Developmental Abnormalities of the Gonad and Abnormal Sex Hormone Concentrations in Juvenile Alligators from Contaminated and Control Lakes in Florida

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The reproductive development of alligators from a contaminated and a control lake in central Florida was examined. Lake Apopka is adjacent to an EPA Superfund site, listed due to an extensive spill of dicofol and DDT or its metabolites. These compounds can act as estrogens. Contaminants in the lake also have been derived from extensive agricultural activities around the lake that continue today and a sewage treatment facility associated with the city of Winter Garden, Florida. We examined the hypothesis that an estrogenic contaminant has caused the current failure in recruitment of alligators on Lake Apopka. Supporting data include the following: At 6 months of age, female alligators from Lake Apopka had plasma estradiol-17β concentrations almost two times greater than normal females from the control lake, Lake Woodruff. The Apopka females exhibited abnormal ovarian morphology with large numbers of polyovular follicles and polynuclear oocytes. Male juvenile alligators had significantly depressed plasma testosterone concentrations comparable to levels observed in normal Lake Woodruff females but more than three times lower than normal Lake Woodruff males. Additionally, males from Lake Apopka had poorly organized testes and abnormally small phalli. The differences between lakes and sexes in plasma hormone concentrations of juvenile alligators remain even after stimulation with luteinizing hormone. Our data suggest that the gonads of juveniles from Lake Apopka have been permanently modified *in ovo*, so that normal steroidogenesis is not possible, and thus normal sexual maturation is unlikely. *Key words:* alligators, estrogens, gonadal abnormalities, reptiles, testosterone. *Environ Health Perspect* 102:680–688 (1994)

Reproductive success, an animal's likelihood to produce offspring that will reproduce in the next generation, is an essential component of the life history of any species. Biotic and abiotic factors can modify embryonic or neonatal survival, differentiation, and growth, thus dramatically influencing an organism's reproductive success. Such abiotic factors as photoperiod, precipitation, and ambient temperature modify embryonic survival, neonatal growth, and age at sexual maturity (*1*). The major biotic factors influencing embryonic or neonatal survival and growth include parental and ecological factors. For example, the genetic makeup of offspring and the nutritional reserves stored in an oocyte influence embtyonic survival. Female age and density-related stress have also been shown to influence embryonic survival and neonatal growth in alligators (*2–5*).

The American alligator (*Alligator mississippiensis*) was listed as an endangered species in the early 1970s, but populations throughout the southeastern United States grew rapidly after federal protection was instituted (*6–8*). Woodward and Moore (*8*) have reported that populations in Florida are stable or continue to grow in some wetland areas. However, one study population, that on Lake Apopka (Lake and Orange Counties, Florida), showed a dramatic decline during the 1980s that continues to the present (Fig. 1). Population density on Lake Apopka today continues at one-tenth of that reported in the late 1970s (*9,10*). Lake Apopka is the third largest freshwater lake (12,500 ha) in the state of Florida and has been designated as one of Florida's most polluted lakes owing to nutrient and pesticide levels (*11,12*). Historically, contaminants and nutrients in the lake have been derived from extensive agricultural activities around the lake that continue today, a sewage treatment facility associated with the city of Winter Garden, Florida, and a major pesticide spill from the former Tower Chemical Company. The Tower Chemical Company, adjacent to Lake Apopka, is registered as an EPA Superfund site due to an extensive spill of a pesticide mixture composed primarily of dicofol but having as much as 15% DDT and its metabolites DDD, DDE, and chloro-DDT) (*13*) and sulfuric acid in 1980 (U.S. EPA, unpublished report). Studies indicate that dicofol as well as DDT and its metabolites have the ability to act as estrogens, binding to and activating estrogen receptors (*14*) and inducing estrogen-dependent cellular growth (*15*). Jennings et al. (*9*) proposed that the major cause of the population crash on Lake Apopka was reproductive failure. Xenobiotic compounds with estrogenic activity could significantly modify both embryonic development and adult reproductive function. A previous study has identified elevated levels of the contaminant *p,p′*-DDE in alligator eggs from Lake Apopka (collected during 1984–1985) when compared to eggs from two other lakes (*16*), but this study could not directly correlate elevated organochlorine compounds with poor egg viability. The levels observed (5.8 ppm wet weight) are above the concentrations known to adversely influence avian eggs and embryos (*17*).

The detrimental effects of environmental contamination on reproductive and endocrine function have been reported in the scientific and popular literature for many years. Although causal agents and effects are known in some cases, the underlying mechanism(s) are still poorly understood [see numerous reviews in Colborn and Clement (*18*)]. In general, reproductive disorders reported to date involve such factors as reduced fertility, reduced hatchability, reduced viability of offspring, modified hormone activity, or

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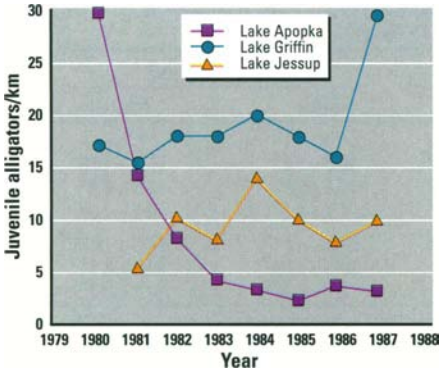

Figure 1. Estimated numbers of juvenile (30–122 cm) alligators per kilometer of shore line on Lakes Apopka, Griffin, and Jessup, Florida, during 1980–1987. Estimates were derived from log-transformed night-light counts, adjusted for water level, and presented on an untransformed scale. Redrawn from Woodward et al. (*10*).

altered adult sexual behavior. There are also reports of poor growth, wasting, and lower rates of neonatal activity (*18*). Recent studies reporting significantly depressed viability rates for alligator eggs from Lake Apopka (*10,21,22*) suggest that a contaminant problem exists on this lake.

The current studies have examined several specific aspects of the biology of alligator eggs and neonates from Lake Apopka as well as from a control area, Lake Woodruff National Wildlife Refuge. We examined viability of eggs from Lake Apopka as well as gonadal development (anatomy and endocrine function) of neonates from both lake systems. We hypothesized that even if viability is depressed for eggs from Lake Apopka, healthy young exhibiting normal growth and reproductive development could be produced. Population studies have shown that due to the long (20 year) reproductive life of female alligators (*2,23*) and high adult female survival, relatively few young need be recruited each year to maintain a viable, stable population (*24*). Why then does the density of juvenile alligators on Lake Apopka remain low? Can we expect a population increase in the next few years, once juveniles currently growing on Lake Apopka reach sexual maturity? Or could there be subtle damage of the reproductive system that would preclude these young alligators from subsequently producing viable offspring? Recent studies suggest that exposure to low concentrations of estrogenic xenobiotic compounds during embryonic development can permanently alter the morphology and physiology of the mammalian reproductive system (*14,19,20,25*). Our studies have examined the comparative reproductive anatomy and endocrinology of neonatal and juvenile alligators obtained from either a lake contaminated with high levels of organochlorine compounds (Lake Apopka) or a relatively uncontaminated control lake, the Lake Woodruff National Wildlife Refuge.

Methods

Eggs and Neonatal Care

We collected alligator eggs from 25 nests on each of two lakes, Lakes Apopka and Woodruff, during June and July 1992. Complete clutches were collected on Lake Apopka, but due to permit restrictions limiting the removal of animals and their products from federal wildlife refuges, only two viable eggs and all dead eggs were collected from nests at Lake Woodruff National Wildlife Refuge. Lake Apopka is adjacent to an EPA Superfund site (listing due to a pesticide spill in 1980 from the Tower Chemical Company). In contrast, the control area, Lake Woodruff, is part of the Lake Woodruff National Wildlife Refuge, a relatively pristine area with little direct agricultural or development pressure. Eggs collected previously from Lake Apopka showed elevated DDE concentrations. The contaminant levels in the eggs appear to be significantly greater than those reported as background environmental levels (EPA, unpublished report), suggesting that the contaminants in the eggs are the result of bioaccumulation and transfer of these factors into the eggs by the female.

Sex determination is temperature dependent in alligators. High temperatures (≥33°C) produce all males, whereas lower incubation temperatures (≤30°C) produce all females (*26*). All alligator eggs were collected before the developmental period during which sex determination occurs (average egg age 12 days), and eggs were incubated at a temperature (30.5°C) known to produce approximately a 1:1 sex ratio. We positioned eggs in plastic pans (61 × 36 × 13 cm) on 5 cm of natural nest material with additional nest material cushioning layers of eggs when required. Eggs were covered with natural nesting materials (2–4 cm). During transportation of clutches, care was taken to avoid excessive vibration or shock, which could cause detachment of the embryonic membranes from the shell and subsequent mortality (*2*). Woodward et al. (*27*) found that careful handling and transport of alligator eggs avoided such mortality. At the incubator, all intact eggs were transilluminated (candled) to check for viability and egg-band development (*27*).

The incubator housing clutches was an insulated 7.3 m × 3.7 m portable building. Humidity and temperature were controlled by a heat/cool humidifier unit. Mean relative humidity readings for 3-hr intervals were 95 ± 1% (measured by wet bulb thermometer). Individual trays within each rack were inspected twice daily to ensure adequate moisture content for each clutch. Target temperature within the incubator was 30.5°C and was monitored with mercury thermometers and a multi-probe thermocouple system (Atkins Technical, Gainesville, Florida). Eight probes were systematically positioned within the nesting medium of sphagnum and in the general circulation of the incubator; temperatures averaged 30.7 ± 0.4°C.

We maintained eggs within their clutch in the same order as collected. A maximum of three clutches per tray, separated by boards, were nestled in sphagnum moss and placed in the incubator. Trays of eggs were covered with 50% shade cloth to allow air circulation but maintain the integrity of the clutch and neonates after hatching. We placed clutches in trays or pans with a minimum of 2.5 cm of sphagnum insulation on the sides, top and bottom. To minimize premature hatching of alligators by audible cueing from adjacent clutches, we grouped clutches with similar hatch dates together.

After hatching, neonatal alligators were maintained in an insulated building. Most hatchlings were retained until they were actively feeding (approximately 10 days). After yolk absorption was complete, hatchlings were web-tagged with sequentially numbered #1 Monel tags between the 3rd and 4th toes of the right rear foot. We recorded data on physical abnormalities, weakness, and death. Mortality was recorded for all clutches, and weight (nearest 0.1 g), total length (TL; to the nearest 1 mm), and snout vent length (SVL; to the nearest 1 mm) were recorded for all live hatchlings.

Within 10 days of hatching, 25 robust neonates from each lake ($N = 50$ neonates total) were chosen and transferred to the Sante Fe Teaching Zoo, Gainesville, Florida, where they were housed in an outdoor enclosure (30 × 10 m). This enclosure was covered with wire mesh to prevent predation, but animals experienced natural fluctuations in photoperiod and temperature. Animals were fed *ad libitum* daily with commercial alligator chow (Burris Mill and Feed Inc., Franklinton, LA). The water in the pool, contained within the enclosure, was changed daily. We visually examined animals daily for general health (i.e., activity level, feeding activity) without disturbing the young. If an animal exhibited poor health, such as significant loss of weight or obvious skin infections, we removed it from the enclosure and the experiment and medically treated it. During the 6-month period the animals were housed at the zoo, they were measured and weighed every 2 weeks.

Table 1. Body measurements (mean ± SE) of neonatal alligators obtained in 1992 as eggs from Lakes Apopka and Woodruff and hatched in captivity

Measure	Lake Apopka	Lake Woodruff
n (male/female)	5/11	9/13
Total length (cm)	34.5 ± 2.1	34.0 ± 1.3
Snout vent length (cm)	17.6 ± 1.0	17.1 ± 0.6
Weight (g)	158.0 ± 28.4	138.0 ± 17.8

Table 2. Mean (± SE) plasma concentrations (pg/ml) of estradiol-17β (E_2) and testosterone (T) in alligators 6 months of age before or after leutinizing hormone (LH) challenge[a]

	Lake Apopka		Lake Woodruff	
Sample	Male	Female	Male	Female
n	5	11	9	13
Plasma E_2	36.2 ± 4.9^a	118.1 ± 19.7^c	40.3 ± 8.8^a	76.2 ± 10.3^b
Plasma T	13.4 ± 3.9^a	13.1 ± 1.7^a	50.4 ± 9.0^b	18.5 ± 3.9^a
Plasma E/T	3.9 ± 1.1^{b,c}	11.3 ± 2.6^d	1.1 ± 0.3^a	6.1 ± 1.3^c
LH Plasma E_2	118.6 ± 32.5^b	170.7 ± 14.8^c	62.3 ± 12.7^a	82.0 ± 14.3^{a,b}
LH Plasma T	15.8 ± 3.1^a	12.3 ± 2.9^a	44.5 ± 12.5^c	28.1 ± 8.1^b
LH Plasma E/T	8.3 ± 2.7^b	20.9 ± 5.6^c	2.1 ± 0.8^a	14.1 ± 4.7^{b,c}

[a]E/T ratio is calculated by dividing the E_2 concentration by the T concentration for each animal. Sex was determined by histological examination of the gonads. Values with different superscripted letters within a row of data are signficantly different (ANOVA, $p < 0.05$ per comparison). Note that the E/T ratios reported in the table are calculated for each individual and then averaged; thus, the value reported is not the same as that calculated by dividing the means reported in the table for plasma E_2 and T concentrations.

Plasma Hormone Determinations

After 6 months of growth, all animals were measured, weighed and had a 1-ml blood sample obtained from the caudal blood vessels between 1000 and 1200 hr; all samples were obtained within 10 min of capture. Immediately after taking the blood sample, we injected each animal intraperentoneally with luteinizing hormone (LH; 10 IU; NIH; Lot S-19). A second injection of LH (10 IU) was given the next day (1000–1100 hr). Twenty-four hours after the second injection, we obtained a second 1-ml blood sample. All heparinized blood samples were stored on ice until centrifugation, which occurred within 2 hr of collection; the plasma obtained was snap-frozen in liquid nitrogen. We analyzed aliquots of the plasma samples for plasma estradiol-17β and testosterone concentrations using validated radioimmunoassays (see below).

All animals were killed after the second blood sample was obtained. Both gonads were removed intact, and the left gonad was fixed in Bouin's fixative for standard histological examination (the right gonad was cultured, and data from this study will be presented elsewhere). After fixation, intact gonads were dehydrated, embedded in paraffin, and serially sectioned at 7 µm thickness. We stained mounted sections with either alcian blue counterstained with hematoxylin and eosin or the trichrome stain of Harris. General morphology of the gonad was examined and used to determine sex of the animal. We compared gonadal sex to that assigned using external morphological features, principally the presence of a penis in males. Abnormalities in gonadal morphology were described after examining the histological preparations. Every third slide was examined throughout the set of serial sections.

Radioimmunoassays

We analyzed plasma samples from neonatal alligators for estradiol and testosterone using radioimmunoassay (RIA) procedures. For estradiol determination, samples (100 µl) were extracted with 5 ml diethyl ether before RIA analysis. Each sample was analyzed in duplicate and corrected for an extraction efficiency of 92 ± 2.4 %. Standard curves were prepared in buffer with known amounts of radioinert estradiol (1, 5, 10, 25, 50, 100, 250, 500 and 1000 pg/ml). The minimum concentration per tube that was distinguishable from zero was 5.2 pg/ml. Cross-reactivities of the estradiol antiserum (supplied courtesy of R.L. Butcher, West Virginia University; characterized by T.S. Gross) with other steroids were 11.2% for estrone, 1.7% for estriol, <1.0% for estradiol-17α, and 0.1% for all other steroids examined. A pooled sample (approximately 250 pg/ml) was assayed serially in 10, 25, 50, 75, and 100-µl volumes (final volume of 100 µl with charcoal-stripped plasma). This inhibition curve was parallel to the standard curve, with the test for homogeneity of regression indicating that the curves did not differ. Further characterization of the assay involved measuring known amounts (1, 2, 5, 10, 25, 50, 100, 250 and 500 pg/ml) of estradiol in 100 µl charcoal-stripped plasma [Y=1.23 + 1.03X; Y= amount of estradiol measured (pg); X= amount of estradiol added (pg); r^2= 0.9151]. Interassay and intra-assay coefficients of variation were 7.8 and 9.2%, respectively.

For testosterone determinations, we also extracted samples (100 µl) with 5 ml diethyl ether before RIA analysis. Each sample was analyzed in duplicate and corrected for the extraction efficiency of 84 ± 3.3%. Standard curves were prepared in buffer with known amounts of radioinert testosterone (1, 5, 10, 25, 50, 100, 250, 500 and 1000 pg/ml). The minimum concentration per tube that was distinguishable from zero was 7.9 pg/ml. Cross-reactivities of the testosterone antiserum (purchased from ICN Biomedicals, Inc., Wilmington, DE; lot 07-189016) with other steroids were 18.75% for 5α-dihydrotestosterone, 3.0% for 5α-androstenediol, <1.0% for androstenedione, and 0.1% for all other steroids examined. A pooled sample (approximately 180 pg/ml) was assayed serially in 10, 25, 50, 75, and 100 µl volumes (final volume of 100 µl with charcoal-stripped plasma). This inhibition curve was parallel to the standard curve, with the test for homogeneity of regression indicating that the curves did not differ. The assay was further characterized by measuring known amounts (1, 2, 5, 10, 25, 50, 100, 250 and 500 pg/ml) of testosterone in 100 µl charcoal-stripped plasma [Y=3.40 + 0.95X; Y= amount of testosterone measured (pg); X= amount of testosterone added (pg); r^2= 0.8763]. Interassay and intra-assay coefficients of variation were 8.7 and 10.3 %, respectively.

Statistics

We tested for differences in hormone concentrations between sexes and lakes by two-way ANOVA (Stat-view II, Abacus Concepts, Inc., Berkeley, CA, 1988). Where significant (p<0.05) variation existed, Scheffe's F-tests were performed. Student's t-tests were used to compare differences in hatchling weights and lengths. A repeated-measures ANOVA was performed to determine differences in growth patterns between lakes and among clutches. Ratio data (estrogen/testosterone ratios) were arcsin-transformed before significance testing to achieve homogeniety of variance (*27*).

Results

Mean clutch size (± 1 SE) for Lake Apopka in 1992 was 47.8 ± 1.3, with an average of 13.9 ± 3.5 hatchlings produced per clutch. Viability is calculated as the number of eggs that produce a viable hatchling as a percentage of the number of eggs laid. Clutch viability was depressed for clutches from Lake Apopka collected in 1992 when compared to viability rates reported from other lakes in other years, which averaged 51.5 % (*21*). Mean viability (28.3 ± 7%) for the 24 Lake Apopka clutches incubated was not different from that observed during the previous 2 years [1990 = 30.8%; 1991 = 28.6%: (*21*)] on Lake Apopka (note that 1 clutch collected from Lake

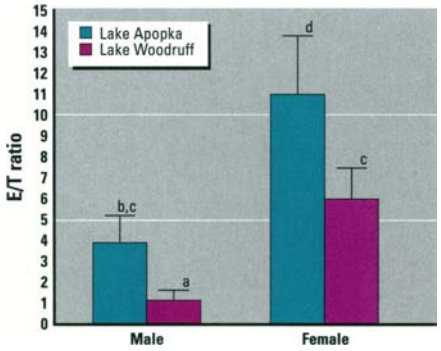

Figure 2. Mean (± SE) estrogen/testosterone ratios for male and female juvenile alligators from Lakes Apopka and Woodruff. Bars with different letters are significantly different.

Apopka in 1992 was not incubated because all eggs were dead upon collection from the nest). However, this mean misrepresents actual viability of many clutches from Lake Apopka, as we observed that these clutches either produced many offspring or no offspring. Of the 24 clutches of eggs incubated, 8 clutches produced no viable offspring, whereas 5 clutches exhibited viability rates of 75% or greater. Neonatal survival was reduced for young from Lake Apopka with 41% mortality within 10 days after hatching, whereas mortality for neonates from Lake Woodruff was less than 1%. There was no significant difference, however, in the length or weight of neonates at hatching when the two lakes were compared (Table 1).

During the 6 months young alligators were raised in captivity, 9 of the original 25 neonates from Lake Apopka died, whereas 2 of the 25 hatchlings from Lake Woodruff died. An additional neonate from Lake Woodruff was removed from the study due to an eye infection that required treatment. For neonates from Lake Apopka that survived, no significant difference was detected in body weight or total length after 6 months of growth when compared to Lake Woodruff neonates. The only major effect noted was that the clutch from which a neonate hatched significantly influenced its growth. A repeated-measures ANOVA, examining the effect of clutch on hatchling growth, indicated a strongly positive effect (SVL: $F = 5.756$, $df = 19, 564$; $p < 0.0001$; weight: $F = 5.445$; $df = 19, 560$; $p < 0.0001$).

Female juvenile alligators from Lake Apopka had significantly higher plasma estradiol-17β (E_2) concentrations ($F = 15.08$; $df = 1, 34$; $p = 0.005$) when compared to either females from Lake Woodruff or to males from either lake (Table 2). In contrast, male alligators from Lake Woodruff exhibited a mean plasma testosterone (T) concentration that was almost four times higher than those of males from Lake Apopka or of females from either lake ($F = 8.39$; $df = 1, 34$; $p = 0.0065$; Table 2). Males from Lake Apopka had a mean plasma T concentration that was not significantly different from that of females from either lake (Table 2). The E_2 and T data can be expressed as a ratio that represents the steroid milieu circulating in the animal. Male and female juvenile alligators (sex determined by gonadal histology) from Lake Woodruff have unstimulated, normal plasma E/T ratios, whereas the females and males from Lake Apopka have significantly elevated plasma E/T ratios ($F = 4.46$; $df = 1, 34$; $p = 0.042$; Fig. 2).

After gonadal stimulation with two injections of LH, a similar relationship was observed in circulating hormone concentrations (Table 2) and E/T ratios, with Lake Woodruff alligators having ratios of 2.05 for males and 14.1 for females, whereas males from Lake Apopka exhibit a ratio of 8.3 and females a ratio of 20.9. It is important to note that the E/T ratio in both Apopka and Woodruff males increased between nonstimulated and stimulated, indicating that the testes responded by synthesizing larger quantities of E_2 than T. However, the responsiveness of the males from Lake Apopka (3.3 times increase in E_2 synthesis) was greater than that observed from Lake Woodruff males (1.5 times) and from females of either lake (Lake Apopka females = 1.4 times; Lake Woodruff females = 1.1 times). These data indicate that all animals were sensitive to LH stimulation, but males from Lake Apopka were more responsive to LH stimulation and responded by making large amounts of E_2. Even though females from Lake Apopka exhibited the poorest response to LH stimulation, they exhibited the highest unstimulated and stimulated plasma concentrations of E_2 (Table 2).

Histological examination of the gonad confirmed the sex of all animals assigned by external morphological features, except four animals from Lake Apopka. In two cases, animals identified as females, owing to the lack of a penis, were subsequently observed to have testes based on histological structure. Additionally, two animals were identified as males, owing to the presence of a penislike structure (enlarged clitoral development?) but had histologically identifiable ovarian tissue.

Histological examination of the ovaries and testes obtained from Lake Woodruff animals revealed normal structures consistent with the age of the animals examined. Females had ovaries characterized by a surface epithelium composed of squamous cells overlying an ovarian cortex with many primary follicles (Fig. 3). The ovarian follicles at this stage consisted of a single or double layer of granulosa cells surrounding a single oocyte with a distinct nucleus. The nucleus of some of the larger follicles was almost completely surrounded by a crescent of granular material (Fig. 3B) previously identified as a Balbiani body (*28*). The presence of the Balbiani body was not ubiquitous among the follicles. Lying below the cortex was a region of loose connective tissue and internal ovarian epithelium which surrounded a series of lacunae (Fig. 3). Some of the lacunae contained eosinophilic secretory material of unknown composition or source. Lacunae are a normal morphological feature of the crocodilian ovary and represent an organized medullary region of the ovary. Although the general ovarian histology observed in the females from Lake Apopka was similar to that described above for animals from Lake Woodruff (Fig. 3), significant cytological differences were noted (Fig. 4). The most notable distinction was the presence of large numbers of polyovular follicles and polynuclear oocytes (Fig. 4B,C,D) in ovaries from Lake Apopka females, which were never seen in ovarian tissue obtained from Lake Woodruff females. It was common to find as many as three to four oocytes per follicle and two or three nuclei per oocyte. Every ovary obtained from a Lake Apopka female exhibited this condition, whereas we saw no polyovular follicles or polynuclear oocytes in ovaries from Lake Woodruff females.

The testes from Lake Woodruff males were histologically characterized by the presence of well-formed seminiferous tubules lined with germ cells at early stages of development, and Sertoli cells (Fig. 5A). Mitotic activity was noted in many of the germ cells. The testes from Lake Apopka males had poorly organized seminiferous tubules (Fig. 5B,C), many of which were lined by a cuboidal epithelium not observed in testes of males from Lake Woodruff. Additionally, within the seminiferous tubules of Apopka males, there were cells with bar-shaped nuclei unlike anything observed in the testes obtained from Lake Woodruff males (Fig. 5B). The significance of these structures is unknown; to our knowledge, they have not been reported previously in reptilian testicular tissue. They may represent aberrant or premature meiotic elements.

Discussion

Alligator eggs and neonates from Lake Apopka differ significantly from those obtained from Lake Woodruff. First, embryos and neonates within the first 10 days of life from Lake Apopka exhibit high mortality rates. Although the neonates from Lake Apopka show no difference in

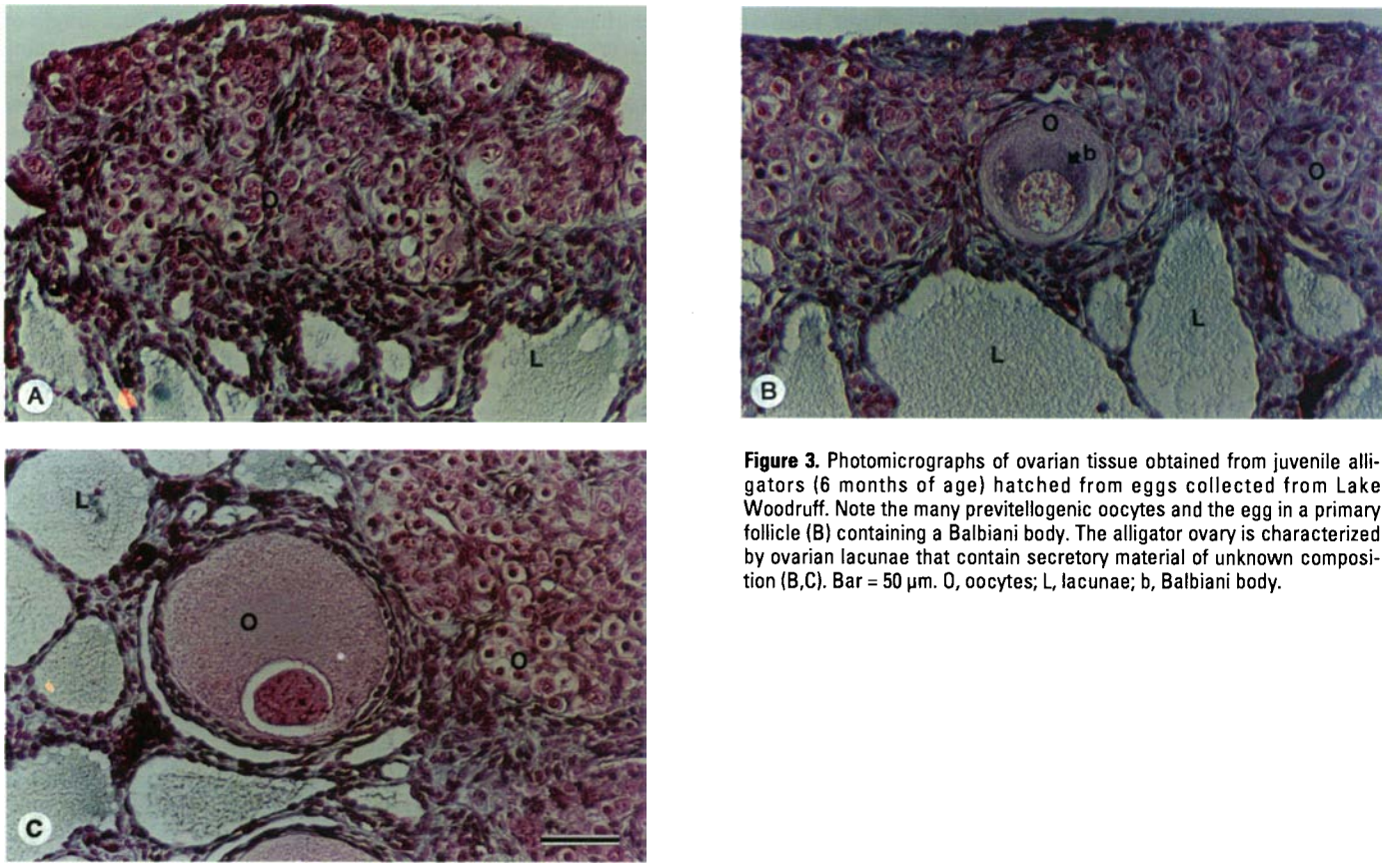

Figure 3. Photomicrographs of ovarian tissue obtained from juvenile alligators (6 months of age) hatched from eggs collected from Lake Woodruff. Note the many previtellogenic oocytes and the egg in a primary follicle (B) containing a Balbiani body. The alligator ovary is characterized by ovarian lacunae that contain secretory material of unknown composition (B,C). Bar = 50 µm. O, oocytes; L, lacunae; b, Balbiani body.

growth during the first 6 months of life, they do exhibit significantly different plasma sex steroid (testosterone and estradiol) concentrations. The modified hormone concentrations correspond with differences in the histological appearance of the gonads. Females from Lake Apopka exhibit ovaries containing large numbers of polyovular follicles and polynuclear oocytes. Testes from males show poorly organized seminiferous tubules.

The viability of alligator eggs has been studied under laboratory conditions for various populations throughout the United States. Alligator eggs removed from the wild and incubated under laboratory conditions can yield viability rates greater than 80% (*3*). However, significant variation among lakes in the viability of alligator eggs (under controlled laboratory conditions) has been reported (*21,22*). Clutches have been collected in the past from Lake Apopka and average 18.3% hatchability as opposed to over 57% from six other lakes studied over the last 5 years (n=1215 clutches, incubated under control conditions) (*21*). Throughout the 1980s, clutches from Lake Apopka consistently have had the lowest viability of any reported, ranging from a high of 54% in 1983 to a low of 3.9% in 1988 (*10,21,22*). A mean viability of 54% in 1983 is the highest currently reported for Lake Apopka, although clutch viability may have been higher before the 1980s. By combining all the available data (*10,21,22*; this study), annual viabilities for clutches from Lake Apopka average 23.2% (n=182 nests) for the years 1983–1986 and 1988–1992. This is substantially lower than any other lake system previously studied in Florida.

Depressed egg viability can be the result of several independent factors or the combined effects of many factors. Several hypotheses have been suggested to explain the elevated embryonic mortality in alligators reported for Lake Apopka (*10,21,22*). First, female alligators from Lake Apopka may produce eggs lacking specific biotic factors required for embryonic development and survival, such as trace amino acids, fatty acids, or ions, as a result of a shortage in the female's dietary supply (*29*). However, at the present time there is no conclusive data to support or reject this possibility. Second, females from Lake Apopka could have genetic abnormalities that contribute to the production of abnormal eggs or embryos with genetic defects. Third, the nesting environment (material composition of nests) could induce embryonic mortality. Fourth, shifts in the age structure of the breeding population, (e.g., many young and/or old females) can depress egg viability, as can density-related stress of the adult population (*4*). Finally, direct effects of xenobiotic factors can contribute to the production of abnormal eggs, embryonic mortality, and neonatal death (*18*).

Studies examining the developmental genetics of reptiles are consistent with the hypothesis that abnormalities could cause death, but no information is available concerning the genetics of adult, juvenile, and embryonic alligators from any of the lakes we have studied to date in Florida. Many studies have suggested a possible correlation between allozyme genotype and differential survival to environmental contaminants or toxins in natural and/or experimental populations of aquatic organisms (*30*). These observations suggest that the population genetics of the alligators from Lake Apopka may vary from those of non- or less-contaminated lakes, but again no data are available to test this assumption. Moreover, no studies are available linking environmental contaminants directly or indirectly to genetic abnormalities in reptiles that would contribute to embryonic mortality.

A previous study (*22*) examined the nesting environment on seven lakes in Florida and noted no major differences in embryonic viability when nest materials were compared with embryonic viability. Lake Apopka has many nests composed of *Sagittaria*, a type of nest vegetation associated with lower nest heights and more shallow caviry depths. However, no relationship was noted between viability and

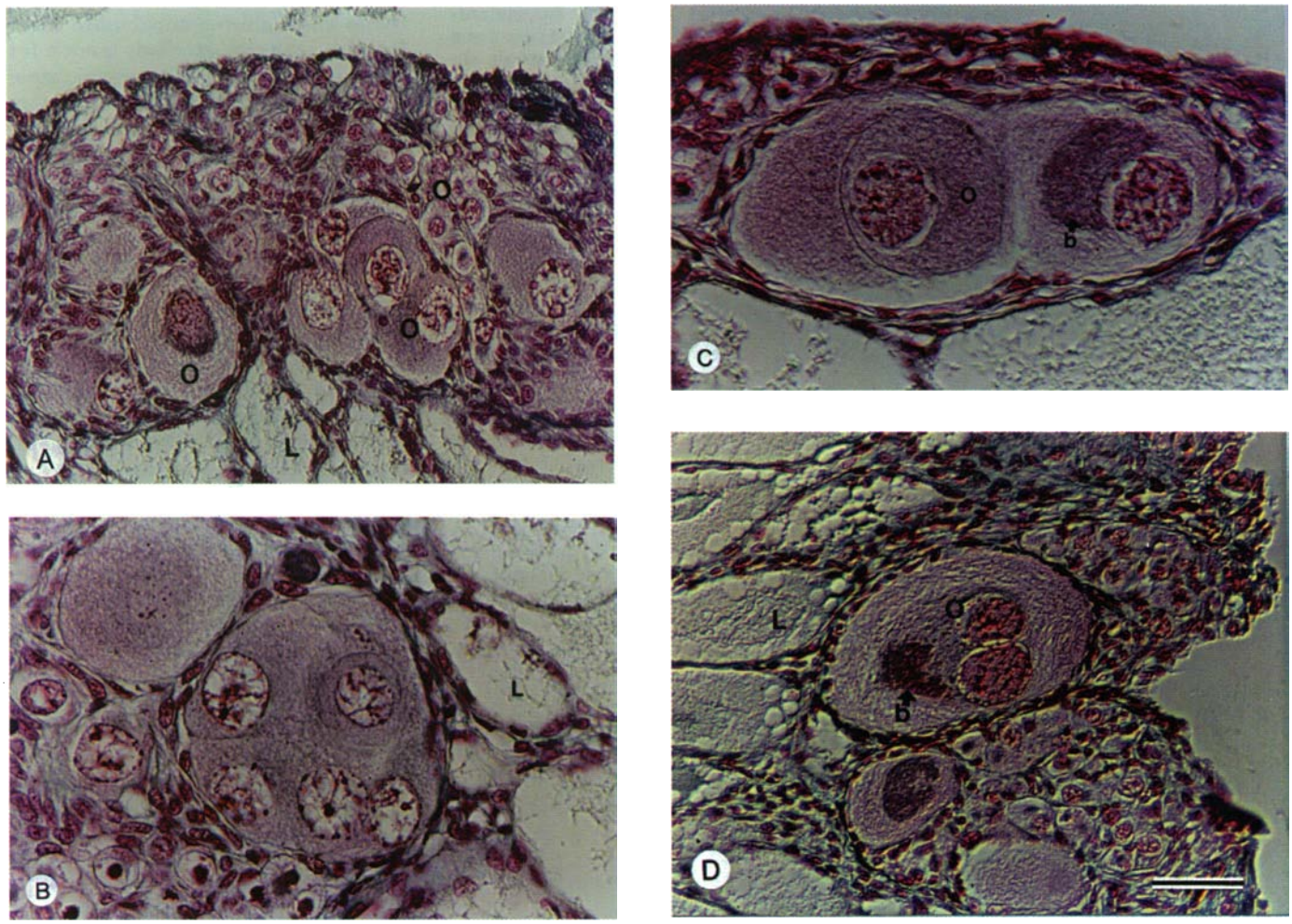

Figure 4. Photomicrographs of ovarian tissue obtained from juvenile alligators (6 months of age) hatched from eggs collected from Lake Apopka. The general morphology is similar to that seen in tissue from animals from the control lake, but histological examination reveals a large numbers of polyovular (A,B,C) follicles and polynuclear (B,D) oocytes. Bar = 50 μm (A,D) and 25 μm (B,C). O, oocytes; L, lacunae; b, Balbiani body.

any nest parameter measured (*22*). The study by Woodward et al. (*10*) concluded that neither stress induced by elevated population density nor age structure of the breeding population at Lake Apopka appears to contribute significantly to the poor egg viability observed. They suggested that the strong association between the 1980 Tower Chemical Co. spill and the subsequent rapid decline in the juvenile population and egg viability could provide the best clue to the reproductive failure reported for Lake Apopka. These observations, combined with studies showing elevated levels of the contaminant *p,p'*-DDE in alligator eggs (collected during 1984–1985) from Lake Apopka (*16*), suggest that contaminants are a major contributor to low egg viability.

We suggest that xenobiotic compounds modify gonadal development and function in alligator neonates and are also responsible for reduced embryonic viability. Almost nothing is known concerning the effects of xenobiotic agents on reptiles. The few studies available suggest that 1) reptiles exhibit a sensitivity to contaminants similar to that reported for birds and mammals (*31*), 2) reptiles bioaccumulate and biomagnify contaminants to levels equal to or greater than that reported for birds and mammals (*32–34*), and 3) reptiles exhibit a higher incidence of embryonic mortality and deformity with elevated concentrations of various contaminants (*35*). The suite of reproductive disorders reported to date in vertebrates exposed to xenobiotic compounds include reduced fertility, reduced hatchability, reduced viability of offspring, impaired hormone activity, structural abnormalities of the reproductive tract and/or altered adult sexual behavior (*18,36*). Our study provides evidence of reduced hatchability, reduced viability of offspring, and endocrine demasculinization of males or superfeminization of female alligators.

Many reptilian species exhibit unique facets of sexual determination. Many crocodilian (including alligators) and turtle species exhibit environmentally determined sex. Genetic factors associated with sex determination in reptiles, and thus gonadal development, are poorly understood but the role of temperature and sex steroids has been examined (*37–39*). In alligators, the temperature of incubation at specific critical periods of embryonic development triggers the determination of sex (*37*). Incubation temperature induces an all-or-none response, so that embryos are males or females and there are few intersexes (*40*).

Alligators and several turtle species can exhibit sex reversal (male to female) if developing embryos are exposed to an estrogenic compound during a specific period of time, usually the second third of the embryonic developmental period. Specifically, estrogen treatment of alligator (*41*) and turtle (*42–44*) eggs incubated at male-producing temperatures can cause sex reversal and the production of apparently normal females. Crews et al. (*44*) found that estradiol benzoate (EB) or an estrogen agonist (R2858) were equally effective in inducing turtle embryos to develop as females when eggs were incubated at male-producing temperatures. Further, they

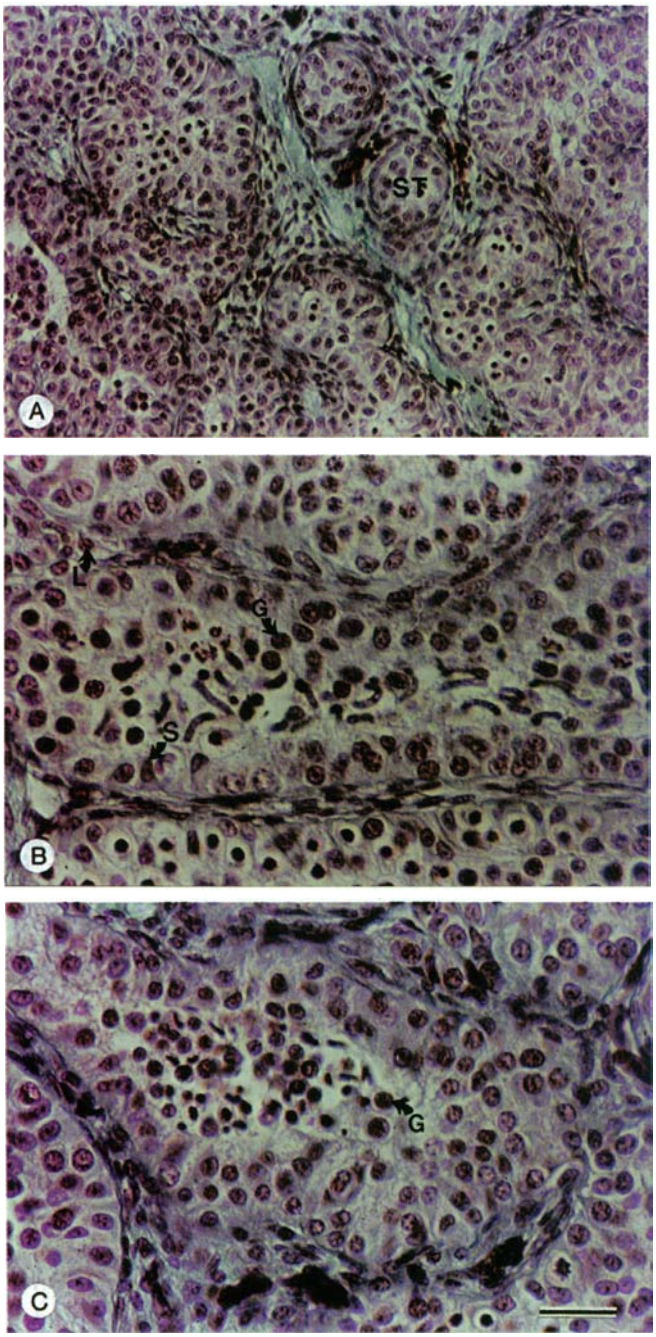

Figure 5. Photomicrographs of testicular tissue obtained from juvenile alligators (6 months of age) hatched from eggs collected from Lakes Woodruff (A) and Apopka (B,C). Tissue obtained from Lake Woodruff animals had well-organized seminiferous tubules with clearly defined Leydig and spermatogonia. Some of the germ cells display mitotic activity indicating early stages of spermatogenesis (A). In contrast, testes from Lake Apopka males had poorly organized tubules and cellular structures (bar-shaped structures) of unknown function or origin (B,C) indicated by the arrows. Bar = 50 μm (A) and 25 μm (B,C). G, germ cell; L, Leydig cell; S, Sertoli cell; ST, seminiferous tubules.

found that some of the EB-treated turtle embryos exhibited hypertrophied oviducts (*44*). As in turtles, alligator eggs incubated at a male-producing temperature and treated with 100 μg or 300 μg EB/egg induced sex reversal in 100% of the eggs so treated (*41*). Intersex individuals are produced when the concentration of an estrogenic compound is below a specific threshold. With intermediate treatment levels, an embryo may exhibit Müllerian (female) ducts and a testis or even an ovotestis. Turtle eggs exposed by painting or injecting them with estradiol or other estrogenic compounds (PCBs; Bergeron and Crews, personal communication) show such partial reversal. Bergeron and Crews (personal communication) observed that 2′,4′,5′-trichloro-4-biphenylol induced 100% sex reversal (based on histological examination of gonads and internal ducts) in the red-eared turtle (*Chrysemys nelsoni*), whereas treatment with 2′,3′,4′,5′-tetrachloro-4-biphenylol stimulated total sex reversal in 50% of the embryos and partial sex reversal (intersex) in 21% of the embryos. Interestingly, turtle neonates (*Chrysemys nelsoni*) from Lake Apopka have either apparently normal ovaries or ovotestes (intersex) (Gross and Guillette, unpublished data). Moreover, amniotic fluid concentrations of E_2 and T indicate that no turtle hatchling from Lake Apopka produces a normal androgen synthesis pattern (Gross and Guillette, unpublished data). Reptiles represent excellent models for determining the extent of estrogenic xenobiotic contamination in an ecosystem owing to the apparent lability of sex determination in response to the presence of estrogen or estrogenlike compounds.

Determination of sex reversal in all the laboratory studies on turtles and alligators reported above is based on histological observation of the gonad and reproductive tracts, but no reports are available on the steroidogenic capabilities of these sex-reversed individuals at hatching or at later stages in life. Our observations that gonadal morphology and plasma hormone concentrations in male and female alligators are both modified provide further support for the hypothesis that the depressed population on Lake Apopka is contaminant induced. Circulating sex steroid concentrations in both male and female alligators from Lake Apopka are significantly different when compared to those of a control population from Lake Woodruff. As in other vertebrates, the reptilian gonad synthesizes sex steroids in a sexually dimorphic pattern. Adult males are characterized by elevated androgens, primarily testosterone, whereas females exhibit elevated estradiol-17β during the follicular growth phase of the reproductive cycle (*45*). As with mammals and birds, steroids appear essential for normal development of the reproductive tract in reptiles, including alligators (*46,47*), and a recent study has reported sexual dimorphism in circulating testosterone concentrations of juvenile green sea turtles (*48*). A sexually dimorphic pattern in plasma or allantoic fluid sex steroid concentrations is obvious at birth in alligators (Gross and Guillette, unpublished data).

Male alligators, 6 months of age, hatched from eggs collected on Lake Apopka had significantly depressed plasma T concentrations when compared to males from the control population from Lake Woodruff. Previous studies have reported significantly depressed plasma androgen levels in various mammalian species after experimental exposure of adult males to various estrogenic xenobiotic contaminants or phytoestrogens (*49–51*). However, these compounds are thought to have a greater direct impact on the developing reproductive system than on the adult system; thus, embryonic exposure may induce significant modifications in male anatomy and physiology (*52–54*).

The mechanisms by which xenobiotic chemicals alter steroidogenesis are still under study, but those identified include: 1) reduced gonadotropin-releasing hormone (GnRH) synthesis from the hypothalamus, 2) reduced LH release from the pituitary, 3) reduced availability of the pre-

cursor cholesterol, 4) modification of the enzymes required for steroidogenesis (e.g., aromatase, cytochrome $P450_{scc}$), and 5) modifications of the cellular receptor numbers and function (*51*). Peterson et al. (*20*) reported a decrease in plasma testosterone concentration in rats exposed *in utero* to small concentrations (0.064 mg/kg) of dioxin. They also reported a 95% reduction in plasma concentrations of LH, the hormone responsible for stimulating testicular steroidogenesis. Similarly, male rodents exposed neonatally to diethylstilbestrol (DES) or *o,p'*-DDT exhibit significantly reduced plasma LH concentrations in response to a GnRH challenge (*55*). As expected, the effective dose varies with the compound used; in the case of the phytoestrogen genistein, some doses can increase LH release, whereas other doses depress plasma LH concentrations (*56*). One mechanism by which the xenobiotic dioxin modifies plasma androgen concentrations in adult male rodents is to inhibit testicular cholesterol mobilization while leaving unaltered cellular concentrations of cytochrome $P450_{scc}$, responsible for the conversion of cholesterol to pregnenolone, the initial step in gonadal steroidogenesis (*51*). Interestingly, this effect is organ specific, as adrenal steroidogenesis is apparently altered after dioxin exposure by reduction of this cytochrome (*57,58*). These studies in adult rodents provide clues to possible loci affected by *in utero* exposure, but mechanisms by which steroidogenesis is altered after embryonic or neonatal exposure to estrogenic xenobiotics are still under study. Further studies must examine gonadal steroidogenesis in other wildlife, as our data suggest that a modification of gonadal androgen and estrogen synthesis has occurred.

In addition to the differences in viability and plasma sex steroid concentrations, we noted significant modifications in gonadal morphology. Both polyovular ovarian follicles and polynuclear oocytes were observed. Polyovular follicles do appear spontaneously in vertebrates and have been reported in a variety of mammals (*25*). Treatment of female rodents, either pre- or postnatally, with DES increases both the frequency of polyovular follicles and the number of females exhibiting this condition (*25*). Polyovular follicles stimulated with gonadotropin ovulated and were capable of fertilization, although the number of eggs fertilized was significantly lower than for uniovular follicles (*59,60*). Further, fertilized ova from DES-induced polyovular follicles will develop into blastulae and continue to develop to implantation-stage embryos, but the frequency of embryos reaching this stage is significantly reduced compared to embryos derived from uniovular follicles (*61*). In species exposed to xenobiotic estrogens, reduced viability of fertilized eggs, owing to their release from polyovular follicles as reported above, can contribute to the low hatching rates reported for various wildlife species. In fact, Masson (*22*) reported that most embryonic mortality occurred in alligator eggs before oviposition, i.e., during the blastula to late gastrula stages of embryonic development.

In conclusion, our data support the hypothesis that contamination of a wildlife population with an estrogenic xenobiotic has the potential to significantly alter embryonic sexual development and thereby significantly depress subsequent reproductive success. These compounds can work at various biological levels, from outright mortality of eggs and adults to more subtle effects such as modification of the steroidogenic capacity of the gonad. These studies have only begun to address what we believe could be a serious, widespread threat to wildlife populations. Our observations also stress the need for more complete examination of the reproductive functioning and health of other species, including humans, living adjacent to Lake Apopka. Future studies of natural populations exposed to xenobiotic estrogens must include an approach that goes from gene to ecosystem, so that the mechanisms by which these xenobiotics influence an individual can be addressed as well as determine the consequences to a population.

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