

## Changes in Femoral Morphology During Egg-laying in *Alligator mississippiensis*

CAROLE S. WINK AND RUTH M. ELSEY

Department of Anatomy, Louisiana State University Medical Center, New Orleans, Louisiana 70112 (C.S.W.), and Louisiana Department of Wildlife and Fisheries, Rockefeller Wildlife Refuge, Grand Chenier, Louisiana 70643 (R.M.E.)

**ABSTRACT** Birds and many reptiles are egg-layers. Birds provide calcium for the formation of eggshells by resorbing medullary bone, which is laid down before ovulation. Turtles do not possess this mechanism and resorb structural bone to form eggshells. Femora from three groups of alligators (egg-laying females; quiescent, immature, or barren females; and males) were examined to determine if alligators, which are closely related to birds in evolution, resorb structural bone during the formation of eggshells as do turtles. Microradiographs of cross sections from femoral mid-shafts were analyzed for porosity, and the robusticity index of each femur was determined. Scanning electron micrographs of anorganic endosteal and periosteal femoral surfaces were analyzed to determine numbers of entrances of vascular canals, numbers of lacunae of osteoblasts, and types of femoral surfaces. Femora from egg-laying females were significantly less robust than those of other females or males, and sections of bone from the egg-layers were significantly more porous than those of the other groups. Scanning electron microscopy of anorganic femoral endosteal surfaces from egg-laying females revealed significantly more resorption areas when compared with males or non egg-laying females. Periosteal surfaces from egg-layers had significantly more resting and less bone-forming surface than those from the other groups. Results indicated that apposition of periosteal bone may have been reduced in egg-layers, and that egg-laying alligators, like turtles, resorb endosteal structural bone, which may be used as a source of calcium for the formation of eggshells.

Birds have evolved a mechanism that provides calcium for the formation of eggshells without imposing a burden on the female's skeletal calcium reserves. Preovulatory estrogen-dependent hypercalcemia and medullary bone formation are followed by resorption of this endosteal bone in correlation with the formation of the calcareous eggshell (Simkiss, '67; Taylor et al., '71). Turtles and alligators also lay eggs and also exhibit pre-ovulatory hypercalcemia associated with the formation of eggshells (Edgren, '60; Simkiss, '61; Lance et al., '83; and Magliola, '84). Turtles, however, do not lay down medullary bone prior to forming eggshells; instead they rely on resorption of structural bone to provide calcium for eggshells (Edgren, '60; Simkiss, '61, and Magliola, '84). Prosser and Suzuki ('68) elicited hypercal-

cemia in young caimans by injecting them with estradiol valerate. They observed no proliferation of medullary bone in the estrogen-treated caimans, indicating that crocodilians, like turtles, may not have evolved a mechanism for skeleton-sparing during the formation of eggshells. The purpose of the present study was to determine if the crocodilian *Alligator mississippiensis* resorbs structural bone during the formation of eggshells.

### MATERIALS AND METHODS

Femora were collected in the field from three groups of freshly killed alligators: 1) egg-laying females; 2) quiescent, barren, or immature females; and 3) males. Femora were chosen because Ferguson ('82) 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). Baited hooks were set at nest sites to ensure catching egg-producing females, and males and control females were caught with nooses at night as described by Chabreck ('63). Laparotomies were performed on all females to verify reproductive status.

#### *Robusticity of bones*

Femora were cleaned and placed in an 85°C water bath for 1 week to divest them of soft tissue. Then they were defatted and dehydrated through a graded series of alcohols and ethers and finally dried in a vacuum oven. Bones were weighed and lengths measured with a vernier calipers. The robusticity of the femora was determined using the formula:

$$\text{robusticity index} = \frac{\text{bone length}}{3\sqrt{\text{bone weight}}}$$

This robusticity or ponderal index was first proposed by Riesenfeld ('72) and has been used to indicate the density or robusticity of a bone as a whole (Riesenfeld, '75, '81; Simon, '84). The lower the index, the denser or more robust the bone; the higher the index, the less dense or more porous the bone.

#### *Porosity of bone sections*

After robusticity indices were determined, pieces 2 cm long were cut from the middle of each femoral shaft. This mid-point on the shaft was determined by measuring the total length of the bone from the femoral head to the lateral condyle and dividing by two. The 2-cm femoral pieces, in turn, were cut in half. The proximal half was processed for SEM, and the distal half was embedded in Ward's Bioplastic. Cross sections 200  $\mu\text{m}$  thick were cut from each embedded piece of femoral shaft with a Bronwill Hard Tissue Cutting Machine and a diamond saw. The fifth section from each embedded piece of bone was selected for microradiography so that each femoral shaft was sampled approximately 1 mm distal to the middle of the bone. Microradiographs of the plastic sections (one from each femur) were made with a Faxitron X-ray machine and Kodak High Resolution

Plates 649-0. After routine processing the microradiographs were projected with a microprojector and drawn at 20 $\times$ . Each drawing was analyzed for porosity with a Numonics Electronic Graphics Calculator. The total area of section was determined by marking around the periphery of the entire cross section (excluding the marrow cavity). Total area unoccupied by bone was determined by marking around the "holes" or radiolucent areas in the bone and adding them together. Porosity was calculated as:

$$\text{percent area unoccupied by bone} = \frac{\text{total area unoccupied by bone}}{\text{total area of section}} \times 100$$

The higher the percent area unoccupied by bone, the greater the porosity of the section.

#### *Scanning electron microscopy*

The proximal portions of pieces of femoral shaft were split into anterior and posterior halves and soaked in 5.25% sodium hypochlorite (Clorox) overnight to render them anorganic (Boyde and Hobdell, '68). Then they were rinsed and dehydrated, critical point dried, and glued on stubs for SEM as described previously (Wink, '82). Anterior portions were glued to the stub endosteal surface up, and posterior portions were glued periosteal surface up. All specimens were sputter coated with gold palladium and observed at 20 kV in a JEOL T-300 SEM. Starting at the proximal cut edge of each bone specimen at a magnification of 75 $\times$ , entrances of vascular canals were counted in ten locations along the middle of each femoral segment. Each location was separated by a distance of half the SEM display screen to avoid overlap (Dempster et al., '79). The lacunae of osteoblasts were also counted at a magnification of 350 $\times$  in 20 locations along the middle of each specimen as described above. Then tracings were made of each of the 20 locations at 500 $\times$ . Using the Numonics Electronics Graphics Calculator, the total area ( $\text{mm}^2$ ) of each type of bone surface within the tracing was determined, and the percent area of each type of bone surface was calculated by:

$$\frac{\text{area of type of bone surface}}{\text{total area of tracing}} \times 100$$

Types of anorganic bone surface have been described extensively by others (Boyde and Hobdell, '69a,b; Jones and Boyde, '70; Dempster et al., '79; Wink, '82). In the present study the following types of surface were analyzed: 1) *forming*—surfaces composed of mineral nodules, indicative of bone forming surfaces in various stages of mineralization; 2) *resting*—surfaces composed of mineralized collagen fibers or bundles of fibers, indicative of completely mineralized resting bone surfaces; and 3) *resorbing*—surfaces showing depressions or pits with scalloped edges, indicative of bone matrix that had undergone osteoclastic resorption. All data were analyzed by Student's t test.

### RESULTS

Male alligators had femora that were more robust ( $P < 0.001$ ) than femora from either group of females (Table 1). Egg-laying alligators had femora that were significantly less robust ( $P < 0.05$ ) than those of the non egg-laying females. Femoral bone sections from egg-layers were significantly more porous than those of males ( $P < 0.01$ ) and non egg-laying females ( $P < 0.001$ ) (Table 1; Figs. 1,

2). Anorganic femoral endosteal surfaces from egg-laying alligators had significantly more ( $P < 0.05$ ) resorbing surface than those of the non egg-laying females (Table 2; Figs. 3, 4), and periosteal surfaces from egg-layers had significantly more resting and less forming surface ( $P < 0.02$ ) than periosteal surfaces from non egg-laying females (Table 3; Figs. 5, 6). There were no significant differences in the other parameters among the groups.

### DISCUSSION

Edgren ('60) reported a decreased density in the femur, tibia, and fibula of the egg-laying female musk turtle, *Sternothaerus odoratus* (Latreille), which coincided with the period of eggshell calcification. In another species of turtle, *Chrysemys picta*, Suzuki ('63) noted a thinning of the femoral cortex of females with shelled oviducal eggs compared to those without oviducal eggs. The present study indicates that another reptile, *Alligator mississippiensis*, also undergoes a reduction in femoral density at the time of egg-laying. Studies by others (Boyde and Hobdell, '68, '69a,b; Jones and Boyde, '70;

TABLE 1. Femoral robusticity index and porosity of femoral diaphyseal cross sections (mean  $\pm$  SD)

Group	Robusticity index (No.)	% Area of cross section unoccupied by bone (porosity)
Egg-laying females	4.33 $\pm$ 0.12* (5)	5.67 $\pm$ 1.74**** (7)
Non egg-laying females	4.21 $\pm$ 0.06 (6)	1.48 $\pm$ 1.32 (5)
Males	3.79 $\pm$ 0.14** (4)	2.10 $\pm$ 1.23 (3)

\*Significantly different from non egg-laying females,  $P < .05$ .

\*\*Significantly different from both groups of females,  $P < .001$ .

\*\*\*Significantly different from males,  $P < 0.01$ .

\*\*\*\*Significantly different from non egg-laying females,  $P < .001$ .

TABLE 2. Vascular canal entrances, osteoblast lacunae, and types of bone surface from femoral anorganic endosteal surfaces (mean  $\pm$  SD)

Group	No.	No. of vascular canal entrances	No. of osteoblast lacunae	Types of bone surface (%)		
				Resting	Forming	Resorbing
Egg-laying females	4	16 $\pm$ 6	35 $\pm$ 15	25.24 $\pm$ 30.39	29.00 $\pm$ 18.55	45.75 $\pm$ 25.00*
Non egg-laying females	4	18 $\pm$ 6	68 $\pm$ 55	51.33 $\pm$ 44.64	41.67 $\pm$ 35.50	7.00 $\pm$ 11.27

\*Significantly different from non egg-laying females ( $P < .05$ ).

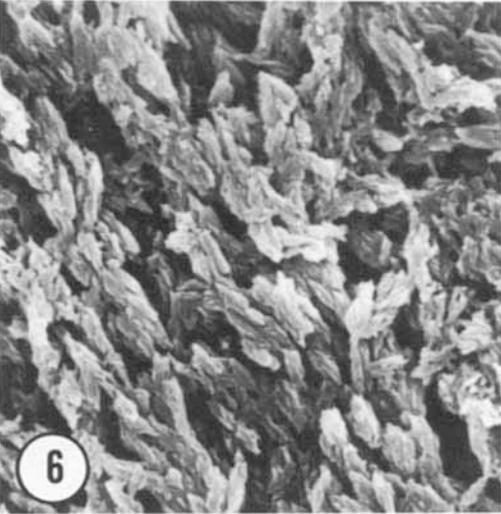
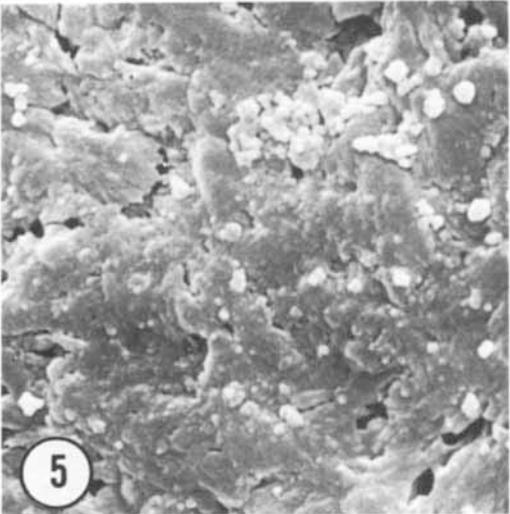
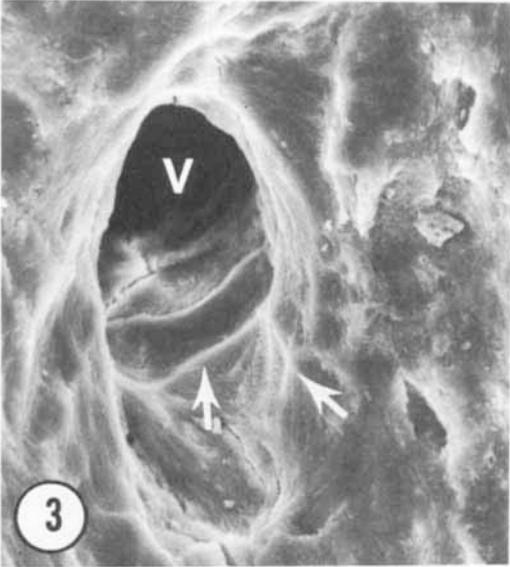
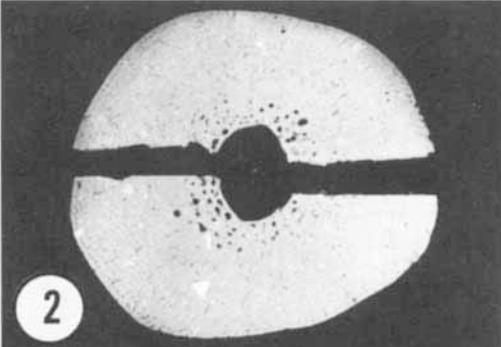
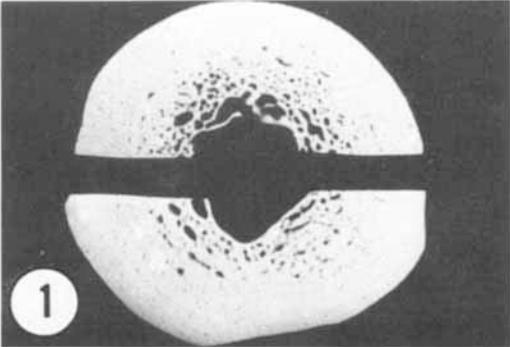


TABLE 3. Vascular canal entrances, osteoblast lacunae, and types of bone surface from femoral anorganic periosteal surfaces (mean  $\pm$  SD)

Group	No.	No. of vascular canal entrances	No. of osteoblast lacunae	Types of bone surface (%)		
				Resting	Forming	Resorbing
Egg-laying females	4	30 $\pm$ 6	71 $\pm$ 34	89.00 $\pm$ 7.63*	11.00 $\pm$ 7.53*	0 0
Non egg-laying females	4	27 $\pm$ 3	86 $\pm$ 10	49.75 $\pm$ 29.92	50.25 $\pm$ 11.27	0 0

\*Significantly different from non egg-laying females ( $P < .02$ ).

Dempster et al., '79) have shown that activity states of bony surfaces can be diagnosed by direct examination of the anorganic surfaces in the SEM; i.e., surfaces demonstrating areas of resorption are losing bone faster than resting or forming surfaces. In the present study, endosteal surfaces from femora of egg-layers had more resorbing surface than the other groups, indicating an increased rate of bone loss at the endosteal surface. Furthermore, microradiographs of femoral diaphyseal cross sections revealed increased porosity in the egg-layers, indicating increased resorption along the vascular canals in the endosteal bone. In addition, apposition of periosteal bone may have been reduced in the egg-layers. Bony surfaces demonstrating areas of formation are laying down bone faster than surfaces of resting bone. Since egg-layers had periosteal surfaces with less forming surface than those of other groups, it may be that appositional bone growth in egg-layers was reduced. This apparent decreased formation and increased resorption of femoral bone in the egg-laying alligator may be explained, in part, by that fact that in a single night (Joanen, '69) the female alligator lays a large number of eggs (40),

which have strong, thick shells. The outer, densely calcified layer of the shell is 100–200  $\mu$ m thick (Ferguson, '82); in many species of birds with eggs of comparable size, the outer layer is only 1–20  $\mu$ m thick (Becking, '75; Tyler, '69). This heavy calcium demand on the alligator probably cannot be met by diet alone, particularly since the female has a reduced food intake during breeding and egg-laying (Joanen, personal communication), and thus cannot provide much calcium for eggshells through diet. Apparently, in the alligator, structural bone of the femur (and probably other bones) is used to provide mineral for the formation of eggshells. Alligators, like turtles, may not have evolved a skeleton-sparing mechanism for the formation of eggshells. However, they may not need such a mechanism and may be able to afford to lose structural bone while forming eggshells because their bones are normally very dense when compared to those of other animals. The femoral robusticity index of the non egg-laying female alligators in this study is  $4.21 \pm 0.06$ ; in adult female Fisher rats it is  $4.43 \pm 0.10$  (Riesenfeld, '81); and in the chicken it is  $5.20 \pm 0.36$  (Wink, personal observation).

In conclusion, our study indicates that the egg-laying alligator, like the turtle, loses femoral bone that may be used as a source of calcium for the formation of eggshells.

#### ACKNOWLEDGMENTS

The authors wish to thank Ted Joanen and Larry McNease of the Louisiana Department of Wildlife and Fisheries for assistance in sampling the alligators. Appreciation is also extended to Valentine Lance for his advice and suggestions throughout this study. We also wish to thank Mr. Garbis Kerimian of the Anatomy Department of Louisiana State University Medical Center for making the photographs. The project was funded in part by the Louisiana Department of Wildlife and Fisheries.

Figs. 1, 2. Microradiographs of femoral cross sections from 1) egg-laying female, and 2) non egg-laying female. Note porosity of endosteal bone in section from egg-layer.  $\times 5$ .

Fig. 3. Endosteal surface from femoral mid-shaft of egg-laying alligator. Note resorbing surface (arrows) around vascular canal (V).  $\times 500$ .

Fig. 4. Endosteal surface from femoral mid-shaft of non egg-laying female. Note forming surface (arrows) around vascular canal (V).  $\times 750$ .

Fig. 5. Periosteal surface from femoral mid-shaft of egg-laying female. Resting bone.  $\times 5,000$ .

Fig. 6. Periosteal surface from femoral mid-shaft of non egg-laying female. Forming surface.  $\times 5,000$ .

## LITERATURE CITED

- Becking, J.H. (1975) The ultrastructure of the avian eggshell. *Ibis* 117:143-151.
- Boyde, A., and M.H. Hobdell (1968) A scanning electron microscope study of mammalian bone surfaces. *J. Dent. Res.* 47:1006 (Abstract).
- Boyde, A., and M.H. Hobdell (1969a) A scanning electron microscope study of primary membrane bone. *Z. Zellforsch.* 99:98-108.
- Boyde, A., and M.H. Hobdell (1969b) Scanning electron microscopy of lamellar bone. *Z. Zellforsch.* 93:213-231.
- Chabreck, R.H. (1963) Methods of capturing, marking, and sexing alligators. *Proc. Southeast. Assoc. Game Fish Comm.* 17:47-50.
- Dempster, D.W., H.Y. Elder, and D.A. Smith (1979) Scanning electron microscopy of rachetic rat bone. O'Hare, Illinois: SEM/1979 IIT Research Institute SEM, Inc. AMF, 6066, pp. 513-520.
- Edgren, R.A. (1960) A seasonal change in bone density in female musk turtles, *Sternotherus odoratus* (Latreille). *Comp. Biochem. Physiol.* 1:213-217.
- Ferguson, M.W.J. (1982) The structure and composition of the eggshell and embryonic membrane of *Alligator mississippiensis*. *Trans. Zool. Soc. Lond.* 36:99-152.
- Joanen, T. (1969) Nesting ecology of alligators in Louisiana. *Proc. Southeast. Assoc. Game Fish Comm.* 23:141-151.
- Jones, S.J., and A. Boyde (1970) Experimental studies on the interpretation of bone surfaces studied with the SEM. Chicago, Illinois: SEM/1970 IIT Research Institute, pp. 193-200.
- Lance, V., T. Joanen, and L. McNease (1983) Selenium, vitamin E, and trace elements in plasma of wild and farm-reared alligators during the reproductive cycle. *Can. J. Zool.* 61:1744-1751.
- Magliola, L. (1984) The effects of estrogen on skeletal calcium metabolism and on plasma parameters of vitellogenesis in the male, three-toed box turtle (*Terrapene carolina triumquais*). *Gen. Comp. Endocrinol.* 54:162-170.
- Prosser, R.L., and H.K. Suzuki (1968) The effects of estradiol valerate on the serum and bone of hatchling and juvenile caiman crocodiles (*Caimen sclerops*). *Comp. Biochem. Physiol.* 25:529-534.
- Riesenfeld, A. (1972) Metatarsal robusticity in bipedal rats. *Am. J. Phys. Anthropol.* 36:229-234.
- Riesenfeld, A. (1975) Endocrine control of skeletal robusticity. *Acta Anat.* 91:481-499.
- Riesenfeld, A. (1981) Age changes of bone size and mass in two strains of senescent rats. *Acta Anat.* 109:64-69.
- Simkiss, K. (1961) The influence of large doses of oestrogens upon the structure of the bones of some reptiles. *Nature* 190:1217-1218.
- Simkiss, K. (1967) Calcium in Reproductive Physiology. New York: Reinhold.
- Simon, M.R. (1984) The rat as an animal model for the study of senile idiopathic osteoporosis. *Acta Anat.* 119:248-250.
- Suzuki, H.K. (1963) Studies on the osseous system of the slider turtle. *Ann. NY Acad. Sci.* 109:351-410.
- Taylor, T.G., K. Simkiss, and D.A. Stringer (1971) The skeleton: its structure and metabolism. In D.J. Bell and B.M. Freeman (eds): *Physiology and Biochemistry of the Domestic Fowl*. Vol. 2. New York: Academic Press, pp. 621-640.
- Tyler, C. (1969) Avian eggshells: their structure and characteristics. *Int. Rev. Gen. Exp. Zool.* 4:81-130.
- Wink, C.S. (1982) Scanning electron microscopy of castor rat bones. *Calcif. Tiss. Res.* 34:547-552.