Implications of flexible-shelled eggs in a Cretaceous choristoderan reptile

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Flexible, or soft-shelled, eggs are almost unknown in the fossil record, leaving large gaps in our knowledge of the reproductive biology of many tetrapod clades. Here, we report two flexible-shelled eggs of the hyphalosaurid choristodere *Hyphalosaurus baitaigouensis* from the Early Cretaceous of China, one containing an embryo and the second associated with a neonate. Choristoderes are an enigmatic group of aquatic reptiles that survived the K–T extinction but died out in the Miocene. Hyphalosaurids, a specialized clade of Choristodera, resemble miniature plesiosaurs and are considered to be primarily aquatic in habit. Scanning electron microscopy of samples from the eggs reveals a thin, non-columnar external mineralized layer characterized by rounded nodes and tentatively identified poorly structured irregular pores, with an underlying amorphous layer presumably representing decomposed protein fibrils. While the relationships of Choristodera remain controversial, eggshell microstructure more closely resembles that of Lepidosauromorpha (the lineage including lizards) as opposed to that of Archosauromorpha (the lineage including birds and crocodiles).

Keywords: eggshell; embryo; paleontology; fossil; reptile

1. INTRODUCTION

Fossil eggs and embryos from dinosaurs, crocodilians and turtles provide unique insight into the evolutionary history of these groups, including evidence for reproductive behaviour (Norell *et al.* 1994a; Chiappe *et al.* 2004; Ji *et al.* 2004; Sato *et al.* 2005; Grellet-Tinner *et al.* 2006), growth strategy (Norell *et al.* 1994b; Chiappe *et al.* 2004; Wang & Zhou 2004; Grellet-Tinner *et al.* 2006; Balanoff & Rowe 2007; Balanoff *et al.* 2008) and phylogeny (Zelenitsky & Modesto 2003; Zhou & Zhang 2004; Zelenitsky & Therrien 2008). Yet, flexible thin-shelled vertebrate eggs, like those laid by lizards and snakes, are known from only a handful of fossil specimens from two clades: pterosaurs and choristoderes (Chiappe *et al.* 2004; Ji *et al.* 2004; Wang & Zhou 2004).

Two remarkably well-preserved flexible-shelled eggs were recently recovered from the Early Cretaceous Yixian Formation in Liaoning Province, China. One egg (Liaoning Paleontological Museum of China, Shenyang: LPM-R169; figure 1a) contains an embryo and the second egg (LPM-R168; figure 1b) is associated with an apparently partially hatched neonate of the long-necked choristodere *Hyphalosaurus baitaigouensis*. Choristoderes are a morphologically diverse but species-poor clade of aquatic diapsid reptiles that are common in the Cretaceous of China. The presence of 24 cervical vertebrae identifies the neonate to the species *H. baitaigouensis* (Gao & Ksepka 2008). The rhomboid shape of the interclavicle is also diagnostic for at least adults of *H. baitaigouensis*, though it must be noted that shape

changes in this bone over ontogeny are not completely understood in *Hyphalosaurus*. The closely related species *Hyphalosaurus lingyuanensis* possesses only 19 cervical vertebrae (Gao *et al.* 1999) and has a poorly developed anteromedial process of the interclavicle (Gao & Ksepka 2008), and no other tetrapod known from the Yixian Formation approaches a cervical count of 24. Development of the skeleton is in concordance with that expected from a neonate. All exposed skull elements, including the frontal, maxilla and jugal, remain unfused. Multiple cervical and caudal vertebrae are rotated so as to expose the anterior or posterior surface of the centrum, showing that the notochord canal remains open throughout the vertebral column. Ribs are wide and are ossified over most of their lengths, but the gastralia are extremely slender and much smaller relative to the ribs than in more mature individuals of *Hyphalosaurus*. The exposed humerus, radius, ulna, tibia, fibula and phalanges exhibit complete ossification only at midshaft, taking the form of cylinders with lighter coloration indicating less complete ossification at the proximal and distal extremities. Articular surfaces of all forelimb and hindlimb elements are completely absent and were presumably still cartilaginous at this ontogenetic stage. No ossified carpal or tarsal elements are identifiable, indicating that these elements were apparently still completely cartilaginous as well. In total body size, the neonate is the smallest *H. baitaigouensis* specimen we have observed and would have measured approximately 60 mm from the snout to the tip of the tail. Adults of this species up to 1.1 m in length have been reported (Gao & Ksepka 2008; figure 1c).

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Figure 1. *Hyphalosaurus baitaigouensis* eggs and hatchling. (a) Unhatched egg with embryo (LPM-R169) and (b) hatched egg with associated neonate (LPM-R168), both to same scale. Semi-transparent outline in (a) indicates approximate position of embryo. Close-ups in (b) show details of skeleton including open notochordal canals (nc) in cervical vertebrae, shape of interclavicle (ic) and poor ossification of tibia (t) and fibula (f). Scale bar, 10 mm. (c) Relative size of eggs, neonate and adult *H. baitaigouensis*. Scale bar, 5 mm.

Egg shape appears to be largely intact in the hatched egg, with minor distortion caused by the exit of the neonate. As preserved, the egg measures approximately 20 mm from pole to pole and is 15 mm wide at midpoint. In the unhatched egg, the outline of an embryo is discernable. The embryo is oriented with its trunk addressed to one side of the egg, and with its head and tail curled at the poles. Although details of osteology cannot be deciphered, the observable proportions of the neck, trunk and tail are present only in *Hyphalosaurus* among taxa reported from the Yixian Formation. Based on the proportions of the embryo, size of the egg and agreement in eggshell structure, the unhatched egg can confidently be referred to *Hyphalosaurus* and most likely belongs to *H. baitaigouensis*, which is the only long-necked choristodere known from the locality. Maximum egg length from pole to pole in this egg is 19 mm, and maximum width is 14 mm. In order to gain insight into eggshell ultrastructure, we sampled both the hatched and unhatched eggs for scanning electron microscopy (SEM).

2. MATERIAL AND METHODS

(a) Provenance and preparation of specimens

The eggs were recovered from beds exposed at Toutai near Yizhou and acquired by the Liaoning Paleontological Museum of China (Shenyang). Strata near Yizhou, which

have also yielded the holotype of *H. baitaigouensis*, are from the upper part of the Yixian Formation (Wang *et al.* 2004). Radiometric dates indicate that the Yixian Formation has a chronological range of 122.3–129.7 Ma (Smith *et al.* 1995; Chang *et al.* 2009).

To verify the authenticity of the specimens, we prepared through the matrix underlying the unhatched egg to reach fresh eggshell surface. The unhatched egg is three dimensional and eggshell exposed in this way is identical in texture and colour to that exposed on the split slab, indicating that the genuine nature of the shell material has not been artificially altered. The size and morphology of the skeleton associated with the hatched egg is consistent with that expected for a neonate (see above).

(b) Scanning electron microscopy

Eggshell samples were collected from both eggs. Samples were mounted onto aluminium stubs using carbon tape and sputter-coated with a thin layer of gold–palladium compound. Microstructures were investigated and images were collected using a Hitachi S-4700 SEM at working voltages of 10 kV (figure 2a,b,h,i) and 5 kV (figure 2d,e,g).

3. RESULTS

SEM of the samples from both eggs reveals details of eggshell ultrastructure (figure 2a,b,d,e,g–i). In cross-section (figure 2g), the external mineralized layer of the

Figure 2. Details of fossil eggshell from SEM imaging. External surface of sample from hatched egg (LPM-R168) viewed from (a) directly above and (b) an oblique (45°) angle. External surface of sample from fossil unhatched egg (LPM-R169) viewed from (d) directly above and (e) an oblique (45°) angle. External surface of eggshell from (c) the extant scincid lizard *Lampropholis* sp. and (f) the extant agamid lizard *Physignathus lesueurii* are shown for comparison. Additional samples of hatched fossil egg from oblique (45°) angle showing (g) average thickness of external mineralized layer (el), (h) thickness of sample including amorphous layer (am) and (i) possible pore opening (p). Scale bar, (a,c,f,g,i) 10 μm ; (b,d,e,h) 20 μm . Images (c,f) adapted from Osborne & Thompson (2005).

egg is very thin ($<10\ \mu\text{m}$). On the surface, the mineralized layer is covered by irregularly spaced circular nodes ranging from 10–50 μm in diameter (figure 2a,b,d,e,h,i). Whether the ornamenting nodes are part of the outer layer or formed by underlying structures cannot be determined with certainty. Regardless, regularly spaced, structured pores appear to have been absent. Although several possible pores can be identified on the shell surface (figure 2i), these are sparse and not arranged in any clear pattern. It remains possible that these openings could instead represent damage. A thick layer of irregular structure occurs below the external mineralized layer (figure 2h). This layer probably represents the degraded fibrous portion of the eggshell—the destruction of protein fibrils during fossilization is not unexpected given their composition.

Eggshell surface ornamentation is pervasive and varies markedly in Lepidosauromorpha. Microscale ornamentation may include nodes, grooves, globules and florets (Packard *et al.* 1982a,b; Packard & Hirsch 1986; Schleich & Kästle 1988; Osborne & Thompson 2005). Some scincid eggshells (Osborne & Thompson 2005) show a pattern of protruding calcite nodes similar to, but nearly an order of magnitude smaller than, those of the *H. baitaigouensis* eggs (figure 2c). In some gekkonids and chameleonids, nodes overlapping the size range seen in *H. baitaigouensis* are formed by underlying spheroid structures creating relief on the outer calcite layer (Schleich & Kästle 1988).

Phylogenetic placement of the Choristodera remains unresolved, in part because their highly derived morphology hinders comparisons. Current competing hypotheses place the Choristodera as the sister taxon to Lepidosauromorpha (Müller 2003), as basal members of Archosauromorpha (de Braga & Rieppel 1997) or as basal reptiles that diverged before the Lepidosauromorpha–Archosauromorpha split (Evans & Hecht 1993; Gauthier 1994; Gao & Fox 1998; Hill 2005). Eggshell characters observed in *Hyphalosaurus* are most consistent with those of Lepidosauromorpha. A thin external layer is present, and regularly arranged pores are absent in the *H. baitaigouensis* eggshell. The mineralized external portion of extant lepidosaurian eggshell is likewise typically limited to a thin calcareous layer or isolated calcareous aggregates (Schleich & Kästle 1988; Packard & DeMarco 1991). Highly structured pores are absent in lepidosaurian eggs, though irregular pore-like openings may facilitate gas exchange (Packard & Hirsch 1986). The New Zealand tuatara *Sphenodon punctatus* lays flexible-shelled eggs that likewise lack structured pores but are unique among extant amniotes in possessing calcareous columns that are interwoven with the protein fibrils (Packard *et al.* 1982a). In contrast to the eggs of lepidosaurians, crocodile, non-avian dinosaur and bird eggs are all characterized by a rigid shell formed from a thick outer layer of inverted calcareous wedges, with organized pore openings positioned between the calcareous

wedges and patent on the surface (Schleich & Kästle 1988; Packard & DeMarco 1991; Mikhailov 1992; Zhou & Zhang 2004). Pterosaur eggshell also shows calcareous wedges similar to other archosaurs, though the external calcareous layer is relatively thin (approx. 30 µm) (Chiappe *et al.* 2004). Most turtles lay eggs with rigid shells, though members of several clades lay flexible-shelled eggs (Ewert 1979). Flexible turtle eggshells also possess thick aragonitic units; however, these units are more loosely arranged than those in rigid turtle eggshells (Packard & Hirsch 1986).

Though the structure of choristoderan eggshell shares many features uniquely with extant lepidosaurian eggshell, polarization of these characters is problematic owing to the lack of unambiguous fossil eggshells from basal amniotes and a lack of consensus on the phylogenetic position of turtles. Extant amphibians provide no useful data on polarity as they do not produce a solid mineralized eggshell. Under proposed phylogenies of Amniota where Testudines are sister taxon to Lepidosauromorpha or are basal to Archosauromorpha + Lepidosauromorpha, a thick layer of wedge-like calcareous shell units that do not interweave with the underlying fibrils would be optimized as the primitive condition for Diapsida. Under these topologies, a thin mineralized eggshell layer and lack of structured pores are most parsimoniously optimized by considering Choristodera and Lepidosauromorpha sister taxa. However, if Testudines and Archosauromorpha are instead considered sister taxa, the primitive eggshell condition for Diapsida becomes ambiguous. In this phylogenetic scenario, the eggshell characteristics shared by Choristodera and Lepidosauromorpha could simply be primitive. Character evidence from eggshell structure would thus merely fail to support a close relationship between Choristodera and Archosauromorpha, but not preclude Choristodera occupying a position basal to the Archosauria—Testudines split or the Archosauromorpha—Lepidosauromorpha split. We recognize that eggshell morphology provides only one source of character data, but nonetheless is worth including along with molecular and skeletal evidence in addressing the perpetually controversial higher level phylogenetic position of Choristodera.

Details of the egg structure also provide insight into choristodere ecology. Eggshell performs several vital functions, such as protecting the embryo, facilitating gas exchange, providing calcium for skeletal growth and maintaining chemical equilibrium (Deeming & Ferguson 1991 and references therein). Extant oviparous vertebrates employ a variety of incubation strategies including direct incubation by the parents, deposition in soil, litter or deep sand nests and fixation of eggs to rocks and trees. Incubation strategy is dictated mainly by water vapour conductance properties (Deeming 2006), and calcification is negatively correlated with conductance (Deeming & Thompson 1991). Most flexible-shelled eggs have high mass-specific water vapour conductance values ($>100 \text{ mg H}_2\text{O d}^{-1} \text{ Torr}^{-1} \text{ g}^{-1}$) and increase greatly in mass over incubation (Deeming & Unwin 2004). In contrast, rigid eggshells in crocodiles, birds, turtles and gekkonid squamates have much lower conductance values and tend to lose a small percentage of mass over incubation (Deeming & Unwin 2004). The thin external layer of the fossil eggshell

implies high conductance values and a requirement of significant moisture for successful incubation. *Hyphalosaurus baitaigouensis* probably deposited its eggs in closed nests near the shoreline to avoid desiccation. Also of interest are the small egg size and the hatchling body size relative to adults of the species (figure 1c). Juvenile specimens of *H. baitaigouensis* are exceedingly common in the Yixian Formation, while adults of the largest size cohort are rare. Our findings are consistent with small hatchling size and low survivorship for *H. baitaigouensis*, as seen in most extant semi-aquatic reptiles.

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