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Research paper

Feminizing effects of exposure to Corexit-enhanced water-accommodated fraction of crude oil *in vitro* on sex determination in *Alligator mississippiensis*

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ABSTRACT

Deepwater Horizon spilled over 200 million gallons of oil into the waters of the Gulf of Mexico in 2010. In an effort to contain the spill, chemical dispersants were applied to minimize the amount of oil reaching coastal shorelines. However, the biological impacts of chemically-dispersed oil are not well characterized, and there is a particular lack of knowledge concerning sublethal long-term effects of exposure. This study examined potential estrogenic effects of CWAF, Corexit 9500-enhanced water-accommodated fraction of oil, by examining its effect on estrogen receptors and sex determination in the American alligator, *Alligator mississippiensis*. The alligator exhibits temperature-dependent sex determination which is modulated by estrogen signals, and exposure to 17β-estradiol (E_2) and estrogenic compounds *in ovo* during the thermosensitive period of embryonic development can induce ovarian development at a male-producing temperature (MPT). CWAF induced transactivation up to 50% of the maximum induction by E_2 via alligator estrogen receptors *in vitro*. To determine potential endocrine-disrupting effects of exposure directly on the gonad, gonad-adrenal-mesonephric (GAM) organ complexes were isolated from embryos one day prior to the thermosensitive period and exposed to E_2, CWAF, or medium alone *in vitro* for 8–16 days at MPT. Both CWAF and E_2 exposure induced a significant increase in female ratios. CWAF exposure suppressed GAM mRNA abundances of anti-Müllerian hormone (*AMH*), sex determining region Y-box 9, and aromatase, whereas E_2 exposure suppressed *AMH* and increased Forkhead box protein L2 mRNA abundances in GAM. These results indicate that the observed endocrine-disrupting effects of CWAF are not solely estrogenically mediated, and further investigations are required.

1. Introduction

The Deepwater Horizon oil spill released over 200 million gallons of MC252 (Mississippi Canyon Block 252) crude oil into the waters of the Gulf of Mexico from the time of the explosion of the Deepwater Horizon oil rig in April 2010 until the wellhead was finally capped in July 2010 (Ramseur, 2010). In an effort to mitigate the effects of the spill and prevent the oil from reaching the coastline, approximately 2.1 million gallons of chemical dispersants (mainly Corexit® 9500) were applied both at the surface of the water and subsurface at the wellhead (Kujawinski et al., 2011). Ingestion of crude oil has been shown to negatively impact fertility indices in male and female rats following both short-term and long-term exposures (Adedara et al., 2014; Raji and Hart, 2012) and cause histopathological and hormonal disrupting effects in rabbit ovaries (Okoye et al., 2014). In addition, both crude and refined oils have demonstrated estrogenic activity in mammalian recombinant cell assays (Vrabie et al., 2011). Some chemical dispersants have also shown estrogenic activity in transactivation assays *in vitro* using a human liver hepatoma cell line (Judson

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et al., 2010). Thus, the need for additional work evaluating potential long-term sublethal effects of crude oil, dispersants, and oil/dispersant mixtures is imperative and will provide for more informed decisions regarding dispersant usage in the unfortunate event of a future oil spill disaster.

Estrogenic endocrine disruption has been well-documented in wildlife (Edwards et al., 2006; Guillette and Gunderson, 2001). Perhaps some of the most striking evidence of the tremendous impact of endocrine-disrupting contaminants (EDCs) on wildlife comes from documented effects on alligator health (Crain et al., 1997; Guillette et al., 1995; Guillette et al., 1999). The effects of organochlorine contaminant exposures on sex hormone levels and sexual differentiation have been extensively studied in the alligator population at Lake Apopka (Apopka, FL, USA), which was heavily contaminated with dichlorodiphenyltrichloroethane (DDT) and its metabolites (*p,p′*-dichlorodiphenyldichloroethylene (DDE) and *p,p′*-dichlorodiphenyldichloroethane (DDD)) following a spill from a pesticide production plant in the 1980s. A dramatic decrease in this alligator population prompted additional research revealing that juvenile alligators from this site displayed abnormal plasma sex steroid concentrations (lowered testosterone concentrations in males and elevated estradiol concentrations in both males and females) as well as morphologically reduced phallus size in males and abnormal ovarian morphology with increased numbers of polyovular follicles (multi-oocyte follicles) in females (Guillette et al., 1994).

All crocodilians, many turtle species, and some lizards exhibit temperature-dependent sex determination (TSD), a type of environmental sex determination in which the temperature of the developing embryo determines its sex (Ferguson and Joanen, 1982). Although the precise molecular mechanisms for TSD have not been completely delineated, estrogen signaling plays a crucial role in this process. Indeed, administration of exogenous 17β-estradiol (E_2) is capable of inducing ovarian development at a male-producing temperature (MPT) in several reptilian species (Bull et al., 1988; Crews et al., 1991), and an estrogen receptor 1 (ESR1) selective agonist is sufficient to induce female development at MPT in the American alligator (Kohno et al., 2015). Although the specific mechanisms by which temperature is transmitted as a driving signal in sex determination are not clear, several studies support the ability of isolated gonads to sense temperature in *in vitro* experiments performed in the TSD reptiles (Moreno-Mendoza et al., 2001; Shoemaker-Daly et al., 2010; Sifuentes-Romero et al., 2013). In addition, one study has also demonstrated that this temperature sensitivity of isolated gonads can be overridden by treatment with exogenous estradiol *in vitro* (Matsumoto et al., 2013). Thus, dosing experiments on isolated gonads can reveal insight into the ability of exogenous compounds to affect sex determination and sex ratios in the TSD species.

The American alligator, *Alligator mississippiensis*, is a long-lived apex predator exhibiting high site fidelity, which makes it an excellent model for studies examining long-term sublethal effects of contaminants. TSD in the alligator also provides a sensitive readout for environmental endocrine disruption. Exposure to exogenous estrogenic compounds during the thermosensitive period (TSP) of development results in sex reversal and skewed sex ratios in the American alligator (Crain et al., 1997, 1999; Matter et al., 1998; Milnes et al., 2005). This chemical-induced ovarian development is believed to occur through interaction with steroid receptors or alteration in steroidogenic enzyme activity via ESR1 (Kohno et al., 2015; Milnes et al., 2005).

The main objective of this study was to assess the endocrine-disrupting potential of Corexit 9500-enhanced water-accommodated fraction of oil (CWAF) by examining its effects on estrogen signaling and gene expression during the TSP of sex determination in the American alligator. Specific aims included characterizing estrogenic activity of CWAF on *A. mississippiensis* estrogen receptors ESR1 and ESR2 using *in vitro* transactivation reporter gene assays, establishing an *in vitro* gonad organ culture system which is sensitive to estrogen exposure in sex determination and differentiation, determining whether CWAF exposure is capable of inducing ovarian development and/or skewing gonadal sex ratios toward female in isolated gonad-adrenal-mesonephric (GAM) complexes cultured at MPT 33.5 °C with CWAF, and examining the expression patterns of several genes putatively involved in the sex determination and differentiation processes to compare CWAF exposure with E_2 exposure on isolated cultured GAM complexes during the TSP of sex determination at MPT.

2. Materials and methods

2.1. Animals

Alligator eggs were collected from ten nests at Lake Woodruff National Wildlife Refuge, Florida in June 2010 and 2015 under scientific collecting permits (#SPGS10-44 and #SPGS13-08) issued by the Florida Fish and Wildlife Conservation Commission. Eggs were transported in their original nest material to Hollings Marine Laboratory (Charleston, SC, USA) where they were incubated in damp sphagnum moss at a pivotal temperature (PVT, 32.5 °C). Each egg was weighed and candled to assess egg fertility. One or two fertilized eggs from each clutch were opened to determine developmental stage of the embryos based on morphological criteria (Ferguson, 1985). Eggs were then maintained in damp sphagnum moss at female-producing temperature (FPT, 30 °C) or MPT (33.5 °C) of sex determination until dissections. Bins were misted daily, and temperatures were monitored continuously using Onset HOBO TidbiT data loggers and software (Onset, Bourne, MA). Timing of exposure at developmental stage was estimated by using a published equation (Kohno and Guillette, 2013). One group of eggs was incubated at FPT until embryonic stage 19, at which point eggs were split into two incubating temperatures, MPT and FPT, and sampled over the course of 36 days as previously described (Yatsu et al., 2016).

All embryonic exposure and animal handling procedures conformed to the guidelines set forth by the Institutional Animal Care and Use Committee at the Medical University of South Carolina and were conducted in compliance with an approved animal protocol (AR#3069).

2.2. CWAF preparation

A modified CWAF appropriate for use in cell-based assays and organ culture was prepared by combining phenol red-free Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Gibco™, ThermoFisher Scientific, Waltham, MA), Mississippi Canyon Block 252 (MC252) crude oil and COREXIT® 9500 (Nalco, Wallisellen, Switzerland) in a 200:20:1 ratio by volume. The mixture was stirred overnight and allowed to separate by gravity for an additional 12 h, and then the aqueous layer was collected as CWAF (Temkin et al., 2015).

2.3. In vitro transactivation assays

In order to characterize *A. mississippiensis* estrogen receptor activation by E_2 (Sigma-Aldrich, St. Louis, MO) and CWAF, *in vitro* transactivation assays were performed closely following the method detailed in Katsu et al. (2010). Human embryonic kidney 293T (HEK293T, ATCC, Manassas, VA) cells were cultured in phenol-red free Dulbecco's Modified Eagle Medium (Sigma-Aldrich) with charcoal/dextran-treated 10% fetal bovine serum

(Hyclone, GE Healthcare Life Sciences, Pittsburgh, PA) and 1x penicillin-streptomycin antibiotic mixture (Gibco™ ThermoFisher Scientific) under sterile conditions at 37 °C and 5% CO_2. HEK 293T cells were seeded in 96-well plates at 1×10^4 cells/well in the above-described medium without antibiotic. Twenty-four h after seeding, cells were transfected with plasmids containing cloned *A. mississippiensis* ESR1 or ESR2 cDNA in pcDNA 3.1 (Invitrogen, Carlsbad, CA) (19 ng/well), the reporter construct of the firefly luciferase gene containing 4× estrogen response element (ERE) in pGL4 (Promega, Madison, WI) (38 ng/well), and 10 ng/well pRL-TK (Promega) containing the *Renilla reniformis* luciferase gene which is used as an internal control to normalize variation in transfection efficiency, using the Fugene HD transfection reagent (0.17 μL/well; Promega). Four h after transfection, cells were exposed to either E_2 in concentrations of 10^{-14} M to 10^{-8} M, 0.1% DMSO (vehicle control for E_2 treatments), CWAF (at 1:50, 1:500, and 1:5000 dilution levels), or medium alone (for CWAF negative control). Forty-four h after exposure to the test compounds, luciferase luminescence signal was detected using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol on a MicroBeta2 Plate Counter (Perkin-Elmer, Waltham, MA). All transfections were performed in triplicate, and each experiment was repeated at least three times.

2.4. Dissection and organ culture

Eggs were dissected one day prior to the predicted beginning of the thermosensitive period (TSP) at stage 21 using the equations detailed previously (Kohno and Guillette, 2013). Due to the structural difficulty of the alligator gonad, it was impossible to culture and analyze isolated gonads at that stage; therefore, GAM complexes were dissected from *A. mississippiensis* embryos (Kohno and Guillette, 2013). For organ culture experiments, one GAM from each embryo was cultured for 8 days while the other GAM was cultured for 16 days under the same treatment. The isolated GAMs were immediately placed onto 0.45 μm Omnipore™ membrane filters (Millipore Sigma, Billerica, MA) after a rinse with phosphate-buffered saline (PBS), and filters were floated in 24-well plates containing 500 μL of the appropriate experimental medium for culture under sterile conditions. The basic medium for all treatment groups was prepared as a modified cell culture medium containing phenol red-free Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Gibco®, ThermoFisher Scientific) with 10% charcoal/dextran-treated fetal bovine serum (Hyclone), 0.5% Peni cillin/Streptomycin/Fungizone Antibiotic-Antimycotic (Gibco®, ThermoFisher Scientific), 1% Glutamax (Gibco®, ThermoFisher Scientific), 1% NEEA (MEM Non-Essential Amino Acids Solution; Gibco®, ThermoFisher Scientific), and 0.1 mM β-Mercaptoethanol (Fisher Scientific). Tissue culture experiments consisted of six different treatment groups: a control group containing the medium described above, a vehicle control group for estradiol treatments containing the basal medium and 0.1% DMSO, three different concentrations of E_2 (10^{-10}, 10^{-8}, and 10^{-6} M) dissolved in 0.1% DMSO and medium, and a 1:50 CWAF dilution prepared with medium. GAMs were incubated at 33.5 °C (MPT) and cultured for either 8 or 16 days, corresponding to the middle and end of the TSP *in ovo*, respectively. Media changes were performed every 48 h during culture. At the end of the culture period, GAMs were collected and placed into *RNAlater*, shaken overnight at 4 °C, and then stored at −30 °C until subsequent RNA extractions.

2.5. In ovo dissections

In addition to organ culture samples, GAMs were also dissected from embryos from each experimental clutch and immediately placed into *RNAlater* for evaluation of gene expression levels at the start of organ culture one day prior to the predicted beginning of the TSP at stage 21. Additionally, GAMs were dissected from embryos from *in ovo* control groups incubated at both 30 °C (FPT) and 33.5 °C (MPT) at the equivalent developmental time points (stages 23 and 24, corresponding to halfway through and at the end of the TSP) to serve as positive control references for mRNA expression. Dissection timing for the FPT *in ovo* group was conducted 10 and 20 days after stage 21, which corresponds to developmental stages 23 and 24 at FPT.

One group of eggs was dissected at 0, 3, 6, 12, 18, 24, 30, and 36 days after developmental stage 19 incubated at FPT or MPT (Yatsu et al., 2015, 2016).

2.6. RNA extractions, cDNA synthesis, and qPCR

Total RNA was isolated using a modified acid guanidinium thiocyanate-phenol-chloroform (AGPC) extraction method (Sambrook et al., 2006) followed by spin-column purification steps (Epoch Life Science, Missouri City, TX) with DNase-I treatment (5 Prime, Gaithersburg, MD). Total RNA quantity was verified by optical density measurements using NanoDrop 1000 (ThermoFisher Scientific, Wilmington, DE), and quality was checked using denaturing agarose gel electrophoresis (Sambrook et al., 2006). Total RNA (0.5 μg) was reverse-transcribed into cDNA using the Applied Biosystems High Capacity cDNA Reverse Transcription kit in a 20 μL reaction (ThermoFisher Scientific, Waltham, MA). Quantitative real-time PCR (qPCR) was conducted using the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) with a homemade SYBR Green reaction mix containing 20 mM Tris-HCl (pH 7.75), 50 mM KCl, 3 mM $MgCl_2$, 0.5% Glycerol, 0.5% Tween-20, 0.5x SYBR Green-I (Invitrogen), 0.2 mM dNTP mix, and 0.01 U/μL Ampli Taq Gold (Applied Biosystems, Foster City, CA) with 0.2 μM each primer. Each reaction was performed with an initial heat denaturing period at 95 °C for 5 min, 35–45 cycles of amplification with denaturing at 95 °C for 15 s and annealing/extension at the appropriate temperature for 1 min, followed by a final 10 s at 95 °C and subsequent melting curve from 65 °C to 95 °C to verify product specificity. Primer sequences and annealing temperatures for each primer pair are given in Supplemental Table S1. Absolute quantification of gene expression was performed using standard curves obtained from standard samples containing known concentrations of the target plasmid for each gene (Kohno et al., 2003). Expression levels were normalized to a normalization factor equal to the geometric mean of beta-actin (*ACTB*) and eukaryotic elongation factor 1 alpha 1 (*EEF1A1*) gene expression levels, which was identified as the best stability value combination between three housekeeping genes, *ACTB*, *EEF1A1*, and ribosomal protein L8 (*RPL8*), across all experimental groups using the NormFinder software program (Andersen et al., 2004) (Supplemental Table S2).

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software Inc., La Jolla, CA). Dose response curves for *in vitro* transactivation assay data were estimated using the four-parameter logistic curve. Gonadal sex from mRNA expression data was determined by the ratio of gonadal aromatase (*CYP19A1*) and anti-Müllerian hormone (*AMH*) mRNA abundances, where *CYP19A1* > *AMH* = female and *CYP19A1* < *AMH* = male (Kohno et al., 2015; McCoy et al., 2015). Sex ratios were analyzed using one-sided Fisher's exact tests to compare the effects of medium vs. CWAF treatment and Chi Square tests followed by one-sided Fisher's exact tests with Bonferroni corrections to compare the effects of 0.1% DMSO vs. E_2 treatments. Abundance of mRNA levels for *CYP19A1* and *AMH* as well as forkhead box protein L2 (*FOXL2*), proliferating cell nuclear antigen (*PCNA*), sex determining

region Y-box 9 (*SOX9*), and steroidogenic factor 1 (*SF1*) were analyzed using two-way ANOVAs with treatment and time as factors for *in vitro* samples and temperature and time as factors for *in ovo* samples with Sidak and Dunnett multiple comparison post-hoc tests comparisons as appropriate. All expression data were checked for normality and transformed as appropriate prior to statistical tests.

3. Results

3.1. In vitro transactivation assays

Exposure to CWAF at 1:50 and 1:500 dilutions significantly stimulated transcriptional activations of *A. mississippiensis* estrogen receptors ESR1 and ESR2 *in vitro* compared to controls ($p < .0001$ for both receptors, Supplemental Fig. S1). The relative induction of CWAF exposure was higher on ESR2 than on ESR1, whereas the inductions in the controls were not significantly different between the two ESRs (Supplemental Fig. S1). When activation levels of CWAF were interpolated onto a dose-response curve for the endogenous ligand E_2, CWAF was shown to reach approximately 30% and 50% of the maximum induction by E_2 on *A. mississippiensis* ESR1 and ESR2, respectively (Fig. 1 and Supplemental Fig. S1). CWAF at 1:50 dilution was equivalent to 0.7×10^{-11} M and 1.0×10^{-11} M E_2 via alligator ESR1 and ESR2, respectively (Fig. 1).

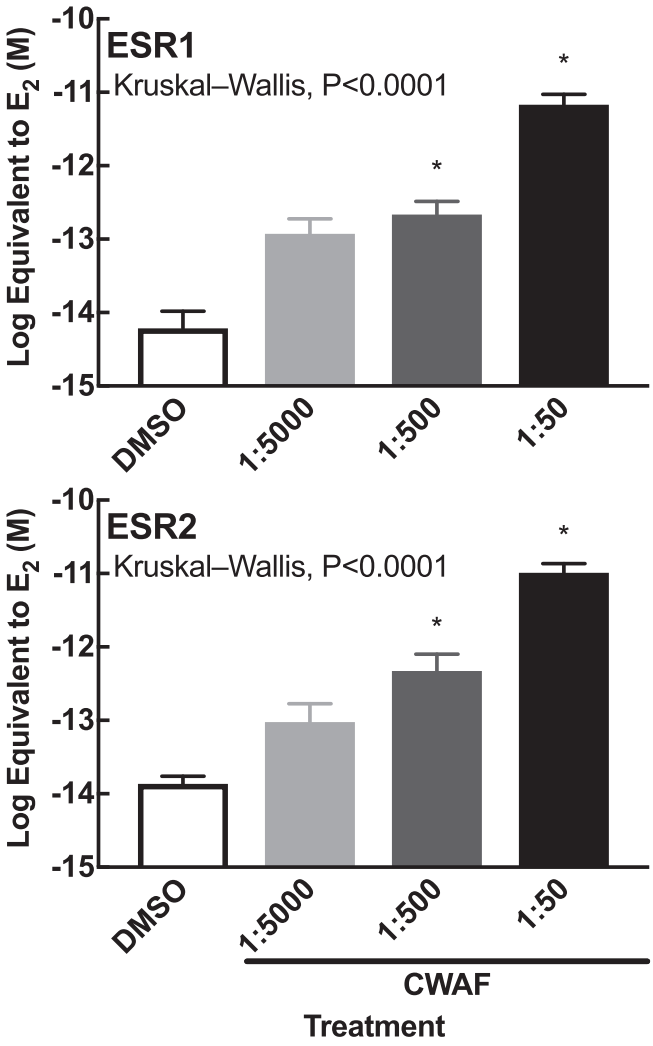

Fig. 1. Estrogen dose equivalent values of CWAF at 1:50, 1:500, and 1:5000 dilutions on alligator ESR1 and ESR2 in the transactivation assays. Data are normalized to the vehicle control (0.1% DMSO for E_2, basal media for CWAF) and maximum induction by E_2 for each assay and shown as mean ± SEM. Each assay was repeated three times in triplicate.

3.2. Abundances of ESRs mRNA during TSD

Both *ESR1* and *ESR2* mRNA were expressed in gonadal RNA with *ESR1* being higher than *ESR2* in general (Fig. 2). *ESR1* mRNA abundances peaked at stage 23 of FPT, whereas *ESR2* mRNA abundances peaked at later stages 26-27 of FPT (Fig. 2). Number of days after stage 19 affected *ESR1* mRNA abundances, while egg incubation temperature affected *ESR2* mRNA abundances with an interaction effect of number of days and temperature (Fig. 2).

3.3. Sex ratios

3.3.1. In ovo

Using a *CYP19A1/AMH* ratio of >1 = female and <1 = male as a criterion for establishing gonadal sex, 100% females were seen by stage 24 at FPT *in ovo*. Prior to this developmental time point, *CYP19A1/AMH* ratios for all samples were <1 regardless of incubation temperature (Supplemental Fig. S2).

3.3.2. In vitro

E_2 exposure *in vitro* for 8 and 16 days significantly altered the sex ratio ($p = .0244$ and .0046, respectively, Chi-square tests), with a significantly higher number of samples displaying a female-like pattern following exposure to 10^{-6} M E_2 relative to the DMSO control after 8 days of culture ($p = .0083$, one-sided Fisher's exact test) and following exposure to both 10^{-6} and 10^{-8} M E_2 relative to the control after 16 days of culture ($p = .0092$ for both concentrations, one-sided Fisher's exact tests, Fig. 3). After 16 days of exposure, CWAF also significantly affected the sex ratio with a higher number of samples displaying a female-like pattern ($p = .0164$, one-sided Fisher's exact test), but the change in sex ratio was not significant after 8 days of exposure (Fig. 3).

3.4. Gene expression in GAM

3.4.1. In ovo

Both developmental stage and egg incubation temperature significantly affected mRNA abundances of anti-Müllerian hormone (*AMH*), gonadal aromatase (*CYP19A1*), forkhead box protein L2 (*FOXL2*), proliferating cell nuclear antigen (*PCNA*), and sex determining region Y-box 9 (*SOX9*) in GAMs ($p < .05$, 2-way ANOVA), although only effect of stage on mRNA abundance of *SF1* was significant in GAMs ($p < .001$, 2-way ANOVA) (Fig. 4). Additionally, there were significant interactive effects between temperature and stage for *AMH, CYP19A1, FOXL2, and PCNA* mRNA abundances (Fig. 4). Abundances of *AMH* and *SOX9* mRNA showed a sexually dimorphic pattern (male > female) at stage 24, with increased *AMH* mRNA abundance under MPT at stages 23 and 24 and decreased *SOX9* mRNA abundance under FPT at stage 23 as compared with that in initial MPT stage 20 (Fig. 4). In contrast, abundances of *FOXL2* and *CYP19A1* mRNA showed sexual dimorphism and were increased at FPT by stage 24 (Fig. 4). Abundances of *FOXL2* mRNA in GAMs at stage 23 and 24 were significantly increased at FPT as compared with that at initial MPT stage 20 expression, with a sexually dimorphic pattern at stage 24 (Fig. 4). *CYP19A1* mRNA abundance in GAMs showed an interesting initial decrease from stage 20 at MPT to stage 23 at FPT, but then increased sharply at FPT and showed sexual dimorphism at stage 24 (Fig. 4). *PCNA* mRNA abundance in GAMS showed a sexually dimporphic pattern at both stages 23 and 24 with mRNA abundances at MPT at both stages significantly decreased from initial

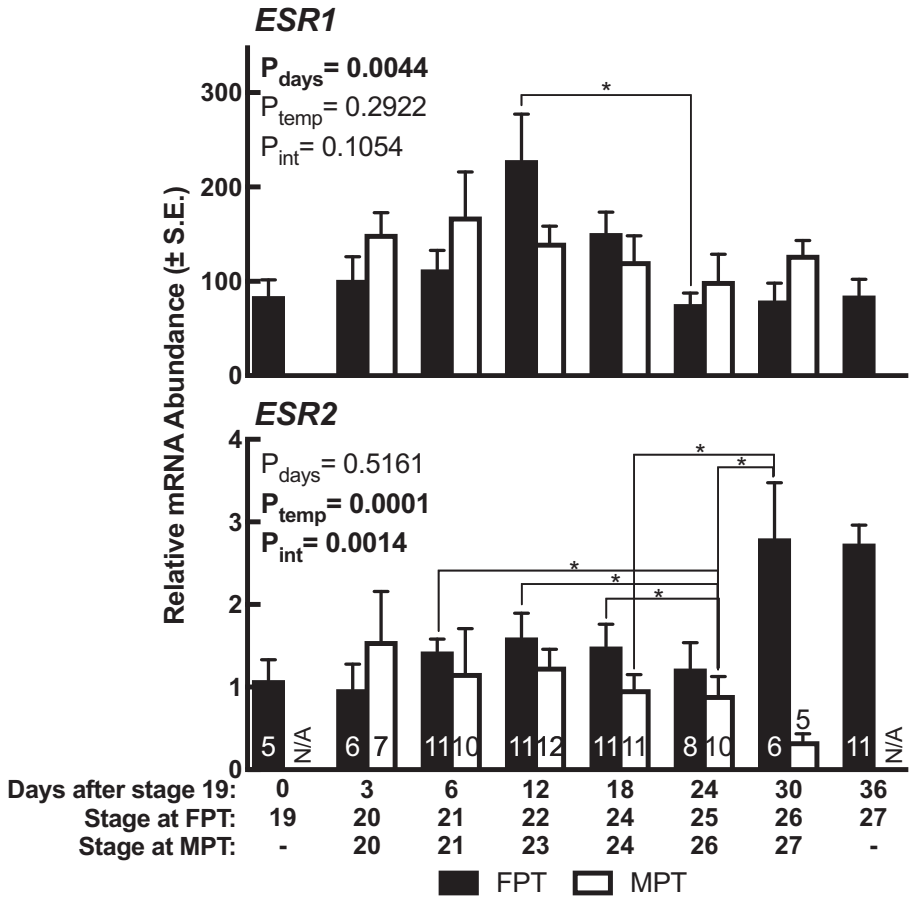

Fig. 2. Abundances of *ESR1* and *ESR2* mRNA in gonad at stage 19 and later *in ovo* incubated at female- or male-producing temperature (FPT or MPT). Asterisks (*) indicate a significant difference between samples. Data presented are mean ± SEM.

stage 20 MPT abundance (Fig. 4). Abundances of *SF1* mRNA in GAMs significantly increased at both MPT and FPT at stage 24 as compared with that in stage 20 at MPT without sexual dimorphism (Fig. 4).

3.4.2. In vitro, E_2 exposure

AMH and *FOXL2* mRNA abundances in cultured GAMs were significantly affected by E_2 exposure ($p < .0001$ and $p = .0375$, respectively) following two-way ANOVAs with treatment and time as factors and Dunnett and Sidak multiple comparison post hoc tests (Fig. 5). E_2 exposure at 10^{-6} M for both 8 and 16 days and at 10^{-8} M for 16 days significantly suppressed mRNA abundances of *AMH* in cultured GAMs, while post-hoc comparisons for *FOXL2* did not provide significant differences in pairwise comparisons (Fig. 5). Duration of culture significantly decreased *AMH* and *SOX9* mRNA abundances in GAMs ($p = .0012$ and $p < .0001$) (Fig. 5). Both *PCNA* and *SF1* mRNA abundances showed no significant differences (Supplemental Fig. S3). There were no significant interactive effects of treatment and time for any of the genes examined.

3.4.3. In vitro, CWAF exposure

CWAF exposure for both 8 and 16 days reduced mRNA abundances of *AMH*, *SOX9*, and *CYP19A1* in GAMs as compared to that in controls by two-way ANOVAs with treatment and time as factors and Sidak multiple comparison post hoc tests ($p < .0001$ for all, Fig. 5). Duration of culture significantly affected *CYP19A1* and *SOX9* mRNA abundances ($p = .0175$ and .0124, respectively), with increased *CYP19A1* and decreased *SOX9* mRNA abundances from 8 to 16 days of culture (Fig. 5). Both *PCNA* and *SF1* mRNA abundances showed no significant differences (Supplemental Fig. S3). There were no significant interactive effects of treatment and time for any of the genes examined.

4. Discussion

CWAF exposure at 1:50 dilution activated ESR1 and ESR2 by approximately 30% and 50% of maximum induction respectively, equivalent to E_2 at 10^{-11} M range. Although CWAF exposure at 1:50 might be not an environmentally relevant concentration, the present study revealed a feminizing effect of CWAF in the organ culture experiments. Thus, further work examining the endocrine disrupting potential of CWAF is merited, and was the basis for design of the *in vitro* organ culture exposure experiment in the present study.

The ratio of the sexually dimorphic gonadal mRNA abundances of *CYP19A1* and *AMH* has been used as a criterion to assess sex in embryonic alligators following the TSP (Kohno et al., 2015). However, use of this ratio as a diagnostic tool to establish gonadal sex inherently relies on the sexually dimorphic pattern of these two genes, which is not established until after the sex determination process is initiated. Our results revealed that *in ovo*, the ratio of *CYP19A1/AMH* in GAMs was less than 1 regardless of temperature until an increase of *CYP19A1* at FPT at stage 24.

In ovo, the increased *CYP19A1/AMH* ratio at FPT by stage 24 occurs due to higher *CYP19A1* and lower *AMH* mRNA abundances in GAM complexes at this developmental stage. However, the increase in this ratio in GAMs in *in vitro* organ culture with E_2 or CWAF exposure was largely due to decreased *AMH* rather than

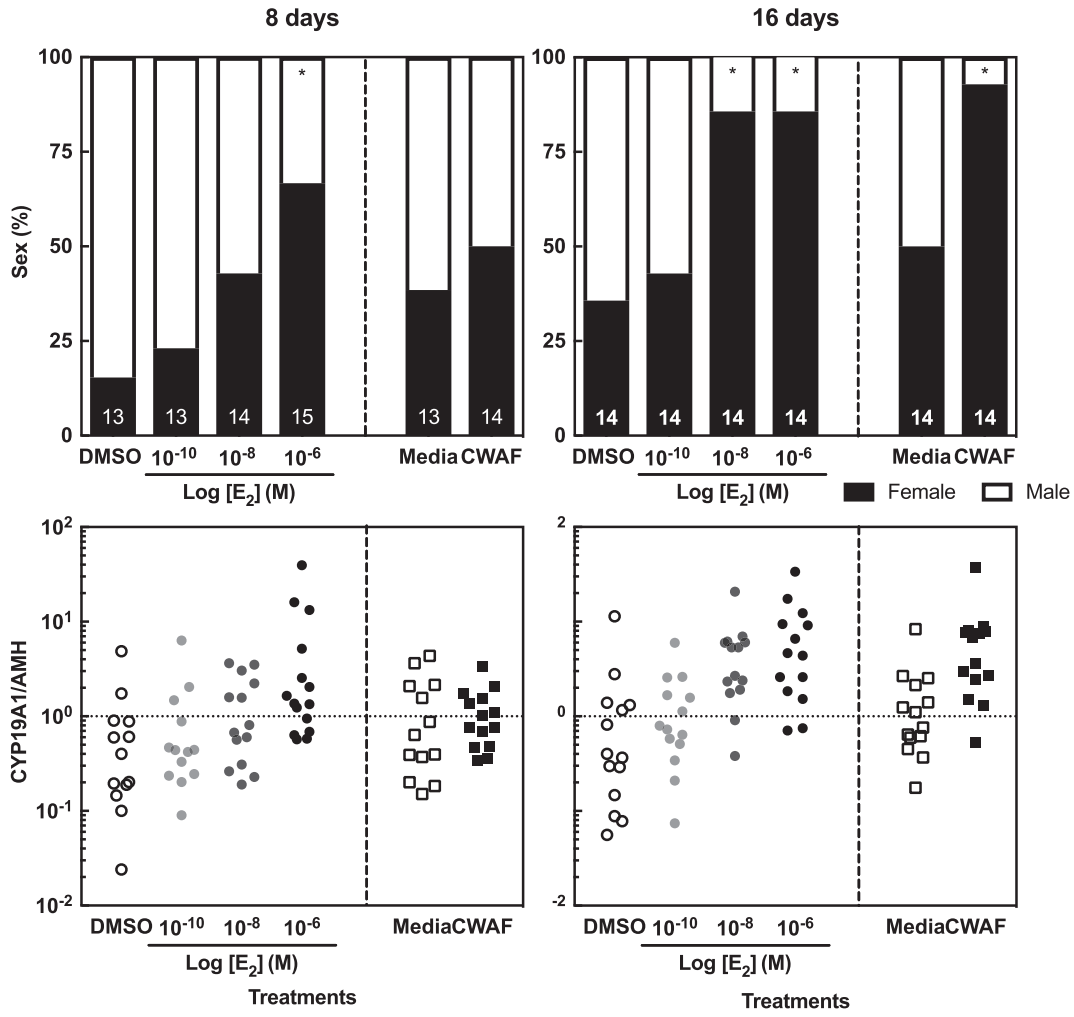

Fig. 3. Sex ratios based on mRNA abundance ratios for E_2 or CWAF-exposed GAMs at days 8 and 16 of culture, corresponding to developmental stages 23 and 24 *in ovo* at MPT. GAMs were cultured at a male-producing temperature (MPT, 33.5 °C) and exposed to either 10^{-10}, 10^{-8}, or 10^{-6} M 17β-estradiol (E_2) or 0.1% DMSO vehicle control for E_2 treatments or a 1:50 CWAF dilution or media alone for CWAF treatments. Gonadal sex was discriminated by the sexually dimorphic pattern of gonadal aromatase (*CYP19A1*) and anti-Müllerian hormone (*AMH*) mRNA abundances, with the male pattern defined by lower abundance of *CYP19A1* mRNA and higher abundance of *AMH* (*CYP19A1/AMH* <1 = male) and the female pattern defined by higher *CYP19A1* and lower *AMH* mRNA abundances (*CYP19A1/AMH* ratio >1 = female). The logarithmic y-axis for *CYP19A1/AMH* ratio data shows fold differences, and asterisks indicate a significant difference in sex ratios by Fisher's exact tests for comparisons to the control with Bonferroni corrections for multiple comparisons at each stage ($p < .0167$). Numbers in bars indicate sample size per treatment.

increased *CYP19A1*, as mRNA abundances of *CYP19A1* in GAM complexes were not significantly affected by E_2 exposure and were decreased following CWAF exposure. Nevertheless, *AMH* suppression in GAMs following E_2 and CWAF exposure during the TSP did result in significant changes in sex ratios from control groups in the present study. These results show that it is *AMH* mRNA abundances and not *AMH/CYP19A1* ratios that are important for distinguishing male vs. female development in GAMs *in vitro*.

While the gene network involved in the sex determination process is quite conserved across vertebrates, the timing, function, and relationships among the genes involved are often inconsistent. Results revealed important differences in the timing of increased expression between *in ovo* and *in vitro* cultured GAMs, with several genes failing to show the increases in transcriptome abundance *in vitro* that were observed *in ovo*, as well as differences in response patterns following E_2 and CWAF exposure.

Sex determining region Y-box 9 (*SOX9*) and anti-Müllerian hormone (*AMH*) are known early players in sex determination and differentiation in both mammals and TSD organisms (Shoemaker and Crews, 2009). *AMH* is a transforming growth factor secreted by Sertoli cells and is involved in the regression of the Müllerian duct during the sex determination process (Rey et al., 2003), while *SOX9* is a transcription factor involved in testis development during sex determination (Koopman, 1999). In mammals, *SOX9* is required for the initiation of *AMH* transcription (Nef and Parada, 2000); however, previous work in the American alligator revealed that *AMH* expression preceded that of *SOX9* during testis differentiation in this species, indicating a lack of dependency on *SOX9* for initiation of *AMH* transcription and potentially other differences in the role and regulation of this gene in crocodilians (Urushitani et al., 2011; Western et al., 1999). Further details as to the interactive and/or regulatory relationship between these two genes across TSD organisms remains unclear, as other work has documented preferential organization of *SOX9* expression in the testis of the red-eared slider turtle *T. scripta* occurring early in the TSP before sexually dimorphic *AMH* expression (Shoemaker et al., 2007). However, in terms of transcript abundance, results of the present *in ovo* study support previous findings documenting an increase of *AMH* before *SOX9* in GAM of *A. mississippiensis*.

In vitro, both genes failed to show the increase observed *in ovo* within the time frame used for GAM culture. Previous work on cultured gonads in *T. scripta* found that *in vitro* molecular interactions

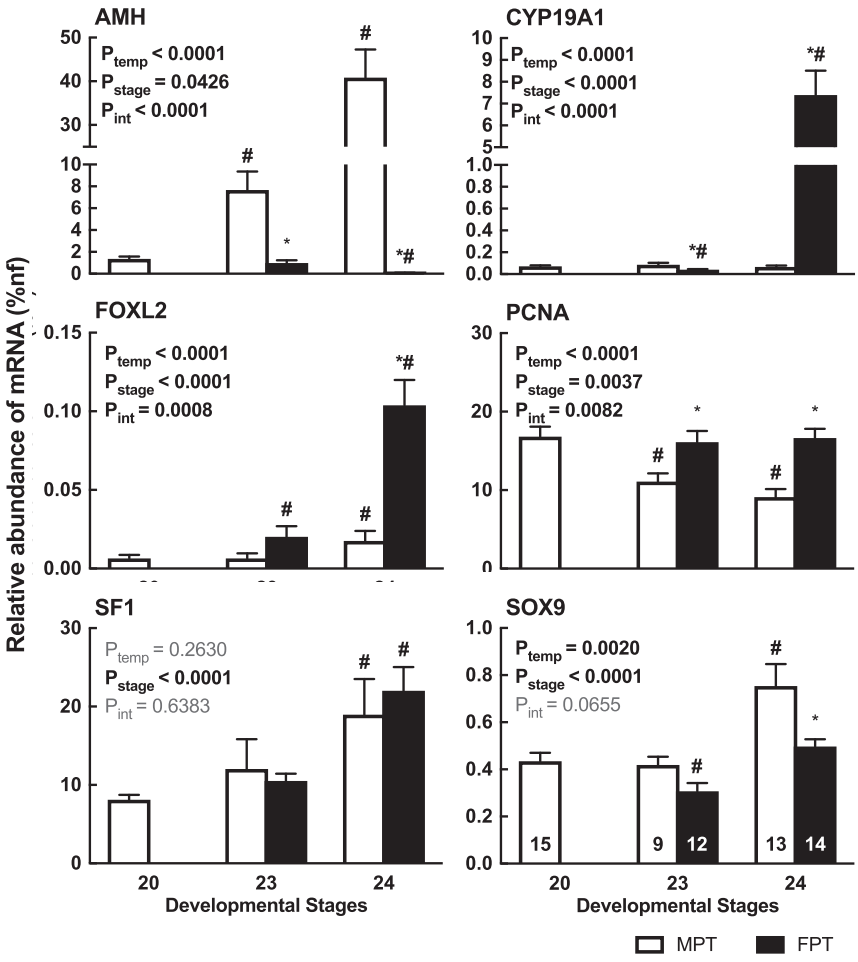

Fig. 4. Abundances of *AMH*, *CYP19A1*, *FOXL2*, *PCNA*, *SF1* and *SOX9* mRNA in GAMs developed *in ovo*. Asterisks (*) indicate a significant difference at FPT compared to MPT at the same developmental stage, while hash marks (#) indicate a significant difference due to stage at either temperature as compared to initial stage 20 MPT. Data presented are mean ± SEM.

proceeded normally for at least 8 and up to 20 days depending on the gene examined; however, developmental cellular interactions were affected by isolation from neighboring tissues (Shoemaker-Daly et al., 2010). While whole GAMs were cultured in this study, it is still possible that separation from the developing embryo could impede the progression of development, particularly through impairment of neuroendocrine feedback processes. Future work to identify circulating factors required for GAM differentiation *in vitro* should be performed in order to more closely match *in ovo* developmental conditions. These genes are still useful markers for estrogenic and/or contaminant-based exposure, however, as E_2 exposure reduced *AMH* mRNA abundances in GAMs and CWAF exposure significantly suppressed mRNA abundances of both *AMH* and *SOX9* in GAMs. The lack of effect of E_2 exposure on *SOX9* transcript abundance in GAMs *in vitro* is surprising, as gonadal expression of this gene is decreased following E_2 exposure *in ovo* at MPT during TSD in *T. scripta* (Barske and Capel, 2010; Matsumoto et al., 2013), and previous research has demonstrated that *SOX9* is endogenously regulated by temperature in cultured *T. scripta* gonads (Shoemaker-Daly et al., 2010). However, *SOX9* expression is not restricted to the gonad, and it is possible that E_2-mediated changes in gonadal expression may have been masked by expression in adrenal and mesonephric tissue (Ramsey and Crews, 2007). *AMH* transcript abundance in GAMs was significantly reduced following E_2 exposure, which is consistent with previous work documenting *AMH* repression in fish species following exposure to 17α-ethinylestradiol (EE_2) both during development in zebrafish (Schulz et al., 2007) as well as during estrogen-mediated (EE_2) sex reversal in rainbow trout (Vizziano-Cantonnet et al., 2008). The reduced transcript abundance of both *SOX9* and *AMH*, which are critically involved in the male sex determination and differentiation pathways, following CWAF exposure in GAMs indicates potential for feminization and consequences for sex ratios and reproductive capabilities in developmentally exposed wildlife populations, although further research would need to be conducted to determine the concentrations and duration of exposure necessary to induce this effect in order to assess environmental relevance.

In ovo, both *FOXL2* and *CYP19A1* displayed a significant increase at FPT by the end of the TSP at developmental stage 24 in GAMs, with increased *FOXL2* mRNA abundance relative to stage 20 MPT levels occurring by stage 23. *CYP19A1* is an enzyme involved in the conversion of androgens to estrogens and is critically involved in ovarian development at FPT in organisms with TSD (Pieau et al., 1999), while *FOXL2* is a transcription factor involved in early ovarian differentiation (Pannetier et al., 2006). *FOXL2* is a direct transcriptional activator of the *CYP19* gene acting through an ovarian-specific promoter region in developing goat ovaries (Pannetier et al., 2006), and expression studies have revealed spatio-temporal co-localization of the two gene products in developing chicken and rainbow trout ovaries (Baron et al., 2004; Govoroun et al., 2004). Our results indicate that a *FOXL2* increase precedes that of *CYP19A1* at FPT in alligator GAMs, which is consistent with the potential for transcriptional activation.

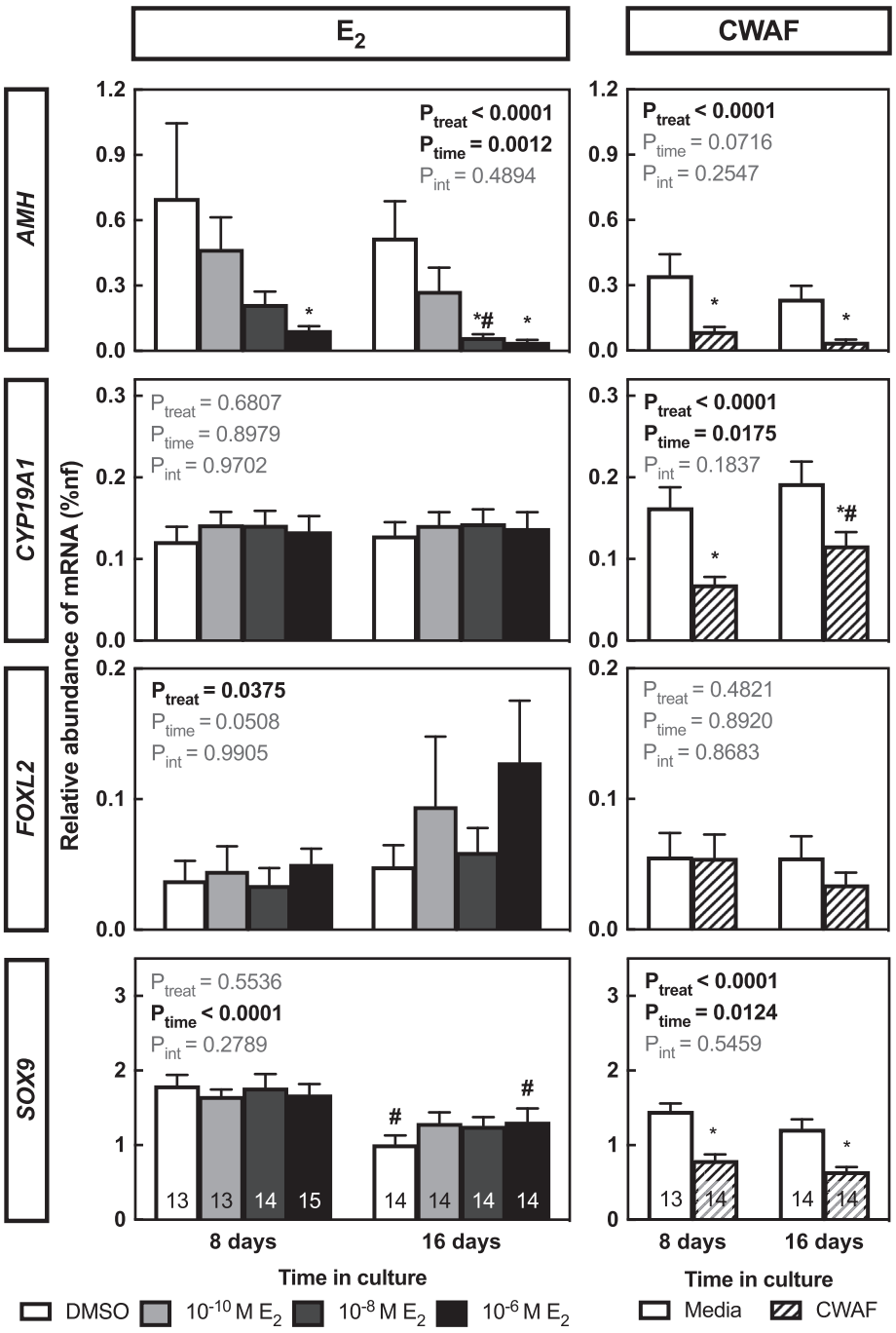

Fig. 5. Abundances of *AMH*, *CYP19A1*, *FOXL2* and *SOX9* mRNA in cultured GAMs with E_2 or CWAF exposure *in vitro*. Asterisks (*) indicate a significant difference due to treatment at the same developmental time point, while hash marks (#) indicate a significant difference due to time within the same treatment group. Data presented are mean ± SEM.

In rainbow trout, gonadal *FOXL2* expression is modulated by estrogen exposure, with a rapid increase of *FOXL2a* occurring following estrogen (EE_2) treatment in genetic males, leading to a proposed short positive feedback loop between this gene and estrogen in this species (Baron et al., 2004; Vizziano-Cantonnet et al., 2008). In contrast, *CYP19A1* expression was not affected by estrogen exposure in this species or in the European sea bass (Navarro-Martin et al., 2009; Vizziano-Cantonnet et al., 2008), suggesting that estrogen-induced sex reversal is not mediated through the action of this gene. The significant effect of E_2 exposure on *FOXL2* but not *CYP19A1* expression in *in vitro* cultured GAMs in the current study is consistent with these previously published findings.

In contrast to E_2 exposure, CWAF exposure in *in vitro* cultured GAMs displayed a repressive effect on *FOXL2* and *CYP19A1* mRNA abundances, although this observed result was only significant for *CYP19A1*. Studies examining the consequences of EDC exposure on the expression of *FOXL2* have been limited, although one study found significantly increased *FOXL2* gene expression following BPA exposure in the rare minnow, *Gobiocypris rarus* (Wang et al., 2012). In contrast, the effects of EDC exposure on *CYP19A1* expression have been more extensively studied. In the American alligator, gonadal *CYP19A1* expression levels were documented to be lower in juvenile alligators from a contaminated lake (Lake Apopka, FL) than from a reference site (Lake Woodruff, FL) (Kohno et al.,

2008), and in zebrafish, concentration-specific decreases in brain *CYP19A1a* mRNA abundances were documented following exposure to water-soluble fractions (WSF) of crude oil (Arukwe et al., 2008). The reduced *CYP19A1* mRNA abundances following CWAF exposure in isolated GAMs in the current study are consistent with these previously published results and indicate potential implications for endogenous estrogen synthesis in exposed organisms.

5. Conclusions

While gonadal *AMH/CYP19A1* ratios are a good indicator of sex determination in the American alligator *in ovo*, *AMH* alone is a potentially good indicator of sex determination in cultured GAMs *in vitro*. However, cellular features of male/female differentiation should be examined to determine if this process is occurring *in vitro* during the time frame used in the present study. The significant effects on sex ratios and the alterations in mRNA abundances following E_2 and CWAF exposures suggest that this organ culture approach might be a valuable method to assess effects of estrogenic exposures on sex determination during the TSP. Additionally, the observed estrogen receptor activation, effect on sex ratios, and significant decreases in mRNA abundances of several key genes involved in vertebrate sex determination and differentiation after CWAF exposure indicate that exposure to substance(s) in CWAF may impact development and reproduction, and identification of the active components in this complex mixture would provide insight into more specific mechanistic details of observed endocrine-disrupting activity. The different response patterns to E_2 versus CWAF in cultured GAMs suggest that while this substance activates *A. mississippiensis* estrogen receptors in *in vitro* transactivation assays, physiological responses are likely not solely estrogenically mediated. Future work examining the consequences of exposure in other species under environmentally relevant conditions is merited, and would lead to more informed decisions regarding dispersant usage as a response effort in oil spill disasters.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.ygcen.2017.11.019.

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