Parental Exposure to Pesticides and Poor Clutch Viability in American Alligators

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In central Florida, alligators (*Alligator mississippiensis*) inhabiting lakes contaminated with organochlorine pesticides (OCPs) produce eggs that have high OCP concentrations and low clutch viability (proportion of eggs in a clutch that yield a live hatchling) compared to those from less contaminated lakes (reference lakes). However, a clear dose—response relationship has not been established between OCPs and poor clutch viability. In order to better elucidate a cause and effect relationship between OCP exposure and clutch viability, we conducted concurrent field and laboratory studies. Our field study reaffirmed that eggs of wild alligators from OCP-contaminated lakes and wetlands continue to have lower clutch viability and higher OCP burdens than eggs from reference lakes. Our field study also demonstrated that OCP egg burdens were strongly correlated with clutch viability for some of the OCP-contaminated sites, but not all. To better test causal relationships, a parental exposure study was conducted using captive adult alligators. Our laboratory study demonstrated that dietary exposure of captive alligators to an ecologically relevant OCP mixture caused alligators to produce eggs with higher OCP burdens and reduced clutch viability, as compared to the captive-control population. The experimentally induced egg burdens and clutch viability reductions were similar to those of wild alligators from OCP-contaminated sites. Our field and laboratory results suggest parental OCP exposure may be contributing to low clutch viability in wild alligators inhabiting OCP-contaminated habitats, raising some concern for endangered crocodilians living in OCP-contaminated habitats.

Introduction

In the southeastern United States, aquatic ecosystems have experienced habitat loss, degradation, alterations in water quality, and in some cases, significant declines in biodiversity due to increases in land development and associated anthropogenic impacts. A case-in-point is the Ocklawaha River Basin (ORB) in central Florida. Within the ORB, the State of Florida has listed 54 waterbodies as impaired, with impaired waters defined as waters not meeting water quality standards or supporting their designated uses (*1*). In addition, American alligators (*Alligator mississippiensis*) from several of the impaired waterbodies in the ORB have exhibited poor clutch viability (number of live hatchlings/fecundity expressed as % ± SE) (*2*), abnormal reproductive hormone concentrations (*3*), and unexplained adult mortality (*4*). For example, alligator eggs from Lake Apopka exhibited clutch viability as low as 4% during the mid 1980s (*5*). These rates were far below those observed in other Florida lakes, including Lake Woodruff National Wildlife Refuge (79%), Orange Lake (82%), and the Everglades Water Conservation Areas (65−75%) (*2*, *5*, *6*).

A few examples of the common water quality impairments for ORB waters include nutrient enrichment, oxygen depletion, increased cyanobacteria/cyanotoxin production, and eutrophication. Although eutrophication, oxygen depletion, and increased cyanotoxin production may affect alligator ecology, via alteration of prey base, and lead to altered reproductive health, these factors are known in areas that have high and low alligator clutch viability. For example, nutrients and oxygen depletion are the major causes of water quality impairment for both Orange Lake and Lake Apopka (*1*).

One factor that varies between areas that have high and low alligator clutch viability is exposure to organochlorine pesticides (OCPs). In one case, a chemical spill originating from a chemical manufacturing plant in 1980 along the south shore of Lake Apopka (*7*) was temporally associated with a decline in reproductive success and consequent alligator population decline on Lake Apopka during the early 1980s (*5*). Furthermore, low clutch viability and higher OCP burdens in eggs have also been associated with nests along the northern and northwestern shore of Lake Apopka, proximal to reclaimed agricultural areas (*8*). Downstream of Lake Apopka, similar associations between lower clutch viability, higher OCP burdens, and reclaimed agricultural areas have been observed on Lake Griffin and its adjacent wetland restoration area, Emeralda Marsh Conservation Area (EMCA) (*9*).

Understanding the relationship between OCPs and low clutch viability in alligators is important for the ecology of Florida's wetland ecosystems, and may provide insight regarding the risk OCPs pose to endangered crocodilians that inhabit areas where OCPs are still used. The link to overall wetland ecology stems from the alligator's position as the apex predator and the habitats (alligator holes) they create, which provide refugia for other species during periods of low rainfall. Furthermore, large-scale wetland restoration is occurring across Florida, with a significant portion of restoration occurring on lands where OCPs were historically applied and elevated levels have been found in soils and fish (*10*). With these concerns in mind, the overall objective of the present study was to examine the relationship between clutch viability and OCP burdens in eggs of alligators by conducting concurrent field and laboratory studies.

Materials and Methods

Field Study. Lakes Apopka (N 28° 35′, W 81° 39′), Griffin (N 28° 53′, W 81° 46′), and EMCA (N 28° 55′, W 81° 47′) were selected as OCP-contaminated sites. Orange Lake and Lake

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Lochloosa (N 29° 30′, W 82° 09′) were selected and treated as a single reference site because alligators can move easily between the lakes due to their close proximity and their hydrological connection. These contaminated and reference sites were selected because previous studies had indicated differential OCP contamination among these sites (*10*, *11*). Alligator nests were located via aerial (helicopter) and ground surveys (airboat). All eggs from each clutch were collected and placed in a polypropylene pan with nest substrate to cushion and insulate the eggs. Clutches were then transported to the U.S. Geological Survey's Center for Aquatic Resources Studies (Gainesville, FL) where they were evaluated for embryonic viability using a bright-light candling procedure. Viable eggs were nested in pans containing moist, clean sphagnum moss and incubated at 30.5 °C and ~98% humidity in an incubation building. One or two eggs were opened from each clutch to identify the embryonic stage of development at the time of collection, and to collect yolk samples for measurement of OCP burdens. Egg yolks were screened for 30 different OCP analytes using previously described GC/MS methods (*12*).

For each clutch, initial data on the following parameters were collected: total clutch mass, total numbers of eggs (fecundity), number of damaged eggs (breached eggshell and eggshell membranes), unbanded eggs, dead banded eggs, and live banded eggs. An unbanded egg refers to either an unfertilized egg or an egg in which the embryo died before attaching to the inner eggshell membrane. Under bright-light candling, embryonic attachment (via extraembryonic membranes) to the inner eggshell membrane forms a visible opaque spot on the dorsal portion of an otherwise translucent egg, with attachment occurring within 24 h of oviposition (*13*). As the embryo and extraembryonic membranes grow, the opaque spot develops into a lateral band around the egg's equator. Should embryonic death occur before attachment, autolysis of the minuscule embryo often precludes confident determination of fertilization status. After attachment occurs, the visible spot or band will persist after embryonic autolysis occurs.

Eggs were candled every 10 days to monitor mortality throughout development. Upon candling, eggs with viable embryos have a red-pink luminescence which is caused by oxygenated blood flowing through the chorioallantoic vessels. Eggs with dead embryos have an orange-yellow luminescence indicating autolysis of vessels and other tissues. Candling more frequently would have increased risk of mortality from handling and less frequently would have decreased ability to bracket the period when death occurred. For data analysis, we classified mortality as either early embryo mortality (Day 1−Day 35) or late embryo mortality (post-Day 35). Our reasons were that organogenesis is a period of development when embryos are especially susceptible to toxicants (*14*) and it occurs in alligators mainly during the first 35 days of the ~70 day incubation period (*13*).

Captive Experiment. Adult alligators ($n = 29$) were obtained from JungleLand Zoo (Kissimmee, FL) and Gatorland Zoo (Orlando, FL). Adults had been hatched and reared in captivity at the zoos, which have been producing alligators for commercial and entertainment purposes for several decades. During their lives, the adults had been exposed to similar environmental conditions and diets, mainly consisting of horsemeat and bone-in chicken. However, the level of prior OCP exposure, via maternal transfer or dietary contamination, was unknown and may be a confounding factor.

Thirteen male and 14 female adult alligators were randomly assigned to breeding groups at a ratio of 1 male: 1 female, except for one large pen which housed two females and one male. Mean total lengths (mean ± SE) for males and females were 2.53 ± 0.15 m and 2.23 ± 0.18 m, respectively. Mean body masses of males and females were 70 ± 13.1 kg and 46 ± 13.8 kg, respectively. Each group was housed in wetland enclosures consisting of vegetated terrestrial habitat (500 m^2) and aquatic habitat (400 m^2, mean water depth ~1.2 m). Seven breeding pairs received OCP treatments, and six served as controls. Two extra OCP-treated females were housed apart from the breeding groups in order to monitor bioaccumulation of OCPs and health status via monthly blood assessments of hematocrit, glucose, and total protein (*15*).

Dose calculations were based on OCP concentrations in alligator yolks collected from contaminated sites in Florida and avian maternal transfer rates (*16*). The dosing regime was designed to coincide with oocyte development and yolk formation (vitellogenesis), which for alligators begins in early fall and continues through late spring (*17*, *18*).

In September 2001, the females assigned to the OCP-treatment groups were initially dosed with a mixture of chlordane (Chem Service, West Chester, PA), *p*,*p*′-DDE (Acros Organics, Morris Plains, NJ), toxaphene (Chem Service, West Chester, PA), and dieldrin (Sigma-Aldrich, St. Louis, MO) via one intramuscular and one intraperitoneal injection. Dosage rates were 36.5 mg/kg body weight (BW) for *p*,*p*′-DDE, 2.6 mg/kg BW for toxaphene, 2.5 mg/kg BW for chlordane, and 8.4 mg/kg BW for dieldrin. These four OCP analytes were chosen because they were found in almost every clutch collected from OCP-contaminated sites and accounted for >90% of total OCP mass in these clutches (*9*). Control females received the same volume of olive oil.

Oral doses also consisted of a mixture of *p*,*p*′-DDE (0.18 mg/kg BW), toxaphene (0.13 mg/kg BW), chlordane (0.014 mg/kg BW), and dieldrin (0.018 mg/kg BW). The process of estimating oral doses began by taking the highest egg yolk concentration for each of the selected OCP analytes measured in wild clutches during 2000 and dividing those values by a maternal transfer rate of 23%, which was an estimated rate based on avian values (*16*). This calculation produced an estimated total OCP dose that a female needed to ingest in order to achieve desired OCP egg yolk burdens. The total dose was then divided into six weekly oral doses, so that exposure would occur similarly throughout the approximate 1.5-month period when follicular maturation, ovulation, and copulation occurred.

Oral dosing began in April 2002, with males and females from the OCP-treated groups being dosed weekly until their winter fast began in late October. The rationale for all males and females of the OCP-treatment groups to both receive oral doses was that a male might occasionally take the female's ration. Although males did not receive the same initial OCP injections as females, oral dosing of males and females improved ecological relevance and ensured all OCP-treated individuals received similar exposure opportunities during the spring reproductive period.

The weekly feed ration consisted of a whole domestic rabbit (2−2.3 kg rabbit/female alligator and 2.7−2.9 kg rabbit/male alligator) that was purchased frozen from a commercial exotic pet food company (The Gourmet Rodent, Archer, FL). Each rabbit was allowed to thaw overnight, supplemented with 0.25 kg of fish feed (Aquamax Fingerling Starter 300, PMI Nutrition International, St. Louis, MO) and a multivitamin (Mazuri Vita-Zu mammal tablet, PMI Nutrition International, St. Louis, MO). To incorporate the OCP mixture into the diet, OCPs were solubilized in reagent grade olive oil (total mixture volume per weekly dose = 8 mL), and this mixture (8 mL) was then injected into the pericardial cavity of the rabbit carcass.

The pattern of animals receiving oral doses from April to October continued through 2003 and 2004. When females in breeding groups began nesting, eggs were collected and incubated using methods described above (*15*). To determine degree of clutch exposure, two egg yolks from each clutch were screened for OCPs using GC/MS (*12*).

TABLE 1. Summary of Clutch Parameters and Site Comparisons for Clutches of American Alligator Eggs Collected during 2000−2002 from Reference and OCP-Contaminated Sites in Central Florida

	reference site	OCP-contaminated sites		
parameter[a]	Orange/Lochloosa	Apopka	EMCA	Griffin
no. clutches	44	31	46	47
clutch viability (%)	70 ± 3.9 A	51 ± 5.8 B	48 ± 5.5 B	44 ± 4.9 B
damaged eggs (%)	2 ± 1.4 A	2 ± 0.6 A	5 ± 1.3 B	4 ± 1.8 AB
unbanded eggs (%)	11 ± 2.2 A	21 ± 4.9 A	14 ± 3.7 A	17 ± 3.2 A
early emb. mort. (%)	12 ± 2.7 A	15 ± 4.2 AB	23 ± 3.9 B	22 ± 3.9 B
late emb. mort. (%)	6 ± 1.7 A	12 ± 3.5 B	10 ± 2.4 B	13 ± 3.1 B

[a] Values indicate mean ± standard error of mean. Values with different letters indicate significant differences ($P < 0.05$). Clutch viability = no. of eggs yielding a live hatchling/fecundity × 100; damaged eggs = no. damaged eggs/fecundity × 100; unbanded eggs = no. of unbanded eggs/fecundity × 100; early emb. mort. = no. of embryonic deaths on or before developmental day 35/fecundity × 100; and late emb. mort. = no. of embryonic deaths post dev. day 35/fecundity × 100. EMCA represents Emeralda Marsh Conservation Area, a wetland restoration area adjacent to Lake Griffin.

TABLE 2. Organochlorine Pesticide Concentrations (ng OCP/g Egg Yolk Wet Weight) for American Alligator Eggs Collected during 2000−2002 from Reference and OCP-Contaminated Sites in Central Florida

	reference site	OCP-contaminated sites		
parameter[a]	Orange/Lochloosa	Apopka	EMCA	Griffin
no. clutches	19	23	31	42
aldrin	0.75 (ND) A	4 ± 0.3 B	2 ± 0.3 C	0.75 (ND) A
methoxychlor	0.75 (ND) A	8 ± 1 B	9 ± 1 B	17 ± 0.3 C
dieldrin	4 ± 0.5 A	344 ± 80.9 B	142 ± 20.4 C	23 ± 3.8 D
heptachlor epoxide	3 ± 0.8 A	17 ± 5.6 B	7 ± 1.4 C	7 ± 1 C
cis-chlordane	2 ± 0.2 A	43 ± 7.6 B	90 ± 13 C	11 ± 0.9 D
cis-nonachlor	5 ± 0.6 A	88 ± 27.3 B	66 ± 9.7 B	18 ± 1.6 C
oxychlordane	4 ± 1 A	51 ± 14.6 B	23 ± 3.8 C	10 ± 1.3 D
p,p′-DDE	74 ± 12 A	5794 ± 1795 B	8069 ± 1402 B	271 ± 31.3 C
p,p′-DDD	2 ± 0.2 A	42 ± 8.5 B	1289 ± 196 C	7 ± 0.9 D
p,p′-DDT	0.75 (ND) A	9 ± 2.1 BC	12 ± 1.2 B	5 ± 0.8 C
o,p′-DDD	0.75 (ND) A	5 ± 0.7 B	37 ± 5.1 C	1 ± 0 B
o,p′--DDT	1 ± 0 A	11 ± 1.9 B	170 ± 162 B	4 ± 0.3 C
trans-chlordane	3 ± 0.7 A	8 ± 1.5 B	25 ± 3.3 C	2 ± 0.2 A
toxaphene	1500 (ND) A	2738 ± 225 B	6865 ± 552 C	3043 ± 426 B
trans-nonachlor	8 ± 1.6 A	212 ± 66.9 B	191 ± 30.5 B	36 ± 4.7 C
∑OCPs	102 ± 16 A	7582 ± 2008 B	15,480 ± 2265 B	1169 ± 423 C
N*o*. OCPs	9 ± 0.3 A	13 ± 0.3 B	14 ± 0.2 C	11 ± 0.1 D

[a] Values indicate mean ± standard error of mean. Values with different letters indicate significant differences ($P < 0.05$). (ND) after value indicates analyte was not detected. Values preceding ND indicate limit of detection. EMCA represents Emeralda Marsh Conservation Area, a wetland restoration area adjacent to Lake Griffin.

Statistical Analysis. For statistical analysis of data gathered during the field study, specific OCP analytes were not included if quantifiable concentrations were not found in at least 5% of all clutches. Numerical data were log-transformed [ln(x)], while proportional data were arcsine square root transformed to meet statistical assumptions. ANOVA (*19*) and the Tukey test (*19*) were used for comparisons of adult female and clutch characteristics (viability and OCP burdens) among sites ($\alpha \leq 0.05$).

Relationships between OCP analyte concentrations in eggs and clutch viability parameters were independently examined for each site to reduce confounding factors associated with potential differences in population dynamics and genetics among sites. For each site, detrended correspondence analysis (DCA) (*20*) was used to initially evaluate data structure. DCA results indicated that a direct gradient, multivariate linear analysis, redundancy analysis (RDA) (*21*) was appropriate since the gradient lengths of the DCA ordination axes were equal to or less than 2 standard deviations (*22*).

For the captive exposure study, treated versus control comparisons were evaluated using T-tests for clutch viability parameters and OCP egg yolk concentrations, except for chlordane. Wilcoxon two-sample tests were used to compare chlordane egg yolk concentrations because data did not meet the statistical assumptions for parametric tests (*19*). All other numerical data (log-transformed [ln(x)]) and proportional data (arcsine square root) were transformed to meet statistical assumptions for parametric tests. All values in results and discussion section are expressed as mean ± SE, unless otherwise noted.

Results and Discussion

From 2000 to 2002, a total of 168 clutches of alligator eggs were collected from the study sites (Table 1). Significant differences ($P < 0.05$) were determined among sites with respect to clutch viability, damaged eggs, early embryo mortality, and late embryo mortality (Table 1). No significant differences were observed or detected among sites with respect to percentage of unbanded eggs.

Clutches from reference lakes had greater clutch viability and lower late embryo mortality rates compared to OCP-contaminated sites. Except for Lake Apopka, reference clutches had lower early embryo mortality rates than the OCP-contaminated sites. With respect to damaged eggs, clutches from EMCA had greater rates than those of the reference sites (Table 1).

Of the 168 clutches, 115 clutches were randomly selected and screened for 30 different OCP analytes (Table 2). Significant differences were detected among sites with respect to individual OCP concentrations in egg yolks, total OCP concentrations in egg yolks, and number of OCPs detected at measurable levels (Table 2). Egg yolks from reference site clutches had significantly lower total concentrations and a lower number of analytes detected at measurable levels. All individual OCP analyte concentrations in egg yolks of clutches from reference lakes were significantly less than those of the OCP-contaminated sites, with one exception. Aldrin and trans-chlordane egg yolk concentrations of reference clutches did not significantly differ from Lake Griffin (Table 2).

Although these results show that eggs from OCP-contaminated sites have higher OCP egg yolk burdens and lower clutch viability, lower clutch viability may be related to other site-specific factors. Therefore, we examined the relationship between OCP egg yolk burdens and clutch viability among clutches within each site in an attempt to control for some of the extraneous factors that differed between sites. Multivariate analysis (redundancy analysis) showed that OCP burdens explained 39% of variation in clutch viability for Lake Apopka, 21% for Lake Griffin clutches, 9% for EMCA clutches, and 0% for the reference site clutches. The primary individual OCP analytes negatively associated with clutch viability among all sites were chlordane, dieldrin, *p*,*p*′-DDE, and toxaphene. Regardless of site, the relatively low (<40%) amount of variation in clutch viability explained by OCP egg yolk burdens suggests other site-specific and/or population-specific factors may have affected clutch viability. In addition, these differences may suggest the presence of a threshold-type dose−response pattern, which has been reported in mammalian developmental toxicity studies (*14*).

The concurrent laboratory study showed that over the initial 10 months of exposure, the behavior and health status of the two extra females appeared to be normal for captive alligators (*15*). For the breeding pairs, no significant differences were detected between treatment groups with respect to same-sex length and weight comparisons of adults ($P >$ 0.4 for total length and mass).

Four of the six control females produced nine clutches from 2002 to 2004. Control female #1 (CF1) produced a single clutch in 2002, CF2 produced two clutches (2003, 2004), and CF3 and CF4 produced three clutches each (2002−2004). CF5 and CF6 did not produce any clutches. For the OCP-treated group, three of the seven treated females (TF) produced seven clutches from 2002 to 2004. TF1 produced two clutches (2002, 2004), TF3 produced two clutches (2003, 2004), and TF4 produced three clutches (2002−2004). The remaining treated females (TF2, TF5−TF7) did not produce any clutches.

Given the limited sample size available for year-to-year comparisons, it is difficult to make any conclusions as to whether there were any biologically or statistically significant effects associated with exposure duration. Nonetheless, we did compare clutch variables among years for each group. No significant differences were observed among years for fecundity, clutch mass, clutch viability, rate of unbanded eggs, or early and late embryo mortality for the treated group. In addition, no significant differences were observed among years concerning yolk lipid percentage or OCP analyte concentrations for the treated group. Similar results were found for the control group, with the exception that early embryo mortality was significantly higher in 2002 clutches ($n = 3$, 26 ± 6%) compared to 2003 ($n = 3$, 0 ± 0%) and 2004 clutches ($n = 3$, 8 ± 4%).

Comparisons between control ($n = 9$) and OCP-treated clutches ($n = 7$) indicated that clutch viability, unbanded egg rates, total OCP concentrations in eggs, and lipid content in yolk varied between control and OCP-treated groups. Specifically, clutch viability for the control group was 35% higher than the treated group, and the treated group had a 42% higher incidence of unbanded eggs (Figure 1). Total OCP burdens in yolks from the control group (50 ± 3.6 ng/g) were less than those of the treated group (13,300 ± 2666 ng/g), and even those of wild alligators from the reference

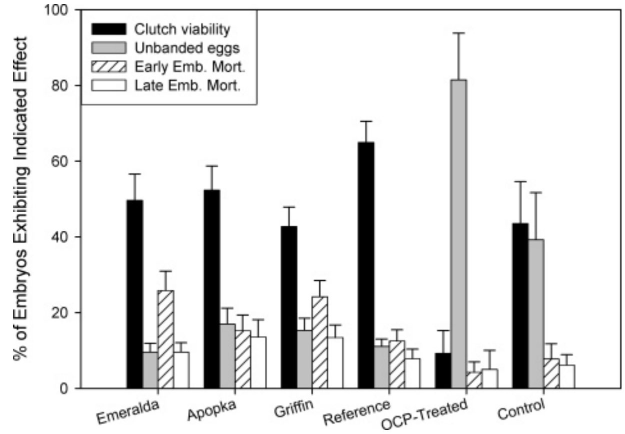

FIGURE 1. Clutch survival parameters for wild and captive alligator clutches collected during field (2000−2002) and laboratory studies (2001−2004). Bars represent mean and error bars represent standard error of mean.

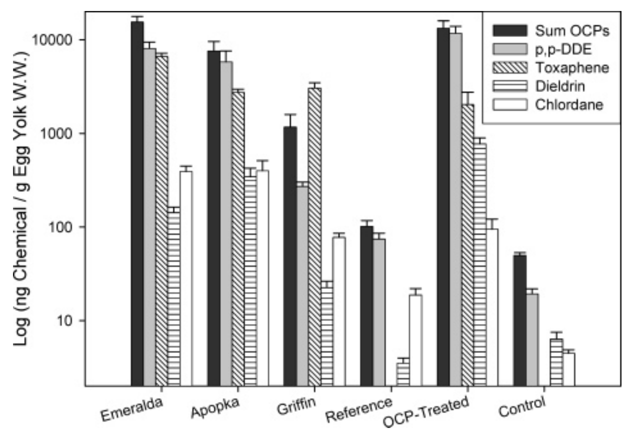

FIGURE 2. Organochlorine pesticide concentrations in clutches of wild and captive alligator clutches collected during field (2000−2002) and laboratory studies (2001−2004). W.W. represents wet weight, bars represent mean, and error bars represent standard error of mean.

site (102 ± 15.5 ng/g). Importantly, the treated group's yolk burdens fell within the range of the OCP concentrations observed in eggs of wild alligators from OCP-contaminated sites (Figure 2). Lipid content of eggs of alligators from the treated group (22 ± 0.7%) was significantly greater than those of eggs of the control group (19 ± 0.7%). Other reports suggest that alterations in lipid and fatty acid content of alligator eggs may be associated with higher embryo mortality (*23*, *24*) and altered lipid metabolism and transport have been shown in catfish (*25*) and mink (*26*) exposed to organochlorines. Because the treated and control groups received similar diets, differences in yolk lipid content suggest that OCP exposure may have altered lipid metabolism and/or follicular deposition.

Results from our laboratory and field studies were similar in that wild and captive alligators that were exposed to OCPs had lower clutch viabilities (Figure 1) and higher egg yolk OCP concentrations (Figure 2) compared to lesser-exposed cohorts. Differences between studies were that reductions in clutch viability in populations from OCP-contaminated sites were primarily due to increases in early and late embryo mortality, while higher rates of unbanded eggs were the primary reason for lower clutch viability in the captive population.

Whether infertility or very early embryo mortality (*27*) caused the higher incidence of unbanded eggs in the OCP-treated group is unknown. With regard to infertility, a factor to consider is that male alligators from the OCP-treated group were also dosed, which suggests the possibility that infertility may be related to alteration of male reproductive function. Another factor to consider is the stress of captivity, given that the percentage of unbanded eggs was 3-fold higher in the control group than in wild clutches from the reference site (Figure 1). Thus, stress due to captivity is a potential confounding factor in these studies and may have exacerbated the effects of OCP exposure.

Our laboratory study is the first to induce elevated egg yolk OCP concentrations via maternal treatment in alligators. Our results confirm that OCPs are maternally transferred to the yolk. Importantly, the captive exposure study demonstrates that alligators exposed to ecologically relevant concentrations of OCPs have lower clutch viability compared to a similar control group. Last, the combined results of our laboratory and field studies strongly suggest that parental OCP exposure may deleteriously affect clutch viability in wild alligator populations. Although American alligators are not endangered, 17 species of crocodilians are listed as rare or endangered (*28*), and many inhabit areas where OCPs are still used to control disease vectors and insect pests (*29*). Our study raises some concern as to whether OCP exposure may or may not be affecting these endangered populations. We hope our findings encourage future researchers and resource managers to pair field studies with ecologically relevant laboratory experiments when investigating the effects of contaminants on wildlife populations.

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Supporting Information Available

Additional figures and tables of data. This material is available free of charge via the Internet at http://pubs.acs.org.

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