Morphology of Shells From Viable and Nonviable Eggs of the Chinese Alligator (Alligator sinensis)

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ABSTRACT The morphology of eggshells from hatched eggs of captive Chinese alligators (Alligator sinensis) was compared with that of shells from eggs with early embryonic death and with the morphology of eggshells from the American alligator (Alligator mississippiensis). Pieces of shells were examined in the scanning electron microscope. Parameters examined included: numbers of open pores on the outer surfaces, total shell thickness, and thickness of the outer densely calcified and inner mammillary layers. Results indicate that shells from Chinese and American alligator eggs with early embryonic death have a thicker outer densely calcified layer than do shells from hatched eggs or full-term embryos. Also, eggshells from Chinese alligator eggs with dead embryos have fewer open pores on the outer surface than do shells from hatched eggs, as has been reported earlier for the American alligator (Wink et al., '90).

The Chinese alligator (Alligator sinensis) is an endangered species (Goombridge, '82), occurring naturally along a 300–400-km stretch of the Yangtze River and its tributaries in Anhui, Jiangsu, and Zhejiang provinces in the People's Republic of China (Wang, '62; Huang, '81). Chinese alligators, like other crocodilians, had been successfully maintained in many zoological park collections throughout the world (Davenport, '82), but after their numbers began to decline, a propagation program was initiated in 1963 by the New York Zoological Society to maintain the species (Behler et al., '82). The crocodilians did not breed in the New York Zoological Park, so in 1976 they were moved to a more suitable habitat at the Rockefeller Wildlife Refuge in Louisiana (Behler, '77). In 1979, the Chinese government also initiated a conservation program for the Chinese alligator, which included the establishment of a captive breeding research center (Webb and Vernon, '92). Since then successful breeding, nesting, egg-laying, and hatching have occurred at both facilities.

Although Alligator mississippiensis mate, construct nests, and deposit eggs in captivity, their hatching rate remains lower than that for wild alligators, i.e., it is 30%–60% rather than 90% (McNeese, '87). Similarly, the hatching rate for captive Chinese alligators at Rockefeller Wildlife Refuge is only 34% (Elsey, pers. comm.); the wild hatching rate is unknown.

It is possible that poor hatchability may be associated with abnormalities of the eggshell. Alligator mississippiensis show a decrease in porosity of eggshells from eggs with early embryonic death when compared to those having held living embryos (Wink et al., '90a). No similar comparative studies have sampled eggshells of the Chinese alligators. Therefore, this study attempts to compare the morphology of eggshells from successfully hatching Chinese alligator eggs with those of eggs that did not hatch.

MATERIALS AND METHODS

Egg collection

Eggshells used in this study came from eggs of A. sinensis, collected from nests constructed in seminatural pens by the captive female alligator at Rockefeller Wildlife Refuge (Grand Chenier, LA), 1988–1991. Fertile eggs were incubated in humid environmental chambers as previously described (Joanen...
and McNease, '76, '77, '81); here, they hatched after 70–72 days. Eggs that had not hatched because the embryo had died (usually within 2–3 weeks after laying) were also collected. Shells and eggs were transported to New Orleans for evaluation.

**Scanning electron microscopy (SEM)**

Pieces, ~ 1 cm², were cut from the shell at the middle and from both ends of each egg. The eggshell membrane was peeled off each piece, and pieces of shell were soaked in sodium hypochlorite (Clorox) for 2 min, rinsed in distilled H₂O, and dehydrated in a series of alcohols. Pieces from each sample of shell were oriented on the SEM stubs either with the fractured radial surface up or the outer surface up. All samples of shell were sputter-coated with gold palladium and viewed at 10Kv in a JEOL T-300 SEM. At a magnification of X55, open unobstructed pores on the outer surface of the shell were counted on four consecutive SEM display screens (33 mm²) as previously described (Wink et al., '90a). Pores that were occluded were not counted. Small pores were examined at higher magnification to determine if they were true pores or only surface defects.

In addition, pieces of shell from the middle of captive *A. mississippiensis* eggs with early embryonic death and from eggs with full-term viable embryos (removed from the incubator just before hatching) (Wink et al., '90a,b) were oriented radial to the surface and processed as above. Micrographs document each radial surface of shells from both Chinese and American alligators. Total thickness of each piece of shell is measured on the micrographs.

**Eggshell layers**

The eggshell of crocodilians has previously been characterized. Ferguson ('82) presented an extensive description of eggshell of *A. mississippiensis*, Grine and Kitching ('87) and Zhao and Huang ('86) described those of *A. sinensis* and *Crocodileus niloticus*. In *A. mississippiensis* and *C. niloticus*, the shell consists of an outer densely calcified layer composed of vertically stacked calcite crystals, a honeycomb layer of horizontally stacked crystals, an organic layer, and a mammillary layer. The inner mammillary layer consists of cones of calcite crystals that have their bases on the organic layer and their tips on the eggshell membrane. The crystals radiate outward from the tips of the mammillary cores. The eggshell of *A. sinensis* consists of an inner cone (mammillary) layer and an outer columnar layer (Zhao and Huang, '87). Pores extend from the outer surface through the calcified layers to the inner surface to end between the cones, as they do in *A. mississippiensis* and *C. niloticus*. Stepped erosion craters occur on the outer surface of the shells in all three species. The thickness of the outer densely calcified and inner mammillary layers was determined from the micrographs of the radial surfaces of shell (Fig. 1). Differences among group means were analyzed by Student's t-test (Sokal and Rohlff, '73).

**RESULTS**

Table 1 summarizes the results of t-tests on measurements between the two categories of Chinese alligator eggshells. There were significantly fewer open pores on the outer surface of shells from the middle and ends of eggs with early embryonic death (EED) when compared with those of successfully hatched eggs. Most pores on outer eggshell surfaces of hatched eggs appeared to be open and unobstructed (Fig. 2A), whereas many of those on shells from eggs with EED were either partially or completely obstructed with material (Fig. 2B). Total thickness of eggshells does not differ between hatched and EED groups. However, pieces from the ends of shells from the hatched eggs are significantly thinner than those from the middle of the shells. Both the middle and ends of shells from the EED group have significantly thicker outer densely calcified layers than do those of the hatched group. Mammillary layer thickness does not differ between the groups.

Table 2 summarizes the results of t-tests between the two categories each of eggshells of *A.* and of *A. mississippiensis*. Eggshells of *A. sinensis* are significantly thinner than those of *A. mississippiensis*. In both species, the outer, densely calcified layer of shell is significantly thicker in shells from eggs with EED than in shells from eggs that have hatched (Chinese) or eggs with full-term embryos (American), (Figs. 3, 4).

**Alligator sinensis** externally resembles the larger *A. mississippiensis*, but rarely exceeds a maximum total length of 2.0 m (Behler et al., '82). Mating, nest-building, and egg-laying behavior are similar in both species, although Chinese alligators are much more secretive than American alligators (Joanen et al., '80). Both nests and eggs are smaller in *A. sinensis* than in *A. mississippiensis*.
Fig. 1. *Alligator sinensis.* Diagram of fractured radial surface of an eggshell from a hatched egg. Outer surface of shell is at top of diagram; inner surface is at bottom. Erosion craters (E) occur on the outer surface of the shell and can be seen continuing into a pore (P), which penetrates through all layers to the inner surface of the shell. The rhombohedral calcite crystals of each shell layer are enlarged with their a, b, and c axes indicated with arrows.

The outer densely calcified layer (ODC) is the outermost layer of shell where the crystals are oriented with their c axes at right angles to the surface of the shell. In the next layer, the honeycomb layer (H), crystals are oriented with their b faces at right angles to the shell surface. The inner mammillary layer (M) contains crystals oriented radially (Ferguson, '82).

**TABLE 1.** Alligator sinensis: Numbers of open pores on outer eggshell surfaces, total eggshell thickness, and thickness of layers of shell from the middle and ends of hatched eggs and from eggs with dead embryos (mean ± SD).

<table>
<thead>
<tr>
<th>Group</th>
<th>Location of sample</th>
<th>Pores on outer surface</th>
<th>Total thickness (μm)</th>
<th>ODC thickness (μm)</th>
<th>Mammillary layer thickness (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hatched M</td>
<td>(10) 6.11 ± 1.5</td>
<td>(10) 343.31 ± 24.73</td>
<td>73.80 ± 25.14</td>
<td>70.36 ± 17.05</td>
<td></td>
</tr>
<tr>
<td>EED4</td>
<td>(11) 3.18 ± 2.1*</td>
<td>(12) 342.80 ± 52.90</td>
<td>157.70 ± 29.0*</td>
<td>70.00 ± 14.85</td>
<td></td>
</tr>
<tr>
<td>Hatched E</td>
<td>(10) 8.60 ± 5.74</td>
<td>(08) 305.71 ± 34.09**</td>
<td>66.14 ± 16.49</td>
<td>66.14 ± 16.46</td>
<td></td>
</tr>
<tr>
<td>EED</td>
<td>(10) 3.60 ± 4.1**</td>
<td>(07) 324.29 ± 57.91</td>
<td>104.00 ± 34.33*</td>
<td>75.57 ± 19.79</td>
<td></td>
</tr>
</tbody>
</table>

1M = middle of egg; E = end of egg.
2( ) = number of eggs.
3ODC = outer densely calcified layer.
4Early embryonic death.
*Significantly different from hatched, P < 0.05.
**Significantly different from hatched, middle, P < 0.05.
Fig. 2. *Alligator sinensis*. Scanning electron micrograph (SEM) of outer surface of eggshells. Arrows indicate pores. E, erosion craters, SD, surface defect. A. Hatched shell with open pores. ×70. B. Shell from egg with early embryonic death with occluded pores. ×100.

Table 3 summarizes measurements made on the eggshells of three crocodilian species, *A. mississippiensis*, *A. sinensis*, and *Crocodylus niloticus*. Measurements were made on pieces from the middle of the shell. In the present study, eggshells from Chinese alligators were more fragile and more difficult to handle than those of the American alligator, possibly due to their overall smaller size relative to American alligator eggs. This fragility

(Joanen et al., '80; Ferguson, '82; Zhao and Huang, '86).

Table 3 summarizes measurements made on the eggshells of three crocodilian species, *A. mississippiensis*, *A. sinensis*, and *Crocodylus niloticus*. Measurements were made on
Fig. 3. Alligator mississippiensis. SEM of radially fractured face of an eggshell from an egg with full-term embryo. Outer surface of shell is at top of micrograph. E, erosion craters; H, honeycomb layer; M, mammillary layer; ODC, outer densely calcified layer; P, pore. A. Low power (×150) view of entire thickness of shell similar to that in Figure 1. Arrow marks boundary between ODC and H. B. Higher power (×1000) of boundary between ODC and H. Upper arrow indicates crystals of ODC with c axes oriented perpendicular to surface. Lower arrow indicates crystals of H with crystals oriented with b axes perpendicular to surface.

may also have been due to the fact that the total shell thickness and thickness of the outer densely calcified (ODC) layer were less in the Chinese alligator eggshell when compared to the American alligator eggshell.

DISCUSSION

Eggshells from eggs with viable embryos

Zhao and Huang ('86) reported the total thickness of the eggshell of A. sinensis to be 400–500 m, which is greater than we observed in this study (300–380 m). However, the eggshells they sampled were from different times during incubation, whereas our samples were all taken late, after hatching or at the end of the incubation period. In some crocodilians it has been shown that eggshells degrade as incubation progresses (Jenkins, '75; Ferguson, '82; Packard and Packard, '89) so that eggshells at the beginning of incubation period are thicker than those at the end of the incubation period (Wink et al., '90a). This may explain the differences in our measurements. Zhao and Huang ('86) also reported that the inner cone (mammillary) layer in the eggshell of A. sinensis was 200–250 m thick, whereas our measurements indicated a mammillary thickness of 40–86 m (including the organic layer), which is closer to Ferguson's ('82) values of 30–40 m (including the organic layer) in A. mississippiensis. These measurements may differ partly because the crystals of the mammillary cones radiate out and upward from the center of each mammilla to become gradually replaced by the horizontally oriented crystals of the honeycomb layer (Ferguson, '82; Grine and Kitching, '87). The measurements in the present study were made from the tip of each mammilla to the apparent boundary between radially directed crystals and horizontal crystals (Fig. 5). However, as the crystals of the mammillary cones gradually give way to the those of the honeycomb layer, it is possible that Zhao and Huang ('86) placed the boundary between these layers somewhat higher.

Eggshells from eggs with dead embryos

A previous study (Wink et al., '90a) reported that eggshells of A. mississippiensis from eggs with early embryonic death (EED) have fewer open pores and more rough, knobby, calcareous deposits on their outer surfaces than do those of eggs with viable,
Fig. 4. *Alligator sinensis.* SEM of radially fractured face of an eggshell with early embryonic death. Outer surface is at top of micrograph. H, honeycomb layer; M, mammillary layer; ODC, outer densely calcified layer. A. Low power (×200) Arrow marks boundary between ODC and H. B. Higher power (×1000) of boundary between ODC and H. Upper arrow indicates crystals of ODC; lower arrow indicates crystals of H.

full-term embryos. In this study, we saw similar phenomena in *A. sinensis.* However, for the present study we measured the total thickness of the shell and the thickness of the shell layers rather than assigning each egg a “deposit score,” denoting rough or smooth surface (Wink et al., '90a). The results of both methods of evaluation indicate that in both species the outer, densely calcified layer (ODC) of the shells in eggs with EED is abnormally thickened, possible occluding pores on the outer surface of the eggs and reducing viability of the embryos. The total thickness of shells in eggs with EED is not significantly different from that of “normal” eggshells of both species, indicating that extraneous calcareous deposits on the outer surface of the shells do not add to total thickness. Instead, as the ODC increases, the thickness of the honeycomb layer immediately below is reduced in shells from eggs with EED.

Ferguson ('82) and Wink et al. ('90a) have observed no differences in the thickness of eggshell pieces from the middle and ends of eggs from American alligators with full-term embryos. However, the present study shows shell pieces from the ends of hatched eggs to

### TABLE 2. *Alligator mississippiensis* and *Alligator sinensis*: Total eggshell thickness and thickness of component layers from the middle of eggs (mean ± SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>Total thickness (μm)</th>
<th>ODC thickness (μm)</th>
<th>Mammillary layer thickness (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chinese, hatched</td>
<td>(10) 343.31 ± 24.73*</td>
<td>73.80 ± 25.141*</td>
<td>70.36 ± 17.05</td>
</tr>
<tr>
<td>Chinese, EED</td>
<td>(12) 342.80 ± 52.90</td>
<td>157.70 ± 29.08**</td>
<td>70.00 ± 14.68</td>
</tr>
<tr>
<td>American full-term embryo</td>
<td>(08) 397.60 ± 31.604</td>
<td>127.60 ± 43.56</td>
<td>68.40 ± 17.78</td>
</tr>
<tr>
<td>American, EED</td>
<td>(07) 410.10 ± 25.804</td>
<td>236.00 ± 34.50*</td>
<td>68.10 ± 15.50</td>
</tr>
</tbody>
</table>

1) = number of eggs.
2) Outer densely calcified layer.
3) Early embryonic death.
4) Reported by Wink et al., '90a.
5) Significantly different from American, full-term embryo, P < 0.05.
6) Significantly different from Chinese, hatched, P < 0.05.
be thinner than those from the middle. The number of pores on the outer surface does not differ significantly between the two regions of shells. The ODC and mammillary layers were slightly thinner in the end pieces than in the middle, but not significantly so; the small cumulative differences may have been responsible for the significant differences seen in total thickness.

In conclusion, it appears that some eggs of both captive *A. mississippiensis* and *A. sinensis* show fewer open pores on the outer surface of the eggshell and a thicker outer densely calcified shell layer and that these are associated with early embryonic death. It is not known if similar eggshell morphology and early embryonic death occur in wild populations of Chinese alligators because their critically low numbers and secretive habits have limited study of this species in the wild.

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**TABLE 3. Total thickness of eggshells and thickness of shell layers (µm) from three crocodilians**

<table>
<thead>
<tr>
<th>Investigator</th>
<th>Crocodilian</th>
<th>Total shell thickness</th>
<th>Outer densely calcified layer</th>
<th>Honeycomb layer</th>
<th>Mammillary layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferguson ('82)</td>
<td><em>Alligator mississippiensis</em></td>
<td>450–650</td>
<td>100–200</td>
<td>300–400</td>
<td>20–30</td>
</tr>
<tr>
<td>Zhao and Huang ('86)</td>
<td><em>Alligator sinensis</em></td>
<td>400–500</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wink and Elsey</td>
<td><em>Alligator sinensis</em></td>
<td>300–380</td>
<td>40–120</td>
<td>170–220</td>
<td>40–86</td>
</tr>
<tr>
<td>Grine and Kitching ('87)</td>
<td><em>Crocodylus niloticus</em></td>
<td>450</td>
<td>20–25</td>
<td>250</td>
<td>180</td>
</tr>
</tbody>
</table>

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**Fig. 5. Alligator sinensis. SEM of the boundary between the honeycomb layer (H) and the mammillary layer (M) on the radial surface of an eggshell from a hatched egg. Radially directed crystals (arrowhead) of M gradually give way to horizontally directed crystals (arrow) of H. (×500).**
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LITERATURE CITED