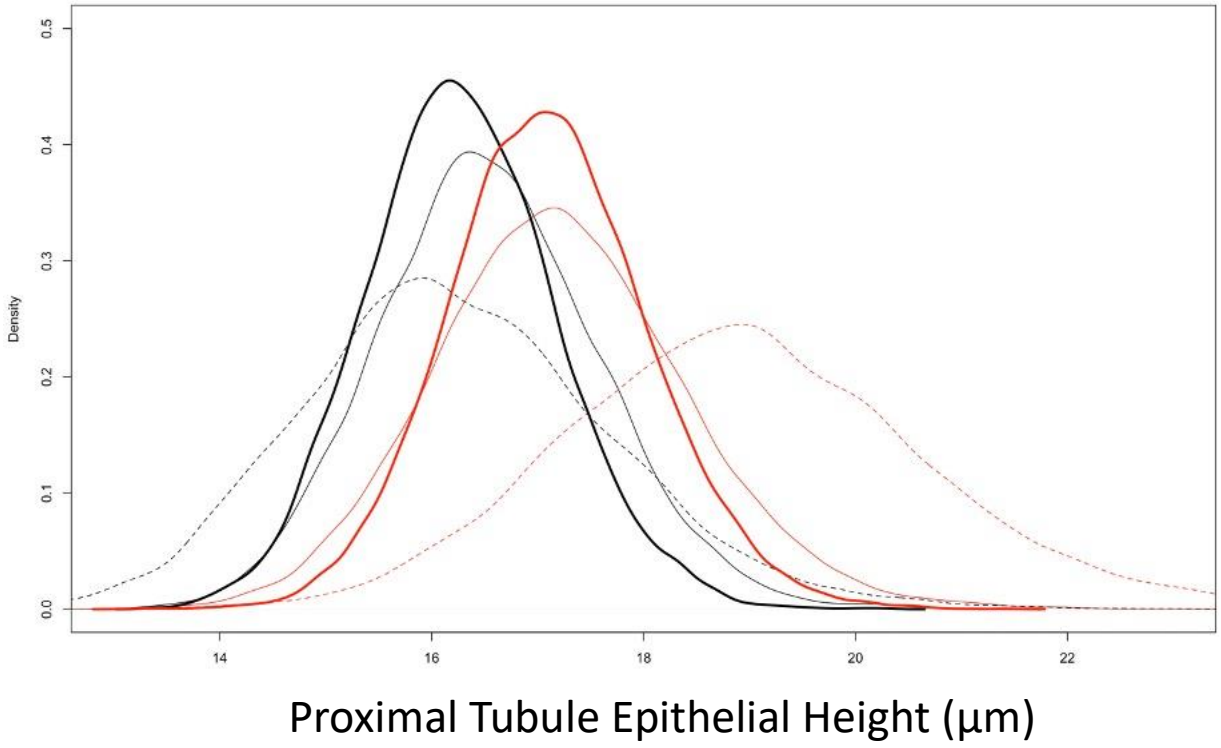

Figure 23. Bayesian posterior distributions of proximal tubule epithelial height (kidney) for small (total length = 48cm) Mississippi (MS) male American alligators. Black = inland animals. Red = coastal animals. Thin solid line = 0 ppt. Dotted line = 10 ppt. Thick solid line = 20 ppt. X-axis represents proximal tubule epithelial height (µm).

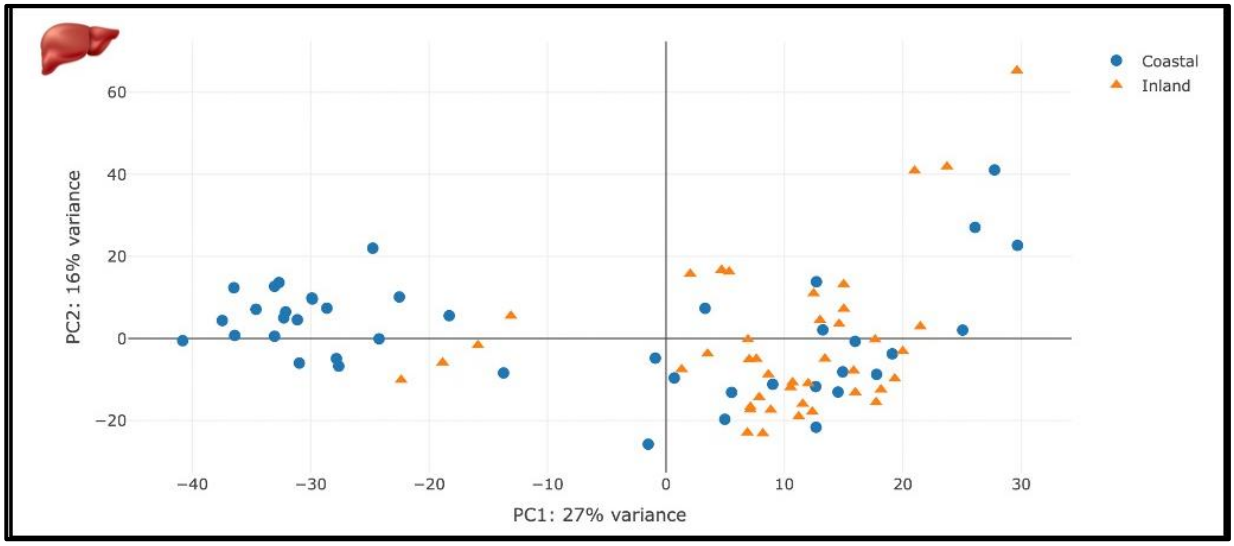

Figure 24. PCA of general expression patterns across habitats from transcripts in the liver of American alligators (*Alligator mississippiensis*). Coastal samples are represented by blue circles and inland samples are represented by orange triangles. Notice the cluster of coastal samples on the lefthand side of the plot and the cluster of inland samples on the righthand side of the plot.

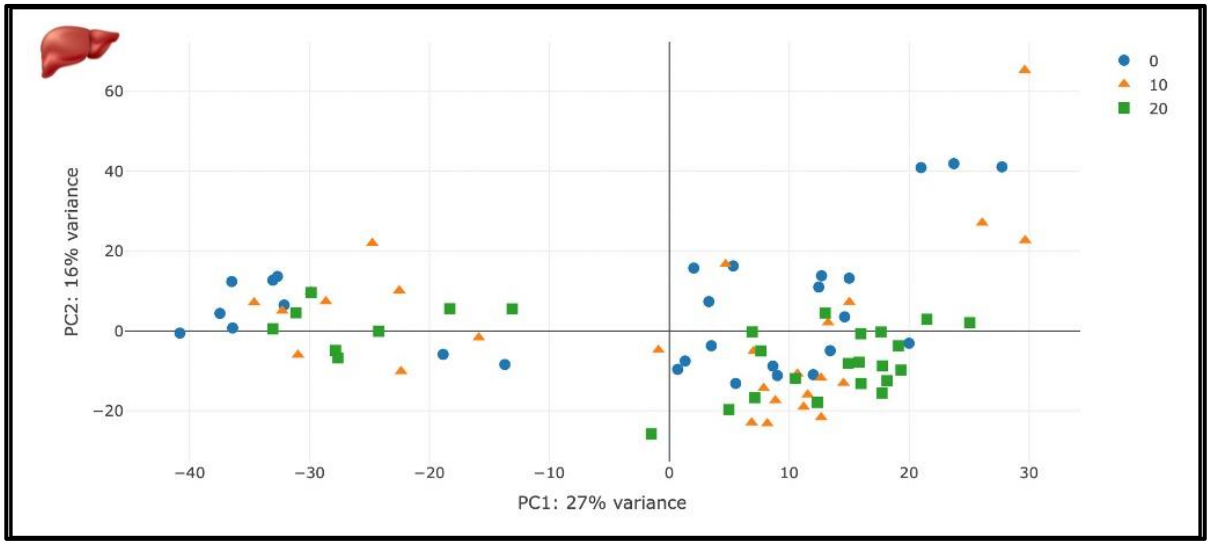

Figure 25. PCA of general expression patterns across salinities from transcripts in the liver of American alligators (*Alligator mississippiensis*). 0 ppt samples are represented by blue circles, 10 ppt samples are represented by orange triangles, and 20 ppt samples are represented by green squares. There is not a clear pattern of clustering regarding salinity.

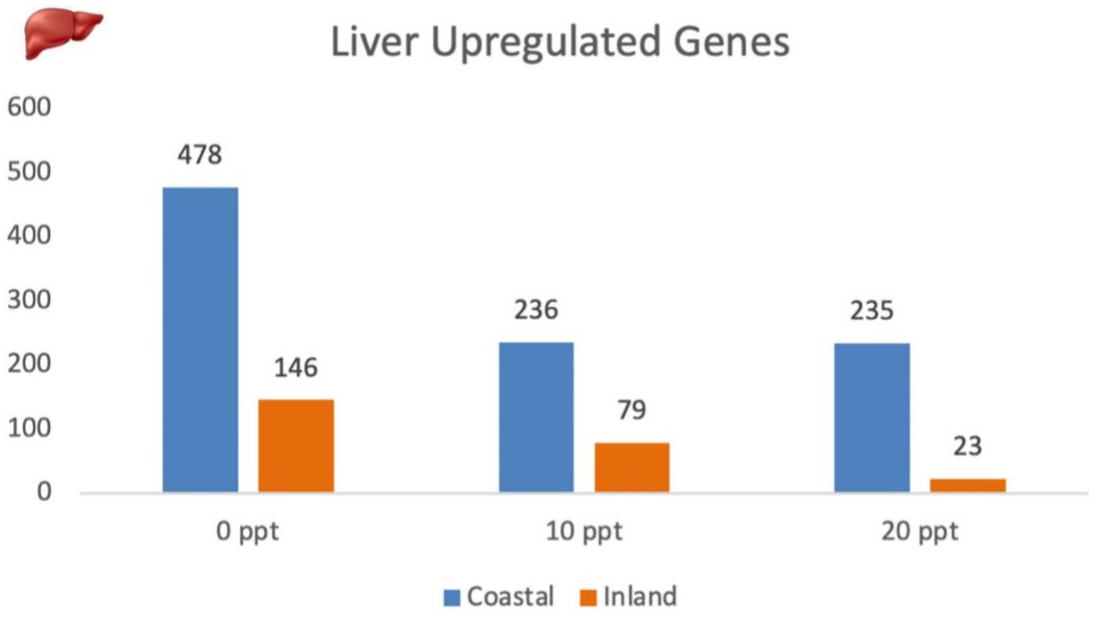

Figure 26. Barplot of number of upregulated genes in the liver of American alligators (*Alligator mississippiensis*) exposed to three different salinities (0, 10, and 20 ppt) for two weeks. Coastal alligators have more upregulated genes than inland alligators in each of the three salinities.

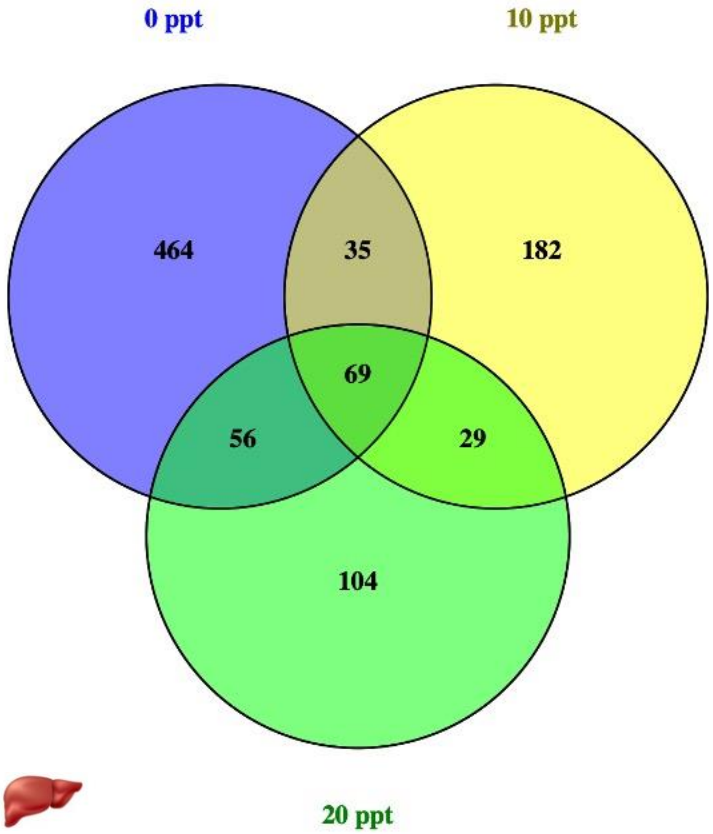

Figure 27. Venn diagram showing the number of differentially expressed genes (DEGs) in the liver transcriptome of American alligators (*Alligator mississippiensis*) shared and not shared across salinities (0, 10, and 20 ppt). Total number of DEGs = 939.

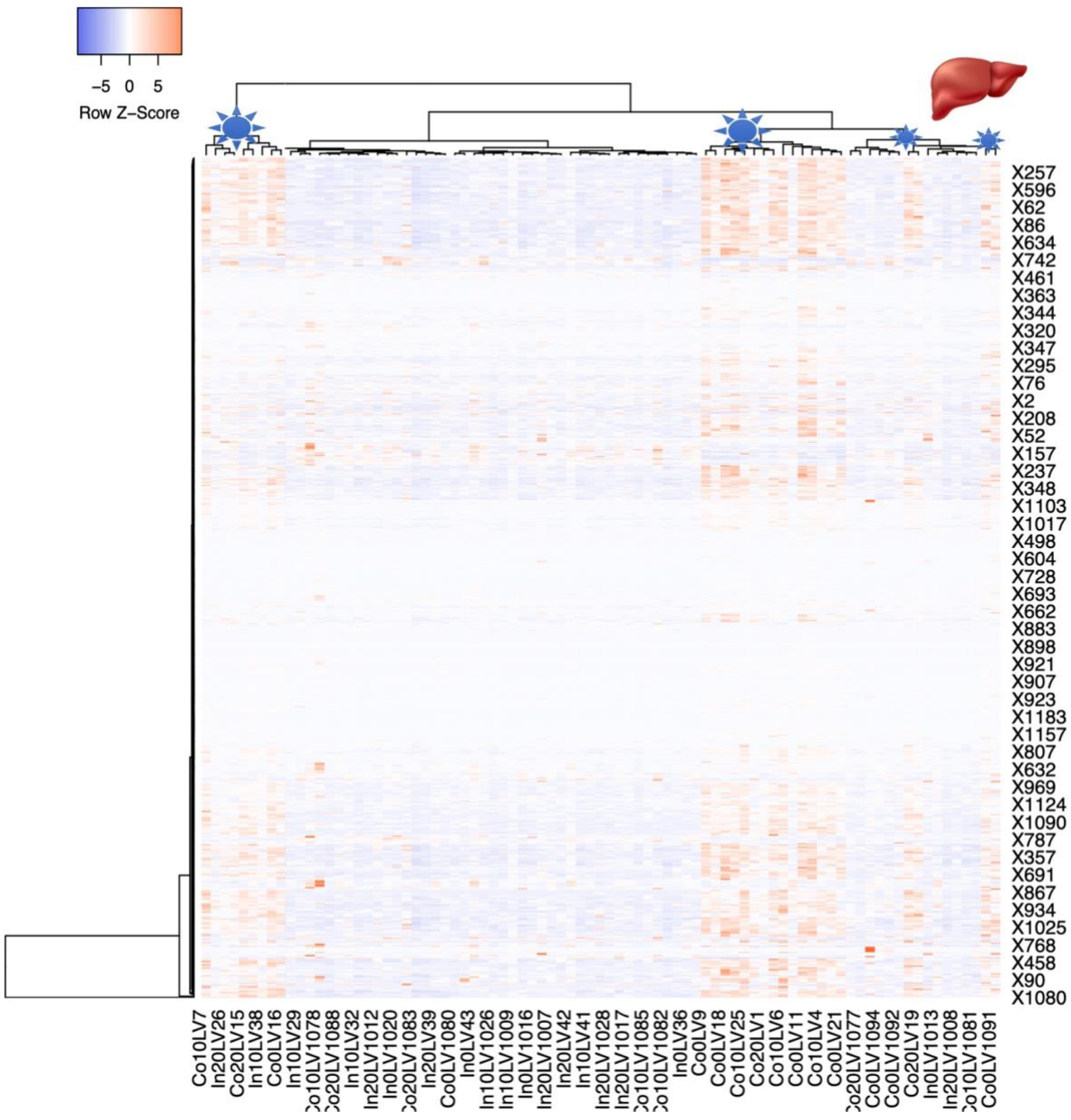

Figure 28. Dendrogram showing similar expression profiles across differentially expressed genes (DEGs) from liver transcriptomes of American alligators (*Alligator mississippiensis*) kept in varying salinities for two weeks. Y-axis is list of genes. X-axis is list of samples. Red = gene is upregulated. Blue = gene is downregulated. Dendrogram at top groups samples by similarity of expression profile. Stars indicate monophyletic groups of upregulation.

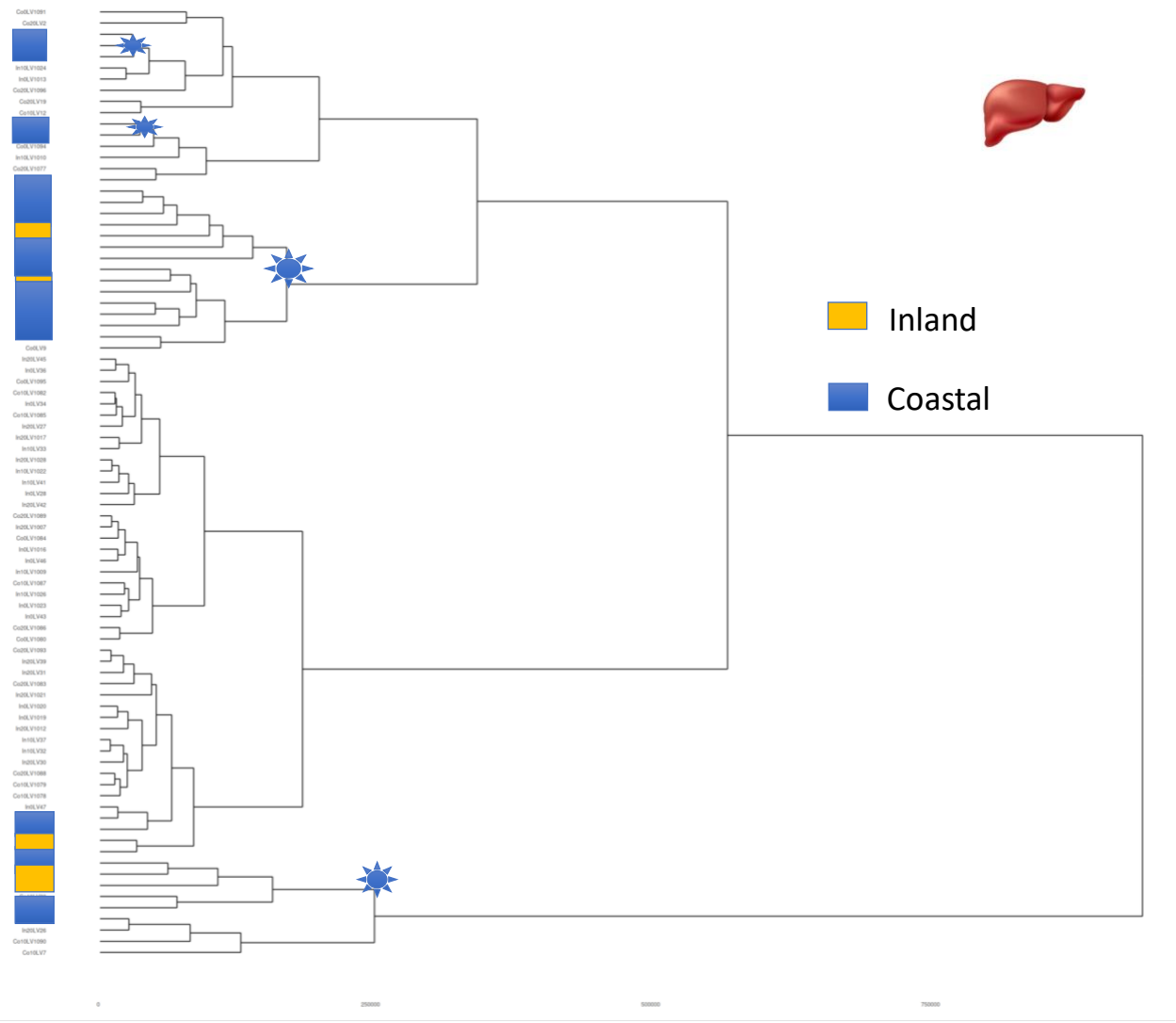

Figure 29. Dendrogram showing similar expression profiles across differentially expressed genes (DEGs) from liver transcriptomes of American alligators (*Alligator mississippiensis*) kept in varying salinities for two weeks. Y-axis is list of genes. Blue boxes are coastal alligators, while yellow boxes are inland alligators. While we see clear clustering of similar expression patterns, they don't fall into clear clades in terms of coastal and inland.

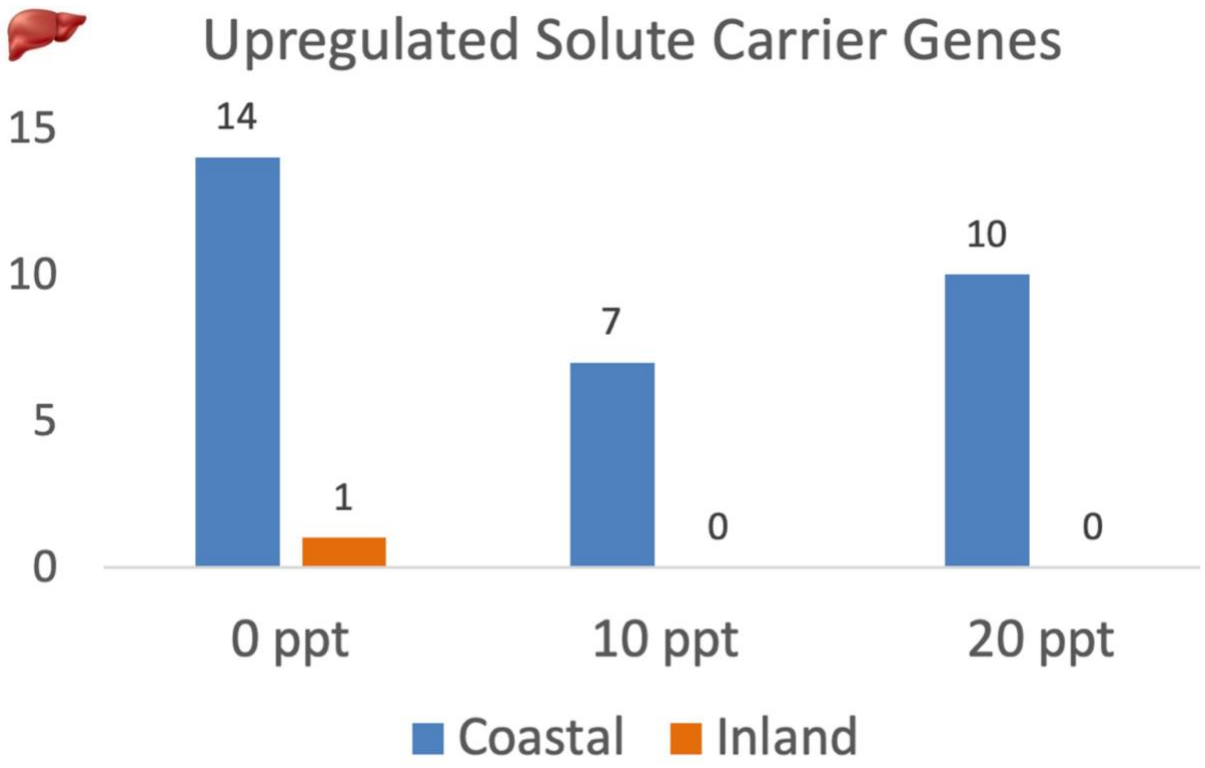

Figure 30. Barplot of number of upregulated solute carrier genes in the liver of American alligators (*Alligator mississippiensis*) exposed to three different salinities (0, 10, and 20 ppt) for two weeks. Coastal alligators have more upregulated solute carrier genes than inland alligators in each of the three salinities.

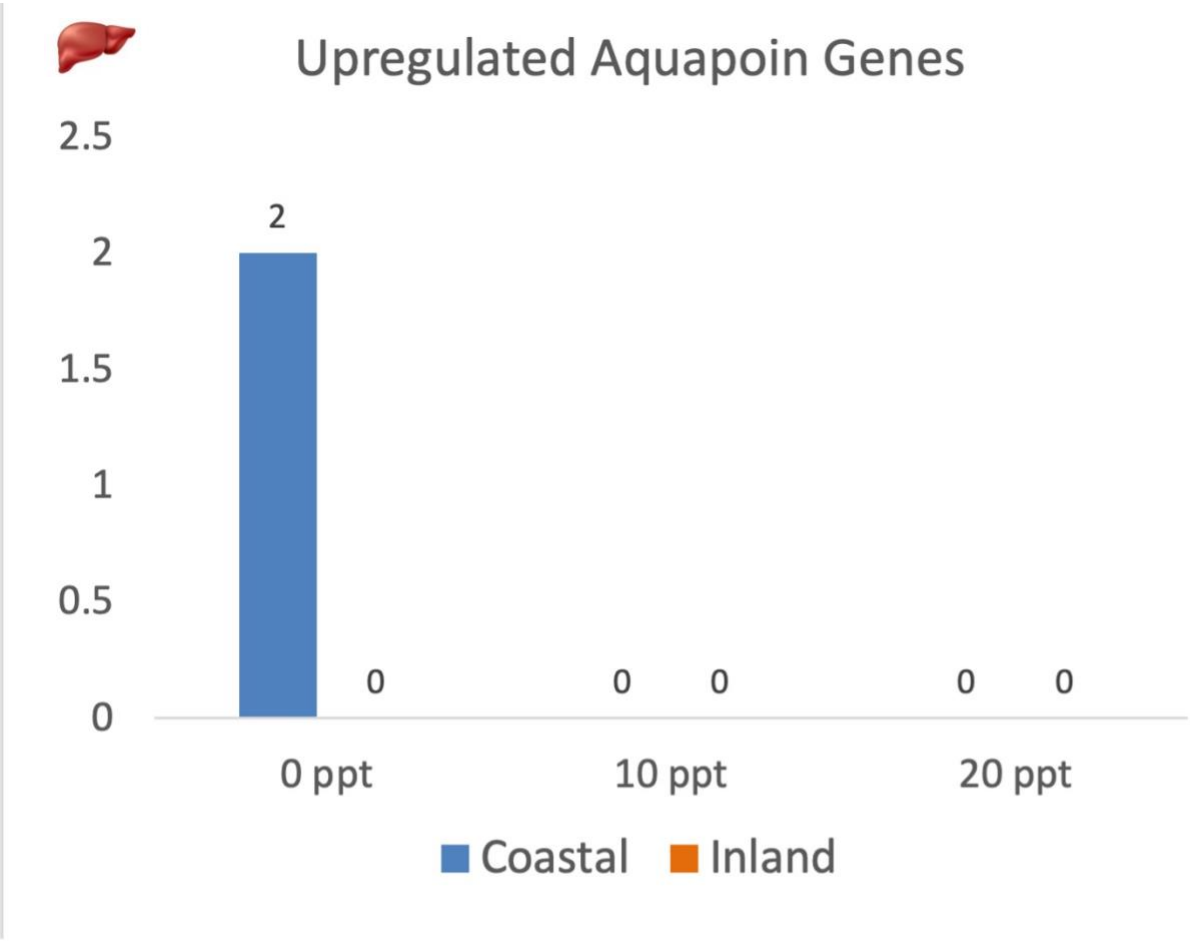

Figure 31. Barplot of number of upregulated aquaporin genes in the liver of American alligators (*Alligator mississippiensis*) exposed to three different salinities (0, 10, and 20 ppt) for two weeks. Coastal alligators have more upregulated aquaporin genes than inland alligators at 0 ppt salinity.

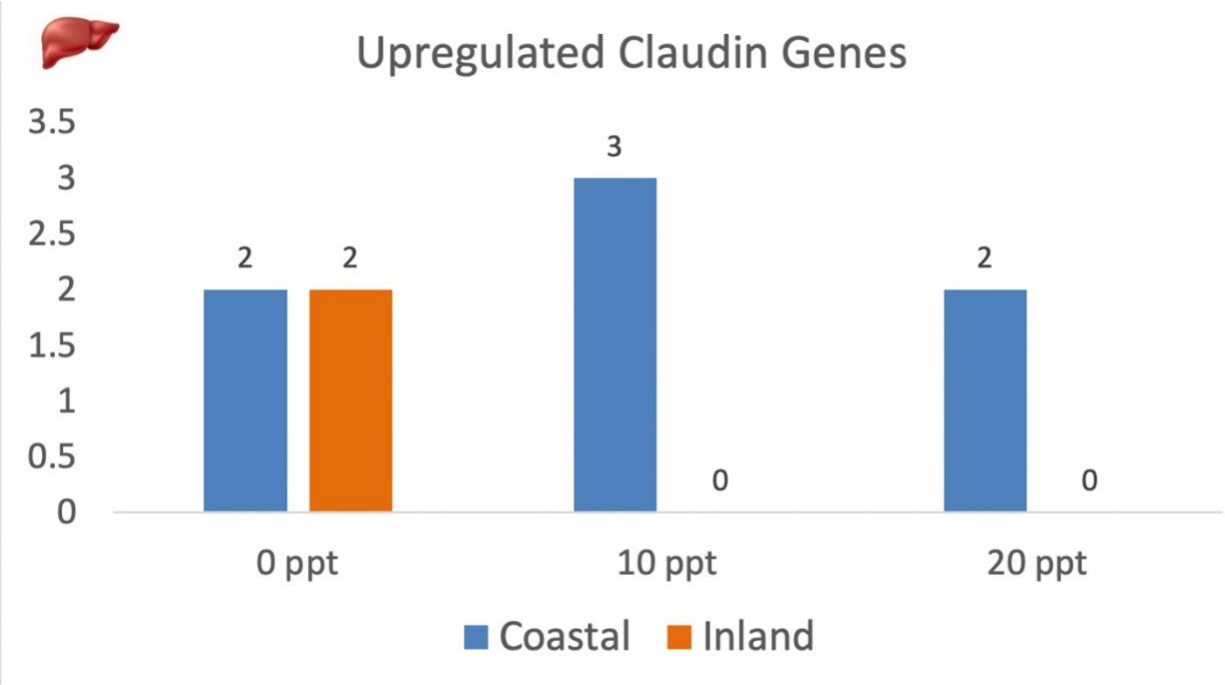

Figure 32. Barplot of number of upregulated claudin genes in the liver of American alligators (*Alligator mississippiensis*) exposed to three different salinities (0, 10, and 20 ppt) for two weeks. Coastal and inland alligators upregulated the same amount of claudin genes at 0 ppt, while coastal alligators upregulated more claudin genes at 10 ppt and 20 ppt salinity.

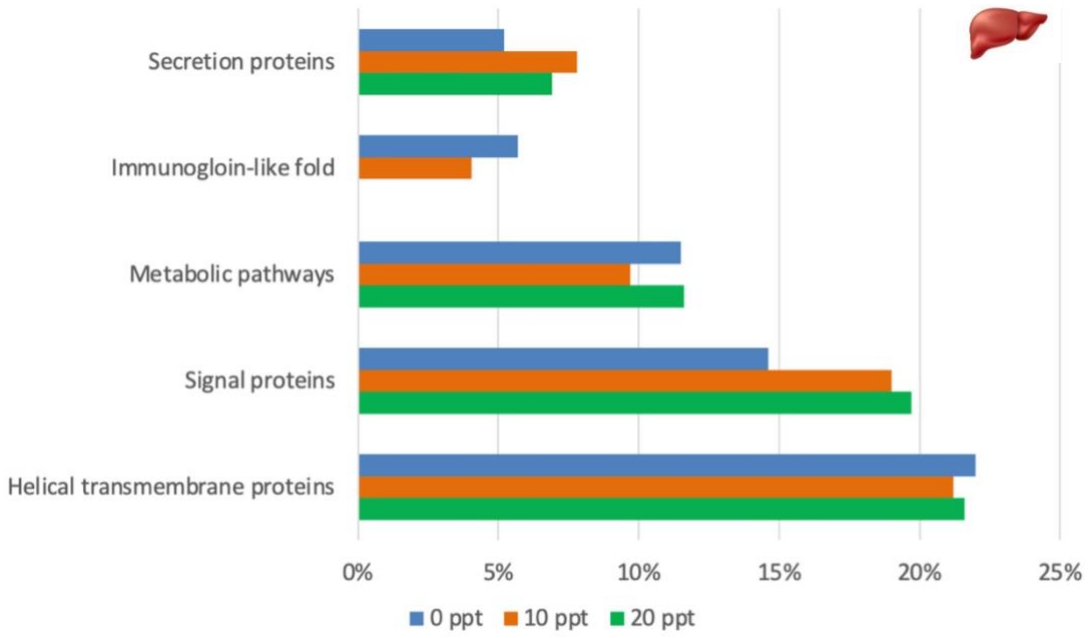

Figure 33. Barplot of top gene ontology (GO) terms for differentially expressed genes (DEGs) between coastal and inland liver transcriptomes of American alligators (*Alligator mississippiensis*) exposed to three different salinities (0, 10, and 20 ppt) for two weeks.

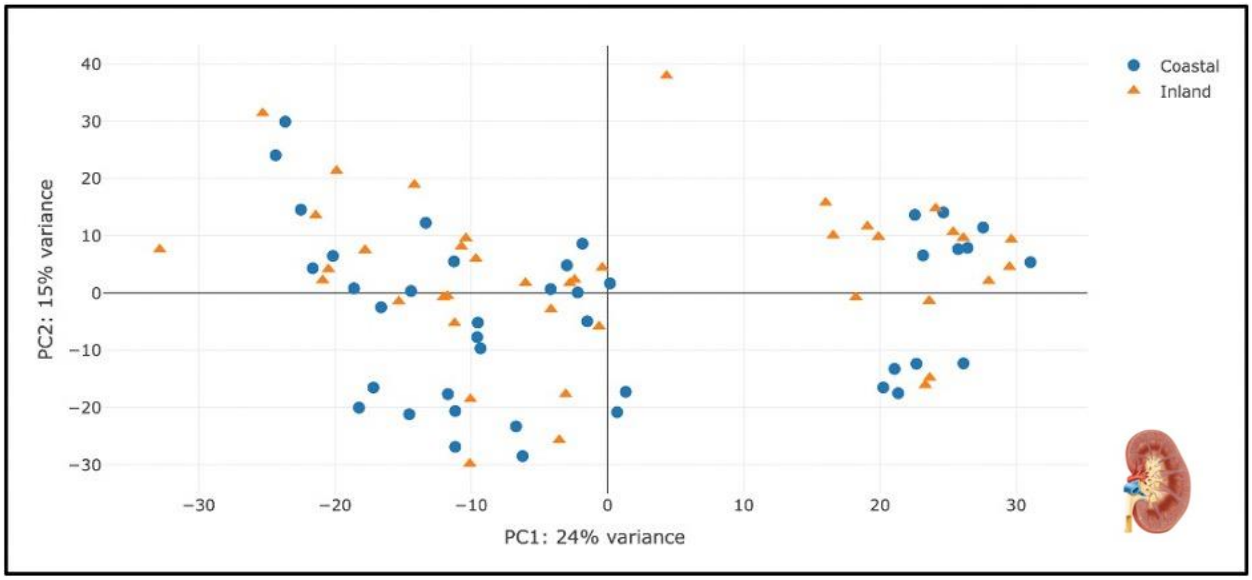

Figure 34. PCA of general expression patterns across habitats from transcripts in the kidney of American alligators (*Alligator mississippiensis*) exposed to three different salinities (0, 10, and 20 ppt) for two weeks. Coastal samples are represented by blue circles and inland samples are represented by orange triangles. There is not a clear pattern of clustering regarding habitat.

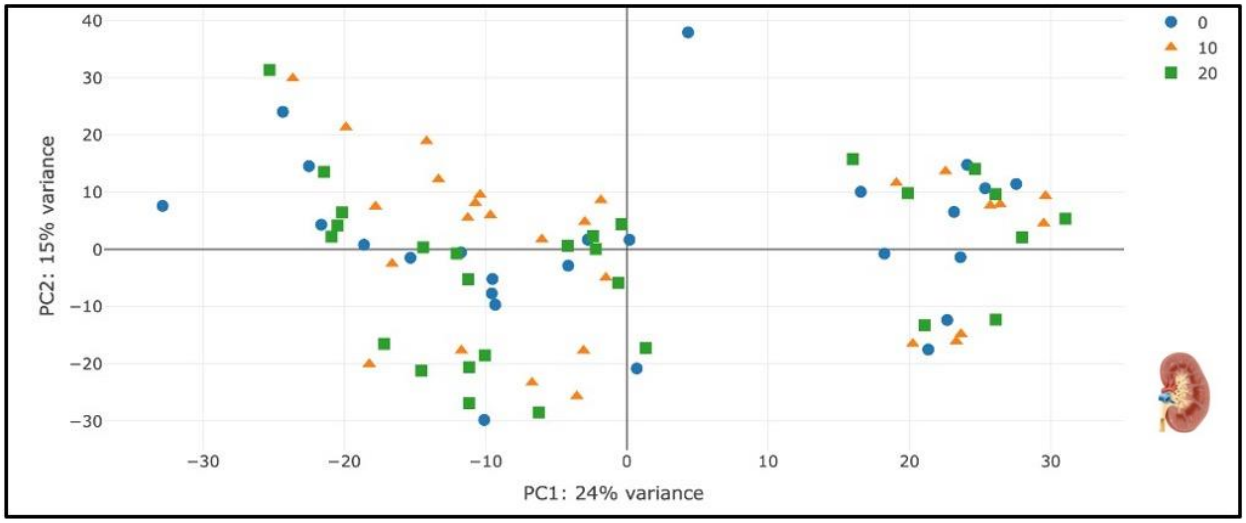

Figure 35. PCA of general expression patterns across salinities from transcripts in the kidney of American alligators (*Alligator mississippiensis*) exposed to three different salinities (0, 10, and 20 ppt) for two weeks. 0 ppt samples are represented by blue circles, 10 ppt samples are represented by orange triangles, and 20 ppt samples are represented by green squares. There is not a clear pattern of clustering regarding salinity.

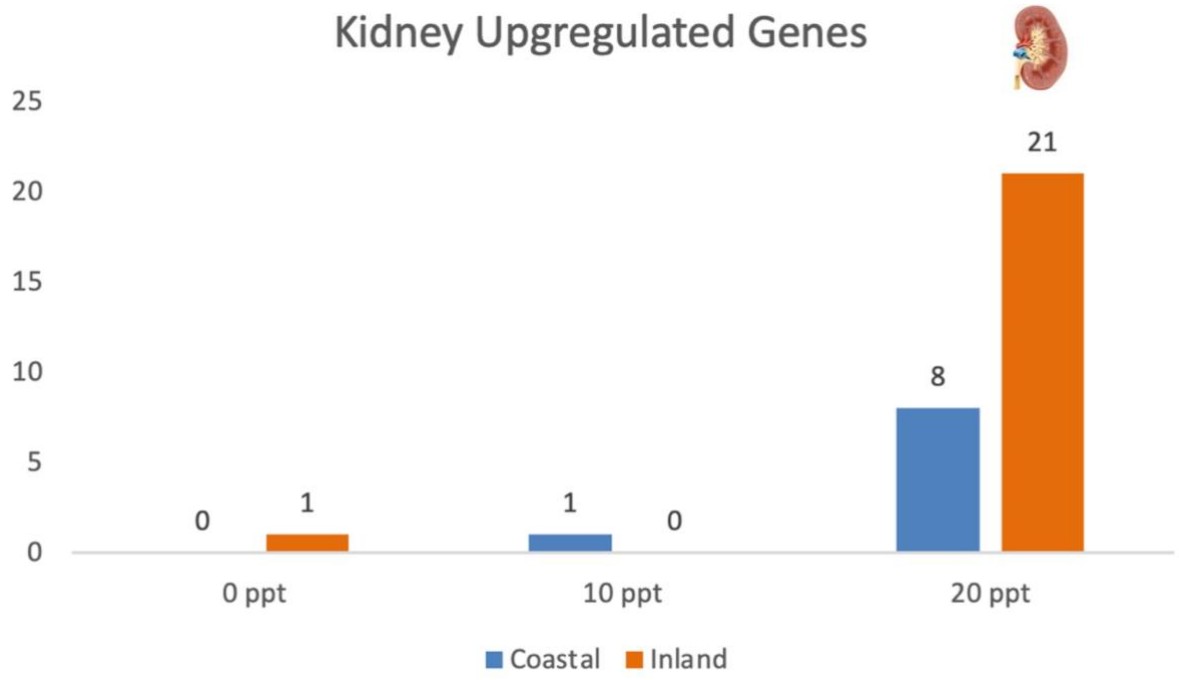

Figure 36. Barplot of number of upregulated genes in the kidney of American alligators (*Alligator mississippiensis*) exposed to three different salinities (0, 10, and 20 ppt) for two weeks. Coastal and inland alligators upregulated the same amount of genes at 0 ppt and 10 ppt, while coastal alligators upregulated more genes at 20 ppt salinity.

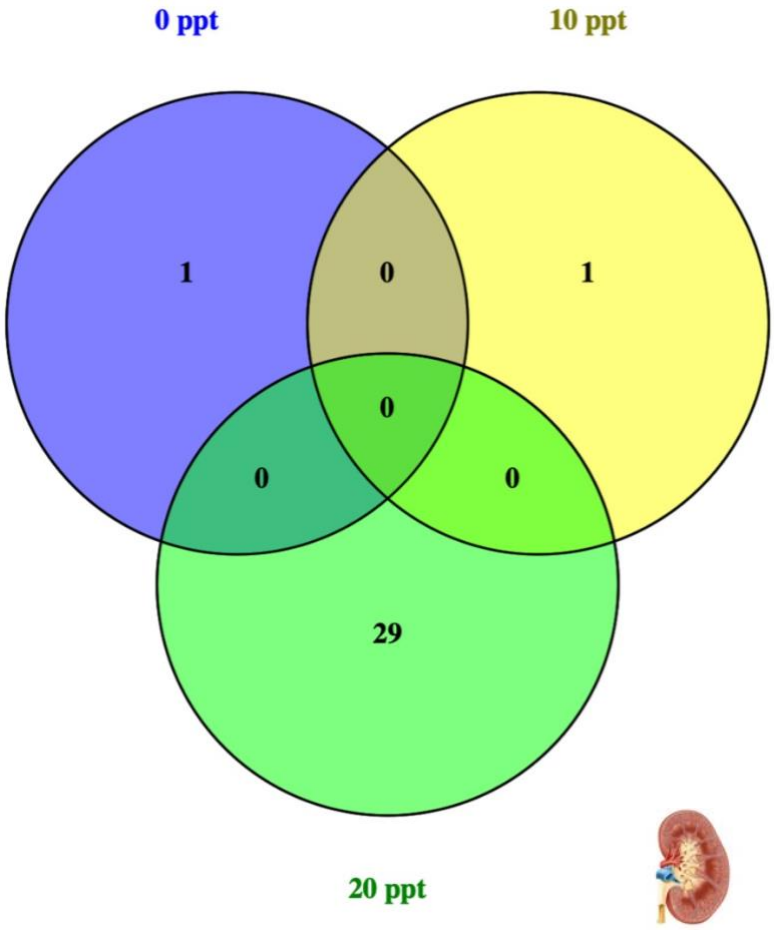

Figure 37. Venn diagram showing the number of differentially expressed genes (DEGs) in the kidney transcriptome of American alligators (*Alligator mississippiensis*) shared and not shared across salinities (0, 10, and 20 ppt). Total number of DEGs = 31.

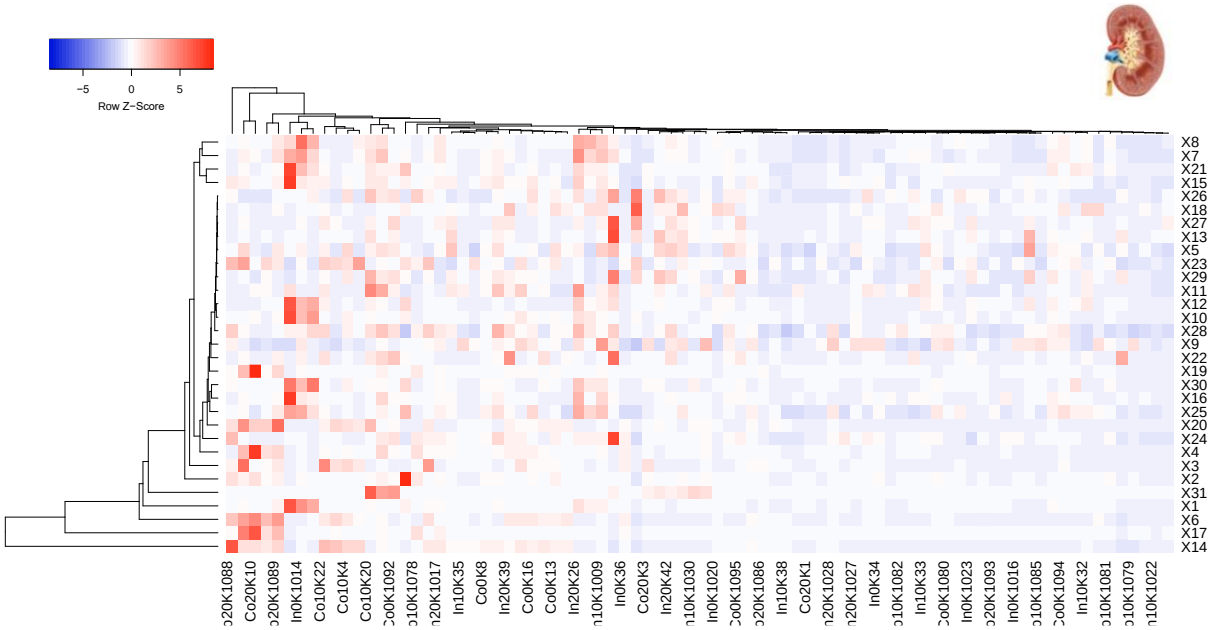

Figure 38. Dendrogram showing similar expression profiles across differentially expressed genes (DEGs) from kidney transcriptomes of American alligators (*Alligator mississippiensis*) kept in varying salinities for two weeks. Y-axis is list of genes. X-axis is list of samples. Red = gene is upregulated. Blue = gene is downregulated. There is no strong pattern grouping samples together.

APPENDIX B: TABLES

Table 1. Populations used with general genetic statistics and location.

Population #	Population Name	Sample Size	Nucleotide Diversity (π)	Observed Heterozygosity	Expected Heterozygosity	Longitude	Latitude
1	Southeastern Texas	9	0.10796	0.09678	0.09768	-96.837542	28.659104
2	Chambers County, Texas	4	0.1363	0.12574	0.10991	-94.53477	29.735733
3	Tyler County, Texas	6	0.14008	0.11184	0.12503	-94.197008	30.864168
4	Oklahoma and southwestern Arkansas	6	0.15449	0.1104	0.13611	-94.115433	33.6362
5	Natchitoches Parish, Louisiana	2	0.15124	0.13233	0.10943	-93.112481	31.761807
6	Rockefeller National Wildlife Refuge, Louisiana	8	0.15881	0.16196	0.14864	-92.819198	29.729936
7	Cypress Bayo, Arkansas	1	0.11914	0.11914	0.05957	-91.886995	35.025185
8	Lodge Corner, Arkansas	4	0.082573	0.06879	0.05658	-91.543105	34.291824

Population #	Population Name	Sample Size	Nucleotide Diversity (π)	Observed Heterozygosity	Expected Heterozygosity	Longitude	Latitude
9	Pendleton, Arkansas	6	0.20373	0.15601	0.15008	-91.38258	33.980313
10	Northeast Louisiana	3	0.16328	0.14073	0.12417	-91.667679	32.228075
11	Central Louisiana	2	0.15567	0.1415	0.09323	-91.886846	31.515046
12	South Louisiana	8	0.17178	0.13593	0.15881	-91.423166	30.238864
13	Adams County, Mississippi	3	0.16539	0.15161	0.13591	-91.517689	31.216461
14	Lake Bruin, Louisiana	12	0.158	0.14912	0.15044	-91.217849	31.95229
15	Warren County, Mississippi	9	0.16852	0.15339	0.15789	-90.839026	32.46702
16	Northwest Mississippi	6	0.17081	0.17743	0.15589	-90.277567	33.2638
17	Ross Barnett Reservoir, Mississippi	8	0.18731	0.17102	0.17506	-90.013887	32.468088
18	Peral River, Mississippi	2	0.18321	0.1423	0.13641	-90.084229	31.609504

Population #	Population Name	Sample Size	Nucleotide Diversity (π)	Observed Heterozygosity	Expected Heterozygosity	Longitude	Latitude
19	Plaquemine Parish, Louisiana	12	0.15975	0.11603	0.13526	-89.939472	29.755816
20	Tombigbee River, Mississippi	3	0.1884	0.1685	0.1553	-88.46508	33.496508
21	Coastal Mississippi	11	0.214	0.19988	0.20374	-89.096797	30.394308
22	Fort Morgan, Alabama	8	0.21327	0.20413	0.19954	-88.024135	30.225934
23	Mobile Bay, Alabama	4	0.21292	0.20546	0.18505	-87.926638	30.634074
24	Alabama River, Alabama	6	0.20736	0.16074	0.18859	-87.329756	32.137554
25	North Florida	8	0.039535	0.03953	0.01977	-84.228563	30.077011
26	Southwest Georgia	5	0.25479	0.25365	0.22764	-83.791576	31.386578
27	Lee County, Florida	10	0.22498	0.19845	0.2123	-81.864258	26.660205
28	Everglades National Park, Florida	4	0.2186	0.20853	0.19014	-80.682192	25.553731

Population #	Population Name	Sample Size	Nucleotide Diversity (π)	Observed Heterozygosity	Expected Heterozygosity	Longitude	Latitude
29	Lake Okeechobee, Florida	7	0.23651	0.2123	0.21846	-80.897353	26.940693
30	Loxahatchee Wildlife Reserve, Florida	10	0.22817	0.21354	0.21635	-80.216209	26.501547
31	Welaka, Florida	7	0.24076	0.22108	0.22264	-81.663523	29.483999
32	Palm Coast, Florida	9	0.23059	0.21782	0.21719	-81.229316	29.538453
33	Altamaha River, Georgia	5	0.24027	0.23184	0.21511	-81.851118	31.667895
34	Northeast Georgia	7	0.24965	0.21244	0.23091	-82.088633	33.374133
35	Savannah River, Georgia	4	0.24289	0.22834	0.21101	-81.13412	32.323344
36	Southern Coastal Region, South Carolina	7	0.18722	0.13119	0.16006	-80.692084	32.778625

Population #	Population Name	Sample Size	Nucleotide Diversity (π)	Observed Heterozygosity	Expected Heterozygosity	Longitude	Latitude
37	Central South Carolina	3	0.17832	0.15051	0.14103	-80.114567	33.3037
38	Georgetown County, South Carolina	2	0.15387	0.11816	0.11327	-79.266221	33.356305
39	Lake Waccamaw, North Carolina	8	0.17238	0.15493	0.15932	-78.55198	34.30655
40	Coastal North Carolina	8	0.1714	0.13801	0.14691	-77.994755	33.996358

Table 2. Number of SNPs and individuals per STRUCTURE dataset.

Populations run through STRUCTURE	**Number of SNPs**	**Number of individuals**
All 40 range-wide	4,263	241
Western (OK, AR, TX, LA, MS, AL)	15,370	108
West of MS River	840	78
MS + AL	1,645	36
Eastern (FL, GA, SC, NC)	21,287	80
Florida	53,571	64
Peninsular Florida	54,459	52
Georgia + Carolinas	90,920	35
Alabama + North Florida + Georgia	71,984	51
Alabama + North Florida + Peninsular Florida	52,312	82
Georgia + North Florida + Peninsular Florida	68,891	85

Table 3. AIC values for the east coast dataset (Florida-Georgia-Carolinas). Boxplot of 100 likelihoods found Models O and R were equally likely. Based on AIC R (highlighted) is the superior model.

Model	delta Likelihood	AIC
A	5751.11	65,874
B	10375.731	87,171
C	10358	87,090
D	10647	88,422
E	5545	64,927
F	5364	64,093
G	5125	62,996
H	5133	63,033
J	5311	63,852
K	5338	63,979
L	10301	86,831
M	10419	87,374
O	5115.711	62,949
P	5119.433	62,967
Q	5117.609	62,958
R	5114.408	62,943

Table 4. AIC values for range-wide dataset (West-Alabama-Florida). Boxplot of 100 likelihoods found Models E and R were equally likely. Based on AIC model R (highlighted) is the superior model.

Model	delta Likelihood	AIC
A	99426.36	616,851
B	118200.281	703,308
C	117391.316	699,583
D	118866.533	706,376
E	91404.981	579,913
F	91392.33	579,855
G	90521.972	575,847
H	90661.97	576,491
J	91595.529	580,793
K	90276.503	574,718
L	117574.235	700,425
M	118181.752	703,223
O	91061.808	578,333
P	91062.935	578,338
Q	91019.286	578,137
R	91213.54	579,031

Table 5. Expected heterozygosity for five crocodilian species, including *Alligator mississippiensis* (this study). Our average heterozygosity is comparable to that found in other crocodilians.

Species	**Expected Heterozygosity**	**Reference**
Crocodylus johnstoni	0.077–0.084	(Cao et al. 2020)
Crocodylus porosus	avg. = 0.25	(Fukuda et al. 2022)
C. moreletti	avg. = 0.13	(Pacheco-Sierra et al. 2018)
C. moreletti	avg. = 0.19	(António De Lemos Barão Da Nóbrega 2021)
A. mississippiensis	0.03953 - 0.253 [avg. = 0.16]	This study

Table 6. Sample sizes per treatment and location for histology measurements from kidney (n = 46) and liver (n = 46) tissue from American alligators.

	0 ppt	10 ppt	20 ppt
Inland LA	5	2	5
Coastal LA	3	4	6
Inland MS	2	2	5
Coastal MS	2	4	4

Table 7. WAIC models run for all 12 responses measured. Model #4 was the best model across all 12 characters measured.

1.Response ~ intercept + habitat + salinity_ten + salinity_twenty + (1|individual)
2. Response ~ intercept + habitat + salinity_ten + salinity_twenty + (1|individual) + habitat:salinity_ten + habitat:salinity_twenty
3. Response ~ intercept + habitat + salinity_ten + salinity_twenty + (1|individual) + sex + state + length
4. Response ~ intercept + habitat + salinity_ten + salinity_twenty + (1|individual) + sex + state + length + habitat:salinity_ten + habitat:salinity_twenty
5. Response ~ intercept + habitat + salinity_ten + salinity_twenty + (1|individual) + sex + state + length + habitat:salinity_ten + habitat:salinity_twenty + state:salinity_ten + state:salinity_twenty

Table 8. Coefficients of fixed factors from best model for behavior (binary, on stand or in the water) and plasma sodium (mmol/L) measurements. For plasma sodium, 10 ppt and 20 ppt coefficients were not calculated because salinity was modeled as a continuous variable. Reference Level = Inland, 0 ppt, male, Louisiana.

	On Stand	Plasma Sodium
Coastal	-1.161	0.019
10 ppt	-0.695	-
20 ppt	-0.32	-
Female	-	0.0115
Total Length	-0.015	-0.0021
Mississippi	-0.072	-0.0706
Coastal:10 ppt	0.541	-0.0333
Coastal:20 ppt	1.16	0.0233

Table 9. Coefficients of fixed factors from best model for liver histology measurements (µm). Reference Level = Inland, 0 ppt, male, Louisiana.

	Central Vein Diameter	Bile Duct Diameter	Hepatic Artery Diameter	Portal Vein Diameter
Coastal	0.109	-0.24	-0.202	-0.298
10 ppt	-0.004	-0.378	-0.112	-0.115
20 ppt	-0.022	-0.23	-0.138	-0.22
Female	0.06	0.165	0.202	0.322
Total Length	-0.005	0.008	0.009	0.008
Mississippi	-0.146	0.299	0.217	0.22
Coastal:10 ppt	-0.074	0.502	0.258	0.175
Coastal:20 ppt	-0.253	0.193	0.031	0.064

Table 10. Coefficients of fixed factors from best model for kidney histology measurements (µm). Reference Level = Inland, 0 ppt, male, Louisiana.

	Renal Corpuscle Diameter	Glomeruli Diameter	Distal Tubule Diameter	Distal Tubule Epithelial Height	Proximal Tubule Diameter	Proximal Tubule Epithelial Height
Coastal	0.056	0.071	-0.023	-0.017	0.036	0.04
10 ppt	-0.062	-0.1	0.0	-0.089	-0.021	-0.023
20 ppt	0.063	0.076	0.075	0.016	-0.014	-0.15
Female	0.03	0.011	-0.112	-0.224	-0.047	-0.031
Total Length	0.013	0.013	0.002	-0.005	0.001	-0.002
Mississippi	0.218	0.232	0.069	-0.121	0.044	-0.029
Coastal:10 ppt	0.102	0.15	0.079	0.246	0.111	0.148
Coastal:20 ppt	0.012	0.001	0.209	0.103	0.053	0.01

Table 11. Number of reads per sample before and after mapping to the reference genome for both tissues. Liver = 84 samples. Kidney = 83 samples.

	BEFORE mapping to ref. genome - Liver	**AFTER mapping to ref. genome - Liver**
Average	23.2 million	8.63 million
Std. Dev.	2.81 million	1.47 million
Minimum	17.0 million	1.56 million
Maximum	29.0 million	11.0 million
	BEFORE mapping to ref. genome - Kidney	**AFTER mapping to ref. genome - Kidney**
Average	21.5 million	16.2 million
Std. Dev.	6.61 million	5.00 million
Minimum	9.14 million	7.02 million
Maximum	52.2 million	40.2 million

Table 12. Top Gene Ontology (GO) terms for differentially expressed genes (DEGs) in liver transcriptomes of coastal and inland populations of American alligators. Alligators were exposed to three different salinities (0, 10 and 20 ppt).

Top GO Terms	0 ppt	10 ppt	20 ppt
1	helical transmembrane proteins (22%)	helical transmembrane proteins (21.2%)	helical transmembrane proteins (21.6%)
2	signal (14.6%)	signal (19%)	signal (19.7%)
3	metabolic pathways (11.5%)	metabolic pathways (9.7%)	metabolic pathways (11.6%)
4	ATP binding (8.6%)	repeat (9.3%)	hydrolase (8.1%)
5	extracellular region (5.7%)	secreted (7.8%)	secreted (6.9%)
6	immunoglobulin-like fold (5.7%)	extracellular region (6.5%)	extracellular region (5.4%)
7	secreted (5.2%)	immunoglobulin-like fold (6.5%)	COMPBIAS:Pro residues (5.4%)
8	nucleotide-binding (3.9%)	DOMAIN:Ig-like (4.0%)	protease (3.9%)
9	ATP-binding (3.9%)	Immunoglobulin-like domain (4.05%)	extracellular space (2.7%)
10	oxidoreductase (3.5%)	epidermal growth factor-like domain (3.7%)	iron (2.7%)

Table 13. Top gene ontology (GO) terms for differentially expressed genes (DEGs) between coastal and inland kidney transcriptomes of American alligators (*Alligator mississippiensis*) exposed to three different salinities (0, 10, and 20 ppt) for two weeks. All GO terms were equally prevalent. Six of the top GO terms were part of GO terms we hypothesized to be significant: solute carriers and metabolism.

Top GO Terms
Hydrolase
mitochondrial ATP transmembrane transport
mitochondrial inner membrane
mitochondrial substrate/solute carrier
mitochondrial carrier protein
cytidine deaminase-like
DOMAIN: CMP/dCMP-type deaminase
CMP/dCMP deaminase, zinc binding
lipoprotein metabolic process
Apolipoprotein L
lipid binding
adenine nucleotide translocator 1