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RESEARCH ARTICLE

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Oil dispersant Corexit 9500 is weakly estrogenic, but does not skew the sex ratio in *Alligator mississippiensis*

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Abstract

During the Deepwater Horizon oil spill, vast quantities of a chemical dispersant Corexit 9500 were applied in remediation efforts. In addition to the acute toxicity, it is essential to evaluate Corexit further with a broader scope of long-term sublethal endocrine endpoints. The American alligator (*Alligator mississippiensis*) is an excellent organism for such an endeavor. It exhibits temperature-dependent sex determination, in which egg incubation temperatures during a thermosensitive period (TSP) in embryonic development determine the sex of embryos. Estrogen signals play a critical role in this process. For example, a single exposure to exogenous estrogen during the TSP overrides the effects of temperature and leads to skewed sex ratios. At a concentration of 100 ppm, Corexit significantly induced transcriptional activity of both alligator nuclear estrogen receptors 1 and 2 in vitro in reporter gene assays. To investigate the estrogenic effects of Corexit on gonadal development, alligator eggs were exposed to Corexit at environmentally relevant concentrations (0.25, 2.5 and 25 ppm) before the TSP in ovo. Exposure to Corexit at 0.25 and 25 ppm significantly delayed hatching and growth. Corexit exposure at any treatment level did not affect sex ratios or testicular mRNA abundance as measured at 1-week post-hatching, suggesting that the combination of Corexit components did not synergize enough to induce ovarian development in ovo. These results point to a need for further investigations on individual and combined components of Corexit to understand better their long-term effects on the development and reproductive health of alligators and other coastal aquatic wildlife.

KEYWORDS

American alligator, Corexit 9500, endocrine disruption, estrogen receptor, oil dispersant, sex determination

1 | INTRODUCTION

1.1 | Dispersant usage in the Deepwater Horizon oil spill

The Deepwater Horizon oil spill, one of the world's most massive marine oil spills in history, resulted in the release of approximately 4.9 million barrels (205.8 million gallons) of Mississippi Canyon block 252 (MC252) crude oil into the Gulf of Mexico between April 20 and July 15, 2010 (Griffiths, 2012; McNutt et al., 2012; Ramseur, 2010). A chemical dispersant was approved for use in reducing the impacts of oil reaching shorelines. An estimated 2.1 million gallons of dispersant, primarily Corexit EC9500A (hereafter called Corexit), were applied to the sea surface and at the wellhead oil source (Kujawinski et al., 2011). The unprecedented extensive use of Corexit during the Deepwater Horizon oil spill has prompted research into its impacts on marine and coastal ecosystems. Corexit is a complex mixture of surfactants and hydrocarbon-based solvents, which decreases the interfacial tension between oil and water and thereby enhances the distribution of oil into smaller droplets. This effect reduces the deposition of oil on shoreline habitats and presumably aids in the biodegradation of oil in the water column, although it is toxic to oil-eating bacteria (Azwell et al., 2011; Kujawinski et al., 2011). However, there are also downsides and risks of applying Corexit in the environment.

1.2 | Endocrine activity of Corexit 9500

Not only can Corexit induce toxicity in aquatic organisms, but the chemical dispersion of crude oil also makes polycyclic aromatic hydrocarbons and other hydrophobic oil components more bioavailable and dispersed within the water column (Hemmer, Barron, & Greene, 2011; Ramachandran, Hodson, Khan, & Lee, 2004). Although there have been multiple studies in a variety of species focusing on the toxicity of Corexit and other dispersants (Almeda, Hyatt, & Buskey, 2014; Hemmer et al., 2011; Toyota, McNabb, Spyropoulos, Iguchi, & Kohno, 2017; Wooten, Finch, & Smith, 2012), studies on sublethal and long-term effects of Corexit have been limited.

Dispersants carry multiple candidates that have been linked to various forms of endocrine disruptors. In vitro cell-based assays were used to analyze endocrine activities in Corexit 9500 and eight other oil dispersants. Estrogenic activity via human estrogen receptor 1 (ESR1) was reported for some dispersants but not identified for Corexit (Judson et al., 2010). Dioctyl sodium sulfosuccinate (DOSS; CAS no. 577-11-7) and Span 80 (CAS no. 1338-43-8), major components of Corexit, have obesogenic activity, which activates the peroxisome proliferator-activated receptor gamma (Temkin et al., 2015) and retinoid X receptor alpha (Bowers, Temkin, Guillette, Baatz, & Spyropoulos, 2016) signaling pathway, respectively. The massive release and potential for the use of Corexit in future oil spills mandate further evaluation of the potential endocrine disruption and long-term effects of Corexit.

1.3 | American alligator as a model of endocrine disruption

As a long-lived top predator with high site fidelity, the American alligator (*Alligator mississippiensis*) is known to experience endocrine disruption in the wild and thus serves as a good sentinel species for studying environmental impacts of potential endocrine disruptors (Kohno & Guillette, 2013; Milnes & Guillette, 2008). Exposure to endocrine-disrupting contaminants (EDCs) can occur either directly or indirectly through food sources or from the mother's yolk deposit as a form of developmental exposure (Milnes, Bryan, Medina, Gunderson, & Guillette, 2005). Alligators are oviparous and exhibit highly synchronous and seasonal reproductive activity. A sexually mature female is capable of producing a single clutch of eggs during a reproductive cycle with oviposition occurring in June or July (Joanen & McNease, 1989; Lance, 1989). This synchronous egg deposition allows for the collection of a large number of eggs and adequate sample sizes for use in exposure experiments in ovo.

All crocodilian species, including alligators, exhibit temperature-dependent sex determination (TSD), in which ambient temperature during a thermosensitive period (TSP) in embryonic development determines the sex of the embryo. In alligators specifically, egg incubation at 30°C (female-producing temperature) during the TSP produces 100% females, whereas egg incubation at 33.0-33.5°C (male-producing temperature, MPT) during the TSP produces 100% males (Ferguson & Joanen, 1983; Lang & Andrews, 1994; McCoy, Parrott, Rainwater, Wilkinson, & Guillette, 2015). The mechanism whereby temperature drives sex determination in alligators is not completely understood. However, developing alligator embryos exhibit high sensitivity to estrogenic EDCs during sex determination and differentiation. Exposure to estrogen during the TSP can override the effects of temperature and induce ovarian development at MPT via ESR1 (Kohno et al., 2015). Several studies have shown ovarian development at MPT and skewed sex ratios in alligators if developing embryos are exposed to an estrogenic EDC during the TSP (Crain, Guillette, Rooney, & Pickford, 1997; Matter, McMurry, Anthony, & Dickerson, 1998). Differences have been observed between estrogen-induced females at MPT and temperature-produced females at female-producing temperature. For example, there are differences in both the brain and the gonadal activities of aromatase (*CYP19A1*), a critical steroidogenic enzyme that converts androgens to estrogens (Milnes, Roberts, & Guillette, 2002). Hence, the American alligator is an excellent model for assessing long-term endocrine effects resulting from exposure to environmental contaminants during embryonic development. In this study, TSD in alligators was used as a bioassay for comparison with in vitro cell-based assays and to examine potential estrogenic and developmental long-term effects of Corexit.

2 | MATERIALS AND METHODS

2.1 | Preparation of chemical reagents and transactivation assay

A transactivation assay (luciferase reporter gene assay with nuclear hormone receptor) was used to determine the interactions of 17β-estradiol (E_2) and Corexit on ESR1 and ESR2 in *A. mississippiensis* according to a previously published method (Katsu et al., 2010). Various dilutions of COREXIT® EC9500A (Nalco Environmental Solutions LLC) were prepared with phenol red-free Dulbecco's modified Eagle medium (DMEM; Sigma-Aldrich). E_2 (Sigma-Aldrich) was diluted in dimethylsulfoxide (DMSO; Sigma-Aldrich) to final concentrations ranging from 10^{-11} to 10^{-5} M. The concentration of DMSO in the culture medium was 0.1%.

Human Embryonic Kidney 293T (HEK 293T; American Type Culture Collection) cells were maintained in phenol red-free DMEM supplemented with 10% HyClone™ charcoal/dextran-treated fetal bovine serum (GE Healthcare Life Sciences) and 1× penicillin-streptomycin antibiotic mixture (Gibco™, Thermo Fisher Scientific) under sterile conditions at 37°C and 5% CO_2. For the transactivation assays, HEK 293T cells were seeded in 96-well plates at 1×10^4 cells per well in the above-described medium without antibiotic. Twenty-four hours after seeding cells, the cells were transfected with plasmids containing cloned *A. mississippiensis* ESR1 or ESR2 in pcDNA3.1 (19 ng/well; Invitrogen), the reporter construct of the firefly luciferase gene containing four estrogen response elements (GGATCnnnAATCG) in pGL4.23 (38 ng/well; Promega), and pRL-TK (10 ng/well; Promega) using Fugene HD transfection reagent (0.17 μL/well; Promega). pRL-TK contained the *Renilla reniformis* luciferase gene with the thymidine kinase promoter of herpes simplex virus and was used as an internal control to normalize variation in transfection efficiency. Four hours after transfection, the cells were exposed to E_2 in concentrations ranging from 10^{-14} to 10^{-8} M, 0.1% DMSO (vehicle control for E_2 treatments), Corexit in concentrations of 1, 10 and 100 ppm, or medium alone (vehicle control for Corexit treatments). The luciferase activity of the cells was measured using the Dual-Luciferase Reporter Assay System (Promega) at 44 hours after exposure to the test compounds on a MicroBeta TriLux Microplate Scintillation and Luminescence Counter (PerkinElmer). Receptor activation of firefly (*Photinus pyralis*) luciferase activity was normalized by the reference luciferase, sea pansy (*R. reniformis*) luciferase activity. Each concentration was tested in triplicate, and each assay was repeated at least three times.

2.2 | Egg collection and processing

Alligator eggs were collected from 10 nests at Lake Woodruff National Wildlife Refuge, Florida on June 24, 2014 under a scientific collecting permit (no. SPGS13-08) issued by the Florida Fish and Wildlife Conservation Commission. Lake Woodruff has relatively low contamination of persistent organic pollutants and has alligators that exhibit healthy reproductive activity. Therefore, this lake has been used as a reference site in previous studies of alligator reproductive biology and development (Guillette et al., 2000; Milnes & Guillette, 2008). Eggs were transported in their original nesting material to Hollings Marine Laboratory, Charleston, SC, USA where they were transferred into damp *Sphagnum* moss and incubated at 32.5°C (intermediate/pivotal temperature of sex determination). Each egg was weighed and candled to assess egg fertility. One or two fertilized eggs from each clutch were dissected to determine the developmental stage of the embryos in each clutch based on morphological criteria defined by Ferguson's staging (Ferguson, 1985, 1987). All fertilized eggs were then maintained in damp *Sphagnum* moss at an intermediate temperature (32.5°C) of sex determination. Timing of exposure at developmental stage 19 was estimated using the following equation: stage = $9.440 \times \log_e$ (days old) − 10.968 (Kohno & Guillette, 2013).

2.3 | Corexit exposure in ovo

All embryonic exposures and animal handling procedures were approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina (AR no. 3069).

Three concentrations of Corexit were tested: 0.25, 2.5 and 25 parts per million (ppm). Corexit was diluted with DMEM, which also served as a vehicle control treatment at 0.5 ppm based on egg weight. E_2 dissolved in 95% ethanol (EtOH) and administered at 0.5 ppm based on egg weight served as a positive control because it can induce 100% production of females at an MPT (Kohno & Guillette, 2013), and 95% EtOH at 0.25 ppm served as a second vehicle control treatment.

At developmental stage 19, before the traditional TSP, ≥18 eggs from each of 10 clutches (182 eggs total) were systematically randomized among six treatment groups (0.25 ppm Corexit, 2.5 ppm Corexit, 25 ppm Corexit, E_2, DMEM vehicle control and EtOH vehicle control) with at least 30 eggs in each treatment. The treatment was delivered to the alligator embryo by "painting" on the eggshell, which is a technique of applying the chemical topically to transport it inside the eggshell (Crews, Bull, & Wibbels, 1991). To avoid contamination between treatment groups, the eggs were organized into separate treatment bins after exposure. All eggs were set up to be incubated continuously at 32.5°C. During incubation, the temperature of each treatment bin was monitored every day by recording the temperature with HOBO temperature loggers (Onset) every 10 minutes. Although the aim was for incubation at 32.5°C, actual average incubation temperatures during the TSP for each clutch within each treatment group ranged from 32.8 to 33.5°C (a male-based temperature range). Therefore, eggs from particular known clutches in some of the treatment groups were unintentionally incubated at male-biased temperatures during the TSP, rather than the targeted intermediate temperature and served as controls.

2.4 | Animal husbandry and tissue collection

Upon hatching, animals were double web-tagged with unique identification numbers and housed in tanks in a temperature-controlled

animal room with an ambient temperature of 32.5°C and 14:10 hour light/dark cycle for 1 week. No food was provided during the experimental period because alligators subsist off internalized maternal yolk for the first 2 weeks post-hatching.

Morphometrics, including body weight, total length, snout-vent length (SVL), tail girth, head length, snout length and snout width, were measured at hatch. At 1 week of age, following collection of blood from the dorsal post-cranial sinus, animals were killed by a lethal dose of pentobarbital at 100 mg/kg body weight administered into the sinus (Myburgh et al., 2014). During the necropsy, morphometrics were again measured, and gonad-adrenal-mesonephros complexes (GAMs) were isolated. The tissues were immediately preserved in either Davidson's fixative for histological analysis or RNA*later*® for mRNA analysis.

2.5 | Histological analysis of the gonad

Histology was conducted to analyze tissue morphological characteristics and determine sex ratios of the treatment and control groups. The GAM tissues preserved in Davidson's fixative were transferred to 70% EtOH, processed to dehydrate and clear the tissues using a Leica ASP300 automatic tissue processor (Leica Biosystems), and embedded in paraffin wax for the cross-sections. The embedded tissues were then sectioned at 7 μm thickness and were stained following a standard protocol for hematoxylin and eosin staining. To determine gonadal sex ratios, ovaries were identified by the presence of germinal epithelium and lacunae, and testes were recognized by the presence of seminiferous tubules (Moore et al., 2010).

2.6 | RNA isolation and quantitative polymerase chain reaction

Patterns of mRNA abundances in the gonadal tissues were analyzed using quantitative polymerase chain reaction (qPCR) to confirm gonadal sex ratios and compare abundances of sexually dimorphic genes. Before qPCR, each gonadal tissue was dissected from each GAM complex under a dissecting microscope and preserved in RNA*later*. RNA was isolated from the gonadal tissues using a single-step acid guanidinium thiocyanate-phenol-chloroform method (Chomczynski & Sacchi, 2006) followed by a clean-up with the SV Total RNA Isolation System, including DNase-I treatment (Promega). RNA quantity and purity were evaluated by measuring optical density with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific), and quality of the RNA was assessed by denaturing agarose gel electrophoresis (Sambrook, Russell, & Sambrook, 2006). Isolated total RNA (660 ng) was reverse transcribed into cDNA in 15 μL reaction volumes using a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific).

All qPCR reactions were conducted using a C1000 Thermal Cycler equipped with a CFX96 real-time PCR detection system (Bio-Rad), and data were analyzed by CFX Manager software (version 3.1; Bio-Rad). Reactions were performed in triplicate reaction volumes of 15 μL with SYBR Green reaction mix containing 20 mM Tris-HCl (pH 7.75), 50 mM KCl, 3 mM $MgCl_2$, 4% DMSO, 0.5× SYBR Green I (Thermo Fisher Scientific), 0.5% glycerol, 0.5% Tween 20 (Sigma-Aldrich), 0.2 mM deoxynucleotide mix (Thermo Fisher Scientific), 0.01 U/μL AmpliTaq Gold (Thermo Fisher Scientific) and 0.2 μM primer mix. Each reaction was conducted with an initial enzyme-activating/heat denature period at 95°C for 5 minutes followed by 45 cycles of 15 seconds denaturing at 95°C and 1 minute annealing and extension at the optimized temperature for the primer pair (Table S1; see Supporting Information).

For each gene, mRNA abundances were calculated as number of copies/μL by comparing with a standard curve obtained from serial dilutions of target-containing plasmid DNA of known concentration (Kohno, Kamishima, & Iguchi, 2003). Each target mRNA abundance was normalized by a normalization factor obtained using the NormFinder software (Andersen, Jensen, & Orntoft, 2004). Analyses of the stabilities of three internal control mRNA abundance genes (ribosomal protein L8 [*RPL8*], eukaryotic elongation translation factor 1 [*EEF1*] and β-actin) identified *RPL8* and *EEF1* as the best combination of two genes (Table S2; see Supporting Information). The geometric mean of *RPL8* and *EEF1* mRNA abundances was used as the normalization factor.

To confirm sex ratios, gonadal mRNA abundances of aromatase (*CYP19A1*) and anti-Müllerian hormone (*AMH*) were analyzed at 1 week of age. Testes were characterized by *CYP19A1*<*AMH*, whereas the ovarian pattern was defined as *CYP19A1*>*AMH* (Kohno et al., 2015; McCoy et al., 2015).

2.7 | Statistical analysis

The transactivation, timing of hatch, morphometric and qPCR data were analyzed using one-way analysis of variance, analysis of covariance (ANCOVA) or Kruskal-Wallis tests followed by Dunnett's or Dunn's multiple comparison tests, respectively, depending on the normality of data distributions and homogeneity of standard deviations of the data. Data are presented as bar charts (mean ± standard error) or box plots with the median, and first and third quartiles ±1.5 interquartile range with outliers. All samples were collected and analyzed in a blinded fashion.

Morphometrics of animals exposed to E_2 were compared with the EtOH vehicle control using an unpaired *t*-test for normally distributed samples or Mann-Whitney for samples with non-normal distribution. Fulton's condition factor was calculated using 100 × weight ÷ $(SVL)^3$ (Heincke, 1908). Morphometric data were analyzed using ANCOVA with SVL as a covariant, and data are represented as adjusted mean ± standard error.

Sex ratios determined from both gonadal morphology and gene expression were analyzed using Fisher's exact tests comparing Corexit and E_2 treatments to DMEM and EtOH, respectively, with Bonferroni corrections.

Statistical analyses were conducted with Prism 8.0.2 (GraphPad Software), JMP Pro 13.2.0 (SAS Institute) and SSPS Statistics 25 (IBM Corp.).

3 | RESULTS

3.1 | Estrogenicity of Corexit in vitro

Transactivation via each ESR requires both the receptor and estrogen response element constructs in transfected cells (Figure S1; see Supporting Information). Exposure of co-transfected cells to Corexit significantly induced transactivation via both ESR1 and ESR2 in vitro, and 100 ppm Corexit significantly induced transcriptional activation of ESR2 compared with the control (Figure 1). The estrogen dose equivalent (EEQ) of 100 ppm Corexit via ESR1 was significantly greater than the EEQ via ESR2 based on a dose-response curve of E_2, although ESR2 was more sensitive to E_2 than ESR1 based on the EC_{50} values (Figure 1 and Table 1). The EEQs of 100 ppm Corexit were 4.509×10^{-13} M (0.123 parts per trillion, ppt) via ESR1 and 0.606×10^{-13} M (0.017 ppt) via ESR2 (Figure 1 and Table 1). Cells transfected with *ESR1* or *ESR2* were also exposed to Corexit at 1, 10 and 100 ppm along with 10^{-11} M (2.7 ppt) E_2, the EC_{50} of E_2 via both ESR1 and ESR2. Corexit did not inhibit or enhance the transactivation induced by 10^{-11} M (2.7 ppt) E_2 at any of these doses in vitro (Figure S2; see Supporting Information).

3.2 | Effects of Corexit exposure in ovo on hatch success and timing

Clutch effects were statistically not significant due to small sample sizes, although there might be potential differences in response to Corexit exposures among clutches. Each treatment used one to three eggs from nine to 10 clutches (Data S1; see Supporting Information).

Corexit exposure in ovo significantly influenced hatching success among all treatment groups. In particular, exposure to 0.25 ppm Corexit significantly increased hatching success relative to the DMEM vehicle control (Figure 2). E_2 exposure did not affect hatching success in comparison with the EtOH vehicle control (Figure 2).

Exposure to 0.25 ppm Corexit delayed the timing of hatch as compared with the DMEM control, whereas there were no significant differences among other treatment groups, including E_2 (Figure 3). Animals exposed to 0.25 ppm Corexit hatched 1.8 days later than eggs in the DMEM control group (Figure 3). Such delays in hatching may or may not be linked to sex determination, but are changes in a development worthy of noting and of further investigation.

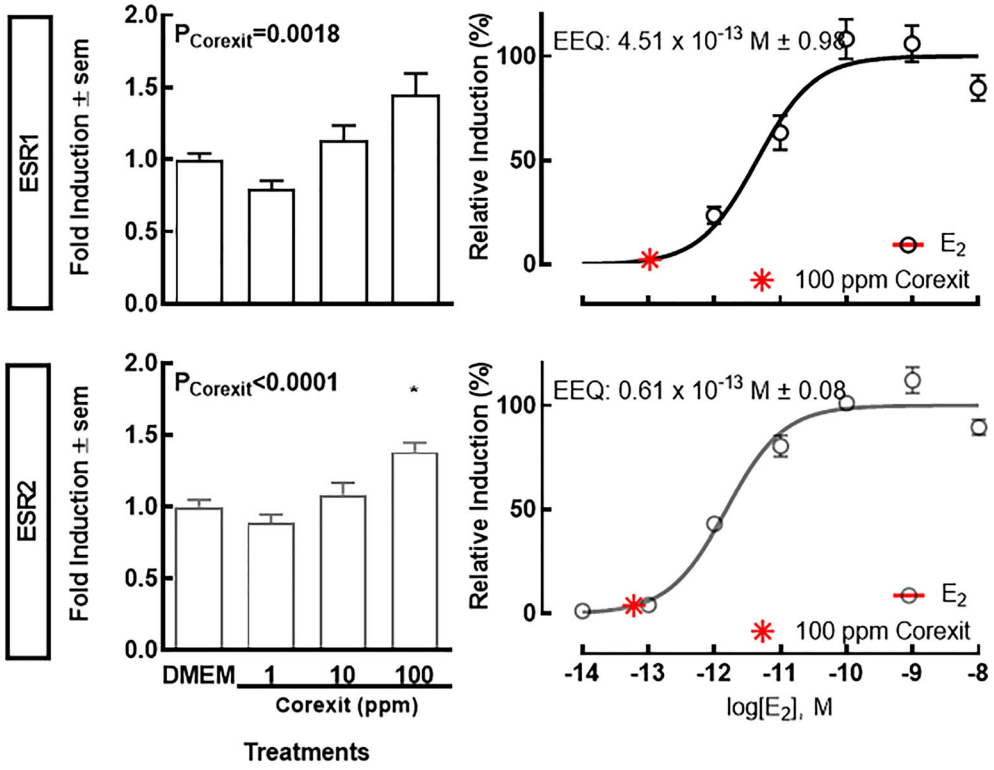

FIGURE 1 Transactivation of alligator ESR1 and ESR2 after exposure to Corexit in vitro. Transactivation was measured as luciferase activity 44 h after exposure. Corexit exposure significantly affected transactivation via both ESR1 and ESR2. Corexit significantly induced transactivation via ESR2 at 100 ppm. Based on a dose-response curve of E_2, the EEQ of 100 ppm Corexit via ESR1 was significantly greater than via ESR2. Data are normalized to vehicle (DMEM) levels and shown as bar charts with the mean ± SEM. $P_{Corexit}$ indicates effects of exposure in ANOVA. *Statistical difference from DMEM control ($P < .05$). Each assay was repeated three times in triplicate. DMEM, Dulbecco's modified Eagle medium; E_2, 17β- estradiol; EEQ, estrogen dose equivalent; ESR1, estrogen receptor 1 (ERα); ESR2, estrogen receptor 2 (ERβ) [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 1 Dose-response curve of E_2 and EEQ of 100 ppm Corexit

	E_2				100 ppm Corexit	
Receptor	EC_{50}	(95% CI)	Goodness of fit (R^2)	Replicates test (*P*-value)	Mean of EEQ	(95% CI)
ESR1	4.452×10^{-12}	$(2.128\text{-}8.688 \times 10^{-12})$	0.7679	.0286	4.509×10^{-13}	$(1.406\text{-}7.611 \times 10^{-13})$
ESR2	1.508×10^{-12}	$(1.007\text{-}2.329 \times 10^{-12})$	0.8976	.0004	0.606×10^{-13}	$(0.432\text{-}0.780 \times 10^{-13})$

CI, confidence interval; E_2, 17β-estradiol; EEQ, estrogen-dose equivalent; ESR, estrogen receptor.

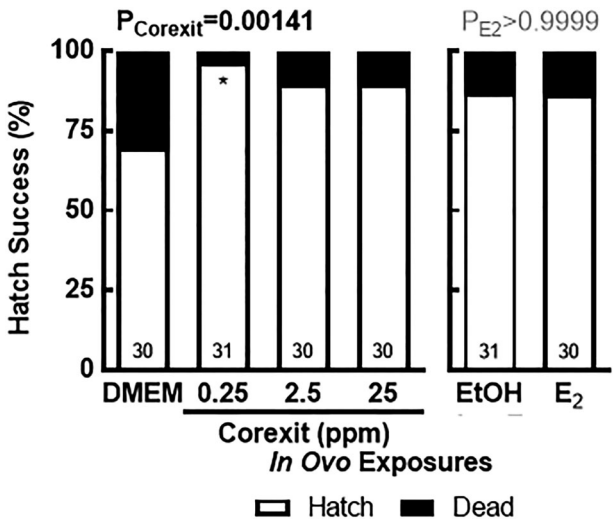

FIGURE 2 Hatch success after in ovo exposure to Corexit or E_2 at developmental stage 19. Exposure to 2.5 ppm Corexit increased hatch success ($P = .0057$), whereas other treatments did not alter it. Asterisks above bars indicate a statistical difference from the vehicle control, DMEM, as determined by Fisher's exact test. Number of eggs in each treatment group is shown at the base of each bar. Corexit at 0.25, 2.5 and 25 ppm. DMEM, vehicle Dulbecco's modified Eagle medium at 0.5 ppm; E_2, 17β-estradiol at 0.5 ppm; EtOH, vehicle ethanol at 0.25 ppm

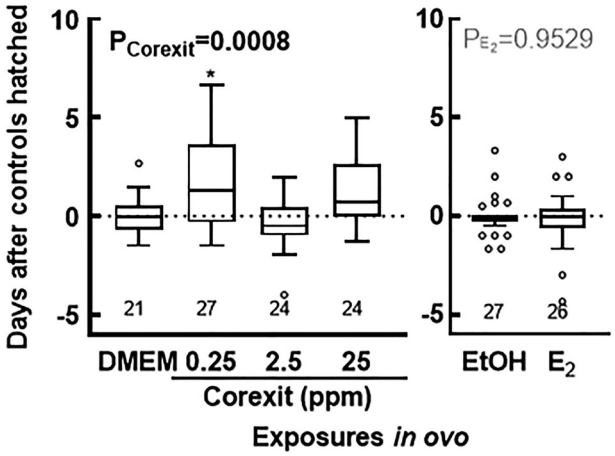

FIGURE 3 Timing of hatch after in ovo exposure to Corexit or E_2 at developmental stage 19. A significant hatch delay of 1.8 days relative to the vehicle control, DMEM, was observed for eggs exposed to Corexit at 0.25 ppm ($P < .0001$). Data are shown as box plots with the median, and first and third quartiles ±1.5 interquartile range with outliers. *Statistical difference from DMEM control. Corexit at 0.25, 2.5 and 25 ppm. DMEM, vehicle Dulbecco's modified Eagle medium at 0.5 ppm; E_2, 17β-estradiol at 0.5 ppm; EtOH, vehicle ethanol at 0.25 ppm

3.3 | Effects of Corexit exposure in ovo on morphometrics

In general, the morphometric size was larger for the Corexit-exposed groups than the DMEM control group. Animals exposed to Corexit at 0.25 and 25 ppm were significantly larger in their body weight, total length, SVL and snout width at hatching than those exposed to the vehicle DMEM, and this may be linked to their delayed hatching (Table 2A). At 1 week of age, morphometrics of the Corexit 0.25 and 25 ppm-exposed groups differed from the DMEM control group in body weight, total length, SVL, head length and snout width (Table 2 B). Tail girth, head length and width, and snout length and width at both hatch and 1 week of age were analyzed using ANCOVA with each SVL as a covariant.

The percentages of increase or decrease between the morphometric measurements recorded at hatching and 1 week of age were also analyzed. Most of the morphometrics increased after 1 week, except body weight and tail girth. A reduction of body weight in the Corexit 0.25 ppm-exposed group was significantly greater than in the DMEM control group (Table 2C).

Morphometrics of the E_2-exposed group were analyzed separately from the Corexit treatments and compared with the EtOH vehicle control due to the difference in the vehicle (Table 2 right). Exposure to E_2 reduced tail girth, head length, head width and snout width at hatching (Table 2A). At 1 week of age, the E_2-exposure group exhibited greater SVL, whereas tail girth, head length, head width, snout length and snout width were less than the EtOH control group (Table 2B). Exposure to E_2 in ovo significantly enhanced the growth of head width from hatching to 1 week of age, compared with the EtOH control (Table 2C).

3.4 | Effects of Corexit exposure in ovo on sex determination and differentiation

The Müllerian duct was present in all but one of the individuals exposed to E_2, significantly more than in the vehicle EtOH group, whereas no significant differences in the existence of Müllerian ducts were observed among the Corexit-exposed groups (Figure S3; see Supporting Information). Although the existence of the Müllerian duct is an indicator of phenotypic sex, gonadal morphology is the gold standard for sexing animals in general. Thus, gonadal morphologies of

TABLE 2 Effects of Corexit exposure in ovo on morphometrics at hatch-out and 1 week of age, and the percentage change between the two measurements. A, Hatch-out. B, 1 week of age. C, Percentage change

		Corexit (ppm)						
Measurement	**DMEM (*n* = 21)**	**0.25 (*n* = 30)**	**2.5 (*n* = 27)**	**25 (*n* = 27)**	**Probability**	**EtOH (*n* = 27)**	**E_2 (*n* = 26)**	**Probability**
A, Hatch-out								
Body weight (g)	50.6 ± 0.8	**56.3 ± 0.8***	**53.6 ± 0.7***	**55.6 ± 0.8***	**< 0.0001***	55.9 ± 0.7	54.8 ± 0.8	0.2780
Total length (cm)	23.3 ± 0.2	**24.2 ± 0.2***	23.9 ± 0.2	**24.4 ± 0.2***	**0.0005***	24.2 ± 0. 2	24.6 ± 0.2	0.2227
SVL (cm)	11.5 ± 0.1	**11.9 ± 0.1***	11.8 ± 0.1	**11.9 ± 0.1***	**0.0035***	11.8 ± 0.1	12.1 ± 0.1	0.0694
Tail girth (mm)	48.1 ± 0.4	48.5 ± 0.3	48.4 ± 0.3	48.4 ± 0.3	0.8983	49.3 ± 0.4	**47.5 ± 0.4***	**0.0017***
Head length (mm)	37.0 ± 0.2	37.2 ± 0.1	37.2 ± 0.2	37.1 ± 0.2	0.7587	37.5 ± 0.2	**36.6 ± 0.2***	**0.0003***
Head width (mm)	20.8 ± 0.1	21.2 ± 0.1	21.1 ± 0.1	21.1 ± 0.1	0.1334	21.5 ± 0.1	**20.7 ± 0.1***	**< 0.0001***
Snout length (mm)	15.1 ± 0.2	15.4 ± 0.2	15.3 ± 0.2	15.1 ± 0.2	0.6382	15.7 ± 0.2	15.2 ± 0.2	0.0624
Snout width (mm)	15.8 ± 0.2	**16.4 ± 0.1***	16.1 ± 0.1	**16.4 ± 0.1***	**0.0213***	16.7 ± 0.2	**16.1 ± 0.2***	**0.0038***
B, 1 week of age								
Body weight (g)	48.1 ± 0.8	**52.5 ± 0.9***	50.6 ± 0.7	**52.0 ± 0.8***	**0.0017***	52.3 ± 0.7	51.4 ± 0.8	0.4054
Total length (cm)	25.2 ± 0.2	**26.2 ± 0.2***	25.8 ± 0.2	**26.1 ± 0.2***	**0.0028***	26.2 ± 0.2	26.3 ± 0.2	0.4841
SVL (cm)	12.5 ± 0.1	**12.8 ± 0.1***	12.7 ± 0.1	**12.8 ± 0.1***	**0.0127***	12.8 ± 0.1	**13.1 ± 0.1***	**0.0332***
Tail girth (cm)	47.3 ± 0.4	47.5 ± 0.3	48.2 ± 0.3	47.9 ± 0.3	0.2646	48.7 ± 0.2	**47.0 ± 0.2***	**0.0042***
Head length (mm)	37.9 ± 0.2	**38.7 ± 0.1***	38.3 ± 0.2	**38.5 ± 0.2***	**0.0155***	38.9 ± 0.1	**38.0 ± 0.2***	**0.0008***
Head width (mm)	20.9 ± 0.2	**21.6 ± 0.1***	21.3 ± 0.1	21.3 ± 0.1	**0.0201***	21.6 ± 0.1	**21.2 ± 0.2***	**0.0328***
Snout length (mm)	15.9 ± 0.2	15.8 ± 0.1	15.9 ± 0.1	16.0 ± 0.1	0.7690	16.3 ± 0.1	**15.5 ± 0.1***	**0.0004***
Snout width (mm)	16.8 ± 0.1	**17.4 ± 0.1***	17.1 ± 0.1	**17.4 ± 0.1***	**0.0093***	17.6 ± 0.4	**16.7 ± 0.4***	**< 0.0001***
C, % Change								
Body weight	−4.97 ± 0.6	**−6.91 ± 0.5***	−5.72 ± 0.5	−6.34 ± 0.4	**0.0390***	−6.47 ± 0.3	−6.31 ± 0.5	0.7862
Total length	8.05 ± 0.5	7.54 ± 0.4	7.69 ± 0.3	6.9 ± 0.3	0.1686	8.16 ± 0.3	7.31 ± 0.5	0.1364
SVL	8.42 ± 0.6	7.38 ± 0.6	7.19 ± 0.6	7.48 ± 0.5	0.5695	8.43 ± 0.8	8.39 ± 0.6	0.7878
Tail girth	−0.01 ± 0.9	−2.20 ± 0.6	−0.23 ± 0.9	−1.09 ± 0.6	0.1449	−1.00 ± 0.8	−1.23 ± 0.7	0.8280
Head length index	3.14 ± 0.3	3.85 ± 0.3	3.12 ± 0.4	3.73 ± 0.3	0.2072	3.80 ± 0.3	3.93 ± 0.3	0.7529
Head width index	1.29 ± 0.6	1.65 ± 0.5	1.33 ± 0.6	1.41 ± 0.7	0.9709	**0.69 ± 0.4**	**1.95 ± 0.4***	**0.0222***
Snout length index	6.24 ± 1.4	3.05 ± 1.3	3.79 ± 1.3	6.03 ± 1.2	0.1347	3.06 ± 1.4	2.30 ± 1.3	0.9122
Snout width index	8.59 ± 1.3	5.82 ± 1.1	6.85 ± 1.1	6.15 ± 0.8	0.3243	5.87 ± 1.1	4.15 ± 1.2	0.2872

DMEM, Dulbecco's modified Eagle medium; E_2, 17β-estradiol; EtOH, ethanol; SVL, snout vent length

*Bold values with asterisks are significantly different from the respective vehicle control (DMEM or EtOH). Tail girth (cm), head length (mm), head width (mm), snout length (mm) and snout width (mm) were adjusted by each SVL in ANCOVA. Data are shown as mean ± SEM. DMEM, vehicle, at 0.5 ppm; Corexit 0.25, 2.5 and 25 ppm based on the egg weights; E2, 17β-estradiol at 0.5 ppm; EtOH, vehicle ethanol at 0.25 ppm

testis and ovary were analyzed (Figure 4A and 4B). No exposure-specific alterations in morphology were identified. E_2 exposure induced more ovaries than EtOH exposure, based on histological analyses, whereas Corexit exposure did not alter sex ratios, as compared with the vehicle control, DMEM.

Eggs incubated at an intermediate temperature during the TSP would have been expected to produce sex ratios of 50:50 male/female in control groups. Instead, the incubation temperatures of eggs were higher (male-biased temperatures and MPTs) during the TSP; therefore, both of the vehicle controls exhibited male biases of 76.2% and 85.2% male in the DMEM and EtOH groups, respectively (Figure 4C). Thus, exposure to E_2 did result in the expected ratio of 100% females but did show anticipated increases in light of the male-biased temperatures during the TSP.

3.5 | Effects of Corexit exposure in ovo on gonadal mRNA abundances

Exposure to E_2 significantly induced the female pattern of mRNA abundances compared with the vehicle control, EtOH (Figure 5), whereas there were no significant differences among Corexit treatments compared with the vehicle control, DMEM (Figure 5). Owing to the small sample sizes of females, only genes involved in male gonadal differentiation were analyzed among all male individuals. Relative testicular

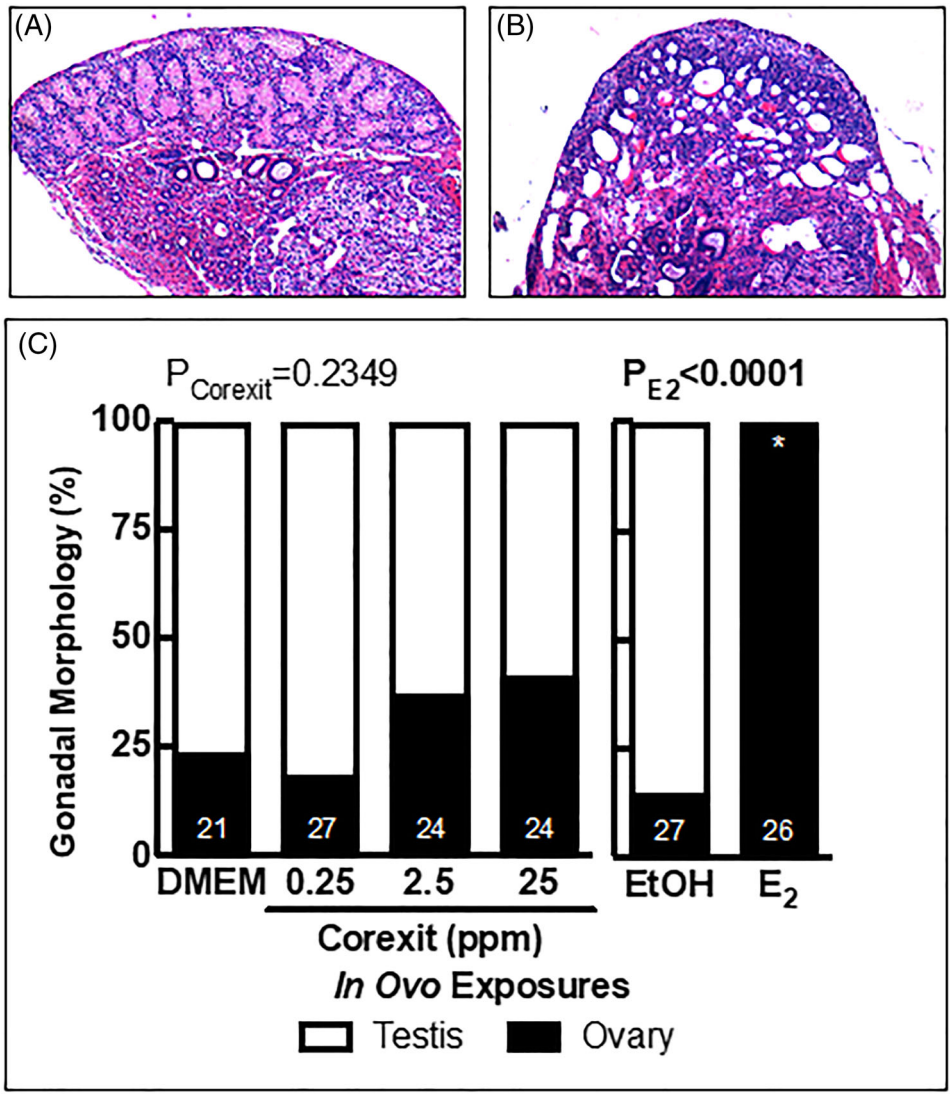

FIGURE 4 Effects of in ovo exposure to Corexit or E_2 on gonadal morphology. A, Testis. B, Ovary. C, Sex ratios. Representative photographs of the testis and ovary, and sex ratios based on histological analysis of gonadal morphology at 1 week of age after exposure to Corexit and E_2 at developmental stage 19. There were significantly more embryos with ovaries in the E_2-exposed group compared with the vehicle control, EtOH, whereas there were no statistically significant differences among Corexit-exposed groups compared with the vehicle control, DMEM. *Statistical difference from EtOH as determined by Fisher's exact test. Corexit at 0.25, 2.5 and 25 ppm. DMEM, vehicle Dulbecco's modified Eagle medium at 0.5 ppm; E_2, 17β-estradiol at 0.5 ppm; EtOH, vehicle ethanol at 0.25 ppm

mRNA abundances were in the following order from lowest to highest: sex-determining region Y-box 9 (*SOX9*), anti-Müllerian hormone (*AMH*), and *doublesex*- and *mab-3*-related transcription factor (*DMRT1*) (Figure S4; see Supporting Information). Corexit exposure in ovo did not significantly alter testicular mRNA abundances of *SOX9*, *AMH* or *DMRT1* at any of the tested concentrations (Figure S4; see Supporting Information).

4 | DISCUSSION

4.1 | Estrogenicity of Corexit in vitro

Significant cytotoxicity was not observed in our experiments at 100 ppm, although assays specific to cytotoxicity or proliferation were not performed. Published work shows that the LC_{50} of Corexit 9500A on HEK293 cells was 93 ppm without the serum (Zheng et al., 2014), but LC_{50} of Corexit was 200 ppm with serum on human airway BEAS-2B epithelial cells (Shi, Roy-Engel, & Wang, 2013). Temkin et al. (2015) conducted experiments similar to ours using Corexit at 50 ppm on HEK293T cells without serum and did not observe significant cytotoxicity. Therefore, serum in the cell culture medium of our experiments is consistent with the lack of observable cytotoxicity of Corexit at 100 ppm in the present study.

We identified estrogenic activities of Corexit via alligator ESR1 and ESR2 in vitro, which is noteworthy because the estrogenic activity of Corexit was not observed using human ESR1 in vitro (Judson et al., 2010). This difference may be due to varying sensitivities of ESR subtypes among species, as previously reported (Kohno et al., 2018;

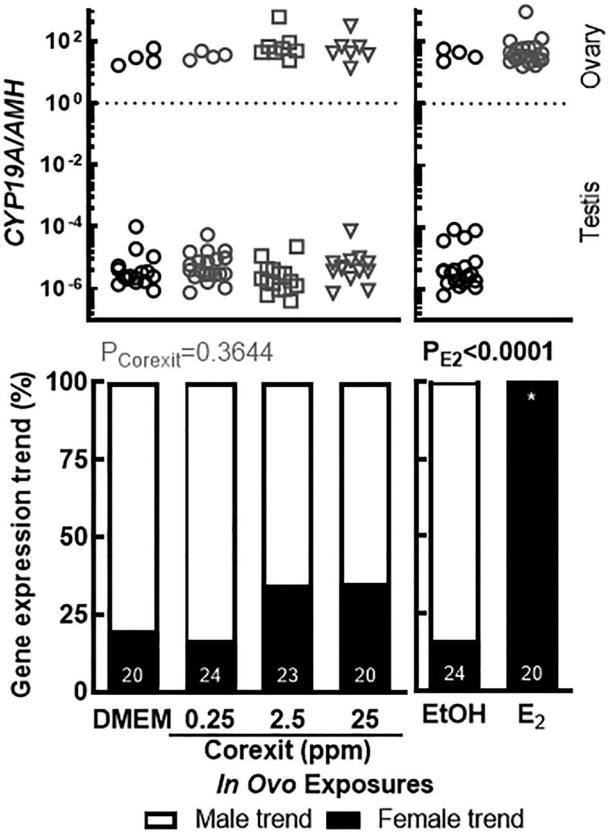

FIGURE 5 Discrimination of gonads using the sexually dimorphic pattern of gonadal mRNA abundances of *CYP19A1* and *AMH* at 1 week of age after exposure to Corexit or E_2 at developmental stage 19. Male pattern was defined as *CYP19A1*<*AMH*, whereas female pattern was defined as *CYP19A1*>*AMH*. Exposure to E_2 significantly induced the female pattern of mRNA abundances compared with the vehicle control, EtOH ($P < .0001$). There were no significant differences among Corexit treatments compared with vehicle control, DMEM. Corexit at 0.25, 2.5 and 25 ppm. *Statistical difference from EtOH as determined by Fisher's exact test. *AMH*, anti-Müllerian hormone; *CYP19A1*, aromatase; DMEM, vehicle Dulbecco's modified Eagle medium at 0.5 ppm; E_2, 17β-estradiol at 0.5 ppm; EtOH, vehicle ethanol at 0.25 ppm

Miyagawa et al., 2014). In vitro, exposure to Corexit at 100 ppm induced activation of ESR1, which is known to play a critical role in the ovarian development in the American alligator (Kohno et al., 2015). While the estrogenic activity of Corexit on alligator ESRs in vitro is much smaller scale than E_2, this highlights that the diversity of receptor sensitivity and exposure routes need to be considered in future studies.

4.2 | Effects of Corexit exposure in ovo on hatch success and timing

DOSS and Span 80, which are major components of Corexit, have polar surface areas of 118 and 96.2 angstroms squared (Å^2), respectively, based on the PubChem database (Kim et al., 2019). As compounds with polar surface areas less than 140 Å^2 are likely completely permeable to the cell membrane (Clark, 2003), these major components of Corexit most likely can pass through the egg membrane in painting experiments.

Altered hatch success rates and timing in the DMEM-exposed controls were observed and might be artificial because we diluted all dosing solutions of Corexit with DMEM, a well-established culture medium. This hatch success rate was lower than the 87.1% hatch success rate in the other vehicle control in this study (EtOH), which is lower than a 99.4% hatch rate observed in a previous study (Joanen & McNease, 1977). Painting mallard duck (*Anas platyrhynchos*) eggs with much higher doses (≥380 ppm) of Corexit 9500 significantly decreased hatch success rates (Wooten et al., 2012). However, in the present study, eggs were exposed to Corexit at much lower concentrations (≤25 ppm), levels that are close to environmentally relevant concentrations. Although there were no apparent signs of damage or mishandling of eggs, the low control hatch success could be attributed to some external factor associated with the collection site to which all eggs were exposed. One potential explanation for the altered hatch timing is early or accelerated hatching of some of the eggs in response to mechanical stimulation from handling or movements of other hatching embryos, which has been reported in alligators and three other species of crocodilians (Doody, 2011). In captivity, Nile crocodile (*Crocodylus niloticus*) embryos have been reported to hatch prematurely because of vocalization of hatchlings from adjacent clutches (Blake, 1974). We had experiences of mass hatch-out in the American alligator during thunderstorms, which might be due to low pressure (data not shown).

4.3 | Effects of Corexit exposure in ovo on gonadal sex

The highest concentration of Corexit painted on the eggs in our study was 25 ppm. Based on the in vitro results, 25 ppm Corexit is equivalent to 1.13×10^{-13} M or 0.03 ppt E_2 based on alligator ESR1 stimulation. Previous work from our group demonstrated that exposure to E_2 in ovo at 0.05 ppm was required to skew the sex ratio significantly at an MPT in the American alligator (Kohno et al., 2015). Therefore, it is possible that the level of estrogenic activity induced by Corexit was not enough to override the testicular-forming effects of the incubation temperature used in these experiments. Although there was a significant trend observed of an increasing number of females with these concentrations of Corexit using Kendall's tau test (18.5%, 37.5% and 41.7% female for 0.25, 2.5 and 25 ppm, respectively), none of the sex ratios in the Corexit-exposed groups were significantly different from the DMEM vehicle control group (23.8% female). Perhaps, the trend toward an increased number of embryos with ovaries in the dose-response manner could have become significant if an intermediate temperature had been achieved during the TSP. The embryo has a variety of nuclear hormone receptors, and the estrogenicity of Corexit's complex compound mixture might be masked by cross-talk involving other nuclear hormone receptors in ovo. Corexit activates retinoid X receptor alpha and peroxisome proliferator-activated receptor gamma (Bowers et al., 2016; Temkin et al., 2015), which can suppress estrogen signals in mammals (Nuñez et al., 1997; Segars et al., 1993).

Although gonadal morphology and sex ratios were not significantly affected by Corexit exposure, Corexit might have exhibited estrogenic activity on gonadal function. Patterns of gonadal mRNA abundance were analyzed among males for sexually dimorphic genes involved in male gonadal differentiation. Contrary to predicted outcomes, none of the mRNA abundance patterns of testicular genes were affected by Corexit exposure. Previous studies have documented the effects of estrogens on expression of these genes. Exposure to 17α-ethinylestradiol suppressed expression of both *AMH* and *DMRT1* in zebrafish (*Danio rerio*) (Schulz et al., 2007). Endogenous estrogen can feminize bipotential gonads by inhibiting expression of *SOX9*, which is a potential mechanism involved in ovarian development at MPT after exposure to environmental estrogens in *Trachemys scripta* (Barske & Capel, 2010). However, in the present study, only male genes in the male samples were analyzed due to the small sample sizes of females in each group.

Further investigation is needed to elucidate the potential effects of Corexit exposure on hatch timing of alligator eggs and confirm whether exposure to low-dose Corexit indeed leads to delayed hatching, as data from the present study argues. In the wild, delayed hatching of some of the eggs in a nest could have severe consequences for the hatchlings. When young alligators hatch and begin vocalizing, typically the mother assists by removing the nest material and digging them out of the nest (Joanen, 1969). If some of the eggs in a clutch have not hatched by this point, they could be left behind without maternal assistance and protection and could be more susceptible to predators. Therefore, the significant effects of Corexit exposure on hatch timing that we observed could have serious implications for hatchling survival in wild populations of alligators, as well as for marine reptiles in general.

The release of Corexit during the Deepwater Horizon oil spill and continued use of this dispersant in ongoing and future oil spills mandate assessing the long-term effects of Corexit on aquatic species. Moreover, eggs of aquatic amniotes have the potential to be exposed to Corexit continuously or multiple times via contaminated environmental water during their development in the wild. The present study aimed to evaluate whether Corexit had estrogenic activity and the potential to disrupt estrogen signaling and gonadal development in the American alligator. Transactivation assays revealed potential estrogenic activity for Corexit. The in ovo single Corexit exposure study aimed to clarify whether Corexit has the potential to skew sex ratios by inducing ovarian development during TSD. DOSS, which is a stable component of Corexit, was detected 1.5-6.3 ppm in the sediment from the Gulf Mexico in 2010 and 2013 (Perkins, Joye, & Field, 2017). In addition, the Corexit-enhanced water-accommodated fraction of crude oil showed higher estrogenicity than Corexit alone and may affect sex determination in alligators (Williams et al., 2018). Further investigations are needed to clarify and understand the potential endocrine disruption and long-term effects of Corexit in aquatic species.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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