# Egg Turning During Incubation has no Effect Upon the Growth of Embryos of *Alligator mississippiensis*

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**Abstract**

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Alligator eggs are not turned during incubation, instead the embryo adheres to the top inside of the shell. Turning is alleged to shear off the embryo and kill it. Avian egg turning allegedly facilitates embryonic development by stimulating growth of the area vasculosa and minimizing the effects of unstirred yolk and albumen layers.

From day 10 to day 45 of incubation, alligator eggs were experimentally turned, gently, through ± 60° in an hourly cycle. This turning regime killed only 6 out of 25 embryos. Compared with unturned controls, no significant effects were observed on the growth, production of extra-embryonic fluids or utilization of albumen and yolk for those embryos that survived turning. The protein concentration of amniotic fluid at various stages of alligator development was examined in eggs incubated at 30 and 33°C. The fluid contained very little protein (max <8 mg) at any time: the protein concentration did not change consistently as development progressed. Differences in response to egg turning in birds and reptiles may be associated with the length of the incubation period, the protein content of the albumen and the mechanism of albumen utilization.

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## Introduction

Egg turning is an important aspect of development in avian and reptilian eggs. In birds, egg turning is essential: failure to turn has major deleterious effects on embryonic growth, growth of the extra-embryonic membranes, utilization of albumen, and hatchability (Tazawa 1980; Tullett & Deeming 1987; Deeming *et al*. 1987; Deeming 1989*a–c*). By contrast, reptile eggs are not turned during incubation, and in crocodilians and many turtles, movement of eggs causes high mortality (Limpus *et al*. 1979; Parmenter 1980; Ferguson 1985), whilst other turtle and squamate eggs are relatively unaffected by turning (Ewert 1979, 1985; Marcellini & Davies 1982; Feldman 1983). The reason for such differences between birds and reptiles is unclear but may be related to the differing patterns of early development in the two groups.

In bird eggs, failure to turn increases the incidence of premature adhesion of the extra-embryonic membranes to the shell membrane (New 1957). In reptiles, however, such adhesions to the shell membrane are normal (Ferguson 1985). Rapid turning of reptile eggs appears to generate shearing forces that rip the extra-embryonic membranes, particularly the allantois, and kill the embryo (Ferguson 1982, 1985; Webb *et al*. 1987*a, b*). There are critical periods when avian and reptilian embryos are sensitive to turning. In the fowl (*Gallus gallus*), turning is required from 3 to 7 days of incubation (New 1957;

Deeming *et al*. 1987; Deeming 1989*a*). In crocodilians and sea turtles, sudden rotation of eggs after 12 h and before 20 days of incubation is deleterious (Parmenter 1980; Ferguson 1985). Hence, care is needed when collecting and transporting alligator and sea turtle eggs during this period (Bustard 1972; Raj 1976; Ferguson 1982, 1985).

Egg turning in the fowl stimulates the expansion of the area vasculosa and the chorio-allantoic membrane during the first half of incubation (Tyrrell *et al*. 1954; Tullett & Deeming 1987; Deeming 1989*c*). Deeming (1989*c*) suggested that movement stimulates the growth of blood vessels in the area vasculosa thereby maximizing the area of this membrane available for yolk absorption. Any reduction in the vascular area may reduce yolk uptake and prevent maximal growth of the embryo during later stages of development (Deeming 1989*c*). Following the observation that eggs of *Alligator mississippiensis* could be gently rotated on day 10 of incubation without significant effects on embryonic mortality (Deeming & Ferguson unpublished) the possibility arose to test this hypothesis in eggs that were not normally turned. Regular experimental turning of alligator eggs should stimulate growth of the area vasculosa, and destroy unstirred layers that may be present in the albumen and yolk (Deeming *et al*. 1987), thereby accelerating embryonic and extra-embryonic growth and development.

Failure to turn fowl eggs restricts and retards utilization of albumen during development. Normally, albumen is translocated into the amniotic fluid, is swallowed by the embryo and enters the yolk sac (Oegema & Jourdian 1974). Failure to turn eggs produces small embryos with

an excess of residual albumen (Tazawa 1980; Tullett & Deeming 1987; Deeming 1989a). The process of movement of albumen into the amniotic sac is affected by turning: in unturned eggs less amniotic fluid is present on days 14 and 16 of incubation (Deeming 1989a) and there is retarded movement of protein into the amniotic fluid (Deeming 1991). By contrast, Ferguson (1982) suggested that egg albumen in alligators is taken up directly by the chorion. Perhaps absorption of albumen in alligators is less critical than in birds. Therefore, the second part of this report describes the protein concentration of amniotic fluid of alligator embryos during development.

## Materials and Methods

Eggs of *Alligator mississippiensis* were collected immediately after laying from four clutches in wild nests and transported by air to the U.K. as described by Deeming & Ferguson (1989). On arrival in the laboratory (day 3) the eggs were weighed and set in an incubator at 30°C accurate to 0.01°C (Vinden Scientific), and at 100% humidity. On day 10 of incubation (equivalent to day 3 in the fowl) each egg was candled to assess viability and to locate the embryo which had adhered to the shell membranes. Each egg was then slowly rotated to position the embryo at its upper surface. Viable eggs were placed, embryo uppermost, in a Polyhatch incubator (Brinsea Products) at 30°C and a relative humidity as close to 100% as possible [measured at 99.9% during incubation using an electronic hygrometer (Soar Corporation, Japan)].

Twenty-five eggs were placed on the baseplate of the incubator and were restrained by metal rods which lay perpendicular to the direction of travel of the baseplate. When the baseplate moved from side to side the eggs, and hence the embryos, were rolled backwards and forwards through an angle of ±60°. For 15 min the plate moved slowly from left to right; after a further 15 min the plate took 15 min to move from right to left. At the end of an hourly cycle the egg had returned to its original orientation. As controls, 16 eggs were placed on top of metal rods, placed close to each other in the incubator, and were not turned during incubation.

On day 45 of incubation (equivalent to day 13 in the fowl), the eggs were removed from the incubator, weighed (in grams), their length and breadth were recorded (in cm) and the maximum length of the opaque band in the eggshell (Ferguson 1982) was measured using calipers. Embryonic viability was assessed by candling: dead eggs were opened to assess the age of embryonic death, whilst viable eggs were placed on ice for 1 h prior to opening. The allantoic and amniotic fluids were decanted from the egg and weighed; the embryo, yolk sac and albumen were then removed from the shell and all components were weighed. The stage of development of each embryo was assessed using the criteria of Ferguson (1985).

In addition, samples of the amniotic fluid were removed from eggs in the study described by Deeming & Ferguson (1989). Total protein concentration was determined by a colorimetric method using Coomassie Blue (Pierce and Warriner). A 0.1 cm³ sample of amniotic fluid was mixed with 5 cm³ of protein assay reagent and the absorbance at a wavelength of 595 nm of the resulting solution was determined by spectroscopy. Samples were assayed from eggs incubated for 20, 28, 36, 44 and 52 days at incubation temperatures of 30 and 33°C.

## Results

Turning alligator eggs from days 10 to 45 of incubation killed 6 out of 25 embryos (24%). Embryonic mortality was, however, unrelated to the onset of turning: two of the embryos died after 11 days of incubation, two embryos died about day 20 and two embryos died about day 40. All embryos in unturned eggs survived to 45 days.

Egg mass, the physical dimensions and rate of water loss during incubation were not significantly different between the two groups, although the unturned, control eggs were slightly smaller (Table 1). The length of the

Table 1. *The effects of egg turning on embryonic growth and utilization of albumen and yolk in eggs of* Alligator mississippiensis *incubated at 30°C. Residues (membranes and blood) were calculated by difference. Values are means (X) and standard errors (S.E.)*

| Variable | Turned | | Unturned | | |
|---|---|---|---|---|---|
| | X | S.E. | X | S.E. | |
| Number of eggs | 25 | | 16 | | |
| Surviving eggs on day 45 | 19 | | 16 | | |
| Initial egg mass (g) | 78.93 | 5.21 | 76.48 | 4.15 | NS |
| Daily water loss (g day⁻¹) | 0.163 | 0.131 | 0.157 | 0.069 | NS |
| Egg length (cm) | 7.42 | 0.19 | 7.43 | 0.25 | NS |
| Egg breadth (cm) | 4.21 | 0.11 | 4.13 | 0.09 | * |
| Opaque band length (cm) | 6.90 | 0.53 | 6.84 | 0.68 | NS |
| Allantoic fluid (g) | 7.50 | 1.84 | 8.78 | 1.67 | * |
| Amniotic fluid (g) | 2.59 | 0.83 | 2.85 | 0.71 | NS |
| Albumen (g) | 5.29 | 2.53 | 5.30 | 2.45 | NS |
| Yolk sac (g) | 33.89 | 3.92 | 30.48 | 4.10 | * |
| Shell (g) | 11.01 | 0.82 | 10.60 | 0.90 | NS |
| Residues (g) | 5.63 | 1.10 | 5.28 | 1.18 | NS |
| Embryo (g) | 6.19 | 0.63 | 6.60 | 0.99 | NS |

Significance level: * = P<0.05; NS = not significant, as assessed using the two-sample *t*-test.

opaque band was unaffected by turning. Egg turning did not significantly affect the mass of the extra-embryonic fluids or albumen (Table 1). The significant difference in the mass of the yolk sac was almost certainly due to the difference in initial egg mass. Embryo mass on day 45 of incubation was unaffected by egg turning (Table 1). All of the embryos were at stage 23 of development on day 45 of incubation (Ferguson 1985).

Total protein concentrations in the amniotic fluid of alligator eggs at incubation temperatures of 30 and 33°C are shown in Table 2. The amount of protein in amniotic fluid was significantly higher at 33 than at 30°C ($F_{1,24} = 6.13$, $P<0.05$) but the absolute concentrations were very low. As amniotic fluid attained a maximum mass of 4 g it is unlikely that the total protein in amniotic fluid was more than 8 mg (assuming a density close to unity). Although days of incubation had a significant effect on protein concentration ($F_{4,24} = 3.21$, $P<0.05$) there was no significant effect of time and there was significant interaction between the two variables ($F_{2,24} = 0.73$, $P>0.05$).

Table 2. *Protein concentrations (mg cm⁻³) in the amniotic fluid of eggs of* Alligator mississippiensis *incubated at 30 and 33°C*

| | Temperature | | | | | |
|---|---|---|---|---|---|---|
| | 30°C | | | 33°C | | |
| Day | N | X | S.E. | N | X | S.E. |
| 20 | N* | N | N | 3 | 1.16 | 0.32 |
| 28 | 3 | 0.66 | 0.08 | 4 | 1.31 | 0.24 |
| 36 | 4 | 0.87 | 0.20 | 4 | 1.07 | 0.34 |
| 44 | 4 | 0.55 | 0.14 | 4 | 1.38 | 0.44 |
| 52 | 4 | 1.82 | 0.24 | N | N | N |

*N = not determined.

## Discussion

Previous data on the effects of egg turning in reptiles suggested that mortality in the experiment described here would be complete. However, egg turning throughout much of the incubation period, including the sensitive period (Ferguson 1985), caused only 24% mortality of alligator embryos. This unexpected result almost certainly reflects the importance of the method of egg turning upon embryonic mortality in reptiles.

In birds eggs, during the early stages of development the embryo lies on top of the yolk sac and is unattached to the shell membrane. The yolk sac acts in a turning couple when the egg is rotated: the embryo moves to the top of the egg owing to the lower density of sub-embryonic fluid compared with the denser yolk which sinks to the bottom of the egg (New 1957). During early development, alligator embryos adhere firmly to both the shell membrane and the yolk sac. Given the similarities in the pattern of formation and composition of sub-embryonic fluid in birds and crocodilians (Manolis et al. 1987) a similar turning couple is almost certainly present in alligator eggs. Violent rotation would, therefore, cause the sub-embryonic fluid to move rapidly towards the top of the egg. This produces a shearing movement which rips the extra-embryonic membranes, particularly the allantois, around stages 9–10 (Ferguson 1985; Webb et al. 1987a, b). Complete inversion of alligator eggs, regardless of the speed, kills the embryo by squashing it under the weight of the overlying yolk (Ferguson 1985). The present study has shown that a high proportion of alligator embryos can survive slow, gentle rotation of the eggs, which does not completely invert them.

Egg turning in birds stimulates growth of vascular membranes: both the area vasculosa and the chorio-allantois are small in unturned eggs (Tyrrell et al. 1954; Tullett & Deeming 1987; Deeming 1989c). Deeming (1989c) suggested that egg turning stimulates growth of the area vasculosa by causing differential increases in blood pressure. A reduced size of the area vasculosa during the first third of incubation was associated with reduced embryonic growth later in incubation (Deeming 1989c). The present study tested whether experimental turning would accelerate growth of the vascular chorio-allantois (by similar mechanisms) and then later embryonic growth in alligators. Day 10 of incubation was chosen as the start of the egg turning treatment so as to coincide with the equivalent start stage of the critical period for turning in the fowl (New 1957; Deeming et al. 1987; Deeming 1989a, c). The size of the opaque band in the alligator eggshell is related to the extent of growth of the vascular chorio-allantois (Ferguson 1982, 1985; Webb et al. 1987a, b). Egg turning did not affect expansion of the opaque band by day 45 which implies that the growth of the chorio-allantois was unaffected by the treatment.

Alligator egg turning did not affect embryonic growth nor the utilization of albumen or yolk. This observation highlights an important difference between embryonic development in birds and crocodilians. Some indication of the nature of this difference comes from the patterns of albumen utilization in the fowl and alligator. In the fowl, albumen is enclosed by the chorio-allantois, which

forms an albumen sac. On day 12 of incubation albumen begins to move through the sero-amniotic connection into the amniotic fluid. The embryo swallows this fluid and albumen appears in its gut and eventually in the yolk sac (Oegema & Jourdian 1974). During development of the alligator embryo there is no significant change in the protein concentration of amniotic fluid. Ferguson (1982) reported that albumen is probably absorbed directly by the chorionic epithelium, although this has yet to be confirmed experimentally. The low protein content of the alligator albumen and allantoic fluid are in keeping with such an uptake mechanism, which would transport principally water, ions and low molecular weight molecules.

By contrast to the negligible effects on growth, albumen utilization etc. of turning alligator eggs, failure to turn avian eggs during incubation has many effects which retard embryonic growth and prevent normal utilization of albumen (Deeming 1991). Such effects are manifested by large amounts of residual albumen in unhatched unturned eggs (Tullett & Deeming 1987), retarded albumen utilization (Deeming 1989a), small changes in the mass of amniotic fluid (Deeming 1989a) and the delayed appearance of protein in the amniotic fluid (Deeming 1991). Of the many differences between development in birds and crocodilians there are two that may be important in explaining the different turning requirements of bird and crocodilian eggs: (1) the length of the incubation period and (2) the composition of the albumen.

Although the eggs of the fowl and the crocodilians are of similar initial mass, 60–70 g in the fowl compared with 65–90 g in crocodilians (Burton & Tullett 1985; Ferguson 1985), the length of the incubation period is some 3–5 times longer in crocodilians (65–100 days) than it is in the fowl (21 days). In addition, the composition of the albumen deposited in the eggs of the fowl and crocodilians is very different. In a 72 g fowl egg, albumen forms 65.71% of the initial mass of the egg contents (Burton & Tullett 1985) compared with 53.84% of the contents of an alligator egg of the same mass (Manolis et al. 1987). The albumen of the fowl egg has 12% solids (7.7 g) almost all of which is protein (Romanoff 1967). By contrast, the albumen of *Crocodylus porosus* eggs (data for *A. mississippiensis* are unavailable but can be considered to be very similar) is only 5% solids (approximately 1.7 g) (Burley et al. 1987).

Therefore, the fowl embryo has a short incubation period in which to utilize a large amount of albumen protein. That this protein is an important source of nutrients for the embryo has been clearly demonstrated (Betz 1968; Deeming 1989b). If the embryo is unable to utilize all of this protein, as is the case in an unturned egg, then its growth suffers and it is unable to hatch within the normal incubation period. By contrast, the alligator embryo has a long incubation period in which to utilize a small amount of albumen protein. Failure to utilize this small amount of protein probably has few effects on the overall nutrition and growth of the alligator embryo. Alligator albumen may be more important in providing a water source for normal development than a protein nutrient source.

This experiment also highlights the importance of investigating the detailed composition of albumen in avian

and crocodilian eggs. Perhaps avian albumen contains proteins (e.g. growth factors) required to initiate and sustain rapid embryonic development and growth; such proteins may be absent in alligator albumen. One could therefore envisage a progression of albumen evolution in parallel with decreased reliance on extrinsic water for successful incubation, through to acceleration of embryonic growth and development at higher incubation temperatures and shorter incubation times. In squamates and turtles, water is absorbed by the egg during incubation and the amount of albumen is related to eggshell structure: parchment-shelled eggs have very little albumen present at oviposition whereas in rigid-shelled eggs albumen forms a larger, if variable, proportion of the egg contents (Tracy & Snell 1985). In crocodilian eggs, albumen appears to have a primary role as a water reservoir, but in avian eggs albumen also contains larger amounts of protein. These proteins may have a nutritional or growth promoting role, correlated with a concomitant decrease in the incubation time and an increase in incubation temperature. Parallel mechanisms, e.g. egg turning, may have evolved to ensure the rapid and complete utilization of these egg components.

In conclusion, the short incubation period of bird eggs, in association with a high incubation temperature, may be too short to allow absorption of all the protein-rich albumen via the chorion which appears to be the mechanism of albumen utilization in crocodilians. Evolution of egg turning in birds tends to ensure that all of the protein-rich albumen, invested into the egg at oviposition, is utilized completely and quickly by the embryo (Deeming 1991).

## Acknowledgements

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