THE EFFECTS OF ESTRADIOL ON PLASMA CALCIUM AND FEMORAL BONE STRUCTURE IN ALLIGATORS (ALLIGATOR MISSISSIPPIENSIS)

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Abstract—1. The effects of exogenous estradiol on plasma calcium and femoral bone structure were studied in young male and female alligators.
2. Males and females responded in the same manner to estrogen treatment.
3. Eight days after the initial injection, plasma calcium was significantly greater in experimentals than in controls.
4. No changes in femoral bone structure were observed.

INTRODUCTION

Birds and many reptiles are egg-layers. In birds, pre-ovulatory, estrogen-dependent hypercalcemia and medullary bone proliferation are followed by resorption of this endosteal bone which can be correlated with formation of the calcareous eggshell (Simkiss, 1967; Taylor et al., 1971). Estrogen treatment elicits hypercalcemia and endosteal bone formation in birds (Bloom et al., 1941), and hypercalcemia without endosteal bone formation in lower reptiles, such as turtles (Simkiss, 1961; Magliola, 1984). Turtles, apparently, have not evolved the skeleton-sparing mechanism of pre-ovulatory bone formation to form eggshells; they resorb structural bone during eggshell formation (Edgren, 1960; Simkiss, 1961; Suzuki, 1963; Magliola, 1984). It has been shown that breeding female alligators, like other egg-laying vertebrates, exhibit a pre-ovulatory hypercalcemia associated with eggshell formation (Lance et al., 1983). Recent studies in our laboratory have indicated that the alligator also resorbs structural bone during eggshell formation (Elsey and Wink, 1985). It is not known, however, how the skeleton and plasma calcium levels of alligators respond to exogenous estrogen treatment. Alligators, which are closely related to birds in evolution, may, like birds, develop hypercalcemia and form endosteal bone in response to estrogen treatment in addition to resorbing structural bone during eggshell formation, as do turtles. Therefore, the purpose of this study was to determine the effects of estrogen treatment on plasma calcium levels and femoral bone morphology of the alligator.

MATERIALS AND METHODS

Animals

Ten young alligators (Alligator mississippiensis) (3.5 years old) were obtained from a commercial alligator farm (Sauros Inc., Bell City, LA). These alligators had been hatched from artificially incubated alligator eggs collected from Rockefeller Wildlife Refuge (Joanen and McNease, 1977). The animals were sexed by palpating within the cloaca and noting the presence or absence of a penis. They were then divided into two groups, with three females and two males in each group. They were maintained at Rockefeller Wildlife Refuge, Grand Chenier, LA, in controlled environmental chambers (Joanen and McNease, 1977) and fed a diet of ground nutria (Myocastor coypus). An initial 5 ml blood sample was taken from each animal by cardiac puncture with a heparinized syringe. One group (three females, two males) was injected with 5 mg estradiol dissolved in 1 ml sesame oil (experimental). The other group of animals (three females, two males) was injected with 1 ml sesame oil only (controls). Injections were given intramuscularly at three sites (tail and each lower extremity). Every 8 days for the next month, blood samples were taken from each animal, and each animal was re-injected with estradiol, or vehicle, as above. Blood samples were immediately centrifuged and the plasma was removed and frozen for later assay.

At the end of 1 month (after four injections) the animals were killed by a blow to the head and the spinal cords severed. Alligators were weighed and body lengths were measured. Necropsies were performed, and livers, ovaries, oviducts, testes and penes were removed and weighed. Femora were also removed and frozen for later analysis. Femora were chosen because Ferguson (1982) showed that the femur undergoes less remodeling during aging than other bones sampled (tibia, fibula, humerus, radius, ulna, mandible, vertebrae, frontal, maxilla, palatine, pterygoid, nasal, jugal, ribs and dorsal neck scutes).

Plasma calcium determinations

Plasma calcium levels were determined by atomic absorption with a 305B Perkin-Elmer atomic absorption spectrophotometer.

Femur robusticity index

Femora were thawed and placed in an 85°C water bath for 1 week to remove soft tissue. They were then defatted and dehydrated in a series of alcohols and ethers and finally dried in a vacuum oven as described previously (Wink and Felts, 1980). They were weighed, and lengths were measured with vernier calipers. Bone robustness was determined using the formula:

$$\text{robusticity index} = \frac{\text{bone length}}{\sqrt{\text{bonet weight}}}$$
This robusticity or ponderal index was first proposed by Riesenfeld (1972) and has been used to indicate the robustness or density of a bone as a whole (Riesenfeld, 1975, 1981; Simon, 1984). The higher the index number, the less dense the bone; the lower the number, the more robust or denser the bone.

**Femoral morphometrics**

Sections of bone 2 cm long were cut from the middle of each dried femoral shaft and embedded in Ward’s Bioplastic. Cross sections of 200 μm thickness were cut from each piece using a Bronwill Hard Tissue Cutting Machine and a diamond saw. Microradiographs of the femoral cross sections were made with a Faxitron X-ray machine and Kodak high resolution plates (649-0). The microradiographs were processed routinely, projected with a microprojector and drawn at 20 x magnification. Drawings of bone sections were analyzed with a Numonics Electronic Calculator for total area, area occupied by bone (mm²), percentage area occupied by bone, area occupied by marrow cavity (mm²) and percentage area occupied by marrow cavity. Total area was obtained by marking around the perimeter of the enlarged drawing of each bone section. Area occupied by bone was obtained by marking around bone in the section (excluding marrow cavity and "holes" or radiolucent areas in the bone). Area of marrow cavity was obtained by marking around the marrow cavity of each section. The percentage areas occupied are obtained using:

\[
\text{percentage area occupied by bone} = \frac{\text{area occupied by bone}}{\text{total area}}
\]

\[
\text{percentage area of marrow cavity} = \frac{\text{area of marrow cavity}}{\text{total area}}
\]

Data were analyzed by Student’s t-test.

**RESULTS**

There were no significant differences in body weight, body length, liver weight or gonad weight between the sexes (male and female control animals or male and female experimental animals). There were no significant differences in body weight, body length or gonad weight between experimental and controls (Table 1). Oviduct weight was significantly greater (although not significantly) than in the controls, no increase in the medulla. Prosser and Suzuki (1968) injected another crocodilian, *Caiman sclerops*, with estrogen and observed a gradual hypercalcemic response that did not become significant until 3 weeks after treatment was begun. Also, there were no morphological changes in the bones. However, the investigators used hatchlings and young juvenile caimans for their studies and noted that the immaturity of the animals may have been responsible for the low response to estrogen.

**DISCUSSION**

It is well known that in egg-laying vertebrates the developing ovarian follicle secretes estradiol, which stimulates the liver to synthesize vitellogenin, a calcium-binding lipophosphoprotein (Ho et al., 1982). Similarly, estradiol injections caused synthesis of vitellogenin in a crocodilian, *Caiman latirostris*, (Van Brunt and Menzies, 1971). In the present study estrogen treatment induced liver hypertrophy in male and female alligators as it did in snakes (Dessauer and Fox, 1959) and lizards (Callard et al., 1972; Callard and Klotz, 1973; Hahn, 1967). This liver hypertrophy was probably secondary to vitellogenin synthesis. Our study also confirms the findings of Forbes (1938), who observed an extreme hypertrophy of the oviducts of female alligators injected with estrogen, and no differences in the penes of estrogen-treated males when compared to those of control males. Histological studies (Forbes, 1938) of the gonads from estrogen-treated animals showed hypertrophy of both ovarian and testicular cortices with no changes seen in the medullas. Gonad weights in the experimental alligators of our study were greater (although not significantly) than in the controls, which may have reflected cortical hypertrophy with no increase in the medulla.

**Table 1. Effects of estrogen on body weight and length, weight of gonads, penis, oviduct, liver and liver weight/body weight (mean ± SD)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (kg)</th>
<th>Body length (cm)</th>
<th>Gonad weight (g)</th>
<th>Penis weight (g)</th>
<th>Oviduct weight (g)</th>
<th>Liver weight (g)</th>
<th>Liver weight/body weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental</td>
<td>7.81 ± 0.85</td>
<td>132.33 ± 3.73</td>
<td>4.70 ± 1.67</td>
<td>5.80 ± 0.85</td>
<td>204.40 ± 58.10*</td>
<td>136.18 ± 26.80*</td>
<td>1.73 ± 0.23*</td>
</tr>
<tr>
<td>(Estrogen-treated)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>7.69 ± 0.79</td>
<td>135.38 ± 2.79</td>
<td>3.56 ± 1.27</td>
<td>7.45 ± 6.86</td>
<td>9.83 ± 4.70</td>
<td>90.7 ± 15.20</td>
<td>1.17 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>(N)</td>
<td></td>
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</table>

*Significantly different from controls, *P* < 0.01.

<table>
<thead>
<tr>
<th>N</th>
<th>Robusticity index</th>
<th>Total Area mm²</th>
<th>% Marrow cavity</th>
<th>% Bone</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>4.07 ± 0.13</td>
<td>282.25 ± 14.25</td>
<td>6.04 ± 2.03</td>
<td>91.40 ± 1.76</td>
</tr>
<tr>
<td>5</td>
<td>4.00 ± 0.13</td>
<td>293.45 ± 27.46</td>
<td>5.43 ± 2.15</td>
<td>91.48 ± 2.49</td>
</tr>
</tbody>
</table>

**Table 2. Effects of estrogen on femoral robusticity index, total area of femoral microradiographs, and percentage area occupied by marrow cavity and bone (mean ± SD)**

<table>
<thead>
<tr>
<th>Group</th>
<th>Robusticity index</th>
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</tr>
</tbody>
</table>

(N) = number of animals.
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Fig. 1. Microradiograph (4 x magnification) of femoral cross sections from (A) estrogen-treated female and (B) control female. Note similarity of marrow cavities and endosteal resorption areas.

Treatment. Budy et al. (1952) also attributed lack of endosteal bone formation, after estrogen treatment, to age in newborn rats. In the present study, we used older (3.5 years old) sexually immature alligators, and the hypercalcemic response was much more rapid (at 8 days). Eight days was the earliest time we sampled blood after the initial injection; there may have been a significant difference in calcium levels between experimentals and controls before 8 days. Suzuki and Prosser (1968) reported significant differences in serum calcium between estrogen-treated adult lizards and controls after 5 days.

We could see no evidence of medullary bone proliferation in the experimental alligators. Marrow cavity areas were the same in both groups and no "extra" bone filled in resorption areas of the endosteal bone. It is unlikely that there was medullary bone proliferation in the femur at any other site than the one we sampled at midshaft, since the robusticity indices of the femora did not differ between the groups.

In conclusion, it appears that the alligator, like lower reptiles, such as turtles, lizards and snakes, responds to estrogen treatment with hypercalcemia, and, unlike the bird, has not evolved the skeleton-sparing mechanism of estrogen-dependent, pre-ovulatory medullary bone proliferation for the formation of egg shells.

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REFERENCES


