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Chronic incidental lead ingestion in a group of captive-reared alligators (*Alligator mississippiensis*): Possible contribution to reproductive failure

Valentine A. Lance [a,*], Thomas R. Horn [a], Ruth M. Elsey [b], Ann de Peyster [a]

[a] *Graduate School of Public Health, San Diego State University, San Diego, CA 92182-4162, USA*
[b] *Louisiana Department of Wildlife and Fisheries, Rockefeller Wildlife Refuge, 5476 Grand Chenier Highway, Grand Chenier, LA 70643, USA*

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Abstract

An American alligator (*Alligator mississippiensis*) breeding facility using male and female alligators raised from artificially incubated eggs was established in 1975. These alligators first reproduced at 6 years of age as compared to 10–12 years in wild alligators, but the eggs produced showed a lower hatching rate than those collected from the wild. By age 21 reproduction had failed almost completely. The alligators were sacrificed and tissues collected at necropsy from 44 captive and 15 wild animals and assayed for metals. Results showed that captive alligators had significantly higher tissue levels of lead than wild alligators. Cadmium did not differ between wild and captive and selenium was 50% higher in wild than captive alligator kidneys. Bone lead in captive alligators was 252,443 ± 20,462 ng/g. High yolk lead was suggested as a probable cause for early embryonic death in alligator eggs. The high tissue lead levels in captive alligators was attributed to long-term consumption of nutria (*Myocastor coypus*) meat contaminated with lead shot. Liver, ovary, and testis were assayed for lipid peroxidation using the thiobarbituric acid (TBA) test. Captive alligators had 3.6 fold increased TBA-reactive materials in the liver tissue compared to wild. Lipid peroxidation was strongly suspected as having been enhanced by consumption of rancid nutria meat containing lead.

Keywords: Alligator; *Alligator mississippiensis*; Reproduction; Yolk; Lead; Cadmium; Selenium; Lipid peroxidation; Embryo

1. Introduction

The American alligator (*Alligator mississippiensis*) is an important economic resource in the southeastern U.S. where, due to protection and sound management practices it is now abundant. In Louisiana, and to a lesser extent in some adjacent states, many thousands of wild alligators are harvested in an annual sanctioned hunt, but large numbers of alligators are also raised on farms for meat and leather products (Elsey et al., 1994). These ranched alligators are raised from eggs collected in the wild and artificially incubated. Although captive-reared juvenile alligators have higher growth rates than wild alligators (Chabreck and Joanen, 1979; Coulson et al., 1973), and are usually healthy and free of disease, as adults they are less fertile and their eggs have lower hatchability than those of wild alligators incubated under identical conditions (Lance et al., 1983; Elsey et al., 1993). The reasons for poor reproduction in captive alligators are unknown. Investigators have studied a number of possible causes for this failure including overcrowding stress (Elsey et al., 1990), trace element and vitamin E deficiencies (Lance et al., 1983), unsaturated long-chain fatty acid deficiency (Noble et al., 1993), and hyperlipidemia (Lance et al., 2001). Although captive alligators did have significantly higher concentrations of saturated fat and significantly lower concentrations of long-chain fatty acids in both the blood and egg yolk of adult females than in those of wild alligators, these differences were not sufficient to explain the range of pathologies seen in the captive animals.

The Louisiana Department of Wildlife and Fisheries (LDWF) established an experimental alligator breeding facility in 1975 using male and female alligators raised from artificially incubated eggs (Elsey et al., 1993). These captive-reared alligators first reproduced at 6 years of age as compared to 10–12 years estimated for wild alligators. The alligators were fed a diet of nutria meat (*Myocastor coypus*). By 1994 the captive alligators were approximately 21 years of age and

* Corresponding author. Tel.: +1 619 594 3690; fax: +1 919 594 6112.
E-mail address: lvalenti@sunstroke.sdsu.edu (V.A. Lance).

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reproduction had declined dramatically. Clutch size remained high at 30–45 eggs per female, but nesting rates (number of females nesting), egg fertility and percent hatching were all extremely low. By 1998 the majority of the few seemingly fertile eggs recovered from nests within the enclosures died in the first weeks of incubation (normal incubation takes 65–75 days). Additionally many eggs were misshapen, poorly calcified, or infertile. Wild alligators nested successfully in the area immediately adjacent to the pens suggesting that the reproductive pathology was not due to local environmental factors.

Given these results the LWDF decided to abandon the experimental breeding program and sacrifice the remaining stock of alligators. We used this opportunity to collect tissues from these animals during necropsy.

The goal of this study was to explore possible additional dietary factors that could be contributing to the low fertility rates in the captive alligators by analyzing tissues collected at necropsy. In particular we wished to examine the TBA-reactive (thiobarbituric acid) products in liver and gonads as an indicator of lipid peroxidation, a known cause of reproductive failure in egg-laying vertebrates (Van Fleet, 1980). Frozen nutria meat stored for 6 to 12 months undergoes oxidation and has higher TBA-reactive products, rancidity and peroxide values compared to fresh nutria meat despite being held at −30 °C (Elsey et al., 1994). A prolonged diet such as described, high in lipid peroxides, is a known cause of reproductive failure (Van Vleet and Ferrans, 1992).

Based on a recent publication of lead poisoning in farmed alligators fed nutria meat (Camus et al., 1998) we were alerted to the possibility that the nutria meat fed to the captive alligators was contaminated with lead shot. During the necropsies many pellets, later confirmed to be lead, were found in the stomachs of the captive alligators. As lead is a known cause of reproductive pathology, particularly in birds (see review by Hui, 2002), we extended our study to include analysis of lead in the tissues collected. Cadmium and selenium, known to cause reproductive problems when present in high concentrations (Hui, 2002), were also measured in selected tissues.

2. Materials and methods

2.1. Animals

A total of 44 captive-reared and 15 wild alligators were used (Table 1). The wild alligators were nuisance animals trapped by LDWF personnel in aquaculture ponds on the refuge or close to houses outside the refuge. Alligators of 2 m and more are perhaps 10 years old, and those greater than 2.5 m are about 20 years old. Males grow faster and reach a greater length than females (Chabreck and Joanen, 1979).

All the necropsies were carried out between May and August 1999. Whole body mass and total length were recorded for each alligator (Table 1), and tissues were collected and fixed in 10% buffered formalin for histopathologic examination (R. Pappendick, unpublished data). Samples from kidney, liver, testis, and ovary were collected and immediately immersed in liquid nitrogen, and subsequently stored on dry ice. The frozen tissues were shipped on dry ice to San Diego and stored at −80 °C until assayed. The yolk and bone samples were stored at −20 °C.

Table 1
Body mass and length of alligators

	n	Body mass (kg)	Range (kg)	Body length (cm)	Range (cm)
Wild male	8	34±7*	21–50	214±13	161–279
Captive male	16	217±21	54–279	353±11	246–404
Wild female	7	29±3	19–42	207±5	185–226
Captive female	28	85±4	73–132	270±4	187–295

* For this and subsequent tables all values are presented as mean and standard error of the mean.

2.2. Metals analysis

A total of 41 bone, 41 kidney, 31 liver, and 16 yolk samples were analyzed for metals. Bone and yolk were analyzed for lead only. Triplicate samples of ~0.2 g were assayed from each tissue. A section of bone about 2 mm thick was taken from the middle of the shaft of the femur and a triangular section cut from this disc (~0.2 g) for analysis. The method of Burger et al. (2000) was used with slight modification to determine Pb, Cd, and Se. Approximately 0.2 g (wet mass) of tissue was added 5 ml of concentrated nitric acid in Parr 4782 Teflon bombs. The bomb was sealed and placed in a Panasonic microwave oven (Matsushita Corp., Franklin Park, IL) for 30 s. Following digestion the bomb was allowed to cool for 30 min, and the digestate was then transferred to an acid-washed polyethylene screw-cap bottle. The digested samples were analyzed using a Perkin Elmer 600 graphite furnace atomic absorption spectrometer. As most of the ovarian follicles had desiccated slightly during prolonged storage, the yolk material was expressed from the follicles and lyophilized prior to digestion. Yolk was frozen on dry ice and placed in a lyophilizer (FTS Systems, Inc., Stone Ridge, NY) overnight. The dried yolk was then digested in nitric acid as above. The results of the yolk analysis are expressed as ng/g dry mass and all other analyses expressed as ng/g wet mass.

Five random samples of metal pellets (weighing ~5 g each), recovered from the alligator stomachs, were placed in acid-washed polyethylene bottles with 10 ml of concentrated nitric acid. These five samples were allowed to sit for 4 days at room temperature until they had dissolved, and then were analyzed.

Reference bovine liver tissue (National Institute of Standards and Testing) was tested for all three metals, and known amounts of each of the three metals (100 μg/l for lead and selenium and 5 μg/l for cadmium) were added to three samples of liver and kidney to check for recovery. Recoveries for spiked samples for all three metals were consistently greater than 85%, and estimates of metals in the bovine liver standard varied from 97% to 104%.

Table 2
Lead in alligator tissues

Tissue		n	Lead (ppm)	Range
Bone	Captive	30	252.43±20.46*	95.96–530.80
	Wild	11	7.98±3.33	0–31.22
Liver	Captive	26	12.50±1.50	2.12–31.88
	Wild	5	0.54±0.30	0.04–1.68
Kidney	Captive	27	4.97±1.28	1.91–7.06
	Wild	14	0.60±0.30	0.03–0.89
Yolk	Captive	11	17.33±2.75	3.37–26.43
	Wild	5	0.74±0.43	0–1.97

* For all comparisons of captive vs. wild, $p<0.05$.

2.3. Thiobarbituric acid analysis of tissue lipid peroxidation

All reagents were obtained from Sigma Aldrich (St. Louis, MO, USA). Forty-five samples of tissue (24 liver, 8 testis, and 13 ovary) were analyzed for lipid peroxidation using the thiobarbituric acid (TBA) test (Wilbur et al., 1949). After thawing, the tissues were kept on ice throughout the analysis. Tris buffer, 4 mM (0.1 M Tris-acetate, 0.1 M KCl, 1 mM ethylenediaminetetraacetic acid (EDTA)) was added at 4:1 volume to weight before homogenization. Next, 1 ml of 20% trichloroacetic acid was added to 2 ml of the homogenate, followed by the addition of 2 ml of 0.67% TBA to a glass screw-top tube (16 × 100 ml). Tubes, with loosened caps, were placed in a boiling water bath for 10 min, allowed to cool and then centrifuged at 2400 × g for 15 min. The supernatant was transferred to a cuvette and the absorbance read at 530 nm on a Perkin Elmer Lambda 3A spectrophotometer.

2.4. Statistics

Results were analyzed using Microsoft Excel® 97. Normal distribution was confirmed by checking data for each measurement using the "normdist" function. Meeting this assumption we then applied the unpaired Student's t-test to compare differences between wild and captive alligators (Gad, 1998).

3. Results

3.1. Metals

3.1.1. Lead

Analysis of pellets found in the stomachs of captive alligators confirmed the presence of lead in all five samples tested. The mass of metal pellets in the stomach contents varied from 1.21 to 51.24 g/individual. The highest amount was found in a large male. No pellets were found in the stomach contents of wild alligators.

A significant difference was evident in lead concentrations between the captive and wild alligators in all tissues (Table 2). Lead concentrations in bone samples were higher than in all other tissues. There were no differences in lead concentrations in any tissues between males and females.

Table 3
Cadmium in alligator tissues

Tissue		n	Cadmium (ppb)	Range
Liver	Captive male	7	34±13*	12–110
	Captive female	20	23±3	13–60
	Wild male	3	46±16	14–64
	Wild female	2	17±1	16–18
Kidney	Captive male	8	16±3	3–29
	Captive female	19	33±4	12–86
	Wild male	7	24±8	6–67
	Wild female	6	30±15	12–103

* No significant differences for all comparisons of captive vs. wild.

Table 4
Selenium in alligator tissues

Tissue		n	Selenium (ppm)	Range
Liver	Captive	27	0.92±0.04*	0.63–1.47
	Wild	5	1.39±0.10	1.18–1.65
Kidney	Captive	27	1.44±0.07**	0.77–2.22
	Wild	14	2.00±0.21	1.13–3.65

* Wild vs. captive, $p>0.05$.
** Wild vs. captive, $p<0.05$.

3.2. Cadmium

Tissue cadmium concentrations were not significantly different between wild and captive alligators (Table 3).

3.3. Selenium

Selenium concentrations in the kidneys of wild alligators were significantly higher than in kidneys of captive alligators ($p<0.05$). There was no significant difference between wild and captive liver samples (Table 4).

3.4. Lipid peroxidation

Significantly more TBA-reactive material was detected in the livers from captive alligators than from wild alligators (Fig. 1). Mean values for TBA-reactive material in ovary or testis

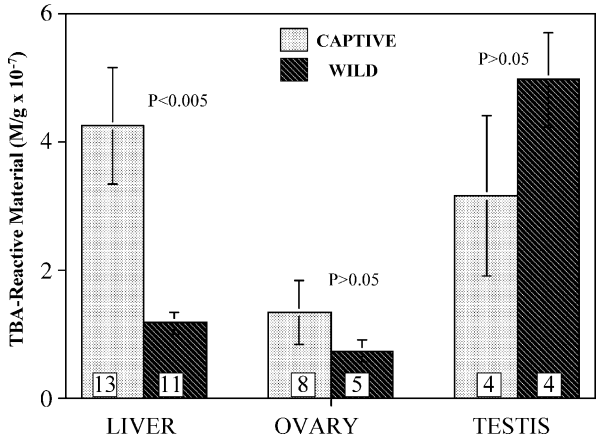

Fig. 1. TBA-reactive material in the liver, ovary and testis of alligators is presented as means and standard errors of the means, and is shown as bars (dark shading indicates specimens from wild alligators and lighter shading, specimens from captive alligators). The numbers at the base of each bar indicate the sample size in each group. Statistical significance is shown at the top of each pair of bars.

from captive alligators were not significantly different from those of wild alligators.

TBA values in testis samples were higher than the ovary (Fig. 1).

4. Discussion

The most significant finding in this study was the extremely high concentration of lead in bone, liver, kidney and yolk of the captive-reared alligators. Lead levels in the bone of captive alligators were the highest yet recorded for any vertebrate and suggest long-term accumulation. In humans more than 95% of the lead burden is incorporated into the hydroxyapatite matrix of bone (O'Flaherty, 1992), and may remain there for decades (Nilsson et al., 1991; Aufderheide and Wittmers, 1992). The extremely high lead content of the alligator femurs would argue for a similar mechanism operating in these animals exposed to lead over a long period. The nutria carcasses used to feed the alligators were obtained from local fur trappers, but had not been examined for lead contamination. Our results suggest that ingestion of lead from these carcasses had been occurring over many years, but we have no information on the degree of contamination. A study of the radial distribution of lead in the femurs from these alligators (Seltzer et al., in press) showing elevated lead in concentric rings, similar to growth rings, supports such an interpretation.

Curiously, only one of the captive alligators (an emaciated female, blind in one eye from an old injury) showed any overt signs of debility, whereas in the study of Camus et al. (1998), six 1-year-old farm-raised alligators died and several others showed signs of lethargy and inanition after ingesting lead-contaminated nutria meat. One-year-old farm-reared alligators weigh from about 4 to 7.5 kg (R. Elsey, unpublished data) whereas the mean body mass of the alligators in our study was 85 kg for the females and 217 kg for the males. The greater body mass of the older alligators might have been in some way protective, i.e. they consumed small doses of lead as a percent of body weight over a much longer period, sequestered the lead into bone, and thus showed no signs of lead toxicosis. In addition, the rate of feeding of the small farmed alligators could have been a factor. In most alligator farms the animals are fed on a daily basis during summer and winter, whereas the adult alligators in this study were fed only once a week and were not fed during the winter months. Lead levels in six kidney samples reported by Camus et al. (1998) ranged from 0.4 to 5.6 ppm, whereas lead in only two liver samples examined were 0.37 and 0.46 ppm, significantly lower than the levels found in this study. Contrary to the study by Camus et al. (1998) we found significantly higher lead concentrations in liver than in kidney (12.5 ppm vs. 4.9 ppm).

In a recent study in juvenile crocodiles, however, it was suggested that even animals of relatively small body mass possess a high resistance to lead (Hammerton et al., 2003). In this experiment young farm-reared crocodiles, *Crocodylus porosus*, (body mass 17–26 kg) were fed a known amount of lead shot (from 1.7 to 3.76 g) and then monitored for 20 weeks. None of the treated crocodiles showed any clinical signs of lead toxicity and all continued to increase in length and body mass despite blood lead concentrations as high as 5 mg/l. The authors concluded that crocodilians might possess a relatively high degree of resistance to lead toxicosis (Hammerton et al., 2003). The mass of the lead pellets recovered from the stomachs of the alligators in our study ranged from 1.2 to 51.2 g, a greater amount of lead than in the study by Hammerton et al. (2003), but, as in their study, there were no obvious signs of lead toxicosis. Even if we take into account the enormous difference in body mass between the captive alligators and the crocodiles, the amount of lead per kg body mass is still about one-half to two-fold higher in the alligators. When the alligators in our study were sacrificed, we were unaware of the presence of lead in the stomachs and failed to collect blood for lead analysis and are unable to make any comparison with the study on the young crocodiles.

A study of another long-lived reptile, the box turtle, *Terrepene carolina*, chronically exposed to effluent from a smelter, reported lead concentrations in bone (femur) of four individuals from 21.7 ppm to 135.8 ppm (Beresford et al., 1981). Liver and kidney had lead concentrations from 9.1 to 46.9 and 8.4 to 52.2 ppm, respectively. While the bone lead concentrations in the turtle were much lower than what we found in captive alligators, the liver and kidney levels were much higher. There was no information available on reproduction or any pathology in these animals.

Whether the lead was directly affecting reproduction in the captive alligators is still unclear, but the lead concentrations in the yolk of preovulatory follicles suggest that any developing embryos would be exposed to potentially toxic levels of the metal. A large body of literature exists on the toxicity of ingested lead in wild birds (Hui, 2002), and an abundance of experimental evidence demonstrates that relatively low lead concentrations in the yolk of bird eggs leads to embryonic mortality, without, however, any measurable effects on the adults (Birge and Roberts, 1976). Lead concentrations of 5 ppm in the yolk of chicken eggs resulted in greater than 75% mortality in chicken embryos, and at 10 ppm resulted in 100% embryonic mortality (Birge and Roberts, 1976). If we assume that the water content of alligator egg yolk is similar to that of chicken (~50%) (Etches, 1995, M. J. Packard personal communication), and we correct for water loss using this number for the dried yolk, the values are still clearly at a level likely to cause the death of the embryo (Table 2). It is therefore highly likely that the early embryonic death seen in many of the eggs from the captive alligators was due to exposure to exceptionally high lead concentrations in the yolk.

The concentrations of lead in yolk of wild alligators seen in this study are in the same range as those reported in alligator eggs from lakes in central Florida (Heinz et al., 1991) and similar to those reported for egg yolk from crocodiles (*C. porosus*) in northern Australia (Manolis et al., 2002) but significantly lower than those reported from crocodiles (*C. acutus*) in southern Florida (Stoneburner and Kushlan, 1984) and crocodiles (*Crocodylus niloticus*) in Zimbabwe (Phelps et al., 1986). The unusually high lead levels in crocodile eggs in Zimbabwe were attributed to river water from drainage basins

composed of granite rock formations with high lead content. There is no information on the hatching success of eggs collected from these river systems. Although the yolk lead levels in southern Florida indicate significant environmental contamination compared to southern Louisiana (Darbonne and Heagler, 1998), they are still orders of magnitude lower than those seen in the yolk from the captive alligators in this study and are unlikely to have any serious effects on the embryos.

No significant differences in cadmium levels between captive and wild alligators were seen in the tissues examined (Table 3), and the cadmium levels were similar to those found in other crocodilians (Campbell, 2003; Almli et al., 2005) and turtles (Burger and Gibbons, 1998) and are unlikely to have resulted in any significant pathology.

In a previous publication, we reported differences in plasma selenium concentrations between wild and captive alligators (Lance et al., 1983), and attributed the higher selenium in wild alligators to a significant proportion of fish in the diet, a rich source of selenium (Ganapathy et al., 1978). A similar result was found in this study. Nutria meat is very low in selenium (Lance and Elsey, 1983) and probably accounts for the significantly lower selenium levels in the tissue of captive alligators compared to those of wild alligators. It is, however, unlikely that selenium deficiency was contributing to the reproductive problems of the alligators. Selenium deficiency in domestic animals is defined as blood concentrations below 0.05 ppm (Van Fleet, 1980), a far lower value than the 0.185 ppm reported earlier for this captive population (Lance et al., 1983). Selenium concentrations in the wild alligators sampled in this study were considerably higher than those reported for alligator populations in Florida (Delaney et al., 1988), but similar to those reported for *C. porosus* in northern Australia (Manolis et al., 2002) and *C. niloticus* in Zambia (Almli et al., 2005).

The high tissue lipid peroxidation values in the captive alligators are consistent with previous studies on this population. Not only were tissue lipid peroxide levels higher than in wild alligators, but specific long chain fatty acids (in particular the C22:6n3, C20:4n6 and C20:5n3 acids) were significantly lower in both adult blood plasma and in egg-yolk of captive alligators (Noble et al., 1993; Lance et al., 2001). These same fatty acids were also very low in the nutria meat fed to the alligators (Noble et al., 1993).

Lead-induced lipid peroxidation in chicks has been well documented (Donaldson, 1991; Knowles and Donaldson, 1996; Somashekaraiah et al., 1992) and similar lead-induced activity is likely to have exacerbated the effects of a diet high in peroxidizable lipid in the alligators. The captive alligators also had significantly higher plasma triacylglycerols, cholesterol, and phospholipids than wild alligators (Lance et al., 2001). Blood plasma with low concentrations of unsaturated long chain fatty acids and extremely high cholesterol, however, has been documented in captive Nile crocodiles that were purported to have reproduced successfully (Mopurgo and Gelman, 1991).

In conclusion, our results suggest that incidental dietary intake of lead shot and long-term consumption of rancid nutria meat contributed significantly to reproductive failure in these captive alligators. While it is likely that the high concentration of lead in the yolk of captive alligators contributed to early embryonic death, other pathologies noted at necropsy including incomplete eggshell formation and oviductal inflammation are probably due to the effects of lipid peroxides (Van Vleet and Ferrans, 1992). Additional factors compounding these insults were crowding, sub-optimum nesting material (Elsey et al., 1990), low levels of essential fatty acids (Noble et al., 1993), obesity, and hyperlipidemia (Lance et al., 2001).

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