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NECROPSY FINDINGS IN AMERICAN ALLIGATOR LATE-STAGE EMBRYOS AND HATCHLINGS FROM NORTHCENTRAL FLORIDA LAKES CONTAMINATED WITH ORGANOCHLORINE PESTICIDES

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ABSTRACT: Increased American alligator (*Alligator mississippiensis*) embryo and neonatal mortality has been reported from several northcentral Florida lakes contaminated with old-use organochlorine pesticides (OCPs). However, a clear relationship among these contaminants and egg viability has not been established, suggesting the involvement of additional factors in these mortalities. Thus, the main objective of this study was to determine the ultimate cause of mortality of American alligator late-stage embryos and hatchlings through the conduction of detailed pathological examinations, and to evaluate better the role of OCPs in these mortalities. Between 2000 and 2001, 236 dead alligators were necropsied at or near hatching (after ~65 days of artificial incubation and up to 1 mo of age posthatch). Dead animals were collected from 18 clutches ranging in viability from 0% to 95%. Total OCP concentrations in yolk ranged from ~100 to 52,000 μg/kg, wet weight. The most common gross findings were generalized edema (34%) and organ hyperemia (29%), followed by severe emaciation (14%) and gross deformities (3%). Histopathologic examination revealed lesions in 35% of the animals, with over half of the cases being pneumonia, pulmonary edema, and atelectasis. Within and across clutches, dead embryos and hatchlings compared with their live cohorts were significantly smaller and lighter. Although alterations in growth and development were not related to yolk OCPs, there was an increase in prevalence of histologic lesions in clutches with high OCPs. Overall, these results indicate that general growth retardation and respiratory abnormalities were a major contributing factor in observed mortalities and that contaminants may increase the susceptibility of animals to developing certain pathologic conditions.

Key words: *Alligator mississippiensis*, contaminants, embryos, hatchlings, histopathology, necropsy, toxicology.

INTRODUCTION

Beginning in the 1940s and continuing through the 1980s, aquatic systems from the upper Ocklawaha River basin in northcentral Florida have suffered habitat loss and population decline of several wildlife species. Indeed, much of the marsh and wetland area surrounding this chain of lakes has been diked and drained for agricultural use or muck farming. The removal of thousands of hectares of shallow lake bottom has resulted in the loss of spawning habitat for several species of sport fish (Benton et al., 1991). In addition to the loss of habitat, this chain of lakes has been polluted by the leaching of old-use organochlorinated pesticides (OCPs) from muck farm soils, sewage discharge, and by ongoing agricultural sources that release nutrient- and pesticide-rich irrigation water (Marburger et al., 2002).

In the last decade, several reports have linked OCPs to endocrine-disrupting effects and population declines in American alligators (*Alligator mississippiensis*) inhabiting northcentral Florida lakes. Reported endocrine-disrupting effects in alligators from Lake Apopka have included altered secondary sex characteristics,

endocrine status, and sex differentiation in hatchlings and juveniles (Gross et al., 1994; Guillette et al., 1994, 1995, 1999a, b). More recently, increased alligator mortalities have also been described from another Florida lake, Lake Griffin. Since 1997, over 300 adult alligators from this lake have been found either dead or lethargic and unresponsive (Schoeb et al., 2002). Although the ultimate cause(s) for these mortalities has not been determined, detailed clinical and pathological examinations have indicated that the nervous system was primarily affected.

To understand better why populations of American alligators are declining at these sites, a series of field and laboratory studies have been conducted over the last decade by this and other research groups. Field studies have shown significant exposure of alligators to OCPs, especially in animals inhabiting reflooded muck-farm environments (Rauschenberger et al., 2004; Sepúlveda et al., 2004). The most notable finding so far has been the observation that these populations produce embryos that die before or soon after hatching (Woodward et al., 1993; Masson, 1995; Giroux, 1998; Rauschenberger et al., 2004). Thus, our overall working hypothesis is that one or more OCPs found at these sites are causing decreased early-life-stage survivorship in animals, and that this decline is the likely cause for the low recruitment observed at these sites. To date, however, a clear relationship among OCPs and early-life-stage mortality has not been established. Furthermore, it is not clear if effects on offspring quality and survival are the result of direct exposure of early-life stages to OCPs accumulated in the yolk through maternal transfer and/or to impairment of the parent's ability to produce quality eggs and sperm due to OCPs or to other environmental factors.

The main objective of this study was to determine the ultimate cause of mortality of American alligator late-stage embryos and hatchlings through the conduction of detailed pathological examinations, and to evaluate better the role of OCPs in these mortalities.

MATERIALS AND METHODS

Sites and animal collections

As part of a long-term health-monitoring program of American alligator populations by our laboratory, egg clutches were collected from several northcentral Florida lakes over the course of two nesting seasons (June/July 2000 and 2001). Lakes Apopka (28°37′N, 81°37′W), Griffin (28°50′N, 81°51′W), and Lochloosa (29°27′N, 82°10′W) and the Emeralda Marsh Conservation Area (28°57′N, 81°48′W), a reclaimed/flooded agricultural marsh east of Lake Griffin, were selected as collection sites. Prior studies by our laboratory (conducted between 1999 and 2002) have indicated vastly different levels of OCP exposure and early-life-stage mortality across these sites. Lake Lochloosa was selected as the reference site because of its low level of OCPs (overall mean for total OCPs in yolk of 231±30 μg/kg) and high clutch viability, defined as the number of live hatchlings/number of eggs in the clutch (71±26%) (Rauschenberger et al., 2004). Lake Griffin was selected as an intermediate OCP-exposure site because yolk concentrations average 4,414±617 μg/kg, and Lake Apopka and Emeralda were selected as high OCP-exposure sites because yolk concentrations average 15,911±1,786 μg/kg and 15,238±1,787 μg/kg, respectively (Rauschenberger et al., 2004). In addition, clutch success on Lakes Apopka and Griffin and on Emeralda are below those observed at the reference Lochloosa site (51±31% , 44±33%, and 52±35%, respectively) (Rauschenberger et al., 2004).

Nests were located by aerial survey (helicopter) and/or from the ground (airboat). Prior to their collection, eggs were marked on the top surface with a pencil. Because alligator embryos attach to the top surface of the egg very early during development, marking them prevents from positioning the embryo on the bottom surface of the egg and causing asphyxiation. Complete clutches were transported to the laboratory in plastic pans (48 × 36 × 17 cm) filled with the original nest substrate material and covered with lids that had holes for ventilation. Upon arrival (<8 hr after collection), eggs were candled, and, if viable, placed with the pencil markings facing up in the same plastic tubs. Original nest material was removed from each pan and replaced with sphagnum moss as substrate. Clutches were incubated in an artificial in-

cubation building (7 × 4 m) at a temperature and relative humidity of 30–33 C and 88–92%, respectively. This intermediate incubation temperature will normally result in a 1 : 1 male : female sex ratio (Ferguson and Joanen, 1982).

Eggs were classified as nonviable if they were cracked, lacked a noticeable band around the equatorial center of the egg (also referred to as an unbanded egg), or were banded but contained a dead embryo. The latter eggs are easily distinguishable from live-banded eggs because, when candled, blood vessels from the chrorioallontoid membrane are usually absent and contents appear dark due to a decline in light transmittance. In addition, one viable egg was randomly selected from each clutch, opened, and the embryo collected to determine the age of the clutch. Yolks from these eggs were also collected for later toxicologic analyses (see below).

Monitoring of embryo and hatchling survival

During the course of the ~65-day incubation period, clutches were monitored for embryo survival by candling eggs approximately every 2 wk. Although many embryos died earlier during development, data from these animals are not presented here because it is the subject of a separate publication. Rather, this study focused on determining cause of death in full-term embryos and hatchlings. Full-term or late-stage dead embryos (days 56–65 of age) were those produced from eggs that failed to hatch within a 2-day period after the last egg of a particular clutch had hatched. Animals that died in the process of hatching were also included in this group. In most cases, these were hatchlings that had begun cracking the eggshell and had their heads partially or totally out of the egg. These late-stage embryos were fully necropsied as described below.

Hatchling survival was also monitored for up to 4 wk of age. Two days posthatch, live hatchlings were weighed and measured, tagged, and transferred to indoor rectangular (137 cm × 76 cm × 61 cm) or circular (122 cm × 76 cm) holding tanks. Animals were housed by clutch in groups of 10, thus available floor space was ~0.1 m^2 per hatchling. Tanks were filled with clean well water to a depth of ~10 cm, provided with a heating lamp set up for a light cycle of 12 hr light:12 hr dark, and kept clean of debris by rinsing them with well water every other day. As tanks were maintained on a slope, animals had access to both dry and wet environments. Ambient and water temperatures ranged from 28 to 35 C. Tanks were checked daily for the presence of dead hatchlings, which were measured and necropsied as explained below. Animals were not fed during the first 2 wk posthatch because they were expected to use their yolk reserves during this time. At 2 wk of age, hatchlings were fed a pelleted diet (AquaXcelTM4710, Burris Mill & Feed, Inc., Franklinton, Louisiana, USA) every other day ad libitum. At the end of this monitoring period, survivors were returned to their original nest location using geographical positioning system (GPS) information gathered at the time of egg collection. Overall, clutch production was calculated as the total number of hatchlings that survived 1 mo posthatch over the total number of viable eggs set for incubation times 100.

Animal measurements and necropsies

Prior to necropsy, dead late-stage embryos and hatchlings were weighed to the nearest 0.1 g, and head length (HL; base of the skull to tip of the nose), total length (TL; tip of the nose to tip of the tail), and snout-vent-length (SVL; proximal end of vent to tip of snout) were measured to the nearest 0.1 mm with digital calipers. Necropsies were thorough and consisted of measuring several organ weights as well as collecting tissues for histopathologic and microbiologic analyses. Liver, heart, kidneys, lungs, yolk, and fat body were excised from all animals and weighed to the nearest 0.01 g. In crocodilians, the fat body is a morphologically distinct structure located in the coelomic cavity, immediately posterior to the liver. Because body weights differed significantly both within and across clutches, organ weights were corrected by differences in body weight through the calculation of organosomatic indexes. These indices are commonly employed in aquaculture studies that have examined effects of different environmental stressors on the health of aquatic organisms (e.g. Shoemaker et al., 2003; Sang and Fotedar, 2004). Organosomatic indexes were calculated by dividing the weight of the organ by the weight of the animal and multiplying the resulting number by 100. Tissues routinely collected for histopathologic analyses included brain, spinal cord, trachea, lungs, heart, esophagus, stomach, small and large intestines, pancreas, liver, spleen, kidneys, thyroid, thymus, adrenal glands, and tail muscle. In some instances, blood and tissue swabs were also collected for microbiologic analyses. These procedures are explained in more detail below. Because animals were hatched under controlled captive conditions,

it was considered unlikely that they would have been parasitized, and thus routine parasite screens were not conducted. In addition, no signs of parasitism were observed during the necropsies.

Histopathology, histochemistry, and indirect immunohistochemistry

Histopathologic analyses were conducted at the Departments of Pathobiology and Clinical Studies, New Bolton Center, University of Pennsylvania. Formalin-fixed (10% buffered formalin) and trimmed tissues were embedded in paraffin, sectioned at 5 μm, mounted on glass slides, air dried, and stained with Mayer's hematoxylin and eosin (H&E). Selected tissues with lesions were also examined with Gram stain, for better bacterial characterization and with Giemsa stain for *Mycoplasma*-like organisms and Chlamydiaceae elementary-body identification. Positive controls included the trachea of chickens infected with *Mycoplasma gallisepticum* and the liver of a bird infected with *Chlamydophila psittaci.* Selected tissue sections with visible bacteria and inflammation where stained with indirect immunohistochemistry to detect *Chlamydophila* spp. and *Listeria monocytogenes*, using primary polyclonal antibodies against specific epitopes of these bacteria. Positive controls included avian tissues infected with *Chlamydophila* spp. and sheep brain infected with *L. monocytogenes*. Negative controls included specific pathogen-free tissues and the omission of the primary antibody.

Microbiology

Samples for aerobic/anaerobic bacterial and fungal cultures were collected at necropsy (heart, lung, liver, intestines, and cloaca) from a subset of the animals using CulturetteS-wabs® (Becton-Dickinson, Franklin Lakes, New Jersey, USA). Prior to the exposure of organs, the skin was scrubbed with cotton soaked in 70% ethanol. The skin was then cut with a sterile blade, and using flamed scissors and forceps, organs of interest were exposed and a swab sample collected. In addition, blood samples were collected antemortem from the occipital sinuses of sick and dying hatchlings using a sterile 1-ml tuberculin syringe fitted with a 27-gauge 1/2-inch needle after vigorous scrubbing of the skin with cotton soaked in 70% ethanol.

Cultures were performed at the University of Florida's College of Veterinary Medicine Microbiology laboratory. All samples were plated to the following media: Columbia 5% sheep blood, MacConkey, Columbia CNA, Mycobiotic and Emmons Sab Dex agar. Cultures were incubated at 37 C and examined at either 24 and 48 hr (aerobic) or 48 hr (anaerobic) postinoculation. Anaerobic cultures were conducted using a Bio-Bag™ Type A (Becton Dickinson). Blood samples were incubated at 37 C for 30 days and plated to the above media at 24 hr, 4 days, and 21 days postinoculation. Bacterial colonies were identified by Gram staining, morphology, and/or commercial biochemical testing (API®, bio-Meriéux, Hazelwood, Missouri, USA; and RapIDÖ ANA II Panel®, Remel, Lenexa, Kansas, USA). Fungal cultures were held for 30 days at 32 C and isolates were identified using scotch-tape preparations.

Toxicology

One viable egg was randomly collected from each clutch, opened, and yolk contents stored in glass amber vials at −20 C until analyzed for OCPs as described in Rauschenberger et al. (2004). Previous work done in our laboratory has shown that OCP concentrations in alligator yolk does not differ across eggs within a particular clutch (Gross, unpubl. data). Thus, yolk OCP concentrations in a randomly selected egg are representative of the whole clutch. Briefly, yolks were first homogenized and a 2–5-g sample extracted into ethyl acetate. Samples were purified using C18 and NH2 SPE (solid-phase extraction) cartridges, and pesticide concentration determined using a gas chromatograph (Hewlett Packard HP-6890, Wilmington, Delaware, USA) and a mass spectrometer (HP 5973) in electron impact mode according to U.S. Environment Protection Agency (EPA) method 8270C (USEPA, 1990). Identification of all analytes and quantitation for toxaphene was conducted in full-scan mode, where all ions are monitored. To improve sensitivity, selected ion monitoring (SIM) was used for the quantitation of all other analytes, except kepone. Samples were analyzed at least three times. For quantitation, a five-point standard curve was prepared for each analyte ($R^2 \geq 0.99$). Fresh curves were analyzed with each set of 20 samples. Each standard and sample was fortified to contain a deuterated internal standard, 5 μl of US-108 (120 μg/ml; Ultra Scientific), added just before analysis. All samples also contained a surrogate, 2 μg/ml of tetrachloroxylene (Ultra Scientific) added after homogenization. Duplicate quality-control samples were prepared and analyzed with every 20 samples (typically at a level of 1.00 or 2.50 μg/ml of γ-BHC, heptachlor, aldrin, dieldrin, endrin, and p, p′-DDT) with an

TABLE 1. Summary of clutch quality information and necropsies performed on American alligator late-stage embryos ($n=97$) and hatchlings ($n=139$) during the 2000 and 2001 reproductive seasons in north-central Florida, U.S.A.

Clutch ID	Site	Collection date	Clutch size/No. eggs incubated[a]	Embryo age at start of incubation (days)	No. dead embryos early/mid development[b] (not necropsied)	No. dead embryos late development[c] (all necropsied)	No. dead hatchlings (all necropsied)	Clutch production (%)[d]
	APOPKA ($n=56$)							
AP351		26 June 2000	40/27	14	11	0	2	52
AP365		27 June 2000	49/41	6	3	1	37	0
AP003		26 June 2001	46/22	22	3	1	2	73
AP013		26 June 2001	49/21	3	5	1	0	71
AP020		26 June 2001	55/32	19	4	2	1	78
AP217		27 June 2001	38/17	22	2	2	0	76
AP373		28 June 2001	48/27	8	6	0	7	52
	GRIFFIN ($n=121$)							
GR051[e]		03 July 2000	43/28	13	7	18	1	0
GR052		03 July 2000	46/13	9	9	1	0	23
GR412		30 June 2000	49/46	17	11	2	0	72
GR421[f]		01 July 2000	56/47	8	12	29	4	0
GR423[f]		03 July 2000	39/36	19	3	15	16	0
GR428		29 June 2000	53/39	18	7	2	3	69
GR431		01 July 2000	52/50	11	3	1	10	72
GR015		01 July 2001	43/11	8	8	3	0	0
GR210		30 June 2001	44/21	14	4	1	9	33
GR211		30 June 2001	24/08	15	2	0	1	63
GR212		30 June 2001	47/22	12	16	0	3	14
GR216		01 July 2001	55/27	8	5	1	1	74
	EMERALDA ($n=56$)							
EM305		30 June 2000	46/44	16	0	4	35	11
EM001		10 July 2001	49/41	19	20	5	1	37
EM002		10 July 2001	54/52	31	1	3	1	90
EM013		10 July 2001	52/26	29	19	0	7	0

TABLE 1. Continued.

Clutch ID	Site	Collection date	Clutch size/No. eggs incubated[a]	Embryo age at start of incubation (days)	No. dead embryos early/mid development[b] (not necropsied)	No. dead embryos late development[c] (all necropsied)	No. dead hatchlings (all necropsied)	Clutch production (%)[d]
	LOCHLOSSA ($n=3$)							
LO003		29 June 2001	46/22	21	7	2	0	59
LO700		39 June 2001	38/19	11	0	1	0	95

[a] Not all eggs collected were incubated. In most cases, the number of eggs removed represents the number of nonviable eggs (i.e., unbanded, banded dead, or damaged); however, eggs were also removed for other studies.

[b] Early/mid embryonic mortality: ~days 1–35/36–57 days of age.

[c] Late embryonic mortality: ~days 58–65 of age (approximately last week of incubation).

[d] Clutch production (%): total number of live 4-wk-old hatchlings over total number of viable eggs incubated times 100.

[e] Two additional animals (late development embryo and hatchling) died but were not necropsied because of advanced autolysis.

[f] Two additional hatchlings died but were not necropsied because of advanced autolysis.

acceptable recovery ranging of 70–30%. Repeated analyses were conducted as allowed by matrix interferences and sample availability. Pesticide concentrations in this study are presented as nonlipid normalized wet-weight values.

Statistical analyses

Statistical analyses were performed using SAS 9 software (SAS, 2002). Two-way analyses of covariance (ANCOVA) (PROC GLM) were used to test the effects of site and animal age (full-term embryo vs. hatchling) or animal condition (live vs. dead) on the dependent variables (body measurements and organosomatic indices). Clutch identification was used as a covariate in these analyses. Total OCPs were compared across sites using a one-way analysis of variance (ANOVA). The ANOVAs were followed by pairwise comparisons using Tukey's multiple comparison tests. The frequency of abnormalities was compared across sites and levels of OCP contamination using chi-square tests (PROC FREQ). Data are reported as mean±SEM unless otherwise indicated. Statistically significant differences were declared at $P \leq 0.05$.

RESULTS

Two thousand one hundred fourteen late-stage embryos and hatchlings belonging to 76 clutches were examined during the reproductive seasons of 2000 and 2001. Clutches were collected from four locations in northcentral Florida: Lakes Apopka ($n=19$), Griffin ($n=22$), Emeralda Marsh ($n=22$), and Lochloosa ($n=13$). Of these animals, 236 (11%) died either at the approximate time of hatching or within a month posthatch and were necropsied (Table 1). Late developmental and posthatch mortality of necropsied alligators differed across sites and was highest in Lake Griffin (121 animals from 12 clutches, 24%), followed by Emeralda (56 animals from four clutches, 9%), Lake Apopka (56 animals from seven clutches, 7%), and Lochloosa (three animals from two clutches, 1%).

Clutch production was also calculated for the subset of clutches from which animals were necropsied and averaged 45±7% (range of 0–95%) (Table 1). Early

through midincubation embryo mortality (up to day ~57 of incubation) was included for this calculation, although, as already discussed, these embryos were not further examined as part of this study. As expected, clutch production followed an opposite trend to that of embryo and hatchling mortality (necropsied animals only) and was lowest in Griffin and Emeralda (33±10% and 35±20%, respectively), intermediate in Apopka (57±10%), and highest in Lochloosa (78±18%) (Table 1).

The developmental stage at which these mortalities occurred also differed across sites, with dead animals being composed of mostly full-term embryos (60%) in the case of Lake Griffin or hatchlings (79–88%) in the case of Lake Apopka and Emeralda. All three dead animals from Lochloosa were late-stage embryos (Table 1).

Comparisons of body measurements and organosomatic indices between late-stage embryos and hatchlings within and across sites are summarized in Table 2. Regardless of site, there was an ~7% increase in HL, SVL, and TL between late-stage embryos and hatchlings (from 34 to 36, 11 to 12, and 22 to 24 cm, respectively). In terms of organosomatic indices, some did not change with age (fat body and kidney, 0.35–0.49 and 1.0–1.1%, respectively), whereas others decreased (yolk somatic index, overall decrease of 51%, from 18% to 8.7%) or increased (liver: 13%, 2.8–3.2%; lung: 43%, 0.8–1.4%; and heart: 25%, 0.6–0.8%). Changes in body weight and organosomatic indices between both age classes, however, were not consistent across sites. For instance, declines in body weight between late-stage embryos and hatchlings were only observed in Griffin animals (decline of 15%, from 48 to 41 g). Similarly, declines in yolk somatic index were most pronounced in Emeralda (65%), and Griffin (49%) animals compared to Apopka (24%) (Table 2). A similar but opposite trend was observed with lung and heart somatic indices, with overall increases of 45 and 26% and of 43 and 25% for Griffin and Emeralda, respectively, compared with an increase of only 15 and 7% for the same indices in Apopka alligators.

Across-site comparisons of body and organ measurements by age category are also summarized in Table 2. Lochloosa animals were not included in these analyses because of small sample size. From this comparison, differences in body measurements across sites were most evident in hatchlings compared with late-stage embryos. Indeed, the size of late-stage embryos did not differ among the three sites studied (overall means of 48 g for body weight and 34, 11, and 23 cm for HL, SVL, and TL, respectively), whereas hatchlings from Lake Apopka were significantly heavier when compared with hatchlings from Lake Griffin and Emeralda (50 vs. 42 g). Although Apopka hatchlings were also larger (HL: 37 vs. 36 cm; TL: 25 vs. 24 cm), these 1-cm differences might not be biologically significant. The SVL in hatchlings did not differ across sites (overall mean of 12 cm). There was an opposite trend in organosomatic indices across sites between late-stage embryos and hatchlings. For instance, liver, kidney, and heart somatic indices did not differ in late-stage embryos from the different sites (overall means of 2.8, 1.0, and 0.6%, respectively), but were increased in hatchlings from Griffin compared with Apopka and Emeralda for liver and kidney somatic indices (3.4 vs. 3% and 1.3 vs. 1%) and from Griffin and Emeralda compared with Apopka for heart somatic index (0.8 vs. 0.6%). In contrast, fat somatic index differed across sites only in late-stage embryos (overall means of 0.8, 0.3, and 0.2% for Apopka, Griffin, and Emeralda, respectively) with hatchlings having an overall mean of 0.5% regardless of site. A similar trend was observed for lung somatic index (overall means of 1.1% for Apopka and of 0.8% for Griffin and

TABLE 2. Summary (mean±SEM) of body measurements and organosomatic indices (organ weight/body weight×100) in American alligator late-stage embryos and hatchlings necropsied during the course of this study, by site. Significant differences between late-stage embryos and hatchlings and across sites are denoted by lower and upper case letters, respectively ($P=0.02$ to < 0.0001; $F=3$–50; $R^2=0.3$–0.9). Clutch was used as a covariate in these analyses. Because of small sample size, Lochloosa animals were not included in these comparisons.

	Apopka		Griffin		Emeralda		Lochloosa
Parameters	Late embryo	Hatchling	Late embryo	Hatchling	Late embryo	Hatchling	Late embryo
Number of clutches	5	5	10	9	3	4	2
Number of animals	7	49	73	48	12	44	3
Body weight (g)	49 ± 4^{aA}	50 ± 1^{aA}	48 ± 0.6^{aA}	41 ± 1.3^{bB}	44 ± 3^{aA}	43 ± 0.8^{aB}	54±0.2
Head length (cm)	34 ± 1^{aA}	37 ± 0.2^{bA}	34 ± 0.1^{aA}	36 ± 0.3^{bB}	33 ± 0.4^{aA}	36 ± 0.2^{bB}	34±2.4
Snout–vent length (cm)	11 ± 0.3^{aA}	12 ± 0.1^{bA}	11 ± 0.07^{aA}	12 ± 0.1^{bA}	11 ± 0.2^{aA}	12 ± 0.1^{bA}	11±2
Total length (cm)	23 ± 0.6^{aA}	25 ± 0.6^{bA}	22 ± 0.2^{aA}	24 ± 0.2^{bB}	22 ± 0.4^{aA}	24 ± 0.2^{bB}	22±2
Fat body index (%)	0.76 ± 0.2^{aA}	0.48 ± 0.02^{aA}	0.33 ± 0.04^{aB}	0.46 ± 0.04^{aA}	0.22 ± 0.04^{aC}	0.58 ± 0.1^{aA}	0.35±0.4
Yolk somatic index (%)	11 ± 2^{aA}	8.4 ± 0.6^{bA}	18 ± 0.7^{aA}	9.2 ± 1^{bA}	24 ± 7^{aB}	8.4 ± 0.8^{bA}	25
Liver somatic index (%)	2.4 ± 0.2^{aA}	3.0 ± 0.1^{bA}	2.9 ± 0.1^{aA}	3.4 ± 0.1^{bB}	2.7 ± 0.2^{aA}	3.1 ± 0.1^{bB}	1.56
Kidney somatic index (%)	1.2 ± 0.3^{aA}	1.1 ± 0.06^{aA}	1.0 ± 0.04^{aA}	1.3 ± 0.06^{aB}	0.97 ± 0.1^{aA}	0.94 ± 0.05^{aA}	0.65
Lung somatic index (%)	1.1 ± 0.3^{aA}	1.3 ± 0.07^{bA}	0.82 ± 0.04^{aB}	1.5 ± 0.09^{bA}	0.86 ± 0.07^{aAB}	1.5 ± 0.06^{bA}	0.29
Heart somatic index (%)	0.63 ± 0.1^{aA}	0.68 ± 0.04^{bA}	0.60 ± 0.02^{aA}	0.81 ± 0.05^{bB}	0.64 ± 0.1^{aA}	0.85 ± 0.04^{bB}	0.74

Emeralda late-stage embryos, with no differences across sites for hatchlings [1.4%]). Finally, yolk somatic index was increased significantly in late-stage embryos from Emeralda (24%) compared with same-age alligators from Lakes Griffin (18%) and Apopka (11%) and did not differ across sites in hatchlings (overall mean of 8.7%) (Table 2).

Differences in body measurements between live (2 days posthatch) and dead hatchlings (late-stage embryos) are presented in Figure 1. From this comparison, there was a significant decline in body weights in dead hatchlings from Griffin (a 23% decline, from 52 to 40 g) and Emeralda (a 16% decline, from 51 to 43 g) compared with their live counterparts ($P<0.0001$, $F=32$, df=5). There were no differences in body weights between dead and live Apopka hatchlings (52 and 50 g, respectively). Head length was decreased in dead hatchlings regardless of site ($P<0.0001$, $F=19$, df=5), although this decline was most evident again in Griffin and Emeralda (a 2.5% decline, from 36.5 to 35.6 cm, and a 5% decline, from 37.4 to 35.6 cm, respectively) compared with Apopka animals (a 1.6% decline, from 37.1 to 36.5 cm). There were no differences in TL or SVL between dead and live hatchlings.

Several abnormalities were observed during gross examination of dead animals

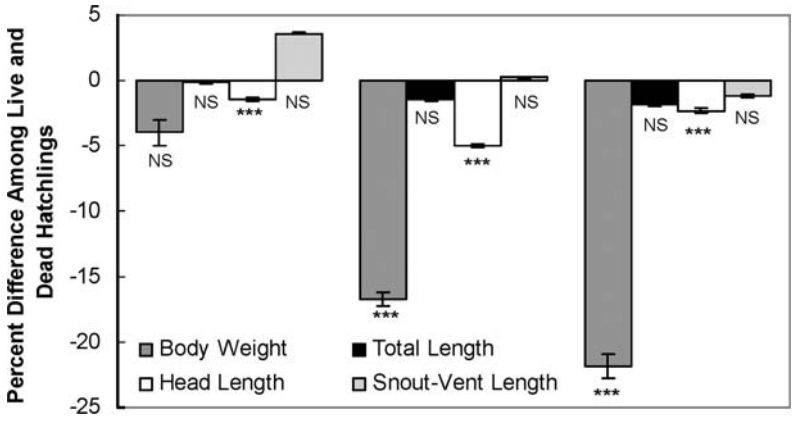

FIGURE 1. Percent differences in body measurements between live and dead hatchlings, by site. Clutch was used as a covariate in these analyses. Asterisks denote significant differences between age categories and NS=not significant. Total of animals examined: Apopka=144, Emeralda=111, and Griffin=180.

(Fig. 2). The most prevalent condition was the presence of edema (80 animals affected, which corresponds to an overall prevalence of 34%), which was either localized internally around the heart and lungs (32% of the edema cases) or abdominal cavity (ascites, 9%), and/or externally affecting limbs (30%) and jaw and neck (29%). Internal edema was evidenced as a layer of white opaque fibrinous material surrounding the lungs and heart. The second most prevalent abnormality was hyperemic or congested organs (73 animals, 29% prevalence), with 80% of cases having hyperemia of the yolk sac (43%) and lungs (37%), and the remaining 20% having hyperemia of the kidneys (8%), gastrointestinal tract (7%), and liver (5%). Severely emaciated alligators were observed at a prevalence of 14%, with over half of the cases diagnosed in Griffin animals. Gross deformities were observed at a very low prevalence (eight animals, 3% prevalence) and consisted of vertebral (scoliosis) and tail (kinked) abnormalities at 31% prevalence each; the presence of extra digits (23%); and conjoined twins (15%). Supernumerary digits consisted of up to three extra digits/limb, and most cases were animals that had abnormal forelimbs (i.e., curved, edematous, or abnormal size). Conjoined twins were attached to each other through the yolk sac and were late-stage embryos that died before hatching. Approximately 9% of the dead animals examined were also diagnosed with other abnormalities, which included oral pin-point, white granulomas (47%); gout, evidenced as the presence of enlarged kidneys and whitish cream prominent tubular contents (33%); liver and lung granular foci of 1–2 mm in diameter (8 and 4%, respectively); and heart petechiae (8%).

Histologic lesions were prevalent and occurred in close to a third of the animals examined (83 animals, corresponding to a 35% prevalence). The percent occurrence of histologic lesions differed across sites, and was approximately half as prevalent in Griffin (23%) compared with Apopka and Emeralda (44 and 39%, respectively) animals (Fig. 2). Over half of the histopathologic changes were lung lesions, mostly heterophilic pneumonias (30%), considered of bacterial origin because of microscopic observation of bacterial organisms, with or without the presence of edema (16%) and atelectasis (9%). Other lesions included the presence of hepatocellular lipidosis, or fatty liver (12%); presence of bacteria in the blood or septicemia (8%); and generalized inflammations of the gastrointestinal tract

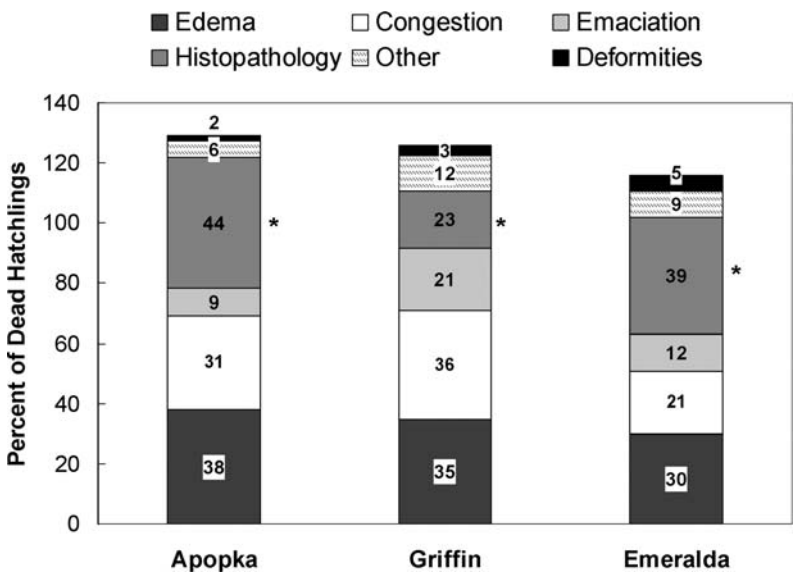

FIGURE 2. Differences in the percent distribution of dead late-stage embryos and hatchlings into the six main categories of abnormalities discovered during the course of this study across sites. Numbers inside bars indicate percent cases. Lochloosa animals were not included in this comparison because of small sample size. Apopka and Emeralda had a higher prevalence of animals with histological lesions compared with Griffin ($P<0.0009$, $X^2=14$, df=2). Note that some animals fell in more than one category.

(gastritis and enteritis, 7%) and kidneys (nephritis, 6%). Most cases of fatty livers were diagnosed during necropsy and were characterized by the presence of yellow tan and friable livers. Other categories of lesions (overall prevalence of 12%) included three cases of tracheitis; two cases of epicarditis and thymus heterophilic infiltration; and one case each of steatitis, leptomeningitis, splenitis, hepatitis, and mineralization of renal tubules. The bacteria observed were all Gram-negative coccobacilli. There were no significant lesions suggesting the presence of Chlamydiaceae or viral infection. In particular, no elementary bodies and no viral inclusion bodies were identified.

The number of animals examined for microbiologic endpoints was limited to 14 animals belonging to six clutches. Of 10 blood samples analyzed, eight were positive for one or more of the following bacterial organisms: *Aeromonas hydrophila, Serratia marcescens, Bacillus* sp., *Bacteroides* sp., *Clostridium* sp., *Staphylococcus* sp., and a Gram-negative rod. *Corynebacterium* sp. was isolated from one of four lung samples. From this same clutch, this organism plus *Clostridium* sp. was also isolated from the liver. One animal from Lake Griffin was examined for *Salmonella* and the results were negative. No fungal organisms were isolated in this study.

There were significant differences in total yolk OCP concentrations across sites. Yolks from Emeralda contained approximately 25 and 2 times the OCPs compared with Griffin and Apopka (31,763±3,777 μg/kg compared with 1,242±481 μg/kg and 13,240±5,046 μg/kg, respectively) ($P<0.0001$, $F=28$, df=2). Regardless of site, over 95% of the OCP mixture was composed of DDT and related metabolites (mostly p,p′-DDE) and of cyclodiene pesticides (mainly dieldrin and chlordanes). To determine the relationship between exposure to high concentrations of pesticides and the occurrence of different gross and histologic abnormalities, animals were divided into three groups based on degree of

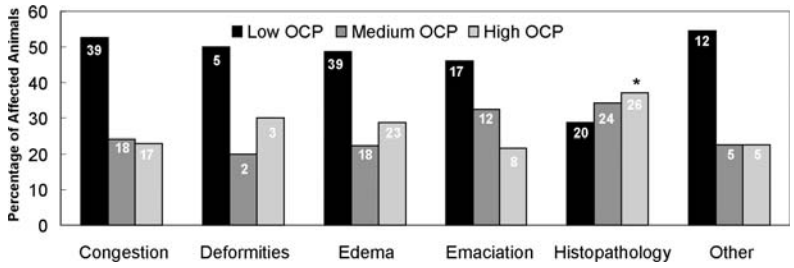

FIGURE 3. Percent distribution of affected animals in relation to organochlorine pesticide (OCP) concentrations. Animals were divided into three categories of pesticide contamination: low=100–900 μg/kg, $n=11$ clutches; medium=1,500–8,500 μg/kg, $n=8$ clutches; and high=26,000–52,000 μg/kg, $n=5$ clutches. With the exception of histopathology ($P=0.004$, $X^2=11$, df=2), the prevalence of the different abnormalities was not related to high OCP concentrations.

contamination: low (100–900 μg/kg, $n=11$ clutches), medium (1,500–8,500 μg/kg, $n=8$ clutches), and high (26,000–52,000 μg/kg, $n=5$ clutches) OCP body burdens. The result of this analysis is shown in Figure 3. Except for a slight increase in the prevalence of histologic lesions with degree of OCP contamination, there was no other significant association between high pesticide burdens and presence of abnormalities.

DISCUSSION

Effects of site on survival and growth of early-life stages

In this study, late embryonic and hatchling mortality differed across sites and was highest in Lake Griffin (24%), compared with Emeralda (9%), Apopka (7%), and Lochloosa (1%). Lochloosa and Apopka clutches were of high (78%) and intermediate (57%) quality, respectively, in terms of percentage of hatchlings that survived 1 mo posthatch. Although clutches from Emeralda experienced only about a third of the late-stage embryo and hatchling mortality compared with Griffin, clutch productivity was about the same for both sites (34%). This was due to a higher rate of embryo deaths in the Emeralda site during the first weeks of incubation. A decreased survival in early life stages of American alligators from polluted sites is consistent with previous reports (Masson, 1995; Giroux, 1998; Sepúlveda et al., 2004), although viability values in clutches from Lake Apopka in the present study were higher than reported in the early 1990s by Woodward et al. (1993). Causes for low clutch viability could be related to either external and/or internal factors, such as flooding and desiccation of eggs; altered eggshell, yolk, or albumen quality; infertility; and/or increased zygote and embryonic rates.

Hatchlings from Apopka and Lochloosa not only survived at an increased rate during the first month of age compared with Griffin and Emeralda animals, but were also significantly larger at the time of hatching. This difference in weight was true for both dead and live hatchlings. In addition, there were significant site differences in organ size and energy allocation and utilization (Lochloosa animals were not included in this analysis because of small sample size). An increased rate in yolk utilization during embryonic development in Apopka animals could explain their larger size at the time of hatching. Indeed, yolk somatic indices were almost doubled in late-stage embryos from Griffin and Emeralda (21%) compared with same-age alligators from Lake Apopka (11%). In contrast, animals from Griffin and Emeralda hatched with a larger yolk sac that declined in size at a much higher rate during the first month posthatch (49% to 65% decline) compared with Apopka alligators (24% decline). In addi-

tion, Apopka alligators hatched with about four times the amount of body fat reserves compared with Griffin and Emeralda animals. Thus, increased survival during the first weeks posthatch in Apopka animals could be explained by a larger body size coupled with an increase in body fat reserves at the time of hatching.

An increased body size in hatchlings from Lake Apopka is consistent with previous reports. Richey (2001) also reported significant differences in body size at hatching, with lowest values in Griffin and Emeralda and highest in Apopka animals. Milnes et al. (2001) examined hatchlings from three lakes in northcentral Florida (Woodruff, Orange, and Apopka) and hypothesized that lake effects resulting from several environmental (such as food and resource availability, nutrient levels, and contaminant levels) and demographic (such as age) factors, and not genetic differences, affect hatchling size through influences on the maternal contribution to the embryonic environment. A difference in survival and hatchling size across sites in the present study supports this lake-effect hypothesis and the idea of a differential preovulatory parental investment in alligators inhabiting different lakes.

Recent studies in different species of reptiles have shown that both body size and amount of yolk reserves at the time of hatching are important predictors of early survival, and that these two traits usually are negatively correlated to each other (Congdon et al., 1999). Increased hatchling survival has been linked to larger yolk reserves at the time of hatching because of a delay in time to starvation during the transition to an exogenous feeding regime (Congdon and Gibbons, 1985, 1989). In addition, because yolk is an important source of immunoglobulins in egg-laying vertebrates (Picchietti et al., 2001), a more complete utilization of these reserves during embryonic development should allow the newly hatched animals to better deal with pathogens, increasing the chances for survival. Larger yolk reserves at the time of hatching, however, imply that a decreased conversion of egg material for embryo growth and development has taken place during incubation, with the resulting production of smaller hatchlings (Congdon et al., 1999). Results from the present study support the bigger-is-better hypothesis because larger hatchlings survived for longer periods, despite their smaller yolk sac at the time of hatching. Because larger hatchlings also had proportionally larger fat bodies, this additional source of energy should also be considered as an important predictor of early survival in reptiles.

Growth retardation in dead embryos and hatchlings

Results from this study indicate general growth impairment for sick and dying hatchlings compared with live counterparts. Differences in size between live and dead hatchlings were more pronounced in animals from Griffin and Emeralda compared with Apopka. Indeed, over half of the cases of severe emaciation were diagnosed in Griffin animals. Causes for decreased growth are unknown at this time, but could be related to alterations in the nutritional composition of eggs in animals inhabiting polluted sites, as discussed in more detail later.

Gross pathology, histopathology, and microbiology

In this study, the most prevalent pathologic changes among dead animals were the presence of edema (overall prevalence of 34%), hyperemic organs (29%), inflammation (30%), and gross deformities (3%). The cause for the generalized edema could be related to the presence of bacterial infections because abnormal retention of fluids has been described in several species of crocodilians with septicemia. For instance, swelling of limbs and abdomen was reported in Indian crocodiles (*Gavialis gangeticus*) infected with *Clostridium* sp. and *Planococcus* sp.

(Misra et al., 1993). Hydropericardium, ascites, and lung edema have also been reported from Nile crocodiles (*Crocodylus niloticus*) infected with *Chlamydia* sp. (Huchzermeyer et al., 1994), and American alligators infected with *Mycoplasma alligatoris* suffered from periocular, facial, neck, and limb edema (Clippinger et al., 2000). Generalized edema could also result from exposure to organochlorine contaminants, as discussed in more detail later.

Histologic lesions were prevalent, occurring in close to a third of the animals examined. However, percent occurrence of lesions differed across sites and was approximately half as prevalent in Griffin compared with Apopka and Emeralda animals. Over half of the histopathologic cases were lung lesions, mostly heterophilic pneumonias of bacterial origin with or without the presence of edema and atelectasis. Other histopathologic findings included the presence of liver lipidosis or fatty liver; septicemia; gastritis and enteritis; and nephritis.

In the present study, several opportunistic bacterial organisms were isolated, including *Aeromonas hydrophila, Serratia marcescens, Bacillus* sp., *Bacteroides* sp., and *Staphylococcus* sp. from blood, *Corynebacterium* sp. from lung, and *Clostridium* sp. from blood and liver. These are all recognized pathogens in reptiles and other vertebrates. No fungal organisms were isolated or morphologically identified in this study. Bacterial septicemias, with particular involvement of certain organs, are a frequent cause of illness and mortality in crocodilian hatchlings. In most cases, Gram-negative bacteria are involved, including *Aeromonas hydrophila, Salmonella* spp., and less often *Clostridium septicum, Edwarsiella tarda, Escherichia coli, Klebsiella* sp., *Pasteurella multocida, Providencia rettgeri, Pseudomonas* spp., and *Staphylococcus* sp. (Ladds and Sims, 1990; Huchzermeyer, 1991; Buenviaje et al., 1994; Ladds et al., 1996; Camus and Hawke, 2002). In Florida, both *A. hydrophila* and *A. shigelloides* have been implicated as the cause of mortality of free-ranging American alligators (Shotts et al., 1972). However, *A. hydrophila* has also been isolated from apparently healthy American alligators (Gorden et al., 1979).

Because the number of samples analyzed for the presence of bacteria and fungi was limited, some pathogenic agents might have been missed during this study. Nevertheless, most cases of pneumonia and tissue inflammation probably had bacterial involvement because of the microscopic observation of heterophilic infiltration and bacteria in the affected organs. Whether bacteria were the primary cause of the problem or the result of an opportunistic invasion and later infection remains unknown at this time. In at least three cases, pneumonia was probably triggered after inhalation of yolk contents (inhalation pneumonia) by full-term embryos. These cases were diagnosed histologically by the presence of a proteinaceous yolk-like material in the airways of affected animals. There were no lesions or inclusions suggestive of accumulation of viral and/or viral-induced cell proteins to indicate the presence of a productive viral infection. Sources of bacteria in the present study are unknown at this time but may be related to opportunistic organisms present in the environment and/or endogenous intestinal bacteria that were able to cross the mucosal barrier and cause disease (Huchzermeyer, 2002).

The low prevalence of deformities observed in the present study (3%) is in agreement with reports from other crocodilian species. For instance, in the Indian gharial (*Gavialis gangeticus*), deformities were detected in 6% of 1,061 hatchlings examined and 80% of them consisted of defects to the vertebral column (Singh and Bustard, 1982). Similar low prevalences of deformities were reported by Boede and Sogbe (2000) in American (*Crocodylus acutus*) and Orinoco (*C. intermedius*) hatchling crocodiles (1–42 days). These authors reported ~5% deformities to the

tail, fore limbs, and maxillary bones. Although most researchers agree that these deformities are of congenital origin, some have considered these abnormalities to be due to exposure of embryos to extreme temperature and humidity conditions (Ayarzaguena, 1990; Blanco-Márquez, 1997).

The presence of enlarged, pale, fatty livers was a relatively common finding in the present study. Because in reptiles, accumulation of fat in liver is just another way of allocating energy for times of need (i.e., hibernation and fasting), the mere presence of intrahepatic lipid can be quite normal and not synonymous of a pathologic condition (Divers and Cooper, 2000). Hepatic lipidosis, however, has also been reported in association with infectious diseases such as adenovirus and chlamydiosis (Huchzermeyer et al., 1994; Huchzermeyer, 2002).

Relationship between OCPs and abnormalities

Yolk OCP concentrations reported in this study (7,062±1,245 μg/kg) are well within ranges considered toxic for many species of egg-laying vertebrates (Jarvinen and Ankley, 1999). However, except for a slight increase in the prevalence of histologic lesions with increased OCP contamination, there was no other significant association between high pesticide burdens and presence of abnormalities.

In American alligators, results have clearly demonstrated reduced egg hatchability for eggs collected from OCP-contaminated sites. Nonetheless, OCP concentrations in eggs do not correlate with embryo survival rates, suggesting the potential for OCP-maternal-mediated effects on egg quality and/or the involvement of other environmental factors. For other egg-laying vertebrate species, there are numerous laboratory and field studies linking OCP exposure to early-life-stage mortality. OCPs can induce early-life-stage mortality through three mechanisms: 1) direct toxicity to developing embryos, 2) altered maternal vitellogenesis and oogenesis leading to changes in egg composition, and/or 3) immunosuppression. Direct toxicity of chlorinated organics has been extensively characterized after exposure of early-life stages of fish to aryl hydrocarbon receptor (AhR) agonists (mostly dioxins and polychlorinated biphenyls [PCBs], but also OCPs) (Smith and Cole, 1973; Monod, 1985; Guiney et al., 1997; Henry et al., 1997). In these studies, mortality was associated with yolk sac and pericardial edema, craniofacial alterations, and severe and generalized hemovascular damage. An interesting finding from these studies is that the same effects occurred regardless of exposure route (i.e., maternal transfer, waterborne, or injections), suggesting these AhR agonists exert their effects on early-life stages through nonmaternal-mediated mechanisms. In fish-eating birds, exposure to organochlorine contaminants also induces embryotoxicity, a condition known as Great Lakes embryo mortality, edema, and deformity syndrome (GLEMEDS), and characterized by increased embryo mortality, growth retardation, subcutaneous pericardial and peritoneal edema, and congenital deformities of the bill and limbs (Gilbertson et al., 1991). This syndrome closely resembles chick edema disease observed in chickens after *in ovo* exposure of hens to dioxins. A second mode of action of OCPs is through an interference with vitellogenesis and oogenesis in adult females, leading to changes in egg composition that are later translated in decreased embryo viability. The types, amounts, and relative proportions of lipids and fatty acids, protein, water, vitamins, and minerals present in eggs have all been identified as crucial for the normal development of embryos. As vitellogenins are the major source of nutrition for the developing embryo and also serve as transporters of hormones and antibodies for all egg-laying vertebrates (Babin, 1992; Picchietti et al., 2001), a decline in circulating levels of these proteins in OCP-exposed females could

result in alterations in egg composition and thus in embryo survival. Alterations of fatty-acid profiles in yolk (Pushpanjali et al., 2000; Stanton et al., 2003) and of protein contents in albumin (Fernie et al., 2003) have been reported in avian eggs exposed to several chlorinated organics. A final mechanism by which OCPs could increase mortality in early-life stages is through imunosuppression. In the present study, the presence of mixed bacterial infections of multiple organ systems suggest imunosuppression and enhanced susceptibility to opportunistic pathogens. As already discussed, imunosuppression and increased susceptibility to infectious agents could have been the result of stress related to captive conditions. Indeed, in juvenile American alligators, stress has been shown to alter plasma corticosterone concentrations and white blood-cell parameters (Lance and Elsey, 1999). In the present study, immunosuppression could also have been related to exposure to OCPs. A significant positive relationship between total egg OCPs and the incidence of histological lesions, most of which included inflammation of lungs and other major organs, supports this link.

There are conflicting reports on the effects of OCPs on the immune function of American alligators. Gross (1997) found decreased antibody responses and hypoplastic lymphoid tissue and bone marrow in Apopka hatchlings, and Rooney (1998) found that juvenile Apopka alligators had smaller thymic medullary/cortical ratios and smaller splenic lymphocyte sheaths. Richey (2001), however, exposed alligator eggs to OCPs and found no evidence of immunosuppression as determined by a battery of tests that measured both cellular and humoral immune function.

CONCLUSIONS

Lesions in dead full-term embryos and hatchlings included generalized edema, organ congestion and inflammation, bacterial infections, growth retardation and emaciation, and developmental defects. Within and across clutches, dead embryos and hatchlings compared with their live cohorts were significantly smaller and lighter. Although alterations in growth and development were not related to yolk OCPs, there was an increase in the prevalence of histopathologic lesions in clutches with high OCP. Overall, these results indicate that general growth retardation and respiratory abnormalities were a major contributing factor in the observed mortalities and that contaminants might be increasing the susceptibility of animals to develop certain pathologic conditions.

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