ECO-EVOLUTIONARY DYNAMICS OF DEVELOPMENTAL PLASTICITY IN THE AMERICAN ALLIGATOR, A SPECIES WITH TEMPERATURE-DEPENDENT SEX DETERMINATION

by

CHRISTOPHER RICHARD SMAGA

(Under the Direction of Benjamin B. Parrott)

ABSTRACT

Developmental plasticity, the irreversible modification of phenotypes in response to the developmental environment, carries critical implications for ecology, evolution, and conservation. In some cases, responses to the developmental environment can be beneficial, increasing organismal fitness. However, when developmental environments are altered by human activities, normal developmental trajectories can become disrupted, resulting in negative fitness outcomes. Despite their significance, however, the ecological and evolutionary dynamics of adaptive and disruptive plasticity in natural systems are not well understood. In this dissertation, I utilize unique attributes of the American alligator (*Alligator mississippiensis*) to integrate developmental plasticity into eco-evolutionary contexts, seeking to connect proximate mechanisms to ultimate outcomes in nature. In Chapter 2, I focus on the disruptive effects of environmental contaminants, testing how maternally deposited hormones and contaminants contribute to gonadal gene expression. I provide novel insight into how reproductive development is altered in contaminated populations and support a non-trivial role of maternally deposited hormones in driving offspring sexual development. Chapters 3-6 center on plasticity in response to incubation temperature and temperature-dependent sex determination (TSD). In Chapter 3, I assess the evolutionary potential for developmental plasticity to drive morphological differences across populations, revealing variable responses to incubation temperature across northern and southern population pairs that are associated with phenotypic divergence. Building off these findings, I then investigate divergence in the molecular pathways associated with TSD across those populations in Chapter 4. My results support unique evolutionary processes acting on TSD genes and highlight several candidate genes for its adaptive evolution. In Chapter 5, I identify time-dependent relationships between incubation temperature, hatchling phenotypes, and post-release traits that contribute to temperature-dependent survival outcomes supported to drive the adaptive evolution of TSD. Finally, in Chapter 6, I evaluate the use of blood gene expression patterns to non-lethally sex hatchling alligators. I demonstrate promising potential for using gene expression to predict natural sex ratios in TSD species, which will aide in incorporating TSD into ecological frameworks and assessing population responses to environmental change. When viewed altogether, my dissertation contributes novel mechanistic, ecological, and evolutionary insights into developmental plasticity as it occurs in nature.

INDEX WORDS: developmental plasticity, polyphenism, temperature-dependent sex determination

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DEDICATION

I dedicate this dissertation to my family: Janet, Jeff, Lauren, Megan and Olivia Smaga, and Abby, Luke, Anna and Brooke Reiser.

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CHAPTER 1

INTRODUCTION

Developmental plasticity, ecology, and evolution

Interactions between organisms and their environment form the foundation of ecology and evolutionary biology. Of particular interest is the environment experienced during embryonic development, which serves as a ubiquitous source of non-genetic phenotypic variation through a phenomenon known as developmental plasticity (West-Eberhard, 1989, 2003). Developmental plasticity can manifest as a continuous response to an environmental gradient or as discrete, alternative phenotypes patterned through a threshold response, often referred to as polyphenisms (Nijhout, 2003). Both carry critical implications for organismal fitness (DeWitt et al., 1998; Ghalambor et al., 2007). For instance, when developmental cues predict later life environments, plasticity can be adaptive by facilitating phenotype-environment matching (Brakefield & Reitsma, 1991; Sword et al., 2000). Contrarily, when developmental environments are altered by human activities, such as anthropogenic contaminant deposition, normal developmental trajectories can become disrupted, resulting in maladaptive outcomes (Gore et al., 2015; Guillette et al., 1995). Over the last several decades, the importance of developmental plasticity has been increasingly realized across ecological, evolutionary, and biomedical sciences, serving as a key driver of life history variation (Kohno et al., 2014; Miura, 2005), both facilitating and inhibiting adaptive evolution (Moczek, 2015; Moczek et al., 2011; Smallegange, 2022), and contributing to the onset of later-life disease (Barker, 2001; De Boo & Harding, 2006). Yet, despite these implications, several fundamental questions remain unanswered about the mechanisms that govern adaptive and disruptive responses to the developmental environment and how they operate in complex, natural systems. These include: 1) *How does exposure to contaminants disrupt reproductive health under ecologically relevant conditions?* 2) *How do responses to the developmental environment vary across ecological scales, and what are the underlying evolutionary processes?* 3) *How does developmental plasticity contribute to evolutionary novelty?* and 4) *What are the conservation implications of developmental plasticity in the face of rapid environmental change?* This dissertation uses a combination of field and lab-based experimental designs, molecular techniques, bioinformatic analyses, and ecological and evolutionary theory to address these questions. The primary goal is to connect the biological, molecular, and physiological mechanisms underlying embryo-environment interactions to ecological and evolutionary outcomes in nature. I accomplish this through two phenomena: the disruptive effects of anthropogenic contaminants on reproductive health and the adaptive role of incubation temperature in determining sex.

Anthropogenic contaminants and reproductive health

Around the globe, anthropogenic contaminants have crucially altered the natural environments in which organisms live, and exposure to these compounds is now commonplace for both wildlife and humans (Gore et al., 2015; Marlatt et al., 2022). Of particular concern are compounds that interact with endogenous hormone signaling, termed endocrine disrupting compounds (EDCs). Developmental exposure to EDCs is associated with a range of alterations to reproductive form and function, including altered timing of reproductive events, reduced fertility, and cancer in humans (Balabanič et al., 2011; Grindler et al., 2015; Messerlian et al., 2018), as well as reduced fertility, sex ratio biases, and population declines in animal populations (Guillette et al., 1996; Guillette & Moore, 2006; Marlatt et al., 2022). However, despite their widespread occurrence, our mechanistic understanding of contaminant-driven reproductive health outcomes is almost exclusively derived from experiments in laboratory models (e.g., cell cultures, rodents). Such studies point towards potential mechanisms and generate a strong hypothetical framework (Amir et al., 2021), but critically, they lack the necessary ecological and evolutionary contexts in which exposures typically occur. This raises a fundamental question: how do EDCs disrupt reproductive development and function in nature? Animal populations provide an exceptional opportunity to address this question, which I take advantage of in Chapter 2.

Incubation temperature and sex determination

Temperature-dependent sex determination (TSD) is a classic polyphenism in which thermal signals during a specific developmental window irreversibly determine sex (Charnier, 1966; Valenzuela & Lance, 2004). Found in many fish and reptiles, including all crocodilians, several turtles, and some lizards, TSD is theoretically and empirically supported to be an adaptive response to incubation temperature plasticity due to its sex-dependent effects on offspring fitness (Bock et al., 2023; Bókony et al., 2019; Charnov & Bull, 1977; Katona et al., 2021; Leivesley & Rollinson, 2024; Schwanz et al., 2016; Shine, 1999; Warner & Shine, 2008b). However, how TSD operates in ecological and evolutionary contexts is only beginning to be understood. For example, responses to incubation temperature are expected to exhibit local adaptation in response to divergent nest temperatures across populations (Pezaro et al., 2017), but this has only been empirically tested in a few species (Carter et al., 2019; Ewert et al., 2005) and the molecular basis of variation in TSD remains largely unexplored. Further, despite empirical support for its adaptive value in a few taxa (Bock et al., 2023; Conover, 1984; Warner & Shine, 2008b), the biological and physiological mechanisms mediating the fitness benefits associated with TSD are not well known. Identifying the selective targets linking incubation temperature to sex determination and sex-specific fitness is necessary for a comprehensive understanding of how TSD originated and its evolutionary potential on contemporary timescales. Additionally, most of the work on TSD has been conducted using constant incubation temperatures in the lab, which are not representative of natural nest conditions (Bowden et al., 2014). Knowledge of the influence of natural thermal environments is essential for assessing how sex ratio variation associated with TSD contributes to population dynamics, particularly responses to rapid environmental change (Mitchell & Janzen, 2010). Yet, progress on this front has been constrained by the lack of reliable methods to non-lethally sex juveniles of TSD species. These limitations collectively demonstrate a need for integrative studies that examine how TSD operates in ecological and evolutionary contexts, which is the primary focus of Chapters 3-6.

American alligators as models of adaptive and disruptive developmental plasticity

To address the above knowledge gaps, I utilize the American alligator (*Alligator mississippiensis*) as a model system. Alligators are oviparous reptiles that inhabit much of the southeastern United States and are well-suited models for understanding developmental plasticity in both adaptive and disruptive contexts. Like humans, alligators are long-lived, apex predators that bioaccumulate contaminants throughout life, which they can then pass into egg yolk and expose to their developing offspring (Nilsen et al., 2020; Rauschenberger et al., 2007). Consequently, alligators have served as ecological models of reproductive health for decades and provided some of the first insight into the negative consequences of EDC exposure in natural populations (Guillette et al., 1994; Hale et al., 2019, 2022; Hale & Parrott, 2020; Milnes et al., 2005, 2008; Moore et al., 2010, 2011; Moore, Roark, et al., 2012). Importantly, alligators also utilize TSD, with incubation temperatures around 33°C producing male-biased sex ratios, and warmer and cooler temperatures producing female-biased sex ratios (Ferguson & Joanen, 1983). Prior research on TSD in alligators has yielded critical insight into its underlying mechanisms, evolutionary benefit, and conservation implications (Bock, Hale, et al., 2020; Bock, Lowers, et al., 2020; Bock et al., 2023; Parrott, Kohno, et al., 2014; Yatsu, Miyagawa, Kohno, Parrott, et al., 2016; Yatsu, Miyagawa, Kohno, Saito, et al., 2016), making this species particularly well-suited for exploring outstanding questions about how TSD operates in natural contexts. Leveraging the above attributes, this dissertation integrates developmental plasticity into ecological and evolutionary frameworks, making critical advances in the fields of endocrine disruption, TSD, and crocodilian conservation.

Dissertation outline

An outline of the primary objectives and projects covered in the six chapters of this work is shown in **Figure 1.1**. As an appendix to this introductory chapter (Appendix A), I review the literature on both adaptive and disruptive influences of the developmental environment on ovarian development in reptiles and amphibians. I identify several key questions relating to ovarian development in these understudied groups, stressing the need to better understand the basic biological events associated with sexual development before assessing how they are modified in adaptive or maladaptive ways. In Chapter 2, I investigate molecular pathways responsible for endocrine disruption in natural populations, testing how maternally deposited hormones and contaminants, both independently and together, contribute to hatchling gonadal gene expression. My findings not only provide insight into how reproductive development is altered in populations impacted by EDC exposure but support a non-trivial role of maternally deposited hormones in driving offspring gonadal development. Chapters 3 and 4 examine how responses to incubation temperature vary across alligator populations in the context of both fitness-related traits and TSD. In Chapter 3, I demonstrate the evolutionary potential of incubation temperature plasticity to drive morphological differences between populations. I then assess how both neutral and selective processes shape divergence in TSD at the molecular level across those populations in Chapter 4, identifying several candidates for its adaptive evolution. Together, results from these two chapters provide critical insight into the origins, divergence, and adaptation of developmental plasticity in vertebrates, which remains understudied relative to insect systems. In Chapter 5, I explore the biological mechanisms underlying support for the adaptive evolution of TSD in alligators under the Sex-Specific Survival to Maturity hypothesis (STM; Schwanz et al., 2016). The STM relies on thermosensitive survival of juveniles in combination with sex-biases in age at maturity. Using compiled, longitudinal data, I identify time-dependent relationships between incubation temperature, hatchling traits, and post-release phenotypes that are associated with survival probability. My findings advance our understanding of how development plasticity contributes to evolutionary change, particularly the evolution of sex determining systems. Finally, in Chapter 6, I assess the ability of blood gene expression patterns to distinguish sex in hatchling alligators. I identify several candidate genes with minimal overlap in expression between males and females that can be reliably used to predict sex. My results thus provide a necessary basis for the development of a targeted assay that will facilitate monitoring of population sex ratios, which will be particularly useful for linking natural nest temperatures to primary sex ratios and predicting population responses to environmental change. Tying the above chapters together, I conclude with broader implications of my work and future directions building off my work that will be particularly fruitful in pushing the field of developmental plasticity forward in the context of ecology and evolution.

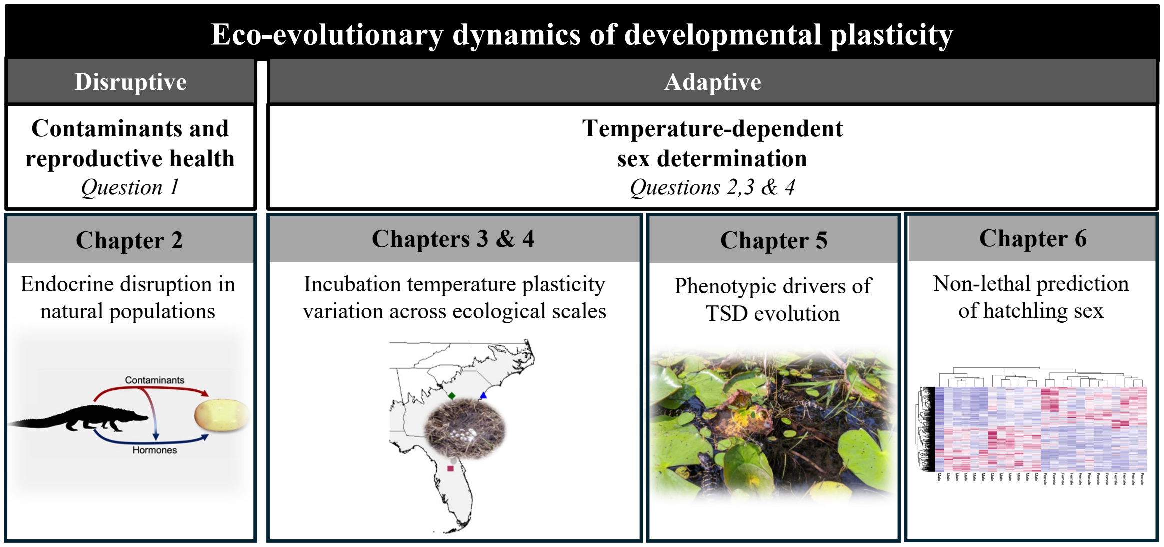

Figure 1.1: Dissertation overview.

CHAPTER 2

MATERNAL DEPOSITION OF HORMONES AND CONTAMINANTS SHAPE THE GONADAL TRANSCRIPTOME IN AMERICAN ALLIGATORS[1]

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[1] Smaga, C. R., Bock, S. L., Johnson, J. M., Paitz, R. T., Letter, A., Deem, V., Brunell, A., & Parrott, B. B. (2025). *Proceedings of the Royal Society B: Biological Sciences*, *292*(2039), 20242105. https://doi.org/10.1098/rspb.2024.2105. Reprinted here with permission of publisher.

Abstract

Environmental conditions influence the maternal deposition of hormones into eggs, which is hypothesized to adaptively modify developmental outcomes in offspring. However, most ecosystems harbor environmental contaminants capable of disrupting endocrine signaling, and maternal exposure to these compounds has the potential to further alter offspring traits. Studies rarely examine maternally derived hormones and contaminants along with offspring phentoypes, and we know little about their interrelationships and potential interactions. Here, we measure yolk concentrations of 24 endocrine disrupting compounds (EDCs) and 28 steroid hormones along with gonadal transcriptomes from two populations of the American alligator (*Alligator mississippiensis*) that differ in reproductive development and exposure to EDCs. Using a network-based approach, we identify gene expression modules associated with hormones and contaminants independently, in combination, or by potential indirect influences of EDCs on maternal hormone deposition. We find that yolk concentrations of both 17β-estradiol and etiocholanolone differ across populations and explain substantial variation in gene expression. We further provide evidence for the indirect effect of the pesticide, methoxychlor, on gonadal gene expression through its relationship with 17β-estradiol. Our results reveal novel pathways by which maternal exposure to environmental contaminants interacts with hormone provisioning to affect offspring sexual development.

Introduction

Maternal provisioning of nutrients and signaling molecules is a critical determinant of offspring traits. Specifically, the maternal deposition of steroid hormones is associated with a range of developmental outcomes in oviparous vertebrates, such as post-natal growth (Hayward & Wingfield, 2004), behavior (Eising & Groothuis, 2003; von Engelhardt et al., 2005), immune function (Navara et al., 2005; Sandell et al., 2009) and sexual development (Bowden et al., 2000; Rutkowska & Cichoń, 2006). The concentrations of maternally derived hormones in egg yolk vary with seasonality (Bowden et al., 2000; Jenni-Eiermann et al., 2020), social environment (Müller et al., 2002), diet (Morosinotto et al., 2016; Rutstein et al., 2005), and temperature (Lessells et al., 2016), and thus mechanistically link maternal environmental conditions and hatchling phenotypes (Dufty et al., 2002). Yet, despite their contribution to patterns of phenotypic variation, the transcriptional pathways that mediate the effects of yolk steroids and the extent to which those pathways are co-regulated by other components of the developmental environment remains largely unknown.

In addition to the deposition of hormones, maternal exposure to anthropogenic contaminants that interact with the endocrine system, often referred to as endocrine disrupting compounds (EDCs), can affect the reproductive development of their offspring (Gore et al., 2015; Marlatt et al., 2022). For example, hatchlings from environments contaminated by EDCs often display impaired reproductive development, with effects ranging from outright sex reversal to more subtle perturbations to gonadal form and function (Marlatt et al., 2022; Smaga et al., 2022). Similar to maternally deposited hormones, EDCs can be offloaded into egg yolk and interact with embryos directly by binding to nuclear hormone receptors or altering the metabolism or synthesis of endogenous hormones (Clairardin et al., 2013; Y. Li et al., 2013). Such direct effects are often the primary hypothesis explaining differences in reproductive development between contaminated and reference populations (Berg et al., 1999; L. J. Guillette et al., 1994). Alternatively, EDCs can act through indirect mechanisms by altering the maternal deposition of steroid hormones themselves. While the latter has received relatively little attention with reports in only a few species, the presence of indirect pathways is supported by correlations between concentrations of contaminants and hormones in egg yolk (Jouanneau et al., 2023; Verboven et al., 2008). However, because few studies measure hormones, contaminants and hatchling gonadal phenotypes together, our mechanistic understanding of how these components interact to modify gonadal development is limited.

American alligators (*Alligator mississippiensis*) offer an insightful system to investigate the combined influence of maternal hormones and contaminants on reproductive development. As in many reptile and amphibian species, alligator sex determination and gonadal differentiation are exquisitely sensitive to endocrine cues (Kohno et al., 2014; Smaga et al., 2022). Additionally, alligators are long lived, apex predators that bioaccumulate contaminants throughout life, passing them into egg yolk during reproduction (Guillette et al., 2000; Johnson et al., 2023), and have been extensively used as ecological models for EDC research (Guillette et al., 2000). Specifically, alligators from a contaminated lake in Florida, Lake Apopka (AP), display a suite of reproductive abnormalities when compared to a nearby reference lake, Lake Woodruff (WO), including altered gonadal steroidogenesis and circulating sex steroid hormone concentrations, reduced male phallus size, and impediments to ovarian follicle development (Guillette Jr. et al., 1996; Guillette et al., 1994; Hale et al., 2019, 2022; Hale & Parrott, 2020; Milnes et al., 2005, 2008). Whereas WO has been minimally impacted by anthropogenic disturbance, AP has a well-documented history of environmental contamination stemming from agricultural inputs and a chemical spill event, resulting in elevated concentrations of organochlorine pesticides (OCPs), which are known EDCs (Woodward et al., 2011).

Reproductive abnormalities in AP alligators have embryonic origins that stem from altered gonadal development (Hale et al., 2022; Moore et al., 2010). When considered together with studies reporting minimal genetic differentiation between WO and AP (Ryberg et al., 2002), it has been hypothesized that maternally deposited EDCs are the primary cause. Recently, Hale et al. (Hale & Parrott, 2020) reported extensive divergence in ovarian transcriptomes in juvenile alligators from WO and AP that were collected as eggs and incubated and reared under common garden conditions to isolate maternal effects. To gain insight into the underlying mechanisms, embryos from WO were treated with 17β-estradiol during gonadal development to mimic the estrogen receptor activating ability of OCP mixtures in AP, which largely recapitulated differences in ovarian transcriptome profiles and reduced follicle counts observed in AP alligators (Bolger et al., 1998; Hale & Parrott, 2020; Vonier et al., 1996). While these findings support the role of altered embryonic estrogen signaling in the disruption of reproductive development at AP, the specific compounds and underlying mechanisms responsible are not resolved. Interestingly, juvenile alligators from AP also display altered circulating levels of sex steroids (Guillette et al., 1994), suggesting disrupted endocrine regulation that is likely to have impacts for hormone deposition in eggs at reproductive maturity. However, the potential contribution of maternal hormone deposition, either independently or in combiniation with EDCs, to sexual development at AP has not been widely considered.

Here, we rely on natural variation in environmental contamination between AP and WO to investigate the contributions of maternally deposited steroid hormones and contaminants, both independently and together, to variation in reproductive development (**Figure 2.1a**). By quantifying the concentrations of 24 EDCs and 28 steroid hormones in egg yolk, we first characterize differences in the developmental endocrine environment across populations. We then sequence clutch-matched hatchling gonadal transcriptomes and assemble them into co-correlated modules to assess their relationships with contaminant and hormone concentrations that differ between populations. Specifically, we identify gene expression modules associated with either hormones or contaminants, both independently and together, and those consistent with regulation by indirect actions of EDCs through their alteration to maternal hormone deposition (**Figure 2.1b**). Based on previous studies at WO and AP, we predict that maternally deposited 17β-estradiol will differ across populations, be associated with EDC concentrations, and explain population divergence in gonadal gene expression.

Materials and methods

Experimental design

Egg collection, incubation and hatchling husbandry were carried out as previously described (Smaga et al., 2024). In June of 2021, we collected 8 clutches of alligator eggs from both AP and WO within two weeks of oviposition. We then transferred eggs to dampened, sphagnum moss in commercial incubators (model I36NLC; Percival Scientific, Perry, IA, USA). Alligators display temperature-dependent sex determination, with males produced under warmer incubation temperatures and females produced at cooler temperatures (Bock, Lowers, et al., 2020; Ferguson & Joanen, 1983), so we initially incubated eggs at an intermediate temperature producing both sexes (32 °C). Upon reaching Ferguson stage 15, which represents the opening of the thermosensitive period of sex determination (Ferguson & Joanen, 1983; McCoy et al., 2015), we randomly assigned eggs to either a constant male-promoting (33.5 °C) or female-promoting (29.5 °C) temperature (MPT and FPT, respectively). At embryonic stage 20, we collected approximately 8 mL of egg yolk from each of 3-4 eggs/clutch from the FPT treatment and stored it at -20 °C. We only collected yolk from FPT eggs; however, previous research has shown that temperature-dependent differences in egg yolk concentrations of hormones do not occur until stage 21 (Conley et al., 1997). Remaining eggs were incubated at MPT and FPT until hatching. Hatchlings were kept under common garden conditions until day 10, on which we euthanized hatchlings via cervical severance and pithing and dissected gonadal-adrenal-mesonephros complexes, fixing them in RNAlater and storing them at -80 °C. We later dissociated gonads from the gonadal-adrenal-mesonephros complex under a dissecting scope and stored them at -80 °C.

Steroid hormone and contaminant quantification and analysis

We quantified concentrations of contaminants and hormones from matched stage 20 egg yolks (for details, see Supplementary Methods). For contaminant quantification, 16 OCPs and 8 polychlorinated biphenyls (PCBs) were quantified at the University of Georgia's Center for Applied Isotope Studies for using gas chromatography-mass spectrometry (GC-MS; Agilent Technologies, Inc., Palo Alto, CA, USA). We sampled 1 egg/clutch as previous reports have demonstrated that variation in contaminant loads within a clutch is minimal relative to those observed across clutches (Heinz et al., 1991; Van den Steen et al., 2006). However, we also measured an additional two eggs from two clutches from AP and one clutch from WO to estimate intra-clutch variability. For steroid hormones, 28 compounds from 2-3 eggs per clutch were quantified at the Metabolomics Laboratory of the Roy J. Carver Biotechnology Center at the University of Illinois using the protocol used by (Merrill et al., 2019). Specific compounds and corresponding limits of detection (LOD) are reported in Table 2.S1.

Prior to analysis, we removed contaminants or hormones below the LOD in >50% of samples. We additionally removed one outlier OCP sample from one of the clutches in which multiple eggs were measured. We tested for population differences in contaminant concentrations by conducting Mann-Whitney U-Tests, using average values for clutches with multiple measurements. We analyzed hormone concentrations using linear mixed effect models (LMMs), with population as a predictor and clutch as a random intercept to account for inter-clutch variation. Population comparisons were used as a filter to reduce the number of compounds in downstream analyses, so we did not correct p-values for multiple testing and retained contaminants or hormones with unadjusted $p\text{-values} < 0.05$. As an overall measure of population differences in hormones and contaminants, we conducted a principal component analysis (PCA) of hormones and contaminants that differed across populations.

RNA extraction, sequencing and alignment

We extracted RNA from hatchling gonads (see Supplementary Methods) and total RNA (> 30 ng/µL) from one individual from each clutch and incubation temperature treatment across each of 6 clutches/population (n = 24) was sent to Novogene (Sacramento, CA, USA) for quality control (all RNA integrity scores > 7.4) and sequencing. Samples were poly(A) enriched, and directional mRNA libraries were prepared and sequenced on an Illumina NovaSeq6000 instrument (paired end 150 bp reads, Illumina, San Diego, CA, USA). Using FastQC (Andrews, 2010) and MultiQC (Ewels et al., 2016), we assessed the quality of raw reads and trimmed adaptor sequences using TrimGalore! (Krueger, 2015) with a stringency level of 3. We then aligned them to the alligator reference genome (ASM28112v4) using Hisat2 (Kim et al., 2019), sorting the resulting SAM files and converting them to BAM format using SAMtools (Danecek et al., 2021). We input BAM files into R (R Core Team 2024, version 4.3.1) using the function BamFileList from the RSamTools package (Morgan, 2024). Using the GenomicFeatures package (Lawrence et al., 2013), we generated exon-by-gene coordinates from the alligator genome annotation (ASM28112v4; (Rice et al., 2017)) with the ‘makeTxDbFromGFF’ function and counted reads per gene from the aligned BAM files using the ‘summarizeOverlaps’ function (mode = ‘Union’) in the GenomicAlignments (Lawrence et al., 2013) package. As a substantial proportion of the transcripts (n = 10,927) detected were identified as uncharacterized loci in the alligator genome annotation (e.g., LOC genes), we employed a previously described approach (Hale & Parrott, 2020), which annotated 5,657 of these loci (see Supplementary Methods).

Assembling co-correlation network gene modules

We characterized gene expression patterns using weighted gene co-correlation network analysis (WGCNA; (Langfelder & Horvath, 2008)). WGCNA assembles genes into modules based on their correlated expression patterns. Gene module metrics (e.g., eigengenes) can then be tested for associations with predictor variables, which reduces the multiple testing burden compared to individual gene approaches, often providing more power and interpretability. First, we only retained genes with greater than 1 count per million (CPM) in more than 5 libraries. We then normalized raw counts from the remaining genes using the trimmed mean of m-values (TMM) in DESeq2 (Love et al., 2014). After visualizing TMM values with a PCA, we removed two outliers corresponding to a WO MPT sample that clustered with the rest of the FPT samples and an AP MPT sample that clustered between MPT and FPT (Figure 2.S1). After re-normalizing the remaining libraries, we used a variance-stabilizing transformation in DESeq2 to transform TMM read counts and determined the soft thresholding power using ‘PickSoftThreshold’ in WGCNA. We then built the network including all filtered genes in a single run using the ‘blockwiseModules’ function with the following parameters: maxBlockSize = 17,732, power = 6, minModuleSize = 20, corType = bicor, networkType = signed. Using the ‘moduleEigengenes’ function, we extracted module eigengenes (the first principal component of module gene expression) as representations of module expression.

Relationships between gene expression, hormones, and contaminants

We tested whether module eigengenes were associated with components of the developmental environment that differed across populations (4 hormones, 10 contaminants, sum of OCPs, sum of PCBs, and PC1 of all 14 compounds). We first assessed relationships between the developmental environment variables and module eigengenes using the ‘moduleTraitCor’ and ‘moduleTraitPvalue’ functions in WGNCA. However, since this approach does not account for differences between incubation temperatures or across populations, we constructed individual linear models (LMs) for each significant correlation, including population, temperature and hormone or contaminant as predictors.

We classified modules based on their relationships to hormone and contaminant concentrations (**Figure 2.1b**). If a module was significantly associated with either hormones or contaminants alone, we considered it directly and independently influenced by that class of compound. For modules associated with both hormones and contaminants together, we identified potential indirect effects of EDCs on maternal hormone deposition by testing for relationships between contaminant and hormone concentrations using LMs, with hormone as the response and contaminant as the predictor. If contaminant concentrations were predictive of hormone concentrations and their directionality was consistent with their effects on gene expression, we considered that module consistent with being indirectly influenced by that contaminant through the respective maternal hormone.

Functional annotation of gene expression modules

We performed enrichment tests for genes comprising modules significantly associated with hormones or contaminants using Gene Ontology (GO) biological process (BP) and molecular function (MF) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with the function ‘gost’ in the R package gprofiler2 (Kolberg et al., 2020). Prior to enrichment tests, we created a custom alligator background using all expressed, annotated genes in the dataset that passed filtering and converted gene names to human counterparts using the 'gconvert' function (final background = 13,555 genes).

Statistical analysis

We conducted all statistical tests in R using the packages dplyr and tidyr (Wickham et al., 2023) for data manipulation. For LMs and LMMs, we used the packages lme4 (Bates et al., 2025) and lmerTest (Kuznetsova et al., 2017), respectively, and evaluated model assumptions of residual normality and homoscedasticity visually with quantile-quantile and residual vs fitted plots, respectively. For models that failed to meet assumptions, we transformed response variables using log or cube-root transformations. For PCAs, we used the 'pcromp' function in R (scale = TRUE, center = TRUE). In all analyses, we used a p-value < 0.05 for significance. All plots and data visualizations were made using ggplot2 (Wickham, 2016).

Results

Population differences in contaminant and steroid hormone concentrations

After filtering, we retained 16 OCPs, 4 PCBs and 16 hormones from 7 clutches at AP and 8 clutches at WO. The average intraclutch coefficient of variation (CV) for the 3 clutches with multiple eggs measured for OCP concentrations was 0.48, while the average interclutch CV across all clutches was 1.22. The average intraclutch CV for the 3 clutches with multiple eggs measured for PCBs was 0.59 and the average interclutch CV across all clutches was 1.42. For hormones, the average intraclutch CV was 0.49, while the average interclutch CV across all clutches was 0.77. Yolk concentrations of four hormones were significantly different between populations with both 5β-tetrahydrocortisol (β = 0.119, p = 0.013) and estrone (β = 7.696, p = 0.029) significantly reduced at AP when compared to WO, and 17β-estradiol (β = -0.363, p = 0.039) and etiocholanolone (β = -0.240, p = 0.038) significantly elevated (Table 2.S2; **Figure 2.2a**). Additionally, we identified 8 OCPs (epoxyheptachlor, endosulfan I, endosulfan II, p',p'-DDE, endrin aldehyde, DDT, endrin ketone, and methoxychlor) and 2 PCBs (trichlorobiphenyl and tetrachlorobiphenyl) that were significantly elevated in egg yolk at AP (Table 2.S3; **Figure 2.2b, c**). We did not detect any contaminant present in yolks at higher concentrations in WO. Consistent with historical data (Rauschenberger et al., 2007), p',p'-DDE made up a significant proportion of OCPs at AP, followed by methoxychlor (**Figure 2.2c**). We also found that summed values of both OCPs and PCBs were significantly elevated at AP (Table 2.S3; **Figure 2.2b, c**). When all 4 hormones, 8 OCPs and 2 PCBs above were examined together in a PCA, we observed separation by population across the first principal component (PC1), which explained 46.38% of the variation (**Figure 2.2d**).

Weighted gene co-correlation network analysis

RNA sequencing produced between 39 and 98 million reads per sample and alignment rates ranged from 45.32% to 91.01% ($\bar{x}$ = 82.18%). After CPM filtering for expressed genes, we retained 17,732 genes for downstream analyses. We identified nine co-expression modules and one 'orphan' module (Grey) comprised of genes lacking correlated expression patterns. The number of genes forming each module ranged from 155 to 5,323 genes (Figure 2.S2).

Associations of hormones and contaminants with gene expression modules

We identified 40 significant correlations spanning 6 modules, 2 hormones and 4 contaminants, in addition to the sum of all OCPs and PCBs and PC1 (Figure 2.S3). After controlling for population and temperature using LMs, we retained associations between 6 modules, 2 contaminants and 2 hormones (Table 2.1).

For three co-expression modules, we found significant relationships with hormone concentrations independent of contaminants (**Figure 2.3a**). The Green module was positively associated with both 17β-estradiol (β = 0.328, p = 0.015, $R^2 = 0.31$) and etiocholanolone (β = 1.724, p = 0.001, $R^2 = 0.47$; Table 2.1; **Figure 2.3b**). Green module genes were enriched for several GO and KEGG terms including the BPs ‘macromolecule biosynthetic process’, ‘nitrogen compound metabolic process’, and ‘RNA metabolic process’, the MFs ‘nucleic acid binding’, ‘RNA binding’, and ‘organic cyclic compound binding’, and the KEGG pathways ‘Spliceosome’, ‘Amyotrophic lateral sclerosis’, and ‘ATP-dependent chromatin remodeling’ (**Figure 2.3b**). Similarly, we detected positive associations between the Magenta module and concentrations of 17β-estradiol and etiocholanolone (17β-estradiol: β = 0.244, p = 0.045, $R^2 = 0.41$; etiocholanolone: β = 0.483, p = 0.011, $R^2 = 0.49$; Table 2.1, **Figure 2.3c**), with enrichment for the BPs ‘RNA metabolic process’ and ‘regulation of RNA metabolic process’ (**Figure 2.3c**). Lasty, the Pink module was negatively associated with 17β-estradiol (β = -0.295, p = 0.024, $R^2 = 0.35$; Table 2.1; **Figure 2.3d)**, with enrichment in the BPs ‘RNA splicing’, ‘mRNA processing’, and ‘RNA processing’, along with the KEGG pathway ‘Nucleocytoplasmic transport’ (**Figure 2.3d**).

Two modules were significantly associated with both hormones and contaminants independently (i.e., no significant relationship between the contaminants and hormones; Table 2.2; **Figure 2.4a**). The Black module was positively associated with 17β-estradiol, etiocholanolone, and endosulfan II (17β-estradiol: $\beta = 0.270$, $p = 0.017$, $R^2 = 0.52$; etiocholanolone: $\beta = 1.256$, $p = 0.005$, $R^2 = 0.57$; endosulfan II: $\beta = 0.065$, $p = 0.047$, $R^2 = 0.47$; Table 2.1; **Figure 2.4b**). Genes in the Black module were enriched for the BPs ‘translation’, ‘peptide biosynthetic process’ and ‘amide biosynthetic process’, the MFs ‘RNA binding’, ‘translation regulator activity, nucleic acid binding’, and ‘translation regulator activity’, and the KEGG pathways ‘Alzheimer disease’ and ‘Parkinson disease’ (**Figure 2.4b**). Alternatively, the Red module was negatively associated with 17β-estradiol, etiocholanolone, and endosulfan II (17β-estradiol: $\beta = -0.289$, $p = 0.030$, $R^2 = 0.31$; etiocholanolone: $\beta = -0.472$, $p = 0.028$, $R^2 = 0.32$; endosulfan II: $\beta = -0.079$, $p = 0.039$, $R^2 = 0.29$; Table 2.1; **Figure 2.4c**). Red module genes were enriched for the BPs ‘RNA processing’, ‘RNA splicing’, and ‘RNA splicing, via transesterification’, and the KEGG pathways ‘Spliceosome’ and ‘Nucleotide excision repair’ (**Figure 2.4d**).

Only the Grey module was significantly associated with both hormones and contaminants and showed evidence consistent with a potential indirect effect of EDCs on maternal hormone deposition (**Figure 2.5a**). This module was negatively associated with both 17β-estradiol ($\beta = -0.514$, $p = 0.033$, $R^2 = 0.49$) and methoxychlor ($\beta = -0.009$, $p = 0.035$, $R^2 = 0.48$; Table 2.1; **Figure 2.5b**). Additionally, we identified a positive relationship between concentrations of 17β-estradiol and methoxychlor in egg yolks ($\beta = 0.010$, $p = 0.008$, $R^2 = 0.54$; Table 2.2; **Figure 2.5c**). Despite consisting of genes whose expression is not co-correlated, Grey module genes were enriched for the BPs ‘ribonucleoprotein complex biogenesis’, ‘ribosome biogenesis’, and ‘nucleic acid metabolic process’ as well as the KEGG pathway ‘Ribosome biogenesis in eukaryotes’ (**Figure 2.5b**).

Discussion

Maternal resource provisioning is critical for successful reproduction and has the potential to connect maternal environmental conditions to offspring traits (Groothuis et al., 2005; Mousseau & Fox, 1998). However, in affected ecosystems, exposure to environmental contaminants can disrupt the physiological state of mothers, compromising hormone provisioning, and said contaminants can also be offloaded into eggs, directly impacting embryos (Gore et al., 2015; Muñoz & Vermeiren, 2020). Here, we found that concentrations of both hormones and EDCs in egg yolks differed across populations and contributed, both independently and in combination, to broad scale variation in gonadal transcriptomes. Specifically, the insecticide, endosulfan II, along with the steroids 17β-estradiol and etiocholanolone were all associated with the Black and Red gene expression modules; however, concentrations of endosulfan II were not correlated with either 17β-estradiol or etiocholanolone within egg yolks, supporting independent effects of each. In contrast, expression of the Grey module was negatively correlated with both methoxychlor and 17β-estradiol, and yolk concentrations of methoxychlor were positively correlated with 17β-estradiol, suggesting that maternal exposure to methoxychlor potentially leads to increases of 17β-estradiol deposition. While EDC-altered deposition of maternal hormones has only rarely been explored and evidence of indirect effects of EDCs through maternal hormone transfer is mixed (French et al., 2001; Jouanneau et al., 2023; Verboven et al., 2008), these results support the presence of “indirect effects” in affected populations. However, such indirect effects are challenging to experimentally isolate and causal relationships cannot be definitively resolved given the observational nature of our study. Nonetheless, our findings reveal the importance of maternal contributions to the developmental environment, as maternal provisioning of steroid hormones and EDCs explained a large proportion of transcriptional variation in developing gonads.

Over half (6 of 10) of the assembled expression modules (comprising 31.6% of expressed genes) were associated with yolk concentrations of 17β-estradiol, suggesting that maternally deposited estrogen exerts persistent and broad influences on transcriptional programs within the developing gonad. On one hand, this isn’t surprising as dosing studies and genetic manipulations to estrogen receptors and its synthesis enzyme, aromatase, have demonstrated widespread influences of embryonic estrogen signaling on vertebrate sex determination and gonadal differentiation (Britt et al., 2001; Britt & Findlay, 2003; Canesini et al., 2018; Dupont et al., 2000; Guiguen et al., 2010; Piprek et al., 2012). However, our understanding of the specific role of maternally deposited estrogen in these processes is more limited. For maternal steroid hormones to elicit their effects, they must be uptaken by embryos and interact with hormone receptors (von Engelhardt et al., 2009). In alligators, concentrations of 17β-estradiol in yolk do not change from stage 16 through 21, but then decline rapidly from stage 21 to 23 of development (Conley et al., 1997), and this decline coincides with gonadal differentiation, the onset of ovarian aromatase expression (Parrott, Kohno, et al., 2014), and the expression of estrogen receptors in the gonads (Hale & Parrott, 2020; Smith &

Joss, 1993). In both birds and reptiles, declines in maternally derived 17β-estradiol concentrations have been shown to be the result of embryonic metabolism (Paitz et al., 2020; Paitz & Bowden, 2008; von Engelhardt et al., 2009). The ultimate fate of steroid hormone metabolites, including those of 17β-estradiol, is not well understood, but they may be inactive and serve no function or be uptaken by embryos, where they either can serve independent or more specific functions than their precursors or be converted back into active forms to be utilized later in development (Paitz & Bowden, 2008). Whereas concentrations of 17β-estradiol were elevated at AP, we found that estrone, an intermediate metabolite of 17β-estradiol metabolism, was elevated at WO. Estrone concentrations were not associated with gene expression modules, and together, these findings are consistent with 17β-estradiol metabolism as an inactivation pathway in the alligator, which may be reduced at AP relative to WO. Reduced metabolism of 17β-estradiol during a period when the gonads are receptive to estrogen is likely to have consequences for sexual development, which our results show persist well after sex determination is complete.

In the context of previous work at AP and WO, the reproductive perturbations observed in AP alligators have embryonic origins and persist at least into juvenile stages (Guillette et al., 1996; Guillette et al., 1994; Milnes et al., 2005, 2008; Moore et al., 2010, 2011; Moore, Forouhar, et al., 2012). Disrupted estrogen signaling is hypothesized to underpin these alterations as treating WO embryos with 17β-estradiol broadly recapitulates the ovarian transcriptional patterns and defects in folliculogenesis observed in AP juveniles (Hale et al., 2019; Hale & Parrott, 2020). Interestingly, these experiments treated with 17β-estradiol prior to the initiation of gonadal estrogen synthesis in the embryonic ovary. Given that both nuclear estrogen receptors were expressed at the stage of treatment, the authors proposed that the induction of developmentally precocious estrogen signaling by maternally derived EDCs is the precipitating event driving reproductive phenotypes in AP alligators. Here, we found that concentrations of 17β-estradiol are elevated in yolks from AP prior to endogenous 17β-estradiol synthesis in the gonad, and that in at least one instance, they are directly associated with EDC concentrations. Relative to EDCs, which are generally weak estrogen receptor agonists (Blair et al., 2000; Bolger et al., 1998; Guillette et al., 2002), EDC-mediated increases of maternally deposited 17β-estradiol would provide a more potent source of precocious estrogen signaling. However, given that the Grey module does not consist of co-expressed genes, it is difficult to interpret the molecular pathways involved. It is possible that elevated estradiol has broad transcriptional effects on a gene by gene basis, or that it alters relationships among co-expressed genes. Nonetheless, our findings raise the possibility that elevated concentrations of maternally transferred 17β-estradiol in egg yolk drive the altered transcriptional profiles in juvenile AP alligator gonads; however, we cannot rule out direct effects of EDCs acting either independently or in combination with endogenous hormones.

Our screening of steroids and environmental contaminants for relationships with transcriptome patterning revealed unexpected relationships between endosulfan II, an insecticide banned in the United States due to human health concerns (Menezes et al., 2017), and the Red and Black gene expression modules. These results were unexpected because endosulfan II contributes relatively little to differences in OCP concentrations between AP and WO, which are primarily driven by p’,p’-DDE and methoxychlor. The

Red and Black modules were also positively correlated to concentrations of 17β-estradiol, suggesting that endosulfan II might act through an estrogenic mechanism. Whereas the binding affinity of endosulfan II to estrogen receptors is very weak (Silva & Gammon, 2009), this compound has been shown to influence steroid hormone concentrations by altering the expression of steroidogenic enzymes (Yan et al., 2019). However, concentrations of endosulfan II and 17β-estradiol were not correlated in egg yolks, suggesting that their associations to transcriptional variation is not due to the indirect effects of endosulfan II on hormone deposition. Instead, we hypothesize that maternally deposited endosulfan II and 17β-estradiol converge to affect estrogen-mediated transcriptional effects, albeit through different molecular mechanisms.

Our approach for detecting associations between hormones and EDCs that differ across populations and gonadal gene expression has some important limitations. First, previous research in both alligators and other taxa suggests that intraclutch variation in EDCs is limited relative to interclutch variation (Heinz et al., 1991; Van den Steen et al., 2006). In our study, variation in hormone and contaminant concentrations were greater across clutches than within as expected, but some contaminants varied by up to 166% across eggs within a clutch. Thus, using a single egg to estimate clutch-wide concentrations contributes additional variation to the data, likely reducing model sensitivity. Second, our approach centered on identifying relationships between maternally deposited hormones, contaminants, and offspring transcriptomes occurring in natural populations; yet, the observational nature of our experimental design limits our ability to make causal inferences. For instance, we did not assess the extent to which egg yolk concentrations of contaminants or hormones are representative of maternal levels.

However, previous reports have demonstrated relationships between concentrations of EDCs in egg yolks and experimental, dietary exposure of maternal alligators (Rauschenberger et al., 2007), and studies in birds have demonstrated relationships between maternally deposited hormones and circulating levels (Williams et al., 2005). Lastly, maternally deposited hormone concentrations are dynamic during development (Conley et al., 1997), and we only measured them at a single timepoint. In line with this, our quantification of contaminants and hormones and measurement of gene expression were temporally separated (stage 20 vs 10-days post-hatch), which may have reduced our ability to detect associations. Future work assessing concentrations of hormones, contaminants and gene expression at multiple, matched time points during development is likely to provide additional insight into how maternally deposited components of the developmental endocrine environment interact with gene expression dynamics.

In summary, by measuring comprehensive panels of EDCs and steroid hormones across populations together with gonadal gene expression, we identified specific molecular components of the developmental environment, representing both classes of compounds, that explain substantial variation in the gonadal transcriptome. In particular, our results point to an especially important role of maternally deposited 17β-estradiol independently and jointly with EDCs. Additionally, we found evidence for indirect effects of EDCs on gonadal development through alterations to maternal 17β-estradiol deposition. Taken together, our findings not only provide insight into the mechanisms underlying altered reproductive development in populations impacted by EDC exposure, but also support a non-trivial role of maternally deposited hormones in driving offspring gonadal development.

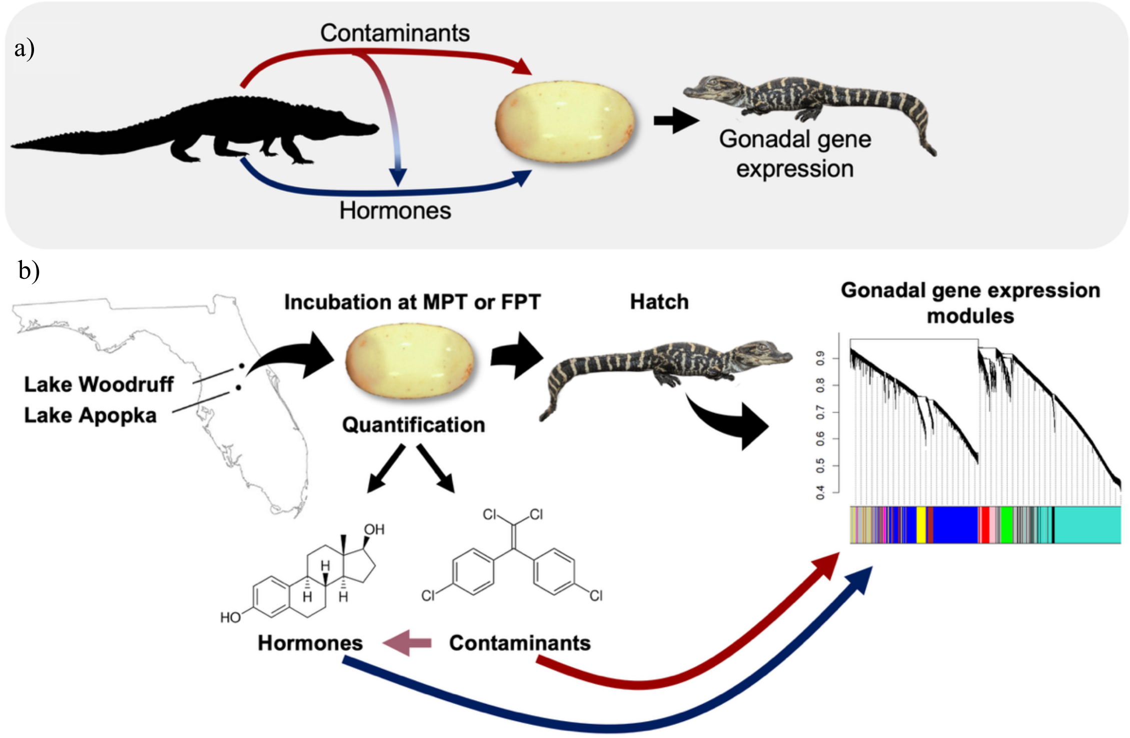


Figure 2.1: (a) Conceptual diagram demonstrating the contributions of maternally deposited hormones and contaminants, both independently and together, to hatchling gonadal gene expression variation. (b) Schematic of experimental design. Independent clutches of alligator eggs were collected from Lake Apopka and Lake Woodruff and incubated at either a male- or female-promoting temperature (MPT and FPT, respectively). Steroid hormones and contaminants were quantified in a subset of egg yolks from each clutch, while the remaining eggs were allowed to hatch. Relationships between hatchling gonadal transcriptomes and yolk concentrations of contaminants and hormones were used to identify gene expression modules associated independently with hormones or contaminants, with both jointly, or by the potential indirect effect of contaminants on maternal hormone deposition.

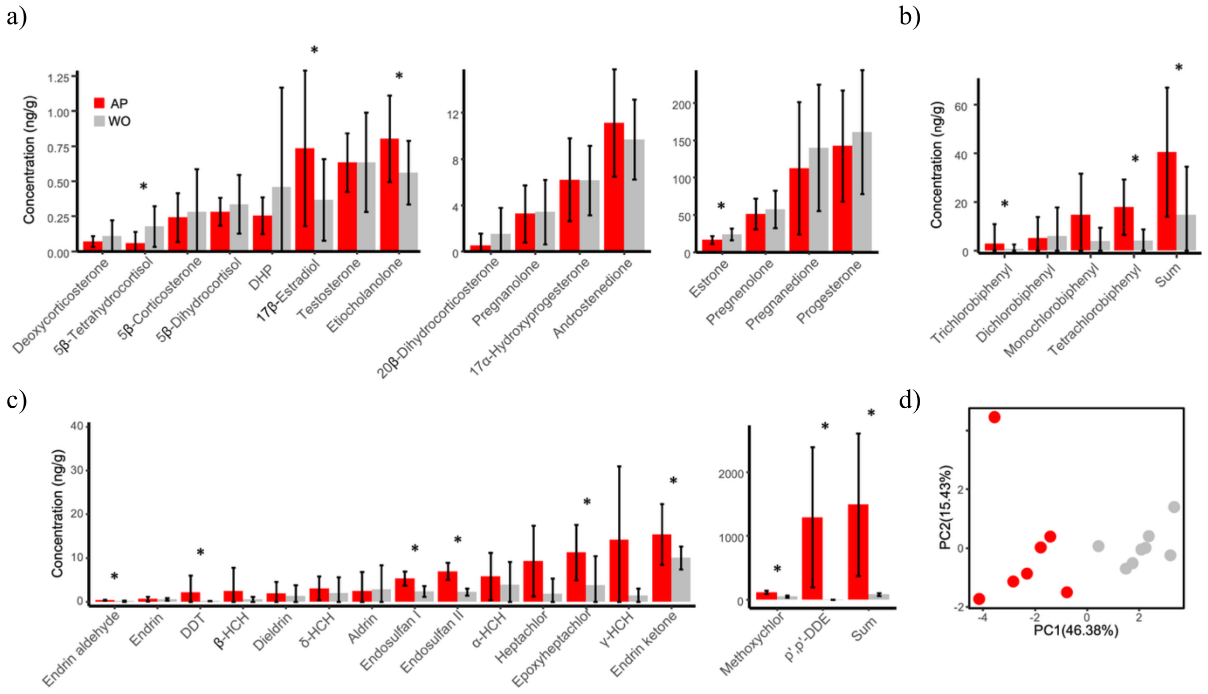


Figure 2.2: Concentrations of contaminants and maternally deposited hormones in egg yolks across populations. (a) Steroid hormones, (b) polychlorinated biphenyls (PCBs), (c) organochlorine pesticides (OCPs), (d) principal component analysis (PCA) of individual hormones, OCPs and PCBs that significantly differed across populations. Asterisks denote statistical significance. Red bars and points represent values from AP and grey represent WO. DHP: dihydrotestosterone; HCH: hexachlorocyclohexane.

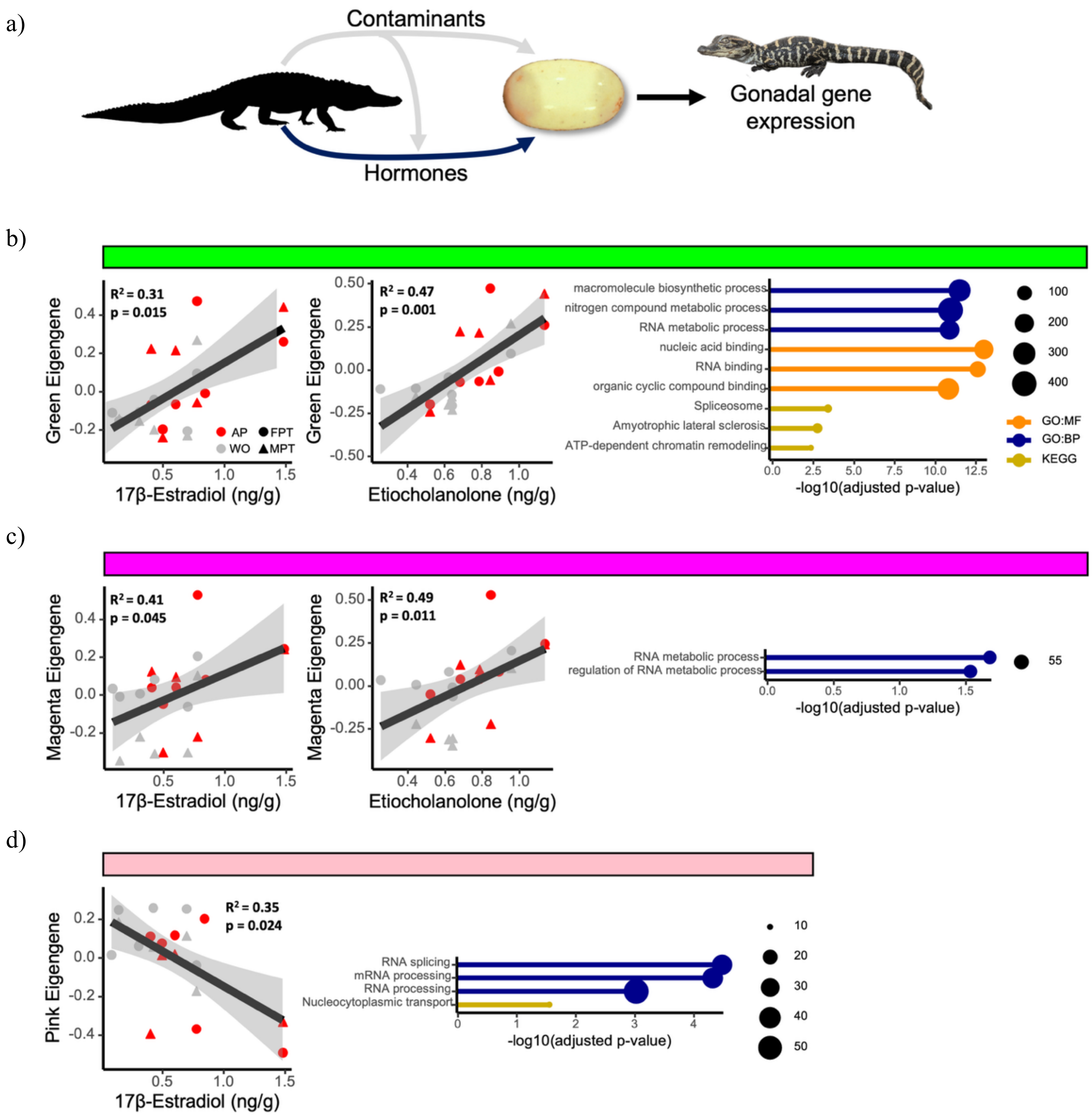


Figure 2.3: (a) Gene expression modules associated with hormone concentrations independent of contaminants in egg yolks. (b) Green module association with 17β-estradiol and etiocholanolone. (c) Magenta module association with 17β–estradiol and etiocholanolone. (d) Pink module association with 17β-estradiol. Corresponding GO and KEGG terms for each module are included. Red points represent values from AP and grey represent WO. Circles represent FPT animals and triangles represent MPT animals. Sizes of points for GO and KEGG terms represent the number of genes.

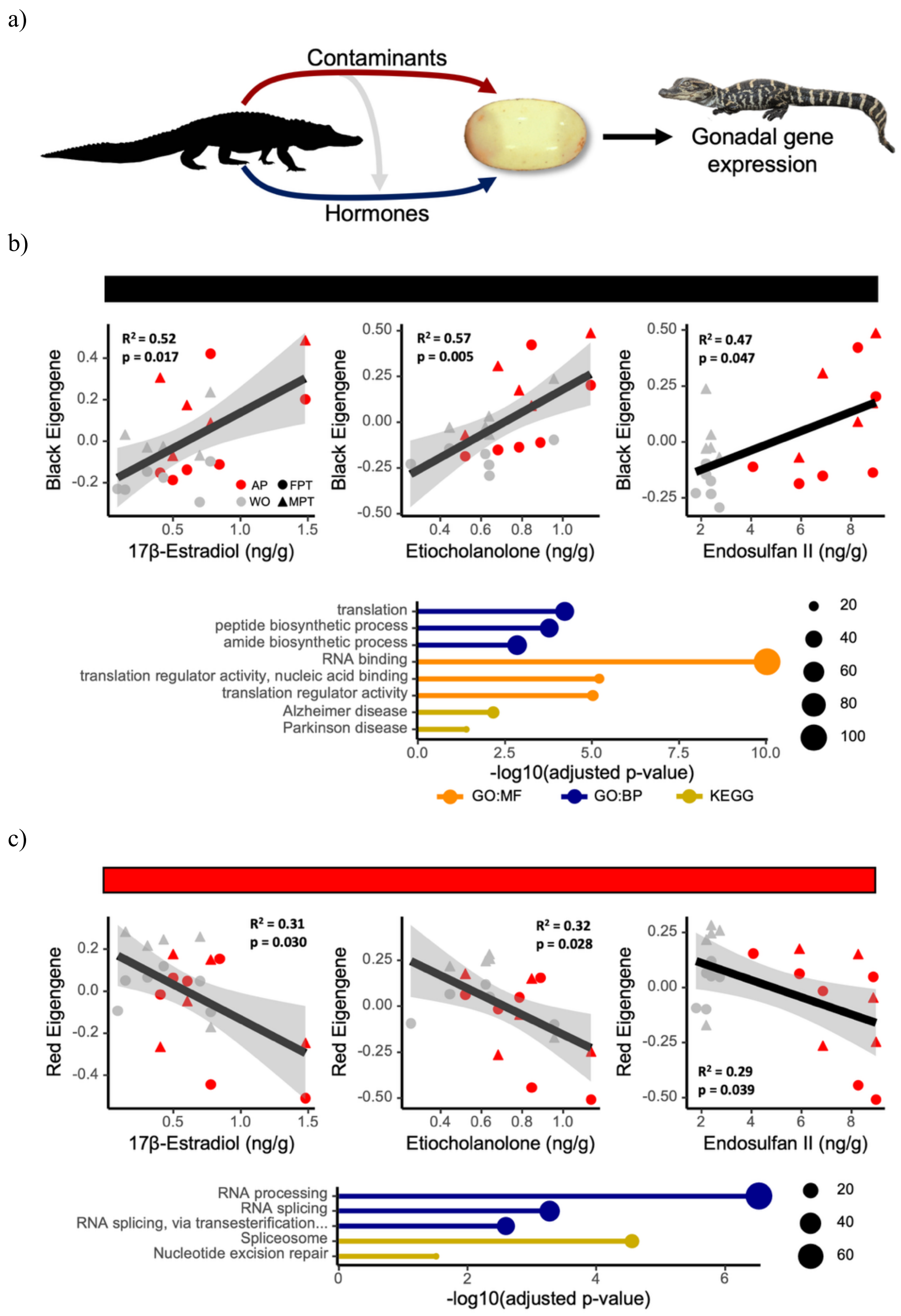


Figure 2.4: (a) Gene expression modules associated with concentrations of both contaminants and hormones in egg yolks. (b) Black module associations with 17β-estradiol, etiocholanolone and endosulfan II. (c) Red module associations with 17β-estradiol, etiocholanolone and endosulfan II. Corresponding GO and KEGG terms for each module are included. Red points represent values from AP and grey represent WO. Circles represent FPT animals and triangles represent MPT animals. Sizes of points for GO and KEGG terms represent the number of genes.

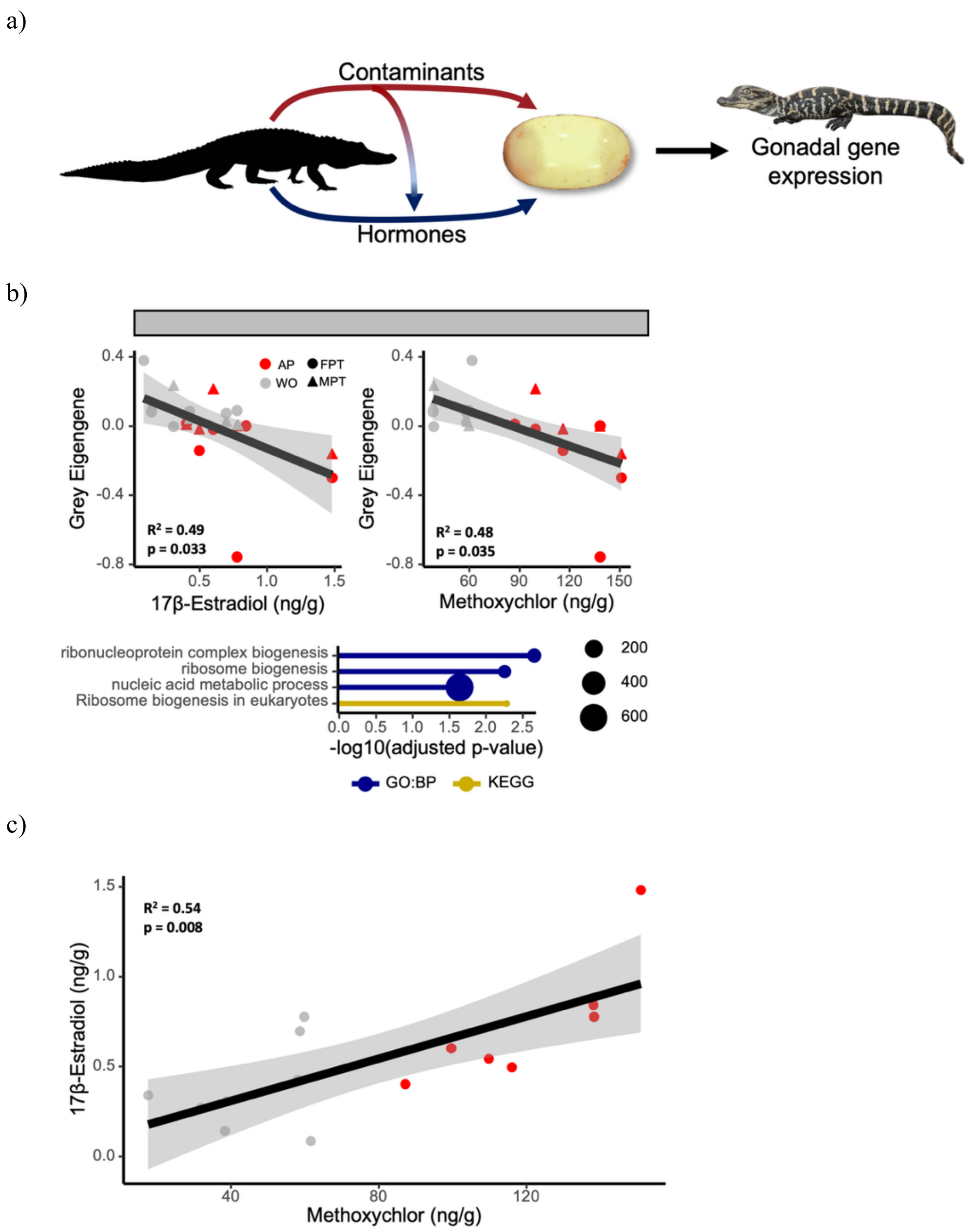


Figure 2.5: (a) Gene modules consistent with indirect effects of EDCs through maternal hormone deposition. (b) Grey module association with concentrations of methoxychlor and 17β-estradiol measured in yolks, enriched GO and KEGG terms are indicated. (c) Positive association between concentrations of methoxychlor and 17β-estradiol in egg yolks. Red points represent values from AP and grey represent WO. Circles represent FPT animals and triangles represent MPT animals. Sizes of points for GO and KEGG terms represent the number of genes.

Table 2.1: Associations between module eigengenes and the developmental environment for those with significant Pearson correlations, with significant results bolded. SE: standard error; PCB: polychlorinated biphenyl.

Module	Compound	Temp. β	Temp. SE	Temp. p-value	Pop. β	Pop. SE	Pop. p-value	Compound β	Compound SE	Compound p-value	R2
Black	**Endosulfan II**	**0.185**	**0.069**	**0.016**	**0.149**	**0.171**	**0.397**	**0.065**	**0.031**	**0.047**	**0.467**
	17β-Estradiol	**0.202**	**0.065**	**0.006**	**-0.098**	**0.073**	**0.192**	**0.270**	**0.102**	**0.017**	**0.518**
	Etiocholanolone*	**0.516**	**0.155**	**0.004**	**-0.21**	**0.170**	**0.231**	**1.256**	**0.395**	**0.005**	**0.568**
	Methoxychlor	0.218	0.069	0.006	0.074	0.149	0.627	0.004	0.002	0.065	0.450
	PC1	0.203	0.074	0.013	0.005	0.186	0.978	-0.044	0.039	0.278	0.375
	Tetrachlorobiphenyl	0.205	0.070	0.009	-0.04	0.103	0.700	0.010	0.005	0.069	0.447
Green	Endosulfan II	-0.006	0.084	0.944	0.210	0.207	0.325	0.076	0.037	0.057	0.219
	17β-Estradiol	**0.013**	**0.078**	**0.866**	**-0.071**	**0.087**	**0.423**	**0.328**	**0.122**	**0.015**	**0.312**
	Etiocholanolone*	**0.067**	**0.172**	**0.699**	**-0.086**	**0.188**	**0.653**	**1.724**	**0.437**	**0.001**	**0.467**
	Methoxychlor	0.031	0.086	0.719	0.091	0.184	0.626	0.004	0.002	0.117	0.164
	PC1	0.015	0.088	0.868	0.062	0.223	0.784	-0.055	0.047	0.257	0.107
	Tetrachlorobiphenyl	0.018	0.086	0.834	-0.030	0.127	0.813	0.010	0.006	0.133	0.155
Grey	Endosulfan I*	0.188	0.154	0.237	0.310	0.25	0.231	-0.079	0.064	0.233	0.385
	Endosulfan II*	0.215	0.157	0.188	0.181	0.39	0.649	-0.073	0.07	0.311	0.371
	17β-Estradiol*	**0.202**	**0.140**	**0.168**	**0.387**	**0.157**	**0.024**	**-0.514**	**0.222**	**0.033**	**0.486**
	Etiocholanolone*	0.202	0.153	0.203	0.462	0.167	0.013	-0.510	0.389	0.206	0.391
	Methoxychlor*	**0.166**	**0.141**	**0.254**	**-0.061**	**0.304**	**0.843**	**-0.009**	**0.004**	**0.035**	**0.482**
	p’,p’-DDE*	0.211	0.145	0.164	0.297	0.194	0.144	0	0	0.064	0.451
	PC1*	0.205	0.148	0.182	-0.055	0.372	0.884	0.140	0.079	0.092	0.433
	Sum PCBs*	0.202	0.153	0.203	0.380	0.201	0.076	-0.007	0.005	0.205	0.391
	Tetrachlorobiphenyl*	0.195	0.146	0.198	0.253	0.216	0.255	-0.020	0.011	0.075	0.443
Magenta	**17β-Estradiol**	**-0.215**	**0.072**	**0.008**	**-0.070**	**0.081**	**0.394**	**0.244**	**0.114**	**0.045**	**0.409**
	Etiocholanolone	**-0.222**	**0.067**	**0.004**	**-0.063**	**0.073**	**0.402**	**0.483**	**0.171**	**0.011**	**0.485**
	Methoxychlor*	-0.440	0.208	0.049	-0.103	0.448	0.820	0.003	0.006	0.559	0.183
	PC1	-0.214	0.079	0.014	0.021	0.199	0.917	-0.039	0.042	0.362	0.291
	Tetrachlorobiphenyl	-0.211	0.078	0.015	-0.060	0.115	0.608	0.006	0.006	0.308	0.299

Pink	Endosulfan I	-0.084	0.085	0.338	0.077	0.138	0.585	-0.039	0.035	0.289	0.177
	Endosulfan II	-0.060	0.082	0.473	-0.127	0.203	0.54	-0.063	0.036	0.101	0.248
	Endrin ketone	-0.078	0.085	0.373	0.132	0.104	0.218	-0.008	0.007	0.308	0.173
	17β-Estradiol	**-0.076**	**0.076**	**0.331**	**0.100**	**0.085**	**0.255**	**-0.295**	**0.120**	**0.024**	**0.345**
	Etiocholanolone	-0.072	0.079	0.373	0.122	0.086	0.175	-0.411	0.201	0.055	0.288
	Methoxychlor	-0.093	0.081	0.262	-0.085	0.173	0.629	-0.004	0.002	0.084	0.260
	PC1	-0.078	0.086	0.375	0.013	0.216	0.953	0.042	0.046	0.369	0.162
	Tetrachlorobiphenyl	-0.081	0.084	0.351	0.086	0.124	0.496	-0.007	0.006	0.249	0.187
Red	**Endosulfan II**	**0.140**	**0.08**	**0.096**	**-0.234**	**0.197**	**0.251**	**-0.079**	**0.035**	**0.039**	**0.292**
	17β-Estradiol	**0.119**	**0.078**	**0.144**	**0.075**	**0.087**	**0.397**	**-0.289**	**0.123**	**0.030**	**0.309**
	Etiocholanolone	**0.124**	**0.077**	**0.127**	**0.085**	**0.085**	**0.331**	**-0.472**	**0.197**	**0.028**	**0.315**
	Methoxychlor*	0.113	0.234	0.634	-0.020	0.504	0.968	-0.005	0.007	0.431	0.003
	PC1	0.118	0.086	0.188	-0.056	0.217	0.799	0.052	0.046	0.272	0.157
	Tetrachlorobiphenyl	0.114	0.086	0.200	0.058	0.126	0.651	-0.007	0.006	0.248	0.163

*Cube-root transformation

Table 2.2: Model results for associations between contaminants and hormones, with significant results bolded. EDC: endocrine disrupting compound; SE: standard error.

	EDC β	**EDC SE**	**EDC p-value**	**Pop. β**	**Pop. SE**	**Pop. p-value**	**R2**
17β-Estradiol							
Endosulfan II	0.083	0.065	0.23	0.028	0.339	0.936	0.257
Methoxychlor	**0.010**	**0.003**	**0.008**	**0.418**	**0.274**	**0.154**	**0.538**
Etiocholanolone							
Endosulfan II	0.046	0.043	0.313	-0.028	0.225	0.905	0.244

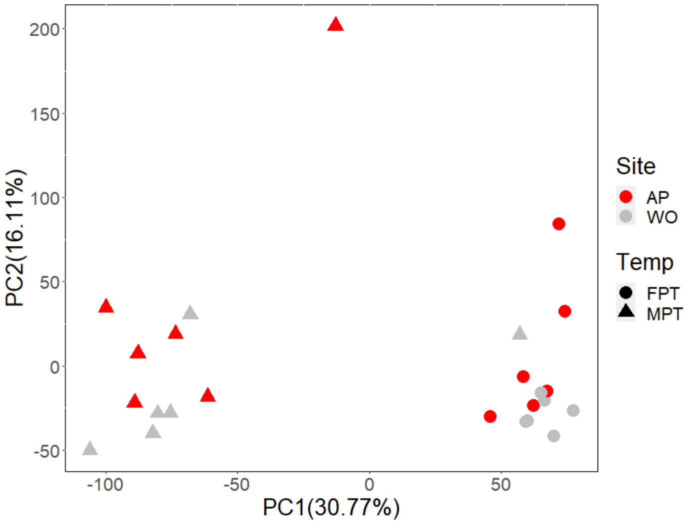


Figure 2.S1: Principal component analysis of expression values for all genes, showing libraries removed as outliers (circled samples).

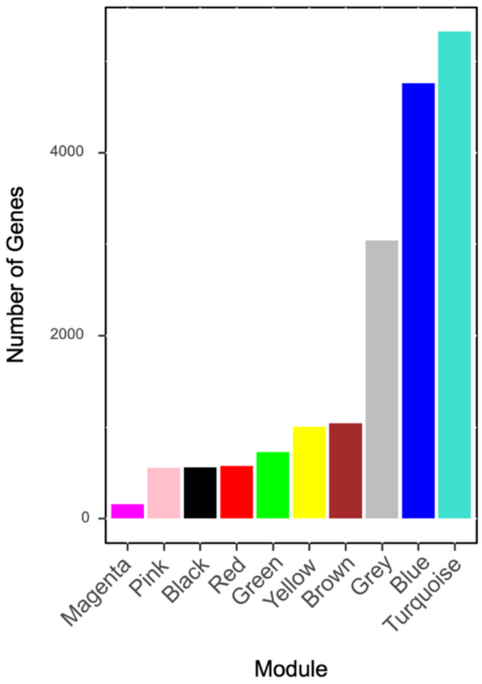
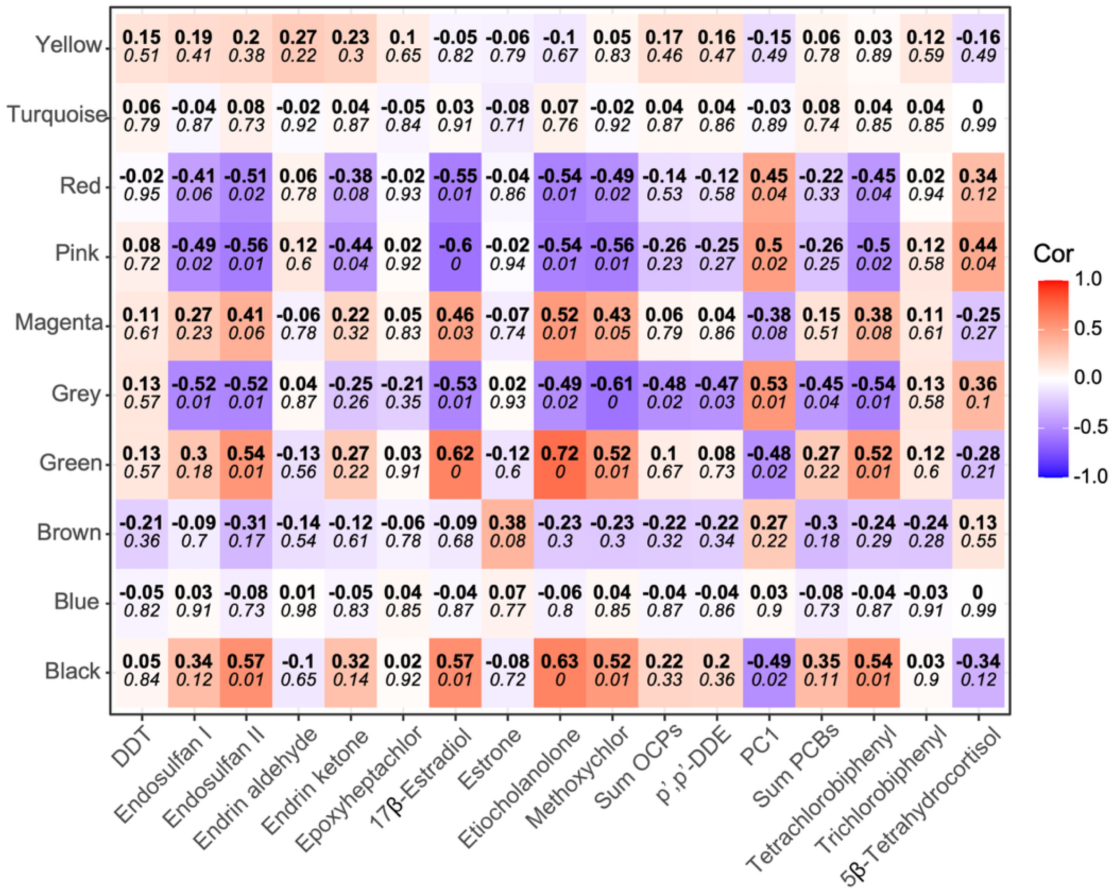


Figure 2.S2: Bar plot showing the number of genes placed in each module assembled from the WGCNA.



	DDT	Endosulfan I	Endosulfan II	Endrin aldehyde	Endrin ketone	Epoxyheptachlor	17β-Estradiol	Estrone	Etiocholanolone	Methoxychlor	Sum OCPs	p',p'-DDE	PC1	Sum PCBs	Tetrachlorobiphenyl	Trichlorobiphenyl	5β-Tetrahydrocortisol
Yellow	**0.15** *0.51*	**0.19** *0.41*	**0.2** *0.38*	**0.27** *0.22*	**0.23** *0.3*	**0.1** *0.65*	**-0.05** *0.82*	**-0.06** *0.79*	**-0.1** *0.67*	**0.05** *0.83*	**0.17** *0.46*	**0.16** *0.47*	**-0.15** *0.49*	**0.06** *0.78*	**0.03** *0.89*	**0.12** *0.59*	**-0.16** *0.49*
Turquoise	**0.06** *0.79*	**-0.04** *0.87*	**0.08** *0.73*	**-0.02** *0.92*	**0.04** *0.87*	**-0.05** *0.84*	**0.03** *0.91*	**-0.08** *0.71*	**0.07** *0.76*	**-0.02** *0.92*	**0.04** *0.87*	**0.04** *0.86*	**-0.03** *0.89*	**0.08** *0.74*	**0.04** *0.85*	**0.04** *0.85*	**0** *0.99*
Red	**-0.02** *0.95*	**-0.41** *0.06*	**-0.51** *0.02*	**0.06** *0.78*	**-0.38** *0.08*	**-0.02** *0.93*	**-0.55** *0.01*	**-0.04** *0.86*	**-0.54** *0.01*	**-0.49** *0.02*	**-0.14** *0.53*	**-0.12** *0.58*	**0.45** *0.04*	**-0.22** *0.33*	**-0.45** *0.04*	**0.02** *0.94*	**0.34** *0.12*
Pink	**0.08** *0.72*	**-0.49** *0.02*	**-0.56** *0.01*	**0.12** *0.6*	**-0.44** *0.04*	**0.02** *0.92*	**-0.6** *0*	**-0.02** *0.94*	**-0.54** *0.01*	**-0.56** *0.01*	**-0.26** *0.23*	**-0.25** *0.27*	**0.5** *0.02*	**-0.26** *0.25*	**-0.5** *0.02*	**0.12** *0.58*	**0.44** *0.04*
Magenta	**0.11** *0.61*	**0.27** *0.23*	**0.41** *0.06*	**-0.06** *0.78*	**0.22** *0.32*	**0.05** *0.83*	**0.46** *0.03*	**-0.07** *0.74*	**0.52** *0.01*	**0.43** *0.05*	**0.06** *0.79*	**0.04** *0.86*	**-0.38** *0.08*	**0.15** *0.51*	**0.38** *0.08*	**0.11** *0.61*	**-0.25** *0.27*
Grey	**0.13** *0.57*	**-0.52** *0.01*	**-0.52** *0.01*	**0.04** *0.87*	**-0.25** *0.26*	**-0.21** *0.35*	**-0.53** *0.01*	**0.02** *0.93*	**-0.49** *0.02*	**-0.61** *0*	**-0.48** *0.02*	**-0.47** *0.03*	**0.53** *0.01*	**-0.45** *0.04*	**-0.54** *0.01*	**0.13** *0.58*	**0.36** *0.1*
Green	**0.13** *0.57*	**0.3** *0.18*	**0.54** *0.01*	**-0.13** *0.56*	**0.27** *0.22*	**0.03** *0.91*	**0.62** *0*	**-0.12** *0.6*	**0.72** *0*	**0.52** *0.01*	**0.1** *0.67*	**0.08** *0.73*	**-0.48** *0.02*	**0.27** *0.22*	**0.52** *0.01*	**0.12** *0.6*	**-0.28** *0.21*
Brown	**-0.21** *0.36*	**-0.09** *0.7*	**-0.31** *0.17*	**-0.14** *0.54*	**-0.12** *0.61*	**-0.06** *0.78*	**-0.09** *0.68*	**0.38** *0.08*	**-0.23** *0.3*	**-0.23** *0.3*	**-0.22** *0.32*	**-0.22** *0.34*	**0.27** *0.22*	**-0.3** *0.18*	**-0.24** *0.29*	**-0.24** *0.28*	**0.13** *0.55*
Blue	**-0.05** *0.82*	**0.03** *0.91*	**-0.08** *0.73*	**0.01** *0.98*	**-0.05** *0.83*	**0.04** *0.85*	**-0.04** *0.87*	**0.07** *0.77*	**-0.06** *0.8*	**0.04** *0.85*	**-0.04** *0.87*	**-0.04** *0.86*	**0.03** *0.9*	**-0.08** *0.73*	**-0.04** *0.87*	**-0.03** *0.91*	**0** *0.99*
Black	**0.05** *0.84*	**0.34** *0.12*	**0.57** *0.01*	**-0.1** *0.65*	**0.32** *0.14*	**0.02** *0.92*	**0.57** *0.01*	**-0.08** *0.72*	**0.63** *0*	**0.52** *0.01*	**0.22** *0.33*	**0.2** *0.36*	**-0.49** *0.02*	**0.35** *0.11*	**0.54** *0.01*	**0.03** *0.9*	**-0.34** *0.12*

Figure 2.S3: Heatmap of Pearson correlations between module eigengene values and hormones and contaminants that differed across populations. Top, bolded numbers are correlation values; bottom, italicized numbers are p-values.

Table 2.S1: All contaminants and hormones measured in egg yolk with limit of detection (LOD) values. HCH: Hexachlorocyclohexane; DHP: Dihydroprogesterone

Compound	LOD (ng/g)
a-HCH	0.1
b-HCH	0.1
y-HCH	0.1
d-HCH	0.1
Heptachlor	0.1
Aldrin	0.1
Epoxyheptachlor	0.2
Endosulfan I	0.5
p',p’-DDE	0.5
Dieldrin	0.2
Endrin	0.2
Endosulfan II	0.5
Endrin aldehyde	0.1
DDT	0.1
Endrin ketone	0.5
Methoxychlor	0.5
Monochlorobiphenyl	0.8
Dichlorobiphenyl	0.8
Trichlorobiphenyl	0.8
Tetrachlorobiphenyl	0.8
Pentachlorobiphenyl	0.8
Hexachlorobiphenyl	0.8
Heptachlorobiphenyl	0.8
Octachlorobiphenyl	0.8
DHP	0.2
20b-DHP	0.2
Cortisone	0.5
Cortisol	0.5
Estrone	2.0
17β-estradiol	10
Progesterone	0.2
Etiocholanolone	10
Testosterone	0.2
Androstenedione	0.2
5b-dihydrocortisol	2.0
5b-tetrahydrocortisol	2.0
b-cortol	200
5b-dihydrocortisone	0.5
Pregnanedione	0.5
Pregnenolone	2.0
17a-hydroxyprogesterone	0.2

Deoxycorticosterone	1.0
11-deoxycortisol	0.2
Corticosterone	0.2
11-ketotestosterone	0.5
17a-hydroxypregnenolone	10
11b-hydroxypresterone	1.0
5b-tetrahydrocorticosterone	50
11-tetrahydrocorticosterone	2.0
5b-corticosterone	2.0
20b-dihydrocorticosterone	0.5
Pregnanolone	0.5

Table 2.S2: Model results for hormone concentrations across populations, with significant results bolded. SE: standard Error; DHP: dihydroprogesterone.

Hormone	**Pop. β**	**Pop. SE**	**Clutch**	**Clutch SE**	**Resid.**	**Resid. SE**	**p-value**
DHP*	0.261	0.212	0.0470	0.216	0.354	0.595	0.238
Estrone	**7.696**	**3.133**	**31.211**	**5.587**	**15.847**	**3.981**	**0.029**
17β-Estradiol	**-0.363**	**0.158**	**0.0400**	**0.201**	**0.153**	**0.392**	**0.039**
Androstenedione	-1.300	1.814	9.5900	3.097	7.885	2.808	0.487
Etiocholanolone	**-0.240**	**0.104**	**0.0230**	**0.151**	**0.051**	**0.227**	**0.038**
Progesterone	20.401	30.26	1838.60	42.88	4625.93	68.014	0.512
Testosterone*	-0.041	0.167	0.073	0.271	0.09	0.301	0.808
5β-Corticosterone*	0.039	0.076	0	0	0.063	0.251	0.605
20β-Dihydrocorticosterone*	0.915	0.541	0.381	0.618	2.087	1.445	0.114
5β-Tetrahydrocortisol$^+$	**0.276**	**0.079**	**0.009**	**0.093**	**0.043**	**0.209**	**0.004**
5β-Dihydrocortisol*	0.107	0.164	0.036	0.191	0.189	0.434	0.528
Pregnanolone*	-0.024	0.256	0.113	0.336	0.389	0.624	0.925
Pregnenolone*	0.106	0.167	0.059	0.244	0.133	0.364	0.540
17a-Hydroxyprogesterone*	0.100	0.234	0.148	0.384	0.167	0.408	0.677
Deoxycorticosterone	0.039	0.032	0.002	0.042	0.006	0.076	0.240
Pregnanedione*	0.271	0.290	0.191	0.437	0.362	0.602	0.367

*Log transformation.; $^+$cube-root transformation

Table 2.S3: Test results for contaminant concentrations across populations, with significant results bolded. OCP: organochlorine pesticide; PCB: polychlorinated biphenyl; HCH: hexachlorocyclohexane.

Contaminant	**W**	**p-value**
OCPs		
α-HCH	34	0.524
β-HCH	36	0.350
γ-HCH	41.5	0.129
δ-HCH	43	0.093
Heptachlor	41	0.148
Aldrin	26	0.862
Epoxyheptachlor	**48**	**0.024**
Endosulfan I	**52**	**0.007**
p',p' -DDE	**56**	**0.001**
Dieldrin	23	0.640
Endrin	36	0.385
Endosulfan II	**56**	**0.001**
Endrin aldehyde	**48**	**0.023**
DDT	**56**	**0.001**
Endrin ketone	**47**	**0.032**
Methoxychlor	**56**	**0.001**
OCP Sum	**56**	**0.001**
PCBs		
Monochlorobiphenyl	44	0.072
Dichlorobiphenyl	40	0.182
Trichlorobiphenyl	**45**	**0.049**
Tetrachlorobiphenyl	**51**	**0.009**
PCB Sum	**46**	**0.043**

CHAPTER 3

THE INFLUENCE OF INCUBATION TEMPERATURE ON OFFSPRING TRAITS VARIES ACROSS NORTHERN AND SOUTHERN POPULATIONS OF THE AMERICAN ALLIGATOR (*ALLIGATOR MISSISSIPPIENSIS*)[2]

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[2] Smaga, C. R., Bock, S. L., Johnson, J. M., Rainwater, T., Singh, R., Deem, V., Letter, A., Brunell, A., & Parrott, B. B. (2024). *Ecology and Evolution*, *14*(2), e10915. https://doi.org/10.1002/ece3.10915. Reprinted here with permission of publisher.

Abstract

Maternal provisioning and the developmental environment are fundamental determinants of offspring traits, particularly in oviparous species. However, the extent to which embryonic responses to these factors differ across populations to drive phenotypic variation is not well understood. Here, we examine the contributions of maternal provisioning and incubation temperature to hatchling morphological and metabolic traits across four populations of the American alligator (*Alligator mississippiensis*), encompassing a large portion of the species' latitudinal range. Our results show that whereas the influence of egg mass is generally consistent across populations, responses to incubation temperature show population-level variation in several traits, including mass, head length, head width and residual yolk mass. Additionally, the influence of incubation temperature on developmental rate is greater at northern populations, while the allocation of maternal resources towards fat body mass is greater at southern populations. Overall, our results suggest that responses to incubation temperature, relative to maternal provisioning, are a larger source of interpopulation phenotypic variation and may contribute to the local adaptation of populations.

Introduction

Developmental plasticity, the expression of alternative phenotypes under different environmental conditions, is a fundamental driver of phenotypic variation across organismal and population level scales. Organismal responses to the developmental environment can be adaptive, neutral, or mal-adaptive (Forsman, 2015; Ghalambor et al., 2007), having important implications for both ecology and evolution (Miner et al., 2005;

West-Eberhard, 1989, 2003). For example, when conditions experienced during development provide reliable cues of later life environments, developmental plasticity can be adaptive by maximizing phenotype-environment matching (Nettle & Bateson, 2015; Pfennig, 1990). Alternatively, environments that disrupt normal developmental processes can lead to plastic responses with negative effects on fitness (Barker, 2001; Guillette et al., 1995), while physical constraints on development can give rise to plasticity that is neutral with respect to fitness (Ghalambor et al., 2007; Gotthard & Nylin, 1995). Regardless of their adaptive value, many embryonic responses to the developmental environment have a heritable, genetic basis and can vary, suggesting they can evolve under novel selective pressures (Pigliucci, 2005).

In oviparous vertebrates, maternal provisioning of nutrients and signaling molecules is critical for proper development and can be a major determinant of offspring traits (Groothuis et al., 2005; Radder et al., 2007; Van Dyke & Griffith, 2018). Complex biological and ecological factors, including maternal diet (Royle et al., 2003; Warner & Lovern, 2014), stress (McCormick, 1998; Saino et al., 2005), and age (Beamonte-Barrientos et al., 2010; Urvik et al., 2018) can influence the quantity and quality of resources provisioned to embryos (Moore et al., 2019; Mousseau & Fox, 1998). However, other components of the developmental environment can influence how maternal resources are utilized by developing embryos (Brown et al., 2011; Du and Shine, 2022, 2008; Mueller et al., 2015; Shine and Brown, 2002). For example, egg mass is a primary determinant of hatchling mass (Deeming & Birchard, 2007), but incubation temperature has been shown to influence diverse hatchling phenotypes across many species (While et al., 2018). This includes modifying the efficiency by which maternal resources are converted into somatic tissue (Bock et al., 2021; Marshall et al., 2020; Pettersen et al., 2019) and how those resources are allocated to specific phenotypes (Flatt, 2001; Telemeco et al., 2010). However, despite the importance of maternal provisioning and incubation temperature in modifying hatchling phenotypes, the extent to which responses to these factors vary across populations is not well resolved (but see (Bodensteiner et al., 2019; Orizaola & Laurila, 2009, 2016; Richter-Boix et al., 2015)).

When viewed through the lens of Developmental Cost Theory (DCT, Marshall et al., 2020), the influence of incubation temperature on maternal resource use represents a fundamental developmental constraint (Gotthard and Nylin, 1995). According to DCT, the energy required for development can be quantified as the product of development rate and metabolic rate (Pettersen et al., 2019). Whereas temperature affects both developmental and metabolic rates, differences in their temperature-dependence results in an optimal temperature at which developmental cost is minimized. As a result, environmental temperatures typically encountered by embryos in nature are tightly correlated to species-specific thermal optima that minimize developmental cost (Marshall et al., 2020; Pettersen et al., 2019). Deviations from these optima are predicted to decrease developmental efficiency and result in reduced size, growth, and energy reserves of individuals. Importantly, responses of metabolic rate and developmental rate to temperature can be decoupled (Pettersen, 2020; Williams et al., 2016). Therefore, thermal dependencies of metabolic and/or development rate can evolve independently, allowing selection to modify the temperature at which developmental cost is minimized under novel thermal environments (Pettersen, Ruuskanen, et al., 2023).

In species with broad geographic ranges, divergent climatic conditions have the potential to exert novel selective pressures on traits influenced by the developmental environment (Conover & Schultz, 1995; Kawecki & Ebert, 2004; Merilä et al., 2000; Orizaola & Laurila, 2009). Populations inhabiting high altitudes and latitudes are often exposed to colder temperatures (Angilletta, 2009), which impose novel thermal constraints on development. To compensate, populations can adapt by altering the thermal sensitivity of developmental processes. For instance, in oviparous reptiles, cooler incubation temperatures can result in longer incubation duration. Embryos from high altitude and latitude populations compensate by displaying faster development rates when compared to those from lower altitudes or latitudes under identical incubation temperatures (Du et al., 2010a; Pettersen, 2020), regardless of egg size (Storm & Angilletta, 2007). These opposing effects of genetic and environmental influences on developmental rate, known as counter-gradient variation (Conover & Schultz, 1995), are thought to reduce the cost of development and allow more time for offspring to acquire resources prior to colder, harsher winters (Olsson & Shine, 1997; Pettersen, 2020). Similarly, high altitude populations of wall lizards (*Podacris uralis*) have been shown to allocate more maternal resources towards somatic tissue relative to low altitude populations when raised at a common temperature (Pettersen, Ruuskanen, et al., 2023). However, our understanding of the extent to which populations vary in how maternal provisioning and incubation temperature shape fitness-related traits in taxonomically diverse species is limited (While et al., 2018).

In the present study, we test whether populations vary in embryonic responses to maternal provisioning and incubation temperature in the American alligator (*Alligator* *mississippiensis*). The alligator’s latitudinal range extends from southern Florida to northeastern North Carolina (Elsey et al., 2019), providing potential for local adaptation of phenotypic responses to the developmental environment. Few studies have examined variation in nest temperatures across the alligator’s range, but comparisons between a northern and southern population did not find significant differences in mean nest temperature (Bock, Lowers, et al., 2020). However, this was based on only three years of overlap between populations, and within each year, the mean nest temperature of southern populations was greater than that of northern populations (Bock, Lowers, et al., 2020). Cooler temperatures at northern latitudes would presumably decrease developmental rate, increase the cost of development and delay hatching dates, reducing time for resource acquisition prior to winter (Olsson & Shine, 1997). Despite these potential differences, there is little information on how responses to the developmental environment vary across the alligator’s range.

Like many turtles and some lizards, alligators display temperature-dependent sex determination (TSD), in which thermal signals experienced during a discrete developmental window determine sex, along with additional phenotypic traits (Allsteadt & Lang, 1995; Bock et al., 2021; Kohno et al., 2014; McCoy et al., 2016). Specifically, incubations at warmer, male-promoting temperatures (MPT) reduce developmental costs, producing larger hatchlings with greater residual yolk reserves when compared to incubations at cooler female-promoting temperatures (FPT, Allsteadt and Lang, 1995; Bock et al., 2023, 2021). Recent reports demonstrate that temperature-sensitive traits, including body mass index (BMI) and snout-vent length (SVL), are associated with higher juvenile survival at MPT in the alligator (Bock et al., 2023; Johnson et al., 2023).

However, these appear to be context dependent as the relationship between phenotypic traits and survival varies across years (Bock et al., 2023). Nonetheless, given that warmer, MPT appears to be the optimum developmental temperature in this species, we hypothesize that northern populations, presumably exposed to cooler temperatures, will show compensatory responses to incubation temperature. Using a common garden incubation and grow out design, we resolve the relative influences of incubation temperature and maternal provisioning on aspects of developmental cost (hatchling mass and incubation duration), along with other morphological (SVL, tail girth (TG), head length (HL), head width (HW), BMI) and metabolic (10-day growth, residual yolk mass and fat body mass) traits across populations. We predict that northern populations will display greater mass and developmental and growth rates relative to southern populations at cooler incubation temperatures. Additionally, we predict that northern populations will have increased residual energy reserves (residual yolk mass and fat body mass), decreasing the need to acquire resources after development prior to winter.

Methods

Experimental design and data collection

In June and July of 2021, 7-8 clutches (eggs from one nest originating from the same female) of alligator eggs were collected from each of four, geographically distinct populations (total n=1,378), including Par Pond on the United States Department of Energy's Savannah River Site in Aiken, South Carolina (South Carolina West, SCW), Tom Yawkey Wildlife Center in Georgetown, South Carolina (South Carolina East, SCE), Lake Woodruff National Wildlife Refuge in De Leon Springs, Florida (Florida

East, FLE), and Lake Apopka in Apopka, Florida (Florida West, FLW; **Figure 3.1a**). After locating nests by helicopter or airboat, all eggs were removed from a nest cavity within two weeks of oviposition. Eggs were placed in plastic bus pans with nesting material from natural nests and driven back to the University of Georgia's Savannah River Ecology Laboratory (SREL) in Aiken, SC (within 4-24 hours after egg collection), where they were individually weighed and 1-2 eggs from each clutch were staged according to Ferguson (1985) to determine stage at collection. The remaining eggs were transferred into new bus pans with dampened sphagnum moss and kept in commercial incubators (model I36NLC, Percival Scientific, Perry, IA, USA) at 32°C, an intermediate temperature that produces mixed sex ratios (Lang & Andrews, 1994). During this period, eggs were misted twice daily, and bins were rotated once daily within each incubator to limit the effect of intra-incubator temperature variation. Incubator temperatures were also monitored with HOBO TidbiT® v2 Temp Loggers (Onset, Bourne, MA, USA).

Embryonic stage 15 (occurring approximately 15 days post-oviposition), just prior to the opening of the thermosensitive period of sex determination (McCoy et al., 2015), was predicted based on the stage of eggs at collection and eggs from each population were randomly assigned in a split-clutch design to one of two temperature treatments: a constant MPT (33.5°C) or a constant FPT (29.5°C). Since full clutches were collected for multiple studies, a random subset of 3-10 eggs/clutch/temperature/site were chosen at this time to raise until hatch for this experiment. Throughout the entire incubation period, eggs were continually monitored as above. While it is increasingly noted that constant temperatures may not be reflective of natural nest conditions (Bowden et al., 2014; Hall & Warner, 2020), the temperatures utilized here have been previously examined in the alligator with known effects on hatchling phenotypes, providing a basis with which to compare our results.

Once embryos pierced the eggshell (“pipped”), the date was recorded, and eggs were placed in glass Mason jars (one egg/jar) with damp, sphagnum moss. Embryos were given 48 hours to hatch from the egg before being assisted if they did not hatch on their own. Once fully hatched, individuals were weighed using a digital balance (±0.01g) and SVL and TG were measured using a flexible ruler (±0.1 mm), and HL and HW were measured using calipers (±1 mm). Hatchlings were then individually marked using unique, numbered toe tags and transported to large, indoor, fiberglass holding tanks where they were held at the SREL aquatic animal facility for 10-days. The aquatic animal holding facility is a semi-climate controlled building with translucent fiberglass ceilings, mimicking natural light cycles and maintaining temperatures between 21 and 29°C (Johnson et al., 2023; Tuberville et al., 2016). During this period, hatching alligators relied on maternal yolk reserves and were not fed (Allsteadt & Lang, 1995). Water was changed daily (using tap water), and hatchlings were monitored visually twice daily for overall health and survival. At 10-days post-hatch (10-DPH), hatchlings were remeasured, euthanized via cervical severance and pithing, and dissected to obtain residual yolk mass and fat body mass. Phenotypes analyzed included morphological traits of mass, SVL, TG, HL, HW and body condition (BMI: mass/2*SVL) at hatch, and metabolic traits including incubation duration (measured in days from stage 15 to pip), change in morphological traits between 10-DPH and hatch (Δ mass Δ BMI, Δ SVL, Δ TG), residual yolk mass, and fat body mass. All experiments were approved by the University of Georgia Animal Care and Use Committee (A2021 05-007-Y3-A0) and collections were carried out under permits from the South Carolina Department of Natural Resources (SC-08-2021) and Florida Fish and Wildlife Conservation Commission (SPGS-18-33).

Statistical analysis

All statistical analyses were conducted in RStudio (R Core Team 2021, version 4.1.2) and all models were built using the *lme4* package (Bates et al. 2015). Model assumptions of residual normality and homoscedasticity were checked visually via residual vs fitted and Q-Q plots, with log transformations made for residual yolk and fat body mass to best meet assumptions. To compare initial egg mass across populations, we used a linear mixed-effects model (LMM) including a fixed-effect of site and random intercepts to control for clutch effects. To determine whether hatch probability or survival to 10-DPH differed across temperatures or sites, we used a generalized linear-mixed model with a binomial distribution including temperature, site, and their interaction as fixed effects, including random intercepts of clutches nested within sites. Post-hoc pairwise comparisons were conducted using the *emmeans* package (Lenth et al. 2023) with Kenward-Roger degrees of freedom and correcting for multiple testing using Tukey's method.

To test for differences in the relative contributions of egg mass and incubation temperature to phenotypic traits across populations, we constructed separate LMMs for every phenotype at each site. In every model, we included fixed effects of egg mass and incubation temperature, while controlling for clutch effects using random intercepts. We then compared model estimates across populations by extracting beta values (i.e., effect size estimates) and 95% confidence intervals (CIs) using the confint function in R. Model beta estimates in which CIs did not overlap zero or another population were considered statistically significant.

To further examine how embryos respond to temperature and maternal provisioning across populations, we used the *ggeffects* package (Lüdecke 2018) to predict temperature-specific mean values of each phenotype at a common egg mass, corresponding to the average egg mass across the dataset ($\bar{x}$ = 82.75g, SD = 9.99), from each population-specific model. By comparing egg mass-corrected mean phenotypes across temperatures and populations, we were able to determine whether populations differed in mean trait values irrespective of egg mass at either or both temperatures and whether variation in the influence of incubation temperature was driven by phenotypic differences at 29.5°C, 33.5°C, or both. Mean values in which 95% CIs did not overlap were considered statistically significant.

Given that populations can also vary in how maternal resources are allocated towards particular phenotypes, we compared ratios of SVL, TG, HL, HW, residual yolk mass and fat body mass to hatchling mass across populations within and across temperatures using LMMs. For this analysis, we included temperature, site, and their interaction as predictors, along with egg mass as a covariate, controlling for clutches nested within sites using random intercepts. We then compared predicted mean values from the model within and among temperatures across populations using the *emmeans* package. Values in which CIs did not overlap were considered statistically significant. We used ratios of traits to hatchling mass instead of egg mass for this analysis because there were significant differences in temperature-specific mass across populations (see below), and as a result, differences in the ratio of traits to egg mass would be confounded by population-specific effects of temperature on mass and may not represent differences in the allocation of maternal resources towards specific phenotypes. All figures were created using the R package *ggplot2* (Wickham 2016).

Results

Egg mass and survival

Egg masses at the two southern populations (FLW: $\bar{x} = 85.1$, SE: 3.08; FLE: $\bar{x} = 86.9$, SE: 3.29) were greater relative to the two northern populations (SCW: $\bar{x} = 74.9$, SE: 3.31 and SCE: $\bar{x} = 80.9$, SE: 3.08), but only a nearly significant difference was observed between SCW and FLE ($\beta = -11.98$, $t = -2.57$, $p = 0.073$; **Figure 3.1b**). Whereas hatch rates were lower at 29.5°C (59.2%) compared to 33.5°C (82.9%; $\beta = 1.12$, $z = 2.08$, $p = 0.038$), differences were not observed between sites at either temperature (all pairwise $p > 0.23$). There were also no differences in survival between sites (all pairwise $p =1$) or temperatures ($p = 1$) during the 10-day growth period, with 79 (94%) and 114 (94%) animals surviving at 29.5°C and 33.5°C, respectively. Final sample sizes of surviving individuals by temperature, clutch, and site are shown in Table 3.1.

Morphological traits

Both egg mass and temperature exerted positive effects on hatchling mass across all populations (Table 3.2). However, whereas the influence of egg mass did not differ across sites (**Figure 3.2a**), temperature more strongly affected hatchling mass at SCE compared to the other three populations (SCW: $\beta = 2.223$, CI = (0.035, 4.637); SCE: $\beta = 6.948$, CI = (5.758, 8.224); FLW: $\beta = 2.755$, CI = (1.314, 4.255); FLE: $\beta = 3.242$, CI = (1.355, 5.004); **Figure 3.2b**). In addition, there was a trend for a greater influence of incubation temperature on SVL at the northern populations relative to the southern populations, with the influence of temperature on SVL not significant in the latter (SCW: β = 0.438, CI = (0.058, 0.817); SCE: β = 0.799, CI = (0.557, 1.041); FLW: β = 0.136, CI = (-0.135, 0.395); FLE: β = 0.117, CI = (-0.127, 0.361); **Figure 3.2c**). Across other morphological traits, the influence of temperature was variable in both direction and magnitude, with significant differences between SCE and FLE for TG (SCE: β = 0.186, CI = (0.060, 0.314); FLE: β = -0.091, CI = (-0.183, -0.004)), HL (SCE: β = 1.382, CI = (0.938, 1.825); FLE: β = 0.001, CI = (-0.527, -0.462)), and HW (SCE: β = 0.176, CI = (-0.192, 0.544); FLE: β = -0.670, CI = (-0.968, -0.373)). Meanwhile, the influence of egg mass was not different across populations for any trait (Table 3.2).

We next examined the extent to which morphological phenotypes varied across populations within a temperature, including whether differences in the influence of incubation temperature were driven by variation at 33.5°C, 29.5°C, or both by comparing model means under a common egg mass. There were significant differences in trait values between at least two populations for all morphological traits after controlling for egg mass differences, with interpopulation variation in morphological traits occurring primarily at 29.5°C (Table 3.S1). For instance, the influence of incubation temperature on mass of SCE hatchlings was primarily driven by a reduction in mass at 29.5°C relative to the other populations (SCW: x̄ = 51.41, CI = (48.60, 54.22); SCE: x̄ = 47.49, CI = (46.48, 48.50); FLW: x̄ = 51.54, CI = (49.38, 53.69); FLE: x̄ = 52.80, CI = (51.16, 54.45); **Figure 3.2b**). This pattern was mostly consistent across additional traits that were differentially impacted by incubation temperature. Both HL (SCW: x̄ = 35.78, CI = (34.89, 36.67); SCE: x̄ = 34.46, CI = (34.12, 34.81); FLW: x̄ = 35.87, CI = (35.41, 36.34); FLE: x̄ = 36.90, CI = (36.47, 37.32)) and HW (SCW: x̄ = 21.10, CI = (20.38, 21.83); SCE: x̄ = 20.01, CI = (19.72, 20.29); FLW: x̄ = 20.82, CI = (20.42, 21.22); FLE: x̄ = 21.24, CI = (21.01, 21.47)) were reduced at SCE relative to the other populations and TG was reduced at SCE relative to FLE (SCE: x̄ = 4.54, CI = (4.44, 4.64); FLE: x̄ = 4.93, CI = (4.82, 5.04)). The exception was SVL, which appeared to involve differences at both 29.5°C (SCW: x̄ = 11.88, CI = (11.45, 12.31); SCE: x̄ = 11.57, CI = (11.38, 11.75); FLW: x̄ = 11.88, CI = (11.61, 12.16); FLE: x̄ = 12.12, CI = (11.93, 12.31)) and 33.5°C (SCW: x̄ = 12.32, CI = (12.01, 12.63); SCE: x̄ = 12.37, CI = (12.21, 12.53); FLW: x̄ = 12.02, CI = (11.79, 12.25); FLE: x̄ = 12.23, CI = (12.06, 12.40); **Figure 3.2c**). Ratios of morphological traits to hatchling mass showed no significant differences across populations at either temperature (Table 3.S1).

Metabolic traits

As with morphological traits, we also examined the effect of egg mass and incubation temperature on metabolic traits across populations. As egg mass increased, Δmass decreased at the two southern populations, but had no effect in northern populations (Table 3.2). However, comparison of beta values across sites showed only a significant difference between FLW and SCW (FLW: β = -0.086, CI = (-0.120, -0.053); SCW: β = 0.009, CI = (-0.048, 0.065)). A positive influence of egg mass on residual yolk mass was observed across all populations except for FLW, but differences across populations were not significant. Incubation temperature did not affect Δmass or ΔBMI at any population, but exerted negative influences on ΔSVL at SCE and fat body mass at all populations (Table 3.2, **Figure 3.3a**). On the other hand, there was a significantly positive influence of incubation temperature on ΔTG and residual yolk mass (Table 3.2,

Figure 3.3b) in at least one population. Whereas the effect sizes of temperature on ΔSVL and ΔTG did not differ across sites, the influence of temperature on residual yolk mass and fat body mass did. Compared to FLE, the influence of temperature was larger at SCE for fat body mass (SCE: β = -0.766, CI = (-0.877, -0.659); FLE: β = -0.528, CI = (-0.644, -0.415); Table 3.2; **Figure 3.3a**) while the opposite was true for residual yolk mass (SCE: β = 0.149, CI = (-0.031, 0.316); FLE: β = 0.594, CI = (0.457, 0.735); Table 3.2; **Figure 3.3b**).

When comparing metabolic phenotypes across populations after correcting for egg mass, we found significant differences in fat body mass between SCE and both FLE and FLW at 29.5°C (SCW: x̄ = 0.23, CI = (0.17, 0.31); SCE: x̄ = 0.21, CI = (0.19, 0.24); FLW: x̄ = 0.32, CI = (0.27, 0.37); FLE: x̄ = 0.30, CI = (0.26, 0.36)) and 33.5°C (SCW: x̄ = 0.13, CI = (0.10, 0.16); SCE: x̄ = 0.10, CI = (0.09, 0.11); FLW: x̄ = 0.16, CI = (0.14, 0.18); FLE: x̄ = 0.18, CI = (0.15, 0.21)), with a trend for smaller fat body masses at the northern populations (**Figure 3.3a**; Table 3.S1). Consistent with the decreased influence of incubation temperature on residual yolk mass at SCE, animals from 29.5°C at SCE had significantly higher residual yolk mass compared to FLE (SCE: x̄ = 3.76, CI = (3.21, 4.40)), FLE: x̄ = 2.55, CI = (2.15, 3.02)); **Figure 3.3b**; Table 3.S1). Upon examination of the mass-corrected allocation of maternal resources towards metabolic phenotypes, there were significant differences for both residual yolk mass and fat body mass across populations. Animals from the southern populations tended to allocate more resources towards fat body mass than the northern populations at both 29.5°C (SCW: x̄ = 0.0045, CI = (0.0036, 0.0054); SCE: x̄ = 0.0045, CI = (0.0038, 00052); FLW: x̄ = 0.0063, CI = (0.0055, 0.0070; FLE: x̄ = 0.0061, CI = (0.0054, 0.0068)) and 33.5°C (SCW: x̄ = 0.0023, CI = (0.0016, 0.0031); SCE: x̄ = 0.0018, CI = (0.0012, 00025); FLW: x̄ = 0.0030, CI = (0.0024, 0.0036; FLE: x̄ = 0.0033, CI = (0.0026, 0.0039); **Figure 3.3c**), and animals from SCE at 29.5°C allocated more resources towards residual yolk mass relative to SCW and FLE (SCW: x̄ = 0.0129, CI = (0.0078, 0.0179); SCE: x̄ = 0.0272, CI = (0.0233, 0.0311); FLW: x̄ = 0.0215, CI = (0.0175, 0.0255; FLE: x̄ = 0.0179, CI = (0.0140, 0.0217); **Figure 3.3d**).

There was no significant influence of egg mass on incubation duration at any population, whereas incubation temperature had a negative influence on incubation duration across all sites (**Figure 3.4**). The influence of temperature was greater at the northern populations than at the southern populations (SCW: β = -14.16, CI = (-15.22, -13.15); SCE: β = -13.07, CI = (-13.51, -12.62); FLW: β = -10.86, CI = (-11.95, -9.71); FLE: β = -11.08, CI = (-12.02, -10.14), driven by comparatively shorter incubation periods at 33.5°C (SCW: x̄ = 45.19, CI = (44.30, 46.08); SCE: x̄ = 45.18, CI = (44.52, 45.85); FLW: x̄ = 46.05, CI = (45.14, 46.96); FLE: x̄ = 46.79, CI = (45.45, 48.14)) and longer incubation periods at 29.5°C (SCW: x̄ = 59.34, CI = (58.12, 60.57); SCE: x̄ = 58.25, CI = (57.56, 58.94); FLW: x̄ = 56.91, CI = (55.70, 58.11); FLE: x̄ = 57.87, CI = (56.45, 59.29); **Figure 3.4**). However, differences across sites within temperatures were not significant (Table 3.S1).

Discussion

Patterns of population-level variation in embryonic responses to maternal provisioning and environmental factors have the potential to inform how the developmental environment contributes to evolutionary change. We observed that, generally, the influence of maternal provisioning on hatchling traits did not vary across populations; however, incubation temperature exerted population-specific effects on both morphological and metabolic traits. This may be explained by a constrained relationship between egg mass and hatch mass (Deeming & Birchard, 2007), which is expected to be under strong selection as hatchling mass is often an important component of survival and fitness (Ronget et al., 2018; Stearns, 2000). Rather than alter this relationship, selection instead tends to act on aspects of maternal allocation, such as egg size and number, to best match population-specific conditions (Angilletta et al., 2004; Sinervo, 1990). On the other hand, responses to incubation temperature may be in part the result of differences in natural nest temperatures across populations (Bock, Lowers, et al., 2020; Du et al., 2019). Such differences likely select for embryonic responses to temperature that reduce developmental cost and decrease the need to acquire resource prior to colder, harsher winters at northern latitudes (Pettersen, Ruuskanen, et al., 2023). Our results suggest that plastic responses to incubation temperature, but not maternal provisions, vary across populations and have potential to be modified by selection.

The four populations examined in this study encompassed a large proportion of the alligator's latitudinal range, with two populations from the northern extent and two populations from the southern extent. While not statistically significant, we observed a trend for smaller egg masses at the northern populations relative to the southern populations. In crocodylians, egg mass scales with maternal body size (Larriera et al., 2004), and differences in maternal size might underlie population differences observed here. In mammals, animals from high latitudes tend to be larger than those from low latitudes in a pattern known as Bergmann's rule (Blackburn et al., 1999), and while this seems to hold in turtles and birds, it does not in other reptiles, such as squamates (Ashton, 2002; Ashton & Feldman, 2003) and has not been examined in crocodylians. On the other hand, trade-offs between offspring size and number have been shown to vary, with fewer, larger offspring favored in colder environments and later in the reproductive season (Angilletta et al., 2004; Hall et al., 2020). Nonetheless, larger egg sizes at southern populations does not support either of these hypotheses. Alternatively, allometric relationships between maternal size and egg mass can be altered by environmental conditions, such as salinity stress (Murray et al., 2013). Given the lack of information on nesting females here, it remains unknown whether differences in egg size are the result of variation in maternal size across populations (maximum size or age at reproduction), population-specific allometric relationships, or differences in maternal allocation and is an interesting area of future research.

We hypothesized that northern populations would show evidence of adaptation to cooler environments by altering embryonic responses to temperature, resulting in faster development and increased mass and growth at cool incubation temperatures. However, only a few traits showed evidence of latitudinal patterns. We found that incubation duration was more strongly influenced by incubation temperature at the northern populations relative to the southern populations. Specifically, embryos from northern populations developed slightly slower at cooler temperatures and faster at warmer temperatures compared to southern populations. Latitudinal differences in incubation duration have been shown in several species and generally follow one of two patterns: co-gradient variation, in which cooler populations development more slowly relative to warmer populations and counter-gradient variation, in which cooler populations development more quickly than warmer populations (Conover & Schultz, 1995; Pettersen, 2020). While our differences within temperatures were not significant, they followed patterns of both co-gradient variation (at 29.5°C) and counter-gradient variation (at 33.5°C), which only partially support our predictions. Similar results have been shown in Asian pond turtles (*Mauremys mutica*; Zhao et al., 2015) and may suggest that the mechanisms responsible for variation in incubation duration across populations are temperature specific. Alternatively, increased plasticity of developmental rate at northern populations may allow embryos to take advantage of warm conditions when they do arise under natural thermal regimes, reducing development time and the cost of development and resulting in earlier hatching. Additional experiments incorporating more incubation treatments and populations are needed to more completely discern how the relationship between temperature and developmental rate differs across populations as well as the underlying mechanisms responsible. We also observed that southern populations tended to allocate more resources towards fat body mass than northern populations at both incubation temperatures, opposite our predictions. The role of the fat body in alligators is not known, and further work examining its function, including how fat body size/mass early in life might impact survival and later life fitness, is needed to more fully appreciate the potential consequences of this pattern.

Apart from latitudinal trends, there were several differences in the influence of incubation temperature between population pairs, specifically between SCE and other populations and primarily driven by temperature's influence on hatchling mass. In alligators, animals incubated at 33.5°C have been previously shown to be larger in mass than those at 29.5°C (Bock et al., 2021), which was upheld across all of our populations.

However, at SCE, the reduction of hatchling mass at 29.5°C was particularly pronounced and appeared to drive additional phenotypic differences. Hatchling mass relative to egg mass reflects the efficiency by which maternal resources are converted into hatchling tissue and is likely a product of the energetic cost of embryonic development (Pettersen et al., 2019). The reduction in mass at SCE at 29.5°C relative to the other sites suggests that development at SCE was particularly inefficient at 29.5°C. Across our populations, SCE is the only coastal site, which may put additional stressors on embryos and breeding females (Albecker & McCoy, 2017). Indeed, the salinity of the incubation environment has been shown previously to have a negative effect on hatchling mass (Bower et al., 2013). However, we only saw an effect at 29.5°C and while differential responses to incubation temperature under salinity stress have been reported (e.g., Hudak and Dybdahl 2023), the extent to which egg yolks from SCE have increased salinity, if at all, relative to our other populations is unknown. Interestingly, animals incubated at 29.5°C at SCE also tended to have residual yolk reserves that were larger or equivalent to other populations after controlling for mass. This may be driven by a reduced rate of yolk assimilation during development or may suggest an increased importance of residual yolk mass under cooler temperatures at SCE, despite reduction in overall size (Murphy et al., 2020; Radder et al., 2004).

The lack of latitudinal trends in most of the morphological and metabolic traits examined here suggests that latitude may not be the best or only microclimatic proxy within which to understand variation in responses to the developmental environment, particularly incubation temperature. A similar lack of latitudinal patterns in response to incubation temperature was shown across several populations of painted turtles

(*Chrysemys picta*), another TSD species (Bodensteiner et al., 2019). These results may be driven by microhabitat population differences in temperature that are not represented by latitude. On the other hand, maternal nest site choice can be an important driver of nest temperatures and may vary across populations (Du et al., 2023; Warner & Shine, 2008a). This can result in similar nest temperatures despite different environmental temperatures (Bodensteiner et al., 2023), and would reduce or eliminate selective pressures for differential responses to incubation temperature. More work is needed to understand how nest temperatures vary across the alligator's range and the role of maternal nest site choice. Another possible reason for the lack a latitudinal patterns is population-specific, non-thermal microclimatic variables (i.e., salinity) that can influence thermal reaction norms. Additionally, other maternal effects, such as yolk composition and deposition of hormones and anthropogenic contaminants, may, in addition to temperature, influence phenotype (Bae et al., 2021; Du et al., 2010b; Groothuis et al., 2005), but were not considered here. Further, since our design focused on incubation temperatures that produce nearly 100% males or females, population variation at each temperature may have been driven by sex differences that would not be explained by latitude. While previous work has shown that phenotypic differences between incubation temperatures are the result of temperature and not sex (Bock et al., 2023), whether sex differences exist across populations irrespective of temperature remains an open question. Future work examining the latter and the role of additional aspects of the developmental environment as potential drivers of variable responses to temperature across populations and the consistency of such effects across years will be particularly informative.

One important component not examined in this study is the role of genetics in shaping trait variation across populations. Specifically, high gene flow between populations can limit the ability of selection to drive local adaptation, rendering the differences observed across our populations unlikely to have a genetic basis or be adaptive (Kawecki & Ebert, 2004; Stamp & Hadfield, 2020). Limited information on population structure of alligators exists, but work utilizing microsatellites has shown that populations generally follow an isolation by distance model: genetic differences between FLW and FLE are relatively low, forming a group with other FL and GA populations, but separate from Louisiana and Texas populations (Davis et al., 2002; Ryberg et al., 2002). Meanwhile, one population examined in SC (Santee Coastal Reserve) was shown to be genetically distinct from both of the latter groups (Davis et al., 2002). These results suggest that there is gene flow between FLW and FLE but limited connectively between them and our northern populations. Given this information, it is likely that FLE and FLW are more closely related genetically than to SCW or SCE, and that genetic distances between FLW and FLE are likely reduced relative to those between SCW and SCE. This aligns with our results as we observed differences in both incubation duration and mass-corrected fat body mass between northern and southern population pairs. Further, while there were no differences between FLE and FLW for any trait, SCE differed from all other populations in response to temperature for mass, showing additional population-specific differences in other traits, usually between SCE and a southern population. However, further work on the genetic structure of these populations is needed to understand the genetic basis of the differences observed, which is critical if they are to be adaptive or modified by selection.

Conclusions

Overall, we found variation in developmental plasticity to incubation temperature for morphological and metabolic phenotypes across populations of alligators. In contrast, the influence of maternal provisioning on hatchling traits was mostly consistent across populations. While the adaptive value of variable plastic responses to incubation temperature was not explicitly tested, variation across populations may suggest evolutionary potential. However, the lack of information on environmental differences between populations, differential selective pressures acting on hatchling alligators, and the genetic basis of the differences observed prevents drawing broad conclusions. Determining the causes of these differences, including the developmental mechanisms involved, would provide important insight into how components of the developmental environment and embryonic responses to them influence intraspecific variation and may contribute to adaptive evolutionary change.

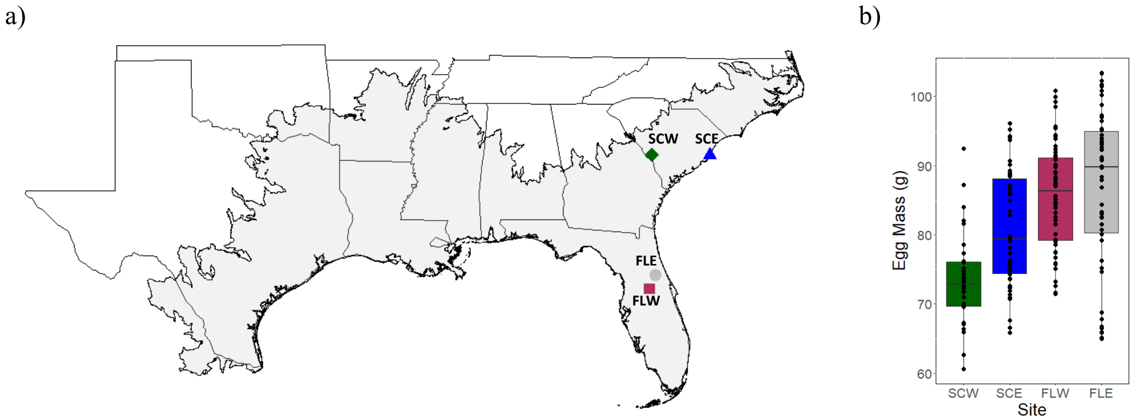

Figure 3.1: Geography and egg size of sampled populations. (a) Map showing the geographic range of the American alligator and sampled populations. (b) Egg mass variation across populations.

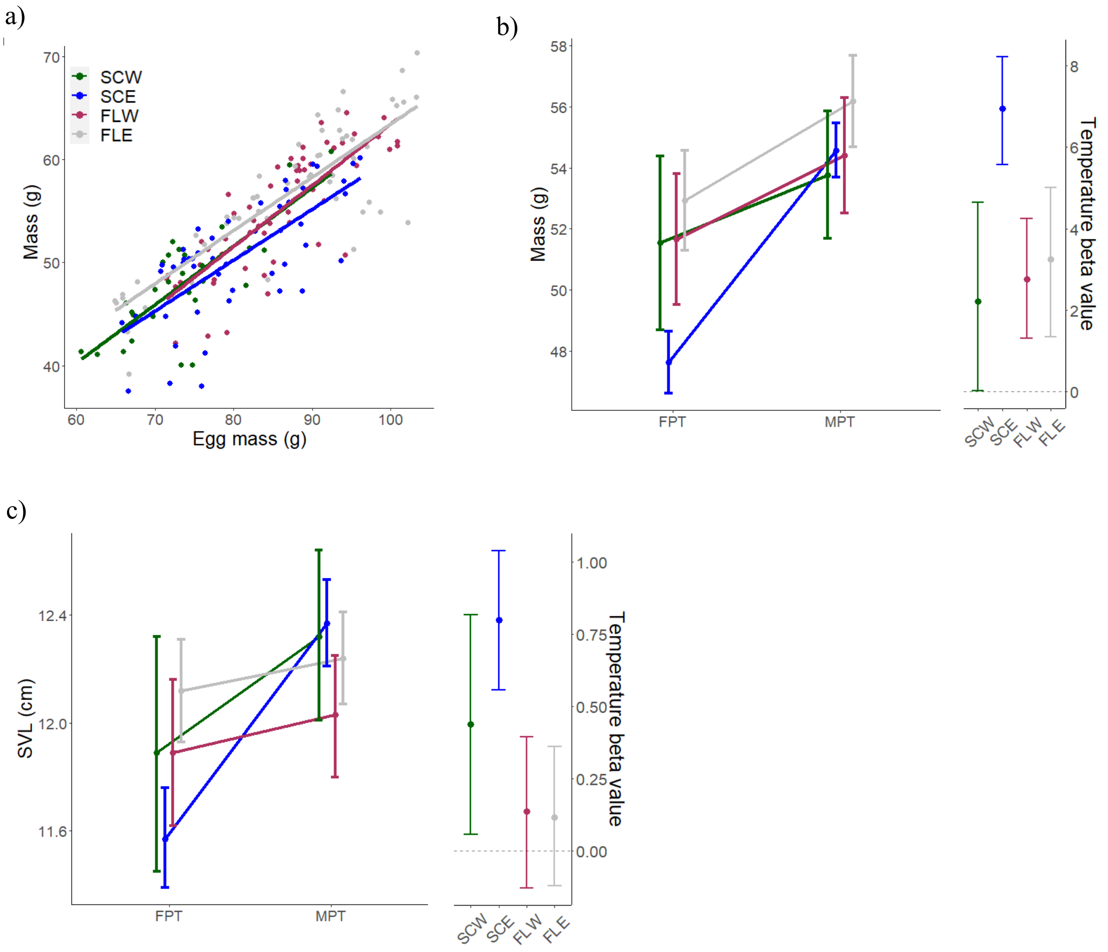

Figure 3.2: Population variation in the influence of egg mass and temperature on morphological traits, showing (a) the relationship between egg mass and hatchling mass, (b) hatchling mass and (c) snout-vent-length (SVL). In (b) and (c), plotted values are model means under a common egg mass (83g). Error bars represent 95% confidence intervals.

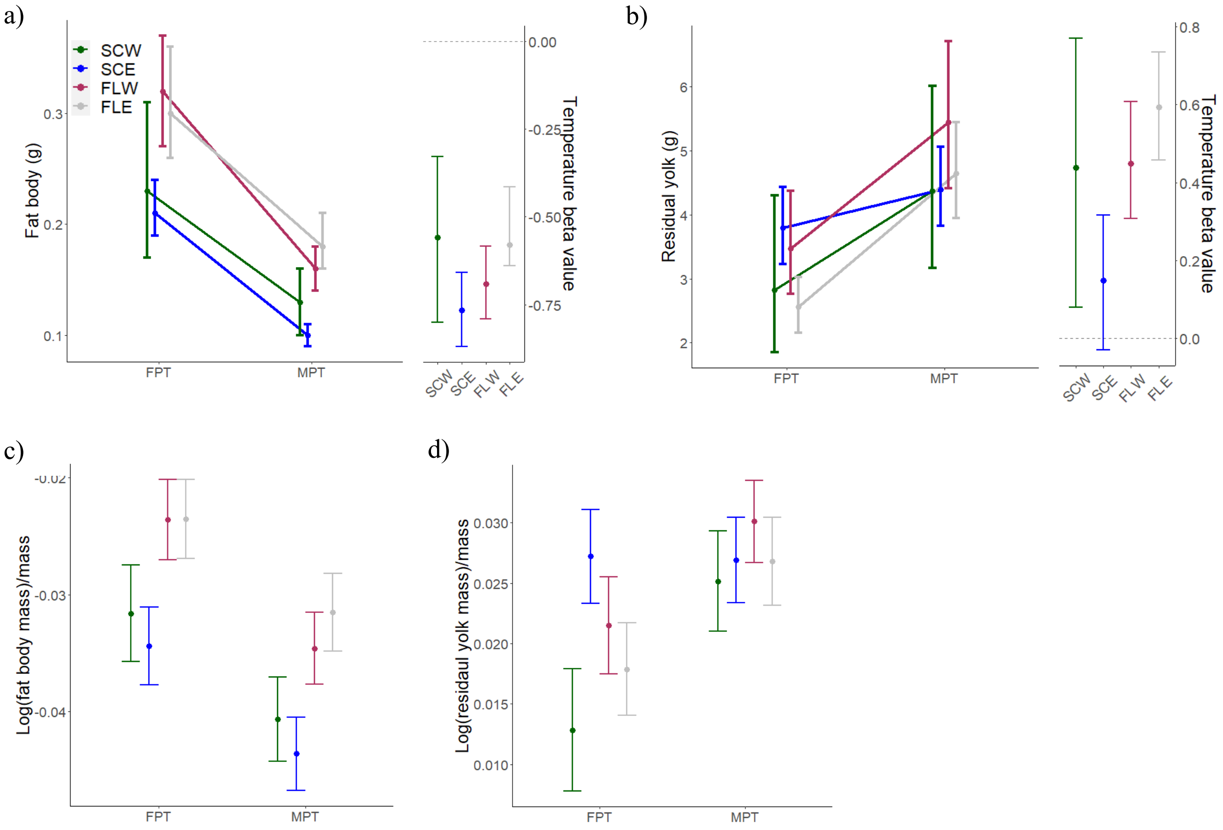

Figure 3.3: Population variation in metabolic traits and the influence of incubation temperature, showing (a) fat body mass, (b) residual yolk mass, (c) mass-specific fat body mass, and (d) mass-specific residual yolk mass. Error bars represent 95% confidence intervals.

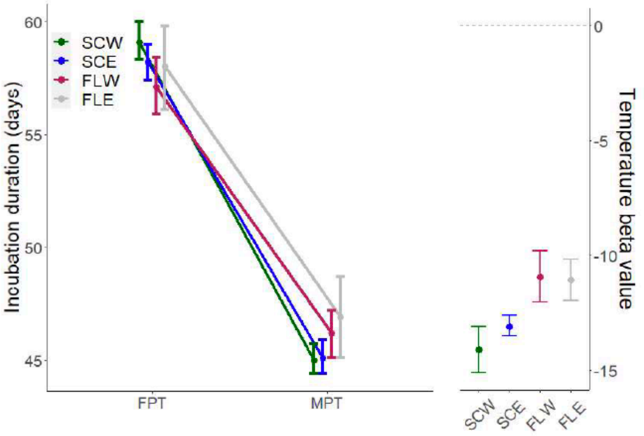

Figure 3.4: Population variation in the influence of incubation temperature on incubation duration. Error bars represent 95% confidence intervals.

Table 3.1: Final sample sizes by temperature and clutch

	AP		SR		WO		YK	
Clutch	FPT	MPT	FPT	MPT	FPT	MPT	FPT	MPT
1	5	4	2	3	6	3	4	5
2	3	4	2	3	5	5	2	3
3	1	2	1	3	2	5	3	5
4	4	5	2	3	4	4	2	4
5	4	5	3	3	4	5	2	4
6	4	8	2	3	3	5	4	2
7	0	3	0	2	0	0	1	2
8	0	5	-	-	2	4	2	2
Total	*21*	*36*	*12*	*20*	*26*	*31*	*20*	*27*

Table 3.2: Model results for the influence of incubation temperature and egg mass on phenotypes at each population. Superscripts for each beta value denote significant differences between sites within each phenotype. Phenotypes in which at least one population comparison was significant are bolded.

Phenotype	Site	Egg mass β	Egg mass CI	Temp β	Temp CI	N	R2
Mass	SR	0.523^{a}	0.349, 0.698	2.223^{a}	0.035, 4.64	31	0.631
Mass	YK	0.551^{a}	0.464, 0.633	6.948^{b}	5.578, 8.224	48	0.858
Mass	AP	0.477^{a}	0.298, 0.664	2.755^{a}	1.314, 4.255	57	0.569
Mass	WO	0.515^{a}	0.409, 0.619	3.242^{a}	1.355, 5.004	54	0.745
SVL	SR	0.019^{a}	-0.008, 0.046	0.438^{ab}	0.058, 0.817	29	0.251
SVL	YK	0.029^{a}	0.014, 0.043	0.799^{b}	0.557, 1.041	49	0.508
SVL	AP	0.031^{a}	0.009, 0.054	0.136^{a}	-0.135, 0.395	57	0.2
SVL	WO	0.025^{a}	0.014, 0.036	0.117^{a}	-0.127, 0.361	54	0.282
TG	SR	0.018^{a}	0.004, 0.032	-0.041^{ab}	-0.232, 0.150	32	0.162
TG	YK	0.015^{a}	0.008, 0.023	0.186^{b}	0.060, 0.314	49	0.298
TG	AP	0.016^{a}	0.002, 0.030	-0.024^{ab}	-0.143, 0.091	58	0.174
TG	WO	0.018^{a}	0.010, 0.025	-0.091^{a}	-0.183, -0.004	54	0.531
Head Length	SR	0.038^{a}	-0.018, 0.094	0.72^{ab}	-0.047, 1.488	32	0.181
Head Length	YK	0.051^{a}	0.024, 0.078	1.382^{b}	0.938, 1.825	50	0.474
Head Length	AP	0.070^{a}	0.034, 0.107	0.563^{ab}	0.009, 1.067	58	0.283
Head Length	WO	0.049^{a}	0.021, 0.075	0.001^{a}	-0.527, 0.462	54	0.262
Head Width	SR	0.037^{a}	-0.008, 0.083	-0.460^{ab}	-1.088, 0.167	32	0.102
Head Width	YK	0.030^{a}	0.007, 0.052	0.176^{b}	-0.192, 0.544	50	0.125
Head Width	AP	0.037^{a}	0.004, 0.070	-0.217^{ab}	-0.582, 0.124	58	0.151
Head Width	WO	0.042^{a}	0.029, 0.055	-0.670^{a}	-0.968, -0.373	54	0.52
BMI	SR	0.018^{a}	0.013, 0.023	0.044^{a}	-0.022, 0.123	28	0.686
BMI	YK	0.018^{a}	0.014, 0.021	0.162^{a}	0.116, 0.208	47	0.777
BMI	AP	0.010^{a}	0.002, 0.019	0.086^{a}	0.027, 0.148	56	0.176
BMI	WO	0.017^{a}	0.013, 0.021	0.113^{a}	0.042, 0.179	54	0.682
Delta mass	SR	0.009^{a}	-0.048, 0.065	-1.060^{a}	-1.861, -0.265	31	0.184
Delta mass	YK	-0.010^{ab}	-0.059, 0.041	-0.643^{a}	-1.255, -0.107	48	0.081
Delta mass	AP	-0.086^{b}	-0.120, -0.053	-0.112^{a}	-0.644, 0.420	56	0.318
Delta mass	WO	-0.062^{ab}	-0.101, -0.022	-0.783^{a}	-1.421, -0.121	53	0.282
Delta SVL	SR	0.006^{a}	-0.016, 0.022	-0.225^{a}	-0.503, 0.025	29	0.084
Delta SVL	YK	-0.003^{a}	-0.019, 0.013	-0.280^{a}	-0.481, -0.093	49	0.126
Delta SVL	AP	0.003^{a}	-0.016, 0.021	0.065^{a}	-0.178, 0.275	56	0.01
Delta SVL	WO	0.005^{a}	-0.005, 0.014	-0.027^{a}	-0.217, 0.165	54	0.021
Delta TG	SR	0.006^{a}	-0.001, 0.013	0.107^{a}	0.011, 0.204	32	0.242
Delta TG	YK	0.005^{a}	-0.001, 0.010	0.060^{a}	-0.036, 0.156	49	0.067
Delta TG	AP	0^{a}	-0.010, 0.009	0.089^{a}	-0.010, 0.200	58	0.044
Delta TG	WO	0^{a}	-0.004, 0.003	0.141^{a}	0.062, 0.220	54	0.185
Delta BMI	SR	-0.002^{a}	-0.005, 0.002	-0.009^{a}	-0.047, 0.034	28	0.046
Delta BMI	YK	-0.001^{a}	-0.004, 0.002	0.021^{a}	-0.019, 0.062	47	0.041
Delta BMI	AP	-0.004^{a}	-0.008, 0	-0.017^{a}	-0.067, 0.037	55	0.104
Delta BMI	WO	-0.003^{a}	-0.005, 0	-0.016^{a}	-0.073, 0.042	54	0.091

Log(residual yolk)	SR	0.038^{a}	0.009, 0.066	0.438^{ab}	0.080, 0.771	31	0.393
Log(residual yolk)	YK	0.026^{a}	0.013, 0.040	0.149^{b}	-0.031, 0.316	50	0.318
Log(residual yolk)	AP	0.007^{a}	-0.010, 0.025	0.449^{ab}	0.307, 0.607	58	0.275
Log(residual yolk)	WO	0.017^{a}	0.006, 0.029	0.594^{a}	0.457, 0.735	54	0.575
Log(fat body)	SR	0.018^{a}	-0.005, 0.039	-0.558^{a}	-0.805, -0.328	31	0.391
Log(fat body)	YK	0.017^{a}	0.007, 0.027	-0.766^{a}	-0.877, -0.659	50	0.8
Log(fat body)	AP	0.007^{a}	-0.005, 0.019	-0.691^{a}	-0.790, -0.584	58	0.646
Log(fat body)	WO	0.004^{a}	-0.006, 0.015	-0.058^{a}	-0.644, -0.415	54	0.507
Duration	SR	-	-	-14.1^{a}	-15.12, -13.12	32	0.961
Duration	YK	-	-	-13.1^{a}	-13.52, -12.62	50	0.971
Duration	AP	-	-	-10.9^{b}	-12.05, -9.81	53	0.861
Duration	WO	-	-	-11.1^{b}	-11.98, -10.18	45	0.85

Table 3.S1: Predicted mean values of traits at a common egg mass (83g)

Site	Phenotype	Temp	Mean	CI
SR	Mass	FPT	51.54	48.69, 54.38
SR	Mass	MPT	53.76	51.68, 55.85
YK	Mass	FPT	47.63	46.61, 48.64
YK	Mass	MPT	54.57	53.69, 55.46
AP	Mass	FPT	51.66	49.51, 53.8
AP	Mass	MPT	54.41	52.52, 56.3
WO	Mass	FPT	52.93	51.29, 54.57
WO	Mass	MPT	56.17	54.67, 57.68
SR	SVL	FPT	11.89	11.45, 12.32
SR	SVL	MPT	12.32	12.01, 12.64
YK	SVL	FPT	11.57	11.39, 11.76
YK	SVL	MPT	12.37	12.21, 12.53
AP	SVL	FPT	11.89	11.62, 12.16
AP	SVL	MPT	12.03	11.80, 12.25
WO	SVL	FPT	12.12	11.93, 12.31
WO	SVL	MPT	12.24	12.07, 12.41
SR	TG	FPT	4.74	4.52, 4.97
SR	TG	MPT	4.7	4.54, 4.86
YK	TG	FPT	4.55	4.44, 4.65
YK	TG	MPT	4.73	4.65, 4.82
AP	TG	FPT	4.8	4.64, 4.97
AP	TG	MPT	4.78	4.63, 4.93
WO	TG	FPT	4.94	4.83, 5.04
WO	TG	MPT	4.84	4.74, 4.95
SR	Head length	FPT	35.79	34.89, 36.69
SR	Head length	MPT	36.51	35.86, 37.16
YK	Head length	FPT	34.48	34.13, 34.82
YK	Head length	MPT	35.86	35.56, 36.15
AP	Head length	FPT	35.89	35.43, 36.35
AP	Head length	MPT	36.45	36.08, 36.83
WO	Head length	FPT	36.91	36.49, 37.33
WO	Head length	MPT	36.91	36.52, 37.30
SR	Head width	FPT	21.11	20.38, 21.85
SR	Head width	MPT	20.65	20.12, 21.18
YK	Head width	FPT	20.01	19.73, 20.30
YK	Head width	MPT	20.19	19.95, 20.43
AP	Head width	FPT	20.83	20.43, 21.22
AP	Head width	MPT	20.61	20.27, 20.95
WO	Head width	FPT	21.25	21.02, 21.48
WO	Head width	MPT	20.58	20.38, 20.79
SR	BMI	FPT	2.14	2.05, 2.23
SR	BMI	MPT	2.18	2.12, 2.25
YK	BMI	FPT	2.05	2.01, 2.09
YK	BMI	MPT	2.21	2.17, 2.25

AP	BMI	FPT	2.18	2.06, 2.31
AP	BMI	MPT	2.27	2.15, 2.38
WO	BMI	FPT	2.18	2.12, 2.24
WO	BMI	MPT	2.29	2.23, 2.35
SR	Δ mass	FPT	-2.14	-3.08, -1.21
SR	Δ mass	MPT	-3.21	-3.86, -2.55
YK	Δ mass	FPT	-2.63	-3.21, -2.06
YK	Δ mass	MPT	-3.28	-3.80, -2.75
AP	Δ mass	FPT	-2.79	-3.23, -2.36
AP	Δ mass	MPT	-2.9	-3.25, -2.56
WO	Δ mass	FPT	-2.62	-3.23, -2.01
WO	Δ mass	MPT	-3.41	-3.98, -2.83
SR	Δ SVL	FPT	0.71	0.30, 1.03
SR	Δ SVL	MPT	0.49	0.24, 0.73
YK	Δ SVL	FPT	0.76	0.58, 0.94
YK	Δ SVL	MPT	0.48	0.32, 0.65
AP	Δ SVL	FPT	0.65	0.44, 0.86
AP	Δ SVL	MPT	0.71	0.54, 0.89
WO	Δ SVL	FPT	0.64	0.48, 0.80
WO	Δ SVL	MPT	0.61	0.46, 0.76
SR	Δ TG	FPT	-0.1	-0.22, 0.01
SR	Δ TG	MPT	0	-0.08, 0.08
YK	Δ TG	FPT	-0.04	-0.11, 0.04
YK	Δ TG	MPT	0.02	-0.04, 0.09
AP	Δ TG	FPT	-0.04	-0.15, 0.06
AP	Δ TG	MPT	0.05	-0.04, 0.14
WO	Δ TG	FPT	-0.07	-0.13, -0.01
WO	Δ TG	MPT	0.07	0.02, 0.13
SR	Δ BMI	FPT	-0.21	-0.27, -0.15
SR	Δ BMI	MPT	-0.22	-0.27, -0.17
YK	Δ BMI	FPT	-0.24	-0.28, -0.20
YK	Δ BMI	MPT	-0.22	-0.25, -0.18
AP	Δ BMI	FPT	-0.22	-0.28, -0.17
AP	Δ BMI	MPT	-0.24	-0.28, -0.20
WO	Δ BMI	FPT	-0.21	-0.26, -0.17
WO	Δ BMI	MPT	-0.23	-0.27, -0.19
SR	Duration	FPT	59.35	58.11, 60.58
SR	Duration	MPT	45.19	44.29, 46.10
YK	Duration	FPT	58.26	57.56, 58.95
YK	Duration	MPT	45.19	44.52, 45.86
AP	Duration	FPT	56.92	55.73, 58.11
AP	Duration	MPT	46.06	45.16, 46.97
WO	Duration	FPT	57.88	56.47, 59.29
WO	Duration	MPT	46.80	45.46, 48.15
SR	Residual yolk	FPT	2.82	1.85, 4.30
SR	Residual yolk	MPT	4.37	3.17, 6.01

YK	Residual yolk	FPT	3.79	3.23, 4.43
YK	Residual yolk	MPT	4.39	3.82, 5.06
AP	Residual yolk	FPT	3.47	2.76, 4.37
AP	Residual yolk	MPT	5.44	4.41, 6.71
WO	Residual yolk	FPT	2.56	2.16, 3.03
WO	Residual yolk	MPT	4.64	3.95, 5.44
SR	Fat body	FPT	0.23	0.17, 0.31
SR	Fat body	MPT	0.13	0.1, 0.16
YK	Fat body	FPT	0.21	0.19, 0.24
YK	Fat body	MPT	0.1	0.09, 0.11
AP	Fat body	FPT	0.32	0.27, 0.37
AP	Fat body	MPT	0.16	0.14, 0.18
WO	Fat body	FPT	0.3	0.26, 0.36
WO	Fat body	MPT	0.18	0.16, 0.21

CHAPTER 4

POSITIVE AND RELAXED SELECTION SHAPE POPULATION DIVERGENCE IN THE MOLECULAR PATHWAYS UNDERLYING TEMPERATURE-DEPENDENT SEX DETERMINATION[3]

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[3] Smaga, C. R., Bock, S. L., Johnson, J. M., Wares, J.P., Rainwater, T., Singh, R., Deem, V., Letter, A., Brunell, A., & Parrott, B. B. Submitted to *Molecular Biology and Evolution*.

Abstract:

Developmental polyphenisms contribute to phenotypic diversity across ecological scales. Whereas our mechanistic understanding of these plastic responses has advanced in recent years, how they evolve at the molecular level is less understood. Temperature-dependent sex determination (TSD) in vertebrates provides a robust system to investigate the evolutionary processes shaping molecular variation in polyphenic traits. In theory, populations of TSD species are expected to locally adapt to divergent nest temperatures through positive selection on the genetic pathways underlying TSD. However, conditionally expressed genes, such as those involved in polyphenisms, may also experience relaxed selection, which can hinder adaptive evolution. To investigate the contributions of these competing processes, we examine variation in gene expression and genic sequence of TSD associated genes across latitudinal populations of the American alligator (*Alligator mississippiensis*). We show that TSD genes experience increased evolutionary rates relative to non-TSD genes, particularly those sensitive to temperature and not endocrine signaling. We further demonstrate that neutral processes, likely the result of relaxed selection due to context dependency, are the predominant driver. Among the subset of genes that show evidence of positive selection, we identify several targets in both gene expression patterns and genic sequences. These include genes involved in meiosis and those associated with calcium signaling, which is hypothesized to play a role in embryonic sensitivity to incubation temperature during TSD. When viewed collectively, our results reveal novel insights into the contemporary evolution of polyphenism mechanisms while highlighting novel candidate genes for the adaptive evolution of TSD across populations.

Introduction

The irreversible modification of developmental trajectories in response to environmental cues is a ubiquitous source of phenotypic diversity across ecological scales (Moczek et al., 2011; West-Eberhard, 1989, 2003). Developmentally plastic responses realized across two or more discrete trait types (e.g., sex, ecomorphs) are referred to as polyphenisms (Nijhout, 2003), which can harbor important eco-evolutionary implications (Ghalambor et al., 2007; Smallegange, 2022). For instance, polyphenic development can result in individuals that are better suited for later life environments through predictive cues during development (Brakefield et al., 1996; Laforsch & Tollrian, 2004), facilitate the evolution of traits in novel environments by exposing cryptic phenotypes to selection (Suzuki & Nijhout, 2006), and influence critical life history decisions, such as the subdivision of castes in insects or determination of sex in vertebrates (Kohno et al., 2014; Miura, 2005). Integrative approaches from developmental biology, genomics, and in some cases, functional genetics, have revealed the identity and functional role of specific genes involved in polyphenic development (Brisson et al., 2010; Czerwinski et al., 2016; Daniels et al., 2014; Schrader et al., 2015; Whiteley et al., 2021; Yatsu, Miyagawa, Kohno, Parrott, et al., 2016), demonstrating complex interactions between epigenetic modifications, transcriptional networks, and endocrine signaling that collectively translate environmental cues into alternative developmental pathways (Projecto-Garcia et al., 2017). However, despite these advancements, our understanding of how such pathways originate, diverge, and adapt remains limited and largely restricted to a few model insect systems.

Temperature-dependent sex determination (TSD) is a taxonomically widespread and robust developmental polyphenism in vertebrates, where thermal cues experienced during specific windows of embryonic development irreversibly determine sex (Bachtrog et al., 2014; Valenzuela & Lance, 2004). Early work in reptiles demonstrated a critical role of estrogen signaling, showing that treatment of embryos incubated at male-promoting temperatures (MPT) with 17β-estradiol prior to sexual differentiation can induce female development across diverse species (Bull et al., 1988; Kohno et al., 2015). Subsequent transcriptional profiling experiments revealed a core set of genetic pathways linking incubation temperature to endocrine signaling and sexual differentiation. These include genes involved in cellular thermosensitivity, temperature-specific epigenetic modifications, and vertebrate sexual development (Bock, Hale, et al., 2020; Castelli et al., 2020; Czerwinski et al., 2016; Deveson et al., 2017; Ge et al., 2017, 2018; Matsumoto et al., 2016; Parrott, Kohno, et al., 2014; Whiteley et al., 2021; Yatsu, Miyagawa, Kohno, Parrott, et al., 2016). Additionally, genetic manipulation of temperature responsive genes established their critical role in temperature sensitivity but dispensability for proper sexual development. For instance, knockdown of *KDM6B*, a chromatin modifier that is among the first genes to respond to incubation temperature, is sufficient to produce females at MPT in turtles. However, overexpression of one of its targets, the conserved testis-biased transcription factor *DMRT1*, is capable of rescuing male fate (Ge et al., 2017, 2018). When taken together, these findings revealed a temporal and functional hierarchy underlying TSD that relies on temperature cues acting upstream of endocrine signals to determine sex (Barske & Capel, 2008; Wibbels et al., 1991). Utilizing a series of hormone and temperature manipulations, Bock (2023) recently decoupled these processes in the American alligator (*Alligator mississippiensis*), identifying genes that respond solely to upstream temperature cues, to both temperature and endocrine signals, and solely to downstream endocrine signals (**Figure 4.1a**). Thus, we know not only the identity of many genes involved in TSD, but their origins during development, providing a powerful system to investigate polyphenism evolution at the molecular level.

The relationship between incubation temperature and sex ratios exhibits heritable variation at the clutch and population level, and theory suggests that TSD should evolve across populations in response to divergent nest temperatures through negative, frequency-dependent selection to produce the rarer sex (Bull, 1982; Janzen, 1992; Krueger & Janzen, 2023; McGaugh et al., n.d.; Pezaro et al., 2017; Rhen & Lang, 1998). In such a case, local adaptation of TSD would occur through positive selection on TSD associated genes (Kawecki & Ebert, 2004; Savolainen et al., 2013). However, sex-biased and polyphenism-associated genes experience relaxed selection owing to their context-dependent expression (divergent utilization across alternative morphs), which increases rates of neutral evolution and can inhibit adaptive evolution (Dapper & Wade, 2020; Schrader et al., 2017; Snell-Rood et al., 2010; Van Dyken & Wade, 2010). Thus, whereas positive selection favoring local adaptation may be a driving force, the evolution of TSD is likely limited by evolutionary constraints on adaptation associated with context-dependency. However, because distinct subsets of genes are associated with environmental sensitivity versus alternative morph generation (Bock 2023; Bui and Ragsdale 2019; Casasa, Zattara, et al. 2020), the relative roles of positive and relaxed selection may differ depending on a gene’s position in the hierarchy. For instance, genes responding solely to temperature cues during TSD are expressed in all individuals prior to sexual fate commitment, potentially reducing their context-dependency. They are also directly responding to selective pressures (nest temperatures) that differ across populations, which may increase the strength of positive selection (Ghalambor et al. 2007; **Figure 4.1a**). On the other hand, genes responding to endocrine signals likely display higher degrees of context-dependency due to testis or ovary-specific functions and, further, may be under increased evolutionary constraint because of their critical functions in gonadal development, akin to “kernel” genes in developmental biology (Hinman and Davidson 2007; **Figure 4.1a**). Yet, despite observed divergence in TSD patterns across populations (Carter et al., 2019; Ewert et al., 2005; González et al., 2019), the relative roles of positive and relaxed selection in shaping variation at the molecular level have not been investigated.

Here we assess the contributions of positive and relaxed selection in shaping TSD gene expression and genic sequence divergence across populations of the American alligator. The alligator range extends from southern Florida to northern North Carolina, and given that ambient environmental temperatures are a predominant driver of nest temperatures (Bock et al. 2020), we reasoned that populations across this latitudinal range likely experience selective pressure for local adaptation. Consistent with this idea, differential responses to incubation temperature across northern and southern alligator populations have been shown previously (González et al., 2019; Smaga et al., 2024). Using a factorial design, we first measure hatchling gonadal transcriptomes in response to incubation temperature across four populations, two northern and two southern, that span a large portion of the alligator’s latitudinal range. We then examine the evolutionary processes responsible for variation in genic sequence and expression of TSD associated genes. We predict that TSD associated genes as a group will show increased evolutionary rates relative to non-TSD associated genes, that endocrine-sensitive genes will show increased evolutionary rates compared to temperature-responsive genes, and that increased evolutionary rates in each of the latter will be primarily driven by neutral processes associated with their level of context-dependency. However, we also predict temperature-patterned genes will be more likely to be under positive selection relative to endocrine-sensitive genes. In efforts to more explicitly investigate divergence in TSD pathways, we further identify genes that are differentially expressed between northern and southern population pairs and test whether they are enriched in functional components of the TSD hierarchy. We then explore genes with the strongest signatures of selection in expression and genic sequence to identify potential targets for the adaptive evolution of TSD. Our results not only reveal novel candidates that may influence TSD outcomes across populations but add to limited empirical data on the contemporary evolution of developmental polyphenisms.

Results

Predicted nest temperatures differ between northern and southern population pairs

Differences in nest temperatures across populations are expected to exert selective pressures on genes involved in TSD (Pezaro et al., 2017). Prior work demonstrated that average daily maximum air temperature explains the majority of variation within nest cavities, and using this model, we estimated nest temperatures at each of our four focal populations over the last 70 years from compiled weather station data (Bock et al. 2020; **Figure 4.1b**). Nest temperature estimates for southern population pairs were significantly higher than northern pairs (two-sided t-test: t = -2.09, p = 0.038; southern: x̄ = 32.94°C, northern: x̄ = 32.76°C; **Figure 4.1b**), supporting potential for adaptive divergence.

Incubation temperature exerts significant influences on gonadal gene expression

We collected and incubated eggs from 6 clutches at each population at either a MPT or female-promoting temperature (FPT) and sequenced gonadal transcriptomes of one individual per clutch, temperature, and population at 10-days post-hatch (10DPH; **Figure 4.1b**). Final sample sizes after removing outlier sequencing libraries are shown in Table 4.1. For individual genes, 6,064 (34.8%) of the 18,364 passing filtering were differentially expressed (false discovery rate (FDR) < 0.05, log2 fold change (logFC) > 0.58) between all MPT and FPT individuals (hereafter, TSD genes). Of these, 2,262 were upregulated at MPT, and 3,802 were upregulated at FPT (**Figure 4.2a**). The MPT-biased genes displaying the greatest statistical significance included *GPAM* (glycerol-3-phosphate acyltransferase, logFC = 3.38), the known testis-promoting gene *AMH* (anti-mullerian hormone, logFC = 11.11), and the TGF-beta superfamily member *INHA* (inhibin subunit alpha, logFC = 9.89). MPT-biased genes were enriched for the molecular function (MF) ‘extracellular matrix binding’, the biological processes (BPs) ‘system development’, ‘multicellular organism development’, and ‘anatomical structure morphogenesis’, the KEGG pathways ‘steroid biosynthesis’, ‘axon guidance’, and ‘lysosome’, and regulation by the transcription factors (TFs) *SUZ12* (polycomb repressive complex 2 subunit), *AR* (androgen receptor), and *SMAD4* (SMAD family member 4; **Figure 4.2b**). At FPT, upregulated genes included *GREB1* (growth-regulated estrogen receptor binding 1; logFC = 7.09), the ovary-biased steroidogenic enzyme *CYP19A1* (aromatase; logFC = 13.95), and the transcription factor *FOXL2* (forkhead box

L2; logFC = 9.31). FPT-biased genes were enriched for the BPs ‘molecular transducer activity’, ‘signaling receptor activity’, and ‘signaling receptor binding’, the MFs ‘multicellular organismal process’, ‘developmental process’, and ‘anatomical structure development’, the KEGG pathways ‘calcium signaling pathway’, ‘ECM-receptor interaction’, and ‘P13K-Akt signaling pathway’, and regulation by the TFs *SUZ12*, *EZH2* (Enhancer of zeste homolog 2), and *REST* (RE1-Silencing Transcription Factor; **Figure 2b**). A full list of enrichment terms for MPT- and FPT-biased genes is included in Tables 4.S1-S4.

Of the 15 co-expression modules (excluding one orphan module) constructed in the network-based approach, 9 modules, containing 65.8% of expressed genes, were associated with incubation temperature (hereafter, TSD modules; Table S5). Module identities, sizes (number of genes), and hub genes are shown in **Figures 4.2c, d**. Together with the differentially expressed gene (DEG) analysis, these results align with prior reports demonstrating substantial effects of incubation temperature on gonadal transcriptomes (Yatsu et al. 2016; Bock 2023).

Temperature-patterned genes exhibit reduced context-dependency and network connectivity

Distinct subsets of genes are associated with environmental sensitivity versus alternative morph generation during polyphenic development (Bui & Ragsdale, 2019; Casasa, Biddle, et al., 2020), and recently, Bock (2023) used a combination of temperature and endocrine treatments in alligator embryos to decouple these in TSD, parsing the gonadal transcriptome into genes patterned by temperature cues alone, the joint effects of temperature and endocrine signals, or endocrine signals independent of temperature (**Figure 4.1a**). Using these gene lists, we categorized the detected TSD genes into three functional groups: those patterned by temperature (temperature-patterned), by temperature and estrogen (estrogen-sensitive), and those patterned by estrogen (estrogen-patterned; Figure 4.S1). With these lists, which comprise 81.3% of TSD genes during embryonic development, we were able to annotate 750 (12.4%) of TSD genes in our 10DPH dataset (**Figure 4.2e**). Temperature-patterned genes showed reduced logFC between MPT and FPT animals relative to both estrogen-sensitive (Dunn's Test (DT); $Z = 12.71$, $p < 1.6e-36$) and estrogen-patterned (DT; $Z = 3.3$, $p < 3.2e-3$) genes (**Figure 4.2f**), and similarly, displayed reduced intramodular network connectivity relative to both estrogen-sensitive (DT; $Z = 5.19$, $p < 6.5e-7$) and estrogen-patterned (DT; $Z = 15.37$, $p < 8.2e-53$) genes (**Figure 4.2g**). To ensure this was not an artifact of different sampling periods (embryonic versus 10DPH), we also compared logFC values among groups during embryonic development using results from Bock (2023), which showed similar patterns between temperature-patterned and estrogen-sensitive but not estrogen-patterned genes (Figure 4.S2). Overall, these results are consistent with increased context-dependency and functional involvement of estrogen-sensitive and estrogen-patterned genes compared to temperature-patterned genes.

TSD genes display increased evolutionary rates relative to non-TSD genes due to neutral processes associated with context-dependency

Genes underlying developmental polyphenisms often evolve more rapidly than constitutively expressed genes, which is hypothesized to be driven by relaxed selection due to their context-dependent expression (Dapper & Wade, 2020; Schrader et al., 2017; Van Dyken & Wade, 2010). We thus hypothesized that TSD genes would display increased evolutionary rates relative to non-TSD genes and that estrogen-sensitive and estrogen-patterned genes would display increased evolutionary rates relative to temperature-patterned genes. To test this hypothesis, we compared genic sequence and expression level variation within and across populations as metrics for evolutionary rates. For gene expression, we calculated the coefficient of variation (CV) within and across populations for each gene separately at MPT and FPT. For genic regions, we estimated gene-level nucleotide diversity within (pi) and across (Dxy) populations using high quality SNPs (29,391) and invariant sites (666,867) derived from transcriptome reads. There was significantly more variation in expression for TSD genes within (Wilcoxon Test (WT); W = 75169605, p-value < 2.2e-16) and across (WT; W = 74454683, p-value < 2.2e-16) populations relative to non-TSD genes (**Figure 4.3a**). Similarly, for genic regions, both pi (WT: W = 10028630, p < 0.001) and Dxy (WT: W = 10030498, p < 0.001) were significantly greater in TSD compared to non-TSD genes (**Figure 4.3b**). Across the TSD hierarchy, expression of temperature-patterned genes was significantly more variable within and across populations than estrogen-sensitive (DT; Within: Z = 12.2, p = 1.2e-33; Across: Z = 11.5, p = 3.9e-30) and estrogen-patterned (DT; Within: Z = 4.7, p = 8.5e-6; Across: Z = 4.5, p = 2.0e-5) genes (**Figure 4.3c**). However, there were no differences between temperature-patterned, estrogen-sensitive and estrogen-patterned genes in pi or Dxy (DT; all pairwise p = 1; **Figure 4.3d**).

To further investigate the roles of neutral processes (expected under relaxed selection) and positive selection in driving increased evolutionary rates of TSD and temperature patterned genes, we created a background of Fst values using only synonymous SNPs (n = 7,056) to represent neutral divergence between northern and southern population pairs. Outliers of this distribution, corresponding to the top 2.5% of Fst values (Fst = 0.437), were considered candidates putatively under positive selection whereas the remaining values were considered neutral (Leinonen et al., 2013; Narum & Hess, 2011; Whitlock & Lotterhos, 2015). We then estimated latitudinal divergence in genic regions with gene-wise Fst values calculated from all SNPs and gene expression using Pst (see Methods). We then compared the proportion of unique genes with Fst or Pst values falling within neutral expectations (Pst or Fst < 0.437) between TSD and non-TSD genes and between temperature-patterned, estrogen-sensitive and estrogen-patterned TSD genes. Based on previous theoretical and empirical work (Dapper & Wade, 2020; Khaitovich et al., 2005), we expected evolutionary differences between TSD and non-TSD genes to be primarily driven by relaxed selection and neutral processes, resulting in similar proportions of neutrally evolving genes between TSD and non-TSD groups. However, given their involvement in thermosensitivity, we also predicted that within TSD genes, temperature-patterned genes would show an increased influence of positive selection relative to estrogen-sensitive and estrogen-patterned genes, resulting in a greater proportion lying outside of neutral expectations. In partial contrast to our predictions, significantly fewer TSD genes followed neutral expectations relative to non-TSD genes for expression (Fisher's Exact Test (FT); odds ratio (OR) = 0.82, p < 0.001; **Figure 4.3e**); however, the distributions of Pst values were similar between groups (**Figure 4.3e**). The same was true for genic regions, but without any significant difference in the proportion of neutrally evolving genes between TSD and non-TSD genes (FT; OR = 0.93, p = 0.83**;** **Figure 4.3f**). Further, there were no differences in the proportion of neutrally evolving genes between temperature-patterned, estrogen-sensitive and estrogen-patterned groups for either expression or genic regions (FT; all pairwise $p > 0.4$; **Figure 4.3e,f**).

The above results suggest a substantial role of neutral processes as a driver of increased TSD gene evolution. To further assess the relationship between context-dependency and relaxed selection, we tested whether sequence and expression diversity of TSD genes scaled with expression differences between FPT and MPT. For each metric of variation, we modeled the effect of the absolute logFC between MPT and FPT of TSD genes, while controlling for each gene's logCPM and length (Schrader et al. 2017). Given differences in evolutionary rates between temperature-patterned relative to estrogen-sensitive and estrogen-patterned genes, we predicted that the influence of context-dependency may differ between these groups, so we also tested for an interaction between a gene's position in the TSD hierarchy and logFC in each model. We found that both the intra ($p < 2e-16$) and interpopulation ($p < 2e-16$) CV of TSD genes were positively associated with logFC (**Figure 4.3g**; Table 4.S6). Similarly, both pi ($p < 0.01$) and Dxy ($p = 0.014$) of TSD genes displayed a positive relationship with their corresponding logFC (**Figure 4.3g**; Table 4.S6). Interestingly, the influence of context-dependency on gene expression differed among the hierarchy, being stronger in temperature-patterned genes relative to estrogen-sensitive genes both within (trend contrast $p = 0.002$) and across (trend contrast $p < 0.0001$) populations (Table 4.S6; **Figure 4.3g**). The influence of context-dependency on estrogen-sensitive genes was also reduced relative to estrogen-patterned genes across populations (trend contrast $p < 0.001$; Table 4.S6; **Figure 4.3g**). For genic regions, there were no differences in the influence of context-dependency between temperature-patterned, estrogen-sensitive, and estrogen-patterned genes (all pairwise trend contrasts p > 0.14; Table 4.S6). Taken together, these results are consistent with increased evolutionary rates of TSD genes, driven predominately by relaxed selection associated with context-dependency. However, they also support a slightly stronger role of positive selection on TSD gene expression as a driver of population divergence.

Gene expression divergence across latitude is enriched for temperature-patterned TSD genes involved in meiosis

To further investigate potentially adaptive divergence in gene expression between the two northern and southern population pairs, we conducted separate DEG analyses for MPT and FPT individuals. Of the 506 unique genes differently expressed across population pairs at either temperature, 328 (65%) were TSD genes (hereafter, latitudinal TSD genes). Similar to observations of increased evolutionary rates, TSD genes were more likely to be differently expressed across population pairs when compared to non-TSD genes (FT; OR = 3.89, p < 2.2e-16; **Figure 4.4a**). A majority of latitudinal TSD genes were among FPT individuals (n = 255), followed by those among MPT individuals (n = 54), across both (n = 12), and those with expression showing an interaction between temperature and latitude (n = 7) (**Figure 4.4b**; Figure 4.S3). While there was no enrichment for genes variable at MPT, both temperatures, or displaying an interaction, latitudinal TSD genes among FPT individuals biased towards southern population pairs were significantly enriched for BPs related to meiosis, including ‘meiotic cell cycle’, ‘meiotic cell cycle process’, and ‘male gamete generation’ (**Figure 4.4b**; Table 4.S7). There were no differences in the proportion of latitudinal TSD genes that are temperature-patterned, estrogen-sensitive, or estrogen-patterned (FET; all pairwise $p > 0.09$; **Figure 4.4c**).

Among the co-expression modules, latitudinal TSD genes showed reduced intramodular connectivity relative to other TSD genes (WT; $W = 1349194$, $p < 2.2e-16$), suggesting they are less tightly connected to gonadal transcriptional networks (Figure 4.S4). Latitudinal TSD genes were also overrepresented in the Greenyellow module, which contained 41.2% of latitudinal TSD genes (**Figure 4.4d**). The Greenyellow module was also the only TSD module that showed a significant association with latitude, being upregulated at both MPT and FPT in northern populations (Table 4.S8, **Figure 4.4e**). Like latitudinal TSD genes, Greenyellow module genes were enriched for meiotic processes including the BPs ‘sexual reproduction’, ‘male gamete generation’, and ‘gamete generation’. They were also enriched for the MFs ‘helicase activity’, ‘catalytic activity, acting on a nucleic acid’, and ‘ATP-dependent activity, acting on RNA’ (**Figure 4.4e;** Table 4.S9). Further, the hub gene of this module, *SYCP3*, is a conserved marker of germ cells undergoing homologous recombination (Syrjänen et al., 2014). When placed in the context of the TSD hierarchy, the Greenyellow module showed a significant enrichment for temperature-patterned genes (FET; $OR = 2.76$, $p < 0.001$) and a reduction in estrogen-sensitive genes (FET; $OR = 0.17$, $p = 0.049$) than expected by chance, and contained no estrogen-patterned genes (**Figure 4.4f**). Taken together with the DEG analyses, these results suggest that gene expression divergence between northern and southern population pairs is enriched for genes with functional roles in meiotic processes, particularly among FPT individuals, and patterned by temperature.

Meiotic processes are enriched for positive selection on gene expression

While the DEG analysis identified candidate genes with expression specific to each population pair, we sought to further investigate latitudinal TSD genes that showed the strongest signatures of selection in expression. Specifically, we focused on those that were latitudinal TSD genes and had Pst values outside of neutral expectations (Pst > 0.437). Among the 328 latitudinal TSD genes, 214 (65.2%) fit this criterion (**Figure 4.4g;** Table 4.S10). Of these, 179 were across FPT individuals, 35 across MPT individuals, and none were shared across both temperatures. The top candidates included *CO6A6* (collagen type VI alpha 6; Pst = 0.73), *LINGO3* (leucine rich repeat and Ig domain containing 3; Pst = 0.73), and *NLRP3* (NLR family pyrin domain containing 3; Pst = 0.69) at FPT and *K2C8* (keratin 8; Pst = 0.70), *LOC106737513* (Pst = 0.67), and *THSD7B* (Thrombospondin Type 1 Domain Containing 7B; Pst = 0.67) at MPT. While there was no functional enrichment for outliers at MPT, FPT outlier genes retained enrichment in meiotic processes including the BPs ‘meiotic cell cycle’, ‘meiotic cell cycle process, and ‘meiotic nuclear division’ (Table 4.S11).

To further explore the expression of the above outlier genes in the context of the period of embryonic thermosensitivity during TSD, we used a previously published timeseries of gene expression dynamics during TSD in the alligator (Yatsu, Miyagawa, Kohno, Parrott, et al., 2016). We identified genes differentially expressed between MPT and FPT at stage 19 of embryogenesis and 3-, 6-, and 12-days post-stage 19, which encompasses a large portion of the thermosensitive period (TSP) during which sex is labile in the alligator (Ferguson & Joanen, 1983; McCoy et al., 2015). Of the 229 outlier latitudinal TSD genes, 9 showed significant differential expression during the TSP

(*COL9A1*, *FBXO47*, *ISX*, *LOC109283552*, *MYL4*, *MYO18B*, *TEX14, TRI47,* and *ZN420;* **Figure 4.4h**). Interestingly, *TRI47* was also among the highest Pst outliers at both MPT and FPT (**Figure 4.4g**). Many of these genes show bias towards MPT prior to 12-days post-stage 19, suggesting involvement in early responses to incubation temperature before sexual fate commitment. Thus, these results suggest not only that divergence in TSD gene expression associated with meiosis shows evidence of positive selection, but, at least in part, may be driven by divergent expression patterns originating during early periods of thermosensitivity.

Genetic variation in calcium signaling genes show signatures of positive selection

Lastly, we sought to identify TSD gene sequences with the strongest signatures of selection. There were 27 TSD genes with outlier Fst values (**Figure 4.5a**; Table 4.2), the most divergent gene being the steroid biosynthesis enzyme *CP1B1* (cytochrome p450 family 1 subfamily B member 1; Fst = 0.84). Interestingly, among the top 10 genes with the highest Fst values were two genes involved in calcium signaling, which is thought to play a role in initial temperature responses during TSD (Castelli et al., 2020; Weber et al., 2020). These included *KCNN3* (potassium calcium-activated channel subfamily N member 3; Fst = 0.76) and *CARHSP1* (calcium regulated heat stable protein 1; Fst = 0.59; **Figure 4.5a**). Of the 27 genic outlier TSD genes, 24 were expressed during the TSP but none showed differential expression during any stage (Figure 4.S5). Unsurprisingly due to the biased nature of RNA-sequencing, many of the SNPs were annotated as 3' UTR variants. However, three genes, *CADPS2* (calcium dependent secretion activator 2), *IGSF9* (immunoglobulin superfamily member 9), and *IRS4* (insulin receptor substrate 4), contained putative missense mutations (Tabel 4.2). While the functional role of the above genes in TSD specifically is not well-known, they represent several candidates, especially those involved in calcium signaling, that are expressed early in TSD and warrant further investigation in the context of mechanism and adaptive evolution.

Limited overlap between genic region and expression outliers

Two TSD genes, *RNAS1* (ribonuclease 1) and *AIG1* (androgen induced gene 1), were outliers in both expression and sequence divergence (**Figure 4.5b**). Although initially unannotated, *RNAS1* is predicted to be a ribonuclease based on similarity to other reptiles. It contained 5 SNPs, 4 of which were in the 3' UTR. We examined whether *RNAS1* variants were associated with expression using a two-way ANOVA with genotype, temperature and their interaction, and found identical, significant associations between genotype and expression level for all SNPs (all $p < 0.001$; **Figure 4.5c**), likely due to high linkage. *AIG1* contained two SNPs annotated as intronic, neither of which showed an association between genotype and expression level (both $p = 0.19$; **Figure 4.5d**). Ultimately, these results suggest little overlap in regulatory and genic region divergence of TSD genes across populations.

Discussion

Our results revealed increased evolutionary rates in the sequences and expression of TSD relative to non-TSD genes, which we show are consistent with a primary role of relaxed selection. Furthermore, we found that evolutionary patterns in gene expression differ within the TSD hierarchy, with upstream temperature-patterned genes showing increased evolutionary rates and more relaxed selection than their more downstream, estrogen-sensitive counterparts. However, we also found support for an increased influence of positive selection on TSD genes as a driver of expression divergence. Of genes showing signatures of positive selection, we identified several TSD gene candidates involved in meiosis, calcium signaling, and those differentially expressed early during the TSP that warrant further investigation regarding their mechanistic role in TSD and its adaptive evolution across populations. When taken altogether, our results provide insight into how both neutral processes arising from context-dependency and positive selection in response to divergent environments shape TSD evolution at the population level.

Genes associated with developmental polyphenisms have been shown to experience more rapid evolution relative to their constitutively expressed counterparts (Snell-Rood et al., 2011; Van Dyken & Wade, 2010). Whether this is a cause or consequence of polyphenic development is a subject of debate, but increasing evidence points towards a significant role of relaxed selection due to their context-dependent expression (Dapper & Wade, 2020; Schrader et al., 2017; Snell-Rood et al., 2010). Here, we show that the same is true for TSD genes as both gene expression and genetic variation were greater within and across populations for TSD relative to non-TSD genes, a characteristic of relaxed selection (Dapper & Wade, 2020). These observations are contrary to expectations under increased positive selection, which would result in decreased variation within but increased variation across populations (McDonald & Kreitman, 1991; Nielsen, 2005). We also observed nearly identical distributions of Fst and Pst divergence values between TSD and non-TSD genes and a positive association between evolutionary rates and a measure of context-dependency. Notably, however, we did find an increased proportion of TSD genes for which their expression is under putative positive selection. Nonetheless, the difference was small, encompassing relatively few genes, suggesting that, overall, relaxed selection is the dominant force driving the rapid evolution of TSD genes.

Our finding of relaxed selection on TSD genes may have implications for the origin of TSD at the macroevolutionary scale. It has been suggested that relaxed selection on polyphenism associated genes may be an ancestral condition reflective of reduced evolutionary constraint (Helanterä & Uller, 2014). The accumulation of genetic variation in response to relaxed selection can acquire functionality and then be co-opted for novel processes, including polyphenic development (Hunt et al., 2011; True & Carroll, 2002). Indeed, in both spadefoot toads (*Spea spp.*) and fire ants (*Solenopsis invicta*), polyphenism associated genes display more rapid evolution relative to constitutively expressed genes, even in ancestral taxa lacking alternative morphs (Hunt et al., 2011; Leichty et al., 2012). Additionally, studies have shown that the evolution of polyphenisms across species relies in part on the rewiring, modification, and eventual co-option of ancient, environmentally sensitive gene networks with morphological development (Casasa, Biddle, et al., 2020; Casasa, Zattara, et al., 2020). We hypothesize that increased evolutionary rates and relaxed selection on expression of temperature-patterned genes relative to estrogen-sensitive and estrogen-patterned genes, if ancestral, may have allowed their co-option with sex determination pathways during the origin of TSD. In support of this idea, genetic variation in *CIRBP* (cold-inducible RNA binding protein), a conserved environmentally responsive gene (Corre & Lebreton, 2024), has been associated with variation in sex ratios in response to temperature in a TSD species (Schroeder et al., 2016). Yet, macroevolutionary studies on TSD in crocodilians are hindered by the lack of an outgroup with genotypic sex determination (all crocodilians have TSD; (Lang & Andrews, 1994)); however, both turtles and lizards show transitions in TSD over evolutionary time (Gamble et al., 2015; Janzen & Phillips, 2006), making them promising models for phylogenetic comparative methods that investigate the evolutionary dynamics of temperature-patterned loci prior to and after the evolution TSD. Such comparisons are likely to provide critical insight into the long-standing question of how molecular pathways associated with temperature sensitivity became coupled with sexual development.

Contrary to our prediction, of the TSD genes showing evidence of positive selection, temperature-patterned TSD genes were not overrepresented. Rather, we found that the expression of temperature-patterned genes showed increased variation within and across populations, indicative of more relaxed selection, and a stronger relationship with context-dependency than their estrogen-sensitive counterparts. We hypothesize that the expression of estrogen-sensitive genes is under increased constraint due to critical functions in sexual differentiation, reducing the effect of context-dependency on their evolution. Meanwhile, temperature genes, while involved in sex determination, are not required for gonadal differentiation (Ge et al., 2017, 2018) and are thus subject to increased neutral evolution from their context-dependent expression. Alternatively, given the diverse roles that estrogen signaling plays outside of sexual differentiation (Moggs & Orphanides, 2001), alterations to estrogen-sensitive gene expression may have more pleiotropic consequences, resulting in increased stabilizing selection (McGuigan et al., 2014). Ultimately, the consequences of relaxed selection on TSD genes are unclear but it may limit the ability of TSD to locally adapt by requiring strong selective pressures and long timeframes. This may partially explain the weak or lack of associations observed between nest temperatures or latitude and TSD patterns expected under adaptive evolution (Carter et al., 2019). It is also likely important to consider when modeling population dynamics under environmental change that is suspected to elevate nest temperatures, skew sex ratios, and eventually drive population declines (Janzen 1994; Bock et al. 2020).

DEGs between northern and southern populations pairs at FPT were enriched in meiosis-related processes and in loci patterned by temperature, suggesting an association between incubation temperature, germ cell dynamics, and TSD evolution. Increased variation among FPT animals compared to MPT, while unexpected, makes sense given meiosis occurs at hatch in females but later in life in males (Smaga et al., 2022). Interestingly, divergence in meiosis gene expression exceeded neutral expectations, potentially suggesting adaptive origins. One possible explanation is differences in ontogeny between northern and southern population pairs, resulting in divergent timing of meiotic progression. Countergradient variation (Conover & Schultz, 1995) is common in reptiles, where cooler populations exhibit more rapid development at common temperatures than warmer populations (Pettersen, 2020). It is possible that northern populations display increased rates of gonadal development at FPT, resulting in more advanced ovarian differentiation and meiosis at hatch. This could serve as an adaptation for cooler temperature populations to take advantage of more stable thermal conditions during development with sufficient resources (e.g., egg yolk) for germ cell/oocyte development. While developmental rates between the population pairs examined here do not appear to differ (Smaga et al., 2024), rates of somatic and gonadal development can be decoupled (Burraco et al., 2023; Ryan & Semlitsch, 1998). Germ cells are present during embryonic periods in alligators but appear to undergo more rapid division between late embryonic stages and hatching (Moore et al., 2008, 2009). However, beyond that, our knowledge of meiosis in alligators, and reptiles more generally, remains limited (Smaga et al., 2022). Future work examining the timing of meiotic events and the associated gene expression changes between temperatures and across populations will be particularly informative.

Three of the identified 27 genic sequence outliers are associated with calcium signaling, which is proposed to connect incubation temperature to early cellular responses during TSD (Castelli et al., 2020; Weber et al., 2020). None of these three genes showed differences in expression in response to incubation temperature during the TSP, which is consistent with a role in mediating cellular sensitivity to calcium. Of these, *KCNN3* is one of three genes in its family with well-characterized roles in action potentials of neurons (Stocker, 2004). Interestingly, another member of this family, *KCNN2,* has been associated with early responses to temperature-induced sex reversal in central bearded dragons (*Pogona vitticeps*; Whiteley et al., 2021). *CARHSP1* is also known to have thermosensitive actions (Nishioka et al., 2022) and has been associated with stabilizing tumor necrosis factor mRNA (Pfeiffer et al., 2011), which regulates *SF1* (steroidogenic factor 1) and *AMH* (anti-mullerian hormone) through NF-kB signaling, both of which serve critical functions in sexual development and TSD (Capel, 2017; Hong et al., 2003). Another outlier, *CADPS2,* is a calcium sensor protein with roles in vesicle trafficking and exocytosis, but with no known function related to TSD (Cisternas et al., 2003). The most divergent genic region gene was *CP1B1*, a steroidogenesis enzyme primarily associated with metabolizing xenobiotics. However, it can also metabolize and be regulated by estradiol (Li et al., 2000; Tsuchiya et al., 2004), which, aside from its role in sex determination and differentiation, has been associated with germ cell numbers and proliferation dynamics (Leavy et al., 2017; Pentikäinen et al., 2000). It is thus interesting to consider that genetic variation in *CP1B1* may underly some of the gene expression differences across populations associated with meiosis. Lastly, of the two genes overlapping as expression and genic sequence outliers, *RNASE1* stood out as allelic variation corresponded to expression variation. The function of *RNASE1* in reptiles is not well understood (Nitto et al., 2005), but it is known to play diverse functions in mammals (Garnett & and Raines, 2022). Given their ability to regulate RNA and the thermosensitivity of alligator *RNASE1*, it is possible ribonucleases may serve as candidates for regulating gene expression in a temperature-dependent manner during TSD. Obviously, the proposed functions of the above genes in TSD are only speculative; however, they represent several exciting candidates for further investigation in the context of both TSD mechanisms and adaptive evolution.

Notably, our study had some important limitations. First, we only measured gene expression at one timepoint, 10DPH. While we categorized genes differentially expressed between incubation temperatures as TSD genes, many could reflect sex differences independent of temperature (i.e., they represent sex-biases independent of sex determining mechanisms). We also utilized annotations for the TSD hierarchy that were determined in embryonic gonads during stage 26, shortly after sex determination is complete. Future work comparing embryonic gene expression patterns throughout the TSP across populations will be needed to more finely identify TSD genes that vary across populations and is likely to provide further functional relevance in the context of TSD mechanisms. Second, while we predicted nest temperature differences between northern and southern population pairs, sex ratio variation in response to incubation temperature has not been empirically tested (but see González et al. 2019). In fact, very few, robust studies on sex ratio reaction norms across a TSD species' range have been conducted (Carter et al., 2019; Ewert et al., 2005). Thus, a more comprehensive understanding of how nest temperatures and reaction norms vary across the alligator's range is necessary before drawing strong conclusions about the evolution of TSD. Third, our approach for identifying outliers, particularly in expression, is prone to limitations. Most notably, gene expression variation across populations, even under common garden conditions, may be driven by non-genetic factors, such as maternal effects or contaminants (Smaga et al., 2025), that we did not account for here. However, studies suggest that gene expression is moderately heritable (Ouwens et al., 2020), and our paired design helps eliminate population-specific factors that would drive differential expression. Nonetheless, while we assume expression divergence is the result of cis or trans-acting genetic variance, further genome sequencing would be required to confirm this.

Despite its limitations, our investigation is the first to examine molecular variation in TSD across populations at the genomic level and one of only a few to do so in polyphenic systems more generally. We not only confirm theoretical expectations of TSD and polyphenism gene evolution but identify several candidates that warrant further investigation regarding its adaptive evolution. Challenges associated with performing gene manipulation experiments in TSD species has hindered our understanding of specific functional roles of genes; however, recent studies have shown promise in knocking down and overexpressing genes involved in TSD (Ge et al., 2017, 2018). We suspect that such studies will become more common in the future, and in combination with eco-evolutionary approaches such as the one presented here will be instrumental in deciphering the evolutionary origins and adaptation of TSD, among other polyphenic systems.

Materials and Methods:

Experimental design

We collected eggs from two northern and two southern populations spanning a large proportion of the alligator's latitudinal range. Lake Apopka (AP; Apopka, FL, USA) and Lake Woodruff (WO; DeLand, FL, USA) are located approximately 35 km apart in central Florida while Par Pond on the Savannah River Site (SR; Aiken, SC, USA) and Tom Yawkey Wildlife Center (YK; Georgetown, SC, USA), are 210 km apart in South Carolina (**Figure 4.1b**). We collected 7-8 clutches of eggs in June and July of 2021 from natural nests within two weeks of oviposition from each population and transported them to the Savannah River Ecology Lab in Aiken, SC, USA. Details on egg collection, transportation, incubation, and hatchling husbandry for the animals used here have been previously reported (Smaga et al., 2024). Briefly, we divided eggs in a factorial design by clutch and population into two temperature treatments, a constant MPT (33.5°C) and a constant FPT (29.5°C) at Ferguson stage 15, prior to the start of the thermosensitive period of sex determination (Ferguson & Joanen, 1983). Upon hatching, we kept hatchlings for 10 days under common garden conditions, when we necropsied hatchlings to obtain gonadal-adrenal-mesonephros complexes (GAMs) and stored them in RNAlater at -80°C. Gonads were later dissociated from GAMs under a dissecting microscope and stored at -80°C in RNAlater. All egg collection and animal husbandry were carried out under permits from the South Carolina Department of Natural Resources (SC-08-2021) and Florida Fish and Wildlife Conservation Commission (SPGS-18-33) and approval from the University of Georgia's Animal Care and Use Committee (A2021 05-007-Y3-A0).

Nest temperature predictions

We estimated alligator nest temperatures for each population based on prior work showing that mean daily maximum temperature explains 76-80% of variation in alligator nest temperatures across years (Bock et al, 2020). Bock et al (2020) examined two populations (FL and SC), one of which had small sample size (4 years), so we averaged coefficients between the two population models reported. We then compiled mean daily maximum temperatures from weather stations in the counties of each of our four populations from 1950-2020 during the nesting season (June 15 – August 15) from the National Oceanic and Atmospheric Administration using their Climate Data Online tool (https://www.ncei.noaa.gov/cdo-web). Using the averaged equation (mean nest temperature = average daily maximum temperature*0.7531 + 8.3416), we predicted nest temperatures at each of our four populations for each year (Table 4.S12) and compared northern and southern population nest temperatures using a two-sample t-test in R (R Core Team, 2024; version 2024.04.2).

Nucleic acid extraction, RNA sequencing, and read alignment

We extracted RNA from gonads using a modified version of the Promega SV Total RNA Isolation System protocol (modifications described in Smaga et al., 2025), followed by an ethanol precipitation of RNA using sodium acetate. We shipped total RNA from one individual from each of 6 clutches from each population and incubation temperature group (N = 48) to Novogene (Sacramento, CA, USA) for further QC and directional library preparation and sequencing on an Illumina NovaSeq6000 (paired-end, 150bp reads; Illumina, San Diego, CA, USA).

We assessed raw sequencing reads using FastQC and MultiQC for quality scores and adaptor contamination (Andrews, 2010). We then trimmed adaptor sequences using TrimGalore! (stringency = 3; Krueger 2015). Trimmed sequences were aligned to the alligator reference genome (ASM28112v40; Rice et al. 2017) using Hisat2 (Kim et al., 2019) and the resulting SAM files were converted into BAM format, indexed, and sorted using SAMtools (Morgan, 2024). Alignment rates ranged from 29 to 91% ($\bar{x} = 81.2$, SD = 11.3), with the total number of mapped reads ranging from 24 to 51 million. We generated per gene read counts based on aligned BAM files using the GenomicFeatures and GenomicAlignments packages (Lawrence et al., 2013) in R as described previously (Smaga et al., 2025).

Annotation of uncharacterized loci

A substantial proportion of transcripts (n = 10,927) were identified as uncharacterized loci in the alligator genome annotation (e.g., LOC genes). To characterize these loci, we employed a previously described approach (Hale et al., 2019; Smaga et al., 2025) to assemble and merge transcripts across libraries using Stringtie, extract FASTA sequences with GffRead, and annotate them using BLAST+ against the UniProt Swiss-Prot protein database. We identified the top gene hits first based on e-value and then by percent identity, and we retained the top gene hit for each sequence as the new annotation. With this approach, we annotated 5,657 of 10,927 uncharacterized loci.

TSD gene and module identification

We identified differently expressed genes (DEGs) between incubation temperature treatments using edgeR (Robinson et al., 2010). Raw gene counts were imported into R with the DGEList function from Rsamtools (Morgan, 2024), defining group as the temperature and population origin of each sample (e.g., WO_MPT). Counts were then filtered to retain only those genes with a count per million (CPM) greater than 1 in at least 6 samples (the smallest number of samples per temperature and population). After visualizing CPM values with a PCA using the prcomp function in R (center =TRUE, scale=TRUE), one MPT sample clustered with the FPT samples and was removed as an outlier for all future analyses (Figure 4.S6). We then re-filtered genes for a CPM of 1 in at least 5 samples. After library normalization using the calcNormFactors function, we built a design matrix from sample groups and estimated dispersion using estimateGLMRobustDisp with default parameters. We fit a quasi-likelihood negative binomial generalized log-linear model based on the above design matrix and identified DEGs from a pairwise test between all MPT and all FPT samples using glmQLFTest, specifying contrasts from the design matrix and assigning each population an equal weight (25%). Genes with a false-discovery rate (FDR) < 0.05 and a log2 fold-change (logFC) > 0.58 (corresponding to a 1.5x difference) were considered TSD genes.

We also used weighted gene co-correlation network analysis (WGCNA; Langfelder and Horvath 2008) to identify modules of genes for which expression was correlated. We normalized raw gene counts passing the above filtering using the trimmed-mean-of-m-values and vst-transformed them using DESeq2 (Love et al., 2014). With the WGCNA package, we determined the optimal soft-thresholing power with the pickSoftThreshold function (networkType = “signed”, corFnc = “bicor”), and in a single run, constructed the network using blockwiseModules (power = 9, minModuleSize = 30, corType = bicor, networkType = signed, maxPOutliers = 0.05, TOMType = signed, reassignThreshold = 1e-6, mergeCutHeight = 0.15). We then summarized gene expression from each module as the first principal component of expression in that module using the moduleEigengenes function. We calculated intramodular connectivity of each gene using the intramodularConnectivity.fromExpr function, and the most highly connected “hub” gene for each module was determined using ChooseTopHubInEachModule. Using Wilcoxon Tests, we compared eigengenes for each module between all MPT and FPT individuals. The resulting p-values were corrected for multiple testing using Bonferroni’s method and modules were considered TSD modules if the adjusted p-value was < 0.05.

We tested TSD genes and modules for enrichment in Gene Ontology molecular functions (MFs) and biological processes (BPs), along with KEGG pathways, against a custom background of all expressed genes in the dataset using the gprofiler2 R package (Kolberg et al., 2020). Prior to enrichment tests, gene names were converted to human counterparts using the ggconvert function. Additionally, we used enrichR (Kuleshov et al., 2016) to test for transcription factor (TF) regulation enrichment using the same gene lists and background as above.

Categorization of TSD genes along the TSD hierarchy

We used gene lists from Bock (2023) to categorize 10DPH TSD genes into groups according to their origins during development based on overlaps between hormone and incubation temperature treatment contrasts (Figure 4.S1). These included temperature-patterned genes (those in which temperature but not estrogen is necessary or sufficient for their expression), estrogen-sensitive genes (those in which estrogen and temperature are necessary or sufficient for their expression), and estrogen-patterned genes (those in which estrogen but not temperature is necessary or sufficient for their expression). We compared connectivity values and absolute logFC among TSD hierarchy groups using one-way Kruskal-Wallis tests follow by post-hoc Dunn's Tests using the R package FSA (Ogle et al., 2025) with Bonferroni p-value correction.

Calling genic variants from transcriptome reads

Using the Genome Analysis Toolkit (GATK) best practices for calling short variants from RNAsequencing data (https://gatk.broadinstitute.org), we called single nucleotide polymorphisms (SNPs) from the transcriptome reads. We first processed aligned BAM files using Mark Duplicates to identify duplicate reads and SplitNCigarReads to deal with splice junctions. We then called variants on a per-sample basis using HaplotypeCaller with the --GVCF option. We combined called variants from each sample and performed joint genotyping using the GenotypeGVCFs function, reporting both variant and invariant covered sites with the option –all-sites. We subsequently hard-filtered covered sites based on GATK recommendations using the VariantFiltration function with the following parameters: QualByDepth < 2, QUAL < 30, StrandOddsRatio > 3.0, FisherStrand > 60.0, RMSMappingQuality < 40.0, MappingQualityRankSumTest < -12.5, and ReadPosRankSumTest < -8.0. We conducted further filtering using VCFtools (Danecek et al., 2011) separately for variant and invariant sites, based on previous filtering recommendations (Song et al., 2016), which included removing variants with a minor-allele frequency < 0.05 (variant sites only), minimum genotype depth < 8, and minimum genotype quality < 20. We also removed variants that were fixed for non-reference alleles. Lastly, we removed variants absent in more than 12.5% of individuals, which corresponds to a maximum of half (6) of the individuals from any population. Using pairwise_genetic_diff (method = "nei") in the R package vcfR (Knaus & Grünwald, 2017), we calculated each SNP's Fst value between northern and southern population pairs.

Comparing evolutionary processes between TSD and non-TSD genes

To measure gene expression variability within populations, we calculated each gene's coefficient of variation (CV; standard deviation/mean) separately for MPT and FPT across all individuals originating from each population. Similarly, to measure variability in gene expression across populations, we calculated each gene's CV at MPT and FPT across all four populations. We used CV as a measure of variation as it allows for assessing variability while accounting for a gene's mean expression. Using Wilcoxon Tests, we compared intrapopulation and interpopulation CVs of TSD and non-TSD genes. For comparing genic sequence variation between TSD and non-TSD genes, we computed nucleotide diversity (pi) and absolute divergence (Dxy) of our populations using Pixy (Korunes & Samuk, 2021). Rather than only incorporating variable sites, Pixy requires both variable and invariant sites as input, increasing the accuracy of diversity estimates in the presence of missing data (Korunes & Samuk, 2021). We used the VCF file containing all sites passing filtering (variable and invariable) and exon-by-gene coordinates as inputs to calculate gene-level statistics. To ensure robust estimates of genetic diversity despite the incomplete genomic coverage of RNAsequencing reads, we removed genes that were covered by less than 5 sites from the analysis. We then used Wilcoxon Tests to compare pi and Dxy between TSD and non-TSD genes. Although estimates of pi and Dxy derived from transcriptomic data are biased due to incomplete coverage of the genome, our primary interest was in comparing across gene groups within our study rather than broader comparisons across studies. To further compare gene expression CV and metrics of genetic diversity between temperature-patterned, estrogen-sensitive, and estrogen-patterned gene groups, we used Kruskal-Wallis tests followed by post-hoc pairwise Dunn’s Tests with Bonferroni p-value correction.

To further assess the relative contributions of neutral and selective processes towards the increased evolution of TSD genes, we generated a neutral background using the Variant Effect Predictor tool (McLaren et al., 2016) to annotate all SNPs and subset for only synonymous variants. We then calculated gene-level estimates of neutral divergence between northern and southern populations by averaging individual synonymous SNP Fst values within each gene. To estimate divergence in expression, we calculated Pst, an estimate of Qst (Brommer, 2011), between northern and southern population pairs for each gene at MPT and FPT separately using the PStat (S. B. D. Silva & Silva, 2018) R package. Estimates of Pst are sensitive to the value c/h^2, where c represents the amount of variation across populations due to additive genetic effects and h^2 represents heritability. To assess how c/h^2 influences Pst values, we randomly selected 1,000 genes and compared their Pst distribution across values of c/h^2. We then plotted the resulting distributions onto the neutral genetic distribution of Fst values and chose a value of c/h^2 that best fit the neutral expectation (Figure 4.S7). While the limitations of utilizing Pst values in place of Qst are well-documented (Pujol et al., 2008), this approach ensures a conservative method for distinguishing between genes evolving neutrally and those putatively under positive selection. We removed 3 genes from the MPT Pst estimates, as they had zero counts in MPT individuals. For genic sequence divergence, we calculated gene-level Fst using all filtered SNPs. We then compared the proportion of loci overlapping neutral divergence (Fst or Pst < 0.437) between TSD and non-TSD and between temperature-patterned, estrogen-sensitive, and estrogen patterned genes using pairwise Fisher's Exact tests.

To assess the influence of context-dependency, we built separate models for each metric of variation that included an interaction between logFC between MPT and FPT and a gene's position in the TSD hierarchy (temperature-patterned, estrogen-sensitive, estrogen patterned, or other), along with gene length and logCPM as covariates. To test for differences in the relationship between logFC and variation between hierarchy groups, we conducted pairwise comparisons of trends (slopes) using the emmeans package (Lenth et al., 2025). We removed the interaction term from models in which pairwise comparisons were not significant to maximize the accuracy of estimates. Predictors were considered significant if they had a p-value < 0.05.

Identification of latitudinal TSD genes

We identified differentially expressed genes between northern and southern population pairs using separate DEG tests for animals incubated at MPT or FPT and for the interaction between population pair (northern or southern) and temperature. We used the glmQLFTest function in edgeR, specifying contrasts according to the above design matrix. Each individual population of each pair was weighted equally in every contrast. Genes were considered significantly variable between population pairs if they had an FDR < 0.05 and a logFC > 0.58. We then compared the proportion of population variable genes overlapping TSD genes to those not overlapping TSD genes using a Fisher's Exact test.

We categorized latitudinal TSD genes as variable at MPT, FPT, both or through an interaction between incubation temperature and population. If a gene was significant for the interaction and also variable at FPT, MPT or both, it was only counted in the interaction group. For each type of variation (MPT, FPT, both, interaction), we conducted separate BP, MF, KEGG and TF regulation enrichment tests for each group according to their bias towards northern or southern population pairs against a background of all TSD genes in the dataset.

To identify TSD modules that varied across northern and southern populations, we fit two-way ANOVAs for each module's eigengene, including population group (southern or northern), temperature, and their interaction as factors in R. Several models failed to meet assumptions of normality and heteroscedasticity, even with transformation. To validate their results, 1,000 bootstraps were performed for each model to estimate p-values using the ANOVA.boot function in the lmboot (Heyman, 2019) package. For every model, the significance of the predictors did not change under this approach, and the values from the original ANOVA are reported. Predictors were considered statistically significant if $p < 0.05$ after Bonferroni correction for multiple testing.

We conduced enrichment tests for MFs, BPs, KEGG pathways, and TFs for TSD modules that differed between northern and southern population pairs as above, using a background of all expressed TSD genes in the dataset.

Enrichment in divergence across the TSD hierarchy

We tested whether the proportion of genes overlapping latitudinal TSD genes differed between temperature-patterned, estrogen-sensitive, and estrogen-patterned genes using separate Fisher's Exact tests for pairwise comparisons under each type of variation (FPT, MPT, both, interaction). Further, we tested for enrichment of temperature-patterned, estrogen-sensitive, and estrogen-patterned genes in modules that varied across populations by comparing the proportion of genes in those modules within each category relative to their background proportion in the filtered dataset.

Characterizing candidate genes under selection

We tested expression and genic sequence outlier genes for BP, MF, KEGG pathway, and TF enrichment as above, utilizing a background of all TSD genes in the dataset. For assessing outlier expression during the thermosensitive period (TSP) of sex determination during embryonic development, we downloaded raw sequencing reads from (Yatsu, Miyagawa, Kohno, Parrott, et al., 2016). In their experiment, they sampled alligator gonads 3-, 6-, and 12-days post stage 19 of development, which includes a large portion of the alligator TSP. We first trimmed and aligned reads to the alligator reference genome, counted those overlapping genes, and imported them into R as above. While the original dataset included samples after 12-days post stage 19, we removed them from our analysis because they lacked replication. We also removed genes with less than 1 CPM in less than 3 samples. Similar to above, we used the glmQLFTest function in edgeR to identify DEGs after filtering for only outlier genes in expression or genic sequence (256 total genes). This allowed us to minimize the multiple testing burden associated with DEG analyses compared to using the entire gene set. We specified contrasts according to a design matrix that included incubation temperature and timepoint (days post stage 19) and identified genes with an $FDR < 0.05$ and $logFC > 0.58$ between temperatures at any timepoint.

To assess relationships between SNPs and expression of outlier genes in both Pst and Fst, we extracted individual genotypes using the extract.gt function in the vcfR package in R and built two-way ANOVAs for each SNP with SNP genotype and incubation temperature as the predictors and expression in CPM as the response. Genotype was considered significant if the $p\text{-value} < 0.05$.

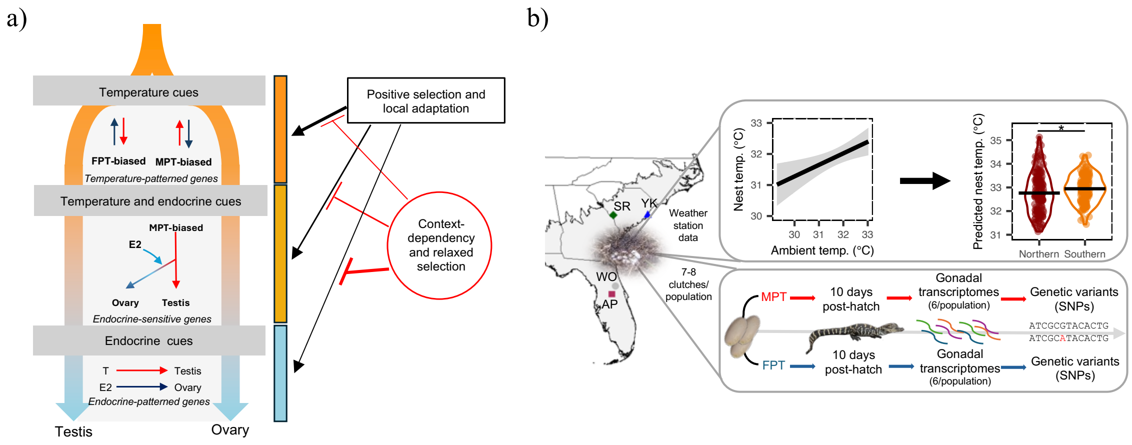

Figure 4.1: Conceptual/hypothetical framework and experimental design. (a) The TSD hierarchy and a conceptual framework for its evolution. Genes responding solely by temperature are likely to experience increased positive selection and reduced context-dependency than those responding to endocrine cues, rendering them more likely to contribute to the local adaptation of TSD. (b) Experimental design, showing the four populations examined and predicted nest temperature differences between northern and southern population pairs. MPT: male-promoting temperature, FPT: female-promoting temperature, E2: 17β-estradiol, T: testosterone, AP: Lake Apopka, WO: Lake Woodruff, SR: Par Pond on the Savannah River Site, YK: Yawkey Wildlife Center. Asterisks denote statistical significance: * $p < 0.05$.

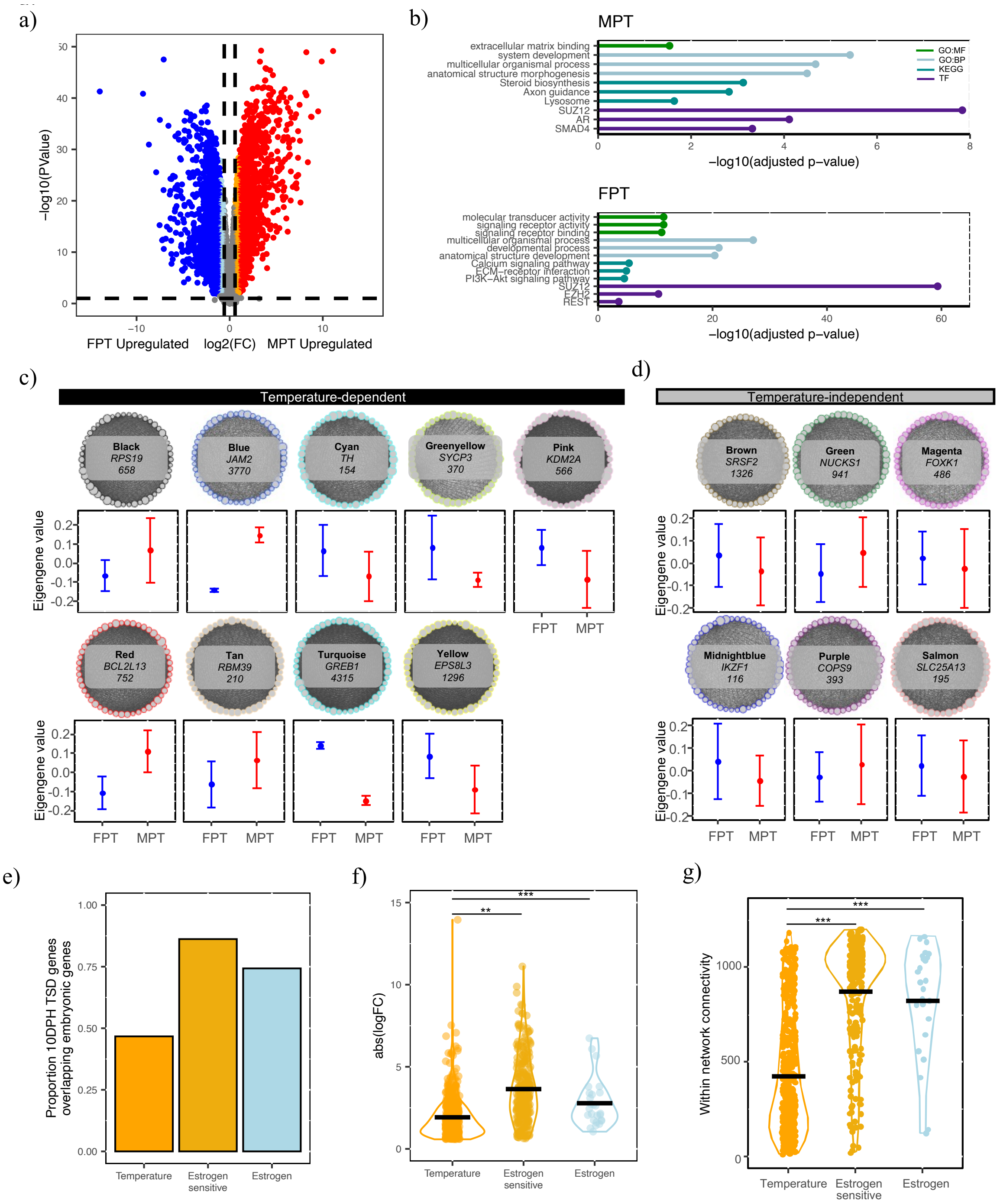

Figure 4.2: Characterization of TSD at 10DPH. (a) Individual gene responses to incubation temperature across all populations. (b) Enriched gene ontology, KEGG pathway, and transcription factor regulation enrichment terms of TSD genes. (c,d) WGCNA modules significantly associated (c) and not associated (d) with incubation temperature, with their hub gene and size shown. (e) Proportion of TSD genes at 10DPH annotated based on the TSD hierarchy from embryonic gonads. (f, g) Absolute value of the log2 fold change (f) and intramodular connectivity (g) of temperature-patterned relative to estrogen-sensitive and estrogen-patterned genes. Asterisks denote statistical significance: ** $p < 0.01$, *** $p < 0.001$.

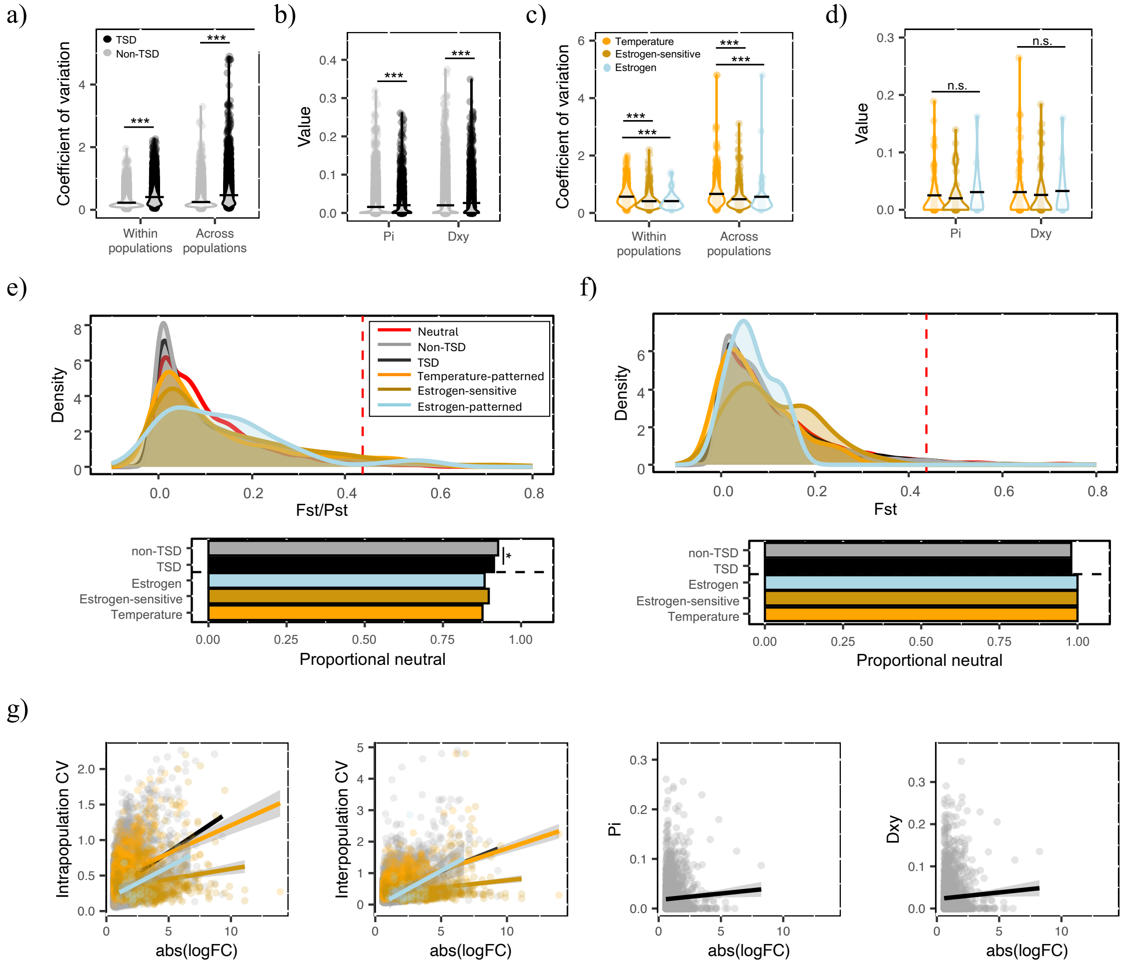

Figure 4.3: Evolutionary dynamics of TSD and non-TSD genes. (a) Expression variation between TSD and non-TSD genes within and across populations. (b) Genetic diversity within and across populations for TSD and non-TSD genes. (c) Expression variation between temperature-patterned, estrogen-sensitive, and estrogen-patterned TSD genes within and across populations. (d) Genetic diversity between temperature-patterned, estrogen-sensitive, and estrogen-patterned TSD genes within and across populations. (e) Distribution of gene expression divergence relative to neutral expectations, showing the proportion of each gene category evolving neutrally; (f) Same as (d) for genic regions. (g) Associations between context-dependency (logFC) and within and across population variation in expression or genic sequence. Distinct regression lines are shown only for those gene groups with significant differences (slopes) in response to logFC. Asterisks denote statistical significance: *** $p < 0.001$.

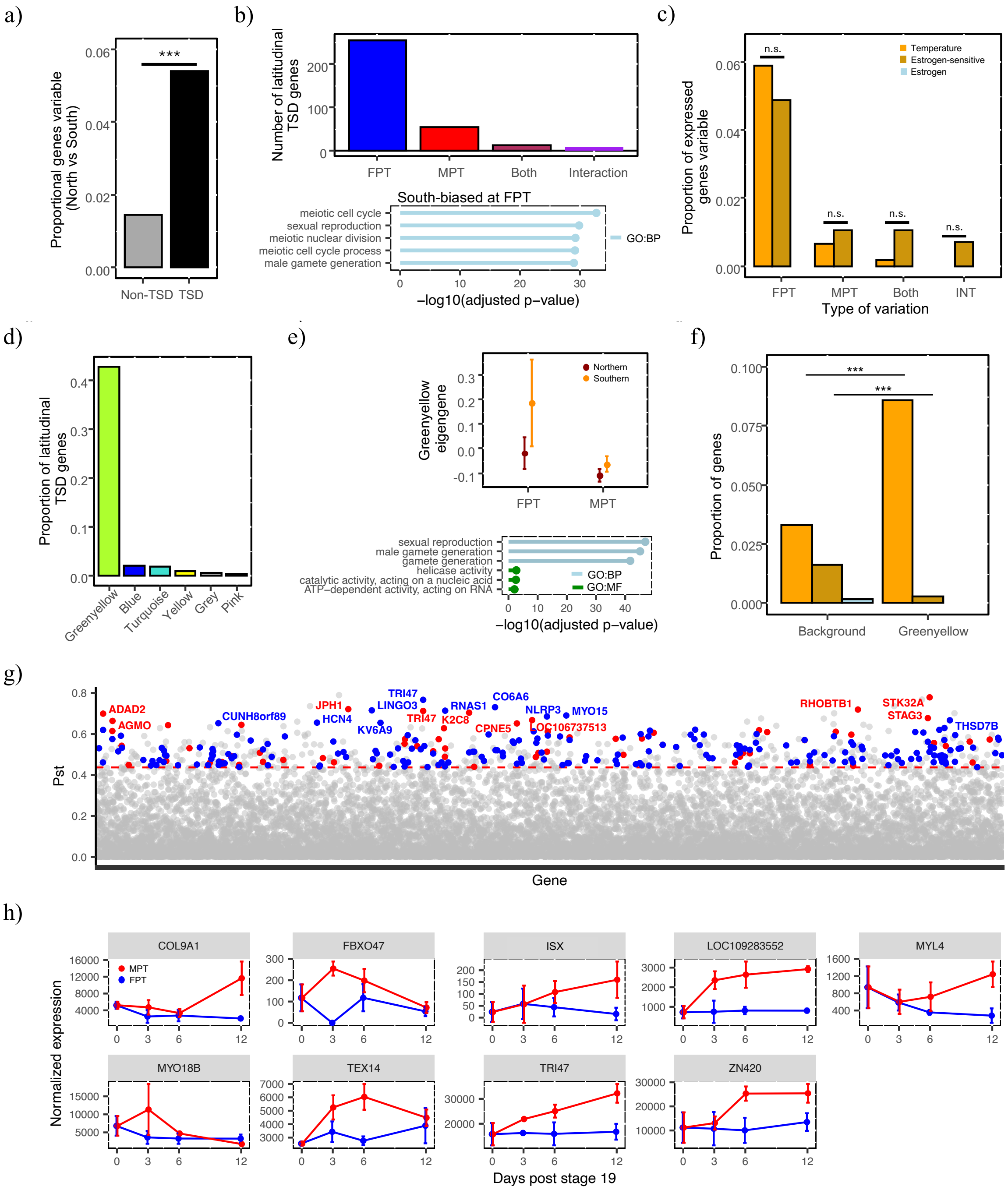

Figure 4.4: Latitudinal variation in TSD genes. (a) Relative proportion of TSD and non-TSD genes variable between northern and southern populations. (b) Variation between northern and southern populations by group, showing enriched terms of south-biased genes at FPT. (c) Relative proportion of temperature-patterned, estrogen-sensitive, and estrogen-patterned latitudinal TSD genes by type of variation. (d) Proportion of latitudinal TSD genes in each module. (e) Differential expression of the Greenyellow module between northern and southern population pairs, with functional enrichment of the Greenyellow module genes shown below. (f) Relative proportion of temperature-patterned, estrogen-sensitive, and estrogen-patterned genes within the Greenyellow

module relative to the background. (g) Outlier latitudinal TSD genes showing signatures of selection (red points = MPT, blue points = FPT), with the top 10 genes at MPT and FPT labeled. Red, dashed line shows cutoff of Pst > 0.437. (h) Expression of genes showing significant temperature responses during the alligator TSP, based on data from Yatsu et al. 2014. Asterisks denote statistical significance: * $p < 0.05$, *** $p < 0.001$.

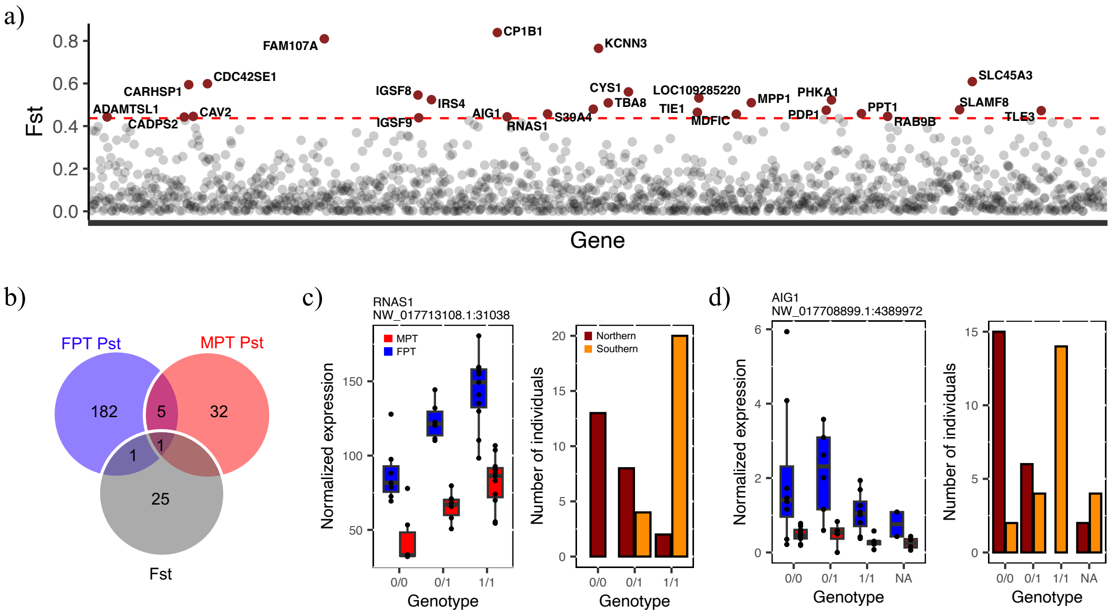

Figure 4.5: (a) Genic sequence outliers between northern and southern population pairs. Red, dashed line shows cutoff at Fst > 0.437. (b) Overlap between expression and genic region outliers. (c) Representative association between SNP genotypes, populations, and RNAS1 expression. (d) Representative association between SNP genotypes, populations, and expression of AIG1.

Table 4.1: Final sample sizes by clutch, temperature and population

	Lake Apopka		Lake Woodruff		Par Pond		Yawkey Wildlife Center	
Clutch #	*MPT*	*FPT*	*MPT*	*FPT*	*MPT*	*FPT*	*MPT*	*FPT*
1	1	1	0	1	1	1	1	1
2	1	1	1	1	1	1	1	1
3	1	1	1	1	1	1	1	1
4	1	1	1	1	1	1	1	1
5	1	1	1	1	1	1	1	1
6	1	1	1	1	1	1	1	1
Total	*6*	*6*	*5*	*6*	*6*	*6*	*6*	*6*

Table 4.2: Coding sequence TSD gene outliers, showing their Fst values, number of SNPs, and putative annotations. Some SNPs may have more than one annotation.

Gene	Description	Fst	# SNPs	SNP Annotations
CP1B1 (LOC102560255)	Cytochrome P450 Family 1 Subfamily B Member 1	0.839	1	3'UTR
FAM107A	Family With Sequence Similarity 107 Member A	0.810	4	3'UTR, synonymous
KCNN3 (LOC102575209)	Potassium Calcium-Activated Channel Subfamily N Member 3	0.765	2	3'UTR
SLC45A3	Solute Carrier Family 45 Member 3	0.609	5	3'UTR
CDC42SE1	CDC42 Small Effector 1	0.599	5	3'UTR
CARHSP1	Calcium Regulated Heat Stable Protein 1	0.594	1	3'UTR
CYS1 (LOC106737915)	Cystin 1	0.560	1	Non-coding exon
IGSF8	Immunoglobulin Superfamily Member 8	0.546	9	Synonymous, 3' UTR
LOC109285220	*NA*	0.532	2	3'UTR, non-coding exon
IRS4	Insulin Receptor Substrate 4	0.524	8	Missense, downstream, upstream, 3'UTR, synonymous
PHKA1	Phosphorylase Kinase Regulatory Subunit Alpha 1	0.523	1	Intron, non-coding exon
MPP1	MAGUK p55 scaffold protein 1	0.510	1	3'UTR, downstream
TBA8 (LOC102576504)	Tubulin Alpha 8	0.509	4	Intron, synonymous
S39A4 (LOC102574341)	Solute Carrier Family 39 Member 4	0.479	1	Synonymous
SLAMF8	SLAM family member 8	0.477	1	3'UTR
PDP1	Pyruvate Dehydrogenase Phosphatase Catalytic Subunit 1	0.475	1	3'UTR
TLE3	TLE Family Member 3, Transcriptional Corepressor	0.473	4	Synonymous
TIE1 (LOC109285085)	Tyrosine Kinase With Immunoglobulin Like And EGF Like Domains 1	0.465	5	Synonymous, upstream, 3'UTR

PPT1	Palmitoyl-Protein Thioesterase 1	0.459	2	3' UTR, downstream
RNAS1 (LOC102567125)	Ribonuclease A Family Member 1	0.457	5	3' UTR, synonymous
MDFIC	MyoD Family Inhibitor Domain Containing	0.456	1	3'UTR
CAV2	Caveolin 2	0.446	1	3'UTR
RAB9B	RAB9B, Member RAS Oncogene Family	0.446	1	3'UTR
AIG1 (LOC102561415)	Androgen Induced 1	0.443	2	Intron
ADAMTSL1	ADAMTS Like 1	0.443	1	3' UTR, downstream
CADPS2	Calcium Dependent Secretion Activator 2	0.442	1	Missense
IGSF9	Immunoglobulin Superfamily Member 9	0.439	2	Missense, synonymous

Figure 4.S1: Gene lists from Bock (2023) used to annotate TSD genes according to the TSD hierarchy. Overlaps of DEGs between FPT-AI and MPT and MPT-E2 and MPT were compared to those from FPT and FPT-AI and MPT and MPT-E2. Genes present in both contrasts were considered estrogen-sensitive, while genes unique to each of the other contrasts were considered temperature-patterned or estrogen-patterned.

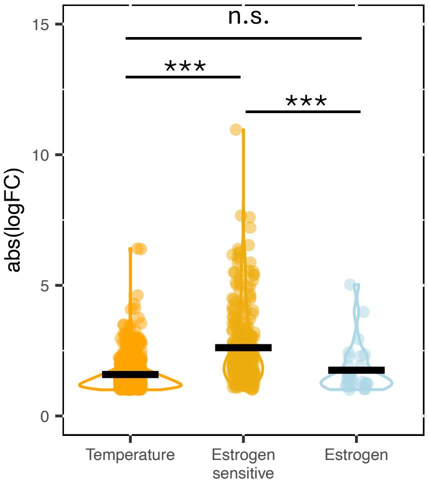

Figure 4.S2: Log$_2$ fold-change values of TSD genes based on expression from stage 26 gonads from Bock (2023).

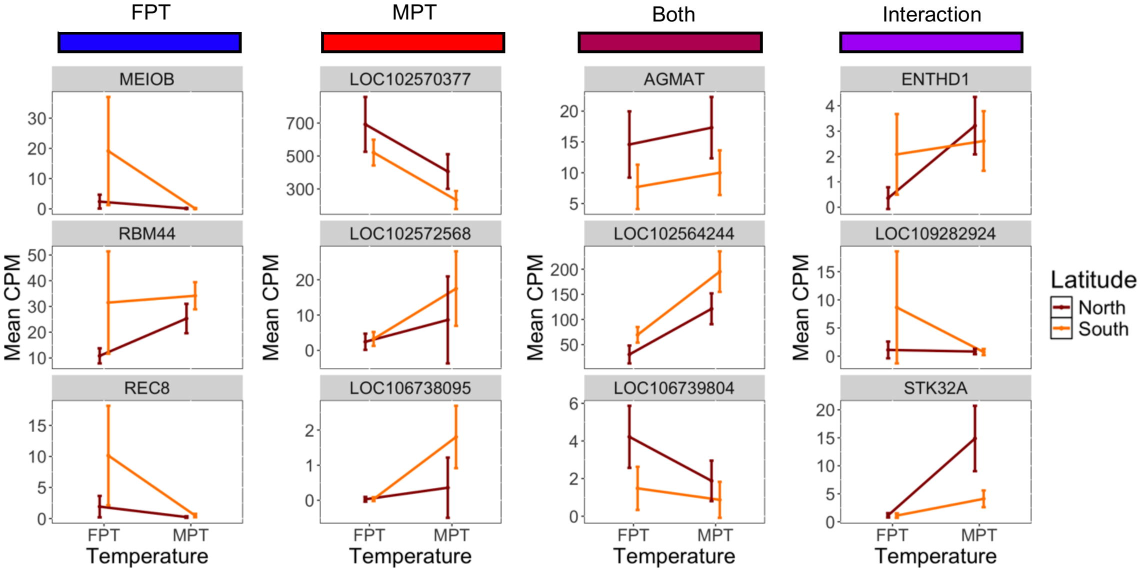

Figure 4.S3: Examples of DEGs between northern and southern populations for each type of variation.

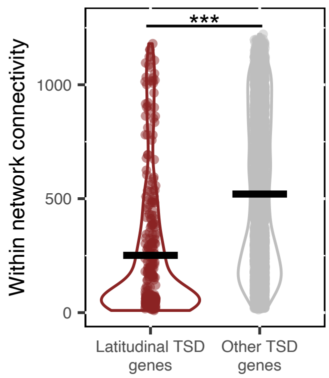

Figure 4.S4: Intramodular connectivity of latitudinal TSD genes relative to non-latitudinal TSD genes.

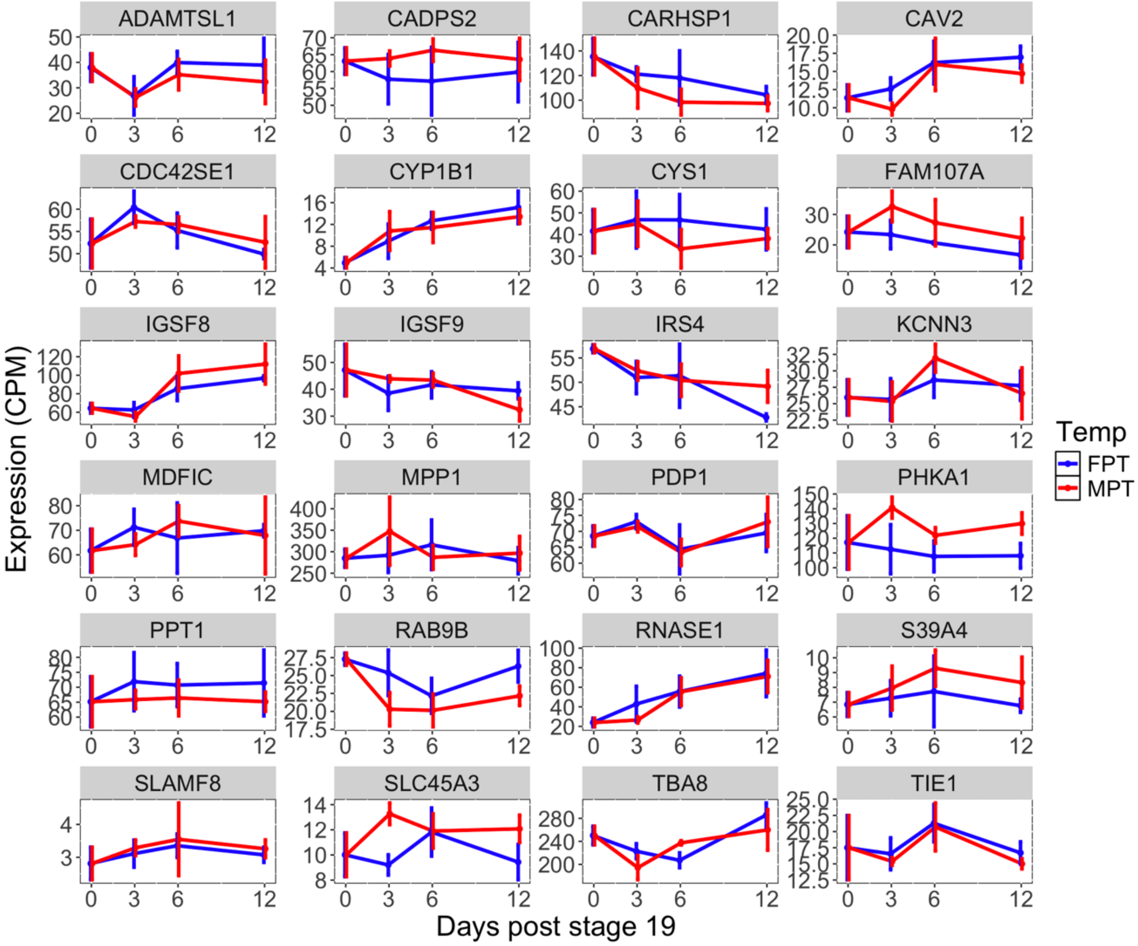

Figure 4.S5: Expression of genic sequence outlier TSD genes during the TSP based on data from Yatsu et al. 2016.

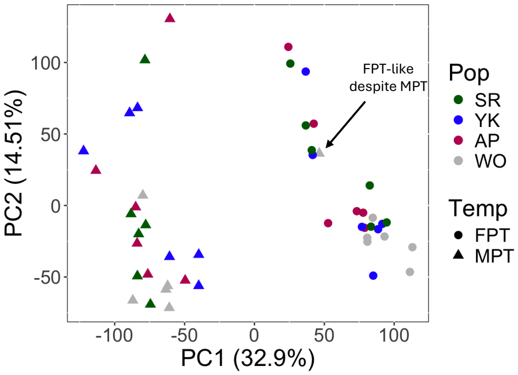

Figure 4.S6: Principal component analysis of normalized read counts across all samples sequenced. FPT-like MPT sample removed is shown by the arrow.

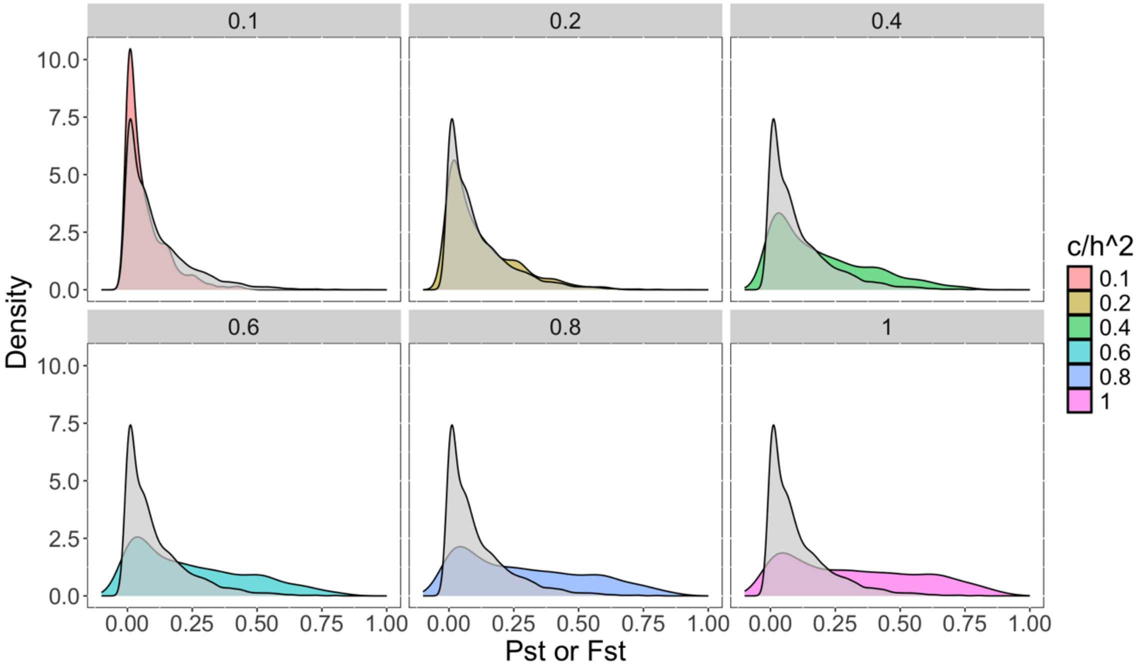

Figure 4.S7: Distributions of Pst values for varying levels of c/h2. The value of 0.2 was chosen as it best matches the distribution of neutral SNP Fst values between northern and southern population pairs (shown in grey).

Table 4.S1: GO/KEGG enrichment for MPT biased genes

Term	Adjusted p-value	Term size	Query size	Intersect size	Term ID	Source
system development	1.52e-06	3482	1536	487	0048731	BP
multicellular organism development	1.26e-05	4024	1536	545	0007275	BP
anatomical structure morphogenesis	2.37e-05	2399	1536	350	0009653	BP
anatomical structure development	7.33e-05	4940	1536	645	0048856	BP
glial cell differentiation	0.0005960	242	1536	57	0010001	BP
multicellular organismal process	0.0007272	5950	1536	751	0032501	BP
cell-cell signaling	0.0021532	1494	1536	227	0007267	BP
animal organ development	0.0021740	2601	1536	363	0048513	BP
intracellular signaling cassette	0.0026721	1609	1536	241	0141124	BP
lipid metabolic process	0.0027743	1101	1536	176	0006629	BP
developmental process	0.0032752	5330	1536	677	0032502	BP
cholesterol metabolic process	0.0039168	108	1536	32	0008203	BP
nervous system development	0.0049274	2238	1536	317	0007399	BP
axon ensheathment	0.0074218	144	1536	38	0008366	BP
ensheathment of neurons	0.0074218	144	1536	38	0007272	BP
sterol metabolic process	0.0079249	111	1536	32	0016125	BP
regulation of localization	0.0111103	1757	1536	256	0032879	BP
myelination	0.0152528	142	1536	37	0042552	BP
secondary alcohol metabolic process	0.019291	115	1536	32	1902652	BP
regulation of biological quality	0.0207762	2412	1536	334	0065008	BP
regulation of transport	0.0379974	1388	1536	207	0051049	BP
cell communication	0.0435068	5107	1536	643	0007154	BP
cellular anatomical entity morphogenesis	0.0447026	733	1536	122	0032989	BP

Steroid biosynthesis	0.0007629	15	1536	9	00100	KEGG
Axon guidance	0.0015443	170	1536	38	04360	KEGG
Lysosome	0.0232810	126	1536	28	04142	KEGG
KEGG root term	0.0241922	6218	1536	744	00000	KEGG
Circadian entrainment	0.0265991	84	1536	21	04713	KEGG
ECM-receptor interaction	0.0373841	80	1536	20	04512	KEGG

Table 4.S2: GO/KEGG enrichment for FPT-biased genes

Term name	Adjust p-value	Term size	Query size	Intersect size	Term ID	Source
multicellular organismal process	3.00E-33	5950	2418	1307	0032501	BP
developmental process	2.65E-21	5330	2418	1145	0032502	BP
anatomical structure development	1.15E-20	4940	2418	1072	0048856	BP
cell differentiation	2.09E-19	3651	2418	829	0030154	BP
cellular developmental process	2.29E-19	3652	2418	829	0048869	BP
cell adhesion	1.22E-17	1203	2418	335	0007155	BP
system development	1.27E-16	3482	2418	784	0048731	BP
regulation of multicellular organismal process	1.57E-16	2487	2418	593	0051239	BP
multicellular organism development	2.99E-16	4024	2418	883	0007275	BP
anatomical structure morphogenesis	1.11E-15	2399	2418	572	0009653	BP
tissue development	2.30E-12	1717	2418	422	0009888	BP
circulatory system process	4.07E-12	511	2418	163	0003013	BP
cell development	1.23E-11	2451	2418	563	0048468	BP
cell communication	2.79E-11	5107	2418	1051	0007154	BP
monoatomic ion transport	3.73E-11	1028	2418	275	0006811	BP
positive regulation of multicellular organismal process	5.17E-11	1371	2418	346	0051240	BP
signaling	7.13E-11	5037	2418	1036	0023052	BP
cell-cell adhesion	8.81E-11	716	2418	206	0098609	BP
locomotion	1.20E-10	1018	2418	271	0040011	BP
system process	1.31E-10	1636	2418	398	0003008	BP
metal ion transport	1.63E-10	747	2418	212	0030001	BP
monoatomic cation transport	1.13E-09	852	2418	232	0006812	BP
cell migration	2.04E-09	1267	2418	318	0016477	BP
anatomical structure formation involved in morphogenesis	2.84E-09	1062	2418	275	0048646	BP
blood circulation	6.73E-09	442	2418	138	0008015	BP
transmembrane transport	1.93E-08	1268	2418	314	0055085	BP

cell motility	2.56E-08	1424	2418	345	0048870	BP
tube development	4.51E-08	994	2418	256	0035295	BP
animal organ development	5.24E-08	2601	2418	572	0048513	BP
regulation of developmental process	5.45E-08	2135	2418	483	0050793	BP
regulation of cell population proliferation	5.62E-08	1407	2418	340	0042127	BP
regulation of locomotion	6.18E-08	887	2418	233	0040012	BP
blood vessel development	6.36E-08	650	2418	182	0001568	BP
vasculature development	1.16E-07	677	2418	187	0001944	BP
monoatomic ion transmembrane transport	1.20E-07	845	2418	223	0034220	BP
epithelium development	1.26E-07	1022	2418	260	0060429	BP
inorganic ion transmembrane transport	1.40E-07	757	2418	204	0098660	BP
tube morphogenesis	1.57E-07	800	2418	213	0035239	BP
leukocyte migration	1.61E-07	290	2418	98	0050900	BP
regulation of cell migration	2.99E-07	805	2418	213	0030334	BP
vascular process in circulatory system	3.39E-07	233	2418	83	0003018	BP
response to stimulus	4.45E-07	6690	2418	1299	0050896	BP
cell-cell signaling	4.53E-07	1494	2418	353	0007267	BP
cell population proliferation	4.68E-07	1682	2418	390	0008283	BP
positive regulation of developmental process	5.43E-07	1167	2418	287	0051094	BP
cell junction organization	6.13E-07	675	2418	184	0034330	BP
cell junction assembly	7.18E-07	398	2418	122	0034329	BP
circulatory system development	8.17E-07	1029	2418	258	0072359	BP
animal organ morphogenesis	8.60E-07	952	2418	242	0009887	BP
regulation of cell motility	1.02E-06	848	2418	220	2000145	BP
blood vessel morphogenesis	1.02E-06	564	2418	159	0048514	BP

inorganic cation transmembrane transport	1.52E-06	677	2418	183	0098662	BP
signal transduction	2.08E-06	4613	2418	930	0007165	BP
monoatomic cation transmembrane transport	2.27E-06	694	2418	186	0098655	BP
nervous system development	3.97E-06	2238	2418	492	0007399	BP
regulation of cell adhesion	4.56E-06	648	2418	175	0030155	BP
response to bacterium	9.12E-06	430	2418	126	0009617	BP
cell surface receptor signaling pathway	1.33E-05	2292	2418	499	0007166	BP
regulation of multicellular organismal development	1.57E-05	1236	2418	294	2000026	BP
regulation of biological quality	1.68E-05	2412	2418	521	0065008	BP
inorganic ion import across plasma membrane	2.26E-05	101	2418	44	0099587	BP
inorganic cation import across plasma membrane	2.26E-05	101	2418	44	0098659	BP
kidney development	2.53E-05	297	2418	94	0001822	BP
epithelial cell differentiation	2.58E-05	582	2418	158	0030855	BP
renal system development	2.81E-05	306	2418	96	0072001	BP
inflammatory response	4.27E-05	609	2418	163	0006954	BP
regulation of cell differentiation	4.62E-05	1364	2418	317	0045595	BP
chemotaxis	4.88E-05	352	2418	106	0006935	BP
taxis	6.94E-05	354	2418	106	0042330	BP
import into cell	7.35E-05	789	2418	200	0098657	BP
response to chemical	8.73E-05	2921	2418	611	0042221	BP
tissue morphogenesis	9.23E-05	559	2418	151	0048729	BP
regulation of blood circulation	1.13E-04	225	2418	75	1903522	BP
skeletal system development	1.31E-04	497	2418	137	0001501	BP

G protein-coupled receptor signaling pathway	2.19E-04	741	2418	188	0007186	BP
import across plasma membrane	3.06E-04	161	2418	58	0098739	BP
calcium ion transport	3.81E-04	373	2418	108	0006816	BP
leukocyte chemotaxis	3.98E-04	162	2418	58	0030595	BP
immune system process	4.53E-04	1806	2418	397	0002376	BP
cellular response to stimulus	5.34E-04	5744	2418	1110	0051716	BP
embryonic morphogenesis	5.66E-04	573	2418	151	0048598	BP
regulation of system process	5.74E-04	480	2418	131	0044057	BP
negative regulation of multicellular organismal process	7.17E-04	943	2418	227	0051241	BP
regulation of body fluid levels	7.62E-04	311	2418	93	0050878	BP
ameboidal-type cell migration	9.07E-04	392	2418	111	0001667	BP
cell chemotaxis	1.03E-03	227	2418	73	0060326	BP
organic anion transport	1.20E-03	349	2418	101	0015711	BP
angiogenesis	1.23E-03	481	2418	130	0001525	BP
cell activation	1.29E-03	870	2418	211	0001775	BP
extracellular structure organization	1.39E-03	297	2418	89	0043062	BP
external encapsulating structure organization	1.39E-03	297	2418	89	0045229	BP
morphogenesis of an epithelium	1.78E-03	456	2418	124	0002009	BP
positive regulation of cell differentiation	1.89E-03	761	2418	188	0045597	BP
sodium ion transport	2.09E-03	193	2418	64	0006814	BP
cell-cell adhesion via plasma-membrane adhesion molecules	2.18E-03	185	2418	62	0098742	BP
extracellular matrix organization	2.49E-03	296	2418	88	0030198	BP
epithelial cell proliferation	2.81E-03	386	2418	108	0050673	BP
regulation of epithelial cell proliferation	2.93E-03	319	2418	93	0050678	BP
positive regulation of cell adhesion	2.95E-03	400	2418	111	0045785	BP

calcium ion import	2.96E-03	72	2418	32	0070509	BP
renal system process	3.20E-03	97	2418	39	0003014	BP
response to lipid	3.36E-03	752	2418	185	0033993	BP
cellular response to vascular endothelial growth factor stimulus	3.53E-03	59	2418	28	0035924	BP
striated muscle contraction	4.72E-03	164	2418	56	0006941	BP
nephron development	6.06E-03	149	2418	52	0072006	BP
biological regulation	6.43E-03	9072	2418	1667	0065007	BP
positive regulation of cell population proliferation	6.54E-03	798	2418	193	0008284	BP
heart contraction	6.67E-03	211	2418	67	0060047	BP
regulation of heart contraction	7.10E-03	178	2418	59	0008016	BP
kidney epithelium development	7.73E-03	146	2418	51	0072073	BP
neurogenesis	7.85E-03	1591	2418	348	0022008	BP
male meiotic nuclear division	9.44E-03	45	2418	23	0007140	BP
calcium ion import into cytosol	9.98E-03	36	2418	20	1902656	BP
cell-cell junction organization	1.07E-02	184	2418	60	0045216	BP
leukocyte activation	1.18E-02	745	2418	181	0045321	BP
cellular response to chemical stimulus	1.25E-02	2144	2418	451	0070887	BP
monoatomic cation homeostasis	1.29E-02	509	2418	132	0055080	BP
regulation of cell-cell adhesion	1.52E-02	375	2418	103	0022407	BP
regulation of leukocyte migration	1.74E-02	178	2418	58	0002685	BP
organic acid transport	1.98E-02	282	2418	82	0015849	BP
heart process	2.03E-02	221	2418	68	0003015	BP
monoatomic ion homeostasis	2.13E-02	518	2418	133	0050801	BP
response to lipopolysaccharide	2.43E-02	248	2418	74	0032496	BP
regulation of immune system process	2.46E-02	1151	2418	260	0002682	BP
regulation of anatomical structure morphogenesis	2.54E-02	753	2418	181	0022603	BP

regulation of striated muscle contraction	2.54E-02	85	2418	34	0006942	BP
secretion	2.66E-02	823	2418	195	0046903	BP
inorganic ion homeostasis	2.70E-02	449	2418	118	0098771	BP
calcium ion import across plasma membrane	3.28E-02	35	2418	19	0098703	BP
homophilic cell adhesion via plasma membrane adhesion molecules	3.31E-02	97	2418	37	0007156	BP
endothelial cell migration	3.33E-02	198	2418	62	0043542	BP
carboxylic acid transport	3.45E-02	281	2418	81	0046942	BP
sodium ion transmembrane transport	4.25E-02	137	2418	47	0035725	BP
chemical homeostasis	4.48E-02	864	2418	202	0048878	BP
homologous chromosome pairing at meiosis	4.68E-02	45	2418	22	0007129	BP
leukocyte cell-cell adhesion	4.72E-02	310	2418	87	0007159	BP
molecular transducer activity	3.79E-12	891	2418	249	0060089	MF
signaling receptor activity	3.79E-12	891	2418	249	0038023	MF
signaling receptor binding	5.89E-12	1151	2418	304	0005102	MF
transporter activity	2.35E-09	1008	2418	264	0005215	MF
transmembrane signaling receptor activity	4.23E-09	723	2418	202	0004888	MF
inorganic molecular entity transmembrane transporter activity	2.00E-08	566	2418	165	0015318	MF
transmembrane transporter activity	2.76E-08	909	2418	239	0022857	MF
metal ion transmembrane transporter activity	5.89E-08	385	2418	122	0046873	MF
inorganic cation transmembrane transporter activity	9.03E-08	469	2418	141	0022890	MF

monoatomic ion transmembrane transporter activity	1.61E-06	599	2418	166	0015075	MF
signaling receptor regulator activity	1.63E-06	368	2418	114	0030545	MF
monoatomic cation transmembrane transporter activity	1.91E-06	496	2418	143	0008324	MF
passive transmembrane transporter activity	2.35E-06	422	2418	126	0022803	MF
channel activity	4.21E-06	421	2418	125	0015267	MF
voltage-gated monoatomic cation channel activity	1.12E-05	142	2418	56	0022843	MF
gated channel activity	1.31E-05	277	2418	90	0022836	MF
glycosaminoglycan binding	1.64E-05	189	2418	68	0005539	MF
monoatomic cation channel activity	8.17E-05	286	2418	90	0005261	MF
monoatomic ion channel activity	9.82E-05	378	2418	111	0005216	MF
signaling receptor activator activity	1.19E-04	344	2418	103	0030546	MF
voltage-gated monoatomic ion channel activity	1.46E-04	166	2418	60	0005244	MF
calcium ion binding	1.63E-04	554	2418	149	0005509	MF
cytokine activity	1.76E-04	136	2418	52	0005125	MF
receptor ligand activity	1.92E-04	338	2418	101	0048018	MF
voltage-gated channel activity	2.46E-04	168	2418	60	0022832	MF
sodium ion transmembrane transporter activity	1.07E-03	127	2418	48	0015081	MF
organic anion transmembrane transporter activity	5.68E-03	206	2418	66	0008514	MF
lipid binding	1.32E-02	702	2418	172	0008289	MF
solute:sodium symporter activity	1.97E-02	63	2418	28	0015370	MF
heparin binding	4.25E-02	137	2418	47	0008201	MF
Calcium signaling pathway	4.03E-06	229	2418	73	04020	KEGG
ECM-receptor interaction	1.16E-05	80	2418	34	04512	KEGG

PI3K-Akt signaling pathway	2.69E-05	318	2418	91	04151	KEGG
Cell adhesion molecules	4.79E-04	115	2418	40	04514	KEGG
Cytokine-cytokine receptor interaction	5.11E-04	186	2418	57	04060	KEGG
Neuroactive ligand-receptor interaction	6.13E-04	303	2418	83	04080	KEGG
MAPK signaling pathway	2.69E-03	277	2418	75	04010	KEGG
Renin secretion	9.92E-03	60	2418	23	04924	KEGG
Ras signaling pathway	1.07E-02	209	2418	58	04014	KEGG
Hematopoietic cell lineage	1.78E-02	62	2418	23	04640	KEGG
Inflammatory mediator regulation of TRP channels	2.36E-02	87	2418	29	04750	KEGG
Rap1 signaling pathway	2.40E-02	201	2418	55	04015	KEGG
Pathways in cancer	2.49E-02	466	2418	110	05200	KEGG
Viral protein interaction with cytokine and cytokine receptor	2.72E-02	52	2418	20	04061	KEGG

Table 4.S3: Transcription factor regulation enrichment at FPT

Term	Overlap	Adjusted P-value	Odds Ratio	Combined Score
SUZ12 CHEA	506/1483	4.5707E-60	2.848	402.324
EZH2 CHEA	80/210	2.8684E-11	2.993	84.473
EZH2 ENCODE	79/255	2.0403E-06	2.173	36.18
REST ENCODE	87/313	6.331E-05	1.862	24.067
REST CHEA	244/1100	0.00024777	1.396	15.823
SMAD4 CHEA	126/526	0.00101205	1.526	14.872
SUZ12 ENCODE	28/80	0.00165503	2.58	23.48
TP63 CHEA	225/1086	0.02173673	1.268	8.106

Table 4.S4: Transcription factor enrichment at MPT

Term	**Overlap**	**Adjusted P-value**	**Odds Ratio**	**Combined Score**
SUZ12 CHEA	240/1483	1.47E-08	1.656	37.57
AR CHEA	152/956	7.76E-05	1.579	21.184
SMAD4 CHEA	90/526	4.82E-04	1.703	19.043
SALL4 CHEA	57/321	4.97E-03	1.767	15.127

Table 4.S5: Wilcox Test results for modules in response to temperature

Module	**W**	**p-value**	**Adjusted p-value**
Black	**86**	**2.122E-05**	**0.0003**
Blue	**0**	**0.000E+00**	**0.0000**
Brown	366	5.630E-02	0.8445
Cyan	**452**	**9.801E-05**	**0.0015**
Green	167	1.993E-02	0.2990
Greenyellow	**495**	**4.513E-07**	**0.0000**
Magenta	343	1.584E-01	2.3760
Midnightblue	374	3.715E-02	0.5573
Pink	**475**	**7.162E-06**	**0.0001**
Purple	165	1.768E-02	0.2652
Red	**36**	**1.210E-08**	**0.0000**
Salmon	324	3.152E-01	4.7280
Tan	**130**	**1.518E-03**	**0.0228**
Turquoise	**552**	**0.000E+00**	**0.0000**
Yellow	**469**	**1.491E-05**	**0.0002**

Table 4.S6: Model results for associations with context-dependency

Metric	Predictor	Estimate	Std Error	t-value	p-value
Intra CV	**abs(logFC)**	**8.31E-02**	**2.30E-03**	**3.61E+01**	**<2E-16**
	Other	**8.31E-02**	**2.30E-03**	**-**	-
	Temperature	**7.41E-02**	**5.34E-03**	**-**	-
	Estrogen-sensitive	**4.96E-02**	**4.18E-03**	**-**	-
	Estrogen	**4.20E-02**	**1.98E-02**	**-**	-
	logCPM	**-6.35E-02**	**6.98E-04**	**-9.09E+01**	**<2E-16**
	Length	1.04E-08	1.75E-08	5.90E-01	5.55E-01
Inter CV	**abs(logFC)**	**1.25E-01**	**3.10E-03**	**4.02E+01**	**<2E-16**
	Other	**1.25E-01**	**3.10E-03**	**-**	-
	Temperature	**1.35E-01**	**6.78E-03**	**-**	-
	Estrogen-sensitive	**6.65E-02**	**5.76E-03**	**-**	-
	Estrogen	**1.68E-01**	**2.57E-02**	**-**	-
	logCPM	**-7.34E-02**	**9.78E-04**	**-7.51E+01**	**<2e-16**
	Length	-2.72E-08	2.46E-08	-1.10E+00	2.70E-01
Pi	**abs(logFC)**	**3.94E-03**	**1.49E-03**	**2.64E+00**	**8.46E-03**
	logCPM	-5.90E-04	4.04E-04	-1.46E+00	1.44E-01
	Length	**-1.62E-08**	**7.90E-09**	**-2.04E+00**	**4.11E-02**
Dxy	**abs(logFC)**	**4.72E-03**	**1.92E-03**	**2.46E+00**	1.39E-02
	logCPM	-2.36E-04	5.18E-04	-4.55E-01	6.49E-01
	Length	-1.76E-08	1.01E-08	-1.74E+00	8.21E-02

Table 4.S7: GO/KEGG enrichment for south-biased FPT genes

Term name	Adjusted p-value	Term size	Query size	Intersect size	Term ID	Source
meiotic cell cycle	1.68E-39	72	143	42	51321	BP
sexual reproduction	2.31E-34	238	143	61	19953	BP
meiotic cell cycle process	3.19E-32	64	143	36	1903046	BP
meiotic nuclear division	5.80E-32	60	143	35	140013	BP
male gamete generation	2.45E-31	144	143	48	48232	BP
gamete generation	1.54E-27	188	143	50	7276	BP
meiosis I cell cycle process	1.71E-27	45	143	29	61982	BP
spermatogenesis	3.22E-27	138	143	44	7283	BP
meiosis I	5.02E-27	42	143	28	7127	BP
cellular process involved in reproduction in multicellular organism	1.63E-26	119	143	41	22412	BP
multicellular organismal reproductive process	8.16E-26	223	143	52	48609	BP
multicellular organism reproduction	2.09E-24	237	143	52	32504	BP
reproductive process	1.13E-23	381	143	63	22414	BP
reproduction	2.88E-23	387	143	63	3	BP
nuclear division	3.33E-23	94	143	35	280	BP
organelle fission	1.32E-21	103	143	35	48285	BP
homologous chromosome pairing at meiosis	8.85E-21	25	143	20	7129	BP
homologous chromosome segregation	3.72E-20	26	143	20	45143	BP
chromosome organization involved in meiotic cell cycle	1.39E-19	27	143	20	70192	BP
meiotic chromosome segregation	4.73E-19	28	143	20	45132	BP
germ cell development	7.14E-19	97	143	32	7281	BP
developmental process involved in reproduction	1.95E-17	261	143	47	3006	BP

reciprocal homologous recombination	7.35E-16	17	143	15	140527	BP
homologous recombination	7.35E-16	17	143	15	35825	BP
reciprocal meiotic recombination	7.35E-16	17	143	15	7131	BP
chromosome organization	2.62E-15	74	143	26	51276	BP
cell cycle process	6.60E-15	222	143	41	22402	BP
chromosome segregation	5.63E-14	54	143	22	7059	BP
cell cycle	5.63E-14	313	143	47	7049	BP
nuclear chromosome segregation	1.02E-13	49	143	21	98813	BP
male meiotic nuclear division	1.07E-13	24	143	16	7140	BP
DNA recombination	4.04E-12	44	143	19	6310	BP
spermatid differentiation	1.82E-11	47	143	19	48515	BP
spermatid development	1.45E-10	45	143	18	7286	BP
DNA metabolic process	2.58E-09	133	143	27	6259	BP
female gamete generation	1.28E-08	56	143	18	7292	BP
oogenesis	2.15E-08	43	143	16	48477	BP
synaptonemal complex organization	3.68E-08	13	143	10	70193	BP
synaptonemal complex assembly	3.68E-08	13	143	10	7130	BP
male meiosis I	8.34E-07	12	143	9	7141	BP
organelle organization	1.65E-05	711	143	57	6996	BP
DNA repair	9.73E-05	61	143	15	6281	BP
nucleic acid metabolic process	4.05E-04	792	143	58	90304	BP
meiotic DNA double-strand break formation	2.90E-03	5	143	5	42138	BP
chiasma assembly	2.90E-03	5	143	5	51026	BP
double-strand break repair	2.24E-02	29	143	9	6302	BP
nucleobase-containing compound metabolic process	2.67E-02	908	143	59	6139	BP
DNA damage response	2.83E-02	103	143	16	6974	BP

Table 4.S8: ANOVA results for gene expression modules across population pairs

Module	Temp. SS	Temp. MS	Temp. F	Temp. adjust p-value	Lat. SS	Lat. MS	Lat. F	Lat. adjust p-value	Int. SS	Int. MS	Int. F	Int. adjust p-value
Black	0.21	0.21	11.46	2.29E-02	0.003	0.003	0.155	1.00E+00	0.001	0.001	0.038	1.00E+00
Blue	0.964	0.964	1168.13	1.25E-31	0.001	0.001	0.782	1.00E+00	0	0	0.261	1.00E+00
Cyan	0.211	0.211	11.61	2.15E-02	0.006	0.006	0.329	1.00E+00	0.001	0.001	0.028	1.00E+00
Greenyellow	**0.339**	**0.339**	**36.36**	**4.95E-06**	**0.186**	**0.186**	**19.959**	**8.50E-04**	**0.075**	**0.075**	**8.008**	**1.06E-01**
Pink	0.319	0.319	20.67	6.59E-04	0.017	0.017	1.071	1.00E+00	0	0	0.002	1.00E+00
Red	0.559	0.559	56.27	3.65E-08	0.012	0.012	1.251	1.00E+00	0.001	0.001	0.086	1.00E+00
Tan	0.189	0.189	10.98	2.80E-02	0.067	0.067	3.88	8.30E-01	0.007	0.007	0.42	1.00E+00
Turquoise	0.981	0.981	2210.74	1.97E-37	0	0	0.294	1.00E+00	0	0	0.19	1.00E+00
Yellow	0.354	0.354	25.58	1.26E-04	0.046	0.046	3.312	1.00E+00	0.005	0.005	0.394	1.00E+00

Table 4.S9: Greenyellow module GO/KEGG enrichment

Term name	**Adjusted p-value**	**Term size**	**Query size**	**Intersect size**	**Term ID**	**Source**
sexual reproduction	1.22E-48	238	128	69	19953	BP
male gamete generation	1.47E-45	144	128	56	48232	BP
meiotic cell cycle	1.16E-43	72	128	43	51321	BP
gamete generation	4.22E-42	188	128	59	7276	BP
spermatogenesis	2.57E-39	138	128	51	7283	BP
multicellular organismal reproductive process	1.61E-38	223	128	60	48609	BP
cellular process involved in reproduction in multicellular organism	3.13E-37	119	128	47	22412	BP
multicellular organism reproduction	8.52E-37	237	128	60	32504	BP
meiotic cell cycle process	6.42E-36	64	128	37	1903046	BP
reproductive process	6.77E-36	381	128	71	22414	BP
meiotic nuclear division	1.24E-35	60	128	36	140013	BP
reproduction	2.10E-35	387	128	71	3	BP
developmental process involved in reproduction	1.40E-30	261	128	57	3006	BP
meiosis I cell cycle process	4.94E-29	45	128	29	61982	BP
meiosis I	1.66E-28	42	128	28	7127	BP
nuclear division	1.93E-26	94	128	36	280	BP
male meiotic nuclear division	9.13E-25	24	128	21	7140	BP
organelle fission	9.19E-25	103	128	36	48285	BP
germ cell development	8.17E-22	97	128	33	7281	BP
cell cycle process	6.17E-19	222	128	43	22402	BP
meiotic chromosome segregation	3.48E-18	28	128	19	45132	BP
cell cycle	4.21E-18	313	128	49	7049	BP
homologous chromosome pairing at meiosis	9.13E-18	25	128	18	7129	BP
homologous chromosome segregation	2.89E-17	26	128	18	45143	BP
chromosome organization involved in meiotic cell cycle	8.44E-17	27	128	18	70192	BP

spermatid differentiation	3.09E-15	47	128	21	48515	BP
homologous recombination	2.15E-14	17	128	14	35825	BP
reciprocal homologous recombination	2.15E-14	17	128	14	140527	BP
reciprocal meiotic recombination	2.15E-14	17	128	14	7131	BP
spermatid development	2.90E-14	45	128	20	7286	BP
chromosome organization	4.61E-14	74	128	24	51276	BP
chromosome segregation	1.06E-13	54	128	21	7059	BP
nuclear chromosome segregation	2.33E-13	49	128	20	98813	BP
male meiosis I	1.57E-11	12	128	11	7141	BP
DNA recombination	2.69E-10	44	128	17	6310	BP
synaptonemal complex assembly	1.18E-08	13	128	10	7130	BP
synaptonemal complex organization	1.18E-08	13	128	10	70193	BP
female gamete generation	2.77E-08	56	128	17	7292	BP
retrotransposon silencing	3.01E-07	12	128	9	10526	BP
piRNA processing	3.01E-07	12	128	9	34587	BP
retrotransposition	3.01E-07	12	128	9	32197	BP
DNA metabolic process	7.25E-07	133	128	23	6259	BP
transposition	9.53E-07	13	128	9	32196	BP
nucleic acid metabolic process	2.15E-06	792	128	58	90304	BP
organelle organization	3.86E-06	711	128	54	6996	BP
regulatory ncRNA-mediated gene silencing	6.87E-06	26	128	11	31047	BP
oogenesis	1.88E-05	43	128	13	48477	BP
regulatory ncRNA processing	5.65E-05	18	128	9	70918	BP
RNA processing	7.25E-05	88	128	17	6396	BP
nucleobase-containing compound metabolic process	2.02E-04	908	128	59	6139	BP
cellular aromatic compound metabolic process	2.41E-04	962	128	61	6725	BP
retrotransposon silencing by	3.51E-04	7	128	6	141005	BP

heterochromatin formation						
heterocycle metabolic process	3.91E-04	948	128	60	46483	BP
organic cyclic compound metabolic process	1.65E-03	1034	128	62	1901360	BP
siRNA-mediated retrotransposon silencing by heterochromatin formation	1.65E-03	5	128	5	141007	BP
chiasma assembly	1.65E-03	5	128	5	51026	BP
DNA repair	2.06E-03	61	128	13	6281	BP
cellular nitrogen compound metabolic process	2.26E-03	1016	128	61	34641	BP
catalytic activity, acting on a nucleic acid	1.97E-04	51	128	13	140640	MF
helicase activity	2.32E-03	13	128	7	4386	MF
ATP-dependent activity, acting on RNA	9.66E-03	6	128	5	8186	MF
RNA helicase activity	9.66E-03	6	128	5	3724	MF

Table 4.S10: Latitudinal TSD gene Pst outliers

Gene	Pst	Temp
CO6A6	0.731	FPT
LINGO3	0.715	FPT
K2C8	0.704	MPT
NLRP3	0.685	FPT
LOC106737513	0.668	MPT
THSD7B	0.667	FPT
AGMO	0.664	MPT
HCN4	0.655	FPT
KV6A9	0.655	FPT
CUNH8orf89	0.653	FPT
EBF2	0.645	MPT
CCDC63	0.643	MPT
TERB2	0.629	FPT
UCP2	0.628	MPT
LOC109285400	0.621	FPT
TBR1	0.62	FPT
MYRIP	0.619	MPT
LOC109283608	0.616	FPT
LOC106737564	0.613	FPT
LOC106738095	0.612	MPT
PTH2R	0.611	MPT
SNX31	0.61	FPT
NEU4	0.609	MPT
SQLE	0.607	FPT
LOC109283708	0.606	MPT
LOC109283184	0.6	FPT
MYO18B	0.6	FPT
COSA1	0.598	FPT
LOC109285853	0.597	FPT
DPEP1	0.596	FPT
FZD6	0.596	FPT
TMEM139	0.595	FPT
MYO15	0.594	FPT
MUC3A	0.592	FPT
ANHX	0.591	FPT
PSPH	0.589	FPT
FSTL4	0.588	FPT

DHE4	0.588	FPT
LOC109283530	0.588	FPT
C2C2L	0.584	MPT
ZMYM1	0.582	FPT
YIPF7	0.582	FPT
RO52	0.578	FPT
ALPL	0.578	MPT
LOC109282056	0.577	MPT
TRI39	0.575	MPT
RTJK	0.575	FPT
VIT	0.573	MPT
ZAR1L	0.572	FPT
TERB1	0.571	FPT
SLP1	0.569	FPT
MRC2	0.569	FPT
MYH7	0.564	FPT
IGFALS	0.563	FPT
STAG3	0.562	FPT
RBM44	0.56	FPT
MROH5	0.557	FPT
MYL4	0.557	FPT
LOC102576232	0.556	FPT
FNDC4	0.553	MPT
FAM71E1	0.551	FPT
LOC109284059	0.547	MPT
REC8	0.547	FPT
LOC109285596	0.546	FPT
MCM8	0.545	FPT
OPRK1	0.544	FPT
ANKRD24	0.543	MPT
TRI25	0.541	MPT
PITX3	0.541	FPT
MEI4	0.541	FPT
RNF17	0.538	FPT
DPEP1	0.537	FPT
STRA8	0.537	FPT
COL9A1	0.534	FPT
TRIM50	0.534	FPT
ANKRD31	0.533	FPT
CAPZA3	0.532	FPT

TMEM59L	0.531	FPT
ABI3BP	0.53	FPT
LIPM	0.53	FPT
RNAS1	0.53	MPT
RFOX2	0.529	FPT
MLC1	0.529	FPT
TMEM217	0.528	FPT
CUNH16orf89	0.527	FPT
MZB1	0.526	FPT
FAM162B	0.525	FPT
CRY2	0.525	FPT
MEI1	0.525	FPT
RNF212B	0.525	FPT
MSH5	0.523	FPT
CYT	0.521	FPT
PLPL1	0.521	FPT
TIGAR	0.521	FPT
CATSPERG	0.519	FPT
AOXC	0.518	FPT
CUNH19orf57	0.516	FPT
LOC109283544	0.516	FPT
ALG13	0.515	FPT
SLC25A31	0.515	FPT
CA185	0.514	FPT
STK31	0.513	FPT
ISX	0.512	FPT
PLCXD2	0.51	FPT
RAD51AP2	0.51	FPT
C27C1	0.509	FPT
TCP4	0.509	FPT
FA2H	0.508	MPT
CD20	0.508	MPT
MEIOB	0.508	FPT
MGAT4C	0.507	MPT
WNT2	0.506	FPT
IL5RA	0.505	FPT
DMRTD	0.505	FPT
TBA3	0.505	FPT
CUNH14orf39	0.502	FPT
DHX32	0.502	FPT

ITIH3	0.5	FPT
LOC109282137	0.5	FPT
MC5R	0.499	FPT
CC2D2B	0.498	FPT
LOC109282373	0.498	MPT
LOC109285930	0.498	FPT
ZNF541	0.497	FPT
CUNH18orf63	0.496	FPT
TDRD1	0.496	FPT
SGO2	0.495	FPT
SKAP1	0.495	FPT
SYCP3	0.495	FPT
CCDC155	0.494	FPT
PIWIL1	0.494	FPT
DLK1	0.493	MPT
TBX15	0.493	FPT
DNAH8	0.492	FPT
LOC109280640	0.492	FPT
LOC102574968	0.491	FPT
TESMIN	0.49	FPT
FBXO47	0.489	FPT
ADPRH	0.489	MPT
CAD18	0.489	FPT
ITIH6	0.488	FPT
LOC106737694	0.488	MPT
DMC1	0.482	FPT
LOC109286094	0.482	FPT
LOC109286235	0.481	FPT
S27A6	0.48	FPT
CUNH12orf40	0.479	FPT
SPATA22	0.479	FPT
RBM46	0.478	FPT
RNF212	0.478	FPT
SMC1B	0.478	FPT
LIPI	0.477	FPT
SLC26A8	0.477	FPT
LOC106738021	0.476	FPT
TCTE3	0.476	FPT
FRMPD3	0.474	MPT
B3GNT5	0.473	FPT

CALR3	0.472	FPT
S6OS1	0.471	FPT
PRSS54	0.471	FPT
LOC109280587	0.47	FPT
BTBD18	0.469	FPT
CYC	0.469	FPT
AIG1	0.468	FPT
CUNHXorf58	0.466	FPT
PKD2L2	0.466	FPT
TDRD15	0.464	FPT
TEX14	0.464	FPT
TMEM116	0.464	FPT
CATSPERB	0.463	FPT
PDCL2	0.463	FPT
RAD21L1	0.463	FPT
IGSF10	0.462	MPT
D42E2	0.462	FPT
CX6B2	0.462	FPT
SYCE3	0.462	FPT
ADAD1	0.461	FPT
ZN420	0.461	FPT
MAS1	0.461	MPT
REC114	0.461	FPT
TRIM16	0.461	MPT
PANX2	0.46	FPT
CUNH10orf105	0.459	MPT
VIPR1	0.459	FPT
SLC38A11	0.459	FPT
LOC106739893	0.458	FPT
S6OS1	0.458	FPT
LOC109283131	0.458	FPT
DDX25	0.457	FPT
DAZL	0.456	FPT
TEX30	0.455	FPT
LOC109282119	0.453	FPT
LOC109282966	0.452	FPT
COL11A2	0.451	FPT
AQP10	0.449	MPT
CTCFL	0.449	FPT
BUCKY	0.449	FPT

R51A2	0.449	FPT
CACNA1I	0.448	FPT
CDK3	0.448	FPT
CUNH9orf84	0.448	FPT
FAM83A	0.448	FPT
SHCBP1L	0.448	FPT
MPZ	0.447	FPT
BRDT	0.446	FPT
HHIP	0.446	FPT
LOC109283552	0.446	FPT
MROH5	0.444	FPT
HFM1	0.441	FPT
MGT4C	0.44	FPT
QRFPR	0.44	MPT
MOV10L1	0.44	FPT
TDH	0.439	FPT
TSPAN16	0.438	FPT

Table 4.S11: Latitudinal TSD gene Pst outlier GO/KEGG enrichment

Term name	**Adjusted p-value**	**Term size**	**Query size**	**Intersect size**	**Term ID**	**Source**	**Temp**
meiotic cell cycle	3.70E-29	72	123	34	51321	BP	FPT
meiotic cell cycle process	1.87E-23	64	123	29	1903046	BP	FPT
meiotic nuclear division	6.15E-23	60	123	28	140013	BP	FPT
sexual reproduction	1.82E-19	238	123	44	19953	BP	FPT
meiosis I cell cycle process	1.18E-17	45	123	22	61982	BP	FPT
meiosis I	6.23E-17	42	123	21	7127	BP	FPT
nuclear division	1.72E-16	94	123	28	280	BP	FPT
male gamete generation	2.73E-16	144	123	33	48232	BP	FPT
organelle fission	2.77E-15	103	123	28	48285	BP	FPT
gamete generation	2.07E-14	188	123	35	7276	BP	FPT
cellular process involved in reproduction in multicellular organism	1.95E-13	119	123	28	22412	BP	FPT
multicellular organismal reproductive process	8.01E-13	223	123	36	48609	BP	FPT
spermatogenesis	1.25E-12	138	123	29	7283	BP	FPT
multicellular organism reproduction	6.27E-12	237	123	36	32504	BP	FPT
reproductive process	7.47E-12	381	123	45	22414	BP	FPT
reproduction	1.39E-11	387	123	45	3	BP	FPT
male meiotic nuclear division	2.88E-11	24	123	14	7140	BP	FPT

homologous chromosome pairing at meiosis	6.37E-11	25	123	14	7129	BP	FPT
homologous chromosome segregation	1.34E-10	26	123	14	45143	BP	FPT
chromosome organization involved in meiotic cell cycle	2.72E-10	27	123	14	70192	BP	FPT
meiotic chromosome segregation	5.30E-10	28	123	14	45132	BP	FPT
chromosome organization	1.12E-09	74	123	20	51276	BP	FPT
cell cycle process	2.16E-09	222	123	32	22402	BP	FPT
cell cycle	8.90E-09	313	123	37	7049	BP	FPT
reciprocal homologous recombination	9.00E-09	17	123	11	140527	BP	FPT
reciprocal meiotic recombination	9.00E-09	17	123	11	7131	BP	FPT
homologous recombination	9.00E-09	17	123	11	35825	BP	FPT
DNA recombination	5.69E-08	44	123	15	6310	BP	FPT
chromosome segregation	1.14E-07	54	123	16	7059	BP	FPT
developmental process involved in reproduction	2.18E-07	261	123	32	3006	BP	FPT
nuclear chromosome segregation	3.42E-07	49	123	15	98813	BP	FPT
germ cell development	2.68E-06	97	123	19	7281	BP	FPT
female gamete generation	4.16E-04	56	123	13	7292	BP	FPT
male meiosis I	8.32E-04	12	123	7	7141	BP	FPT

DNA metabolic process	4.31E-03	133	123	18	6259	BP	FPT
oogenesis	2.38E-02	43	123	10	48477	BP	FPT

Table 4.S12: Nest temperature predictions

Year	Lake Apopka, FL (Orange County, FL)	Lake Woodruff, FL (Volusia County, FL)	Yawkey Wildlife Center, Georgetown, SC (Georgetown County, SC)	Savannah River Site (Barnwell County, SC)
1950	33.85	32.84	32.36	32.64
1951	33.54	33.01	32.88	32.81
1952	33.74	33.16	33.73	33.77
1953	33.43	32.67	32.64	33.03
1954	32.92	32.91	33.46	34.1
1955	32.91	32.31	31.99	31.86
1956	33.69	32.93	32.65	33.79
1957	32.93	32.57	32.2	33.04
1958	33.19	32.85	31.65	33.03
1959	32.48	32.3	31.73	32.62
1960	32.92	32.61	32.01	33.06
1961	33.38	32.52	31.34	32.29
1962	33.32	32.8	31.89	33.21
1963	33.31	33.03	31.52	32.49
1964	33.2	32.72	31.14	31.59
1965	32.07	31.61	31.42	31.4
1966	32.19	32.11	31.9	32.66
1967	32.65	32.14	31.31	31.94
1968	32.16	32.25	32.58	33.34
1969	33.6	33.12	32.72	32.78
1970	33.15	33.31	33.29	33.32
1971	33.33	32.62	32.02	32.22
1972	33.56	32.64	32.15	32.45
1973	33.54	32.71	32.06	32.51
1974	32.18	31.76	31.45	31.53
1975	32.88	32.32	31.71	31.78
1976	33.34	32.38	31.81	32.68
1977	33.2	33.63	33.62	34.31
1978	33.04	32.49	32.66	33.03
1979	33.42	32.2	32.44	32.16
1980	33.75	33.59	33.63	34.18
1981	34.32	33.74	33.18	33.27
1982	33.14	32.87	31.76	32.05
1983	33.04	33.04	33.02	33.31
1984	32.5	32.42	32.45	32.52

1985	32.99	32.16	31.96	32.76
1986	33.14	32.76	33.64	35.13
1987	34.21	33.02	33.17	33.88
1988	32.68	32.42	32.66	33.98
1989	33.62	33.06	32.09	32.58
1990	33.55	32.8	33.12	34.38
1991	33.46	33.04	32.85	33.1
1992	33.57	33.08	32.63	33.23
1993	33.74	32.95	33.64	34.78
1994	32.72	32.15	32.46	32.86
1995	32.67	32	31.88	33.34
1996	33.29	32.06	31.98	33.63
1997	33.3	33.03	31.66	33.37
1998	34.36	33.99	33.31	34.31
1999	33.11	32.83	32.47	33.58
2000	33.52	32.65	31.92	34.27
2001	32.45	31.98	31.87	32.76
2002	32.08	31.44	32.12	33.7
2003	32.61	31.69	31.81	31.66
2004	33.33	32.79	32.04	32.58
2005	33.32	32.32	32.38	32.47
2006	33.48	32.42	32.48	32.78
2007	33.66	32.66	32.67	32.94
2008	32.99	32.34	32.47	33.46
2009	33.6	32.97	32.55	33.38
2010	33.98	33.23	33.18	34.28
2011	33.7	33.01	33.54	34.9
2012	32.87	32.34	32.62	33.48
2013	32.72	32.41	31.26	31.76
2014	33.53	32.69	31.59	33.08
2015	33.61	33.21	33.42	34.55
2016	33.87	33.48	33.28	34.47
2017	33.5	32.36	31.97	32.86
2018	33.58	32.42	32.42	33.3
2019		32.63	32.66	34.05
2020		32.85	31.83	33.81
Median	*33.32*	*32.67*	*32.42*	*33.06*
Mean	*33.22724638*	*32.66788732*	*32.39394366*	*33.12985915*

CHAPTER 5

ORGANISMAL TRAITS CONNECTING INCUBATION TEMPERATURE TO JUVENILE SURVIVAL: IMPLICATIONS FOR THE EVOLUTION OF TEMPERATURE-DEPENDENT SEX DETERMINATION[4]

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[4] Smaga, C. R., Bock, S. L., Johnson, Rainwater, T., Singh, R., & Parrott, B. B. To be submitted to *Journal of Animal Ecology*.

Abstract

1. Understanding the ecological and evolutionary drivers of diverse sex determining systems is a major goal of biology. In particular, the adaptive value of temperature-dependent sex determination (TSD) has fascinated scientists since its discovery.
2. Recent work supports the Sex-Specific Survival to Maturity (STM) hypothesis, an extension of the Charnov-Bull model, which suggests that thermosensitive survival of juveniles in combination with sex-biases in age at maturity can drive the adaptive evolution of TSD. However, the biological mechanisms linking incubation temperature to survival are not well understood.
3. Developmental efficiency (DE), a proxy for developmental cost (the product of incubation duration and metabolic rate), is hypothesized to contribute to morphological variation underlying temperature differences in survivorship, but empirical tests of associations between DE, early-life phenotypes, and temperature-dependent survival are lacking.
4. The American alligator (*Alligator mississippiensis*) is a model TSD species that shows support for the STM. Using several, independent years of mark-release-recapture data in alligators, we investigated the contributions of DE and other organismal traits (morphological and metabolic phenotypes) to temperature-dependent survivorship.
5. Incubation temperature exerted persistent effects on early-life growth and size, including in traits that were not thermosensitive at hatch.

6. The influence of incubation temperature on survival was partially mediated by DE through its effect on post-release phenotypes. However, DE and phenotypic traits only accounted for a small portion of incubation temperature's total influence, the rest mediated by unmeasured factors.
7. Our study highlights the importance of longitudinal data for understanding how developmental conditions such as incubation temperature contribute to evolutionary change, including the evolution of TSD in the alligator.

Introduction

The ecological and evolutionary drivers of diverse sex determining strategies observed across the tree of life have intrigued scientists for decades (Fisher, 1930; Bull, 1985; Schwanz et al., 2013; Bachtrog et al., 2014). Whereas many species utilize sex chromosomes, others rely on environmental cues in a phenomenon referred to as environmental sex determination (ESD). Many reptiles and fishes display the most common form of ESD, in which temperatures experienced during specific developmental windows determines sexual fate (Charnier, 1966; Crews et al., 1994; Kohno et al., 2014; Lang & Andrews, 1994; Valenzuela & Lance, 2004). Several theories have attempted to explain the evolutionary benefit of temperature-dependent sex determination (TSD), mostly derived from the Charnov-Bull model, which posits that TSD is evolutionarily favored when incubation temperature exerts sex-dependent effects on offspring fitness (Charnov & Bull, 1977; Shine, 1999). Studies directly measuring reproductive success in short lived species have provided empirical support for the Charnov-Bull model by demonstrating sex-specific effects of incubation temperature on fecundity (Conover, 1984; Warner & Shine, 2008b). However, many TSD species are long lived, which presents challenges for resolving the influence of incubation temperature on lifetime fecundity. Further, direct links between incubation temperature and reproduction are more difficult to imagine in longer-lived species due to the length of time separating development and reproductive maturity.

In 2016, Schwanz et al. (Schwanz et al., 2016) proposed a novel extension of the Charnov-Bull theory that relies on incubation temperature affecting juvenile survival rather than lifetime reproductive success. In this model, termed the Sex-Specific Survival to Maturity hypothesis (STM), TSD is evolutionarily favored over genotypic sex determination (GSD) when two conditions are met: i) incubation temperature influences juvenile survival independent of sex and ii) the age at maturity differs between the sexes. Under these circumstances, the sex that matures later disproportionally benefits from incubation temperatures that increase early life survival. Perhaps the best support for the STM comes from experimental approaches that demonstrate enhanced survival at the incubation temperatures that produce the later-maturing sex in both a turtle and a crocodilian (Bock et al., 2023; Leivesley & Rollinson, 2024), independent of sex when tested (Bock et al., 2023). This is augmented by additional comparative studies showing that TSD species display significantly greater age differentials at maturity than species with GSD (Bókony et al., 2019) and exhibit higher levels of sexual size dimorphism (Katona et al., 2021), a potential correlate of age at first reproduction. Despite this support, however, our understanding of the development-by-environment interactions that link incubation temperature to juvenile survival that ultimately underlies the evolution of TSD remains limited.

It was recently hypothesized that developmental cost, measured as the product of metabolic rate and incubation duration (Marshall et al., 2020), serves as an overarching mechanism underlying variation in morphological phenotypes that collectively contribute to temperature-dependent survival outcomes (Bock et al., 2023). Both incubation duration and metabolic rate are sensitive to incubation temperature, but differences in their thermodependencies result in an optimum temperature at which maternal resources are most efficiently converted into offspring mass (Pettersen et al., 2019). Minimizing developmental cost is evolutionarily favored both within and across species (Marshall et al., 2020; Pettersen, 2020), suggesting sub-optimal developmental temperatures likely impose fitness costs for individuals. In line with this, direct outcomes of developmental cost, namely hatchling and residual yolk mass after correcting for egg mass, have been shown to be beneficial in some cases (Kissner & Weatherhead, 2005; Murphy et al., 2020; Radder et al., 2004). Yet, their sufficiency to explain temperature-dependent survival remains unclear.

American alligators (*Alligator mississippiensis*) are long-lived reptiles that utilize TSD and were among the first species to demonstrate empirical support for the STM hypothesis (Bock et al., 2023). Incubating eggs at intermediate temperatures typically results in male offspring, whereas females are produced at both cooler and warmer incubation temperatures (**Figure 5.1a**; Ferguson & Joanen, 1983). Importantly, males and females exhibit a stark bias in age at first reproduction, with male alligators siring their first clutch almost a decade after females first reproduce (males: 24 years, females: 16 years; Wilkinson et al., 2016; Zajdel et al., 2019). Recent work from our research group showed that, consistent with predictions of the STM, hatchlings produced at male-promoting temperatures (MPTs) have enhanced survival in the wild compared to those incubated at both cool and warm female-promoting temperatures (FPTs; **Figure 5.1b**; Bock et al., 2023; Johnson et al., 2023). This effect of incubation temperature on survival is independent of sex as eggs incubated at MPT and sex reversed by estrogen treatment have higher survival probability than FPT controls (Bock et al., 2023). Interestingly, animals incubated at MPT also show reduced development cost relative to FPT (Bock et al., 2023), which results in increased mass at hatch and greater reserves of residual yolk (Bock et al., 2021; Smaga et al., 2024). Previous attempts to establish links between specific hatchling phenotypes and survival in alligators have produced equivocal results, with inconsistent effects that vary across years and experiments (Bock et al., 2023; Johnson et al., 2023). However, these studies only examined traits at hatch and survival at two timepoints (pre- and post-winter), and did not consider time-dependent relationships between incubation temperature, developmental cost, later-life phenotypes, and survivorship.

Here, we utilize multi-year field data to further investigate how organismal traits (e.g., morphological and metabolic phenotypes) contribute to temperature-dependent survivorship in alligators. We hypothesize that reduced developmental cost at MPT is advantageous for survival, predicting that hatchlings incubated at MPT will exhibit enhanced post-release growth associated with developmental cost that is positively linked to survival probability. To test this, we first analyze several independent years of release-mark-recapture data to identify how incubation temperature influences post-release phenotypes over time. We then explicitly examine mechanistic pathways linking incubation temperature, a metric of developmental cost, post-release phenotypes, and survival utilizing structural equation models (SEMs). When viewed collectively, our findings provide novel insight into how development-by-environment interactions influence subsequent survival and contribute to evolutionary outcomes associated with sex determining strategies.

Materials and Methods

Study design and data processing

To investigate relationships between incubation temperature, phenotype, and survival in the wild, we utilized three years of mark-release-recapture data (Table 5.1; Bock et al. 2023; Johnson et al. 2023). Data from Bock et al. consisted of three years (2019, 2020, and 2021) in which eggs were incubated at FPT or MPT, released, and recaptured monthly for 1 year. The 2019 experiment took place on Par Pond on the Savanah River Site in Aiken, SC, while the 2020 and 2021 experiments took place at Yawkey Wildlife Center in Georgetown, SC. In the 2020 experiment, eggs were further manipulated by treating MPT or FPT eggs with either estradiol or vehicle, allowing the decoupling of incubation temperature and sex while controlling for hormone treatment. Additionally, the experiment from 2021 also included incubations at a high female promoting temperature (HFPT) in addition to MPT and FPT. Johnson et al. (2023) utilized similar methods, incubating at MPT or FPT, and took place in 2021 at Par Pond on the Savannah River Site. In both studies, hatchlings were re-measured at each recapture event for mass using spring scales, snout-vent length (SVL) using a ruler, tail girth (TG) using a flexible tape measure, and head length (HL) and head width (HW) using calipers.

We compiled all survival/recapture data from the above studies and inspected it to remove any individuals lacking critical information along with trait values at any time point that were noticeably due to human error or outliers. The latter were identified visually by plotting hatchling traits against days post-hatch (DPH) at capture. Further, we ensured that any hatchling observed at a later timepoint that was not captured at previous timepoints was recorded as alive at all prior timepoints.

Based on incubation temperature, we split all hatchlings into two groups. Hatchlings incubated at either 29°C (FPT in 2019 from Bock et al. 2023) or 29.5°C (all other FPT) were considered FPT and those incubated at 33.5°, MPT. Animals incubated at HFPT in 2021 were removed from the dataset. Given the lack of differences in survival between vehicle and estrogen-treated FPT hatchlings and between MPT and estrogen-treated MPT hatchlings, we grouped them together with the rest of the FPT and MPT groups, respectively. Final sample sizes of released hatchlings by year, population, temperature group, and treatment are shown in Table 5.1.

To condense variation in recapture dates across years and populations, we split the data into 6 discrete time intervals, corresponding to the number of DPH of hatchlings at recapture. These included release-60, 61-120, 121-240, 241-300 and 301-360DPH (hereafter, timepoints A-E, respectively; **Figure 5.1b**). The third timepoint was longer because it spanned winter months during which no recapture efforts were conducted. With this setup, each time interval represents the survival status of any recaptured individual during that period, whereas the phenotypic traits associated with that timepoint represent those measured at hatch or any previous recapture event. For instance, timepoint A represents survival from release to 60DPH and phenotypic values during this period include those measured at hatch or any other measurement directly prior to a recapture before 60DPH. This binning approach allowed us to detect non-linear changes in hatchling phenotypes over time and their associations with survival at high resolution while retaining sufficient sample sizes at each timepoint.

Assessing the thermosensitivity of hatchling traits and survival

We first used the compiled dataset to confirm previous results demonstrating an influence of incubation temperature on hatchling phenotypes and survival through the first year of life. We chose three traits to examine that describe the overall condition of hatchling alligators: mass, SVL, and TG. Using linear mixed-effect models (LMMs) in the lme4 package (Bates et al., 2025) in R (R Core Team, 2024, version 2024.04.2) with temperature and egg mass as predictors and random intercepts of origin (the year and population origin of each individual) and clutch nested within origin, we tested whether each phenotype was different between incubation temperatures. We then used Cox-proportional hazards mixed models in the coxme R package (Therneau, 2009) to assess the influence of incubation temperature on survival curves generated from time-to-death data, which we estimated based on the last day post-hatch an individual was observed. Individuals that remained alive after one year were right censored. Phenology and age at release are also known to influence survival (Perez-Heydrich et al., 2012; Warner & Shine, 2007) and differed across individuals, so within the model, we included Julian day of release and DPH at release, along with temperature group as predictors. As nested random effects are not available for coxme, we included a random intercept that denoted the year, population, and clutch combination of each individual.

Modeling hatchling traits over time in response to incubation temperature

To assess how incubation temperature influences post-release traits over time, we fit separate LMMs for each phenotype, including an interaction between incubation temperature and timepoint as a predictor, DPH at measurement and egg mass as covariates, and origin and clutch nested within origin as random intercepts. We then used the emmeans package (Lenth et al., 2025) to conduct pairwise comparisons between temperatures at each timepoint, adjusting p-values using the Sidak method. Additionally, we tested for temperature differences in mean phenotypic change between consecutive timepoints using the contrast function in emmeans with Kenward-Roger degrees of freedom and Bonferroni p-value correction (2 tests, adjusted p = 0.025). It is important to note that due to the nature of the data, timepoint designations for traits reflect their values at recaptures prior to their survival status at that timepoint.

Identifying trait-survival associations over time

To identify how thermosensitive traits are associated with survival over time and test the hypothesis that developmental cost serves as a mechanistic link between incubation temperature, phenotypes, and survival, we utilized SEMs. SEMs allow for the simultaneous testing of multiple mechanistic pathways to explain variation in a response variable of interest, including direct and indirect effects mediated by intermediate variables. In this case, we were interested in the direct effects of incubation temperature on survival as well as indirect effects mediated by DE and/or the measured traits. We exclusively focused on timepoints A-C, as these included both MPT and FPT individuals (see **Results**). As data on embryonic metabolic rates and incubation durations were not available, we quantified a metric of developmental cost, termed developmental efficiency

(DE), for all alive individuals as the residuals of a linear model of hatchling mass on egg mass. Due to strong correlations and statistical non-independence among our phenotypes of interest (measured values of mass, SVL, TG and their delta values between consecutive captures), we calculated the first two principal components of separate principal component analyses (PCAs) at each timepoint as representative of major axes of phenotypic variation using the prcomp function in R (center = TRUE, scale = TRUE). We did not include delta values in the release-60DPH period, as most individuals during this period were not captured more than once prior to noting their survival status. Finally, using the piecewiseSEM (Lefcheck et al., 2024) and lme4 packages in R, we fit separate SEMs at each timepoint using centered and scaled values for each variable. Each SEM included the following sub models:

i. a linear model of incubation temperature on DE
ii. a LMM of incubation temperature and DE on PC1, with time between recaptures and DPH at measurement as covariates and origin and clutch nested within origin as random intercepts
iii. a LMM of incubation temperature and DE on PC2, with PC1, time between recaptures, and DPH at measurement as covariates and origin and clutch nested within origin as random intercepts
iv. a generalized linear mixed model (family = binomial(link = “logit”); 1 = alive, 0 = dead)) of PC1, PC2, incubation temperature, and DE on survival, with time between recaptures and DPH at measurement as covariates and origin and clutch nested within origin as random intercepts.

Given the relatively small sample sizes and unequal representation of MPT and FPT individuals combined with a relatively large number of predictors, particularly at later timepoints, we used 1,000 parametric bootstraps with the bootMer function in the lme4 package to obtain more robust estimates of predictor significance in the survival models prior to incorporating them into the SEM. From the SEM summary, we then extracted the standardized path coefficients. To estimate the proportion of temperature's influence on survival mediated by significant indirect pathways ($p < 0.05$ for all paths), we multiplied the coefficients along the path and divided by the total influence of incubation temperature on survival (the sum of all of incubation temperature's direct and indirect paths regardless of significance).

Statistical analyses and figure preparation

All statistical analyses were conducted in R using R Studio and with a p-value cutoff of $p \leq 0.05$ unless otherwise noted. We used the packages dplyr and tdyr (Wickham et al., 2023) for data preparation and manipulation and ggplot2 for visualization (Wickham, 2016).

Results

Incubation temperature exerts strong influences on hatchling traits and survival in the wild

Results from linear models controlling for egg mass indicated a significant effect of incubation temperature on mass ($\beta = 2.520 \pm 0.211$, $p < 2e-16$) but not SVL ($\beta = 0.030 \pm 0.030$, $p = 0.321$) or TG ($\beta = -0.021 \pm 0.015$, $p < 0.154$), with animals from MPT weighing 4.5% more on average than those from FPT (**Figure 5.1c-e**). In our Cox-proportional hazards model for survival, we found a significant effect of incubation temperature (hazard-ratio = -0.946 ± 0.261, $p < 0.001$), with animals from MPT 38.8% less likely to die at any timepoint than those from FPT (**Figure 5.1f**). We also found a significant negative effect of release DPH (hazard-ratio = -0.209 ± 0.039, $p < 0.0001$), but no effect of Julian day of release (hazard-ratio = -0.019 ± 0.019, $p = 0.313$). These results corroborate previous work demonstrating a strong influence of incubation temperature on hatchling mass and survival probability.

Incubation temperature influences post-release phenotypes

Due to the lack of surviving FPT individuals at later timepoints, we limited our analysis of incubation temperature differences in post-release traits to timepoints A-C. We found a persistent effect of incubation temperature on all three phenotypes, including how they changed between timepoints. Similar to trait patterns observed at hatching, animals incubated at MPT had significantly higher mass prior to timepoint A survival, whereas no differences between incubation temperatures were observed for TG or SVL (**Figure 5.2a-c**; Table 5.2). Animals incubated at FPT lost mass on average between timepoints A and B, while animals incubated at MPT generally maintained their mass (**Figure 5.2a;** Table 5.2). This was not the case between timepoints B and C, where average changes in mass were similar between incubation temperatures (**Figure 5.2a**; Table 5.2). The SVL of individuals incubated at MPT showed a significantly greater average increase between timepoints A and B, resulting in longer SVL prior to timepoint B survival that persisted through timepoint C (**Figure 5.2b**; Table 5.2). However, change in SVL between timepoints B and C was not different between incubation temperatures (**Figure 5.2b**; Table 5.2). Results from TG mirrored those from SVL, with animals from

MPT losing less TG on average between timepoints A and B, resulting in larger TGs prior to timepoint B survival that persisted through timepoint C (**Figure 5.2c**; Table 5.2). These results suggest incubation temperature exerts persistent effects on post-release growth, particularly between timepoints A and B.

Developmental efficiently partially mediates incubation temperature's influence on survival

In all SEMs, p-values of predictors in survival models aligned with bootstrapped 95% confidence intervals, suggesting robust measures of significance. Thus, p-values reported are from the original models. Model results for each SEM, including raw estimates with standard errors, are shown in Tables 5.S1-3. The estimates reported below are the standardized estimates for interpretable comparisons.

Timepoint A

PC1 (73.89% of variation) was negatively associated with all traits, while PC2 (17.26% of variation) was negatively associated with TG and mass but strongly positively associated with SVL (**Figure 5.3a**). After excluding individuals with missing data, the SEM included 585 observations and demonstrated moderate fit to the data based on Chi-Squared test ($\chi^2 = 3.69$, $p = 0.60$) and Fisher's C statistic ($C = 19.29$, $p = 0.04$). We found a positive, direct effect of incubation temperature on both survival (estimate = 0.354, p = < 0.001) and DE (estimate = 0.380, $p < 0.001$). DE, in turn, negatively influenced PC1 (estimate = -0.415, $p < 0.001$). However, there were no significant relationships between PC1, PC2 or DE and survival (**Figure 5.3a**).

Timepoint B

All 6 phenotypes (mass, SVL, TG, Δmass, ΔSVL, and ΔTG) were negatively associated with PC1 (55.95% of variation), while PC2 (16.71% of variation) was negatively associated with mass, SVL and TG and positively associated with their delta values (**Figure 5.3b**). The SEM included 85 observations and was well fit to the data (Chi-Squared: $\chi^2 = 1.09$, p = 0.955; Fisher's C: C = 12.17, p = 0.274). Incubation temperature again had a direct positive effect on both survival (estimate = 0.393, p = 0.049) and DE (estimate = 0.278, p = 0.01). DE further negatively influenced both PC1 (estimate = -0.173, p = 0.010) and PC2 (estimate = -0.343, p < 0.001). PC1 was also significantly negatively associated with survival (estimate = -0.620, p = 0.021), which supports an indirect influence of incubation temperature on survival through DE and PC1 (**Figure 5.3b**). This is in line with our hypothesis that elevated DE results in increased size and growth that are beneficial for survivorship. The latter indirect path contributed 7.88% to temperature's total influence.

Timepoint C

Like prior timepoints, PC1 (56.81% of variation) was negatively associated with all traits. On the other hand, PC2 (19.70% of variation) was positively associated with ΔTG and Δmass, negatively associated with ΔSVL and SVL, and showed little association with mass and TG (**Figure 5.3c**). The SEM contained 54 observations and was well fit to the data according to both Chi-Squared ($\chi^2 = 3.26$, p = 0.66) and Fisher's C (C = 11.89, p = 0.29). Interestingly, there was no significant direct effect of incubation temperature on survival during this period (estimate = -0.920, p = 0.097). However, incubation temperature still positively influenced DE (estimate = 0.426, p = 0.001), which was negatively associated with PC1 (estimate = -0.121, p = 0.016). PC1 was also directly and negatively influenced by incubation temperature (estimate = -0.148, p = 0.030) and further, negatively associated with survival (estimate = -1.126, p = 0.016). This again supports a persistent indirect effect of incubation temperature on survival through DE and PC1, which accounted for 8.60% of temperature's total influence. An additional 24.66% was explained by the path between incubation temperature, PC1, and survival, independent of DE.

Discussion

Despite its discovery over 60 years ago, the evolutionary benefit of TSD has remained elusive. Recent evidence supports the STM hypothesis in American alligators (Bock et al., 2023); yet the mechanisms connecting incubation temperature to juvenile survival are largely unknown. Identifying organismal traits associated with variation in survivorship is challenging due to complex interactions between morphological, metabolic, and performance-based phenotypes, as well as ecological processes (Arnold, 1983; Calsbeek & Irschick, 2007). In the case of TSD and the STM, however, survival must be mediated by a specific subset of traits that are robustly and consistently influenced by incubation temperature, either as a single, advantageous phenotype or a series of interrelated phenotypes that each provide a benefit under a particular ecological condition. Here, we demonstrate that incubation temperature has lasting effects on post-release phenotypes that are associated with survival probability in alligators. Specifically, animals at MPT display greater early-life growth, resulting in larger sizes that are positively associated with survival during timepoints B and C. We show further support for temperature mediated differences in developmental cost as an underlying mechanism, as growth and size at MPT were significantly associated with DE. Interestingly, however, DE associated phenotypic variation only explained a relatively small portion of temperature's influence on survival, the remaining mediated by unknown factors. Taken altogether, our results suggest that temperature driven, time-dependent relationships involving DE and hatchling size contribute to the evolution of TSD under the STM. However, currently unmeasured traits, such as those associated with predator avoidance, are likely critical, as a large portion of incubation temperature's influence on survival remained unexplained.

We found lasting effects of incubation temperature on all analyzed phenotypes post-release. Between timepoints A and B in particular, animals at MPT maintained similar masses and grew more in SVL on average relative to FPT animals, who lost mass and grew less in SVL. These differences were maintained several months later and suggest persistent, early-life effects of incubation temperature, even when absent at hatch. Similar results have been found in tuatara (Nelson et al., 2004) and Cuban rock iguanas (Alberts et al., 1997), in which incubation temperature differences do not arise until later in life. This is also consistent with a previous meta-analysis demonstrating that in reptiles, the effects of incubation temperature don't diminish over time, and in some cases, even increase in magnitude (Noble et al., 2018). Interestingly, crocodilians exhibit substantial sexual size dimorphism, where males reach larger sizes as adults compared to females (Platt et al., 2011; Platt et al., 2009; Wilkinson et al., 2016). Reproductive success is correlated to size in male alligators (Zajdel et al., 2019), and whereas the STM hypothesis relies on an effect of incubation temperature on juvenile survival (Schwanz et al., 2016), persistent effects of MPT on growth and size could also be more beneficial for males relative to females. This, in turn, would provide an additional selective pressure for coupling incubation temperature and sex (e.g., Conover, 1984), potentially independent of survival. The extent to which sexual dimorphism in alligators is a long-term consequence of incubation temperature or other sex-specific factors (Badyaev, 2002; Cox et al., 2009) remains to be tested, but in our dataset, there were minimal differences in mass, SVL or TG between vehicle and estrogen-treated (sex-reversed) MPT animals across timepoints, suggesting little effect of sex (data not shown). Nonetheless, our results demonstrate the critical importance of longitudinal studies for understanding how developmental conditions such as incubation temperature may contribute to later life phenotypes, sometimes independent of differences at hatch.

Post-release traits were significantly associated with DE at all timepoints and positively related to survival during timepoints B and C, supporting a role for developmental cost in mediating the effects of incubation temperature on survivorship. In fact, our mediation analysis demonstrated that after accounting for DE, the direct effect of incubation temperature on phenotypes during timepoints A and B was no longer significant, suggesting most of the phenotypic effects of incubation temperature we observed can be attributed to incubation temperature’s influence on DE. The mechanisms connecting DE to later-life phenotypes are not known but may be driven by increased residual yolk, which provides a nutrient-rich source of energy likely facilitating increased and longer periods of growth in MPT relative to FPT animals (Murphy et al., 2020; Radder et al., 2004). An additional, non-mutually exclusive explanation is that increased metabolic efficiency during development at MPT persists post-hatch, resulting in more efficient utilization of yolk and other acquired resources towards growth. Indeed, incubation temperature is known to have lasting effects on the resting metabolic rates of other reptiles (Noble et al., 2018; O'Steen & Janzen, 1999; Singh et al., 2020), although the consequences of such differences are likely complex (Burton et al., 2011; Norin & Metcalfe, 2019). We suspect variation in temperature-dependent developmental cost serves as an overarching explanation for fitness consequences associated with thermal developmental plasticity of morphological phenotypes in reptiles more broadly. However, future work is needed across additional taxa to establish causal links between developmental cost, phenotypic variation, and survival.

We also observed a strong, direct effect of incubation temperature on survival independent of DE or other measured phenotypes during timepoints A and B. As in many reptiles, hatchling crocodilians subsist on residual yolk for the first several weeks to months post-hatch (Allsteadt & Lang, 1995; Fischer et al., 1991; Whitehead, 1990). We hypothesize that during timepoint A, animals are almost solely reliant on residual yolk, reducing the need to forage, which is likely to carry elevated predation risks. As a result, morphological phenotypes are less important. Rather, temperature-dependent, behavioral traits associated with optimal yolk utilization (i.e., thermopreference for metabolic efficiency) are more critical. Indeed, previous work demonstrated that hatchlings produced from incubations at FPT show preference for warmer areas compared to those incubated at MPT, which may reflect requirements for warmer temperatures supporting optimal yolk metabolism (Johnson et al., 2023). If these preferences translate to increased basking behaviors, it could leave individuals incubated at FPT more susceptible to predation, potentially independent of size. Interestingly, animals incubated at FPT also displayed increased freeze time in the presence of a decoy predator (Johnson et al., 2023), which may further represent differences in the trade-off between predation risk and thermoregulation for optimal yolk metabolism between MPT and FPT. Notably, however, few studies, have explicitly examined the functional importance and early-life utilization of residual yolk (but see Murphy et al., 2020; Radder et al., 2004, 2007). Thus, how it influences early-life foraging behaviors and, in turn, predation likelihood, is largely unknown. Nevertheless, by timepoint B, we predict that most individuals have utilized their yolk and become free foraging. The influence of incubation temperature on size through DE becomes more important because larger animals forage more efficiently and, when doing so, may be less likely to be predated upon due to increased escape ability and gape limitation of predators (Mittelbach, 1981; Verwaijen et al., 2002). Such context-dependent relationships between traits and survival have been identified in various taxa, where the direction, magnitude, or significance of trait-survival associations varies across space, time, and ecological context (Civantos & Forsman, 2000; Congdon et al., 1999; Janzen et al., 2007; Kissner & Weatherhead, 2005; Olsson & Madsen, 2001; Warner & Shine, 2007). Future work in alligators manipulating both organismal traits (e.g., developmental cost) and ecological pressures (e.g., predation), coupled with observations of behavior in nature, will likely be particularly informative.

Our approach for identifying mechanisms underlying temperature-dependent survival had a few important limitations. First, due to differential mortality, the sample sizes of FPT animals were lower than those at MPT, especially during later timepoints. Such imbalances can reduce the reliability of model estimates, particularly for traits on survival. Further, survivorship bias can complicate interpretations about causation. Our use of bootstrapping to confirm significance of estimates on survival and utilization of an SEM framework alleviates some of these concerns but does not fully account for such limitations in our data structure. Second, the survival data from wild-released hatchlings came from constant incubations in the lab, which are not representative of fluctuating temperatures experienced in natural nests (Bowden et al., 2014; Les et al., 2007; Pettersen, Nord, et al., 2023). Further work investigating the influence of more naturalistic incubation conditions on patterns of DE, growth, and survival in alligators is needed if we are to tie such effects to the evolution of TSD.

Given that the evolution of TSD under the STM relies on sex-specific selection acting on thermosensitive, survival-linked phenotypic variation, understanding both the organismal biology and ecological processes responsible for temperature-dependent survival is critical. By analyzing longitudinal survival data across several years in the wild, we identified organismal factors contributing to temperature-dependent survival in a species with TSD. Our results strengthen support for the STM and highlight developmental cost as a key mechanism linking incubation temperature to survival. However, our results also demonstrate the need to understand relationships between incubation temperature and additional traits, specifically behavioral and metabolic phenotypes associated with predation risk, that are likely critical targets of selection linking incubation temperature to sex-specific fitness and the evolution of sex-determining systems.

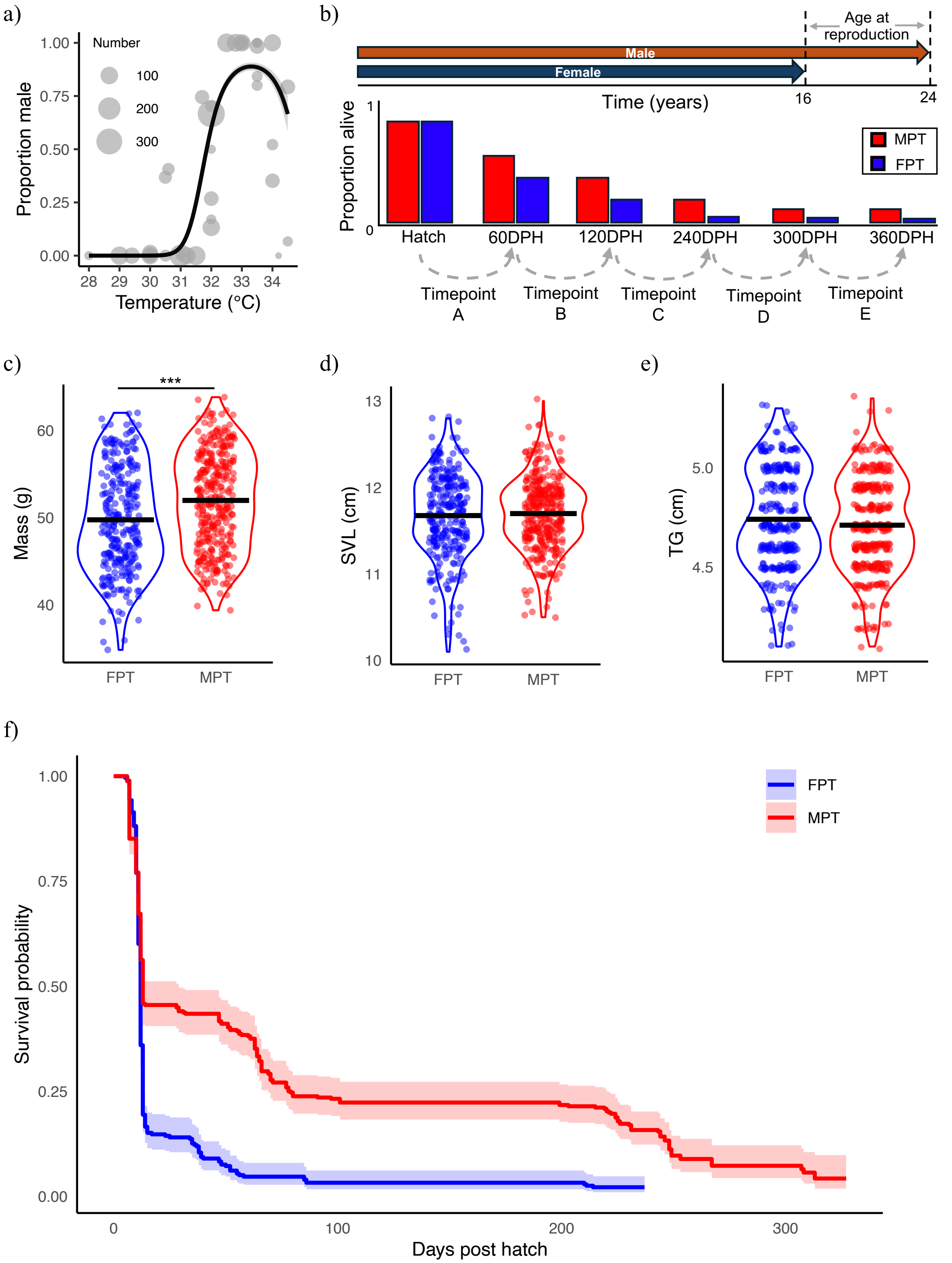

Figure 5.1: The influence of incubation temperature on hatchling traits and survival during the first year of life. (a) Reaction norm of incubation temperature and sex ratios in alligators, modified from Bock et al. 2022. Point size indicates sample size. (b) Conceptual framework of the STM in alligators, showing sampling timepoints categorized in this study. (c) Hatchling mass. (d) Hatchling snout-vent length (SVL). (e) Hatchling tail girth (TG). (f) Early-life survival during the first year of life. DPH: days post-hatch.

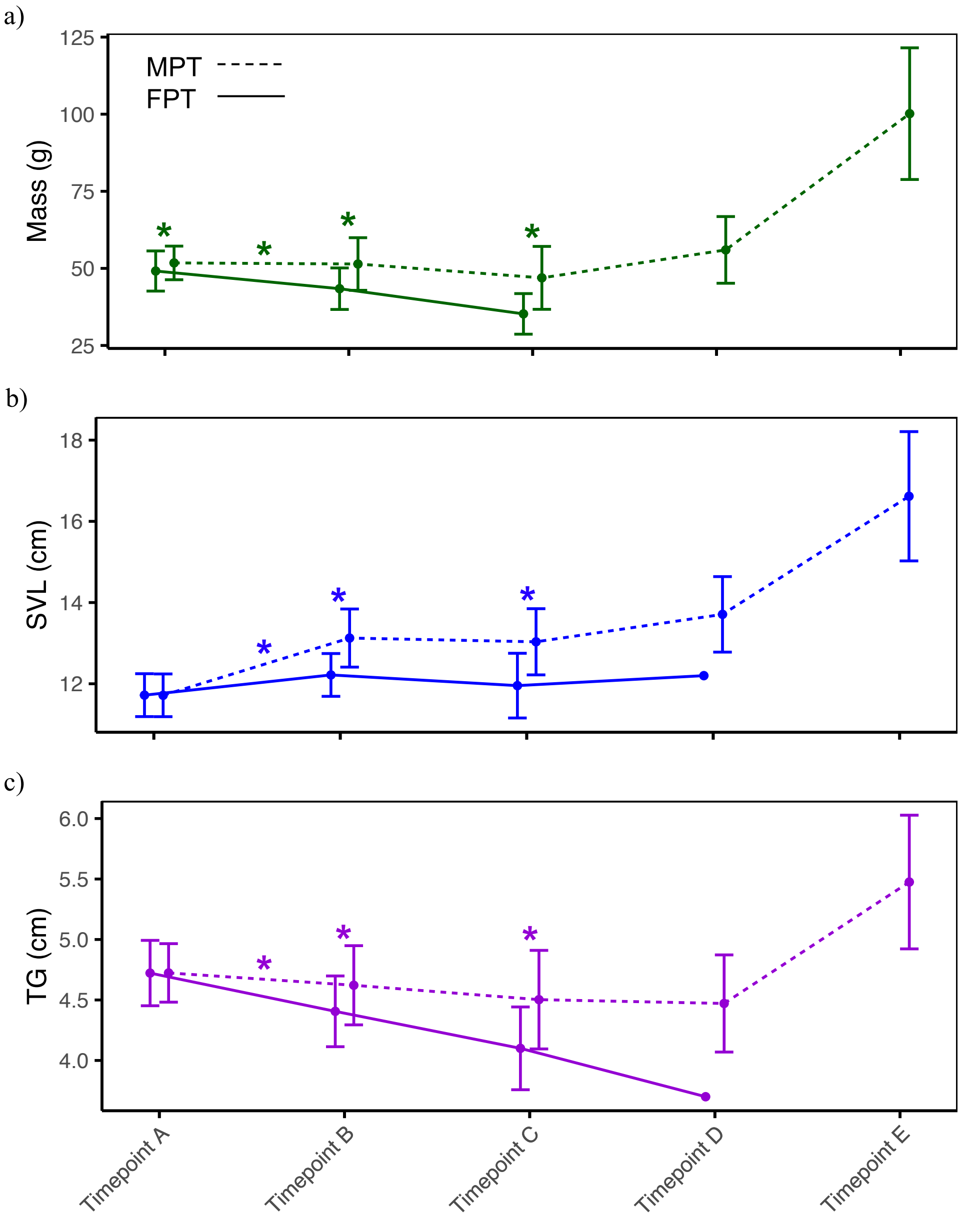

Figure 5.2: The influence of incubation temperature on post-release phenotypes. (a) Mass. (b) snout-vent length (SVL). (c) tail girth (TG). Solid lines represent female-promoting temperatures (FPT), dashed lines represent male-promoting temperatures (MPT). Error bars represent standard errors. Asterisks denote statistical significance between incubation temperatures in model means at each timepoint or between consecutive timepoints.

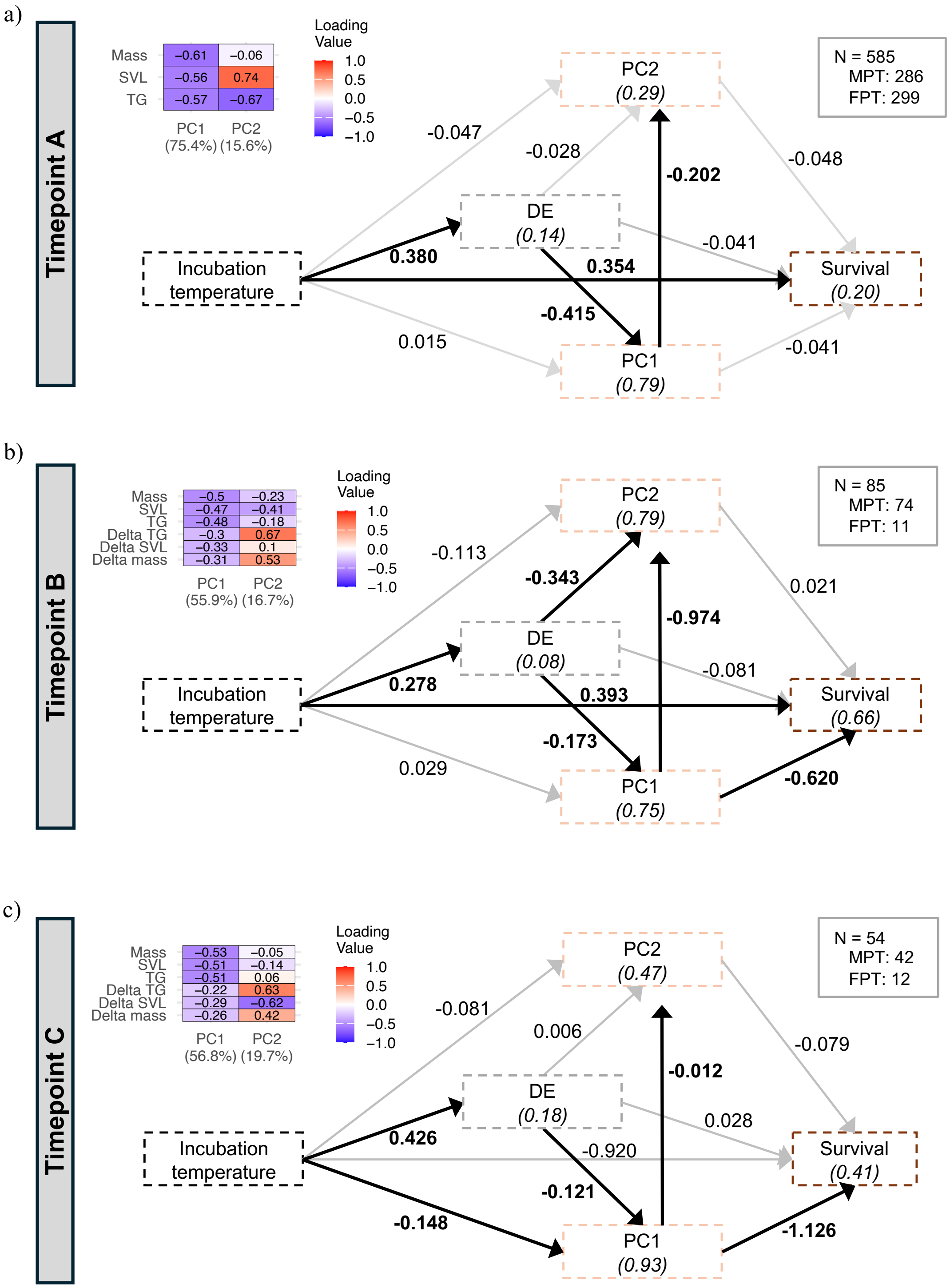

Figure 5.3: Structural equation models of mechanistic relationships between incubation temperature and survival. (a) Timepoint A (b) Timepoint B. (c) Timepoint C. For each timepoint, heatmaps display loadings of traits on the first two principal components. Boxes in top right corners show sample sizes by incubation temperature. Numbers along paths represent standardized coefficients, with significant paths bolded. Numbers in each box in italics represent estimated conditional R^2 for each response variable.

Table 5.1: Samples size for wild-released individuals

Year	**Population**	**Treatment**	**MPT**	**FPT**	***Total***
2019	PAR Pond	-	60	38	98
2020	Yawkey Wildlife Center	E2	72	70	142
		VEH	64	69	133
2021	Yawkey Wildlife Center	-	51	33	84
2021	PAR Pond	-	91	72	163
Total	-	-	338	282	*620*

Table 5.2: Model estimates for incubation temperature's influence on post-release traits and their change over time. SE: Standard error.

Timepoint	**Phenotype**	**Temperature estimate**	**SE**	**Adjusted p-value**
Release-60DPH	**Mass**	**-2.820**	**0.456**	**< 0.0001**
61-120DPH	**Mass**	**-7.410**	**1.440**	**< 0.0001**
121-240DPH	**Mass**	**-7.810**	**1.580**	**< 0.0001**
(Release-61DPH) - (61-120DPH)	**Mass**	**-4.596**	**1.490**	**0.004**
(61-120DPH) - (121-240DPH)	Mass	0.399	2.070	1
Release-60DPH	SVL	0.003	0.046	1
61-120DPH	**SVL**	**-0.887**	**0.147**	**< 0.0001**
121-240DPH	**SVL**	**-1.020**	**0.144**	**< 0.0001**
(Release-61DPH) - (61-120DPH)	**SVL**	**-0.889**	**0.151**	**< 0.0001**
(61-120DPH) - (121-240DPH)	SVL	0.130	0.198	1
Release-60DPH	TG	0.006	0.019	0.995
61-120DPH	**TG**	**-0.221**	**0.059**	**0.0008**
121-240DPH	**TG**	**-0.254**	**0.058**	**< 0.0001**
(Release-61DPH) - (61-120DPH)	**TG**	**-0.227**	**0.060**	**0.0004**
(61-120DPH) - (121-240DPH)	TG	0.033	0.078	1

Table 5.S1: SEM results for timepoint A

Response	Predictor	Estimate	Std.Error	DF	Crit.Value	p-value	Std.Estimate
DE	**Temperature**	**0.7593**	**0.0766**	**585**	**9.9155**	**0**	**0.3799**
PC1	Temperature	0.0301	0.1566	35.59	0.1923	0.8486	0.0151
PC1	**DE**	**-0.4145**	**0.0218**	**555.86**	**-19.0549**	**0**	**-0.4145**
PC1	**DPH at capture**	**0.089**	**0.0441**	**54.47**	**2.0168**	**0.0487**	**0.089**
PC1	Time between captures	0.1506	0.1294	32.37	1.1637	0.2531	0.1506
PC2	Temperature	-0.0937	0.1284	9.40	-0.7296	0.4834	-0.0469
PC2	DE	-0.0283	0.0476	496.46	-0.5937	0.553	-0.0283
PC2	**PC1**	**-0.2024**	**0.0614**	**106.85**	**-3.2941**	**0.0013**	**-0.2024**
PC2	Time between captures	0.0296	0.0931	5.00	0.3176	0.7636	0.0296
PC2	**DPH at capture**	**0.4317**	**0.0479**	**38.57**	**9.0072**	**0**	**0.4317**
Survival	**Temperature**	**1.4454**	**0.4002**	**585**	**3.6122**	**0.0003**	**0.3535**
Survival	DE	-0.0843	0.1307	585	-0.6452	0.5188	-0.0412
Survival	PC1	-0.0828	0.1314	585	-0.63	0.5287	-0.0405
Survival	PC2	-0.0985	0.1113	585	-0.885	0.3761	-0.0481
Survival	Time between captures	-0.0643	0.2716	585	-0.2367	0.8129	-0.0314
Survival	Release DPH	0.2217	0.1681	585	1.3189	0.1872	0.1083
Survival	DPH at capture	0.1101	0.1372	585	0.8025	0.4223	0.0538

Table 5.S2: SEM results for timepoint B

Response	Predictor	Estimate	Std.Error	DF	Crit.Value	P.Value	Std.Estimate
DE	**Temperature**	**0.8217**	**0.3123**	**83**	**2.631**	**0.0101**	**0.2775**
PC1	Temperature	0.087	0.3751	14.71	0.232	0.8197	0.0294
PC1	**DE**	**-0.1731**	**0.0651**	**66.63**	**-2.6597**	**0.0098**	**-0.1731**
PC1	DPH at measurement	-0.2908	0.1821	9.71	-1.5971	0.1422	-0.2908
PC1	**Time between recaptures**	**0.6001**	**0.2529**	**8.82**	**2.373**	**0.0422**	**0.6001**
PC2	Temperature	-0.3346	0.4581	3.24	-0.7305	0.5143	-0.113
PC2	**DE**	**-0.3423**	**0.0787**	**55.77**	**-4.3497**	**0.0001**	**-0.3423**
PC2	**PC1**	**-0.9735**	**0.1383**	**70.78**	**-7.0386**	**0**	**-0.9735**
PC2	Time between recaptures	0.4373	0.3215	1.99	1.3604	0.3072	0.4373
PC2	DPH at measurement	-0.0425	0.2289	2.10	-0.1858	0.869	-0.0425
Survival	**Time between recaptures**	**3.1891**	**1.6263**	**85**	**1.961**	**0.0499**	**0.393**
Survival	DE	-0.223	0.3692	85	-0.6039	0.5459	-0.0814
Survival	**PC1**	**-1.6989**	**0.7417**	**85**	**-2.2906**	**0.022**	**-0.6201**
Survival	PC2	0.0582	0.4496	85	0.1294	0.8971	0.0212
Survival	**Time between recaptures**	**1.7873**	**0.8202**	**85**	**2.1791**	**0.0293**	**0.6524**
Survival	Release DPH	-0.4762	0.449	85	-1.0608	0.2888	-0.1738
Survival	DPH at measurement	-0.0151	0.5742	85	-0.0262	0.9791	-0.0055

Table 5.S3: SEM results for timepoint C

Response	Predictor	Estimate	Std.Error	DF	Crit.Value	P.Value	Std.Estimate
DE	**Temperature**	**1.0155**	**0.299**	**52**	**3.3969**	**0.0013**	**0.4262**
PC1	**Temperature**	**-0.3531**	**0.1576**	**47.85**	**-2.2397**	**0.0298**	**-0.1482**
PC1	**DE**	**-0.1213**	**0.0483**	**46.79**	**-2.5107**	**0.0156**	**-0.1213**
PC1	DPH at measurement	0.5217	0.5586	44.66	0.934	0.3553	0.5217
PC1	Time between recaptures	0.3316	0.668	44.28	0.4964	0.6221	0.3316
PC2	Temperature	-0.193	0.4282	43.65	-0.4507	0.6545	-0.081
PC2	DE	0.0062	0.1507	47.94	0.0408	0.9676	0.0062
PC2	PC1	-0.0124	0.3789	39.33	-0.0327	0.9741	-0.0124
PC2	Time between recaptures	-0.2936	1.5932	12.88	-0.1843	0.8566	-0.2936
PC2	DPH at measurement	-0.5184	1.3247	12.00	-0.3913	0.7024	-0.5184
Survival	Temperature	-5.1679	3.1159	54	-1.6586	0.0972	-0.9199
Survival	DE	0.0654	0.445	54	0.147	0.8831	0.0277
Survival	**PC1**	**-2.6556**	**1.1069**	**54**	**-2.3992**	**0.0164**	**-1.1264**
Survival	PC2	-0.1857	0.4402	54	-0.4219	0.6731	-0.0788
Survival	Time between recaptures	-3.2074	3.5364	54	-0.907	0.3644	-1.3604
Survival	Release DPH	-2.1534	1.1941	54	-1.8033	0.0713	-0.9134
Survival	DPH at measurement	-1.7353	2.8521	54	-0.6084	0.5429	-0.7361

CHAPTER 6

EVALUATING GENE EXPRESSION PATTERNS IN BLOOD AS A TOOL TO NON-LETHALLY SEX HATCHLING AMERICAN ALLIGATORS (*ALLIGATOR MISSIPPIENSIS*), A SPECIES WITH TEMPERATURE-DEPENDENT SEX DETERMINATION[5]

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[5] Smaga, C. R. & Parrott, B. B. To be submitted to *Molecular Ecology.*

Abstract

Knowledge of sex ratio variation in natural populations is critical for understanding ecological and evolutionary dynamics, including conservation outcomes in imperiled species. However, in taxa with temperature-dependent sex determination (TSD), links between nest temperatures, sex ratios, and population dynamics are not well-established, in part due to the lack of reliable, non-lethal methods for sexing hatchlings. To address this, we investigate whether gene expression patterns in blood can distinguish sex of hatchling American alligators (*Alligator mississippiensis*). We detect hundreds of genes differentially expressed between males and females, many of which are regulated by sex-specific transcription factors and potentially linked to sex-steroid hormone signaling. We further reveal both shared and tissue-specific responses to sex in blood and gonads, providing insight into how sex differences are established in somatic tissues in species lacking sex chromosomes. Most importantly, however, we identify several genes in blood showing little or no overlap in expression between males and females, suggesting they can be reliably used to predict sex. Our results thus provide a necessary starting point for the development of targeted approaches to predict sex using blood gene expression, which will enable further integration of TSD into eco-evolutionary frameworks and improve predictions of population responses to environmental change.

Introduction

Sex ratio variation in natural populations has critical implications for ecology, evolution, and conservation (Donald, 2007; Waples, 2024; West et al., 2002). Whereas most organisms utilize sex chromosomes to determine sex, others rely almost entirely on environmental factors (Bachtrog et al., 2014). Particularly common across many reptile species is temperature-dependent sex determination (TSD), where the temperature experienced during a critical developmental window determines sex (Valenzuela & Lance, 2004). In recent years, our understanding of the molecular mechanisms and evolutionary significance of TSD in reptiles has improved significantly (Bock, Hale, et al., 2020; Bock et al., 2023; Czerwinski et al., 2016; Deveson et al., 2017; Ge et al., 2017, 2018; Kohno et al., 2014; Parrott, Kohno, et al., 2014; Weber & Capel, 2021; Yatsu, Miyagawa, Kohno, Parrott, et al., 2016). However, knowledge of links between natural nest temperatures, primary sex ratios, and population dynamics in nature remains much more limited. For instance, because most of the work on TSD has utilized constant incubation temperatures in the lab, less is known about how fluctuating thermal profiles experienced in natural nests influence sex ratio outcomes (Bowden et al., 2014; Breitenbach et al., 2020). Furthermore, whereas several studies have predicted sex ratio skews and potential population declines under increasing global temperatures (Bock, Lowers, et al., 2020; Hays et al., 2023; Jensen et al., 2018), empirical data on how primary sex ratios vary over space and time is scarce. Such information is critical for incorporating TSD into eco-evolutionarily frameworks, including predicting conservation outcomes in the face of rapid environmental change.

One hinderance to understanding natural sex ratio variation associated with TSD that has received increased attention is the lack of easy to implement, reliable, and non-lethal methods of sexing hatchling reptiles that utilize it (Schwarzkopf & Brooks, 1985; Tezak et al., 2020; Valenzuela et al., 2004). In many TSD species, secondary sex characteristics do not manifest until later in life, and studies frequently rely on nest temperature data to predict primary sex ratios in the wild (Bock, Lowers, et al., 2020; Carter et al., 2019; Escobedo-Galván et al., 2016; Hays et al., 2023; Mitchell et al., 2008; Santidrián Tomillo et al., 2015). These methods require knowledge of population-specific reaction norms, development rates, and/or periods of thermosensitivity during gonadal development (e.g., Georges et al., 1994), and, even when those are known, frequently fail to adequately capture sex ratio variation (Carter et al., 2019; Massey et al., 2019; Mitchell et al., 2008). As a result, lethal sampling and inspection of the gonads remains the primary, confirmatory way to determine hatchling sex in many TSD species (Bock et al., 2021; Janzen, 1994; Schwarzkopf & Brooks, 1985). This poses obvious ethical issues when incorporating large samples sizes needed for population or species-level analyses (Wilson & Hardy, 2002). It is further hindered by the listed status of several TSD taxa, including many turtles and crocodilians (IUCN 2025).

Previous studies have shown promise in using minimally invasive, molecular techniques to accurately predict sex in some TSD species. For instance, Western blotting was successfully used to sex red-eared slider (*Trachemys scripta*) and loggerhead sea turtles (*Caretta caretta*) by measuring anti-mullerian hormone concentrations in plasma (Tezak et al., 2020). Similarly, ELISA-based measurement of plasma testosterone concentrations was capable of distinguishing sex in hatchling desert tortoises (*Gopherus agassizii*; Walden et al., 2023). In American alligators (*Alligator mississippiensis*), DNA methylation patterns in hatchling blood can accurately predict both sex and incubation temperature independently (Bock et al., 2022). However, each of the above methods has their own set of limitations that prevent their broad implementation across species and in an applied context. For example, Western blotting requires validated, sometimes species-specific antibodies and is inefficient when measuring large numbers of individuals. On the other hand, predictive models using sex-specific methylation patterns in blood utilize several genome-specific loci with small effect size, necessitating the development of complex, targeted qPCR assays prior to widespread utilization. While these methods still represent viable options, further investigation into accurate and non-lethal approaches to sex hatchlings of many TSD species is warranted.

Gene expression patterns in blood provide currently untapped potential for measuring several components of reptilian phenotype. Reptile red blood cells are nucleated and transcriptionally active, expressing genes involved in stress response, oxidative stress, insulin and insulin-like signaling, and mitochondrial function (Chiari & Galtier, 2011; Waits et al., 2020). Changes in the expression of these genes can be reflective of both intrinsic and extrinsic processes including aging (Perez-Gomez et al., 2020), growth (Baker et al., 1993; DeVol et al., 1990), immune function (Shaffer et al., 2001), and exposure to environmental stressors (de Nadal et al., 2011). Interestingly, several of the above physiological traits are also known to exhibit sex-specific patterns (Bronikowski et al., 2022; Geffroy & Douhard, 2019; Klein & Flanagan, 2016; Shealy et al., 2025). If present and detectable through gene expression in hatchlings, such differences may serve as a robust biomarker of sex in TSD species. Yet, few reptile blood transcriptomes have been sequenced to date, and the extent to which sex influences gene expression patterns in hatchling blood is largely unknown.

In this study, we assess the ability of gene expression patterns in blood to distinguish sex in hatchling American alligators. Alligators are long-lived, TSD reptiles that inhabit much of the southeastern United States. Males are produced at incubation temperatures around 33°C, whereas females are produced at cooler and warmer incubation temperatures (Ferguson & Joanen, 1983). Accurate identification of primary sex ratios in nature are especially relevant in this species, as alligators have become a powerful model system for understanding TSD from both mechanistic and evolutionary perspectives (Bock, Hale, et al., 2020; Bock et al., 2023; McCoy et al., 2015, 2016; Parrott, Kohno, et al., 2014; Smaga et al., 2024; Smith & Joss, 1993). Furthermore, as in many other TSD species, alligators are expected to experience sex ratio skews as global temperatures increase, potentially threatening population persistence (Bock, Lowers, et al., 2020). Utilizing hatchlings spanning male- and female-promoting incubation temperatures (MPT and FPT, respectively) across four populations, we sequence the blood transcriptomes of 24 individuals. We first characterize gene expression differences in blood between males and females. We then compare patterns of sexual dimorphism in blood to previously published data in gonads. Finally, we identify a subset of loci in blood with minimal or no overlap in expression between the sexes, which we propose as candidate genes that can be reliably used to predict the sex of novel individuals. Our results demonstrate exciting potential for the use of gene expression patterns of just a few genes to predict sex in a TSD species that, with the development of targeted qPCR approaches, would be suitable for large scale ecological and conservation studies.

Materials and Methods

Experimental design and samples

To investigate sex-specific gene expression patterns in blood, we utilized individuals incubated at MPT (33.5°C) or FPT (29.5°C) across four alligator populations and dissected at 10-days post-hatch from Smaga et al. (2024). The four populations included Lake Apopka (Apopka, FL, USA), Lake Woodruff (DeLand, FL, USA), Par Pond on the Savannah River Site (Aiken, SC, USA) and Tom Yawkey Wildlife Center (Georgetown, SC, USA). Prior to dissection, 1 mL blood was taken from the post-occipital sinus of each individual and immediately placed in a heparin tube on ice. Whole blood was then spun at 1000 rcf for 10 minutes at 4°C to separate plasma. After removing plasma, 2 mL of RNAlater was added to each tube containing blood cells and the mixture was stored at -20°C. As these individuals were dissected, their sex was confirmed based on the presence or absence of oviducts.

We chose a subset of 24 total individuals of confirmed sex spanning MPT and FPT across three clutches from each population for RNA sequencing (Table 6.1). Notably, these individuals also had their gonadal transcriptomes sequenced in Chapter 4, which adds an additional confirmation of sex based on gonadal gene expression and allowed us to directly compare sexually dimorphic gene expression between blood and gonads.

Nucleic acid extraction and transcriptome sequencing

We extracted RNA from blood cells using a modified version of the Promega SV Total RNA Isolation System (Promega; Madison, WI) protocol as reported in (Smaga et al., 2025), including an RNA precipitation using sodium acetate. Prior to extraction, we spun down 200-400 ul of blood cell-RNAlater mixture at 4°C for 10 minutes at 16,000 x g and removed the RNAlater supernatant. After extraction, we measured RNA for concentration and purity using a Nanodrop One (Thermo Fisher Scientific, Waltham, MA) and sent >1.2 ug of total RNA to Novogene (Sacramento, CA, USA) for further quality control and sequencing (RIN: $\bar{x} = 5.28 \pm 1.29$). Samples were poly(A) enriched, and directional mRNA libraries were prepared and sequenced on an Illumina NovaSeq6000 instrument (paired end 150 bp reads, Illumina, San Diego, CA, USA).

Alignment and read counting

We inspected raw reads using FastQC and MultiQC and removed low quality bases and adaptor sequences using TrimmGalore! (F. Krueger, 2015) with a stringency of 3. Using a recently updated sequence of the alligator genome (rAllMis1; RefSeq: GCF_030867095.1), we aligned reads using Hisat2 (Kim et al., 2019). We then indexed and sorted the resulting SAM files and converted them to BAM format using SAMtools (Danecek et al., 2021). Finally, we input BAM files into R using RSamTools (Morgan, 2024) and counted reads overlapping exon coordinates (generated from the makeTxDbFromGFF function in GenomicFeatures (Lawrence et al., 2013)) using the summarizeOverlaps function (mode = ‘Union’) in the GenomicAlignments (Lawrence et al., 2013) package as reported previously (Smaga et al., 2025).

Identification of genes differentially expressed between the sexes

Prior to assessing sex differences, we removed genes whose expression was < 1 count per million (CPM) in more than 12 individuals. As an initial examination of expression across sexes and populations, we visualized expression patterns in CPM using a principal component analysis (PCA) with the prcomp function in R (center = TRUE, scale = TRUE; R Core Team 2024). We then identified differentially expressed genes (DEGs) between males and females using the edgeR package (Robinson et al., 2010). Specifically, we normalized library sizes using the calcNormFactors function, estimated dispersion using the stimateGLMRobustDisp function, and fitted a negative binomial generalized log-linear model using a design matrix consisting of the incubation temperature and population origin of each sample with the glmQLFit function. Using the glmQLFTest function, we conducted Quasi-Likelihood F-Tests between the sexes, weighting each population equally in the contrast. We considered genes with an FDR < 0.05 and log2FC > 0.58 as significant sex DEGs. We performed hierarchical clustering of sex DEGs and visualized the resulting heatmap using the pheatmap function in the R package pheatmap (Kolde, 2019).

Functional enrichment of sex DEGs

We tested for functional enrichment of sex DEGs using Gene Ontology biological process (BP) and molecular function (MF) terms, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and transcription factor regulation annotations (TFs) using the gprofiler2 (Kolberg et al., 2020) and enrichR (database = 'ENCODE_and_ChEA_Consensus_TFs_from_ChIP-X'; Kuleshov et al., 2016) packages in R. For GO and KEGG terms, we used a custom background of all expressed genes in the dataset. For transcription factors enrichment with enrichR, we confirmed significance by comparing the proportion of genes regulated by significant TFs in the DEG set relative to the background gene set using Fisher's Exact Tests and Bonferroni-corrected p-values. Prior to enrichment tests, we converted gene names into human gene counterparts using the gconvert function in gprofiler2.

Enrichment of hormone response elements in sex DEG promoters

The sex steroids estradiol and testosterone play fundamental roles in establishing morphological and physiological differences between the sexes (Wells, 2007). To further assess their role in sex DEG expression, we compared the proportion of sex DEGs containing predicted binding sites of the primary sex hormone receptors estrogen-receptor 1 (ESR1), estrogen-receptor 2 (ESR2), and androgen receptor (AR) in their promoters relative to the rest of the genes expressed in blood. We defined promoters as the region encompassing 2,000 bp upstream and 200 bp downstream of the transcription start site, which we extracted using the promoters function in GenomicFeatures. Using the FIMO function in MEME suite with JASPAR position weight matrices for each receptor (ESR1: MA0112.1; ESR2: MA0258.1; AR: AR_0007.2) as input and a p-value threshold of 5.0e-6, we predicted binding sites of each hormone receptor in the alligator genome. We then calculated the proportion of male-biased, female-biased, and non-biased (background) genes containing receptor motifs in their promoters using the intersect function in BEDTools (Quinlan & Hall, 2010). Finally, we tested whether each of the three transcription factor binding sites were enriched in male- or female-biased genes relative to the background gene set using Fisher's Exact Tests.

Comparison of sex differences between blood and gonad

To compare patterns of sexual dimorphism between gonads and blood, we used published read counts from the gonads of our 24 individuals (Chapter 4). We employed the same methods as above to identify sex DEGs in gonads using edgeR. We then compared the proportion of sex DEGs relative to the total number of expressed genes in blood and gonad using Fisher's Exact Tests. We further compared the absolute log2FC between gonad and blood sex DEGs using a Wilcoxon Test. Finally, we identified overlaps in the identities of DEGs between gonad and blood, considering each gene's directionality in each tissue (male versus female bias).

Identifying candidate genes for sex prediction

To identify candidate genes that could be used to reliably predict sex, we visually examined expression in CPM of the top 20 significant DEGs with a log2FC > 2. We then calculated cutoff values between the sexes by taking the average of the minimum counts in the higher expressed sex and the maximum counts in the lower expressed sex for each gene.

Data manipulation and statistical analysis

We conducted all statistical tests in R using the packages dplyr and tidyr for data manipulation (Wickham et al., 2023). For all analyses, we used a p-value < 0.05 for significance, unless otherwise noted. We used the R package ggplot2 (Wickham, 2016) for data visualizations.

Results

Sex-specific gene expression patterns in blood

Our PCA incorporating all 12,060 genes passing filtering showed no significant separation between the sexes along either PC1 or PC2 (**Figure 6.1**). However, our DEG analysis identified 667 sex DEGs (**Figure 6.2a,** Table 6.S1). Of these, similar numbers of genes were male-biased (357; 53.5%) and female-biased (310; 46.5%). The top male-biased genes by FDR included *RNPEP*, *LOC109282028*, *LOC102559531*, *RLN3*, and *LOC102573155*, while the top female-biased genes by FDR included *POU6F1*, *LOC106739510*, *KEF_r01*, *HOOK2*, and *IQSEC1*. Hierarchal clustering of sex DEGs showed two primary clusters, one including half of the male individuals and other splitting the rest of the male individuals from all female individuals (**Figure 6.2b**). We found little interpretable enrichment of male- or female-biased genes for GO or KEGG terms, with male-biased genes being enriched for the KEGG pathway 'DNA replication' and female-biased genes being enriched for the BP 'cytoplasmic translation', the MFs 'structural constituent of the ribosome', and the KEGG pathways 'Ribosome' and 'Coronavirus disease – COVID-19'. However, both male- and female-biased genes showed significant enrichment for several, mostly non-overlapping, transcription factors (**Figure 6.2c**). The most significant were *GATA1*, *E2F4*, and *ZMIZ1* in males and *BRCA1*, *CREB1*, and *TAF1* in females. Only *NFYB* and *RUNX1* were shared between both sexes (**Figure 6.2c**). Interestingly, male but not female-biased genes were more likely to contain ESR1 motifs in their promoters (male: odds ratio = 1.43, p = 0.04; female: odds ratio = 0.84, p = 0.53; **Figure 6.2d**). A similar, although non-significant, trend was apparent for ESR2 (male: odds ratio = 1.40, p = 0.10; female: odds ratio = 0.99, p = 1; **Figure 6.2d**). On the other hand, female but not male-biased genes were more likely to contain AR motifs (male: odds ratio = 1.14, p = 0.61; female: odds ratio = 2.01, p = 0.02; **Figure 6.2d**).

Comparison of gonad and blood gene expression dimorphism

Among genes expressed in the gonad (16,589), a significantly greater proportion were sex DEGs when compared to blood (odds ratio = 0.14, p < 2.2e-16; **Figure 6.3a**). Gonadal sex DEGs also displayed a significantly greater absolute log2FC between the sexes (W = 1363214, p = 4.92e-14; **Figure 6.3b**). When comparing the identities of sex

DEGs between blood and gonad, 142 were common across both tissues (**Figure 6.3c,** Table 6.S2). Interestingly, more than half of these (82, 57.7%) were in the opposite direction while 60 (42.2%) were in the same direction (**Figure 6.3c**). The top sex DEGs shared and in the same direction between blood and gonad by average FDR were *CLSTN3*, *BICD1*, and *RNPEP*. The top genes showing opposite directionality were *GALR3*, *CACNG7*, and *LOC102571426*.

Identification of candidate, sex-predictive loci

Density plots of the top 20 sex DEGs in blood by FDR and with a log2FC greater than 2 are shown in Figure 6.S1. A subset of these genes showed no or minimal overlap between the sexes, suggesting their expression can reliably distinguish between males and females (**Figure 6.4**). These included *LOC102559531*, *LOC102563321*, *LOC106739510*, *POU6F1*, and *RNPEP*.

Discussion

Accurate, cost-effective, and easy to implement tools for sexing hatchling reptiles with TSD are critical for understanding the consequences of sex ratio variation in natural populations, including responses to rapid climate change (Mitchell & Janzen, 2010). Here, we demonstrated that gene expression patterns in hatchling alligator blood harbor significant sex differences. Although we did not find much functional enrichment of sex DEGs, male- and female-biased genes were enriched for regulation by distinct transcription factors, indicative of divergent physiological processes. Further, both estrogen and androgen appeared to play an important role in establishing sex differences, as their receptor binding sites were more likely to reside in promoters of sex DEGs.

Perhaps most importantly, several of the most significant sex DEGs displayed little or no overlap in expression between males and females, suggesting they can reliably be used to determine the sex of novel individuals. Thus, our results suggest that with the development of targeted qPCR approaches, blood gene expression has the potential to provide a suitable method for large scale ecological and conservation studies predicting alligator hatchling sex ratios.

We detected 667 genes differentially expressed between the sexes, corresponding to 5.53% of all genes expressed in blood. Male- and female-biased genes showed enrichment for regulation by mostly non-overlapping transcription factors, suggesting sex-specific regulatory mechanisms. Similar results have been observed in other somatic tissues in mammals (Lopes-Ramos et al., 2020; Oliva et al., 2020), and are supported, in part, to be the result of sex-biased hormone signaling (Blencowe et al., 2022). In line with this, we found that sex DEGs in blood were more likely to contain ESR1 and AR motifs within their promoters, supporting regulation by estrogen and testosterone, respectively. Interestingly, however, female-biased genes were associated with androgen motifs and male-biased genes with estrogen motifs, suggesting a predominant role of negative but not positive regulation by androgens and estrogens. Links between sex-steroid hormone signaling and gene expression patterns of somatic tissues have been established in some taxa (Blencowe et al., 2022; Haakensen et al., 2011; Pataky et al., 2023) but are comparatively lacking in TSD reptiles. Yet, plasma concentrations of both testosterone and estradiol have been shown to differ between the sexes in hatchlings (Xia et al., 2011), sometimes robustly enough to reliably distinguish them (Walden et al., 2023). In alligators specifically, estradiol concentrations in hatchling female plasma are higher than in males (Medler & Lance, 1998), but extensive overlap makes it unsuitable for predicting sex (C. Smaga; unpublished data). Nonetheless, we suspect that estrogen and/or testosterone signaling plays a role in establishing sexually dimorphic expression patterns of blood cells, similar to their role in gonadal gene expression (Bock, 2023; Canesini et al., 2018; Kohno et al., 2015). However, future work assessing relationships between circulating hormones in plasma and somatic tissue gene expression is needed to confirm this.

The 667 (5.53%) sex DEGs in blood observed here was in stark contrast to the 4,981 (30%) sex DEGs in gonads. Few studies have compared sexually dimorphic gene expression across tissues in a TSD species (but see Martínez-Pacheco et al., 2024), but similar results were reported when comparing differentially methylated sites between the sexes in alligator blood and gonads (Bock et al., 2022). This is not surprising as the gonad is the site of primary sex determination and critical driver of secondary sex characteristics (Capel, 2017). Interestingly, while several genes were sex-specific in both tissues, only 60 (42.2%), were in the same direction. We suspect these genes may be those that are exquisitely sensitive to sex steroids and coordinate sex-specific functions that are not confined to any one tissue, such as whole organism growth, immune function, or metabolism (Lopes-Ramos et al., 2020; Oliva et al., 2020). They may also serve as interesting candidates for regulating aspects of sexual size dimorphism in alligators. A comparatively greater proportion of genes (58.8%) showed discordant directions between tissues, suggesting regulatory mechanisms outside of sex-steroid signaling. In mammals, this is thought to occur through sex-specific regulatory marks in part established through cell-autonomous sexual identity (Bear & Monteiro, 2013; Gatev et al., 2021; Wijchers &

Festenstein, 2011). Given that TSD species lack sex chromosomes, we suspect similar mechanisms occur but are driven instead by tissue-specific epigenetic patterning in response to incubation temperature during development (Matsumoto et al., 2013; Parrott, Bowden, et al., 2014; Parrott, Kohno, et al., 2014). Further work incorporating additional tissues will be particularly informative for understanding the causes and consequences of sex, tissue, and their interaction on gene expression dynamics of somatic tissues in TSD taxa. Such information is likely to provide key insight into how sexual dimorphic traits, such as ageing, growth, and immune function, are established in the absence of sex chromosomes.

We identified several candidate genes that serve as targets for developing a qPCR-based assay to predict sex in alligators. Namely, *LOC102559531*, *LOC102563321*, *LOC106739510*, *POU6F1*, and *RNPEP* displayed very little or no overlap between the sexes, allowing hard expression cutoffs to distinguish males and females. We suspect the ratio of only two genes, one male- and one female-biased, will be sufficient to reliably sex animals using qPCR, which would allow relatively rapid screening of large numbers of individuals. Such a method also requires very little starting material, as we observed suitable concentrations of RNA using only a small volume of blood-cell-RNAlater mixture. The patterns observed here may be specific to alligators. However, sex-specific gene expression dynamics of somatic tissues are highly conserved across mammals (Naqvi et al., 2019), and we suspect that several sex DEGs identified here are consistent across additional TSD species. If true, a broad assay utilizing a few candidate loci could be developed that would allow non-lethal sexing across several TSD taxa, addressing a major limitation of currently available sexing methods that rely on species-specific markers.

Our results provide a necessary basis and proof of concept for future exploration but are not without their limitations. Although we used known-sex individuals, we cannot rule out that the differences observed are the result of incubation temperature and not sex. This may not be a major issue, as it is probable that sex and temperature are rarely decoupled in the wild. However, further investigation into how additional temperatures, such as those that produce both sexes, and more naturalist, fluctuating temperatures may reduce or diminish the differences between sexes observed here is needed prior to implementing this method (Massey & Hutchings, 2021; McCoy et al., 2016; Noble et al., 2018). We also recognize that the development, validation and optimization of primers and PCR conditions is required before being utilized in a conservation or ecological context. Despite these limitations, however, our findings suggest that measuring hatchling blood gene expression may be a valid method for sexing hatchling alligators and potentially additional reptiles with TSD. They also reveal interesting insight regarding how sexually dimorphic gene expression patterns may be established in somatic tissues for species lacking sex chromosomes. While the development of targeted methods will be required, we suspect, once validated, they will be particularly valuable for studying TSD in nature, including how sex ratios vary, both adaptively or maladaptively, under natural and human induced change.

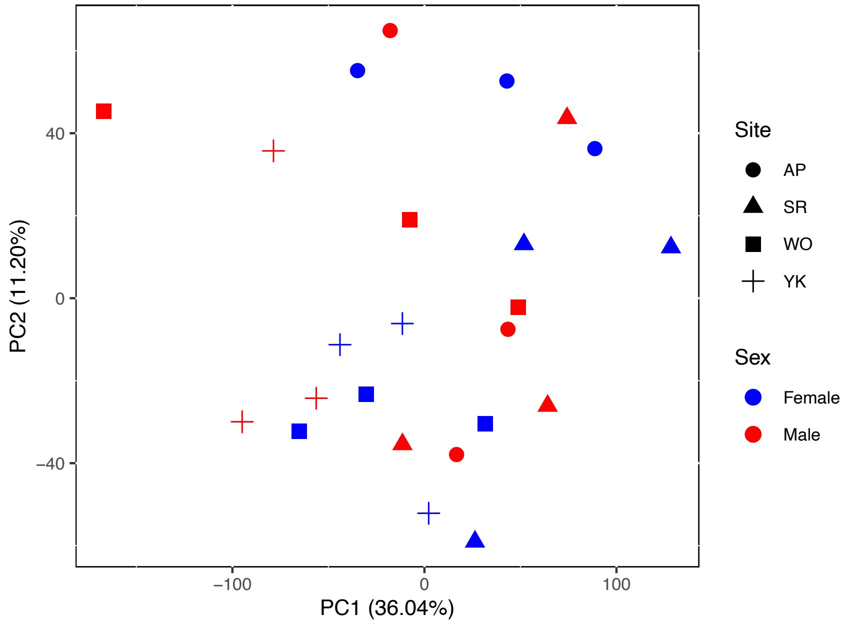

Figure 6.1: Principal component analysis of 12,060 genes expressed in hatchling blood. AP: Lake Apopka, WO: Lake Woodruff, SR: Par Pond on the Savannah River Site, YK: Yawkey Wildlife Center.

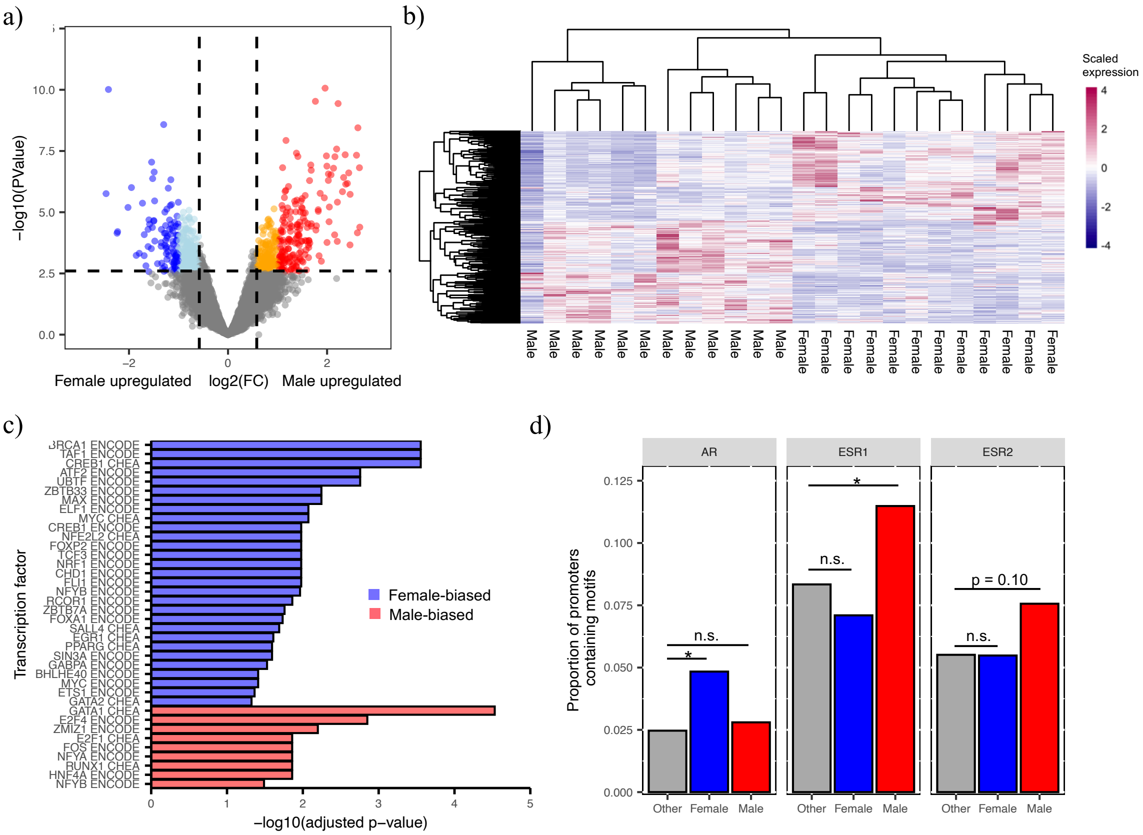

Figure 6.2: Characterization of sex differences in blood gene expression. (a) Volcano plot showing male- and female-biased sex DEGs, labeled in red and blue, respectively. (b) Heatmap of sex DEGs, showing clustering according to scaled expression level. (c) Transcription factor regulation enrichment of sex DEGs, separated by sex bias. (d) Enrichment of hormone response elements within promoters of sex DEGs, separated by sex bias. Asterisk denotes statistical significance: * $p < 0.05$.

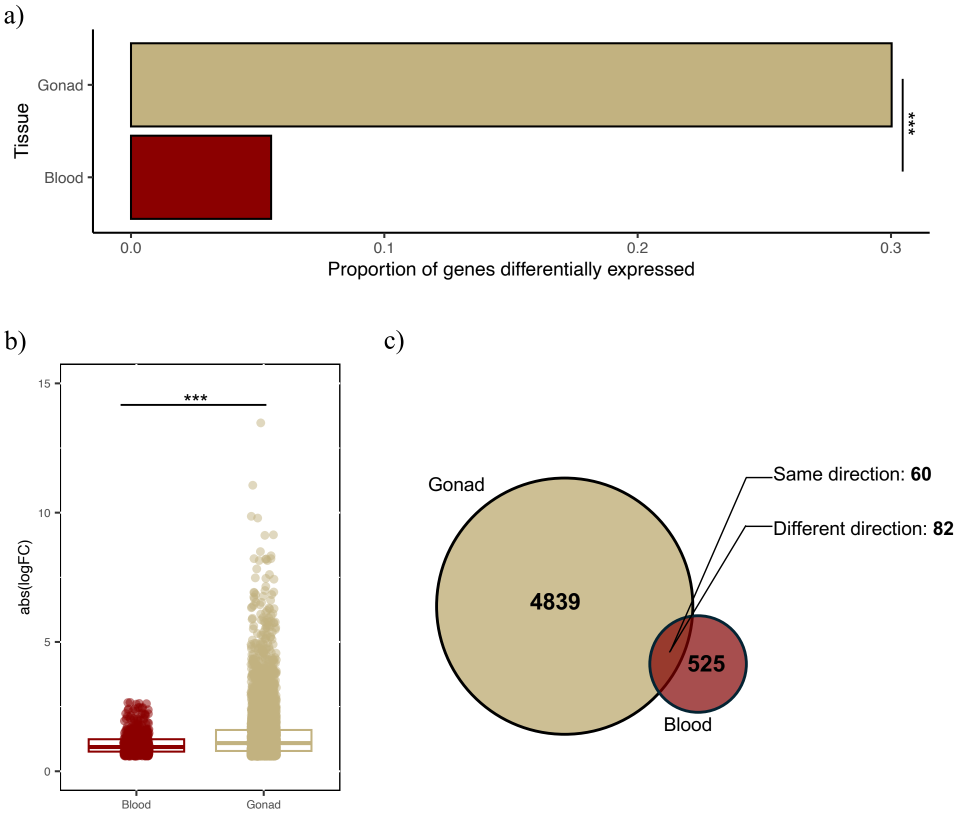

Figure 6.3: Comparison of sexual dimorphism between gonad and blood. (a) Proportion of expressed genes showing sex-specific patterns in blood relative to gonad. (b) Log_2 fold-change of blood and gonad sex DEGs. (c) Identity overlap of sex DEGs in blood and gonad, with overlapping genes separated by directionality. Asterisks denote statistical significance: *** $p < 0.001$.

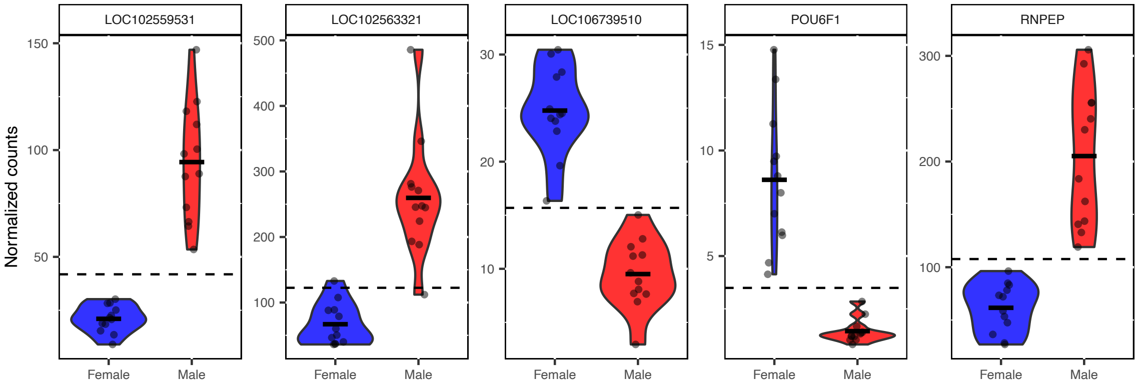

Figure 6.4: Candidate genes for sex prediction using qPCR approaches. Black, dashed line indicates cutoff value calculated as the average of the minimum expression of the higher expressed sex and the maximum expression of the lower expressed sex. Crossbars indicate sex-specific mean.

Table 6.1: Final sample sizes by clutch, temperature and population

	Lake Apopka		Lake Woodruff		Par Pond		Yawkey Wildlife Center	
Clutch #	*MPT*	*FPT*	*MPT*	*FPT*	*MPT*	*FPT*	*MPT*	*FPT*
1	1	1	0	1	1	1	1	1
2	1	1	1	1	1	1	1	1
3	1	1	1	1	1	1	1	1
Total	*3*	*3*	*3*	*3*	*3*	*3*	*3*	*3*

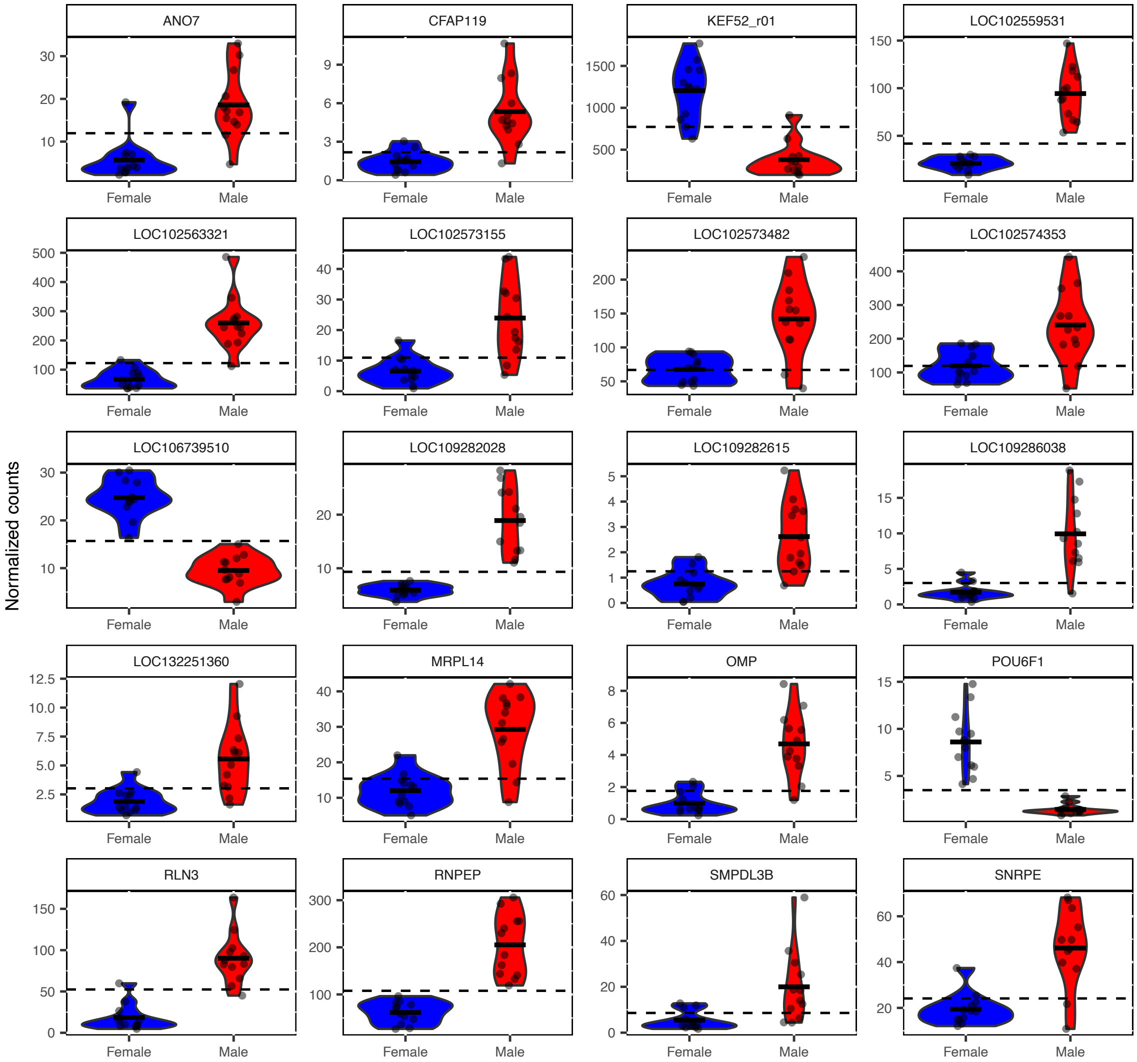

Figure 6.S1: Top 20 most significant sex DEGs by FDR with log2FC > 2. Crossbars indicate sex-specific mean.

Table 6.S1: Significant sex DEGs in blood

Gene	logFC	logCPM	F	PValue	FDR	Bias
RNPEP	1.96	7.128	114.364	8.61E-11	5.86E-07	Male
POU6F1	-2.414	2.327	113.133	9.72E-11	5.86E-07	Female
LOC109282028	1.764	3.668	101.295	2.97E-10	1.10E-06	Male
LOC102559531	2.223	5.773	99.207	3.65E-10	1.10E-06	Male
LOC106739510	-1.299	4.062	81.151	2.64E-09	6.36E-06	Female
RLN3	2.622	5.786	78.626	3.57E-09	7.18E-06	Male
LOC102573482	1.171	6.667	69.278	1.18E-08	2.03E-05	Male
LOC102573155	2.193	3.926	63.454	2.63E-08	3.97E-05	Male
SNRPE	1.381	5.013	60.801	3.87E-08	4.67E-05	Male
LOC109282615	2.366	0.753	59.385	4.51E-08	4.67E-05	Male
OMP	2.595	1.59	59.575	4.71E-08	4.67E-05	Male
SMPDL3B	1.987	3.588	59.056	5.01E-08	4.67E-05	Male
MRPL14	1.411	4.351	58.679	5.30E-08	4.67E-05	Male
LOC102563321	2.158	7.406	58.532	5.42E-08	4.67E-05	Male
KEF52_r01	-1.542	9.621	55.235	9.03E-08	7.26E-05	Female
LOC132251360	1.671	1.888	53.585	1.18E-07	8.89E-05	Male
CFAP119	1.996	1.797	52.919	1.31E-07	9.32E-05	Male
LOC102574353	1.103	7.444	52.012	1.52E-07	9.64E-05	Male
LOC109286038	2.654	2.505	51.996	1.53E-07	9.64E-05	Male
ANO7	2.131	3.659	51.7	1.60E-07	9.64E-05	Male
LOC102568143	1.68	7.328	50.522	1.94E-07	1.12E-04	Male
HOOK2	-1.491	5.762	49.548	2.29E-07	1.26E-04	Female
LOC132248260	2.437	1.075	49.183	2.49E-07	1.30E-04	Male
SPR	2.027	6.186	48.507	2.74E-07	1.38E-04	Male
LOC132244622	2.08	1.686	48.097	2.97E-07	1.43E-04	Male
GPR182	2.335	5.762	46.605	3.82E-07	1.77E-04	Male
IQSEC1	-1.522	2.608	46.026	4.24E-07	1.90E-04	Female
INAFM2	-1.154	4.212	45.443	4.71E-07	2.03E-04	Female
LOC109283324	2.026	3.108	45.066	5.04E-07	2.06E-04	Male
HHLA2	1.647	3.294	44.975	5.12E-07	2.06E-04	Male
PPP1R1B	2.41	1.358	43.626	6.67E-07	2.59E-04	Male
SFRP1	2.377	1.417	43.223	7.21E-07	2.72E-04	Male
LOC102571710	1.221	4.646	41.99	8.92E-07	3.26E-04	Male
ZNF395	-1.95	6.474	41.465	9.85E-07	3.50E-04	Female
MPP1	-1.206	6.23	41.155	1.05E-06	3.60E-04	Female
VAMP5	1.167	4.72	40.102	1.28E-06	4.30E-04	Male
LOC106737625	1.153	8.519	39.614	1.41E-06	4.60E-04	Male
LOC109282453	2.311	1.256	39.548	1.45E-06	4.60E-04	Male

PDZK1IP1	1.861	2.844	38.688	1.70E-06	5.25E-04	Male
NAV2	-2.462	1.562	38.483	1.75E-06	5.27E-04	Female
LOC109283711	2.466	1.421	38.27	1.86E-06	5.48E-04	Male
ECE2	1.559	7.313	37.695	2.07E-06	5.96E-04	Male
LOC132252489	1.139	2.573	36.74	2.52E-06	7.08E-04	Male
LOC106737548	1.944	5.031	36.622	2.59E-06	7.09E-04	Male
SYT3	1.496	6.043	36.262	2.79E-06	7.47E-04	Male
WNK4	1.39	8.056	35.6	3.20E-06	8.39E-04	Male
GNMT	-1.482	4.322	35.234	3.46E-06	8.70E-04	Female
KNTC1	1.476	6.824	35.184	3.49E-06	8.70E-04	Male
MTFR1L	1.346	7.993	35.129	3.54E-06	8.70E-04	Male
SNORC	2.209	1.521	34.743	3.89E-06	9.30E-04	Male
RPS27A	-1.003	10.79	34.63	3.93E-06	9.30E-04	Female
NEO1	-1.727	1.586	34.274	4.28E-06	9.92E-04	Female
P4HA2	1.364	6.377	33.806	4.70E-06	1.07E-03	Male
CEBPB	-1.141	5.693	33.641	4.87E-06	1.09E-03	Female
LOC102564916	1.089	5.03	33.262	5.29E-06	1.15E-03	Male
WBP2	-1.168	3.239	33.217	5.35E-06	1.15E-03	Female
GRM4	2.046	0.432	32.572	6.14E-06	1.30E-03	Male
LYL1	1.485	8.949	32.453	6.33E-06	1.31E-03	Male
GOLM1	-2.019	2.226	32.427	6.39E-06	1.31E-03	Female
SBK1	1.203	5.01	32.211	6.68E-06	1.34E-03	Male
GALR3	1.5	1.579	32.049	6.95E-06	1.38E-03	Male
PIM1	0.929	6.166	31.815	7.30E-06	1.41E-03	Male
BICD1	1.107	5.527	31.779	7.36E-06	1.41E-03	Male
FLVCR2	-1.235	2.533	31.716	7.48E-06	1.41E-03	Female
ARMH4	1.822	3.99	31.467	7.91E-06	1.47E-03	Male
TNKS2	-0.788	3.686	31.245	8.31E-06	1.52E-03	Female
PLEKHH2	1.822	4.468	30.792	9.22E-06	1.65E-03	Male
LOC102574840	1.368	2.05	30.634	9.57E-06	1.65E-03	Male
TET3	1.262	5.504	30.614	9.61E-06	1.65E-03	Male
POMT1	1.459	5.529	30.557	9.73E-06	1.65E-03	Male
RPL23A	-1.276	10.873	30.468	9.93E-06	1.65E-03	Female
PIK3IP1	-0.901	8.6	30.46	9.95E-06	1.65E-03	Female
KCNJ14	1.18	3.422	30.438	1.00E-05	1.65E-03	Male
LOC109282345	1.561	4.377	30.179	1.06E-05	1.73E-03	Male
LOC102564321	1.521	1.604	30.127	1.08E-05	1.73E-03	Male
DNAAF3	1.165	4.263	29.364	1.29E-05	2.04E-03	Male
AOPEP	-1.029	2.386	28.877	1.44E-05	2.26E-03	Female
RPL10A	-0.782	10.637	28.484	1.59E-05	2.45E-03	Female

LOC106737704	1.121	7.943	28.346	1.64E-05	2.48E-03	Male
HNRNPUL2	-0.79	9.867	28.337	1.64E-05	2.48E-03	Female
YAF2	-0.965	2.897	28.286	1.66E-05	2.48E-03	Female
GTF2IRD1	1.522	0.662	28.233	1.70E-05	2.50E-03	Male
DAZAP2	-0.906	5.212	28.145	1.72E-05	2.50E-03	Female
DCPS	1.257	5.688	28.039	1.77E-05	2.53E-03	Male
SAP18	-0.781	7.451	27.755	1.89E-05	2.67E-03	Female
DOK2	0.816	7.342	27.724	1.91E-05	2.67E-03	Male
LOC102569416	-0.681	7.026	27.685	1.92E-05	2.67E-03	Female
PIK3CB	-1.597	3.434	27.412	2.06E-05	2.78E-03	Female
HDAC6	0.805	9.283	27.393	2.07E-05	2.78E-03	Male
C2H11orf16	-1.18	2.654	27.383	2.07E-05	2.78E-03	Female
CENPV	1.104	2.616	27.263	2.13E-05	2.82E-03	Male
LOC102573729	-0.856	8.934	27.227	2.15E-05	2.82E-03	Female
FOSL1	-1.498	2.025	27.177	2.18E-05	2.83E-03	Female
RARA	0.841	6.216	26.954	2.30E-05	2.93E-03	Male
SLC45A3	-1.499	2.521	26.936	2.32E-05	2.93E-03	Female
ID3	1.552	6.885	26.903	2.33E-05	2.93E-03	Male
SPIRE2	2.041	1.04	26.739	2.45E-05	3.05E-03	Male
LOC102572053	1.446	8.79	26.562	2.54E-05	3.13E-03	Male
XPA	-1.08	3.239	26.483	2.59E-05	3.13E-03	Female
UHRF2	-0.738	3.778	26.471	2.60E-05	3.13E-03	Female
PC	1.141	5.403	26.299	2.71E-05	3.24E-03	Male
HEXIM1	0.871	8.463	26.062	2.88E-05	3.38E-03	Male
ART4	1.61	5.17	26.048	2.89E-05	3.38E-03	Male
LOC109284085	1.682	2.09	25.98	2.94E-05	3.41E-03	Male
TECPR2	-1.008	5.067	25.862	3.03E-05	3.48E-03	Female
EXOC1L	-1.583	0.721	25.829	3.08E-05	3.50E-03	Female
ZFYVE21	-1.149	5.732	25.75	3.12E-05	3.50E-03	Female
CXXC5	1.247	1.429	25.738	3.13E-05	3.50E-03	Male
PANX3	1.393	2.647	25.65	3.20E-05	3.53E-03	Male
STX1B	1.409	1.167	25.629	3.22E-05	3.53E-03	Male
RBM6	-0.879	6.098	25.529	3.30E-05	3.58E-03	Female
LOC106738720	0.812	4.535	25.491	3.33E-05	3.58E-03	Male
LOC109282180	-1.26	6.352	25.288	3.51E-05	3.74E-03	Female
ASIC4	1.462	7.255	25.211	3.58E-05	3.76E-03	Male
LOC102576565	1.786	2.695	25.188	3.60E-05	3.76E-03	Male
ILF2	0.782	8.196	25.169	3.61E-05	3.76E-03	Male
POLE	-1.052	4.356	25.087	3.69E-05	3.81E-03	Female
FAM178B	1.683	0.45	25.089	3.74E-05	3.82E-03	Male

LOC102570282	1.118	2.617	25.006	3.77E-05	3.82E-03	Male
IRF7	1.285	4.78	24.954	3.82E-05	3.83E-03	Male
MANF	0.829	3.81	24.935	3.84E-05	3.83E-03	Male
SMTNL2	1.389	4.705	24.811	3.97E-05	3.89E-03	Male
RBM45	-1.012	2.837	24.809	3.97E-05	3.89E-03	Female
NPSR1	2.661	0.508	24.432	4.09E-05	3.98E-03	Male
LOC109285558	1.6	0.618	24.638	4.20E-05	4.02E-03	Male
FGFR1	-1.527	3.186	24.578	4.21E-05	4.02E-03	Female
IKZF1	0.851	4.648	24.557	4.23E-05	4.02E-03	Male
CCNI	0.687	7.416	24.526	4.27E-05	4.02E-03	Male
TMEM217	1.244	0.961	24.485	4.33E-05	4.05E-03	Male
IGF2BP2	-1.13	3.981	24.41	4.40E-05	4.08E-03	Female
GEMIN8	-0.798	4.11	24.38	4.44E-05	4.08E-03	Female
ARID1B	-0.846	3.405	24.331	4.49E-05	4.11E-03	Female
BSN	-0.954	2.506	24.103	4.77E-05	4.33E-03	Female
GADD45B	1.446	9.141	24.017	4.88E-05	4.39E-03	Male
SRGAP2	-1.177	3.551	23.962	4.95E-05	4.39E-03	Female
GPCPD1	-1.614	3.959	23.916	5.01E-05	4.39E-03	Female
KIF13A	-0.826	3.595	23.891	5.04E-05	4.39E-03	Female
MN1	-1.345	0.821	23.926	5.04E-05	4.39E-03	Female
ANXA7	-0.857	5.708	23.88	5.06E-05	4.39E-03	Female
CEL	1.07	4.287	23.689	5.32E-05	4.58E-03	Male
LOC102560752	1.774	1.464	23.678	5.37E-05	4.59E-03	Male
HIC2	0.722	4.571	23.571	5.49E-05	4.66E-03	Male
GTF2H4	-0.758	4.584	23.523	5.56E-05	4.69E-03	Female
ZFAND5	-1.116	5.103	23.432	5.70E-05	4.75E-03	Female
ATP9B	-0.794	3.033	23.423	5.71E-05	4.75E-03	Female
LOC106738130	0.959	6.168	23.363	5.80E-05	4.79E-03	Male
LOC132248595	-0.695	6.507	23.322	5.87E-05	4.81E-03	Female
ZFAND2A	1.071	5.077	23.282	5.93E-05	4.83E-03	Male
CRB3	1.262	3.154	23.221	6.03E-05	4.88E-03	Male
RALGDS	0.91	5.823	23.114	6.20E-05	4.98E-03	Male
DNAJC12	-2.229	1.406	23.137	6.23E-05	4.98E-03	Female
RUNX1T1	1.108	3.834	23.035	6.34E-05	5.02E-03	Male
GBA1	-0.903	4.019	23.015	6.37E-05	5.02E-03	Female
ACP5	1.398	6.081	22.976	6.44E-05	5.04E-03	Male
GABARAPL2	-0.708	7.992	22.786	6.78E-05	5.24E-03	Female
PANK4	-0.899	5.669	22.756	6.83E-05	5.24E-03	Female
LOC102573401	-0.842	6.862	22.743	6.86E-05	5.24E-03	Female
CACNA1F	1.071	4.849	22.742	6.86E-05	5.24E-03	Male

LOC106738157	2.612	2.209	22.723	6.94E-05	5.26E-03	Male
CIAO2A	0.961	6.598	22.638	7.06E-05	5.32E-03	Male
WDSUB1	-1.287	3.699	22.576	7.18E-05	5.36E-03	Female
MYLK3	-2.24	2.749	22.572	7.20E-05	5.36E-03	Female
SLC24A5	-1.262	1.193	22.527	7.31E-05	5.41E-03	Female
PDIA4	0.708	4.077	22.228	7.89E-05	5.80E-03	Male
PBX4	-0.898	6.284	22.208	7.94E-05	5.80E-03	Female
KEF52_r02	-1.159	11.136	22.172	8.02E-05	5.82E-03	Female
MED16	0.859	8.15	22.079	8.22E-05	5.90E-03	Male
DISP3	-1.691	4.87	22.076	8.23E-05	5.90E-03	Female
LOC106738337	1.431	1.246	22.069	8.27E-05	5.90E-03	Male
PTCH2	2.153	1.686	22.004	8.40E-05	5.96E-03	Male
UBXN2A	-1.015	3.961	21.928	8.57E-05	6.05E-03	Female
KCNF1	-1.044	4.553	21.876	8.70E-05	6.06E-03	Female
LOC132250906	1.411	0.32	21.908	8.70E-05	6.06E-03	Male
MISP3	1.433	1.424	21.732	9.07E-05	6.26E-03	Male
TBL1X	-1.024	3.162	21.72	9.08E-05	6.26E-03	Female
NEK7	-0.981	4.237	21.671	9.21E-05	6.31E-03	Female
TMEM86A	-1.224	1.717	21.616	9.37E-05	6.38E-03	Female
C8H17orf80	-0.731	4.63	21.545	9.53E-05	6.46E-03	Female
PHB2	-0.595	8.974	21.411	9.90E-05	6.63E-03	Female
LOC109280221	0.953	1.401	21.379	1.00E-04	6.63E-03	Male
SELENOH	0.966	3.746	21.368	1.00E-04	6.63E-03	Male
HECA	-0.973	4.791	21.362	1.00E-04	6.63E-03	Female
LOC102576504	0.938	7.172	21.353	1.01E-04	6.63E-03	Male
DCAF15	0.985	7.201	21.212	1.05E-04	6.86E-03	Male
JPH2	0.983	6.027	21.119	1.07E-04	7.00E-03	Male
LOC109281289	1.387	2.972	21.073	1.09E-04	7.06E-03	Male
EDC3	-0.841	4.992	20.982	1.12E-04	7.20E-03	Female
JUP	-1.233	4.252	20.937	1.13E-04	7.26E-03	Female
PIGU	-0.823	3.534	20.889	1.15E-04	7.31E-03	Female
MAP1LC3A	-0.891	8.565	20.852	1.16E-04	7.35E-03	Female
PORCN	0.94	6.229	20.802	1.17E-04	7.39E-03	Male
RPL34	-0.678	7.242	20.797	1.18E-04	7.39E-03	Female
LOC102564219	-0.959	3.993	20.744	1.19E-04	7.46E-03	Female
DUSP22	0.992	8.229	20.648	1.23E-04	7.63E-03	Male
TMEM86B	1.367	0.694	20.614	1.25E-04	7.70E-03	Male
TRPV1	1.043	2.412	20.545	1.26E-04	7.70E-03	Male
KISS1R	1.233	3.194	20.537	1.27E-04	7.70E-03	Male
AIMP1	-0.635	8.225	20.531	1.27E-04	7.70E-03	Female

LOC102559575	1.226	1.929	20.53	1.27E-04	7.70E-03	Male
LOC102568113	1.109	6.593	20.241	1.38E-04	8.32E-03	Male
SLC41A3	1.249	2.721	20.214	1.39E-04	8.32E-03	Male
ALOX5	-1.03	3.114	20.194	1.40E-04	8.32E-03	Female
SMYD4	-0.884	5.036	20.188	1.40E-04	8.32E-03	Female
PEAK1	-1.258	4.049	20.139	1.42E-04	8.33E-03	Female
VMP1	-0.989	5.264	20.138	1.42E-04	8.33E-03	Female
LOC106737721	1.634	4.246	20.108	1.43E-04	8.33E-03	Male
LOC102565447	1.562	5.091	20.1	1.44E-04	8.33E-03	Male
EIF3B	0.619	7.727	20.083	1.44E-04	8.33E-03	Male
LOC132249606	0.905	7.575	20.082	1.44E-04	8.33E-03	Male
LOC102559498	1.633	9.613	20.067	1.45E-04	8.33E-03	Male
LOC106738142	-1.049	4.148	20.036	1.46E-04	8.36E-03	Female
POLR1E	-0.75	6.125	19.99	1.48E-04	8.41E-03	Female
LOC102574067	1.59	1.191	19.966	1.50E-04	8.41E-03	Male
GPT	-1.479	5.32	19.929	1.51E-04	8.41E-03	Female
CCNI2	-0.842	6.156	19.918	1.51E-04	8.41E-03	Female
AQP1	1.027	7.267	19.907	1.52E-04	8.41E-03	Male
ZNF423	1.49	2.709	19.909	1.52E-04	8.41E-03	Male
E2F2	1.066	5.842	19.89	1.53E-04	8.41E-03	Male
TBC1D31	-1.114	3.6	19.865	1.54E-04	8.41E-03	Female
MCM4	0.797	4.153	19.862	1.54E-04	8.41E-03	Male
SLC35F5	-0.978	2.903	19.859	1.54E-04	8.41E-03	Female
CDC42BPG	1.484	1.461	19.853	1.55E-04	8.41E-03	Male
ABI3BP	2.206	1.245	19.845	1.56E-04	8.41E-03	Male
FMNL3	0.969	7.471	19.715	1.61E-04	8.63E-03	Male
PRX	1.27	1.17	19.715	1.61E-04	8.63E-03	Male
EHD1	1.216	3.424	19.684	1.62E-04	8.65E-03	Male
AFF1	0.953	5.473	19.644	1.64E-04	8.71E-03	Male
LOC132251227	0.766	6.086	19.618	1.65E-04	8.74E-03	Male
ENTPD7	-0.771	6.86	19.58	1.67E-04	8.79E-03	Female
RHOBTB3	1.999	1.443	19.495	1.72E-04	8.98E-03	Male
PCGF3	-0.882	3.761	19.467	1.73E-04	8.98E-03	Female
POLM	1.136	7.096	19.466	1.73E-04	8.98E-03	Male
TTC7B	0.9	3.586	19.45	1.74E-04	8.98E-03	Male
DMPK	-1.349	2.214	19.426	1.75E-04	8.99E-03	Female
NDUFA9	0.667	4.276	19.418	1.75E-04	8.99E-03	Male
RPL35	-0.751	11.565	19.358	1.78E-04	9.11E-03	Female
CDC42SE1	0.707	8.607	19.333	1.80E-04	9.14E-03	Male
HMOX1	1.652	5.807	19.275	1.83E-04	9.26E-03	Male

TRIM69	1.36	2.604	19.242	1.84E-04	9.31E-03	Male
GTF3C2	-0.804	5.836	19.222	1.86E-04	9.32E-03	Female
HOMER3	1.406	4.837	19.192	1.87E-04	9.33E-03	Male
LOC109281397	0.83	6.744	19.192	1.87E-04	9.33E-03	Male
DENND5B	-0.918	4.232	19.087	1.93E-04	9.58E-03	Female
CSNK1G1	-0.798	3.453	19	1.98E-04	9.79E-03	Female
PGAP3	0.712	6.933	18.976	2.00E-04	9.82E-03	Male
ATPAF2	0.82	5.723	18.857	2.07E-04	1.01E-02	Male
MRPL52	-1.103	6.182	18.842	2.08E-04	1.01E-02	Female
CALCOCO1	-0.815	5.139	18.804	2.10E-04	1.02E-02	Female
ATP2B4	0.762	5.455	18.72	2.15E-04	1.04E-02	Male
LOC132250780	2.463	2.22	18.572	2.17E-04	1.04E-02	Male
PARD6A	0.76	4.789	18.691	2.17E-04	1.04E-02	Male
LOC109285227	1.622	5.593	18.677	2.18E-04	1.04E-02	Male
STIM2	-0.731	2.222	18.672	2.18E-04	1.04E-02	Female
SNX25	-1.276	3.17	18.646	2.20E-04	1.04E-02	Female
FGF17	1.437	1.626	18.621	2.22E-04	1.05E-02	Male
MACF1	0.774	8.75	18.595	2.23E-04	1.05E-02	Male
TPRG1L	-0.832	5.528	18.591	2.24E-04	1.05E-02	Female
ERI1	-0.645	4.338	18.498	2.30E-04	1.07E-02	Female
GPANK1	-0.623	4.727	18.452	2.33E-04	1.08E-02	Female
ARL3	-0.695	4.158	18.441	2.34E-04	1.08E-02	Female
LOC106738229	0.928	6.16	18.381	2.38E-04	1.10E-02	Male
ALDH9A1	-0.729	6.063	18.334	2.42E-04	1.11E-02	Female
TSHZ1	-1.35	1.265	18.286	2.47E-04	1.13E-02	Female
SLC6A16	0.841	4.481	18.21	2.51E-04	1.14E-02	Male
ITSN1	-1.613	4.653	18.176	2.53E-04	1.15E-02	Female
RANBP10	0.754	7.038	18.145	2.56E-04	1.15E-02	Male
LOC102574636	-1.242	3.889	18.144	2.56E-04	1.15E-02	Female
RBM7	-0.689	4.984	18.056	2.63E-04	1.18E-02	Female
USP50	-1.03	5.125	18.027	2.65E-04	1.18E-02	Female
LOC102559475	1.048	5.098	18.021	2.66E-04	1.18E-02	Male
RPL28	-0.861	10.707	17.998	2.68E-04	1.19E-02	Female
USP54	-0.979	1.947	17.932	2.73E-04	1.20E-02	Female
DNAH10	0.929	1.512	17.931	2.73E-04	1.20E-02	Male
LOC102563162	1.631	0.613	17.929	2.75E-04	1.21E-02	Male
HCN3	-0.804	5.314	17.87	2.78E-04	1.22E-02	Female
LOC102562945	0.616	5.278	17.767	2.87E-04	1.25E-02	Male
LOC132248243	-0.851	3.609	17.744	2.89E-04	1.25E-02	Female
DRAM2	0.89	3.501	17.734	2.90E-04	1.25E-02	Male

BATF3	-1.146	1.535	17.736	2.90E-04	1.25E-02	Female
LOC132245930	-1.254	2.987	17.72	2.91E-04	1.25E-02	Female
TGIF2	-0.849	2.849	17.688	2.94E-04	1.26E-02	Female
ATP11C	-0.726	3.764	17.595	3.03E-04	1.29E-02	Female
DCAF6	-0.727	4.358	17.558	3.06E-04	1.30E-02	Female
LOC106737488	-1.548	0.405	17.579	3.07E-04	1.30E-02	Female
PLXND1	1.22	0.81	17.519	3.11E-04	1.31E-02	Male
BPHL	1.067	0.782	17.504	3.12E-04	1.31E-02	Male
ATP13A5	1.217	5.976	17.496	3.12E-04	1.31E-02	Male
ABT1	0.616	7.206	17.422	3.19E-04	1.33E-02	Male
TCF7L2	-0.94	2.774	17.376	3.24E-04	1.35E-02	Female
NDFIP1	-0.673	7.038	17.359	3.25E-04	1.35E-02	Female
EIF4EBP3	1.165	6.223	17.353	3.26E-04	1.35E-02	Male
MCM3	0.815	4.001	17.309	3.31E-04	1.36E-02	Male
LOC102574957	0.863	3.448	17.26	3.36E-04	1.38E-02	Male
DDIT4	-0.995	4.917	17.214	3.40E-04	1.39E-02	Female
NLK	0.737	3.18	17.197	3.42E-04	1.39E-02	Male
SLC25A39	0.981	6.38	17.166	3.45E-04	1.40E-02	Male
LOC102572093	1.576	1.872	17.145	3.48E-04	1.41E-02	Male
RPLP2	-0.68	10.976	17.053	3.58E-04	1.44E-02	Female
PRRC1	-0.669	3.801	16.895	3.76E-04	1.51E-02	Female
NDUFC1	0.682	4.199	16.802	3.87E-04	1.55E-02	Male
LOC102559494	-0.689	4.591	16.789	3.89E-04	1.55E-02	Female
MORC3	-0.732	3.785	16.774	3.91E-04	1.55E-02	Female
R3HDM4	0.837	11.121	16.754	3.93E-04	1.56E-02	Male
LOC102562601	1.1	0.455	16.757	3.94E-04	1.56E-02	Male
AP5M1	-0.699	5.095	16.724	3.97E-04	1.56E-02	Female
PLEKHA6	0.945	5.143	16.712	3.98E-04	1.56E-02	Male
ZHX1	-1.215	1.354	16.692	4.02E-04	1.56E-02	Female
SERTAD1	1.471	4.647	16.654	4.05E-04	1.56E-02	Male
ARRDC1	1.14	3.307	16.65	4.06E-04	1.56E-02	Male
BICRA	-0.959	3.718	16.647	4.06E-04	1.56E-02	Female
RPL14	-0.734	11.861	16.647	4.06E-04	1.56E-02	Female
IKZF5	-0.853	3.431	16.633	4.08E-04	1.56E-02	Female
SKA3	0.642	3.755	16.622	4.10E-04	1.56E-02	Male
LOC102570732	-0.871	4.181	16.619	4.10E-04	1.56E-02	Female
LOC132250788	-1.097	4.477	16.605	4.12E-04	1.57E-02	Female
LOC109284725	-0.753	3.578	16.595	4.13E-04	1.57E-02	Female
NOC4L	0.753	3.817	16.537	4.21E-04	1.59E-02	Male
CDK9	-0.635	5.205	16.465	4.30E-04	1.62E-02	Female

GTPBP1	0.602	5.588	16.439	4.34E-04	1.63E-02	Male
MARCHF8	-0.931	5.667	16.437	4.34E-04	1.63E-02	Female
FAM219A	-0.837	1.474	16.381	4.42E-04	1.64E-02	Female
CDKN1C	-0.822	4.939	16.373	4.43E-04	1.64E-02	Female
LOC102561902	1.098	8.087	16.372	4.43E-04	1.64E-02	Male
VAMP8	0.738	6.362	16.363	4.44E-04	1.64E-02	Male
LOC109283614	0.834	1.72	16.318	4.51E-04	1.66E-02	Male
MTMR10	-0.778	2.526	16.29	4.55E-04	1.67E-02	Female
SYNGR3	-1.034	1.353	16.223	4.66E-04	1.70E-02	Female
AAGAB	-0.656	6.196	16.214	4.66E-04	1.70E-02	Female
CAP2	1.44	1.855	16.184	4.71E-04	1.71E-02	Male
GCNA	0.806	5.107	16.151	4.75E-04	1.71E-02	Male
NOS1	-1.792	1.44	16.163	4.76E-04	1.71E-02	Female
STK11IP	-0.698	4.851	16.14	4.77E-04	1.71E-02	Female
LOC109281023	-1.09	0.342	16.119	4.83E-04	1.73E-02	Female
DCLRE1A	0.912	2.434	16.073	4.88E-04	1.74E-02	Male
LOC109285037	-0.864	1.143	16.06	4.91E-04	1.74E-02	Female
CPEB3	-1.246	1.11	16.058	4.91E-04	1.74E-02	Female
ALDH6A1	-0.874	3.475	16.005	4.98E-04	1.76E-02	Female
USP25	-0.937	3.56	16.002	4.99E-04	1.76E-02	Female
ECE1	-0.982	1.917	15.902	5.15E-04	1.81E-02	Female
MAMDC4	0.797	4.261	15.883	5.18E-04	1.82E-02	Male
PALM3	1.554	0.085	15.821	5.32E-04	1.86E-02	Male
CKAP2	1.317	2.578	15.742	5.42E-04	1.89E-02	Male
LOC132250789	-1.01	3.149	15.735	5.44E-04	1.89E-02	Female
ZAR1	1.062	2.899	15.724	5.45E-04	1.89E-02	Male
LOC106738055	1.157	5.653	15.656	5.57E-04	1.93E-02	Male
LOC102567736	0.88	3.46	15.62	5.64E-04	1.94E-02	Male
PTPN2	0.593	4.62	15.59	5.70E-04	1.96E-02	Male
CRYBG3	1.299	0.71	15.547	5.80E-04	1.98E-02	Male
PDLIM4	-1.853	0.633	15.548	5.81E-04	1.98E-02	Female
COPB2	-0.649	4.511	15.522	5.82E-04	1.98E-02	Female
ANKRD9	-1.05	2.929	15.48	5.90E-04	1.99E-02	Female
FNBP1	0.771	7.826	15.473	5.92E-04	1.99E-02	Male
GGT5	-1.195	3.458	15.469	5.92E-04	1.99E-02	Female
LOC132252173	1.064	2.171	15.459	5.94E-04	1.99E-02	Male
ASPA	-1.693	0.686	15.473	5.95E-04	1.99E-02	Female
GLCE	-1.06	2.118	15.455	5.95E-04	1.99E-02	Female
LOC102577101	0.602	5.2	15.445	5.97E-04	1.99E-02	Male
SMCO3	1.839	0.378	15.447	6.01E-04	2.00E-02	Male

TET1	-0.807	2.517	15.414	6.03E-04	2.00E-02	Female
LOC132250709	-0.995	3.881	15.387	6.08E-04	2.01E-02	Female
FAM184A	-0.833	4.265	15.375	6.11E-04	2.01E-02	Female
POLD4	0.823	6.28	15.355	6.15E-04	2.02E-02	Male
LOC102569402	-1.041	0.938	15.322	6.23E-04	2.04E-02	Female
NCBP1	-1.064	4.929	15.313	6.23E-04	2.04E-02	Female
ZGPAT	0.663	4.548	15.296	6.27E-04	2.04E-02	Male
LOC106739203	0.797	4.465	15.261	6.34E-04	2.06E-02	Male
CBLB	-0.674	3.61	15.254	6.35E-04	2.06E-02	Female
RPA2	0.754	4.7	15.252	6.36E-04	2.06E-02	Male
IFT57	0.658	3.219	15.24	6.38E-04	2.06E-02	Male
RIT1	-0.671	5.668	15.196	6.47E-04	2.08E-02	Female
LARP1	0.692	5.664	15.166	6.54E-04	2.09E-02	Male
GPR150	1.252	4.679	15.145	6.59E-04	2.09E-02	Male
INTS2	-0.626	3.207	15.141	6.59E-04	2.09E-02	Female
UAP1	0.943	2.406	15.133	6.61E-04	2.09E-02	Male
NDC80	1.094	2.043	15.128	6.62E-04	2.09E-02	Male
CATSPER4	1.039	3.418	15.127	6.62E-04	2.09E-02	Male
CFAP126	1.102	1.354	15.111	6.66E-04	2.09E-02	Male
PARPBP	1.044	1.088	15.098	6.70E-04	2.10E-02	Male
CBX8	-0.763	5.796	15.042	6.81E-04	2.13E-02	Female
LOC102566452	-1.021	2.72	14.995	6.92E-04	2.16E-02	Female
RPL39	-0.599	10.783	14.953	7.02E-04	2.17E-02	Female
LOC102557808	-0.715	2.974	14.948	7.03E-04	2.17E-02	Female
TP53BP2	1.438	1.854	14.921	7.10E-04	2.17E-02	Male
FAP	-0.904	3.47	14.915	7.10E-04	2.17E-02	Female
CDIP1	0.91	4.331	14.915	7.10E-04	2.17E-02	Male
RPS13	-0.639	10.531	14.906	7.12E-04	2.17E-02	Female
ENSA	0.597	5.082	14.903	7.13E-04	2.17E-02	Male
AMIGO1	1.626	0.197	14.921	7.14E-04	2.17E-02	Male
CACNG7	-0.767	4.326	14.895	7.15E-04	2.17E-02	Female
VTI1A	-0.848	1.811	14.873	7.21E-04	2.17E-02	Female
EML6	1.347	0.884	14.877	7.21E-04	2.17E-02	Male
LIMD2	0.711	6.642	14.865	7.22E-04	2.17E-02	Male
EPHA10	-1.054	4.341	14.85	7.26E-04	2.18E-02	Female
CLDN15	0.822	3.054	14.825	7.32E-04	2.19E-02	Male
PROSER2	1.244	2.271	14.772	7.45E-04	2.22E-02	Male
MAP2K6	0.725	3.172	14.738	7.53E-04	2.24E-02	Male
PIGN	-0.895	2.007	14.711	7.60E-04	2.25E-02	Female
RFFL	-0.739	2.181	14.709	7.61E-04	2.25E-02	Female

SRD5A1	0.889	1.348	14.689	7.66E-04	2.26E-02	Male
PTRH2	-0.622	3.603	14.682	7.67E-04	2.26E-02	Female
SLC7A5	1.086	6.378	14.68	7.68E-04	2.26E-02	Male
LOC102558907	-0.64	4.713	14.654	7.74E-04	2.27E-02	Female
SNAPC1	-0.685	4.479	14.638	7.79E-04	2.28E-02	Female
PCMTD1	-0.82	5.318	14.622	7.83E-04	2.29E-02	Female
IL27RA	0.867	6.981	14.605	7.87E-04	2.29E-02	Male
CSE1L	0.667	3.059	14.595	7.90E-04	2.29E-02	Male
ASPH	-0.891	1.139	14.596	7.92E-04	2.29E-02	Female
HMBS	0.75	7.58	14.569	7.97E-04	2.29E-02	Male
SHCBP1	1.163	1.872	14.571	7.97E-04	2.29E-02	Male
CREG1	0.871	8.86	14.56	7.99E-04	2.29E-02	Male
LOC132248244	-0.764	4.039	14.532	8.07E-04	2.31E-02	Female
PPP1R10	0.751	6.298	14.512	8.12E-04	2.32E-02	Male
LIF	1.459	0.543	14.533	8.12E-04	2.32E-02	Male
CCDC24	1.197	1.994	14.484	8.20E-04	2.33E-02	Male
LOC109283695	0.678	3.073	14.477	8.22E-04	2.33E-02	Male
PABPN1	0.669	5.74	14.461	8.26E-04	2.34E-02	Male
LOC102566561	0.834	6.716	14.442	8.31E-04	2.34E-02	Male
HEBP1	1.041	7.132	14.425	8.36E-04	2.35E-02	Male
LOC106738577	-1.235	2.106	14.404	8.42E-04	2.36E-02	Female
PELO	-0.588	4.434	14.397	8.44E-04	2.36E-02	Female
LMNA	0.789	8.267	14.275	8.79E-04	2.44E-02	Male
ANXA11	-0.625	6.253	14.273	8.80E-04	2.44E-02	Female
SMYD1	-0.761	2.946	14.261	8.84E-04	2.44E-02	Female
PPM1D	-0.725	3.958	14.25	8.87E-04	2.44E-02	Female
DIPK2B	0.743	8.5	14.241	8.89E-04	2.44E-02	Male
NFIX	1.385	4.094	14.241	8.89E-04	2.44E-02	Male
PFKFB4	-0.774	3.65	14.228	8.93E-04	2.44E-02	Female
ME3	1.06	3.138	14.195	9.03E-04	2.46E-02	Male
CDC20	1.125	4.039	14.137	9.21E-04	2.50E-02	Male
LOC102570590	-0.908	3.502	14.114	9.28E-04	2.51E-02	Female
VPS18	-0.636	4.641	14.104	9.31E-04	2.51E-02	Female
LOC109286161	1.145	3.115	14.103	9.32E-04	2.51E-02	Male
RAPGEF1	-0.842	3.973	14.03	9.55E-04	2.57E-02	Female
KNL1	0.913	2.758	14.027	9.56E-04	2.57E-02	Male
LOC132244013	-1.076	0.631	14.028	9.60E-04	2.57E-02	Female
LOC132245936	1.494	0.596	13.97	9.69E-04	2.59E-02	Male
PTPRN	-1.001	1.653	13.979	9.72E-04	2.59E-02	Female
LOC102568370	-0.718	2.207	13.958	9.79E-04	2.60E-02	Female

LOC132246647	0.948	2.812	13.879	1.01E-03	2.66E-02	Male
LOC102558474	1.885	0.462	13.888	1.01E-03	2.66E-02	Male
TSPAN6	-0.772	2.22	13.86	1.01E-03	2.66E-02	Female
OPHN1	-0.798	5.716	13.845	1.02E-03	2.67E-02	Female
LOC132250791	-0.638	4.673	13.828	1.02E-03	2.68E-02	Female
EBF4	0.987	5.908	13.817	1.03E-03	2.69E-02	Male
LOC102559184	1.257	5.288	13.797	1.03E-03	2.70E-02	Male
LOC109283710	1.052	1.996	13.789	1.04E-03	2.70E-02	Male
MFSD12	0.901	5.099	13.777	1.04E-03	2.70E-02	Male
RPL37A	-0.864	9.901	13.765	1.04E-03	2.70E-02	Female
KDSR	0.83	5.521	13.742	1.05E-03	2.72E-02	Male
BHLHE40	-1.13	3.161	13.729	1.06E-03	2.72E-02	Female
LOC102566371	-0.911	2.375	13.727	1.06E-03	2.72E-02	Female
SLC35E4	-1.043	1.161	13.689	1.07E-03	2.76E-02	Female
DRG2	0.609	8.205	13.662	1.08E-03	2.77E-02	Male
LOC109282009	1.514	1.555	13.655	1.09E-03	2.78E-02	Male
LOC102563395	1.401	1.822	13.639	1.09E-03	2.78E-02	Male
LOC132245805	-1.087	3.485	13.614	1.10E-03	2.80E-02	Female
BACH1	0.909	7.986	13.591	1.11E-03	2.81E-02	Male
MIPOL1	-0.844	3.512	13.59	1.11E-03	2.81E-02	Female
UBE2D3	-0.641	9.22	13.583	1.11E-03	2.81E-02	Female
LOC102565156	0.766	6.687	13.576	1.11E-03	2.81E-02	Male
LOC109284225	1.232	1.156	13.568	1.12E-03	2.82E-02	Male
LOC132251390	1.14	1.333	13.536	1.13E-03	2.84E-02	Male
TMOD4	0.795	9.058	13.528	1.13E-03	2.84E-02	Male
TBC1D15	-0.868	6.556	13.514	1.14E-03	2.85E-02	Female
SCYL2	-0.747	3.55	13.51	1.14E-03	2.85E-02	Female
TMEM181	-0.747	4.224	13.475	1.15E-03	2.88E-02	Female
SGF29	-0.862	6.241	13.468	1.16E-03	2.88E-02	Female
SOBP	-1.021	0.345	13.448	1.17E-03	2.89E-02	Female
MTFR2	1.136	1.5	13.434	1.17E-03	2.89E-02	Male
LOC102569714	0.84	5.941	13.429	1.17E-03	2.89E-02	Male
FUZ	0.994	1.962	13.428	1.17E-03	2.89E-02	Male
KIF2C	0.902	4.668	13.375	1.19E-03	2.93E-02	Male
KLHL29	-1.337	0.692	13.381	1.20E-03	2.94E-02	Female
FOSL2	-0.768	2.989	13.361	1.20E-03	2.94E-02	Female
SH2D6	0.833	5.649	13.341	1.21E-03	2.95E-02	Male
XKR8	0.769	3.167	13.331	1.21E-03	2.96E-02	Male
SLC18A2	-0.817	4.049	13.316	1.22E-03	2.96E-02	Female
LUC7L	-0.591	6.193	13.315	1.22E-03	2.96E-02	Female

LOC102574531	0.638	8.399	13.305	1.22E-03	2.96E-02	Male
MINDY3	0.761	4.627	13.292	1.23E-03	2.97E-02	Male
TRIM66	0.768	1.848	13.241	1.25E-03	3.01E-02	Male
LOC102574395	-1.423	2.645	13.217	1.26E-03	3.03E-02	Female
RNF128	-0.896	3.255	13.208	1.27E-03	3.03E-02	Female
GABBR1	-0.812	1.573	13.206	1.27E-03	3.03E-02	Female
ENDOG	-0.712	1.797	13.194	1.27E-03	3.03E-02	Female
CHMP1B	-0.616	4.581	13.178	1.28E-03	3.04E-02	Female
MYLIP	-1.095	3.369	13.148	1.29E-03	3.06E-02	Female
FAM120C	0.677	6.144	13.147	1.29E-03	3.06E-02	Male
ANAPC4	-0.742	4.463	13.136	1.30E-03	3.06E-02	Female
VPS8	-0.58	3.29	13.113	1.31E-03	3.08E-02	Female
TMEM104	-0.782	3.188	13.109	1.31E-03	3.08E-02	Female
TFRC	0.704	10.198	13.102	1.31E-03	3.08E-02	Male
WBP2NL	-0.69	8.57	13.094	1.32E-03	3.08E-02	Female
KEF52_t10	1.321	1.876	13.096	1.32E-03	3.08E-02	Male
KLF16	0.697	6.871	13.091	1.32E-03	3.08E-02	Male
OSGIN1	1.01	3.925	13.061	1.33E-03	3.10E-02	Male
LOC102566761	0.845	1.069	13.032	1.35E-03	3.13E-02	Male
RPS8	-0.682	11.18	13.014	1.35E-03	3.14E-02	Female
RALGAPA2	0.626	3.665	12.994	1.36E-03	3.16E-02	Male
YIF1B	-0.786	9.947	12.968	1.38E-03	3.18E-02	Female
GGCX	0.689	6.139	12.956	1.38E-03	3.19E-02	Male
ADAP2	-0.625	3.244	12.934	1.39E-03	3.21E-02	Female
PBX1	0.676	3.147	12.885	1.42E-03	3.25E-02	Male
TSACC	0.948	1.213	12.848	1.44E-03	3.28E-02	Male
HCN2	1.031	2.564	12.835	1.44E-03	3.28E-02	Male
SLC30A10	0.891	6.194	12.833	1.44E-03	3.28E-02	Male
RRM2	1.059	3.413	12.821	1.45E-03	3.29E-02	Male
LOC132251715	1.073	0.824	12.788	1.47E-03	3.32E-02	Male
MITF	-0.711	2.194	12.778	1.47E-03	3.32E-02	Female
LOC132246428	0.954	1.765	12.753	1.49E-03	3.34E-02	Male
CLSTN3	0.799	3.795	12.729	1.50E-03	3.35E-02	Male
BET1L	-0.607	4.204	12.724	1.50E-03	3.35E-02	Female
UBA52	-0.735	10.928	12.718	1.50E-03	3.35E-02	Female
PIAS2	-0.745	6.003	12.717	1.50E-03	3.35E-02	Female
RAPGEFL1	1.019	1.212	12.7	1.51E-03	3.36E-02	Male
CGRRF1	-0.741	2.978	12.692	1.52E-03	3.36E-02	Female
PTTG1	0.786	3.108	12.688	1.52E-03	3.36E-02	Male
C13H1orf159	0.915	1.536	12.681	1.52E-03	3.36E-02	Male

KPNA2	0.631	4.856	12.67	1.53E-03	3.37E-02	Male
ZFAND6	-1.087	7.796	12.662	1.53E-03	3.37E-02	Female
LOC132251611	-1.657	1.983	12.296	1.54E-03	3.39E-02	Female
HES2	1.302	1.772	12.639	1.55E-03	3.39E-02	Male
ME2	0.785	3.613	12.632	1.55E-03	3.39E-02	Male
VPS37C	-0.778	4.874	12.608	1.56E-03	3.41E-02	Female
UBE2E1	0.684	3.442	12.58	1.58E-03	3.43E-02	Male
SLC22A31	0.987	3.455	12.574	1.58E-03	3.43E-02	Male
EAF1	-0.813	3.386	12.548	1.60E-03	3.46E-02	Female
LOC102571833	1.382	6.487	12.532	1.60E-03	3.47E-02	Male
APOC1	1.368	5.161	12.5	1.62E-03	3.50E-02	Male
KIFAP3	-0.84	1.622	12.475	1.64E-03	3.52E-02	Female
PRKCG	-1.061	0.883	12.476	1.64E-03	3.52E-02	Female
LOC102566394	-1.281	2.585	12.454	1.65E-03	3.52E-02	Female
LOC102561222	0.818	3.574	12.445	1.66E-03	3.52E-02	Male
CDT1	0.955	2.861	12.421	1.67E-03	3.52E-02	Male
MCM5	0.865	4.098	12.419	1.67E-03	3.52E-02	Male
LFNG	0.595	6.027	12.417	1.67E-03	3.52E-02	Male
YME1L1	-0.755	5.533	12.413	1.67E-03	3.52E-02	Female
CHCHD7	0.584	5.485	12.392	1.69E-03	3.54E-02	Male
ZBTB14	-0.796	1.515	12.377	1.70E-03	3.56E-02	Female
LOC102574910	-0.925	0.792	12.363	1.71E-03	3.57E-02	Female
MTHFR	-0.589	5.712	12.353	1.71E-03	3.57E-02	Female
TLCD3A	-0.724	2.711	12.31	1.74E-03	3.61E-02	Female
LOC102565912	0.69	5.24	12.281	1.75E-03	3.64E-02	Male
LOC106738633	-1.064	7.763	12.248	1.78E-03	3.68E-02	Female
SLC6A4	-0.979	0.764	12.184	1.82E-03	3.77E-02	Female
ANKIB1	-0.799	2.266	12.159	1.83E-03	3.78E-02	Female
OFD1	-0.814	1.99	12.157	1.84E-03	3.78E-02	Female
LOC106738398	-0.916	2.754	12.147	1.84E-03	3.78E-02	Female
ATOSA	-0.92	5.211	12.129	1.85E-03	3.80E-02	Female
ADGRG2	1.332	0.629	12.103	1.88E-03	3.83E-02	Male
AMMECR1L	0.587	4.656	12.089	1.88E-03	3.84E-02	Male
RPL22	-0.597	5.149	12.061	1.90E-03	3.86E-02	Female
LOC106738082	-0.875	2.078	12.059	1.90E-03	3.86E-02	Female
SERINC3	-0.624	8.428	12.036	1.92E-03	3.88E-02	Female
LOC132250448	1.135	0.62	12.02	1.93E-03	3.90E-02	Male
MCM6	0.699	3.151	12.003	1.94E-03	3.90E-02	Male
ATRIP	0.677	2.253	11.984	1.95E-03	3.93E-02	Male
LOC132245823	-0.622	3.072	11.975	1.96E-03	3.93E-02	Female

LOC102561537	0.693	2.834	11.961	1.97E-03	3.93E-02	Male
ENTREP3	-1.075	1.226	11.966	1.97E-03	3.93E-02	Female
HYLS1	0.68	2.776	11.956	1.97E-03	3.93E-02	Male
KLF15	-1.319	3.381	11.956	1.97E-03	3.93E-02	Female
AHCY	0.723	4.811	11.924	2.00E-03	3.97E-02	Male
TMC4	0.7	3.193	11.907	2.01E-03	3.98E-02	Male
PNMT	0.669	5.713	11.905	2.01E-03	3.98E-02	Male
LOC106737317	1.091	4.152	11.864	2.04E-03	4.03E-02	Male
SLC1A4	0.592	6.108	11.848	2.05E-03	4.05E-02	Male
GPAT3	-0.638	4.312	11.831	2.06E-03	4.07E-02	Female
RHBDD1	-0.604	3.944	11.795	2.09E-03	4.09E-02	Female
SLC1A5	0.71	3.876	11.788	2.10E-03	4.09E-02	Male
LOC102563438	-0.794	4.23	11.782	2.10E-03	4.09E-02	Female
CDON	0.841	0.775	11.787	2.10E-03	4.09E-02	Male
PTS	-0.704	2.378	11.778	2.10E-03	4.09E-02	Female
PDE8A	-0.761	3.603	11.77	2.11E-03	4.10E-02	Female
MLH1	-0.937	1.233	11.758	2.12E-03	4.10E-02	Female
APLF	-0.618	3.267	11.753	2.12E-03	4.10E-02	Female
PURA	-0.689	2.855	11.752	2.12E-03	4.10E-02	Female
ATP5F1B	0.697	8.703	11.742	2.13E-03	4.11E-02	Male
YWHAH	0.629	4.209	11.733	2.14E-03	4.12E-02	Male
USP21	-0.58	5.379	11.713	2.15E-03	4.14E-02	Female
RPL17	-0.594	9.751	11.706	2.16E-03	4.14E-02	Female
FNIP1	-0.868	3.779	11.705	2.16E-03	4.14E-02	Female
DHRS3	-0.981	2.753	11.692	2.17E-03	4.15E-02	Female
LOC102557982	1.274	6.079	11.69	2.17E-03	4.15E-02	Male
LOC132251925	1.27	0.232	11.696	2.18E-03	4.15E-02	Male
SCAMP5	-0.75	4.722	11.681	2.18E-03	4.15E-02	Female
LTBP3	1.096	0.712	11.673	2.19E-03	4.15E-02	Male
SGK3	-0.841	3.144	11.667	2.19E-03	4.15E-02	Female
UROD	1.115	7.798	11.657	2.20E-03	4.16E-02	Male
LOC102571335	-1.034	3.701	11.655	2.20E-03	4.16E-02	Female
CNTFR	-1.132	0.058	11.652	2.21E-03	4.17E-02	Female
MALSU1	0.711	2.572	11.634	2.22E-03	4.18E-02	Male
LOC102570701	0.846	4.386	11.631	2.22E-03	4.18E-02	Male
COMMD1	-0.776	6.103	11.615	2.23E-03	4.19E-02	Female
RPL19	-0.603	11.948	11.606	2.24E-03	4.19E-02	Female
RFWD3	0.648	4.741	11.604	2.24E-03	4.19E-02	Male
TMEM38A	-0.912	3.361	11.601	2.24E-03	4.19E-02	Female
ACSL1	-0.628	3.572	11.593	2.25E-03	4.19E-02	Female

LOC109281168	1.096	1.508	11.592	2.25E-03	4.19E-02	Male
TK1	1.039	6.021	11.589	2.25E-03	4.19E-02	Male
LY75	-0.606	2.9	11.558	2.28E-03	4.23E-02	Female
LOC132250140	1.583	2.709	11.551	2.29E-03	4.23E-02	Male
TMEM158	-1.253	1.102	11.51	2.33E-03	4.29E-02	Female
CHSY1	-0.587	3.212	11.495	2.33E-03	4.29E-02	Female
CDK20	0.781	8.79	11.493	2.33E-03	4.29E-02	Male
TPST2	0.593	4.361	11.483	2.34E-03	4.30E-02	Male
GFI1B	0.793	7.36	11.45	2.37E-03	4.34E-02	Male
LOC106737871	1.349	0.749	11.459	2.37E-03	4.34E-02	Male
PTPN4	-0.943	3.113	11.438	2.38E-03	4.35E-02	Female
SKIL	-0.906	2.061	11.433	2.39E-03	4.35E-02	Female
MARCHF2	-0.733	0.995	11.431	2.39E-03	4.35E-02	Female
HDC	-1.028	3.125	11.424	2.39E-03	4.35E-02	Female
ACLY	0.621	3.602	11.401	2.41E-03	4.38E-02	Male
ATF5	0.836	4.131	11.388	2.43E-03	4.39E-02	Male
RUNDC3A	1.447	1.258	11.377	2.44E-03	4.41E-02	Male
LOC132243217	-0.85	3.557	11.319	2.49E-03	4.46E-02	Female
NAP1L4	-0.669	7.149	11.317	2.49E-03	4.46E-02	Female
ACSS1	0.762	4.263	11.312	2.49E-03	4.46E-02	Male
KCND2	1.157	0.757	11.317	2.49E-03	4.46E-02	Male
BSG	0.685	8.68	11.311	2.50E-03	4.46E-02	Male
SND1	-0.732	8.862	11.294	2.51E-03	4.47E-02	Female
LOC132251612	-1.086	1.758	11.293	2.52E-03	4.47E-02	Female
EPOR	0.683	11.175	11.283	2.52E-03	4.47E-02	Male
SEC22C	-0.627	2.923	11.282	2.52E-03	4.47E-02	Female
PLPP4	0.797	4.614	11.281	2.52E-03	4.47E-02	Male
HHIPL2	1.429	0.982	11.26	2.55E-03	4.49E-02	Male
SEC61A2	-0.664	3.447	11.252	2.55E-03	4.49E-02	Female
QRICH2	0.691	1.625	11.232	2.57E-03	4.51E-02	Male
MPI	-0.629	3.384	11.228	2.57E-03	4.51E-02	Female
ZBTB48	0.599	5.066	11.213	2.59E-03	4.52E-02	Male
EEF1A2	-1.292	0.692	11.218	2.59E-03	4.53E-02	Female
HAUS2	0.619	3.23	11.186	2.61E-03	4.55E-02	Male
AGXT2	-0.917	1.943	11.169	2.63E-03	4.58E-02	Female
FBXL13	-1.599	2.118	11.132	2.67E-03	4.63E-02	Female
MRPL38	0.586	4.313	11.116	2.68E-03	4.63E-02	Male
MIOX	1.244	7.095	11.115	2.68E-03	4.63E-02	Male
OSBPL6	0.85	5.119	11.111	2.69E-03	4.63E-02	Male
SPTB	0.698	9.6	11.101	2.70E-03	4.63E-02	Male

FBXO48	-0.628	2.688	11.1	2.70E-03	4.63E-02	Female
TGFBRAP1	-0.62	3.922	11.1	2.70E-03	4.63E-02	Female
TRPT1	-0.977	5.724	11.093	2.70E-03	4.63E-02	Female
RAB44	0.761	6.741	11.084	2.71E-03	4.64E-02	Male
ZFYVE16	-0.803	6.001	11.069	2.73E-03	4.66E-02	Female
LOC106740288	-0.636	5.317	11.064	2.73E-03	4.66E-02	Female
LOC102575929	0.629	2.489	11.041	2.76E-03	4.70E-02	Male
THADA	0.742	4.581	11.03	2.77E-03	4.71E-02	Male
SMKR1	0.812	2.949	11.029	2.77E-03	4.71E-02	Male
ABI1	-0.589	5.149	10.994	2.81E-03	4.74E-02	Female
WDFY2	-0.718	1.449	10.992	2.81E-03	4.74E-02	Female
GIPC1	0.663	3.489	10.984	2.82E-03	4.74E-02	Male
FAU	-0.667	10.433	10.982	2.82E-03	4.74E-02	Female
THUMPD1	-0.841	3.551	10.969	2.83E-03	4.75E-02	Female
LONP2	-0.79	3.031	10.962	2.84E-03	4.76E-02	Female
AZIN2	1.048	5.096	10.944	2.86E-03	4.77E-02	Male
NR3C1	-0.924	5.785	10.934	2.87E-03	4.78E-02	Female
TUBD1	-0.681	2.617	10.925	2.88E-03	4.79E-02	Female
LOC102571426	1.133	4.031	10.923	2.88E-03	4.79E-02	Male
CALR3	0.717	1.803	10.91	2.90E-03	4.80E-02	Male
ATP5MC1	0.811	4.904	10.887	2.92E-03	4.84E-02	Male
TMEM164	1.02	5.36	10.872	2.94E-03	4.85E-02	Male
RNF227	0.917	3.911	10.819	3.00E-03	4.94E-02	Male
ITFG2	-0.662	6.219	10.806	3.01E-03	4.95E-02	Female
TMEM269	-0.798	4.848	10.786	3.03E-03	4.97E-02	Female
SNCAIP	-0.993	0.524	10.792	3.04E-03	4.97E-02	Female
LOC132243832	-1.066	0.798	10.788	3.04E-03	4.97E-02	Female
LOC132251166	0.99	1.472	10.779	3.05E-03	4.97E-02	Male
DIS3L	-0.718	2.1	10.773	3.05E-03	4.97E-02	Female

Table 6.S2: Sex DEGs shared between blood and gonads

	Gonad					Blood				
Gene	**logFC**	**Log CPM**	**F**	**Pvalue**	**FDR**	**logFC**	**Log CPM**	**F**	**Pvalue**	**FDR**
ABI3BP	-1.294	5.562	74.189	1.26E-08	9.93E-08	2.206	1.245	19.845	1.56E-04	8.41E-03
ACP5	-0.759	3.654	47.6	5.16E-07	2.81E-06	1.398	6.081	22.976	6.44E-05	5.04E-03
ADGRG2	-0.816	3.343	53.347	2.08E-07	1.23E-06	1.332	0.629	12.103	1.88E-03	3.83E-02
AHCY	-1.08	8.675	37.362	3.22E-06	1.47E-05	0.723	4.811	11.924	2.00E-03	3.97E-02
ALOX5	-0.633	2.896	7.03	1.43E-02	2.71E-02	-1.03	3.114	20.194	1.40E-04	8.32E-03
AMIGO1	-1.281	3.779	45.94	6.81E-07	3.61E-06	1.626	0.197	14.921	7.14E-04	2.17E-02
ANKIB1	1.006	6.991	134.44	4.87E-11	6.59E-10	-0.799	2.266	12.159	1.83E-03	3.78E-02
ANKRD9	1.232	3.236	79.282	7.01E-09	5.80E-08	-1.05	2.929	15.48	5.90E-04	1.99E-02
ANO7	-0.899	1.45	14.89	8.09E-04	2.09E-03	2.131	3.659	51.7	1.60E-07	9.64E-05
APOC1	-0.655	4.811	18.111	3.02E-04	8.71E-04	1.368	5.161	12.5	1.62E-03	3.50E-02
AQP1	-1.364	5.725	253.929	7.49E-14	1.98E-12	1.027	7.267	19.907	1.52E-04	8.41E-03
ARRDC1	-0.742	4.646	108.112	4.00E-10	4.36E-09	1.14	3.307	16.65	4.06E-04	1.56E-02
ASPA	-1.794	2.658	40.066	1.93E-06	9.21E-06	-1.693	0.686	15.473	5.95E-04	1.99E-02
ATF5	1.59	7.355	210.187	5.37E-13	1.14E-11	0.836	4.131	11.388	2.43E-03	4.39E-02
BICD1	1.672	6.361	350.63	2.43E-15	9.60E-14	1.107	5.527	31.779	7.36E-06	1.41E-03
CACNG7	1.951	7.26	640.657	3.42E-18	3.32E-16	-0.767	4.326	14.895	7.15E-04	2.17E-02
CALR3	-0.951	2.735	35.532	4.62E-06	2.03E-05	0.717	1.803	10.91	2.90E-03	4.80E-02
CAP2	0.959	4.248	100.454	8.00E-10	8.15E-09	1.44	1.855	16.184	4.71E-04	1.71E-02
CBLB	0.701	5.353	93.988	1.49E-09	1.42E-08	-0.674	3.61	15.254	6.35E-04	2.06E-02
CDKN1C	-0.977	5.672	63.305	5.00E-08	3.43E-07	-0.822	4.939	16.373	4.43E-04	1.64E-02
CEBPB	-1.475	3.189	112.937	2.64E-10	3.02E-09	-1.141	5.693	33.641	4.87E-06	1.09E-03
CGRRF1	0.633	4.947	52.371	2.41E-07	1.42E-06	-0.741	2.978	12.692	1.52E-03	3.36E-02
CLSTN3	1.59	7.479	683.971	1.66E-18	1.86E-16	0.799	3.795	12.729	1.50E-03	3.35E-02

CPEB3	0.595	2.532	9.472	5.36E-03	1.14E-02	-1.246	1.11	16.058	4.91E-04	1.74E-02
CRB3	-1.183	1.882	42.669	1.20E-06	5.99E-06	1.262	3.154	23.221	6.03E-05	4.88E-03
CREG1	1.028	5.37	147.992	1.89E-11	2.82E-10	0.871	8.86	14.56	7.99E-04	2.29E-02
CXXC5	0.888	5.287	197.504	1.02E-12	2.04E-11	1.247	1.429	25.738	3.13E-05	3.50E-03
DENND5B	0.619	5.214	67.188	3.00E-08	2.17E-07	-0.918	4.232	19.087	1.93E-04	9.58E-03
DISP3	-0.642	4.467	33.995	6.30E-06	2.68E-05	-1.691	4.87	22.076	8.23E-05	5.90E-03
DNAH10	-0.739	4.515	35.622	4.53E-06	1.99E-05	0.929	1.512	17.931	2.73E-04	1.20E-02
ECE1	1.117	7.685	117.453	1.81E-10	2.16E-09	-0.982	1.917	15.902	5.15E-04	1.81E-02
ECE2	1.279	3.542	80.577	6.06E-09	5.11E-08	1.559	7.313	37.695	2.07E-06	5.96E-04
EEF1A2	-0.661	8.976	25.427	4.30E-05	1.50E-04	-1.292	0.692	11.218	2.59E-03	4.53E-02
EML6	0.928	3.346	46.748	5.95E-07	3.19E-06	1.347	0.884	14.877	7.21E-04	2.17E-02
EPHA10	-0.594	3.652	17.639	3.47E-04	9.86E-04	-1.054	4.341	14.85	7.26E-04	2.18E-02
FAP	-1.605	2.843	46.093	6.64E-07	3.53E-06	-0.904	3.47	14.915	7.10E-04	2.17E-02
FGF17	3.04	2.031	223.278	2.96E-13	6.85E-12	1.437	1.626	18.621	2.22E-04	1.05E-02
FLVCR2	-1.666	5.066	296.527	1.46E-14	4.72E-13	-1.235	2.533	31.716	7.48E-06	1.41E-03
FNIP1	-0.777	4.25	60.614	7.22E-08	4.75E-07	-0.868	3.779	11.705	2.16E-03	4.14E-02
FOSL2	-1.408	6.066	181.517	2.42E-12	4.46E-11	-0.768	2.989	13.361	1.20E-03	2.94E-02
GABBR1	0.811	4.33	60.674	7.16E-08	4.72E-07	-0.812	1.573	13.206	1.27E-03	3.03E-02
GADD45B	0.62	4.644	18.561	2.65E-04	7.74E-04	1.446	9.141	24.017	4.88E-05	4.39E-03
GALR3	-4.511	3.185	586.847	1.01E-17	8.22E-16	1.5	1.579	32.049	6.95E-06	1.38E-03
GEMIN8	0.684	5.566	95.828	1.24E-09	1.22E-08	-0.798	4.11	24.38	4.44E-05	4.08E-03
GGT5	0.601	8.597	60.805	7.03E-08	4.64E-07	-1.195	3.458	15.469	5.92E-04	1.99E-02
GLCE	-1.324	6.053	160.832	8.21E-12	1.33E-10	-1.06	2.118	15.455	5.95E-04	1.99E-02
GOLM1	-0.901	6.394	166.737	5.71E-12	9.62E-11	-2.019	2.226	32.427	6.39E-06	1.31E-03
GPR182	-1.483	3.533	58.298	1.00E-07	6.38E-07	2.335	5.762	46.605	3.82E-07	1.77E-04
GTF2IRD1	-0.602	4.628	52.124	2.50E-07	1.47E-06	1.522	0.662	28.233	1.70E-05	2.50E-03
HDC	0.719	1.567	8.205	8.81E-03	1.77E-02	-1.028	3.125	11.424	2.39E-03	4.35E-02

HHIPL2	-1.468	4.118	135.952	4.36E-11	5.98E-10	1.429	0.982	11.26	2.55E-03	4.49E-02
HMOX1	-0.808	3.324	50.757	3.10E-07	1.78E-06	1.652	5.807	19.275	1.83E-04	9.26E-03
HYLS1	-0.594	3.013	23.146	7.62E-05	2.52E-04	0.68	2.776	11.956	1.97E-03	3.93E-02
ID3	0.774	8.705	53.434	2.05E-07	1.22E-06	1.552	6.885	26.903	2.33E-05	2.93E-03
IFT57	0.726	5.928	91.073	1.99E-09	1.84E-08	0.658	3.219	15.24	6.38E-04	2.06E-02
IL27RA	-0.811	2.315	30.273	1.39E-05	5.47E-05	0.867	6.981	14.605	7.87E-04	2.29E-02
JPH2	-1.048	2.454	21.652	1.13E-04	3.58E-04	0.983	6.027	21.119	1.07E-04	7.00E-03
KCND2	0.749	0.691	8.521	7.78E-03	1.58E-02	1.157	0.757	11.317	2.49E-03	4.46E-02
KCNF1	-1.673	2.265	128.386	7.68E-11	9.89E-10	-1.044	4.553	21.876	8.70E-05	6.06E-03
KCNJ14	-1.843	2.213	116.031	2.05E-10	2.41E-09	1.18	3.422	30.438	1.00E-05	1.65E-03
LFNG	-0.953	5.695	93.03	1.63E-09	1.55E-08	0.595	6.027	12.417	1.67E-03	3.52E-02
LOC102558474	-1.034	2.139	19.74	1.90E-04	5.75E-04	1.885	0.462	13.888	1.01E-03	2.66E-02
LOC102559184	-2.315	5.318	455.214	1.45E-16	8.53E-15	1.257	5.288	13.797	1.03E-03	2.70E-02
LOC102561902	1.832	7.541	263.055	5.16E-14	1.43E-12	1.098	8.087	16.372	4.43E-04	1.64E-02
LOC102562601	-0.968	1.263	29.436	1.69E-05	6.49E-05	1.1	0.455	16.757	3.94E-04	1.56E-02
LOC102563438	-2.141	2.219	53.69	1.98E-07	1.18E-06	-0.794	4.23	11.782	2.10E-03	4.09E-02
LOC102564219	1.043	5.006	153.982	1.27E-11	1.97E-10	-0.959	3.993	20.744	1.19E-04	7.46E-03
LOC102564916	-0.836	4.224	46.477	6.22E-07	3.32E-06	1.089	5.03	33.262	5.29E-06	1.15E-03
LOC102565156	-0.817	2.719	43.081	1.12E-06	5.60E-06	0.766	6.687	13.576	1.11E-03	2.81E-02
LOC102566452	-1.602	3.14	108.114	4.00E-10	4.36E-09	-1.021	2.72	14.995	6.92E-04	2.16E-02
LOC102566561	-1.051	6.104	146.747	2.05E-11	3.03E-10	0.834	6.716	14.442	8.31E-04	2.34E-02
LOC102568370	-1.239	3.203	35.428	4.71E-06	2.07E-05	-0.718	2.207	13.958	9.79E-04	2.60E-02
LOC102570282	-1.676	5.077	312.88	8.22E-15	2.88E-13	1.118	2.617	25.006	3.77E-05	3.82E-03
LOC102570701	0.614	6.794	26.878	3.03E-05	1.10E-04	0.846	4.386	11.631	2.22E-03	4.18E-02
LOC102571426	-2.653	6.703	670.111	2.08E-18	2.19E-16	1.133	4.031	10.923	2.88E-03	4.79E-02
LOC102571710	-0.626	4.014	28.331	2.16E-05	8.09E-05	1.221	4.646	41.99	8.92E-07	3.26E-04
LOC102572093	-0.598	0.709	6.852	1.55E-02	2.90E-02	1.576	1.872	17.145	3.48E-04	1.41E-02

LOC102574636	-1.785	0.521	47.853	4.94E-07	2.70E-06	-1.242	3.889	18.144	2.56E-04	1.15E-02
LOC102574910	-1.121	1.598	38.4	2.64E-06	1.23E-05	-0.925	0.792	12.363	1.71E-03	3.57E-02
LOC102576504	0.672	7.094	69.958	2.12E-08	1.58E-07	0.938	7.172	21.353	1.01E-04	6.63E-03
LOC106737488	-1.178	1.052	7.05	1.42E-02	2.69E-02	-1.548	0.405	17.579	3.07E-04	1.30E-02
LOC106737548	0.777	4.02	56.141	1.37E-07	8.46E-07	1.944	5.031	36.622	2.59E-06	7.09E-04
LOC106738055	-0.869	0.862	8.35	8.32E-03	1.68E-02	1.157	5.653	15.656	5.57E-04	1.93E-02
LOC106738337	1.703	2.761	117.717	1.78E-10	2.12E-09	1.431	1.246	22.069	8.27E-05	5.90E-03
LOC109281289	-1.101	0.67	14.678	8.69E-04	2.23E-03	1.387	2.972	21.073	1.09E-04	7.06E-03
LOC109281397	1.996	4.705	183.637	2.15E-12	4.01E-11	0.83	6.744	19.192	1.87E-04	9.33E-03
LOC109285037	0.596	0.816	12.599	1.73E-03	4.12E-03	-0.864	1.143	16.06	4.91E-04	1.74E-02
MACF1	0.906	8.519	89.92	2.24E-09	2.06E-08	0.774	8.75	18.595	2.23E-04	1.05E-02
MAP1LC3A	0.97	6.669	166.131	5.93E-12	9.91E-11	-0.891	8.565	20.852	1.16E-04	7.35E-03
ME3	-0.887	5.714	60.817	7.02E-08	4.64E-07	1.06	3.138	14.195	9.03E-04	2.46E-02
MFSD12	-1.993	4.527	287.491	2.02E-14	6.26E-13	0.901	5.099	13.777	1.04E-03	2.70E-02
MIOX	-3.236	1.688	36.396	4.06E-06	1.80E-05	1.244	7.095	11.115	2.68E-03	4.63E-02
MITF	0.864	3.653	55.363	1.53E-07	9.41E-07	-0.711	2.194	12.778	1.47E-03	3.32E-02
MORC3	0.838	6.494	127.473	8.19E-11	1.05E-09	-0.732	3.785	16.774	3.91E-04	1.55E-02
MPP1	-1.029	7.459	49.906	3.55E-07	2.00E-06	-1.206	6.23	41.155	1.05E-06	3.60E-04
MYLIP	0.986	5.132	116.349	1.98E-10	2.35E-09	-1.095	3.369	13.148	1.29E-03	3.06E-02
MYLK3	-0.647	0.672	6.857	1.54E-02	2.89E-02	-2.24	2.749	22.572	7.20E-05	5.36E-03
NEO1	-0.704	6.805	78.554	7.61E-09	6.26E-08	-1.727	1.586	34.274	4.28E-06	9.92E-04
NLK	0.828	4.689	174.568	3.59E-12	6.37E-11	0.737	3.18	17.197	3.42E-04	1.39E-02
NOS1	-1.252	4.206	49.941	3.53E-07	1.99E-06	-1.792	1.44	16.163	4.76E-04	1.71E-02
NR3C1	-0.94	5.112	65.522	3.73E-08	2.64E-07	-0.924	5.785	10.934	2.87E-03	4.78E-02
OSGIN1	-1.811	3.452	82.895	4.70E-09	4.05E-08	1.01	3.925	13.061	1.33E-03	3.10E-02
PARD6A	0.685	3.088	19.001	2.34E-04	6.93E-04	0.76	4.789	18.691	2.17E-04	1.04E-02
PCMTD1	0.884	6.212	155.479	1.15E-11	1.81E-10	-0.82	5.318	14.622	7.83E-04	2.29E-02

PDZK1IP1	-1.432	3.379	17.633	3.48E-04	9.87E-04	1.861	2.844	38.688	1.70E-06	5.25E-04
PIK3CB	-1.457	4.241	191.661	1.39E-12	2.71E-11	-1.597	3.434	27.412	2.06E-05	2.78E-03
PIK3IP1	1.052	7.722	154.028	1.27E-11	1.97E-10	-0.901	8.6	30.46	9.95E-06	1.65E-03
PLPP4	0.652	1.688	9.326	5.67E-03	1.20E-02	0.797	4.614	11.281	2.52E-03	4.47E-02
PLXND1	-1.037	7.005	66.38	3.33E-08	2.39E-07	1.22	0.81	17.519	3.11E-04	1.31E-02
PRKCG	2.584	2.667	177.502	3.09E-12	5.55E-11	-1.061	0.883	12.476	1.64E-03	3.52E-02
PTCH2	2.082	5.726	251.26	8.36E-14	2.19E-12	2.153	1.686	22.004	8.40E-05	5.96E-03
RHOBTB3	1.653	5.18	163.508	6.96E-12	1.14E-10	1.999	1.443	19.495	1.72E-04	8.98E-03
RNF128	-1.034	5.146	56.037	1.39E-07	8.58E-07	-0.896	3.255	13.208	1.27E-03	3.03E-02
RNPEP	0.978	7.166	127.547	8.15E-11	1.04E-09	1.96	7.128	114.36	8.61E-11	5.86E-07
RPL23A	0.638	9.894	36.091	4.13E-06	1.83E-05	-1.276	10.87	30.47	9.93E-06	1.65E-03
RPL28	1.18	9.803	113.542	2.51E-10	2.89E-09	-0.861	10.71	17.998	2.68E-04	1.19E-02
RPS13	0.776	9.928	98.142	9.94E-10	9.92E-09	-0.639	10.53	14.906	7.12E-04	2.17E-02
SERINC3	0.63	8.471	68.636	2.50E-08	1.84E-07	-0.624	8.428	12.036	1.92E-03	3.88E-02
SLC22A31	-0.686	0.462	7.749	1.06E-02	2.08E-02	0.987	3.455	12.574	1.58E-03	3.43E-02
SLC24A5	-0.814	3.297	26.964	2.97E-05	1.08E-04	-1.262	1.193	22.527	7.31E-05	5.41E-03
SLC30A10	-2.862	0.689	90.735	1.98E-09	1.84E-08	0.891	6.194	12.833	1.44E-03	3.28E-02
SLC45A3	0.717	5.123	75.498	1.08E-08	8.60E-08	-1.499	2.521	26.936	2.32E-05	2.93E-03
SLC6A4	-0.794	2.117	12.659	1.69E-03	4.05E-03	-0.979	0.764	12.184	1.82E-03	3.77E-02
SLC7A5	-1.029	5.755	138.619	3.60E-11	5.05E-10	1.086	6.378	14.68	7.68E-04	2.26E-02
SMCO3	-1.31	0.628	23.073	7.85E-05	2.59E-04	1.839	0.378	15.447	6.01E-04	2.00E-02
SMPDL3B	1.21	5.173	104.707	5.41E-10	5.73E-09	1.987	3.588	59.056	5.01E-08	4.67E-05
SMTNL2	-1.926	4.922	321.838	6.08E-15	2.23E-13	1.389	4.705	24.811	3.97E-05	3.89E-03
SNX25	0.791	5.055	109.233	3.62E-10	3.99E-09	-1.276	3.17	18.646	2.20E-04	1.04E-02
SOBP	0.794	5.783	84.596	3.90E-09	3.41E-08	-1.021	0.345	13.448	1.17E-03	2.89E-02
SPR	0.786	5.438	112.826	2.66E-10	3.04E-09	2.027	6.186	48.507	2.74E-07	1.38E-04
SPTB	-1.799	6.433	223.645	2.82E-13	6.57E-12	0.698	9.6	11.101	2.70E-03	4.63E-02

SYT3	-1.277	1.267	42.584	1.23E-06	6.09E-06	1.496	6.043	36.262	2.79E-06	7.47E-04
TBC1D15	0.956	6.023	294.515	1.56E-14	4.98E-13	-0.868	6.556	13.514	1.14E-03	2.85E-02
TFRC	-1.538	6.731	382.029	9.65E-16	4.27E-14	0.704	10.20	13.102	1.31E-03	3.08E-02
TMEM158	-1.078	5.659	101.056	7.56E-10	7.74E-09	-1.253	1.102	11.51	2.33E-03	4.29E-02
TMEM164	-0.603	5.116	68.917	2.41E-08	1.78E-07	1.02	5.36	10.872	2.94E-03	4.85E-02
TMEM217	-0.86	1.599	8.522	7.77E-03	1.58E-02	1.244	0.961	24.485	4.33E-05	4.05E-03
TP53BP2	-0.806	5.973	242.826	1.20E-13	3.05E-12	1.438	1.854	14.921	7.10E-04	2.17E-02
TPRG1L	0.647	6.591	75.307	1.11E-08	8.77E-08	-0.832	5.528	18.591	2.24E-04	1.05E-02
TSHZ1	0.582	5.637	29.642	1.60E-05	6.20E-05	-1.35	1.265	18.286	2.47E-04	1.13E-02
UBXN2A	-0.58	4.606	49.37	3.87E-07	2.16E-06	-1.015	3.961	21.928	8.57E-05	6.05E-03
XKR8	2.056	3.593	362.853	1.68E-15	7.02E-14	0.769	3.167	13.331	1.21E-03	2.96E-02

DISCUSSION AND CONCLUSIONS

Developmental plasticity has critical implications for ecology, evolution and conservation (Donelson et al., 2023; Fox et al., 2019; Ghalambor et al., 2007; Miner et al., 2005; Moczek et al., 2011; West-Eberhard, 2003). However, despite its widespread consequences, our knowledge of how responses to the developmental environment operate under the complexities of nature remains limited. In this dissertation, I integrated developmental plasticity into eco-evolutionary frameworks utilizing the American alligator as a model system. I demonstrated novel pathways through which environmental contaminants impact reproductive health in natural populations, including a non-trivial role of maternally derived hormones. I further identified both neutral and selective evolutionary processes shaping population divergence in the molecular pathways underlying TSD, highlighting several candidate genes for its adaptive evolution. Through additional exploration of a hypothesis for the adaptive value of TSD, I revealed persistent effects of incubation temperature on post-release phenotypes that contribute to survival outcomes and the evolution of sex determining systems. Finally, I demonstrated the use of gene expression patterns in blood to sex hatching alligators, which will facilitate understanding TSD-associated sex ratio variation in ecological and conservation contexts. Collectively, these studies contribute novel insights into the ecological, evolutionary, and conservation implications of developmental plasticity in natural systems.

Results from each of my chapters open exciting avenues for future research. In Chapter 2, my finding that elevated maternally deposited estradiol may contribute to altered reproductive development at AP raises intriguing questions about the causes and consequences of maternal hormone deposition. Namely, what are mechanisms responsible for increased deposition of estradiol at AP? And how do increases in estradiol availability during different stages of development affect later life reproductive phenotypes? Future experimental work will be instrumental for addressing these questions. In Chapters 3 and 4, I identified population-level morphological divergence linked to incubation temperature, as well as candidate genes for the adaptive evolution of TSD. However, I did not explicitly examine how these findings relate to variation in TSD reaction norms across populations. Very few studies have robustly examined sex ratio responses to incubation temperature across a species' range (Carter et al., 2019; Ewert et al., 2005; González et al., 2019). Assessing this variation in our four populations and linking it to the gene expression and genic sequence outliers we identified has potential to provide significant insight into TSD from both mechanistic and evolutionary perspectives. Results from Chapter 5 highlighted developmental cost is an important driver of temperature-dependent survival associated with the adaptive evolution of TSD in alligators. Yet, they also implicated additional effects of incubation temperature independent of the phenotypes measured. Relatively little is known about how incubation temperature influences behavioral phenotypes in reptiles or how these behaviors may contribute to survival outcomes (Burger, 1991; Flores et al., 1994; Johnson et al., 2023). Follow-up experiments testing how incubation temperature impacts hatchling behaviors under differential ecological conditions will be especially informative for further linking incubation temperature to sex-specific fitness and, in turn, the evolution of TSD. Finally, Chapter 6 provided a necessary proof of concept and first step for non-lethally sexing hatchling alligators using blood gene expression. However, to implement this method in ecological settings, further work developing targeted qPCR approaches, ideally across multiple species, is necessary. Such a tool would provide an unprecedented ability to link natural nest temperatures to sex ratios and population dynamics, with implications for basic biology and applied conservation. Although these future research directions may be specific to my study system, I believe they are likely to have broader implications for developmental plasticity, TSD, and crocodilian conservation.

The research presented in this dissertation is not without its limitations. First, all incubation experiments utilized constant temperatures known to produce males or females. While constant temperatures are insightful for isolating incubation temperature's influence, they are not representative of natural nests, which experience fluctuations on diel, weekly, and monthly time scales (Bock, Lowers, et al., 2020; Bowden et al., 2014). Although there is a growing interest in the role thermal fluctuations play in developmental plasticity of reptiles (Les et al., 2007; Raynal et al., 2022), our understanding of their consequences relative to laboratory settings remains limited. Second, the alligator provides an insightful system to understand developmental plasticity in eco-evolutionary contexts but its longevity and reproductive ecology present challenges for experimental manipulation and causative inference. This is particularly relevant for Chapters 2, 4, and 5, where our interpretations rely on expected, but not verified, causative relationships. Establishing causation in long-lived species is notoriously difficult, but likely to be partially alleviated by continually advancing genomic tools and long-term datasets (e.g., Ge et al., 2017, 2018; Wilkinson et al., 2016). Despite these limitations, however, I believe the trade-offs made in each chapter represent an acceptable balance between ecological relevance, feasibility, and interpretability.

By taking an integrative approach bridging the diverse fields of developmental and molecular biology, bioinformatics, ecology, and evolution across biological levels of organization, this dissertation contributes unique mechanistic and conceptual insights into how embryos interact with their environment. Previous research on developmental plasticity has made significant contributions to the ecological, evolutionary and biomedical sciences. It is my hope that in some small way, my dissertation facilitates a deeper understanding of such organism-environment interactions as they occur in nature and perhaps fostering a few new and creative ways to think about biology.

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APPENDIX A1

SEX DETERMINATION AND OVARIAN DEVELOPMENT IN REPTILES AND AMPHIBIANS: FROM GENETIC PATHWAYS TO ENVIRONMENTAL INFLUENCES[6]

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[6] Smaga, C. R., Bock, S. L., Johnson, J. M., & Parrott, B. B. (2022). *Sexual Development, 1–21*. https://doi.org/10.1159/000526009. Reprinted here with permission of publisher.

Abstract

Background: reptiles and amphibians provide untapped potential for discovering how a diversity of genetic pathways and environmental conditions are incorporated into developmental processes that can lead to similar functional outcomes. These groups display a multitude of reproductive strategies, and whereas many attributes are conserved within groups and even across vertebrates, several aspects of sexual development show considerable variation.

Summary: in this review, we focus our attention on the development of the reptilian and amphibian ovary. First, we review and describe the events leading to ovarian development, including sex determination and ovarian maturation, through a comparative lens. We then describe how these events are influenced by environmental factors, focusing on temperature and exposure to anthropogenic chemicals. Lastly, we identify critical knowledge gaps and future research directions that will be crucial to moving forward in our understanding of ovarian development and the influences of the environment in reptiles and amphibians.

Key messages: reptiles and amphibians provide excellent models for understanding the diversity of sex determination strategies and reproductive development. However, a greater understanding of the basic biology of these systems is necessary for deciphering the adaptive and potentially disruptive implications of embryo-by-environment interactions in a rapidly changing world.

Introduction

The environment experienced by an organism during development can have profound organizational and ecological effects, some of which persist throughout life (West-Eberhard et al., 1989; Miner et al., 2005). This ability to alter phenotype in response to environmental cues (i.e., phenotypic plasticity) can be adaptive in several contexts, but also leaves organisms sensitive and vulnerable to changing or altered environmental conditions (DeWitt et al., 1998). Reptiles and amphibians display a broad range of reproductive strategies, spanning oviparous to viviparous, lecithtrophic to matrotrophic, and environmental to genotypic sex determination (Thompson and Speake, 2003; Blackburn, 2015; Bachtrong, 2014). Relative to other vertebrate groups, this diversity is unparalleled and provides fertile ground for comparative developmental studies. Yet, the potential for understanding how environmental cues are incorporated into diverse developmental programs in adaptive and disruptive contexts remains relatively untapped in these groups. Arguably, one of most important developmental processes for adult reproductive fitness is the proper organization of the germ-cell containing gonad. The decision to develop a testis or ovary requires precise coordination of genetic and endocrine signaling pathways in response to intrinsic and extrinsic cues (Capel, 2017), and sexual development in reptiles and amphibians provides unique opportunities for disentangling how divergent pathways arising from conserved genes can lead to similar functional outcomes under varied environments. However, understanding this diversity from both proximate and ultimate perspectives requires extensive knowledge of the basic biological events that occur within an organism in an adaptive context, which is lagging in these groups, especially relative to mammals.

Basic comparative development

Mechanisms of sex determination

Ovaries and testes emerge during development from common bipotential primordia. A remarkable diversity of mechanisms exists to initially steer the bipotential gonad toward the ovarian versus testicular fate, in a process known as primary sex determination. Reptiles and amphibians uniquely exemplify this diversity with every major sex-determining mechanism (SDM) represented in these groups, including female heterogametic (ZW) sex chromosomes (e.g., majority of snakes (Matsubara et al., 2006), African clawed frog (Yoshimoto et al., 2010)), male heterogametic (XY) sex chromosomes (e.g., some lizards (Gamble et al., 2014, 2015), boas and pythons (Gamble et al., 2017)), polygenic sex determination (e.g., some amphibians (Miura, 2018; Nakamura, 2009; Ruiz-Garciá et al., 2021)), and environmental sex determination (e.g., some squamates (Charnier, 1966; Holleley et al., 2015), many turtles (Bull, 1980), all crocodilians (Lang and Andrews, 1994), and tuatara (Mitchell et al., 2006)) (reviewed extensively elsewhere; for example, see (Bachtrog et al., 2014; Capel, 2017; Valenzuela and Lance, 2004)). The distribution of different SDMs across the phylogeny of reptiles and amphibians suggests that evolutionary transitions between SDMs occur frequently and sometimes rapidly (Bachtrog et al., 2014; Janzen and Phillips, 2006; Jeffries et al., 2018; Pokorná and Kratochvíl, 2009). For example, a study of the SDMs of 12 gecko species found evidence for 17 to 25 transitions between XX/XY, ZZ/ZW, and TSD systems (Gamble et al., 2015). Transitions between sex determination systems also occur within species, as is shown in the frog species *Rana rugosa* which exhibits different SDMs (XX/XY and ZZ/ZW) across different populations in Japan (Miura, 2008). Thus, this group provides opportunities to gain novel insights into variation in the transcriptional networks and cellular processes underlying early ovarian development as well as the evolutionary forces shaping this variation.

The lens through which mechanisms of sex determination in reptiles and amphibians are examined has largely been shaped by studies of the mammalian XY and avian ZW systems. Both of these SDMs are evolutionarily stable, highly canalized, and under the control of a single 'master' sex-determining locus (*e.g., Sry* in mammals and *DMRT1* in birds (Capel, 2017; Graves, 2016; Koopman et al., 1991; Sinclair et al., 1990; Smith et al., 2009). This contributed to the idea that vertebrate sex determination broadly invokes the same transcriptional hierarchy, and variation in SDMs across species is limited to the initial upstream 'switch' operating at the top of this hierarchy (Crews and Bull, 2009; Mclaren, 1988; Wilkins, 1995). However, subsequent work in reptiles and amphibians is revealing a much more complex picture (Czerwinski et al., 2016; Deveson et al., 2017; Shoemaker and Crews, 2009; Yatsu et al., 2016). In fact, sex determination often involves nuanced interactions between multiple loci and environmental signals in these taxa (Bachtrog et al., 2014; Barske and Capel, 2008; Deveson et al., 2017; Holleley et al., 2015; Lambert et al., 2018, 2019; Pen et al., 2010). Variation exists at multiple points in the transcriptional networks underlying sexual fate commitment and gonadal differentiation, and the key unifying property of these networks is not their hierarchical nature, but rather a robust mutual antagonism (i.e., genes promoting ovarian fate simultaneously inhibit those promoting testis fate, and vice versa) which permits network flexibility (Adolfi et al., 2021; Capel, 2017; Crews and Bull, 2009; Herpin and Schartl, 2015).

Despite the profound differences in SDMs between mammals, birds, reptiles, and amphibians, many of the same genes or gene families have been recruited to serve in the transcriptional networks underlying sex determination across taxa, albeit in varying configurations. Doublesex and mab-3 (DM) domain genes, named for their association with sex determination in *Drosophila melanogaster* and *Caenorhabditis elegans* and characterized by a zinc-finger DNA binding motif, are perhaps the best examples of this phenomenon (Matson and Zarkower, 2012). A paralogue of the DM-domain gene *DMRT1*, which determines sex in birds through a Z-linked dosage-sensitive mechanism (Smith et al., 2009), resides on the W-chromosome in the African clawed frog (*Xenopus laevis*) and serves as the decisive regulatory signal in this species directing gonadal development toward the ovarian fate (Yoshimoto et al., 2008). *X. laevis* also possesses an autosomal copy of *DMRT1*, which directs testis development in the absence of the W-linked copy (DM-W). The DM-W locus encodes a truncated protein lacking key functional domains and is thought to antagonize the masculinizing function of the autosomal copy of *DMRT1* through an underlying dominant negative mechanism (Matson and Zarkower, 2012; Okada et al., 2009). In the red-eared slider turtle (*Trachemys scripta*), a species relying solely on temperature to determine gonadal fate, *DMRT1* expression responds to incubation temperature early in the thermosensitive period and is necessary for testis development. Interruption of *DMRT1* expression in *T. scripta* embryos at male-promoting temperatures results in the bipotential gonad proceeding towards the ovarian fate (Ge et al., 2017). Even among mammals, including humans and mice, *DMRT1* serves a critical role in promoting testis differentiation and maintaining testicular fate into adulthood through its antagonism of the ovarian-specific transcription factor, *FOXL2* (Capel, 2017; Krentz et al., 2009; Matson et al., 2011; Raymond et al., 2000).

Many of the genes that show evidence of conserved upstream roles in sex determination across vertebrates are involved in promoting testis fate (e.g., DM-domain genes, Sox genes, *AMH*), and in their absence ovarian development proceeds (Herpin and Schartl, 2015). There are currently no well-substantiated explanations for this pattern, but what is clear is that development of an ovary requires robust repression of the transcriptional networks promoting testis fate, and vice versa. The interaction of DM-domain genes and Sox genes with the transcription factor forkhead box L2 (*FOXL2*) and Wnt signaling (particularly the WNT4/R-spondin 1/ß-catenin pathway) typifies this mutual antagonism. During mammalian sex determination, *WNT4* and *RSPO1* (R-spondin 1) are expressed in the somatic progenitors of the gonad (which will eventually take on the fate of either granulosa cells or Sertoli cells) and trigger stabilization and translocation of cytoplasmic ß-catenin into the nucleus (Rotgers et al., 2018). Following this initiation of the WNT4/R-spondin 1/ß-catenin pathway, expression of *FOXL2* is upregulated leading somatic progenitors to take on the fate of granulosa cells and ultimately promoting ovarian differentiation (Rotgers et al., 2018). Interruption of *Wnt4, Rspo1,* or *Ctnnb1* (ß-catenin) function in mice leads to upregulation of *Sox9* and partial ovarian to testicular fate reversal (Chassot et al., 2008; Jeays-Ward et al., 2003; Rotgers et al., 2018; Vainio et al., 1999). Further, deletion of *Foxl2* in adult mouse ovaries leads to upregulation of *Dmrt1* and *Sox9* and transdifferentiation of the ovary to testicular morphology, implicating *Foxl2* in the active maintenance of ovarian fate into adulthood (similar to the previously discussed role of *Dmrt1* in testis maintenance)(Uhlenhaut et al., 2009).

The role of *FOXL2* and the WNT4/R-spondin 1/ß-catenin pathway in promoting ovarian development and antagonizing the masculinizing actions of *DMRT1* and *SOX9* appears to be at least partially conserved among reptiles and amphibians. In several reptiles with TSD including *T. scripta* and *C. serpentina* (Rhen et al., 2007, 2021), *RSPO1* and *FOXL2* exhibit upregulation at female-promoting temperatures during the thermosensitive period. Further, in *T. scripta*, ectopic activation of canonical Wnt signaling at male-promoting temperatures results in partial sex-reversal of the gonadal medulla, however inhibition of Wnt signaling does not sex-reverse differentiating ovaries suggesting this signaling pathway is not necessary for ovarian development in this species (Mork and Capel, 2013). In the American alligator (*Alligator mississippiensis*), another species with TSD, *RSPO1* does not exhibit differential expression during the thermosensitive period, though both *WNT4* and *FOXL2* are upregulated at female-promoting temperatures (Rice et al., 2017; Yatsu et al., 2016). In six anuran species (*X. laevis, Bombina bombina*, *Bufo viridis*, *Hyla arborea*, *Rana arvalis*, and *Rana temporaria*) with genetic sex determination (GSD), *RSPO1* expression is upregulated in females during sex determination (Piprek et al., 2013). In the common Indian garden lizard (*Calotes versicolor*), a species with GSD but for which the mechanism is poorly understood, both *FOXL2* and *RSPO1* are expressed in the developing ovary and proteins appear to be localized in pre-granulosa cells (Priyanka et al., 2018). The transcriptional networks underlying sex determination in reptiles and amphibians appear to rely on genes from conserved gene families with known roles in vertebrate sex determination, particularly through the antagonistic actions of testis-promoting DM-domain genes/Sox genes and ovary-promoting FOXL2 and the WNT4/R-spondin 1/ß-catenin pathway. It should be noted, however, that our view remains limited. For example, in *X. laevis*, *DMRT1* contains two promotors, one of which controls germ cell numbers in both sexes, and when knocked down, leads to female-to-male sex-reversal in a subset of frogs (Mawaribuchi et al., 2017).There is also substantial evidence that other genes with conserved roles in sex determination do not necessarily follow the same temporal trajectory during development in these taxa as they do in the well-characterized systems of model organisms (i.e., SOX9 expression in mammals versus reptiles with TSD)(Western et al., 1999). This suggests that as we increasingly move away from candidate-gene approaches and instead apply more unbiased sequencing approaches to examine the reproductive development of reptile and amphibian species, we are likely to uncover a more varied and nuanced perspective on the diversity of vertebrate sex determination.

Dual functions of transcriptional pathways in sex determination and gonadal differentiation

The connections between variation in transcriptional networks underlying sex determination and variation in processes governing the morphological differentiation of the ovary and testis across species are far from clear, especially in reptiles and amphibians. One complication that clouds understanding of these connections is the fact that many of the same genes that have been co-opted to serve as regulators of sex determination have dual functions in the morphological differentiation and/or gonadal function of both sexes. Further, the roles of these genes in morphological differentiation are not necessarily dependent upon their roles in sex determination. For example, *SOX9* does not appear to play an upstream role in sex determination in *Xenopus tropicalis*, as it does in mammals, but *SOX9* does appear to serve important roles in gonadal function. In this species, *SOX9* is upregulated after gonadal differentiation in both sexes, however in the testis, *SOX9* is restricted to the nuclei of Sertoli cells, while in the ovary, *SOX9* is first localized in the nuclei of previtellogenic oocytes and then later is localized to vitellogenic oocytes (el Jamil et al., 2008; Vining et al., 2021). Further, closely related species with very little variation in adult ovarian and testicular morphology/function can rely on vastly different systems of sex determination (e.g., clades of turtles with both TSD and GSD species (Valenzuela and Lance, 2004)). Few studies in reptiles and amphibians have simultaneously characterized the time-course of transcriptional and cellular events during sex determination (Yao and Capel, 2005), yet research of this nature is likely to yield critical insights into ways in which transcriptional variation during sex determination may relate to variation in cellular processes underlying gonadogenesis and differentiation. For example, the somatic precursors to granulosa or Sertoli cells in the bipotential gonad are largely considered to be the site of primary sex determination in many vertebrates, yet the role of germ cells in this process has been relatively neglected, though germ cell-specific genes have been implicated in contributing to sex determination in some species, such as the TSD species, *Mauremys mutica* (Liu et al., 2021). How might reptiles and amphibians differ in the site of primary sex determination within the bipotential gonad, and how might this influence the behavior of different cell populations during gonadogenesis? Are there patterns of cellular organization or gonadal structure that link species relying on similar SDMs? These and many other related questions await further inquiry.

The role of estrogen signaling in the developing ovary

Development of the ovary in reptiles and amphibians largely appears to converge on the production of estrogen, which generally serves as a conserved readout for commitment to the ovarian fate. One of the earliest pieces of evidence for this came from the demonstration of reptile and amphibian embryos' sensitivity to the influence of exogenous estrogen exposure (Bull et al., 1988; Hayes, 1998). Reptiles with TSD provide some of the clearest examples of this sensitivity. Early experiments in *T. scripta*, *A. mississippiensis*, *C. serpentina, E. macularius,* and *Trionyx spiniferus* revealed that embryos incubated at male-promoting temperatures and exposed to exogenous 17ß-estradiol (E2) during the thermosensitive period developed ovaries (Bull et al., 1988; Crews et al., 1989; Kohno et al., 2015; Rhen and Lang, 1994; Wibbels et al., 1991). These findings were followed by the discovery that expression of the gene *CYP19A1* which encodes aromatase, the enzyme that converts testosterone to estrogen, is upregulated at female-promoting temperatures in these species, though the timing of this upregulation varies. In *C. serpentina*, *CYP19A1* exhibits delayed upregulation in response to female-promoting temperatures suggesting the influence of estrogen may be limited to ovarian differentiation rather than initial sex determination (Rhen et al., 2007; Rhen and Schroeder, 2010). Similarly, *CYP19A1* expression and aromatase activity is not upregulated until late in the thermosensitive period (Stage 23-24) in *A. mississippiensis* (Milnes et al., 2002; Parrott et al., 2014). In *T. scripta*, *CYP19A1* expression is upregulated in the middle of the thermosensitive period (stage 18), though in some studies its expression appears to precede that of *FOXL2* (Bieser and Wibbels, 2014; Czerwinski et al., 2016; Ramsey et al., 2007; Shoemaker et al., 2007). Given this evidence, it remains unclear whether aromatase and, in turn estrogen production, plays an upstream role in sex determination in some reptile and amphibian species initiating the ovarian transcriptional network, or if it is primarily involved in canalizing ovarian fate and differentiation.

Estrogen is clearly a powerful suppressor of the testicular transcriptional network (Barske and Capel, 2010), yet *CYP19A1* expression tends to lag temporally in its upregulation at female-promoting temperatures relative to the upregulation of testis-promoting genes such as *DMRT1* at male-promoting temperatures during TSD (Czerwinski et al., 2016; Ge et al., 2017). Further, in *T. scripta* if *DMRT1* expression is interrupted at male-promoting temperatures, *CYP19A1* expression increases and ovarian development proceeds (Ge et al., 2017, 2018), yet it remains unclear what upregulates *CYP19A1* expression in the absence of a female-promoting temperature cue. Clearly, there is much more to be learned about the role of estrogen in regulating sex determination and ovarian differentiation in reptiles and amphibians. As it relates to the structure and function of the ovary, sex determination systems in reptiles and amphibians seem to be divergent means to a similar end, though subtle species differences in ovarian development and cellular architecture undoubtedly harbor interesting comparative insights.

Differentiation and maturation of the ovary

After primary sex is determined, many of the morphological aspects of reptilian and amphibian ovarian development are broadly conserved across groups. Similar to mammals, the ovary differentiates into two parts: a cortex and medulla. The cortex contains actively proliferating germ cells that become organized into nests that are connected by intercellular bridges, while the medulla contains dense connective tissue that regresses during maturation. Nests of germ cells proliferate in the cortex until they enter meiosis, becoming oocytes. Oocytes in the diplotene stage become surrounded by somatic (pre-granulosa) cells, break from nests, and become fully enveloped by granulosa cells, followed by the theca layer. The resulting follicles continue to enlarge and mature, bulging into the medulla and becoming vacuolated. Vitellogenesis proceeds as the follicles fill with yolk platelets prior to ovulation and await fertilization. Several papers have described these events in detail for specific species in each taxa (Crocodilia: Moore et al., 2008, 2010; Uribe and Guillette, 2000; Calderon et al., 2004; Testudines: Pérez-Bermúdez et al., 2012; Nainan et al., 2009; Callebaut et al., 1997; Squamata: Aldokhi et al., 2019; Delssin et al., 2019; Hernández-Franyutti et al., 2005; Doddamani, 1994; Vieira et al., 2010; Anura: Ogielska and Kotusz, 2004; Piprek et al., 2017; Dumont, 1972; Caudata: Mendoza-Cruz et al., 2017; Chardard et al., 2003; Chardard and Dournon, 1999). For the purposes of this review, the remainder of this section will focus primarily on the major differences that exist across groups and important gaps that must be addressed to gain a comprehensive understanding of ovarian development in reptiles and amphibians.

Histological differences in both the cortex and medulla are evident when looking comparatively across groups. For example, unlike reptiles in which the entire ovary is a single unit, anuran and caudate ovaries contain several sacs, each of which consists of a cortex and medulla (Ogielska and Kotusz, 2004, Uribe, 2003). While the presence of germinal nests in the cortex is ubiquitous across groups, the number of nests varies substantially. Crocodilians, turtles, and frogs generally have several (Callebaut et al., 1997; Uribe and Guillette, 2000; Ogielska and Kotusz, 2004; Moore et al., 2008; Pérez-Bermúdez et al., 2012), while most squamates have one or two, but as many as six (Jones et al., 1982; Aldokhi et al., 2019). It has been hypothesized that germinal nest number is related to clutch size and frequency of reproduction (Guraya, 1989). This was examined across several lizard species, in which Radder et al. (2008) found a significant relationship between germinal bed number and clutch size, but not reproductive mode, brood frequency, or number of clutches per year. Whether variation exists in other groups is unknown, but additional comparative studies have the potential to further link aspects of ovarian development (e.g., germinal nest number) to variation in reproductive ecology and life-history.

The ovarian medulla in crocodilians and turtles develops a system of large lacunae that continually increase in size during ovarian maturation (Callebaut et al., 1997; Uribe and Guillette, 2000; Moore et al., 2009; Pérez-Bermúdez et al., 2012). Anurans and caudates also develop a similar central cavity within each ovarian sac, termed a lumen (Ogielska and Kotusz, 2004, Uribe, 2003), while squamates lack such a structure (Aldokhi et al., 2019; Delssin et al., 2019). Ovarian lacunae are also evident in birds, although their size and number vary both within and across taxonomic groups (Nainan et al., 2010; Pérez-Bermúdez et al., 2012; Uribe and Guillette, 2000). Their function is unknown, but proposed hypotheses include allowing space for follicular growth, providing a supportive structure for follicles, and nourishing growing oocytes (Pérez-Bermúdez et al., 2012). More work is needed to understand the consequences and function of ovarian lacunae and why these structures are present in some but not all groups.

Other intriguing, histological differences also exist during later stage follicular maturation. For example, in crocodilians (Uribe and Guillette, 2000; Moore et al., 2008), testudines (Callebaut et al., 1997; Nainan et al., 2010; Pérez-Bermúdez et al., 2012), anurans (Dumont, 1972) and caudates (Uribe, 2009) a single or few layers of homogenous granulosa cells surround the oocyte and remain relatively unchanged throughout, whereas lizards (Delssin et al., 2019; Aldokhi et al., 2019) and snakes (Tumkiratiwong et al., 2012) begin with a single layer of cells termed small cells that develop into pyriform cells, with an intermediate cell stage, giving the appearance of three layers that eventually regress back to a single layer. In squamates, intercellular bridges between small cells and the growing oocyte have been observed that are believed to play a nutritive role in nourishing the oocyte (Andreuiccetti, 1992). Such bridges have not been observed in other groups, but abundant microvilli reaching from follicular cells to the oocyte suggest material transfer also occurs in turtles (Nainan et al., 2010; Pérez-Bermúdez et al., 2012), anurans (Konduktorovaa and Luchinskayaa, 2013; Dumont, 1972), and caudates (Uribe, 2003).

At the onset of vitellogenesis, yolk deposition occurs within the oocyte and the animal and vegetal poles become distinguishable. Details have been described in crocodilians (Calderón et al., 2004; Uribe and Guillette, 2000), turtles (Callebaut et al., 1997; Nainan et al., 2010), anurans (Dumont, 1972), and caudates (Uribe, 2003). Generally, vacuoles appear at the periphery of the oocyte and yolk spheres accumulate, mostly within the vacuoles. As yolk deposition continues, yolk platelets form and distribute non-randomly within the oocyte, appearing smaller at the periphery and center and larger in the intermediary region. In crocodilians (Calderón et al., 2004; Uribe and Guillette, 2000) and turtles (Nainan et al., 2010), yolk deposition has been compared to birds, in which it occurs in three successive stages (primordial, intermediate, and late yolk). However, it appears that differences exist even within groups and no such comparisons have been made in amphibians or squamates. Unsurprisingly, in matrotrophic-like squamates harboring varying degrees of placental development, yolk deposition is reduced, with fewer yolk droplets (Hernández-Franyutti et al., 2005; Vieira et al., 2010).

A defining feature of the ovary in vertebrates is the early transition of germ cells from a mitotic to meiotic state as opposed to at sexual maturation in testes. Interestingly, the details of this transition in reptiles and amphibians remain largely unknown. The timing of oogonia to oocyte transition appears to be species specific in squamates, where it can be initiated either during embryonic development (Doddamani, 1994) or early in juvenile life (Antonio-Rubio et al., 2015; Delssin et al., 2019). On the other hand, turtles and crocodilians show early-stage oocytes at birth (Moore et al., 2008; Rhen et al., 2015), suggesting it begins during late embryonic stages. In anurans, the oogonia to oocyte transition has been observed during hindfoot development (Gosner stages 36-40) and during late larval development (Ogielska and Kotusz, 2004; Wallacides et al., 2009). However, as this process has only been studied in a few species, drawing overarching conclusions with respect to taxonomic diversity is difficult. Further, the proximate mechanisms underlying the mitotic to meiotic transition in reptiles and amphibians are largely unknown. The current consensus in mammals is that retinoic acid signaling from somatic cells upregulates *STRA8* expression, leading to meiotic entry (Bowles et al., 2006; Wang et al., 2017). This idea was recently challenged by Vernet et al. (2020) who showed that *STRA8* expression is reduced in mice lacking retinoic acid receptors, but meiosis progresses normally, suggesting other factors also play a role. Nonetheless, the role of retinoic acid is conserved in chicken and fish (Feng et al., 2015; Li et al., 2016; Smith et al., 2008), with both *STRA8* dependent and independent pathways existing in the latter (Feng et al., 2015). As for reptiles and amphibians, the role of retinoic acid has only been examined in the salamander *Pleurodeles waltl* in which exogenous retinoic acid triggers meiotic transition (Wallacides et al., 2009). While this indicates that retinoic acid performs a conserved role in the mitotic to meiotic transition across vertebrates, a time-series comparison examining retinoic acid, *STRA8* expression and germ cell behavior during embryonic and early post-natal life across several species would be an intriguing new direction of inquiry into this critical aspect of ovarian development.

Unlike mammals, in which all oogonia enter meiosis early in life, in most reptiles and amphibians, the adult ovary retains nests of mitotic oogonia which can serve as an active supply of new oocytes. This includes crocodilians, turtles, squamates, and caudates (Callebaut et al., 1997; Uribe and Guillette, 2000; Uribe, 2009; Nainan et al., 2010; Aldoki et al., 2019). This unique feature opens interesting questions about reproductive senescence in these groups, since it appears the oocyte pool has the potential to evade depletion. How new oogonia are selected to enter meiosis throughout life is unknown, as studies specifically examining this aspect of germ cells in adult animals are nonexistent. Interestingly, anurans are the exception and, like mammals, form a definitive pool of diplotene oocytes during the juvenile stages, a subset of which are recruited each breeding cycle (Ogielska et al., 2013, Callen et al., 1986). Primary oogonia become restricted to germ patches after the juvenile period and no longer contribute to the pool of oocytes, degenerating thereafter (Ogielska et al., 2013). Once again, due to the small number of species examined, it is difficult to say if this is a defining feature of anurans or if variation exists within groups, which would raise interesting evolutionary questions about the costs and benefits of a regenerating oocyte pool.

Another important aspect of ovarian maturation, the recruitment of primary follicles from the primordial follicle pool of oocytes, is an area of active research in mammals and also almost completely unknown in reptiles and amphibians. After entering meiosis, oocytes break from nests and enter a quiescent state until they are recruited as primary follicles. This involves complex interactions between many factors, including several members of the TGF-β superfamily, such as *AMH*, inhibins, activins, BMPs, and GDFs. Excellent reviews have been published on this process in mammals (Findlay et al., 2002; Skinner et al., 2005; Trombly et al., 2009; Wang et al., 2017), and it appears a multitude of coordinated interactions are required for proper nest breakdown and follicle recruitment. Very few data exist in reptiles and none in amphibians. In *A. mississippiensis*, activin, follistatin, and aromatase follow similar expression profiles in ovaries during the first five months of life, starting with elevated expression during early post-natal life as germ cells leave nests and form primary follicles. This is followed by a decrease in expression coinciding with later stage follicle formation (Moore et al. 2008, 2010a). In contrast, *GDF9* and *PCNA* expression increases during this time and correlates with follicle maturation and somatic cell proliferation (Moore et al., 2010a). While these patterns are largely consistent with what is observed in mammals, in which proper ratios of activin and estrogen are required for normal follicle formation (Trombly et al., 2009), only a few of the key signaling components have been examined in a single species and our understanding of the molecular mechanisms driving such processes are far from resolved. Once primary follicles are formed, in several reptile species, administration of mammalian FSH leads to increased growth and maturation in sexually immature animals (e.g., Jones et al., 1975; Moore et al., 2012a; Hale et al., 2019), again suggesting conserved mechanisms of late-stage follicle maturation. Early work showed that the granulosa layer is likely responsible for ovarian responses to gonadotropins, as isolated granulosa, but not thecal cells, produced progesterone under FSH stimulation (Crews and Licht, 1975). However, the technique used to separate cells in the latter study prevented complete separation of theca and granulosa cell layers. Future work should focus on linking molecular mechanisms to the histological events characterizing reptilian and amphibian folliculogenesis, including the role of different cell types in this process.

Environmental influences on the development of the ovary

Temperature and climate influence sex determination and ovarian development

In the context of ongoing shifts in global thermal regimes, an understanding of the intricate connections between temperature, ovarian development, and reproductive performance in reptiles and amphibians will provide critical information towards efforts to predict and potentially mitigate adverse population-level consequences of rapid environmental change (Benard, 2015; Bock et al., 2020a; Janzen, 1994; Jensen et al., 2018). In addition, interactions between thermal cues and reproductive development in reptiles and amphibians provide models in which to investigate how environmental variation is integrated into the biology of the ovary in adaptive contexts. Temperature can influence the ovarian function of reptiles and amphibians across the entire ontogeny of an organism – from the initial decision to develop an ovary during sex determination in embryos (Bull, 1980; Capel, 2017) to the initiation of folliculogenesis and oogenesis during the breeding season in adults (James and Shine, 1985; Lance, 1989; Licht, 1973; Marion, 1970; Pancharatna and Patil, 1997; Sarkar et al., 1996). The molecular mechanisms by which thermal cues are translated into biological responses which direct ovarian determination, maturation, and function remain poorly understood, however emerging evidence, particularly from studies of species with TSD, continues to shed light on these fundamental processes (Bock et al., 2020b; Carter et al., 2018; Ge et al., 2018; McCoy et al., 2016; Weber et al., 2020).

Thermal plasticity of reptile and amphibian sex determination exists along a continuum between TSD systems, in which temperature cues dictate whether to initiate ovarian or testis development, and GSD systems, in which genetic signals are the sole determinant of gonadal fate (Barske and Capel, 2008; Sarre et al., 2004). Growing evidence suggests many species likely fall somewhere in between these two extremes, with gene-by-environment interactions shaping the developmental trajectory of the bipotential gonad (Holleley et al., 2015, 2016; Mork et al., 2014; Pen et al., 2010; Radder et al., 2008). For example, in TSD species, it is possible that cryptic genetic influences may be present that bias sex determination towards a male or female fate. When gonads from embryos incubated at a pivotal temperature (producing both sexes) in red-eared slider are removed and cultured separately at the pivotal temperature, matched gonads show a predisposition towards the same sexual fate (Mork et al. 2014). Additionally, sex ratios from incubation at identical temperatures vary across clutches and populations along latitudinal clines, suggesting factors other than just temperature contribute to sex determination in some species (Ewert et al. 2005; Rhen and Lang 1998). However, whether these results are due to genetic variation, cryptic GSD influences, or other maternal effects such as yolk steroid hormones (Bowden et al. 2000) requires further inquiry. On the other hand, temperature can exert major influences on GSD systems. In the central bearded dragon, *Pogona vitticeps*, sex is generally determined by sex micro-chromosomes in a ZZ/ZW system (Ezaz et al., 2005), however incubation temperatures above ~32˚C can override this genetic system to produce phenotypic females with a ZZ chromosomal complement (Quinn et al., 2007). Sex-reversed ZZ females possess functional ovaries with similar transcriptional profiles to those of normal ZW females (Deveson et al., 2017) and can reproduce with ZZ males to produce offspring whose sex is determined solely by temperature (Holleley et al., 2015). Accordingly, high temperature-induced sex reversal can trigger a rapid transition from GSD to TSD, and this has been demonstrated in wild populations of *P. vitticeps* (Holleley et al., 2015, 2016). Temperature-induced sex reversal also occurs in the scincid lizard, *Bassiana duperreyi*, though in this case, low temperatures override an XX/XY system (Radder et al., 2008). Intriguingly, there is even evidence for gene-by-environment interactions driving offspring sex in a live-bearing lizard, the snow skink (*Niveoscincus ocellatus*), which inhabits an elevational gradient conferring vastly different microclimates (Pen et al., 2010). In highland populations of *N. ocellatus*, offspring sex is determined by a genetic system, while in lowland populations offspring sex ratio depends on maternal gestation temperature (Pen et al., 2010; Wapstra et al., 2004). This population variation in sex determination system is hypothesized to have arisen via evolution of the threshold for temperature-induced sex reversal resulting from climate-related selective pressures (Pen et al., 2010). The specific mechanisms by which temperature interacts with genotype to direct the sexual trajectory of an embryo in these species remain largely unresolved, however increased application of high-throughput sequencing approaches in these systems (Deveson et al., 2017; Whiteley et al., 2021) point to unique discoveries on the horizon.

Evidence for temperature-induced sex reversal has also been found in certain amphibian species including two newts (*Pleurodeles poireti* and *P. waltl*) (Dournon et al., 1990), wood frog (*Rana sylvatica*) (Lambert et al., 2018), and green frog (*Rana clamitans*) (Lambert et al., 2019). When exposed to different temperature treatments between 19˚C and 34˚C, *R. sylvatica* tadpoles exhibited increasingly male-biased sex ratios at higher temperatures, with only males produced at the highest temperature treatments (Lambert et al., 2018). Interestingly, offspring sex ratio exhibited a linear relationship with rearing temperature in this species (Lambert et al., 2018). This pattern stands in sharp contrast to the sigmoidal pattern of most reptile temperature-by-sex ratio reaction norms in which mixed sex ratios only tend to result from a narrow range of temperatures (termed the transitional range of temperatures (TRT) (Valenzuela and Lance, 2004)). Such differences in temperature-by-sex ratio reaction norms between reptiles and amphibians raise the possibility that fundamentally different mechanisms mediate temperature influences on sexual development in these taxa. The linear thermal reaction norm of *R. sylvatica* resembles the reaction norms of several fish species with temperature-induced sex reversal, some of which have implicated germ cell-related mechanisms in mediating temperature effects on sex ratio (Adolfi et al., 2019; Nakamura et al., 2012; Ospina-Alvarez and Piferrer, 2008; Siegfried and Nüsslein-Volhard, 2008; Slanchev et al., 2005). Further experiments are needed to understand the mechanisms underlying amphibian temperature-induced sex reversal and their relationship to variation in thermal reaction norms across species.

Among reptiles with TSD, comparisons of transcriptional, post-transcriptional, and epigenetic patterns across species have shed light on the underlying mechanisms by which temperature is translated into a sex-determining signal during development. Current models suggest chromatin modifiers and epigenetic modifications function at the interface between ancient cellular thermo-sensors that translate temperature into cellular signals and conserved transcriptional networks regulating sex determination (Weber and Capel, 2021). This includes interactions between calcium ion flux, redox status and epigenetic mechanisms regulating gene expression (Castelli et al., 2020). Two genes encoding chromatin modifiers in the *Jumonji* family, *KDM6B* and *JARID2*, are among the first to respond to temperature during the thermosensitive period in both *T. scripta* and *A. mississippiensis* (Czerwinski et al., 2016; Yatsu et al., 2016). *KDM6B* encodes a histone demethylase which removes the repressive histone 3 lysine 27 trimethylation (H3K27me3) mark to activate the transcription of its targets (Agger et al., 2007; Lan et al., 2007), while *JARID2* encodes a component of the polycomb repressive complex 2 (PRC2) which functions in the localization of this complex to its target genes for silencing via the addition of H3K27me3 (Kaneko et al., 2014; Landeira and Fisher, 2011; Peng et al., 2009; da Rocha et al., 2014; Sanulli et al., 2015). In *T. scripta*, when *KDM6B* function is interrupted via RNA interference, embryos incubated at male-promoting temperatures develop ovaries and exhibit increased H3K27me3 at the promoter of *DMRT1*, a key regulator of testis development (Ge et al., 2018). This provided the first demonstration of a functional role for higher order epigenetic processes in regulating the expression of genes with conserved roles in vertebrate sex determination in response to temperature (Georges and Holleley, 2018). Though the expression of *KDM6B* and *JARID2* is upregulated in both *A. mississippiensis* and *T. scripta* embryos at 'low' temperatures (~26-30˚C), in *A. mississippiensis* these temperatures promote ovarian development while in *T. scripta* they promote testis development (Bock et al., 2020b; Yatsu et al., 2016). This suggests that the function and localization of these chromatin modifiers likely depends upon the genomic context in which they operate, though the target loci of JARID2 and KDM6B have yet to be elucidated across different TSD species. Other epigenetic processes, including DNA methylation, have also been implicated in TSD (Matsumoto et al., 2013; Navarro-Martín et al., 2011; Parrott et al., 2014), and it is likely these mechanisms operate in a coordinated manner to shape the dynamic epigenome during sex determination, as has been demonstrated in other key developmental processes (DiGiacomo et al., 2013; Potok et al., 2013).

Beyond sex determination, developmental temperature can exert lasting influences on other phenotypic traits in reptiles and amphibians with persistent consequences for adult reproductive function (Singh et al., 2020). At the transcriptional level, evidence for temperature-related intersexual and intrasexual variation in reptiles and amphibians is sparse. However, in *A. mississippiensis*, incubation temperature was shown to influence the degree of sexually dimorphic gonadal gene expression observed in hatchlings (McCoy et al., 2016). In particular, the magnitude of sexually dimorphic expression of *SOX9* and *AMH* tended to be greater in hatchlings resulting from higher incubation temperatures (34˚C) compared to those derived from lower incubation temperatures (32˚C) (McCoy et al., 2016). It is unclear whether these temperature-related differences in intersexual transcriptional variation persist into adulthood and, if so, whether they have consequences for the functioning of the adult ovary and testis. Even so, it is intriguing to consider that the early developmental thermal plasticity characteristic of many reptile and amphibian species may shape patterns of adult reproductive function. In *E. macularius*, incubation temperature has been shown to not only determine offspring sex, but also influence intrasexual variation in growth, female fertility (as indicated by the proportion of infertile eggs laid), and the ratio of circulating plasma androgens to estrogens (Crews et al., 1998). Further, in the jacky dragon (*Amphibolurus muricatus*), both females and males reared in a seminatural field enclosure exhibited intrasexual differences in lifetime reproductive success related to incubation temperature (Warner and Shine, 2008). The mechanisms underlying this persistent temperature-related variation in reproductive endpoints remain unclear, and future studies examining the contributions of both direct effects of developmental temperature on organizational processes shaping gonadal physiology as well as indirect effects mediated by temperature effects on correlated phenotypic traits (e.g., body size) will be highly informative.

Anthropogenic contaminants alter normal ovarian trajectories

Exposure to anthropogenic contaminants is now a common experience for nearly all life on earth (Gore et al., 2015), and the environmental sensitivity of reptile and amphibian reproductive development appears to convey a distinct vulnerability to many of these compounds (Crews et al., 1995; Guillette, 2006; Orton and Tyler., 2015). Specific impacts of exposure to environmental contaminants on ovarian biology in these taxa range from outright sex reversal to more nuanced effects on ovarian function detected at the molecular and cellular levels. For example, alterations to gene expression networks and disrupted folliculogenesis are connected to systemic effects on circulating steroid hormone levels and reduced fertility in exposed individuals (Sifakis et al., 2017). Perhaps the most common and widely studied of these contaminants are endocrine disrupting compounds (EDCs), which are capable of interacting with steroid hormone receptors, especially the estrogen receptors (Kuiper et al., 1997; Bolger et al., 1998; Kiyama and Wada-Kiyama, 2015). Mirroring the underlying complexity of the endocrine system, EDCs, in addition to interacting with hormone receptors, have also been shown to affect hepatic biotransformation of steroid hormones and inhibit steroid hormone synthesis (Fisher, 2004).

Given the central role of estrogen signaling in sex determination, a commonly assessed impact of EDC exposure on reptiles and amphibians is their ability to cause sex-reversal. Yet, research has shown that sex-reversal resulting from exposure to exogenous hormones and their mimics may not invoke the same mechanisms that determine sex in their absence. For example, the natural hormone (E2) is commonly used as a positive control for EDCs and sex reversal (Matsumoto et al., 2014; Jandegian et al., 2015), which has been shown in alligators to occur via stimulation of ESR1 (Kohno et al., 2015; Doheny et al., 2016). In *Caiman latirostris*, embryos sex-reversed by E2 exhibit altered timing and expression levels of aromatase, *ESR1*, and progesterone receptor, as well as changes in apoptosis and proliferation dynamics during later stages of development compared to normal females (Canesini et al., 2018). In the same species, sex-reversal by E2 and by bisphenol A (BPA) results in females with reduced late-stage follicles (Stoker et al., 2008). In the sea turtle *Lepidochelys olivacea*, sex reversal by E2 at male-producing temperatures leads to small, underdeveloped ovaries with decreased cellular proliferation, delayed downregulation of *SOX9* and upregulation of aromatase, and precocious upregulation of *FOXL2* (Díaz-Hernández et al., 2015). In *T. scripta*, exposure to high doses of PCBs leads to sex reversal. While sex-reversed animals show similar expression of key ovarian genes, including *FOXL2*, *CYP19A1*, and *RSPO1*, the response is delayed and less robust, and methylation patterns established at the aromatase promoter do not follow a normal female pattern, but remain similar to control males (Matsumoto et al., 2014). These results suggest sex-reversed females have ovaries with altered morphology and function compared to normal females. Thus, population-level impacts of contaminant-induced sex-reversal in nature may extend beyond skewed sex ratios, and also encompass impaired fertility and reproductive success. A list of environmentally relevant compounds known to cause complete sex-reversal with references can be found in Table A.1. The remainder of this section will focus on instances of altered ovarian development from laboratory experimental studies and well-developed natural systems.

Laboratory exposures

Laboratory studies evaluating reptiles and amphibians treated with EDCs are useful for determining potential impacts of anthropogenic chemicals but are difficult to draw overarching conclusions from because of variation in the route, developmental timing, and duration of exposure. Further, exposures in controlled settings often lack the environmental and ecological complexity present in nature. Despite these limitations, such studies tend to be favored for amphibian ecotoxicology and have shown that, at both a histological and gross morphological scale, contaminants generally negatively impact oogenesis and reproductive capability. Estrogenic compounds have been found to slow development in *X. laevis*, *Anaxyrus terrestris*, *Hyla versicolor*, *Rana pipien*s and *Rana sphenocephalus* and have negative downstream impacts on female reproductive health (Tavera-Mendoza et al., 2009; Storrs and Semlitsch, 2008; Hayes et al., 2003). A wealth of research (and controversy) has focused on atrazine, one of the most used pesticides in the United States (Rohr, 2021). Exposure to this compound has been reported to induce complete feminization, testicular oocytes in males, delayed gonadal development, as well as extraneous gonads in *R. pipiens* (Hayes et al., 2002, 2003, 2010). Atrazine exposure during sexual differentiation in *X. laevis* also leads to higher rates of primary and secondary oocyte atresia (Tavera-Mendoza et al., 2009). It is suspected that atrazine induces aromatase gene expression and increases conversion of androgens to estrogens, driving such abnormalities (Hayes et al., 2002). Exposure to other pesticides such as triadimefon (and its metabolites) and methoxychlor can cause increased oocyte abscission and inhibit oogenesis altogether (Pickford and Morris, 1999; Zhang et al., 2020). Additional alterations include increased oocyte atresia in *R. sylvatica* exposed to flavone (Mackenzie et al., 2003) and inhibited oviduct development in *X. tropicalis* exposed to progestin or ethynylestradiol (Kvarnryd et al., 2011; Gyllenhammar et al., 2009b). BPA and 17α-ethinyl estradiol (EE2) also generally cause feminization in a number of amphibian species as well as upregulation of vitellogenin, a biomarker of estrogen exposure (for an in-depth review on EE2 and BPA's effects, see Bhandari et al. (2015)).

Similar to these cases in amphibians, laboratory studies have also been instrumental in gauging the potential impact of environmental contaminants on ovarian development and function in reptiles. For example, *C. latirostris* exposed to atrazine or BPA as embryos, or injected with the same dose of BPA as juveniles, show advanced stages of ovarian and oviductal development, determined via histological examination or gene expression (Stoker et al., 2008; Galoppo et al., 2017). Both studies used E2 as a positive control with many of the same effects, including sex-reversal at higher doses with opposite effects at the low dose treatments. This suggests that disrupted estrogen signaling is likely responsible for the altered phenotypes and that the effects are dose-dependent and non-monotonic. Mechanistic reasons for the opposite effects of low versus high doses are unknown. Other alterations to ovarian morphology have been observed in embryonic lizards exposed to maternal atrazine (Parsley et al., 2015a), diethylbestrol (Parsley et al., 2015b), or injected with cadmium (Simoniello et al., 2010). These exposures often induced altered cellular structure of the ovary and disrupted oogenesis and folliculogenesis. In one of these cases (Simoniello et al., 2010), the effects were more similar to FSH exposure than E2, which were used as controls.

Altogether, while these laboratory studies provide evidence of the ability of EDCs to disrupt ovarian development, results are complicated by differences in dosage, timing of exposure, and organismal variation. While including controls such as FSH and E2 has provided important insights into mechanisms of action, we are still lacking an understanding of which specific pathways are impacted, largely due to a gap in our understanding of how such processes operate in the context of normal ovarian development and function. Moving forward, resolving the multifaceted roles estrogen signaling plays in ovarian development in reptiles and amphibians is needed. Mechanistic studies examining the molecular and cellular dynamics, rather than observational exposure experiments are likely to provide key insights into how EDCs interact with ovarian development to affect downstream reproductive function.

Natural exposures

Wild populations exposed to EDCs provide a unique opportunity to understand their consequences in natural settings, which is more directly applicable to discovering the ecological impacts of these compounds. One of the earliest observations of altered ovarian development in polluted environments is the *A. mississippiensis* population at Lake Apopka, FL. Extensive inputs of organochlorine pesticides from agricultural practices and an industrial spill event has led to sustained exposure detectable not only in plasma of alligators but also in egg yolks (Heinz et al., 1991). Following observations of reduced juvenile recruitment at the population level, abnormalities to ovarian follicles, such as multi-oocytic follicles and polyovular follicles, were first reported in juvenile alligators inhabiting the lake (Guillette et al., 1994). Subsequent studies using a combination of field collections and lab incubations revealed that disruptions in steroidogenic and TGF-ß signaling pathways along with impeded folliculogenesis occurring in juvenile ovaries likely stemmed from earlier embryonic exposure due to the maternal deposition of these contaminants in yolks (Moore et al. 2010b, 2011, 2012b; Hale et al., 2019; Hale and Parrott., 2020). Altogether, these and other reports from Lake Apopka suggest that embryonic exposure to EDCs alters transcriptional networks involved in early oogenesis and folliculogenesis and that these perturbations persist into juvenile life and perhaps beyond.

The mechanisms by which maternally deposited EDCs interact with ovarian development to affect reproductive function are not well resolved in natural populations. Based on the proclivity of many EDCs, including those at Lake Apopka, to activate estrogen receptors *in vitro*, it is suggested that disruptions to estrogen signaling during gonadal differentiation are responsible. This idea is supported by observations that EDCs and their metabolites are capable of competitively binding estrogen and progesterone receptors in alligator oviducts (Vonier et al., 1996). However, in this study and others, EDCs typically exhibit weak agonism of nuclear estrogen receptors, with activation on par with E2 only observed at high concentrations (Vonier et al., 1996; Bolger et al., 1998; Guillette et al., 2002). Interestingly, when alligator eggs collected from a reference lake are treated with E2 prior to the start of ovarian estrogen synthesis (measured by *CYP19A1* expression), the ovarian transcriptome in resulting juvenile alligators broadly mirrors those measured in alligators from Lake Apopka (Hale et al., 2019; Hale and Parrott, 2020). Additionally, impediments to folliculogenesis observed in ovaries of Lake Apopka alligators, including severe decreases in stage III follicles and increases in germ cell nests were also induced by embryonic treatment with E2 (Hale and Parrott, 2020). These findings support an alternative hypothesis explaining disrupted ovarian development in Lake Apopka alligators in which EDCs induce weak, but developmentally precocious estrogen signaling to impact germ cell behavior in the early differentiation of the ovary. This hypothesis is also consistent with previous reports demonstrating that key pathways involved in germ cell nest breakdown and folliculogenesis (e.g., activin, inhibin, follistatin) are altered in Lake Apopka ovaries (Moore et al., 2010b, 2012b). In this model, the precocious timing of estrogen signaling, rather than a simple increase in estrogenic activity, is the precipitating event. However, further investigations examining the influence of precocious estrogen signaling on early germ cell behavior, including their initial proliferation within the gonad, the mitotic to meiotic transition, primordial follicle formation and recruitment, and follicle maturation as well as the genomic changes occurring during each of these events are needed.

Another example of a wild population with ovarian defects in reptiles occurs at Moody Pond, MA, where painted turtles (*Chrysemys picta*) are exposed to various heavy metals and organic compounds during development. When compared to a control site, ovaries in Moody Pond turtles harbor fewer small and large follicles (Rie et al., 2005), and adult females have lower vitellogenin and an abated response to gonadotropin stimulation (Kitana et al., 2006). When eggs collected from this same site are raised in the lab, they have higher levels of oocyte apoptosis than the control population (Kitana and Callard, 2008). This same study measured cadmium content in eggs from Moody Pond and exposed control *T. scripta* eggs to relevant doses, which resulted in elevated oocyte apoptosis, suggesting embryonically exposed turtles originating from Moody Pond have reduced reproductive ability via reductions in oocyte number and abated response to FSH.

Complementary studies of contaminants' effects on ovarian development in wild amphibian populations are less detailed, but have been reported. For example, Hayes et al. (2003) observed slowed gonadal development, testicular oogenesis, and even oocyte growth in male leopard frogs (*R. pipiens*) in multiple populations across several states exposed to the pesticide atrazine. In suburban ponds with increased levels of EDC contamination, a greater proportion of females in populations of *R. clamitans* were observed, suggestive of skews in population sex ratios (Lambert et al., 2015). Nemesházi et al. (2020) also observed that agricultural areas harbored an increased prevalence of female-to-male sex reversal in agile frog (*Rana dalmatina*) populations in north-central Hungary. Mink frog (*Rana septentrionalis*) populations exposed to E2 at an experimental lake in Ontario, Canada exhibited higher rates of individuals with intersex gonads whereas no intersex individuals were observed in green frogs (*R. clamitans)* occupying the same habitat (Park and Kidd, 2005). These studies have focused on gonadal aberrations and sex-reversal, and reveal the potential impact of environmental contaminants occurring at population scales. Whereas our understanding of the underlying proximate mechanisms in these systems are generally lacking, they have great potential to reveal the extent to which contaminant-mediated impacts on reproduction affect populations and ecological communities.

Summary and future directions

Reptiles and amphibians offer excellent models in which perspectives and approaches from biological, ecological, and evolutionary fields can be applied to understand the diversity of sex determination strategies and reproductive development. Whereas many facets of ovarian development appear broadly conserved, we identify here several characteristics in which differences are observed both within these taxa and/or in comparison to other vertebrate clades. In many cases, simple descriptions of fundamental biological processes involved in sex determination, ovarian differentiation, and germ cell biology are lacking, despite their necessity to better gauge the extent of existing variation and to resolve the attendant taxonomic relationships. In other cases, especially species in which ovarian development is relatively well studied, connecting ovarian biology to taxon-specific aspects of life history and ecology has the potential to reveal key ecological factors and evolutionary pressures that drive diversity in reproductive development. Recent work in TSD reptiles is leading to exciting breakthroughs regarding the molecular mechanisms translating thermal cues into biological responses, but how these pathways respond to and operate within complex and dynamic environments is a critical question moving forward. In addition, whether the pathways identified in TSD reptiles also mediate the environmental sensitivity of sex determination and ovarian development in amphibians and across other SDMs remains unknown. Further, the extent to which TSD is represented across amphibians more broadly is an important question for conservation efforts in a rapidly changing world. Similarly, well-developed model systems for elucidating the influence of anthropogenic compounds on reproductive development are present in these groups and offer great potential for deciphering the impact of contaminants in natural settings. Lastly, the returns of past advances in unbiased sequencing approaches are reflected by new discoveries in comparative reproductive biology that would never have occurred relying on candidate gene/pathway approaches. This is especially true in reptiles and amphibians, in which a lack of technological and funding resources historically constrained approaches to comparisons of genetic pathways to mammalian models. The increasing availability of such approaches in these species opens up new, intriguing opportunities for answering fundamental questions connecting basic biology with environmental factors. In closing, we propose a set of outstanding and critical questions, which subjectively represent some of the most pressing issues that the field might consider moving forward (**Figure A1.1**).

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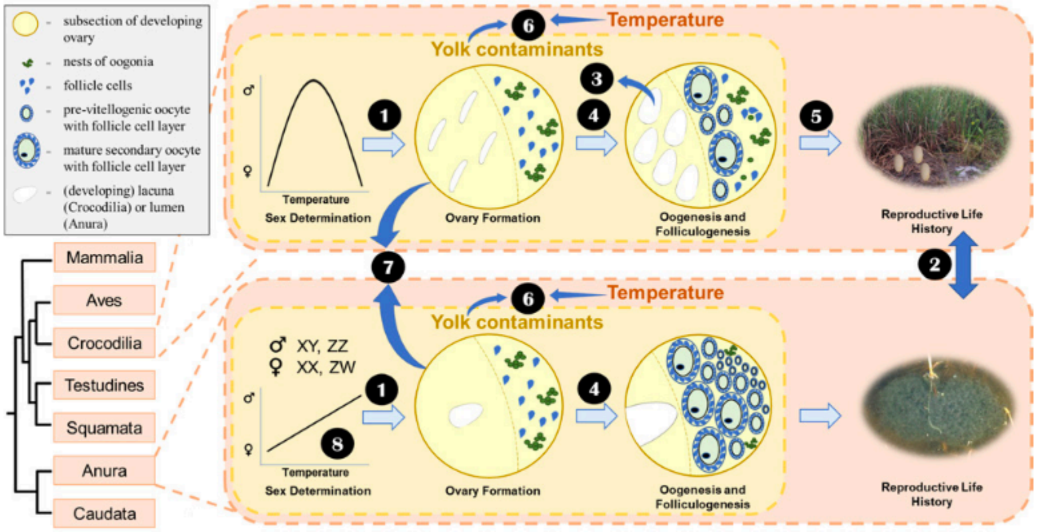

① In which cell types does primary sex determination occur across reptiles and amphibians? Are patterns of cellular organization and/or gonadal structure linked to SDMs across species?

② Is variation in ovarian development (e.g., germinal nest number) linked to inter-specific differences in reproductive ecology and life-history?

③ What is the function(s) of ovarian lacunae and why are these structures present in some but not all groups?

④ What are the pathways that mediate the mitotic to meiotic transition in reptilian and amphibian germ cells?

⑤ What are the evolutionary costs and benefits of a regenerating oocyte pool?

⑥ How are transient environmental cues integrated into robust developmental responses and what are the downstream consequences on intrasexual variation in reproductive function?

⑦ What is the role of estrogen signaling on germ cell biology within the differentiating ovary and how important is the developmental timing of these events?

⑧ What are mechanisms responsible for environmental sex determination in amphibians?

Figure A.1: Illustration of differences in sex determination and ovarian development in the context of emerging and outstanding questions in the field.

Table A.1: Compounds inducing sex-reversal in reptiles and amphibians

Taxa	Species	Compound	Type	Study
Crocodilia	*Alligator mississippiensis*	17β -estradiol	Hormone	Bull et al., 1988
	Alligator mississippiensis	Corexit 9500	Oil dispersant	Williams et al., 2018
	Alligator mississippiensis	Dicofol	Pesticide	Rooney, 1998
	Alligator mississippiensis	trans-Nonachlor	Pesticide	Rooney, 1998
	Alligator mississippiensis	p,p'-DDD	Pesticide	Rooney, 1998
	Alligator mississippiensis	p,p'-DDE	Pesticide	Matter et al., 1998
	Alligator mississippiensis	2,3,7,8-tetrachlorodibenzo-p-dioxin	Polychlorinated biphenol	Matter et al., 1998
	Alligator mississippiensis	ethynylestradiol	Synthetic hormone	Matter et al., 1998
	*Alligator mississippiensis**	17-αmethyltestosterone	Synthetic androgen	Murray et al., 2016
	Caiman latirostris	Bisphenol A	BPA	Stoker et al., 2008
Squamata	*Calotes versicolor**	Testosterone	Hormone	Ganesh and Raman, 1985
	Eublepharis mecularius	17β -estradiol	Horomone	Bull et al., 1988
Testudines	*Chrysemys picta*	Bisphenol A	BPA	Jandegian et al., 2015
	*Emys orbicularis**	Letrozole	Aromatase Inhibitor	Richard-Mercier et al., 1995
	Trachemys scripta	2',4',6'-Trichloro-4-biphenylol	PCB	Crews et al., 1995
	Trachemys scripta	2',3',4',5'-Tetrachloro-4-biphenylol	PCB	Crews et al., 1995
	Trachemys scripta	cis-Nonachlor	Pesticide	Willingham and Crews, 1999
	Trachemys scripta	p,p'-DDE	Pesticide	Willingham and Crews, 1999
	Trachemys scripta	chlordane	Pesticide	Willingham and Crews, 1999
	Trachemys scripta	trans-Nonachlor	Pesticide	Willingham and Crews, 1999
	Trachemys scripta	aroclor	PCB	Willingham and Crews, 1999

	Trachemys scripta	4-hydroxy-2,4,6-trichlorobiphenyl	PCB	Matsumoto et al., 2014
	Trachemys scripta	4MA	Reductase inhibitor	Crews and Bergeron, 1994
	Trachemys scripta	MK906	Reductase inhibitor	Crews and Bergeron, 1994
	Trionyx spiniferus	17β -estradiol	Hormone	Bull et al., 1988
Anura	*Acris crepitans**	PCB/PCDF (general)	PCB/PCDF	Reeder et al., 1998
	Lithobates pipiens	17α-ethinylestradiol	Hormone	Hogan et al., 2008; Mackenzie et al., 2003
	Lithobates pipiens	Atrazine	Pesticide	Langlois et al., 2010; Orton et al., 2006; Hayes et al., 2003
	Lithobates pipiens	DE-71	PBDE	Schmidt et al., 2011
	*Lithobates pipiens**	ICI 182780	Anti-estrogen	Mackenzie et al., 2003
	Lithobates pipiens	Nonlyphenol	Surfactant	Mackenzie et al., 2003
	Lithobates pipiens	PCB-70	PCB	Jofré and Karasov 2008
	Lithobates pipiens	PCB-101	PCB	Jofré and Karasov 2008
	Lithobates pipiens	Sodium nitrate	Fertilizer	Orton et al., 2006
	Lithobates sylvatica	17α-ethinylestradiol	Synthetic hormone	Tompsett et al., 2013
	*Lithobates sylvatica**	Clover root exudate	Phytoestrogen	Lambert, 2015
	*Pelophylax nigromaculatus**	17β-trenbolone	Hormone	Li et al., 2015
	Rana rugosa	Dibutyl phthalate	Plasticizer	Ohtani et al., 2000
	Rana temporaria	17α-ethinylestradiol	Synthetic hormone	Pettersson and Berg, 2007
	Xenopus laevis	3-*t*-butyl-4-hydroxyanisol	Preservative	Kloas et al., 1999
	Xenopus laevis	4-octylphenol	Surfactant	Kloas et al., 1999

Xenopus laevis	Atrazine	Pesticide	Hayes et al., 2002, 2010
Xenopus laevis	Bisphenol A	BPA	Levy et al., 2004; Kloas et al., 1999
Xenopus laevis	Nonlyphenol	Surfactant	Kloas et al., 1999
Xenopus [Silurana] tropicalis	17α-ethinylestradiol	Synthetic hormone	Hirawaka et al., 2012; Gyllenhammar et al., 2009a,b; Berg et al., 2009; Pettersson and Berg 2007; Pettersson et al., 2006
*Xenopus [Silurana] tropicalis**	17β-trenbolone	Hormone	Olmstead et al., 2012