Estrogen Receptor 1 (ESR1; ERα), not ESR2 (ERβ), Modulates Estrogen-Induced Sex Reversal in the American Alligator, a Species With Temperature-Dependent Sex Determination

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All crocodilians and many turtles exhibit temperature-dependent sex determination where the temperature of the incubated egg, during a thermo-sensitive period (TSP), determines the sex of the offspring. Estrogens play a critical role in sex determination in crocodilians and turtles, as it likely does in most nonmammalian vertebrates. Indeed, administration of estrogens during the TSP induces male to female sex reversal at a male-producing temperature (MPT). However, it is not clear how estrogens override the influence of temperature during sex determination in these species. Most vertebrates have 2 forms of nuclear estrogen receptor (ESR): ESR1 (ERα) and ESR2 (ERβ). However, there is no direct evidence concerning which ESR is involved in sex determination, because a specific agonist or antagonist for each ESR has not been tested in nonmammalian species. We identified specific pharmaceutical agonists for each ESR using an in vitro transactivation assay employing American alligator ESR1 and ESR2; these were 4,4′,4′′-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT) and 7-bromo-2-(4-hydroxyphenyl)-1,3-benzoxazol-5-ol (WAY 200070), respectively. Alligator eggs were exposed to PPT or WAY 200070 at a MPT just before the TSP, and their sex was examined at the last stage of embryonic development. Estradiol-17β and PPT, but not WAY 200070, induced sex reversal at a MPT. PPT-exposed embryos exposed to the highest dose (5.0 μg/g egg weight) exhibited enlargement and advanced differentiation of the Müllerian duct. These results indicate that ESR1 is likely the principal ESR involved in sex reversal as well as embryonic Müllerian duct survival and growth in American alligators. (*Endocrinology* 156: 1887–1899, 2015)

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Abbreviations: *AMH*, anti-Müllerian hormone; *CYP19*, cytochrome P450–19; *DAX1*, dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1; DDE, Dichlorodiphenyldichloroethylene; DDT, Dichlorodiphenyldichloroethane; DES, diethylstilbestrol; DMSO, Dimethyl sulfoxide; DPN, 2,3-bis(4-hydroxyphenyl)-propionitrile; E_2, 17β-estradiol; EDC, endocrine disrupting contaminant; *EEF1*, eukaryotic elongation factor-1; ESR, estrogen receptor; EtOH, ethanol; E.W., egg weight; FERb 033, 2-chloro-3′-fluoro-3,4′-dihydroxy-[1,1-biphenyl]-4-carboxaldehyde oxime; *FOXL2*, forkhead box L2; FPT, female-producing temperature; *FST*, follistatin; GAM, gonad-adrenal-mesonephros; *GDF9*, growth and differentiation factor 9; ICI-182780, 7α,17β-[9-[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol; LBD, ligand-binding domain; MPT, male-producing temperature; PCB, Polychlorinated biphenyl; PPT, 4,4′,4′′-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol; Q-PCR, quantitative real-time PCR; *RPL8*, ribosomal protein L8; TSD, temperature-dependent sex determination; SOX9, sex determining region Y-box 9; TSP, thermo-sensitive period; WAY 200070, 7-bromo-2-(4-hydroxyphenyl)-1,3-benzoxazol-5-ol.

All crocodilian and many turtle species exhibit temperature-dependent sex determination (TSD), where the ambient temperature during a thermo-sensitive period (TSP) of egg incubation (developmental stages 21–24 in the American alligator, *Alligator mississippiensis*) determines the sex of the offspring (1). Estrogens play a critical role in sex determination in crocodilians and turtles, as it likely does in most nonmammalian vertebrates. Indeed, administration of estrogens during the TSP overrides a male-producing temperature (MPT) in crocodilians and various turtles, amphibians, and teleost fish (2–5). In general, timing of the sensitivity to estrogens, especially 17β-estradiol (E_2), coincides with the TSP in 2 turtle species (6, 7).

Embryonic exposure to estrogenic compounds can alter sex determination in nonmammalian species, but does it also lead to other health consequences as it does in mammals? There are clear mechanistic differences between sex determination, differentiation, and maintenance in mammals: sex determination and differentiation are driven by sex chromosomes (eg, *sex-determining region Y* gene), whereas sex maintenance has more potential to be affected by environmental signals. In contrast, sex determination, differentiation, and maintenance have not been totally distinguished in TSD species due to the lack of sex chromosome, although some studies indicated differences between the temperature- and exogenous estrogen-induced receptor signaling pathway and DNA methylation of cytochrome P450–19 (*CYP19*) promoter in TSD species (8–10). Although exposure to estrogenic compounds does not influence sex determination in mammals, there is clear evidence from the public health disaster involving embryonic exposure to the pharmaceutical estrogen diethylstilbestrol (DES) that in utero estrogen exposure is associated with health risks such as breast cancer, clear cell adenocarcinoma of vagina and cervix, reproductive tract structural differences, pregnancy complications, and infertility in women as well as noncancerous epididymal cysts in men (11). Some environmental contaminants have been identified as estrogenic in action and included in the endocrine disruptors (12–14). Thus, it is important to understand the mechanisms by which estrogenic compounds influence embryonic development.

The American alligator is a long-lived organism that serves as a top predator in aquatic freshwater and estuarine ecosystems in the Southeastern United States. Individuals exhibit limited geographical ranges. Thus, the alligator is considered a sentinel species to investigate local, long-term environmental health, including issues such as bioaccumulation/biomagnification of environmental contaminates. Indeed, we have evaluated various endocrine and reproductive alterations associated with environmental contaminants and the American alligator is considered an important sentinel of environmental health (15, 16).

Sensitivity to temperature and exogenous chemicals during the TSP could provide a powerful model to investigate the impact of estrogenic endocrine disrupting contaminants (EDCs) as well as climate change due to alter global temperatures. In ovo exposure to exogenous estrogens, pharmaceutical estrogens, or EDCs such as transnonachlor, *p,p'*-Dichlorodiphenyldichloroethylene (DDE) and Polychlorinated biphenyl (PCB), at environmentally relevant concentration, can induce sexual reverse in red-eared turtles (17–21). Likewise, endogenous or pharmaceutical estrogens as well as in ovo exposure to *p,p'*-Dichlorodiphenyldichloroethane (DDD), *o,p'*-DDE, or *p,p'*-DDE skews sex ratios in the American alligator (18, 22–26). However, it is still not clear how endogenous or pharmaceutical estrogens or even EDCs override or alter TSD. The effect of estrogens during TSD indicates that they act locally and directly on the gonad to induce ovarian differentiation as documented with in vitro studies in turtles (27, 28). Although cell migration from the mesonephros into the gonad is important for the differentiation of functional testicular seminiferous tubules (29), the gonadal tissue itself can "sense" temperature and estrogenic compounds, which can initiate sex determination without mesonephric cell migrations (27, 28).

Estrogen-induced SOX9 suppression during turtle TSP can be delayed by administration of the estrogen receptor (ESR) antagonist, ICI-182780 (7α,17β-[9-[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol) (30), which has potent antiestrogenic activity and binds to both ESR subtypes in mammals (31–33). These observations indicate that E_2 has the potential to regulate the suppression of SOX9 during natural ovarian determination in at least 1 species with TSD. Most vertebrates studied to date, except sharks (34), have 2 forms of the nuclear ESRs, ESR1 (ERα) and ESR2 (ERβ), which have different ligand specificity, distribution, and function (35). In the red-eared turtle, localization and timing of ESRs expression suggests that ESR1 and ESR2 play important roles in sex determination and differentiation, respectively (10, 36). *ESR1* mRNA abundance in the turtle gonad peaks late in the TSP, stage 19 in both female-producing temperature (FPT) and MPT, whereas *ESR2* mRNA concentration in the gonad gradually increases after TSP, stage 23 at FPT as detected by quantitative real-time PCR (Q-PCR) (10). Although the pattern of ESR localization under differing temperatures regimens was not so clear, differential shifts of each ESR after E_2 administration in the turtle indicated potentially different roles at each developmental stage (10). Exogenous E_2 exposure rapidly reduced *ESR1* in the ovarian medulla and

stayed on the boundary of medulla and cortex, whereas *ESR2* responded to E_2 slowly and was expressed in the cortex during later stages based on visualization with in situ hybridization (10). Thus, ESR1 and ESR2 could be involved in sex determination and differentiation of ovary development in the red-eared slider turtle sequentially, and this suggests that ESR1 could play important roles in exogenous E_2-induced sex reversal in TSD amniotes. Further detailed investigation is required to understand estrogen signaling during normal TSD as well as during estrogen-induced sex reversal in crocodilian species.

Here, we report on studies using pharmaceutical estrogen agonists that have specific affinities for each ESR in mammals. We tested each on ESR1 and ESR2 clones from the American alligator using an in vitro transactivation assay. We then exposed eggs exogenously to E_2, or the ESR specific agonists before the TSP and examined their potential to sex reverse embryos incubated at a MPT.

Materials and Methods

Transactivation assay

The expression plasmids of the American alligator *ESRs* in the pcDNA3.1 vector (Life Technologies), and the luciferase reporter plasmid containing 4 estrogen-responsive elements were prepared as described previously (37). To exclude possible differences in affinity of each ESR for a common ERE, a fusion protein of GAL4 and ligand-binding domain (LBD) (including the hinge region and F-domain) of each ESR was also tested. Each partial cDNA was subcloned into pBIND vector (Promega) as describe previous (38), and pG*5luc* vector (Promega), including 5 GAL4-binging site, was used as the reporter construct. Hormonal transcriptional activity of the HEK293 cells transfected with the expression and reporter constructs using FuGene transfection reagent (Promega) was measured by the luciferase reporter gene assay as previously described (37, 38). E_2 was purchased from Sigma-Aldrich, and all ESR agonists, 4,4′,4′′-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT), 7-bromo-2-(4-hydroxyphenyl)-1,3-benzoxazol-5-ol (WAY 200070), 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN), and 2-chloro-3′-fluoro-3,4′-dihydroxy-[1,1-biphenyl]-4-carboxaldehyde oxime (FERb 033), were purchased from Tocris Bioscience. Each estrogenic compound was dissolved in Dimethyl sulfoxide (DMSO) (Sigma-Aldrich), serially diluted with DMSO, and added to the culture media at 0.1% DMSO. Luciferase activity was measured as chemiluminescence activity using the Dual-Luciferase Reporter Assay System (Promega). Luminescence was measured using a Turner Designs Luminometer TD-20/20 (Promega). All transfections were performed in triplicate. Estrogenic activity of each ligand was determined and ranked based on it EC_{50} calculated in fold inductions from the dose-response curve.

Incubation and treatment of eggs

All field and laboratory work were conducted under permits from the Florida Fish and Wildlife Conservation Commission and Unites States Fish and Wildlife Service. Five clutches of alligator eggs were collected from the Lake Woodruff National Wildlife Refuge, and 2 clutches were collected from the Lake Apopka, Florida in June, 2010 within the first 2 weeks after oviposition. Lake Woodruff has relatively low chemical contamination of persistent organic pollutants, and American alligators exhibit healthy reproductive activity; this lake serves as a reference site for many studies of alligator reproductive biology and development, whereas the Lake Apopka is contaminated with agricultural compounds (15, 39). At least 1 egg from each clutch was examined to determine the developmental stage of the embryo based on criteria described by Ferguson (40). Eggs were treated by applying 0.5 μL of solvent with or without the active compound onto the surface of the eggshell at developmental stage 19; the TSP for sex determination in the American alligator is developmental stages from 21 through 24 (23, 25). Eggs were then incubated at 33.5°C, the temperature at which 100% males (MPT) are produced or at 30.0°C the incubation temperature that produces 100% females (FPT) (1, 23). All treatments were administrated with 0.5 μL of 95% ethanol (EtOH) per gram of egg weight (E.W.): E_2 at 0.05, 0.5, 5, 50, and 500 ng/g E.W., PPT or WAY 200070 at 0.5 ng, 50 ng and 5 μg/g E.W., with doses equivalent to 0.05 parts per billion–5 parts per million based on E.W. The highest doses were equivalent to 1.8×10^{-6}M E_2, 1.3×10^{-5}M PPT, and 1.6×10^{-5}M WAY 200070, based on E.W. Eggs were incubated in wet sphagnum moss (see details in

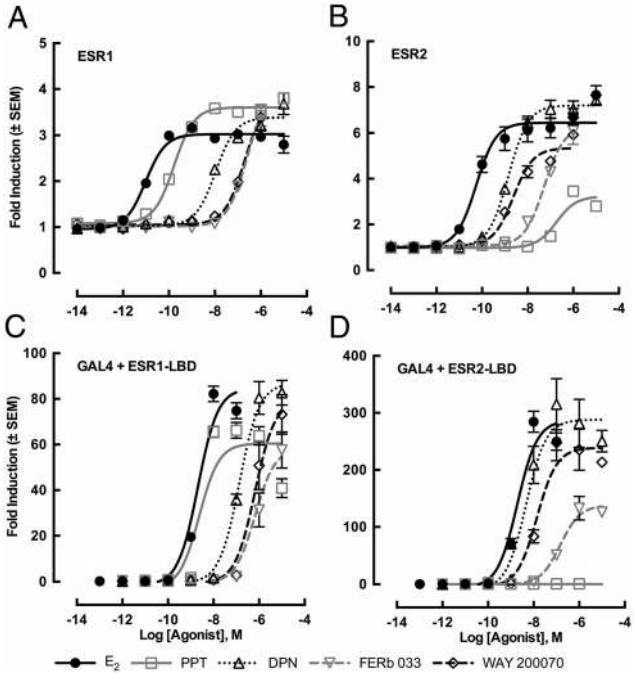

Figure 1. Transcriptional activities of alligator ESR1 (A and C) or ESR2 (B and D) for various ESR ligands. HEK293 cells were transiently transfected with the 4 estrogen-responsive elements containing vector together and an alligator ESR expression vector (A and B) or were transfected with pG5*luc* vector containing 5 GAL4 binding sites and LBD of each ESR in pBIND vector, including the GAL4 DNA-binding domain (C and D). Cells were incubated with serial concentrations of E_2, PPT, DPN, FERb 033, or WAY 200070 at 10^{-14}M–10^{-5}M. Data were represented as fold inductions of luciferase signals as compared with vehicle (DMSO) controls. PPT and WAY 200070 showed the highest specificity on ESR1 and ESR2, respectively. Each column represents the mean of triplicate determinations, and vertical bars represent the mean ± 1 SEM.

Refs. 23, 25). Developmental stage was predicted based on an equation developed for the pattern of embryonic development at these temperatures (25). Necropsy was performed at developmental stage 27, which was 1 stage before hatching. The gonad-adrenal-mesonephros (GAM) complexes were dissected free of other tissues and preserved in Bouin's fixative or RNA*later* (Life Technologies).

Morphology of Müllerian duct and GAM

The GAM from each animal was removed with the Müllerian duct and fixed in Bouin's fixative overnight. After overnight fixation, the tissue was transferred to 70% ethanol. Gross morphology of Müllerian duct-GAM complex was observed with a stereomicroscope, and pictures of the complex were obtained digitally. The presence or absence of the Müllerian duct was determined. A fixed GAM from each animal was embedded in paraffin, sectioned at 7 μm, and stained with hematoxylin and eosin for microscopic examination and assignment of sex. Testes were identified by the presence of developed medullary sex cords and seminiferous tubules, whereas ovaries were characterized by the presence of germ cells in the cortex and lacunae in the medulla as described previously (41).

RNA isolation and quantitative RT-PCR

Gonadal tissue was dissected free (using a stereomicroscope) from other tissues for each GAM preserved in RNA*later*, and gonadal RNA was isolated using the total RNA Isolation system (Promega) as described previously (42). Using the isolated total RNA, cDNA was obtained using a iScript cDNA Synthesis kit (Bio-Rad). Q-PCR was conducted using a CFX96 real-time PCR detection system (Bio-Rad) with homebrew SYBR Green reaction mixture, which contained 20mM Tris-HCl (pH 7.75), 50mM KCl, 3mM $MgCl_2$, 4% DMSO, 0.5× SYBR Green I (Invitrogen), 0.5% glycerol, 0.5% Tween 20 (Sigma-Aldrich), 0.2mM deoxynucleotide mix (Thermo Scientific), 0.01-U/μL AmpliTaq Gold (Applied Biosystems), 0.2μM primer mix, and 1/25 vol of cDNA. The cycle-threshold values were compared with a standard curve obtained from serial dilutions of the plasmid DNA, whose concentration was known and which contained the target cDNA. The plasmids were diluted with TE buffer (10mM Tris-HCl and 1mM EDTA; pH 8.0) plus 5 ng/μL of yeast tRNA as described previously (42, 43). The raw data were obtained as number of copies/μL, and each value was normalized by a normalization factor, which was obtained by using Norm Finder (44). Normalization factor was the geographic mean of *eukaryotic elongation factor-1* (*EEF1*) and *ribosomal protein L8* (*RPL8*) mRNA abundance, whose stability was the best combination of 2 genes in 4 internal control genes, *EEF1*, *RPL8*, *β-actin*, and *18S ribosomal RNA*, in Norm Finder (see Supplemental Table 1). PCR primer sequences and annealing temperatures are shown in Supplemental Table 2 below. mRNA abundance was also analyzed using the ΔΔ Ct method with efficiency correction (45). Q-PCR efficiency was estimated by CFX Manager Software (version 3.1; Bio-Rad). Intra- and interassay coefficient of variations for all 3 assays was less than 5% in cycle-threshold values.

Statistical analyses

EC_{50} values of various estrogenic compounds in the in vitro ESR transactivation assays were estimated by nonlinear best-fit models using 3 parameters for fitting the curve. χ^2 tests were used to compare sex ratios among treatment groups and/or egg-incubation temperatures with Bonferroni corrections. Concentrations of mRNA were compared by one-way ANOVA followed by Dennett's comparison, with the EtOH treatment group incubated at MPT serving as the control group. In the cases where groups showed nonnormal distributions, a Kruskal-Wallis test followed by Dunn's comparison was used to evaluate the treatment effects. Effects of egg-incubation temperature on mRNA abundance was evaluated by unpaired *t* test for normally distributed samples, or by Mann-Whitney for samples without normal distribution or equal deviations. Outliers were detected and removed using the robust regression and outliers removal method at Q = 0.1%. Each value is shown as a mean ± 1 SEM. Statistical analyses were conducted using Prism 6.0b (GraphPad Software) and JMP 9.0.2 (SAS Institute) for Mac. Cluster analysis of ovarian mRNA abundance was conducted with GENE-E software (Broad Institute).

Table 1. Specificity of ESR Agonists on Full-Length of ESR or Gal4-Ligand Binding Domain of ESR Fusion Protein in Transactivation

	ESR1 Full Length on ERE				ESR2 Full Length on ERE			
	EC_{50} (M)	RP (%)	RE (%)	ESR1/ESR2 of EC_{50}	EC_{50} (M)	RP (%)	RE (%)	ESR2/ESR1 of EC_{50}
E_2	9.66×10^{-12}	100.0	100.0	0.2	5.55×10^{-11}	100.0	100.0	5.7
PPT	1.67×10^{-10}	5.8	128.3	0.0	1.83×10^{-7}	0.0	41.5	1093.0
DPN	1.08×10^{-8}	0.1	117.6	7.6	1.42×10^{-9}	3.9	113.6	0.1
FERb 033	2.53×10^{-7}	0.0	148.9	4.7	5.38×10^{-8}	0.1	96.9	0.2
WAY 200070	1.94×10^{-7}	0.0	138.9	82.8	2.35×10^{-9}	2.4	79.7	0.0
	ESR1 LBD-GAL4				**ESR2 LBD-GAL4**			
	EC_{50} (M)	RP (%)	RE (%)	ESR1/ESR2 of EC_{50}	EC_{50} (M)	RP (%)	RE (%)	ESR2/ESR1 of EC_{50}
E_2	2.08×10^{-9}	100.0	100.0	1.07	1.93×10^{-9}	100.0	100.0	0.93
PPT	2.37×10^{-9}	87.6	71.5	N/A	N.D.	N/A	N.D.	N/A
DPN	1.36×10^{-7}	1.5	102.5	29.12	4.66×10^{-9}	41.5	100.5	0.03
FERb 033	1.13×10^{-6}	0.2	76.0	5.84	1.49×10^{-7}	1.3	48.1	0.13
WAY 200070	6.62×10^{-7}	0.3	93.8	47.17	1.40×10^{-8}	13.8	83.3	0.02

RP, relative potency = (EC_{50} of E_2/EC_{50} of other agonist) × 100; RE, relative efficacy = maximum response % of E_2 found at any doses.

Results and Discussion

Specificities of ESR agonists in vitro

ESR agonists induced transcriptional activity in the in vitro ESR transactivation assay via full-length receptor on ERE with EC_{50} values ranked as follows: for ESR1, E_2 > PPT > DPN > FERb 033 = WAY 200070; and for ESR2, E_2 > DPN = WAY 200070 > FERb 033 > PPT (Figure 1, A and B, and Table 1). As seen with the assay using the full-length receptor, each ESR revealed a similar specificity to ESR agonists when the fusion protein of GAL4 and ESR LBD were used, although the sensitivities to each agonist were lowered (Figure 1, C and D, and Table 1). Thus, the specificity of each ligand was dependent on the LBD affinity to the ligand rather than the selectivity of the ERE. This reduction in ligand sensitivity was also observed in a previous study of sex steroid hormone receptors in the slider turtle (38). This shift in sensitivity could be caused due to lack of the activation function 1 domain in the A-B domain of ESR. PPT displayed great specificity for alligator ESR1. Although DPN and WAY 200070 showed similar transactivation activity on alligator ESR2, WAY 200070 exhibited significantly less cross-reactivity on ESR1 than DPN; therefore, we used WAY 200070 as a specific agonist for alligator ESR2 (Figure 1 and Table 1). Studies examining the specificity of pharmaceutical ESR agonists in mammalian rodents showed that DPN exhibited the highest specificity for ESR2 (46, 47), whereas alligator ESR2 displayed better specificity for WAY 200070. Although alligator ESR2 has a predicted amino acid identity similar to 90%–92% with that of the ligand binding region of mammalian ESR2 (48), specific characteristics of ESR2 binding affinity or transactivation are different as noted here.

Gross anatomy. Effects of in ovo exposure to estrogens during the TSP at different incubation temperatures

Expanded Müllerian ducts, enlarged cloaca, and a thickened pubis symphysis were observed after exposure to 5-μg PPT/g E.W. at a MPT, whereas these alterations were not seen after exposure to 2 lower doses of PPT, nor after exposure to any of the doses of E_2 or WAY 200070 (Figures 2 and 3), although E_2 did sex reverse embryos incubated at MPT (see below). In the present study, ovarian anti-Müllerian hormone (*AMH*) mRNA abundances were not altered by treatments in ovo; thus, it is unlikely that a suppressed AMH signal would relate to the PPT-induced hyperplasia. As expected, Müllerian ducts were identified in the embryos incubated at FPT but not in most of the embryos incubated at MPT (Figure 3). Regression of the Müllerian duct, which is mainly regulated by AMH

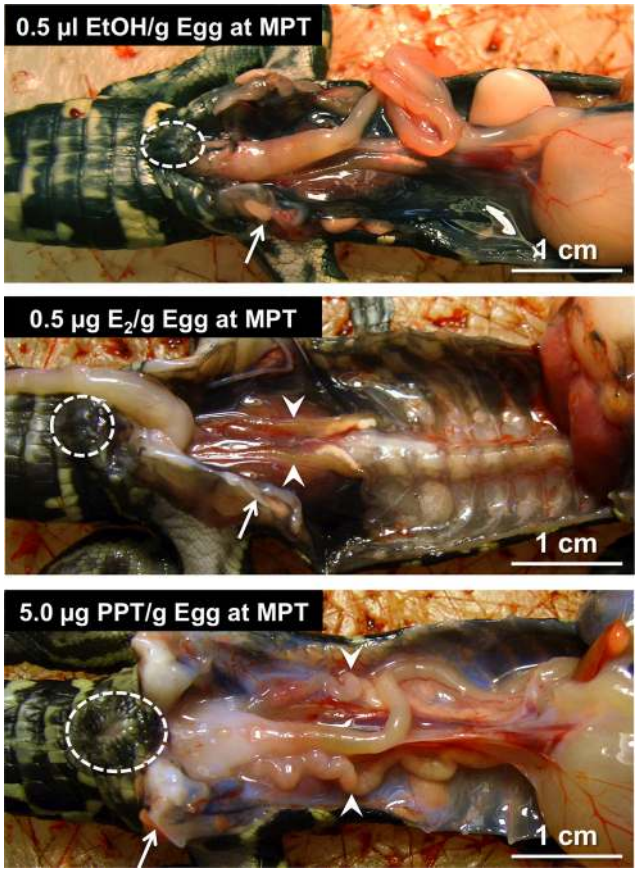

Figure 2. Gross morphology around the abdominal cavity in embryonic American alligator at developmental stage 27. Embryo at developmental stage 19 was exposed to 0.5-μL 95% ethanol/g E.W. (top panel), 0.5-μg E_2/g E.W. (E_2) (middle panel), or 5.0 μg/g E.W. of PPT (bottom panel) at MPT. PPT induced enlarged cloaca, pelvic bone, and Müllerian duct. Circle, arrow, and arrowhead indicate cloaca, pelvic bone, and Müllerian duct, respectively.

(49), was interrupted by exposure to PPT (0.5 and 5 μg/g E.W.) or E_2 (0.05 and 0.5 μg/g E.W.) but not by WAY 200070 exposure (Supplemental Figure 1). Thus, an estrogenic signal via ESR1 plays a critical role in the survival of the Müllerian duct.

Gonadal histology. Effects of in ovo exposure to estrogens during the TSP at different incubation temperatures

Based on histological characteristics, sex reversal was significantly induced under 2 of the experimental conditions. Embryos exposed to the higher doses of E_2 and PPT (0.05–0.5 μg/g and 0.5–5.0 μg/g E.W., respectively) and incubated at a MPT exhibited ovarian, vs testicular, development in all the exposed embryos ($P < .0001$ and $P = .0027$, respectively), when compared with exposure to EtOH (vehicle) control at MPT ($P = .0027$) (Figure 4). All other treatments, except EtOH at FPT control ($P < .0001$), showed no significant difference in their gonadal sex ratio within a treatment group when compared with

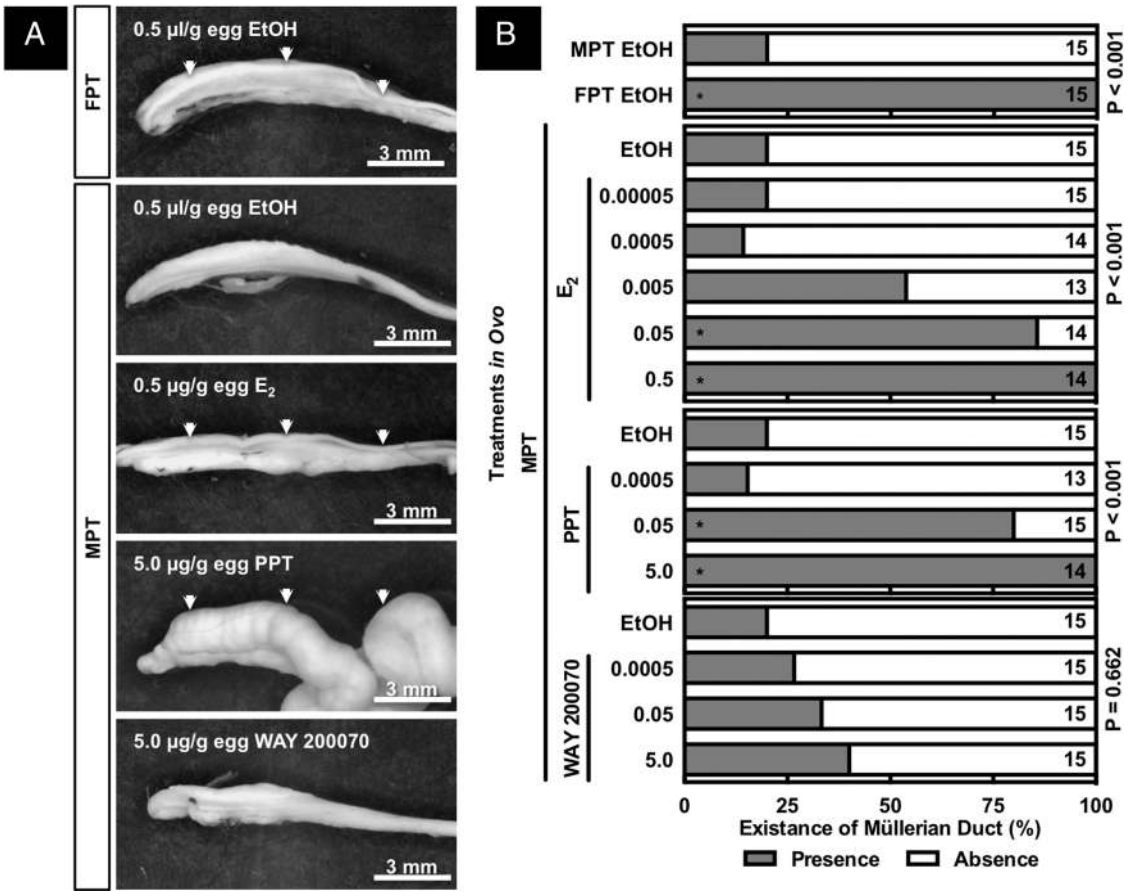

Figure 3. Gross morphology of GAM complex and Müllerian duct (A) isolated from embryonic American alligator at developmental stage 27. Embryo at developmental stage 19 was exposed to 0.5 μL/g E.W. of 95% ethanol, various doses of E_2, PPT, or WAY 200070 at FPT or MPT. Existences of Müllerian duct were observed and counted, and data were shown as percentage of Müllerian ductal presence or absence (B). An exposure to E_2 and PPT increased the existence of Müllerian duct at MPT, whereas WAY 200070 exposure did not alter it. Numbers on the graph indicate number of animals. Asterisks indicate significant difference in χ^2 test ($P < .05$).

the EtOH vehicle control at MPT (Figure 4). Gonadal size was obviously smaller in the embryos exposed to 5-μg PPT/g E.W. when compared with the other treatment groups. Ovarian medullary lacunae in embryos exposed to 5-μg PPT/g E.W. were more developed than those observed from embryos exposed to E_2 treatments (Figure 4C). Developmental exposure to E_2 or overexpression of CYP19 was sufficient to induce sex reversal in genetic male chickens, but it did not alter gonadal size or ovarian lacunae development (50, 51). Although sex reversal and hypertrophic Müllerian ducts were induced by DES exposure in male chickens, a smaller gonad also was not observed (52). The smaller gonads and limited development of ovarian lacunae in the alligator neonates observed in this study are hypothesized to be a response to disproportionate activation of ESR1 signaling after PPT exposure.

Estrogen stimulates not only nuclear ESRs but also stimulates membrane bound ESRs (53, 54). Although PPT could induce or enhance rapid reaction via cell membrane bound receptors in mammals (48, 55–58), those effects were highly reduced in ESR1 knockout mice (56). Thus, the estrogen-induced sexual reversal observed in the present study was most likely caused through nuclear ESR1, but further investigations are required to clarify whether nuclear or membrane ESR1, or both, are induced when exogenous estrogens are used to induce sex reversal in the American alligator.

mRNA abundance. Effects of in ovo exposure to estrogens during the TSP at different incubation temperatures

The pattern of mRNA abundance was analyzed by quantitative RT-PCR with known concentrations of serially diluted plasmid DNA as standard samples (see Supplemental Table 2). Gonadal tissues displayed a clear induction of sex-specific mRNA after egg incubation at thermally sensitive temperatures as well as in ovo exposure to the specific agonists for the 2 ESRs. Males showed higher gonadal mRNA abundance of *AMH* compared with *CYP19* (aromatase), whereas female displayed the opposite pattern as observed for other vertebrates (42, 59,

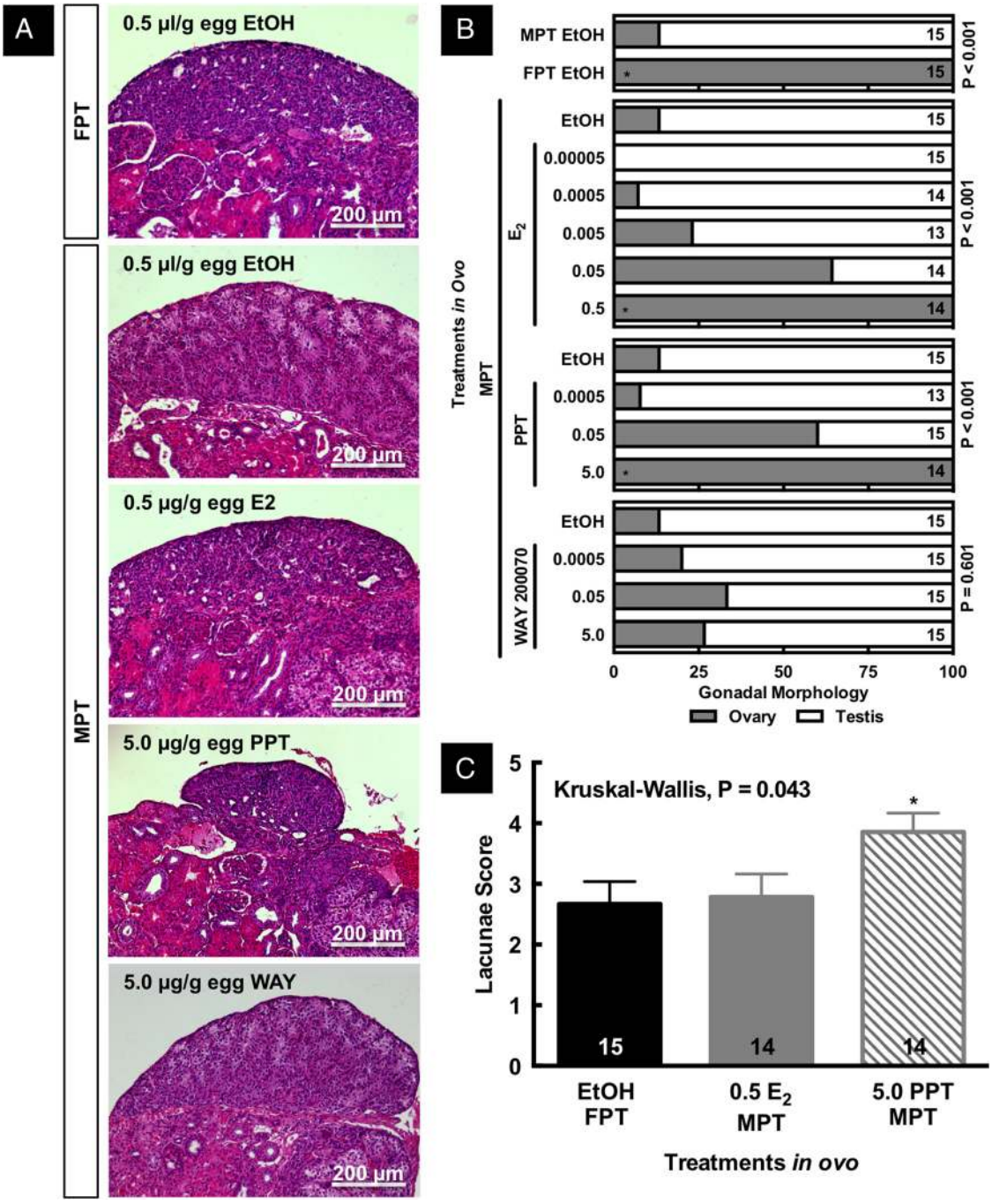

Figure 4. Gonadal histology of embryonic American alligator at developmental stage 27 (A). Embryo at developmental stage 19 was exposed to 0.5 μL/g E.W. of 95% ethanol, various doses of E_2, PPT, or WAY 200070 at FPT or MPT. Gonadal characteristics were observed and counted, and data were shown as percentage of ovarian or testicular characteristics (B) and score of lacunae (C). An exposure to E_2 and PPT induced the ovarian development at MPT, whereas WAY 200070 exposure and control induced the testicular development with seminiferous tubules at MPT. PPT exposure at developmental stage 19 induced higher score of lacunae development. Numbers on the graph indicate number of animals. Asterisks indicate significant difference in χ^2 test or ($P < .05$).

60). Using this sexual dimorphic pattern as a criterion, the sex of embryos was functionally confirmed (Figure 5). Although most embryos displayed consistent results based on the existence of the Müllerian duct, gonadal morphology, and gonadal mRNA pattern, Müllerian duct existence was the least reliable of these 3 sexing criteria as described previously (61). The mRNA expression pattern in 3 embryos did not matched with their gonadal morphology, but they were not observed in a specific treatment group (Figure 5). The gonadal morphology of 1 male displayed an ovarian-like mRNA expression pattern (*CYP19* > *AMH*), whereas 2 females with ovaries exhib-

Table 2. Primers for Q-PCR

Primer Name	Position	Sequence (5′ to 3′)	Annealing Temperature (°C)	GenBank Accession Number
ACTB	209	ATGAGGCCCAAAGCAAAAGA	64.0	DQ421415
	280	CCCAGTTGGTGACAATGCC		
AMH	1483F	AGCAGCTCAACCTCTCTGAGGA	64.0	NM_001287280
	1726R	TAGCAGAAAGCCAGAAGGTGC		
CYP19	1227F	CAGCCAGTTGTGGACTTGATCA	66.4	AY029233
	1305R	TTGTCCCCTTTTTCACAGGATAG		
DAX1	618F	TGCTCTTTCCTTGCTGAGATC	62.0	AF180295
	692R	ACTGTGCCAATGATAGGCCTA		
DMRT1	175F	AGCCCAACTCACTCAACAAG	62.0	XM_006261265
	261R	GATGGAAGGAACATCCTGAA		
EEF1	321F	CGTTCTGGTAAGAAGCTGGA	62.0	XM_006269752
	489R	TGACACCAACAGCAACAGTC		
ESR1	410F	AAGCTGCCCCTTCAACTTTTTA	64.0	AB115909
	481R	TGGACATCCTCTCCCTGCC		
ESR2	34F	AAGACCAGGCGCAAAAGCT	60.7	XM_006265650
	105R	GCCACATTTCATCATTCCCAC		
FOXL2	111F	CATCAGCAAGTTCCCCTTC	60.7	EU848473
	207R	GGGCACCTTGATGAAACAC		
FST	800F	GCCCTACTGGGCAGATCCAT	64.0	DQ010156
	911R	CCTTGAAATCCCACAAGCAT		
GDF9	557F	TCAGTTTCCTCCTCTTCTCCAATT	62.0	XM_006263403
	634R	ACACACTTGGCTAGAAGGATCATTC		
INHA	645F	CAACTGCCACCGCGC	66.4	XM_006261090
	707R	ACAATCCACTTGTCCCAGCC		
RN18S	1082F	GAGTATGGTTGCAAAGCTGAA	66.4	AF173605
	1303R	AGTCTCGTTCGTTATCGGAAT		
RPL8	702F	GGTGTGGCTATGAATCCTGT	62.0	XM_006266675
	827R	ACGACGAGCAGCAATAAGAC		

ACTB, β-actin; DMRT1, doublesex and mab-3-related transcription factor 1; INHA, inhibin α; RN18S, 18S ribosomal RNA.

ited a testicular-like mRNA expression pattern (*CYP19* < *AMH*) (Figure 5). When the mRNA abundances were analyzed by treatment group, it was difficult to evaluate the effects of each treatment due to having both sexes in some treatment groups in which sex reversal occurred in only some of the exposed embryos (Supplemental Figure 1).

Gonadal mRNA abundances of *CYP19*, dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (*DAX1*), follistatin (*FST*), forkhead box L2 (*FOXL2*), growth and differentiation factor 9 (*GDF9*), and *AMH* were compared within the female embryos to evaluate their ovarian function. DAX1 is an orphan member of the nuclear hormone receptor superfamily and plays an important role in ovarian development and regulation of CYP19 (62–64). Fst is produced by granulosa cells in ovary and antagonizes activin signaling, which plays important roles in the feedback loop of the hypothalamic-pituitary-gonadal axis (65). FOXL2 is a transcription factor and master regulator for CYP19 and is critical in ovarian differentiation and maintenance (66, 67). GDF9 is vital for early ovarian follicle development and contributes to testicular germ cell health (68, 69). In previous reports, ovarian FST, GDF9 mRNA expressions were lower in neonates from Lake Apopka (contaminated with agricultural chemicals) as compared with a reference lake, Lake Woodruff (70, 71).

Sex reversed embryos induced by exposure to 5 μg of PPT/g egg showed a significant suppression in gonadal mRNA abundance of *CYP19* (Figure 6A). This result in *CYP19* mRNA abundance was observed using both methods of analyzing abundance, the standard curve method and ΔΔCt method. DNA methylation of the gonadal *SOX9* and *CYP19* promoter is involved in TSD in the American alligator (59). Although DNA methylation of the *CYP19* promoter plays a critical role in TSD of the European sea bass, E_2-induced sex reversal did not altered the DNA methylation level of *CYP19* promoter in the female gonad (9). In red-eared slider turtle, DNA methylation of the gonadal *CYP19* promoter was associated with TSD, whereas PCB-induced sex-reversed hatchlings showed no difference from normal females in gonadal DNA methylation of the *CYP19* promoter (8, 72). We are currently investigating DNA methylation patterns after in ovo PPT or E_2 exposure as well as methylation in American alligator from Lake Apopka. *AMH*, *DAX1*, *FOXL2*, *FST*, and *GDF9* mRNA abundance in the embryonic ovary were not altered by either E_2 or PPT exposure in ovo in this study (Figure 6A). Gonadal mRNA abundances of

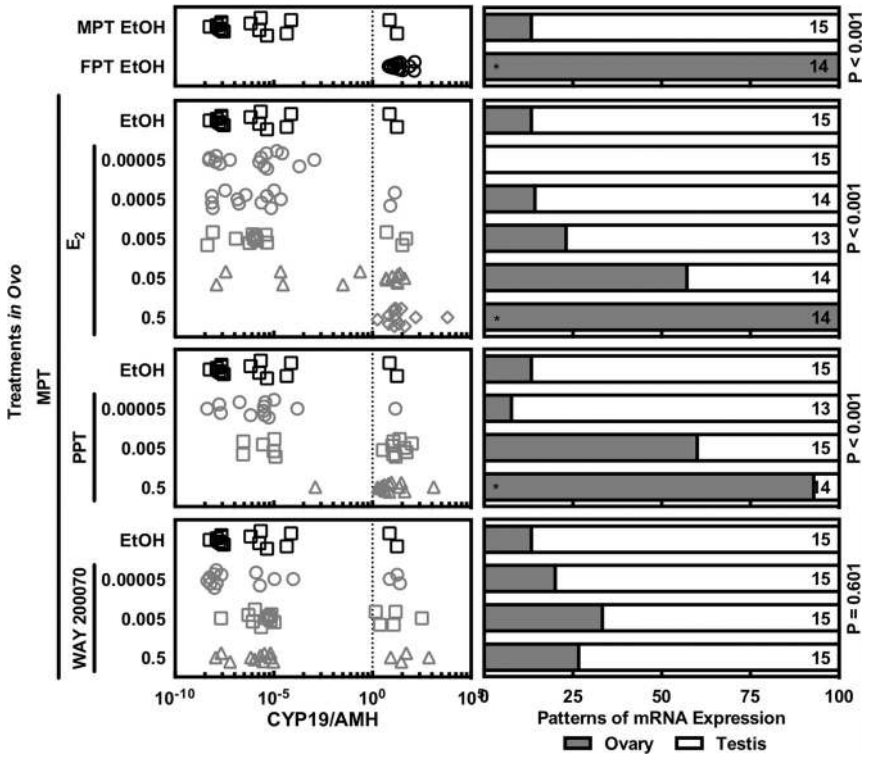

Figure 5. Discrimination of gonads by sexual dimorphic pattern of gonadal mRNA abundances of *CYP19* (aromatase) and *AMH*. Embryo at developmental stage 19 was exposed to 0.5 μL/g E.W. of 95% ethanol, various doses of E_2, PPT, or WAY 200070 at FPT or MPT. Gonadal tissue was speared out from GAM complex at developmental stage 27, and their mRNA abundances were quantified by Q-PCR. Male pattern was defined with lower abundance of *CYP19* mRNA and higher abundance of *AMH*, whereas female pattern was defined with high *CYP19* and low *AMH* mRNA abundances. An exposure to E_2 and PPT induced the female pattern of mRNA abundance at MPT, whereas WAY 200070 exposure and control induced the male pattern of mRNA abundance at MPT. Numbers on the graph indicate number of animals. Asterisks indicate significant difference in χ^2 test ($P < .05$).

CYP19, *DAX1*, *FST*, and *GDF9*, however, did show significant reductions in neonatal or juvenile ovarian tissues obtained from alligators from Lake Apopka when compared with similar tissues from Lake Woodruff (70, 71, 73). Additionally, mRNA abundances of *ESR1*, *ESR2*, *DAX1*, and inhibin-α did not show definitive alteration in the embryonic ovary after PPT exposure in ovo (Supplemental Figures 2 and 3). These differences in mRNA abundances between present and previous study could be due to age differences of animals. Further investigations are required to clarify it.

The contaminants in Lake Apopka such as DDE and PCBs exhibit very low affinities for the alligator ESR1 as compared with E_2 but are present at concentrations (parts per million) that are biologically relevant (L.J.G., unpublished data). Although suppressed *CYP19* expression and enzyme function were previously observed in neonatal or juvenile alligators from Lake Apopka (70, 74), the suppression of *CYP19* mRNA abundance in PPT-exposed gonads could be due to a different pathway from the observed suppression in Apopka alligator ovaries based on the results of the cluster analysis performed using the current dataset at developmental stage 27 (Figure 6B). Based on the mRNA abundances of the genes examined in this study, female embryos were classified into 3 major clusters (Figure 6B). PPT- or E_2-exposed animals made a major cluster, whereas the EtOH vehicle control and Apopka embryos clustered together (Figure 6B). Thus, egg-incubation temperature could induce differences in gonadal function based on the mRNA abundances rather than the developmental exposure to estrogen, PPT, or environmental contaminants in Lake Apopka at developmental stage 27 (Figure 6B). These results indicate that the pattern of mRNA abundance in an individual embryo could be more meaningful than the mean of each treatment group at developmental stage 19. This is coincident with the DNA methylation pattern of *CYP19* in sex-reversed gonads of PCB-exposed red-eared slider turtles, which was correlated with temperature rather than exposure to PCB (8). Sea bass also reveal that E_2 exposure did not alter DNA methylation levels in the *CYP19* promoter in females induced at the same temperature (9). Therefore, temperature could be the primary factor regulating DNA methylation patterns of the gonadal *CYP19* gene during development, although further investigation is required to understand the details of the mechanism driving alteration of DNA methylation patterns in sex-reserved animals.

Enlarged Müllerian duct induced by PPT exposure

Although the Müllerian ducts were identified in some male neonates (61), their presence has been used as a secondary characteristic in sexing female American alligators, especially in developing embryos and neonates. We have, however, observed that some embryos exhibit Müllerian ducts even with testicular tissue: this observation was made mostly in the group exposed to 0.05-μg PPT/g E.W. or 0.005-μg E_2/g E.W., which are below the dose needed to sex reverse the embryo. Thus, the presence of a Müllerian duct cannot be used as a good indicator for gonadal sex (female) in embryos or hatchlings. A hypertrophic Müllerian duct was previously induced by in ovo

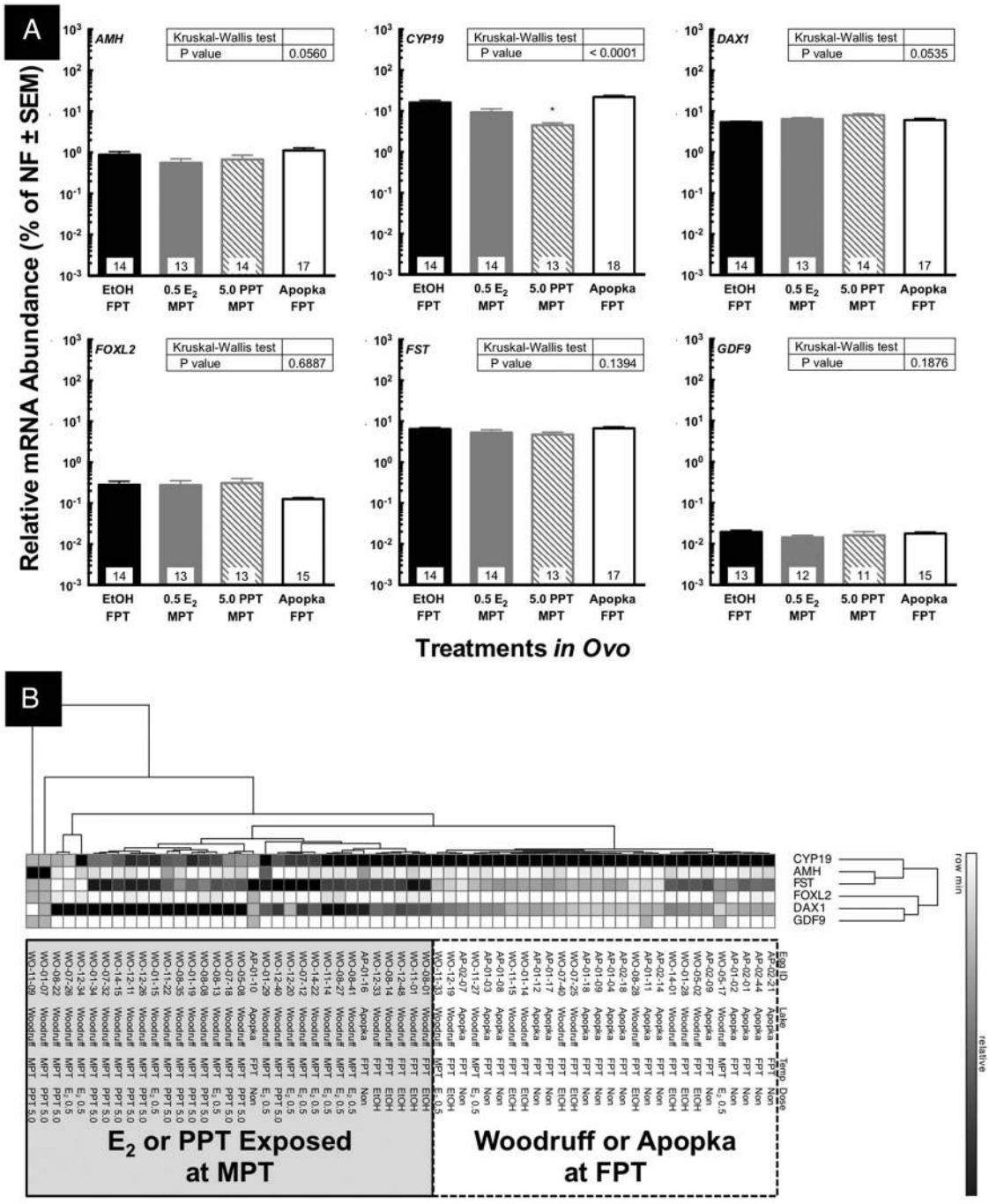

Figure 6. Alterations of gonadal mRNA abundances at developmental stage 27 in female embryos (A). Embryo at developmental stage 19 was exposed to 0.5 μL/g E.W. of 95% ethanol, various doses of E_2, PPT, or WAY 200070 at FPT or MPT. Gonadal tissue was speared out from GAM complex at developmental stage 27, and their mRNA abundances were quantified by Q-PCR. The embryos from Lake Apopka were also analyzed at stage 27 after incubation at FPT without exogenous exposure. *CYP19* (aromatase), *DAX1*, *FST*, *FOXL2*, *GDF9*, and *AMH* were quantified. Asterisks indicate significant difference as compared with vehicle controls (EtOH) at FPT ($P < .05$). Based on the quantified mRNA abundances, the 2-way hierarchical clustering was conducted with GENE-E software (B) and classified the individual embryos into 2 major groups.

exposure to 0.5 mg of norethindrone (19-nor-17α-ethynyltestosterone, a progestin) in the American alligator (75), and the histological characteristics reported were similar to those induced in this study with PPT-induced hyperplasia. Thus, PPT or PPT metabolites might have progestin activity in the American alligator, but this possibility has not been tested. In ovo exposure to estriol or DES (a synthetic estrogen) in chickens (52, 76), and in ovo exposure to estradiol benzoate or testosterone propionate in the snapping turtle, *Chelydra serpentina* (19), induced hypertrophic Müllerian ducts. In the present study, ovarian *AMH* mRNA abundances were not altered by treat-

ments in ovo within females; thus, suppression of AMH action on the Müllerian ducts is unlikely to be related to the observed PPT-induced hyperplasia. However, here, we observed not only hyperplasia after in ovo PPT exposure but regional (anterior-posterior axis) differentiation in the enlarged Müllerian duct with both epithelial and stromal expansions (based on histological analysis) (data not shown) in the American alligator. ESR1 is required to induce hyperplasia and hypertrophy in vaginal epithelium and all uterine compartments in E_2-stimulated mice (77). Thus, hyperplasia of Müllerian ducts is induced via an ESR1 signal and is consistent with previous studies reporting hypertrophic Müllerian ducts in estradiol benzoate-treated snapping turtles (19). Further investigations are needed to understand the mechanisms inducing oviducal hyperplasia or hypertrophy in reptiles.

In conclusion, we have identified that ESR1 plays a central role in sex reversal induced with an exogenous estrogen in the American alligator. We saw no evidence in this study that ESR2 plays a similar role. Although the abundances of *CYP19* and *AMH* mRNA were reversed by PPT or E_2 exposure independent of egg-incubation temperature, ovarian mRNA expression patterns were associated with egg-incubating temperature rather than exposure based on the genes examined in this study and a cluster analysis at developmental stage 19. Further, we have noted that stimulation of ESR1 alone with an exogenous pharmaceutical ligand leads to abnormal maturation and growth (hypertrophy and hyperplasia) of the Müllerian tract of the developing embryo. At this point, we do not know if this phenotype is a response to ESR1 activation above normal stimulation or persistence of the signal with a lack of concomitant ESR2 signaling as would occur if a native endogenous ligand, such as E_2, had been used. Further investigations are needed to understand steroid signaling during ovarian development as well as the possible role of EDCs induced sex reversal or skewed sex ratios via alterations in the ESR1 signaling cascade.

Acknowledgments

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