AMERICAN ALLIGATORS: INTEGRATIVE WILDLIFE MODELS TO EXPLORE THE DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE IN THE ENVIRONMENT

by

MATTHEW DAVID HALE

(Under the Direction of BENJAMIN B. PARROTT)

ABSTRACT

The last century of human development has been characterized by the widespread release of chemical contaminants into the environment, many of which can interfere with the endocrine system. Consistent with the "developmental origins of health and disease" model, embryos are highly sensitive to these endocrine disrupting contaminants (EDCs), and exposures are associated with numerous disease pathologies in adulthood. Despite this, the mechanisms linking developmental EDC exposures to future disease states are not well understood, nor are the consequences of long-term, chronic exposures. Herein, we employ a wildlife model of endocrine disruption, the American alligator, to explore both of these questions.

As long-lived predatory species, alligators may receive multi-decadal exposures to contaminants that bioaccumulate/magnify. Leveraging this utility, we use the alligator to explore effects of a toxic EDC, dioxin, in the offspring of exposed individuals inhabiting a historically contaminated system. Using a molecular biomarker of exposure*, CYP1A2*, we identify contemporary effects of dioxins in embryos, suggesting that offspring of exposed individuals remain susceptible to effects of exposures. We then directly quantify dioxins in alligator egg yolk in this contaminated system and uncover their continued presence decades after initial release.

Next, we investigate mechanisms underlying the developmental origins of altered ovarian function, using a population exposed to high levels of organochlorine pesticides (OCPs). Embryos exposed through yolk and raised under lab settings display persistent suppression of fertility-related genes *ESR2*, *AMH*, and *AHRs*, all of which can be recapitulated in reference animals exposed to estradiol prior to gonadal differentiation. These patterns are characteristic of broader changes in the ovary, as non-targeted approaches reveal over 75% of ovarian genes are affected by developmental OCP exposures, the majority of which are recapitulated in estradiol-exposed reference animals. These changes co-occur with altered ovarian follicle profiles, suggesting that OCPs act as estrogens to disrupt follicle development and program future ovarian function, and that the timing of exposures determines future outcomes.

Collectively, this work highlights the utility of the alligator as an integrative model to study the effects and mechanisms of development contaminant exposures under environmentally relevant settings.

INDEX WORDS: American alligator, endocrine-disrupting contaminants, development, dioxin, ovary, estrogen, liver, environmental health, sentinel species

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MATTHEW DAVID HALE

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MATTHEW DAVID HALE

Major Professor:	Benjamin B. Parrot
Committee:	Doug B. Menke
	Jill A. Anderson
	Travis C. Glenn
	Stacey L. Lance

Electronic Version Approved:
Suzanne Barbour
Dean of the Graduate School
The University of Georgia
August 2019

DEDICATION

To my family and friends, for your tireless support and encouragement; to my mentors, for your patient guidance, and to Louis. J. Guillette, Jr., to whom much is owed.

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Alligator science is team science. I've said this frequently during the last months of my dissertation because it perfectly summarizes how difficult this work would have been, if not for the support of the many friends, family, lab mates, and collaborators that have supported me over the last six years.

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CHAPTER 1

INTRODUCTION

1.1: ***General Introduction***

The past century of human history has been characterized by the rapid development and spread of industrial manufacturing capabilities across the global stage. We have acquired the means to develop and manufacture synthetic chemical compounds at a rapid pace to meet global demands for consumer goods, pharmaceuticals, and industrial products. Unfortunately, our ability to produce these chemicals has outstripped the capacity of regulatory bodies to assess their safety. In the United States alone, more than 85,000 synthetic chemicals are currently registered for use (EPA Toxic Substances Control Act; https://www.epa.gov/tsca-inventory/about-tsca-chemical-substance-inventory), a fraction of which have received some form of toxicological testing[1,2]. And due to both intentional and unintentional release, anthropogenic chemical contaminants are now a ubiquitous presence in diverse ecosystems across the globe that constitute a direct threat to human and ecological health[3].

Our modern perspective of the toxic threat posed by anthropogenic contaminants has been directly informed by population declines and overt pathologies in wildlife exposed *in situ*. Wildlife inhabiting contaminated systems have frequently served as “sentinel species”, by providing the first warning signs for the presence of unknown environmental contaminants[4]. Epitomized by their

inclusion in Rachel Carson's text *Silent Spring*[5], sentinels have informed our understanding of the behavior of anthropogenic contaminants in the environment as well as potential consequences of exposures for organismal and environmental health[6]. This utility has been particularly valuable in the study of a subset of anthropogenic contaminants that can mimic, block, or otherwise interfere with the endogenous functions of endocrine hormones, termed endocrine disruptors (EDCs). By disrupting endocrine signaling, EDCs drive a diverse suite of pathologies in endocrine-regulated processes, including reproductive failure, immune suppression, and disrupted thyroid function[7,8]. Further, wildlife sentinels have been fundamental to the understanding that developing embryos are uniquely sensitive to the detrimental influences of EDC exposure. Endocrine hormones endogenously regulate tissue growth and development; interference with these processes by EDCs drives shifts in functional trajectories that ultimately contribute to adult health and disease[9,10].

Despite the overall utility of wildlife models, the nature of complex exposures in ecologically-relevant settings frequently precludes causal investigations of exposure effects and ultimately limits findings to powerful, yet associative, relationships in humans and wildlife[11,12]. Traditional laboratory models have historically filled this gap, but are typically employed in controlled settings with a reductionist approach to exposures. These toxicological models have informed our understanding of mechanisms, but nonetheless trade experimental control for environmental relevance and ultimately fail to capture the complexity associated with real-world, long-term exposures. The research

presented herein is devoted to leveraging the unique life-history and physiological traits of a particular sentinel species, the American alligator (*Alligator mississippiensis*), as an environmental model to explore unanswered questions in ecotoxicology. The alligator is characterized by a long lifespan and high trophic position, making it an ideal top-down predatory model for contaminants that bioaccumulate and biomagnify[13]. Further it exhibits high site fidelity, permitting long-term monitoring of individual systems[14,15]. Lastly, the alligator is exquisitely sensitive to environmental contaminants that can disrupt the function of endogenous hormones and endocrine organs through diverse mechanisms, including hormone mimicry[9,16]. This unique collection of life history and physiological traits places the alligator as an interdisciplinary, integrative model that can be dually employed both to indicate the presence of contaminants as well as to study their effects. Furthermore, given its long life-span and high trophic status, the alligator is uniquely effective among environmental models as a translatable model to inform human health[17].

As will be detailed in chapters that follow, this collective utility has been invaluable in the study of long-lived, persistent organic pollutants (POPs), particularly a subset that interfere with the normal functioning of the endocrine system, endocrine disruptors (EDCs). Herein, we employ the alligator model to address (1) the developmental effects of long-term exposure to a highly toxic and persistent class of POPs, termed dioxins, in a historically contaminated estuary in coastal South Carolina; and (2) the functional ramifications and mechanisms

underlying complex reproductive pathologies induced by EDC exposures during development.

1.2: ***Wildlife Sentinels: The Crocodilian Ecotoxicology Model***

In the decades elapsed since *Silent Spring*, wildlife species have continued to serve as sentinels for environmental pollutants in natural systems globally, and now include invertebrate and vertebrate models ranging from *Daphnia* to marine mammals (for examples, see [17–22]). The efficacy of any particular species as a sentinel is determined by its sensitivity to a given contaminant or stressor, its fidelity to a focal system, and its applicability or relevance to other species and to its ecosystem as a whole[6].

Consistent with these criteria, crocodilians are uniquely effective indicators in the study of environmental health. As a consequence of their longevity[14,23,24] and high trophic status, crocodilians are frequently exposed to high levels of contaminants that can bioaccumulate and biomagnify, including metals and organic contaminants (reviewed in [25]). . Furthermore, as a consequence of their relative site fidelity[14,15], exposures are often limited to constant inputs from a single aquatic system, permitting long-term monitoring[26,27] and longitudinal investigations into the impacts of contaminant exposures[16,28]. All crocodilians are also oviparous, producing a single clutch of eggs in a given year[29] and offloading maternally-derived lipophilic contaminants into eggs as a consequence of yolk provisioning[30]. And for particular contaminants, including organochlorine pesticides (OCPs), the relationship between yolk contaminant burdens and

maternal adipose concentrations is nearly 1:1[31], suggesting that eggs can be used to infer exposure histories and contaminant levels in adults.

Finally, all crocodilian species studied to date employ temperature-dependent sex determination (TSD)[32]; thus their reproductive development is highly sensitive to external environmental cues. This process is integrated in part through the endocrine system, as evidenced by the ability of exogenous estrogens to override the effects of temperature in determining gonadal sex[33–35]. TSD is believed to be adaptive at evolutionary scales[32,36–38], but its integration through endocrine signaling creates opportunities for non-adaptive environmental factors, namely EDCs, to alter gonadal development. Indeed, EDCs, including maternally-derived OCPs, can induce shifts in reproductive development and skew sex ratios (reviewed in [17,39,40]). Ultimately, this last trait renders the crocodilian model as an effective means through which we can detect EDCs in a given system, as well as explore their effects[9] at multiple scales.

1.3: ***Utility of the Crocodilian Model: Case Study of the Lake Apopka Alligator Population***

Given their overall utility, it is perhaps unsurprising that crocodilians are represented in a multitude of studies monitoring levels of diverse contaminants in the environment, including organochlorine pesticides (OCPs)[26,41–43], aromatic hydrocarbons[43–47], metals[41,48–57], perfluorinated compounds[58–62], synthetic endocrine compounds[63], and radionuclides[64]. Nonetheless, despite their realized efficacy as monitors for anthropogenic pollutants (reviewed in [25]), comparatively

few studies have attempted to describe physiological effects of exposures and underlying mechanisms[65] (but see Hotchkiss et al. [12]). One exception to this trend, however, is the American alligator. The alligator has contributed not only as a sentinel for the detection of environmental contaminants across its habitat range in the Southeastern United States, but as a model for the study of their effects on embryonic development and reproductive health. These contributions have been drawn predominantly from a single, well-characterized population inhabiting a contaminated freshwater system in Florida, Lake Apopka (Orange County, FL, USA).

Historically a nationally-renowned bass fishery and tourist destination, Apopka has been subjected to repeated anthropogenic disturbances over the last century, including destruction of marsh habitat on its northern bank, draw-downs, canal building, and impoundment, and repeated chemical treatment for pest control. Further, the lake has received direct municipal runoff and sewage effluent from adjacent towns and agricultural runoff from farming on its North bank (reviewed in [66,67]. Contaminant input to Apopka culminated in a 1980 spill-event of dicofol, a ΣDDT-rich miticide. Collectively, these disturbances have served as drivers of Lake Apopka's rapid degradation[67] from the 1940s-80s, a decline punctuated by the lake's eutrophication, frequent algal blooms, restructuring of vegetation and fish communities, and repeated animal mortality events[66,67].

The story of the American alligator at Apopka began shortly after the 1980 dicofol spill event. Sparked by growing interests in commercial alligator harvests

and egg-collections for ranching, population surveys at multiple lakes identified abnormally few juvenile and hatchling animals at Apopka[68]. These findings were soon followed by observations of rapidly declining clutch viability[69], elevated embryonic mortality[70] and reduced hatchling survival[71]. The timing of these observations in proximity to the spill prompted speculation that contaminant-induced toxicosis was a primary driver, consistent with observations that ΣDDT and other OCPs were present at high levels in eggs[26]. While investigations that followed have revealed that OCPs can explain some, but not all, of poor clutch viability[26,27,30,72], a novel relationship emerged implicating OCPs, which can act as environmental estrogens[73–76], as causative agents in a suite of reproductive abnormalities.

During attempts to describe the cause of reproductive failure at Apopka, it was observed that juveniles of both sexes at that site displayed numerous morphological and physiological abnormalities of the reproductive system[16]. These included abnormal ovarian follicles, disorganized testis morphology, and altered levels of circulating steroid hormones at Apopka relative to juveniles from a reference population[16]. Critically, these observations were made in animals collected as eggs and raised in controlled settings, and thus were attributable to maternally-deposited factors in yolk. Studies that followed expanded these observations to include reduced phallus size[77–79] and disrupted gonadal steroidogenesis[80]. Most recently, it has been observed that lab-raised hatchlings and juveniles from Apopka exhibit suppressed responsiveness to a gonadotropin hormone, follicle stimulating hormone (FSH)[81,82]. Gonadotropins ultimately

control ovarian steroidogenesis, follicle growth, and ovulation across vertebrates, and are thus indispensable to reproduction[83,84].

The collective Apopka phenotype contributed much to a fledgling field of endocrinologists, physiologists, and wildlife biologists studying reproductive abnormalities in a variety of wildlife species in the late 20th century[85]. Exposure to anthropogenic contaminants that could mimic or otherwise alter the normal function of the endocrine system (EDCs), had already been associated with reproductive and developmental abnormalities in mammals, fish, birds, and invertebrates (reviewed in [85–87]). Apopka, however, was uniquely influential in that it was among the first systems linking EDCs in the environment to dramatic reductions in fertility. Furthermore, ovarian abnormalities observed in alligators closely resembled mammalian lab models exposed perinatally to a synthetic, pharmacologic estrogen, diethylstilbestrol (DES)[88,89], indicating that diverse taxa could be impacted through environmental exposures. DES, historically prescribed to pregnant women during the 1940s-70s, has now been causally linked to diverse reproductive pathologies in the children (exposed *in utero*) of mothers taking DES during pregnancy (DES daughter's syndrome; reviewed in [90,91]) via direct activation of the estrogen receptor by DES[92–95] during sensitive windows of embryogenesis.

Similarities between Apopka and mammalian DES models reveal a fundamental connection between exposures and reproductive abnormalities, wherein ubiquitous environmental contaminants, which in isolation may be weak agonists or antagonists for steroid hormone receptors, can collectively influence

reproductive development and induce pathologies in diverse taxa. Further, they highlight a putative mechanism underlying a complex suite of reproductive abnormalities in an environmentally-relevant context and underscore the potential for endocrine-disrupting contaminants to elicit such effects. Nonetheless, despite strong associations, a direct causal link between contaminants in yolk at Apopka and reproductive pathologies has yet to be fully demonstrated, and attempts to recapitulate ovarian abnormalities in reference populations with individual OCPs have been unsuccessful[96,97], suggesting that effects are either an emergent property of this particular contaminant mixture, or that non-contaminant maternal factors may also be contributors[27]. However, the similar effects of a synthetic estrogen, DES, and OCP contaminants at Apopka, suggests that reproductive abnormalities at Apopka are the product of an aberrant estrogenic cue during development. This speculation is supported by observations that contaminants in yolk, including *p,p*-DDE, dieldrin, and toxaphene, are weak estrogens[73–76,98] (reviewed in [99])

1.4: *Novel Applications of the Alligator Model: Dioxin Toxicity in Long-lived Species*

Dioxins, a collective term referring to a number of species of halogenated aromatic hydrocarbons, are among the most damaging anthropogenic chemicals ever produced[100]. Including planar polychlorinated biphenyls (PCBs), dibenzo-furans (PCDFs), and dibenzo-dioxins (PCDDs) they constitute a cornerstone of ecotoxicology, and today exhibit nearly global distributions[101–104] due to their

persistence in the environment and global atmospheric[105,106] and aquatic transport[104,107]. And despite international efforts to curtail their production since the 1980s-90s, they remain threats to environmental and organismal health into the modern day.

Toxicity of this class is best exemplified by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, or TCDD (also referred to as “dioxin”). Despite never being intentionally produced, TCDD has repeatedly entered the environment as an unintended or undetected byproduct of industrial chemical manufacturing, waste incineration, pulp bleaching, or as a contaminant in pesticide mixtures (reviewed in [[100,108]]). It readily adsorbs to organic material in sediments, water, and the atmosphere, and its half-life in organisms can be as long as 7-15 years[109]. Furthermore, it is widely believed to be the most toxic anthropogenic contaminant ever produced, inducing a myriad of pathologies upon exposure and exhibiting one of the lowest LD_{50} values recorded in laboratory models (0.6μg/kg[110]). Effects of acute exposures include embryo mortality[111], teratogenesis[112–114], liver and thymic toxicity[115,116], and skin pathologies[117]. TCDD and related species are also reproductive toxicants and potent endocrine disruptors at developmental and adult stages in both sexes[118–120] (for reviews, see [121–124]).

The toxicity of dioxins, furans, and PCBs is mediated by the aryl hydrocarbon receptor (AHR) signaling pathway[125–128]. The AHR is an orphan nuclear receptor of the basic helix-loop-helix Per/ARNT/SIM (bHLH-PAS) family and is capable of binding dioxins and other co-planar halogenated species. Once activated by ligand-binding, the receptor associates with a binding partner, the

aryl hydrocarbon receptor nuclear translocator (ARNT) in the nucleus and drives expression of inducible targets by targeting a consensus sequence, binding, and recruiting transcriptional activators. Of the dioxin-inducible gene battery, cytochrome p450 enzymes 1A1 and 1A2 (CYP1A1, CYP1A2) are among the best characterized targets and are frequently used as biomarkers of AHR activation by dioxin or other xenobiotic ligands (reviewed in [129]). These CYPs act canonically to metabolize the activating ligand and are directly implicated in toxicity of the activating ligand[130–132].

The toxicity of dioxins is ultimately compounded by their persistence in the environment and exposed organisms, as inhabitants of contaminated systems can experience long-term, chronic exposures. To this effect, our current understanding of the long-term effects of dioxins are generally limited to incidentally-exposed human populations[100], which have revealed complex, latent pathologies in exposed individuals. One of the most informative study systems in this regard is the population of the Seveso, Italy. In 1976, a chemical manufacturing facility producing the herbicide trichlorophenol (TCP), experienced a combination of mechanical and technical failures that resulted in an acute discharge of a liquid and gaseous chemical plume into the atmosphere[133]. The TCP synthesis process is known to produce small quantities of TCDD, and the nature of the failure led to high levels of dioxin being released into the air and surrounding environment[133,134], ultimately contaminating the nearby town of Seveso. In the short-term, acute responses in exposed individuals included chloracne, a hallmark dermatosis induced by dioxin, elevated immune

parameters, and altered liver function (reviewed in [135]). But in the long-term, more nuanced patterns have been revealed. Retrospective cohort studies conducted 20+ years after the 1976 event have consistently uncovered increased risk of cancers and cancer-related mortality, cardiovascular disease and mortality, metabolic syndrome, thyroid dysfunction, and reduced male and female fertility[135]. Further, *in utero* or early-life exposures have been associated with altered sex ratios of offspring, persistent changes to thyroid function, and reduced fertility, collectively indicating that not only are effects persistent, but they may not be realized until later life stages[135]. Lastly, a growing body of evidence is beginning to uncover multi- (F2) and transgenerational (F3) impacts of dioxin in mammals, including reduced fertility[114,136,137], sperm and ovarian follicle abnormalities[114,136,138], and uterine pathologies[139]. Thus, mounting evidence implicating dioxins as drivers of complex, multigenerational pathologies, underscores a clear and pressing need for experimental models with which to investigate effects of chronic, long-term exposures in exposed individuals and their offspring.

Considering the nature of their release throughout history and behavior in abiotic compartments, aquatic systems are likely to act as reservoirs of contamination (reviewed in [140140]), positioning the alligator as an effective model for investigating the consequences of long-term, multigenerational exposures. Traditional toxicological approaches have been highly effective at identifying acute toxic responses to these contaminants, but we currently lack effective

models to study chronic exposures, latent effects of early life exposures, and effects in offspring of chronically exposed individuals.

1.5: Knowledge Gaps in Ecotoxicology: Dissertation Objectives

Modern research paradigms in toxicology and ecotoxicology have been integral to our understanding of the modes of action of EDCs and their effects in exposed organisms and populations. Under traditional investigative frameworks employed in these fields, laboratory models are exposed to single chemicals under controlled settings in order to identify responses and mechanisms in affected tissues. And in environmental settings, forensic investigations in ecotoxicology are employed *post hoc* to probe the causes of pathologies observed in exposed populations. Together, this contaminant-focused investigative paradigm has been used repeatedly to inform regulatory bodies of the risk(s) posed to humans and wildlife alike by contaminants in the environment. However, limitations have emerged in recent years that reflect gaps in this paradigm. These include the inability of traditional exposure models to account for chemical behavior and transformation in the environment; the poor translation of risk criteria derived from effects of laboratory exposures to an ecosystem or community; and the inability to predict effects of environmentally-relevant chemical mixtures from individual constituents[3,141–145]. Conversely, identifying causal agents in affected sentinel wildlife or human populations *in situ* is difficult due to aforementioned complexity of environmental exposures and the limited experimental tractability of wildlife species[11]. As a consequence of this

contaminant-focused paradigm, our understanding of the modes of action through which EDCs drive pathologies has been limited to a handful of mechanisms (i.e., contaminants are estrogenic, androgenic, anti-estrogenic/androgenic, or antithyroid.), and exact cause-and-effect relationships are not commonly fully elucidated[146] (e.g., the role of endocrine-disrupting OCPs as drivers of reproductive failure at Apopka is unclear, and the true cause is likely multi-factorial[27,30])

The research presented in this dissertation positions the alligator as an integrative model to fill the gap between traditional laboratory models and environmental sentinels and to address unanswered questions concerning environmental contaminants. As an oviparous species, the majority of alligator development occurs independently of variable maternal endocrine profiles, and instead is only subject to the influence of maternal hormones and EDCs present in yolk at oviposition. As a consequence, embryos experience relatively stable endocrine profiles throughout development. Further, eggs are amenable to experimental manipulation of the developmental endocrine environment through application of exogenous test substances, permitting investigations of novel stressors against relevant backgrounds of maternally-derived environmental contaminants. Herein, we develop, and then utilize, the alligator model to explore two major questions in ecotoxicology: (1) Does dioxin persist in a long-lived, predatory species and how are long-lived species and their offspring affected by exposures, and (2) What mechanisms underlie contaminant-induced pathologies in an ecologically-relevant context?

In our first objective, we develop and implement transcriptional biomarkers of contaminant exposures to probe the continued presence of dioxins at the Tom Yawkey Wildlife Center Heritage Preserve (YWC; Georgetown County, SC, USA). YWC falls within the Winyah Bay estuary, a system with historically elevated levels of dioxin due to pulp bleaching. Specifically in Chapter 2, we test the hypothesis that ***expression of hepatic biomarker genes, CYP1A1 and CYP1A2, will be significantly elevated in YWC embryos compared to individuals from reference sites lacking historical dioxin contamination.*** Then, to further pursue the relationship between prior contaminant input and hepatic function, we explore the relationship between site of origin and hepatic biomarker expression in lab-raised juveniles. Acting on evidence taken from site-of-origin comparisons in embryos and juveniles, in Chapter 3 we next test the hypothesis that ***effects of site are driven by elevated dioxin and/or dioxin-like contaminants in yolk at YWC***, and secondly, that ***variation in yolk dioxin burdens drive variation in hepatic expression of dioxin-related genes***.

In our second objective, we explore mechanisms underlying persistent disruptions in ovarian function that result from developmental exposure to endocrine-disrupting OCPs. Utilizing a model population in central Florida (Lake Apopka, Orange County, FL, USA) inhabiting a historically disturbed environment, we first seek to determine if a complex mixture of estrogenic OCPs in yolk act directly via estrogen signaling to disrupt gonadal development *in ovo,* reprogramming ovarian function later in life. Specifically in Chapter 4, we first test the primary hypothesis that ***altered ovarian transcription observed in Apopka***

animals is the result of precocious estrogen signaling. Using a targeted, qPCR-based approach we investigate expression of key ovarian genes in lab-raised juveniles from Apopka or a reference site; a subset of reference animals received a single dose of estradiol, an endogenous estrogen, at the bipotential stage of gonadal development, before the acquisition of sex-specific gonadal steroidogenesis. We also employ a gonadotropin-challenge model in this study, wherein juvenile animals can be challenged with follicle-stimulating hormone in order to assess changes in ovarian function. We observed consistent suppression of a suite of fertility-related ovarian genes in both Apopka and estrogen treated reference animals, as well as altered gonadotropin responses. Thus in Chapter 5, to further explore this suppressive phenotype and to more broadly characterize the ovarian response to an external gonadotropin challenge, ***we employ a non-targeted, genomics-based approach to explore the extent of transcriptional programming in the ovary linked to developmental exposures to OCPs or estradiol.***

CHAPTER 2

AHR AND CYP1A EXPRESSION LINK HISTORICAL CONTAMINATION EVENTS TO MODERN DAY DEVELOPMENTAL EFFECTS IN THE AMERICAN ALLIGATOR[1,2]

[1]Hale MD., Galligan TM., Rainwater TR., Moore BC., Wilkinson PM., Guillette LJ., Parrott BB. 2017. AHR and CYP1A expression link historical contamination events to modern day developmental effects in the American alligator. *Environmental Pollution* 230: 1050-1061. Reprinted here with permission of the publisher.

[2]Hale MD., Galligan TM., Rainwater TR., Moore BC., Wilkinson PM., Guillete LJ., Parrott BB. 2018. Corrigendum to "AHR and CYP1A expression link historical contamination events to modern day developmental effects in the American alligator" [Environ. Pollut. 230 (2017) 1050-1061]. *Environmental Pollution* 242: 2096-2098

Abstract

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that initiates a transcriptional pathway responsible for the expression of CYP1A subfamily members, key to the metabolism of xenobiotic compounds. Toxic planar halogenated aromatic hydrocarbons, including dioxin and PCBs, are capable of activating the AHR, and while dioxin and PCB inputs into the environment have been dramatically curbed following strict regulatory efforts in the United States, they persist in the environment and exposures remain relevant today. Little is known regarding the effects that long-term chronic exposures to dioxin or dioxin-like compounds might have on the development and subsequent health of offspring from exposed individuals, nor is much known regarding AHR expression in reptilians. Here, we characterize AHR and CYP1A gene expression in embryonic and juvenile specimen of a long-lived, apex predator, the American alligator (*Alligator mississippiensis*), and investigate variation in gene expression profiles in offspring collected from sites conveying differential exposures to environmental contaminants. Both age- and tissue-dependent patterning of *AHR* isoform expression are detected. We characterize two downstream transcriptional targets of the AHR, *CYP1A1* and *CYP1A2*, and describe conserved elements of their genomic architecture. When comparisons across different sites are made, hepatic expression of *CYP1A2*, a direct target of the AHR, appears elevated in embryos from a site associated with a dioxin point source and previously characterized PCB contamination. Elevated CYP1A2 expression is not persistent, as site-specific variation was absent in juveniles

originating from field-collected eggs but reared under lab conditions. Our results illustrate the patterning of AHR gene expression in a long-lived environmental model species, and indicate a potential contemporary influence of historical contamination. This research presents a novel opportunity to link contamination events to critical genetic pathways during embryonic development, and carries significant potential to inform our understanding of potential health effects in wildlife and humans.

Introduction

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor and orphan nuclear receptor belonging to the basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) superfamily, that plays a role in clearance of xenobiotic compounds[147]. Although historically termed the “dioxin receptor” due to its role in mediating the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and structurally related compounds[148], the AHR functions across a broad array of physiological roles, including immune function, reproduction, and steroid-hormone signaling[149]. Whereas the short-term adverse health effects and developmental impacts resulting from acute exposures to TCDD and structurally related polychlorinated biphenyls (PCBs) have been well documented, the lasting consequences of long-term, persistent exposures on the offspring of long-lived organisms are not as well understood.

Canonically, in the absence of an activating ligand, the AHR is sequestered in the cytosol by a chaperone protein complex[150–153]. Ligand binding

to the cytosolic receptor-chaperone complex promotes shuttling into the nucleus[147,154]. Upon recognition of a short DNA response element (core sequence 5'-GCGTG-3')[155] termed the AHRE (Aryl hydrocarbon response element also referred to as the XRE [xenobiotic response element] or DRE [dioxin response element]), the resulting AHR-ligand complex drives transcription of genes with AHRE-containing promoter regions through recruitment of transcriptional machinery[156].

Cytochrome P450 enzymes, particularly CYP1 enzymes, are inducible targets of the AHR, conserved in a broad range of vertebrate taxa that includes mammals, birds[157], reptiles[158,159], amphibians[160,161], and fish[162]. Among these, the CYP1A subfamily members, CYP1A1 and CYP1A2, are the best-characterized direct targets of AHR activation, and commonly act upon the AHR ligand that initiated signaling. This inducible pathway acts to mediate the hepatic clearance of xenobiotic chemicals through metabolic conversion into water-soluble forms for excretion[163]. However, CYPs are also directly implicated in the toxicity associated with exposure to AHR ligands, particularly planar halogenated aromatic hydrocarbons, including dioxins and coplanar PCBs, and polyaromatic hydrocarbons (PAHs). The toxicity of this latter group is directly attributable to their metabolic products generated from CYP action, which are commonly genotoxic, carcinogenic, or procarcinogenic[164–166]; mechanisms of toxicity for dioxin and other planar halogenated hydrocarbons are less well understood. Additionally, endogenous compounds like lipoxin, tryptamine, and bilirubin[167] are CYP substrates, the CYP1A-mediated metabolism of which can have significant

repercussions. For example, Spink et al.[168] (1998) demonstrated that pre-treatment of human breast cells with TCDD was sufficient to induce CYP1A1- and CYP1B1-mediated metabolism of estradiol-17β, an endogenous steroid hormone, to 4-hydroxyestradiol, a mutagen and pro-mutagen associated with tumor development[169,170].

The American alligator is a long-lived, apex-predator that displays high site-fidelity and a long reproductive life span of decades[14,15]. Despite extensive utilization of this model in environmental studies**,** the AHR-signaling pathway in this species has not been fully described. Work by the Winston laboratory[158,171,172] has established that the alligator demonstrates inducible hepatic CYP protein expression following treatment with AHR ligands. Additionally, three distinct alligator *AHR* sequences were recently cloned and their sensitivity to activation by exogenous ligands characterized through *in vitro* transcriptional activation assays[173]. However, studies investigating isoform-specific gene expression patterning of the AHR and its inducible targets, *CYP1A1* and *CYP 1A2,* are lacking, as are particular functions of the identified AHR isoforms in the alligator. Similarly, investigations addressing how environmental quality might influence this pathway in alligator populations have also not been conducted. The objective of the present study was to describe the tissue distribution of gene expression of three *AHR* isoforms and of two inducible cytochrome-p450s during early life stages in *A. mississippiensis,* and to assess how site-of-origin influences expression of these genes. We investigate expression profiles of these genes in three populations of alligators, collected

from sites conveying very different historical contaminant profiles, the Tom Yawkey Wildlife Center Heritage Preserve, a dioxin/PCB contaminated site; Lake Apopka, FL, an organochlorine pesticide contaminated site; and Lake Woodruff, a reference site.

The Tom Yawkey Wildlife Center Heritage Preserve (YWC) is a wildlife management area located on the north-central coast of South Carolina (Georgetown County) and adjacent to Winyah Bay, an estuary historically characterized by high dioxin contamination[174–176] resulting from nearby industrial activities. In 1988, the U.S. EPA determined levels of dioxin in effluent from the International Paper Co. facility in Georgetown County to be 640 ppq, the highest levels detected nationally in a survey of 103 pulp mill facilities[174]. Levels in blue catfish (*Ictalurus furcatus*) and double-crested cormorant (*Phalacrocorax auritus*) from this system ranged from 87-104 pg/g (ppt)[175] and 17-46 ppt[176], respectively. Additionally, this site is further characterized by high PCB contamination. Cobb et al.[47] (1997) reported mean ΣPCBs at YWC to be 2,459 ± 418 ng/g in alligator egg tissues, which increased to 4,228 ± 1,640 ng/g when the embryo was included in the analysis. A similar study confirmed these observations[46], reporting mean ΣPCBs in alligator eggs at 3,176 ng/g (table 1). Surprisingly, studies addressing potential biological effects this exposure might have on alligators, as well as other local wildlife and human populations, are lacking.

Lake Apopka (AP) in Orange County, Florida is a site with well-characterized organochlorine pesticide (OCP) contamination, and many reports have described reproductive and steroidogenic perturbations associated with

OCP exposure in alligators[16,80,177]. OCPs, including dicofol, chlordane, and toxaphene, are posited to have entered AP both through adjacent agricultural pesticide application and improper holding and disposal of contaminated waste[16,26,178], which resulted in ground and surface water contamination of the lake in the 1980s. High levels of DDE, a xenoestrogenic metabolite of the pesticide DDT, were consistently detected in adipose tissue of adult alligators (30-60 mg/kg), serum of juveniles (7-17 ng/ml)[78], and alligator egg yolk (3.5-5.8 mg/kg)[66] from 1995-2000, when compared to other lakes in Florida. Lake Woodruff (WO; Volusia County, FL), in contrast, is relatively pristine with little agricultural development and low levels of environmental contamination, and is frequently utilized as a reference site in studies examining alligator exposure and response to OCPs[16,81,82,177,179]. PCBs have been detected in alligator tissues, including yolk, at both of these sites, but both sites are characterized by concentrations lower than that detected at YWC (table 2.1).

Sampling from these three sites (YWC, AP, WO) conveying different environmental exposures presents a novel opportunity to link long-term, multi-generational contaminant exposure in these systems to present day manifestations in AHR signaling constituents. Here, our objective is to characterize developmental AHR gene expression and investigate possible site-of-origin effects in a long-lived environmental model, the American alligator (*Alligator mississippiensis*). We seek to link historical environmental quality and long-term contaminant exposure to present day AHR expression, whether it be

through maternal transfer of environmental contaminants through yolk or heritable, persistent alterations to gene expression.

Methods

Embryo Site Comparison and Tissue Distribution

All experiments performed as part of this study on both juvenile and embryonic specimens conformed to guidelines approved by the Institutional Animal Care and Use Committees at the Medical University of South Carolina and the University of Florida. All fieldwork and egg collections were approved and permitted by South Carolina Department of Natural Resources and the Florida Fish and Wildlife Conservation Commission. Eggs were collected after oviposition from all three sites (YWC, AP, WO) in June 2014. Eggs were returned to Hollings Marine Laboratory (Charleston, SC), weighed, candled to ensure viability, and placed in incubators at 32.5°C. A representative embryo from each clutch was necropsied to establish developmental stage[180] (Ferguson 1985). Three eggs were selected from six total clutches from each site (n = 18 eggs per site). Once determined to be viable and approximately staged, eggs were maintained at 32.5°C in sphagnum moss and misted daily. Upon eggs reaching developmental stage 19, incubation temperature was shifted to 30°C (FPT; female-promoting temperature) and maintained until stage 27. Embryos were then extracted from eggs and necropsied, and tissues including liver, heart, kidney, thyroid, brain, small and large intestine, and gonad-adrenal-mesonephros complex (GAM) were isolated and fixed in RNAlater. GAMs were manually

dissected into respective component tissues post-fixation. Liver samples were collected from the medial tip of the right lobe. All tissues excluding liver were placed into RNAlater™ and rocked at 4˚C for 24 hours, then stored at -80˚C until later analysis. Liver samples were diced into 20 mg sections, placed into RNAlater™ and immediately stored at -80˚C.

Juvenile Site Comparison and Tissue Distribution

Moore et al.[82,179] (2012) and Parrott et al.[181] (2014) have described the collection and husbandry of juvenile alligators used in this study. Briefly, animals used for the site comparison of hepatic expression were collected as eggs from nests as described above from AP (6 clutches, n=15), WO (10 clutches, n=17), and YWC (10 clutches; n=20) in June 2011; a representative embryo from each clutch was necropsied for staging. Eggs were maintained at FPT until hatching, at which point animals were marked with specific numbered monel tags on the right-rear foot and transferred to 1000-liter flow-through tanks at Hollings Marine Laboratory. Animals were fed commercial crocodilian food pellets *ad libitum* and sacrificed via intra-venous sodium pentobarbital injection upon reaching 1kg in size. No significant variation was observed in time required to reach this size benchmark across different sites. Animals were promptly necropsied and liver tissue was immediately transferred to RNAlater and stored at -80˚C.

Animals used for quantifying tissue distribution patterns were collected as eggs from nests from Lake Woodruff (4 clutches; n = 11 total) in June 2007, segregated into two groups and maintained at respective FPT (30°C; n =5) or

MPT (male producing temperature; 33°C; n = 6) temperatures until hatching. Hatchlings were marked on the right hindlimb with monel tags as previously described and transferred to temperature controlled animal room tanks at the University of Florida. Water conditions were kept consistent between tanks and temperatures were maintained at 27-31°C. Animals were fed commercial crocodilian food pellets *ad libitum* and sacrificed by IV sodium pentobarbital injection upon reaching 5 months of age. Animals were promptly necropsied and tissues, including adrenal gland, brain, eyelid, kidney, liver, gonads, intestine, and thyroid were immediately transferred to RNAlater and stored at -80°C.

RNA Extraction and Preparation of cDNA

Whole RNA was extracted using a modified APGC[182] (acid-phenol guanidinium thiocyanate) column-purification protocol. Briefly, 20 mg of tissue was thawed and immediately transferred to 1.0 ml denaturing solution (water-saturated acid phenol, guanidinium thiocyanate, 2 M sodium acetate, 1 M sodium citrate, 10% sodium N-lauroyl sarcosine, 14.4 M beta-mercaptoethanol). For larger heterogenous tissues (kidney, brain), whole tissues were minced and homogenized, from which 20 mg were collected. A sterilized stainless-steel bead was added to each sample, and tissues were further homogenized via a Retsch ball mill (30 Hz, 6 minutes). Beads were removed and homogenates were centrifuged at 4°C, 12,000 rcf for 10 minutes. Supernatants were collected, added to a new 1.5 ml microcentrifuge tube and mixed with 0.2 ml 37% chloroform, vigorously shaken, and incubated at room temperature for 3 minutes.

Homogenates were then centrifuged at 4˚C and 12,000 rcf for 15 minutes. Following centrifugation, the upper RNA-containing aqueous layer was collected and mixed in a 1:1 ratio with 100% EtOH, and transferred to a silica-membrane spin column (EconoSpin™; Epoch Life Science; Fort Bend, Texas). Columns were centrifuged at 15,000 rcf for 1 minute at room temperature. Bound RNA was washed (1 M Tris-HCL, potassium acetate, 60% EtOH) and treated with DNase (5Prime DNase I; Gaithersburg, MD). Following DNase treatment, total RNA was eluted in DEPC-treated water. RNA concentrations were assessed via spectrophotometry; RNA quality was checked via banding patterns on a denaturing gel; persistence of genomic DNA contamination was assessed using qRT-PCR with non-intron spanning primers against RNA without reverse transcriptase treatment. RNA was then diluted to 67ng/μl, and 1μg total was used for cDNA synthesis. cDNA was synthesized using iScript reverse transcriptase (BioRad; Hercules, CA) according to the manufacturer's specifications and the resulting cDNA was immediately aliquoted into appropriate volumes for analysis and transferred to -80˚C.

Quantitative Real-time PCR

Gene expression was assessed as previously described[181,183]. Briefly, expression levels were determined using plasmid DNA standard curves containing gene-specific target amplicons of known concentrations. Standard curves were run on each plate to achieve consistency across all samples for a given gene. Samples were run in triplicate and the mean starting quantity

(copies/µl) was then normalized to sample-matched expression of ribosomal protein L8 (*RPL8*). Reactions were performed with 2µl of cDNA, AmpliTaq Gold (Applied Biosystems, Carlsbad, CA) and SYBR Green (Life Technologies, Grand Island, NY). Expression analysis was performed using a C1000 thermal cycler-CFX96 real-time detection system (Bio-Rad, Hercules, CA) and CFX manager software (software v3.0). Primers used and amplicon sizes are reported below in Table 2. Expression values are reported relative to sample-matched reference gene expression. For tissue-distribution comparisons, both non-normalized (raw) and relative expression values are reported.

qPCR Standard Samples

Standard samples for qPCR were generated by using pGEM®-T Vector System II (Promega, Madison, WI) to ligate target amplicons for each *AHR* and *CYP* isoform investigated here. Briefly, ligated amplicons were used to transform competent *E. coli* (One Shot® TOP10, Thermo Fisher Scientific, Waltham, MA). Successful transformations were screened for the target amplicon by performing PCR amplification using M13 F/R primers. Amplified products were run on a 2% TAE agarose gel; migration-distance shifts relative to negative controls (empty plasmid) were assumed to indicate successful ligation of target amplicons. Plasmid products were isolated using Wizard® *Plus* SV DNA Purification System (Miniprep. Promega, Madison, WI), precipitated in 50% polyethylene glycol (PEG), and eluted in 5 ng/µl tRNA TE-buffer solution. Products were sequenced to ensure appropriate target amplicons were successfully ligated. Amplification

efficiency, specificity (off-target amplification), and self-reactivity (primer-dimers) were assessed by running primer sets through temperature gradient protocols spanning 60-70°C against no-template controls (NTC), against target receptor-matched plasmid DNA, and against non-matched plasmids. This effectively addressed a primer set's proclivity to: amplify self (primer-dimer; against NTC), amplify non-target receptor cDNA (against a different plasmid cDNA for non-matched isoform, e.g *AHR1A* primers against full-length *AHR1B* plasmid), and amplify target receptor cDNA (against target-matched plasmid cDNA) across a gradient of possible primer annealing temperatures. Optimal annealing temperatures for primer sets were then selected by choosing the temperature at which optimal amplification efficiency occurred without self-reactivity or off-target amplification.

CYP1A Locus Description

CYP1A loci synteny and genomic architecture were described using UCSC Genome Browser (genome.ucsc.edu). Genomic regions used to assess synteny and putative AHR response elements (DRE/XRE/AHRE) were extracted from the most recent alligator genome assembly (NCBI assembly/406428). Presence and location of AHR core consensus sequences were assessed with MatInspector[184] and Sequence Manipulation Suite[185].

Statistical Analyses

Statistical analyses were performed with GraphPad Prism (v.6) and SPSS (IBM®; v.23). Normalized expression values were arcsine transformed and the

Shapiro-Wilk test (α = 0.05) was used to assess normality of transformed values. Outlier detection and removal was conducted via ROUT (Q=1%). One-way ANOVA (α = 0.05) was used to assess statistical significance of expression differences in embryo and juvenile site comparisons with Tukey's post-hoc multiple comparisons test used where necessary. Non-parametric tests were utilized when necessary. One-way ANOVA (α = 0.05) was also used to assess potential clutch effects within individual clutches in all experiments. No significant effects of clutch were detected. Expression patterns in tissue distribution were not used in statistical tests due to variance of reference gene expression across the tissues assessed. Relative and non-normalized data are reported for all tissues and described qualitatively.

Results

Genomic Architecture of the CYP1A1 and CYP1A2 loci

CYP1A1 and *CYP1A2* in mammals and the avian orthologues, *CYP1A4* and *CYP1A5*[186,187], are two primary transcriptional targets of the AHR. In mammal and bird genomes[157], the loci encoding these two genes lay in close proximity in a head-to-head arrangement. The intergenic region separating each transcription start site harbors multiple AHR binding sites[188,189]. Whereas this genetic arrangement is conserved in mammals and highly similar in birds[157], the *CYP1A1* and *CYP1A2* loci have not been well characterized in reptilian taxa. Based on homology searches, two predicted coding regions with high similarity to *CYP1A1* and *CYP1A2* in other vertebrates were identified in the alligator

genome. These two genes lay in a head-to-head arrangement, similar to the genomic architecture observed in mammals (Fig. 1A). To investigate the conservation of this arrangement in more detail, comparisons of the intergenic region and syntenic relationships with other genes flanking these two genes were made across chicken (*Gallus gallus*), zebra finch (*Taeniopygia guttata*), western clawed frog (*Xenopus tropicalis*), painted turtle (*Chrysemys picta*), mouse (*Mus musculus*) and human. In *A. mississippiensis*, the putative *CYP1A2* gene is upstream of predicted mRNAs encoding CSK and CPLX3-like proteins and downstream of CLK3 and ARID3B-coding regions. On the opposite strand, the putative *CYP1A1* gene is downstream of a predicted *ULK3* mRNA and upstream of predicted coding regions for *EDC3*, *UBL7*, an ACT-like gene, *SEMA7A*, and *CYP11A1*. This core syntenic architecture was similarly observed in all species except western clawed frog, which exhibits a species-specific gene-block upstream of *CYP1A1* and lacks an annotated *CYP1A2* gene. This observation in *X. tropicalis* is supported by work from Goldstone et al.[161] (2007), which identified a single *CYP1A* gene with greater similarity to fish CYP1A sequences when compared to birds and mammals. Despite differing nomenclature, *CYP1A4* and *CYP1A5* as annotated in chicken and zebra finch, are established orthologues of mammalian *CYP1A1* and *CYP1A2*, respectively[157]. The high sequence similarity taken together with the conserved genomic architecture suggests that these two predicted genes are indeed alligator *CYP1A1* and *CYP1A2.* The intergenic region separating these two genes in alligator contain 28 XRE core consensus sequences (GCGTG), potentially mediating AHR-dependent gene transcription

(Fig. 1B). Of these 28 possible XREs, 14 “full” sequences were detected (TNGCGTG).

Tissue distribution of AHR signaling constituent expression

In order to characterize the temporal and spatial patterning of gene expression for AHR-signaling constituents, expression of three recently reported *AHR* isoforms[173] (*AHR1A*, *AHR1B*, *AHR2*), and two CYP1A isoforms, *CYP1A1* and *CYP1A2* were assessed in embryonic and juvenile alligators from the reference site (WO). Across all tissues sampled from late stage alligator embryos (stage 27), *AHR1A* appeared to be the most highly expressed isoform (Fig. 2A), followed by *AHR2* (Fig. 2B) and *AHR1B* (Fig. 2C). Generally, expression of all isoforms was detected highly in liver, kidney, thyroid, and intestine, whereas adrenal gland and ovary consistently displayed low expression of all isoforms. Whereas hepatic expression of *CYP1A2* (Fig. 2D) was detected at high levels, *CYP1A1* expression was not detectable in any of the embryonic tissues examined.

Compared with embryonic expression, all *AHR* isoforms appeared to be expressed at lower relative levels in juvenile animals (Fig. 3). Normalized *AHR1A* (Fig. 3A) expression was highest in kidney and intestine; expression was detected at similar levels in the brain, adrenal gland, liver, and ovary. Expression of *AHR1B* (Fig. 3B) was detected at levels lower than *AHR1A* and *AHR2*. Similar to embryonic patterns, the highest relative expression detected of *AHR1B* was in thyroid. Expression was also detected in the ovary and adrenal gland, which

contrasts to the low levels observed in these embryonic tissues. Expression of *AHR2* (Fig. 3C) was detected at comparable levels in most tissues, excluding particularly high expression in intestine and thyroid. *CYP1A1* was not detectable in any of the tissues assayed. However, *CYP1A2* (Fig. 3D) was detected at high levels in the liver and at lower levels in the kidney and intestine. Although slightly different tissues were assessed in these cohorts, patterning appears similar for these receptors in both embryonic and juvenile animals.

Hepatic AHR constituent expression across sites of variable environmental quality

In order to assess the impacts of site and environmental quality on expression of AHR-signaling constituents, hepatic gene expression of three *AHR* isoforms (*AHR1A, AHR1B, AHR2*) and two CYPs, *CYP1A1,* and *CYP1A2,* were measured in embryonic alligators (stage 27) originating from three sites with different organic contaminant profiles (YWC, AP, WO). Similar to tissue distribution studies, *AHR1A* expression appeared elevated across all sites compared to expression of *AHR1B* and *AHR2*, which were expressed at comparable levels (Fig. 4). There was no observable impact of site on the expression of *AHR1A* (Fig. 4A; $p = 0.1042$, $F[2,45] = 2.379$). However, site did have a statistically significant impact on gene expression of *AHR1B* and *AHR2* (Fig. 4B, $p < 0.0001$, $F[2,39] = 40.65$; Fig. 4C, $p = 0.0071$,$F[2,45] = 5.536$ respectively). Whereas expression of *AHR1B* in YWC and WO embryos was indistinguishable, it appeared significantly elevated in those embryos originating

from AP (α = 0.05; 95% CI WO vs AP: [-2.25 x 10^{-4}, -1.22 x 10^{-4}]; 95% CI YWC vs AP: [-2.1 x 10^{-4},-1.06 x 10^{-4}]). A similar pattern of *AHR2* gene expression was observed, in which hepatic expression in AP embryos was significantly elevated compared to those from WO and YWC (α = 0.05; 95% CI WO vs AP: [-1.96 x 10^{-4}, -1.75x 10^{-6}]; 95% CI YWC vs AP: [-2.26 x 10^{-4}, -2.91 x 10^{-5}]). In regards to AHR transcriptional targets, *CYP1A1* expression was undetectable across all sites. In contrast, hepatic expression of *CYP1A2* was elevated approximately two-fold in embryos originating from YWC ($p = 0.0013$, $F[2,45] = 7.742$) when compared to both WO (α = 0.05; 95% CI: [1.15 x 10^{-3}, 5.96 x 10^{-3}]) and AP (α = 0.05; 95% CI: [8.11 x 10^{-4}, 5.55 x 10^{-3}]). Taken together, these data demonstrate gene expression of AHR signaling constituents varies across sites of differing environmental quality.

Elevated expression of hepatic *CYP1A2* at YWC could be potentially explained by two hypotheses: (1) due to either genetic or otherwise non-contemporary, environmental (e.g., epigenetic) influences, the population of alligators at YWC are characterized by elevated constitutive expression of this enzyme; or, (2) the presence of maternally-deposited AHR-activating contaminants is inducing expression via canonical AHR signaling. Given the historic dioxin and PCB contamination occurring near YWC and the persistent nature of these contaminants, we sought to gain insights between these hypotheses by assessing the expression of AHR signaling constituents in juvenile animals collected as embryos from these three sites, but raised under controlled and identical laboratory conditions. In lab-raised juvenile alligators, site

of origin had no observable impact on *CYP1A2* expression (Fig. 5C, p = 0.2053). Expression of all *AHR* isoforms in juveniles appeared reduced when compared to embryonic expression levels (Fig. 4), and expression of the two detectable isoforms, *AHR1A* (Fig. 5A) and *AHR2* (Fig. 5B) was statistically indistinguishable between sites in juveniles. *CYP1A1* expression levels again fell below the limit of detection for all sites. These data suggest the elevated levels of hepatic *CYP1A2* expression observed in embryonic alligators from YWC do not persist, further implying that alligators from YWC are not characterized by a constitutive elevation of hepatic *CYP1A2* expression.

To further explore the nature of elevated *CYP1A2* expression observed in embryonic animals from YWC and the role of AHR-dependent transcription, relationships between *AHR* and *CYP1A2* expression were assessed using linear regression analyses. Strikingly, whereas significant positive relationships for *AHR1B* (Fig. 6B p = 0.0017, R^2=0.6061) and *AHR2* (Fig. 6C, p=0.0017, R^2=0.574) were detected at YWC, these relationships were absent in WO and AP embryos. These relationships between *AHR* expression and *CYP1A2* expression were not detected in juvenile animals, regardless of site (data not shown). There are multiple explanations that might explain the site-specific presence or absence of these relationships, ranging from AHR auto-induction, for which there are examples in fish[190–192], to possible links between the abundance of activated AHR receptor and its transcriptional targets. While both of these possible mechanisms are dependent upon the presence of activating AHR

ligand, these possibilities have not been tested and little precedent is available for these types of analyses in the AHR literature.

Discussion and Conclusions

Collectively, this study illustrates spatial and temporal gene expression patterning of the aryl hydrocarbon receptor and site-of-origin influences on the expression of CYP1A2, an inducible target. Elevated hepatic expression of *CYP1A2* was observed at a site in close proximity to a historical source of dioxin and PCB contamination (YWC). As alligators are capable of transferring maternal contaminants into egg yolk[30], we hypothesized the observed increase in *CYP1A2* expression to be the result of *in ovo* exposure to maternally-deposited dioxin and other persistent dioxin-like compounds. Supporting this interpretation, elevated expression of *CYP1A2* did not persist in juvenile alligators from YWC raised under controlled laboratory conditions and examined more than four months after putatively being exposed to maternally deposited contaminants in yolk. This finding is consistent with the hypothesis that upregulated *CYP1A2* expression observed in YWC embryos results from acute *in ovo* exposures, rather than from a characteristic inherent to this population. Taken together, these findings along with a well-established history of dioxin and PCB contamination at or near YWC suggest elevated *CYP1A2* levels observed in alligator embryos from this site might be due to maternally deposited contaminants. However, despite these observations, a direct causal link remains lacking and alternative explanations, such as genetic contributions between populations, remain plausible and warrant

further investigation. Furthermore, elevated *CYP1A2* at YWC could remain a legacy effect of exposure in previous generations, perhaps mediated through heritable epigenetic modifications. AHR activation by diverse ligands has an established role in altering epigenetic modifications of the *CYP1A1* gene promoter in mice by displacing HDAC/DNMT complexes[193,194], as part of typical induction of transcription, but more recent reports have uncovered a role for activated AHR in permanently altering both global methylation patterns[195] and promoter-specific patterning in the *Cyp* locus[196]. While these findings have not been extended to assess transgenerational inheritance of these altered epigenetic patterns, they establish a putative mechanism through which prior exposures to AHR-ligands could influence contemporary gene expression.

Possible alternative explanations for the elevated *CYP1A2* expression detected at YWC include genetic differences at the population level. While comparisons of genetic population structure have not been made between YWC, Lake Apopka, and Lake Woodruff explicitly, Davis et al.[197] (2002) reported significant differences in genetic population structure between Lakes Apopka and Woodruff and the Santee Coastal Reserve, a SC coastal population in close proximity to TWC (<20km). Thus, genetic differences could account for the observed elevation in *CYP1A2* expression, but further research would be needed to elucidate how precisely they could account for altered expression reported in embryos but not in juveniles.

Additional contributing factors to the observations made herein include environmental differences between our study sites that do not include historical

contamination events. Lake Woodruff and Lake Apopka are eutrophic and hypereutrophic freshwater systems in north-central Florida, respectively, and while alligators at YWC inhabit freshwater impoundments, the presence of tidal creeks and estuarine riverine systems in close proximity to these impoundments grant YWC alligators opportunity to forage in brackish/marine habitat. Dietary analysis of adult alligators at Lakes Apopka and Woodruff revealed freshwater fish species constituted a large majority of consumed prey species (79.9% at Lake Apopka, 62.2% at Lake Woodruff)[198] while adult alligators of a coastal barrier island population similar to YWC in Georgia (Sapelo Island, GA) consumed upwards of 75% marine vertebrates (fish) and invertebrates[199]. Assuming that foraging habits are similar between YWC and this Georgia coastal population, differences in maternal diet could explain our observations, assuming that diet corresponds to altered ligand profiles in yolk or that differing nutritional status between populations similarly contributes to ligand profiles. The presence of a diverse suite of diet-derived endogenous AHR ligands has been established in humans[200], including flavonoids, indigo-derivatives, indole-containing compounds, and carotenoids. The latter most of these, carotenoids, are nutritive and antioxidant components in yolk, and have been found in the eggs of birds[201] and reptiles, including freshwater turtle[202] and chameleon[203]. A number of studies in birds have indicated that yolk carotenoids correspond closely with maternal dietary intake[201]. It is plausible that differences in diet between YWC, WO, and AP alligators could be responsible for changes in carotenoid levels in yolk, and

that levels of this (or other) endogenous AHR ligands contributes to our observations.

CYP1A1 expression was not detected in our study, and yet exposure to AHR ligands has been shown to induce expression of both *CYP1A1* and *CYP1A2* in embryonic chicken[186,187], herring gull (*Larus arengtatus*)[204], cormorant[205], and mouse[206,207]. Additionally, mammalian *CYP1A1* is characterized by little-to-no basal hepatic expression in the absence of activating ligands, while *CYP1A2* is expressed basally, but is less inducible than *CYP1A1* following exposures to AHR activating ligands[167,206]. This pattern is similarly observed in chicken, where *CYP1A4* is expressed at lower constitutive levels but is significantly more inducible than *CYP1A5*[204]. The lack of detectable *CYP1A1* expression in YWC alligators suggests the absence of an AHR-activating ligand at this site, and would thus require that elevated "basal" *CYP1A2* expression at YWC be a facet of embryonic development particular to that population. An alternative explanation would be that CYP1A regulation in *A. mississippiensis* departs from the observed patterns in mouse or chicken, whereby CYP1A2 adopts the role of the primary inducible hepatic CYP1A enzyme. Head and Kennedy[204] (2007) demonstrated such a reversal in embryonic herring gull hepatocytes, where CYP1A5 was more inducible and expressed at lower basal levels than CYP1A4. Alligators could mirror this regulatory mode, and further studies examining dose-response relationships between AHR agonists and *CYP1A* expression are needed to characterize the details of transcriptional outputs of AHR activation in this species.

Similar to observations at YWC, the elevated hepatic expression of two *AHR* isoforms in embryonic alligators from AP is an interesting finding with potentially complex regulatory underpinnings that might stem from both environmental and/or genetic mechanisms. Studies examining the relationship between *AHR* expression and exposure to OCPs, the major contaminants of concern at AP, are both sparse and contradictory. Wojtowicz et al.[208] (2011) observed that DDE exposure significantly reduced AHR-protein expression in human placental explants, while more recent findings[209] have detailed the ability of DDE to induce *AHR* expression in human peripheral blood mononuclear cells (PBMCs). Earlier work by this group highlights an alternative explanation for elevated *AHR* expression at AP; exposure to DDE is capable of inducing mRNA and protein expression of pro-inflammatory markers, including COX-2, TNF-α, IL-1β, and IL-6 in PBMCs[210,211]. Champion et al.[212] (2013) have separately described the ability of some inflammatory mediators, particularly IL-1β, to drive increased *AHR* gene-expression *in vitro*. This would establish a possible link between developmental DDE exposure and enhanced *AHR* expression through induction of inflammatory mediators at AP.

The tissue-dependent patterning of *AHR*-isoform expression in alligator embryos reported here is consistent with patterns observed in other vertebrate taxa. We observed consistent expression of *AHR1A* in nearly all tissues assessed, with particularly high expression in the liver, kidney, brain, and intestine. Alligator AHR1A is phylogenetically highly similar to tetrapod AHR[173], including avian AHR1 genes and the single mammalian AHR. In the embryonic

mouse, *Ahr* expression displays variability with developmental progression, but generally is detected highly in liver, brain, heart, and adrenal gland[213]. Interestingly, we consistently observed very low *AHR* expression in the adrenal gland, but this disparity could be the result of species- or stage-specific dynamics of expression. Similarly, Walker et al[214]. (2000) reported AHR expression in chick embryonic heart, kidney, and liver. This further suggests developmental expression in alligator closely resembles that observed in other vertebrate taxa. The contrast between moderately high *AHR* expression detected in the mesonephros and the low expression observed in the ovary and adrenal gland is particularly interesting. These tissues form a single developmental complex, the GAM, and the patterning observed indicates possibly distinct regulation of expression in these closely-related and developmentally linked tissues, which further raises the possibility of distinct developmental functions of these receptor isoforms.

We have detected site-of-origin influences on gene expression of AHR signaling constituents: elevated expression of two *AHR* isoforms at a site of high OCP contamination and significantly elevated hepatic expression of *CYP1A2* at a site associated with high dioxin/PCB contamination. The former of these observations is possibly driven by complex mechanisms and requires additional experiments to fully understand. The latter was detected in embryonic alligators from a site immediately adjacent to an aquatic system (Winyah Bay) with high dioxin and PCB contamination. *CYP1A2* is an established biomarker of exposure to dioxin and dioxin-like compounds, and in this context indicates a possible

contemporary effect of historical contamination of Winyah Bay on alligators at YWC. Furthermore, we have characterized the patterning of AHR gene expression in multiple tissues in both embryonic and juvenile alligators, permitting future studies of AHR signaling and possible influences environmental exposures might have on it. Broadly, the effects of long-term dioxin exposure (20-30+ years) are not well understood. Studies investigating the impacts of multigenerational exposure to PCBs and other AHR-activating environmental contaminants in fish have uncovered instances of acquired tolerance. Atlantic tomcod in the Hudson River are postulated to have acquired PCB tolerance through an adaptive six-base deletion in AHR2[215] followed by rapid selection upon this resistant phenotype. These findings are supported by similar work from Reitzel et al.[216] (2014) that implicate adaptive resistance and genetic selection in highly exposed Atlantic killifish populations at the AHR2 locus. However, in species with longer generation times, these adaptive genetic changes would likely not occur fast enough to impart tolerance to individuals or entire populations. The American alligator is an effective model of environmental contaminant exposure in this context, and further studies focused on the mechanisms underlying our observations might potentially inform us of the long-term effects due to dioxin in aquatic systems decades after a contamination event occurs.

The last known quantification of AHR-activating ligands at the YWC was reported in 2002, and reassessing these levels will be crucial to understanding the nature of signaling observed at that site, including elevated *CYP1A2* levels.

Future directions consequently include a direct assessment of dioxins and dioxin-like PCBs in alligator egg yolk from the sites investigated here. Furthermore, we aim to extend our investigation to address liver histopathology of alligator embryos at these sites and in proximal SC coastal populations with differing exposure histories as a means to further support or refute the presence of effects following developmental exposure to AHR-ligands and to characterize *CYP1A* isoform induction following direct treatment of alligators with bona fide AHR-activating ligands. Lastly, considering the possibility of epigenetic differences between our sites resulting from differential exposure history, we hope to characterize methylation patterning in the promoter regions of AHR-responsive genes. Although it will be difficult to parse the effects of exposure in previous generations from current *in ovo* exposure, altered patterning in these animals, if detected, will provide evidence for a persistent influence of exposure to dioxins and other similar compounds in AHR signaling and gene expression.

Table 2.1. Total yolk PCB levels. Levels of total PCB congeners are reported in alligator egg yolk

	Mean ∑PCBs (ppm)	95% CI	Year	Reference
YWC	3.17	1.97, 5.12	2002	Cobb *et al*., 2002
Lake Woodruff	0.97	0.65, 1.29	2000	Woodward *et al*., unpublished data
Lake Apopka	1.57	0.00, 3.29	2000	Woodward *et al*., unpublished data

Table 2.2. qPCR primers and amplicon characteristics**.** Sequence and optimal annealing temperature for qPCR primer pairs are listed for their respective target genes.

Gene	Sequence	Anneal Temp (°C)	Amplicon Size (nt)
AHR1A	GTTACACAAGTTCCAAAACGGT	64	151
AHR1A	GGATGCCAAGTCTGAGAAGG		
AHR1B	CTGTTACTACCTACAAGCCTGACC	68.4	141
AHR1B	GAAACTTCAACCGTCCTTGGAG		
AHR2L	TCCTACCCACGTGAACCAAA	64	135
AHR2L	GGTGAATTCCATGGGAGCATT		
CYP1A1	CATTCTTCCTTTATGCCCTTC	62	229
CYP1A1	CCCAAGCCAAATATCATCACT		
CYP1A2	CCAGAACATTGGCAGAGAGAG	60	128
CYP1A2	TCTTCCAGTGTGGGATTGTG		

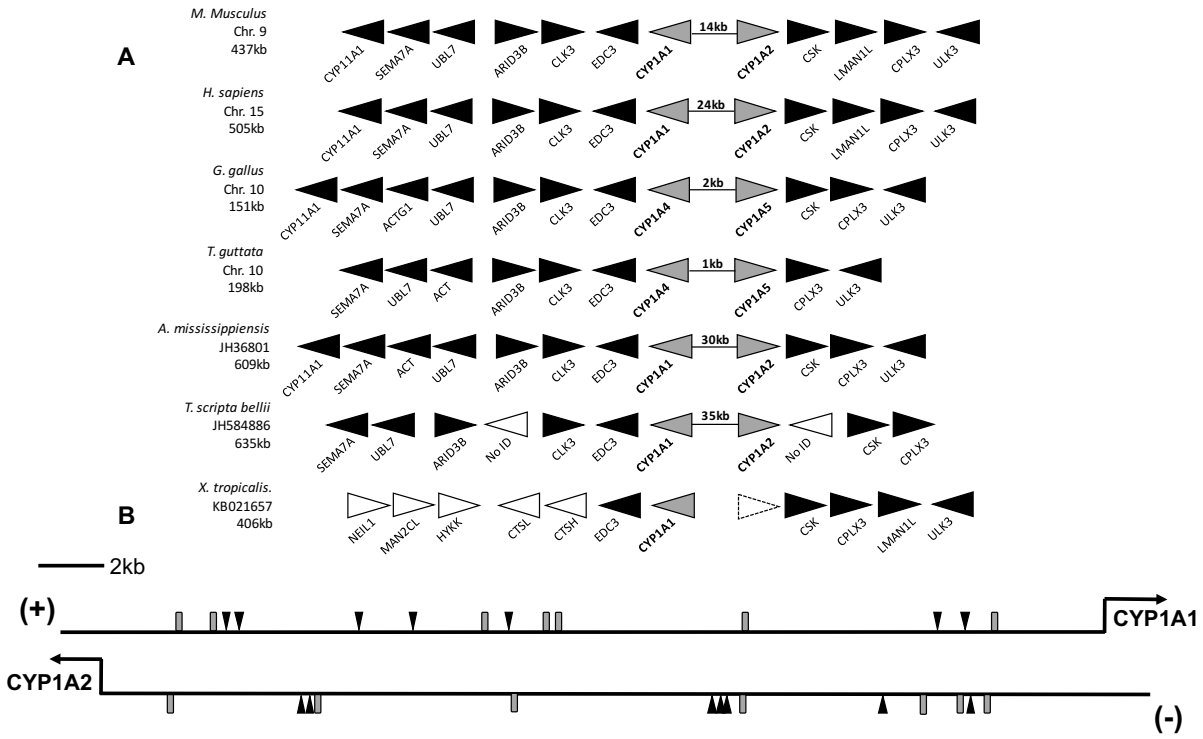


Figure 2.1. Vertebrate CYP1A locus synteny (A) and possible alligator AHR response elements (B). (A) Locus synteny between humans (*H. sapiens*), mouse (*M. musculus*), chicken (*G. gallus*), Zebra finch (*T. guttata*), Painted turtle (*T. scripta bellii*), Western clawed frog (*X. tropicalis*) and American alligator (*A. mississippiensis*) is shown. Arrows denote direction and location of coding sequences surrounding CYP1A1/1A4 and CYP1A2/1A5. In *G. gallus* and *T. guttata*, CYP1A4 and CYP1A5 are orthologous to mammalian CYP1A1 and CYP1A2, respectively. Intergenic distances and gene sizes are not drawn to scale. Locus size is determined by number of base pairs spanning the distance between the first and last genes depicted, i.e CYP11A1 and ULK3. (B) *A. mississippiensis* CYP1A genomic architecture and location of possible AHR response elements (AHRE/XRE/DRE) are shown in grey, using the XRE core consensus sequence GCGTG, for the 30kb intergenic region separating CYP1A1

and CYP1A2. Sequences containing the full XRE, TNGCGTG, are marked in black (triangles).

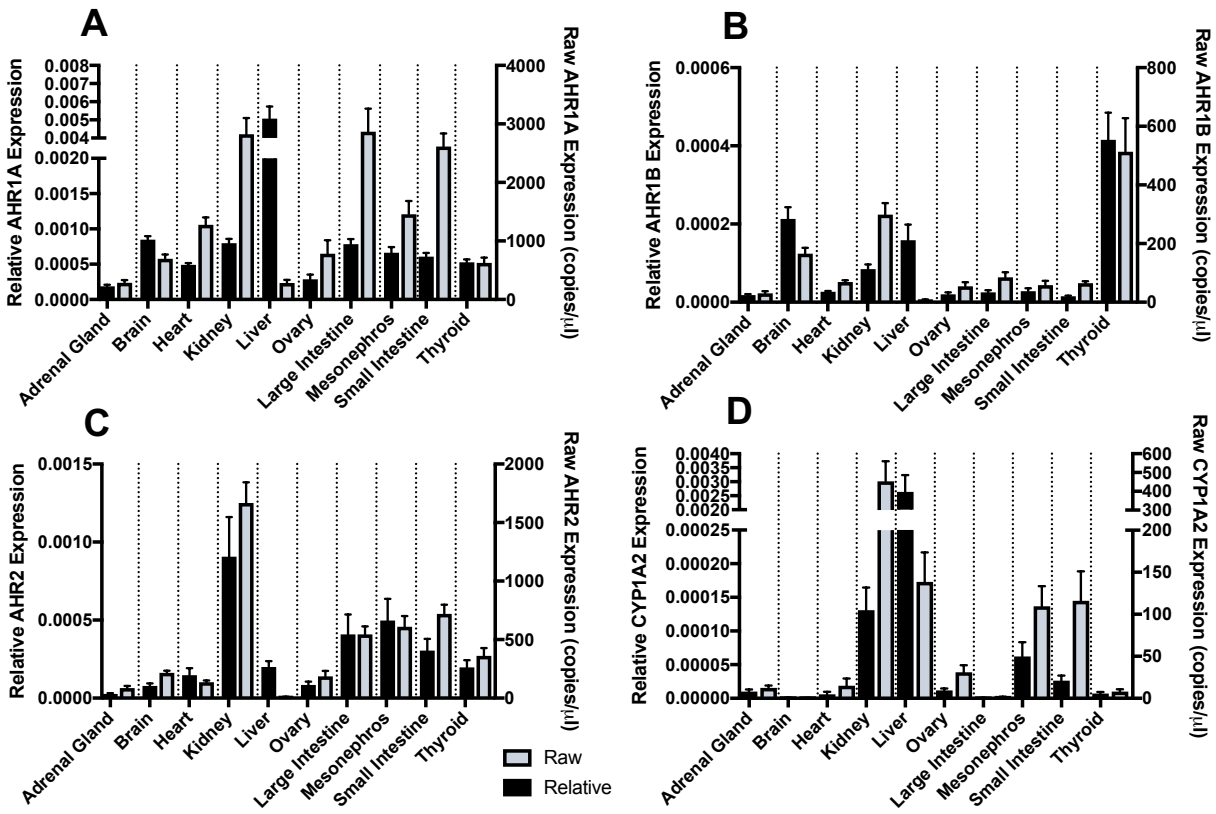


Figure 2.2. Embryonic tissue distribution. Gene expression of three *AHR* isoforms, *AHR1A* (A), *AHR1B* (B), *AHR2* (C), and *CYP1A2* (D) in stage 27 alligator embryos from Lake Woodruff. Raw (grey bars) and relative (black bars) expression is reported ±SEM for the indicated tissues. Relative expression values are raw values normalized to expression of RPL8 (not shown).

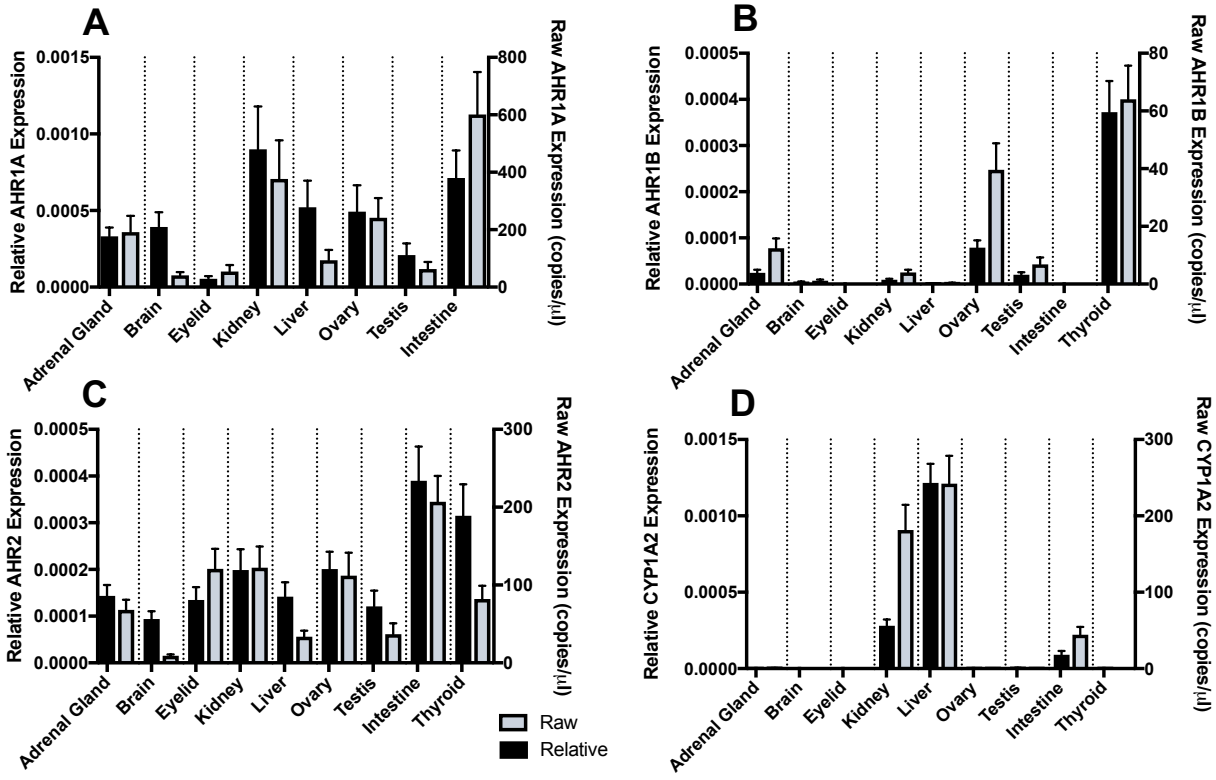


Figure 2.3. Juvenile tissue distribution. Gene expression of three *AHR* isoforms, *AHR1A* (A), *AHR1B* (B), *AHR2* (C), and *CYP1A2* (D) in juvenile alligators raised from hatching under controlled lab conditions from Lake Woodruff. Raw (grey bars) and relative (black bars) expression is reported ±SEM for the indicated tissues. Relative expression values are raw values normalized to expression of ribosomal protein L8 (RPL8; not shown).

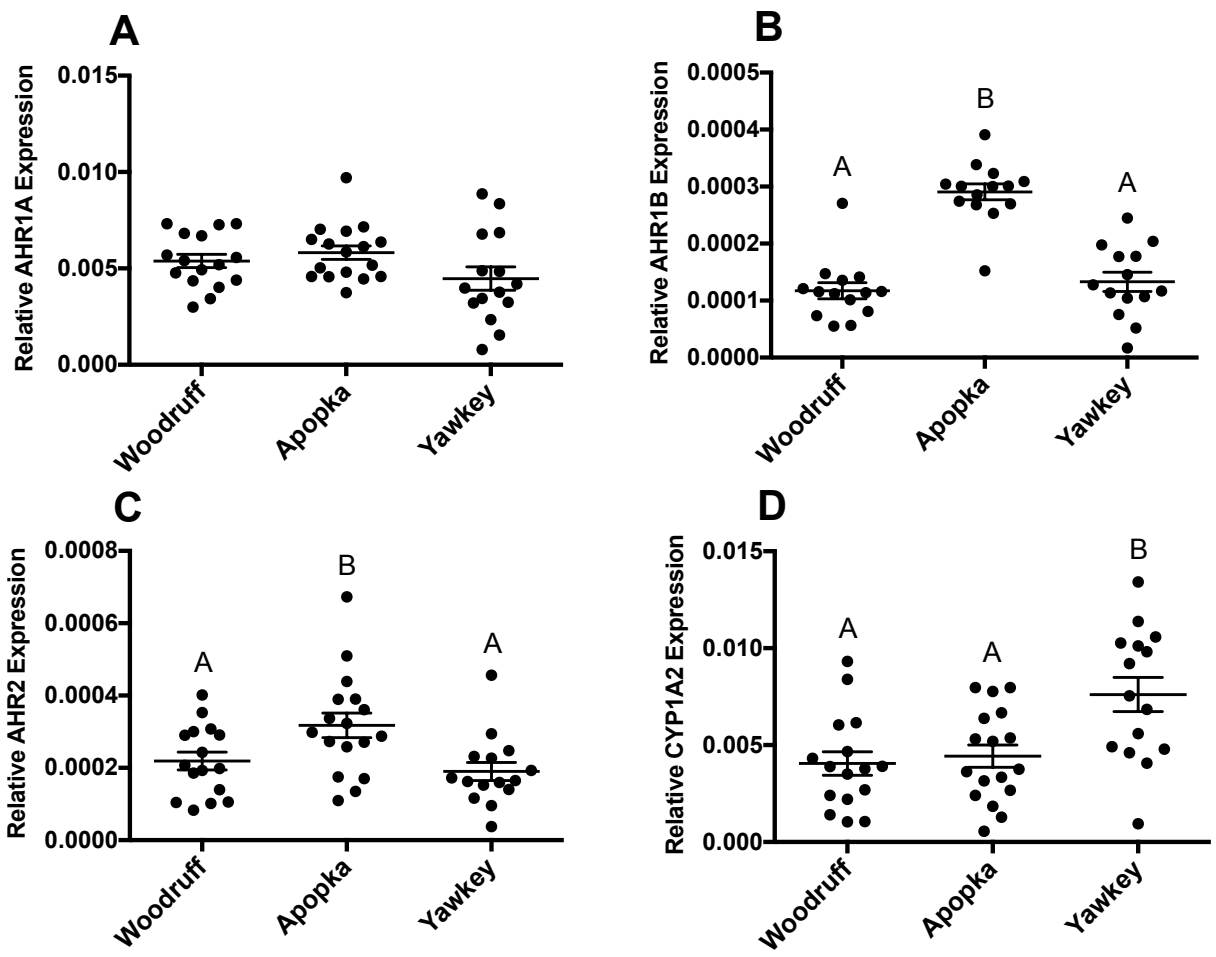


Figure 2.4. Embryonic site comparison**.** Hepatic gene expression of three *AHR* isoforms, *AHR1A* (A), *AHR1B* (B), and *AHR2* (C) and *CYP1A2* (D) in stage 27 alligator embryos from three sites of varying environmental quality. Expression of indicated genes is normalized to expression of RPL8. Bars denote mean expression ±SEM. Letters denote significance between sites within a gene.

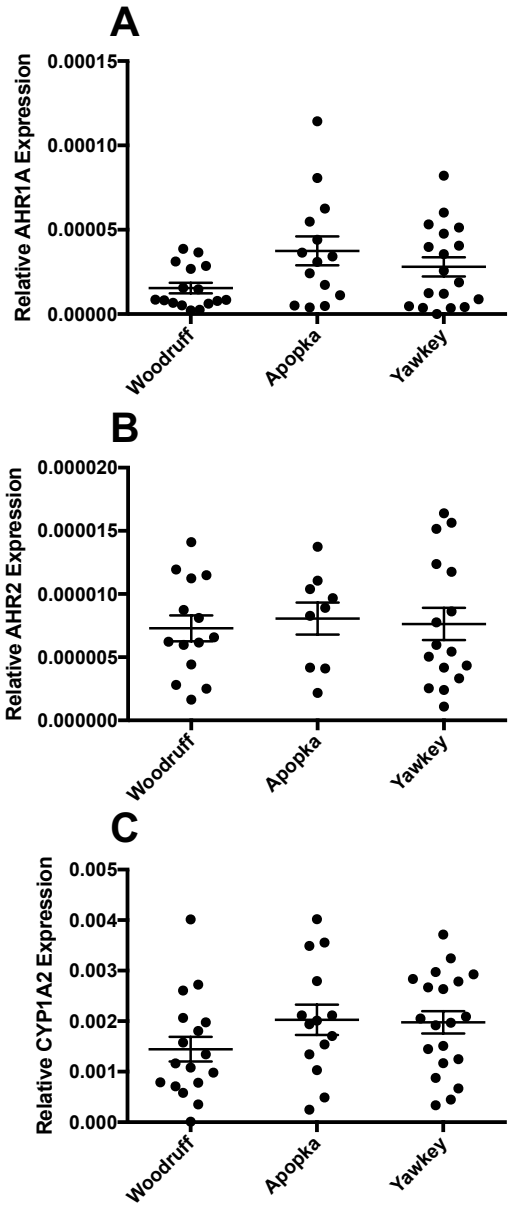


Figure 2.5. Juvenile site comparison**.** Hepatic gene expression of two *AHR* isoforms, *AHR1A* (A), *AHR2* (B), and *CYP1A2* (C) in juvenile alligator from three sites of varying environmental quality, raised under identical laboratory conditions. Expression of indicated genes is normalized to expression of RPL8. Bars denote mean expression ±SEM. Letters denote significance between sites within a gene.

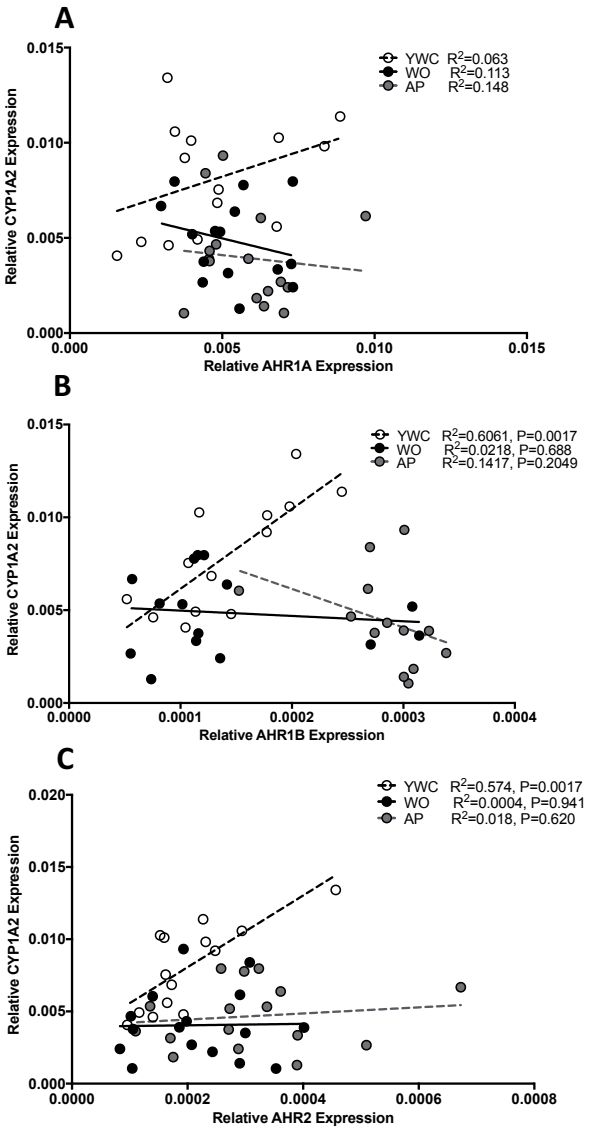


Figure 2.6. Linear regression of normalized hepatic *AHR* isoform and *CYP1A2* gene expression in stage 27 embryos from three sites of varying environmental quality: YWC (open circles, black hashed line), AP (grey circles, grey hashed line), and WO (black circles, black solid line). Significant non-zero slopes were not detected for *AHR1A* expression at any site. Significant relationships were detected for *AHR1B* (P = 0.0017, R^2 = 0.6061) and *AHR2* (P=0.0017, R^2 = 0.5735) in YWC embryos.

CHAPTER 3

THE IMPACT OF MATERNALLY DERIVED DIOXINS ON EMBRYONIC DEVELOPMENT AND HEPATIC AHR SIGNALING IN A LONG-LIVED APEX PREDATOR[2]

[2]Hale MD., Bertucci EM., Rainwater TR., Wilkinson PM., Parrott BB. 2019. The impact of maternally derived dioxins on embryonic development and hepatic AHR signaling in a long-lived apex predator. *Chemosphere* 229: 489-499. Reprinted here with permission of the publisher.

Abstract

Dioxins and related contaminants are highly pervasive in aquatic systems and elicit deleterious effects in exposed organisms. Because dioxins exhibit a proclivity to bioaccumulate, long-lived predatory species are particularly vulnerable to their persistence in the environment. We have previously reported elevated expression of *CYP1A2*, a biomarker of dioxin exposure, in American alligator embryos collected from the Tom Yawkey Wildlife Center (YWC). This coastal population inhabits a system with historical dioxin contamination associated with industrial activities. Herein, we utilize ecological attributes of the alligator to address the persistence of dioxins and furans in yolk and their potential to drive changes in hepatic function. Specifically, we assess variation in expression of AHR signaling components in embryos and its connection to contaminant levels in matched yolk samples. Compared to a reference population, toxicity equivalency levels and total penta-, hexa-, octa-substituted CDDs were elevated at YWC. Contrary to predictions, toxicity equivalency levels were not significantly related to hepatic *AHR1B* or *CYP1A2* expression. However, a significant association was detected between expression of both factors and embryo:yolk mass ratios, wherein decreasing embryo mass was negatively associated with *CYP1A2* but positively associated with *AHR1B*. These findings suggest that variation in embryonic metabolism and developmental progression likely influence AHR signaling and dioxin toxicity in alligators and potentially other oviparous species. While dioxin concentrations observed in alligators in this study are lower than historical values reported for other wildlife

species inhabiting this system, they indicate the continued presence and possible long-term influence of these contaminants in a high trophic status species.

Introduction

Halogenated aromatic hydrocarbons are a class of highly pervasive and toxic environmental contaminants that are capable of eliciting adverse responses in exposed organisms. Among these, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and structurally-related compounds have been shown to be particularly harmful[217]. Due to their long ecological half-life and observed proclivity to bioaccumulate and biomagnify, dioxin and dioxin-like compounds remain among the “dirty dozen” of persistent organic pollutants and, despite curtailed production, continue to pose a threat to the health of humans and wildlife[111,135,218–220]. However, despite an abundance of evidence describing negative effects of acute exposure in model species, there are relatively few studies describing the implications of long-term, chronic dioxin exposure, especially in regard to long-lived vertebrates. In this study, we explore the impacts of embryonic exposure to dioxins in an attempt to connect historical contamination events to modern day biological impacts.

The mechanism underlying dioxin toxicity is generally well described, and requires activation of the xenobiotic-sensing orphan receptor, the aryl hydrocarbon receptor (AHR)[148]. Upon ligand binding of dioxin or structurally-related compounds, the activated AHR dissociates from a host of chaperone proteins in the cytoplasm[150–153] and translocates to the nucleus[147,154], whereupon it associates with the AHR nuclear translocator (ARNT). This AHR-ARNT

complex then binds to cognate response elements in promoter regions of genes, permitting recruitment of transcriptional machinery and, ultimately, transcription[154–156]. Among AHR responsive genes are phase-I cytochrome p450 metabolic enzymes, including members of the CYP1A subfamily, which are implicated in the ultimate mechanism of dioxin toxicity[221]. The American alligator (*Alligator mississippiensis*) possesses an inducible, hepatic CYP-dependent mixed-function oxygenase system that can be activated by canonical AHR ligands 3-methylcholanthrene, benzo[*a*]pyrene, and phenobarbital (reviewed in [222]). Additionally, three *AHR*[173] and two *CYP1A* isoforms originating from distinct loci with associated dioxin-responsive elements (DREs) have been described in the alligator, which confirms expression of this pathway in the liver[223]. However, AHR activation and *CYP1A* transcript induction have yet to be described mechanistically in this species. Similarly, the persistence of dioxin in long-lived sentinel species, such as the alligator, has not been well described.

As a long-lived apex-predatory species exhibiting high site fidelity[14,15] the American alligator is a uniquely effective model for assessing the impact of persistent lipophilic contaminants (i.e., dioxins) on organismal development and reproduction[17]. As such, we have previously used this model to investigate potential effects of historical dioxin contamination in a South Carolina estuarine system[223], and reported significantly elevated hepatic expression of *CYP1A2* in alligator embryos. The Tom Yawkey Wildlife Center Heritage Preserve (YWC) in Georgetown County, South Carolina is a site in immediate proximity to the Sampit River/Winyah Bay system, which is characterized by historically elevated

dioxin and furan levels, likely from industrial point sources[175,176,224]. PCBs have also been reported at high levels in alligator egg yolk in this system[46,47]. Thus, elevated *CYP1A2* expression at YWC is presumed to be a contemporary effect of prior contaminant input in the 1980s and indicative of the continued presence of dioxin and dioxin-like contaminants. In addition, because effects in YWC alligators were observed in embryos exclusively, and not in juveniles raised under contaminant-free laboratory conditions, they implicate maternally-derived contaminants as drivers of altered transcription. However, this study did not examine dioxin levels, and therefore was unable to directly link variation in the hepatic expression of AHR signaling components to the presence of yolk contaminants. As dioxin production was purportedly eliminated in the early 1990s following point source discovery, these findings suggest dioxin persistence might span decades in species of high trophic status.

In the present study, we investigate the persistence of dioxins and furans in alligator egg yolks at YWC, a site with historic dioxin examination. Our objectives are to characterize the persistence of these contaminants in the alligator, and to further describe the role for developmental exposures to drive variation in hepatic function in the context of the aryl hydrocarbon receptor pathway. Specifically, we hypothesize that YWC will exhibit significantly elevated levels of dioxins relative to a reference site and that, within a site, variation in yolk dioxin burdens will be significantly associated with variation in expression of AHR signaling constituents, particularly *CYP1A2*.

Materials and Methods

Animal Husbandry and Tissue Collection

All experimentation conducted in this study met established Institutional Animal Care and Use Committee guidelines at the Medical University of South Carolina and University of Georgia, and all fieldwork and egg collections were approved and permitted by the South Carolina Department of Natural Resources and Florida Fish and Wildlife Conservation Commission. Embryos used in this study were collected as eggs shortly following oviposition at YWC on June 19-30, 2016, and from Lake Woodruff (WO; Volusia County, FL) on June 23-24, 2016. Five eggs each were collected from 4 WO clutches and 44 YWC clutches. When possible, maternal females were captured during nest attendance and plasma was sampled post-oviposition as part of an ongoing study[14] at YWC, but not at WO. Following collection, eggs were immediately weighed and candled to confirm viability, then transferred to artificial nests consisting of damp sphagnum moss in bus pans and incubated at 32°C at the Hollings Marine Laboratory (Charleston, SC, USA). A representative embryo from each clutch was sacrificed prior to transfer to incubators in order to determine developmental stage[180]; eggs were maintained at 32°C until they reached stage 15, at which point they were transferred to 30°C, a female-promoting temperature (FPT) that results in 100% female offspring[183,225]. Stage predictions were determined according to Kohno and Guillette[39]. Embryos were maintained at FPT until reaching stage 27, at which point they were removed from eggs and sacrificed via decapitation. Temperatures within artificial nests were monitored daily throughout incubation

using Onset HOBO TidBit v2 temperature loggers. At sacrifice, whole egg yolks were collected in 50mL polyethylene plastic tubes and frozen at -20°C. Hepatic tissue samples were collected from the medial portion of both lobes, diced into ~200mg pieces, and stored in RNAlater. Liver tissue in RNAlater was rocked overnight on an orbital shaker at 4°C, then frozen and stored at -80°C.

RNA Extraction and cDNA Synthesis

Total RNA was isolated using a modified AGPC (acid guanidinium thiocyanate-phenol-chloroform) method with silica flow-column purification. Approximately 100mg of liver tissue was lysed in 1mL of a lysis buffer (water-saturated acidic-phenol, 2M guanidinium thiocyanate, 95mM sodium acetate, 12mM sodium citrate, 0.24% *N*-lauroyl sarcosine, and 14.4M beta-mercaptoethanol) via a Retcsh ball mill. RNA was isolated from resulting lysate using a chloroform phase extraction, followed by aqueous phase dilution with 100% EtOH and column-binding (EconoSpin™; Epoch Life Science; Fort Bend, TX, USA). RNA was treated with DNase (Omega BioTek; Norcross, GA, USA) prior to elution with ultra-pure DEPC-treated water. Total RNA concentration was assessed via spectrophotometry (Nanodrop ND2000; Thermo Fisher Scientific, Waltham, MA, USA); banding patterns on an electrophoretic denaturing gel were used to assay RNA quality. cDNA synthesis was achieved using 1ug total RNA input and SuperScript III reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) with both random hexamer (35uM; Integrated DNA Technologies, Skokie, IL, USA) and anchored oligo dT priming (25uM; Integrated

DNA Technologies, Skokie, IL, USA). Resulting cDNA was diluted 1:1 with ultra-pure water for use in downstream qPCR analysis.

qPCR Standards and Primers

Intron-spanning primer sets and amplicon-matched plasmid standards used for expression quantification of *CYP1A1*, *CYP1A2*, *AHR1A*, and *ACTB* were designed and prepared as previously described[223]. Briefly, initial primer design for *CYP1A1, CYP1A2,* and *AHR1A* was conducted using predicted *A. mississippiensis* target gene mRNA and genomic sequences (allMis0.2/allMis1; GCA_000281125.1; accessed through UCSC Genome Browser; genome.ucsc.edu) and primer3[226,227]. Intron-spanning primer pairs were used to amplify embryonic hepatic cDNA and resulting amplicons were ligated into a pCR 4-TOPO vector (TOPO-TA Cloning Kit, ThermoFisher Scientific, Waltham, MA). Plasmids were isolated from transformed *E. coli* using Wizard *Plus* SV DNA Purification System (Miniprep, Promega, Madison, WI, USA) following manufacturer's recommended protocol. Purified plasmids were eluted in 5ng/uL tRNA-TE buffer solution and sequenced to verify appropriate target gene amplicons were successfully amplified and ligated. Intron-spanning primer sets and amplicon-matched standard plasmids used herein for analysis of genes *AHR1B* and *AHR2* have been previously reported using this same approach[223]. Similarly, *ACTB* primers and standard plasmids were prepared as described above, but using the pGEM-T Vector System (Promega, Madison, WI, USA).

Amplification efficiency, specificity, and self-reactivity of primers were assessed via PCR reactions against: target-matched amplicons, no-template controls, and alligator liver cDNA across a range of annealing temperatures (60-70°C). Specificity and self-reactivity were assured via the absence of primer-dimers and/or multiple melt-curve peaks. Amplification efficiency and melt curve profiles were analyzed using CFX manager software (software v3.0). Primer sequences, amplicon size, and annealing temperatures are reported in Table 3.1.

Quantitative Real-time PCR

Gene expression analysis was conducted as previously described[181,183]. Target gene expression values were determined via absolute quantitation using amplicon-containing plasmids of known concentrations (copies/μL); interpolation of this standard curve and sample expression values (copies/μL) were determined using the CFX manager software (software v3.0). All samples and standards were run in triplicate. Any triplicate with technical variability (CV) ≤ 40% was discarded. Expression values (copies/uL) were normalized to sample-matched expression values of an internal standard, beta-actin (*ACTB*). PCR reactions were conducted using 2μL of cDNA template in a 50μL total reaction volume, using a homebrew SYBR green reaction mixture that has been described previously[33]. Briefly, reactions were conducted with: 0.2μM primer mix, 50mM KCl, 20mM Tris-HCl, 0.5% glycerol, 0.5% tween-20, 4% DMSO, 3mM $MgCl_2$, 20μM dNTP mix, 0.01U/μL AmpliTaq gold (Applied Biosystems; Carlsbad, CA), and 0.5X SYBR Green (Life Technologies, Grand Island, NY). Individual

triplicate reactions were conducted in 15μL total volume. Gene expression values are reported as relative expression of target gene value normalized to *ACTB* values.

Dioxin/Furan Quantification

PCDDs and PCDFs were extracted from homogenized egg yolks via Soxhlet extraction with methylene chloride as an extraction solvent. Prior to analysis, extracted samples were cleaned using acid/base partitioning and column chromatography. Contaminant quantification was conducted via isotope dilution high-resolution gas chromatography/high-resolution mass spectrometry (HRGC/HRMS; magnetic sector) in cleaned extracts (EPA method 1613B[228]) by Frontier Analytical Laboratories (El Dorado Hills, CA, USA). Toxicity equivalency (TEQ) was calculated using WHO TEQ factors[217]. Complete contaminant values are reported in Appendix 3A Table 1 (S1).

Statistical Analysis

All statistical analyses were performed using GraphPad Prism software (version 7.0b), and JMP Pro (version 13.2). Relative gene expression comparisons across clutches were conducted via Kruskal-Wallis non-parametric 1-way ANOVA on outlier-removed (ROUT method, Q coefficient = 1%) values. For across-clutch comparisons, outlier analysis was conducted to identify outliers within clutches. Only clutches with three or more samples after outlier removal were used in subsequent analyses (clutches with only one or two embryos

represented were excluded). Due to differences in sample sizes between YWC and WO, ANOVAs were conducted for both sites together (i.e., without regard to site) and direct comparisons between sites were not conducted. Expression of internal standard gene *ACTB* did not vary significantly by clutch or by site, as assessed via the methods described above (data not shown). Comparisons of contaminant levels were made using non-lipid adjusted concentrations (pg/g yolk; wet mass) via Mann-Whitney U test. Non-detect values were converted to 1/2 detection limit, where necessary. Any individual congener with fewer than 50% of total values above the detection limit was excluded from analysis. Detection limits are reported in Appendix 3A Table 2. Comparison of TEQ levels by island at YWC was also conducted using Mann-Whitney U tests.

Analysis of gene expression, contaminant values and embryo characteristics were conducted via linear regression, using gene expression as response variables and embryo characteristics (percent embryo mass, contaminant levels, yolk and embryo mass) as predictor variables. Regressions of gene expression and contaminant values utilized lipid-adjusted TEQ values (pg/g lipid) for each sample. Site was initially included as an explanatory variable in models but its effects were non-significant and thus was excluded. Gene expression values were square-root transformed in all regression analyses to achieve normality and homoscedasticity, as assessed via Shapiro-Wilk test and manual inspection of residual distribution. Linear regression analyses were also used to address relationships between maternal snout-vent length (SVL) and

yolk TEQ levels. Yolk TEQ (pg/g lipid) values were transformed via BoxCox function (λ=-0.298).

Results

We first set out to describe natural variation in expression of *CYP1A* and *AHR* isoforms at YWC (Figure 3.1). Expression of *CYP1A2*, but not *CYP1A1*, varied significantly by clutch (Figure 3.1C; approximate p=0.0051; KW=60.19). Median relative *CYP1A2* expression ranged from 0.7506 (S25) to 5.817 (C10) across clutches, an approximate 8-fold enrichment, indicating a high degree of variability. Similarly, expression of *AHR1B*, but neither *AHR1A* nor *AHR2*, varied significantly by clutch (Figure 3.1B; approximate p=0.0284, KW=53.83).

In order to examine the role of yolk contaminants as drivers of hepatic gene expression, we quantified levels of dioxins and dioxin-like furans in egg yolks from YWC and a reference site, WO. Contaminant levels were quantified in yolks from embryos spanning a range of low, intermediate, and high *CYP1A2* expression at YWC (n=12), with the expectation that concentrations would be positively associated with *CYP1A2* transcript abundance. At WO, one representative yolk was analyzed from each clutch. Embryos (YWC n=12; WO n=4; Figure 3.1A, blue circles) selected from each clutch were those closest to the respective clutch average at both sites (i.e., within a given clutch, intermediate CYP1A2-expressing embryos were selected). Generally, contaminant levels (pg/g wet mass) were higher at YWC (Figure 3.2B, Table 3.2) relative to the reference site, WO, and median TEQ values (Figure 3.2A) were

significantly higher at YWC relative to WO (exact p=0.0297, U=6). Similarly, median concentrations (pg/g wet mass) of total PeCDD (exact p=0.0132, U=4), total HxCDD (exact p=0.0418, U=7), and OCDD (exact p=0.0297, U=6) were significantly higher at YWC. And while concentrations of 2,3,7,8-TCDD (exact p=0.0582, U=8) and total TCDD (exact p=0.0582, U=8) appeared higher at YWC, these differences were not significant. A similar non-significant increase was observed for total HpCDD (exact p=0.0582, U=8). In contrast to dioxin and TEQ levels, furans were generally higher at WO than YWC, but only total TCDF concentrations (exact p=0.0297, U=6) were significantly different between sites. Furthermore, these patterns may be driven by the presence of diphenyl ethers at WO (Appendix 3A Table 1). Concentrations of total HpCDF and OCDF consistently fell below detection limits. While investigating spatial patterns of nest distribution at YWC, we observed a spatial clustering of nests into two distinct groups, which are distinguished by island (Figure 3.1A). Interestingly, median yolk TEQ levels were significantly higher in Cat Island nests (Figure 3.1A, inset) than South Island (exact p=0.0177, U=3), suggestive of potential fine-scale geographic differences in contaminant distribution at YWC.

We next sought to describe the relationship between yolk contaminant burden and expression of *CYP1A2* and *AHR1B* in the liver. To this end, embryo-matched hepatic *CYP1A2* and *AHR1B* expression values were regressed against lipid-adjusted TEQ values (pg/g lipid) measured in corresponding yolks with the hypothesis that TEQ and expression values would be positively associated. Unexpectedly, variation in neither *CYP1A2* (Figure 3.33A) nor *AHR1B* (Figure

3.3B) expression levels were significantly associated with TEQ. Thus, in an effort to identify non-contaminant drivers behind this variation, we explored the relationship between embryo and clutch characteristics related to contaminant levels in yolk and hepatic transcription. Specifically, we investigated the effects of clutch size, egg size (mass [g]), and embryo and yolk mass on expression of both genes. As site of origin was not determined to significantly influence the relationship between TEQ and expression levels (data not shown), data from YWC and WO embryos were analyzed together. For all samples with embryo/clutch characteristics and expression data, neither clutch size nor egg size were significantly related to either *CYP1A2* or *AHR1B* (data not shown). In contrast, embryo mass and yolk mass were significant predictors of variation in both *CYP1A2* and *AHR1B* (Figure 3.4). Surprisingly, the direction of effects for both characters differed between the two genes. Expression of *CYP1A2* significantly decreased with increasing yolk mass (Figure 3.4A; $y=-6.616*X + 2.81$, $p<0.0001$, $R^2=0.2115$), while it increased with increasing embryo mass (Figure 3.4B $y=4.155*X - 0.012$, $p=<0.0001$, $R^2=0.23$). In contrast, *AHR1B* receptor expression was positively associated with yolk mass (Figure 3.4C; $y=0.1375*X + 0.0396$, $p<0.0001$, $R^2=0.0931$), but was negatively associated with increasing embryo mass (Figure 3.4D; $y=-0.0736*X + 0.0934$, $p=0.0014$, $R^2=0.0702$). When these analyses were limited to only embryos with yolk contaminant data (n=15; one sample lacked egg mass), these relationships remained significant (Figure 3.4A-D; samples marked as blue dots with boxed insets).

These findings collectively suggest that differences in the relative composition of somatic tissue and yolk across embryos are drivers of variation in *AHR* and *CYP* expression in the liver. While all embryos in this study were sacrificed at developmental stage 27, there is significant clutch variation in percent embryo mass (embryo mass ÷ [embryo mass + yolk mass]) across samples ($p<0.0001$, KW=105.7; Appendix 3A Figure 1), and differences in the ratio of embryo mass and yolk mass (Figure 3.5) might correspond to different contaminant exposure. We hypothesized that embryos with less relative yolk have experienced greater total contaminant exposure due to their increased conversion of yolk into somatic tissue (Figure 5). Thus, percent embryo mass was included as a continuous covariate of yolk lipid-adjusted TEQ (pg/g lipid) in regression analysis with *AHR1B* and *CYP1A2* expression levels. While this approach did marginally improve model fit for both *CYP1A2* (ΔAICc = -8.98) and *AHR1B* (ΔAICc = -6.85, Table 3.4), it did not reveal a significant relationship between contaminant values and gene expression (Table 3.4). In contrast, the best model fit included percent embryo mass alone based on AIC and RMSE criteria (Table 3.3).

Discussion

Dioxins are highly persistent and capable of eliciting a wide range of toxic effects in exposed organisms. Within this class, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin is particularly toxic, and was released into aquatic systems across North America as a byproduct of industrial activities in the mid to late 20th century. In

the present study, we describe elevated dioxin levels in alligators inhabiting a historically contaminated estuarine system in South Carolina (YWC). Relative to a reference site in FL, egg yolk from alligators at YWC have elevated TEQ levels, as well as concentrations of OCDD, and total penta- and hexa-substituted CDDs. A notable exception to this trend includes significant elevation of total TCDF concentration in reference samples. In an attempt to elucidate the role for these contaminants in yolk in driving hepatic gene expression, we employed regression models to address associations between sample matched TEQ concentrations and expression of AHR signaling constituents, *AHR1B* and *CYP1A2,* in the liver of alligator embryos. And while significant relationships between contaminant burdens and gene expression were not detected, we identified a novel relationship between yolk utilization and expression of both genes. Collectively these findings stand to inform both our understanding of the persistence of dioxins in high trophic-level predators and possible drivers of fine-scale differences in sensitivity to dioxins during development. Despite elevated contaminant concentrations at YWC relative to a reference site, dioxin and furan levels reported herein are lower than those previously recorded in the Winyah Bay, SC system (Table 3.5). Historically, levels of 2,3,7,8-TCDD were one to two orders of magnitude greater in perch (*Morone americana*), blue catfish (*Ictalurus furcatus*), and cormorant (*Phalacrocorax auritus*) than in alligator samples examined in this study. This could be plausibly explained by elimination of point source input to Winyah Bay and subsequent reduction in contaminant burdens in exposed individuals over time. This would be consistent with gradual elimination

of dioxin via excretion[229–231] and yolk provisioning[232,233] in resident alligators. This is supported by long-term monitoring of dioxin levels in birds inhabiting contaminated systems, wherein after point source elimination, yolk dioxin levels declined significantly[234,235]. Furthermore, this would suggest that levels in the alligator might have once been comparable to those in other species in Winyah Bay, but a lack of historical samples precludes further speculation. This reduction could also be explained by historical sampling being done in closer proximity to the contaminant point source[175,176].

It is not immediately clear why TEQ levels are higher on Cat Island than South Island, but this pattern could be explained by Cat Island being geographically closer to the contaminant point source (Figure 3.1). In contrast to physical proximity as a driver, recent evidence suggests that female crocodilians exhibit relatively small home ranges and display nest site fidelity[236–239], and additionally, individual size in crocodilians has been demonstrated to modulate both trophic position of prey items and activity space[199,240,241]. Thus, differences in TEQ levels across islands might reflect an underlying segregation of females by size and therefore prey consumption, if those differences result in differential contaminant exposure. Observed spatial variation in contaminant levels might also reflect differences in age among nesting females, with younger, smaller females experiencing less total exposure. Indeed, for those nests at which the maternal female was captured, a positive trend was observed between maternal snout-vent length (SVL) and respective yolk TEQ concentrations (Appendix 3A Figure 2). This relationship was not significant, but is nonetheless suggestive of a

possible link between nesting female characteristics (i.e., age and body size) and contaminant burdens in yolk, wherein older, larger females have higher contaminant burdens due to greater overall exposure duration.

Compared to other systems with known dioxin point sources, concentrations at YWC are relatively low. Dioxin concentrations in eggs of piscivorous birds from the Strait of Georgia (British Columbia, CAN) ranged from 100 and 133 ppt wet mass[234,235] to as high as 3500 ppt lipid mass[242] in the late 1980s and early 90s. Similarly, historic dioxin and dioxin-equivalent levels in eggs of bird species from the Great Lakes basin (USA) are higher than those reported herein. Dioxin-equivalent values from Green Bay, WI (USA) ranged from 11.4 ppt in red-winged blackbird (*Agelaius phoeniceus*) to 214 ppt in Forster's tern (*Sterna forsteri*), 350 ppt in double-crested cormorant (*Phalacrocorax auritus*), and 440 ppt in common tern (*Sterna hirundo*)[243–245]. However, keeping with temporal trends observed in the Strait of Georgia, dioxin concentrations in many of these species declined following elimination of point source input to Green Bay and the Great Lakes basin[246]. Thus, contextualizing levels in alligator egg yolk in terms of other contaminated systems requires either contemporary monitoring in those systems or historical analysis in Winyah Bay.

The relationship between embryo/yolk composition and hepatic *AHR* and *CYP1A2* expression reported in the present study is particularly striking. In an attempt to account for inter-individual variation in yolk utilization, we developed a "realized" exposure model (Figure 3.5), wherein contaminant values were adjusted by percent embryo mass to reflect greater exposure in embryos that

have utilized a larger proportion of yolk. Differences in both CYP and receptor expression might predict fine-scale differences in sensitivity to dioxin and other AHR ligands within a given stage or across individuals and suggests precise regulatory control of this pathway during late developmental stages. Consistent with this hypothesis, a growing body of evidence posits the AHR as a critical regulator of cell proliferation and differentiation[247–253], thus changes in *AHR1B* expression could reflect fine-scale variation in developmental progression within a stage. Additionally, late stages of avian embryonic development are associated with rapid shifts in hepatic expression of genes associated with cell cycle regulation and lipid metabolism[254], and the peri-hatching period is characterized by the rapid accumulation of cholesterol and lipid derivatives in the liver[255,256]. Current evidence suggests that the AHR is involved in regulation of energy homeostasis[257], and the activated AHR has been demonstrated to modulate hepatic transcription of genes involved in lipid metabolism[258]. Thus, increased *AHR1B* expression observed in embryos with more yolk could reflect regulation in response to changing rates of lipid metabolism and growth in late-stage alligator embryos, as they approach hatching. However, disentangling the concomitant regulation of *AHR* and *CYP1A* expression by endogenous developmental processes and contaminant-activated signaling, while potentially informative for our understanding of dioxin exposure dynamics, is beyond the scope of the current study.

To our knowledge, this study provides the first assessment of dioxin and furan levels in a crocodilian. Despite the utility of crocodilians as environmental

models and their likelihood of exposure as long-lived apex predators, dioxin concentrations and effects in these reptiles have been poorly addressed. One study to date has probed the effects of developmental dioxin in the alligator, observing sex reversal and gonadal abnormalities in neonates[259] following exposure. Despite these possible effects, very little is known regarding toxicokinetics of these contaminants in the alligator, including inducibility of the AHR-responsive gene battery that mediates toxicity and clearance (but see [171,172,260,261]). Nonetheless, dioxins are capable of eliciting adverse effects at low doses during development[262–265] and in adulthood following chronic exposure[266,267], suggesting that dioxin and related contaminants might pose a substantive threat in exposed populations. Given the persistent nature of these contaminants and the important ecological role for crocodilians[268], we believe that a broader investigation of the prevalence and effects of dioxin in wild populations is warranted. Furthermore, due to the longevity and high trophic position of alligators, detection of dioxins in alligator yolk stands to inform health risk for understudied human populations coinhabiting historically-contaminated systems, like YWC.

Table 3.1. qPCR primers, annealing temperatures, and amplicon sizes.

Gene	Primer Sequence	Annealing Temperature (ºC)	Amplicon Size
AHR1A	CCAGTTATGCTGACTCCTCAA	62.0	189
	CTGAGGGGGATATGCTTCATT		
AHR1B	CTGTTACTACCTACAAGCCTGACC	68.4	141
	GAAACTTCAACCGTCCTTGGAG		
AHR2	TCCTACCCACGTGAACCAAA	64.0	135
	GGTGAATTCCATGGGAGCATT		
CYP1A1	TCATCAACCAATGGCAAGTCA	64.0	202
	AGTGTGGCCAAGAAGAGGAA		
CYP1A2	ACAGGATCCTCAGTTACCTTCA	64.0	161
	GACAAGGTTGACAATCTTTCCCT		
ACTB	GAGGGTTTTAGGTGTAACTGCTTG	65.0	195
	ACATACTGGCACCGCTTTTC		

Table 3.2. Summary of yolk contaminant burdens by sample. ND denotes values below detection limits

Nest	Site	TEQ	2,3,7,8-TCDD	2,3,7,8-TCDF	OCDD	OCDF	Total HpCDD	Total HpCDF	Total HxCDD	Total HxCDF	Total PeCDD	Total PeCDF	Total TCDD	Total TCDF
W1	WO	4.95	1.64	1.2	7.37	ND	3.14	ND	2.71	13.4	2.27	16.6	1.64	40.2
W3	WO	8.14	2.58	2.28	8.26	ND	ND	3.36	4.56	30.1	4.08	24.9	2.58	46.4
W4	WO	4.25	1.46	1.2	ND	ND	ND	ND	3.33	3.95	1.92	6.47	1.46	13.9
W7	WO	2.88	ND	ND	ND	ND	ND	ND	ND	ND	2.88	ND	ND	9.09
C8	YWC	22.6	5.71	0.912	12.3	ND	3.68	ND	15	5.94	14.6	5.49	5.71	17.2
C10	YWC	17.3	4.1	2.68	10.4	ND	5.13	ND	14.2	ND	10.8	5.45	4.1	7.56
C1	YWC	23.2	6.95	0.995	11.7	ND	6.03	ND	15.5	7.11	13.8	6.46	6.95	11.8
S10	YWC	4.5	1.43	ND	5.86	ND	2.59	ND	2.03	ND	2.85	0.924	1.43	2.68
S12	YWC	2.86	ND	0.998	5.4	ND	ND	ND	1.64	ND	2.59	ND	ND	8.27
S19	YWC	12.8	3.54	ND	7.72	ND	ND	ND	8.25	3.2	7.86	1.82	3.54	6.39
S8	YWC	9.49	3.06	ND	10.7	ND	4.74	ND	6.96	ND	5.7	ND	3.06	8.92
S4	YWC	11.1	4.57	1.38	8.34	ND	3.93	ND	4.01	1.12	5.19	3.83	4.57	9.18
S2	YWC	22.9	5.86	3.83	12.2	ND	5.29	ND	17.1	4.4	13.9	4.7	5.86	17.9
C12	YWC	42.3	8.01	ND	8.69	ND	3.91	ND	32.6	5.51	30.5	2.34	8.01	5.87
S11	YWC	9.71	1.88	0.652	8	ND	4.31	ND	8.69	ND	6.61	0.853	1.88	5.28
C13	YWC	22.4	4.35	1.17	13.2	ND	5.67	ND	18.6	12.3	15.1	1.83	4.35	7.18

Table 3.3. Model selection criteria for hepatic gene expression regression analyses. Measures of model fit are reported for two approaches to modeling the relationship between yolk contaminant burdens and *CYP1A2* or *AHR1B* expression: using yolk TEQ levels as a predictive variable alone or using it with a measure of relative yolk utilization, percent embryo mass. Model criteria are also reported for regression analysis using percent embryo mass alone.

	CYP1A2				AHR1B			
Model Effects	**AICc**	**RMSE**	**Rsquare**	**Rsquare Adj**	**AICc**	**RMSE**	**Rsquare**	**Rsquare Adj**
TEQ (pg/g lipid)	25.41	0.4457	0.0685	0.0019	-67.96	0.0241	0.0014	-0.0699
TEQ (pg/g lipid) + % Embryo mass	16.43	0.3118	0.5766	0.5115	-74.81	0.0180	0.4816	0.4019
% Embryo mass	13.76	0.3096	0.5504	0.5182	-77.92	0.0176	0.4642	0.4260

Table 3.4. Regression model output summary. Output summaries are reported for model effects percent embryo mass and TEQ (pg/g lipid).

	Term	Estimate	Std Error	t Ratio	Prob>|t|
CYP1A2	Intercept	-0.873458	0.561824	-1.55	0.144
	% Embryo mass	3.367317	0.852435	3.95	0.0017
	TEQ (pg/g lipid)	0.183101	0.203784	0.9	0.3853
AHR1B	Intercept	0.179205	0.032452	5.52	<.0001
	% Embryo mass	-0.170886	0.049238	-3.47	0.0041
	TEQ (pg/g lipid)	0.007777	0.011771	0.66	0.5203

Table 3.5. Historical YWC/Winyah Bay dioxin/furan contaminant levels and sources. Dioxin and furan levels from the current study are pg/g wet mass.

Matrix	TCDD (ppt)	TCDF (ppt)	Year	Publication
Sludge	62	161	1987-1988	US EPA 1990a[224]
Softwood pulp	9.2-16	38-52	1987-1988	US EPA 1990a[224]
Hardwood pulp	1.9	7.7	1987-1988	US EPA 1990a[224]
Effluent	0.49-0.64	1.5-1.6	1987-1988	US EPA 1990a[224]
Sediment	>17.3 (TEF)		1992	Coller-Socha 1994 (USACE)[269]
White Perch	15.7-18.2	48.5	1987-1988	US EPA 1990b[175]
Blue Catfish	87.3-107.0	21.0-27.5	1987-1988	US EPA 1990b[175]
Cormorant	19.9-46.7	0.7-1.0	1991	Geitner 1991 (US FWS)[176]
Alligator Yolk	**1.43-8.01**	**0.652-3.83**	**2016**	***Current study***

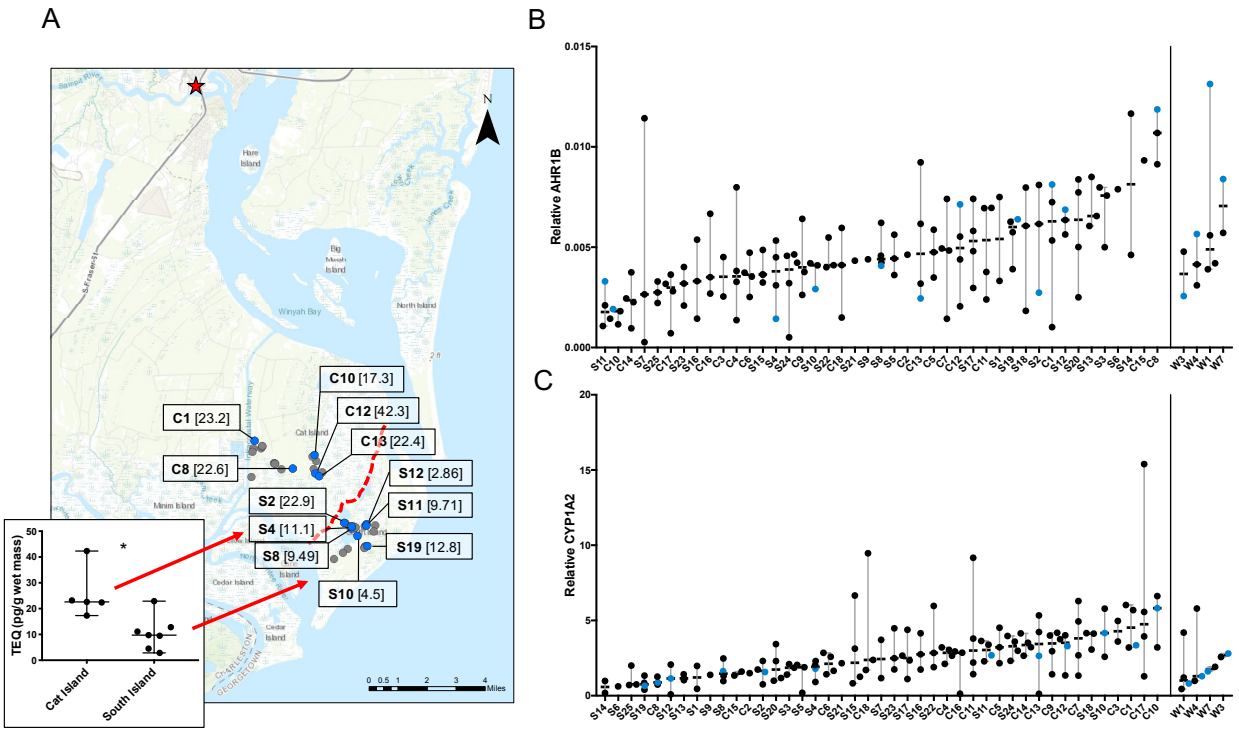


Figure 3.1. Dioxin contaminant burdens and hepatic transcription**.** TEQ (pg/g wet mass) (A) and hepatic transcription levels of *AHR1B* (B) and *CYP1A2* (C) at the Tom Yawkey Wildlife Center Heritage Preserve (YWC) are reported, along with site proximity to a historical contaminant point source (star). Variation by clutch is reported for (A) non-lipid adjusted TEQ. Circles denote nest/clutch locations – blue circles (n=16; 12 at YWC, 4 at WO) represent clutches included in yolk contaminant analyses (one sample per clutch). Qualitative assessment reveals general clustering of nests by island – Mann Whitney U test of median TEQ levels (±95% confidence interval; inset) for each island reveals significant elevation of yolk contaminants on Cat Island relative to South Island. Individual sample (circles) and clutch-ranked median (±95% confidence interval) expression values are reported for AHR1B (B) and CYP1A2 (C). Blue circles designate individuals selected for sample-matched yolk contaminant analysis

from those nests marked in (A). Nests on South Island are denoted by an “S” prefix and Cat Island with “C”. Woodruff nests are designated by “W”.

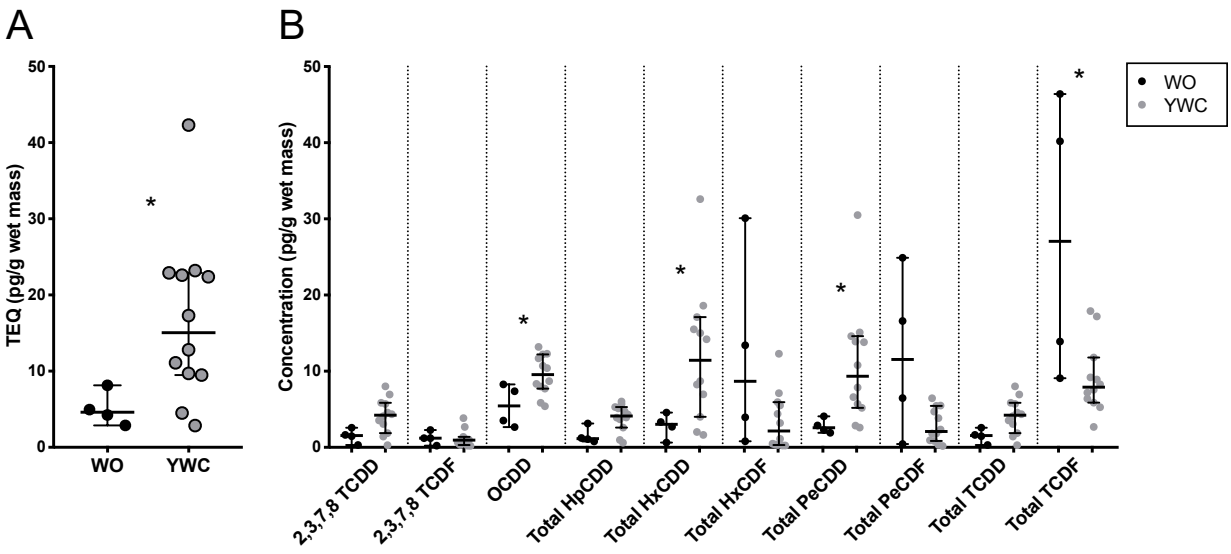


Figure 3.2. Yolk contaminant site comparison. Median and individual TEQ (A) and non-lipid adjusted contaminant concentrations (pg/g wet weight) (B) are grouped by site. Error bars represent 95% confidence intervals. Asterisks denote significant differences between sites ($0.01<p<0.05$). Total HpCDF and OCDF levels were consistently below detection limits

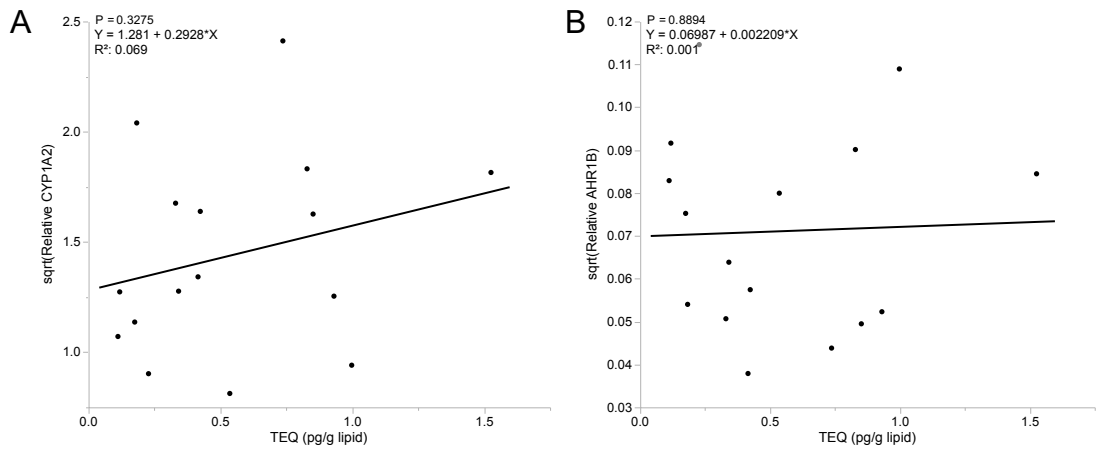


Figure 3.3. Relationship between yolk contaminant burden and normalized hepatic gene expression. Linear regression analysis depict the relationship between *CYP1A2* (A) and *AHR1B* (B) expression in the liver and yolk TEQ levels (pg/g lipid). Significant non-zero slopes were not detected for either gene.

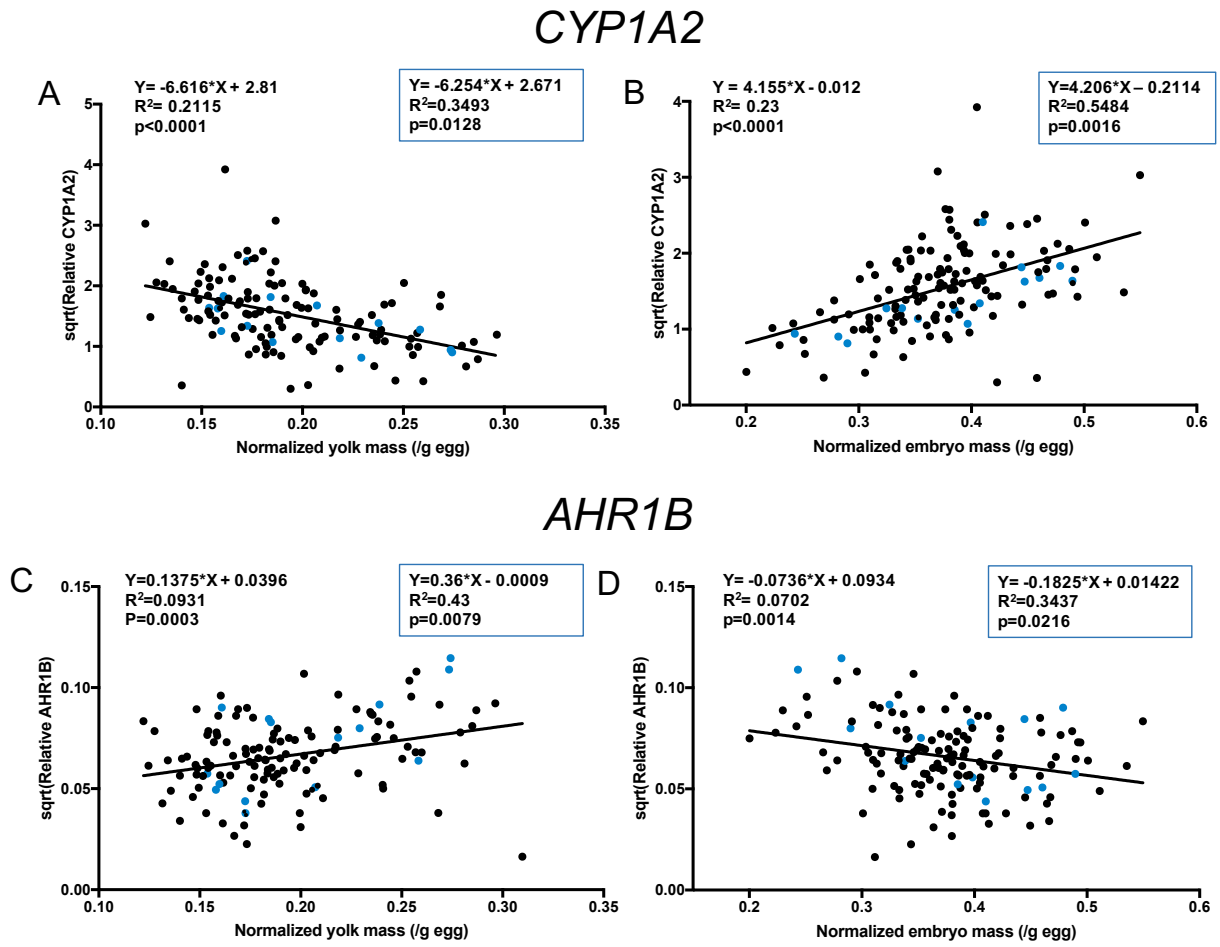


Figure 3.4. Relationship between embryo characteristics. Linear regression analysis depicts the relationship between yolk mass (A,C) and embryo mass (B,D) in hepatic expression of *CYP1A2* and *AHR1B* for all embryos from YWC and WO. Samples with yolk contaminant levels are marked in blue (regression summary for this subset in box insets). Relationships between embryo and yolk mass were statistically significant (α=0.05) for the entire dataset and contaminant subset.

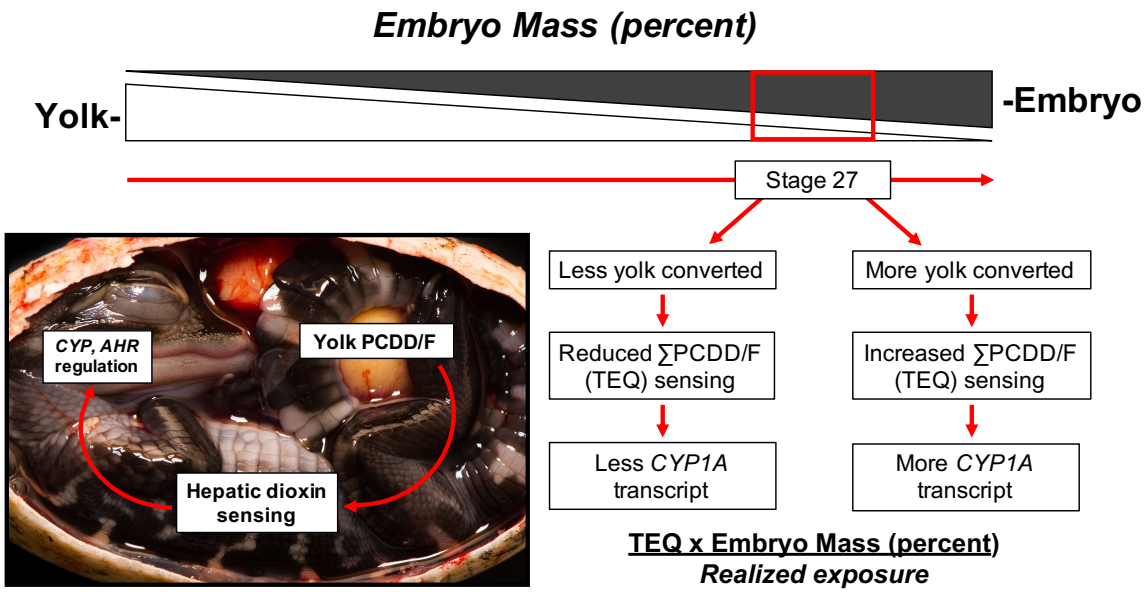


Figure 3.5. Developmental model linking yolk utilization and hepatic AHR signaling and transcription. Variation in the degree of yolk utilization within a single developmental stage corresponds to greater overall contaminant mobilization from yolk to embryonic tissue and thus greater overall contaminant exposure. Therefore, yolk contaminant concentrations and the degree of yolk utilization represent total “realized” exposure. This model informs the use of % embryo mass (mass embryo (g) / [mass embryo (g) + mass yolk (g)]) as a covariate in predictive analysis of yolk contaminant burdens and hepatic gene expression

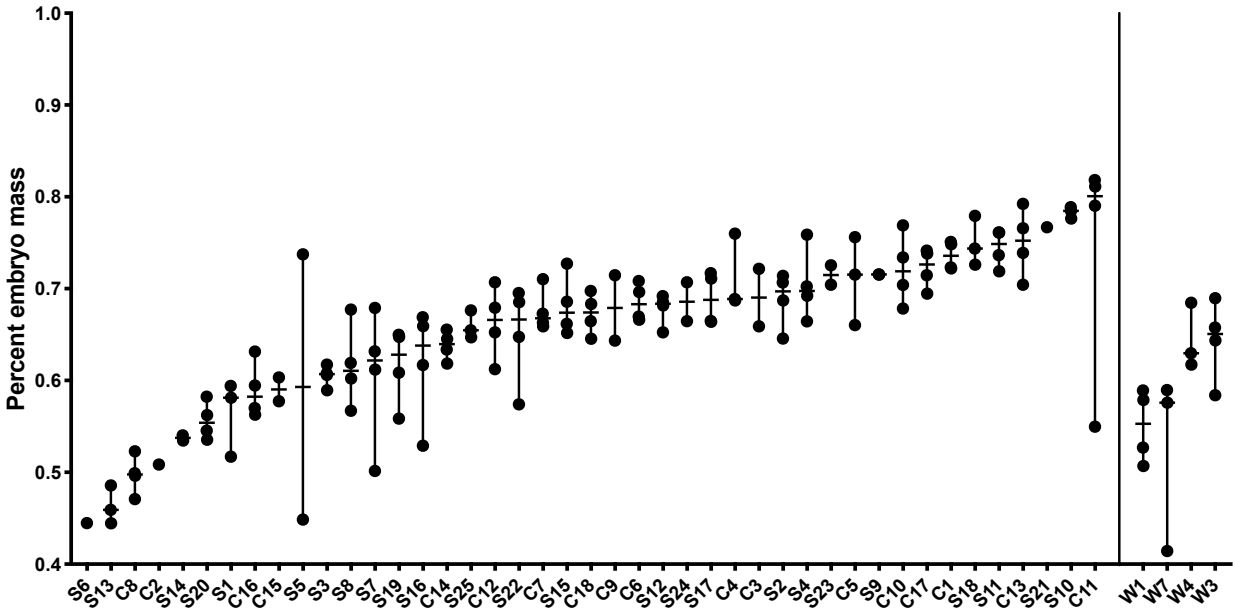
3. Appendix

3A. Table 1. Verbose dioxin/furan congener levels

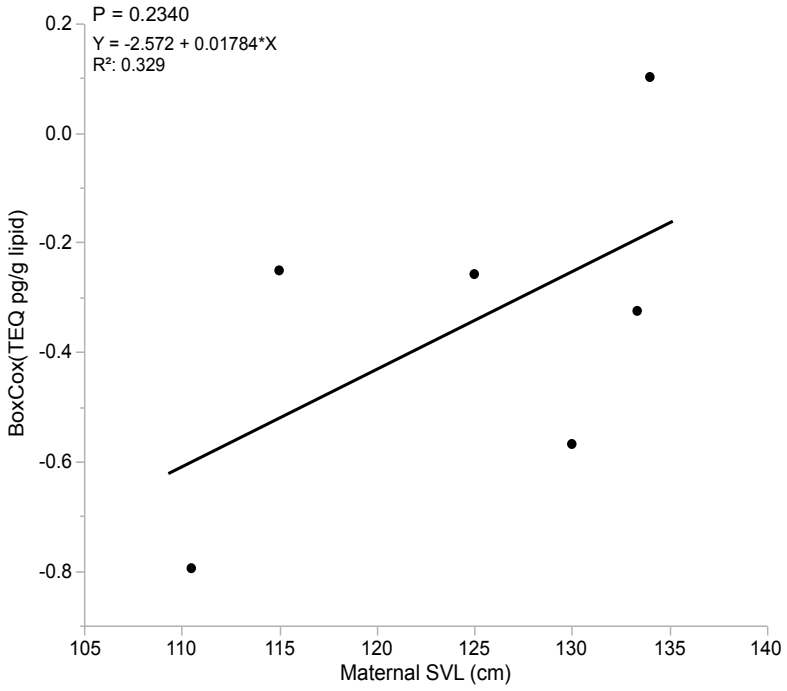
	Site	% Lipids	TEQ	2,3,7,8-TCDD	1,2,3,7,8-PeCDD	1,2,3,4,7,8-HxCDD	1,2,3,6,7,8-HxCDD	1,2,3,7,8,9-HxCDD	1,2,3,4,6,7,8-HpCDD	OCDD	2,3,7,8-TCDF	1,2,3,7,8-PeCDF	2,3,4,7,8-PeCDF	1,2,3,4,7,8-HxCDF	1,2,3,6,7,8-HxCDF	2,3,4,6,7,8-HxCDF	1,2,3,7,8,9-HxCDF	1,2,3,4,6,7,8-HpCDF	1,2,3,4,7,8,9-HpCDF	OCDF	Total TCDD	Total PeCDD	Total HxCDD	Total HpCDD	Total TCDF	Total PeCDF	Total HxCDF	Total HpCDF
W1	WO	21.77	4.95	1.64	2.27	ND	2.71	ND	1.58	7.37	1.2	0.848 (J)	1.16	ND	2.58	ND	ND	ND	ND	ND	1.64	2.27	2.71	3.14	40.2 (D,M)	16.6 (D,M)	13.4	ND
W3	WO	24.67	8.14	2.58	4.08	ND	4.56	ND	ND	8.26	2.28	ND	ND	ND	7.55	ND	ND	3.36	ND	ND	2.58	4.08	4.56	ND	46.4 (D,M)	24.9 (D,M)	30.1 (D,M)	3.36
W4	WO	24.29	4.25	1.46	1.92	ND	3.33	ND	ND	ND	1.2	ND	1.4	ND	ND	ND	ND	ND	ND	ND	1.46	1.92	3.33	ND	13.9 (D,M)	6.47 (D,M)	3.95	ND
W7	WO	24.38	2.88	ND	2.88	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.88	ND	ND	9.09 (D,M)	ND	ND	ND
C8	YWC	22.65	22.6	5.71	14.6	4.28	8.87	1.82	3.68	12.3	0.912	ND	2.31	ND	ND	ND	ND	ND	ND	ND	5.71	14.6	15	3.68	17.2 (D,M)	5.49 (D,M)	5.94	ND
C10	YWC	23.48	17.3	4.1	10.8	5.12	7.21	1.92	3.32	10.4	2.68	0.996	2.06	ND	ND	ND	ND	ND	ND	ND	4.1	10.8	14.2	5.13	7.56 (D,M)	5.45 (D,M)	ND	ND
C1	YWC	28	23.2	6.95	13.8	4.52	8.97	2.02	3.68	11.7	0.995	ND	1.85	ND	1.6	ND	ND	ND	ND	ND	6.95	13.8	15.5	6.03	11.8 (D,M)	6.46 (M)	7.11	ND
S10	YWC	24.64	4.5	1.43	2.85	ND	2.03	ND	1.43	5.86	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.43	2.85	2.03	2.59	2.68 (D)	0.924 (D,M)	ND	ND
S12	YWC	25.67	2.86	ND	2.59	ND	1.64	ND	ND	5.4	0.998	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2.59	1.64	ND	8.27	ND	ND	ND
S19	YWC	23.9	12.8	3.54	7.86	3.02	5.22	ND	ND	7.72	ND	ND	1.82	ND	ND	ND	ND	ND	ND	ND	3.54	7.86	8.25	ND	6.39	1.82 (J)	3.2	ND
S8	YWC	27.8	9.49	3.06	5.7	2.62	4.33	ND	2.78	10.7	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	3.06	5.7	6.96	4.74	8.92	ND	ND	ND
S4	YWC	26.74	11.1	4.57	5.19	ND	4.01	ND	2.22	8.34	1.38	ND	2.61	ND	ND	ND	ND	ND	ND	ND	4.57	5.19	4.01	3.93	9.18 (D,M)	3.83	1.12	ND
S2	YWC	24.61	22.9	5.86	13.9	5.17	9.76	2.19	5.29	12.2	3.83	ND	2.8	ND	1.19	ND	ND	ND	ND	ND	5.86	13.9	17.1	5.29	17.9 (D,M)	4.7 (D,M)	4.4	ND
C12	YWC	27.72	42.3	8.01	30.5	9.62	19.6	3.38	3.91	8.69	ND	ND	1.42	ND	0.975	ND	ND	ND	ND	ND	8.01	30.5	32.6	3.91	5.87 (D,M)	2.34 (D,M)	5.51	ND
S11	YWC	22.92	9.71	1.88	6.61	3.15	4.32	1.22	2.93	8	0.652	ND	0.853	ND	ND	ND	ND	ND	ND	ND	1.88	6.61	8.69	4.31	5.28	0.853	ND	ND
C13	YWC	26.31	22.4	4.35	15.1	5.98	12.6	ND	5.67	13.2	1.17	ND	1.83	ND	3.43	ND	ND	ND	ND	ND	4.35	15.1	18.6	5.67	7.18 (D,M)	1.83	12.3	ND

3A. Table 2**.** Dioxin/furan congener detection limits in alligator egg yolk.

	Site	2,3,7,8-TCDD	1,2,3,7,8-PeCDD	1,2,3,4,7,8-HxCDD	1,2,3,6,7,8-HxCDD	1,2,3,7,8,9-HxCDD	1,2,3,4,6,7,8-HpCDD	OCDD	2,3,7,8-TCDF	1,2,3,7,8-PeCDF	2,3,4,7,8-PeCDF	1,2,3,4,7,8-HxCDF	1,2,3,6,7,8-HxCDF	2,3,4,6,7,8-HxCDF	1,2,3,7,8,9-HxCDF	1,2,3,4,6,7,8-HpCDF	1,2,3,4,7,8,9-HpCDF	OCDF	Total TCDD	Total PeCDD	Total HxCDD	Total HpCDD	Total TCDF	Total PeCDF	Total HxCDF	Total HpCDF
WO1 BETA	WO	--	--	0.752	--	0.675	--	--	--	--	--	0.367	--	0.53	0.565	0.551	0.628	1.43	--	--	--	--	--	--	--	0.628
WO3 GAMMA	WO	--	--	2.63	--	2.34	2.46	--	--	0.712	0.794	0.423	--	0.667	0.766	--	1.23	4.88	--	--	--	2.46	--	--	--	--
WO4 ALPHA	WO	--	--	0.701	--	0.667	1.56	5.33	--	1.21	--	0.514	0.506	0.63	0.823	1.14	1.41	4.21	--	--	--	1.56	--	--	--	1.41
WO7 DELTA	WO	0.618	--	1.25	1.22	1.14	2.14	7.04	0.448	0.656	0.86	0.762	0.751	1.38	1.6	1.37	1.89	7.56	0.618	--	1.25	2.14	--	0.86	1.6	1.89
YK7 DELTA	YWC	--	--	--	--	--	--	--	--	0.481	--	0.397	0.393	0.475	0.486	0.813	0.898	2.15	--	--	--	--	--	--	--	0.898
YK11 DELTA	YWC	--	--	--	--	--	--	--	--	--	--	0.349	0.385	0.432	0.491	0.633	0.721	1.32	--	--	--	--	--	--	0.491	0.721
YK23 DELTA	YWC	--	--	--	--	--	--	--	--	0.828	--	0.345	--	0.412	0.45	0.45	0.473	1.15	--	--	--	--	--	--	--	0.473
BR ALPHA	YWC	--	--	0.58	--	0.554	--	--	0.386	0.376	0.406	0.47	0.487	0.573	0.62	0.495	0.543	1.13	--	--	--	--	--	--	0.62	0.543
YK22 ALPHA	YWC	0.557	--	0.823	--	0.803	1.23	--	--	0.598	0.643	0.586	0.606	0.694	0.747	0.641	0.674	1.14	0.557	--	--	1.23	--	0.643	0.747	0.674
BSB GAMMA	YWC	--	--	--	--	1.24	1.96	--	0.44	0.501	--	0.63	0.59	0.815	0.932	1.24	1.63	2.83	--	--	--	1.96	--	--	--	1.63
YK13 DELTA	YWC	--	--	--	--	1.01	--	--	0.929	0.749	0.383	0.739	0.765	0.981	0.983	0.644	0.653	1.34	--	--	--	--	--	0.383	0.983	0.653
YK18 BETA	YWC	--	--	0.993	--	0.89	--	--	--	0.876	--	0.544	0.55	0.614	0.661	0.44	0.503	1.15	--	--	--	--	--	--	--	0.503
INW DELTA	YWC	--	--	--	--	--	--	--	--	1.04	--	0.624	--	0.715	0.822	0.84	0.862	1.91	--	--	--	--	--	--	--	0.862
YK8 DELTA	YWC	--	--	--	--	--	--	--	0.611	0.412	--	0.477	--	0.549	0.575	0.51	0.499	0.878	--	--	--	--	--	--	--	0.51
YK16 GAMMA	YWC	--	--	--	--	--	--	--	--	0.593	--	0.454	0.432	0.491	0.5	0.482	0.592	1.16	--	--	--	--	--	--	0.5	0.592
PFP BETA	YWC	--	--	--	--	1.7	--	--	--	0.743	--	0.649	--	1.02	1.22	1.28	1.86	4.67	--	--	--	--	--	--	--	1.86



3A. Figure 1**.** Individual sample (circles) and clutch-ranked median (±95% confidence interval) expression values are reported for percent embryo mass (mass embryo (g) / [mass embryo (g) + mass yolk (g)]).



3A. Figure 2**.** Relationship between maternal snout-vent length (SVL) and TEQ levels in egg yolk

CHAPTER 4

EMBRYONIC ESTROGEN EXPOSURE RECAPITULATES PERSISTENT OVARIAN TRANSCRIPTIONAL PROGRAMS IN A MODEL OF ENVIRONMENTAL ENDOCRINE DISRUPTION[3]

[3]Hale MD., McCoy JA., Doheny BM., Galligan TM., Guillette LJ., Parrott BB. 2019. Embryonic estrogen exposure recapitulates persistent ovarian transcriptional programs in a model of environmental endocrine disruption. *Biology of Reproduction* 100(1): 149-161 https://doi.org/10.1093/biolre/ioy165. Reprinted here with permission of the publisher.

Abstract

Estrogens regulate key aspects of sexual determination and differentiation, and exposure to exogenous estrogens can alter ovarian development. Alligators inhabiting Lake Apopka, FL are historically exposed to estrogenic endocrine disrupting contaminants and are characterized by a suite of reproductive abnormalities, including altered ovarian gene expression and abated transcriptional responses to follicle stimulating hormone. Here, we test the hypothesis that disrupting estrogen signaling during gonadal differentiation results in persistent alterations to ovarian gene expression that mirror alterations observed in alligators from Lake Apopka. Alligator embryos collected from a reference site lacking environmental contamination were exposed to estradiol-17 beta or a non-aromatizable androgen *in ovo* and raised to the juvenile stage. Changes in basal and gonadotropin-challenged ovarian gene expression were then compared to Apopka juveniles raised under identical conditions. Assessing basal transcription in untreated reference and Apopka animals revealed a consistent pattern of differential expression of key ovarian genes. For each gene where basal expression differed across sites, *in ovo* estradiol treatment in reference individuals recapitulated patterns observed in Apopka alligators. Among those genes affected by site and estradiol treatment were three aryl hydrocarbon receptor (AHR) isoforms, suggesting that developmental estrogen signaling might program sensitivity to AHR ligands later in life. Treatment with gonadotropins stimulated strong ovarian transcriptional responses, however, the magnitude of responses was not strongly affected by steroid hormone treatment.

Collectively, these findings demonstrate that precocious estrogen signaling in the developing ovary likely underlies altered transcriptional profiles observed in a natural population exposed to endocrine disrupting contaminants.

Introduction

Endocrine signaling acts to coordinate sexual development during embryogenesis and regulate reproductive function in adulthood. During development in non-mammalian vertebrates, estrogens are of particular importance as a key determinant of sexual fate in the differentiating gonad[34,35,270,271]. However, a critical dependence upon estrogen signals presents a vulnerability to the influence of exogenous endocrine cues, including in the form of environmental contaminants. Deleterious effects of environmental endocrine disrupting contaminants (EDCs) have been uncovered in a broad suite of wildlife, including thyroid abnormalities[272–274], production of intersex individuals[275,276], decreased fertility[277–280], and general population decline[279,281,282]. These early studies pioneered our current understanding of endocrine disruption and ultimately contributed to the “developmental origins of health and disease” hypothesis[86,283]. In the present study, we seek to build on these initial observations by investigating the effects of exogenous estrogens on ovarian function in an environmental model of endocrine disruption, the American alligator.

Due to the plasticity and environmental sensitivity of their reproductive development, reptiles are disproportionately represented among early studies

focused on estrogenic EDCs and development. The alligator population inhabiting Lake Apopka (Orange County, FL, USA) is exposed to organochlorine pesticide EDCs and displays a suite of reproductive abnormalities associated with contaminant exposure. Early investigations uncovered disorganized testis morphology and reduced phallus size in juvenile male alligators, as well as an increased incidence of polyovular/multi-oocytic females[16,78]. Juveniles of both sexes also display altered circulating steroid hormone levels and disrupted gonadal steroidogenesis[16,77,80]. More recent analyses have investigated the mechanisms underlying alterations to steroid hormone levels, and have identified corresponding changes in ovarian transcriptional networks of wild-caught juveniles at Lake Apopka[284]. Furthermore, ovarian expression of steroid hormone receptors and *CYP19A1*; activin and inhibin-related signaling factor, follistatin (*FST*); and the G-protein coupled receptor, follicle-stimulating hormone receptor (*FSHR*), all appear affected[81,82,179,285,286]. These latter observations were made in animals originating from field-collected eggs that were incubated and raised under controlled laboratory settings, providing evidence that persistent ovarian phenotypes observed in exposed individuals likely originate during development.

Collective evidence suggests that the suite of abnormalities observed in exposed alligators are due in part to the action of estrogenic organochlorine pesticides (OCPs) eliciting abnormal organizational changes in the developing ovary[9,287]. For example, the increased prevalence of multi-oocytic follicles and other follicular abnormalities observed in alligators at Lake Apopka are similar to those observed in rodent models exposed perinatally to synthetic estrogens or

estrogenic contaminants[88,95,288–291]. The history of contamination at Lake Apopka includes long-term pesticide input from agricultural run-off as well as an industrial spill event of dicofol[292]. Dicofol contains both the pesticide DDT and its metabolite, DDE[293,294], both of which are detected at high levels in alligator egg yolk[26]. These contaminants, as well as other OCPs detected in alligator egg yolk, interact directly with the estrogen receptor *in vitro*[73,295] and skew sex ratios in turtles, both as individual components and in combination[296]. Critically, the most abundant contaminant at Lake Apopka, *p,p*-DDE, is capable of skewing sex ratios of exposed offspring toward a female bias, consistent with an estrogenic mode of action[96,259,296,297].

Treatment with exogenous estrogens can override male-promoting temperatures in reptiles with temperature-dependent sex determination[33,298], implicating estrogen signaling as an integral downstream effector of temperature. However, the persistent disruptions in gene expression observed at Lake Apopka suggest an additional, more nuanced role for estrogen signaling in the embryonic ovary, wherein the developmental endocrine milieu within a sex contributes to the establishment of gene regulatory networks that remain intact into adulthood. This hypothesis is supported by evidence in rodents investigating persistent effects of perinatal exposure to estrogenic chemicals in the female reproductive tract. For example, neonatal estrogen activation has been linked to persistent transcriptional shifts of growth factors, Wnt signaling components, and steroid hormone receptors in the uterus[299], vagina[95,299,300], and ovary[291,301]. Furthermore, epidemiological investigations have uncovered reproductive abnormalities,

reduced fertility, and increased cancer risk associated with fetal exposure to diethylstilbestrol, a pharmaceutical estrogen widely prescribed to pregnant women in the 1940s–70s to prevent miscarriages[90,302,303]. However, empirical demonstrations of causality have not yet been thoroughly explored in heterogeneous populations exposed to environmental estrogens or estrogenic contaminants.

We sought to investigate the role of developmental endocrine signaling in mediating persistent changes in ovarian transcriptional networks and subsequent ovarian function observed in natural alligator populations. Gonadal estrogen biosynthesis does not begin until approximately embryonic stage 21 as evidenced by the timing of aromatase (*CYP19A1*) expression[181,304]. Therefore, it is possible that developmentally precocious estrogen signaling, due to the presence of maternally-deposited estrogenic contaminants, prior to this window might be responsible for the observed abnormalities in alligators from Lake Apopka. We explored this hypothesis by administering estradiol-17β (E_2) prior to stage 21 in animals from Lake Woodruff, a reference site, and assessing whether treatment could recapitulate observed changes in gene expression in juvenile alligators naturally exposed to estrogenic OCPs (Figure 4.1). We also assessed whether ovarian responsiveness to gonadotropins is affected by precocious estrogen signaling by administering follicle-stimulating hormone (FSH) to juvenile alligators. This FSH challenge model permits insight into altered reproductive function in a species that would otherwise require 7–12 years to reach sexual maturity, and has been previously used to uncover abated functional responses

of *CYP19A1,* follistatin (*FST*)*,* and the follicle-stimulating hormone receptor (*FSHR*) in alligators from Lake Apopka[82,179]. Lastly, we addressed the possible estrogen receptor isoform-specific mechanisms that might underlie altered transcriptional profiles in contaminant-exposed juvenile alligators.

Using this model, we uncover a subset of ovarian genes for which expression varies across sites conveying different environmental exposures. In each of these instances, patterns of ovarian gene expression observed in alligators from Lake Apopka are recapitulated in E_2-treated reference animals, suggesting that environmental EDCs at Lake Apopka elicit their effects through precocious activation of estrogen signaling. We further explore the developmental origins of these alterations with dosing experiments that incorporate estrogen receptor isoform-specific agonists, and uncover that suppression of *AHR1A* and anti-müllerian hormone (*AMH*) is mediated through estrogen receptor-α (ESR1) signaling. Our findings indicate a conserved organizational role for developmental estrogen signaling in gene expression patterning in the ovary, and further highlight a means by which environmental EDCs contribute to persistent alterations in ovarian function.

Materials and Methods

Juvenile Experiment: Egg Collection, Incubation, and Dosing

All experiments performed as part of these studies conformed to guidelines approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina. All fieldwork and egg collections were

approved and permitted by the Florida Fish and Wildlife Conservation Commission. Juvenile alligators used in this study were collected as eggs shortly after oviposition from Lakes Apopka (Orange County, FL) and Woodruff (Volusia County, FL) on June 23–35, 2014. Eggs were collected from 17 clutches (102 eggs total) at Lake Woodruff and 6 clutches (102 eggs total) from Lake Apopka. Eggs were weighed, candled to assess viability, and a representative embryo from each clutch was used to assess developmental stage[180].

Following transport and staging, eggs were maintained at 32°C, a temperature that promotes development of both male and female offspring if experienced during the TSP, in damp sphagnum moss until they reached stage 19, as predicted according to Kohno and Guillette[39]. At stage 19, clutches were randomly distributed among treatment groups and dosed by topical application to the egg shell with either 0.5 μg/g egg weight E_2, 250 μg/g egg weight 5α-dihydrotestosterone (DHT), or vehicle (95% ethanol). Doses of E_2 and DHT administered were selected based on their ability to elicit morphological changes during development; 0.5 μg/g E_2 is sufficient to sex reverse alligator embryos incubated at an exclusive male-promoting temperature (MPT) to produce females[33], and 250 μg DHT induces follicular abnormalities that are consistent with polycystic ovarian syndrome in rodent models of prenatal exposure[305,306]. Concentrations of E_2 and DHT applied are much higher than endogenous levels reported in alligator egg yolk (E_2: 2-20ng/g; testosterone: 1-3ng/g[307,308] [DHT has not been investigated in alligator egg yolk]), and are comparable to levels of the most abundant contaminants reported in yolk at AP (e.g., 5.8ppm DDE[26]).

Furthermore, levels of endogenous E_2 and testosterone do not differ between sites[307]. Immediately following dosing, eggs were transferred to 30°C, an exclusive female-promoting temperature (FPT), and maintained through the TSP until hatching. Embryos that failed to complete hatching within 48 hours of pipping were manually assisted by opening the egg to minimize hatchling mortality.

Juvenile Husbandry, FSH Administration, and Necropsy

At hatching, neonates were marked for identification by notching of tail scutes and with numbered Monel tags attached to the webbing between the two middle digits on both hind limbs. Animals were housed indoors at Hollings Marine Laboratory (Charleston, SC) in custom fiberglass aquatic tanks that allowed for basking. Hatchlings were maintained on a 12/12 h light/dark cycle. At two-week intervals, animals were sorted and regrouped in tanks according to size, such that similarly sized animals were housed together. Throughout the grow-out period, tanks were cleaned and water replaced weekly. Animals were fed a commercially available diet for crocodilians (Mazuri Exotic Animal Nutrition, Richmond, IN, USA) according to size class. For approximately two months following hatching, all animals were fed daily. Once reaching two months of age, the largest animals (>132 g) were fed twice weekly; intermediate animals (93–132 g) were fed three times weekly; and the smallest animals (<92 g) were fed daily. During the experiment, animals that failed to thrive (exhibiting either no growth or negative growth between measurement periods) were observed and

excluded from the study. Neither site nor treatment had an effect on failure to thrive as assessed via chi-square test (data not shown).

At the conclusion of the 5-month grow-out period (approximately 151 days; average age = 150.8±1.6 days [SD]), animals ranged in mass from 218–510 g; average mass did not differ significantly among treatment groups and sites (data not shown). At 5 months of age, animals were randomly assigned to one of two treatment groups and were administered the first of four daily injections of either 277.8 μU/g recombinant ovine FSH (Sigma-Aldrich F8174) or vehicle (0.8% sterile saline) via intramuscular injection at the base of the tail. All injections were made between 14:00–16:00 each day. The dose of FSH used in this study has been previously shown to elicit upregulation of canonical FSH-responsive targets in the gonads of 5-month-old alligators[82]. On the fifth day following the initial FSH injections, animals were administered a lethal dose of pentobarbital (0.1 mg/g animal mass) by injection into the postcranial sinus, followed by decapitation. Gonad-adrenal complexes were necropsied, immediately dissociated into individual component tissues, and transferred to RNAlater. Gonads in RNAlater were rocked overnight at 4°C and then stored at -80°C. Final sample sizes of treatment groups are reported in Table 4.1.

Estrogen Receptor Agonists Experiment: Egg Collection, Incubation, and Dosing

Collection and husbandry of alligator embryos used in this study have been previously described[309]. Briefly, embryos were collected as eggs shortly after oviposition from Lake Woodruff (WO, Volusia County, FL) on June 15–21,

2012, from eight clutches. Eggs were weighed, candled to assess viability, and a representative embryo from each clutch was staged according to Ferguson[180]. Viable eggs were transferred to damp sphagnum moss and maintained at 30°C (FPT), until reaching stage 19. Stage predictions were calculated according to Kohno and Guillette [[39]]. At stage 19, embryos from each clutch were randomly distributed among treatment groups and dosed by topical application to the eggshell with either 0.5 μL/g absolute ethanol (vehicle control) or one of three concentrations of E_2 (0.005, 0.05, or 0.5 μg/g egg weight [Sigma-Aldrich, St. Louis, MO, USA]), 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl) trisphenol (PPT; 0.05, 0.5, or 5.0 μg/g egg weight [Tocris Bioscience, Bristol UK]), or 7-bromo-2-(4-hydroxyphenol)-1,3-benzoxazol-5-ol, WAY 200070 (WAY; 0.05, 0.5, or 5.0 μg/g egg weight). All compounds were dissolved in 95% ethanol and filtered prior to use. Following dosing, eggs were maintained at FPT throughout the TSP and until reaching stage 27, at which point they were necropsied, and gonad-adrenal-mesonephros (GAM) complexes were dissected and fixed in RNAlater (Invitrogen, Carlsbad, CA, USA). Once fixed, GAMs were dissociated into component tissues under a dissecting microscope, and gonads were assessed in isolation. Final sample sizes of treatment groups are reported in Table 4.2.

RNA Extraction and cDNA Synthesis

RNA was extracted from juvenile gonadal samples using a modified AGPC (acid guanidinium thiocyanate-phenol-chloroform[182]) column-purification method³³³³³³. Briefly, approximately 10

mg of gonadal tissue was lysed in 1 mL of a denaturing solution containing water-saturated acidic phenol, 2M guanidinium thiocyanate, 95mM sodium acetate, 12mM sodium citrate, 0.24% *N*-lauroyl sarcosine, and 14.4M beta-mercaptoethanol, using a Retsch ball mill and sterilized stainless-steel beads. Phase separation was conducted by adding 0.2 mL of 37% chloroform; RNA was isolated from the aqueous phase following mixing with 100% EtOH and binding to a silica-membrane spin-column (EconoSpin™; Epoch Life Science; Fort Bend, Texas, USA). Column-bound RNA was treated with DNase (5Prime DNase I, Gaithersburg, MD, USA) prior to elution in ultra-pure DEPC-treated water. Concentrations and purity were assessed using spectrophotometry (Nanodrop ND2000; Thermo Fisher Scientific, Waltham, MA, USA) and banding patterns on an electrophoretic denaturing gel. RNA from juvenile gonadal tissue was diluted to 67 ng/μL and a total of 1.5 μg was used for cDNA synthesis using the recommended protocol from the manufacturer (iScript reverse transcriptase kit; Bio-Rad Laboratories, Hercules, CA, USA). Resulting cDNA was diluted 1:2 in ultra-pure water. RNA extractions from embryonic gonadal samples used in this study haves been previously described[309], and were conducted using the same AGPC-column purification method as detailed above. RNA from embryonic gonadal tissue was diluted to 33 ng/μL and cDNA diluted 1:30 in ultra-pure water using the same manufacturer-recommended protocol (iScript reverse transcriptase kit; BioRad).

Quantitative Real-time PCR

Gene expression in both experiments was assessed via absolute quantification, as previously described[181,183] using a C1000 thermal cycler CFX96 real-time detection system (BioRad). qPCR reactions were conducted with 2 μL of cDNA template and a SYBR green reaction mix containing 0.2 μM primer mix, 50 mM KCl, 20 mM Tris-HCl, 0.5% glycerol, 0.5% Tween-20, 4% DMSO, 3 mM $MgCl_2$, 20 μM dNTP mix, 0.01 U/μL AmpliTaq Gold (Applied Biosystems, Inc., Foster City, CA, USA), and 0.5X SYBR Green (Life Technologies, Grand Island, NY, USA). A final volume of 50 μL per sample was run in 15 μL triplicate reactions, and target expression values are reported as the average copies/μL for each triplicate, normalized to a sample-matched internal standard. Juvenile expression values were normalized to the geometric mean of internal standard genes, ribosomal protein L8 (*RPL8*) and eukaryotic translation elongation factor 1 (*EEF1*). Embryonic expression values were normalized to beta-actin (*ACTB*). Expression values for all target and internal standard genes were determined using interpolation on a standard curve comprising gene-matched plasmid standards of known concentrations (copies/μL). Primers used were designed to be intron-spanning, and primer sequences, annealing temperatures, and amplicon information are reported below in Table 4.3. Due to increased technical variation observed at lower template concentrations, different *AHR1A* primer and plasmid standards were used during transcript quantification in juveniles and embryos (i.e., embryonic cDNA was more dilute and a more robust standard set

was developed). Both primer sets target the same region in the putative alligator *AHR1A* sequence.

Statistical Analysis

All data analyses were performed using normalized expression values with GraphPad Prism software (version 7.0b) with $\alpha = 0.05$. Outlier analysis was conducted on normalized data via the ROUT method (Q coefficient = 1%), and data were transformed via either -1*log (all genes excluding *AMH*) or square root (*AMH*) to achieve normality and homoscedasticity. Comparisons of basal (animals not receiving FSH) gene expression values in juveniles were conducted using unpaired *t*-tests (two-tailed) to assess site effects, and one-way ANOVA to assess the influence of developmental exposure among treatment groups. Dunnett's post-hoc tests were used to identify significant differences between vehicle-treated animals from Lake Woodruff and those exposed to either E_2 or DHT. Identification of genes displaying FSH-responsive expression was conducted via unpaired *t*-tests in control alligators from Lake Woodruff, comparing vehicle- and FSH-treated groups. The effects of developmental exposure on the magnitude of expression induction resulting from FSH administration were assessed using induction ratios of square root-transformed mean FSH-treated and mean-vehicle treated expression. Unpaired *t*-tests (two-tailed) were used to address site effects on induction ratios, and one-way ANOVAs were used to address the influence of developmental exposure. For each induction ratio, standard error (SE_Q) was calculated as: $SE_Q = \sqrt{([SE_A^2/x_A^2]}$

+ [SE_B^2/x_B^2]), where x denotes group means. Where necessary, non-parametric tests and Welch's correction were applied to adjust for lack of normality in all treatment groups or in cases of unequal variances, respectively. All figures reported herein are illustrated using non-transformed data, excluding induction ratios (Figures 4.3, 4.4), where ratios are the quotient of square-root transformed FSH and vehicle groups.

Results

Basal Expression in Juveniles

We first assessed the persistent effects of precocious endocrine cues on ovarian gene expression patterns in animals that were not treated with FSH. Target genes included canonical factors related to ovarian function and FSH signaling, *CYP19A1, FST*, and *FSHR*, as well as nuclear hormone receptors estrogen receptor alpha (*ESR1*), estrogen receptor beta (*ESR2),* androgen receptor (*AR*), progesterone receptor (*PR*), and glucocorticoid receptor (*GR*). We also investigated the expression of three aryl hydrocarbon receptor isoforms, *AHR1A, AHR1B*, and *AHR2*, which have been reported in the alligator but have not been described in the context of crocodilian ovarian function or development[173,223], and *AMH*. As the bulk of known contaminants at Lake Apopka have estrogenic properties, we anticipated that E_2 exposure in alligators originating from Lake Woodruff would recapitulate changes observed in alligators from Lake Apopka. No effects of developmental exposure were detected in basal expression of *CYP19A1*, *FST*, or *FSHR*, nor was expression of the nuclear hormone receptors *AR, ESR1, PR, or GR* affected (Figure 4.2). In contrast, the

expression of *AMH* ($t_{8.9}$ = 3.269, *P* = 0.009), *ESR2* (t_{16} = 2.963, *P* = 0.009), and the aryl hydrocarbon receptors *AHR1A* (t_{16} = 2.784, *P* = 0.013), *AHR1B* (t_{13} = 5.396, *P* = 0.0001), and *AHR2* (t_{15} = 11.68, *P* < 0.0001) were significantly suppressed in ovaries from alligators originating from Lake Apopka when compared to those from Lake Woodruff (Figure 4.2). Strikingly, for each gene for which a site effect was observed, developmental E_2 exposure of alligator embryos from Lake Woodruff produced similar expression patterns to those observed in Apopka animals (Figure 4.2). Contrasts between individuals from Lake Woodruff that were treated with either vehicle or E_2 were significant for *AMH* ($F_{2,16}$ = 4.29, *P* = 0.019), *ESR2* ($F_{2,18}$ = 5.23, *P* = 0.013), *AHR1A* ($F_{2,18}$ = 3.622, *P* = 0.039), *AHR1B* ($F_{2,18}$ = 6.181, *P* = 0.004), and *AHR2* ($F_{2,19}$ = 7.709, *P* = 0.001). Surprisingly, DHT-exposed animals did not differ significantly in gene expression from the vehicle-treated reference group. These data suggest that the persistent differences in ovarian gene expression observed in alligators from Lake Apopka and Lake Woodruff are likely due to developmental estrogen signaling.

FSH-responsive Gene Expression in the Juvenile Alligator Ovary

We next sought to identify genes for which ovarian expression is responsive to exogenous FSH challenge. We compared expression patterns in FSH-challenged and vehicle-treated Woodruff control animals (Figure 4.3). As expected, significant upregulation in expression in response to FSH challenge was observed for *CYP19A1* (t_{13}=6.191, *P*<0.0001), *FST* (t_{13}=2.621, *P*=0.0212),

and *FSHR* (t_{13}=3.408, *P*=0.0047), all of which have been previously reported to respond to FSH signals in the alligator[81,82]. Similarly, expression of *ESR2* (t_{13}=2.589, *P*=0.0225), *AHR1B* (t_{11}=3.71, *P*=0.0034), and *AHR2* (t_{13}=3.71, *P*=0.0231) also responded to FSH, however expression of these genes decreased in response to FSH treatment, implicating a role for FSH in downregulating these genes. Transcriptional responses were not detected for *AHR1A*, *GR*, *AMH*, *PR*, *ESR1*, or *AR*. Collectively, elevation of the canonical FSH responsive genes *CYP19A1, FST,* and *FSHR* validated the functionality of the FSH-challenge model.

Effects of Precocious Endocrine Cues on Juvenile FSH-response

Next, we sought to investigate how precocious endocrine cues might alter the functional ovarian response to FSH later in life, as Apopka animals raised under laboratory settings have been previously shown to exhibit abated responsiveness to FSH. Specifically, we anticipated that alligators from Lake Apopka would show suppressed responsiveness in *CYP19A1, FST,* and *FSHR* expression, and hypothesized that animals from Lake Woodruff exposed developmentally to E_2 would mirror these effects. To address this, we compared gene induction ratios of challenged to unchallenged expression for individual exposure groups. Unexpectedly, we were not able to detect a site-of-origin effect in induction ratios for *CYP19A1*, *FST*, or *FSHR* (Figure 4.4). Further, developmental steroid hormone exposure did not appear to impact induction ratios of these genes, indicating that estrogenic signaling in the developing ovary

does not influence responsiveness of these genes within the limitations of our experimental design. However, a site-of-origin effect was detected in the induction of *ESR1* ($t_{26} = 2.151$, $P = 0.041$), wherein animals originating from Lake Apopka appeared to display heightened responsiveness relative to those from Lake Woodruff (Figure 4.4A). Interestingly, estradiol exposure in Woodruff animals recapitulated this effect for *ESR1*, as exposed animals responded positively to FSH, whereas controls were non-responsive ($F_{2,32} = 6.508$, $P = 0.002$; Figure 4.4A). Furthermore, whereas FSH treatment significantly suppressed *AHR1B* expression in alligators from Lake Woodruff, expression appeared elevated in response to FSH treatment in Apopka animals ($t_{20} = 3.191$, $P = 0.004$; Figure 4.4B). However, whereas treatment of Woodruff embryos with E_2 appeared to switch the directionality of *AHR1B* transcriptional response, similar to the pattern observed in Apopka alligators, this effect was not statistically significant ($F_{2,29} = 2.179$, $P = 0.085$). These data suggest that FSH-responsiveness is associated with environmental quality and embryonic estrogen signaling. However, aside from *ESR1*, a clear and robust recapitulation of Apopka profiles was not observed in E_2-exposed Woodruff juveniles, indicating that the causal factors and interactions underlying variability in FSH-mediated transcriptional responses are likely more complex than our experimental design was able to resolve.

Mechanisms of ER-mediated Transcriptional Repression

The persistent changes in basal expression of *ESR2, AMH*, and the *AHRs* following precocious embryonic E_2 exposure raised questions as to the developmental mechanisms underlying these observations. To this end, we collected alligator embryos from Lake Woodruff and exposed them at stage 19 to one of three doses of estrogen receptor-selective agonists: PPT, an ESR1-selective agonist, WAY, an ESR2-selective agonist, or E_2, a non-selective agonist[33] (Figure 4.5A). The selective nature of these compounds was previously demonstrated for alligator ESR1 and ESR2 in receptor activation assays, and additional studies incorporating *in ovo* treatment with PPT and WAY have shown that sex reversal of embryos incubated at MPT and treated with E_2 is mediated by ESR1[33]. Because ESR1 appears to mediate the influence of E_2 on sex determination, we sought to test whether the intrasexual variation observed in response to embryonic E_2 treatment may also be due to ESR1 activation, or whether ESR2 mediates this effect of estrogen treatment. In late-stage embryos (stage 27), we observed a consistent effect of PPT in suppressing the expression of *AHR1A* ($F_{9,89} = 2.191$, $P = 0.029$) and *AMH* ($F_{9,89} = 5.578$, $P < 0.001$) relative to controls that was not observed at any dose of the ESR2 agonist WAY (Figure 4.5). Expression of *AHR1A* (Figure 4.5B) was significantly reduced in both the high (5 μg/g; $P = 0.001$) and medium dose groups (0.5 μg/g; $P = 0.041$) relative to controls, whereas *AMH* was suppressed in the high dose group alone (Figure 4.5E; $P = 0.002$). Collectively, changes in the expression of *AMH* and *AHR1A* in embryos suggest an ESR1-mediated mechanism underlying persistent

transcriptional changes in juveniles. Yet, it remains to be elucidated why the same effect of E_2 observed in juveniles is not similarly observed in embryos, and why suppressive effects of either PPT or E_2 were not detected for *AHR1B*, *AHR2*, and *ESR2*. These observations collectively suggest that the effects of precocious endocrine signaling may vary at particular life stages and only become realized once the ovary develops to a more mature state.

Discussion

Estrogens serve a critical role in ovarian differentiation, development, and function in vertebrates. Estrogenic contaminants have the potential to induce ectopic estrogen receptor-regulated gene expression at inappropriate developmental stages, and EDCs have been widely documented to disrupt ovarian development and function in a diverse array of vertebrate taxa[310–313]. Alligators exposed to high levels of estrogenic OCPs during development exhibit a broad array of reproductive abnormalities that span gross morphological abnormalities[16,77,78], disrupted steroidogenesis[16,80], and persistent alterations in expression of ovarian genes[81,82,285,286,314]. These observations implicate a role for estrogen signaling during development in shaping patterns of gene expression in the adult gonad and highlight a novel means by which the embryonic environment may influence intrasexual variation in reproductive function. In the present study, we showed that developmental exposure to estradiol can recapitulate persistent alterations in ovarian gene expression that are observed in natural populations of alligators exposed to high levels of estrogenic

contaminants, and that this effect is likely to at least in part be mediated through ESR1. This effect was observed in the altered expression of four nuclear receptors, *ESR2* and three *AHR* isoforms, as well as the peptide hormone, *AMH*.

Whereas experiments addressing the functional significance of our observations on both the reproductive abnormalities observed in Lake Apopka alligators and on basic ovarian function are challenging in the alligator, ample evidence from laboratory models suggests that alterations in the transcription of these genes impart substantial consequences on ovarian function. Of the two estrogen receptor isoforms expressed in vertebrate taxa, *ESR2* is expressed predominantly in follicular granulosa cells[315,316], and studies using ESR2-depleted mammalian models have revealed that loss of this receptor is associated with reduced fecundity, retarded follicular development, fewer corpora lutea, and suppressed responsiveness to gonadotropins[317]. Because dysregulated gonadotropin responsiveness has been previously observed in alligators at Lake Apopka, the suppressed *ESR2* expression reported in the present study and in previous studies[286] could plausibly explain this gonadotropin dysregulation in alligators at Lake Apopka. Similarly, disrupted *AMH* expression would be expected to impact ovarian function. During gonadal development in the alligator, administration of exogenous E_2 is sufficient to downregulate *AMH* expression in neonates incubated at MPT, an effect which co-occurs with male-to-female sex reversal[318,319]. However, within the context of typical ovarian development, the implications of estrogen signaling on *AMH* are not well understood, and little is known regarding the persistent transcriptional regulatory

effects of estrogens on *AMH* expression. Limited evidence in female rodents exposed perinatally to exogenous estrogens has demonstrated persistent upregulation of *AMH* expression in adulthood[291,320], but a suppressive role for estrogen signaling has not been similarly described. In contrast to direct transcriptional regulation, it is possible that precocious estrogen activation alters follicle dynamics, which in turn modulate *AMH* levels in the ovary.

Perhaps the most striking finding from the current study is that all three *AHRs* were expressed at lower levels in ovaries from Lake Apopka alligators, and that this pattern was recapitulated in reference animals through embryonic treatment with E_2. Similar to ESR2 and AMH, the AHRs are critical for ovarian function and reproduction, as loss of their expression results in subfertility and reduced ovulation, as well as suppressed *CYP19A1* expression and E_2 biosynthesis upon gonadotropin stimulation[321,322]. In addition, the AHR and estrogen receptor engage in a high degree of regulatory cross-talk, wherein each is capable of both attenuating and amplifying the other's transactivational potential[323]. This suggests that our observations of altered *AHR* expression in Lake Apopka alligators might contribute to altered regulation of estrogen signaling in the ovary, and could further explain the abated *CYP19A1* responsiveness reported in previous studies. However, this latter explanation is contradicted by the relatively robust *CYP19A1* responsiveness observed in the present study, necessitating further investigation to elucidate the functional consequences of suppressed *AHR* expression. Despite this, the AHR pathway contributes to normal ovarian function, as well as mediating responses to

xenobiotic endocrine disrupting contaminants, including the nearly ubiquitous polychlorinated biphenyls (PCBs) and dioxins. Altered expression of these receptors could therefore be expected to result in substantial changes to ovarian responses to these contaminants. Furthermore, whereas some limited evidence suggests that estrogen signaling can alter AHR expression *in vitro*[324] and in extra-gonadal tissues[325,326], a long-term regulatory influence of estrogens has not been demonstrated in the ovary for any species.

The suppressed expression of *AHR1A* and *AMH* in stage 27 alligator embryos following exposure to PPT suggests that altered transcription in the juvenile ovary is likely mediated in part by actions of ESR1. The underlying mechanisms by which developmental estrogen signaling and ESR1 activation might transmit changes incurred during development to transcription at juvenile or adult stages is particularly interesting. Developmental activation of the estrogen receptor could induce a persistent, stable change in epigenetic patterning and the chromatin accessibility of affected genes (e.g., ligand-bound ESR1 recruits DNA methylation machinery to the *AHR1A* promoter, leading to stable promoter hypermethylation and persistent suppression). This hypothesis is supported by a growing body of evidence implicating epigenetic modifications as a means to stably control gene transcription in the gonad[68]. In many species with temperature-dependent sex determination, including the alligator, sexually dimorphic promoter methylation regulates expression of male- and female-promoting factors, including *DMRT1, SOX9,* and *CYP19A1*[181,327–330]*.* These sexually dimorphic patterns are established during development in response to

incubation temperature, and appear to be plastic during sex determination[329,330]. However, DNA methylation patterning can be altered by EDCs[331,332], implicating a native role for the endocrine system in establishing the epigenome that can be co-opted or otherwise disrupted by endocrine-active contaminants. This epigenetic disruptive potential has been demonstrated in rats exposed perinatally to the environmental estrogen methoxychlor. Exposed rats exhibited hypermethylation of the ESR2 promoter and suppressed receptor expression in the ovary, along with a suite of reproductive abnormalities in adulthood that mirror ESR2-depleted models[301,312]. Similarly, perinatal exposure to bisphenol A (BPA), an estrogenic chemical present in plastics, has been demonstrated to elicit persistent downregulation of estrogen receptor expression in the rat testis, concomitant with promoter hypermethylation[333]. Furthermore, estrogen signaling has been implicated in the regulation of histone modifiers[334–336], including recent evidence in a TSD turtle species, *Trachemys scripta,* wherein estrogen suppresses expression of *Kdm6b*, a histone demethylase associated with activation of the crucial male-promoting gene *Dmrt1* during sex determination[336]. Collectively, these observations suggest that estrogens regulate epigenetic patterning during development, and that estrogenic EDCs might induce changes to this patterning that impart functional consequences for adult reproductive function in both sexes.

Given the contaminants present at Lake Apopka and their modes of action, we set out to explicitly address the effects of precocious estrogen signaling in the alligator ovary. Despite this focus, it is intriguing that we failed to

observe clear effects of exposure to a non-aromatizable androgen, DHT. Evidence in mammals suggests that androgen excess during development can induce persistent organizational shifts in the ovary, and associated pathologies include altered steroidogenesis and polycystic ovarian syndrome in humans and laboratory models[337,338]. Persistent dysregulation of transcription following AR activation has been poorly studied in non-mammalian systems; however, in the chicken, AR is expressed at high levels in the early ovary[339] and its loss is associated with disrupted gonadal development[340]. These observations are consistent with a role for disrupted androgen signaling in shaping the functional trajectory of the ovary. Similarly, exposure to 17-α methyltestosterone, another non-aromatizable androgen, can induce formation of testis-like characteristics in alligator embryos incubated at FPT[341], further suggesting a key role for androgens in gonadal differentiation in reptiles and birds. However, the consequences of precocious AR activation have not been well described in either of these systems. The lack of any overt effects of DHT in the current study suggests that the AR does not participate in early development of the gonad, but more research is necessary to support this observation.

It is likely that the implications of exposure to EDCs in the alligator covaries with the timing of exposure, as has been suggested in human reproductive disorders[310]. This raises the possibility that topical applications of E_2 and DHT in the current study do not completely recapitulate exposure dynamics to maternally-derived contaminants in yolk at AP. In light of this, our observations resolve some degree of variation in reproductive outcomes by linking

contaminant-driven estrogen signaling to a steroidogenically-precocious window in gonadal development. In conclusion, observed changes in expression of *ESR2, AHR1A, AHR1B, AHR2,* and *AMH* indicate that the nature of the embryonic environment is intimately linked to gonadal function later in life and that exposure to exogenous estrogens at particular developmental windows can induce persistent changes in critical transcriptional programs. Although additional research is necessary to assess the functional consequences of these changes, the implications of these findings are potentially broad and stand to inform our understanding of the developmental origins of adult reproductive disease in wildlife and humans alike. Furthermore, these observations expand the putative functions for estrogen signaling in the developing ovary to include the establishment of patterns of gene expression and regulation.

Table 4.1. Juvenile experiment treatment group summary. Summary of stage 19 treatment and follicle-stimulating hormone (FSH)-challenge groups in juvenile study, including sample sizes following exclusion of animals that failed to thrive.

Site	Stage 19 Treatment	5-month Treatment	Total n
Woodruff	DHT	FSH	7
	(250 μg/g)	Vehicle	5
	E_2	FSH	7
	(0.5 μg/g)	Vehicle	9
	EtOH	FSH	7
	(0.5 μL/g)	Vehicle	8
Apopka	EtOH	FSH	8
	(0.5 μL/g)	Vehicle	10

Table 4.2. Embryo experiment treatment group summary. Summary of stage 19 treatment with ER-selective agonists, including dosage and sample sizes

Site	Stage 19 Treatment	Dose (μg/g)	Total n
Woodruff	E_2	High (0.5)	10
		Medium (0.05)	10
		Low (0.005)	10
	PPT	High (5.0)	9
		Medium (0.5)	11
		Low (0.05)	11
	WAY	High (5.0)	11
		Medium (0.5)	11
		Low (0.05)	11
	EtOH	0.5 (μL/g)	10

Table 4.3. qPCR primers, annealing temperatures, and amplicons.

Gene	Primer Sequence (5'-3')	Annealing Temperature (°C)	Amplicon Size (bp)
CYP19A1	GCAGCCCTTACTTGAGATGG TGGACTAGGGCAATGAGAGC	64	114
FST	GCCTACTGGGCAGATCCAT CCTTGAAATCCCACAAGCAT	64	111
FSHR	GAAATTACCAAACGAGGTTTTTCAA GGGCAGGAAACTGATTCTTGTC	60	87
AMH	AGTGAGCCAGGAGAGAACCA TCCAGGATAAAACACCAGCA	62	152
AR	GCCAGACTCCTTCTCCAACC TCTCCATCCCATGGCGAAAA	62	177
ESR1	AAGCTGCCCCTTCAACTTTTTA TGGACATCCTCTCCCTGCC	64	71
ESR2	CCAAAGAGCCCATGGTGTGA ACCATTTGCAATGGGACTTGT	64	114
PR	AGCAGTTGGATTGCGCCAGAA TCAGTGCCCGAGACTGAAGA	64	143
GR	CGTTGGACTGCTGAATTCCTTT AAAAAACTGTCCCGCATGCC	64	103
AHR1A juvenile	GTTACACAAGTTCCAAAACGGT GGATGCCAAGTCTGAGAAGG	64	151
AHR1A	CCAGTTATGCTGACTCCTCAA	64	189

embryo	CTGAGGGGGATATGCTTCATT		
AHR1B	CTGTTACTACCTACAAGCCTGACC GAAACTTCAACCGTCCTTGGAG	68.4	141
AHR2	TCCTACCCACGTGAACCAAA GGTGAATTCCATGGGAGCATT	64	135
RPL8	CTCTCACAATCCTGAAACCAA GTTTGTCAATACGACCTCCAC	62	116
EEF1	CGTTCTGGTAAGAAGCTGGA TGACACCAACAGCAACAGTC	62	168
ACTB	GAGGGTTTTAGGTGTAACTGCTTG ACATACTGGCACCGCTTTTC	62	195

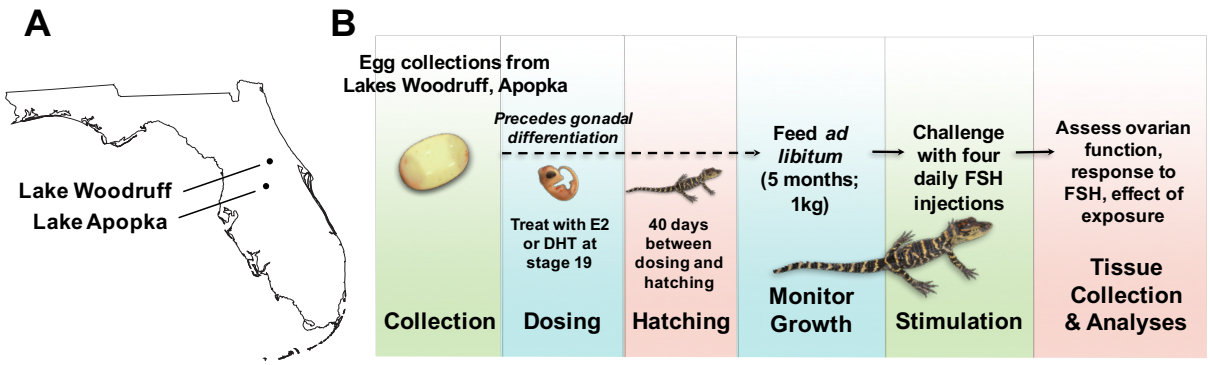


Figure 4.1. Juvenile study sampling locations and experimental design. Embryos used in this study were collected from one of two locations: (A) Lake Woodruff, a reference site with low historical presence of environmental contaminants, and Lake Apopka, a site characterized by long-term input of anthropogenic contaminants owing to its proximity to agriculture and an acute spill event. (B) Embryos from these sites were exposed to one of three treatments at stage 19, prior to gonadal sex determination: estradiol-17β (E2); dihydrotestosterone (DHT), a nonaromatizable androgen; or a vehicle control. Embryos were then allowed to hatch and were raised to approximately five months of age, at which point they were administered either ovine follicle-stimulating hormone (FSH) or a vehicle control once daily for 4 days prior to necropsy.

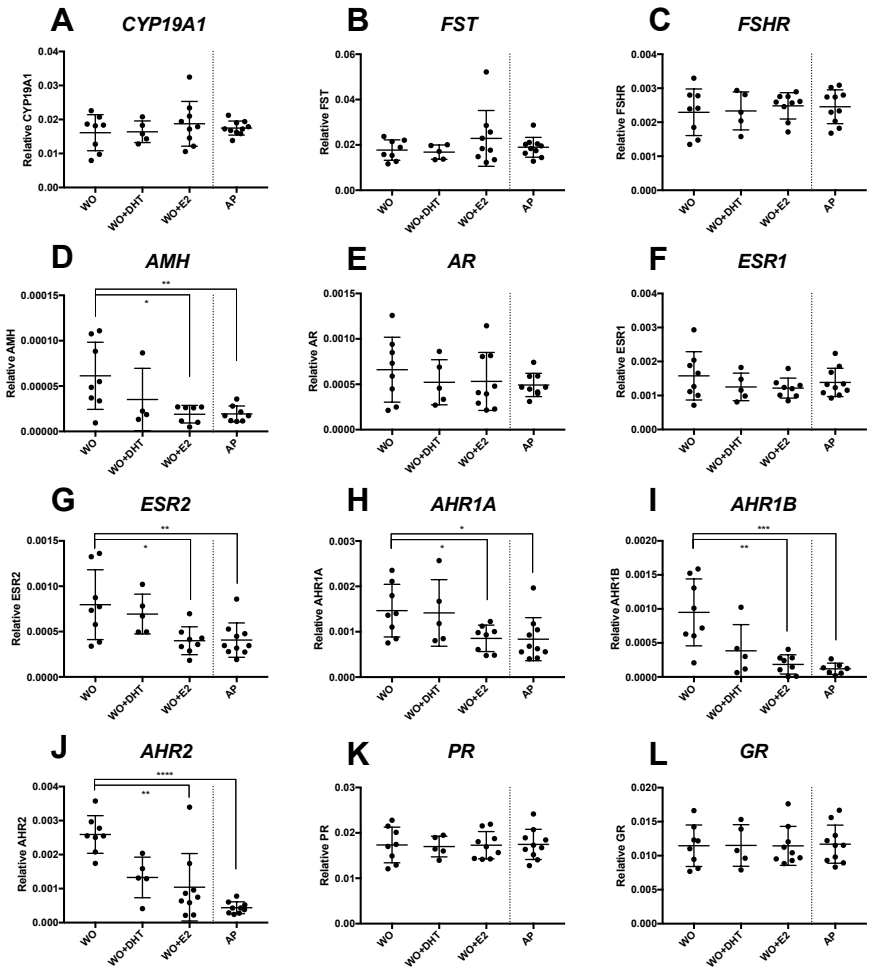


Figure 4.2. Precocious endocrine exposures and basal gene expression. The influence of precocious estrogen or androgen exposure on basal ovarian gene expression (absent gonadotropin challenge) in juvenile alligators from Lake Woodruff (WO) and Lake Apopka (AP) is reported for (A) *CYP19A1*, (B) *FST*, (C) *FSHR*, (D) *AMH*, (E) *AR*, (F) *ESR1*, (G) *ESR2*, (H) *AHR1A*, (I) *AHR1B*, (J) *AHR2*, (K) *PR*, and (L) *GR*. Brackets denote treatment groups that differ significantly from vehicle-exposed reference animals and asterisks denote P-value magnitude (**** $P \leq 0.0001$; *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$) Points represent individual animals, whereas bars indicate group means ± SD.

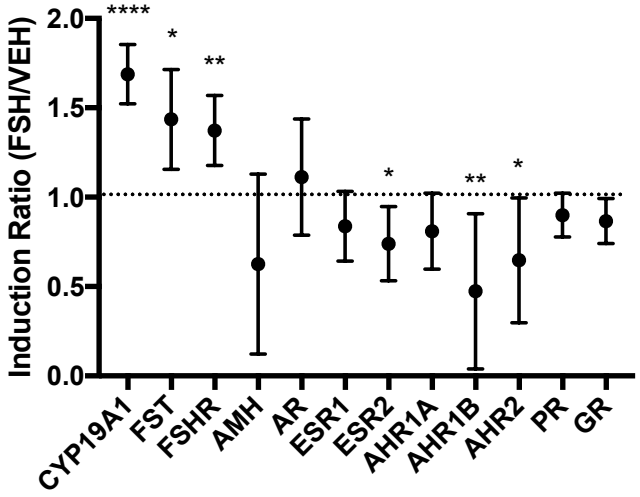


Figure 4.3. FSH-driven transcriptional responses. Induction ratios (expression levels in FSH-treated animals/expression levels in vehicle-treated animals; FSH/VEH) of targeted ovarian genes with 95% CI are reported in vehicle-exposed reference animals from Lake Woodruff. An induction ratio of 1, indicated by the dotted line, denotes no effect of FSH challenge. Asterisks above bars denote significantly responsive genes and P-value magnitude (**** $P \leq 0.0001$; *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$).

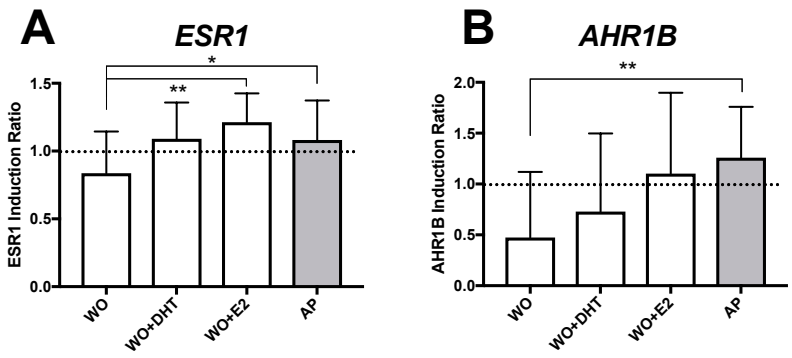


Figure 4.4. Precocious endocrine cues and FSH responses**.** The ability of precocious endocrine cues to alter gene expression in the FSH-challenged juvenile ovary is reported as induction ratios (FSH/VEH ± SD) for (A) *ESR1* and (B) *AHR1B.* Brackets denote treatment groups that differ significantly from vehicle-exposed reference animals and asterisks denote P-value magnitude (**** $P \leq 0.0001$; *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$).

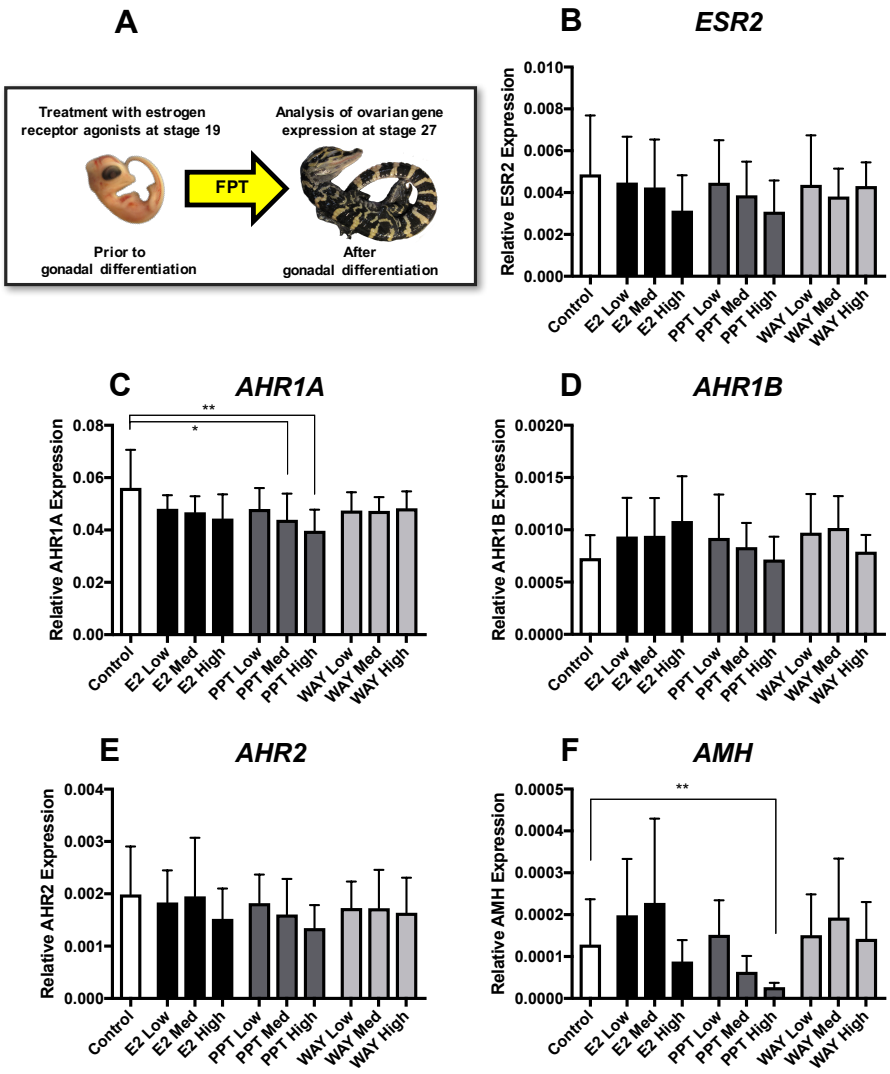


Figure 4.5. Ovarian responses to ER-selective agonists. The influence of exposure to estrogen receptor-selective agonists on gene expression in the embryonic ovary (A) is reported (group means ± SD) for (B) *ESR2*, (C) *AHR1A*, (D) *AHR1B*, (E) *AHR2*, and (F) *AMH*, genes that were persistently altered in the juvenile ovary. Agonists include E_2, which is non-selective, PPT, an ESR1-selective agonist, or WAY, an ESR2-selective agonist. Brackets denote significant differences relative to controls and asterisks denote P-value magnitude (**** $P \leq 0.0001$; *** $P \leq 0.001$; ** $P \leq 0.01$; * $P \leq 0.05$).

CHAPTER 5

THE ROLE OF PRECOCIOUS ESTROGEN SIGNALS DURING DEVELOPMENT AS DRIVERS OF ALTERED OVARIAN FUNCTION IN A WILDLIFE MODEL OF ENVIRONMENTAL HEALTH[4]

[4]Hale MD., Parrott BB. To be submitted to *Proceedings of the National Academy of Sciences of the United States of America*

Abstract

The introduction of anthropogenic chemicals into natural systems reflects one of the most influential sources driving environmental degradation over the last century. Of these, special concern has developed in recent decades over a group of contaminants that can interfere with normal functioning of the endocrine system, or endocrine disrupting contaminants (EDCs). By mimicking, antagonizing, or otherwise disrupting the function of endogenous endocrine hormones, EDCs can elicit numerous deleterious effects in exposed organisms. However, the mechanistic underpinnings of contaminant-induced pathologies remain poorly described and foundational knowledge is derived from associative epidemiological studies in exposed human or wildlife populations. In an effort to directly elucidate mechanisms underlying reproductive pathologies resulting from EDC exposure in environmentally-relevant settings, we employ a well-characterized wildlife model of endocrine disruption, the American alligator, wherein developmental exposures to endocrine-disrupting organochlorine pesticides (OCPs) are associated with a suite of reproductive abnormalities. Utilizing a non-targeted, genomics-based approach, we describe the ability of precocious estrogen signaling to recapitulate broad patterns of functional divergence in the ovary associated with environmental contaminant exposure. Specifically, lab-raised juvenile alligators exposed *in ovo* to OCPs exhibit broad shifts in ovarian transcription of genes related to cytoskeletal features and proliferation; these shifts are overwhelmingly recapitulated in reference animals exposed to an acute dose of estradiol during the bipotential stage, prior to

acquisition of sex-specific gonadal steroidogenesis. Transcriptional changes in both OCP- and estradiol-treated animals are further enriched for regulatory elements belonging to the polycomb group of proteins, suggesting that patterns might originate in an endocrine-dictated epigenetic landscape during development. Further, concomitant changes in follicle density are observable in both the OCP and estradiol exposed ovary and transcriptional shifts are closely associated with follicle numbers, indicating that follicle dynamics are both highly sensitive and drive functional regimes in the ovary. Collectively, this work elucidates potential mechanisms underlying contaminant-induced pathologies in an environmentally relevant context and ultimately informs our understanding of the basic functions of the endocrine system during development.

Introduction

The last century of human industrial development has been characterized by the widespread production and release of anthropogenic chemical pollutants into natural systems. In the last three decades, mounting concern has developed regarding the ability of a subset of these environmental pollutants to interfere with the normal functioning of the endocrine system, termed endocrine disrupting chemicals (EDCs). The disruptive mechanisms of EDCs are diverse, including hormone mimicry or blocking, disruption of hormone synthesis or clearance, or altered transport throughout the body[10,85]. Of these, the proclivity of contaminants to disrupt estrogen signaling, particularly to mimic the function of endogenous estrogens, is among the best studied modes of action for EDCs[146]. Because

estrogens regulate patterns of proliferation and differentiation during embryonic stages, inappropriate activation of signaling can induce dramatic shifts in tissue patterning that persist into later life stages. Consistent with Barker and colleagues' "developmental origins of health and disease" model (DOHaD)[342–344], these shifts are associated with adult disease and disease susceptibility, particularly in endocrine-regulated tissues such as the reproductive system[9,310]. For example, EDC exposure during development is linked to numerous reproductive disorders in women, including endometriosis, polycystic ovarian syndrome, uterine fibroids, breast cancer, and premature ovarian failure (reviewed in [310]), and in men, including hypospadias, testicular and prostate cancer, and cryptorchidism (reviewed in [345]).

The precise mechanisms underlying reproductive pathologies induced by estrogenic EDCs are not fully understood, but are thought to involve altered patterns of cellular differentiation and proliferation caused by inappropriate activation of the estrogen receptor and expression of estrogen-regulated genes[91,346]. For example, women exposed *in utero* to the non-steroidal estrogen diethylstilbestrol (DES) commonly develop uterine and vaginal morphological abnormalities, including rare cervical and vaginal cancers (reviewed in [90,347]). In rodent DES models, these perinatal exposures induce spatiotemporal shifts in the expression of estrogen-regulated HOX and *Wnt* factors, which control regional specification in the developing reproductive system; these shifts underlie abnormal development of the uterus and vagina, and are concomitant with DES-induced epithelial hyperproliferation in the vagina[92,300,348–353]. Furthermore,

transcriptional shifts in HOX expression as well other estrogen-responsive genes induced by DES are accompanied by DNA methylation changes[348,349,354,355]. Critically, reproductive abnormalities and underlying epigenetic shifts are similarly induced by other non-steroidal estrogens[301,332,356–358], suggesting a common mode of action for estrogenic EDCs in shaping the patterns of cellular differentiation and imprinting expression of proliferation or differentiation-related genes through epigenetic modifications.

Despite the fundamental utility of laboratory models for the investigation of mechanisms behind EDC-induced pathologies, they can present challenges to assessments of risk for particular contaminants. Chiefly, traditional approaches in toxicology investigate the effects of EDCs in isolation and then attempt to inform risk assessment in humans or other species in a contaminant-centric paradigm. However, this focus on singular contaminants can fail to accurately represent complex contaminant mixtures that occur under environmental settings[141,144,145]. These relative shortcomings in traditional models highlight the necessity of ecologically-relevant research models to explore EDC-induced pathologies. Consistent with the sentinel species concept[6], wildlife species exposed *in situ* have repeatedly served as models for environmental health to fill this gap[16,277,278,359]. Among wildlife models, a population of American alligators inhabiting a highly contaminated freshwater system, Lake Apopka, in central Florida (Orange County, FL) has contributed much to our current understanding of how contaminant exposure during embryonic stages determines future reproductive health. There, developmental exposures to a complex mixture of

estrogenic endocrine disrupting organochlorine pesticides (OCPs)[26,30] are associated with a suite of reproductive abnormalities, including ovarian and testicular morphological abnormalities[16] and shifts in gonadal transcription that persist into juvenile stages[28,81,82,179,284]. Reproductive pathologies at Apopka have been repeatedly been observed in juvenile animals collected as eggs and raised under laboratory settings, implicating exposure to EDCs *in ovo* as causative agents[16,28,82].

In the present study, we sought to leverage the utility of the alligator as an environmental model to probe the functional consequences of developmental EDC exposures in naturally exposed organisms, and to elucidate the mechanisms underlying complex contaminant-induced pathologies. Specifically, we employ a non-biased, genomics-based approach to describe functional divergence in the ovarian transcriptome associated with estrogenic EDCs exposure at Apopka in lab-raised juvenile animals (Figure 5.1). To further explore population-level divergence in a functional context, we utilize a gonadotropin-challenge model[28,81,82], wherein juveniles can be stimulated with exogenous follicle-stimulating hormone (FSH) to probe functional consequences of contaminant exposures *in ovo*. Lastly, we investigate the ability of a single precocious dose of estradiol-17β during a bipotential of gonadal development in reference animals to recapitulate functional shifts observed in juveniles exposed to estrogenic OCPs at Apopka.

Methods

Animal Husbandry, FSH Administration, and Sample Collection

All experiments described herein were reviewed and approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina (Charleston, SC) and alligator egg collections were approved and permitted by the Florida Fish and Wildlife Conservation Commission. Husbandry for animals used in this study has been previously described[28,360] in detail. Briefly, alligator embryos were collected as eggs from two sites in Florida, Lake Apopka (AP; Orange county, FL) and Lake Woodruff NWR (WO; Volusia county, FL). Both locations and populations are well-characterized; Lake Apopka is characterized by high levels of organochlorine pesticide (OCP) contaminants, the product of long-term agricultural runoff and an acute spill event of dicofol, a ΣDDT and metabolite-containing miticide, in the 1980s. These contaminants are detectable at high levels in adult and juvenile alligators, and in egg yolk[16,26,66,78]. In contrast, Lake Woodruff NWR is a relatively pristine site with minimal anthropogenic disturbance or contaminant input, and is used herein as a reference site[16,77,81,286,297,314,361]. Eggs were collected from both sites in June 2014, candled to assess viability, staged[39,180], and transferred to artificial nests of damp sphagnum moss at the Hollings Marine Laboratory (Charleston, SC). At AP, 102 eggs total were collected from 6 clutches; at WO, 102 eggs total were collected from 17 clutches. Eggs were maintained at 32°C until stage 19, at which stage they were randomly distributed among three treatment groups and dosed with either 0.5μg/g (egg weight) estradiol-17β (E2), 250μg/g

dihydrotestosterone (DHT), or vehicle control (95% EtOH). WO-DHT treated and AP-DHT and E2 treated animals were not used in any analyses presented herein.

Following treatment, embryos were incubated at 30°C, a temperature which produces exclusively females, until hatching. Upon hatching, animals were individually marked with numbered monel tags between the middle digits on both hindlimbs and transferred to indoor fiberglass tanks at the Hollings Marine Laboratory that permit both basking and swimming. Neonates were maintained on 12/12 light dark cycles and fed *ad libitum* for approximately two months following hatching. To ensure that neonates were housed with similarly-sized individuals, all animals were weighed and sorted by mass every two weeks. Once reaching two months of age, animals were switched to a feeding schedule based on mass to ensure relative homogeneity of animal size. Upon reaching five months of age, animals across all treatment groups were further divided into two treatment groups and were dosed once daily for four days with either 277 μU/g recombinant ovine FSH or vehicle control (0.8% sterile saline) via intramuscular injections at the base of the tail. On the fifth day following initial treatment, animals were euthanized with 0.1 mg/g pentobarbital followed by decapitation. Ovaries were then necropsied and weighed; the right ovary was fixed in RNAlater, rocked for 12hr on an orbital shaker at 4°C, then frozen at -80°C. The left ovary was fixed in 4% formaldehyde (10% NBF) for 24hr and stored at 4°C in 70% ethanol. Total RNA was isolated from RNAlater-fixed right

ovaries using a modified AGPC extraction with silica column purification[28] for RNAseq analysis.

RNAseq Read and Count Data Generation

Alligator RNAseq library preparation and sequencing was conducted by the Georgia Genomics and Bioinformatics Core at the University of Georgia. Briefly, 1-2μg total RNA (n=7 libraries per treatment group) were assessed for quality via Agilent 2100 Bioanalyzer (RIN $\geq$ 7.80, average = 8.6) and then diluted to a common concentration. Libraries were prepared from poly(A)-enriched mRNA using the KAPA Stranded mRNA-Seq kit for Illumina platforms (KAPA Biosystems, Cape Town, South Africa), and were sequenced on an Illumina NextSeq (150 cycles, 75-bp paired end reads) in two individual sequencing runs. Sample libraries used in (1) assessment of population-specific responsiveness to FSH, (2) expression differences in the non-challenged ovary across populations, (3) and FSH-by-population interactions were prepared and sequenced simultaneously (WO-FSH, WO-VEH, AP-FSH, and AP-VEH). Sample libraries used in (4) assessment of the effects of precocious estrogen signaling were prepared and sequenced independently of the previous run (WO-VEH and WO-E2-VEH). Identical WO-VEH samples were sequenced in both experiments, but libraries were prepared independently (i.e libraries were not resequenced but were generated de-novo). Read quality was assessed via FastQC (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc) and MultiQC (1.5)[362].

Following QA, reads from both sequencing runs were determined to lack adapter contamination (<0.1% in all samples) and exhibited mean read quality Phred scores > 30 and therefore were not subjected to quality trimming. Fastq reads were aligned to the most recent alligator genome assembly (ASM28112v4)[363] via Hisat2 (2.1.0)[364] with parameter –dta and guidance parameters -ss and -exon enabled during index generation; splice site and exon coordinate information were generated from alligator Refseq annotations (GCF_000281125.3, annotation release 102) using BEDtools (2.26.0)[365]. Resulting alignments were coordinate-sorted and converted to .bam format via Samtools (1.6)[366]. Once sorted, count matrices were generated using R packages GenomicFeatures[367] and GenomicAlignments[367]. GenomicFeatures function “makeTxDbFromGFF” was used to generate exon-by-gene coordinates for the alligator assembly, which were then used in GenomicAlignments to generate feature counts from sorted alignments, using function “summarizeOverlaps” (parameters mode= “Union”, fragments=TRUE, singleEnd=FALSE, ignore.strand=FALSE). This generated a count matrix with 24848 unique genes in both sequencing runs.

Histology and Ovarian Morphology

PFA-fixed ovaries were bisected on their transverse plane and halves were paraffin embedded and sectioned at 4μm thickness. Sections were H&E stained and imaged, then composite-stitched using a Keyence BZ-X710 at 10X or 20X magnification. Images were analyzed in ImageJ (1.52a). Briefly, total

cortical area was measured using the freehand selection tool. Stage III oocytes were identified by a pink basophilic cytoplasm and by the presence of a complete follicular granulosa cell layer and one or more surrounding thecal cells[368,369]. Follicle diameter was measured for each object on its longest axis. When possible, images from both ovarian halves were analyzed, and resulting measures averaged to represent each ovary; one representative section or pair of sections were analyzed from each sample. Follicle density is reported as the number of late stage II or stage III follicles normalized to total cortical area. As the majority of follicles observed were definitively stage III (85.9%) and late stage II were rare, both stages were combined into a single measure of follicle count per unit cortical area. Furthermore, average follicle diameter, a tool for differentiating stage II and III follicles, did not differ significantly by site or FSH (data not shown), indicating that groups did not differ in relative proportion of these two follicle types.

Differential Gene Expression and Follicle Analysis

Identification of differentially expressed genes was conducted using R (R Core Team; www.R-project.org; version 1.1.456) package edgeR[370–373]. In the first experiment describing population-level effects between AP and WO (no E_2-treated animals), low expression genes (CPM<1, minimum number of expressing libraries = 7) were removed prior to analysis (18,435 genes passing filtering). In the second experiment comparing WO controls and WO-E_2 animals, library sizes were approximately doubled, thus filtering was relaxed (CPM<0.5, minimum

number of expressing libraries = 7), retaining 19,230 genes for analysis; multi-dimensional scaling (MDS) and principal components analysis was used to identify and remove two WO-FSH treated samples, given their relative distance from remaining libraries in that treatment group and high BCV values (Appendix 5A Figure 1; n=5 after removal). Histological examination of these samples revealed that they exhibited abnormally low follicle counts for WO samples. Following filtering in both experiments, libraries were TMM normalized to adjust for composition biases and fit to a negative binomial model ("glmQLFit", robust=TRUE). Hypothesis testing was conducted with planned linear contrasts via quasi-likelihood F-test ("glmQLFTest"). Genes with an FDR-adjusted p-value <0.05 were considered significant. Overlap of DEGs between WO and AP basal contrasts and WO-E_2 exposure was assessed by identifying presence/absence of significantly affected genes across experiments; significant recapitulation of genes elevated and suppressed at AP and in E_2-exposed WO animals was determined using the hypergeometric distribution (α=0.05). Because total features passing low-expression filters differed slightly between experiments (n=18,435 vs n=19,230), only shared features passing filtering in both experiments were used in recapitulation analyses (n=18,360 shared genes). Overlap between gene lists was identified using Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

Total body mass at necropsy (g) and follicle density (counts per unit area) data were analyzed with 2-way ANOVA in GraphPad Prism (version 8.0.1). To account for zero values in follicle density data (ovarian samples with no

observable stage III oocytes), a small nominal value (1.0×10^{-8}) was added for all samples. Homogeneity of variances across treatment groups was confirmed via Bartlett test ($\alpha = 0.05$). Normality was confirmed via manual inspection of residual distributions and confirmed via Shapiro-Wilk test of normality ($\alpha=0.05$). All treatment groups for the three metrics assessed met both assumptions. Post-hoc tests were conducted via Tukey's multiple comparisons test within FSH groups (FSH-treated and non-challenged groups independently). Mass at necropsy did not differ among groups (Appendix 5A Figure 2)

Annotation of Uncharacterized Loci

During DEG analyses, we identified a large proportion of uncharacterized loci in RefSeq annotations (approximately 30% of genes passing filtering), many of which were consequential for functional enrichment (e.g *CYP19A1* [LOC102566432], *CYP17A1* [LOC102567971], and *CYP11A1* [LOC102569028]). To confirm the identity of these genes, read alignments were assembled into transcripts using StringTie (1.3.3) in a 2-pass assembly approach[374,375], merging transcripts across all libraries (parameter –merge). Fasta sequences were then extracted from the StringTie merged assembly via the GFFread utility in Cufflinks (2.2.1)[376] and used in NCBI Blastx (https://blast.ncbi.nlm.nih.gov/Blast.cgi) against the Uniprot Swiss-prot database[377] (e-value cutoff $1e^{-5}$). Top hits according to e-value for each unannotated transcript were used as evidence for gene identity in downstream analyses. Any LOC loci that was not identified via this approach was excluded from downstream analyses.

Functional Enrichment of DEGs: Transcription Factors, Gene Ontology, and KEGG Enrichment

Functional annotation of GO terms (biological process), KEGG pathways, and enriched transcription factors for affected genes was conducted using gProfiler[378] and Enrichr[379,380]. Analyses were limited to genes changing by at least two-fold between treatment groups. Transcription factor enrichment was assessed using the ChIP-X Enrichment Analysis (ChEA 2016) database[381] through Enrichr, using an adjusted p-value < 0.05 to identify significantly enriched terms. To confirm these results in an alligator ovary-specific context, enrichment in affected genes lists (DEGs at least two-fold responsive) was compared to total enrichment in all annotated ovarian genes passing filtering (background) using Fisher's exact tests ($\alpha = 0.05$) (Appendix 5A Table 1 [AP-responsive] and Table 2 [E2-responsive]). Gene ontology and KEGG pathway analysis was conducted using unordered enrichment testing in gProfiler (version r1760_e93_eg40), with a Benjamini-Hochberg FDR-adjusted alpha, and strong hierarchical filtering against an alligator ovary-specific background. Prior to enrichment testing, alligator annotations were converted to human Ensembl gene IDs. Electronic annotations were excluded.

Gene Expression Clustering and Trait Association Analyses

Associations between gene expression patterns and follicle densities were assessed using two independent approaches using R package psych in RStudio. First, read counts passing filtering in both experiments were vst transformed

using package DESeq2[382]. Pearson correlation coefficients between sample follicle densities and expression values were determined using psych package function “corr.test” with an FDR-adjusted p-value (α=0.05). Correlative associations were also explored using weighted gene cluster network analysis via WGCNA[383]. Briefly, read counts from 26 libraries generated in the first sequencing run were filtered to remove low expression genes and vst-transformed as described above. Pairwise Pearson correlations were then calculated in WGCNA for transformed read counts to generate a signed regulatory network using the blockwiseModules function (parameters: soft threshold = 17; mergeCutHeight = 0.15; minModuleSize=30; maxBlockSize = 18435). This hierarchical clustering analysis yielded a cluster dendrogram (Appendix 5A Figure 3) comprised of 12 co-expressed gene modules ranging in size from 6469 to 35 and 1 module of orphaned (unassigned) genes. Principal components analysis was then conducted to extract eigengenes (PC1) for each gene module. Following extraction, significant (α = 0.05) associations between module eigengenes and follicle density were investigated using linear mixed-effects models in R (package “nlme”), using follicle density, site (AP/WO), and their interaction as main effects and FSH as a random effect. Homogeneity of variance and normal residual distributions were investigated for each model using Bartlett tests and Shapiro-Wilk goodness of fit tests, respectively. The best variance-covariance structure for each model was determined by AIC values; models with lowest AIC scores passing normality and homogeneity tests were selected for further analysis. Presence/absence of DEGs within each module

were then used to assess relationships between differential gene expression and follicle density; membership in a module with an eigengene highly correlated with follicle density was taken as evidence that expression was associated with follicular profiles.

Results

Population Divergence in the Resting Ovary

To first address shifts in ovarian function across populations, we investigated transcriptomic divergence in the resting ovary at WO and AP, absent any gonadotropin challenge. We identified an unexpectedly large effect of population (Figure 5.2B), with approximately 75% of detectable genes differing significantly between sites (FDR < 0.05; green and blue markers). Functional enrichment of strongly suppressed genes at AP (≥ two-fold reduction relative to WO control animals; green markers) revealed striking enrichment of Polycomb group proteins (PcG) (Figure 5.2B; 5A Table 1), particularly components of polycomb repressive complex 2 (PRC2). This included core components SUZ12, EZH2, and EED, as well as accessory factors JARID2 and MTF2. Consistent with the role of PRCs in establishing repressive transcriptional patterns, this enrichment was only observed in genes suppressed at AP. Interestingly, suppressed genes were also significantly enriched for direct targets of estrogen receptor-α (ERα/ESR1), suggesting that developmental contaminant exposures are capable of eliciting persistent functional shifts in the ovary via both direct (ESR1) and indirect (PRC) estrogen-mediated processes.

In an attempt to place transcriptomic shifts in a functional context, we assessed enrichment in strongly-affected DEGs for gene ontology (biological process) and KEGG terms (figure 5.2C; 5A Table 2,3). In suppressed genes at AP, we detected consistent enrichment of terms related to cell cycle progression and mitosis (GO:BP terms: G1/S transition of mitotic cell, DNA replication; KEGG pathway term: cell cycle), as well as development and morphogenesis (GO:BP terms: nervous system development, neural retina development, embryonic morphogenesis). In contrast, strongly upregulated genes at AP were enriched for ciliary and cytoskeletal terms (GP:BP terms: cilium movement, axoneme assembly), as well as extracellular matrix-related processes (GO:BP term extracellular matrix organization; KEGG pathway term: ECM-receptor interaction). Collectively, these data suggest that developmental contaminant exposures are capable of inducing broad patterns of divergence in gene networks controlling cellular proliferation and development that persist into later life stages.

Population Divergence in Ovarian Gonadotropin Responsiveness

To further explore associations between developmental contaminant exposure and ovarian function, we compared responsiveness to a gonadotropin challenge in juveniles from both sites. In both populations, FSH administration triggered broad transcriptional responses in the ovary, including canonical FSH-responsive genes *CYP19A1, INHA*, and *FST*[81,82,179]. However, despite sharing a core of responsive genes, both populations possessed uniquely-responsive gene

batteries (figure 3B). Furthermore, the magnitude of uniquely responsive genes at AP was much greater compared to WO (figure 5.3A,B); specifically, AP animals appeared to have gained negative responsiveness in over 2900 genes.

To place these changes in a functional context, we investigated FSH responsiveness in the cellular pathway controlling ovarian steroidogenesis in response to FSH, as developmental EDC exposure can induce changes in gonadal hormone synthesis[16,80] By querying the presence or absence of significantly responsive genes in this pathway, we sought to uncover potentially novel consequences of developmental contaminant exposure in gonadotropin-induced steroid hormone production (Figure 5.4). Specifically, we found that while both populations respond positively to FSH by upregulating *CYP17A1, CYP11A1, HSD17B1, CYP19A1, FSHR*, and *PKA* signaling components (Figure 5.4; blue boxes), AP animals exhibit unique responses in this pathway. Expression of *PLA2* (Figure 5.4; blue star) is significantly increased at AP, while *COX2* and *IGF1R* (Figure 5.4; yellow stars) are significantly reduced. Interestingly, both *PLA2* and *COX2* are critical components in the biosynthesis of prostaglandins, which are integral to ovulation[384]. Upregulation of *PLA2* at AP would suggest greater production of arachidonic acid from membrane phospholipids; in contrast, suppressed expression of *COX2* could be indicative of reduced prostaglandin synthesis. Similarly, *IGF1R*, the cognate receptor for insulin-like growth factor 1 (IGF-1), is a critical to ovarian function, necessary for granulosa cell proliferation basally and in response to estrogen[385] and furthermore is required for granulosa cell responsiveness to FSH[386].

Recapitulation of Apopka Ovarian Transcriptomes by Estrogen

We next sought to explore potential mechanisms underlying population-level divergence of the ovarian transcriptome. Specifically, many OCPs at AP are capable of acting as environmental estrogens[73,96,259,295], leading us to hypothesize that the presence of these contaminants in alligator egg yolk might constitute an aberrant estrogenic signal during early gonadal development that ultimately contribute to altered ovarian function later in life. To test this hypothesis, we exposed WO alligators to estradiol-17β (E2) at stage 19, which precedes the onset of gonadal steroidogenesis in the alligator[181,304]. Thus, we directly assessed the ability of a precocious estrogenic cue to recapitulate AP transcriptional programming and observed a large degree of overlap. Similar to patterns observed at the population level, estrogen-treated WO animals were highly dissimilar from control animals (figure 5.4A), with over 70% of ovarian genes detected as differentially expressed between the two groups (FDR<0.05). Strikingly, estrogen treatment was capable of significantly recapitulating AP transcriptional programs, inducing persistent upregulation of over 76% of the same genes elevated at AP and downregulation of over 77% of suppressed AP genes (figure 5.4B). These patterns remain significant in highly responsive (>two-fold affected) genes (39.3% highly upregulated, 57.7% highly downregulated; Appendix 5A Figure 4). Furthermore, highly responsive genes were consistently enriched for the same factors as AP genes; enrichment for PRC components as regulatory factors in genes suppressed with E2 treatment. We also detected the same functional enrichment of cell cycle pathways (GO:BP term: regulation of

transcription involved in G1/S transition of mitotic cell cycle; KEGG pathway term: cell cycle) in suppressed genes, as well as recapitulation of ciliary functional terms in elevated genes (GO:BP term: cilium movement). Thus, despite AP and E_2-treated WO animals maintaining some degree of uniquely-affected genes, accompanying unique functional enrichment (figure 5.2B, Appendix 5A Figure 5), consistent signals related to cell cycle progression and proliferation and cytoskeletal features detected in both treatments.

Altered Ovarian Follicular Profiles

Considering evidence that exposure to estrogenic compounds during development can disrupt follicle development[16,88,89,387,388], we next sought to investigate follicular profiles in AP and E_2-treated WO animals. Interestingly, we observed a marked reduction in the relative density of pre-vitellogenic, stage III follicles in AP animals compared to WO controls ($p<0.0001$; $F_{2,35}=22.44$). Within non-challenged (VEH) groups, both AP (adjusted $p=0.0001$) and E2-treated WO animals (adjusted $p=0.0025$) exhibited significantly lower density of stage III oocytes than WO controls, but were indistinguishable from one another (adjusted $p=0.5641$). These patterns were generally consistent in FSH treated animals; AP animals exhibited significantly reduced follicle density than WO controls (adjusted $p=0.0003$). However, E2-treated WO animals were statistically indistinguishable from both groups.

Association Between Ovarian Transcriptome Dynamics and Follicular Profiles

In an attempt to explore possible associations between altered follicular profiles and ovarian transcription, we employed WGCNA to identify clusters of co-expressed genes, then looked for significant associations between these gene clusters and follicle counts. Clustering analyses in WO and AP FSH-treated and non-challenged animals identified 12 modules of significantly correlated genes (Table 5.1, Appendix 5A Figure 3) that ranged in size from 6469 to 35 member genes. Regressing the first principle component for each of these modules with follicle density revealed that 10 were significantly correlated with density after controlling for effects of site (WO vs AP) and FSH (FSH vs VEH); 3 of these were positively associated with follicle density and 7 of were negatively associated. These significantly associated modules accounted for 17296, or 93.8%, of detectable ovarian genes, suggesting that transcriptional networks in the ovary are tightly associated with follicle development. Assessing DEG membership in each module revealed that the majority of genes suppressed at AP (FDR < 0.05; log2fc > 1) were positively associated with follicle density (Figure 5.5A), with most genes falling into one of two modules (turquoise, red). In contrast, genes elevated at AP (FDR < 0.05; log2fc < -1) were consistently found in modules negatively associated with follicle density (blue, brown, green, green-yellow, yellow, pink, tan, salmon). Consistent with prior observations, E2-responsive genes in treated-WO animals mirrored membership patterns observed in DEGs at AP.

Interestingly, module analysis revealed distinct patterns within AP and WO FSH responses. Comparing genes responding positively to FSH challenge, both sites shared overlapping membership in modules both positively and negatively correlated with follicle density (Table 5.1;blue, yellow, and black modules). However, consistent with overall increased responsiveness observed at AP, a group of positively associated genes were detected at AP exclusively (red). This same pattern was observed in genes downregulated at AP following FSH challenge; both sites exhibited patterns of shared membership, however, unique modules were detected (Table 5.1; green and pink modules). Overall, the degree of membership for differentially expressed genes in the FSH-challenged ovary in modules significantly associated with follicle density confirmed evidence that ovarian transcriptional networks are highly reflective of underlying follicular profiles.

Lastly, to confirm the association between DEGs and follicle densities, particularly in the resting ovary, we employed pairwise correlations between follicle density and expression values. Consistent with our WGCNA approach, we observed that the bulk of differentially expressed genes were highly correlated with follicle density (figure 5.5B). Unsurprisingly, the bulk of genes upregulated in AP and WO-E2 treated animals were negatively associated with follicle density, while most genes suppressed in these groups were positively associated with follicle density.

Discussion

In the present study, by employing a non-targeted, transcriptomics-based approach, we have: (1) elucidated broad divergence in the ovary after developmental contaminant exposure; (2) uncovered functional pathways and signatures of epigenetic modifiers within patterns of divergence; and (3) described a possible mechanism behind reproductive pathologies at Apopka, wherein EDCs might precociously activate estrogen signaling at a bipotential stage. Few studies to date have employed transcriptomics to investigate organismal responses to contaminants in environmentally-relevant settings (but see [389,390]) and fewer still have addressed effects of developmental exposures that persist into later life stages (but see [391]). Nonetheless, the proclivity of environmental endocrine disruptors to shape future ovarian function is well supported (for reviews, see [310,392–394]), and hinges upon the ability of EDCs to activate or disrupt pathways controlling differentiation and proliferation in the embryonic gonad.

The magnitude of population-level differences reported herein are generally greater than those found in similar studies. Recent evidence from the European eel (*Anguilla anguilla*) investigating population-level transcriptome differences following OCP exposure during gonadal development reported that approximately 2% of ovarian transcripts were differentially regulated between populations[395]. Similarly, female Queen conch (*Strombus gigas*) exposed to EDC contaminant mixtures containing tributyltin (TBT) exhibit approximately 5% of total ovarian transcripts dysregulated at high pollution sites relative to controls[396].

This would suggest that the magnitude of effects associated with contaminant exposure in the present study is either unique to the specific contaminant milieu present at Lake Apopka or reflects an inherent heightened sensitivity to exposures during development in the alligator (e.g., developmental exposures elicit greater shifts, or alligators are more sensitive than other aquatic models). In support of the former hypothesis, adult Florida largemouth bass (*Micropterus salmoides floridanus*) stocked in experimental ponds at Lake Apopka exhibit a similarly large proportion of dysregulated transcripts, some 22% of total ovarian genes, while concomitantly accumulating high levels of the same OCP contaminants present in alligator egg yolk[397]. This would suggest that population-level effects reported herein might be unique to the Apopka contaminant milieu. However, the degree of transcriptomic effects observed in the present study in estrogen-treated control animals is generally greater than similar studies investigating steroid hormone exposures in isolation[398–402], supporting the hypothesis that the timing of exposures is critical to outcomes[9], and that alligator development is particularly sensitive to endocrine cues.

The recapitulation of Apopka transcriptional effects by precocious estrogen treatment in reference animals directly is suggestive of both a mechanism and the timing for effects of contaminant exposures. As evinced by the ontogeny of gonadal aromatase expression, estrogen biosynthesis is limited until the onset of sex determination, approximately 2-4 stages following estradiol exposure used herein[181,403,404]. This would imply that the early gonad is maintained in an estrogen-naïve state, but remains capable of responding to

estrogenic cues. Furthermore, this would suggest that activation of signaling is fundamentally linked to either germ cell or somatic cell behavior in the early gonad. Consistent with this hypothesis, prior investigations of reproductive abnormalities at Apopka have uncovered increased prevalence of multioocytic follicles (MOFs)[16], an abnormal follicular state linked to precocious estrogen-mediated induction of TGF-β signaling components, follistatin (*FST*) and inhibin-α (*INHA*), that regulate granulosa cell proliferation, germ-cell nest breakdown and follicle assembly during the peri-hatching period[287]. However, this is the first report of reduced numbers of stage III follicles at Apopka, which suggests that estrogen might influence follicle development at an earlier stage than previously hypothesized by limiting germ cell proliferation or compromising growth or differentiation of oocytes. Limited evidence exists describing primordial germ cell (PGCs) behavior in the alligator, but work by Smith and Joss[405] indicates the presence of PGCs in the cortex of the undifferentiated gonad as early as stage 20, one stage after experimental estrogen dosing in the current study. Concomitantly, estrogen receptors are actively expressed in the early gonad in birds[406–408], turtle[409–411], eutherian mammals[412], and marsupials[413]; furthermore data from mammalian studies supports receptor expression by PGCs themselves[412,413]. This would collectively suggest that PGCs are present in the gonad during precocious estradiol treatment and are capable of responding to receptor activation.

Taken together, this evidence links exposure to EDCs or exogenous estradiol to patterns of germ cell behavior in the undifferentiated gonad. This

connection would be consistent with regulatory control of proliferation by estrogen in the differentiated ovary[414], but has not been well-explored during earlier stages of development. Non-genomic estrogen signaling can drive PGC proliferation in mouse[412,415] and chicken[408]. Similarly, treatment of Broad-snouted caiman (*Caiman latirostris*) embryos with exogenous estradiol has been linked to increases in the proportion of stage III follicles in hatchlings[416]. It is not immediately clear why estradiol/estrogenic OCPs in the present study were associated with reductions, rather than increases, in stage III follicles given findings in other studies, but differences might reflect additional changes that occur between early PGC proliferation and future follicle assembly or growth. In this vein, the effects and relative contributions of precocious versus contemporary endocrine signaling (i.e., effects attributable to differences in circulating estrogen levels at 5 months) in the present study need to be elucidated individually to fully differentiate direct versus indirect effects of precocious estrogen signaling. Apopka alligators have been repeatedly characterized as having altered levels of plasma steroid hormones[16,81,297,417]; if this pattern is repeated in the current study as well, these differences could contribute to follicle dynamics.

Consistent enrichment of polycomb components in genes affected at both Apopka and in estrogen-treated reference animals suggests that epigenetic mechanisms contribute to EDC-induced reproductive pathologies in the alligator. In this fashion, precocious estrogen signaling could represent a mistimed regulatory cue that ultimately disrupts the “normal” ontogeny of epigenetic

patterning during development. Consistent with this hypothesis, estrogen signaling can induce expression of *EZH2*, the H3K27 histone methyltransferase component of the polycomb repressive complex 2 (PRC2), via genomic ER signaling, while also suppressing EZH2 activity through non-genomic signaling[334,418,419]. In addition, estrogen signaling can regulate EZH2-PRC2 activity by inducing expression of the lncRNA HOTAIR, which contributes to PRC2 occupancy of chromatin[420,421]. Furthermore, this regulatory function is not limited to endogenous estrogens and can be similarly engaged by non-steroidal estrogens DES, BPA, and genistein[334,418,419]. These findings would suggest that, in addition to altered DNA methylation patterns resulting from developmental estrogen exposure[332], changes to histone modifications also contribute to reproductive pathologies induced by EDCs. Lastly, enrichment of PRC components raises intriguing questions regarding the endogenous function and ontogeny of epigenetic regulators during development, particularly in the context of endocrine regulation. Recent evidence in a species of turtle with TSD has revealed that the most proximal factor responsive to temperature in the bipotential gonad is *KDM6B*, the regulatory counterpart to EZH2 that removes H3K27 methylation to activate gene expression[336]. In contrast to EZH2 however, expression of *KDM6B* is suppressed by estrogen, which would suggest that estrogen signaling might represent a crucial hinge in the balance of suppressive versus permissive epigenetic modifications.

Herein, we provide the first experimental description of a potential mechanism underlying reproductive abnormalities associated with EDC exposure

at Lake Apopka. Prior attempts to recapitulate ovarian pathologies observed *in situ* with singular EDC components (e.g., toxaphene[97], DDE[96]) have been generally unsuccessful, suggesting that individual compounds fail to elicit some minimum threshold of estrogenic activity. However, additional work is needed to fully support that EDCs at Apopka are acting through the estrogen receptor (e.g., partial inhibition of estrogen receptor activation to ameliorate effects). Nonetheless, these findings support an estrogenic mode of action for contaminants to act as drivers of adult reproductive disease. Furthermore, the close association between follicle densities and transcriptional networks suggests that development of the ovarian follicle might be acutely sensitive to these mistimed estrogen cues. Given the conservation of endocrine function across vertebrate taxa, these results should inform our understanding of the developmental origins of reproductive function and the threats posed to environmental and organismal health by EDCs.

Table 5.1. WGCNA module analysis, model output, and module-DEG membership. Mixed-effects linear models were used to investigate the relationship between 13 correlated gene clusters (first principal component, or eigengene, of each cluster), or modules, and follicle density. Main model effects include follicle density (follicle_den), site (AP vs. WO) and the interaction between follicle density and site (interaction). FSH-challenge status included as a random effect. Differentially expressed genes in both the resting and FSH challenged ovary were assessed for membership in individual modules to identify genes significantly affected due to differences in follicular profiles. E2 DEGs, while not included during initial module detection, were assessed for module membership as well.

			Model Effects p-value			Gene number overlap							
Module	Color	ModuleGenes	Follicle_den	Site	Interaction	AP Elevated (log2FC<-1)	AP Suppressed (log2FC>1)	E2 Elevated (log2FC<-1)	E2 Suppressed (log2FC>1)	WO FSH Up (all)	WO FSH Down (all)	AP FSH Up (all)	AP FSH Down (all)
0	grey*	529	**0.0377 (-)**	0.8526	0.2646	3	0	4	1	2	1	0	31
1	turquoise	6469	**0.0003 (+)**	**0.0011**	**0.0054 (-)**	0	1734	0	1377	0	100	32	622
2	blue	4607	**0.0009 (-)**	**0.0011**	**0.0081 (+)**	1136	0	773	0	846	0	460	92
3	brown	1926	**0.0005 (-)**	**0.0052**	**0.0142 (+)**	454	0	247	1	0	93	0	1419
4	yellow	1463	**0.0007 (-)**	**0.0022**	**0.0028 (+)**	42	0	55	0	992	0	1240	0
5	green	947	**0.0007 (-)**	0.6664	0.7318	584	0	246	0	1	0	0	619
6	red	814	**0.0002 (+)**	0.0737	**0.0447 (-)**	0	29	0	15	0	0	505	0
7	black	703	**0.0154 (+)**	0.6446	0.5677	0	3	0	2	115	0	587	0
8	pink	289	**0.0096 (-)**	0.0712	0.085	84	0	36	0	0	0	0	18
9	magenta	254	0.0919	0.9754	0.3844	0	0	0	0	0	2	0	186
10	purple	225	0.6553	0.651	0.8861	0	0	1	0	0	52	0	210
11	green-	131	0.0918	0.9073	0.6478	63	0	95	0	5	0	0	9
12	tan	43	**0.002 (-)**	0.2278	**0.0343 (+)**	31	0	27	0	0	0	0	0
13	salmon	35	**0.0062 (-)**	0.1655	**0.0376 (+)**	4	0	15	0	0	0	0	0
					List Size	2403	1766	1639	1473	1955	248	2828	3209

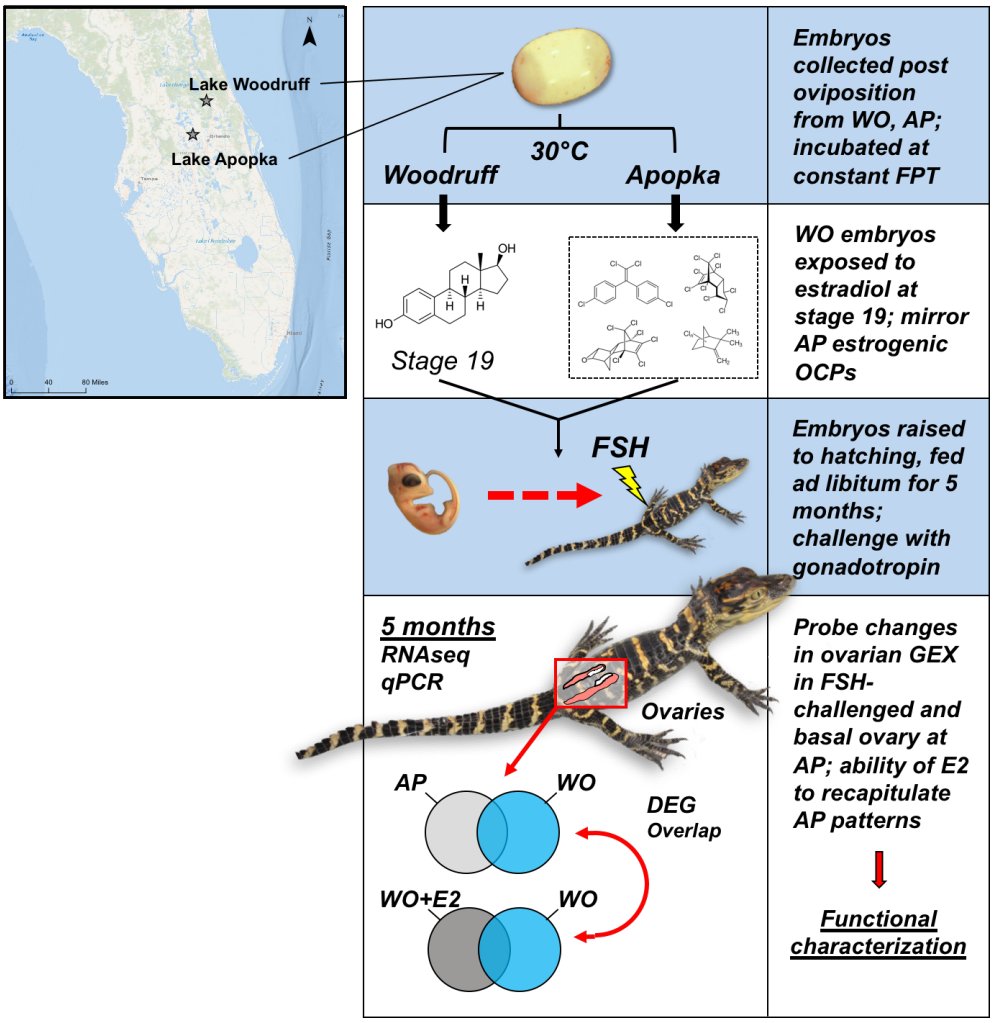


Figure 5.1. Experimental design and sampling. Juvenile alligators used herein were collected as eggs from two lakes in central Florida, Lake Apopka which has experienced long-term organochlorine pesticide input from municipal, agricultural, and industrial sources, and a reference site, Lake Woodruff, which is relatively pristine. Reference embryos were treated at stage 19 with either estradiol-17β or vehicle control in an attempt to recapitulate exposure to estrogenic OCPs at Apopka. Following hatching, animals were raised for five

months. At five months, they were administered either FSH or vehicle controls. The direct ability of estradiol to induce Apopka transcriptomic profiles was assessed by comparing DEGs between Woodruff control animals and Apopka to estradiol-treated Woodruff animals and controls.

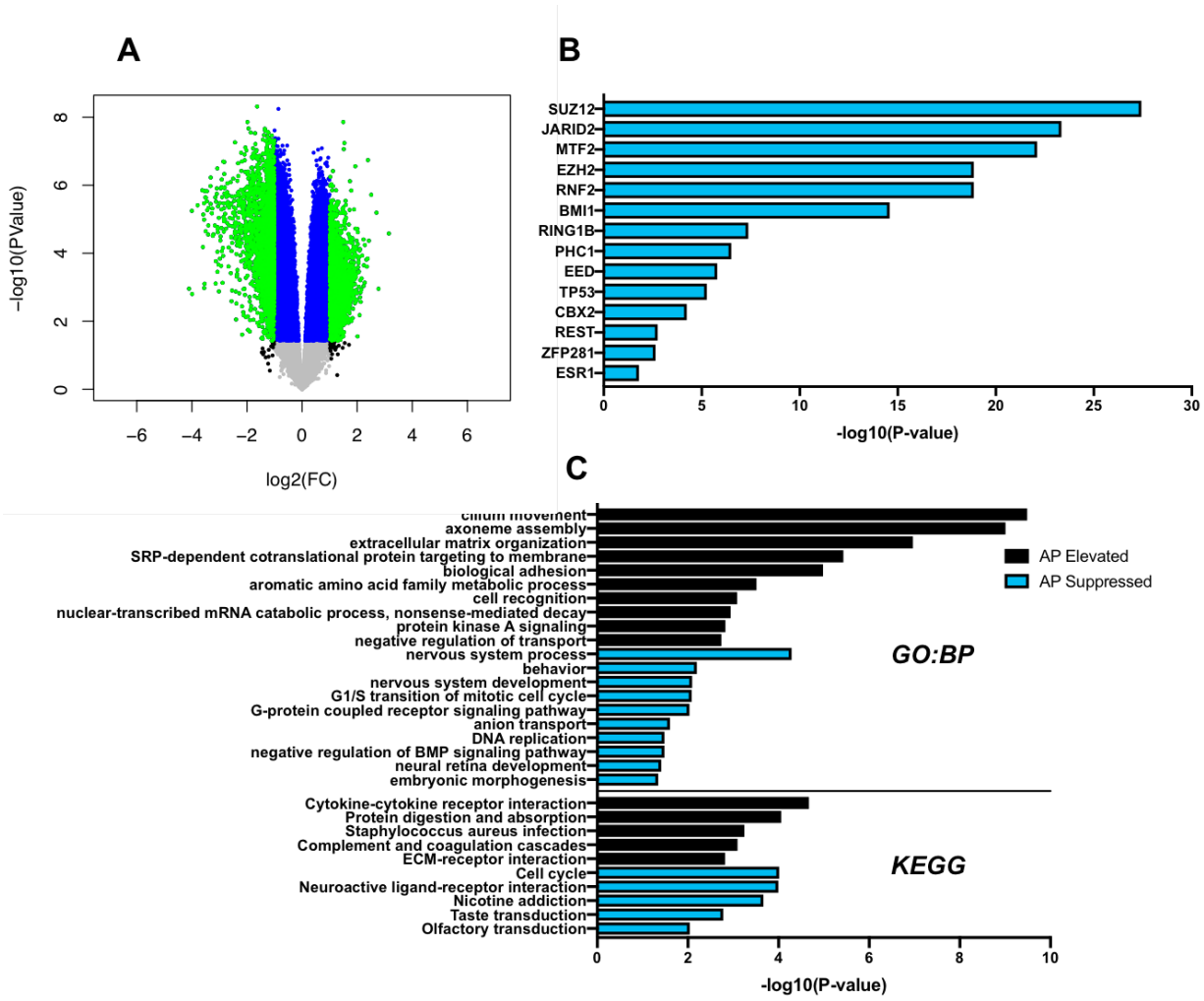


Figure 5.2. Population-level divergence in ovarian transcriptional profiles and functional DEG characterization in the resting ovary. (A) Volcano plot depicting the number, relative effect size, and significance of differentially expressed genes at AP relative to WO controls in the resting ovary. Individual points represent genes; green and blue points are significantly differentially expressed between populations (FDR<0.05). Points with a negative $\log_2$(FC) are significantly elevated at AP. Green points are both significant and are at least two-fold differentially expressed between populations. (B) Significantly enriched transcription factors predicted to regulate highly (>two-fold) suppressed genes at

AP relative to WO controls. Significant enrichment was not detected for highly upregulated genes at AP. (C) Functional enrichment of highly responsive genes at AP (black bars: AP elevated; blue bars: AP suppressed, including GO:biological process and KEGG pathway enrichment).

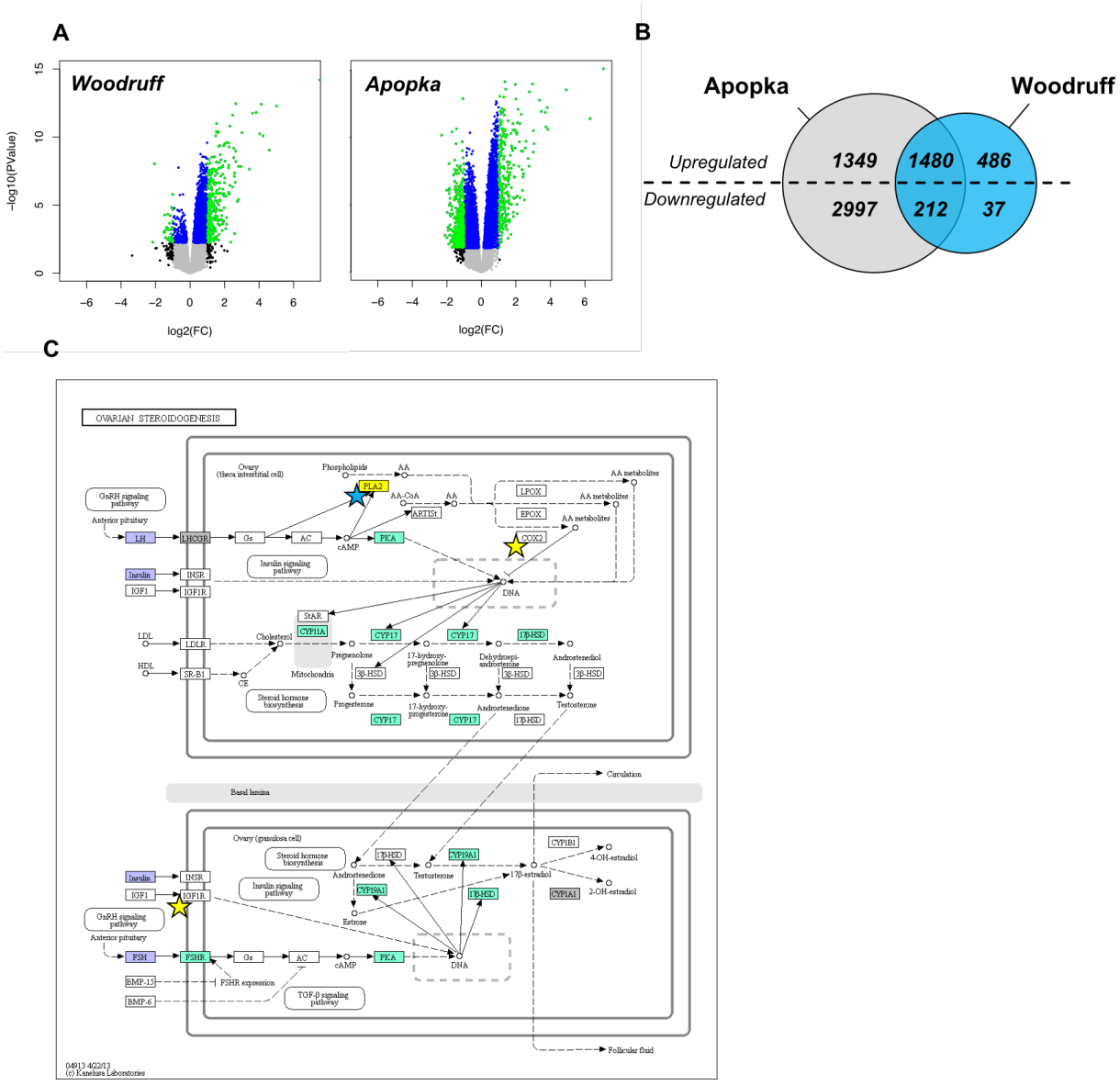


Figure 5.3. Population-level divergence in gonadotropin-challenged ovarian transcriptional profiles. (A) Volcano plots depicting population-specific responses to FSH are depicted; individual points represent genes. Green and blue points are significant (FDR<0.05) while green points are both significant and at least two-fold responsive to FSH challenge. Genes in both populations with a positive log_2(FC) are upregulated by FSH, while negative log_2(FC) values indicate suppression by FSH. (B) Venn diagram depicting shared and unique FSH-

responsive genes at AP and WO. (C) KEGG ovarian steroidogenesis pathway depicting expression patterns in FSH-challenged WO animals. Blue terms correspond to genes significantly upregulated with FSH; yellow terms correspond to significantly downregulated genes; white terms are not significantly different between FSH-challenged and non-challenged animals. Grey terms were not detected and expression of purple terms was not assessed. Any term marked with a star represents a uniquely responsive gene detected at AP; yellow stars denote genes that were uniquely downregulated with FSH administration AP, while blue stars denote genes that were uniquely upregulated at AP. All genes expressed at WO were also expressed at AP, and vice versa.

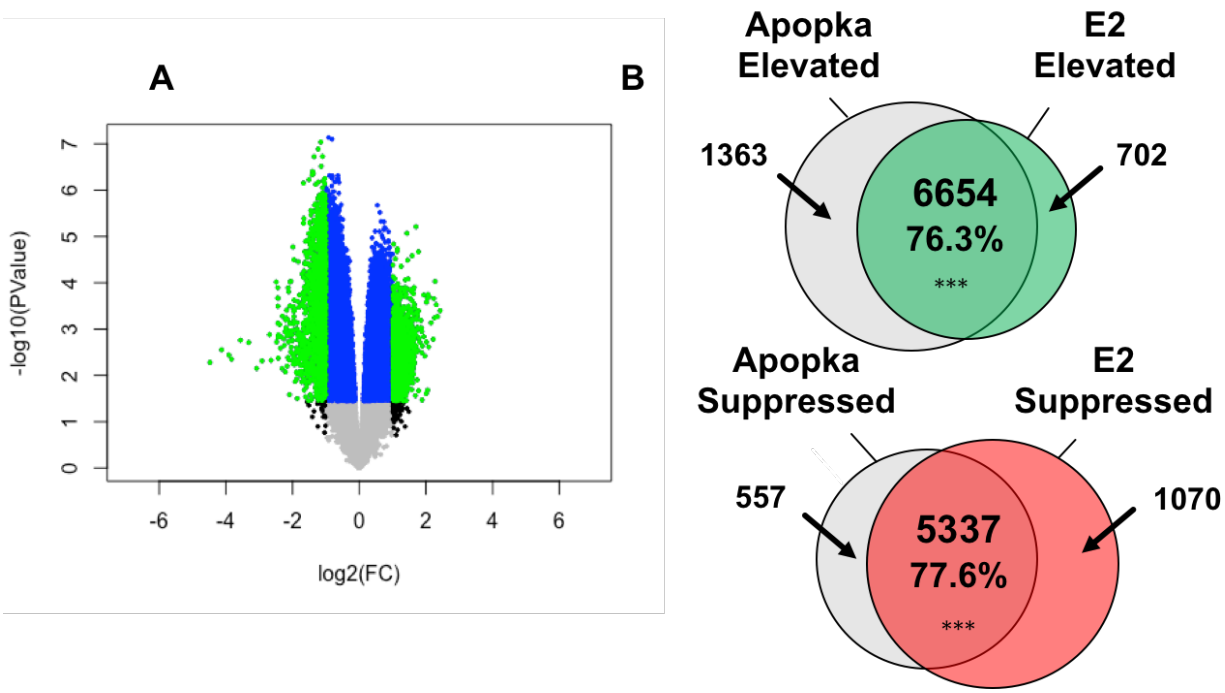


Figure 5.4. Recapitulation of Apopka transcriptional profiles by estradiol. Estradiol exposure prior to gonadal steroidogenesis induces a large magnitude of differential gene expression at WO. (A) Volcano plot depicting DEGs between WO control animals and estradiol-treated WO animals. (B) Overlap between AP-elevated and E2-elevated DEGs, and AP-suppressed and E2 suppressed DEGs. Asterisks denote significance (hypergeometric probability; *** = p-value < 0.0000)

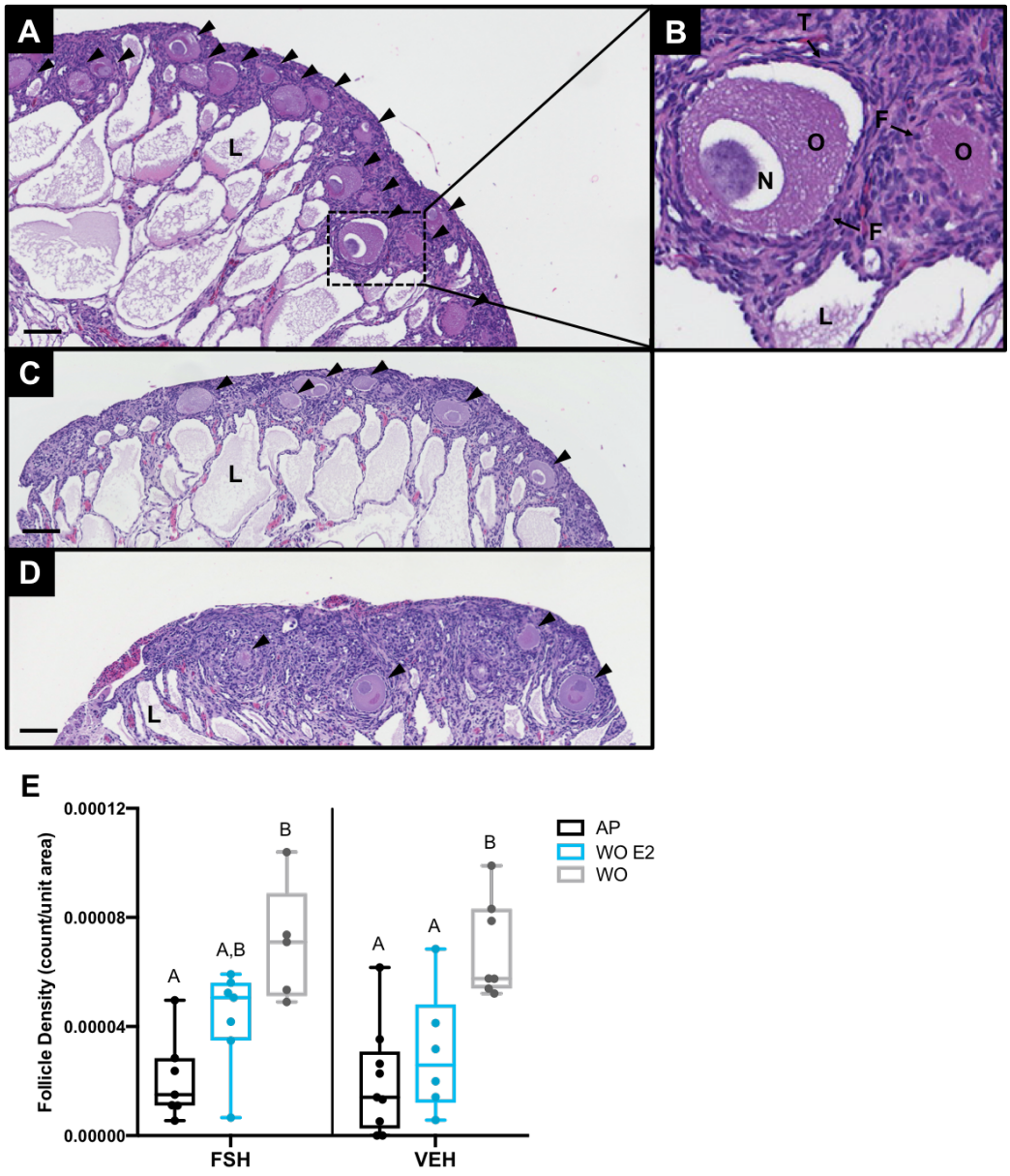


Figure 5.5. Ovarian follicular dynamics associated with precocious developmental endocrine cues. Representative ovarian images from: WO control animals (A, B inset), WO-estradiol treated animals (C); and AP animals (D), depicting cortical SIII follicles (black arrows) and lacunae (L). SIII follicles (B inset) are characterized by large eecentric nuclei (N), basophilic ooplasm, and a complete layer of follicular granulosa (F). Occasional thecal cells (T) are also

observable. Scale bar = 100μm. Sections are composite stitches of multiple images taken at 10x (A,B and D) or 20X (C) using a Keyence BZ-X710. Relative densities of stage III follicles in non-challenged (VEH) and FSH challenged (FSH) ovary in WO controls (WO), AP, and estradiol-treated WO animals (WO+E2). Letters above box plots denote statistical significance within a gonadotropin treatment group (VEH vs. FSH).

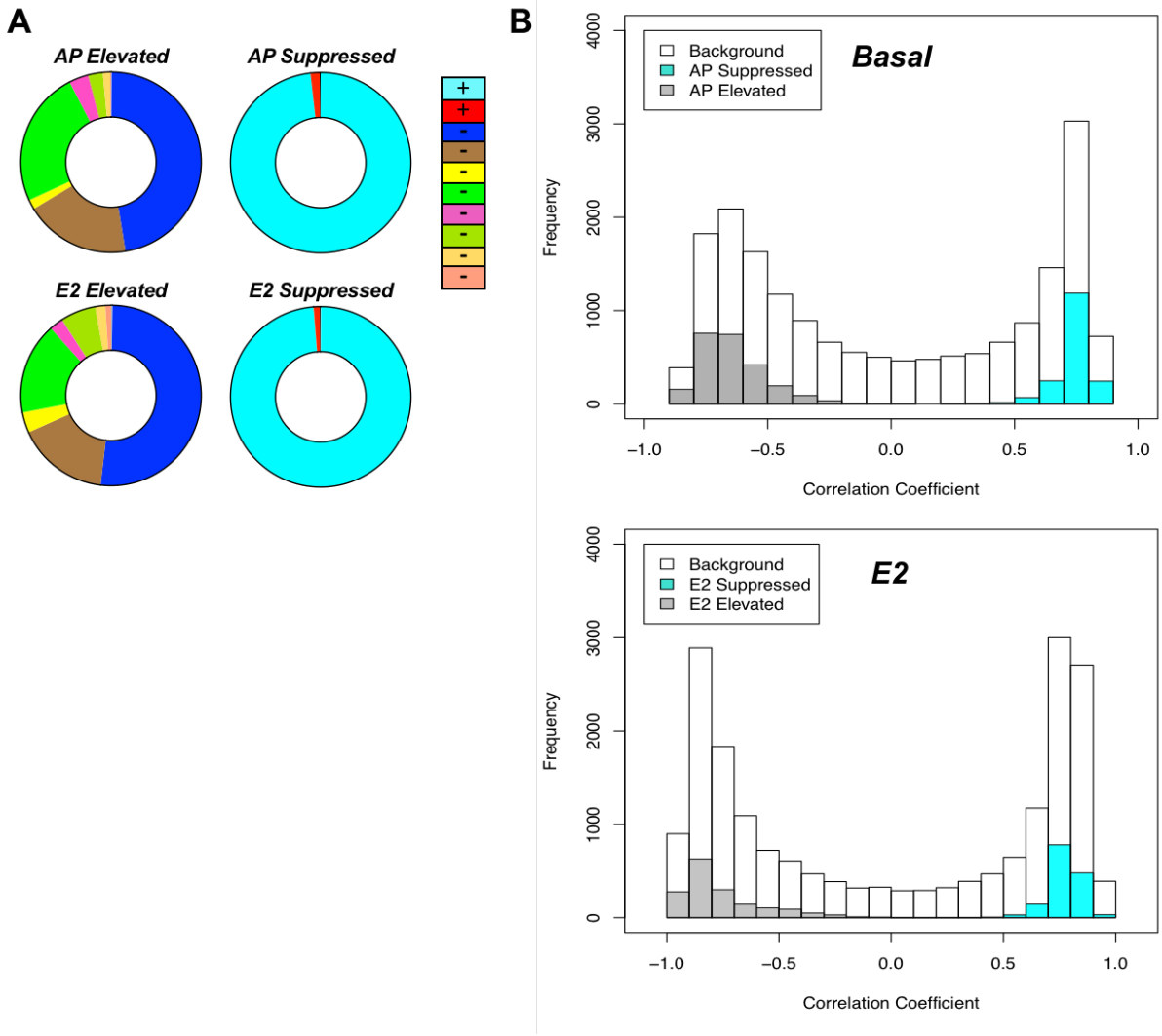


Figure 5.6. Association between follicle dynamics and differential gene expression. (A) DEG membership in each module is depicted by its relative proportion in a given module. The direction of each module's association is depicted in colored legend. Genes suppressed at AP and in E2-treated reference animals belong predominantly to one large module (aquamarine) that is positively associated with follicle density. Similarly, genes elevated at AP and in E2-treated reference animals are predominantly associated with modules that are highly negatively associated with follicle density. These results are confirmed using

pairwise Pearson correlations (B,C) between follicle density and gene expression. Genes highly upregulated at AP and in E2-treated WO animals (grey bars) are consistently negatively correlated with follicle density, while genes highly suppressed at AP and with E2 treatment are consistently positively associated with follicle density. White bars (background) depict the frequency of correlations in all detectable genes in the ovary.

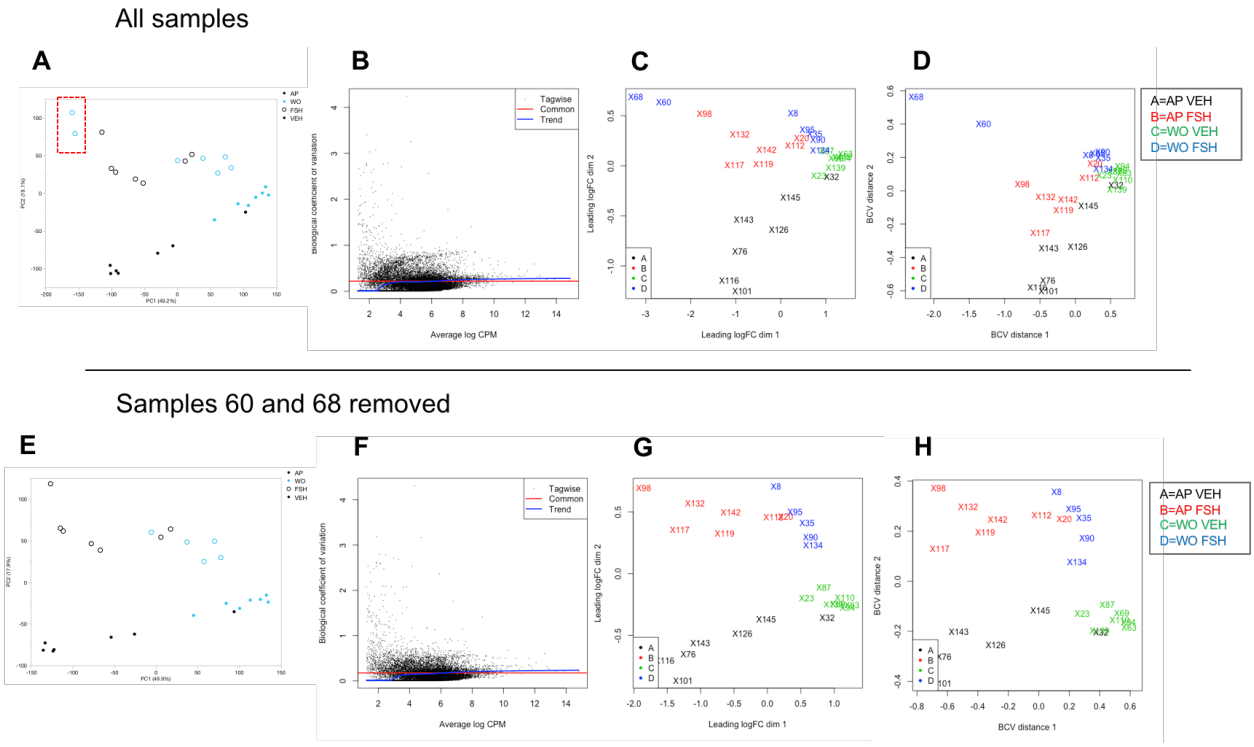
Appendix 5A

5A Table 1. Basal Enrichr transcription factor enrichment confirmation. Significant terms identified in Enrichr were confirmed against an alligator-specific background. Log10Pvalue and adjusted p-value references original Enrichr output identifying significant terms. Group proportion identifies the relative proportion of genes in either significant upregulated (elevated), downregulated (suppressed), or all expressed (background) gene lists. Fisher's exact tests were used to identify significant enrichment relative to background proportions. Asterisks (*) denote enriched terms that are non-significant with a Bonferroni correction (adjusted p-value<0.0167). Red highlighted cells denote a gene list that is overenriched for a given term relative to background, while blue cells denote a gene list that is underenriched relative to background

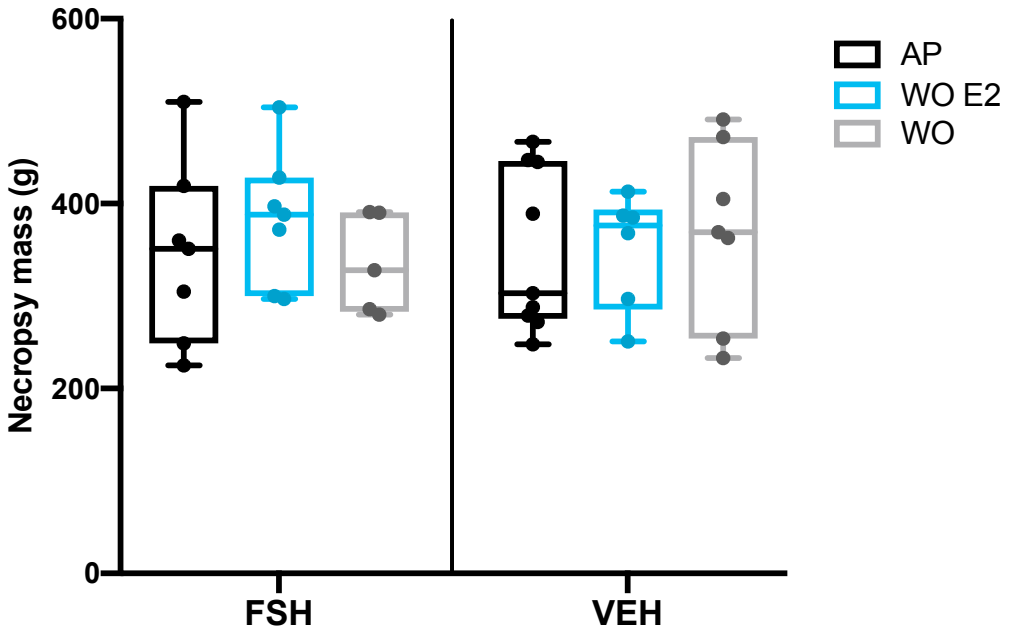
						Group proportion			Unadjusted p-value		
Term	Study_type	Study_cell	Species	log10PValue	Adjusted P-value	**Elevated**	**Suppressed**	**Background**	**B vs E**	**B vs S**	**E vs S**
SUZ12	ChIP-Seq	MESCs	Mouse	27.42635245	3.75E-28	0.2347	0.3581	0.2144	0.0584	**<0.0001**	**<0.0001**
JARID2	ChIP-Seq	MESCs	Mouse	23.34688158	4.50E-24	0.0726	0.1452	0.0616	0.0861	**<0.0001**	**<0.0001**
MTF2	ChIP-Seq	MESCs	Mouse	22.11226973	7.72E-23	0.1555	0.2591	0.1481	0.4221	**<0.0001**	**<0.0001**
EZH2	ChIP-Seq	MESCs	Mouse	18.87867243	1.32E-19	0.0720	0.1386	0.0625	0.1356	**<0.0001**	**<0.0001**
RNF2	ChIP-Seq	MESCs	Mouse	18.87867243	1.32E-19	0.0720	0.1386	0.0625	0.1356	**<0.0001**	**<0.0001**
BMI1	ChIP-Seq	NPCS	Mouse	14.58382368	2.61E-15	0.0569	0.1114	0.0483	0.1329	**<0.0001**	**<0.0001**
RING1B	Chip-Seq	NPCs	Mouse	7.374162261	4.23E-08	0.0774	0.1535	0.1004	**0.0025**	**<0.0001**	**<0.0001**
PHC1	ChIP-ChIP	MESCs	Mouse	6.509155103	3.10E-07	0.0430	0.0825	0.0401	0.5528	**<0.0001**	**<0.0001**
EED	ChIP-ChIP	MESCs	Mouse	5.790157326	1.62E-06	0.0454	0.0743	0.0344	**0.0246***	**<0.0001**	**0.0011**
TP53	ChIP-ChIP	R1E	Mouse	5.257221409	5.53E-06	0.0551	0.0916	0.0520	0.5992	**<0.0001**	**0.0002**
CBX2	Chip-Seq	ESCs	Mouse	4.236066087	5.81E-05	0.0363	0.0701	0.0342	0.6183	**<0.0001**	**<0.0001**
REST	ChIP-Seq	MESCs	Mouse	2.747219938	1.79E-03	0.0962	0.1526	0.1057	0.2517	**<0.0001**	**<0.0001**
ZFP281	Chip-Seq	ESCs	Mouse	2.641340446	2.28E-03	0.1004	0.1328	0.1032	0.7647	**0.0018**	**0.0074**
ESR1	ChIP-Seq	MCF-7	Human	1.794719466	1.60E-02	0.0079	0.0223	0.0126	0.1208	**0.0085**	**0.0018**

5A Table 2. E2 Enrichr transcription factor enrichment confirmation. Significant terms identified in Enrichr were confirmed against an alligator-specific background. Log10Pvalue and adjusted p-value references original Enrichr output identifying significant terms. Group proportion identifies the relative proportion of genes in either significant upregulated (elevated), downregulated (suppressed), or all expressed (background) gene lists. Fisher's exact tests were used to identify significant enrichment relative to background proportions. Asterisks (*) denote enriched terms that are non-significant with a Bonferroni correction (adjusted p-value<0.0167). Red highlighted cells denote a gene list that is overenriched for a given term relative to background, while blue cells denote a gene list that is underenriched relative to background

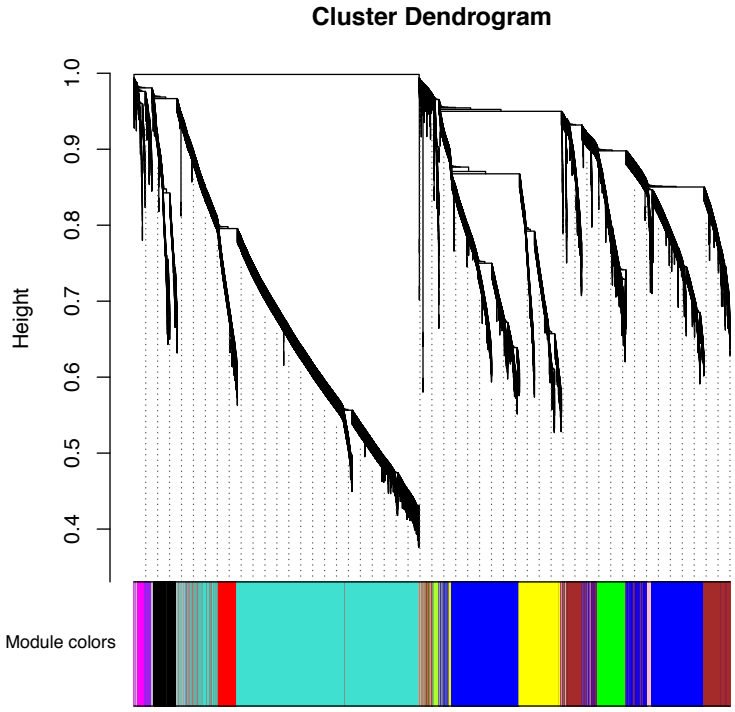
Term	Study_Type	Study_cell	Species	log10PValue	Adjusted	**Suppressed**	**Elevated**	**Background**	**B vs S**	**B vs E**	**E vs S**
MTF2	ChIP-Seq	MESCs	Mouse	22.02343657	9.47E-23	0.2737	0.1637	0.1519	**<0.0001**	0.2982	**<0.0001**
JARID2	ChIP-Seq	MESCs	Mouse	17.01894293	9.57E-18	0.1302	0.0660	0.0558	**<0.0001**	0.1563	**<0.0001**
BMI1	ChIP-Seq	NPCS	Mouse	15.19989119	6.31E-16	0.1211	0.0570	0.0501	**<0.0001**	0.3191	**<0.0001**
EZH2	ChIP-Seq	MESCs	Mouse	14.57262696	2.68E-15	0.1373	0.0841	0.0649	**<0.0001**	**0.017***	**0.0001**
SUZ12	ChIP-ChIP	MESCs	Mouse	6.854716173	1.40E-07	0.1119	0.0759	0.0553	**<0.0001**	**0.0056**	**0.0052**
CBX2	Chip-Seq	ESCs	Mouse	6.089086999	8.15E-07	0.0804	0.0416	0.0357	**<0.0001**	0.3155	**0.0002**
RING1B	Chip-Seq	ESCs	Mouse	5.633995499	2.32E-06	0.1526	0.1031	0.0995	**<0.0001**	0.6776	**0.0008**
EED	ChIP-ChIP	MESCs	Mouse	4.513567452	3.07E-05	0.0743	0.0570	0.0367	**<0.0001**	**0.0014**	0.1109
TP53	ChIP-ChIP	R1E	Mouse	4.084353457	8.23E-05	0.0916	0.0606	0.0539	**<0.0001**	0.336	**0.0078**
PHC1	ChIP-ChIP	MESCs	Mouse	3.999843694	1.00E-04	0.0783	0.0479	0.0417	**<0.0001**	0.3134	**0.0048**
RNF2	ChIP-ChIP	MESCs	Mouse	2.958730445	1.10E-03	0.0926	0.0561	0.0519	**<0.0001**	0.5283	**0.0018**
REST	ChIP-Seq	MESCs	Mouse	2.690031583	2.04E-03	0.1567	0.1076	0.1078	**<0.0001**	>0.9999	**0.0011**
SMAD4	ChIP-Seq	A2780	Human	2.553615502	2.80E-03	0.1628	0.1013	0.1325	**0.009**	**0.0026**	**<0.0001**
P300	ChIP-Seq	ESCs	Human	2.39375688	4.04E-03	0.1353	0.0678	0.0969	**0.0002**	**0.0011**	**<0.0001**
ERG	ChIP-ChIP	JURKAT	Human	2.204273096	6.25E-03	0.0315	0.0154	0.0163	**0.0013**	0.9022	**0.0184***
POU5F1	ChIP-ChIP	HESCs	Human	2.002654777	9.94E-03	0.0509	0.0335	0.0254	**<0.0001**	0.1154	**0.0488***
ESR1	ChIP-Seq	MCF-7	Human	1.366329854	4.30E-02	0.0224	0.0145	0.0122	**0.0119**	0.4814	0.1921



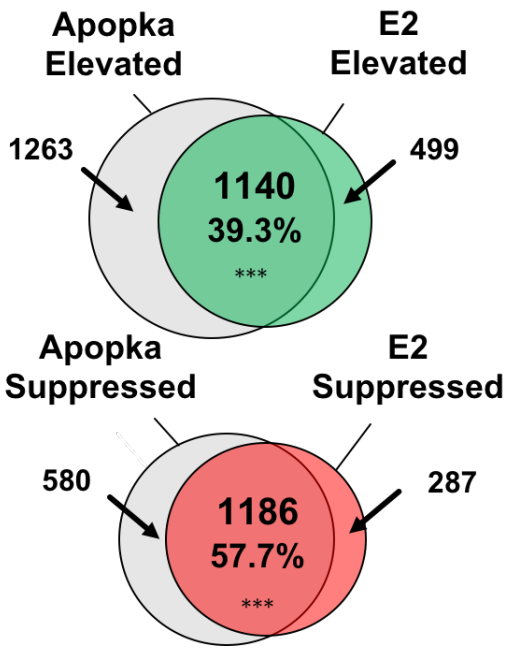
5A Figure 1. Multidimensional scaling (MDS), biological coefficient of variation (BCV) and principal components analyses for log-transformed counts per million (CPM) expression values**.** Principal components analyses and MDS for all reads passing filtering with (A-D) and without (E-H) putative WO-FSH outlier samples 60 and 68 (A; red box inset). PCA using all genes passing filtering identified two FSH-treated WO samples as possible outliers, corresponding to a band of genes with high BCV (B,F). The relative contribution of samples 60 and 68 to overall patterns of variation persist when limited to top 500 most influential genes driving differences in leading logFC (C,G) and BCV (D,H).



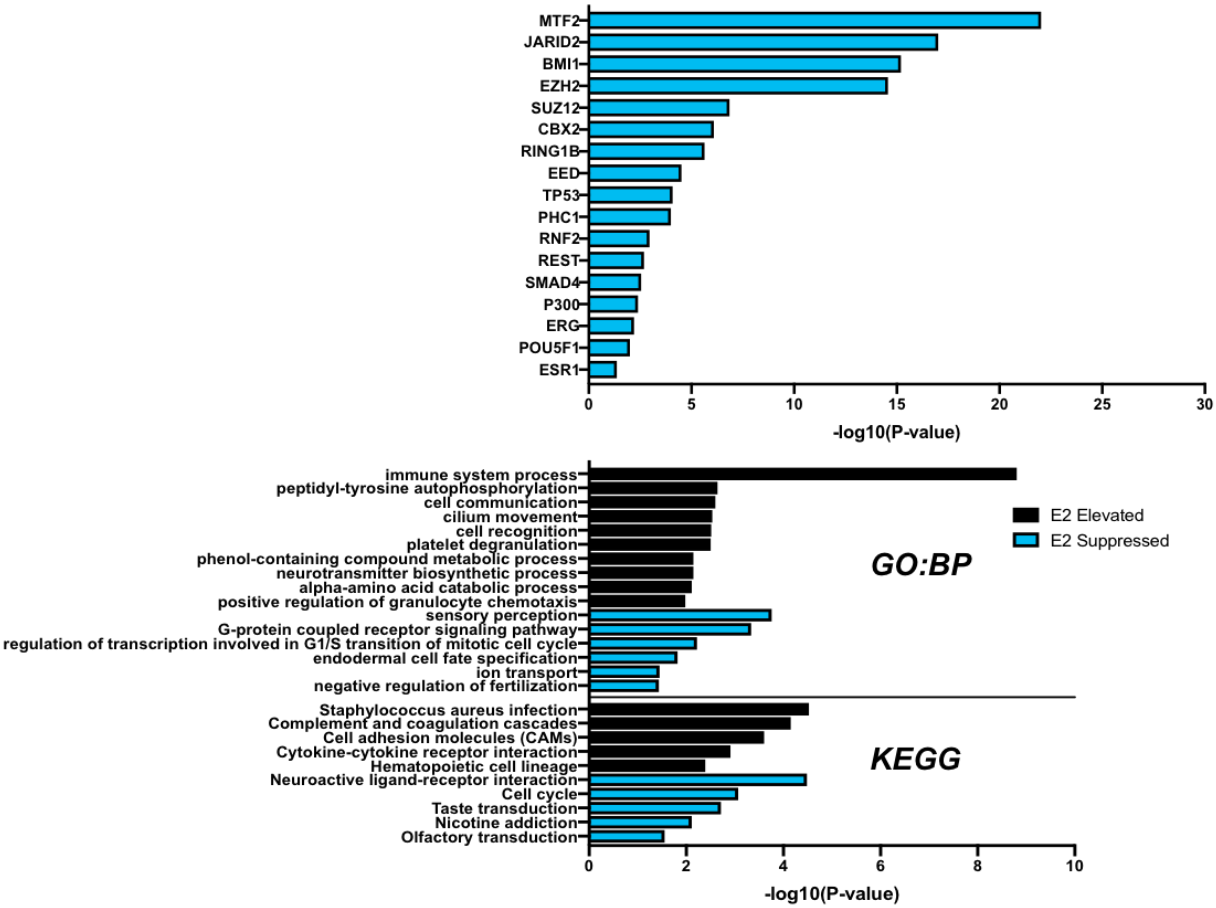
5A Figure 2. Total mass (g) at necropsy. Total mass in Apopka, Woodruff, and E2-treated WO groups with (FSH) and without (VEH) gonadotropin challenge do not differ as assessed via 1-way ANOVA (α=0.05)



5A Figure 3. Weighted gene cluster network analysis (WGCNA) cluster dendrogram. Clustering analysis of all genes passing filtering in Apopka and Woodruff groups with and without gonadotropin challenge revealed 13 coexpression modules (12 significant modules and 1 containing residual, unassigned genes). Read counts were VST transformed prior to analysis using pairwise Pearson correlations and WGCNA function ‘blockwiseModules’.



5A Figure 4. Recapitulation of Apopka transcriptional programs by estradiol. Overlap of highly elevated or suppressed ($log_2FC>|1|$) genes in Apopka or E_2-treated WO animals relative to untreated WO controls is reported. Significant, non-random overlap was assessed via hypergeometric probability distribution ($\alpha=0.05$).



5A Figure 5. Transcription factor and GO/KEGG term enrichment in WO-E_2 affected genes. (A) Significantly enriched transcription factors predicted to regulate highly (>two-fold) suppressed genes in WO-E_2 treated animals relative to WO controls. (B) Functional enrichment of highly responsive genes in WO-E_2 affected genes (black bars: E_2 elevated; blue bars: E_2 suppressed, including GO:biological process and KEGG pathway enrichment).

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

6.1: Dissertation Summary

The overarching goal behind this body of research was to investigate two fundamental questions in toxicology, specifically in the context of environmental endocrine disruptors, using a sentinel species and model of environmental health, the American alligator: 1.) How are the offspring of long-lived organisms affected by long-term dioxin exposures; and 2.) How do disruptions to endocrine signaling during development influence reproductive development and health under environmentally relevant settings. As a long-lived predatory species, the alligator is uniquely suited as a "top-down" indicator for persistent, lipophilic contaminants that can bioaccumulate and biomagnify in the environment. Further, endocrine signals are critical to reproductive development in alligators and crocodilians, and exogenous steroid hormones can induce overt gonadal phenotypes including sex reversal[34]. Thus, alligators are dually effective as sentinels for the detection of environmental contaminants as well as models with which the effects of contaminants, particularly EDCs, can be mechanistically studied..

6.2 Dioxin and the Alligator: Conclusions, Limitations, and Future Directions of Chapters 2 and 3

In Chapters 2 and 3, our overall objective was to explore organismal responses to long-term, chronic dioxin exposures under environmentally-relevant settings, particularly in the offspring of exposed individuals. We employed an alligator population inhabiting a historically-contaminated system as a model in

this investigation because the longevity and trophic status of the alligator makes them particularly susceptible to dioxin accumulation, which subsequently makes them effective models for human exposures. These factors, coupled with the experimental tractability of alligator embryos relative to humans presents a unique opportunity to explore both effects of exposures and underlying mechanisms. To accomplish this, we focused primarily on the signaling pathway that mediates toxicity of dioxins, the AHR pathway.

Because AHR signaling has not been characterized in the alligator, our first task in Chapter 2 was to describe fundamentals of receptor expression and inducible *CYP1A* target genes in order to build a "toolkit" for investigation of dioxin-mediated effects during development. In so doing, we discovered that the alligator possesses two putative *CYP1A* genes and that the liver expresses relatively high levels of these *CYP1A* genes, as well as AHRs. Given the broad conservation of the AHR pathway and CYP genes across vertebrates[157,161], these findings were unsurprising, but nonetheless contributed directly to our knowledge of AHR signaling in alligators and more generally in reptiles. Current knowledge on AHR function in reptiles is scarce, and what is known is derived indirectly from a collection of studies in populations exposed *in situ* to AHR-activating ligands[222]. Moreover, fundamentals of AHR signaling (i.e., which components are expressed and where) is poorly described; thus the "toolkit" developed in this chapter contributes directly to a fundamental understanding of this pathway in vertebrates.

The groundwork established in Chapter 2 directly enabled the subsequent identification of effects of site in the expression of AHR signaling components at YWC and, unexpectedly, at Apopka. By identifying significant upregulation of the dioxin-inducible gene *CYP1A2* in YWC embryos, as well as upregulation of two AHR isoforms in AP embryos, we have uncovered a possible direct link between historical contamination events and contemporary effects in developing embryos, but not juveniles. This would suggest that the offspring of long-lived species continue to be exposed to these contaminants decades after their release. In addition, the significant association between AHR and CYP expression levels in YWC embryos contributed to a novel concept of toxicity, which is that natural variation in AHR expression might predict embryonic responsiveness to dioxins by modulating the induction of the AHR gene battery. And while we did not explore hepatic elevation in AHR expression observed at Apopka further, these observations are intriguing because they too suggest that developmental contaminant responses might vary within in a population. In this case, however, exposure to estrogenic OCPs could drive increased toxicity of dioxins in contaminant mixtures.

Although changes in CYP expression at YWC (and AHRs at Apopka) are suggestive of contaminant-mediated effects, in Chapter 2, we were not able to fully rule-out either possible genetic variation contributing to changes or non-contaminant environmental effects (e.g., other components in yolk). Thus, in an attempt to further support or refute a possible contaminant-driven mechanism behind altered hepatic transcription at YWC, in Chapter 3, we directly quantified

yolk dioxin and furan burdens. Foremost, these efforts directly supported our initial hypothesis by uncovering elevated levels of multiple dioxin species and overall TEQ levels at YWC. These findings strongly support the utility of the YWC population to study the long-term effects of dioxins not only in embryos, but in chronically-exposed adults. The application of this model to mechanistic studies of chronic dioxin toxicity is potentially highly valuable to inform a growing body of human epidemiological data linking chronic and developmental exposures to latent endocrine-related health effects[135]. They also provide the first record of dioxin contamination in a crocodilian. However, findings in Chapter 3 also raise of host of additional questions regarding the fundamentals of AHR signaling in the alligator. Chiefly, although yolk dioxin burdens are elevated at YWC, variation in those levels generally failed to predict variation in *CYP1A2* expression, as well as *AHR1B.* While it is difficult to speculate why these relationships weren't supported without a better fundamental understanding of how dioxins activate the AHR and at what levels, these results suggest that the larger picture of hepatic AHR signaling cannot be explained wholly by yolk dioxin burdens. This supposition is supported by the striking relationships between percent embryo mass (a metric describing conversion of yolk into somatic tissue) and *CYP1A2* and *AHR1B* expression, which suggests a role for fine-scale differences in developmental progression in the peri-hatching period as a driver of hepatic function. Furthermore, PCBs, which can act both as AHR ligands and antagonists, and are elevated at YWC[46,47] could also influence AHR signaling in the liver.

Taken together, the work presented in Chapters 2 and 3 has elucidated the possible consequences of dioxin contamination in the environment for long-lived species. But in so doing, it highlights two major areas where additional research is warranted. The first of these areas regards our practical understanding of AHR signaling and toxicokinetics of dioxin in the alligator. Chiefly, acute dioxin toxicity is poorly described in alligators and crocodilians[222], and we know very little of how AHR signaling contributes to toxicity in the liver or in other tissues. Controlled dosing studies with dioxin in the alligator that employ our AHR "toolkit" would fill in these gaps by identifying the magnitude and duration of CYP induction following exposures. Further, they could identify levels of exposure at which dioxin-activated AHR signaling translates to overt signs of toxicity. This understanding is critical if we are to further explore toxic effects of long-term exposures in the alligator, and would support the tractability of the alligator as a research model. Furthermore, it would provide a foundation for the exploration of possible AHR-mediated adaptive responses to long-term exposures in the alligator, as has been reported in other chronically-exposed vertebrate populations[215,422].

A distinct advantage of the alligator model at YWC is derived from ongoing and long-term monitoring and adult mark-recapture efforts that have taken place there since the 1970s[14,423]. Thus, it is possible to also describe connections between maternal age, transfer of dioxins to yolk, and AHR signaling in offspring for specific individuals. In Chapter 3, we attempt make use of this utility by identifying a positive trend (non-significant) between maternal SVL, which can be

used as a proxy for age[14], and offspring yolk contaminant burdens. This utility is typically reserved for either retrospective or prospective paired mother-child human cohort studies, where experimental investigations of causation are obviously precluded for ethical reasons. But these studies in the alligator offer an opportunity to describe the variation of maternal contributions to offspring contaminant exposures attributable to age.

The second area where additional research is warranted is the exploration of exposure-related pathologies *in situ* at YWC and in Winyah Bay. To date, only a single study has investigated toxic responses to dioxins *in ovo* in a crocodilian. Therein, Matter et al.[424] identify the developing gonad as a sensitive tissue, reporting morphological abnormalities in both the ovary and testis. This is generally corroborated by avian data wherein, in addition to reproductive abnormalities[425], low dose dioxin exposures in chick (*Gallus gallus*) can cause numerous cardiovascular deformities[426–428], hepatic fat accumulation[429], and reduced growth during developmental stages [425,428]. This would indicate that despite a paucity of data in the alligator, predictive endpoints of toxicity are available, including gross morphological abnormalities and altered growth patterns in early life stages.

The utility of the YWC population similarly presents a unique opportunity for investigating effects of exposures on male fertility. Evidence in laboratory models[118–120] and humans[430] alike implicate early-life dioxin exposures in impaired testicular development and sperm defects. However, in long-lived species like humans, the timing of exposures can elicit contradictory effects on

semen quality; exposures during infancy or childhood are associated with reduced sperm numbers and motility, while peripubertal exposures instead increase these parameters[430]. With access to individuals of known approximate age and estimated exposure timing, alligators at YWC present an opportunity to explore this connection in an environmentally-relevant setting and test possible mechanisms. For example, employing a grow out model, as used in Chapter 2, and administering dioxin either *in ovo* or during post-hatching stages, could delineate sperm abnormalities occurring during development versus maturation. Furthermore, this approach could be used to elucidate differing landscapes of dioxin-responsive genetic pathways that ultimately contribute to contradictory effects observed in humans.

Despite advantages of the alligator model at YWC, crocodilians do carry some relative disadvantages and limitations compared to traditional laboratory models. Chiefly, the protracted generation time (8-15 years, approximately[423,431]) and long reproductive lifespan of alligators (upwards of 30 years[14]) precludes transgenerational studies. Furthermore, the difficulty of maintaining captive breeding populations and the cryptic nature of crocodilian copulation in the wild limits controlled mating studies that could be used to identify genetic loci dictating contaminant responses; it also limits the feasibility of genome editing techniques (e.g., Crispr-Cas9) that would be useful in probing the role of AHR signaling components in toxicity. Lastly, the same reasons that make crocodilians effective environmental sentinels of pollution – their trophic status and longevity – consequently makes true “reference” populations difficult to establish, as some

degree environmental contamination is likely to occur in every population. This is exemplified by the detection of elevated furan levels at Woodruff in Chapter 3; this ubiquitous presence of yolk contaminants in alligator studies necessitates proper contextualization of findings. However, this limitation is generally manageable by proper study design and furthermore could be viewed as a boon to the "environmental relevance" of the alligator (e.g., human exposures similarly occur against a contaminated backdrop).

The overall goal of our work at YWC has been to explore the effects and mechanisms of long-term dioxin exposure in the alligator, and then to use these findings to inform risks to environmental health of the Winyah Bay system as a whole. Due to the limited knowledge of AHR signaling and dioxin in the alligator and at YWC, our efforts first had to establish a foundation from which these questions could be addressed. And while those questions of overt chronic dioxin toxicity are ultimately beyond the scope of this dissertation work, our findings provide the inertia for these and many more questions in Winyah Bay.

6.3 Apopka and Precocious Estrogen Signaling: Conclusions, Limitations, and Future Directions of Chapters 4 and 5

In Chapters 4 and 5, our overall objective was to explore the contributions of developmental EDC exposures to future reproductive health and function. Specifically, we sought to elucidate the mechanistic underpinnings of EDC-induced reproductive pathologies in alligators at Lake Apopka. Despite being an influential wildlife model of endocrine disruption, the connections linking OCPs at

Apopka to altered reproductive development have remained mostly associative to date. In an attempt to explore these associations experimentally, we tested the hypothesis that Apopka contaminants collectively act in an estrogenic fashion at inappropriate times during gonadal development. In Chapter 4, we achieved this by exposing embryos from a reference population to a single dose of either estradiol or DHT, a non-aromatizable androgen, at the bipotential stage of gonadal development, then assessed the ability of said exposures to recapitulate Apopka transcriptional patterns at the juvenile stage. The ability of estradiol, but not DHT, to recapitulate persistent patterns of transcriptional suppression in the ovary provided a strong indication that EDCs at Lake Apopka are collectively acting in an estrogenic fashion. The work presented in this chapter provides the first experimental description of a mechanism underlying pathologies at Apopka and reveals that the timing of EDC exposures (i.e., before the gonad acquires the capability for sex-specific steroid hormone production) is crucial to their effects on future reproductive health.

In light of our observations in Chapter 4, in Chapter 5 we sought to probe the full extent of developmental programming attributable to EDC exposures using a non-targeted, genomics-based approach. Furthermore, we sought to reassess the ability of precocious estradiol to recapitulate EDC-induced transcriptional programs. Despite a wealth of data describing specific pathologies and transcriptional shifts in the reproductive tract following EDC exposures, we know relatively little regarding how exposures collectively contribute to altered ovarian function at the genomic level, particularly under environmentally-relevant

contexts. In this fashion, employing non-biased methods permitted us to place changes observed at Apopka in Chapter 4 in a broader functional context. Through this approach, we uncovered a strong association between site of origin and the ovarian transcriptome, with over 75% of detectable ovarian genes differentially expressed between Apopka juveniles and a reference population. Consistent functional patterns were detected within differentially expressed genes: genes suppressed at Apopka were consistently enriched for mitotic pathways and the cell cycle, while genes elevated at Apopka were enriched for pathways relating to cytoskeletal features. Furthermore, we uncovered that transcriptional shifts occurred concomitantly with reductions to late-stage, pre-vitellogenic follicles. Correlations between expression patterns and follicle counts revealed that these reductions were tightly associated with differentially expressed genes, suggesting that the ovarian follicle might be highly sensitive to developmental endocrine cues and represent the predominant transcriptional unit in the ovary. Finally, as described in Chapter 4, precocious exposures to estradiol in reference animals was sufficient to recapitulate the majority of follicular and transcriptional changes observed at Apopka.

Taken together and placed in the context of prior work at Apopka, data in these two chapters suggest three possible models linking altered follicle profiles and transcriptional programs to precocious estrogen signaling (Figure 6.1) in the ovary. The first model (Figure 6.1A) hinges on the ability of precocious estrogen to imprint expression of (1) steroidogenic enzymes, thus disrupting circulating hormone levels, or (2) steroid hormone receptors, thus disrupting endocrine

signaling in target tissues (i.e., ovarian follicles). Apopka animals have been repeatedly characterized by disruptions to gonadal steroidogenesis, both at the transcriptional level and at the level of enzymatic function[16,80,82,177,417]. As a consequence, circulating levels of estrogens and androgens are affected, influencing endocrine-mediated transcriptional networks are estrogen-stimulated follicle development. Under this paradigm, we would anticipate (1) levels of circulating estrogen to be suppressed in Apopka or estradiol-treated reference animals, and, (2) that supplementing juveniles with exogenous estrogens would ameliorate transcriptional shifts and promote increased numbers of stage III follicles. Alternatively, if steroid hormone receptor expression is compromised as has previously been reported previously in Apopka animals and in Chapter 4 (Figure 4.2G)[82,286], circulating estrogen levels might remain suppressed in Apopka/estradiol-treated animals, but supplementation would not be expected to rescue transcription or follicle development. Further, under either scenario, we would expect to observe altered epigenetic patterning of steroidogenic enzymes or steroid hormone receptors. However, given the persistent suppression of *ESR2*, but not *CYP19A1,* in Chapter 4 (Figure 4.2A,G), the former of these possibilities seems more plausible.

In the second model (Figure 6.1B), precocious estrogen signaling acts directly on germ cells in the bipotential gonad, prolonging proliferation and delaying meiotic initiation, leading to reduced densities of stage III oocytes. This model hinges on the ability of estrogen signaling to drive germ cell proliferation and delay meiotic initiation[408,415,432,433], which is an early step in the production of

follicles (primary oocytes) from oogonia[434]. Under this model, precocious estrogen signaling compromises meiotic initiation and reduces the pool of primary oocytes, in turn reducing the proportion of stage III follicles. If correct, we would anticipate that (1) administration of estrogen or estrogenic OCPs at a bipotential stage would induce aberrant germ cell proliferation that (2) estrogen treated animals would exhibit a greater proportion of proliferative germ cells and oogonia (and fewer primary oocytes) at hatching and into juvenile stages. It is also possible that precocious estrogen influences germ cell behavior, but in the opposite fashion as outlined above. Estrogen signaling is capable of inducing expression of inhibin and FST, which antagonize activin signaling, a pro-proliferative germ cell factor[287]. Rather than favoring proliferation and delaying differentiation, precocious estrogen might instead suppress germ cell proliferation. In this case, we would anticipate that estrogen or estrogenic OCPs would reduce germ cell numbers and overall density of oocyte nests.

In the final model (Figure 6.1C), precocious estrogen signals influence follicle assembly or recruitment through altered behavior and proliferation of granulosa cells, as has been suggested by Guillette and Moore[287], rather than affecting germ cells. In the Guillette and Moore model, precocious estrogen signaling could induce mistimed expression of inhibin and follistatin, which antagonize the activity of activin. In addition to its role in germ cell proliferation, activin also promotes proliferation in granulosa cells[287]. Compromised proliferation of granulosa cells in turn could delay development of primary oocytes by slowing germ cell nest breakdown[435], thereby leading to reduced

numbers of stage III follicles in juveniles. It is also possible that estrogens influence granulosa proliferation in a different manner than outlined above. Rather than influencing activin-mediated granulosa proliferation, precocious estrogen signaling might act in a fashion similar to what has been observed in the developing reproductive tract following DES exposures. Therein, DES and other non-steroidal estrogens directly disrupt patterns of HOXA10 expression and methylation in the female reproductive tract, which in turn disrupts regional differentiation and induces vaginal and uterine abnormalities[348,354]. Under this model, precocious estrogen signaling might similarly disrupt expression of HOXA7 expression, which is critical for granulosa growth and proliferation[352] by suppressing GDF-9, an oocyte derived growth factor, which promotes HOXA7 expression in granulosa cells. Interestingly, expression of GDF-9 is persistently suppressed in the ovary of Apopka juveniles, but the etiology of suppressed GDF-9 expression is currently unknown[285,436]. Whether mediated through an inhibin/activin mechanism or through altered HOX expression, under this model, we would anticipate that (1) precocious estrogen signaling would lead to suppressed proliferation of granulosa or pre-granulosa cells and (2) reduce the number of primary oocytes relative to germ cells or oogonial nests.

Together, these three models highlight crucial next steps at Apopka. Foremost, quantification of circulating steroid hormone levels is necessary to delineate direct developmental effects of precocious estrogen signaling (e.g., imprinting or persistent suppression via epigenetic mechanisms; compromised germ/somatic cell proliferation) from indirect effects resulting from altered

circulating hormone levels in juveniles (e.g., alterations to estrogen-regulated follicle growth, transcription). Secondly, describing (1) the ontogeny of estrogen receptor expression in the alligator and (2) the effects of precocious estrogen signaling on somatic (pre-granulosa) and germ cell behavior in the bipotential gonad will help elucidate the etiology of reduced follicle densities in juveniles and affirm the plausibility of effects being mediated through the estrogen receptor. Co-treatment of embryos with an estrogen receptor antagonist at the bipotential stage would also support (or refute) a direct role for the estrogen receptor in mediating effects of precocious signaling.

The potential involvement of the Polycomb epigenetic modifiers in reproductive pathologies at Apopka is intriguing in light of recent evidence that their functional counterpart, the histone demethylase, KDM6B, is the most proximate factor regulating sex determination in TSD species. KDM6B acts to remove H3K27 methylation and activate gene expression, while the polycomb repressive complex 2 adds these marks to repress expression[437,438]. Critically, both KDM6B and the polycomb methylase EZH2 are directly regulated by estrogen signaling but in opposite directions[334,418,419,437], which would suggest that precocious estrogen signaling might disrupt future reproductive function by "tipping" the balance between permissive and repressive chromatin states. However, this role for estrogen signaling in regulation of KDM6B and EZH2 might also suggest that enrichment of polycomb components in differentially expressed genes is the result of differing levels of circulating estrogens in juveniles. Thus, future efforts should be taken to describe both the short-term and long-term

impacts of estrogen signaling on epigenetic patterning in both embryos and juveniles.

At a broader scale, findings in Chapters 4 and 5 raise questions regarding the adaptive potential of the endocrine system during development. Early evolutionary hypotheses explaining the adaptive potential of environmental sex determination, (i.e., Charnov and Bull) established a putative role for estrogen signaling as a driver of female development under environmental conditions expected to maximize female fitness[36]. However, a more nuanced adaptive role for estrogen signaling may be evinced, as has been described for androgens and glucocorticoids in birds. Therein, under variable but predictable environments, maternal females can modulate levels of androgens and glucocorticoids in yolk, which in turn induce physiological and behavioral phenotypes in offspring that are thought to maximize fitness in a given environment[439,440]. This prenatal-sensing/postnatal-response axis forms the backbone of environmental matching and predictive-adaptive response hypotheses[441]. Estrogens, however, have been received relatively little attention in this context. Our data might suggest that changes to maternal allocation of yolk estrogens could promote variation in reproductive development and future function. Indeed, empirical evidence supporting this paradigm has been demonstrated in turtle[442], wherein laying females can influence offspring sex by increasing yolk estradiol levels between clutches in a single year. However, a role for yolk estrogens in promoting variation within a sex has not been demonstrated. And while investigated herein under the context of endocrine disruptors, our research highlights that regulation

(or dysregulation) of endocrine signaling can influence patterns of variability in ovarian function. Clearly, additional research is required to confirm (1) that variation in yolk estrogens promote variable reproductive phenotypes in populations *in situ* and (2) that there is any such adaptive potential to these variable phenotypes. However, these possibilities are nonetheless intriguing and highlight a substantial gap in our current understanding of adaptive benefits of endocrine signaling during development.

Overall, findings reported in these two chapters directly inform our understanding of the developmental origins of health and disease. Consistent with Barker et al.'s DOHaD model, we have uncovered a possible mechanism linking the developmental endocrine environment to future reproductive function in the form of dramatic shifts in ovarian transcription and follicle development. Importantly, these observations have been made in an environmentally-relevant context, implying that these effects are highly probable to occur in populations exposed *in situ*, including humans. Further, we have provided suggestive evidence that a collective developmental burden of weakly estrogenic contaminants is capable of acting in a manner consistent with a potent, endogenous estrogen, estradiol.

6.4 Final Conclusions

In summation, this dissertation work includes two major contributions that span ecotoxicology, endocrinology, and developmental biology. First, we have identified the ability of a long-lived and highly toxic contaminant, dioxin, to influence development decades after its release. In so doing, we have laid the

foundation for future investigative studies of the effects of chronic, long-term exposures. Secondly, we have uncovered a putative mechanism underlying a complex suite of reproductive pathologies in the alligator, and subsequently highlighted a suite of functional consequences of developmental endocrine disruption. And, as outlined above, each of these major contributions is accompanied by a myriad of new questions and avenues of research.

Despite its presentation as two distinct narratives, findings at YWC and Apopka stand to inform a larger, unified understanding of organismal responses to complex contaminant exposures. For example, the observed shifts in AHR expression observed in chapter 2 (reduced hepatic AHR expression in Apopka embryos) and chapter 4 (reduced ovarian AHR expression in Apopka juveniles) suggest that exposures to estrogenic contaminants might program AHR-mediated contaminant responses in the short term and long-term. Furthermore, the AHR and ER engage in complex regulatory crosstalk, wherein each can either antagonize or potentiate the other's signaling through both protein-protein interactions[130,323] and epigenetic regulation of target genes[443]. Thus, future studies at YWC stand to gain by incorporating considerations of dioxin exposures on estrogen-mediated developmental processes, while studies at Apopka stand to gain by exploring the mechanisms of OCP-induced changes in AHR expression. In both instances, investigations might reveal that exposures *in ovo* to AHR or ER ligands modulate contaminant responses later in life, and that early exposures could predispose organisms to reduced sensitivity later in life.

Ultimately, this work underscores the utility of the alligator of an environmentally-relevant developmental model. However, alligators and crocodilians are charismatic, keystone species and thus are integral components of healthy ecosystems[268]. Therefore, the importance of studying the health of crocodilian populations for their own sake should not be overlooked. In the face of ongoing global change, industrialization, and a changing climate, is my hope that the work presented herein can contribute in some small way to the preservation of alligators and crocodilians globally.

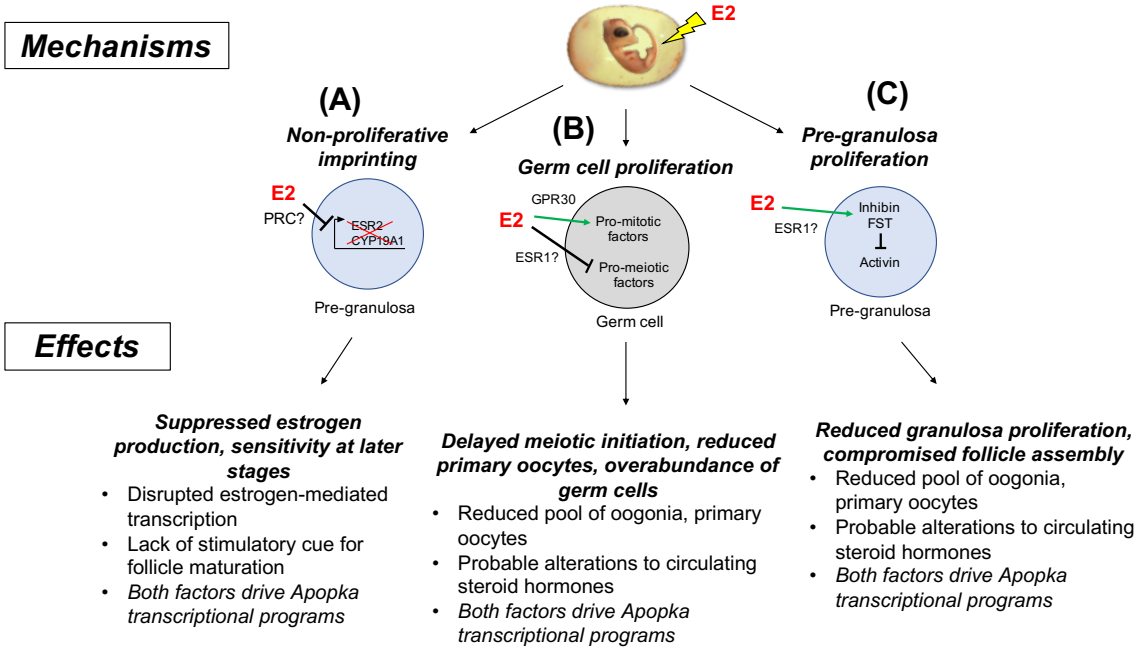


Figure 6.1. Possible mechanisms linking precocious estrogen signaling to future ovarian function. Three possible models describing the link between transcriptional programs/follicle development and precocious estrogen signaling include (A) imprinting of steroidogenic enzymes or hormone receptors, altered germ cell proliferation (B), or altered supporting somatic proliferation. In (A) mistimed estrogenic signals inappropriately activate epigenetic modifiers like polycomb components, leading to imprinting and persistent suppression of steroidgenic factors including CYP19A1 (reported previously [82]) or ESR2 (reported herein). Imprinting leads to either reduced steroidogenesis or sensitivity to paracrine cues, disrupting estrogen-mediated transcription and follicle development. In (B), mistimed cues inappropriately activate expression of pro-mitotic factors via non-genomic estrogen signaling (GPR30) and suppress expression of pro-meiotic factors via (possible) genomic signaling. This leads to

an overabundance of germ cells and a lack of oogonia or primary oocytes. An alternative version of model (B) involves the precocious activation of inhibin and FST expression that antagonizes pro-proliferative activin (not shown). This in turn suppress germ cell proliferation and drives a reduction in oogonia and primary oocytes. In model (C), precocious estrogen signaling acts via a similar mechanism to induce inhibin and FST expression, antagonizing activin and suppressing granulosa proliferation. This compromises germ cell nest breakdown and follicle assembly, driving a reduction in oogonia and primary oocyte numbers. An alternative version of model (C) involves the ability of estrogenic EDCs to suppress expression of GDF-9 (reported previously [285]) via an unknown mechanism. GDF-9 promotes expression of the pro-proliferative granulosa factor HOXA7 (not shown). Suppression of HOXA7 similarly compromises granulosa proliferation and follicle assembly.

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