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Assessing impacts of precocious steroid exposure on thyroid physiology and gene expression patterns in the American alligator (*Alligator mississippiensis*)

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ABSTRACT

The thyroid gland is sensitive to steroid hormone signaling, and many thyroid disrupting contaminants also disrupt steroid hormone homeostasis, presenting the possibility that thyroid disruption may occur through altered steroid hormone signaling. To examine this possibility, we studied short-term and persistent impacts of embryonic sex steroid exposure on thyroid physiology in the American alligator. Alligators from a lake contaminated with endocrine disrupting contaminants (Lake Apopka, FL, USA) have been shown to display characteristics of thyroid and steroid hormone disruption. Previous studies suggest these alterations arise during development and raise the possibility that exposure to maternally deposited contaminants might underlie persistent organizational changes in both thyroidal and reproductive function. Thus, this population provides a system to investigate contaminant-mediated organizational thyroid disruption in an environmentally-relevant context. We assess the developmental expression of genetic pathways involved in thyroid hormone biosynthesis and find that expression of these genes increases prior to hatching. Further, we show that nuclear steroid hormone receptors are also expressed during this period, indicating the developing thyroid is potentially responsive to steroid hormone signaling. We then explore functional roles of steroid signaling during development on subsequent thyroid function in juvenile alligators. We exposed alligator eggs collected from both Lake Apopka and a reference site to 17β-estradiol and a non-aromatizable androgen during embryonic development, and investigated effects of exposure on hatchling morphometrics and thyroidal gene expression profiles at 5 months of age. Steroid hormone treatment did not impact the timing of hatching or hatchling size. Furthermore, treatment with steroid hormones did not result in detectable impacts on thyroid transcriptional programs, suggesting that precocious or excess estrogen and androgen exposure does not influence immediate or long-term thyroidal physiology.

1. Introduction

Thyroid hormones (THs) are critical regulators of essential processes in vertebrates, including growth and development, metabolism, and reproduction (Zoeller et al., 2007). Developmental exposure to common environmental thyroid disrupting compounds can impact immediate and long-term survival and fitness by impairing growth and development, delaying hatching, and diminishing hatch success in alligators, snapping turtles, and birds (Boggs et al., 2013; Chen et al., 2008; Dimond, 1952, 1954; McKernan et al., 2009; Roelens et al., 2005).

Abbreviations: AP, Lake Apopka; AR, androgen receptor; cAMP, cyclic adenosine monophosphate; DHT, dihydrotestosterone; E_2, 17β-estradiol; ESR1, estrogen receptor alpha; ESR2, estrogen receptor beta; FSH, follicle-stimulating hormone; GR, glucocorticoid receptor; I, iodide; NIS, sodium-iodide symporter; OCP, organochlorine pesticide; PDS, pendrin; POP, persistent organic pollutant; PR, progesterone receptor; TH, thyroid hormone; TG, thyroglobulin; TRH, thyrotropin releasing hormone; TSH, thyrotropin (thyroid stimulating hormone); TSHR, thyrotropin receptor; WO, Lake Woodruff

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Many persistent organic pollutants (POPs), including organochlorine pesticides (OCPs), are implicated as thyroid disruptors (Jefferies and French, 1972; Liu et al., 2011; Mayne et al., 2005; Richert and Prahlad, 1972), with their effects typically attributed to altering TH synthesis, metabolism, transport, and/or signaling (Liu et al., 2011), reviewed in (Brucker-Davis, 1998; Pearce and Braverman, 2009). OCPs also disrupt steroid hormone axes across vertebrate taxa through numerous mechanisms, including disruption of steroidogenesis in various tissues (Benguira and Hontela, 2000; Hart et al., 1971; Jonsson et al., 1994; Lund et al., 1988; Wójtowicz et al., 2007a,b; You et al., 2001), disruption of hepatic steroid metabolism (Balazs and Kupfer, 1966; Nowicki and Norman, 1972; Welch et al., 1967; Welch et al., 1971), and direct agonism/antagonism of steroid hormone receptors (Danzo, 1997; Kelce et al., 1995; Nelson et al., 1978; Oien et al., 1997). Importantly, the hypothalamo-pituitary-thyroid (HPT) axis is sensitive to steroid hormone signaling. Within the thyroid, steroid signaling regulates transcription and activity of elements in the TH biosynthesis pathway (del Senno et al., 1989; Furlanetto et al., 1999; Furlanetto et al., 2001; Lima et al., 2006; Lorenz et al., 2018; Xu et al., 2013), as well as thyrocyte proliferation, thyroid growth, and histomorphology (Abdel-Dayem and Elgendy, 2009; Banu et al., 2001a,b, 2002b; de Araujo et al., 2006; Furlanetto et al., 1999; Leatherland, 1985; Lorenz et al., 2018; Manole et al., 2001; Qu et al., 2001; Razia et al., 2006; Sekulic et al., 2007; Sosic-Jurjevic et al., 2006; Xu et al., 2013). Additionally, steroid signaling affects upstream regulation of thyroid function by regulating thyrotropin releasing hormone (TRH) and thyrotropin (TSH) expression in the hypothalamus and anterior pituitary gland, respectively, and by altering thyroidal TSH receptor (TSHR) expression and responsiveness to TSH signaling (Banu et al., 2001a,c, 2002b; Leatherland, 1985). Systemically, steroids also modulate thyroid-mediated signaling by influencing circulating TH concentrations (Abdel-Dayem and Elgendy, 2009; Bottner et al., 2006; Bottner and Wuttke, 2005; Chakraborti and Bhattacharya, 1984; Chen and Walfish, 1978; de Araujo et al., 2006; Kordi and Khazali, 2015; Leatherland, 1985; McCormick et al., 2005; Qu et al., 2001; Uribe et al., 2009). Steroid hormone receptors are expressed in the thyroid in rats, humans, and the American alligator (Banu et al., 2002a; Bermudez et al., 2011; Bottner and Wuttke, 2005; Manole et al., 2001; Stanley et al., 2012; Stanley et al., 2010). Thus, POP-induced thyroid disruption could potentially be mediated through disruption of steroid hormone homeostasis. POPs could both indirectly interfere with thyroid function by altering steroid hormone secretion, transport, or metabolism in extrathyroidal tissues, thus interfering with steroid signaling in the thyroid, or directly by activating or antagonizing thyroidally expressed steroid hormone receptors. To our knowledge, this mechanism of thyroid disruption has not been studied in any species.

American alligators (*Alligator mississippiensis*) inhabiting Lake Apopka (Orange county, Florida, USA), a site contaminated with OCPs (Guillette Jr. et al., 1999; Heinz et al., 1991), exhibit altered thyroidal phenotypes that include diminished neonatal plasma TH concentrations, altered thyroidal histomorphology, and impaired growth (Boggs et al., 2013), as well as disrupted reproductive physiology, including gonadal transcription, steroidogenesis, and systemic steroid hormone homeostasis (Crain et al., 1998; Guillette et al., 1994, 1995, 1996, 1999; Moore et al., 2012). Importantly, these effects are persistent; Boggs et al. (2013) and Moore et al. (2012) demonstrated that these impaired thyroidal and gonadal phenotypes, respectively, remain present in Lake Apopka animals raised under controlled laboratory conditions for months, indicating that *in ovo* OCP exposure induces organizational effects with long-lasting consequences on the alligator endocrine system. Thus, the Lake Apopka alligator population presents a unique opportunity to examine the effects of steroid hormone disruption on both short- and long-term thyroid physiology under realistic exposure conditions.

Here, we investigate the patterns of TH biosynthetic pathway (Fig. 1) gene transcription during embryonic development in the alligator and perform experiments that test functional linkages between precocious steroid hormone signaling and thyroid gene transcription later in life. THs are protein-derived hormones generated from the iodination of tyrosine groups on thyroglobulin (TG). The iodide (I^-) required for this process is transported from circulation into thyrocyte cytoplasm and then from the cytoplasm into the thyroid follicular lumen (where iodination occurs) by sodium-iodide symporter (NIS) and pendrin (PDS), respectively (Fig. 1). Within the lumen, thyroid peroxidase (TPO) catalyzes the iodination of TG tyrosyl residues and the formation of protein-bound forms of THs (Fig. 1). This process is ultimately regulated by TSH; binding of TSH to its cognate receptor, TSHR, stimulates the production and secretion of THs by various mechanisms, including induction of TG, TPO, NIS, and PDS transcription, mediated through cyclic adenosine monophosphate (cAMP; Fig. 1) (Gerard et al., 1989; Kogai et al., 1997; Muscella et al., 2008; Van Heuverswyn et al., 1985). By characterizing transcription of TG, NIS, and PDS, we examine the capacity of the thyroid to generate and concentrate the substrates required for TH production (i.e., TG and I^-). Then, measuring expression of TPO assesses the capacity to produce protein-bound THs from these substrates. We define these collective outcomes as "apparent productive capacity". Lastly, expression of TSHR indicates sensitivity to stimulation from the pituitary.

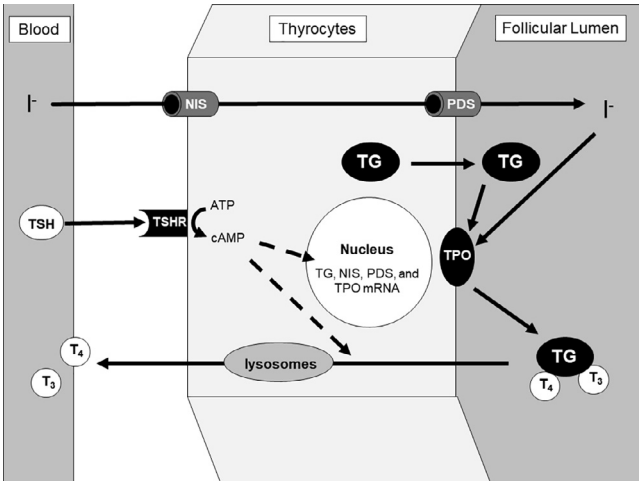

Fig. 1. Thyroid hormone biosynthesis pathway. ATP = adenosine triphosphate, cAMP = cyclic adenosine monophosphate, NIS = sodium-iodide symporter, PDS = pendrin, TG = thyroglobulin, TPO = thyroid peroxidase, TSHR = thyrotropin receptor.

The purpose of this study was to first describe the developmental expression of thyroid hormone biosynthetic pathway and steroid hormone receptor genes, and then examine the persistent effects of embryonic steroid exposure on thyroid function in the American alligator. Using an ecologically relevant model in which persistent thyroidal abnormalities have been linked to OCP exposure, we describe both the native transcriptional patterns of genes related to thyroid function and steroid hormone signaling in the alligator embryo, as well as the functional consequences of embryonic exposure to exogenous steroid hormones on transcription of these genes later in life. While embryonic stages are critical windows of thyroid hormone activity in vertebrates, previous studies of American alligator thyroidal physiology have not described embryonic life stages (Bermudez et al., 2011; Boggs et al., 2013, 2016). In this study we test two competing hypotheses regarding the roles that steroid hormones play in mediating OCP-induced thyroid disruption. Our first hypothesis is that *in ovo* OCP exposure decreases systemic sex steroid hormone signaling in Apopka embryos which disrupts thyroid physiology. In this case, we would expect that treatment with exogenous sex steroids might compensate for the OCP-mediated reduction in steroid hormone signaling and prevent the formation of the abnormal thyroid phenotype in Apopka animals. Alternatively, a

competing hypothesis is that OCP exposure leads to elevated sex steroid signaling at target tissues. In this case, dosing Woodruff alligators with sex steroids might recapitulate the impaired phenotype in Apopka alligators, and therefore, these steroid-dosed Woodruff animals would demonstrate hatch metrics (e.g., timing, success rate) and gene expression profiles comparable to those of vehicle-treated Apopka alligators. Because the mechanism of OCP-mediated thyroid disruption proposed above has not been explored in any species, this study has the potential to influence our understanding of general thyroid physiology and disruption. Additionally, considering the potential implications of such disruption on alligator health and fitness, further examination of thyroid disruption in Lake Apopka is warranted from an ecological perspective as well.

2. Materials and methods

2.1. Animals, treatments, and tissue collection

All experiments performed in this study conformed to guidelines established 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.

2.1.1. Developmental characterization experiment

Six clutches of American alligator eggs were collected in June 2015 from a reference population located in Lake Woodruff National Wildlife Refuge (WO; Volusia County, FL, USA). Eggs were returned to Hollings Marine Laboratory, Charleston, SC where they were assessed for viability by candling. Viable eggs were transferred into artificial nests made of moistened sphagnum moss in plastic bussing bins with drainage holes drilled into the bottoms. The nests were placed into incubators set and pre-warmed to 30 °C, a female-promoting temperature, where they were maintained for the duration of the study. Throughout incubation, nest temperatures were monitored by thermistor (Onset Computer Corporation, Bourne, MA, USA), and nests were lightly moistened with water once every 24 h. Due to limited availability of alligator eggs, we did not have sufficient eggs to study effects following incubation at both male- and female-promoting temperatures. Thus, we selected female-promoting temperature because both Boggs et al. (2013) and Moore et al. (2012) utilized female-promoting temperature in their examinations of thyroidal and gonadal disruption in alligators Lake Apopka.

To determine the developmental stage of each clutch, one viable egg from each clutch was sacrificed the day after the clutch was collected, and the embryo was removed and examined; staging was determined per Ferguson (1985). Using the determined stage, we predicted the date and time at which each clutch would reach later developmental stages of interest when incubated at 30 °C per methods described by Kohno and Guillette Jr. (2013). Stages during the final trimester of incubation were examined (stages 24, 25, 26, and 27) because this period is roughly equivalent to the stage at which snapping turtle and chicken embryos exhibit progressive increases in thyroidal function immediately preceding hatch (Dimond, 1952; Grommen et al., 2011). Additionally, earlier sampling is limited due to difficulties associated with thyroid dissection prior to stage 24. To control for potential maternal effects, clutches were distributed equally across stage groups. Upon reaching the desired stage, embryos were removed from eggs, staged, and sacrificed. Due to imprecision associated with stage predictions, predicted stage did not always align with actual stage at time of collection; this led to an imbalance in the number of embryos in each stage (stage 24, n = 5; stage 25, n = 5, stage 26, n = 5; stage 27, n = 9). Thyroid glands were dissected and fixed in RNALater, then incubated on an orbital shaker at 4 °C for 24 h. After 24 h, excess RNALater was removed, and thyroids were frozen at −20 °C.

2.1.2. Steroid hormone-mediated thyroid disruption experiment

Animals used in this study were collected as eggs from two populations in Florida, Lake Woodruff (WO; Volusia County, FL) and Lake Apopka (AP; Orange County, FL). These two sites have been thoroughly characterized; AP is a site with historical OCP contamination, associated with reproductive and thyroidal abnormalities in alligator, while WO is relatively pristine and has been used extensively as a reference population. At AP, between 9 and 25 eggs were collected from each of 17 clutches (102 eggs total). Unequal collection across clutches was due to variable clutch sizes, and efforts were made to maintain equal representation of clutches across treatment groups. At WO, 6 eggs apiece were collected from each of 17 clutches (102 eggs total) shortly after oviposition and were transported to Hollings Marine Laboratory (Charleston, SC, USA). All collections at both sites were conducted in June 2014. Eggs were transferred to damp sphagnum moss and maintained with daily misting, as described above. Upon arrival, a representative embryo from each clutch was staged, and remaining eggs were transferred to incubators and maintained at 32 °C, a temperature which promotes mixed sex ratios. Embryos were maintained at this temperature until they reached stage 19, which precedes gonadal differentiation, at which time clutches were distributed equally across treatment groups to control for maternal effects, and were topically treated directly onto eggshells with either estradiol-17β (E_2; 0.5 μg/g egg weight), dihydrotestosterone (DHT; 250 μg/g egg weight), or vehicle control (95% ethanol) as previously described (Kohno and Guillette, 2013). Doses of steroid hormones were selected by their established capacity to elicit morphological, and therefore transcriptional, responses in embryonic gonads; 0.5 μg/g E2 is capable of sex-reversing embryos incubated at an exclusive male-promoting temperature to a female fate in alligators (Kohno et al., 2015), while 250 μg/g DHT is capable of eliciting follicular abnormalities similar to polycystic ovarian syndrome (PCOS) in mammalian models (Caldwell et al., 2014; Singh, 2008).

Immediately following dosing at stage 19, embryos were transferred to 30 °C to produce female offspring, where they were maintained until hatching. At hatching, pip date and hatchling morphometrics (mass, total length, and snout-vent-length [SVL]) were recorded. Neonates were weighed and measured, and then marked via two independent methods: dual numbered monel tags applied to the middle-digit webbing of both hindfeet and with unique notching of the tail scutes. Marked hatchlings were then transferred to custom fiberglass aquatic tanks at the Hollings Marine Laboratory, where they were maintained for the remainder of the study, approximately 5 months. Body mass, length, and general body condition were measured and recorded every two weeks following hatching. Following each measurement period, animals were sorted and grouped according to size to limit antagonistic interactions. During the first two months of grow-out period, animals were fed *ad libitum* daily. After two months, in order to achieve relative homogeneity in size of animals, feeding schedules were implemented according to size: juveniles greater than 132 g were fed twice weekly, while animals between 93 and 132 g were feed three times weekly. The smallest animals at the two-month mark, less than 92 g were fed daily. During this grow-out phase, a subset of animals failed to thrive, exhibiting either no growth or negative growth between measurements, and were fed by gavage and thereafter excluded from analysis.

At the conclusion of the grow-out period (approximately 150 ± 4 days), juveniles ranged in mass from 218 and 510 g; average mass did not differ across treatment groups (data not shown). Upon reaching 5 months, animals were euthanized via injection of a lethal dose of pentobarbital (0.1 mg/g animal mass) in the postcranial sinus and decapitated. Thyroids were dissected, fixed in RNALater overnight at 4 °C on an orbital shaker, and then stored at −20 °C. Animals used in this study were shared with an overlapping investigation; for four days prior to euthanasia, all juveniles included in the present study were administered either 4 daily intramuscular injections of sterile saline (0.8%) or recombinant ovine follicle-stimulating hormone (FSH) in the

Table 1
Sample sizes for individual treatment groups in juvenile gene expression analyses.

Gene	Site	Dose	Sample size
TG	AP	DHT	9
		E2	5
		ETOH	9
	WO	DHT	4
		E2	8
		ETOH	8
TSHR	AP	DHT	9
		E2	5
		ETOH	9
	WO	DHT	4
		E2	8
		ETOH	8
TPO	AP	DHT	9
		E2	4
		ETOH	9
	WO	DHT	4
		E2	8
		ETOH	8
NIS	AP	DHT	9
		E2	3
		ETOH	8
	WO	DHT	3
		E2	4
		ETOH	5
PDS	AP	DHT	9
		E2	5
		ETOH	10
	WO	DHT	4
		E2	8
		ETOH	8
AR	AP	DHT	7
		E2	0
		ETOH	6
	WO	DHT	4
		E2	6
		ETOH	6
ESR1	AP	DHT	9
		E2	5
		ETOH	10
	WO	DHT	3
		E2	8
		ETOH	7
GR	AP	DHT	9
		E2	5
		ETOH	10
	WO	DHT	4
		E2	8
		ETOH	7
PR	AP	DHT	8
		E2	5
		ETOH	10
	WO	DHT	4
		E2	8
		ETOH	8

base of the tail for the treatment regime for the overlapping study. For growth analyses in juvenile alligators (see section 2.5 Statistical Analysis), non-gavaged animals from both groups (saline and FSH) were included, as all growth data preceded treatment and inclusion improved statistical power (AP: DHT n = 18, E2 = 12, ETOH = 18; WO: DHT n = 11, E2 = 16, ETOH = 15). However, because of the potentially confounding effects of FSH administration on thyroid gene expression, FSH-treated animals were excluded from qPCR analyses. Because all remaining juveniles received the same saline treatment, effects of treatment do not confound gene expression results. Treatment group sample sizes for gene expression analyses, which were further subjected to outlier removal and filtering are reported in Table 1.

2.2. RNA extraction

Total RNA was collected using an acid-phenol guanidinium thiocyanate column extraction protocol. Briefly, the thyroid tissue was placed into a microcentrifuge tube containing 1.0 mL of denaturing solution (water-saturated acid phenol, 2 M guanidinium thiocyanate, 95 mM sodium acetate, 12 mM sodium citrate, 0.24% sodium N-lauroyl sarcosine, 14.4 M beta-mercaptoethanol) and a sterile stainless-steel homogenizer bead. Tubes were closed and sealed with Parafilm before being loaded onto a bead mill (Retsch Mixer Mill 400) in which tissues were homogenized at 30 Hz for 3 min twice. Parafilm and beads were removed, and homogenates were centrifuged at 4 °C and 12,000 rcf for 10 min. Supernatant was transferred to a microcentrifuge tube containing 200 μL of 37% chloroform. The precipitate and chloroform were mixed by shaking for 15 s, incubated at room temperature for 3 min, and centrifuged at 4 °C and 12,000 rcf for 15 min. After centrifugation, the aqueous phase containing the RNA was removed and added to a tube containing 600 μL of 100% ethanol. The aqueous phase and ethanol were mixed thoroughly by pipetting, and the mixture was loaded onto silica-membrane spin column assemblies (EconoSpin™, Epoch Life Science; Fort Bend, TX). Columns were centrifuged at room temperature and 15,000 rcf for 1 min to bind RNA to the silica membrane. The column was washed twice with a 1 M Tris-HCL/potassium acetate/60% ethanol solution and then treated with DNase (5Prime DNase I, Gaithersburg, MD) for 30 min to degrade genomic DNA. DNase stop solution (2.15 M guanidinium thiocyanate, 4.3 mM Tris-HCl, 57% ethanol) was added to the column to halt the DNase reaction, and the column was washed three more times with the same wash solution as above. RNA was eluted from the column by addition and centrifugation of 30 μL diethylpyrocarbonate (DEPC)-treated water twice. RNA concentrations in the eluent were measured by spectrophotometry (Nanodrop ND2000, ThermoFisher Scientific; Waltham, MA). RNA quality was assessed via gel electrophoresis on a denaturing gel (1.5% agarose, MOPS buffer; 37% formaldehyde); observation of distinct 18S and 28S RNA bands was used as an indicator that RNA was of usable quality. RNA eluent was stored at −20 °C.

2.3. Reverse transcription

2.3.1. Developmental characterization experiment

RNA was diluted to 34.54 ng μL^{-1} in ultrapure water, except one sample which had a concentration below 34.54 ng μL^{-1} and remained undiluted. Reverse transcription (RT) was performed using the Applied Biosystems (Foster City, CA) High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor with slight modification to the manufacturer's protocol – water was omitted from the master mix and 13.2 μL of RNA was used per reaction in lieu of the water, maintaining a reaction volume of 20 μL. The RT reaction was conducted on a BioRad (Hercules, CA) thermal cycler programmed according to the Applied Biosystems RT kit specifications (25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min, 4 °C indefinitely). Following RT, cDNA was diluted 1:5 in ultrapure water. Diluted cDNA was aliquoted in 2 μL quantities for qPCR analysis and stored at −20 °C.

2.3.2. Steroid hormone-mediated thyroid disruption experiment

RNA was diluted to 66.6 ng μL^{-1} in DEPC-treated water. RT was performed using the BioRad iScript™ cDNA Synthesis Kit with minor modification – water was omitted from the master mix and 15 μL of RNA was used per reaction to maintain a reaction volume of 20 μL. The RT reaction was performed on a BioRad thermal cycler programmed according to the RT kit specifications (25 °C for 5 min, 42 °C for 30 min, 85 °C for 5 min, 4 °C indefinitely). cDNA was diluted 1:5 in ultrapure water. Diluted cDNA was aliquoted in 2 μL quantities for qPCR analysis and stored at −20 °C.

Table 2
qPCR oligonucleotide primer sequences and optimal annealing temperatures. For each target, the forward primer is listed first and the reverse primer is listed second.

Target	Primer Sequence	Accession No.	Annealing Temp. (°C)	Amplicon Size (bp)
Thyroglobulin (TG)	TCAATGCCTAGTGCTCAGAAA GCATGATTCTTCTTGCACACA	XM_019481751	64.0	157
Thyroid peroxidase (TPO)	AATGAAAGCACTGAGGGAAGG AGCATCAACTGGCACTTCTG	XM_019492703	66.2	145
Thyrotropin receptor (TSHR)	AAGCTGGATGCTGTCTACCTG TGTTTTCACAGGGGGTAGTTTC	XM_006260866	64.0	204
Sodium-iodide symporter (NIS)	GCATCCACCAGCATCAAC TAAAGGAGGCCTGGAGCA	XM_006272133	64.0	190
Pendrin (PDS)	TCATGCAGGTATGTGATGTTCC TCACCACAACTGTCAGTAATCC	XM_006257819	66.2	150
Estrogen receptor alpha (ESR1)	AAGCTGCCCCTTCAACTTTTTA TGGACATCCTCTCCCTGCC	NM_001287274	64.0	72
Estrogen receptor beta (ESR2)	CCAAAGAGCCCATGGTGTGA ACCATTTGCAATGGGACTTGTG	NM_001287264	64.0	114
Androgen receptor (AR)	GCCAGACTCCTTCTCCAACC TCTCCATCCCATGGCGAAAA	XM_019487877	62.0	178
Progesterone receptor (PR)	AGCAGTTGGATTGCGCCAGAA TCAGTGCCCGAGACTGAAGA	AB115911	64.0	143
Glucocorticoid receptor (GR)	AAAAAACTGTCCCGCATGCC CGTTGGACTGCTGAATTCCTTT	AB701407	64.0	104
Ribosomal protein L8 (RPL8)	CTCTCACAATCCTGAAACCAA GTTTGTCAATACGACCTCCAC	XM_006266675	62.0	122

2.4. Quantitative PCR

Intron-spanning qPCR primers for TG, TSHR, TPO, NIS, PDS, RPL8, CYP19A1, ESR1, ESR2, AR, PR, and GR are listed in Table 2. Plasmid DNA standards for each PCR amplicon were produced and serially diluted to create a standard curve for absolute quantification of target amplicons. To perform the qPCR analysis, a master mix containing 0.01 U/μL AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA), 0.2 μM primer mix, 20 μM dNTPs, 0.5X SYBR® Green dye (Invitrogen, Carlsbad, CA), 50 mM KCl, 20 mM Tris-HCL, 0.5% glycerol, 0.5% tween-20, 4% DMSO, and 3 mM $MgCl_2$ was made for each target gene. 48 μL of master mix was added to and mixed with 2 μL of standard, cDNA, or water (no template control). 15 μL of each mixture was then loaded into each of three sequential wells in a 96-well PCR plate. The PCR reaction was performed on a BioRad (Hercules, CA) CFX96 thermal cycler programed as follows: 95 °C for 5 min then 45 cycles of 95 °C for 15 s and annealing temperature for 1 min. Production of a single amplification product was ensured by melt curve analysis following the PCR reaction. BioRad CFX manager software was used for absolute quantification and melt curve analysis. Standard curves were considered usable if regression analysis indicated $R^2 > 0.98$. Quantification was performed via interpolation on the standard curve, and triplicate values were averaged to provide a single value per sample to be used in statistical analyses. All genes were normalized to reference gene ribosomal protein L8 (RPL8), the expression of which did not vary by developmental stage or treatment. Reported values are normalized expression values averaged by stage or treatment.

2.5. Statistical analysis

For developmental analysis, changes in normalized gene expression by developmental stage were analyzed by one-way ANOVA ($\alpha = 0.05$) with Tukey post-hoc analysis, or Kruskal-Wallis test ($\alpha = 0.05$), if the assumptions of the ANOVA were violated. For the exposure analysis, normalized gene expression values were analyzed via GraphPad Prism (version 7.0b). Within treatment groups, outlier detection and removal was conducted via the ROUT method (Q coefficient = 1%). Following removal, remaining samples were log transformed to achieve normality and homoscedasticity. Effects of site and developmental exposure were analyzed via two-way ANOVA. Effects of site and developmental exposure on time to pip (incubation length) and hatchling mass, total length, and snout-vent length (SVL) were assessed by Kruskal-Wallis test. Incubation length, or time to pip, is reported as the time between reaching stage 19 and pipping. Due to assisted hatching, time to complete hatch was not analyzed. Growth data (mass and total length) were analyzed via linear mixed models in RStudio (RStudio Team, 2016) using package 'nlme' (Pinheiro et al., 2018). Mass and length were log transformed and fit individually using age at measure, site, and dose as main effects and subject ID as a random effect. Due to variation in hatch timing and consequent variation in age at each measurement period, age at measure was defined as days elapsed since hatch for each animal at each respective measurement period, including mass and length at hatch (age = 0). Intercepts were fit individually by subject ID (mass correlation structure = unstructured, based on best AIC score; length correlation structure = ARMA(2,0)). Interactive effects of site and dose were initially included, but effects were non-significant and therefore excluded to maintain model parsimony. Normality was confirmed via manual inspection of residual frequency distribution and Shapiro-Wilk test ($\alpha = 0.05$)

3. Results

3.1. Embryonic expression of TH biosynthesis genes

To examine the apparent productive capacity and sensitivity of the alligator thyroid to TSH during embryonic development we examined expression of key genes involved in thyroid function (Fig. 2). Expression of TG ($F = 8.245$, $p = 0.001$), TPO ($\chi^2 = 8.041$, $p = 0.045$), PDS ($F = 4.414$, $p = 0.017$), and NIS ($F = 7.924$, $p = 0.001$) varied significantly by stage, with expression of each increasing progressively with increasing developmental stage. Conversely, expression of TSHR ($F = 1.039$, $p = 0.397$) did not vary significantly by developmental stage (Fig. 2). These findings suggest that the synthesis of THs might increase during embryonic development and are consistent with roles of THs in regulating embryonic growth and the timing of hatching.

3.2. Expression of steroid hormone receptors in the developing thyroid

We next assessed the expression of steroid hormone receptors in the developing thyroid. Expression of ESR1, AR, PR and GR was detected, but did not exhibit the same pattern of change during development that was observed for genes associated with thyroid function. Significant differences in expression by stage were observed for ESR1 ($F = 3.860$, $p = 0.026$) and AR ($\chi^2 = 14.593$, $p = 0.002$) (Fig. 3); expression of

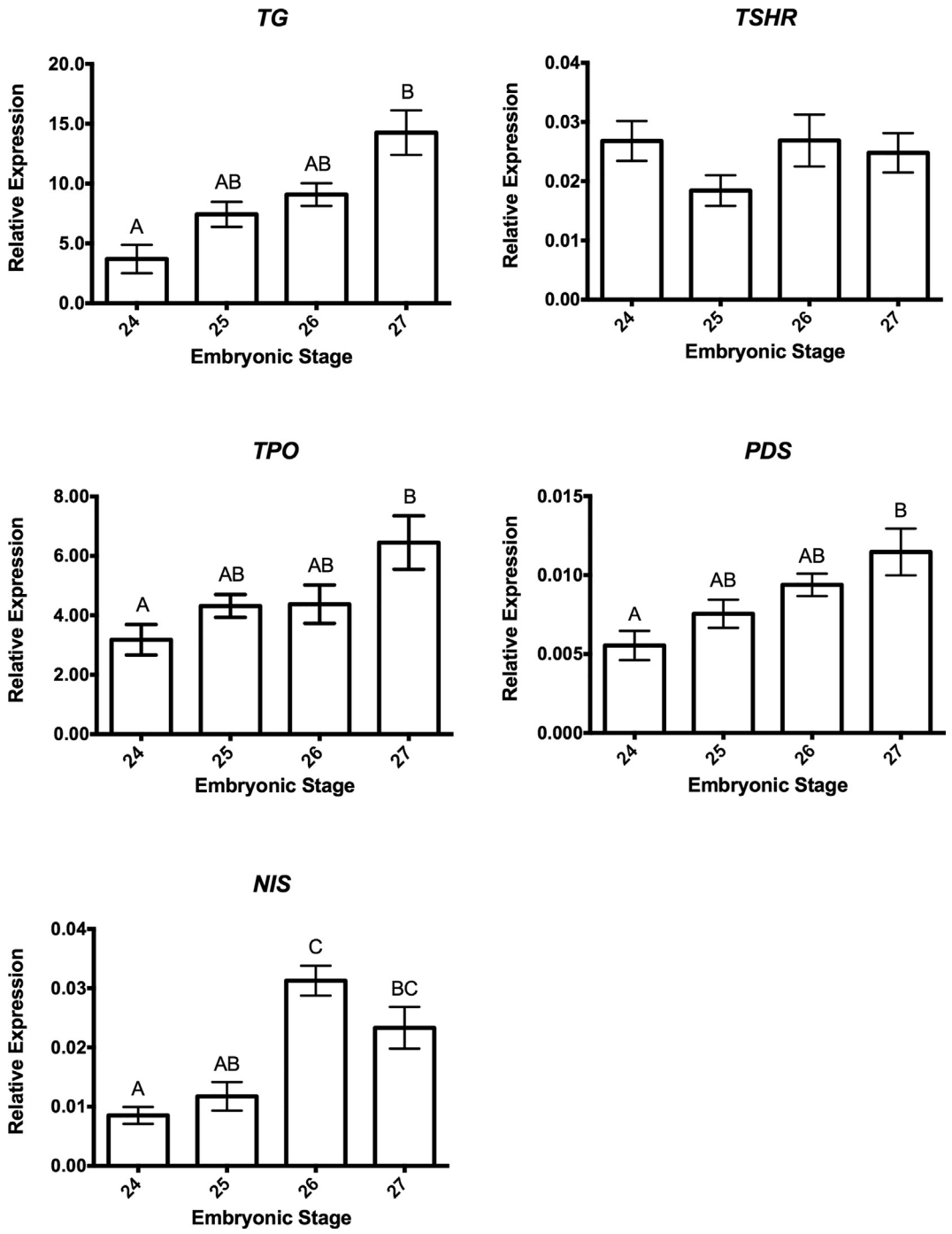

Fig. 2. Developmental patterns of thyroid hormone biosynthesis gene expression during the final trimester of embryonic development displayed as mean relative expression. Bars with different letter headings are significantly different ($p < 0.05$). Error bars indicate standard error of the mean (SEM). (stage 24, n = 5; stage 25, n = 5, stage 26, n = 5; stage 27, n = 9). TG = thyroglobulin, TSHR = thyrotropin receptor, TPO = thyroid peroxidase, PDS = pendrin, NIS = sodium-iodide symporter.

both receptors decreased in late-stage embryos. ESR1 expression was highest at stage 24 and lowest at stage 27, with stages 25 and 26 exhibiting intermediate levels of expression. Stages 24 and 25 demonstrated the highest expression of AR while stages 26 and 27 exhibited the lowest. In comparison, levels of GR ($\chi^2 = 2.569$, $p = 0.463$) and PR ($F = 0.840$, $p = 0.488$) expression did not change throughout development. ESR2 expression was detectable but not quantifiable at any stage (data not shown).

3.3. Steroid hormone-mediated thyroid disruption

We sought to investigate the influence of altered steroid cues *in ovo* on transcription patterns in the thyroid and thyroidally regulated processes. Hatch timing and hatchling length did not vary significantly by treatment group, indicating that developmental thyroid function was not affected. WO animals had significantly lower body mass at hatch than AP animals (Fig. 4; $\chi^2 = 17.2$, $p = 0.0041$). Mass and length growth rates did not vary by site during the first two months of grow-out when all individuals were kept on the same feeding schedule (Table 3). All target genes, except ESR2, were expressed at quantifiable

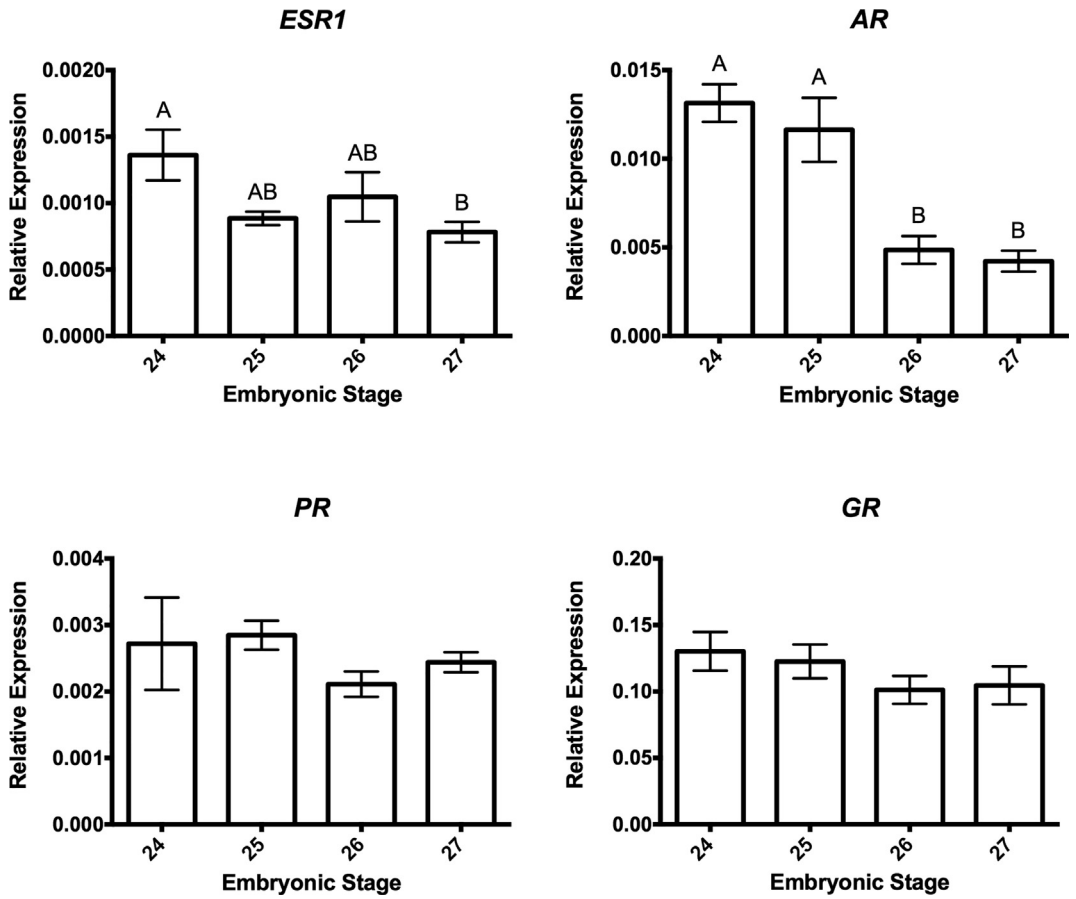

Fig. 3. Developmental patterns of thyroidal expression of steroid hormone receptor genes during the final trimester of embryonic development displayed as mean relative expression. Bars with different letter headings are significantly different ($p < 0.05$). Error bars indicate standard error of the mean (SEM). (stage 24, n = 5; stage 25, n = 5, stage 26, n = 5; stage 27, n = 9). ESR1 = estrogen receptor alpha, AR = androgen receptor, PR = progesterone receptor, GR = glucocorticoid receptor.

levels in the juvenile alligator thyroid. However, neither site of origin nor embryonic steroid exposure significantly affected expression of any target genes, including TH biosynthesis pathway and steroid hormone receptor genes (Figs. 5 and 6).

4. Discussion

The goals of this study were to describe facets of basic embryonic thyroid physiology in the American alligator, and then examine a novel mechanism of thyroid disruption mediated through steroid hormone signaling, using the alligator as a model species. Collectively, our findings stand to inform our understanding of the dynamics of thyroid function in early life stages as well as the ramifications of perturbed endocrine signaling on these dynamics. We demonstrate that the alligator thyroid exhibits a gradual increase in its apparent productive capacity during the final stages of embryonic development, as indicated by the increase in transcription of TG, NIS, PDS, and TPO (Fig. 2). This result is similar to developmental patterns observed in chickens, in which THs serve functional roles in regulating the timing of hatch (Grommen et al., 2011). Importantly, TSHR expression does not vary significantly by developmental stage, suggesting this increase in apparent productive capacity occurs without concomitant changes in sensitivity to TSH, and, instead, the developmental changes in TG, NIS, PDS, and TPO expression are likely produced by elevated secretion of TSH from the anterior pituitary. While the roles that THs play in regulating physiologic processes during development in alligators have not been empirically described, our results suggest that THs regulate early developmental processes in alligators in a manner consistent with other related vertebrates, such as snapping turtles and birds (Chen et al., 2008; Dimond, 1952, 1954; McKernan et al., 2009; Roelens et al., 2005).

To determine whether the embryonic alligator thyroid gland is sensitive to steroid hormone signaling, we described expression of five steroid hormone receptor genes, including estrogen receptor alpha (ESR1) and beta (ESR2), androgen receptor (AR), glucocorticoid receptor (GR), and progesterone receptor (PR), throughout the final trimester of embryonic development in alligator embryos from a reference population, Lake Woodruff. The embryonic alligator thyroid expressed four of five steroid hormone receptors, excluding only ESR2 (Fig. 3). Furthermore, we observed developmental stage-specific expression patterns for ESR1 and AR, with the youngest embryos exhibiting the highest expression of both and the later-stage embryos exhibiting lower expression (Fig. 3). These data indicate a progressive decrease in thyroidal sensitivity to estrogen and androgen signaling during the final stages of embryonic development, whereas sensitivity to glucocorticoids and progestogens remains constant across this same developmental time frame. These findings suggest that the embryonic thyroid is responsive to steroid signaling and that this response might be regulated during a critical window of thyroid development by adjusting expression of steroid hormone receptors. To our knowledge, this is the first study to examine thyroidal sensitivity to steroid hormones during embryonic development in any species; thus, we are unable to compare these findings to steroid nuclear receptor expression patterns across other taxa.

We examined the impact of precocious steroid hormone exposure and disruption by dosing both Apopka and Woodruff embryos with

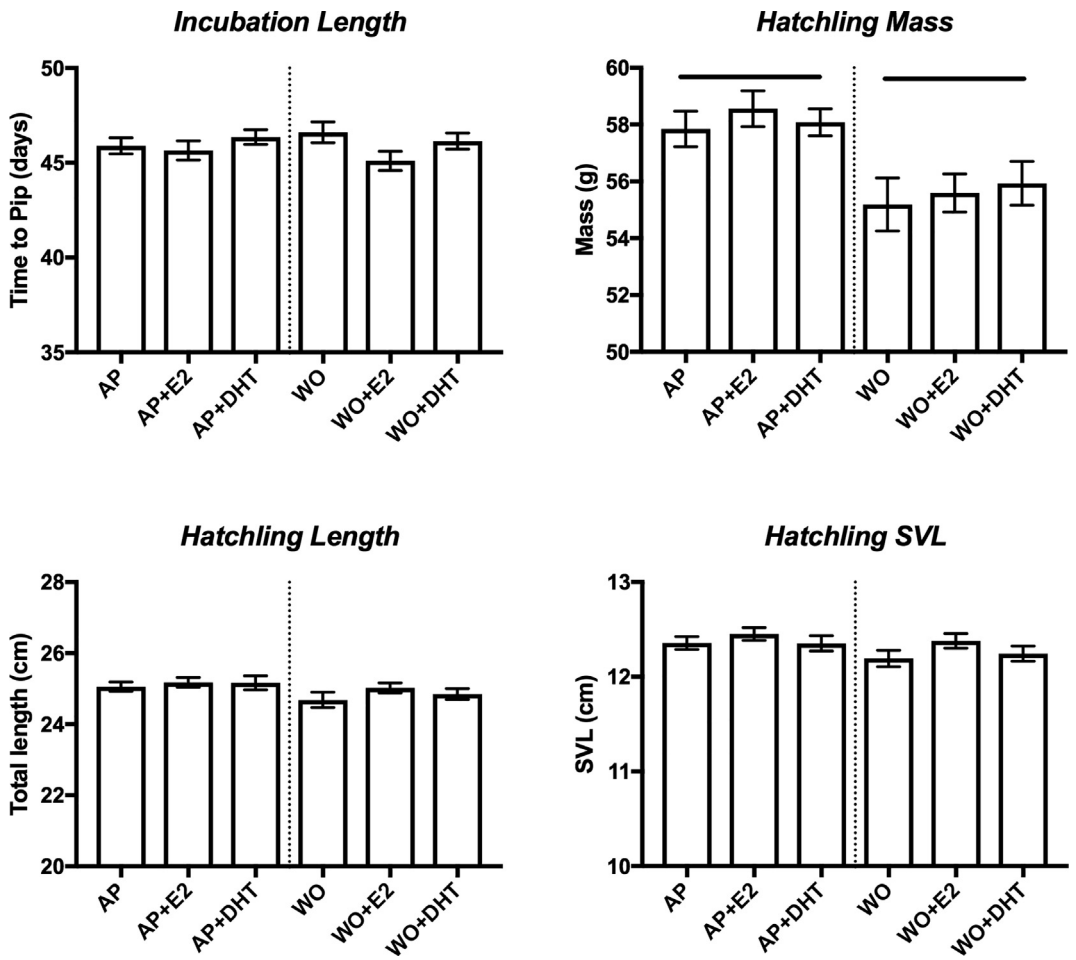

Fig. 4. Hatch metrics for Lake Woodruff (WO) and Lake Apopka (AP) animals exposed to ethanol (EtOH), dihydrotestosterone (DHT), or estradiol (E2) displayed as mean values. Error bars indicate standard error of the mean (SEM). Horizontal lines over bars indicate statistically significant ($p < 0.05$) differences by site. SVL = snout-vent length.

Table 3
Mixed linear model output summary for mass and total length growth in juveniles during the first two months of growth.

		Value	Std. Error	DF	t-value	p-value
Mass (g)	(Intercept)	1.731092	0.012507	359	138.4118	0
	Age	0.006894	0.00012	359	57.60303	0
	SiteWO	0.015533	0.012775	86	1.21587	0.2274
	DoseE2	0.014145	0.016009	86	0.88351	0.3794
	DoseETOH	−0.004264	0.015229	86	−0.28001	0.7801
Length (cm)	(Intercept)	1.40149	0.003718	359	376.907	0
	Age	0.00269	5.54E−05	359	48.5791	0
	SiteWO	0.001522	0.003595	86	0.4233	0.6731
	DoseE2	0.004482	0.004505	86	0.9947	0.3227
	DoseETOH	−0.000653	0.004286	86	−0.1524	0.8792

steroids prior to the onset of gonadal steroidogenesis; this experiment tested two competing hypotheses, as stated in Section 1. We anticipated that precocious sex steroid treatment would either: 1) prevent the expression of impaired thyroidal phenotypes in Lake Apopka alligators, or 2) induce thyroid disruption in Lake Woodruff animals. However, our results do not support either of these hypotheses. Site of origin did have a significant effect on body mass at hatch, with Apopka animals being heavier on average than Woodruff animals, but treatment with exogeneous steroids did not impact hatchling mass, suggesting that this difference is likely not due to embryonic steroid hormone levels. Our findings differ from Boggs et al. (2013), who reported that Woodruff hatchlings were larger than Apopka hatchlings, which may indicate that the apparent thyroid disruption observed by Boggs et al. at Lake Apopka has abated since their study was performed. Alternatively, it may be that hatchling size is influenced by environmental factors that exhibit temporal variation by year. Furthermore, there are no significant differences between gene expression profiles of vehivle control-treated Woodruff and Apopka animals, indicating that apparent productive capacity of the thyroid does not vary between the two sites after a five month grow-out period. Together, these results suggest that there is a minimal effect of site of origin-associated contaminant profiles on thyroid function in juvenile alligators. Our findings are inconsistent of Boggs et al. (2013), but it is important to note that the present study and Boggs et al. examined different metrics associated with thyroidal function.

Overall, our results suggest that precocious sex steroid exposure does not immediately or persistently impact thyroid physiology in the American alligator, which may indicate that the effect of *in ovo* OCP exposure on the organization of the thyroid reported by Boggs et al. (2013) is not mediated through steroid hormones. Considering the role that THs play in regulating hatching and growth (Boggs et al., 2013; Chen et al., 2008; Dimond, 1952, 1954; McKernan et al., 2009; Roelens et al., 2005), had precocious estrogen or androgen exposures impacted immediate thyroid function in the embryonic alligator, we would likely have observed differences in hatch timing and morphometrics in animals from both sites. However, hatch metrics do not vary by treatment. Similarly, if thyroid transcriptional networks are persistently affected by precocious steroid exposure, we would have observed treatment-dependent differences in gene expression profiles at 5 months of age

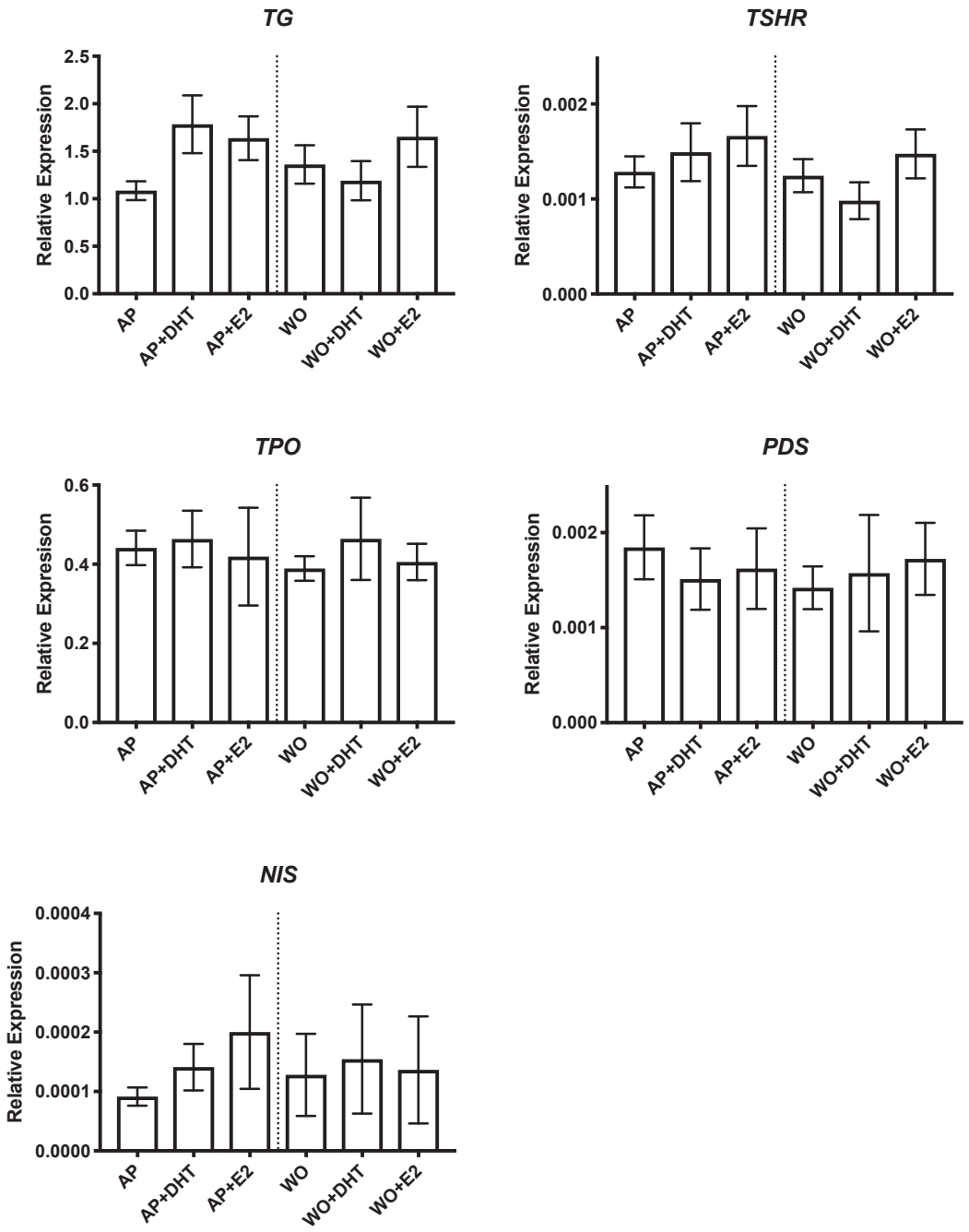

Fig. 5. Expression of thyroid hormone biosynthesis genes in 5-month-old alligators by site and treatment displayed as mean relative expression. Error bars indicate standard error of the mean (SEM). Sample sizes reported in Table 1. TG = thyroglobulin, TSHR = thyrotropin receptor, TPO = thyroid peroxidase, PDS = pendrin, NIS = sodium-iodide symporter.

within each site. However, there are no treatment-dependent differences in gene expression profile. Nonetheless, it is possible that steroid signaling does play an important role in early thyroid function in the alligator, despite these results, considering the body of evidence suggesting that steroids regulate thyroid growth and development (Banu et al., 2001a,b, 2002b; Furlanetto et al., 1999; Manole et al., 2001; Xu et al., 2013). Perhaps our treatment scheme was unsuccessful in delivering steroids during a period of sensitivity, or our doses were insufficient to produce observable changes. We dosed at stage 19 (Ferguson, 1985) to provide a precocious dose of steroids (i.e., before endogenous gonadal steroid hormone secretion commences) but we did not examine thyroidal sensitivity to steroid hormones at stage 19. Therefore, it is possible that the thyroid was not sensitive to androgens and estrogens at stage 19 and the dosed steroids were metabolically deactivated before the onset of thyroidal sensitivity. We therefore cannot definitively conclude that thyroidal physiology in the American alligator is insensitive to steroid hormone signaling at early life stages. Future studies should explore the basic links between the developing thyroid and steroid hormone signaling in the American alligator and other vertebrates to better address the hypothesis that the thyroid is susceptible to indirect disruption mediated through impaired steroid hormone signaling. By reporting important baseline information regarding the developmental abundance of thyroid transcripts related to thyroid function and steroid sensitivity in the American alligator, the present study will facilitate future studies of comparative physiology and endocrine disruption.

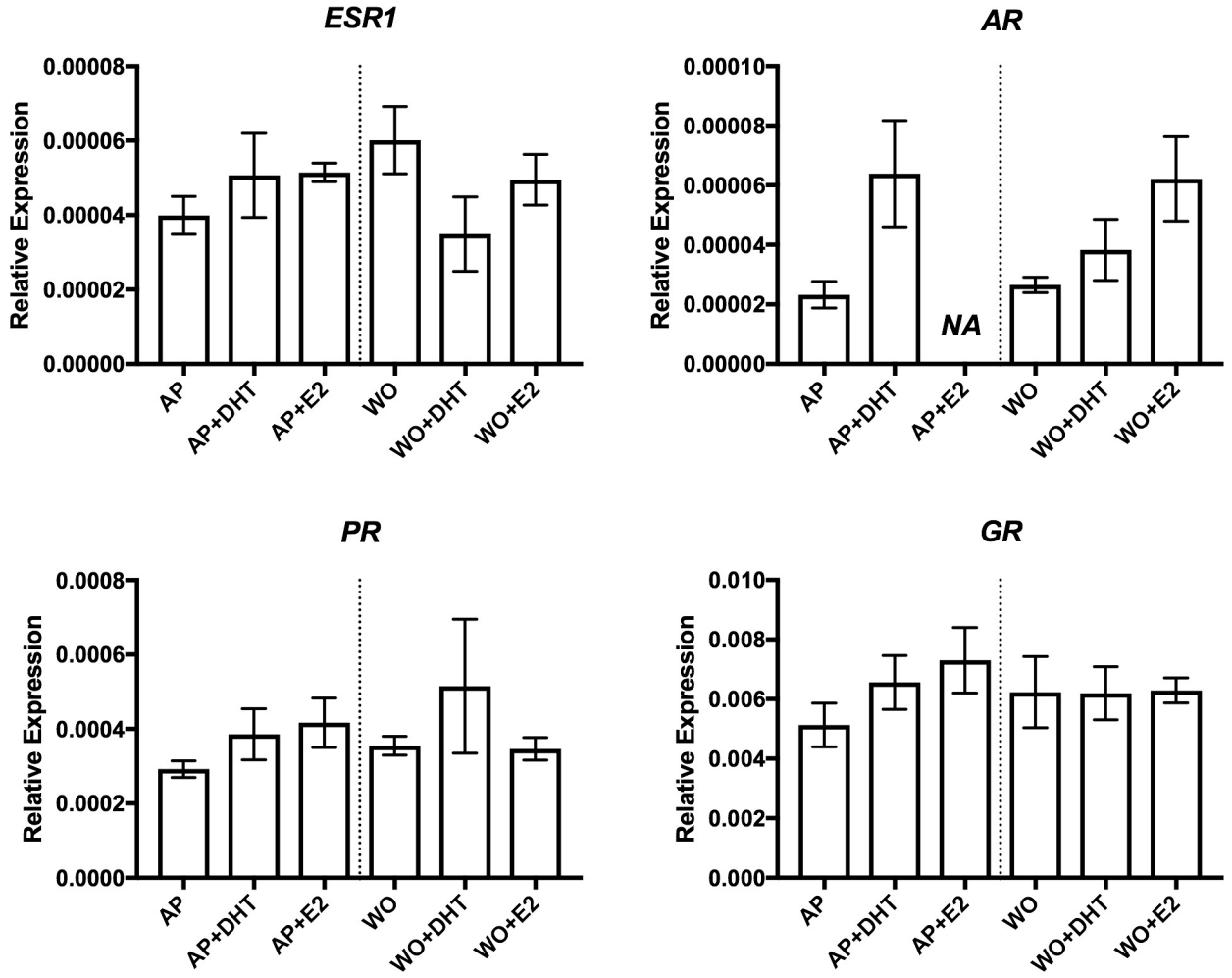

Fig. 6. Thyroidal expression of steroid hormone receptor genes in 5-month-old alligators by site and treatment displayed as mean relative expression. Error bars indicate standard error of the mean (SEM). NA indicates that poor technical replication (i.e., triplicate coefficients of variation exceeded 40% for all samples in this group) precluded reliable data analysis. Sample sizes reported in Table 1. ESR1 = estrogen receptor alpha, AR = androgen receptor, PR = progesterone receptor, GR = glucocorticoid receptor.

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