

Abnormal Bone Composition in Female Juvenile American Alligators from a Pesticide-Polluted Lake (Lake Apopka, Florida)

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Reproductive disorders have been found in pesticide-exposed alligators living in Lake Apopka, Florida (USA). These disorders have been hypothesized to be caused by exposure to endocrine-disruptive estrogen-like contaminants. The aim of this study was to expand our analysis beyond previous studies by investigating whether bone tissue, known to be affected by sex steroid hormones, is a potential target of endocrine disruptors. Long bones from 16 juvenile female alligators from Lake Apopka (pesticide-contaminated lake) and Lake Woodruff (control lake) were evaluated by peripheral quantitative computed tomography. We observed significant differences in bone composition, with female alligators from the contaminated lake having greater trabecular bone mineral density (BMD), total BMD, and trabecular mineral content compared with females from the control lake ($p < 0.05$). Increased trabecular and total BMD measurements suggest that juvenile female alligators from Lake Apopka were exposed to contaminants that created an internal environment more estrogenic than that normally observed. This estrogenic environment could be caused by both natural and anthropogenic compounds. Effects on BMD indicate interference with bone homeostasis. We hypothesize that contaminants present in the lake inhibit the natural and continuous resorption of bone tissue, resulting in increased bone mass. Although this is the only study performed to date examining effects of environmental estrogenic compounds on alligator bones, it supports previous laboratory-based studies in rodents. Further, this study is important in demonstrating that the alterations in morphology and physiology induced in free-ranging individuals living in environments contaminated with endocrine-active compounds are not limited to a few systems or tissues; rather, effects can be observed in many tissues affected by these hormones.

Key words: alligator, bone mineral density (BMD), EDCs, endocrine disruption, Lake Apopka, pesticides, pollution, pQCT. *Environ Health Perspect* 112:359–362 (2004). doi:10.1289/ehp.6524 available via <http://dx.doi.org/> [Online 1 December 2003]

Wildlife is exposed to a wide range of natural and man-made chemicals present in the environment. Extensive ecotoxicologic studies have documented that many of these compounds can affect survival of wildlife populations by altering an individual's ability to fight disease or reproduce (Guillette and Crain 2000). These effects appear to involve various mechanisms of action, including the alteration of cell signaling, leading to endocrine disruption or altered immune system function (for reviews, see Crain and Guillette 1997; Fournier et al. 2000).

Much of the focus on endocrine disruption over the last decade has been on the estrogenic, anti-estrogenic, or anti-androgenic actions of various environmental contaminants (Gray et al. 2001; McLachlan 2001). Further, most studies have examined alterations in the reproductive system. For example, we have reported developmental abnormalities of the gonads and abnormal sex hormone concentrations in both sexes of neonatal and juvenile alligators from pesticide-contaminated Lake Apopka, Florida (USA; for review, see Guillette et al. 2000). Lake Apopka was the recipient of an industrial pesticide spill in 1980 that contaminated the lake with the endocrine-active pesticides DDT [1,1,1-trichloro-2,2-bis(4-chlorophenyl)ethane]

and dicofol (*p,p'*-dichlorodiphenyl-2,2,2-trichloroethanol).

This lake also has received agricultural and stormwater runoff. A declining alligator population in Lake Apopka during the 1980s, coupled with a dramatic decline in successful egg-hatching rates from 1983 to 1988, led to studies that found extensive pesticide contamination in this population with such compounds as dicofol and the DDT metabolite 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (*p,p*-DDE) in alligator eggs, plasma, and tissue (Guillette et al. 1999a; Heinz et al. 1991). We have hypothesized that the reported abnormalities in alligators from Lake Apopka were caused partly by the anti-androgenic and estrogenic actions of the various pesticides found in these animals. Recent studies have extended this work to other systems influenced by sex and stress steroids, such as the immune system. We have observed alterations in immune tissue histology and immune cell function for animals from Lake Apopka (Rooney 1998; Rooney et al. 2003).

Estrogens also exhibit dramatic influences on bone and are essential for bone tissue homeostasis. An increase in estrogenic levels could increase the amount of bone tissue, whereas a decrease in estrogens would cause a

decline such as seen in age-related bone loss in both postmenopausal women and men (Khosla et al. 1998; Kiel et al. 1987). Given the studies published during the last decades, it is obvious that the skeleton could be an important target for a number of endocrine-disrupting chemicals (EDCs; Aulerich et al. 2001; Gierthy et al. 1994; Jämsä et al. 2001; Lind 2000; Lind et al. 1999, 2000a, 2000b; Migliaccio et al. 1996). Because 17β -estradiol and testosterone play an essential role in bone tissue homeostasis, the aim of this study was to examine possible effects on the skeleton of female juvenile alligators from a lake polluted with EDCs.

Materials and Methods

In total, 16 juvenile female American alligators (*Alligator mississippiensis*) were collected at night by hand from two lakes in central Florida approximately 65 km apart: Lake Apopka (pesticide-polluted lake) and Lake Woodruff (reference lake; for further descriptions of these lakes and the organochlorine pollutants identified to date, see Guillette et al. 1999a). The mean ages of the female animals from the reference and the contaminated lakes, respectively, were similar (5.6 vs. 5.2 years; Table 1). Extensive research on these populations over the last decade has shown that animals from Lake Woodruff represent an excellent reference population, with high reproductive rates (Guillette et al. 2000). Importantly, recent studies have reported that animals from Lake Apopka and Lake Woodruff show similar growth patterns (Guillette et al. 2000; Milnes et al. 2002) and are indistinguishable at the population genetics level using modern molecular markers (Davis et al. 1994).

The animals, as part of a larger research effort, were given a lethal dose of sodium

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pentobarbital (Sigma Chemical, St. Louis, MO, USA). The tibia and femur were dissected free from soft tissue and stored at -20°C pending analysis of bone characteristics. The total length of the tibia (the distance from the upper edge of the knee joint to the distal end of the bone) and the femur (the distance from the upper edge of the hip joint to the distal end of the bone) were measured using an electronic caliper to the nearest 0.1 mm (Figure 1).

The bone composition and dimensions were evaluated by peripheral quantitative computed tomography (pQCT; Stratec XCT 960A with software version 5.20; Norland Stratec Medizintechnik GmbH, Birkenfeld, Germany). Precision, long-term stability, linearity, and accuracy of the pQCT bone scanner were evaluated once a day using a validation phantom.

Before measurement, the bones were strapped horizontally on a special device for stability. To evaluate the reproducibility of the pQCT measurements, the coefficient of variation (CV) (mean ± SD) was calculated from 10 repeated measurements with a single sample repositioning before each measurement.

The tibial and femoral diaphyses were scanned at the midpoint (50%; Figure 1) of

the bone, because this area consists almost exclusively of cortical bone. The diaphysis of the tibia was scanned at midshaft using a voxel size of 0.148 × 0.148 × 1.25 mm, whereas a voxel size of 0.197 × 0.197 × 1.25 mm was used for the longer and thicker femur. The scan line was adjusted using the scout view of the pQCT system, and an attenuation threshold of 0.93 cm⁻¹ was used to define cortical bone. For the analyses, we used the total cross-sectional area (including marrow cavity and cortical bone, in square millimeters), cortical area (square millimeters), cortical thickness (millimeters), cortical bone mineral content (milligrams per millimeter), cortical bone mineral density (BMD; milligrams per cubic centimeter), total BMD (milligrams per cubic centimeter), and periosteal circumference (millimeters).

The metaphyses of the tibia and femur were scanned at a point located 11.5 and 16%, respectively, of the total bone length from the proximal tip (Figure 1). This region on tibia and femur was chosen because of its high content of trabecular bone. The scan line was adjusted using the scout view of the pQCT system, and peel mode 2, contour mode 1, threshold 0.400 cm⁻¹, and inner threshold 0.700 cm⁻¹ were used. For the analyses, we used the total cross-sectional area (square millimeters), trabecular area (square millimeters), trabecular bone mineral content (milligrams per millimeter), trabecular BMD (milligrams per cubic centimeter), and total BMD (milligrams per cubic centimeter).

Statistical analysis. The results obtained were evaluated by one-way analysis of variance followed by a post hoc Fisher's protected

least significant difference test. Differences were considered significant at *p* < 0.05 (StatView, version 5.0; SAS Institute Inc., Cary, NC, USA)

Results

The reproducibility of the pQCT measurements from female juvenile alligator tibia and femurs of the bones varied, with a CV from 0.3 to 16.5%. The results from the measurements of the bones are presented in Tables 1–4.

The tibial diaphyseal total BMD in females from the pesticide-polluted Lake Apopka was significantly greater than that of females from Lake Woodruff (Table 1; *p* < 0.05). The tibial trabecular BMD in females from Lake Apopka was also significantly greater (34%) than that of females from the control lake (Table 2; *p* < 0.05). In addition, the trabecular bone mineral content was 75% greater in females from Lake Apopka than that of females from Lake Woodruff (Table 2; *p* < 0.05). The changes in trabecular density and content are visible in the images of the pQCT scans shown in Figure 2.

The femoral metaphyseal total BMD in females from Lake Apopka was 12% greater than that observed in females from Lake Woodruff (Table 4; *p* < 0.05). The femur trabecular BMD was 30% greater in females from Lake Apopka than that in females from Lake Woodruff (Table 4; *p* < 0.05).

Discussion

This study reports differences in bone composition in juvenile female alligators from Lake Apopka, contaminated with a variety of pesticides, pesticide metabolites, and nutrients, relative to that in females of similar age and

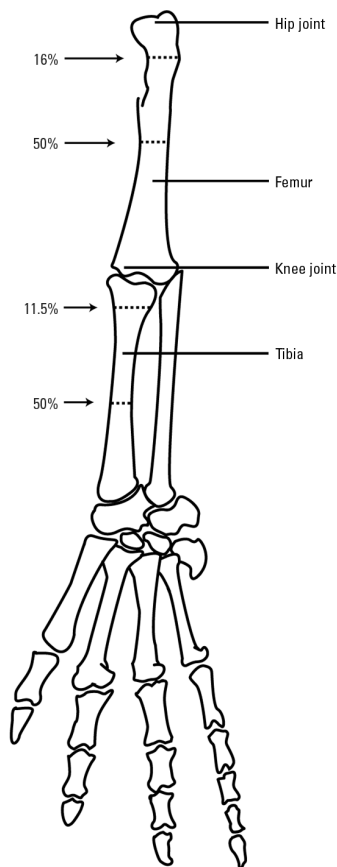


Figure 1. Drawing of alligator leg skeleton showing the measuring points of the pQCT.

Table 1. Tibia, diaphyseal measure point.

Measures	Lake Woodruff (n = 7)	Lake Apopka (n = 9)
Age (year)	5.6 ± 0.8	5.2 ± 0.4
Length of tibia (mm)	52.0 ± 3.7	60.3 ± 3.1
Total cross-sectional area (mm ²)	5.2 ± 0.8	7.1 ± 0.7
Cortical area (mm ²)	4.9 ± 0.7	6.9 ± 0.7
Cortical thickness (mm)	1.0 ± 0.1	1.2 ± 0.1
Cortical bone mineral content (mg/mm)	6.2 ± 0.9	8.9 ± 0.9
Cortical BMD (mg/cm ³)	1288.9 ± 9.3	1242.1 ± 21.1
Total BMD (mg/cm ³)	1145.9 ± 23.3	1230.5 ± 11.8*
Periosteal circumference (mm)	8.0 ± 0.6	9.3 ± 0.5

Results were obtained from pQCT measurements; the measuring point was located at the middle (50%) of the bone (Figure 1) of tibial bone from female alligators (*Alligator mississippiensis*), 3–11 years of age, originating from Lake Woodruff (control lake) and Lake Apopka (contaminated lake). Values are mean ± SE.

**p* < 0.05 compared with controls.

Table 2. Tibia, metaphyseal measure point.

Measures	Lake Woodruff (n = 7)	Lake Apopka (n = 9)
Total cross-sectional area (mm ²)	10.9 ± 1.7	15.5 ± 1.7
Trabecular area (mm ²)	4.3 ± 0.6	6.1 ± 0.8
Trabecular bone mineral content (mg/mm)	0.8 ± 0.1	1.4 ± 0.2*
Trabecular BMD (mg/cm ³)	171.8 ± 12.9	228.2 ± 16.4*
Total BMD (mg/cm ³)	628.6 ± 33.1	678.1 ± 20.5

Results were obtained from pQCT measurements; the measuring point was located at a distance 11.5% of the length from the proximal part of the bone (Figure 1) of tibial bone from female alligators (*Alligator mississippiensis*), 3–11 years of age, originating from Lake Woodruff (control lake) and Lake Apopka (contaminated lake). Values are mean ± SE.

**p* < 0.05 compared with controls.

size from a reference lake, Lake Woodruff. Pronounced effects on tibial and femoral bone were observed in this study. The tibial diaphyseal total BMD and metaphyseal trabecular BMD in juvenile females from Lake Apopka were significantly greater than those observed in juvenile females from Lake Woodruff. A further difference was noted: Females from Lake Apopka exhibited greater tibial metaphyseal trabecular bone mineral content than did females obtained from Lake Woodruff. The femoral metaphyseal trabecular BMD and metaphyseal total BMD were significantly greater in females from the contaminated lake.

Five variables associated with bone structure differed significantly between the two groups of alligators; four of these are metaphyseal variables. Using our analysis, the metaphysis seems to be more sensitive to alteration compared with the diaphysis relative to exposure to the compounds present in Lake Apopka. The basis for these differences is not known, but several hypotheses can be proposed. First, the metaphyseal region of the bone might have a higher metabolic rate than the diaphyseal region because of a higher content of trabecular bone. Second, hormones influence skeletal morphology and physiology, but not all parts of a bone show similar responses. For example, the trabecular part of the long bones is used as an easily accessible calcium storage source for physiologic functions, whereas other skeletal elements are not used as readily. Specifically, previous studies have shown that the long bones of adult female alligators undergo dramatic changes in structure with reproductive activity (Wink and Elsey 1986; Wink et al. 1987). Further, estradiol treatment has been shown to alter bone structure and plasma calcium concentrations in

alligators (Elsey and Wink 1986). We observed that alligators of the size and age used in this study are actually subadult animals that display seasonal variation in plasma sex steroid hormone concentrations and show a pattern similar to that observed in adults, although plasma hormone concentrations vary less than those observed in adults (Rooney 1998).

Given our knowledge of normal physiologic responses in alligators, we hypothesized that contaminants that can alter hormonal homeostasis would influence the morphology and function of systems dependent on these hormones. EDCs can affect different hormonal systems in vertebrates, including alligators, such as *a*) thyroid hormones, which are critical for growth, differentiation, and metabolic regulation (Crain et al. 1998; Hewitt et al. 2002); and *b*) sex steroids, which regulate reproductive functions but have effects throughout the organism (Guillette et al. 1999b; Iguchi et al. 2001). We have previously shown that alligators living in contaminated environments have a number of abnormalities in the reproductive, endocrine, and immune systems, including altered plasma sex steroid concentrations (Guillette et al. 2000; Rooney et al. 2003).

The results of this study suggest that the alligators from Lake Apopka are exposed to estrogenic compounds, because we observed an increase in total and trabecular BMD, a characteristic of estrogen exposure in other vertebrates (Breen et al. 1998). A decrease in BMD would have suggested exposure to anti-estrogenic compounds, with osteoporosis as a result. The animals used in this study were exposed to a complex mixture of chemicals, many of which have the potential to interact with the alligator estrogen receptors (ERs; Guillette et al. 2002; Vonier et al. 1996). Major contaminants

present are DDT and its metabolites, DDE and DDD (Guillette et al. 1999a; Heinz et al. 1991). *o,p'*-DDT is generally considered estrogenic, but the metabolite *p,p'*-DDE has varying effects: It has been reported to be estrogenic or to have no estrogenic action in reptiles (Matter et al. 1998; Podreka et al. 1998; Willingham and Crews 1999). Feminization of the males is thought to be caused by either estrogen (or estrogen-like compounds) binding to ERs causing estrogenic effects, or estrogen blocking androgen receptors causing anti-androgenic effects. Thus, the presence of a compound like *p,p'*-DDE that can have anti-androgenic as well as estrogenic action might give rise to an estrogenic internal environment.

The overall effect of these complex mixtures of compounds with varying actions could depend on life stage, reproductive stage, or stress level. For example, a previous study by Lind et al. (1999) demonstrated that the hormonal activity of a single substance may differ depending on the endocrine conditions within a given tissue or organ. The authors found that in ovariectomized rats, 3,3',4,4',5-pentachlorobiphenyl (PCB-126) caused a decrease in length of the tibia and an increase in BMD, which indicates an estrogenic effect. In sham-operated female rats, however, PCB-126 appeared to exhibit anti-estrogenic activity by impairing the mineralization process of the tibia, as indicated by increased organic content and osteoid surface (Lind et al. 1999).

Table 3. Femur, diaphyseal measure point.

Measures	Lake Woodruff (n = 8)	Lake Apopka (n = 9)
Length of femur (mm)	71.6 ± 6.7	80.2 ± 4.5
Total cross-sectional area (mm ²)	27.4 ± 5.5	35.6 ± 4.1
Cortical area (mm ²)	26.1 ± 5.2	34.4 ± 4.0
Cortical thickness (mm)	2.3 ± 0.3	2.7 ± 0.2
Cortical bone mineral content (mg/mm)	36.8 ± 7.5	47.2 ± 5.3
Cortical BMD (mg/cm ³)	1385.2 ± 13.4	1380.2 ± 8.0
Total BMD (mg/cm ³)	1320.2 ± 18.3	1321.5 ± 7.0
Periosteal circumference (mm)	17.9 ± 1.8	20.8 ± 1.3

Results were obtained from pQCT measurements; the measuring point was located at the middle (50%) of the bone (Figure 1) of femur bone from female alligators (*Alligator mississippiensis*), 3–11 years of age, originating from Lake Woodruff (control lake) and Lake Apopka (contaminated lake). Values are mean ± SE.

Table 4. Femur, metaphyseal measure point.

Measures	Lake Woodruff (n = 8)	Lake Apopka (n = 9)
Total cross-sectional area (mm ²)	67.4 ± 12.3	89.9 ± 10.7
Trabecular area (mm ²)	37.9 ± 6.4	46.6 ± 5.9
Trabecular bone mineral content (mg/mm)	7.1 ± 1.6	10.1 ± 1.3
Trabecular BMD (mg/cm ³)	171.7 ± 15.9	218.3 ± 7.3*
Total BMD (mg/cm ³)	507.3 ± 23.9	567.0 ± 13.3*

Results were obtained from pQCT measurements; the measuring point was located at a distance 16% of the length from the proximal part of the bone (Figure 1) of femur bone from female alligators (*Alligator mississippiensis*), 3–11 years of age, originating from Lake Woodruff (control lake) and Lake Apopka (contaminated lake). Values are mean ± SE.

**p* < 0.05 compared with controls.

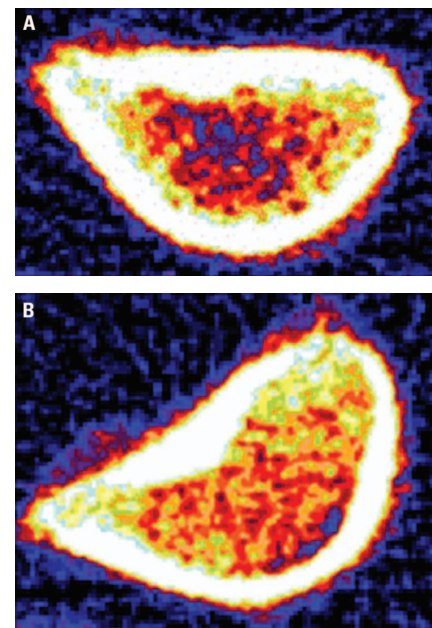


Figure 2. Images from pQCT scans of proximal tibial metaphysis. (A) Lake Woodruff female. (B) Lake Apopka female. The white outer shell is cortical bone with a high BMD, and the inner spongy structures are trabecular bone with a much lower BMD. The blue areas contain bone tissue with a lower mineral density compared with the yellow and red areas.

Most likely, the observed findings on alligator bone tissues are due to either increased plasma levels of 17 β -estradiol (Milnes et al. 2002) or a direct effect of contaminants on processes controlling bone homeostasis. Effects on BMD indicate an interference with the bone remodeling process. A possible mechanism explaining our observations could be the inhibition of osteoclastic activity due to an antagonistic effect via osteoclastic ERs (Bryant et al. 1999; Kameda et al. 1997; Oursler et al. 1991). Another possibility is an effect on both the osteoblasts and osteoclasts resulting in the production and release of transforming growth factor β . This growth factor may mediate the actions of estrogen in bone (Bord et al. 2001) via either a local or systemic mechanism of action (Seibel et al. 1999). Future studies are required to extend the observations reported here and to elucidate the mechanisms by which these effects are induced. Further, although this study has documented effects in the population of juvenile females from Lake Apopka, additional work is needed to examine the ontogenetic development of this response.

Questions to be addressed in the future include determining whether the differences noted here persist in adult females and whether they are found in juvenile and adult males. Although this is the only study performed to date examining the possible relationships between environmental estrogenic compounds and alligator bones, it is important because it demonstrates that the alterations in morphology and physiology induced in free-ranging individuals by environments contaminated with endocrine-active compounds are not limited to just a few systems, such as the reproductive or immune system. Future studies should examine all of the tissues influenced by steroid hormones to determine the complete extent of possible effects of exposure to endocrine disruptors.

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