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Gene expression patterns in juvenile American alligators (*Alligator mississippiensis*) exposed to environmental contaminants

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ABSTRACT

Reproductive and developmental abnormalities have been reported in the American alligator (*Alligator mississippiensis*) population from Lake Apopka, FL, that is chronically exposed to a complex mixture of environmental contaminants. To begin to understand the molecular mechanisms that could lead to the observed abnormalities of the reproductive and endocrine system, we quantified concentrations of the steroid hormones testosterone (T) and estradiol-17β (E_2) and expression of steroid hormone receptors and genes relating to steroidogenesis in gonadal tissue from juvenile alligators from three lakes in Florida using enzyme immunoassay and quantitative real-time polymerase chain reaction. Alterations of ESR2 (estrogen receptor β) and SF1 (steroidogenic factor 1) mRNA expression in male gonadal tissue, without an observed difference in plasma concentrations of T, from the different lakes, begin to provide insight into potential mechanisms underlying the alterations of the reproductive system previously observed. Likewise, alterations in P450 aromatase and DAX1 (dosage-sensitive sex reversal, adrenal hypoplasia congenita critical region on the X chromosome, gene 1) mRNA expression, with elevated plasma E_2 concentrations in females, provide leads to the potential mechanisms modifying folliculogenesis and ovarian development. The investigation of these genes also helps clarify normal endocrine and reproductive system function in the American alligator.

1. Introduction

Chemical contamination of the environment is a global concern. Various environmental contaminants can alter development, reproduction and endocrine physiology in wildlife (Guillette and Gunderson, 2001; Milnes et al., 2006; Tyler et al., 1998). A variety of chemical contaminants have been observed to mimic hormones (with most data, to date, indicating an interaction with nuclear steroid hormone receptors), act as hormone antagonists or alter the synthesis and/or degradation of hormones (Crain and Guillette, 1997; Guillette and Gunderson, 2001; Guillette and Milnes, 2001; McLachlan, 2001). Therefore, an integrated investigation examining circulating hormone concentrations, and expression of genes essential for steroidogenesis and steroid hormone reception (nuclear steroid receptors) would advance our understanding of the possible mechanisms of endocrine disruption. The alligator population from Lake Apopka, FL, has been observed to have reproductive and developmental abnormalities associated with chronic exposure to a variety of environmental contaminants derived from agricultural activity, storm water runoff and a pesticide spill of dicofol (composed of up to 15% DDT) and sulfuric acid in 1980 (Guillette et al., 1999; EPA, unpublished report). Alligators from Lake Apopka have elevated concentrations of various pesticides and their metabolites, as well as heavy metals in their blood and eggs (Guillette et al., 1999; Heinz et al., 1991; Burger et al., 2000). Lake Woodruff National Wildlife Refuge (LWNWR) has been used as the reference lake in our studies as low levels of pesticides have been reported in their blood and eggs and this lake is relatively oligotrophic, with low nutrient levels (Guillette et al., 1999; Guillette and Edwards, 2005). Orange Lake, in contrast, is more eutrophic than LWNWR, although both lakes have low levels of organochlorine pesticides and their metabolites (Guillette and Edwards, 2005).

Reduced plasma concentrations of sex steroids, testosterone (T) or estradiol-17β (E_2) have been observed in juvenile male or female alligators from Lake Apopka relative to similar sized and aged alligators from LWNWR (Guillette et al., 2000). These endocrine abnormalities displayed seasonal variation as well with concentrations of plasma sex steroid hormones, E_2 or T, varying over the spring and summer in juvenile alligators from LWNWR but not in

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animals from Lake Apopka (Rooney et al., 2004). Although we have studied changes in hormones and morphology among various populations of alligators in Florida, the relationship between plasma hormone concentrations and mRNA expression of steroidogenic genes in gonadal tissue has not been investigated.

Sex steroid hormones are essential for normal reproductive activity in both male and female vertebrates and they have direct actions during sex determination in some vertebrates. Estrogen-induced responses occur, at least in part, by interacting with a class of nuclear transcription factors, the estrogen receptors (ESRs) (Nilsson and Gustafsson, 2002). Alligators have two distinct nuclear estrogen receptors, ESR1 (ERα) and ESR2 (ERβ), as found in other amniote vertebrates (Katsu et al., 2004). Further, we have shown that a number of contaminants found in either the blood or eggs of alligators have the ability to bind with the ESR and displace its endogenous ligand, estradiol-17β, suggesting these compounds have the potential to disrupt estrogen signaling (Guillette et al., 2002; Vonier et al., 1996).

Sex steroid hormones are synthesized via steroidogenesis involving various enzymes and regulating factors (Payne and Hales, 2004). One of the key proteins in steroidogenesis is steroidogenic acute regulatory protein (StAR) which regulates the transfer of cholesterol from the cytoplasm to the inner mitochondrial membrane; the initiation of a series of enzyme reactions required for steroidogenesis (Stocco, 2000). StAR gene expression is regulated by steroidogenic factor 1 (SF1) in mammals (Manna et al., 2003). SF1 is expressed in adult steroidogenic tissues where it plays a role in regulating the expression of several steroidogenic enzymes and is an essential factor in vertebrate sex differentiation (Parker and Schimmer, 1997). In the mouse, SF1 co-localizes with DAX1 (dosage-sensitive sex reversal, adrenal hypoplasia congenita critical region on the X chromosome, gene 1) in the steroidogenic cells of the developing gonads, adrenals, hypothalamus and anterior pituitary, indicating that these proteins are important in the endocrine system (Ikeda et al., 1996). DAX1 is an orphan member of the nuclear hormone receptor superfamily of transcription factors, and plays an important role in germ cell development, ovarian determination and ovarian development (Swain et al., 1998, 1996; Yu et al., 1998). DAX1 is also reported to play a role in regulating cytochrome P450 aromatase ($P450_{arom}$) expression; this enzyme catalyzes estrogen production from an androgen substrate, and plays important roles in gonadal development in many vertebrates (Simpson et al., 1994; Wang et al., 2001). Understanding the expression patterns of these genes (SF1, DAX1 or $P450_{arom}$), related to steroidogenesis, in the alligator provides an important advance to our understanding of the molecular mechanisms underlying the endocrine and reproductive alterations due to various environmental contaminants. The aim of this study was to understand the normal and potentially abnormal reproductive and endocrine systems in juvenile alligators from three central Florida lakes known to have differing exposures to various environmental contaminants.

2. Materials and methods

2.1. Animals

Juvenile American alligators (*Alligator mississippiensis*) were collected by hand at night from an airboat. Five females and five males each were collected from Lake Woodruff (29°06′N; 81°25′W), Orange Lake (29°28′N; 82°11′W) and Lake Apopka (28°37′N; 81°37′W), FL, USA, on April 16, 18 and 17, 2002, respectively. The average snout vent length (SVL) was 54.86 ± 1.35 cm in males and 57.24 ± 1.61 cm in females, and the average body weight (BW) was 3.75 ± 0.40 kg in males and 3.91 ± 0.34 kg in females. There was no difference in SVL and BW among groups collected from the three lakes. We have previously examined growth in juvenile alligators from lakes Apopka and Woodruff, using growth rings in the femur, and reported that animals obtained from these lakes did not display significant differences in growth either between lakes or sexes and that males and females between 50 and 60 cm SVL were 5–7 years of age (Milnes et al., 2002).

Blood was collected within 1 min of capture from the post cranial supravertebral vein using a sterile needle and syringe; whole blood was transferred to Vacutainer® tubes containing lithium heparin (BD Technologies, Research Triangle Park, NC), and kept on ice until centrifuged within 4 h. Blood was separated into plasma and blood cells by centrifugation at 1200 × *g* for 15 min at 4 °C. The supernatant (plasma) was stored at −70 °C until analyzed. Animals were killed by a lethal dose of sodium pentobarbital (Sigma Chemical Co., St. Louis, MO), and the testis and ovary were isolated and preserved in RNA*later*® (Ambion, Austin, TX). After incubation of tissues in RNA*later*® at 4 °C overnight, tissues were frozen at −70 °C until used. Animal care and experimental process were approved by the Institutional Animal Care and Use Committee, University of Florida.

2.2. Enzyme immunoassay (EIA)

To analyze steroid hormones, the plasma samples were double extracted using ethyl ether. Five milliliters of ethyl ether were mixed with 0.1 ml (for testosterone [*T*]) or 0.2 ml (for estradiol-17β [E_2]) of plasma for 1 min in a clean glass tube. The sample tube was put in the methanol bath with dry ice at −25 °C to freeze the aqueous phase. The organic phase, including steroid hormones, was transferred to a clean glass tube. The process was repeated to maximize removal of plasma steroids. After extraction, the organic phase, containing the hormones, was evaporated under nitrogen. The plasma concentrations of *T* and E_2 were measured in duplicate by commercial EIA kit according to the instructions provided (Cayman Chemical, Ann Arbor, MI). The extracted steroid hormones were hydrated in 150 μl (*T*) or 250 μl (E_2) of the assay buffer. Then, 50 μl of hydrated samples was use in each assay. The volume for hydration of each sample was adjusted so that the specific binding was in the range of 20–80% binding, which is approximately 6–110 pg/ml for *T* and 10–900 pg/ml for E_2. The specificity of the *T* EIA is 100% for testosterone, 27.4% for 5α-dihydrotestosterone, 18.9% for 5β-dihydrotestosterone, 3.7% for androstenedione, 2.2% for 11-keto testosterone and less than 1% for other steroids tested, whereas the E_2 EIA displayed specificity of 100% for estradiol-17β, 17% for estradiol-3-glucuronide, 4% for estrone and less than 1% for other steroids according to the manufacture's protocols. These assays were validated for the samples extracted from alligator plasma using the series dilution of the extracted plasma samples. The serial diluted samples revealed parallelism with the standard curves provided by the kit, indicating that proteins or other factors in the extracted sample of alligator plasma does not alter the antibody binding to *T* or E_2 in this assay.

2.3. Alligator molecular biology

The cDNAs encoding the estrogen receptors, ESR1 and ESR2 and the progesterone receptor (PR) were cloned previously from the American alligator (Katsu et al., 2004), as were the cDNAs for $P450_{arom}$, DAX1and SF1 (Gabriel et al., 2001; Western et al., 2000).

Table 1
The primers for quantitative real-time PCR of alligator gonadal tissues

Name	Direction	Position (base)	Sequence (5′–3′)	Amplified length (base)	GenBank accession or reference
Ribosomal protein L8	F	–	GGTGTGGCTATGAATCCTGT	–	Katsu et al. (2004)
	R	–	ACGACGAGCAGCAATAAGAC		
ESR1	F	410	AAGCTGCCCCTTCAACTTTTTA	72	AB115909
	R	481	TGGACATCCTCTCCCTGCC		
ESR2	F	34	AAGACCAGGCGCAAAAGCT	72	AB115910
	R	105	GCCACATTTCATCATTCCCAC		
PR	F	50	AAATCCGTAGGAAGAACTGTCCAG	72	AB115911
	R	121	GACCTCCAAGGACCATTCCA		
$P450_{arom}$	F	1227	CAGCCAGTTGTGGACTTGATCA	79	AY029233
	R	1305	TTGTCCCCTTTTTCACAGGATAG		
DAX1	F	618	TGCTCTTTCCTTGCTGAGATC	74	AF180295
	R	692	ACTGTGCCAATGATAGGCCTA		
SF1	F	141	CAGTCTCGATGTGAAATACCTGGA	67	AF180296
	R	207	CGCGTTGGCCTTCTCCT		

F, forward; R, reverse.

2.4. RNA isolation

Total RNA was isolated using the RNeasy total RNA isolation kit (Qiagen, Valencia, CA), and was treated with ribonuclease-free deoxyribonuclease I (DNase I; Qiagen) to remove the contamination of genomic DNA. The concentrations and quality of the RNA samples were estimated by measuring optical density at 260 nm and by formaldehyde gel electrophoresis. After isolation, RNA was stored at −70 °C until analyzed.

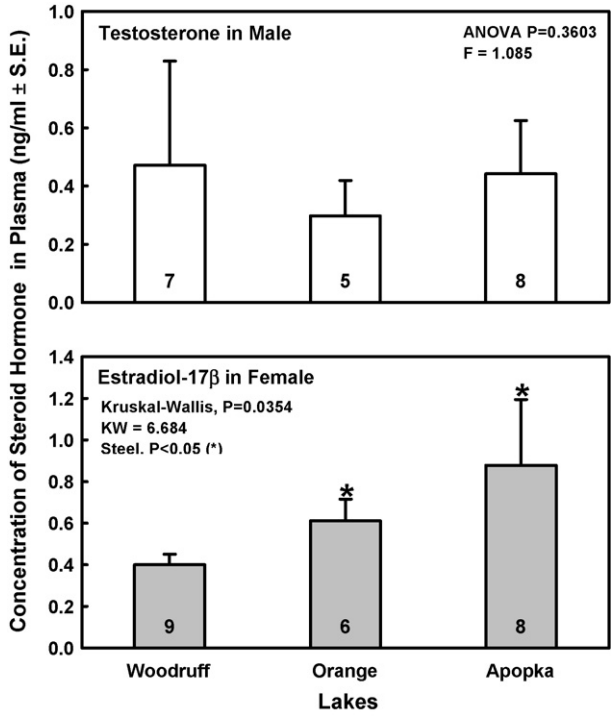

Fig. 1. Plasma testosterone (*T*) concentrations from male juvenile alligators and plasma estradiol-17β (E_2) concentrations from female juvenile alligators obtained from Lake Woodruff (WO), Orange Lake (OR) or Lake Apopka (AP), FL, USA. Female alligators from OR or AP had a trend of elevated plasma E_2 concentrations relative to female alligators from WO, whereas there was no difference in male plasma T concentrations among males from the different lakes.

2.5. Quantitative real-time PCR

Quantitative real-time PCR (Q-PCR) analyses were performed on a Gene Amp 5600 sequence detection system instrument and software with the default setting (Applied Biosystems, Foster City, CA). One microgram of DNase I-treated total RNA was used in 20 μl of RT (reverse transcription) reaction with super script II (Invitrogen, Carlsbad, CA) and random primer (Invitrogen). One microliter of RT product was used as the template DNA in 15 μl of PCR. SYBR Green PCR Master Mix (Applied Biosystems) was used with the specific primer pairs (see Table 1) in triplicate for Q-PCR analyses. The specificity of amplification was confirmed by the dissociation curve method after the reactions. The mRNA expression of each target gene was normalized by an endogenous control gene, ribosomal protein L8, and data were expressed as the mean ± S.E. by calculations with the comparative C_T method (Applied Biosystems, 1997). This calculation method requires a validation that efficiencies of the target gene and reference gene are approximately equal; these calculations were performed and these assays are valid (data not shown).

To compare the mRNA expression levels of ESR1 and ESR2, initial signal density was estimated using the C_T value and the efficiency of amplification in PCR with each primer. The equation for the amplification of the signal in PCR is: $X_n = X_0 \times (1 + E_x)^n$ with X_n = signal density of target molecules at cycle n; X_0 = initial signal density of target molecules; E_x = efficiency of target amplification; n = number of cycles (Rasmussen, 2001). Efficiency of amplification in PCR was calculated by: $E_x = 10^S - 1$ with S = slope in log plot of PCR amplification. Data from 0.1 to 1.0 of arbitrary signal units on the PCR amplification plot were used to estimate the slope. The initial signal density was given by: $X_0 = X_n/(1 + E_x)^n$. At the threshold in Q-PCR, these are defined as: X_n = threshold; $n = C_T$ value in Q-PCR (Ramakers et al., 2003). The initial signal density was divided by the length of target DNA, because SYBR green I bound to double strand DNA depends on length (Schneeberger et al., 1995; Vitzthum et al., 1999).

2.6. Statistical analysis

Data were analyzed by Student's *t*-test or Welch's *t*-test for unpaired groups, or by one-way analysis of variance (ANOVA) for multiple parametric comparisons, Mann–Whitney *U*-tests were also used for comparison of unpaired groups, or Kruskal–Wallis tests and Steel tests were used for the multiple comparisons in non-

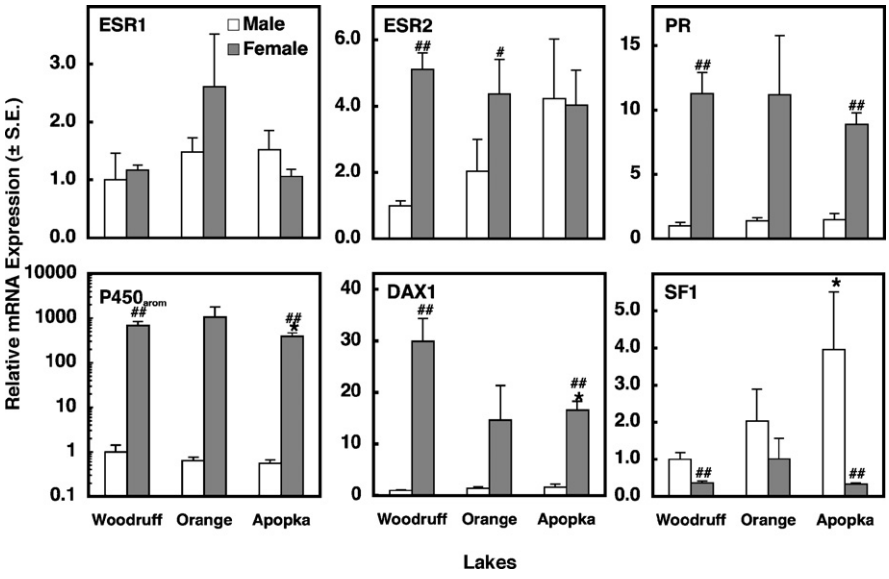

Fig. 2. Comparison of mRNA expression for various genes in the gonads of juvenile alligators comparing sex and lake of origin. Sexual dimorphism in the expression of estrogen receptor 1 (ESR1), progesterone receptor (PR), aromatase ($P450_{arom}$), DAX1 and steroidogenic factor 1 (SF1) mRNA was observed in all juvenile alligators from Lake Woodruff (WO) whereas gonadal expression of ESR2 was not sexually dimorphic. Data were normalized by the expression of ribosomal protein L8 using the comparative C_T ($\Delta\Delta C_T$) method and were expressed as a relative expression to males from WO. Sharp and asterisk symbols indicate significant difference in sex (#, $P<0.05$; ##, $P<0.01$) within a lake, and in comparison to animals of the same sex from the reference lake, WO (*, $P<0.05$).

parametric analyses. To compare the expression level of ESR1 and ESR2 mRNA, paired *T*-tests or Wilcoxon matched pair tests were used in parametric or nonparametric analyses, respectively. Spearman rank correlation test was used to validate the correlations. Data are reported in figures as mean ± standard error (S.E.).

3. Results

3.1. Hormone concentrations in plasma

No difference in plasma concentrations of testosterone (*T*) was observed in males collected from the three lakes (Fig. 1). In contrast, plasma concentrations of estradiol-17β (E_2) exhibited a different pattern among the lakes. Plasma concentrations of E_2 collected from female alligators from Lake Apopka and Orange Lake were elevated compared to values obtained for juvenile females from Lake Woodruff National Wildlife Refuge (LWNWR) (Fig. 1).

3.2. mRNA expression of steroid hormone receptors and steroidogenesis-related genes

Differences of threshold cycle (C_T) value in target genes and an endogenous gene (ribosomal protein L8) were constant when the initial amount of RNA was changed. The slope should be less than 0.1 and was so for all of our analyses. This constancy satisfied the assumption of the $\Delta\Delta C_T$ method in the calculation of the relative quantification of gene expression.

The expression of PR, $P450_{arom}$, DAX1 and SF1 mRNA exhibited sexual dimorphism in animals from LWNWR and Lake Apopka, whereas animals from Orange Lake showed no sexual dimorphism in the expression of these mRNAs (Fig. 2). Sexual dimorphism in the expression of ESR2 mRNA was not observed in juveniles from Lake Apopka, whereas it was seen in alligators from LWNWR and Orange Lake (Fig. 2). Plasma concentrations of T and E_2 were significantly correlated with the expression of ESR2 mRNA in both male and female juvenile alligators from Lake Apopka (Table 2). The expression of $P450_{arom}$ and DAX1 mRNA in females from Lake Apopka was lower than that observed in females from LWNWR (Fig. 2). Males from Lake Apopka exhibited higher expression of SF1 mRNAs when compared with expression observed in males from LWNWR (Fig. 2).

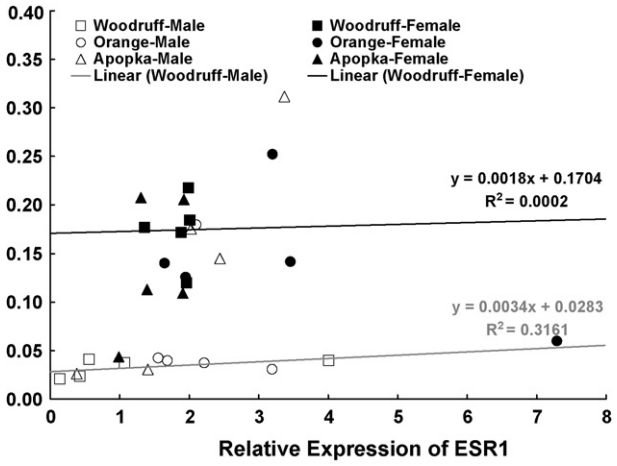

Fig. 3. Comparison of alligator gonadal mRNA expression of ESR1 and ESR2 between sexes and among the lakes. Initial signal density was estimated by using a linear regression approach described in the methods. Data were normalized by the expression of ribosomal protein L8, and were expressed as relative expression to males from Lake Woodruff.

The expression of ESR1 mRNA was higher when compared with ESR2 in both sexes from all lakes ($P<0.01$), however females from LWNWR and Orange Lake had higher levels of ESR2 compared to males whereas this pattern was lost in animals from Lake Apopka (Fig. 3).

4. Discussion

Juvenile alligators, over 40 cm snout vent length, exhibit gonadal steroidogenesis and annual cyclicity in plasma sex steroids (Rooney et al., 2004). Although the plasma concentrations of sex steroids are lower than those seen in adults, these peripubertal animals provide an excellent model system to study alterations in endocrine

Table 2
The correlation of steroid hormone and mRNA expression

Lake		ESR1	ESR2	PR	$P450_{arom}$	DAX1	SF1
Testosterone in males							
WO ($N=5$)	R	−0.030	0.466	−0.741	−0.540	−0.199	0.533
	P	0.481	0.214	0.076	0.174	0.374	0.178
OR ($N=3$)	R	−0.694	0.932	−0.661	0.443	0.784	−0.500
	P	0.256	0.119	0.270	0.354	0.213	0.500
AP ($N=4$)	R	0.743	0.901	−0.595	0.045	−0.565	−0.567
	P	0.129	0.049*	0.203	0.477	0.217	0.216
Estradiol-17β in females							
WO ($N=5$)	R	−0.300	0.455	−0.602	−0.440	0.350	0.025
	P	0.342	0.221	0.141	0.229	0.282	0.484
OR ($N=5$)	R	0.579	0.113	0.770	0.613	0.296	−0.158
	P	0.153	0.428	0.064	0.136	0.315	0.400
AP ($N=5$)	R	−0.019	0.856	−0.071	0.721	0.216	0.292
	P	0.488	0.032	0.455	0.085	0.364	0.317

R, correlation coefficient; P, one-tailed P value.
* Indicate the statistical significance.

function. We compared animals obtained from three central Florida (USA) lakes, and observed that those caught from Lake Apopka and Orange Lake showed alterations in gonadal mRNA expression when compared to the reference population from Lake Woodruff National Wildlife Refuge (LWNWR). We observed elevations in gonadal ESR2 and SF1 mRNA expression in males, and differences in ovarian $P450_{arom}$ and DAX1 mRNA expression in females (see Fig. 4). Further, we observed that animals from Orange Lake exhibited altered gene expression compared to LWNWR but the pattern displayed in juvenile alligators from Lake Apopka was unique when compared to animals from the other two lakes.

The concentrations of sex steroids have been investigated previously in these populations of alligators and we reported that differences can exist in juvenile alligators among the lakes in Florida depending on the time of year the samples are collected (Guillette et al., 2000; Rooney et al., 2004). Our analysis of steroid hormone concentrations in the plasma of juvenile alligators coincides with a previous report that observed seasonal variation (Rooney et al., 2004). That study reported no difference in plasma T concentrations among juvenile males from the same lakes when animals we collected in early May. We observed the same in our animals that were caught in the middle of April. Rooney et al. (2004) suggested that variation in the seasonal pattern of plasma T concentrations could exist among years due to ambient temperature patterns and we conclude that the slight differences we see in our data versus that previous study are due to natural variation in seasonal patterns. The concentration of E_2 in plasma from Orange Lake or Lake Apopka females was higher than that observed from Lake Woodruff and Orange Lake females of similar size and age. Likewise, we observed obvious difference in gonadal gene expression for the enzyme $P450_{arom}$, essential for the conversion of androgens to estrogens. Further, mRNA expression of SF1, which in some species regulates $P450_{arom}$ expression in the ovary, also was not different when ovarian tissue from females from the different lakes was compared.

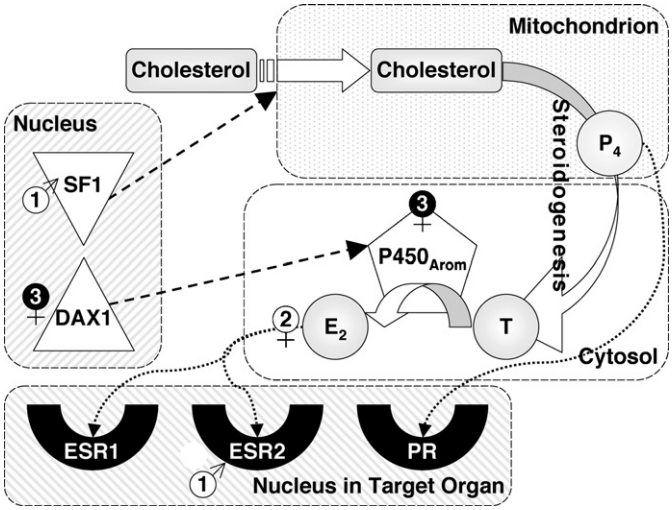

Fig. 4. Interactions of steroid hormones, their receptors and related genes examined in this study of juvenile alligators. Arrows indicate that SF1 or DAX1 regulates the transport of cholesterol or transcription of $P450_{arom}$, respectively. Broken arrows indicate binding the steroid hormone to the receptor. Open or filled numbers indicate the mRNA expression or hormone concentration was higher, or was lower in the contaminated lake (Lake Apopka) than in the reference lake (Lake Woodruff), respectively. Open circle #1 was observed in males, and open circle #2 or filled circle #3 was observed in females. These alterations could be caused by the environmental contaminants in the Lake Apopka.

The expression of ESR2 mRNA was not sexually dimorphic in alligators from Lake Apopka, unlike juveniles from the other lakes, but plasma T and E_2 concentrations and expression of ESR2 were correlated. Although plasma T concentrations in males were similar among the three lakes, expression of ESR2 and SF1 mRNA showed alterations in males from Lake Apopka when compared to males from other lakes. ESR1 is expressed at a higher level than ESR2 in the immature testes of the mouse (Jefferson et al., 2000). The expression of ESR1 was localized only to the Leydig cells, whereas ESR2 was observed in both Leydig and Sertoli cells of the rodent testis (O'Donnell et al., 2001), and ESR2 expressed in Sertoli cells was regulated by E_2 not T (Sneddon et al., 2005). In contrast to the testes, the ovary expresses both ESR1 and ESR2, and ESR2 is higher than ESR1 in immature rodents ovaries (Jefferson et al., 2000). ESR2 is localized in the granulosa cells, whereas ESR1 is expressed in the theca and interstitial cells of the rodent ovary (Mowa and Iwanaga, 2000; Hewitt and Korach, 2002). Therefore, it has been reported that the rodent testis expresses more ESR1 than ESR2, whereas more ESR2 than ESR1 is observed in the rodent ovary. However, juvenile alligator gonads expressed more ESR1 than ESR2 in both male and female gonads, although at this time, we have no knowledge concerning the localization (e.g., gonadal cell type) of ESRs in crocodilian gonads.

The ovary of the juvenile alligator has a structure somewhat unique when compared to rodents, in that it has a large popula-

tion of ovarian lacunae cells that have been hypothesized to give rise to the thecal cells of the developing ovarian follicle, and few granulosa cells (Moore et al., 2008). In our analysis, juvenile alligators clearly expressed more ESR1 than ESR2 in either gonad type, but the patterns were not exactly the same, as females from Lake Woodruff had significantly more ovarian ESR2 expression than seen in testicular tissue. A regression analysis of juveniles from Lake Woodruff revealed a clear difference in the pattern in ESR2 mRNA expression when males and females from the reference site, Lake Woodruff, were compared. Interestingly, three of five males from Lake Apopka and one male of five from Orange Lake exhibited the 'female' ratio pattern of ESR1/ESR2. Although based on a small sample size (due to the protected status of this species), these results suggest that a significant percentage of the male alligators from Lake Apopka had abnormal gonadal expression of ESR1 and ESR2 when compared with the alligators from the reference population, Lake Woodruff. Why might this be of concern? A number of studies examining environmental contaminants with estrogenic activity as well as phytoestrogens, demonstrate that these compounds bind and activate ESR2 more effectively that ESR1, although ESR2 displays a lower affinity for E_2 that ESR1 (Kuiper et al., 1997, 1998; Kostelac et al., 2003). Given our concern that a number of contaminants on Lake Apopka have estrogenic activity, based on previous studies showing an affinity between alligator ER and various contaminants found in alligator plasma or eggs (Guillette et al., 1999; Heinz et al., 1991) as well as estrogenic chemical-induced sex reversal of embryos (Matter et al., 1998; Milnes et al., 2005), the altered relative ratio of ESR1/ESR2 could indicate either higher potential responsiveness to environmental estrogens in males or be a 'signal' that these animals are exposed and ESR2 expression is elevated in a manner different that seen in reference males. Therefore, the different expression levels of each ESR type could contribute to a differing sensitivity of the alligator gonad to estrogenic environmental contaminants, and might be one of the keys to clarifying the gonadal alterations observed in alligators from Lake Apopka.

Plasma E_2 concentrations in juvenile female alligators were slightly different among the three lakes. Gonadal expression of $P450_{arom}$ and DAX1 were reduced in ovarian tissue from females from Lake Apopka compared with females from Lake Woodruff. DAX1 regulates $P450_{arom}$ gene expression, which catalyzes estrogen production from an androgen substrate, in mammals (Simpson et al., 1994; Wang et al., 2001). As the result of negative feedback, gonadal expression of $P450_{arom}$ and DAX1 could be reduced in females by elevated plasma concentrations of E_2. Therefore, alterations in gonadal expression of $P450_{arom}$ and DAX1 could be directly influenced by endogenous plasma E_2 concentrations in females and by exogenous estrogens in both males and females. These results suggest that ESR2, SF1, $P450_{arom}$ and DAX1 are important candidates to help explain the abnormalities previously reported in male and female alligators from Lake Apopka.

In addition to these observations, more study is needed to clarify whether the observed alterations of gene expression represent permanent changes in the expression pattern for these genes. There are observations to indicate apparently permanent alterations in various morphological features in American alligators (Milnes et al., 2004, 2005), however, there is no evidence to indicate that these are due to irreversible alterations in gene expression. Irreversible alterations in mammalian gene expression have been described following embryonic or neonatal exposure to estrogens. For example neonatal estrogen administration induces persistent cell proliferation and cornification of vaginal epithelium independent of endogenous hormones in adult mice (Forsberg, 1979; Iguchi, 1992; McLachlan et al., 1980; Takasugi et al., 1962) and recent studies have supported a permanent alteration in gene expression pattern to support these morphological and physiological changes (Miyagawa et al., 2004). Fetal exposure to the plastic monomer bisphenol A (BPA) also alters androgen receptor density in adult mice (vom Saal et al., 1997).

Our data on gene expression are unique, as previous studies have not examined endogenous mRNA levels for genes associated with steroidogenesis in alligator tissues from contaminated and reference populations. However, future studies must determine to what degree the mRNAs present in these tissues are translated to active protein. Interestingly, we would predict that sex steroid concentrations in the blood should be associated with expression levels of numerous genes, given that the receptors for sex steroids are transcription factors known to alter the expression of hundreds to thousands of genes (Watanabe et al., 2003).

In conclusion, we observed an elevation in SF1 mRNA in testicular tissue from Lake Apopka alligators, and reductions of $P450_{arom}$ and DAX1 mRNA in ovarian tissue from females of the same population. We also noted elevated ESR mRNA expression in male gonadal tissue as well. These results provide target genes for future study as they may be part of the underlying basis for the abnormalities observed in testicular and ovarian biology observed in alligators from pesticide contaminated wetlands in Florida, such as Lake Apopka. Additional experiments, exposing animals to common environmental contaminants during development and post-hatching time periods, would be needed to further examine how changes in gene expression is potentially related to the abnormalities observed, such as multioocytic follicles (Guillette and Moore, 2006). Furthermore, an investigation of DNA methylation of genes encoding alligator ESR2, SF1, $P450_{arom}$ and DAX1 might provide insight into the mechanism(s) by that altered gonadal gene expression occurs in alligators from contaminated environments. These investigations would also clarify basic mechanisms of endogenous endocrine function in the American alligator.

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