THE EVOLUTION OF REPRODUCTION WITHIN TESTUDINATA AS EVIDENCED BY THE FOSSIL RECORD

by

Daniel Ryan Lawver

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ABSTRACT

Although known from every continent except Antarctica and having a fossil record ranging from the Middle Jurassic to the Pleistocene, fossil turtle eggs are relatively understudied. In this dissertation I describe four fossil specimens, interpret paleoecology and conduct cladistic analyses in order to investigate the evolution of turtle reproduction. Fossil eggshell descriptions primarily involve analysis by scanning electron and polarized light microscopy, as well as cathodoluminescence to determine the degree of diagenetic alteration. Carapace lengths and gas conductance are estimated in order to investigate the ecology of the adults that produced fossil turtle eggs and clutches, as well as their incubation environments, respectively. Cladistic analyses of turtle egg and reproductive characters permit assessment of the usefulness of these characters for determining phylogenetic relationships of fossil specimens and the evolution of reproduction in turtles. Specimens described here include 1) *Testudoolithus oosp.* from the Late Cretaceous of Madagascar, 2) a clutch of eggs (some containing late stage embryos and at least one exhibiting multilayer eggshell) from the Late Cretaceous Judith River Formation of Montana and named *Testudoolithus zelenitskyae* oosp. nov., 3) an egg contained within an adult *Basilemys nobilis* from the Late Cretaceous Kaiparowits Formation of Utah, and 4) a clutch of *Meiolania platyceps* eggs from the Pleistocene of Lord Howe Island, Australia. *Meiolania platyceps* eggs are named *Testudoolithus lordhowensis* oosp. nov. and provide valuable information on the origin of aragonite eggshell composition and nesting behaviors.

Cladistic analyses utilizing egg and reproductive characters are rarely performed on taxa outside of Dinosauria. My analyses demonstrate that morphological data produces poorly resolved trees in which only the clades Adocia and Trionychia are resolved and all other turtles form a large polytomy. However, when combined with molecular data, egg and reproductive characters have more resolving potential towards the top of trees. This poor resolution is likely due to homoplasy in the form of character reversals, convergent evolution, and/or from the limited number of informative characters.

CHAPTER ONE

INTRODUCTION

Testudinata is a monophyletic clade consisting of all taxa (stem and crown clade Testudines) containing a 'true' turtle shell that includes a carapace and plastron. Each is made up of multiple elements, with the vertebrae, ribs, cleithrum, and dermal bones forming the carapace, and the clavicles, interclavicles, and gastralia comprising the plastron. This, along with the fact that their limb girdles are concealed within the shell, gives turtles a unique bauplan amongst vertebrates.

Extant turtles occur on all continents except Antarctica and in all of the world's oceans. Turtles are diverse and include 13 extant families divided between two suborders, Pleurodira and Cryptodira, with approximately 317–323 living species (Nicholson et al., 2015). Amongst extant taxa, carapace lengths range from 10 cm to 175 cm in *Homopus signatus* and *Dermochelys coriacea*, respectively, and possibly as large as 330 cm in the extinct taxon *Stupendemys geographicus* (Sánchez-Villagra and Scheyer, 2010).

Turtles are relatively common in the fossil record because of their highly ossified shell, which enhances their preservation potential. Turtles first appear in the fossil record in the Late Triassic (middle Norian) period, whereas intermediate stem taxa (proto turtles) such as *Eunotosaurus africanus, Pappochelys rosinae*, and *Odontochelys semitestacea* are Late Permian to Late Triassic (Carnian) in age. These taxa lack the carapace and plastron, or in the latter, just the carapace (Li et al., 2008; Lyson et al., 2013; Schoch and Sues, 2015).

The phylogenetic position of turtle families within Testudines is relatively well understood (Fig. 1; Crawford et al., 2014), whereas the position of Testudinata in the vertebrate tree of life is extensively debated (as reviewed by Joyce, 2015). Two hypotheses are currently at the forefront of this debate: Testudinata represent the sister taxon to Lepidosauria or, alternatively, Archosauria (Joyce, 2015). Although numerous molecular phylogenetic studies suggest that turtles are likely the sister taxon to Archosauria (Iwabe et al., 2005, Hugall et al., 2007; Shen et al., 2011; Chiari et al., 2012; Crawford et al., 2012; Fong et al., 2012; Shaffer et al., 2013; Wang et al., 2013; Field et al. 2014; Thomson et al. 2014), morphological analyses indicate that they may derive from the line leading to Lepidosauria (Reisz and Laurin, 1991; deBraga and Rieppel, 1997; Lee 1997; Müller, 2004; Lyson et al., 2010; Lee, 2013). Despite their relationship to Testudinata, the intermediate stem taxa *Eunotosaurus africanus, Pappochelys rosinae*, and *Odontochelys semitestacea* provide little support in favor of one hypothesis over the other. While this debate remains very much alive, new fossil discoveries will likely help resolve this problem by providing intermediate morphologies.

In addition to their unique bauplan, turtles are unusual in other respects, including their reproductive physiology. Among extant amniotes, only turtles lay eggs composed of aragonite, whereas calcite comprises the eggshell of all other egg-laying amniotes (Hirsch, 1983). For this reason, unaltered turtle eggs and eggshells are easily recognizable in the fossil record and are known from every continent except Antarctica and range in age from the Jurassic to the Pleistocene (Lawver and Jackson, 2014). Turtle eggs from the Middle Jurassic (Buckman, 1859) correspond roughly to the age of the

turtle crown group, as estimated from the fossil record (Danilov and Parham, 2006; Joyce, 2007; Anquetin et al., 2009) and molecular calibration studies (Joyce, Parham et al., 2013). Considering that the oldest known fully shelled turtle, *Proterochersis robusta*, is from the Late Triassic (Fraas, 1913; Joyce, Schoch et al., 2013), it is apparent that the evolution of aragonite eggshell occurred just before the split of the crown group, or that a substantial gap exists in the fossil record. For this reason, eggs and eggshells from stem turtles are critical for determining when this unique mineral composition evolved.

My thesis includes three primary avenues of research: 1. study of extant turtle reproductive biology, 2. description and diagenetic assessment of fossil turtle eggs, and 3. incorporation of the resulting data into phylogenetic analyses in order to investigate whether turtle egg and eggshell characters are useful in cladistic analyses. Studying extant turtle eggs and clutches assists in interpreting the anatomy, behavior, and ecology of extinct turtles. This is because turtle reproductive physiology and behavior result in biologically significant patterns that are potentially recognizable in the fossil record. Additionally, diagenetic assessment and description of new specimens of turtle eggs, provide valuable information about their diversity and paucity in the fossil record when compared to dinosaur eggs. Finally, this information provides characters for phylogenetic analyses of extant and fossil turtle eggs, aiding in evaluation of previous interpretations and hypotheses about the evolution of turtle reproduction and paleoecology.

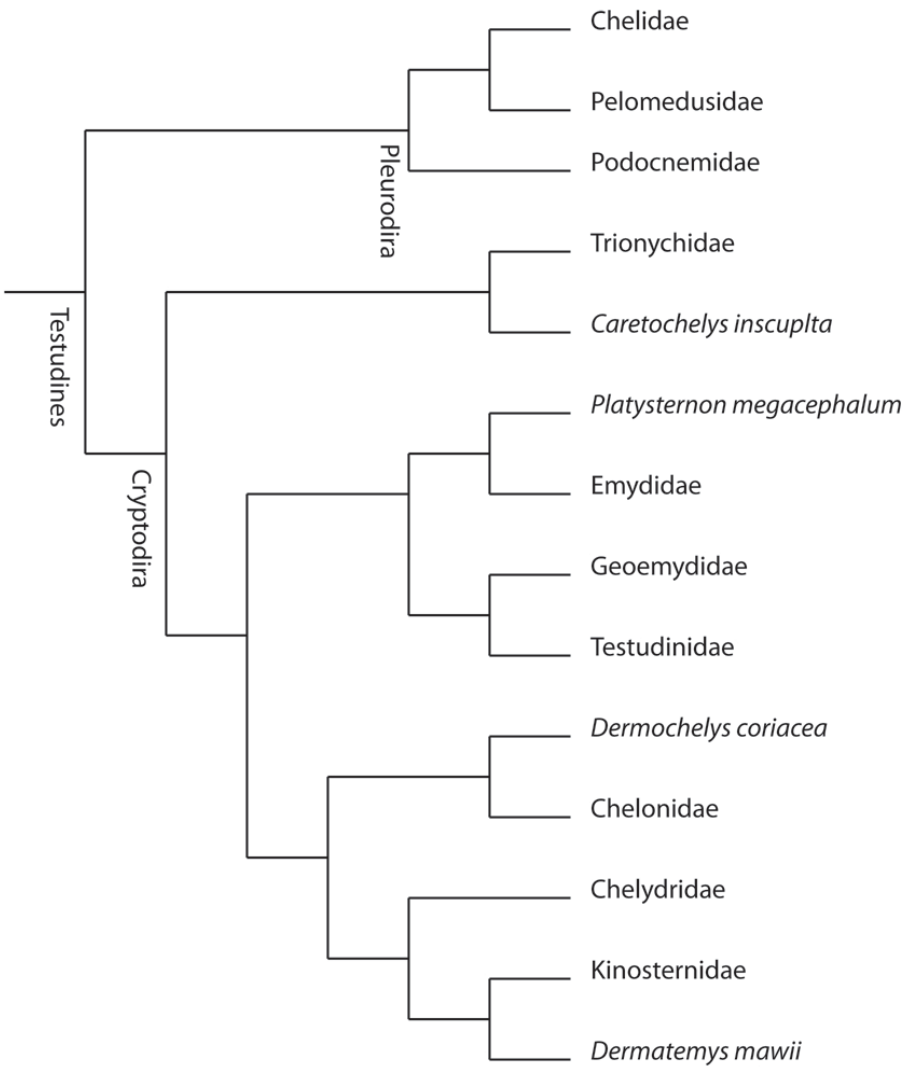


Figure 1. Phylogenetic relationships of extant Testudines. Modified from Crawford et al. (2014).

Dissertation Outline

The research presented here follows the following format: Chapter 2 provides a review of previous work on the fossil record of turtle reproduction, including research on fossil turtle eggs, embryos, nests, and copulating pairs. In this chapter I analyze the global geographic distribution of fossil turtle eggs and eggshell occurrences and reevaluate their

ootaxonomy. Chapter 3 describes fossil turtle eggs from Madagascar, which enhances the sparse fossil record of turtle eggs from Gondwana. Chapter 4 describes a fossil turtle egg clutch with embryos (MOR 710) from the Judith River Formation of Montana. This study provides information about the taphonomy and diagenesis of the fossil eggs. Chapter 5 describes the eggshell microstructure from one of eight eggs preserved inside an adult *Basilemys nobilis* specimen from the Kaiparowitz Formation of Utah. This specimen allows definitive egg assignment to a specific taxon and comparison to two previously described gravid *Adocus* turtles. I also provide information about the reproductive biology and paleoecology of extinct large-bodied turtles. Chapter 6 examines the eggshell microstructure from eggs assigned to the stem turtle *Meiolania platyceps*. This study investigates the origin and evolution of the aragonite eggshell composition present in turtle eggs. Chapter 7 focuses on a phylogenetic analysis of modern and fossil turtle eggs and eggshell described in this dissertation and assesses the usefulness of such characters in phylogenetic reconstructions. Finally, Chapter 8 summarizes the principal findings of my dissertation, with a brief section on future work.

Methods and Materials

All studies include microscopic analysis of eggshell microstructure, using the following techniques. Eggshell fragments were broken in half with one half prepared as radial and/or tangential thin sections (30 μm thick) at the Gabriel Laboratory for Cellular and Molecular Paleontology at the Museum of the Rockies, Montana State University or Spectrum Petrographic, Vancouver, Washington. Thin sections were studied by

transmitted and polarized light microscopy, using a Nikon Eclipse E600 equipped with a digital camera. The other half of each specimen was coated with gold (10 nm) and mounted on an aluminum stub. Specimens were imaged under a JEOL 6100 SEM located at the Image and Chemical Analysis Laboratory, Montana State University. Eggshell microstructural features (e.g., shell thickness, pore width, etc.) were measured with ImageJ image analysis software available from the National Institute of Health (http://imagej.nih.gov/ij/). The remaining methods are specific to individual chapters presented in this dissertation and descriptions of these techniques are provided in the appropriate chapters.

CHAPTER TWO

A REVIEW OF THE FOSSIL RECORD OF TURTLE REPRODUTION: EGGS, EMBRYOS, NESTS, AND COPULATING PAIRS

Contributions of Authors and Co-Author

Manuscript in Chapter 2

Author: Daniel R. Lawver

Contribution: Conceived and implemented the study design. Collected and analyzed data. Wrote first draft of the manuscript.

Co-Author: Frankie D. Jackson

Contributions: Wrote one brief section on egg pathology (p. 24) and provided feedback on early drafts of the manuscript.

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CHAPTER TWO

A review of the fossil record of turtle reproduction: eggs, embryos, nests, and copulating pairs

Daniel R. Lawver and Frankie D. Jackson

Department of Earth Sciences, Montana State University, Bozeman MT 59717 USA

—*email:* danlawver@gmail.com (corresponding author); frankiej@montana.edu

ABSTRACT

The fossil record of turtle reproduction (e.g., eggs, embryos, nests, and copulating pairs) is relatively poor compared to that of dinosaurs. This record extends from the Middle Jurassic to the Pleistocene and specimens are known from every continent except Antarctica. Fossil turtle eggs are recognized as body fossils and confident taxonomic identification at the genus or species level is dependent on embryos preserved within fossil eggs or by eggs found within a gravid female. Cladistic analyses of egg and eggshell characters demonstrate a high degree of homoplasy and only few characters provide a strong phylogenetic signal. Taphonomic studies of fossil turtle eggs are rare but can elucidate size and number of eggs produced by extinct taxa. Pathological fossil turtle eggs are known from a few localities and provide information about physiological or environmental stresses experienced by the gravid female. Fossil turtle eggs are relatively abundant in Asia, Europe, and North America but are poorly represented in Gondwana.

An ootaxonomic review of fossil turtle eggs shows that of 15 named ootaxa, eight are nomina valida, five are nomen nudum and two are junior synonyms of other ootaxa.

KEYWORDS

Testudines, paleoology, Testudoolithidae, Testudoflexoolithidae

Introduction

Turtles are some of the most distinct animals living today and possess unique anatomical and physiological characters, also in regards to their reproduction. For example, unlike all other living amniotes, turtles produce eggs composed of the mineral aragonite, rather than calcite (Hirsch 1983). When preserved in the rock record, this mineral composition allows definitive assignment of fossil eggs and eggshell to Testudines or their stem lineage. Indeed, based on this unique eggshell composition, some authors suggest that hard-shelled eggs evolved independently in turtles from all other amniotes (Carpenter 1999; Sander 2012).

Fossil turtle eggs are known from every continent except Antarctica and range in age from the Jurassic to the Pleistocene. The oldest described turtle eggs were collected from the Middle Jurassic of England (Carruthers 1871). This corresponds roughly to the age of the turtle crown, as estimated from the fossil record (Danilov and Parham 2006; Joyce 2007; Anquetin et al. 2009) or through molecular calibration studies (Joyce et al. 2013). Considering that the oldest known fully shelled amniote (“turtle” in common

parlance), *Proterochersis robusta*, is known from the Late Triassic (Fraas, 1913; Joyce et al. 2013), it is apparent that the presence of aragonitic eggs is either a feature that was acquired just before the split of the crown group, or that a substantial gap exists in the fossil record. In contrast to non-avian dinosaurs, which receive the majority of attention in paleo-oological studies, modern analogs exists for extinct turtle eggs, embryos, nests, and copulating pairs. In addition, extant turtles provide information about growth and development, physiology, anatomy, and paleoecology that are necessary for more accurate interpretations of fossil specimens and localities.

Methods Used in the Study of Fossilized Eggs

Standard preparation techniques for the study of fossil eggshell include examination of thin sections with a polarized light microscope (Quinn 1994) and scanning electronic microscope (SEM), the latter typically being equipped with energy dispersive x-ray (EDX) and backscatter electron imaging (BEI; Tyler 1969; Erben et al. 1979; Carpenter 1999; Schweitzer et al. 2002; Jackson et al. 2002). Depending on the purpose of the study, additional analyses may include cathodoluminescence (CL; England et al. 2006; Grellet-Tinner et al. 2010; Fernández and Matheos 2011), epifluorescence microscopy (Jackson et al. 2010) and x-ray diffraction (Jackson et al. 2002). Structural attributes of eggshell (shell thickness, pore width) are typically measured with image analysis software. Computed tomography (CT) and phase contrast synchrotron microtomography allow high resolution imaging of eggs, which is necessary for identification of *in ovo*

embryonic remains, while avoiding mechanical preparation and potential damage to the specimens (Fernandez et al. 2012). Finally, two recent studies of fossil eggs also include electron backscatter diffraction (EBSD) and/or orientation contrast imaging to "map" the orientation of crystal growth (Grellet-Tinner et al. 2012; Moreno-Azanza et al. 2013a).

Physical Attributes of Turtle Eggs

Turtle eggs range from spherical in large-bodied species to elongate in relatively small-bodied species (Ewert 1979; Iverson and Ewert 1991). Intra- and interspecific variation in egg size, shape, and number of eggs per clutch characterizes modern turtle taxa. For example, younger, smaller females may lay fewer and smaller eggs per clutch than older, larger females (Kuchling 1999). Clutch size ranges from a single large egg in *Platemys platycephala* to over 100 eggs in the leatherback sea turtle (*Dermochelys coriacea*; Ernst and Barbour 1989). Interestingly, *D. coriacea* lays both large and small eggs in a single clutch. The small, yolkless eggs are thought to result from excess albumen (Spotila 2004) or, alternatively, to act as spacers between the larger, viable eggs thereby increasing gas exchange (Gulko and Eckert 2004).

Turtle eggshell consists of a single layer of shell units comprised of needle-like aragonite crystals that originate from an organic core within the shell membrane. The degree of mineralization varies widely among turtle eggs and includes pliable (chelonioids, emydids, and some pleurodires), semi-pliable (chelydrids), and rigid-shelled eggs (carettochelyids, geoemydids, kinosternoids, testudinids, and trionychids). This

variation in eggshell mineralization corresponds to variation in the height:width ratios of the shell units and whether the shell units are loosely or tightly spaced and interlocking in structure (Figure 1). The tightly interlocking structure of rigid-shelled eggs enhances their potential for preservation in the fossil record (Hirsch 1983).

Classification of Fossil Turtle Eggs

Early studies often assigned fossil eggs to turtles based on their size, spherical shape, and eggshell texture (Buckman 1859; Meyer 1860; Carruthers 1871; Hay 1908; Straelen 1928), but '*Oolithes*' *bathonicae* Buckman, 1859 was the only named taxon well into the 20th century. Hirsch (1983) was the first to compare the microstructure of fossil eggs to that of modern turtle eggs, emphasizing the importance of polarized light microscopy and scanning electron microscopy (SEM). Hirsch (1996) later extended the parataxonomy developed for classifying dinosaur eggs to include fossil turtle eggs and started naming fossil turtle oospecies using microstructure characteristics as the basis of his diagnoses. Based on the degree of eggshell mineralization, he established two morphotypes under the Testudoid basic type of eggshell structure: 1) Spheruflexibilis, for soft or pliable eggs, and 2) Spherurigidis, for rigid eggshell; spheru referring to the spherulitic ultrastructure of the shell units. These morphotypes are distinguished by the height:width ratio of their shell units and whether the shell units are tightly interlocking. A single oofamily corresponds to each morphotype, namely Testudoolithidae and Testudoflexoolithidae,

respectively. Currently, at least five valid oogenera and eight valid oospecies have been established for fossil turtle eggs (Table 1).

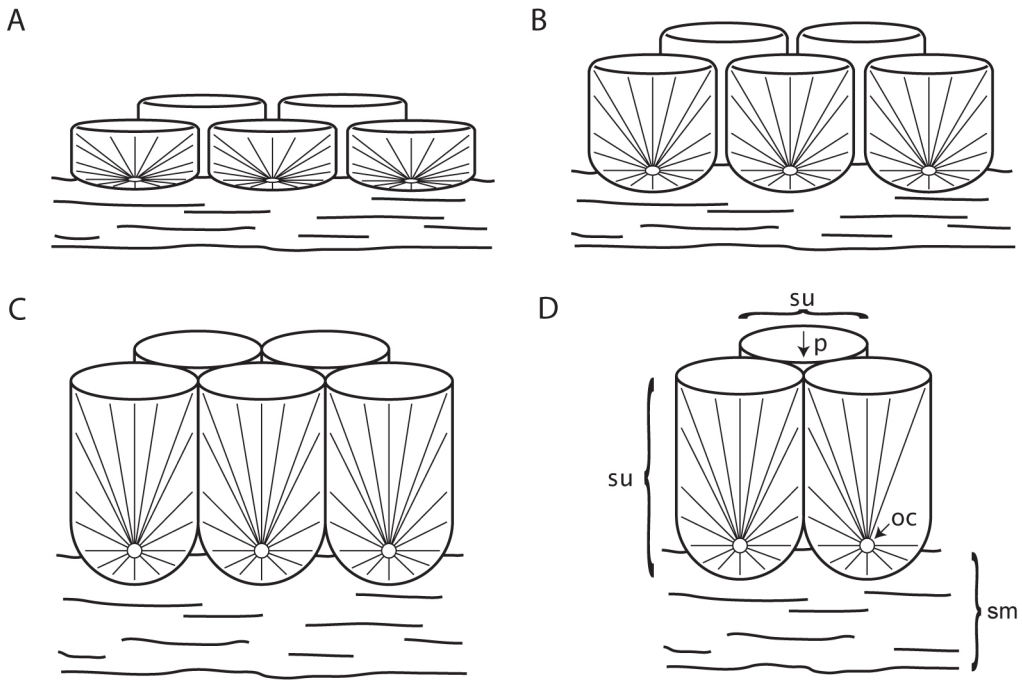


Figure 1. Eggshell microstructure of fossil and modern turtle eggs. A, Pliable-shelled turtle eggs with shell units wider than tall. B, Semipliable turtle eggs with shell units as wide as tall. C, Rigid-shelled turtle eggs with shell units taller than wide. D, Turtle eggshell structure. Abbreviations: oc, organic core; p, pore; sm, shell membrane; su, shell unit. Modified from Hirsch (1983, fig. 2).

It is important to note that Hirsch's (1996) attempt to build an internally coherent nomenclatural system for turtle eggshells that is consistent with the ICZN failed from the beginning, because his scheme demands referral of the previously named oospecies *Oolithes bathonicae* Buckman, 1859 to the new oogenus *Testudoflexoolithus*. Following the rules of the ICZN (1999), *Testudoflexoolithus* is the objective junior synonym of *Oolithes* and should not be used. However, given that Hirsch's (1996) nomenclatural

system has be utilized consistently over the course of the last two decades (e.g., Kohring 1999a; Jackson et al. 2008; Barta et al. 2014), we herein maintain nomenclatural stability by maintaining this system, while formally acknowledging the apparent break with the ICZN.

Taxonomic Assignment of Fossil Turtle Eggs

Numerous authors have attempted to identify the species of turtle that potentially laid a given fossil egg (Gergens 1860; Meyer 1860; Hummel 1929; Kohring 1993; Gaffney 1996; Azevedo et al. 2000). However, definitive identification requires embryonic remains or eggs that occur within a gravid female, because eggshells generally do not provide enough characters to allow diagnosing species (Hirsch 1989). Therefore, all identifications that lack these criteria should be treated with suspicion. In addition, embryos often lack the diagnostic features necessary to identify fossil eggs beyond the genus level. For example, McGee (2012) identified fossil turtle eggs with embryos from Canada as originated from *Adocus* based on eggshell microstructure and morphology of the embryonic bones, but she refrained from identifying the specimens to a particular *Adocus* species because diagnostic morphologies were not present in the embryos, likely due to their early ontogenetic stage. Fossil gravid turtles can potentially provide species-level identification of eggs, depending on the presence of autapomorphic characters and quality of adult preservation. For example, eggs were discovered in an undescribed

Table 1. Valid turtle ootaxa and key features for diagnosis.

Ootaxon	Holotype	Material	Egg shape	Length x Width (mm)	Eggshell thickness	Shell unit height:width
Testudoflexoolithus agassizi	MCZ 2810/HEC 49	Eggshell fragments	-	-	0.06–0.1 mm	1:1 or 2:3
Testudoflexoolithus bathonicae	NHMUK PV OR37987/ HEC 186	An egg imbedded in matrix	Ellipsoidal	48 x 26 mm	0.2-0.25 mm	1:1
Chelonoolithus braemi	Guimarota 98-2	Eggshell fragments	-	-	0.2 mm	1:1
Emydoolithus laiyangensis	IVPP V18544	A nearly complete egg	Elongate	9.1 x 2.2 cm	0.4-0.5 mm	2:1 to 5:1
Haininchelys curiosa	-	Eggshell fragments	-	-	0.25-0.3 mm	1.2:1 to 2.3:1
Testudoolithus hirschi	-	Eggshell fragments	-	-	0.15 mm	3:1
Testudoolithus jiangi	ZMNH M8713	A clutch of 23 eggs	Spherical	35-52 mm	0.7-1.0 mm	2.5:1 to 3:1
Testudoolithus rigidus	UCM 55806/ HEC 425	Half of an egg	Spheroidal	42 x 47 mm	0.22-0.24 mm	2:1

Canadian specimen of *Basilemys variolosa* after the carapace was inadvertently damaged (Braman and Brinkman 2009). Although fossil gravid turtles may provide higher levels of taxonomic identification, incomplete egg formation at the time of death results in thinner than normal eggshell (Zelenitsky et al. 2008; Knell et al. 2011) that may complicate identification.

In addition to incorrect taxonomic identification of fossil turtle eggs, some older literature refers to megaloolithid dinosaur eggs as "testudoid" (Sochava 1971; Erben et al. 1979). These studies explicitly state that the eggs in question are those of dinosaurs and that the term does not reflect taxonomic identification. The word "testudoid" refers to the gross similarity between the eggshell structure of megaloolithid and modern turtle eggs, thus resulting in misleading terminology. Similarly, Mikhailov (1997) provides identical descriptions for both Discretispherulitic (megaloolithid) and testudoid eggs. However, turtle eggs are comprised of aragonite, have a smaller overall egg size and generally a smooth external surface compared to megaloolithid eggs.

Fossil Eggs: Trace Compared with Body Fossils

Fossil eggs are interesting because they do not strictly conform to the traditional definitions of either trace or body fossils but rather are somewhere in between these categories. Some authors (Jain 1989; Lockley and Gillette 1989; Bray and Hirsch 1998) regard fossil eggs and eggshell as trace fossils. This category is defined as any evidence of an organism, excluding body parts, and characterized by organismal behavior

preserved in a substrate, such as footprints, burrows, feeding marks, and coprolites (Martin 2013). Further, Bertling et al. (2006) suggests that trace fossils should be categorized by their morphology alone and should not be influenced by the taxonomic identity of the trace maker; however, others (Simpson 1975; Lockley and Gillette 1989) suggests that a certain level of taxonomic identification is possible.

On the other hand, Martin (2001) views fossil eggs as the preserved remains of an organism itself and therefore, they are body fossils. Here we argue that, of the views presented, the evidence for eggs being body fossils is most convincing because eggshell, similar to the shell of invertebrates, is an essential part of the developing organism (Martin 2001). The chorioallantoic membrane provides a direct connection between the embryo and the egg, which allows for aerobic metabolism and the uptake of calcium by the embryo from the eggshell (Carpenter 1999). In addition, the aragonite structure of the eggshell is under direct genetic control (Rodriguez-Navarro et al. 2007; Dunn et al. 2012) and the embryo, yolk, albumin, membranes, and mineralized eggshell form a single unit. As such, fossil eggs and eggshell offer insight into some of the earliest ontogenetic stages of extinct organisms and are occasionally augmented by data from identifiable embryonic remains and gravid females. In contrast, the preserved structure of a nest and its egg arrangement are trace fossils that provide evidence of parental behavior in the preparation of a suitable environment for embryonic development, sometimes including manipulation of the eggs by the parent (Varricchio et al. 1999).

Cladistic Analysis of Eggs and Eggshell Characters

Several studies advocate the usefulness of egg and eggshell characters in determining phylogenetic relationships among extinct taxa (Mikhailov 1992; Grellet-Tinner 2006; Zelenitsky and Modesto 2003; Grellet-Tinner and Chiappe 2004; Varricchio and Jackson 2004; Jin et al. 2010). However, such analyses are rarely applied to extant taxa in order to test the validity of this approach.

Winkler (2006) mapped eggshell characters onto a composite phylogeny of extant pleurodiran turtles (12 taxa and 13 characters). This study suggests a high degree of homoplasy in the eggs and eggshells of these taxa and only three characters provide a phylogenetic signal. However, the scope of her study was narrow because she only examined pleurodiran turtles from two families (e.g., Chelidae and Pelomedusidae), both of which lay hard-shelled eggs.

Lawver (2012) more recently conducted an expanded cladistic analysis using egg and eggshell characters to assess the phylogenetic relationships of multiple turtle clades (e.g., Chelidae, Kinosternidae, Dermatemydidae, Trionychidae, Bataguridae, and Testudinidae) within Testudines, and also included a fossil turtle egg from the Upper Cretaceous (Campanian) Judith River Formation of Montana, which likely represents an *Adocus* sp. egg because of its microstructural similarities to those from an *in situ* clutch discovered in Alberta, Canada (Zelenitsky et al. 2008). The resulting strict consensus tree contains a fully resolved archosaurian clade with a sister taxon relationship to Testudines. Within Testudines, a clade consisting of the Trionychidae and the fossil turtle egg is

resolved, but all other in-group taxa form a large polytomy. If the *Adocus* sp. identification of the fossil is confirmed, it would suggest that some egg and eggshell characters are potentially informative for diagnosing a clade consisting of trionychids and *Adocus* sp. This clade is otherwise well supported by previous morphological analyses (Gaffney and Meylan 1988; Meylan and Gaffney 1989; Joyce 2007). The results of the study by Lawver (2012) are otherwise consistent with Winkler's (2006) conclusions by demonstrating a high degree of homoplasy in turtle egg characters and only limited use in diagnosing turtle clades.

Nests

Most descriptions of fossil turtle eggs focus on isolated specimens rather than *in situ* eggs and clutches, thus providing little or no information about site taphonomy. Currently, descriptions of *in situ* material include only the three specimens discussed below.

Zelenitsky et al. (2008) describes an *in situ Adocus* sp. egg clutch and gravid female, respectively, from the Upper Cretaceous (Campanian) Oldman and Dinosaur Park formations in Alberta, Canada. The *in situ* clutch covers an oval-shaped area 480 mm x 350 mm and consists of approximately 26 eggs. Zelenitsky et al. (2008) interpreted the sandy siltstone surrounding the clutch as well-drained levee deposits. Twenty-four of the 26 rigid-shelled eggs are crushed, whereas two relatively uncrushed specimens measure about 40 mm x 42 mm in diameter. The eggshell measures 730–810 μm thick.

The second specimen consists of a gravid adult that displays a crushed carapace 310 mm wide by 405 mm long. The carapace contained at least five 35 mm x 40 mm rigid-shelled eggs. The eggshells measure 500–650 μm thick and some preserve permineralized membrane that measures about 1/3 of the eggshell thickness. The substantially thinner eggshell associated with the gravid female, compared to the *in situ* clutch, indicates that the eggs were not fully formed but within days of oviposition (Zelenitsky et al. 2008). Zelenitsky et al. (2008) uses the correlation between body size and clutch size among extant turtle species to estimate 1) the carapace length of the female that laid the *in situ* clutch, and 2) the number of eggs produced by the gravid female. The 26 eggs in the *in situ* clutch suggest an adult carapace length of 495 mm, whereas the gravid female likely produced a smaller clutch of approximately 19 eggs. The latter, however, preserved only five eggs within the specimen. Rigid eggshell is thought to have evolved independently in turtle clades in response to predation, and low pore density of *Adocus* sp. eggs is consistent with low water vapor conductance, which may indicate adaptation in response to dry nesting conditions (Zelenitsky et al. 2008).

Fang et al. (2003) describe two eggs from a turtle clutch from the Lower Cretaceous Tiantai Basin, Zhejiang, China, establishing a new oogenus and oospecies within a new oofamily. Jackson et al. (2008) reassigned the specimens to *Testudoolithus jiangi* and provided information about the clutch, its paleoecology, and depositional environment. These eggs and their rather confusing parataxonomic history are discussed in our Systematic Paleontology section below. Here, we focus on the clutch and sedimentology of the nesting site.

As typical of many specimens from the Tiantai Basin, the clutch was recovered from a construction site and its original size remains unknown. In its current condition, the 27 cm by 47 cm block contains 23 eggs and 4 additional eggs separated from the block during excavation. Sedimentary structures associated with the clutch confirm its original orientation in the stratum and indicate a low flow regime, typical of fluvial environments that characterize many turtle nesting localities today. In addition, the clutch contains three superimposed layers of eggs, suggesting that the female buried the eggs in a relatively deep, excavated hole. Blue-gray reduction "halos" surrounding the eggs and associated burrow and root traces indicate that pedogenic processes occurred near or below the water table.

Based on comparison of egg shape and size to extant taxa, Jackson et al. (2008) propose that the clutch belonged to a Cretaceous turtle of large body size, possibly comparable to large bodied extant testudinids turtles. The thick, rigid eggshell and spherical egg shape likely reduced the possibility of lethal desiccation because the greater pore length and reduced surface area retarded water loss during incubation. In addition, the specimen reveals rare evidence of fungal-animal association in the fossil record (Jackson et al. 2009). A single egg on the periphery of the clutch contains fossil fungal structures, suggesting opportunistic fungal invasion after the egg was compromised and prior to subsequent failure of the clutch as a whole.

Bishop et al. (2011) reports a sea turtle nesting trace that included an egg chamber, egg molds, body pit, and an adult crawlway from the Late Cretaceous (Maastrichtian) Fox Hills Formation in Elbert County, Colorado. The strata containing

the traces consist of 1) a basal sandstone with faint horizontal and cross-bedding; 2) a strongly laminated sequence of heavy mineral layers interbedded with bioturbated sands, which in turn were overlain by 3) anatomizing sandstones with scour-flute casts on their bases; and 4) a thin homogenous interval capped by festoon cross-bedded sandstone. Bishop et al. (2011) interprets the sequence as representing a forebeach, backbeach, and dune field. The sedimentary structures of these nesting traces (Bishop et al. 2011: fig. 13.8 and 13.9) strongly resemble the size and morphology of those produced by modern loggerhead sea turtles at nesting sites monitored by the St. Catherines Island Sea Turtle Conservation Program near Savannah, Georgia (Bishop et al. 2011).

Winkler and Sánchez-Villagra (2006) describe a single layer of abundant turtle eggshells in a coarse, poorly sorted sandy deposit. These eggshells occur within a 600 m^2 area at a locality in the Miocene Urumaco sequence of Venezuela. Based on an *in situ* fragment of an egg, they suggest an elliptical shape and estimate the original size as 56.5 mm x 43.5 mm. This red oxidized sandstone also contained foraminifera, the ichnofossil *Thalassinoides*, and small unidentifiable fragments of bivalve shells, possibly of marine origin. Although providing no evidence for their conclusions, Winkler and Sánchez-Villagra (2006) interpret these eggshell fragments as egg “clusters” or “clutches”. Further, they conclude that turtles deposited their eggs at a colonial nesting site on a beach directly facing the sea or brackish waters, possibly near a river delta or lagoon. Based on a large, poorly preserved carapace within a few centimeters of a purported egg clutch, they tentatively assign the eggshells to *Bairdemys venezuelensis*. The lack of a detailed site description warrants caution with the interpretations of this locality.

Pathological Conditions of Eggs

In extant amniotes that lay hard-shelled eggs, adverse stimuli from physiological or environmental stress occasionally produce prolonged egg retention by the female. One or more eggshell layers may be deposited over the retained egg, producing an unusually thick, multilayered eggshell that typically results in embryonic death (Ewert et al. 1984). Among modern taxa, this condition occurs most commonly in turtles, currently reported in ten species (Jackson and Schmitt 2008). However, reports of similar abnormal conditions in fossil turtle eggshells are rare. Kohring (1998b) reported multilayered turtle eggs from the Miocene of the Czech Republic, and Jackson and Schmitt (2008) document a multilayered egg in a weathered clutch from the Upper Cretaceous (Campanian) Judith River Formation of Montana.

Paleobiogeography

The temporal and geographic distribution of fossil turtle eggs and eggshell suggest two interesting trends. First, there is a trend for the gradual increase in fossil turtle eggs and eggshell occurrences towards the present (Figure 5). This “pull of the recent” may be explained by the fact that the aragonite composition of turtle eggshell is metastable and therefore is likely to be altered with increasing geologic time. However, some specimens from the Jurassic show relatively unaltered aragonite crystals whereas specimens from three to four million year rocks are completely altered (Hirsch 1996). This suggests that certain local environments are more suitable for exceptional preservation for longer

periods of time. Additionally, certain time periods (Campanian and Albian) show an unexpectedly large number of occurrences. This may be explained by collecting biases toward accessible and highly productive localities of this age, or perhaps the preservation potential was higher for aragonite during this period. An exact mechanism for such preservation is unknown at this time.

The Miocene also shows a large peak of occurrences of fossil turtle eggs and eggshell; however, the Miocene represents 17.5 my, which is significantly longer than many other geologic periods. At this time, limited age information for several specimens discussed above prohibit proper analysis using equilibrated time binning.

Second, the geographic distribution of turtle reproduction in the fossil record shows that the vast majority of specimens come from Laurasian continents, with at least 49 occurrences. In contrast, Gondwanan continents have significantly fewer occurrences (approximately 14) of fossil turtle eggs and eggshell. This is likely due to collection biases. Trends such as these have also been observed in the temporal and geographic distributions of dinosaurian ootaxa in which greatest diversity is found in Laurasian continents at the end of the Cretaceous (Varricchio et al. 2011).

Systematic Paleontology

Valid Ootaxa

See Appendix 4 for the hierarchical taxonomy of ootaxa as described in this work.

Testudoflexoolithidae Hirsch, 1996

Diagnosis. Eggshell of the taxon Testudoflexoolithidae is diagnosed by the testudoid basic type, the spheruflexibilis morphotype, loosely abutting shell units, pore canals absent or widely spaced between shell units, and spheroidal to ellipsoidal egg shape.

Testudoflexoolithus Hirsch, 1996

Type oospecies. *Testudoflexoolithus bathonicae* (Buckman, 1859).

Diagnosis. Eggshell of the taxon *Testudoflexoolithus* is diagnosed by loosely abutting shell units, an ellipsoidal egg shape, and 0.06–0.2 mm thick eggshell.

Testudoflexoolithus agassizi Hirsch, 1996

Taxonomic history. *Testudoflexoolithus agassizi* Hirsch, 1996 (new oospecies).

Type material. MCZ 2810/HEC 49 (holotype), eggshell fragments (Hirsch 1996 fig. 3a–d).

Type locality. Florida, USA (see Figure 4); Florida Oolite, Pleistocene (Hirsch 1996).

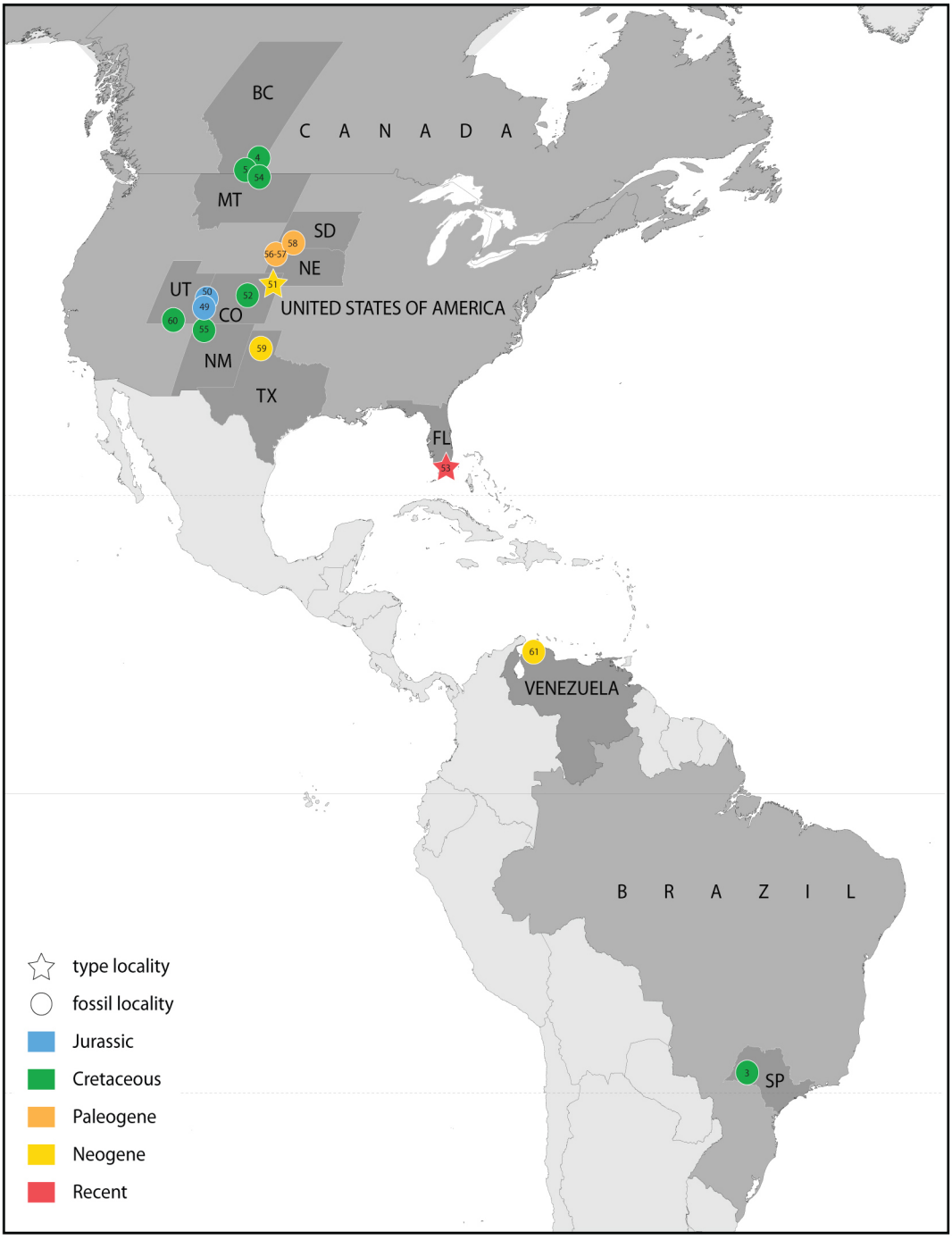


Figure 2. The geographic distribution of valid ootaxa in the New World. Type localities are indicated with stars. Locality numbers are cross-listed in Appendix 3. Abbreviations: BC, British Columbia, Canada; CO, Colorado; FL, Florida; MT, Montana; NE, Nebraska; NM, New Mexico; SD, South Dakota; TX, Texas; UT, Utah, SP, São Paulo State, Brazil. Map provided by Walter Joyce.

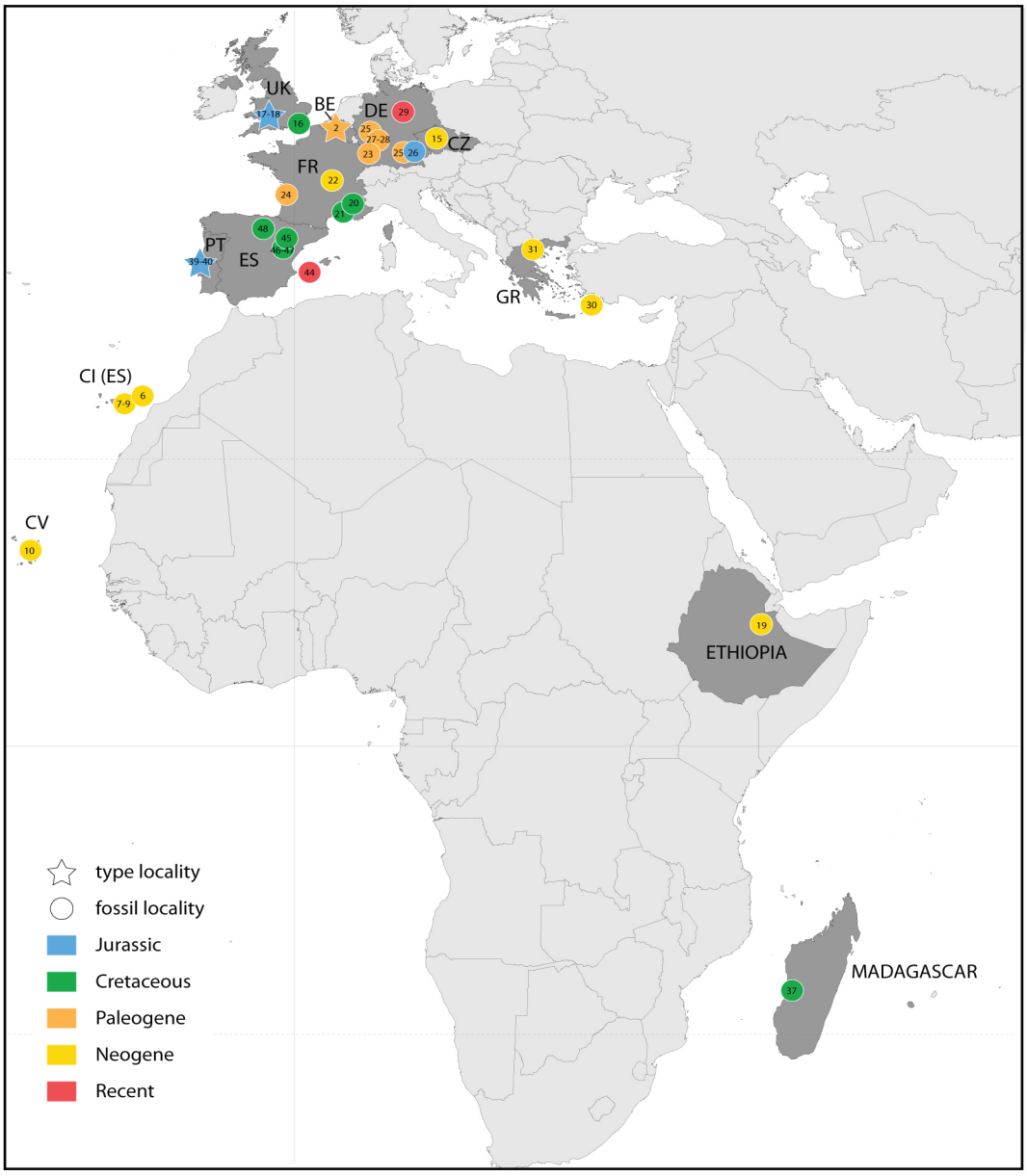


Figure 3. The geographic distribution of valid ootaxa in Europe and Africa. Type localities are indicated with stars. Locality numbers are cross-listed in Appendix 3. Abbreviations: BE, Belgium; CI, Canary Islands; CV, Cape Verde; CZ, Czech Republic; DE, Germany; ES, Spain; FR, France; GR, Greece, PT, Portugal; UK, United Kingdom. Map provided by Walter Joyce.

Diagnosis. Eggshell of the taxon *Testudoflexoolithus agassizi* is diagnosed by a shell unit height:width ratio of 1:1 to 2:3 and 0.06–0.1 mm thick eggshell. *Testudoflexoolithus*

agassizi can be distinguished from *Testudoflexoolithus bathonicae* by a shell thickness that is two or more times thicker.

Comments. The microstructure of *Testudoflexoolithus agassizi* is very similar to that of the extant sea turtle *Lepidochelys kempii* but the egg shape remains unknown (Hirsch 1996). Given the relatively recent age of the fossil and the facies in which they were found, it is plausible that a cheloniid sea turtle produced these specimens. The loosely abutting eggshell units of this oospecies are likely to disassociate from one another after decomposition of the shell membrane. Preservation of this pliable eggshell is therefore rare (Hirsch 1996). Very little locality information is available for this taxon.

Testudoflexoolithus bathonicae (Buckman, 1859)

Taxonomic history. *Oolithes bathonicae* Buckman, 1859 (new oospecies); *Testudoflexoolithus bathonicae* Hirsch 1996 (new combination).

Type material. BMNH 37987/HEC 186 (lectotype), "egg A" of Hirsch (1996), an egg embedded in matrix (Van Straelen 1928, fig. 2; Hirsch 1996, fig. 2a–d); BMNH 37987/HEC 186 (paralectotype), "eggs B" of Hirsch (1996), a second egg found in association with the lectotype (Van Straelen 1928, fig. 2; Hirsch 1996, fig. 2a).

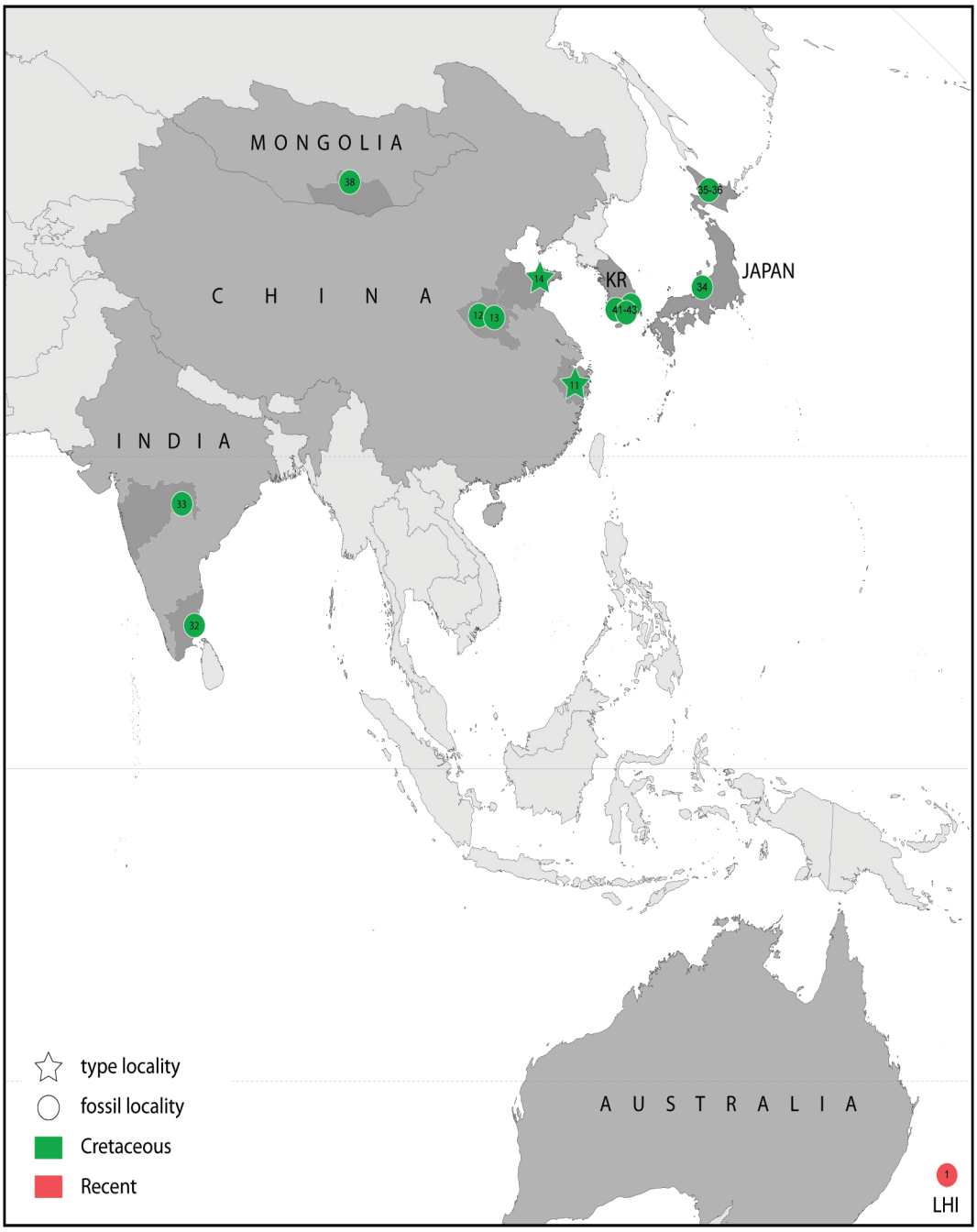


Figure 4. The geographic distribution of valid ootaxa in Asia and Australia. Type localities are indicated with stars. Locality numbers are cross-listed in Appendix 3. Abbreviations: KR, Korea; LHI, Lord Howe Island. Map provided by Walter Joyce.

Type locality. Hare Bushes-Quarry, great Oolite of Cirencester, England (see Figure 2); White Limestone Formation, Bathonian, Middle Jurassic.

Diagnosis. Eggshell of the taxon *Testudoflexoolithus bathonicae* is diagnosed by small (48 mm x 26 mm), ellipsoidal eggs with a shell unit height:width ratio of 1:1 and 0.2–0.25 mm thick eggshell. *Testudoflexoolithus bathonicae* can be distinguished from *T. agassizi* by a shell thickness that is approximately half as thick.

Comments. Buckman (1859) reports fossil eggs from the Middle Jurassic (Bathonian; Riding et al. 1985) Great Oolite of Cirencester (White Limestone Formation), England and named them *Oolithus bathonicae*, which was the first name assigned to a fossil egg. However, Buckman (1859) ruled out a turtle origin for the specimens because they were not spherical in shape. Instead, he suggested that a saurian reptile or perhaps a teleosaurian laid these eggs. It wasn't until 1996 that Hirsch reexamined these specimens and discovered their true identity, assigning the eggs to a turtle, later confirmed by Cox et al. (1999). Hirsch (1996) furthermore referred *O. bathonicae* to the newly created taxon, *Testudoflexoolithus* in an attempt to create an internally consistent parataxonomic nomenclatural system for turtle eggs (see Classification of Fossil Turtle Eggs). Despite the obvious break against the rules of the ICZN, we maintain the internal integrity of the parataxonomic nomenclature developed by Hirsch (1996), who also designated the lectotype of *Testudoflexoolithus bathonicae* from the original type series. The microstructure of *Testudoflexoolithus bathonicae* is similar to recent sea turtle eggs

(Hirsch 1996); however, it is unlikely that a chelonioid turtle produced *T. bathonicae* because this clade did not appear until the Early Cretaceous (Joyce et al. 2013). Additionally, the ellipsoidal shape of *T. bathonicae* differs from the spherical egg shape of all extant sea turtles.

Testudoolithidae Hirsch, 1996

Diagnosis. Eggshell of the taxon Testudoolithidae is diagnosed by the testudoid basic type, the spherurigidis morphotype, presence of a calcareous layer with interlocking shell units, simple and widely spaced pores between shell units, and spheroidal to ellipsoidal egg shape.

Chelonoolithus Kohring, 1998a

Type and only included oospecies. *Chelonoolithus braemi* Kohring, 1998a

Chelonoolithus braemi Kohring, 1998a

Taxonomic history. *Chelonoolithus braemi* Kohring, 1998a (new oospecies).

Type material. FUB Guimarota 98-2 (holotype), eggshell (Kohring 1998a, fig. 1–6).

Type locality. Guimarota Coal Mine, Leiria District, Portugal; Alcobaça Formation, Kimmeridgian, Upper Jurassic (Kohring 1998a).

Diagnosis. Eggshell of the taxon *Chelonoolithus* is diagnosed by a shell unit height:width ratio of 1:1, an eggshell thickness of approximately 0.2 mm, and a flat and smooth (unornamented) exterior surface of the eggshell (Kohring 1998a).

Comments. The shell unit height:width ratio is consistent with that of some extant aquatic taxa such as *Chelydra serpentina,* some *Kinosternon* spp., some trionychids, and *Chelodina longicollis* (Kohring 1998a). The great age of this specimen, however, makes any attribution to one of these taxa implausible (Joyce et al. 2013).

Emydoolithus Wang et al., 2013

Type and only included oospecies. *Emydoolithus laiyangensis* Wang et al., 2013.

Emydoolithus laiyangensis Wang et al., 2013

Taxonomic history. *Emydoolithus laiyangensis* Wang et al., 2013 (new oospecies).

Type material. IVPP V18544 (holotype), a nearly complete egg (Wang et al. 2013, fig. 3a).

Type locality. Jingangkou, Laiyang, Shandong Province, China (see Figure 3); Jingangkou Formation, Upper Cretaceous (Wang et al. 2013).

Diagnosis. Eggshell of the taxon *Emydoolithus laiyangensis* is diagnosed by a symmetrical, elongate, elliptical (9.1 cm x 2.2 cm) egg, eggshell 0.4–0.5 mm thick, shell unit height:width ratio 2:1 to 5:1, 50–60 shell units per square millimeter.

Comments. *Emydoolithus laiyangensis* is similar to *T. jiangi* in microstructure and eggshell thickness but differs in its elongate egg shape (Wang et al. 2013). Wang et al. (2013) compares *E. laiyangensis* to the eggs of *Podocnemis unifilis* but suggests that the eggs belongs to an emydid turtle; however, this identification is unlikely considering the lack of emydid fossils from the Cretaceous, combined with the results of recent molecular calibration studies (Joyce et al. 2013).

Haininchelys Schleich et al., 1988

Type and only included oospecies. *Haininchelys curiosa* Schleich et al., 1988.

Haininchelys curiosa Schleich et al., 1988

Taxonomic history. *Haininchelys curiosa* Schleich et al., 1988 (new oospecies).

Type material. BSPG uncat. (syntype series), eggshell fragments (Schleich et al. 1988, fig. 4a–h).

Type locality. Hainin, Hainaut Province, Belgium; Upper Paleocene (Schleich et al. 1988).

Diagnosis. Eggshell of the taxon *Haininchelys curiosa* is diagnosed by a shell thickness of about 0.25–0.30 mm, a height:width ratio of 1.2:1 to 2.3:1, and primary spherites diameter of 0.02–0.04 mm.

Comments. The microstructure of *Haininchelys curiosa* appears similar to *Testudoolithus rigidus*; however, the pores are funnel-shape, as opposed to the simple, straight pores of *T. rigidus*. The external surface of each shell unit is rounded in *H. curiosa*, similar to the eggshell of *Emydura subglobosa*, which may indicate that a pleurodire produced this type of egg.

Testudoolithus Hirsch, 1996

Type oospecies. *Testudoolithus rigidus* Hirsch, 1996.

Diagnosis. Eggshell of the taxon *Testudoolithus* is diagnosed by spheroidal egg shape and an eggshell thickness between 0.2–0.8 mm.

Testudoolithus hirschi Kohring, 1999a

Taxonomic history. *Testudoolithus hirschi* Kohring, 1999a (new oospecies).

Type material. FUB uncat. eggshell fragments (Kohring 1990a, fig. 1a–f; Kohring 1999a, fig. 62a–c).

Type locality. KM 11 locality, Guimarota Coal Mine, Leiria District, Portugal; Alcobaça Formation, Kimmeridgian, Upper Jurassic (Kohring 1999a).

Diagnosis. Eggshell of the taxon *Testudoolithus hirschi* is diagnosed by slender shell units with a height:width ratio of 3:1 and a shell thickness of 0.15 mm. *Testudoolithus hirschi* can be distinguished from other *Testudoolithus* ootaxa by the extremely thin eggshell.

Comments. Kohring (1999a) did not assign *Testudoolithus hirschi* to a turtle taxon. The slender shell units of *T. hirschi* are similar to those of some kinosternid and geoemydid turtles and may indicate that this oospecies was produced by a cryptodire. Note that the

locality KM 11 refers to the eleventh stratigraphic layer at the Guimarota Coal Mine, which is dominated by limestone and marls (Helmdack 1971).

Testudoolithus jiangi (Fang et al., 2003)

Taxonomic history. *Tiantaioolithus jiangi* Fang et al., 2003 (new oospecies); *Testudoolithus jiangi* Jackson et al., 2008 (new combination).

Type material. ZMNH M8713 (syntype series), a clutch of 23 eggs (Fang et al. 2003, figs. 7–9 pl. 2; Jackson et al. 2008, fig. 2a–c; Jackson et al. 2009, fig. 2)

Type locality. Shuantang Village, Tiantai County, Zhejiang Province, China; Liangtoutang Formation, Albian, Early Cretaceous (Jackson et al. 2008).

Referred material and range. TM 006, Upper Cretaceous, Chichengshan Formation, Shuangtang, Zhejiang Province, China (Jackson et al. 2008).

Diagnosis. Eggshell of the taxon *Testudoolithus jiangi* is diagnosed by spherical eggs approximately 35–52 mm in diameter, a shell thickness of 0.7–1.0 mm, and a shell unit height:width ratio of 2.5:1 to 3:1. *Testudoolithus jiangi* can be distinguished from other *Testudoolithus* ootaxa by an eggshell thickness that is three or more times thicker.

Comments. Fang et al. (2003) erected a new oofamily Testudoolithidae for these specimens but this taxon had previously already been named by Hirsch (1996). This lead Jackson et al. (2008) to propose that Testudoolithidae Fang et al. 2003 is a junior homonym of Testudoolithidae Hirsch, 1996 and that *Tiantaioolithus* Fang et al. 2003 is a junior subjective synonym of *Testudoolithus* Hirsch, 1996. The microstructure differs from *Testudoolithus hirschi* and therefore Jackson et al. (2008) proposed a new combination, *Testudoolithus jiangi*, for the specimens. Jackson et al. (2008) compared *Testudoolithus jiangi* to extant tortoise eggs but did not suggest that a tortoise produced these eggs because of there Cretaceous age. It is likely that the similarity between *Testudoolithus jiangi* and some extant tortoises is due to similar ecology (Jackson et al. 2008).

Testudoolithus rigidus Hirsch, 1996

Taxonomic history. *Testudoolithus rigidus* Hirsch, 1996 (new oospecies).

Type material. UCM 55806/HEC 425 (holotype), half of an egg with well-preserved eggshell (Hirsch 1996, fig. 1a–c).

Type locality. UCM L 87086, North of Stoneham, Weld County, Colorado, USA; Ogallala Formation, Miocene (Hirsch 1996).

Referred material and range. BMNH 47208, Lower Cretaceous, Gault Formation, Folkstone, England (Hirsch 1983); CMNH AL 108, Pliocene, Sidi Hakoma Member of the Hadar Formation, Hominid Site, Afar, Ethiopia (Hirsch 1983); MLP uncat., Pliocene, Calcarenites, Gran Canaria (Hirsch and López-Jurado 1987); MNHN-Mr. Pascal 1871-222, Miocene, Saint-Gérand-le-Puy, Department of Allier, France (Kohring 1993).

Diagnosis. Eggshell of the taxon *Testudoolithus rigidus* is diagnosed by shell unit height:width ratio of about 2:1, eggshell thickness 0.22–0.24 mm, and spheroidal eggs (42 mm x 47 mm). *Testudoolithus rigidus* is distinguished from *T. hirschi* by a smaller height:width ration and differs from *T. jiangi* by eggshell that is a third the thickness.

Comments. Straelen (1928) described a fossil turtle egg from the late Early Cretaceous (Albian; Gale et al. 1996) of England and Hirsch (1983) confirmed this identification. Later, this specimen was assigned to the oospecies *Testudoolithus rigidus* Hirsch, 1996. Hirsch (1996) compared the microstructure of *Testudoolithus rigidus* with that of the extant taxa *Gopherus polyphemus* and *Chelonoidis carbonaria* and determined that *T. rigidus* is more similar to *Gopherus polyphemus* and the eggshell thickness is thinner than *Chelonoidis carbonaria.* Mueller-Töwe et al. (2011) describe five turtle eggs and multiple eggshell fragments from the Pliocene Apolakkia Formation of Rhodes, Greece. Despite some diagenetic alteration, the eggshell microstructure allowed assignment to the oospecies *Testudoolithus rigidus*. Mueller-Töwe et al. (2011) suggest that the eggs where laid by a giant land tortoise.

Invalid and Problematic Ootaxa

Emydidarum ovum Schleich and Kästle 1988

nomen nudum

Material. BSPG 1952 II, 270 isolated eggs and eggshell fragments (Schleich and Kästle 1988, pl. 42. 1–8).

Locality. Gaimersheim, Germany; Chattium, Upper Oligocene (Schleich and Kästle 1988).

Comments. Schleich and Kästle (1988) propose a classification system and zoological nomenclature for fossil eggshell of geckos, lizards, snakes, crocodilians, turtles, and tortoises. However, their system and nomenclature were never widely accepted. Schleich and Kästle (1988) suggested *Emydidarum ovum* as the name for fossil turtle eggs (presumably for emydid eggs) that were difficult to systematically classify. A formal diagnosis and type specimen, however, were never published and *Emydidarum ovum* is therefore herein interpreted as a nomen nudum.

Testudinarum ovum Schleich and Kästle 1988

nomen nudum

Material. Eggshell fragments (Schleich and Kästle 1988, pl. 44. 3–8, pl. 45. 1–8; Schleich et al. 1988, fig. 2-3).

Locality. Pöttmes and Weidorf, Bavaria, Germany; Aragonium, Middle Miocene (Schleich and Kästle 1988).

Comments. See comments above for *Emydidarum ovum* Schleich and Kästle (1988). Schleich and Kästle (1988) suggested the name *Testudinarum ovum* for fossil turtle eggs that were difficult to systematically classify. A formal diagnosis and type specimen were never published, however, and *Testudinarum ovum* is therefore interpreted herein as a nomen nudum. Interestingly, one of these specimens shows purported evidence of a cuticle layer on the exterior eggshell surface (Schleich and Kästle 1988), a feature typical of some avian eggshell.

Testudoolithus magnirigidus Zelenitsky 1995

nomen nudum

Material. TMP 94.16.1A, eggshell fragments (Zelenitsky 1995, plate 8. 1–6, plate 9. 1–6, plate 10. 1–2).

Locality. Wann's Hill Locality, southern Alberta, Canada; Oldman Formation, Campanian, Late Cretaceous (Zelenitsky 1995, fig. 2.1).

Comments. The *in situ* clutch described by Zelenitsky et al. (2008) and McGee (2012) were referred to the name *Testudoolithus magnirigidus.* Eggshell of this oospecies is thicker than *T. rigidus* but comparable in eggshell thickness to *T. jiangi.* However, the shell unit comprising *T. magnirigidus* display a domed external surface and 'lateral feathering' (Zelenitsky et al. 2008), both of which are absent in *T. jiangi* (McGee 2012). Zelenitsky et al. (2008) compared the structure of the eggs from the *in situ* clutch to eggs discovered inside gravid *Adocus* specimens from Alberta and Utah. They concluded that *T. magnirigidus* can be confidently identified as the eggs of an *Adocus* species.

Zelenitsky (1995) named the taxon *Testudoolithus magnirigidus* in her unpublished master's thesis. According to Article 8.1.3 of the fourth edition of the International Code of Zoological Nomenclature (ICZN), species names are only valid if "produced in an edition containing simultaneously obtainable copies by a method that assures numerous identical and durable copies." An unpublished master's thesis does not meet these criteria because they are not widely distributed and therefore *T. magnirigidus* is not a valid taxon according to the ICZN. We believe that *T. magnirigidus* should be formally described and thereby made available. Such validation is important because a high level of homoplasy in turtle eggs (as was documented in the Cladistic Analysis section above) suggests that several different turtle taxa could produce eggs with similar

microstructure as *T. magnirigidus.* Therefore, specimens that are not associated with embryos or gravid females might be incorrectly interpreted as *Adocus* eggs. Additionally, validation would facilitate future comparisons with new specimens.

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Appendix 1

Institutional Abbreviations

BMNH The Natural History Museum, London, United Kingdom

BSPG Bayerische Staatssammlung für Paläontologie and Geologie, Munich, Germany.

CMNH Cleveland Museum of Natural History, Cleveland, Ohio

FUB Institut für Paläontologie, Freie Universität Berlin, Berlin, Germany.

HEC Hirsch Eggshell Catalogue, University of Colorado, Boulder, Colorado

IVPP Institute of Vertebrate Paleontology and Paleoanthropology, Beijing, China

MCZ Museum of Comparative Zoology, Cambridge, Massachusetts

MLP Museum Las Palmas, Gran Canaria, Canary Islands.

MNHN Muséum National d'Histoire Naturelle, Laboratoire de Paléontologie, Paris, France

TM Tiantai Prefecture Museum, Tiantai, Zhejiang Province, China

TMP Royal Tyrrell Museum of Palaentology, Alberta, Canada

UCM University of Colorado Museum, Boulder, Colorado

ZMNH Zhejiang Museum of Natural History, Hangzhou, Zhejiang Province, China

Appendix 2

Named Turtle Oogenera

Chelonoolithus Kohring, 1998a (type oospecies: *Chelonoolithus braemi* Kohring, 1998a)

Emydidarum Schleich and Kästle, 1988 (type oospecies: *Emydidarum ovum* Schleich and Kästle, 1988)

Emydoolithus Wang et al., 2013 (type oospecies: *Emydoolithus laiyangensis* Wang et al., 2013)

Haininchelys Schleich et al., 1988 (type oospecies: *Haininchelys curiosa* Schleich et al., 1988)

Oolithes Buckman, 1859 (type oospecies: *Oolithes bathonicae* Buckman, 1859)

Testudinarum Schleich and Kästle, 1988 (type oospecies: *Testudinarum ovum* Schleich and Kästle, 1988)

Testudinidovum Schleich and Kästle, 1988 (nomen nudum, lacking diagnosis and type species)

Testudinovum Schleich and Kästle, 1988 (nomen nudum, lacking diagnosis and type species)

Testudoflexoolithus Hirsch, 1996 (type oospecies: *Testudoflexoolithus bathonicae* [Buckman, 1859])

Testudoolithus Hirsch, 1996 (type oospecies: *Testudoolithus rigidus* Hirsch, 1996)

Tiantaioolithus Fang et al., 2003 (type oospecies: *Tiantaioolithus jiangi* Fang et al. 2003)

Appendix 3

Biogeographical Summary of Fossil Turtle Eggs, Embryos, Nests, and Copulating Pairs

Numbers in brackets reference Figures 2, 3, and 4.

Australia

[1] Pleistocene; Ned's Beach Formation; Lord Howe Island; *Meiolania platyceps*; eggs and clutches (Anderson 1925; Gaffney 1996)

Comments. Numerous eggs and clutches associated with bones of *Meiolania platyceps* occur in the Pleistocene Ned's Beach Formation of Lord Howe Island, Australia and have been assigned to this taxon (Anderson 1925; Gaffney 1996). This taxonomic assignment seems plausible because the eggs are quite large, approximately 9 cm in diameter (Gaffney 1996). It is also plausible that various sea turtle (Chelonioidea) laid their eggs at some of these localities but no extant chelonioid turtle is known to lay eggs of this size (Ernst and Barbour 1989). *Meiolania platyceps* otherwise represents the only taxon known from Lord Howe Island that was capable of laying eggs of this size. However, these eggs remain undescribed and lack embryonic remains, and therefore, this identification remains tentative for the moment.

Belgium

[2] Late Paleocene; Hainaut Province; *Haininchelys curiosa*; eggs (Schleich et al. 1988)

Brazil

[3] Late Cretaceous (Turonian-Santonian); Adamantina Formation; São Paulo State; ?*Podocnemis*; egg with a possible embryo (Azevedo et al. 2000)

Comments. Azevedo et al. (2000) describe a possible turtle egg and embryo from the Late Cretaceous (Turonian-Santonian) Adamantina Formation of Brazil (Nava and Martinelli 2011). Additionally, the authors tentatively assign the egg to the genus *Podocnemis*. However, the study only included examination of structural features of the outer egg surface and low quality CT scans for embryo identification. Confirmation of their results requires identification of the aragonite eggshell composition and mechanical preparation or use of synchrotron tomography for detailed description of the embryo. It is unlikely that future analysis will confirm the taxonomic attribution of this material to crown group *Podocnemis* because this clade did not diversify until the Tertiary. However, it is plausible that this egg is attributable to the clade Podocnemididae as this taxon is known to have Cretaceous representatives (Gaffney et al. 2011).

Canada

[4] Late Cretaceous, late Campanian; Dinosaur Park Formation; Alberta; *Basilemys variolosa* and *Testudoolithus magnirigidus/Adocus* sp; gravid turtles (Braman and Brinkman 2009; Zelenitsky et al. 2008)

[5] Late Cretaceous, late Campanian; Oldman Formation; Alberta; *Testudoolithus magnirigidus*/*Adocus* sp.; clutch of eggs with embryos (Zelenitsky 1995; Zelenitsky et al. 2008; McGee 2012)

Comments. McGee (2012) provides a detailed description of the morphology of the embryonic remains from a clutch of turtle eggs from the late Campanian Oldman Formation of Alberta using computer tomography. She concurs with Zelenitsky et al.'s (2008) referral to *Adocus* sp. and also concludes that the embryos died at a late stage of development. Additionally, an undescribed specimen of *Basilemys variolosa* from the Upper Cretaceous (late Campanian) Dinosaur Park Formation on display at the Royal Ontario Museum was discovered to contain eggs when the carapace was accidently broken (Braman and Brinkman 2009).

Canary Islands

[6] Miocene and Pliocene; Calcarenites; ?*Geochelone burchardi*; Fuerteventura; eggs (Rothe and Klemmer 1991)

[7] Pliocene; Calcarenites; *Testudoolithus rigidus*/?*Geochelone*; Gran Canaria; egg (Macau-Vilar 1958; López-Jurado 1985; Hirsch and López-Jurado 1987) NOTE: Specimen was subsequently lost.

[8] Tertiary; Calcarenites; ?*Geochelone*; Lanzarote and Fuerteventura; eggs (Hutterer et al. 1997)

[9] Miocene; Calcarenites; Lanzarote; eggs (García-Talavera 1990)

Comments. Fossil turtle eggs have been reported from Miocene and Pliocene deposits from Fuerteventura, Lanzarote, and Gran Canaria, Canary Islands. All of these eggs were assigned to the genus *Geochelone* because of their similarity to eggs of modern *Geochelone* species (Hutterer et al. 1997). However, the eggs from Fuerteventura were most likely laid by *G. burchardi*, the only species of testudinid turtle, fossil or modern known from this island (Rothe and Klemmer 1991).

Cape Verde

[10] Miocene; Maio; eggs (Bebiano 1932)

Comments. Bebiano (1932) reports eight turtle eggs from the Miocene of the Republic of Cape Verde. However, this identification was made using the egg shape and the fact that they were found in embedded in calcareous sands. Further investigation is needed to confirm this identification.

China

[11] Early Cretaceous, Albian and Late Cretaceous, Cenomanian-Turonian; Liangtoutang and Chichengshan formations, respectively; Zhejiang Province; *Testudoolithus jiangi*; egg and clutch (Fang et al. 2003; Jackson et al. 2008; Jin 2009)

[12] Late Cretaceous; Nanchao Formation; ?emydid; eggs with embryos (Cohen et al. 1995)

[13] Cretaceous; Henan Province; egg with embryo (Fang et al. 2009)

[14] Late Cretaceous; Jingangkou Formation; Shandong Province; *Emydoolithus laiyangensis*; egg (Wang et al. 2013)

Comments. Yabe and Ozaki (1929) describe purported fossil turtle eggs from the Cretaceous Tsuantou Formation (Zhang 2009) of Liaoning Province, China. This identification was based on the spheroidal egg shape rather than microstructural features of the eggshell. Wang et al. (2013) recently identified these specimens as those of dinosaurs but did not assign them to a specific oospecies.

Turtle eggs with embryos are also known from the Upper Cretaceous Nanchao Formation of China. These specimens are hypothesized to belong to an emydid turtle; however Cohen et al. (1995) failed to provide a formal description of this material. If this identification proves correct, this specimen would push the fossil record of emydid turtles back 40 million years (Cohen et al. 1995), which appears unlikely considering recent molecular calibration studies (Joyce et al. 2013). Additionally, a turtle egg with a purported embryo was discovered in Xixia County, Henan Province, China (Fang et al. 2009, fig. 1).

Czech Republic

[15] Early Miocene; *Testudoolithus*; eggshell (Kohring 1998b; 1999b)

Comments. Kohring (1998b; 1999b) reports *Testudoolithus* eggshell from the Early Miocene of the Czech Republic. One of these specimens exhibits pathological multi-layered eggshell.

England

[16] Early Cretaceous, Albian; Gault Formation; Kent; *Testudoolithus rigidus*; egg (Straelen 1928; Hirsch 1983; 1996)

[17] Middle Jurassic, Bathonian; White Limestone Formation; Gloucenstershire; *Testudoflexoolithus bathonicae*; eggs (Buckman 1859; Hirsch 1996; Cox et al. 1999)

[18] Middle Jurassic, Bathonian; White Limestone Formation; Gloucenstershire; eggs (Carruthers 1871)

Comments. In addition to the specimens described by Buckman (1859), Carruthers (1871) also describes fossil eggs from the Great Oolite and agrees with Buckman that the eggs were reptilian, but suggests that turtles or pterosaurs laid these eggs. He named two new ootaxa, *Oolithus sphaericus* for specimens from Stonesfield, England and *O. obtusatus* for a single egg from the Isle of Wight. However, Carpenter and Alf (1994) suggest that *O. sphaericus* are actually dinosaur eggs and Mikhailov et al. (1996) categorized *O. sphaericus* and *O. obtusatus* as Incertae sedis. Further study of these specimens is required to determine their true taxonomic identity. Ensom (1997) describes numerous eggshell fragments and discusses their similarity to turtle eggshell described by Kohring (1990b), but ultimately rejects this identification in favor of megaloolithid eggshell, typically assigned to sauropod dinosaurs (Ensom 2002).

Ethiopia

[19] Pliocene; Hadar Formation; Afar; *Testudoolithus rigidus*; eggs (Hirsch 1983)

Comments. Hirsch (1983) discusses two partially and one completely altered turtle eggshell from the Pliocene Hadar Formation (Hominid Site) in the Afar region of Ethiopia. He determines that although aragonite may be completely replaced by calcite, the eggshell can be confidently identified as that of a turtle using polarized light and scanning electron microscopy (Hirsch 1983), presumably because specimens preserve a pseudomorph of the original aragonite structure.

France

[20] Early Cretaceous, late Albian; ?Formation of Salazac; Department of Gard; egg (Masse 1989)

[21] Late Cretaceous; Testudoolithidae; Department of Bouches-du-Rhône; eggshell (Garcia 2000)

[22] Early Miocene; Department of Allier; *Testudoolithus rigidus*/?*Cheirogaster sp*.; eggs (Kohring 1993)

[23] Eocene; Department of Bas-Rhin; eggs (Kuntz 1981)

[24] Tertiary; Department of Lot-et-Garonne; eggs (Brunet 1838)

Comments. Masse (1989) discusses one complete and one partial pliable turtle egg from the Early Cretaceous (late Albian) of France. These specimens are diagenetically altered but the radiating crystals suggest that they were originally composed of aragonite. Garcia (2000) also describes several eggshell fragments from the Late Cretaceous of France and assigns the specimens to Testudoolithidae. Kohring (1993) reports six turtle eggs from the Early Miocene of France and assigns them to *Cheirogaster* sp. This identification is based on size and shape of the eggs and not microstructural features. Kuntz (1981) mentions six oval and smooth turtle eggs from the Eocene of France. Brunet (1838) reports three turtle eggs, measuring approximately 6.0 cm x 2.5 cm from the Tertiary of the Gironde basin.

Germany

[25] Oligocene and Miocene; *Testudinarum ovum*; State of Bavaria; eggs (Schleich and Kästle 1988)

[26] Jurassic; Solnhofen; State of Bavaria; ?Eurysternid; gravid turtle (Joyce and Zelenitsky 2002)

[27] Eocene; Messel; State of Hesse; *Allaeochelys crossesculpta*; copulating pair (Joyce et al. 2012)

[28] Tertiary; *Trionyx gergensi* or *Chelonia mydas*; State of Rhineland-Palantinate; egg (Gergens 1860; Meyer 1860; Hummel 1929)

[29] Pleistocene; State of Saxony-Anhalt; ?*Emys orbicularis*; eggs and embryo (Hemprich 1932)

Comments. Joyce and Zelenitsky (2002) describe a gravid eurysternid turtle from a Late Jurassic limestone concretion discovered near the village of Schamhaupten, Germany. After initial preparation, the concretion was cut into four sections to determine if it was fossiliferous, revealing four circular egg-like objects within a turtle shell. However, investigation of the microstructure revealed complete diagenetic alteration of the eggshell. Further, the eggshell now appears to be

inside out (e.g. the base of the shell units are on the exterior of the eggshell) and the exact mechanism for such preservation remains unknown. The authors conclude that these eggs must be referred to as pseudomorphs because no details of the eggshell microstructure can be ascertained (Joyce and Zelenitsky 2002). Hemprich (1932) discusses turtle eggs (one containing a possible embryo) from the Pleistocene of Germany and assigns them to *Emys orbicularis*; however, this assignment seems to be based on their similarity to the eggs of this extant species and not eggshell microstructure or embryonic remains. Therefore, a detailed systematic description of these specimens is needed. Meyer (1860) mentions turtle eggs from the Tertiary of Mainz, Germany and suggests that they belong to *Trionyx gergensi*, based on egg size and shape. In contrast, Gergens (1860) suggests that these eggs belong to *Chelonia mydas*. Later, Hummel (1929) mentions the same specimens and agrees with Meyer (1860) that they were laid by *Trionyx gergensi*; however, the author does not give his reasoning for this assignment.

Preservation of paired turtle specimens include numerous examples of *Allaeochelys crassesculpta* from the Eocene Messel Pit in Germany. However, Joyce et al. (2012) provides the first definitive evidence that these turtles died while copulating. The authors inferred the gender of the individuals by using body size and tail length (i.e., males are smaller, have longer tails, and lack plastral kinesis). Additional confirmation that these turtles died in the act of mating includes their in-line position and the presence of interlocking tails in some specimens (Joyce et al. 2012). The authors hypothesize that once the male mounted the female, they sank to the bottom of the lake where anoxic conditions resulted in their demise. This is further supported by the fact that some extant aquatic turtles “freeze” while mating and sink to the bottom of open bodies of water (Joyce et al. 2012).

Greece

[30] Pliocene; Apolakkia Formation; Rhodes; *Testudolithus rigidus*/?Giant land tortoises; eggs (Mueller-Töwe et al. 2011)

[31] Late Miocene; Central Macedonia; eggs (Del Campana 1919)

Comments. Del Campana (1919) describes six fossil turtle eggs from the Upper Miocene of Thessaloniki (formerly Salonica), Greece and suggests that these eggs are those of a sea turtle because they occurred in marine sediments. However, microstructural analysis of the eggshell was not preformed.

India

[32] Late Cretaceous; State of Tamil Nadu; egg (Sahni 1957)

[33] Late Cretaceous, Maastrichtian; Lameta Formation; State of Maharashtra; ?Pelomedusid; eggshell and partial clutch (Mohabey 1996; 1998)

Comments. Sahni (1957) reports the first fossil egg from India. This elongate egg measures 49 mm wide x 27 mm long; however, this identification is based on gross morphology and not microstructural features. Further research is necessary to confirm its turtle affinities. Mohabey (1998) reports a partial turtle nest and eggshells from the Lower Cretaceous (Maastrichtian) Lamenta Formation of India and mentions turtle eggs from the Pleistocene of the Narmada Valley. However, Kohring (1999a) questions the turtle origin of the Cretaceous specimens because of the calcite mineralogy and poor quality of the photographs. Additionally, Mohabey (1998) identified a mammillary layer at the basal most portion of the eggshell. This feature is characteristic of avian and non-avian theropods, therefore, assignment of these specimens to a turtle remains questionable (Jackson et al. 2008; Knell et al. 2011) and further analysis is needed.

Japan

[34] Early Cretaceous; Kuwajima Formation; Ishikawa Prefecture; eggshell (Isaji et al. 2006)

[35] Late Cretaceous (Coniacian); Middle and Upper Yezo Group; Hokkaido; ?Protostegid; egg (Fukuda and Obata 1991)

[36] Late Cretaceous (Coniacian); Hokkaido; ?*Chelonia* and ?*Trionychid;* eggs (Obata et al. 1972)

Comments. Isaji et al. (2006) describe turtle eggshells from the Lower Cretaceous Kuwajima Formation of central Japan and suggest that these specimens were laid by an unidentified aquatic turtle. Fukuda and Obata (1991) and Obata et al. (1972) report on turtle eggs from the Late Cretaceous (Turonian-Coniacian) of Hokkaido, Japan. Both specimens preserved two superimposed layers that are thought to represent secondary diagenetic deposits. Additionally, the specimens described by Isaji et al. (2006) also appear to be diagenetically altered because aragonite was not found after staining the thin sections; however, the turtle origin of these specimens are supported by the radiating crystallite in the shell units.

Madagascar

[37] Late Cretaceous (Campanian); Menabe Region; eggs (Lawver 2013)

Comments. Lawver (2013) describes three associated spherical eggs from the Late Cretaceous (Campanian) Morondava basin, western Madagascar and assigns them to *Testudoolithus* oosp. Two of the eggs lack evidence of eggshell in hand sample, and their spherical morphology results from infilling of the eggs by sediment prior to erosion of the eggshell. The third egg preserves eggshell on approximately half of the specimen. Thin sections of the eggshell reveal substantial diagenetic alteration of the original aragonite. However, portions of the lower two-thirds of the eggshell preserve original crystalline structure, thus allowing definitive assignment to Testudines (Lawver 2013). Bright red to orange-red color under cathodoluminescence, suggests replacement of the original aragonite by calcite (Lawver 2013).

Mongolia

[38] Late Cretaceous; Ömnögovi Province; egg with embryo (Mikhailov et al. 1994)

Ologoy-Ulan-Tsav locality of Mongolia. These specimens are important because at least one egg contains embryonic remains, which require further research.

Portugal

[39] Late Jurassic, early Kimmeridgian; Alcobaça Formation; Leiria District; *Testudoolithus hirschi* and *Chelonoolithus braemi*; egg and eggshell (Kohring 1990a, c; 1998a; 1999; 2000)

Comments. Kohring (1990a) reports two different types of turtle eggshell from the Upper Jurassic (early Kimmeridgian) Guimarota mine of Portugal. The author suggests that the specimens belong to cryptodiran turtles because only these turtles are known from the Guimarota mine.

South Korea

[40] Late Cretaceous, Campanian; Seonso Formation; South Jeolla Province; eggs (Huh and Zelenitsky 2002; Lee 2003)

[41] Early Cretaceous, Albian; Jindong Formation; South Gyeongsang Pronvince; eggs (Lee 2003)

[42] Late Cretaceous; Goseong Formation; South Gyeongsang Pronvince; eggs (Yang et al. 2006; Paik et al. 2012)

Comments. Huh and Zelenitsky (2002) report fossil eggs from the Upper Cretaceous (Campanian, Kim et al. 2008) Seonso Formation of South Korea and tentatively identify a partial egg and eggshell fragments as those of a turtle. This identification is based on the small size, eggshell thickness, and smooth outer surface; however, no microstructural features are preserved because the eggshell is completely altered to sparry calcite. Lee (2003) provides limited locality data for these specimens, as well as for undescribed fossil turtle eggs from the late Early Cretaceous (Albian) Jindong Formation of South Korea.

Spain

[43] Pleistocene; *Testudoolithus*; Formentera; eggs (Filella-Subirà et al. 1999)

[44] Early Cretaceous, early Barremian; El Castellar Formation; Teruel Pronvince; *Testudoolithus*, *Testudoflexoolithus*/?Batagurinae, Testudoid, and others; eggshell (Kohring 1990b; Ruiz-Omeñaca et al. 2004)

[45] Early Cretaceous, late Barremian; Cuenca Province; *Testudoolithus*; eggshell (Buscalioni et al. 2008)

[46] Early Cretaceous, Valanginian-Hauterivian; Villanueva de Huerva Formation; Aragon; Testudoolithidae; eggshell (Moreno-Azanza et al. 2008; 2009)

[47] Early Cretaceous, late Berriasian; Leza Formation; La Rioja Province; Testudoolithidae; eggshell (Moreno-Azanza et al. 2013b)

Comments. Kohring (1990b) describes three types of eggshell fragments from the Early Cretaceous (lower Barremian) of Galve, Spain. His Type A eggshell was attributed to a semi-aquatic turtle because its shell unit height:width ratio is 1:1 (Kohring 1990b). The type B eggshell exhibits very large pores that are surrounded by smaller pores, which may be an artifact of diagenesis. Kohring (1990b) suggests that this eggshell type belongs to a batagurid turtle. Type C eggshell was assigned to tortoises because of similar eggshell microstructure (Kohring 1990b). Filella-Subirà et al. (1999) describe a partial clutch of six *Testudoolithus* eggs from the Pleistocene of the Balearic Islands and attributed them to a large tortoise. They calculated that the nest chamber measured 13.5 cm long by 14.5 cm wide and 11 cm high and occurred between 25 and 27 cm deep. They suggest a maximum length of 78.9 cm for the adult female (Filella-Subirà et al. 1999). Buscalioni et al. (2008) briefly describe turtle eggshell fragments from the Early Cretaceous (Upper Barremian) of Spain, which can likely be assigned to *Testudoolithus* based on the aragonite crystalline structure in their figure 10a.

Moreno-Azanza et al. (2008) describe eggshell fragments from the Early Cretaceous (Valanginian-Hauterivian) of Spain and assign the specimens to Testudoolithidae indet. They suggest that a testudinid turtle laid the eggs; however, tortoises did not appear until after the Mesozoic. Moreno-

Azanza et al. (2009) also suggests that these turtle eggshells may represent a new *Testudoolithus* oospecies. Moreno-Azanza et al. (2013b) identify eggshell fragments from the Early Cretaceous (upper Berriasian) of northern Spain as Testudoolithidae and suggest that these specimens may belong to a new oospecies.

United States of America

[48] Late Jurassic, Tithonian; Morrison Formation; Colorado; egg (Bray and Hirsch 1998)

[49] Late Jurassic, Tithonian; Morrison Formation; Colorado; egg (Hirsch et al. 1987)

[50] Oligocene-Miocene; Ogallala Formation; Colorado; *Testudoolithus rigidus/?Stylemys*; egg (Hirsch 1996; Hirsch and Bray 1988)

[51] Late Cretaceous, Maastrichtian; Fox Hills Formation; Colorado; Sea turtle; nest (Bishop et al. 2011)

Comments. Turtle eggshells (Hirsch and Packard 1987; Hirsch et al. 1987) and possible pliable or soft-shelled eggs (Bray and Hirsch 1998) are reported from the Jurassic (late Kimmeridgian-Tithonian; Kowallis et al. 1991) Bushy Basin Member of the Morrison Formation of Colorado; however, this assignment requires additional research. Hirsch and Bray (1988) describe spherical eggs from the Oligocene-Miocene Ogallala Formation of Colorado and discuss how spheroidal owl eggs could be confused with those of some turtles. They determined that avian and turtle eggs cannot be distinguished by macrostructure alone. Further, they identify three of the four eggs as avian, based on their eggshell microstructure, but one may belong to a turtle (Bray and Hirsch 1998).

[52] Pleistocene; Florida Oolite; Florida; *Testudooflexoolithus agassizi*; egg (Hirsch 1996)

Comments. Agassiz discovered flexible turtle eggshell fragments in the Pleistocene Florida Oolite in the 1800s (Hirsch 1996) but they remained undescribed until 1996.

[53] Late Cretaceous, Campanian; Judith River Formation; Montana; eggs with embryos (Clouse 2001; Jackson and Schmitt 2008)

Comments. Clouse (2001) reports a weathered clutch of fossil turtle eggs from the Upper Cretaceous (Campanian) Judith River Formation of Montana. This clutch consists of at least 13 eggs, some of which contain embryonic bones. Jackson and Schmitt (2008) briefly described the eggshell microstructure and demonstrated that the clutch contained at least one pathological, multilayered egg.

[54] Late Cretaceous, Campanian; Fruitland Formation; New Mexico; *Testudoolithus sp*.; eggshell (Tanaka et al. 2011)

Comments. Tanaka et al. (2011) describe numerous isolated turtle eggshell fragments from the Upper Cretaceous (Campanian) Fruitland Formation of New Mexico. They identified the specimens as *Testudoolithus* oosp.

[55] Oligocene; Brule Formation; Nebraska; eggs (Hirsch 1983)

[56] Oligocene; White River Group; Nebraska, ?*Stylemys nebrascensis*; eggs (Hay 1908) NOTE: Specimens were subsequently lost.

[57] Oligocene; Nebraska and South Dakota; eggs (Hirsch and Packard 1987)

Comments. Hirsch (1983) describes at least four altered turtle eggs from the Oligocene Brule Formation of Nebraska. X-ray diffraction analysis shows complete replacement of aragonite by calcite; however, polarized light microscopy reveals the original crystal morphology. Hay (1908) mentions several turtle eggs from the Oligocene White River Group of Nebraska that he identifies as *Stylemys nebrascensis* because this taxon is commonly found in these deposits and few other species are known. This identification remains questionable because the specimens have since

been lost (Hirsch 1996). Additionally, Hirsch and Packard (1987) examined numerous spherical eggs from Nebraska and South Dakota and identified all but two as turtle eggs. The latter they identified as avian, most likely those of owls (Hirsch and Packard 1987).

[58] Late Pliocene; Texas; egg (Brattstrom 1961)

Comments. Brattstrom (1961) briefly mentions a fossil egg associated with skeletal elements from a tortoise in the Late Pliocene of Texas. This specimen measures 49.5 x 48.2 x 46.9 mm in size but has not been systematically described.

[59] Late Cretaceous, Campanian; Kaiparowits Formation; Utah; *Adocus sp*.; gravid turtle (Knell et al. 2011)

Comments. Knell et al. (2011) describe a gravid specimen of *Adocus* from the Upper Cretaceous (Campanian) Kaiparowits Formation of southern Utah. Compared to *Adocus* specimens from Alberta (Zelenitsky et al. 2008), the Utah eggs are smaller, have significantly thinner eggshell and the outer surface displays flat, rather than domed shell units. This likely occurred because the gravid female died at an earlier stage of egg development than the Canadian specimen (Knell et al. 2011).

Venezuela

[60] Late Miocene; Urumaco Formation; Falcón; ?*Bairdemys venezuelensis*; Eggshells (Winkler and Sánchez-Villagra 2006)

Comments. See Nests section above.

Appendix 4

Hierarchical Taxonomy of Turtle Ootaxa

Testudoflexoolithidae Hirsch, 1996

- *Testudoflexoolithus* Hirsch, 1996
  - *Testudoflexoolithus agassizi* Hirsch, 1996
  - *Testudoflexoolithus bathonicae* Buckman, 1859

Testudoolithidae Hirsch, 1996

- *Chelonoolithus* Kohring, 1998
  - *Chelonoolithus braemi* Kohring, 1998
- *Emydoolithus* Wang et al., 2013
  - *Emydoolithus laiyangensis* Wang et al., 2013
- *Haininchelys* Schleich et al., 1988
  - *Haininchelys curiosa* Schleich et al., 1988
- *Testudoolithus* Hirsch, 1996
  - *Testudoolithus hirschi* Kohring, 1999
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CHAPTER THREE

AN OCCURRENCE OF FOSSIL EGGS FROM THE MESOZOIC OF MADAGASCAR AND A DETAILED OBSERVATION OF EGGSHELL MICROSTRUCTURE

Contributions of Author and Co-Authors

Manuscript in Chapter 3

Author: Daniel R. Lawver

Contribution: Conceived and implemented the study design. Collected and analyzed data. Wrote first draft of the manuscript.

Co-Author: Armand H. Rasoamiaramanana

Contributions: Helped with repatriation of the specimens to Madagascar.

Co-Author: Ingmar Werneburg

Contributions: Collected and analyzed CT data and provided feedback on early drafts of the manuscript.

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CHAPTER THREE

AN OCCURRENCE OF FOSSIL EGGS FROM THE MESOZOIC OF MADAGASCAR AND A DETAILED OBSERVATION OF EGGSHELL MICROSTRUCTURE

DANIEL R. LAWVER,[*,1] ARMAND H. RASOAMIARAMANANA,[2] and INGMAR WERNEBURG[3,4]

[1]Department of Earth Sciences, Montana State University, Bozeman, Montana 59717 U.S.A., danlawver@gmail.com;

[2]Département de Paléontologie, Université d' Antananarivo, Antananarivo (101), Madagascar, armandras@yahoo.fr;

[3]Paläontologisches Institut und Museum der Universität Zürich, Karl Schmid Strasse 4, 8006 Zürich, Switzerland, i.werneburg@gmail.com;

[4]Museum für Naturkunde, Leibniz Institut für Evolutions und Biodiversitätsforschung an der Humboldt, Universität zu Berlin, Invalidenstraße 43, 10115 Berlin, Germany

*Corresponding author.

ABSTRACT—Whereas fossil turtle eggs have a near global distribution and range from Middle Jurassic to Pleistocene they are rarely documented from the Mesozoic of Gondwana. Here we report three fossil turtle eggs from the Upper Cretaceous (Campanian) of the Morondava Basin, Madagascar. The spherical eggs range in size from 33.5 mm to 35.5 mm and have an average eggshell thickness of 440 μm. They can be confidently identified as rigid-shelled turtle eggs by the presence of tightly packed shell units composed of radiating acicular crystals and a shell unit height-to-width ratio of 2:1. Lack of associated skeletal remains precludes taxonomic identification of the eggs. Although a large vertebrate fauna has been reported from the Late Cretaceous of Madagascar, these specimens are the first eggs from the Mesozoic of the island.

INTRODUCTION

Turtles are unique amongst amniotes because they lay eggs composed of aragonite instead of calcite. This composition is less stable and is more susceptible to diagenetic alteration (Hirsch, 1983), which may account for the relatively small number of fossil turtle eggs that have been described previously (reviewed by Lawver and Jackson, 2014). Although the vast majority of fossil turtle eggs are described from Laurasian continents, specimens are known from every continent except Antarctica (likely a collection bias) and their temporal distribution ranges from Middle Jurassic to Pleistocene (Lawver and Jackson, 2014).

Turtle eggshell consists of a single layer of shell units made up of needle-like aragonite crystals that originate from an organic core within the shell membrane. The degree of mineralization varies widely among turtle eggs and includes pliable, semi-pliable, and rigid-shelled eggs (Hirsch, 1983). This variation in eggshell mineralization corresponds to variation in the height to width ratios of the shell units and whether the shell units are loosely or tightly spaced and interlocking in structure (Hirsch, 1996). The tightly interlocking structure of rigid-shelled eggs enhances their potential for preservation in the fossil record (Hirsch 1983).

Numerous vertebrate taxa have been described from Upper Cretaceous rocks of Madagascar. This fauna includes: ray-finned fishes, anurans, turtles, snakes, lizards, crocodyliforms, non-avian and avian dinosaurs, and mammals (Krause and Kley, 2010). Although the vast majority of these fossils were collected from the Mahajanga Basin in

northwestern Madagascar, other basins are beginning to gain attention. Recently, an abelisauroid theropod was described from Upper Cretaceous rocks of the Diego Basin in the northern-most point of Madagascar (Farke and Sertich, 2013), as well as fishes, rhynchosaurs, aetosaurs, phytosaurs, dinosaurs, and traversodonts from the Triassic and Jurassic of the Morondava Basin (Burmeister et al., 2006 and references therein). Despite this rich fossil record, no Mesozoic eggs have been previously described from Madagascar. Here, we describe three spherical turtle eggs, referred to as the Malagasy eggs, from the Belo Region of the Morondava Basin of western Madagascar are described.

GEOLOGICAL BACKGROUND

The western region of Madagascar is characterized by three large sedimentary basins that resulted from rifting between Africa and Madagascar, which was initiated as early as the Permo-Triassic. These basins include the Diego and Mahajanga basins to the north and the larger Morondava Basin to the south (Fig. 1; Walaszczyk et al., 2014). During the Middle Jurassic, Madagascar drifted southward until reaching its current position by the Early Cretaceous (Walaszczyk et al., 2014) and by the Late Cretaceous (Turonian and Campanian) increased subsidence and marine transgression resulted in continuous open marine sedimentation in the Morondava Basin. Within the Morondava Basin, the rock units trend approximately north-south and range in age from Late

Carboniferous to Recent, with the oldest units to the east (Nichols and Daly, 1989; Burmeister et al., 2006).

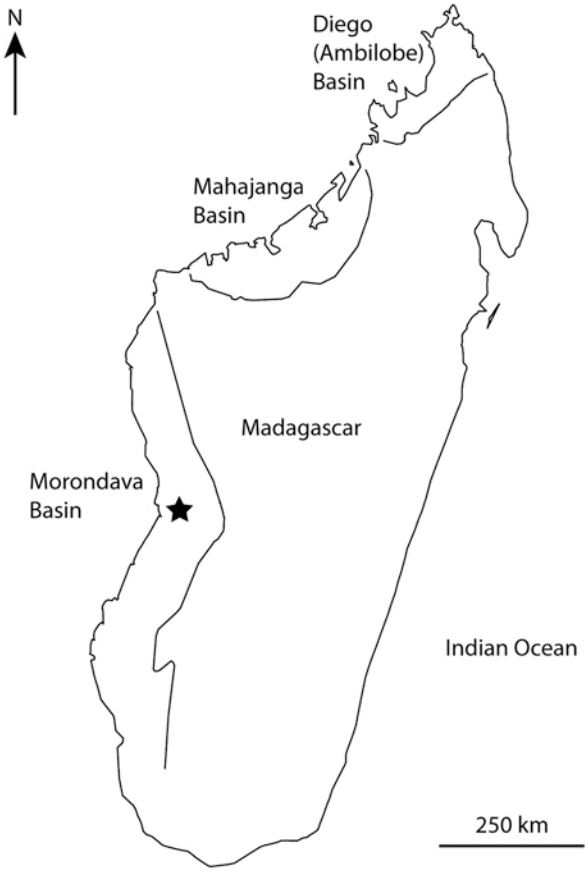


Figure 1. Map of Madagascar showing the general area in the Belo region in which the fossil turtle eggs were discovered, indicated by the star. Modified from Rogers et al. (2000).

The eggs in this study were discovered and collected by amateurs and lack exact locality information. However, all three eggs were found together and in association with scaphite ammonites and the heteromorph ammonite *Bostrichoceras* in marine rocks

suggesting that they come from the Campanian units of the Belo Region near the Tsiribinha River (Richter, 2008; 2010; Walaszczyk et al., 2014).

METHODS

All three eggs were molded and cast prior to removal of eggshell samples for scanning electron microscopy (SEM), cathodoluminescence (CL) analysis, and thin sectioning. Radial and tangential thin sections (30 μm thick) were made following standard procedures and examined under a Nikon Eclipse LV100POL light microscope. Eggshell fragments were mounted on an aluminum stud, coated with 10 nm of gold, and imaged under a JEOL JSM-6100 SEM at 10 kV. Images included the inner surface and radial views of the eggshell. A radial, thick section of eggshell was polished and examined with cathodoluminescence (CL) to assess potential diagenetic alteration. Photomicrographs were taken using a Nikon Digital Sight DS-5Mc camera and microstructural features were measured with the image analysis software ImageJ. Computed tomographic (CT) scans were performed with a GE Phoenix v | tome | x CT-scanner at the Anthropologisches Institut der Universität Tübingen, Germany with a resolution of 50 μm.

Institutional Abbreviations and Repatriation—The specimens are given a provisional collection number of the laboratory collection of Paläontologisches Institut und Museum der Universität Zürich, Switzerland (PIMUZ lab). Under the guidance of Armand H. Rasoamiaramanana, the specimens including thin sections and SEM samples

will be incorporated into the paleontological collection of the Département de Paléontologie at the Université d'Antananarivo in Madagascar. The artificial plaster casts of the eggs are housed at the Department of Earth Sciences, Montana State University, Bozeman MT and accessioned under the collection number VP-0951.

SYSTEMATIC PALEONTOLOGY

OOFAMILY TESTUDOOLITHIDAE Hirsch, 1996

OOGENUS *TESTUDOOLITHUS* Hirsch, 1996

TESTUDOOLITHUS oosp.

Material—PIMUZ lab#2012.IW30, complete egg; PIMUZ lab#2012.IW31 and PIMUZ lab#2012.IW32, natural casts of eggs.

Locality—Belo Region near the Tsiribinha River, Morondava Basin, Madagascar.

Description—Two of three spherical eggs, PIMUZ lab#2012.IW31 and PIMUZ lab#2012.IW32, lack eggshell and measure approximately 35.5 mm and 34.8 mm in diameter, respectively. These eggs are natural casts resulting from sediment infill prior to surface erosion.

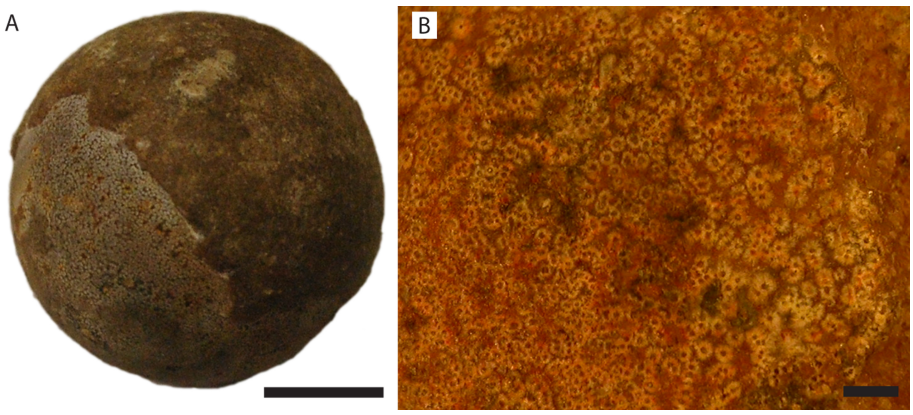


Figure 2. Fossil turtle egg (PIMUZ lab#2012.IW30). A, whole egg with eggshell preserved on approximately half of the specimen; B, close-up of infilling sediment preserving the basal-most portion of shell units and impressions of nuclei. Scale bars equal 1 cm (A) and 1 mm (B).

The third egg (PIMUZ lab#2012.IW30) measures approximately 33.5 mm in diameter (including eggshell) with smooth eggshell preserved on half of the specimen (Fig. 2). In places where the eggshell is missing or removed for sampling, the underlying sediment exhibits impressions of small, circular features that measure approximately 250–500 μm in diameter (Fig. 2B). These features likely represent the basal-most portions of the shell units that broke free while sampling the eggshell for thin section, CL and SEM analysis. This is evident by the fact that these features are made up of radiating crystals that originate from the impression left behind by the organic core. Additionally, the diameters of these circular features correspond to the diameters of the shell units within the eggshell.

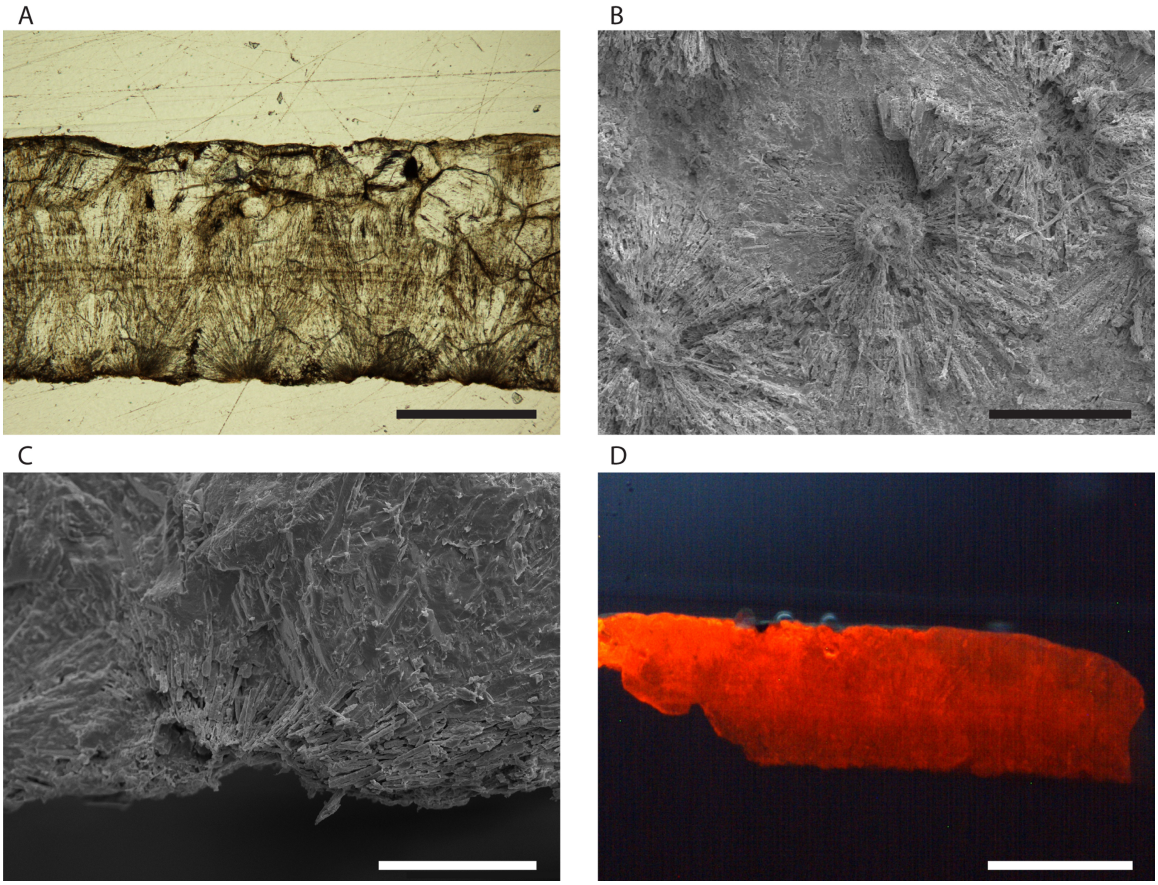


Figure 3. Photomicrographs of fossil eggshell (PIMUZ lab#2012.IW30). A, radial thin section in plane polarized light showing original crystalline morphology on the lower two-thirds of the specimen; B, SEM image of the inner surface showing a preserved neucleation site surrounded by radiating crystrals; C, SEM image in radial view showing needle-like crystals radiating from a nucleation site; D, cathodoluminescense showing red to orange-red fluorescence suggesting calcite or dolomite replacement of the original aragonite composition. Scale bars equal 250 µm (A), 100 µm (B), 50 µm (C), and 150 µm (D).

Eggshell is 440 µm thick with shell units that are tightly packed and have a height to width ratio of 2:1 (Fig. 3A). Pores were not identified in any of the radial sections. The permineralized organic core at the inner shell surface measures 52 µm in diameter (Fig. 3B-C). The lower two-thirds of the eggshell exhibits tight, interlocking acicular crystals that radiate from the former organic core (Fig. 3A-C). Cathodoluminescence imaging

reveals bright orange to orange-red fluorescence over the entire sample (Fig. 3D) and under cross-polarized light, the eggshell displays a blocky extinction pattern (Fig. 4).

DISCUSSION

The nearly spherical, intact eggs suggests that embryos failed to hatch but computer tomographic imaging of PIMUZ lab#2012.IW30 provides no evidence of embryonic remains inside the egg. This suggests that the eggs were either infertile, termination of embryonic development occurred prior to ossification of the skeleton, or the bones failed to preserve.

With the exception of turtles, all other amniotes lay eggs composed of calcite, which has a more tabular microstructure (Mikhailov, 1991). The presence of tightly interlocking, acicular crystals in the lower two-thirds of the eggshell (Fig. 3A) is consistent with the aragonite microstructure of turtle eggshell. Therefore, the eggshell microstructure of the Malagasy eggs clearly suggests assignment to Testudines or their stem lineage.

Comparisons

Hirsch (1996) proposed a parataxonomic classification for fossil eggshells that recognized the oofamilies Testudoolithidae and Testudoflexoolithidae; these categories

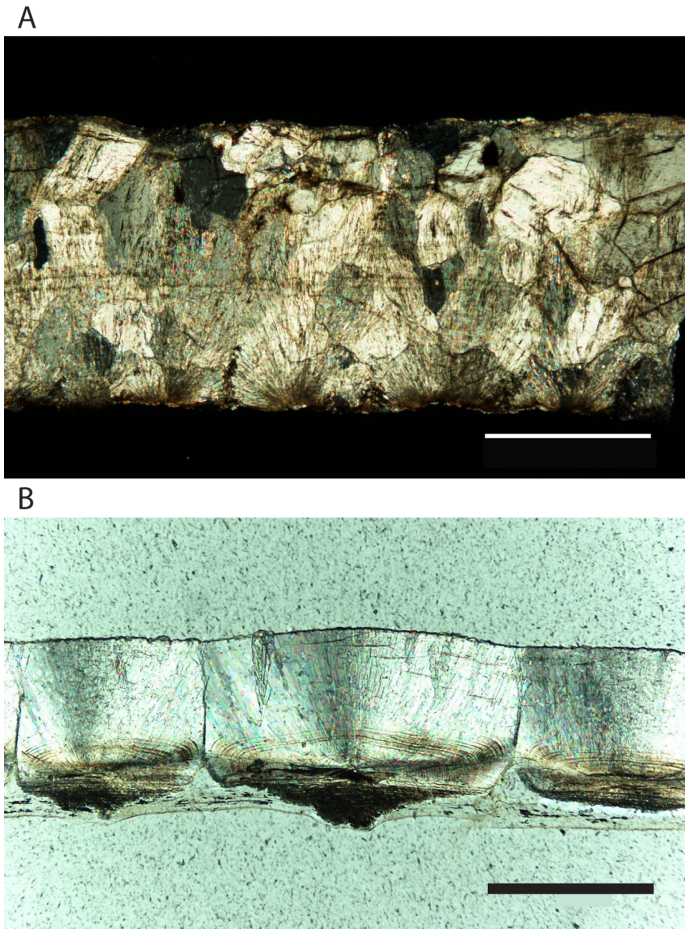


Figure 4. Polarized light microscopy of fossil and modern turtle eggshell. A, altered fossil eggshell (PIMUS lab#2012.IW30) showing blocky and irregular extinction pattern; B, modern turtle eggshell (*Phyrnops gibba*; ES 196) showing the typical extinction pattern of unaltered turtle eggshell. Scale bars equal 250 μm.

are primarily based on their rigid and flexible eggshell structure, respectively. The Malagasy eggs can be distinguished from the oofamily Testudoflexoolithidae based on their tightly packed shell units and a shell unit height to width ratio of 2:1 (Table 1). Among oospecies assigned to the Testudoolithidae, the Malagasy specimens differ from *Chelonoolithus braemi* Kohring, 1998 by a shell unit height to width ratio. They differ from *Emydoolithus laiyangensis* Wang et al., 2013 in overall egg shape but are similar in

eggshell thickness and are within the lower range of height to width ratio of *E. laiyangensis.* They can be distinguished from *Haininchelys curiosa* Schleich et al., 1988 by their greater eggshell thickness. Conversely, the Malagasy eggs have thinner eggshell than *Adocus* eggs described by Zelenitsky et al. (2008). They differ from *Testudoolithus hirschi* Kohring, 1999 and *Testudoolithus jiangi* Jackson et al., 2008 by a smaller shell unit height to width ratio. Finally, the Malagasy eggs are similar to *Testudoolithus rigidus* Hirsch, 1996 by a shell unit height to width ratio of 2:1, but differ in the smaller egg size and thicker eggshell.

We refer the Malagasy eggs to the oofamily Testudoolithidae and the oogenus *Testudoolithus* oosp. However, classification at the oogenus and oospecies level is difficult due to extensive alteration of the specimens. Thin section analysis of the eggshell shows substantial recrystallization, characterized by blocky extinction under crossed polarized light microscopy. This differs from the sweeping extinction pattern characteristic of modern and unaltered fossil turtle eggshell (Fig. 4B). Cathodoluminescence also reveals bright orange to orange-red fluorescence suggesting calcite or dolomite replacement of the original aragonite (Fig. 3D; Marshall, 1988), which prohibits assessment of pore density and gas conductance in these specimens due to recrystallization. Despite this replacement, pseudomorphs of the needle-like aragonite crystals are clearly preserved (Fig. 3A-C). Additionally, Hirsch (1983) and Masse (1989) demonstrate that despite alteration of aragonite to calcite, the structure of fossil turtle eggs can be identifiable when comparing the microstructure of altered specimens to avian and crocodilian eggshell under polarized light and scanning electron microscopy.

TABLE 1. Turtle ootaxa and diagnostic features. Modified from Lawver and Jackson (2014).

Oospecies	Type specimen	Material	Egg shape	Length x Width (mm)	Eggshell thickness (mm)	Shell unit height:width	References
Testudoflexoolithus agassizi	MCZ 2810/HEC 49	Eggshell fragments	-	-	0.06–0.1	1:1 or 2:3	Hirsch, 1996
Testudoflexoolithus bathonicae	BMNH 37983/HEC 186	An egg imbedded in matrix	Ellipsoidal	48 x 26	0.2–0.25	1:1	Hirsch, 1996
Chelonoolithus braemi	Guimarota 98-2	Eggshell fragments	-	-	0.2	1:1	Kohring, 1998
Emydoolithus laiyangensis	IVPP V18544	A nearly complete egg	Elongate	91 x 22	0.4–0.5	2:1–5:1	Wang et al., 2013
Haininchelys curiosa	-	Eggshell fragments	-	-	0.25–0.3	1.2:1–2.3:1	Schleich et al., 1988
Testudoolithus hirschi	-	Eggshell fragments	-	-	0.15	3:1	Kohring 1999
Testudoolithus jiangi	ZMNH M8713	A clutch of 23 eggs	Spherical	35–52	0.7–1.0	2.5:1–3:1	Jackson et al., 2008
Testudoolithus rigidus	UCM 55806/ HEC 425	Half of an egg	Spheroidal	42 x 47	0.22–0.24	2:1	Hirsch, 1996
Adocus eggs	-	Gravid females and clutch with embyros	Spherical	40 x 42	0.73–0.81	2.5:1–3.5:1	Zelenitsky et al., 2008
Malagasy eggs	-	Three eggs	Spherical	33.5–35.5	0.44	2:1	This paper

Additionally, distinguishing altered turtle eggshell from that of lizards should be possible due to their distinctive shell units comprised of jagged columns.

Malagasy Turtles

Late Cretaceous Malagasy turtles are only known from the Maevarano Formation within the Mahajanga Basin of northern Madagascar. To date, no cryptodires have been discovered from the Mesozoic of this island. In contrast, pleurodires are represented by the podocnemideran *Sokatra antitra* Gaffney and Krause, 2011, *Kinkonychelys* sp., and *K. rogersi* Gaffney et al., 2009, cf. *Erymnochelys* sp., and an indeterminate Bothremydid (Gaffney and Forster, 2003), all of which belong to Pan-Podocnemidae (sensu Joyce et al., 2004). However, all specimens are Maastrichtian in age and therefore geologically younger than the Campanian Morondava eggs. Here we present hypotheses for the taxonomic identity of these eggs below.

Taxa that may have laid their eggs in a coastal environment are large terrestrial turtles, protostegids, bothremydids, podocnemidids, or chelonioids (Fig. 5). Neither protostegids nor large terrestrial turtles have been documented from the Mesozoic of Madagascar and therefore can be preliminarily ruled out. Additionally, definitive eggs from bothremydid turtles have not yet been documented; however, their close phylogenetic relationship with podocnemidids (Cadena, 2015) may indicate that they had similar eggs. Although podocnemidid eggs are typically rigid-shelled and elongate, *Podocnemis expansa* lays pliable, spherical eggs similar to extant sea turtles (Ernst and

Barbour, 1989). Therefore, we suggest that it is plausible that either a bothremydid or podocnemidid turtle produced the Malagasy eggs.

Alternatively, the eggs may have been produced by a chelonioid (sea turtle); however, the origin of crown chelonioids is controversial. Hirayama (1998) describes

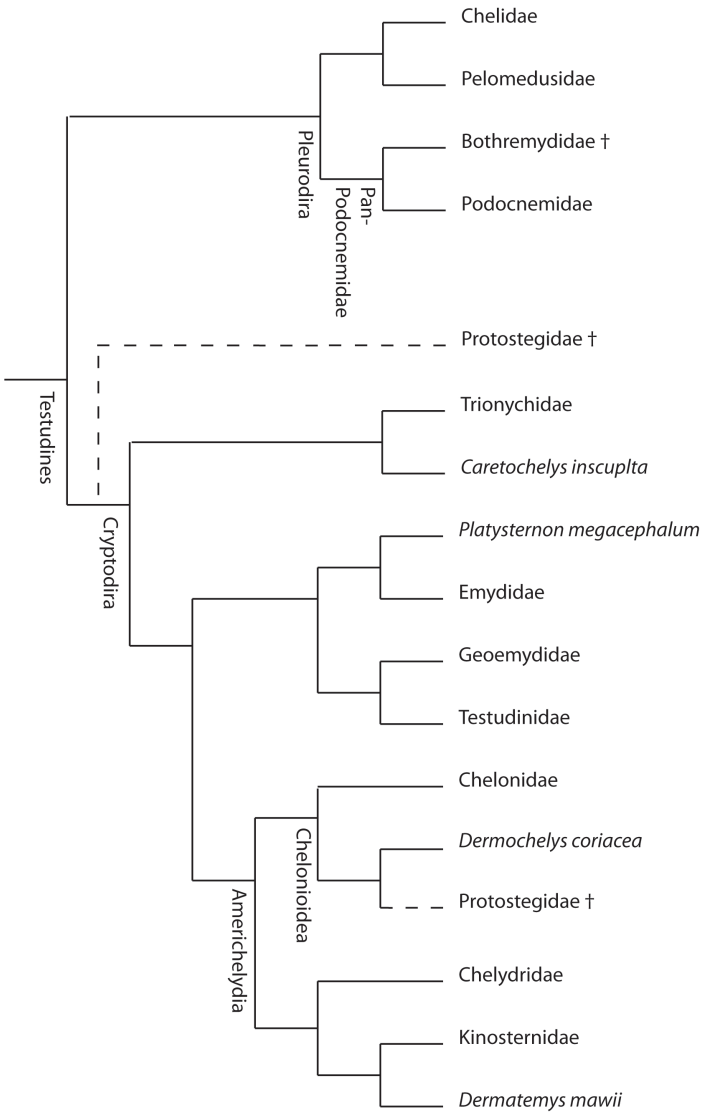


Figure 5. Phylogenetic tree of Testudines. Modified from Joyce (2007), Cadena (2015), and Crawford et al. (2014).

Santachelys gaffneyi from the Early Cretaceous of Brazil as the oldest sea turtle (Chelonioidea) but other researchers dispute this identification and suggest that Chelonioidea did not appear until the Late Cretaceous (Joyce, 2007; Sterli, 2010; Anquetin, 2012). Additionally, molecular divergence analysis places the origin of Chelonioidea as latest Cretaceous (Joyce et al., 2013). Although, no chelonioid skeletal material is documented from the Mesozoic of Madagascar, this absence does not suggest that sea turtles were not present in Madagascar during the Late Cretaceous. Since these specimens were discovered in marine rocks it is tempting to identify them as sea turtle eggs; however, the poorly mineralized eggshell of extant sea turtles consists of loosely organized shell units that differ significantly from the rigid eggshell of the Malagasy eggs (Hirsch, 1996). Despite this difference, it is not known if stem chelonioid turtles laid rigid or pliable-shelled eggs.

Investigation of chelonioid outgroups of other americhelydian turtles may provide information on the microstructure of stem chelonioid eggs. The eggs of chelydrids are spherical and semi-pliable, whereas *Dermatemys mawii* and kinosternids have rigid-shelled eggs with an elongate shape (Ernst and Barbour, 1989). Tracing these characters on to a simplified tree of americhelydian turtle demonstrates that stem chelonioids likely laid spherical eggs and whether these eggs were pliable or rigid is equivocal. Furthermore, turtle eggs of the oospecies *Testudoflexoolithus bathonicae* from the Middle Jurassic (Bathonian) of England have flexible eggshell and closely resemble those of crown chelonioids (Hirsch, 1996) and may have been laid by a stem chelonioid; although,

T. bathonicae differs from crown chelonioid eggs in having an elongate shape. With this in mind, we suggest that it is plausible that a stem chelonioid turtle may have laid the Malagasy specimens but less likely than a bothremydid or podocnemidid origin because only pan-podocneidid turtles are currently known from the Mesozoic of Madagascar.

Definitive taxonomic identification requires the preservation of embryos or preservation of a gravid adult. Finally, evidence for thinning of the eggshell and rounded edges (Oser and Jackson, 2014), as well as the association of these eggs with scaphite ammonites and the heteromorph ammonite *Bostrichoceras* in marine rocks suggests that they were subject to transport from the paleo-nesting environment or reworking during their taphonomic history (Evans, 2012 and references therein).

Jackson et al. (2008) and Zelenitsky et al. (2008) use egg shape and clutch size to estimate the adult body size of the female turtles that laid their specimens. Therefore comparisons of the Malagasy eggs with those of extant turtles may also provide useful paleoecological data. Extant turtles lay eggs that range in shape from spherical in large-bodied taxa to elongate in small-bodied taxa (Ewert, 1979; Elgar and Heaphy, 1989; Iverson and Ewert, 1991). Spherical eggs, which pack into the uterus more efficiently, are associated with taxa that lay large clutches (12 or more eggs) and may help to reduce surface area thereby reducing water loss during incubation (Iverson and Ewert, 1991). Additionally, thicker eggshell and low porousity reduces water vapor conductance. This suggests that the spherical Malagasy eggs with thick eggshell are from a taxon that would have laid a large clutch in a relatively arid environment, similar to that of other Late Cretaceous turtle eggs (Jackson et al., 2008; Zelenitsky et al., 2008). Adult body size

cannot be calculated for the individual that laid the Malagasy eggs due to the incomplete preservation of the clutch.

The Malagasy eggs are only the fourth documented occurrence of Mesozoic turtle eggs from Gondwanan continents, following a single specimen from Brazil (Azevedo et al., 2000) and two from India (Sahni, 1957; Mohabey, 1998). The specimens described by Azevedo et al. (2000) and Sahni (1957) were identified solely by gross morphology and microstructural analysis was not performed. Additionally, some researchers have questioned a turtle origin for the specimens described by Mohabey (1998) because of low quality figures and the identification of a mammillary layer in the bottom of the eggshell (Kohring, 1999; Jackson et al., 2008; Knell et al., 2011). This feature is characteristic of only avian and non-avian theropod eggshell. Therefore, additional microstructural analysis is needed to clarify the identity of these specimens.

CONCLUSIONS

Recent paleontological research of the Mesozoic of Madagascar has documented a great diversity of vertebrates from the Late Cretaceous; however, no fossil eggs have been described to date in this area. Despite significant diagenetic alteration, the eggs described here can be confidently identified to Testudines because of their distinctive microstructure composed of radiating, acicular crystals. Additionally, they can be identified as rigid-shelled turtle eggs by tightly interlocking shell units with a shell unit height to width ratio of 2:1. The lack of associated skeletal remains, however, precludes

taxonomic assignment of the specimens. Future discovery of turtle eggs associated with skeletal remains of embryos or gravid adults may elucidate the taxonomic identification of these eggs.

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CHAPTER FOUR

AN ACCUMULATION OF TURTLE EGGS WITH EMBRYOS FROM THE CAMPANIAN (UPPER CRETACEOUS) JUDITH RIVER FORMATION OF MONTANA

Contributions of Author and Co-Author

Manuscript in Chapter 4

Author: Daniel R. Lawver

Contribution: Conceived and implemented the study design. Collected and analyzed data. Wrote first draft of the manuscript.

Co-Author: Frankie D. Jackson

Contributions: Advised on the study design. Edited and provided feedback on early drafts of the manuscript.

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CHAPTER FOUR

AN ACCUMULATION OF TURTLE EGGS WITH EMBRYOS FROM THE CAMPANIAN (UPPER CRETACEOUS) JUDITH RIVER FORMATION OF MONTANA

DANIEL R. LAWVER,[*,1,2] and FRANKIE D. JACKSON[1]

[1]Department of Earth Sciences, Montana State University, Bozeman, Montana, 59717, U.S.A., danlawver@gmail.com; frankiej@montana.edu

[2]Museum of the Rockies, Bozeman, Montana, 59717, U.S.A.

[*]Corresponding author

ABSTRACT

A weathered accumulation of eggs, interpreted as remnants of a single clutch composed of at least 16 turtle eggs (MOR 710) from the Campanian (Upper Cretaceous) Judith River Formation of north-central Montana, USA, represents a new oospecies *Testudoolithus zelenitskyae*. This ootaxon is diagnosed by the following unique combination of characters: spherical eggs 34–39 mm in diameter, 660–760 µm thick eggshell, shell unit height-to-width ratio of 3.15:1–5.5:1, and domed shell units. Estimated egg mass indicates that the egg-laying adult likely possessed a carapace 35.0–54.4 cm in length. Similarities between *T. zelenitskyae* and *Adocus* sp. eggs, along with comparable body size, suggest that this taxon might have produced MOR 710. One egg exhibits abnormal multilayered eggshell, likely resulting from prolonged egg retention by the female turtle. At least five of these eggs, including the multilayered specimen, preserve embryonic remains that demonstrate a late stage of embryonic development. This suggests that death occurred just prior to hatching.

KEYWORDS:

Testudoolithidae

Testudines

Cryptodira

Ootaxonomy

Multilayered egg

1. INTRODUCTION

Fossil turtle egg clutches, gravid adults, and turtle embryos are relatively rare in the rock record when compared to dinosaurian specimens (Lawver and Jackson, 2014). Jackson, Jin, Varricchio, Azuma, and Jiang (2008) report an in situ turtle egg clutch preserving 23 spherical eggs in three superimposed layers from the Albian (Lower Cretaceous) deposits of Tiantai basin of Zhejiang, China. Zelenitsky, Therrien, Joyce, and Brinkman (2008) describe a clutch of 26 eggs from the Campanian (Upper Cretaceous) Oldman Formation of Alberta, Canada and estimated that a female with carapace length of 49.5 cm produced the clutch. A possible tortoise clutch containing at least five eggs comes from the Pliocene Apolakkia Formation of Rhodes, Greece (Mueller-Töwe et al., 2011), and fossil egg clutches from the Pleistocene of Lord Howe Island, Australia are tentatively assigned to the stem turtle, *Meiolania platyceps* (Anderson, 1925; Gaffney, 1996, Lawver and Jackson, in press).

In contrast to these turtle clutches, fossil gravid turtles provide definitive assignment of eggs to a specific taxon. A gravid turtle and a turtle clutch from the Upper Cretaceous Dinosaur Park and Oldman formations, respectively, show similar eggshell microstructure. Zelenitsky et al. (2008) conclude that both are referable to *Adocus* sp. Likewise, Knell, Jackson, Titus, and Albright III (2011) report portions of two eggs within a gravid *Adocus* sp. from the Campanian (Upper Cretaceous) Kaiparowits Formation of Utah. Additionally, an undescribed gravid *Basilemys variolosa* from the Dinosaur Park Formation of Alberta, Canada preserves at least three eggs that were

discovered when the specimen was inadvertently damaged (Braman and Brinkman, 2009). Finally, an eurysternid specimen from the Late Jurassic of Solnhofen in Germany contains spherical objects that Joyce and Zelenitsky (2002) interpreted as highly altered eggs.

Fossil turtle embryos are known from North America (Zelenitsky, 1995; Clouse, 2001; Jackson and Schmitt, 2008; Zelenitsky et al., 2008; McGee, 2012), Asia (Mikhailov Sabath, and Kurzanov , 1994; Cohen, Cruickshank, Joysey, Manning, and Upchurch, 1995; Fang, Yue, and Ling, 2009), and Europe (Hemprich, 1932). Although, morphological analysis of these specimens could assist in determining their taxonomic affinity, only one specimen has been investigated. Using computerized tomography (micro-CT) McGee (2012) confirmed that *Adocus* sp. produced the clutch from the Oldman Formation, thereby agreeing with the previous identification of Zelenitsky et al. (2008).

Although rare, fossil turtle clutches, gravid females, and embryonic remains provide important information about the evolution of turtle reproduction and paleoecology. Here, we describe a weathered accumulation of turtle eggs (some containing embryos), which we interpret as remnants of a single clutch. The egg accumulation is from the Campanian strata of the Judith River Formation of north-central Montana, USA. Jackson and Schmitt (2008) briefly report the microstructure of the multilayered egg from this clutch when establishing criteria for recognition of egg abnormalities in the fossil record. However, a detailed description of the clutch was beyond the scope of their paper. Jackson and Schmitt (2008) simply referred to this egg

as MOR 710, whereas here it is assigned as MOR 710B. We describe this weathered clutch, assign the eggs to parataxonomy, and discuss the implications for the evolution of turtle reproductive biology.

Institutional Abbreviations: AM, Australian Museum, Sydney, New South Wales, Australia; BMNH, The Natural History Museum, London, United Kingdom; HEC, Hirsch Eggshell Catalogue, University of Colorado, Boulder, Colorado, U.S.A; IVPP, Institute of Vertebrate Paleontology and Paleoanthropology, Beijing, China; LBA, L. Barry Albright field number; MCZ, Museum of Comparative Zoology, Cambridge, Massachusetts, U.S.A; MOR, Museum of the Rockies, Bozeman, Montana, U.S.A; NHMU, Natural History Museum of Utah, Salt Lake City, Utah, U.S.A; TMP, Royal Tyrrell Museum of Paleontology, Alberta, Canada; UCM, University of Colorado Museum, Boulder, Colorado, U.S.A; ZMNH, Zhejiang Museum of Natural History, Hangzhou, Zhejiang Province, China.

2. MATERIALS AND METHODS

The fossil turtle eggs studied are housed at the Museum of the Rockies (MOR) in Bozeman, Montana. Two multilayered eggshell were removed from an egg from MOR 710. One was etched with hydrochloric acid for about five seconds in order to better reveal the fine crystalline structure. Histological radial sections were made through eggshell fragments from both normal eggs (MOR 710A, C), the multilayered specimen (MOR 710B), and an embryonic costal element labeled MOR 710:EB 3 (the exact

location of the section is indeterminate). Additional eggshell thin sections (MOR 710:ES 1–3) came from unspecified eggs within the clutch. Histological procedures follow Lamm (2013). Eggshell fragments were mounted on an aluminum stub, coated with 10 nm of gold, and imaged under a JEOL JSM-6100 scanning electron microscope (SEM) at 10 kV. Images included the inner surface and radial cross sections of the eggshell. Photomicrographs of histological sections of eggshell and bone were taken with a Nikon Digital Sight DS-5Mc camera and microstructural features were measured with the image analysis software ImageJ (Rasband, 1997; http://imagej.nih.gov/ij/). Assessment of potential diagenetic alteration of the eggshell included a Nikon eclipse 50i microscope equipped with cathodoluminescence (CL). Egg mass was calculated using Hoyt's (1979) equation:

$$\text{Mass} = 0.000548 \times LB^2$$

where L is egg length in mm and B is egg breadth in mm. Carapace length for the gravid female turtle that produced MOR 710 is estimated using the positive correlation between egg mass and adult carapace length (Elgar and Heaphy, 1989) and the regression line:

$$y = 0.0568x + 1.5811$$

The latter was derived from 63 species (Elgar and Heaphy, 1989: Appendix), where y is the egg mass and x is carapace length ($r^2 = 0.666$). Note that the regression equation provided in Elgar and Heaphy (1989: Fig. 1) contains a typographical error, which results in unrealistically small carapace lengths.

3. GEOLOGY

The Campanian (Upper Cretaceous) Judith River and Two Medicine formations in north-central Montana consist of eastward thinning, non-marine clastic deposits that record regressive-progradational phases of shoreline migration of the Western Interior Cretaceous Seaway (Fig. 1A, B) (Lorenz, 1981; Rogers, 1998). The temporally and lithostratigraphically correlative formations are now separated by the Sweetgrass arch, a north-south-trending anticline (Fig. 1A, B). The Judith River Formation in eastern Montana includes deposits of a broad lowland coastal alluvial plain (Rogers, 1998). Marine rocks of the Claggett and Bearpaw formations underlie and overlie the formation, respectively (Rogers, 1998). Eberth, Thomas, and Deino (1992) and Goodwin and Deino (1989) dated rocks of the Judith River Formation in central Montana as 74.5 and 78.0 Ma, respectively, which corresponds to the middle to late Campanian (Fig. 1B).

MOR 710 comes from a sandy siltstone in a fining-upward stratigraphic sequence representing overbank deposits at the Egg White Site (MOR locality JR-122L) in Hill County, near Havre, Montana (Clouse, 2001; Fig. 1). This specimen occurred approximately 4 m laterally from a clutch of lambeosaurine eggs preserved beneath a bentonite layer within the so-called upper nesting horizon of the lower nesting ground (Clouse, 2001). According to Clouse (2001), this nesting ground lies in the upper half of the Judith River Formation, deposited during the latter half of the Bearpaw Seaway transgression. Invertebrate fossils within the nesting horizon consist of freshwater and

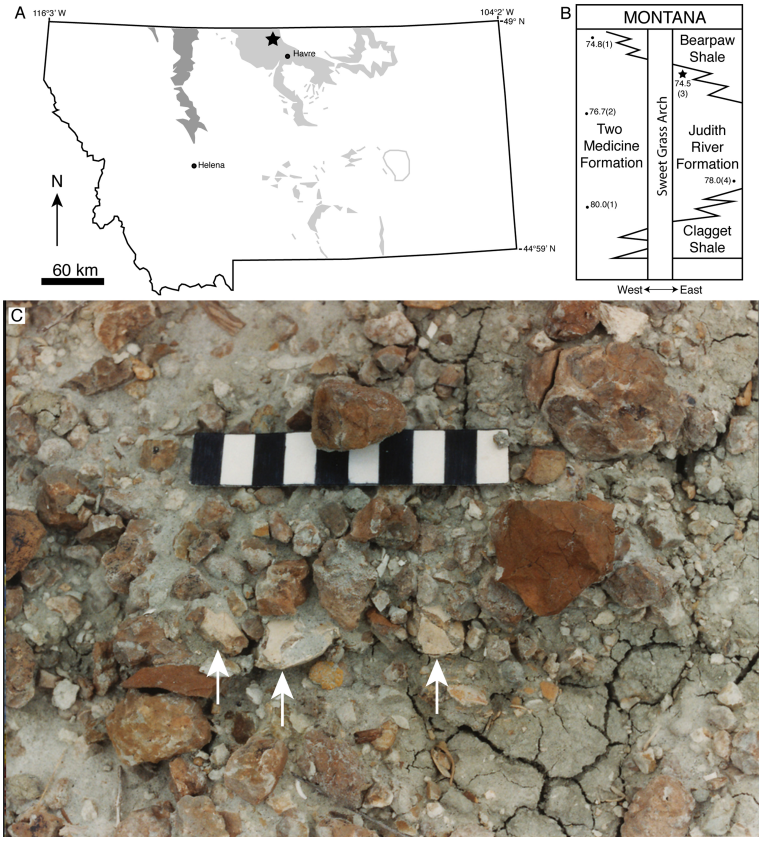


Figure 1. The Campanian Judith River and Two Medicine formations and stratigraphically correlative units of Montana. A, Map of Montana showing exposures of the Judith River (light grey) and Two Medicine (dark grey) formations. Star represent the Egg White Site locality where MOR 710 was collected; B, Geologic cross section of north-central Montana showing the Two Medicine-Judith River clastic wedge with radiometric dates and references (modified from Jackson and Varricchio, 2010). Star represents the approximate location of the Egg White Site; C, Field photograph of MOR 710 showing original site exposed in eroded concretionary layer (photo credit, V. Clouse). Arrows point to three of the eggs. Scale bar equals 10 cm. References: 1, Rogers et al. (1993); 2, Varricchio et al. (2010); 3, Eberth et al. (1992); 4, Goodwin and Deino (1989).

brackish species, including large unionid bivalves, as well as unidentified small bivalves and gastropods (Clouse, 2001). Vertebrate fossils include shed theropod and ornithopod teeth and fragmentary turtle shells referable to *Basilemys* sp., *Adocus* sp., *Aspideretoides* sp., and an undescribed large-bodied terrestrial form.

4. SYSTEMATIC PALEONTOLOGY

Oofamily Testudoolithidae Hirsch, 1996 sensu Jackson et al. 2008

Oogenus *Testudoolithus* Hirsch, 1996 sensu Jackson et al. 2008

Oospecies *Testudoolithus zelenitskyae* oosp. nov.

(Fig. 2–3)

Holotype. MOR 710, the weathered remains of a clutch composed of at least 16 turtle eggs.

Etymology. After Darla Zelenitsky in recognition of her initial description of *Adocus* eggs from Alberta, Canada and continuing contribution to oological research.

Type Locality and Age. MOR locality JR-122L, Egg White Site, north-central Montana, U.S.A, Judith River Formation, Upper Cretaceous (Campanian).

Diagnosis. *Testudoolithus zelenitskyae* differs from all other oospecies in the following unique combination of characters: spherical turtle eggs 34–39 mm in diameter; 660–760 μm thick eggshell; shell unit height-to-width ratio of 3.15:1–5.5:1 and domed shell units.

Distribution. Judith River Formation, Montana, U.S.A. (Jackson and Schmitt, 2008, MOR 710), Oldman and Dinosaur Park formations, Alberta, Canada (Zelenitsky et al., 2008, TMP 1999.63.2 and TMP 2008.27.1, respectively), and Kaiparowits Formation, Utah, U.S.A. (Knell et al., 2011, NHMU 16868 [LBA-06-7]). All these formations are Campanian (Late Cretaceous) in age.



Figure 2. MOR 710A and C, two lithologically compressed eggs with normal eggshell structure. Scale bar equals 1 cm.

Description

Eggs. Eleven beige eggs (Fig. 2) measuring 34–39 mm in diameter occurred on a weathered surface eroded into a concretionary layer (Fig. 1C). The lithostatically compressed eggs are filled by sandy siltstone. These occurred in close association with one well-preserved egg of the same color and four highly crushed, orange eggs, which were found in situ within a sandy siltstone matrix. The similarity of preservation (but not color) and in-filling sediment suggest all eggs came from the same horizon and originally represented a clutch. In addition, all eggs exhibit relatively intact eggshell on both sides and thus appear to be unhatched. We estimate egg mass as 21.5–32.5 g. The weathered condition of the material, however, prevents determination of the original number of eggs, their relative position to one another, and number of egg layers that once comprised the clutch.

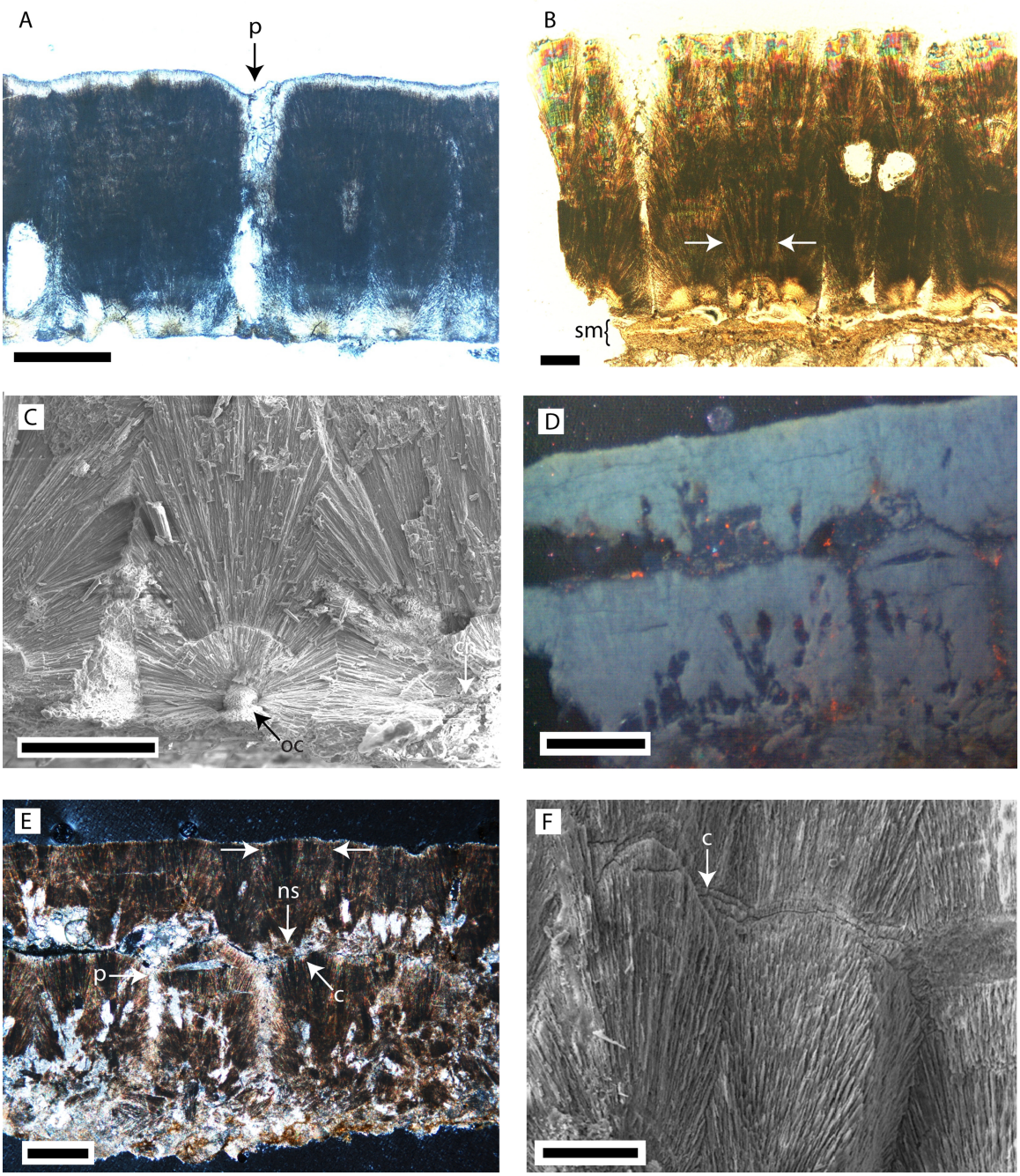


Figure 3. Radial thin sections and SEM images of MOR 710 eggshell. **A**, MOR 710A showing straight tube-like pore. Inner and outer shell surfaces are at the top and bottom of image, respectively. Scale bar equals 250 µm; **B**, MOR 710:ES 1 under cross-polarized light. Note the closely packed, narrow shell units (between horizontal arrows), acicular aragonite crystals, and craters at the inner shell surface. Scale bar equals 100 µm; **C**, radial SEM from MOR 710B with preservation of the former organic core. Scale bar equals 90 µm; **D,** eggshell from MOR 710B under cathodoluminescence showing dull blue luminescence, with bright orange and non-luminescent areas representing alteration

associated with secondary calcite and open fractures, respectively. Scale bar equals 250 μm; **E**, MOR 710B under cross-polarized light showing multilayers comprising the eggshell. Note the close contact of the two eggshells and a rare nucleation site (ns) between layers. Shattered structure and secondary replacement on the left decreases apparent shell thickness. Scale bar equals 250 μm; **F**, SEM micrograph of multilayered eggshell from MOR 710B etched with hydrochloric acid. Note absence of nuclei and close confirmation of inner and outer eggshell layers. Panel F is reprinted from Cretaceous Research, Volume 29, Jackson and Schmitt, 2008; and Scanning, Volume 24, Jackson, Schweitzer, and Schmitt, 2002. Scale bar equals 10 μm. **Abbreviations**: **c**, contact; **cn**, cratered nuclei; **ns**, nucleation site; **oc**, organic core; **p**, pore; **sm**, shell membrane.

Single-layered Eggshell. The 660–760 μm thick eggshell is composed of a single layer of shell units; the latter average 135–240 μm wide, with a height-to-width ratio of 3.15:1–5.5:1 (Fig. 3A, B). Shell units consist of tightly interlocking acicular aragonite crystals that radiate from nucleation sites at the inner shell surface (Fig. 3C). These crystals feather out at their terminal ends and the shell units exhibit domed ornamentation on the external surface. Well-preserved craters, likely resulting from osteogenesis, occur at the base of some but not all shell units and measure 60 μm in diameter (Fig. 3B). A straight tube-like pore that measures 50 μm in diameter occurs between shell units and extends from the inner to the outer eggshell surfaces (Fig. 3A). Under cross-polarized light, the eggshell displays sweeping extinction. Remnants of 46–107 μm-thick shell membrane are preserved below the inner shell surface and consist of two layers, which also characterizes extant turtle eggs (Fig. 3B; Hirsch, 1983).

Multilayered Eggshell. MOR 710B includes two eggshell layers. Preservation within MOR 710B is highly variable. Some specimens display substantial diagenetic alteration, primarily dissolution of aragonite and reprecipitation of calcite (Fig. 3D). The

inner and outer eggshells measure 500–540 μm and 340–380 μm, respectively, with a total thickness of 835–860 μm (Fig. 3E, F). The inner surface of the lower eggshell has a shattered appearance, with splinter-like fragments of eggshell randomly oriented within the secondary mineral matrix. The inner eggshell layer is typically thinner than the single layered eggshell described above, likely due to this alteration. Although sometimes slightly separated from the inner eggshell, the overlying eggshell conforms closely to the external surface of the underlying shell units. Two eggshell fragments from different areas of the egg show no evidence of nucleation sites between the inner and outer eggshell layers under SEM (Fig. 3E and unpublished image). With the exception of one or possibly two nuclei, radial thin section from different fragments also show an absence of nuclei (Fig. 3F). Shell units in the outer eggshell layer have a height-to-width ratio of 1.65:1, and their external surfaces are typically flatter and lack the domed shape of the underlying ones. No pores were observed in the outer eggshell, whereas this layer truncates a pore of the underlying eggshell in at least one area of the shell (Fig. 3E).

Cathodoluminescence imaging of MOR 710B reveals dull blue luminescence throughout the specimen except for small areas of non-luminescence and/or bright orange luminescence that occur primarily between shell units at the inner shell surface, in pores between shell units and within dissolution cavities (Fig. 3D).

Embryonic Remains. At least five eggs contain embryonic remains, including the multilayered specimen (MOR 710B) described above. Although the state of embryonic preservation in MOR 710D prohibits a detailed description, we provide a brief anatomic and histologic assessment based on the available information from this egg.

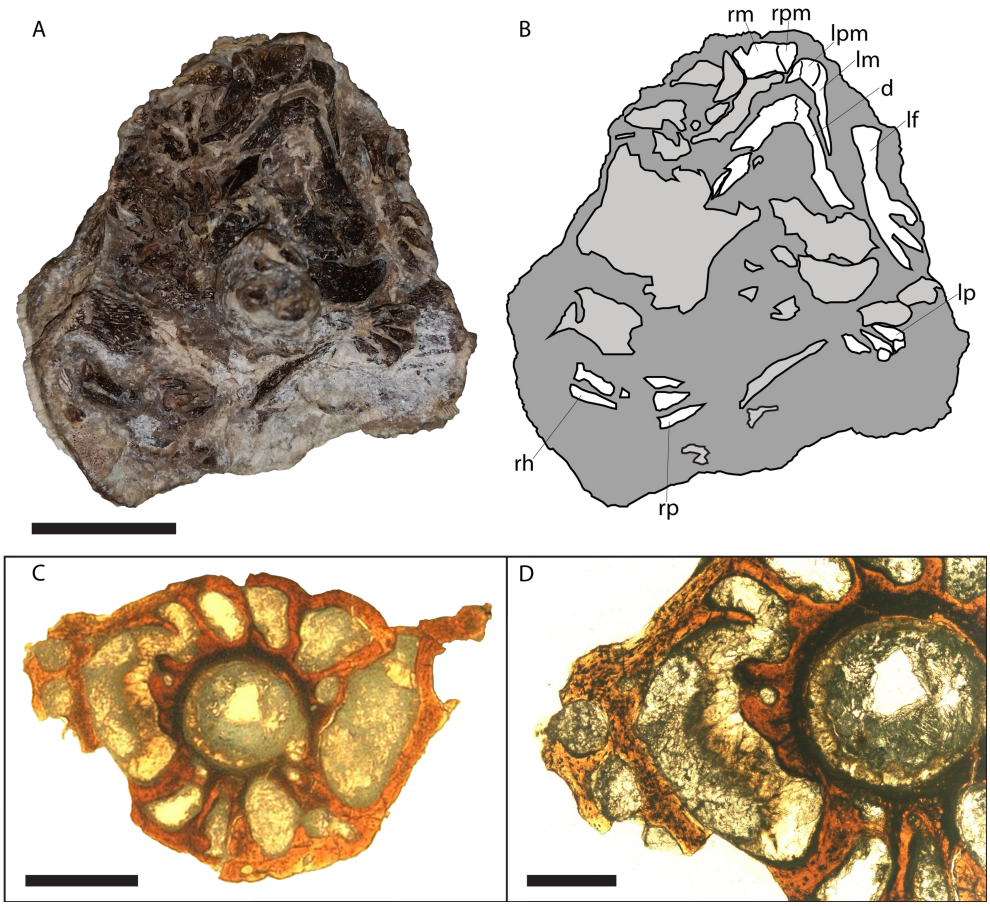


Figure 4. MOR 710D embryo. **A**, Partially prepared egg showing exposed embryonic skeletal elements. Scale bar equals 1 cm; **B**, line drawing of A. White represents identifiable skeletal elements, whereas light grey areas are unidentifiable elements; **C**, histological thin section (MOR 710:EB 3) of a costal element showing the medullary cavity and vascular canals infilled with secondary calcite crystals, as well as trabeculae growing in the posteroanterior direction. Scale bar equals 0.5 mm; **D**, enlargement of C showing numerous randomly arranged osteocyte lacunae. Scale bar equals 0.25 mm. **Abbreviations**: **d**, dentary; **lf**, left forelimb; **lm**, left maxilla; **lp**, left pes; **lpm**, left premaxilla; **rh**, right hindlimb; **rm**, right maxilla; **rp**, right pes; **rpm**, right premaxilla.

Partial preparation of MOR 710D reveals well ossified and partially articulated embryonic skeletal elements that include the skull, lower jaw, fore- and hindlimbs, as well as numerous unidentifiable bones (Fig. 4A, B). The skull and lower jaw are preserved in ventral view. Although, the premaxillae and maxillae are articulated, the

remaining exposed cranial elements are disarticulated and unidentifiable. The triturating surfaces of the upper and lower jaws, as well as the palatal bones are not exposed. The lower jaw includes both dentaries, which exhibit a weakly sutured symphysis. The left forelimb preserves the manus and a possible radius, whereas the hindlimb elements include the right tibia, right fibula, as well as both right and left ungual phalanges. Numerous other unidentifiable postcranial elements are exposed on the partially prepped surface of the egg.

A histological thin section of a costal element (MOR 710:EB 3) shows that the embryonic bone consists of primary woven bone with numerous osteocyte lacunae and bony trabeculae that radiate from the medullary cavity and surround large vascular canals (Fig. 4C, D). There is no evidence of compact bone or bone remodeling. Bone growth dominates in the anteroposterior direction.

5. DISCUSSION

The needle-like aragonite crystals allow definitive assignment of MOR 710 to Testudines (Hirsch, 1983). The generally well-preserved aragonite structure shows some dissolution and calcite replacement, with small areas of bright orange luminescence and areas of non-luminescent. Bright orange luminescence is due to Mn^{2+} incorporation into the calcite crystal (Marshall, 1988), which is generally linked to elevated Mn^{2+} concentrations (Wendler, Wendler, Rose, and Huber, 2012). Areas of non-luminescence may result from increased Fe^{2+}, a main quencher element of CL in carbonates (Barbin,

2000; Boggs and Krinsley, 2006; Götte and Richter, 2009), or possibly because Mn^{2+} levels are below the detection limits (Barbin, 2000; Wendler et al., 2012). Some non-luminescent areas in the eggshell may represent open space in fractures that are unfilled by calcite.

In contrast to these small bright orange and non-luminescent areas, MOR 710B primarily exhibits a dull blue color, similar to CL imaging of extant and recent fossil turtle eggshell (Lawver and Jackson, in press; Lawver, unpublished data). Blue luminescence is thought to occur in the absence of activator elements (Wendler et al. 2012) and has been detected in the aragonite shells of *Nautilus* (Cephalopoda) that are only minimally affected by diagenetic overprint (Barbin et al., 1995). Blue luminescence in MOR 710B also suggests minimal diagenetic recrystallization of the aragonite eggshell.

Parataxonomy

Hirsch's (1996) parataxonomic classification for fossil turtle eggs and eggshells includes two oofamilies, Testudoflexoolithidae and Testudoolithidae. Flexible eggs with loosely abutting shell units comprise the former, whereas the latter includes rigid eggs with tightly packed shell units. The rigid structure of the eggshell excludes MOR 710 from Testudoflexoolithidae (Table 1).

Among oospecies assigned to Testudoolithidae, MOR 710 differs from *Chelonoolithus braemi* Kohring, 1998 and *Haininchelys curiosa* Schleich, Kästle, and van Dyck, 1988 in its thicker eggshell and a shell unit height-to-width ratio of 3.15:1–

5.5:1. MOR 710 can be distinguished from *Emydoolithus laiyangensis* Wang, Wang, Zhao, Zhang, and Jiang, 2013 in overall eggs shape, thicker eggshell, and a greater height-to-width ratio. Thicker eggshell also distinguishes MOR 710 from *Testudoolithus hirschi* Kohring, 1999, and *T. rigidus* Hirsch, 1996. MOR 710 is similar to *Testudoolithus jiangi* Jackson et al., 2008 (Fang, Lu, Jiang and Yang, 2003) and the eggs fall within the ranges of egg size range and eggshell thickness of this ootaxon; however, MOR 710 has a greater shell unit height-to-width ratio.

5.1 Taxonomy

MOR 710 could potentially represent the eggs of one of several turtle taxa known from the Judith River Formation: *Axestemys splendida, Aspideretoides* sp., *Plastomenus* sp., *Basilemys* sp., *Neurankylus eximius, Plesiobaena antiqua*, and *Adocus* sp. (Sahni, 1972; Joyce and Bourque, 2016; Vitek, 2012). The trionychids *Axestemys splendida* and *Plastomenus* sp. seem unlikely candidates because extant trionychid eggs differ from MOR 710 in their thinner eggshell (0.1–0.2 mm), smaller height-to-width ratio, and lack of domed external surfaces (Fig. 5). A gravid *Basilemys variolosa* from the stratigraphically correlative Dinosaur Park Formation in Alberta, Canada could potentially provide identification; however, Braman and Brinkman (2009) failed to describe the eggshell microstructure, thereby precluding comparison to MOR 710. The eggs of *N. eximius* and *P. antiqua* are currently unknown, although *N. eximius* may have been large enough to produce a similar sized clutch as MOR 710. Taxa from the Judith

Table 1: List of turtle ootaxa and their distinguishing characteristics.

Ootaxon	Holotype/Material	Geographic and temporal distribution	Egg shape	Length x Width (mm)	Eggshell thickness (mm)	Shell unit height:width
Testudoflexoolithus agassizi[1]	MCZ 2810/HEC 49: Eggshell fragments	Florida, USA; Pleistocene	-	-	0.06–0.1	1:1 or 2:3
Testudoflexoolithus bathonicae[1,2]	MB(NH)37983/ HEC 186: An egg imbedded in matrix	England; Bathonian, Middle Jurassic	Ellipsoidal	48 x 26	0.2–0.25	1:1
Chelonoolithus braemi[3]	Guimarota 98-2: Eggshell fragments	Portugal; Kimmeridgian, Upper Jurassic	-	-	0.2	1:1
Emydoolithus laiyangensis[4]	IVPP V18544: A nearly complete egg	Shandong Province, China; Upper Cretaceous	Elongate	91 x 22	0.4–0.5	2:1 to 5:1
Haininchelys curiosa[5]	-: Eggshell fragments	Belgium; Upper Paleocene	-	-	0.25–0.3	1.2:1 to 2.3:1
Testudoolithus hirschi[6]	-: Eggshell fragments	Portugal; Kimmeridgian, Upper Jurassic	-	-	0.15	3:1
Testudoolithus jiangi[7,8]	ZMNH M8713: A clutch of 23 eggs	Zhejiang Province, China; Albian, Early Cretaceous	Spherical	35–52	0.7–1.0	2.5:1 to 3:1
Testudoolithus rigidus[1]	UCM 55806/ HEC 425: Half of an egg	U.S.A., Europe, Africa; Lower Cretaceous - Pliocene	Spheroidal	42 x 47	0.22–0.24	2:1
Meiolania platyceps eggs[9]	AM F82183: A clutch of at least 10 eggs	Lord Howe Island, Australia; Pleistocene	Spherical	53.9	0.8	1.2:1
Testudoolithus zelenitskyae	MOR 710: A clutch of at least 16 eggs	U.S.A. and Canada; Campanian, Upper Cretaceous	Spherical	34–39	0.66–0.76	3.15:1 to 5.5:1

Modified from Lawver and Jackson (2014).

References: 1, Hirsch (1996); 2, Buckman (1859); 3, Kohring (1998); 4, Wang et al. (2013); 5, Schleich et al. (1988); 6, Kohring (1999); 7, Fang et al. (2003); 8, Jackson et al. (2008); 9, Lawver and Jackson (2016).

River Group of Alberta include *Judithemys sukhanovi* and *Boremys pulchra*; however, eggs of these taxa are also currently unknown. Additionally, *J. sukhanovi* may have been capable of producing a clutch as large as MOR 710.

The eggshell microstructure of most eggs within MOR 710 is most similar to eggs preserved within a gravid *Adocus* sp. and a fossil egg clutch from Alberta, Canada (Zelenitsky et al., 2008). Similarities include thick eggshell, with shell units taller than wide, and a domed external surface (see Table 2). In her unpublished master's thesis Zelenitsky (1995) named these *Adocus* sp. eggs *Testudoolithus magnirigidus*; however, this name does not meet the criteria of the International Committee on Zoological Nomenclature (Article 8.1.3) that requires simultaneously obtainable copies and wide distribution. Consequently, this oospecies is a nomen nudum (Lawver and Jackson, 2014) and we name the new ootaxon *Testudoolithus zelenitskyae*. A significant amount of homoplasy occurs in extant turtle eggshells (Winkler, 2006; Lawver, 2012) and, therefore, *Testudoolithus zelenitskyae* could potentially belong to another, as yet undescribed extinct taxa of close phylogenetic affinity to *Adocus*. Therefore, we assign the eggs to parataxonomy for better comparison with other egg types as well as allowing for the possibility of homoplastic morphology shared among multiple taxa.

5.2 Embryonic Remains

The embryonic remains provide only limited information. Nevertheless, some morphological and histological comparisons are possible with extant trionychid *Apalone*

spinifera. For example, preservation of anteroposteriorly projecting bony trabeculae of the costal elements (Fig. 4C, D) coincides with the initial expansion of this element in order to form the costal plates of the carapace. In *A. spinifera* this expansion begins no later than Stage 23 (Sheil, 2003), thus suggesting that the fossil embryos obtained 23 of 25 embryonic developmental stages prior to their demise.

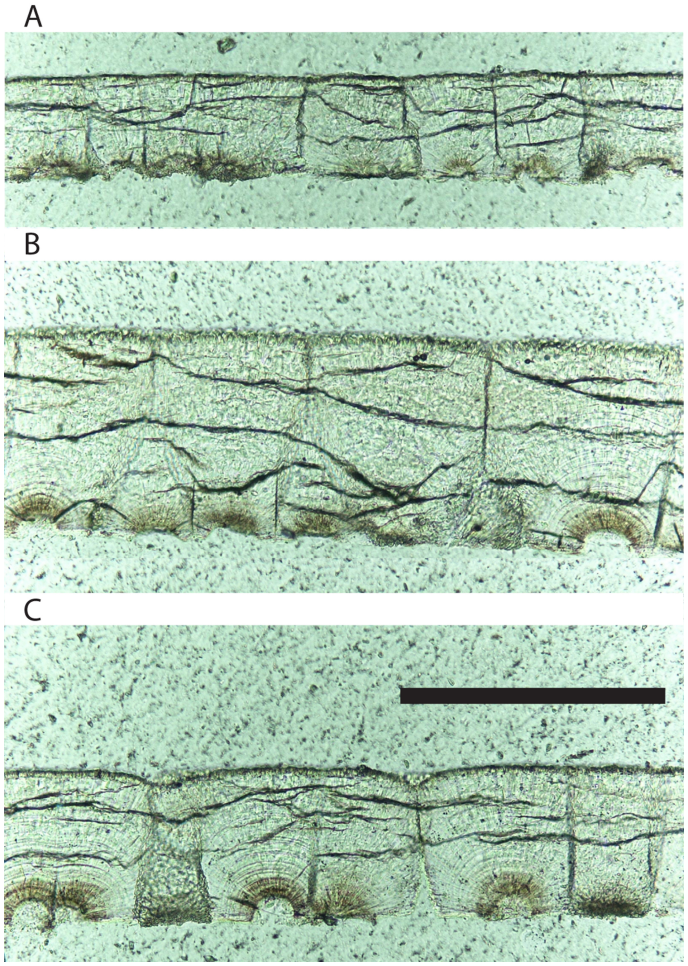


Figure 5. Radial thin sections of extant trionychid eggshell. **A**, *Apalone mutica* eggshell (ES 203); **B**, *Apalone ferox* eggshell (ES 204); **C**, *Apalone spinifera* eggshell (ES 205). All egghshells were photographed at the same scale. Scale bar equals 250 µm.

Table 2: Egg/eggshell characteristics of *Testudoolithus zelenitskyae* and *Adocus* sp. specimens discussed in the text.

Specimen	Material	Formation	Egg shape	Length x Width (mm)	Eggshell thickness (mm)	Shell unit height:width
MOR 710	Weathered clutch of at least 16 eggs	Judith River Formation	Spherical	34–39	0.66–0.76	3.15:1–5.5:1
NHMU 16868 (LBA-06-7)[1]	Gravid *Adocus* sp. preserving at least two eggs	Kaiparowits Formation	Spherical	35	0.25–0.28	2.5:1
TMP 1999.63.2[2]	Gravid *Adocus* sp. preserving at least five eggs	Oldman Formation	Spherical	35–40	0.73–0.81	2.5:1
TMP 2008.27.1[2]	Clutch of at least 26 eggs	Dinosaur Park Formation/	Spherical	40x42 40x43	0.5–0.65	3.5:1

Note: All specimens are Late Cretaceous, Campanian in age.
References: 1, Knell et al. (2011); 2, Zelenitsky et al. (2008).

Additionally, embryos of the more distantly related taxa, *Chelydra serpentina, Macrochelys temmincki,* and *Phrynops hilarii* also possess well-developed phalanges at stage 23 (Sheil, 2005; Bona and Alcalde, 2009; Sheil and Greenbaum, 2005). Finally, the well-ossified embryonic bones that characterize all eggs within MOR 710 also suggest that termination of embryonic development occurred close to hatching.

5.3 Multilayered Eggshell

The multiple eggshell layers of MOR 710B closely resembles a pathological condition reported in extant turtle eggs, whereby one or more additional eggshell layer is deposited over the original egg (Erben, Hoefs, and Wedepohl, 1979; Ewert, Firth, and Nelson, 1984; Jackson and Varricchio, 2003). However, the presence of an embryo in an advanced stage of development in a pathological egg is surprising. Here, we discuss alternative explanations that may account for this unusual occurrence.

First, the additional layer is biological in origin. This multilayered condition is relatively common in extant turtles, currently described for at least nine taxa (Jackson et al., 2004 and reference therein). The additional shell layers result from prolonged retention of one or more eggs in the uterus due to physiological or environmental stresses (Erben et al., 1979; Ewert et al., 1984). The abnormal layer(s) often block the pore canals of the underlying eggshell, and termination of embryonic development often results from asphyxiation. However, in at least one case an extant turtle embryo from a multilayered egg survived to hatching and died shortly thereafter (personal communication, M. Ewert,

May, 2001, Bloomington, IN). If the outer eggshell in MOR 710B resulted from egg retention, the pores in the inner and outer eggshells may have aligned and extended through both layers in some areas of the egg, permitting limited but potentially adequate embryonic gas exchange during embryonic development. For example, Ewert et al. (1984) described a pore in an abnormal *Rhinoclemmys aerolata* eggshell that extended straight through three superimposed layers.

A phenomenon known as egg capping offers an alternative explanation for the second layer in MOR 710B. Although rare in avian species ($\leq$ 1%), a portion of a hatched egg occasionally "caps" an adjacent, unhatched egg (Derrickson and Warkentin, 1991; Verbeek 1996). If this shell fragment is not removed by the adult, the embryo may suffocate. Derrickson and Warkentin (1991) speculated that egg capping might occur more often in clutches with greater hatching asynchrony and egg size variability. Hauber (2003), however, felt that this was not feasible to address because of the lack of published comparative data. Further, Hauber (2003) reports that no eggs in his study (0%) were covered by eggshell from conspecific eggs, whereas egg capping occurred in 33% of eggs from parasitized nests. The parasitic eggs were larger and hatched earlier than the host eggs, thereby increasing the potential for egg capping.

We interpret the multiple layers in MOR 710B as an abnormality for the following reasons. 1) Egg capping is unreported in extant turtles and other reptile eggs, likely because of their incubation in a substrate or vegetation mound, rather than in open nests characteristic of birds. 2) The inner surface of the outer layer of MOR 710B follows the contour of the underlying shell units (Fig. 3E, F) and its outer surface lacks the domed

shape of normal eggshell. 3) None of the eggs in the turtle clutch appear hatched; to the contrary, the eggs contain well-formed embryos that suggest an advanced stage and synchronous embryonic development; missing eggshell most likely resulted from recent weathering. 4) Most importantly, the base of the shell units in the outer eggshell layer lack nucleation sites (Fig. 3E, F). We identified only one or possibly two nuclei in samples viewed in thin section (Fig 3E) and none under SEM. If the second layer in MOR 710B represented a fragment from a previously hatched egg one would expect nucleation sites at the base of each shell unit. These are clearly absent in most areas of the eggshell (Fig. 3E).

As a final note, in extant multilayered eggs, shell membrane may or may not occur between the inner and outer eggshell layers (Ewert et al., 1984). This variation also occurs in Mesozoic multilayered eggshells (Jackson et al., 2008). If present in MOR 710B, decay of the membrane may have provided a conduit for ground water, thus contributing to more extensive recrystallization and precipitation of calcite between the layers in some areas of the egg (Fig. 3E).

5.3 Ecological Inferences

The size, shape, and estimated mass (21.5–32.5 g) of eggs preserved in MOR 710 allow anatomical and ecological interpretations. Extant turtles that lay spherical eggs are generally of large body size (Elgar and Heaphy, 1989) and have large clutches because this shape permits simultaneously shelling of more eggs in the uterus prior to oviposition

(Ewert, 1979; Iverson and Ewert, 1991). We estimate carapace length for the gravid female turtle that produced MOR 710 as 35.0–54.4 cm using the positive correlation between egg mass and adult carapace length (Elgar and Heaphy, 1989). However, it is worth noting that rigid-shelled turtle eggs have a density of 1.126, whereas birds have a density of 1.03–1.09 (Iverson and Ewert, 1991). This indicates that the egg mass and carapace lengths calculated here represent minimum estimates. These egg characteristics (i.e., size, shape, mass) suggest that MOR 710 was produced by a large bodied taxon. This inference is further supported by carapace length estimates of the gravid *Adocus* sp. specimens from Alberta and Utah (Zelenitsky et al., 2008; Knell et al., 2011). The 40.5 cm long Alberta adult preserves at least five eggs, with a diameter of approximately 35–40 mm (Zelenitsky et al., 2008). Likewise the 50–55 cm-long gravid adult from Utah preserves at least two eggs with diameter of 35 mm. Both gravid specimens (e.g., Zelenitsky et al., 2008; Knell et al., 2011) are estimated to have originally contained more eggs than observed.

6. CONCLUSIONS

MOR 710 comes from the Campanian (Upper Cretaceous) Judith River Formation of north-central Montana and consists of a weathered accumulation of eggs interpreted as remnants of a clutch originally composed of at least 16 spherical turtle eggs, at least one of which displays an additional, abnormal eggshell layer. Normal eggs exhibit a 660–760 μm thick eggshell comprised of a single layer of tightly interlocking aragonite shell units

with a height-to-width ratio of 3.15:1–5.5:1. In contrast, the abnormal eggshell includes 500–540 μm and 340–380 μm-thick inner and pathological shell layers, respectively. The pathological layer likely represents a retained egg from the same or different nesting attempt due to physiological or environmental stress experienced by the female turtle. When compared to the extant trionychid, *Apalone spinifera*, the embryonic bones in at least five eggs (including the multilayered specimen) demonstrate a late stage of development. This indicates that these embryos were close to hatching when death occurred. The similarity of the eggshell microstructure between the Judith River eggs and that of *Adocus* sp. (Zelenitsky et al., 2008; Knell et al., 2011) suggests that this taxon might have produced MOR 710. Further descriptions of taxonomically identifiable fossil turtle eggs, such as those from the Canadian gravid *Basilemys variolosa* specimen, will help support or refute this identification.

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CHAPTER FIVE

A GRAVID *BASILEMYS NOBILIS* FROM THE UPPER CRETACEOUS KAIPAROWITS FORMATION, UTAH: EGGSHELL DESCRIPTION AND OOTAXONOMIC CLASSIFICATION

Contribution of Author

Manuscript in Chapter 5

Author: Daniel R. Lawver

Contribution: Conceived and implemented the study design. Collected and analyzed data. Wrote the manuscript.

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DANIEL R. LAWVER

[1]Department of Earth Sciences, Montana State University, Bozeman, Montana, 59717, U.S.A., danlawver@gmail.com

*Corresponding author

ABSTRACT—A *Basilemys nobilis* specimen come from the Upper Cretaceous Kaiparowits Formation within Grand Staircase-Escalante National Monument of southern Utah preserves at least eight eggs within its shell. The partial to nearly complete, spherical eggs are 40 mm in diameter. Eggshell measures 994 μm thick and shell unit height-to-width is 4.4:1. Except for areas on the inner and outer eggshell surfaces, that show dissolution and recrystallization, cathodoluminescence demonstrates that the original aragonite composition is minimally altered. Some pores also exhibit in-filling by secondary calcite. The adult's carapace length is estimated as 58.8 cm long. Similarities with *Adocus* sp. and Trionychia eggs suggest that rigid eggshell and spherical eggs are synapomorphies for these clades and thick eggshell is a synapomorphy of Adocia.

INTRODUCTION

Fossil turtle eggs are known from every continent except Antarctica and range in age from the Middle Jurassic to the Pleistocene (Lawver and Jackson, 2014). Early identification typically relied on egg macrofeatures such as size, shape, and the lack of ornamentation (Buckman, 1859; Carruthers, 1871; Meyer, 1860; Hay, 1908; van Straelen, 1928). In contrast, Hirsch (1983) was the first to use scanning electron microscopy (SEM) and polarized light microscopy to compare the microstructure of fossil eggs with those of modern turtles. This approach exploited the unusual mineral composition of turtle eggshell for initial assessment and taxonomic identification of fossil material.

Turtles are unique amongst amniotes because they lay eggs composed of the mineral aragonite, as opposed to calcite present in all other amniotic eggs. When preserved in the fossil record, this unusual composition allows for definitive identification, at least at higher taxonomic levels (Hirsch, 2983). Several authors, however, have attempted to assign fossil eggs to more specific fossil or extant clades. For example, Hay (1908) compared macrofeatures of fossil eggs from the Oligocene of South Dakota to modern *Gopherus polyphemus* eggs and assigned those specimens to the fossil tortoise *Stylemys nebrascensis*. Similarly, Winkler and Sánchez-Villagra (2006) described rigid turtle eggs from the upper Miocene Urumaco Formation in Venezuela and referred the specimens to the taxonomic species *Bairdemys venezuelensis*.

With a few exceptions where multiple lines of evidence support identification (Lawver and Jackson, 2016), definitive identification requires fossil eggs containing embryonic remains or eggs preserved inside an adult turtle (Lawver and Jackson, 2014). Here, I describe one of eight eggs contained within the shell of a *Basilemys nobilis* specimen from the Kaiparowits Formation of Utah, interpreted previously as a gravid individual (pers. comm. Tyler Lyson, August, 2015). *Basilemys* is a genus of North American crown turtles within the extinct family Nanhsiungchelyidae (Fig. 1).

Taxonomic identification of these eggs is important because it allows eggshell microstructures to be linked to a specific taxon and permits investigation of evolutionary trends within clades. I further assess the referral of this egg to *Basilemys nobilis*, assign the egg to parataxonomy, and discuss its ecological and evolutionary implications. A detailed description of the adult bones and site taphonomy will be described elsewhere.

MATERIALS AND METHODS

In 2014 a field crew from the Denver Museum of Natural Sciences discovered six nearly complete to complete and articulated adult *Basilemys nobilis* skeletons in Grand Staircase-Escalante National Monument in southern Utah. An additional *Basilemys* at this locality contained eight eggs within its shell. Osteological elements associated with this fragmentary specimen included an unspecified number of cervical vertebrae, osteoderms, and pelvic elements.

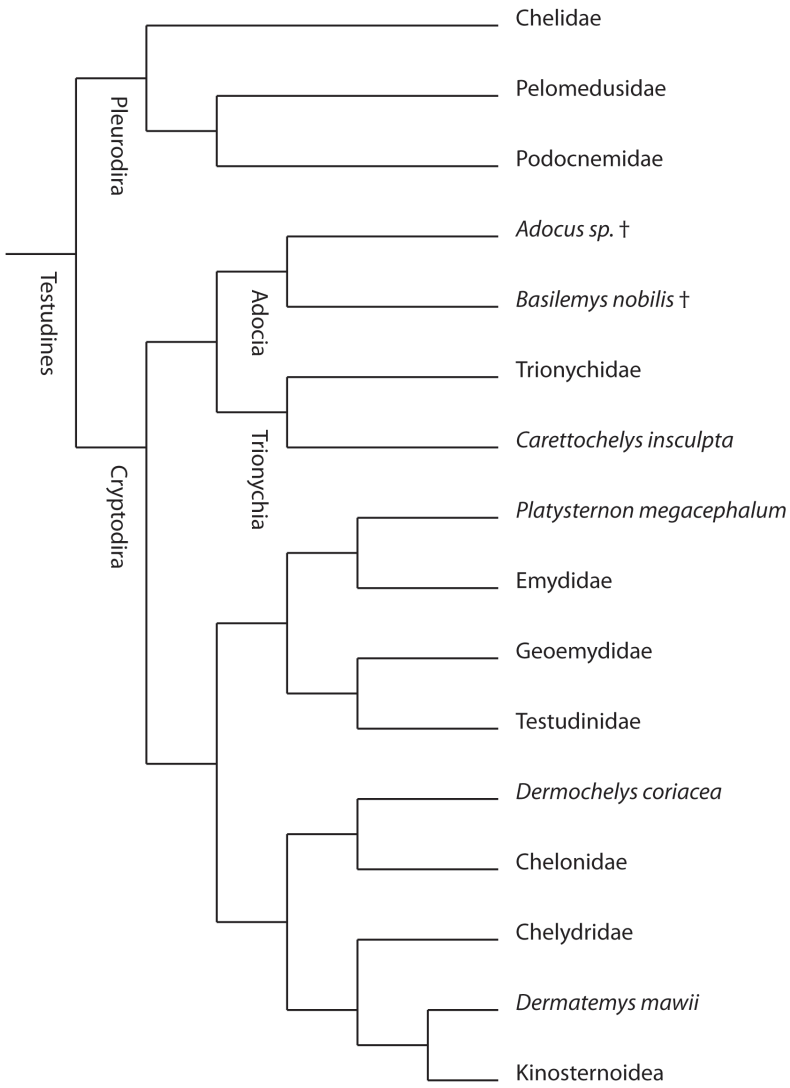


Figure 1. Phylogenetic relationships of Testudines. Modified from Joyce (1997) and Crawford et al. (2014).

Two eggshell fragments (DMNH EPV.91000) were removed from one of eight fossil eggs. These were broken and half of each fragment prepared as a radial thin section (Lamm, 2013) and studied under a Nikon Eclipse polarized light microscope. The second half of each eggshell was etched in acetic acid for about five seconds to better reveal their fine crystalline structures. The samples were then coated with 10 nm of gold and imaged

with a JEOL JSM-6100 SEM at 10 kV. Structural features (e.g., eggshell thickness, shell unit dimensions) were measured with ImageJ analysis software (http://imagej.nih.gov/ij/). Assessment of potential diagenetic alteration of the eggshell included examination under a Nikon Eclipse 50i microscope equipped with cathodoluminescence (CL). Egg mass was calculated using Hoyt's (1979) equation: Mass = 0.000548 x LB^2 where L and B represent egg length and breadth in millimeters. Since the adult material is fragmentary, carapace length for the adult female was estimated using the positive correlation between egg mass and adult carapace length (Elgar and Heaphy, 1989) and the regression line: y = 0.0568 x + 1.5811. The latter was derived from 63 species (Elgar and Heaphy, 1989: Appendix; see also Lawver and Jackson, 2017), where y is the egg mass and x is carapace length (r^2 = 0.666). Other species in the Elgar and Heaphy (1989:Appendix) analysis were omitted from my analysis because of missing data. It should be noted that values on the X and Y axes in Elgar and Heaphy (1989: Fig. 1) are physically impossible (i.e., log egg weight of 10^4 g = 10 kg of egg weight and carapace length of $10^{7.3}$ mm = 25 km). Their regression equation also contains a typographical error that results in unrealistically small carapace lengths (Lawver and Jackson, 2017).

Institutional Abbreviations—AM, Australian Museum, Sydney, New South Wales, Australia; **DMNH**, Denver Museum of Natural History, Denver, Colorado, U.S.A.; **HEC**, Hirsch Eggshell Catalogue, University of Colorado, Boulder, Colorado, U.S.A.; **IVPP**, Institue of Vertebrate Paleontology and Paleoanthropology, Beijing, China; **MCZ**, Museum of Comparative Zoology, Cambridge, Massachusetts, U.S.A.; **MOR**, Museum of the Rockies, Bozeman, Montana, U.S.A.; **NHMU**, Natural History Museum of Utah,

Salt Lake City, Utah, U.S.A.; **NHMUK** (formerly BMNH), The Natural History Museum, London, U.K.; **TMP**, Royal Tyrrell Museum of Paleontology, Alberta, Canada; **UCM**, University of Colorado Museum, Boulder, Colorado, U.S.A.; **ZMNH**, Zhejiang Museum of Natural History, Hangzhou, Zhejliang Province, China.

GEOLOGY

The Upper Cretaceous (late Campanian) Kaiparowits Formation is exposed in southern Utah within the boundaries of Grand Staircase-Escalante National Monument and measures 860 m thick (Fig. 2). Terrestrial rocks of the Wahweap and Canaan Peak formations underlie and overlie the formation, respectively (Titus et al., 2013). The Kaiparowits Formation was deposited in a remarkably short (~76.6–74.5 Ma) interval as a result of the rapidly subsiding Cordilleran foreland basin during the initiation of the Laramide uplift (Roberts, 2007; Roberts et al., 2013). The Kaiparowits Formation represents deposition in relatively wet, sub-humid alluvial to coastal plain environment and, as a result of sea level rise in the Western Interior Seaway, a tidally influenced river and estuarine systems (Roberts, 2007; Roberts et al., 2013). Lohrengel (1969) suggests that this environment resembled the modern Gulf Coast region of the United States. Nine different facies are interpreted for the Kaiparowits Formation including fluvial channel lags, storm-generated shell beds, meandering fluvial channels, anastomosing fluvial channels, crevasse splays and channels, shallow lakes, tidally influenced fluvial channels, floodbasin ponds, lakes and weakly developed paleosols, and backswamps and oxbow

lakes (Roberts, 2007: table 2). The Kaiparowits Formation preserves a vast paleoflora and -fauna that includes aquatic and terrestrial fossil vertebrates, invertebrates, leaves, wood, and palynomorphs, as well as trace fossils (Roberts et al., 2013).

SYSTEMATIC PALEONTOLOGY

Oofamily TESTUDOOLITHIDAE Hirsch, 1996 sensu Jackson et al. 2008

Oogenus *TESTUDOOLITHUS* Hirsch, 1996 sensu Jackson et al. 2008

(Figs. 3–4)

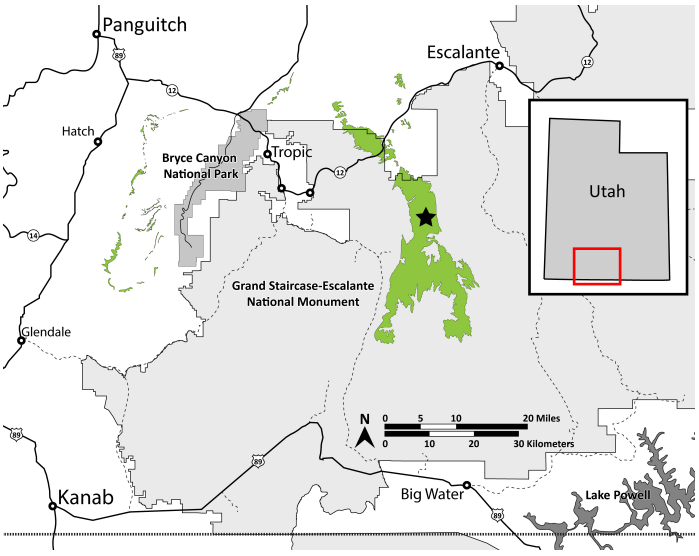


Figure 2. Locality map. Map of Grand Staircase-Escalante National Monument (light grey) showing the esposures of the Kaiparowits Formation in green. The star indicates the Uncle Charlie's Bone Orchard locality DMNH EPV.91000 was discovered. Inset is a map of Utah with box showing the location of Grand Staircase-Escalante National Monument. Map courtousy of Joe Sertich.

Material—(DMNH EPV.91000), eight partial to nearly complete eggs preserved inside of an adult *Basilemys nobilis*.

Locality and Age—Locality DMNH 4891, named Uncle Charlie's Bone Orchard within Grand Staircases-Escalante National Monument, south-central Utah, U.S.A., Upper Cretaceous (late Campanian), Kaiparowits Formation.

Diagnosis—Eggs differ from all other ootaxa in the following unique combination of characters: spherical turtle eggs 40 mm in diameter; 994 μm thick eggshell; shell unit height-to-width ratio of 4.4:1.

Description—The eight spherical eggs preserved within the *Basilemys nobilis* measure approximately 40 mm in diameter (Fig. 3); using Hoyt's (1979) equation, I estimate the original egg mass as 35 g. The 994 μm-thick rigid eggshell consists of a single layer of shell units composed of needle-like crystals of aragonite. Shell units comprising the eggshell are tightly packed with interlocking crystals and their height-to-

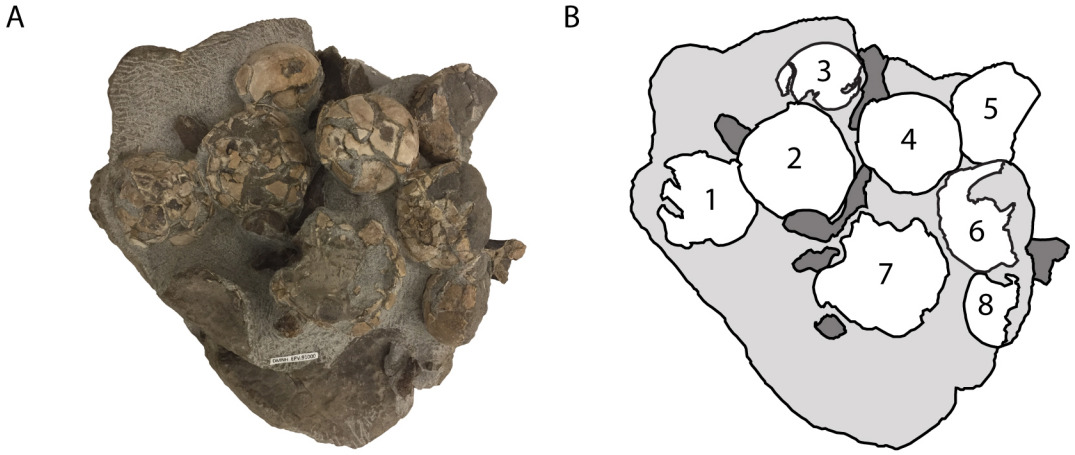


Figure 3. *Basilemys nobilis* eggs. A, photograph of eight partial to near complete eggs. B, line drawing of A. Eggs are numbered 1–8; unidentified bones are shown in dark grey and matrix is light grey.

width ratio equals 4.4:1. Thin sections reveal accretion lines in the lower 20% of the eggshell that are oriented concave-down (Fig. 4A). The eggshell displays a sweeping extinction pattern under crossed polarized light in the lower half and to a lesser extent in the upper half of the eggshell (Fig. 4B), and SEM reveals relatively straight, narrow pores that measure 42 μm in diameter and extend partially through the eggshell. This is likely due to the obliquely cut thin sections. The highly irregular outer eggshell surface likely results from recent weathering. Scanning electron microscopy imaging shows aragonite crystals with more distinct morphology in the outer third of the eggshell (Fig. 4C). The slightly irregular inner eggshell surface exhibits occasional craters or pits that are frequently infilled with authigenic calcite (Fig. 4C, D). These craters, along with pores and areas of the outer eggshell surface, exhibit bright orange to red luminescence under CL (Fig. 4E, F). Most of the eggshell displays a dull blue or dark green luminescence under CL.

DISCUSSION

Despite some alteration of the eggshell, SEM and polarized light microscope examination show needle-like aragonite crystals that allow definitive assignment of DMNH EPV.91000 to Testudines (Hirsch, 1983). The dull blue to dark green luminescence of the eggshell under CL resembles that of extant (Lawver unpublished data) and minimally altered fossil turtle eggshell (Lawver and Jackson, 2016; 2017). Blue luminescence occurs in the absence of activator elements such as Fe^{2+} (Wendler et al., 2012), and green

luminescence is reported from biogenic aragonite present in fossil and recent fish otoliths, marine bivalves, gastropods, cephalopods, and corals (Barbin, 2000; 2013). In contrast, areas of bright orange luminescence result from incorporation of Mn^{2+} into authigenic calcite crystals (Marshall, 1988). The overall appearance of the eggshell differs from completely altered specimens such as Late Cretaceous turtle eggs from Madagascar, which reveal bright orange to red luminescence throughout the entire eggshell (Lawver et al., 2015: Fig. 3D; Chapter 2: Fig. 3D). This suggests that significant diagenetic recrystallization of DMNH EPV.91000 occurred only in areas with bright orange luminescence. The aragonite mineral and the location of the eight eggs inside the shell of a *Basilemys nobilis* further support the previous interpretation of this specimen as a gravid adult.

Definitively identified fossil turtle eggs associated with adults are rare and only two gravid specimens have previously been described in the literature (Table 1). Zelenitsky et al. (2008) report a gravid *Adocus* specimen from the late Campanian Dinosaur Park Formation of Alberta, Canada that preserves at least five eggs. Additionally, the authors used a least-square regression to predict a maximum clutch size of 19 eggs for the gravid *Adocus* and an estimated carapace length of 405 mm (Zelenitsky et al., 2008). Knell et al. (2011) describe a second gravid *Adocus* specimen from the Kaiparowits Formation of Utah that includes at least two eggs. A *Basilemys variolosa* specimen from the Dinosaur Park Formation of Alberta represents the only other published account of a gravid individual; however, the eggshell microstructure of this

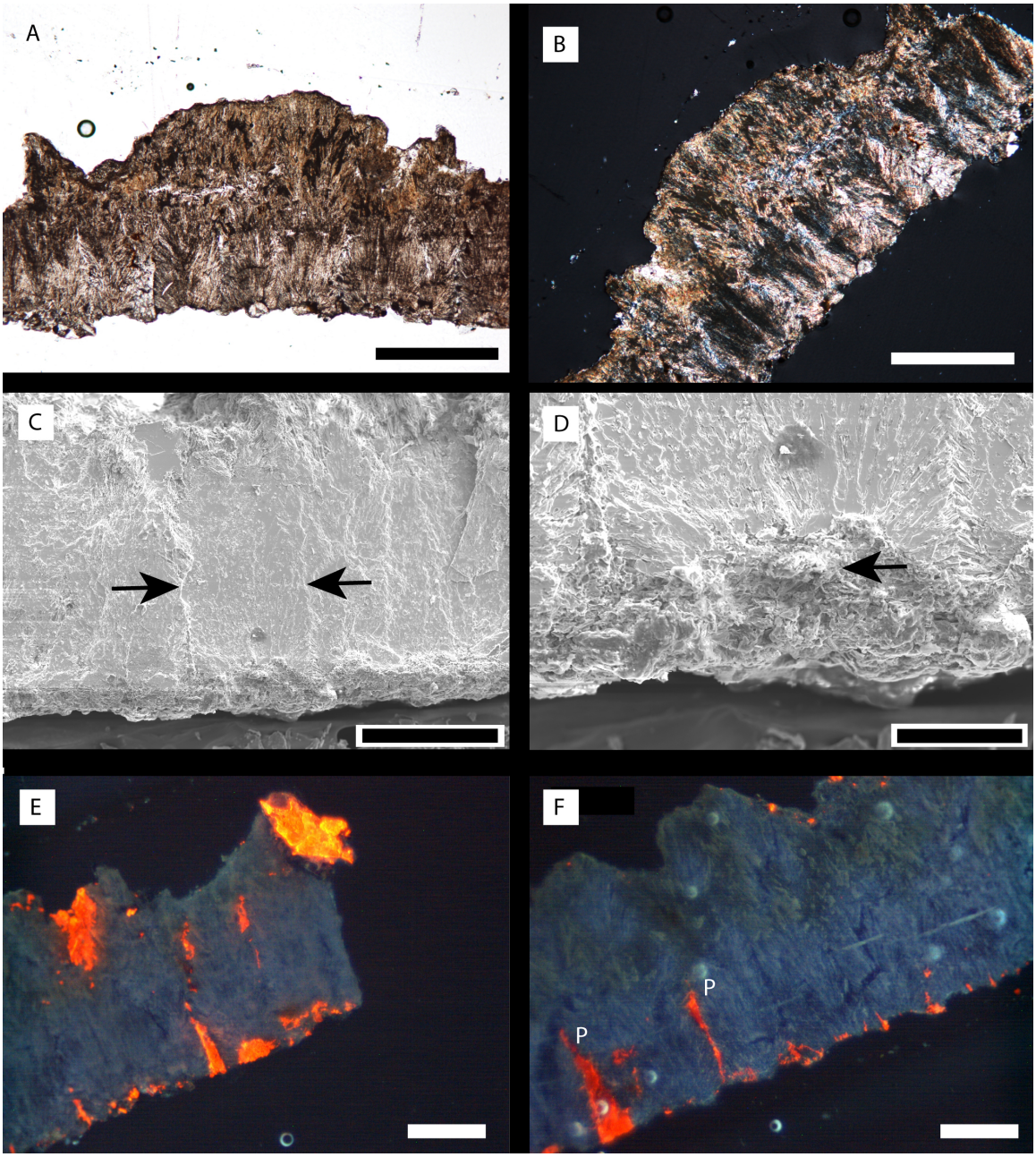


Figure 4. *Basilemys nobilis* eggshell (DMNH EPV.91000) microstructure. A, Radial thin section (DMNH EPV.91000) in plane polar light. Note highly irregular, weathered surface. B, same as A in cross-polarized light and rotated 45° demonstrating sweeping extinction pattern evident in lower portion of the shell. C, SEM radial micrograph of DMNH EPV.91000. D, enlargement of C showing the lower shell unit and possible cavity that contained the former organic core. E, cathodoluminescence showing dull blue and dark green luminescence indicating minimal alteration, and areas of bright orange luminescence indicating replacement by authigenic calcite. F, a second eggshell fragment under cathodoluminescence showing calcite infilled pores (P). Scale bars equal 500 µm (A, B), 200 µm (C), 60 µm (D), 250 µm (E,F).

specimen has yet to be described or assigned to parataxonomy (Braman and Brinkman, 2009).

Parataxonomy

Hirsch's (1996) parataxonomic classification of fossil turtle eggs and eggshells includes two oofamilies, Testudoflexoolithidae and Testudoolithidae. Flexible eggs with loosely abutting shell units comprise the former, whereas the latter includes rigid eggs with tightly packed shell units. The rigid structure of the eggshell excludes the eggs within the *Basilemys nobilis* from Testudoflexoolithidae (Table 2). The following additional characters permit assignment of the eggs to *Testudoolithus*, within the Testudolithidae: spherical shape, shell unit height greater than width, and eggshell thickness greater than 0.2 mm.

Among oospecies assigned to Testudoolithidae, the *Basilemys nobilis* egg examined in this study differs from *Chelonoolithus baemi* Kohring 1998, *Haininchelys curiosa* Schleich et al. 1988, *Testudoolithus hirschi* Kohring 1999, *T. rigidus* Hirsch 1996, and *T. lordhowensis* Lawver and Jackson 2016 by its thicker eggshell and greater height-to-width ratio and *T. zelenitskyae* by its thicker eggshell. The height-to-width ratio of the *Basilemys* egg falls within the range of *Emydoolithus laiyangensis* Wang et al. 2013, but differs in egg shape and eggshell thickness. Additionally, the egg is similar to *Testudoolithus jiangi* Jackson et al. 2008 in eggshell thickness but displays a greater height-to-width ratio. Despite the unique combination of characters present in this specimen (i.e., large, thick-shelled spherical eggs with shell unit height-to-width ratio of

4.42:1), assignment to a new oospecies awaits publication because a doctoral dissertation fails to satisfy the restricting criteria of the International Committee on Zoological Nomenclature (Article 8.1.3), namely, simultaneously obtainable copies and wide distribution.

Physiology and Ecology

In addition to providing the first taxonomic assignment of eggs to *Basilemys nobilis*, the specimen also provides additional information. Although the eggshell is unusually thick, there is no evidence of multiple layers that would indicate a pathological condition (Lawver and Jackson, 2017, Fig. 3D-F). Further, the presence of the eggs inside an adult individual suggests the possibility that death occurred prior to complete eggshell deposition. Therefore, the 994 μm thick eggshell likely represents a minimum thickness. Thinner than normal eggshell resulting from termination of eggshell formation is also described from gravid *Adocus* sp. specimens from Canada and Utah (Zelenitsky et al., 2008; Knell et al., 2011). Nevertheless, *Basilemys nobilis* eggshell exceeds that of all other turtle ootaxa except for *T. jiangi* (0.7–1.0 mm) and an undescribed specimen from China, (1.5 mm, Pers. Comm., F. Jackson, November, 2016).

The presence of dissolution craters at the base of the shell units in the *Basilemys nobilis* eggshell is somewhat surprising. Similar features occur in extant turtle eggs as a result of osteogenesis. This process typically begins after stage 17 of embryonic development in *Apalone spinifera*, *Macrochelys temminckii*, and *Chelydra serpentina* (Sheil, 2003; 2005; Sheil and Greenbaum, 2005) and as late as stage 24 in *Eretmochelys imbricata* (Sheil,

2013). At this stage of development, the embryo begins to mobilize calcium ions from the eggshell, which results in small resorption craters at the base of most shell units. Fossil eggshells preserving these features are often interpreted as representing hatched eggs or that the egg was viable at the time of death but lack embryonic remains due to preservational biases (Sahni et al., 1994; Agnolin et al., 2012). Therefore, because oviposition had not yet occurred, these features in the *Basilemys nobilis* egg likely represent an artifact of eggshell dissolution and/or replacement of the organic core and original aragonite during diagenesis. The bright orange luminescence of the infilling material further supports this interpretation (Fig. 4E,F).

The size, shape, and estimated mass (35 g) of the *Basilemys nobilis* egg allow some anatomical inferences. Extant turtles that produce spherical eggs often exhibit large body size and produce large clutches (Elgar and Heaphy, 1989). This is due to the capability of the uterus in large taxa to hold and shell many spherical eggs at a time (Ewert, 1979; Iverson and Ewert, 1991). Because of the fragmentary condition of the specimen, I used the positive correlation between egg mass and adult carapace length (Elgar and Heaphy, 1989) to estimate a carapace length of 58.8 cm for the gravid *Basilemys*. However, this represents a minimum estimate due to the slightly greater egg density of turtle eggs compared to bird eggs (Lawver and Jackson, 2017). Additionally, this size estimate is relatively small for *Basilemys nobilis* because carapace lengths of nearly one meter typify this species (Hutchison et al., 2013). This may indicate that the specimen was a younger individual or the presence of sexual dimorphism in this taxon,

Table 1: Egg/eggshell characteristics of taxonomically identified specimens.

Specimen	Material/Identification	Formation	Length x width (mm)	Eggshell thickness (mm)	Shell unit height:width
MOR 710[3]	Weathered *Adocus* clutch of at least 16 eggs	Judith River	34–39	0.66–0.76	3.15:1–5.5:1
NHMU 16868 (LBA-06-7)[1]	Gravid *Adocus* sp. preserving at least two eggs	Kaiparowits	35	0.25–0.28	2.5:1
TMP 1999.63.2[2]	Gravid *Adocus* sp. preserving at least five eggs	Oldman	35–40	0.73–0.81	2.5:1
TMP 2008.27.1[2]	*Adocus* clutch of at least 26 eggs	Dinosaur Park	40x42 40x43	0.5–0.65	3.5:1
DMNH EPV.91000	Gravid adult preserving at least eight eggs	Kaiparowits	40	>0.99	4.4:1

Note: All specimens are Late Cretaceous, late Campanian in age and have a spherical egg shape.
References: 1, Knell et al. (2011); 2, Zelenitsky et al. (2008); 3, Lawver and Jackson (2017).

Table 2: List of turtle ootaxa and their distinguishing characteristics.

Ootaxon	Holotype/Material	Geographic and temporal distribution	Egg shape	Length x width (mm)	Eggshell thickness (mm)	Shell unit height:width
Testudoflexoolithus agassizi[1]	MCZ 2810/HEC 49: Eggshell fragments	Florida, USA; Pleistocene	-	-	0.06–0.1	1:1 or 2:3
Testudoflexoolithus bathonicae[1,2]	MB(NH)37983/ HEC 186: An egg imbedded in matrix	England; Bathonian, Middle Jurassic	Ellipsoidal	48 x 26	0.2–0.25	1:1
Chelonoolithus braemi[3]	Guimarota 98-2: Eggshell fragments	Portugal; Kimmeridgian, Upper Jurassic	-	-	0.2	1:1
Emydoolithus laiyangensis[4]	IVPP V18544: A nearly complete egg	Shandong Province, China; Upper Cretaceous	Elongate	91 x 22	0.4–0.5	2:1 to 5:1
Haininchelys curiosa[5]	-: Eggshell fragments	Belgium; Upper Paleocene	-	-	0.25–0.3	1.2:1 to 2.3:1
Testudoolithus hirschi[6]	-: Eggshell fragments	Portugal; Kimmeridgian, Upper Jurassic	-	-	0.15	3:1
Testudoolithus jiangi[7,8]	ZMNH M8713: A clutch of 23 eggs	Zhejiang Province, China; Albian, Early Cretaceous	Spherical	35–52	0.7–1.0	2.5:1 to 3:1
Testudoolithus rigidus[1]	UCM 55806/ HEC 425: Half of an egg	U.S.A., Europe, Africa; Lower Cretaceous - Pliocene	Spheroidal	42 x 47	0.22–0.24	2:1
Testudoolithus lordhowensis[9]	AM F82183: A clutch of at least 10 eggs	Lord Howe Island, Australia; Pleistocene	Spherical	53.9	0.8	1.2:1
Testudoolithus zelenitskyae[10]	MOR 710: A clutch of at least 16 eggs	U.S.A. and Canada; Campanian, Upper Cretaceous	Spherical	34–39	0.66–0.76	3.15:1 to 5.5:1
Basilemys nobilis	A nearly complete egg preserved within a gravid individual	U.S.A.; Campanian, Upper Cretaceous	Spherical	40	0.994	4.42:1

Modified from Lawver and Jackson (2017)
References: 1, Hirsch (1996); 2, Buckman (1859); 3, Kohring (1998); 4, Wang et al. (2013); 5, Schleich et al. (1988); 6, Kohring (1999); 7, Fang et al. (2003); 8, Jackson et al. (2008); 9, Lawver and Jackson (2016); 10, Lawver and Jackson (2017).

characterized by smaller females compared to males. Several extant tortoises (e.g., *Centrochelys sulcata, Chelonoidis nigra, Chersina angulate, Dipsochelys elephantine* as well as others) are often used for comparison with *Basilemys* and convergently exhibit sexual dimorphism (Bonin et al., 2006). *Basilemys* is also tortoise-like in its large, thick and somewhat domed shell, stout limbs covered in osteoderms, and features of its triturating surface. The latter are consistent with an herbivorous diet (Brinkman, 1998; 2003; Knell, 2012).

Evolutionary Relationships

Taxonomic identifications provided by this and other gravid *Basilemys* and *Adocus* adults from Utah (Knell et al., 2011) and Alberta, Canada (Zelenitsky et al., 2008) allow more accurate interpretations of the evolution of reproductive attributes of these and closely related taxa. These two extinct taxa share a close phylogenetic relationship to each other, as well as to the extant clade Trionychia (Carettochelyidea and Trionychidae). *Basilemys nobilis* and *Adocus* sp. eggs have relatively thicker eggshell (994 µm and 660–760 µm, respectively) than *Carettochleys insculpta* and trionychid eggs from the genus *Apalone* (430–450 µm and 88–192 µm, respectively), resulting in greater shell unit height-to-width ratios in the extinct taxa. This thicker eggshell may represent an adaptation to less humid environments of *Basilemys nobilis* and *Adocus,* as proposed by Jackson et al. (2008) for *Testudoolithus jiangi* from China. Therefore, these taxa may have constructed their nests in well-drained floodplains characterized by seasonally dry conditions. In addition, *Basilemys nobilis, Adocus sp.,* and trionychids are characterized

by spherical eggs and rigid eggshell, which may represent synapomorphies for this clade. Future work should include description of the Canadian gravid *Basilemys variolosa* and other recently discovered fossil gravid turtles will further our understanding of the early evolution of reproduction in turtles.

CONCLUSIONS

The *Basilemys nobilis* eggs comes from the Campanian (Upper Cretaceous) Kaiparowits Formation within Grand Staircase-Escalante National Monument of southern Utah and consists of eight partial to nearly complete spherical eggs preserved within the shell of an adult. The 994 μm thick eggshell is comprised of a single layer of tightly interlocking aragonite shell units with a height-to-width ratio of 4.4:1. Cathodoluminescence demonstrates that the majority of the eggshell is minimally altered with dull blue to dark green luminescence, whereas secondary calcite (present at the inner and outer surfaces and within some pores) exhibits bright orange color. The adult carapace length is estimated to be 58.8 cm. Comparison with the closely related *Adocus* sp. eggs from Alberta and Utah and those of extant Trionychia suggest that spherical eggs with rigid eggshell represent a synapomorphy of this clade and thick eggshell a synapomorphy of Adocia. Further description of the Canadian gravid *Basilemys variolosa* specimen will allow comparison of eggshell features within a single genus.

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CHAPTER SIX

A FOSSIL EGG CLUTCH FROM THE STEM TURTLE *MEIOLANIA PLATYCEPS*: IMPLICATIONS FOR THE EVOLUTION OF TURTLE REPRODUCTIVE BIOLOGY

Contributions of Author and Co-Author

Manuscript in Chapter 6

Author: Daniel R. Lawver

Contribution: Conceived and implemented the study design. Collected and analyzed data. Wrote first draft of the manuscript.

Co-Author: Frankie D. Jackson

Contributions: Advised on the study design. Edited and provided feedback on early drafts of the manuscript.

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CHAPTER SIX

A FOSSIL EGG CLUTCH FROM THE STEM TURTLE *MEIOLANIA PLATYCEPS*: IMPLICATIONS FOR THE EVOLUTION OF TURTLE REPRODUCTIVE BIOLOGY

DANIEL R. LAWVER, [*,1] and FRANKIE D. JACKSON[1]

[1]Department of Earth Sciences, Montana State University, Bozeman, Montana, 59717, U.S.A., danlawver@gmail.com; frankiej@montana.edu

[*]Corresponding author

ABSTRACT—A fossil egg clutch from the Pleistocene of Lord Howe Island, Australia belongs to the stem turtle *Meiolania platyceps*, which we assigned to *Testudoolithus lordhowensis* oosp. nov. This ootaxon is diagnosed by the following unique combination of characters: large spherical eggs (53.9 mm diameter), 800 µm thick eggshell, and barrel-shaped shell units with height-to-width ratio of 1.2:1. Thin sections and scanning electron microscopy demonstrate that these eggs are composed of radiating acicular aragonite crystals. This mineral composition first evolved either before the split between Meiolaniformes and crown Testudines or prior to *Proterochersis robusta*, the earliest known stem turtle. A calculated gas conductance of 170.27 mg H_2O day^{-1} $Torr^{-1}$ for *Meiolania platyceps* eggs compares closely with that of two extant tortoise eggs. This value and the presence of at least two superimposed egg layers within the clutch indicate that *Meiolania platyceps* deposited its eggs inside an excavated hole nest. This nesting strategy likely evolved no later than the Early to Middle Jurassic.

INTRODUCTION

Turtles are unique amongst amniotes because their eggshells consist of the mineral aragonite instead of calcite that characterizes the eggshell of all other living and extinct groups (Hirsch, 1983). However, it remains unknown when this unusual eggshell composition first evolved. The oldest fossil turtle eggs come from the Middle Jurassic Great Oolite of England (Buckman, 1859; Hirsch, 1996). These aragonitic eggs correspond roughly to the evolution of the turtle crown group, as estimated from the fossil record (Danilov and Parham, 2006; Joyce, 2007; Anquetin et al., 2009) and molecular calibration (Joyce, Parham et al., 2013). However, it is equally plausible that these Middle Jurassic specimens belonged to a turtle representing either a stem or crown taxon. Therefore, fossil turtle eggs of a stem taxon of known taxonomic affinity would permit more accurate assessment of the origin and evolution of this unique eggshell composition, as well as the evolution of turtle nesting behavior.

Here, we describe a fossil turtle egg clutch from the Pleistocene of Lord Howe Island, Australia. These large spherical eggs occur with the skeletal remains of the stem turtle *Meiolania platyceps* at several localities on the island. This large-bodied (carapace length at least 66 cm) terrestrial taxon is known from hundreds of specimens that represent nearly the entire skeleton. In the absence of other turtle species on Lord Howe Island, Anderson (1925) and Gaffney (1983, 1996) assigned the eggs to *Meiolania platyceps* Owen, 1886.

The objectives of our paper are to: (1) describe the fossil clutch and eggshell microstructure; (2) compare the eggshell structure to modern and fossil turtle eggs in order to assign them to parataxonomy and further test their taxonomic identification; (3) calculate gas conductance for the first time for a fossil turtle egg; and (4) discuss the significance of *Meiolania* eggs in the evolution of turtle reproductive biology. Below, we provide a brief review of turtle taxonomy in order to provide context for the described specimen.

Institutional Abbreviations—AM, Australian Museum, Sydney, New South Wales, Australia; **BMNH**, The Natural History Museum, London, United Kingdom; **ES**, Department of Earth Sciences, Montana State University, Bozeman, Montana, U.S.A.; **HEC**, Hirsch Eggshell Catalogue, University of Colorado, Boulder, Colorado, U.S.A.; **IVPP**, Institute of Vertebrate Paleontology and Paleoanthropology, Beijing, China; **MCZ**, Museum of Comparative Zoology, Cambridge, Massachusetts, U.S.A.; **UCM**, University of Colorado Museum, Boulder, Colorado, U.S.A.; **ZMNH**, Zhejiang Museum of Natural History, Hangzhou, Zhejiang Province, China.

REVIEW OF TURTLE TAXONOMY

The turtle crown clade, Testudines is a monophyletic group consisting of all extant taxa (Joyce, Parham et al., 2013), whereas the turtle stem lineage includes all taxa that are phylogenetically positioned outside of Testudines. Collectively, Testudines and the stem lineage form the clade Testudinata, which is defined as the clade including the

first amniote with a fully developed turtle shell (Fig. 1; Joyce, 2015; Joyce et al., 2004). *Proterochersis robusta* from the Late Triassic (Middle Norian) of Germany represents the oldest stem turtle (Fraas, 1913; Joyce, Schoch et al., 2013). Intermediate stem taxa that lack a fully developed turtle shell (e.g., carapace and plastron) include *Eunotosaurus africanus* Seeley, 1892 and *Pappochelys rosinae* Schoch and Sues, 2015. These

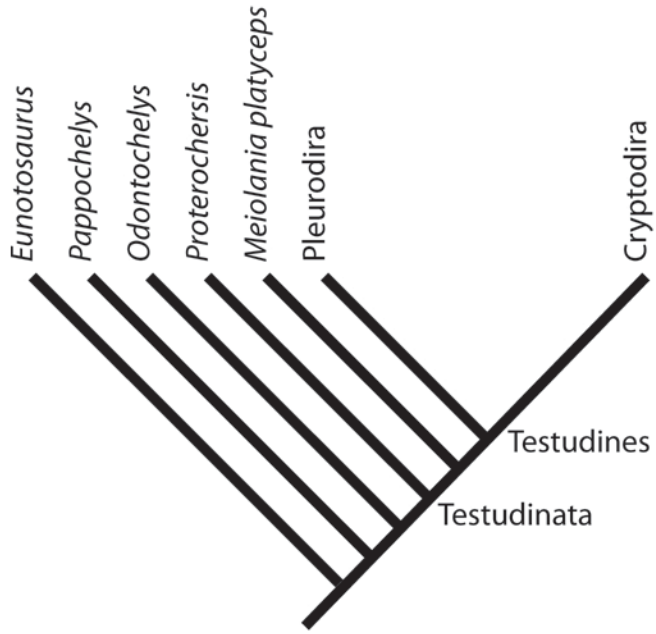


Figure 1. Simplified phylogenetic tree of stem and crown turtle relationships, following Anquetin (2012), Joyce et al. (2011), and Schoch and Sues (2015).

intermediate taxa are phylogenetically outside of Testudinata and come from the Middle Permian (Guadalupian) of South Africa and Late Triassic (Ladinian) of Germany (Lyson et al., 2013; Schoch and Sues, 2015). A third intermediate stem taxon, *Odontochelys semitestacea* Li et al., 2008 lacks only the carapace and comes from the Late Triassic (Norian) of China.

Meiolania platyceps, a member of the clade Meiolaniformes, represents one of the most recent stem turtle extinctions. As defined by Sterli and de la Fuente (2013), Meiolaniformes consists of all taxa more closely related to *Meiolania platyceps* than to Cryptodira (hidden-neck turtles) and Pleurodira (side-neck turtles). Meiolaniformes is typically resolved as either the sister clade to Testudines or to the clade including Testudines and *Kallokibotion bajazidi* (Anquetin, 2012; Joyce et al., 2011; Sterli et al., 2015). Therefore, *Meiolania platyceps* is phylogenetically positioned near the top of the turtle stem lineage (Fig. 1).

MATERIALS AND METHODS

Fifteen eggshell fragments (donated to the Department of Earth Sciences, Montana State University, Bozeman, Montana) came from unspecified eggs within the clutch (AM F82183), which is housed in the Australian Museum Sydney, New South Wales, Australia. These fragments detached from eggs prior to or after collection of the specimen. Radial (ES 538) and tangential (ES 537) thin sections (30 µm thick) were made following standard procedures (Lamm, 2013) and examined under a Nikon Eclipse LV100POL light (Nikon Metrology, Inc., Tokyo, Japan) microscope. Additionally, tangential thin sections were made from eggshells of two extant tortoise (Testudinidae) taxa (*Kinixys erosa,* ES 535 and *Indotestudo elongata*, ES 536) in order to compare their gas conductance values with that calculated from a fossil egg. Assessment of potential diagenetic alteration of *Meiolania* eggshell included a Nikon eclipse 50i (Nikon

Metrology, Inc., Tokyo, Japan) microscope equipped with cathodoluminescence (CL). Glue was removed from seven fossil eggshell fragments and these were mounted on an aluminum stud, coated with 10 nm of gold, and imaged under a JEOL JSM-6100 scanning electron microscope (SEM; JEOL U.S.A., Inc., Peabody, Massachusetts, U.S.A.) at 10 kV. Images include the inner and outer surfaces, as well as radial cross sections of the eggshell. Photomicrographs were taken using a Nikon Digital Sight DS-5Mc camera and microstructural features measured with ImageJ analysis software (Rasband, 1997).

The following simplified equation provided an estimate of gas conductance for both fossil and extant eggs in this study: $G_{H2O} = (2.109 \text{ mg } H_2O \text{ day}^{-1} \text{ Torr}^{-1} \text{ mm}^{-1}) \text{ x } (A_p/L_s)$, where A_p is total pore area in mm^2 and L_s is average pore length in mm (Varricchio et al., 2013). Surface area of *Meiolania platyceps* eggs were calculated using the equation for a sphere: $A = 4 \pi r^2$, where r is the radius. The following equations were used to calculate the surface area of *Kinixys erosa* and *Indotestudo elongata* eggs. Surface area of prolate ellipsoid = $2\pi(1 + A \text{ x } \arcsin(m)/[mB]) \text{ x } B^2$, where A is the radius of the long axis, B is the radius of the short axis, and $m = \sqrt{(1 - [B/A]^2)}$. Surface area of an ellipsoid $\approx 4 \pi([(A^p B^p) + (A^p C^p) + (B^p C^p)]/3)^{1/p}$, where A is the radius of the long axis, B is the radius of the width, C is the radius of the depth, and p = 1.6075. Additionally, to avoid the assumptions of incubation temperature and associated gas constants, we calculated effective porosity by dividing the total pore area by the average pore length (Varricchio et al., 2013). Caverns between shell units were avoided in order to prevent artificial inflation of these calculations. Egg mass was calculated using Hoyt's

(1979) equation: Mass = 0.000548 x LB^2, where L is egg length in mm and B is egg breadth in mm. Note that in general rigid-shelled turtle eggs have a density of 1.126, whereas birds have a density of 1.03–1.09 (Iverson and Ewert, 1991). This indicates that the egg mass and carapace lengths calculated here represent minimum estimates. Taxonomy and terminology used herein follows Joyce, Parham et al. (2013).

GEOGRAPHIC AND GEOLOGIC CONTEXT

Lord Howe Island lies approximately 580 km off of the east coast of Australia, surrounded by the Tasman Sea (Fig. 2) and supports a population of approximately 360 people (Australian Bureau of Statistics, 2011). About 11 km long and 2 km wide, the crescent-shaped island represents the eroded remnants from two episodes of volcanic activity in late Miocene time, approximately 6.9 and 6.3 million years ago (Orchard and Wilson, 1994). Fossil eggs come from several localities on Lord Howe Island (Gaffney, 1983), and the egg clutch described here (AM F82183) was excavated from a cliff at Neds Beach. Sedimentary strata at this locality (formerly known as Neds Beach Calcarenite) are Middle to Late Pleistocene in age. The skeletal carbonate eolianite and beach calcarenite are now divided into two formations based on lithostratigraphy (Brooke et al., 2003). The Searles Point Formation comprises eolianite units bounded by clay-rich paleosols, and outcrops exhibit karst features such as caves and subaerially exposed relict speleothems. The Neds Beach Formation overlies the Searles Point Formation and consists of dune and beach units bounded by weakly developed fossil soil horizons. Other

than fossil eggs and *Meiolania platyceps* bones, avian osteological remains are the only other vertebrate fossils known from the Neds Beach Formation (Gaffney, 1983).

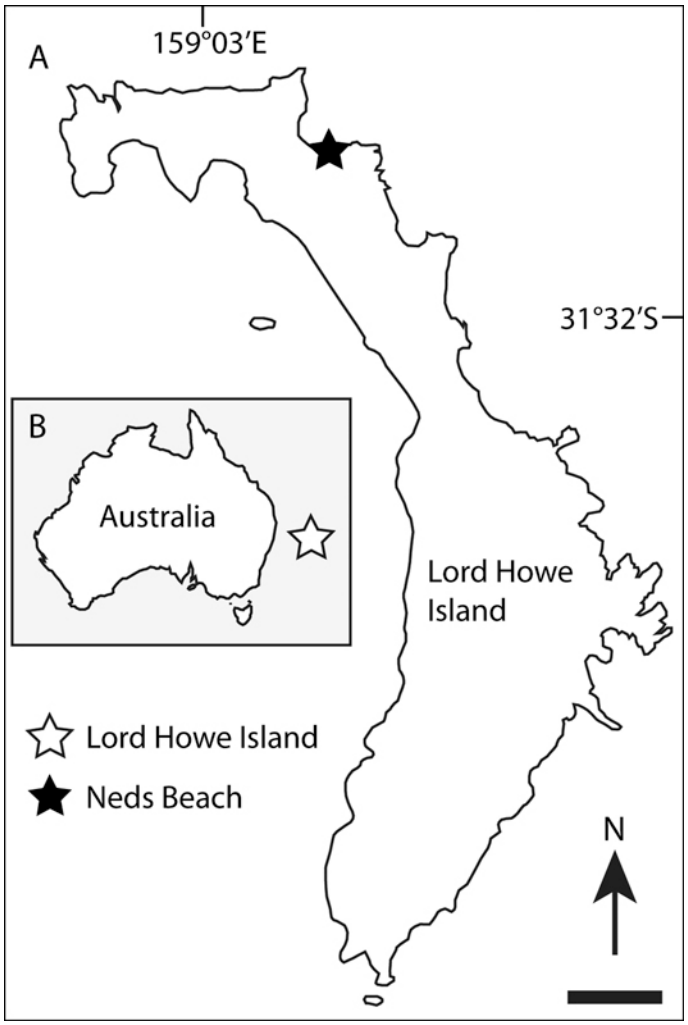


Figure 2. Locality maps. A, map of Lord Howe Island, with black star indicating the Neds Beach locality where AM F82183 was collected. B, map of Australia, with Lord Howe Island indicated by the white star. Scale bar equals 1 km.

SYSTEMATIC PALEONTOLOGY

Oofamily TESTUDOOLITHIDAE Hirsch, 1996 sensu Jackson et al. 2008

Testudoolithidae Fang, Liwu, Yangen, and Liangfeng, 2003:520, pl. II, figs. 7–9.

Oogenus *TESTUDOOLITHUS* Hirsch, 1996 sensu Jackson et al. 2008

Tiantaioolithus Fang, Liwu, Yangen, and Liangfeng, 2003:520, pl. II, figs. 7–9.

Oospecies *TESTUDOOLITHUS LORDHOWENSIS*, oosp. nov.

(Fig. 3)

Holotype—AM F82183, a clutch containing at least 10 eggs.

Etymology—The specific name *lordhowensis* refers to Lord Howe Island where the specimen was collected.

Type Locality and Age—Neds Beach, Lord Howe Island, Australia; Middle to Late Pleistocene, Neds Beach Formation.

Diagnosis—*Testudoolithus lordhowensis* eggs differ from all other oospecies in the following unique combination of characters: large spherical turtle eggs 53.9 mm in diameter; 800 μm thick eggshell and barrel-shaped shell units with a height-to-width ratio of 1.2:1. Differential diagnosis included in the Comparisons section below.

Distribution—Pleistocene, Neds Beach Formation, Lord Howe Island, Australia (Fig. 2).

Description

The 23 cm by 18 cm fossil egg clutch (AM F82183) consists of at least ten large (53.9 mm) spherical eggs that occur in at least two superimposed layers within a calcarenite (Fig. 3A). The 800 µm thick eggshell consists of a single layer of shell units composed of needle-like crystals of aragonite that radiate outward in all directions from nucleation points 75 µm above the inner eggshell surface (Fig. 3B-E). The barrel-shaped shell units are tightly packed and their height-to-width ratio equals 1.2:1. Circular features occasionally occur at the inner eggshell surface and measure about 60–68 µm in diameter; these likely correspond to the size and location of the former organic core (Fig. 3B). Cathodoluminescence of *Meiolania platyceps* eggshell demonstrate a dull blue luminescence, with areas of greatest luminescence within accretion lines (Fig. 3D). A thin layer of glue also luminesces blue at the exterior surface of the eggshell (Fig. 3D). The specimens show no evidence of orange and red luminescence.

In the basal 50–75% of eggshell, caverns or elongate spaces occasionally separate the shell units (Fig. 3B-C). Numerous pores (~1 pore/mm^2) are identified in the tangential thin section and are round to irregular in cross section, with a diameter of 60–320 µm (Fig. 3G). The eggshell exhibits a sweeping extinction pattern under cross-polarized light (Fig. 3C). Accretion lines are concave down in the lower half of the eggshell but transition to parallel horizontal lines in the upper half (Fig. 3B-C). Scanning electron microscopy shows planes of weakness that coincide with these horizontal accretion lines

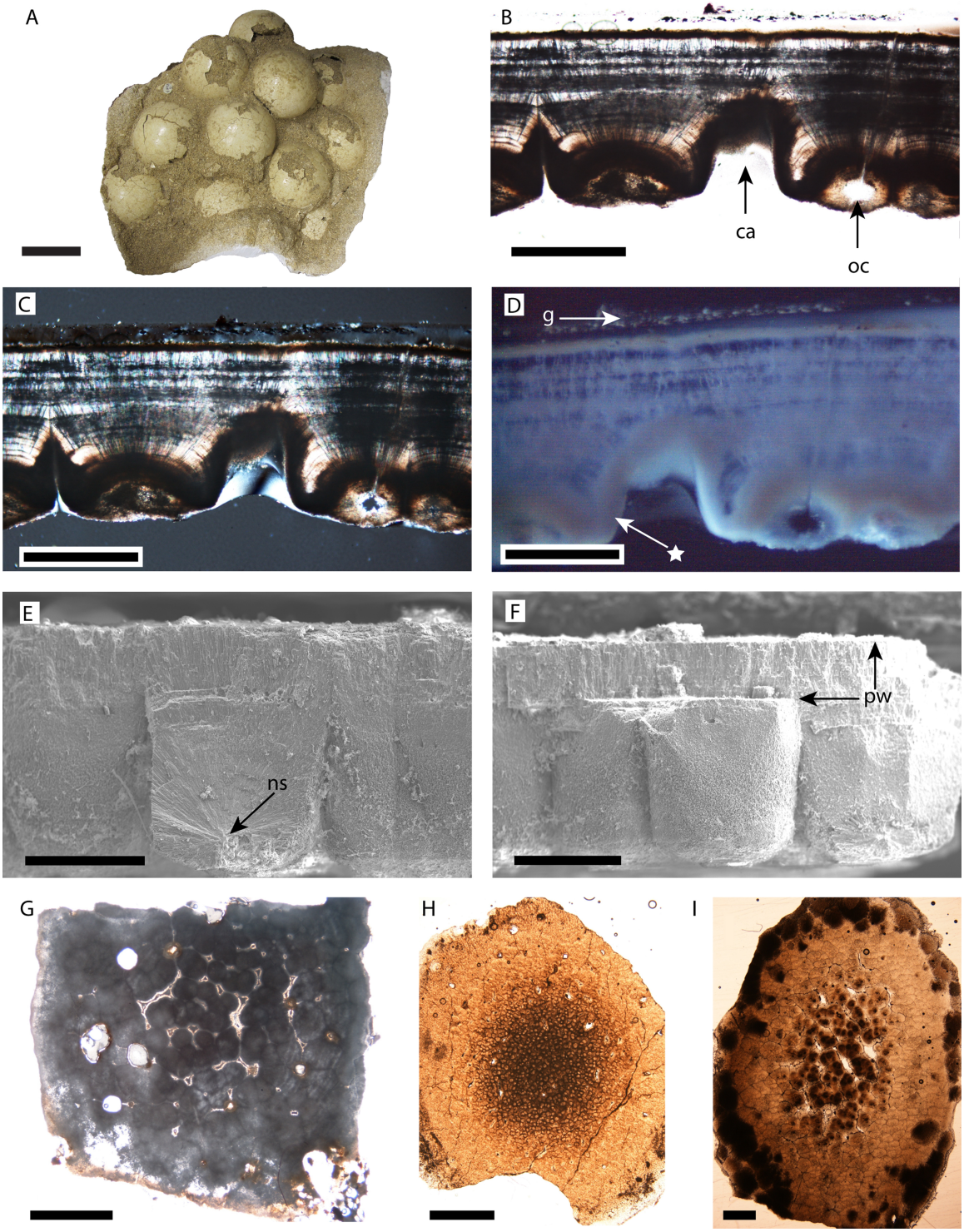


Figure 3. *Testudoolithus lordhowensis* and extant tortoise eggshell. **A**, Egg clutch (AM F82183, prepared upside-down). Scale bar equals 5 cm; **B**, radial thin section (ES 538) in plane polar light. Scale bar equals 500 µm; **C**, same as B in cross-polarized light demonstrating sweeping extinction pattern. Scale bar equals 500 µm; **D**,

cathodoluminescence of *Meiolania platyceps* eggshell showing dull blue luminescence, with darker color often associated with horizontal accretion lines. Reduced luminescence along the inner shell surface may result from increased organic matter or Fe^{2+} and indicated by the star. Scale bar equals 400 μm; **E**, radial SEM micrograph depicting a nucleation site and radiating aragonite crystals within a shell unit. Note minor alteration in center shell unit at the same level as shell separation in E; however, needle-like crystals extend through the upper portion of the eggshell. Scale bar equals 300 μm; **F**, radial SEM micrograph of eggshell exhibiting a plane of weakness associated with parallel accretion lines. Scale bar equals 300 μm; **G**, tangential thin section (ES 537) of *Meiolania* eggshell showing abundant pores. Note that the three large pores on the left reflect greater shell curvature and were excluded from gas conductance calculations. Scale bar equals 2 mm; **H**, tangential thin section (ES 535) of *Kinixys erosa* eggshell showing pores. Scale bar equals 2 mm; **I**, tangential thin section (ES 536) of *Indotestudo elongata* eggshell showing pores. Scale bar equals 1 mm. **Abbreviations**: **ca**, cavern; **g**, glue; **ns**, nucleation site; **oc**, organic core; **pw**, plane of weakness.

and sometimes allow the outermost portion of the eggshell to separate from the main body of the shell (Fig. 3F); however, the reason for this weakness is unclear. Eggshell fragments analyzed with SEM are approximately 185–340 μm thinner than eggshell fragments in thin sections, which may reflect glue removal prior to SEM analysis. Shell units that lack planes of weakness often show alteration of the mineral at approximately the same level as those eggshells that exhibit separation; however, aragonite crystals are continuous throughout the shell thickness with little evidence of diagenetic recrystallization (Fig. 3E, middle shell unit).

Comments—Radial thin sections and SEM of the eggshell reveal a lack of resorption pits or craters at the base of the shell units. In contrast, hatched eggs of extant turtles exhibit a flat inner eggshell surface and a single small pit at the center of each shell unit when viewed from the inner surface. This results from calcium (in the form of aragonite) mobilized by a transepithelial mechanism utilizing the ectodermal cells of the

chorioallantoic membrane (Bilinski et al., 2001). This allows the embryo to utilize calcium and other components of the mineralized eggshell for skeletal ossification. The lack of resorption pits in the fossil eggs indicates that they were likely infertile or embryonic development terminated prior to calcium mobilization for osteogenesis. In extant turtles, clutch failure may be due to predation, microbial invasion, improper incubation temperatures, inadequate gas exchange, or flooding (Tinkle et al., 1981; Standing et al., 1999; Phillott and Parmenter, 2006).

Gas Conductance

A tangential thin section (ES 537) of the fossil eggshell reveals 41 pores in an eggshell fragment approximately 41 mm^2, with a total pore area for the fragment of 0.29 mm^2. This equates to 0.7% pore area for an egg within AM F82183 with a radius of 26.96 mm and a total area of 9129.13 mm^2. Thus, A_p equals 64.59 mm^2, resulting in a G_{H2O} of 170.27 mg H_2O day^{-1} $Torr^{-1}$ and a porosity of 80.73 mm per egg. Egg mass equals 85.9 g. Similar data for tangential thin sections of two extant tortoises **(***Kinixys erosa*, Fig. 3H and *Indotestudo elongata*, Fig. 3I) are provided in Table 1.

Comparisons

Hirsch (1996) proposed a parataxonomic classification for fossil turtle eggs/eggshells that included two oofamilies, Testudoflexoolithidae and Testudoolithidae. Flexible eggs with loosely abutting shell units comprised the former, whereas the later

included rigid eggs with tightly packed shell units. The rigid shells of the Lord Howe Island eggs exclude these specimens from Testudoflexoolithidae (Table 2).

Among oospecies assigned to Testudoolithidae, *Testudoolithus lordhowensis* differs from *Chelonoolithus braemi* Kohring, 1998 by a shell unit height-to-width ratio of 1.2:1 and *Emydoolithus laiyangensis* Wang et al., 2013 in overall eggs shape. *Testudoolithus lordhowensis* is distinguished from *Hainichelys curiosa* Schleich et al., 1988 by its greater eggshell thickness; however, it falls within the range of eggshell height-to-width ratio. *Testudoolithus lordhowensis* also differ from *T. hirschi* Kohring, 1999 and *T. rigidus* Hirsch, 1996 in its thicker eggshell and from all other *Testudolithus* ootaxa by a smaller shell unit height-to-width ratio. Numerous other eggs and eggshells have been assigned to either Testudoolithidae or *Testudoolithus sp.* but all differ from *Testudoolithus lordhowensis* by their overall egg shape, size, eggshell thickness, or shell unit height-to-width ratio (Lawver and Jackson, 2014).

DISCUSSION

Sir Richard Owen erected *Meiolania platyceps* in 1886 based on well-preserved skeletal remains discovered on Lord Howe Island. The carapace of this large bodied, terrestrial taxon measures at least 66 cm in length and its unusual skull exhibits many knob- and horn-like protrusions, with two large, posterolaterally directed, cow-like cranial horns. Traditionally, it was assumed that these horns likely prevented *M. platyceps* from withdrawing its head into the shell; however, Werneburg et al. (2015)

suggest that some degree of head retraction may have been possible, albeit using a different mode than either extant cryptodires or pleurodires. In addition, armored rings and terminal spikes protected the tail.

Anderson (1925) and Gaffney (1983, 1996) identified the Lord Howe Island fossil specimens as *Meiolania platyceps* eggs based on their close association with bones of this species at several localities. Furthermore, *M. platyceps* represents the only fossil turtle currently known from Lord Howe Island, thus further supporting their identification. An alternative hypothesis is that sea turtles laid eggs on these Pleistocene beaches before returning to the sea. However, all sea turtles produce pliable, rather than the rigid-shell eggs, with loosely abutting shell units that are wider than they are tall. Therefore, our examination of the eggshell microstructure rules out a chelonioid origin for the eggs. Comparison of the fossil egg size and mass to those of extant turtle taxa further supports the previous assignment of the fossil eggs to *Meiolania platyceps.* The Lord Howe Island specimens are the largest fossil turtle eggs yet described in the literature (53.9 mm and estimated mass of 85.91 g). Only two extant turtles produce larger eggs: *Geochelone elephantopus* (55–68 mm and 106.88 g) and *G. gigantea* (48–51 mm and 87.10 g) (Elgar and Heaphy, 1989; Ernst and Barbour, 1989; Robeck et al., 1990). Note that the taxonomic history and nomenclature for both taxa are complex and subsequently these taxa are currently named *Chelonoidis nigra* and *Aldabrachelys* (*Testudo*) *gigantea*, respectively (Le et al., 2006; Frazier, 2009). Elgar and Heaphy (1989) demonstrate a significant positive correlation between turtle carapace length and egg mass. Because *Meiolania platyceps* eggs closely approximate the weight of *Aldabrachelys* (*Testudo*)

gigantea eggs, we predict a similar carapace length; therefore, the female that laid the fossil clutch likely had a carapace length of approximately 75 cm. This is slightly longer than a 66 cm carapace length for AM 137122; however, three plausible explanations may explain the size difference: maturity, sexual dimorphism, or simply some individuals of *M. platyceps* attained greater sizes.

Although future discovery of additional terrestrial turtle fossils on the island cannot be ruled out, several factors suggest this may be unlikely. Lord Howe Island measures only 11 km long and 0.6–2.8 km wide. Small islands typically support fewer species than corresponding areas on the mainland because of less habitat diversity (Lack, 1969; Begon et al., 2003). Further, Lord Howe Island lies 580 km from the east coast of Australia, and species richness typically shows an exponential decrease with distance from the mainland (Begon et al., 2003). Rafting and drifting between isolated land masses represent a mechanism of dispersal and likely accounts for the presence of terrestrial tortoise species on islands such as the Galapagos, Seychelles and Lord Howe islands. In the case of giant tortoises (as well as *Meiolania platyceps*) their large body size would preclude the use of all but the very largest of rafts (Gerlach et al., 2006). Finally, some extant tortoises are known to swim; Townsend (1936) reports Galapagos giant tortoises that were swept 32 km into the sea by hurricanes. However, Gerlach et al. (2006) provide the only direct evidence of a giant tortoise (*Aldabrachelys gigantea*) surviving such a sea crossing. Therefore, the dispersal and ability to colonize islands 580 km from the mainland by a large terrestrial turtle such as *Meiolania platyceps* likely represented rare events.

TABLE 1. Gas conductance, porosity, and mass calculations.

Taxon	No. of pores	Area examined (mm^2)	Total pore area (mm^2)	Egg size (mm)	Egg surface area (mm^2)	A_p (mm^2)	G_{H2O} (mg H_2O day^{-1} $Torr^{-1}$)	Porosity (mm)	Mass (g)
Meiolania platyceps	41	41	0.29	53.9	9129.13	64.6	170.27	80.73	85.9
Kinixys erosa	23	63.1	0.28	46.6 x 31.5	4543	20.4	83.6	39.2	25.2
Indotestudo elongata	9	66.35	0.29	48.4 x 35.0 x 31.1	4155.9	18.0	84.2	39.9	28.9

Evolution of Turtle Reproductive Biology

The early evolution of turtle reproductive biology remains perplexing because of the paucity of definitively identified eggs and nests in the rock record. Therefore, an understanding of the evolution of turtle reproduction relies on the study of extant and extinct turtles within the framework of turtle phylogeny. Below, we discuss two aspects of turtle reproductive history: aragonite eggs and turtle nesting behavior.

Aragonite Eggs—Cathodoluminescence of *Meiolania* eggshell reveals a dull blue luminescence, similar to other minimally altered biogenic aragonite present in recent and fossil marine gastropods, cephalopods, and corals (Barbin, 2000). Additionally, the lack or orange and red luminescence indicates that secondary calcite is absent (see Lawver et al., 2015, fig. 3D for comparison). This, along with the needle-like morphology of the crystals composing *Meiolania* eggshell, indicates that diagenetic recrystallization has not occurred and alteration is minimal. Greater luminescence within the accretion lines when compared to the overall eggshell may indicate a change in the rate of aragonite deposition or the chemical environment within the shell gland (uterus) during eggshell formation, as observed in other biogenic aragonite (Barbin, 2000; Boggs and Krinsley, 2006). Reduced luminescence at the inner eggshell surface may represent a higher concentration of organic material (Kusuda et al., 2013) or Fe^{2+} (Barbin, 2000; Boggs and Krinsley, 2006; Götte and Richter, 2009).

Extant turtle eggs vary in their aragonite eggshell structure from pliable to semi-pliable to rigid. The vast majority of fossil turtle eggs described in the literature exhibit

TABLE 2. List of turtle ootaxa and their distinguishing characteristics.

Ootaxon	Holotype	Material	Egg shape	Length x Width (mm)	Eggshell thickness (μm)	Shell unit (height:width)
Testudoflexoolithus agassizi[1]	MCZ 2810/ HEC 49	Eggshell fragments	-	-	60–100	1:1 or 2:3
Testudoflexoolithus bathonicae[1,2]	BM(NH)37983/ HEC 186	An egg imbedded in matrix	Ellipsoidal	48 x 26	200–250	1:1
Chelonoolithus braemi[3]	Guimarota 98-2	Eggshell fragments	-	-	200	1:1
Emydoolithus laiyangensis[4]	IVPP V18544	A nearly complete egg	Elongate	91 x 22	400–500	2:1 to 5:1
Haininchelys curiosa[5]	-	Eggshell fragments	-	-	250–300	1.2:1 to 2.3:1
Testudoolithus hirschi[6]	-	Eggshell fragments	-	-	150	3:1
Testudoolithus jiangi[7,8]	ZMNH M8713	A clutch of 23 eggs	Spherical	35–52	700–1000	2.5:1 to 3:1
Testudoolithus rigidus[1]	UCM 55806/ HEC 425	Half of an egg	Spheroidal	42 x 47	220–240	2:1
Testudoolithus lordhowensis	AM F82183	A clutch of at least 10 eggs	Spheroidal	53.9	800	1.2:1

Modified from Lawver and Jackson (2014).

References: 1, Hirsch (1996); 2, Buckman (1859); 3, Kohring (1998); 4, Wang et al. (2013); 5, Schleich et al. (1988); 6, Kohring (1999); 7, Fang et al. (2003); 8, Jackson et al. (2008).

rigid eggshells and these primarily date to the Late Cretaceous worldwide (Lawver and Jackson, 2014). However, the earliest record comes from the Jurassic Great Oolite of England and the eggs exhibit pliable eggshell comprised of aragonite (Buckman, 1859; Hirsch, 1983). Phylogenetic inferences suggest that leathery (pliable) eggs evolved first, with more calcified eggs evolving independently from leathery eggs (Laurin et al., 2000; Sander, 2012). This suggests that the scarcity of pliable turtle eggshells likely results from a taphonomic bias, namely, increased preservation potential of rigid shelled eggs (Hirsch, 1983; Sander, 2012).

The phylogenetic position of *Meiolania platyceps* indicates that the evolution of aragonite eggshell occurred prior to the split of the crown clade, Testudines. This suggests a minimum age for the evolution of the aragonite eggshell as Early to Middle Jurassic because the split between Meiolaniformes and Testudines likely occurred during this time interval (Sterli et al., 2015). Additionally, this date roughly corresponds to the age of the English Great Oolite eggs. It is unlikely that this character arose independently more than once because aragonite occurs only in turtle eggs and no other taxa within Amniota (Hirsch, 1983; Mikhailov, 1997). Therefore, aragonite turtle eggs probably evolved lower in the stem lineage or prior to the Triassic stem taxon, *Proterochersis robusta*. If the latter proves correct, a substantial gap exists in the fossil record of turtle eggs (Lawver and Jackson, 2014).

Turtle Nesting Behavior—Although behavior is often difficult to interpret from the rock record, fossil eggs and clutches provide valuable evidence about nesting strategies of extinct animals, especially when compared to extant taxa. Calculating gas

conductance for fossil turtle eggs allows a close comparison with living turtles because of the conservative nature of their evolution, i.e., turtles have changed little over time. In contrast, most previous gas conductance studies focus on dinosaur eggs. However, non-avian dinosaurs are vastly different in terms of their behavior, ecology, and reproductive biology than their closest living relatives, crocodilians and birds. Therefore, modern turtle eggs provide an appropriate correlate that permits more rigorous testing of these methods and greater confidence in the results. Below we discuss gas conductance of *Meiolania platyceps* eggs and compare the latter to values obtained from two extant terrestrial species, *Kinixys erosa* and *Indotestudo elongata*.

Nesting behaviors of living turtles range from deep or shallow excavations, to eggs laid on the ground surface, or in vegetation mounds, similar to crocodilians (Ernst and Barbour, 1989; Bonin et al., 2006; Jackson et al., 2015). In extant taxa, the gas conductance of an egg reflects the environment of incubation, thereby providing important insights into the nesting strategy (Ar et al., 1974; Seymour, 1979; Deeming, 2006). For example, most avian eggs are exposed to the atmosphere during incubation and exhibit low water vapor conductance (G_{H2O}) in order to decrease the amount of water lost to evaporation (Seymour, 1979). In contrast, reptiles typically incubate their eggs in high humidity microenvironments, and the eggs generally exhibit higher gas conductance values compared to avian eggs (Seymour, 1979). For this reason, gas conductance calculations are often used to determine the nesting strategy that was employed by extinct vertebrates (Seymour 1979; Williams et al. 1984; Sabath 1991; Deeming 2002, 2006; Sander et al. 2008; Jackson, Varricchio et al. 2008; Varricchio et al., 2013 and references

therein; Tanaka et al., 2015). However, this method has not been applied to the study of fossil turtle eggs.

The calculated gas conductance for *Meiolania platyceps* eggs of 170.27 mg H_2O day^{-1} $Torr^{-1}$ is approximately 10.6 times higher than predicted for a bird egg of equal mass (Ar et al., 1974; Ar and Rahn, 1985), which indicates that these eggs were incubated in a high humidity environment. Similarly, the gas conductance for *Kinixys erosa* and *Indotestudo elongata*, calculated by the same thin section method, are approximately 19.7 and 11.7 times higher, respectively, than bird eggs of equal mass (Table 1). *Kinixys erosa* inhabits humid forest and swampy environments and eggs are deposited in a small nest constructed from dead vegetation and detritus near water (Bonin et al., 2006). *Indotestudo elongata* lives in cool to warm, humid environments that experience frequent precipitation and nests are shallow excavations (Sriprateep et al., 2013).

Brooke et al. (2003) report that deposition of the Neds Beach Formation occurred in humid environment. The gas conductance values from *Meiolania platyceps* eggs and their preservation in at least two superimposed layers demonstrate that the gravid female deposited her eggs in an excavated hole nest. Fossil turtle clutches from the Late Cretaceous of Canada (Zelenitsky et al., 2008) and China (Jackson, Jin et al., 2008) preserve eggs in possibly two and three superimposed layers, respectively, also suggesting a similar nesting strategy. Additionally, Bishop (2011) described the nesting traces of a Late Cretaceous sea turtle, which preserved an egg chamber, egg molds, body pit, and an adult crawlway in the Fox Hills Sandstone of Colorado. The presence of similar nesting behavior in *Meiolania platyceps* suggests a minimum age for hole nests of

Early or Middle Jurassic, prior to the split between Meiolaniformes and Testudines. However, it is highly plausible that this behavior evolved in turtles prior to the Jurassic and therefore represents a plesiomorphic condition for turtles.

CONCLUSIONS

Large, spherical eggs from the Pleistocene of Lord Howe Island, Australia are those of *Meiolania platyceps* and represent the new ootaxon, *Testudoolithus lordhowensis*. Diagnostic features for this new oospecies include the unique combination of characters: large size (53.9 mm), spherical egg shape; 800 μm thick eggshell; barrel-shaped shell units; and a height-to-width of 1.2:1. Egg mass suggests a female carapace length of approximately 75 cm, which is slightly longer than the 66 cm carapace of a mostly complete *M. platyceps* specimen (AM137122) from Lord Howe Island.

These eggs also confirm that the aragonite eggs evolved prior to the split of the turtle crown group, indicating that this unusual eggshell composition evolve no later than the Early or Middle Jurassic. Further, this suggests that aragonite eggs first evolved lower in the stem lineage of turtles or prior to *Proterochersis robusta*.

Estimated gas conductance for *Meiolania platyceps* is approximately 10.6 times higher than the predicted value for a bird egg of equal mass, indicating a humid incubation environment. This conductance value and preservation of the eggs in at least two superimposed layers indicates that the gravid female laid the clutch in an excavated hole nest.

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CHAPTER SEVEN

MODERN AND FOSSIL EGGS AND THEIR ROLE IN HYPOTHESIZING TURTLE PHYLOGENETIC RELATIONSHIPS

Contribution of Author

Manuscript in Chapter 7

Author: Daniel R. Lawver

Contribution: Conceived and implemented the study design. Collected and analyzed data. Wrote the manuscript.

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CHAPTER SEVEN

MODERN AND FOSSIL EGGS AND THEIR ROLE IN HYPOTHESIZING TURTLE PHYLOGENETIC RELATIONSHIPS

DANIEL R. LAWVER

[1]Department of Earth Sciences, Montana State University, Bozeman, Montana, 59717, U.S.A., danlawver@gmail.com

*Corresponding author

ABSTRACT—Cladistic analyses using egg and reproductive characters have traditionally focused on taxa within Dinosauria and few studies have concentrated on extant taxa. Turtles provide an appropriate test of this method because of their relatively conservative evolution. My analyses show that turtle egg and reproductive characters produce poorly resolved trees in morphological-only datasets, whereas combining morphological with molecular data provides greater potential to resolve clades towards the top of the tree. Thick eggshell and spherical egg shape are synapomorphies for Adocia and the clade containing Adocia and Trionychia, respectively. However, many other characters demonstrate homoplasy within Testudines likely due to species' habitats or incubation environments. Other characters are only useful for distinguishing turtles from the outgroup.

INTRODUCTION

Although fossil eggs have been known since the seventeenth century, these specimens were simply viewed as curiosities rather than deemed important enough for rigorous investigation (Carpenter et al., 1994). In the late twentieth century, modern paleoological research began with microscopic examination and comparison to extant taxa. Pre-cladistic investigations utilized schematic tree diagrams and overall similarities between eggs (Zhao, 1979), and Mikhailov (1992) discussed the potential importance of egg and eggshell attributes as phylogenetic data. Later, authors mapped egg and eggshell characters onto existing phylogenetic trees derived from osteological data. However, nearly all previous studies focused on taxa within the Dinosauria (Mikhailov, 1992; Grellet-Tinner, 2000; 2001, 2005; 2006; Zelenitsky, 2004; Zelenitsky and Modesto, 2003; Zelenitsky and Therrien, 2008a, b; Garcia et al., 2006; Sellés, 2012; Tanaka et al., 2011; López-Martínez and Vicens, 2012; Ribeiro et al., 2014; Moreno-Azanza et al., 2009; Barta, 2014; Grellet-Tinner and Chiappe, 2004; Grellet-Tinner and Makovicky. 2006; Varricchio and Jackson, 2004; Jin et al., 2010; Varricchio and Barta, 2015). In contrast, only a few studies have tested the validity of this approach by applying cladistic analyses to eggs of extant species and comparing the results to well-established phylogenies. With the exception of one study (Winkler, 2006) discussed below, these analyses of extant taxa are limited to non-avian theropods, namely ratites (Grellet-Tinner, 2000; Zelenitsky and Modesto, 2003).

Several potential problems may diminish the reliability of studies utilizing extinct and extant specimens. Eggs, eggshell microstructure and other reproductive attributes of avian and non-avian theropod dinosaurs diverged from those of other reptiles and reflect a long and complex history. Some changes potentially impact reproductive characters and should be ordered or more heavily weighted when used in phylogenetic analyses. For example, unlike reptiles that lay eggs en masse, birds produce only one egg at a time, a change likely resulting from the evolution of flight (Varricchio and Jackson, 2016). Although non-avian theropod eggs like those of *Troodon* are large compared to reptile eggs, they are small relative to female body size when compared to those of modern birds. Eggshells attributed to most non-avian theropods consist of two structural layers (e.g., Mikhailov, 1997; Varricchio and Barta, 2015), whereas more derived bird eggs exhibit three or even four structural layers (Zelenitsky and Modesto, 2003; Lawver and Jackson, 2016a, Table 1). Incubation in non-avian theropods varies from full to partial egg burial in a substrate, whereas nearly all birds incubate eggs in open nests.

In addition to these differences in reproductive features, other problems also characterize these studies. Grellet-Tinner (2000) used many qualitative characters, thus providing less resolution among taxa. Interpretations of characters also differed between his study and that of Zelenitsky and Modesto (2003). Whereas, Zelenitsky and Modesto (2003) produce higher resolution trees, their study only includes nine discrete eggshell characters. Additionally, these studies are partially outdated because of more recent molecular analyses (Yuri et al., 2013; Mitchell et al., 2014) that demonstrate differing tree topologies and thus warrant reevaluation. These evolutionary changes suggest that

taxa outside of Theropoda may provide a more appropriate test for assessing the reliability of using reproductive attributes as characters in phylogenetic analyses. Because of their conservative evolution, turtles offer a likely candidate for testing this hypothesis than avian or non-avian theropods.

Only Winkler's (2006) study, however, examines the evolutionary history of turtle egg and eggshell characters. Winkler (2006) traced these characters on a phylogeny of extant pleurodiran turtles that she obtained from Seddon et al. (1997) and modified with information in Georges and Adams (1992). Base on the results, she suggested that only a few characters provided a phylogenetic signal. However, the scope of her study was narrow because Winkler (2006) only examined 12 extant species from two families (e.g., Chelidae and Pleomedusidae). Additionally, the analysis contained only 13 characters and lacked outgroup taxa. Therefore, the study presented here represents the first cladistics analysis using egg and eggshell characters of multiple clades within Testudines. Additionally, this study includes fossil eggs of known taxonomic affinity, five outgroup taxa, and 24 characters, as well as comparison to established phylogenies based on molecular and/or osteological data obtained from the literature. This allows a more rigorous analysis. Finally, I address whether egg and reproductive characters produce less resolution because of inadequate data or result from homoplasy.

METHODS AND MATERIALS

Specimen Preparation

The 22 extant turtle eggs (Table 1) used in this study come from the M. A. Ewert Memorial Turtle Egg Collection in the Department of Earth Sciences, Montana State University. Chicken, crocodilian and emu eggs, the latter donated by the Montana Emu Ranch Company, are also housed in the Department of Earth Sciences, Montana State University. Four fossil specimens were obtained from university and museum collections (Table 2). Modern and fossil eggshell fragments were removed from whole or partial eggs and mounted on an aluminum stub, coated with 10 nm of gold, and imaged under a JEOL JSM-6100 (SEM) at 10 kV. Additionally, 36 thin sections (30 µm thick) were made following standard histological procedures (Lamm, 2013). Photomicrographs of histological sections of eggshell were taken with a Nikon Digital Sight DS-5Mc camera and features measured with ImageJ analytical software (Rasband, 1997; http://imagej.nih.gov/ij/)**.** Images included the inner and outer eggshell surfaces as well as radial cross sections.

Cladistic Analyses

In order to test the validity of using only egg and eggshell characters for hypothesizing turtle phylogenies, I compared the topologies resulting from my analyses to those obtained from the literature. My four analyses included: 1) parsimony analysis consisting of only morphological data; 2) backbone constraint analysis; 3) maximum

likelihood analysis utilizing cytochrome b sequences; and 4) combined parsimony analysis incorporating both morphological and molecular data. Comparative trees were obtained from Crawford et al. (2015) and Joyce et al. (2013). Additionally, Spinks et al. (2004) provide the basis for comparison for Geoemydidae, Le et al., (2006) for Testudinidae, Sprinks et al. (2014) for Kinosternidae, Joyce et al. (2009) for Trionychidae, and Seddon et al. (1997) for Chelidae.

Table 1. Modern and fossil taxa analyzed in the study. Fossil taxa are indicated with *.

Taxon	Specimen Number	Hatched
Phrynops geoffroanus	ES 192	?
Emydura subglobosa	ES 193	?
Elseya novaeguineae	ES 194	?
Platemys platycephala	ES 195	?
Mesoclemmys gibba	ES 196	?
Kinosternon minor	ES 197	Hatched
Kinosternon hirtipes	ES 198	Hatched
Kinosternon scorpioides	ES 199	Hatched
Staurotypus triporcatus	ES 200	?
Kinosternon carinatum	ES 201	No
Dermatemys mawii	ES 202	No
Apalone mutica	ES 203	?
Apalone ferox	ES 204	Hatched
Apalone spinifera	ES 205	Hatched
Rhinoclemmys areolata	ES 206	?
Rhinoclemmys pulcherrima	ES 207	Hatched
Hieremys annandalii	ES 208	?
Heosemys spinosa	ES 209	No
Cistoclemmys flavomarginata	ES 211	Hatched
Kinixyz erosa	ES 212	?
Malacochersus tornieri	ES 213	?
Indotestudo elongata	ES 215	?
Gallus gallus	-	No
Dromaius novaehollandiae	-	No
Alligator mississippiensis	ES 345	?
Crocodylus acutus	ES 415	?
*Adocus sp.**	MOR 710	No

*Basilemys nobilis**	DMNH EPV.91000	-
*Meiolania platyceps**	ES 538	No
*Pachycorioolithus jinyunensis**	JYM F0033	?

Table 2. Locality and age data for fossil taxa.

Taxon	Specimen Number	Locality	Age	Formation
Meiolania platyceps[1]	MOR 710	Lord Howe Island, Australia	Pleistocene	Neds Beach
Basilemys nobilis[2]	DMNH EPV.91000	Utah, U.S.A.	Late Cretaceous	Kaiparowits
Adocus sp.[3]	ES 538	Montana, U.S.A.	Late Cretaceous	Judith River
Pachycorioolithus jinyunensis[4]	JYM F0033	Zhejiang Province, China	Early Cretaceous	Liangtoutang

Refences: 1, Lawver and Jackson (2016); 2, Chapter 5 (this dissertation); 3, Lawver and Jackson (2017); 4, Lawver et al. (2016).

The morphological analysis contains 22 extant turtle taxa that lay rigid-shelled eggs. Additionally, I include three fossil turtle taxa (*Meiolania platyceps, Basilemys nobilis* and *Adocus* sp.) to assess their phylogenetic position. The outgroup consists of two crocodilian and three avian eggs; one of the latter represents a fossil specimen, namely *Pachycorioolithus jinyunensis* (Lawver et al., 2016).

The data matrix was built in *Mesquite* (http://www. Mesquiteproject.org/) and the parsimony analysis performed with *PAUP** (Swafford, 2002). Heuristic searches included 10,000 maximum trees, 100 random addition replicates, holding multiple trees at each step, tree-bisection-reconnection branch swapping using non-minimum trees, and retention of multiple parsimonious trees. Sixteen characters were obtained from the

literature, with modificatons to characters 4, 6, 8, 14, and 23 (see Appendix 1 for details). I included eight new characters to permit a more comprehensive analysis of eggshell (see

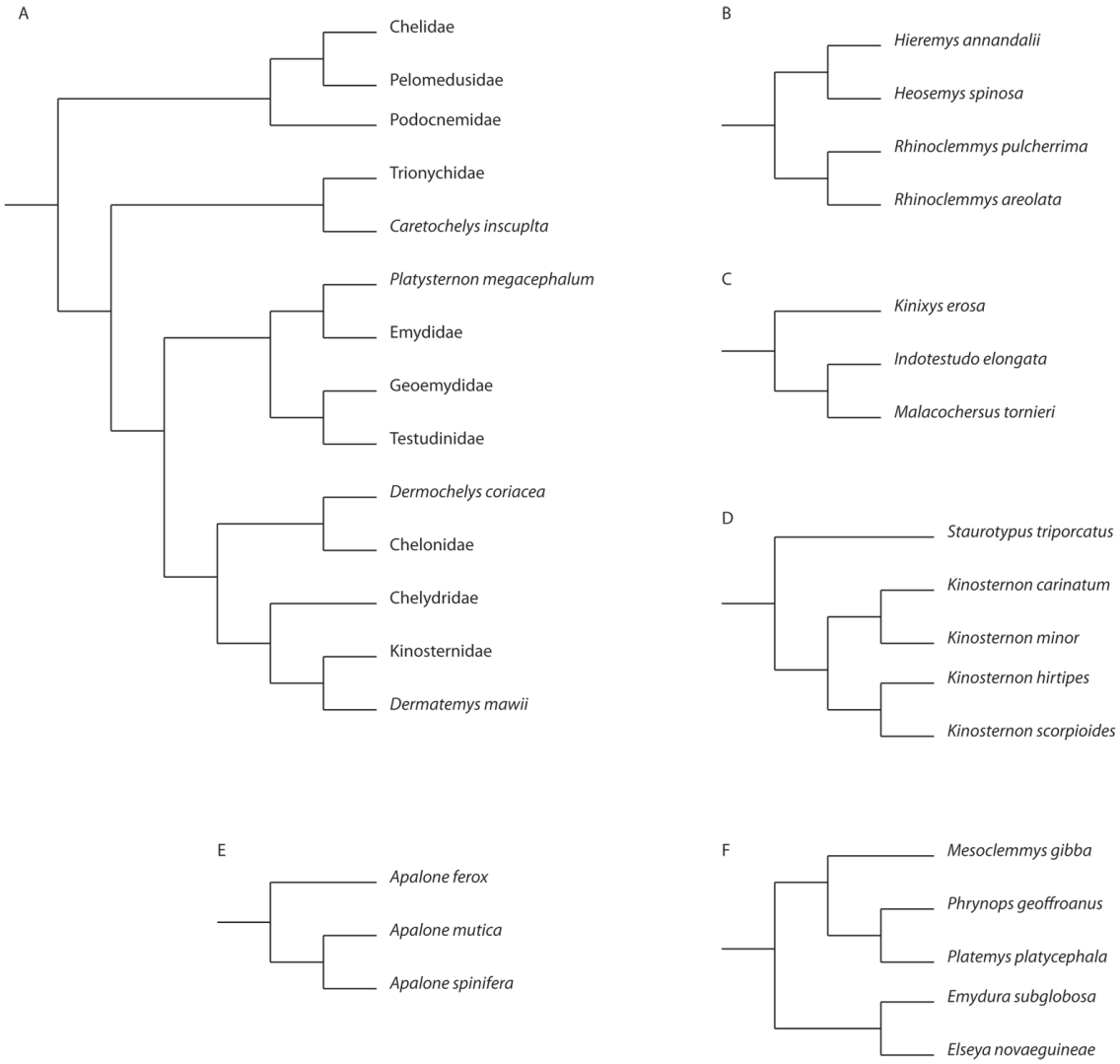


Figure 1. Comparison trees obtained from the literature. A, Testudines (Crawford et al., 2015; Joyce et al., 2013); B, Geoemydidae (Sprinks et al., 2004); C, Testudinidae (Le et al., 2006); D, Kinosternidae (Sprinks et al., 2014); E, Trionychidae (Joyce et al., 2009); F, Chelidae (Seddon et al., 1997).

Appendix 1 and 2 for characters and their coding). Characters 6, 7, 20, 23 were multistate characters and unordered. All characters were equally weighted.

A backbone constraint analysis was implemented in order to test the difference in tree lengths between the most parsimonious tree from the morphological analysis and the accepted topology obtained from the literature. This was achieved by forcing the preferred tree topology of some but not all taxa. For this analysis, extant taxa were constrained at the family level (i.e., all kinosternids were forced into the monophyletic clade, Kinosternidae); however, individual taxa were allowed to change topologies within clades. Additionally, fossil taxa were left unconstrained.

As a control for the morphological analysis, I performed a molecular analysis utilizing mitochondrial cytochrome b sequences. Sequences for 21 extant turtles and four outgroup species were obtained from *GenBank* and aligned in the program *MEGA* (Molecular Evolutionary Genetics Analysis; http://www.megasoftware.net/) using the default settings (Appendix 3). A maximum likelihood analysis was conducted in *MEGA* with the default settings. Following this analysis, I used a combined analysis to assess the possible effects of morphology on tree topology. The data was concatenated in *Mesquite* and a parsimony analysis performed in *PAUP* following the same search specifications as the morphological analysis.

Institutional abbreviations—AM, Australian Museum, Sydney, New South Wales, Australia; DMNH, Denver Museum of Natural History, Denver, Colorado, U.S.A.; ES, Department of Earth Sciences, Montana State University, Bozeman, Montana, U.S.A.; JYM, Jinyun Museum, Jinyun, Zhejiang Province, China; MOR, Museum of the Rockies, Bozeman, Montana, U.S.A.

RESULTS

Phylogenetic Analyses

Microscopic examination led to the recognition of 24 discrete eggshell characters (Appendix 1). The strict consensus tree resolves the ingroup as monophyletic (Fig. 2A; Table 3). Within turtles, *Rhinoclemmys pulchurrima*, and a clade containing *Mesoclemmys gibba, Dermatemys mawii* and *Phrynops geoffroanus* forms a grade leading to a large polytomy containing the remaining ingroup taxa. Within this polytomy two clades are formed, one consisting of *Apalone ferox* and *A. spinosa*, and another including the fossil taxa, *Adocus* sp. and *Basilemys nobilis*. Additionally, a clade consisting of *Hieremys annandalii, Kinosternon scorpoides*, and the fossil taxon *Meiolania platyceps* is resolved with *K. scorpoides* more closely related to *Meiolania platyceps.* The backbone constraint tree has a length of 112 steps (Fig. 2B). *Adocus* sp. and *Basilemys nobilis* are resolved within Testudinidae in 62% of trees and *Meiolania Platyceps* is resolved inside of Geoemydidae in 100% of trees.

Table 3. Analysis statistics.

Analysis	Tree Length	Consistency Index	Retention Index
Morphological	85	0.3294	0.5899
Backbone	112	0.25	0.4173
Combined	3629	0.5569	0.6587

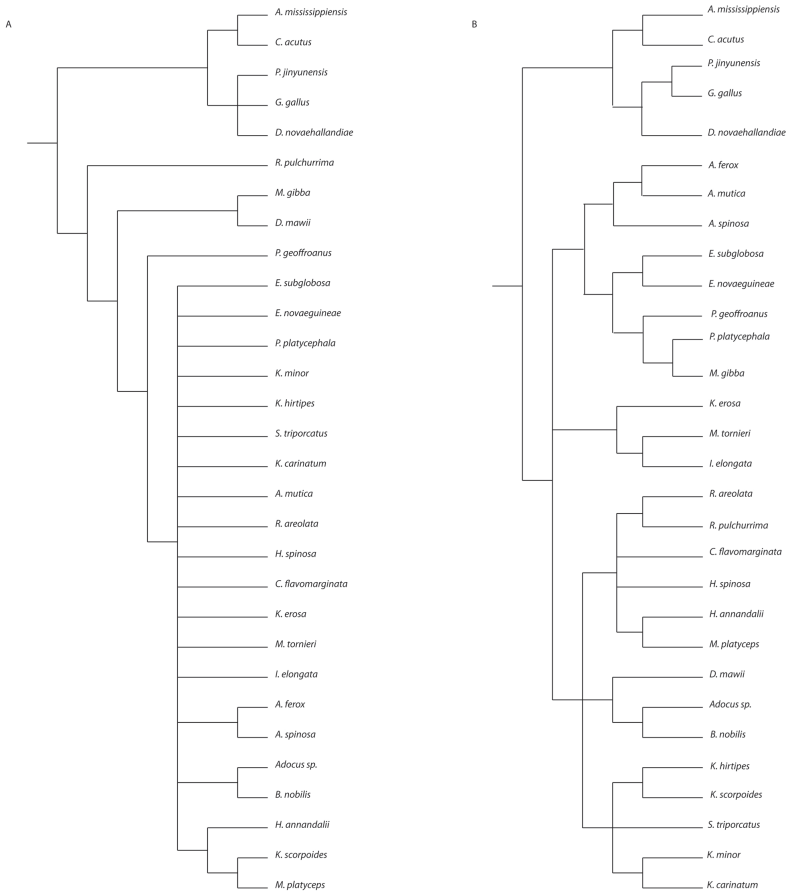


Figure 2. Phylogenetic hypotheses. A, strict consensus tree of egg and eggshell morphological data. B, backbone constraint tree.

The molecular analysis resolves both Pleurodira and Cryptodira clades (Fig. 3A). Pleurodira is represented by the Chelidae, which consists of an Australian clade (*Elseya novaeguineae* and *Emydura subglobosa*), and a South American clade (*Mesoclemmys gibba, Platemys platycephala,* and *Phrynops geoffroanus*). Within Cryptodira, Trionychidae, Kinosternidae, *Dermatemys mawii* form a grade leading to the clade Testuguria (Testudinidae, and Geoemydidae).

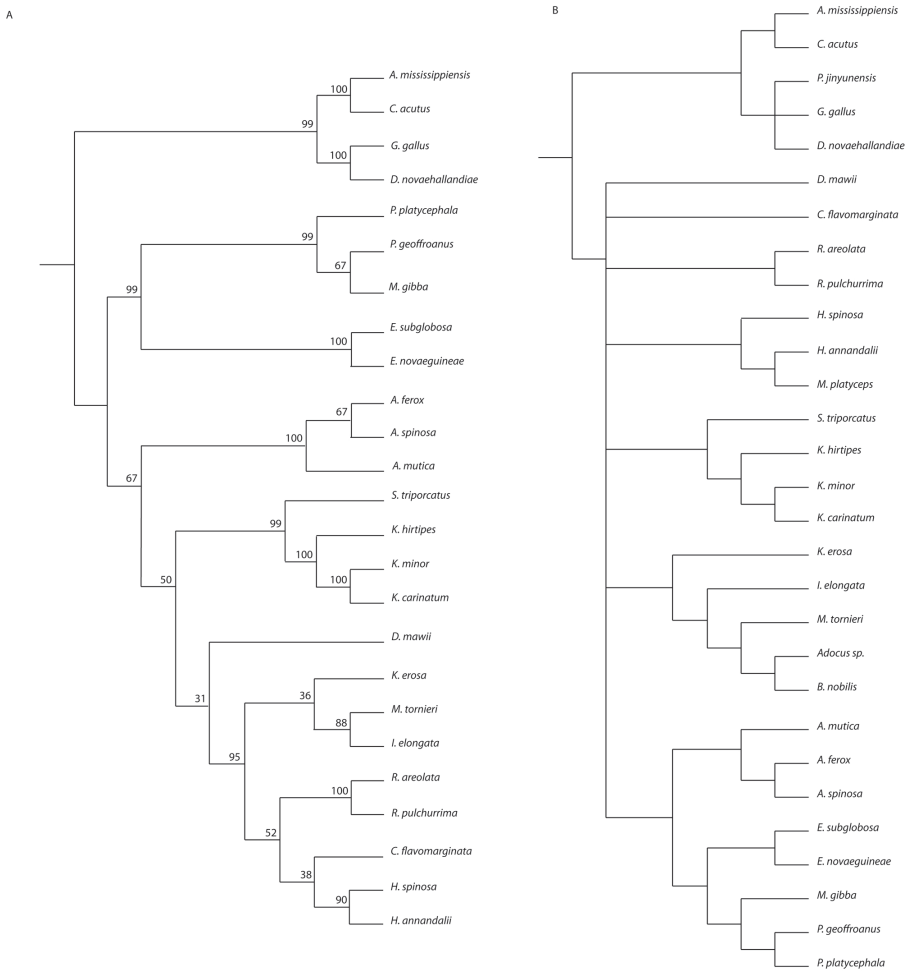


Figure 3. Phylogenetic hypotheses. A, molecular analysis with bootstrap values inferred from 500 replicates; B, combined analysis.

The combined analysis resulted in six most parsimonious trees. The strict consensus tree results in a large turtle polytomy (Fig. 3B). Both Australian and South American clades are resolved within Pleurodira, which forms a clade with Trionychidae. Additionally, the Testudinidae, Kinosternidae, a *Rhinoclemmys* clade and a clade containing *Heosemys spinosa, Hieremys annandalii* and *Meiolania platyceps* are resolved within this large polytomy. The retention index of only the morphological data from this analysis is 0.4091.

DISCUSSION AND CONCLUSIONS

Trionychidae and the extinct clade Adocia represent the only clades resolved in the morphological analysis that reflect accepted taxonomic relationships derived from the literature. Round egg shape and thin eggshell unite *Apalone ferix* and *A. spinifera* within Trionychidae; however, *A. mutica* is excluded from this clade, likely due to its extremely thin eggshell (88 μm). *Adocus sp.* and *Basilemys nobilis* represent Adocia and are united by spherical egg shape and shell unit proportions. The remainder of the turtle taxa are either unresolved or form polyphyletic relationships with distantly related turtle taxa.

The low resolution of the morphological analysis suggests that, with two exceptions (Trionychidae and Adocia), egg and eggshell characters do not provide a strong phylogenetic signal amongst turtle taxa and concurs with the findings of Winkler (2008). This is likely due to homoplasy (convergent evolution or reversals) that results from independent evolution or reversals of character states. The backbone constraint tree is 27 steps longer then the most parsimonious unconstrained tree and thus supports this interpretation. See Appendix 3 for ancestral state reconstruction for each character.

Several aspects of turtle reproductive biology may contribute to the lack of phylogenetic signal. Female body size and choice of nesting site play a major role in determining the attributes of turtle eggs (size, shape, eggshell porosity, and number of eggs per clutch) and ultimately contribute to this homoplasy. For example, taxa with convergently large body size tend to produce large clutches. These taxa also produce spherical eggs because they are more easily packed into the uterus, which permits

simultaneous shelling of more eggs prior to oviposition (Ewert, 1979; Iverson and Ewert, 1991). Additionally, younger, smaller females typically lay fewer and smaller eggs per clutch than older, larger females of the same species (Kuchling, 1999). Such variation occurs within a single clutch in *Dermochelys coriacea,* in which a clutch of 50–100 eggs may contain 10–40 smaller and infertile eggs (Spotila, 2004; Gulko and Eckert, 2004).

Despite providing better resolution than the morphological analysis, the combined analysis results in reduced resolution when compared to the molecular analysis. The molecular analysis produces a tree that only deviates from the accepted topology from the literature by the placement of *Dermatemys mawii* as the sister taxon to the clade Testuguria. In recent studies (Joyce, 2007; Crawford et al., 2015) this species represents the sister taxon to Kinosternidae. Otherwise, this general congruency at the family level indicates that the molecular analysis is an appropriate control for the morphological analyses.

Strengths and Limitations of the Analyses

Many limitations characterize phylogenetic analyses that depend solely on reproductive attributes. These analyses tend to suffer from lack of informative characters as well as excessive missing data and positive identification of fossil specimens due to the lack of embryonic remains. Additionally, intraspecific variation or even variation within a single egg makes scoring characters very difficult. Diagenesis may also alter eggshell thickness, ornamentation and microstructure, resulting in differing

interpretations by researchers. As a result of these problems, phylogenetic relationships among turtles cannot be resolved by using egg and reproductive characters alone.

Barta's (2014) analysis demonstrates that the great diversity of dinosaurian egg types and eggshell structures allow identification of more discrete characters (i.e., 36 characters) than evolutionarily conservative rigid-shelled turtle eggs. For instance, eggshell microstructure (i.e., shell unit height-to-width ratio, ornamentation, spacing of shell units) is less informative than eggshell thickness and egg shape. In fact, many characters included in my analyses are more useful for distinguishing turtle from archosaurian eggs and provide little or no signal for distinguishing among turtle taxa.

My analysis resolved the fossil clade Adocia by uniting *Adocus sp.* and *Basilemys nobilis*; however, placement of these fossil taxa within the tortoise clade is incongruent with previous analyses (Anquetin, 2012; Joyce et al., 2013). This is not surprising in an analysis based solely on egg attributes because of the strong similarities between their eggs. Their distant phylogenetic relationship with testudinids and convergent evolution of thick eggshell likely contributes to this topology. Grellet-Tinner (2000) and Winkler (2006) also concluded that homoplasy was responsible for lack resolution in their analyses but to a lesser extent in the former than the latter. Therefore, cladistics analyses utilizing only egg and reproductive characters should be used with caution until more informative characters are identified.

Despite these limitations, these characters do have the ability to resolve characters towards the top of the phylogeny, as evidence by the relatively high retention index of the combined analysis. This suggests that when combined with other data sets egg and

reproductive characters provide some phylogenetic signal. Finally, fossil turtle egg and eggshell characters should not be the sole evidence used to identify the taxonomic affinity of isolated eggs or eggshell fragments.

FUTURE DIRECTIONS

Detailed descriptions of fossil turtle egg, especially those preserved inside specimens or containing embryos, will provide more taxonomically identifiable egg characters. This will allow more thorough investigation of evolutionary trends within the reproductive biology of turtles. Assessing the extent of diagenetic alteration allows evaluation of biologically important microstructures, which maybe informative in cladistic analyses. Although rare in the fossil record, inclusion of turtle taxa that produce pliable and/or semi-pliable eggs into a cladistics analysis will provide additional characters and may produce better resolution. Additionally, including more outgroup taxa such as squamates could produce interesting results. Discovery of eggs from stem turtles will potentially add to our knowledge of the origin of the unique aragonite composition of turtle eggs.

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Appendix 1. Explanation of characters and character states used in the morphological and combined analyses.

Character 1:

Mineral composition: (0) calcite; (1) aragonite.

Comments: This character was taken from Varricchio and Jackson (2004). All amniotic eggshells are composed of calcium carbonate with calcite being present in most groups (i.e., lepidosaurs, crocodilians, and birds), whereas all turtle eggshells are composed of needle-like crystals of aragonite, a pseudomorph of calcite (Hirsch, 1983). Therefore, aragonite eggshell is an autapomorphy of Testudines. This character is useful for distinguishing between turtles and other amniotic eggshells but not for distinguishing between different clades within Testudines (ingroup).

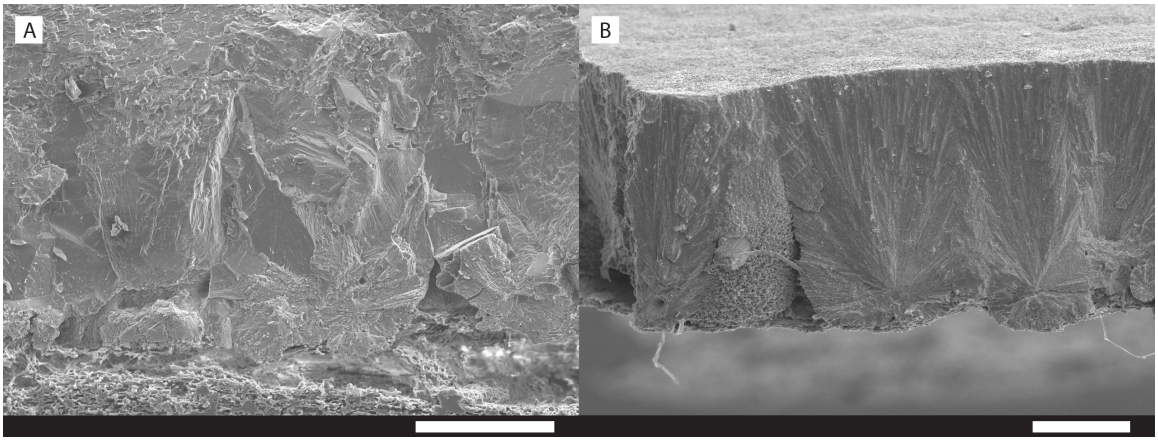


Figure 4. Character 1 Mineral composition. A, calcite (*Dromaius novaehollandiae*). B, aragonite (*Platemys platycephala*). Scale bars equal 100 µm. Note needle-like crystals in B.

Character 2:

Number of eggs laid at a time: (0) multiple eggs en masse; (1) single egg laid at a time.

Comments: All crocodilians and most turtles lay eggs en masse (Grigg and Kirshner, 2015); although capable of laying multiple eggs, some turtles will lay only one egg per clutch (Bonin et al., 2006). All birds produce a single egg over a 24 hour or greater interval, assembling the clutch over days to weeks. Some species delay the onset of incubation until clutch completion.

Character 3:

Number of clutches per year: (0) one; (1) two or more.

Comments: Crocodilians lay only a single clutch per year (Grigg and Kirshner, 2015), whereas most turtles lay multiple clutches (Bonin et al., 2006). Some birds produce a single clutch per year but many taxa are capable of producing two or more clutches in a single breeding season (Cooper et al., 2005).

Character 4:

Egg shape: (0) spherical, length approximately equal to width; (1) elongate eggs, length greater than width.

Comments: Winkler (2006) used a ratio of width-to-length to quantitatively separate this character into three character states. I have modified this character, preserving only two character states. Turtle eggs can range from spherical to elongate (Table 3), whereas all crocodilian and most bird eggs are elongate. Maximum widths and lengths of complete eggs were measured using digital calipers or taken from the literature (Bonin et al., 2006).

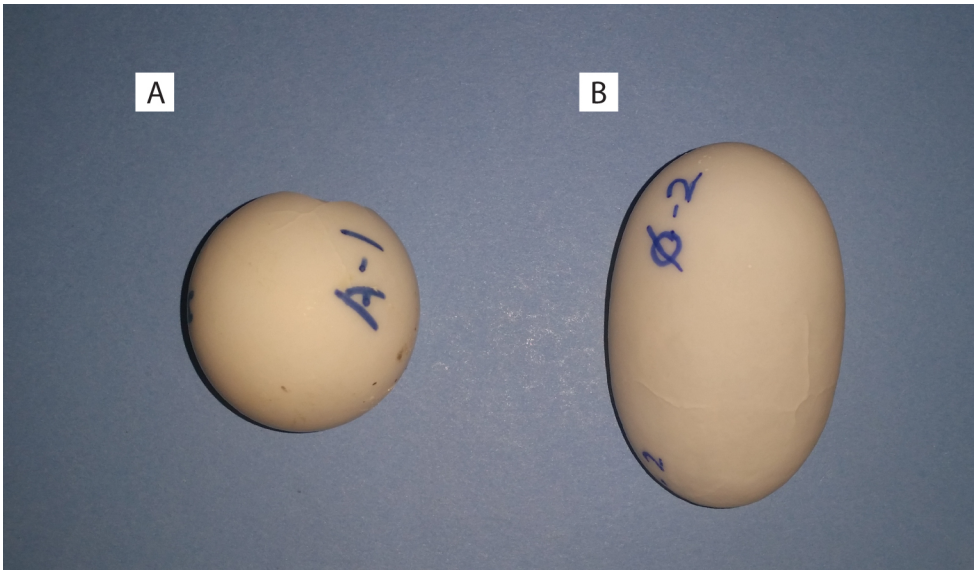


Figure 5. Character 4 Egg shape. A, spherical (*Phrynops geoffroanus*); B, elongate (*Mesoclemmys gibba*).

Table 4. Egg and eggshell characteristics.

Taxon	Egg size (mm)	Egg shape	Eggshell thickness (µm)	Shell unit h:w	Outer surface of shell unit
Phrynops geoffroanus	25–31	Spherical	296	1.35:1	Flat
Emydura subglobosa	31–44 x	Elongate	187	2.1:1	Slightly

	19.5				domed
Elseya novaeguineae	55 x 33	Elliptical	225	1.79:1	Flat
Platemys platycephala	45–50 x 25.5	Oblong	282	1.52:1	Slightly domed
Mesoclemmys gibba	42 x 31	Elongate	507	1.39:1	Flat
Kinosternon minor	28 x 15	Elongate	200	1.5:1	Flat
Kinosternon hirtipes	30 x 17	Elliptical	162	2.6:1	Flat
Kinosternon scorpioides	40x 18	Elongate	316	2.6:1	Flat
Staurotypus triporcatus	40 x 24		284	1.83:1	Flat
Kinosternon carinatum	15 x 28	Elliptical	167	1.98:1	Flat
Dermatemys mawii	60 x 32	Elongate	528	1.54	Flat
Apalone mutica	20–23	Spherical	88	0.91:1	Flat
Apalone ferox	-	Spherical	192	1.4:1	Slightly domed
Apalone spinifera	28	Spherical	138	1.41:1	Slightly domed
Rhinoclemmys areolata	60 x 31		257	2.15:1	Flat
Rhinoclemmys pulcherrima	45 x 28	Elongate	298	0.88:1	Flat
Hieremys annandalii	60 x -	Elongate	448	2.27:1	Flat
Heosemys spinosa	60 x 32	Oblong	347	1.73:1	Flat
Cistoclemmys flavomarginata	42 x 23	Elongate	246	1.62:1	Flat
Kinixyz erosa	45x 35	Oval	529	5.14:1	Flat
Malacochersus tornieri	40x 35	Elongate	197	2.38:1	Slightly domed
Indotestudo elongata	50 x 40	Elliptical	459	2.17:1	Flat
Gallus gallus		Elongate	415	7.2:1	Flat
Dromaius novaehollandiae	150 x 90	Elongate	1056	7.36	Flat
Alligator mississippiensis	-	Elongate	446	1.42:1	Flat
Crocodylus acutus	-	Elongate	593	1.58:1	Flat
Adocus sp.	34–39	Spherical	660–760	3.15–5.5:1	Domed
Basilemys nobilis	40	Spherical	994	4.4:1	?
Meiolania platyceps	53.9	Spherical	800	1.2:1	Flat
Pachycorioolithus jinyunensis	?50 x 32	Elongate	90–166	?	Flat

Character 5:

Egg shape: (0) asymmetrical about the equator; (1) symmetrical about the equator.

Comments: This character was taken from Varricchio and Jackson (2004). Asymmetric eggs (e.g., most bird and some non-avian theropod eggs) are broader at one pole than the other, whereas symmetrical eggs have similar pole dimensions. The symmetry of *Pachycorioolithus jinyunensis* from the Early Cretaceous of China, included in my analysis remains unknown due to the taphonomic loss of one pole. Most turtles and all crocodilian eggs are symmetrical.

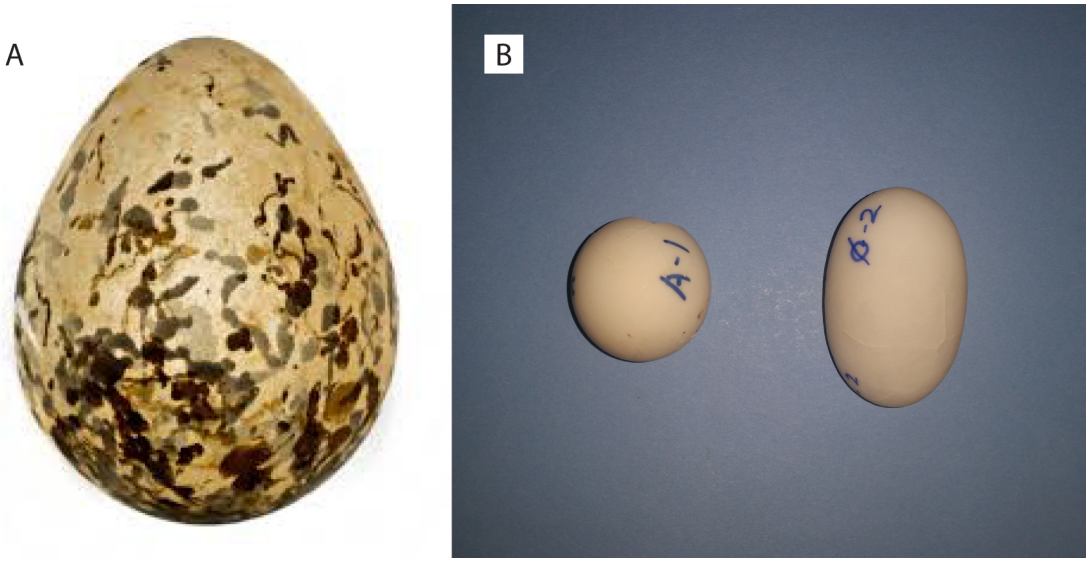


Figure 6. Character 5 Egg shape. A, asymmetrical about the equator; B, symmetrical about the equator (*Phrynops geoffroanus* and *Mesoclemmys gibba*).

Character 6:

Shell unit height-to-width ratio: (0) 0.8 – 1.2; (1) 1.2 – 2; (2) >2.

Comments: Three character states are necessary to show the extreme differences in height-to-width ratio amongst turtles, and therefore this character was modified from Winkler (2006) to include quantitative information. The rigid, tightly abutting shell units are taller than wide, except in *Apalone mutica* and *Rhinoclemmys pulcherrima*, which are wider than tall.

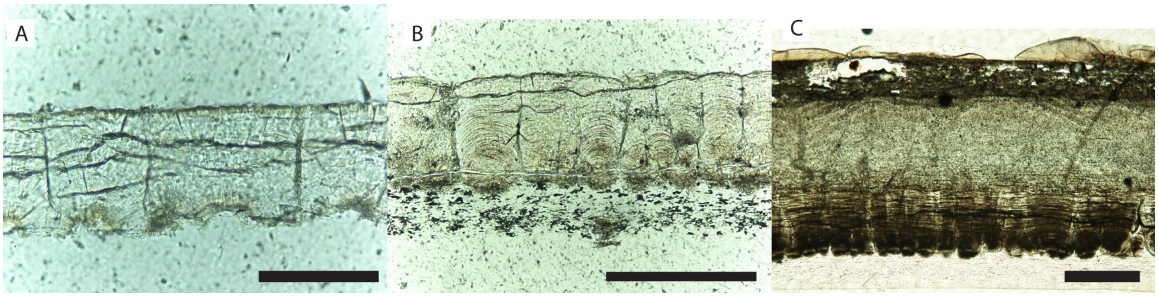


Figure 7. Character 6 Shell unit height-to-width ratio. A, h:w = 0.8 – 1.2; B, h:w = 1.2 – 2; C, h:w greater than 2. Scale bars equal 100 µm (A), 250 µm (B, C).

Character 7:

Eggshell thickness: (0) < 0.2 mm; (1) < 0.4 mm but > 0.2 mm; (2) > 0.4 mm.

Comment: Eggshell thickness ranges from 0.088 mm in *Apalone mutica* eggs to 0.994 mm in *Basilemys nobilis* eggs.

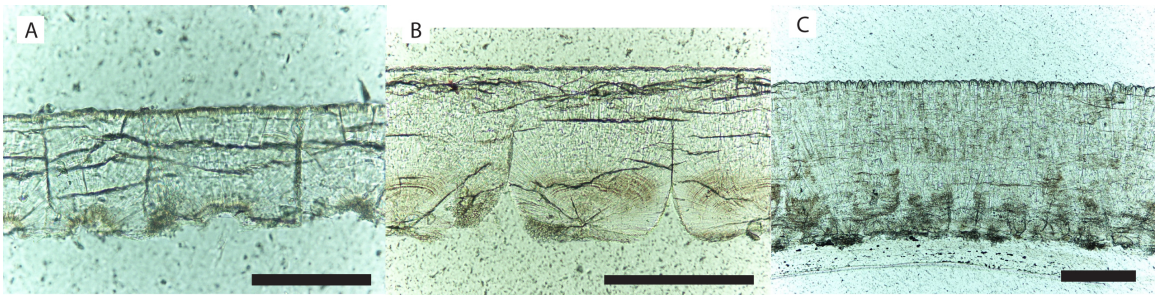


Figure 8. Character 7 Eggshell thickness. A, < 0.2 mm (*Apalone mutica*); B, 0.4–0.2 mm thick (*Phrynops geoffroanus*); C, > 0.4 mm (*Kinixyz erosa*). Scale bars equal 100 µm (A), 250 µm (B, C).

Character 8:

Detailed surface morphology: (0) smooth; (1) ornamented surface.

Comments: This character was modified from Winkler (2006) because of the greater diversity of ornamentation in my study. The shell units' outer surfaces are typically flat, but in some taxa exhibit ornamentation or microscopic texture such as pitting, bumps, or linear features. Different character states for each type of ornamentation are not used here because many are only observed in a single taxon and therefore would not provide a phylogenetic signal. These observations are made under SEM of the outer eggshell surface.

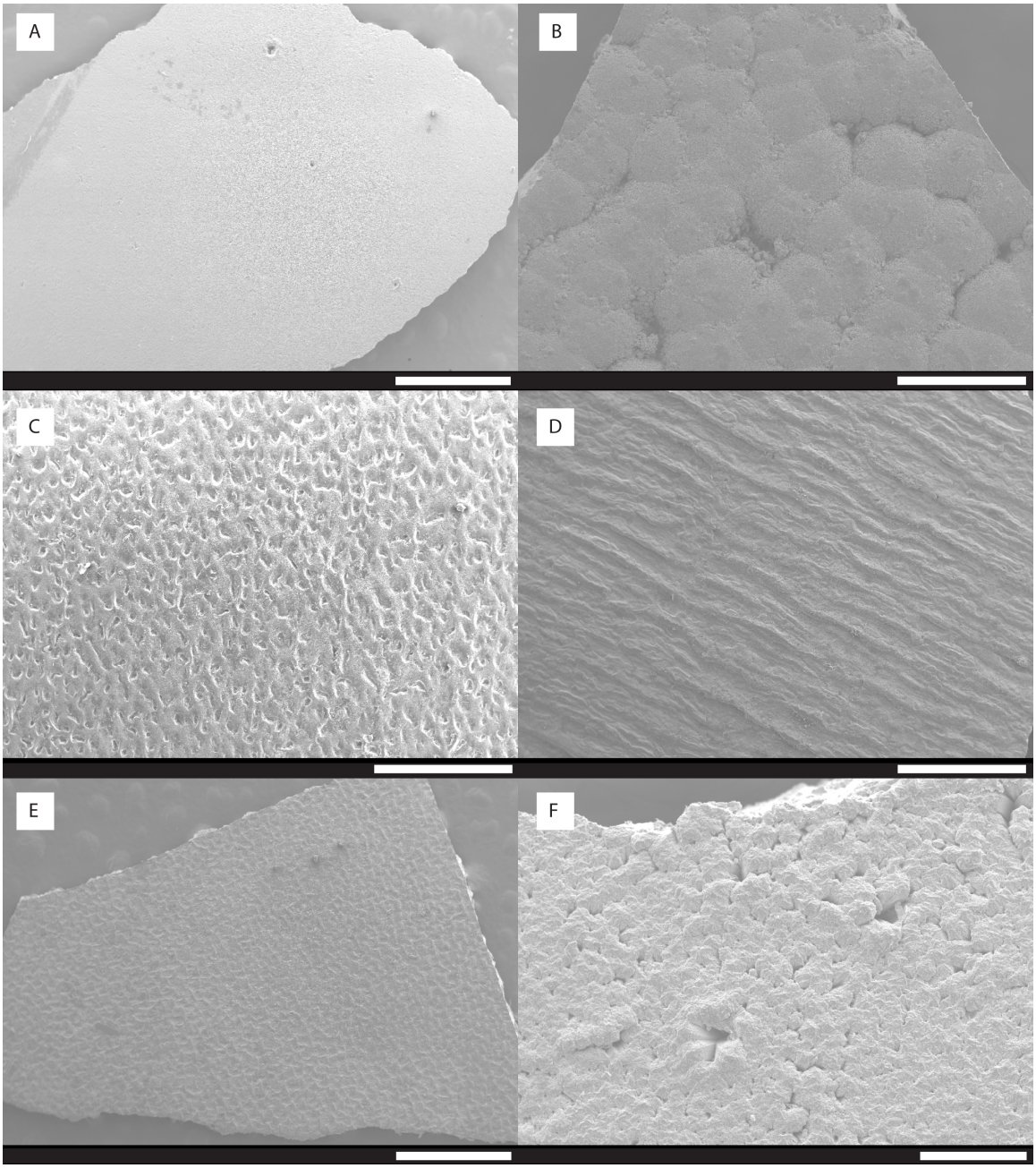


Figure 9 Character 8 Detailed surface morphology. Turtle eggshell ornamentation. A, absent (*Phrynops geoffroanus*); B, present, low domed (*Emydura subglobosa*); C, present, highly pitted and associated with pores (*Staurotypus triporcatus*); D, present, linear ridges (*Dermatemys mawii*); E, present, highly pitted and not associated with pores (*Rhinoclemmys areolata*); F, present, individual aragonite crystals visible on external surface (*Mesoclemmys gibba*). Scale bars equal 100 µm (A, E, F), 200 µm (B), 500 µm (C), 800 µm (D).

Character 9:

External surface of shell unit: (0) shell units not visible; (1) shell unit clearly outlined.

Comments: This character was taken from Winkler (2006). These observations are made under SEM of the outer eggshell surface.

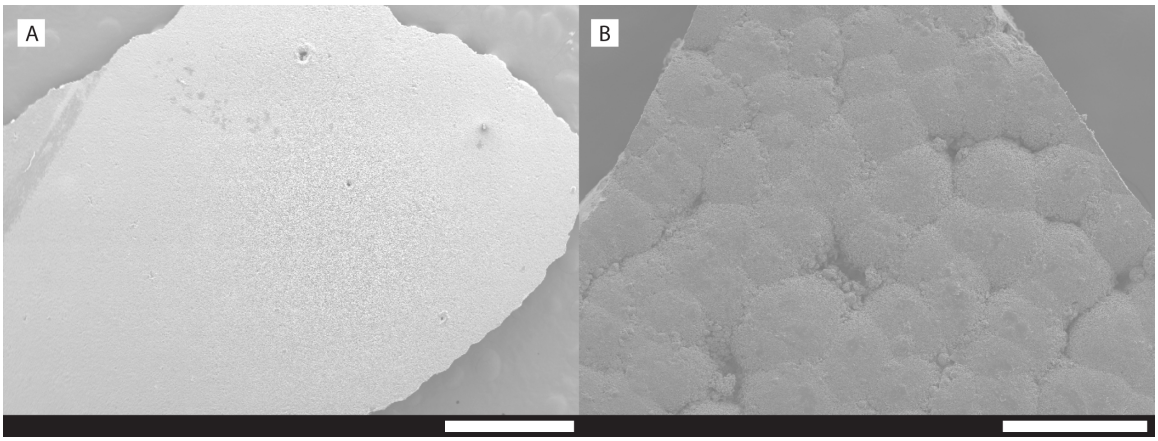


Figure 10. Character 9 External surface of shell unit. A, shell units not visible on external surface (*Phrynops geoffroanus*); B, visible on external surface (*Emydura subglobosa*). Scale bars equal 100 µm (A), 200 µm (B).

Character 10:

External surface of shell unit: (0) flat; (1) domed.

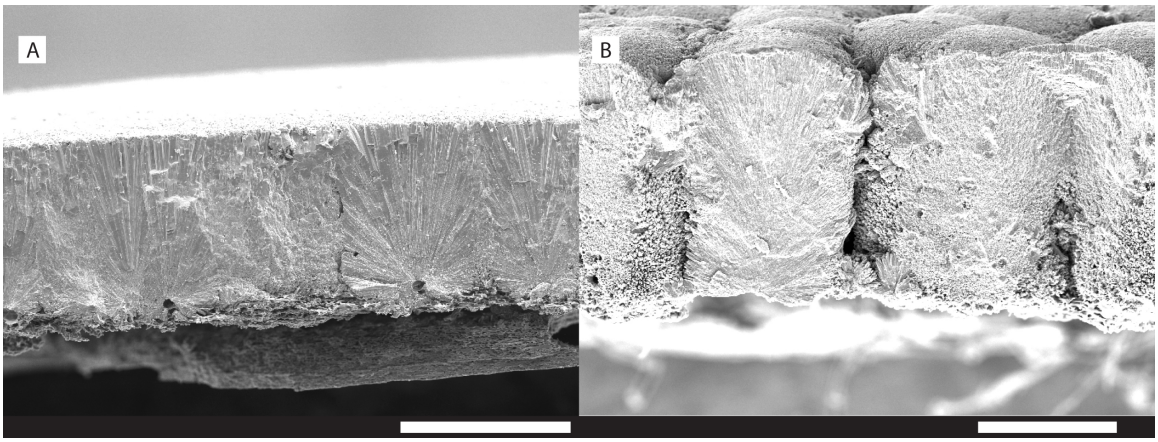


Figure 11. Character 10 External surface of shell unit. A, flat shell unit (*Kinixyz erosa*); B, domed (*Emydura subglobosa*). Scale bars equal 300 µm (A), 90 µm (B).

Character 11:

Accretion lines: (0) absent; (1) present.

Comment: Accretion lines are typically only visible in thin section but *Meiolania platyceps* eggshell exhibits accretion lines in SEM due to diagenetic dissolution along the lines.

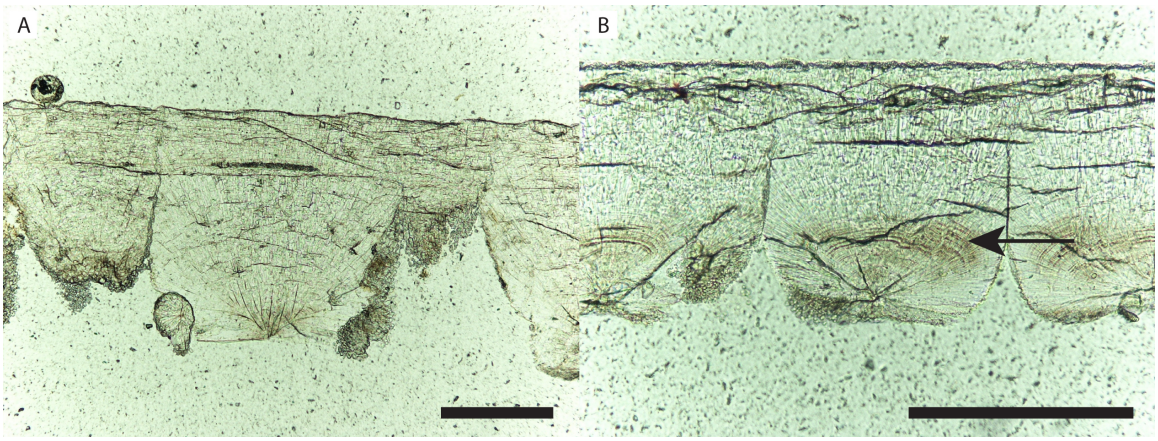


Figure 12. Character 11 Accretion lines. A, accretion lines absent (*Dermatemys mawii*); B, present (*Phrynops geoffroanus*). Scale bars equal 250 μm.

Character 12:

Shape of accretion lines: (0) straight; (1) curved; (2) both.

Comments: This character was taken from Barta (2014). Accretion lines are typically present in the lower half of the eggshell and generally concave down (Table 4). Some taxa have straight, horizontal accretion lines in the top half of the eggshell. Eggshell with only straight accretion lines was not observed indicating that curved lines are potentially a prerequisite for straight lines in turtle eggs.

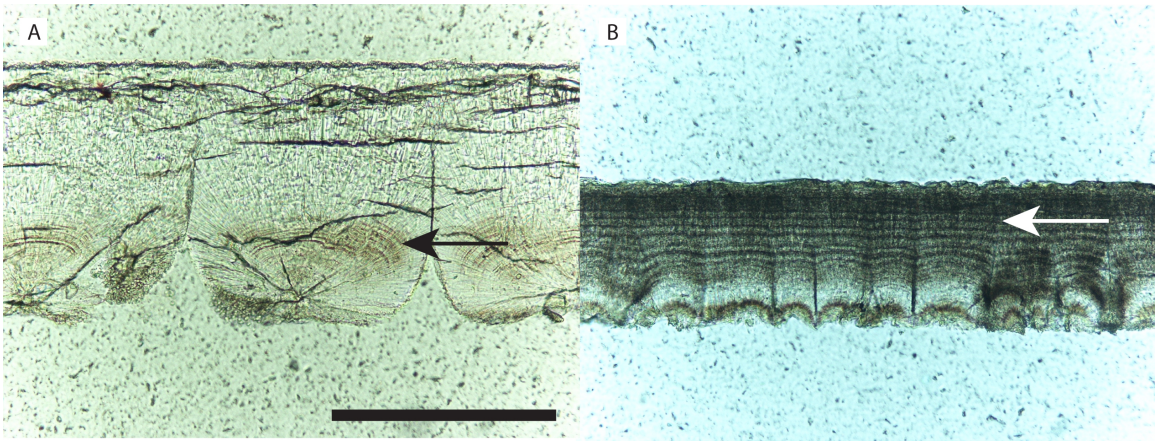


Figure 13. Character 12 Shape of accretion lines. A, curved accretion lines (*Phrynops geoffroanus*); B, straight accretion lines (*Kinosternon hirtipes*). Scale bar equals 250 µm.

Character 13:

Distribution of accretion lines: (0) present in basal half of the eggshell; (1) present throughout eggshell thickness.

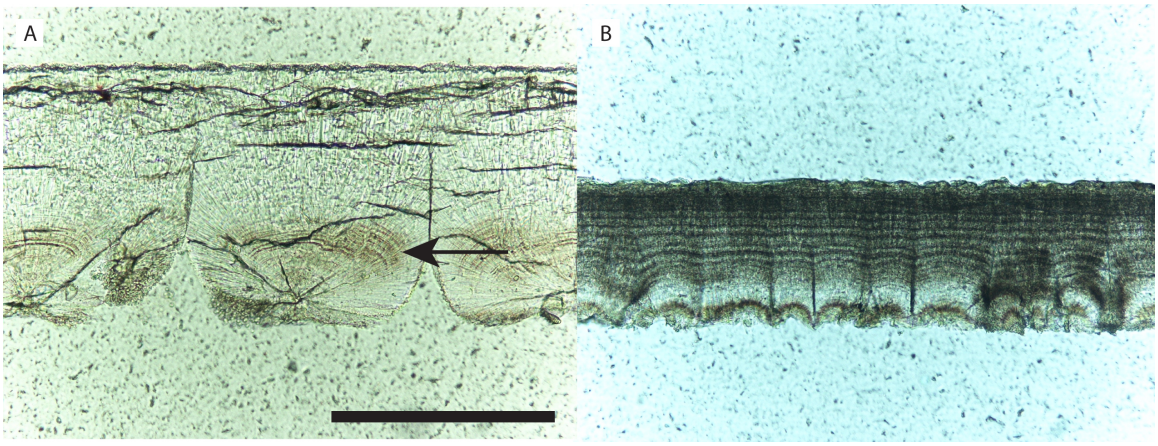


Figure 14. Character 13 Distribution of accretions lines. A, accretion lines in basal half of eggshell (*Phrynops geoffroanus*); B, accretion lines throughout eggshell thickness (*Kinixyz hirtipes*). Scale bar equals 250 µm.

Table 5. Egg and eggshell characteristics.

Taxon	Pore width (μm)	Pore frequency	Accretion line distribution	Accretion line shape
Phrynops geoffroanus	25–60	Infrequent	Bottom half	Curved
Emydura subglobosa	16–51	Frequent	Throughout	Curved
Elseya novaeguineae	10	Frequent	Bottom half	Curved
Platemys platycephala	16	Sparse	Bottom half	Curved
Mesoclemmys gibba	25–45	Infrequent	Bottom half	Curved
Kinosternon minor	-	-	Bottom 60%	Curved
Kinosternon hirtipes	31	Infrequent	Throughout	Curved /Straight
Kinosternon scorpioides	-	-	Bottom 75%	Curved /Straight
Staurotypus triporcatus	20	Frequent	Bottom	Curved
Kinosternon carinatum	3	Infrequent	Bottom 75%	Curved
Dermatemys mawii	3	Infrequent	Absent	-
Apalone mutica	20–50	infrequent	Absent	-
Apalone ferox	47	Frequent	Bottom half	Curved
Apalone spinifera	8	Infrequent	Bottom half	curved
Rhinoclemmys areolata	-	-	Bottom 2/3	Curved
Rhinoclemmys pulcherrima	-	-	Bottom half	Curved
Hieremys annandalii	-	-	Bottom 75%	Curved
Heosemys spinosa	2	Infrequent	Bottom half	Curved
Cistoclemmys flavomarginata	-	-	Absent	-
Kinixyz erosa	30–50	Infrequent	Absent	-
Malacochersus tornieri	-	-	Absent	-
Indotestudo elongata	-	-	Bottom half	Curved
Gallus gallus	13	Infrequent	Absent	-
Dromaius novaehollandiae	45	-	Bottom	Straight
Alligator mississippiensis	74	Frequent	Absent	-
Crocodylus acutus	46	Frequent	Absent	-
Adocus sp.	50	?	Bottom	Curved
Basilemys nobilis	42	?	Bottom	Curved
Meiolania platyceps	60–320	Frequent	Throughout	Curved /Straight
Pachycorioolithus jinyunensis	20–30	Infrequent	Absent	-

Character 14:

Appressed shell units (inner surface): (0) absent, (1) present.

Comments: This character was modified from Winkler (2006) because of the somewhat ambiguous nature of its original description. This character is present if a series of shell units merge without any indication of shall unit boundaries as observed from the inner eggshell surface. Additionally, many specimens that have appressed shell units often exhibit resorption craters that merge together to form long troughs.

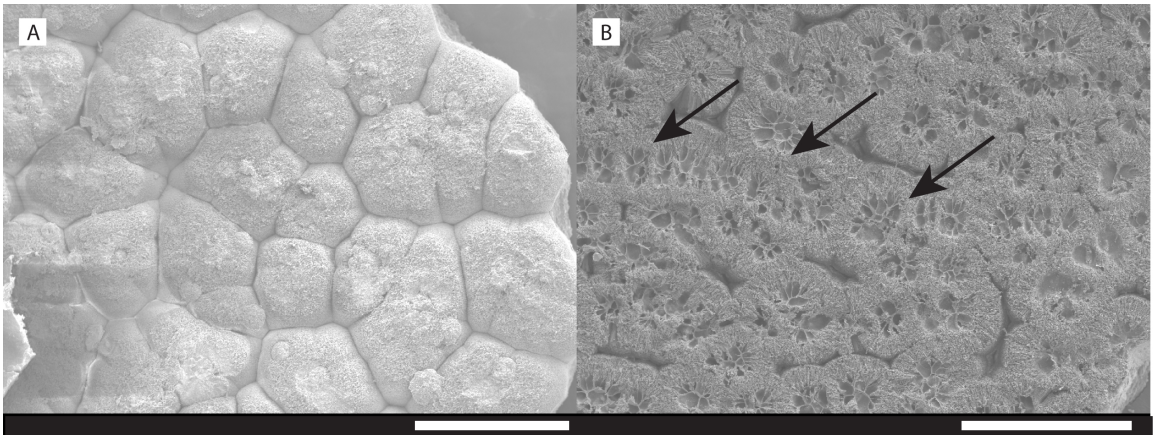


Figure 15. Character 14 Appressed shell units. A, appressed shell units absent (*Mesoclemmys gibba*); B, present (*Kinosternon hirtipes*). Scale bars equal 600 µm (A), 300 µm (B).

Character 15:

Interlocking shell units: (0) distinct shell unit border; (1) indistinct shell unit border.

Comment: This character was taken from Winkler (2006). A distinct shell unit border is defined by a hard line between two adjacent shell units when observed under SEM. When two adjacent shell units lack clear separation, the shell unit border is coded as indistinct.

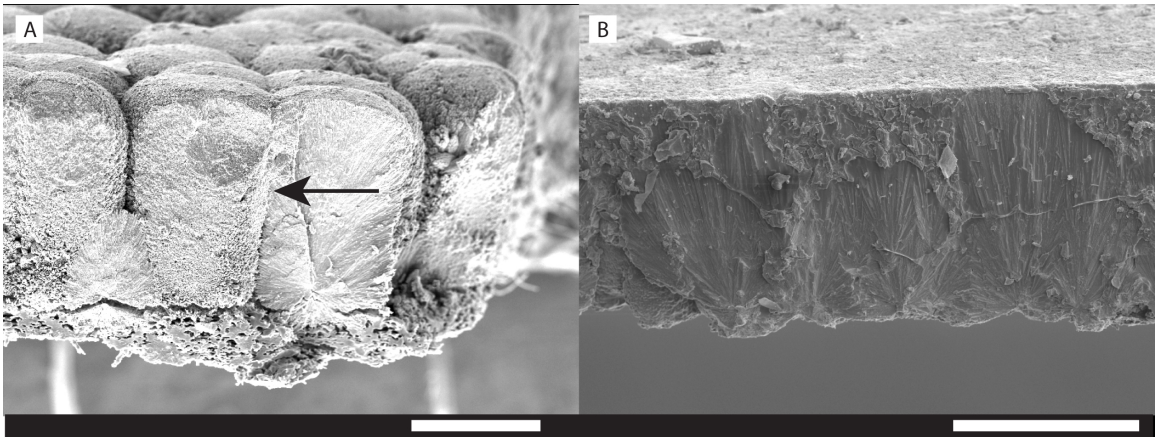


Figure 16. Character 15 interlocking shell units. A, distinct shell unit border (*Emydura subglobosa*); B, indistinct shell unit border (*Phrynops geoffroanus*). Scale bars equal 100 µm (A), 200 µm (B).

Character 16:

Size of inter-unit spaces: (0) small, (1) large.

Comment: This character was taken from Winkler (2006). Small inter-unit spaces extend from the inner to outer surface, to approximately one-fifth of the total shell unit height or further as a slit-like structure. Large inter-unit spaces extend further than 1/5th of the total shell unit height; the space width at the inner shell surface approximates the width of the shell unit base. These features frequently represent the basal-most portion of pore canals.

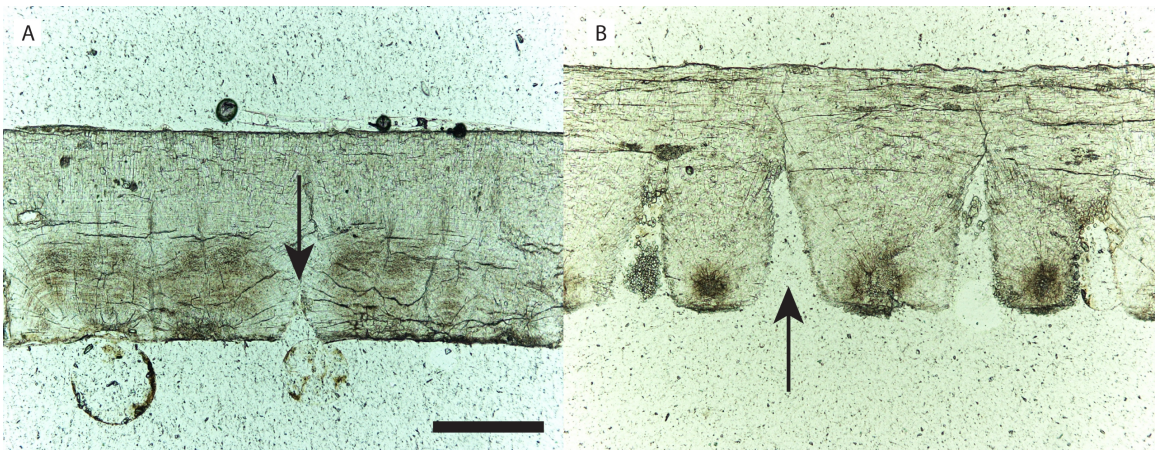


Figure 17. Character 16 Size of inter-unit spaces. A, small inter-unit space (*Indotestudo elongata*); B, large inter-unit space (*Dematemys mawii*). Scale bar equals 250 µm.

Character 17:

Irregular crystal growth between shell units: (0) present, (1) absent.
Comments: This character was taken from Winkler (2006).

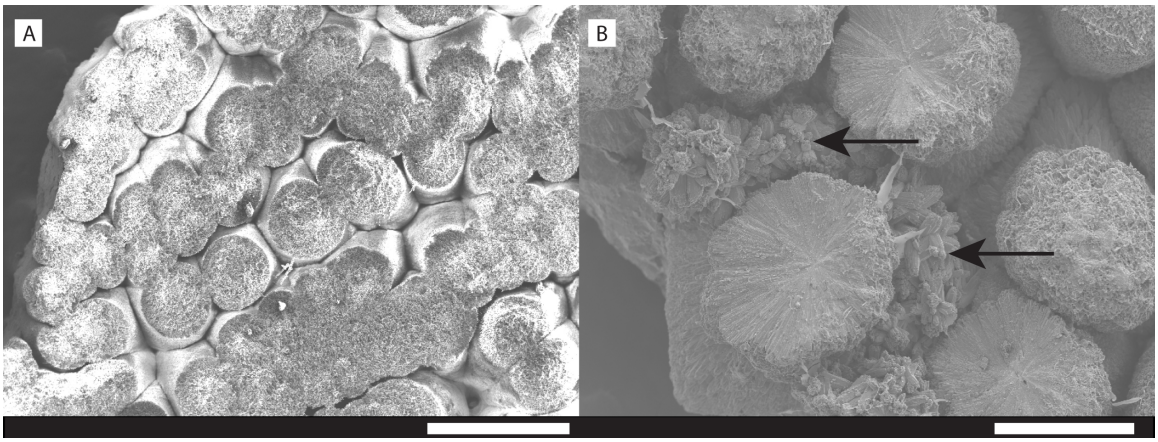


Figure 18. Character 17 Irregular crystal growth. A, irregular crystal growth absent (*Staurotypus triporcatus*); B, present (*Dermatemys mawii*). Scale bars equal 300 µm (A), 500 µm (B).

Character 18:

Development of pore apertures (as seen on outer surface under SEM): (0) absent; (1) present.
Comments: This character was taken from Winkler (2006).

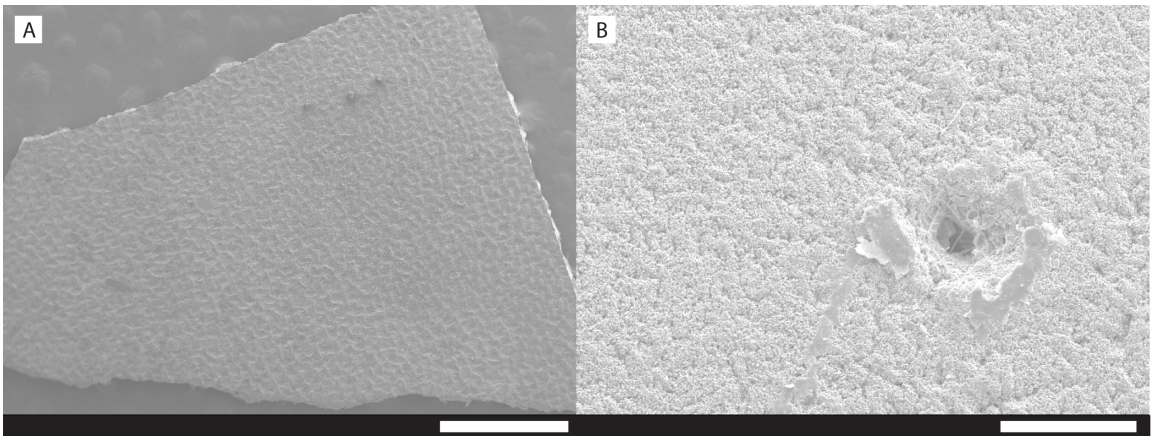


Figure 19. Character 18 Pores. A, pores absent (*Rhinoclemmys areolata*); B, pores present (*Phrynops geoffroanus*). Scale bar equals 100 µm (A), 80 µm (B).

Character 19:

Pore shape: (0) round, (1) oval or flattened.

Comment: Pore shape as seen from the external surface of the eggshell.

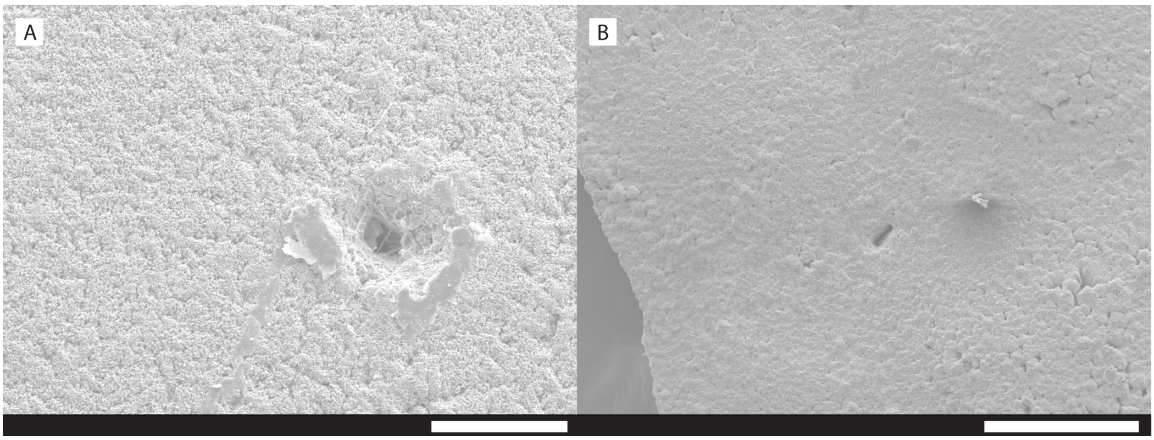


Figure 20. Character 19 Pore shape. A, round pores (*Phrynops geoffroanus*); B, flattened pores (*Mesoclemmys gibba*). Scale bars equal 80 µm (A), 300 µm (B).

Character 20:

Distribution of pore apertures: (0) sparse, (1) frequent, (2) dense.

Comment: This character was taken from Winkler (2006). As in Winkler's (2006) analysis, some specimens were limited to crushed eggs and therefore exact location on the egg from which each eggshell fragment was taken is questionable.

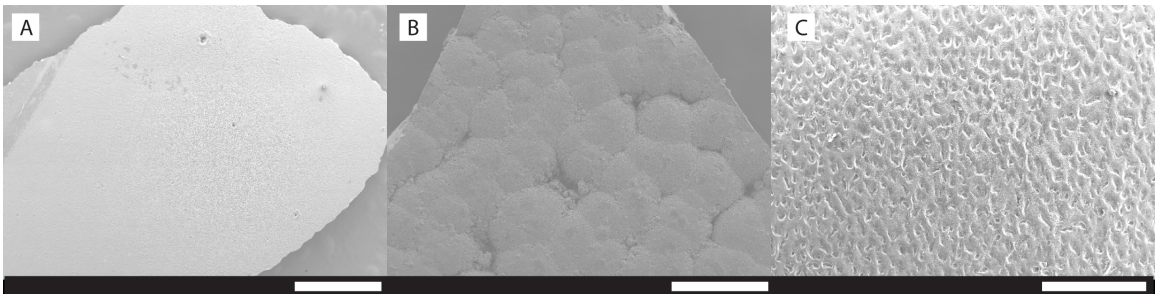


Figure 21. Character 20 Distribution of pores. A, sparse pores (*Phrynops geoffroanus*); B, frequent pores (*Emydura subglobosa*); C, dense pores (*Staurotypus triporcatus*). Scale bars equal 100 µm (A), 200 µm (B), 500 µm (C).

Character 21:

Extinction pattern: (0) sweeping (1) blocky.

Comment: This character was taken from Barta (20014). Sweeping extinction is characteristic of turtle and some non-avian dinosaur eggshells (e.g., *Megaloolithus*) eggshells, whereas blocky extinction is characteristic of avian and crocodilian eggshell. Therefore, this character is only informative in distinguishing turtle eggs from avian and crocodilian eggs.

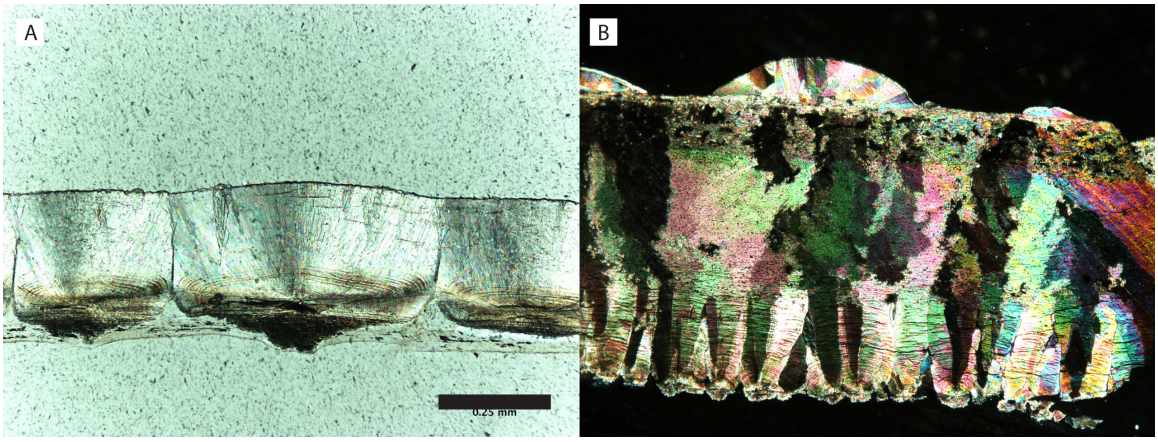


Figure 22. Character 21 Extinction pattern. A, sweeping extinction pattern (*Mesoclemmys gibba*); B, blocky (*Dromaius novaehollandiae*). Scale bar equals 250 µm.

Character 22:

Multiple eggshell layers: (0) absent, (1) present.

Comment: Turtle eggshell is characterized by a single layer of shell units consists of needle-like aragonite crystals that radiate from an organic core that lies within the shell membrane.

Multiple eggshell layers correspond to the mammillary, continuous and external layers in avian eggshell and inner middle and outer layers of crocodilians (Table 5); the homology of these layers remains unknown. This character is informative to distinguish between avian and crocodilian eggshell and other amniotic eggshell; however, it is not informative for chelonians.

Emus and cassowaries contain an additional layer (i.e., resistant zone) that measures 190 µm and lies between the continuous and external layers. This porous layer exhibits flat upper and lower boundaries with the external and continuous layers, respectively. The mammillary layer displays a slightly undulating upper contact with the continuous layer in *G. gallus* and *D. novaehallandiae* but appears flat in *P. jinyunensis.* Additionally, the mammillary layer of *D. novaehallandiae* exhibits horizontal lines representing tabular crystalline structure. The continuous layer features squamatic texture (sensu Mikhailov, 1997) that obscures shell units in *D. novaehallandiae* and *P.*

jinyunensis. The external layer exhibits narrow, vertically oriented prisms in *G. gallus and P. jinyunensis*, respectively. The wide dome-shaped ornamentation of the external layer of *D. novaehallandiae* eggshell spans the width several shell units, thus resembling sagenotuberculate ornamentation (sensu Mikhailov, 1997) in hand sample. This ornamentation consists of calcite crystals that appear to radiate from a central location (Fig. 3B). *Dromaius novaehollandiae* eggshell is highly pigmented. Teal to green color occurs in the valleys between the ornamentation (which corresponds to the external surface of resistant zone) whereas the ornamentation is dark green to black. The extinction pattern of modern and fossil bird eggshell is blocky under crossed polarized light.

In crocodilian eggs the shell units are composed of calcite wedges (sensu Mikhailov, 1997) and large caverns frequently occur between shell units in this layer (Fig. 3C, D). The inner layer exhibits an aggregate of calcite plates (rosettes) that form the basal knobs that provide the nucleation site for shell unit growth. The middle layer is characterized by tabular ultrastructure that forms regular striations. The outer layer also exhibits tabular ultrastructure arranged into vertical columns (Marzola et al., 2014). Shell units are roughly wedge-shaped with the widest region occurring at the outer eggshell surface and light microscopy does not reveal accretion lines. Microscopic examination of the outer eggshell surface reveals a slightly undulating ornamentation due to erosion craters.

Crocodilian eggshell microstructure has been the subject of controversy since the 1980's. Hirsch (1985) and Mikhailov (1991) describe the crocodyloid "basic type" of eggshell structure as consisting of a single-layered eggshell; however, Ferguson (1982) observed multiple structural layers in *Alligator mississippiensis* eggshell. Marzola et al. (2014) confirmed this observation in eggs of this taxon, as well as in *Paleosuchus palpebrosus* and *Crocodylus mindorensis*. Moreno-Azanza et al. (2014) also described three structural layers in the eggshell from an indeterminate krokolithid specimen from the Upper Cretaceous of the Pyrenees. Coding of this character has major consequences for the placement of crocodilians. When the analysis was run with coding for a single structural layer, crocodilians are resolved within the turtle polytomy as the sister taxon to *Meiolania platyceps*. In contrast, coding for multiple structural layers moves the crocodilian eggs outside of Testudines and into the outgroup to form an archosaurian clade, along with Aves, which are consistently resolved outside of Testudines.

Figure 6. Outgroup eggshell structural layer measurements

Taxon	Mammillary layer (µm)	Continuous layer (µm)	External layer (µm)
Gallus gallus	90	215	30
Dromaius novaehollandiae	385	460–490	36–150
Pachycorioolithus jinyunensis	46	46	74
	Inner layer (µm)	Middle layer (µm)	Outer layer (µm)
Alligator mississippiensis	100	134	56
Crocodylus acutus	127	173	136

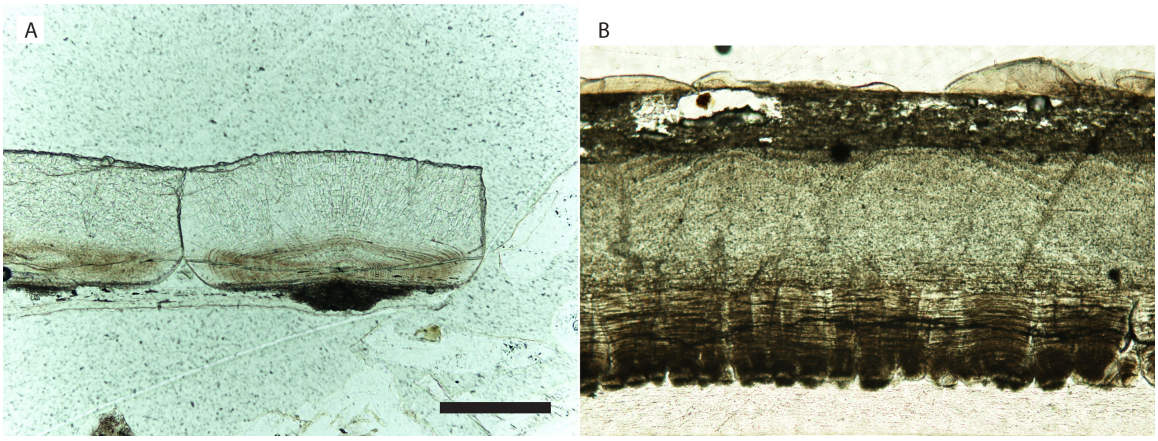


Figure 23. Character 22 Multiple eggshell layers. A, single eggshell layer (*Staurotypus triporcatus*). B, multiple eggshell layers (*Dromaius novaehallandiae*). C, multiple eggshell layers (*Gallus gallus*). D, multiple eggshell layers (*Crocodylus acutus*). Scale bars equal 200 µm (A, D), 500 µm (B), 100 µm (C).

Character 23:

Nest type: (0) buried, (1) vegetative mound, (2) ground surface.

Comments: This character was modified from Barta (2014) to better reflect the nesting habits of Testudines. Turtles are not known to partially bury their eggs in the same way that is observed in some theropod taxa.



Figure 24. Character 23 Nest type. A, buried nest; B, vegetative mound nest; C, surface nest.

Character 24:

Cuticle layer on outer surface: (0) absent, (1) present.

Comment: This character was taken from Varricchio and Jackson (2004). As with most bird eggs, some turtle eggs possess a cuticle on the outer surface of the eggshell. Winkler (2006) also documented this in Pelomedusoides, *Elseya dentate*, and *Erymnochelys madagascariensis.*

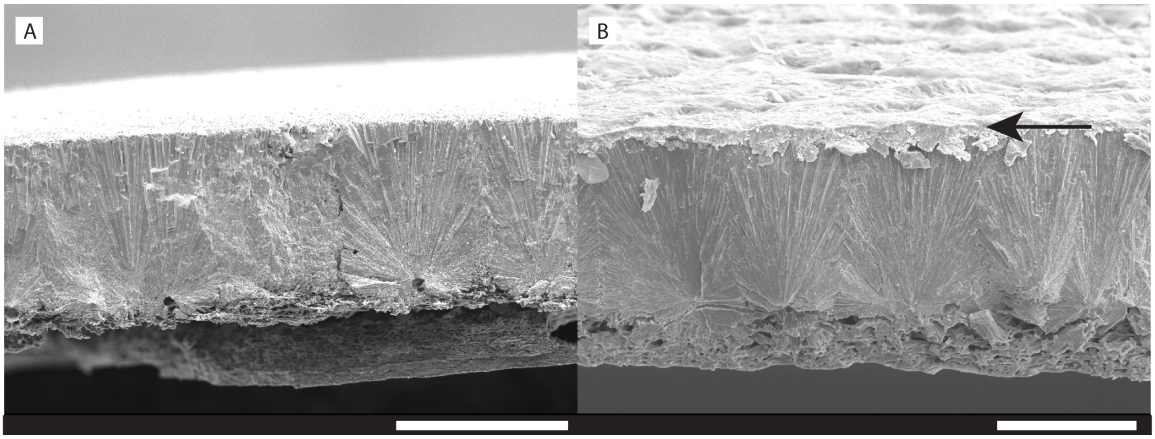


Figure 25. Character 24 Cuticle. A, absent (*Kinixyz erosa*); B, present (*Kinosternon carinatum*). Scale bars equal 300 µm (A), 90 µm (B).

Appendix 2: Data matrix for the morphological and combined analyses.

	10	20	24
Phrynops geoffroanus	10?0101000	1100100100	0000
Emydura subglobosa	10?1110101	1110000111	00?0
Elseya novaeguineae	10?1101000	110?000112	00?0
Platemys platycephala	1111101011	1101000100	00?0
Mesoclemmys (Phrynops) gibba	10?1102000	1100111101	0020
Kinosternon minor	1{0 1}11101000	1101101111	00{0 2}0
Kinosternon hirtipes	1011110000	1211000100	00?0
Kinosternon scorpioides	1011111100	1211100??0	00?0
Staurotypus triporcatus	10?1111100	11010001{0 1}2	00?0
Kinosternon carinatum	1011100000	111?000100	00?1
Dermatemys mawii	1011102000	0—0011100	00{0 2}0
Apalone mutic	1010100000	0--10001{0 1}0	00?0
Apalone ferox	1010100011	1101000101	0010
Apalone spinfera	1010100011	1101000101	0010
Rhinoclemmys areolata	10?1011100	1110001100	00?1
Rhinoclemmys pulcherrima	1011101100	1100110?00	00?0
Hieremys annandalii	10?1112100	1111100?10	00?0
Heosemys spinosa	1{0 1}?1101100	1101000100	00?0
Cistoclemmys flavomarginata	1111101000	0–11000101	00?0
Kinixys erosa	1011112100	0—?000101	0010
Malacochersus tornieri	1111110001	0—1000111	00?0
Indotestudo elongata	1011112100	1101000??0	00?0
Adocus sp.	10?01221?1	110?001???	0000
Basilemys nobilis	1??0122???	110?001???	00??
Meiolania platyceps	10?01021?0	121?110?12	0000
Gallus gallus	0??1?20???	0—?101???	11??
Dromaius novaehollandiae	0111021 0?0	0—?100??0	1121
Pachycorioolithus jinyunensis	0111022100	110?100??0	1120
Alligator mississippiensis	0001101100	0—?010?02	1110
Crocodylus acutus	0001102100	0—?110102	1100

Appendix 3. Ancestral state reconstruction of morphological characters on the preferred tree topology from Joyce (2007) and Crawford et al. (2015). See Appendix 1 for description of character states.

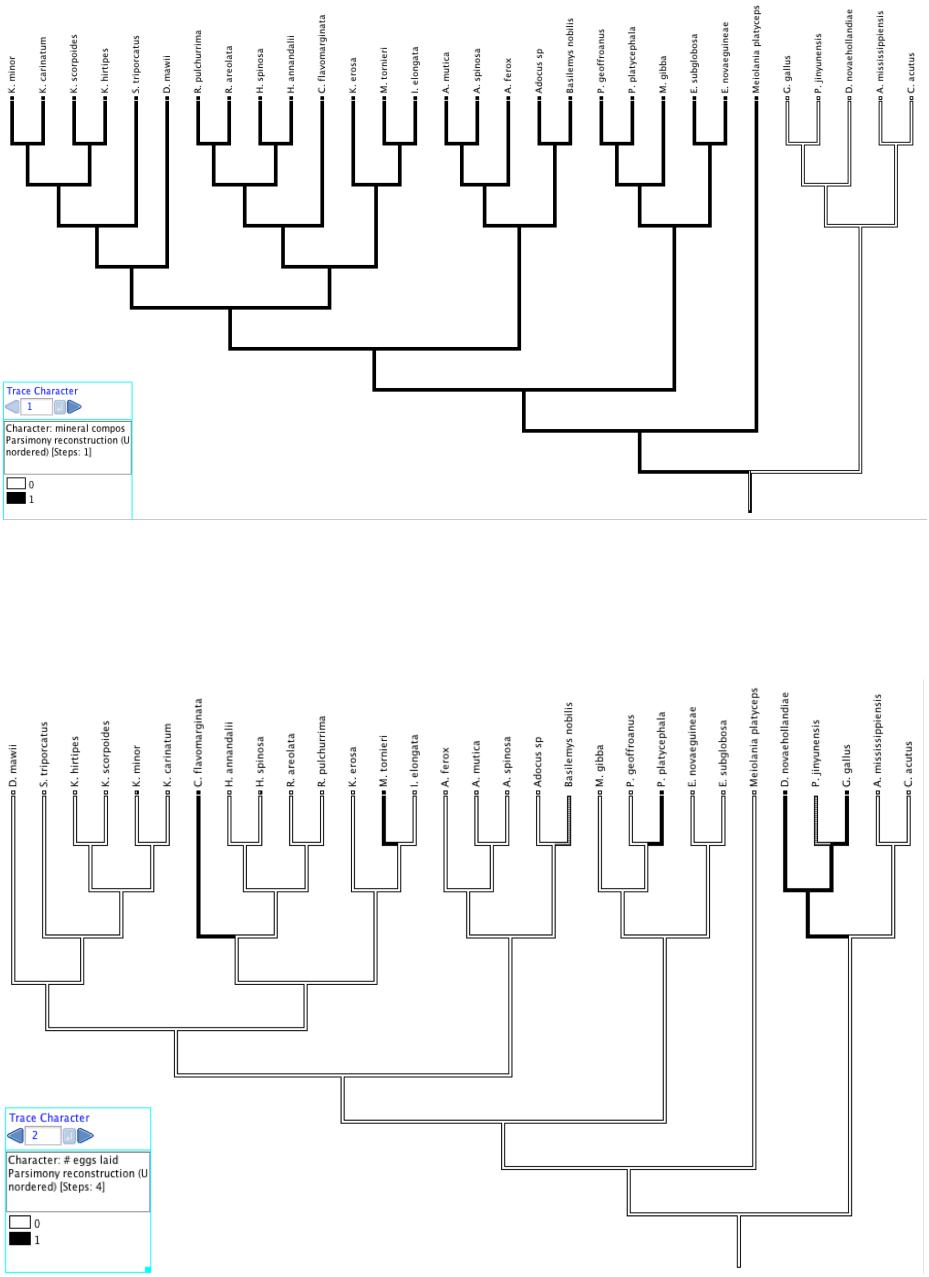


Figure 26. Ancestral state reconstruction of characters 1 and 2.

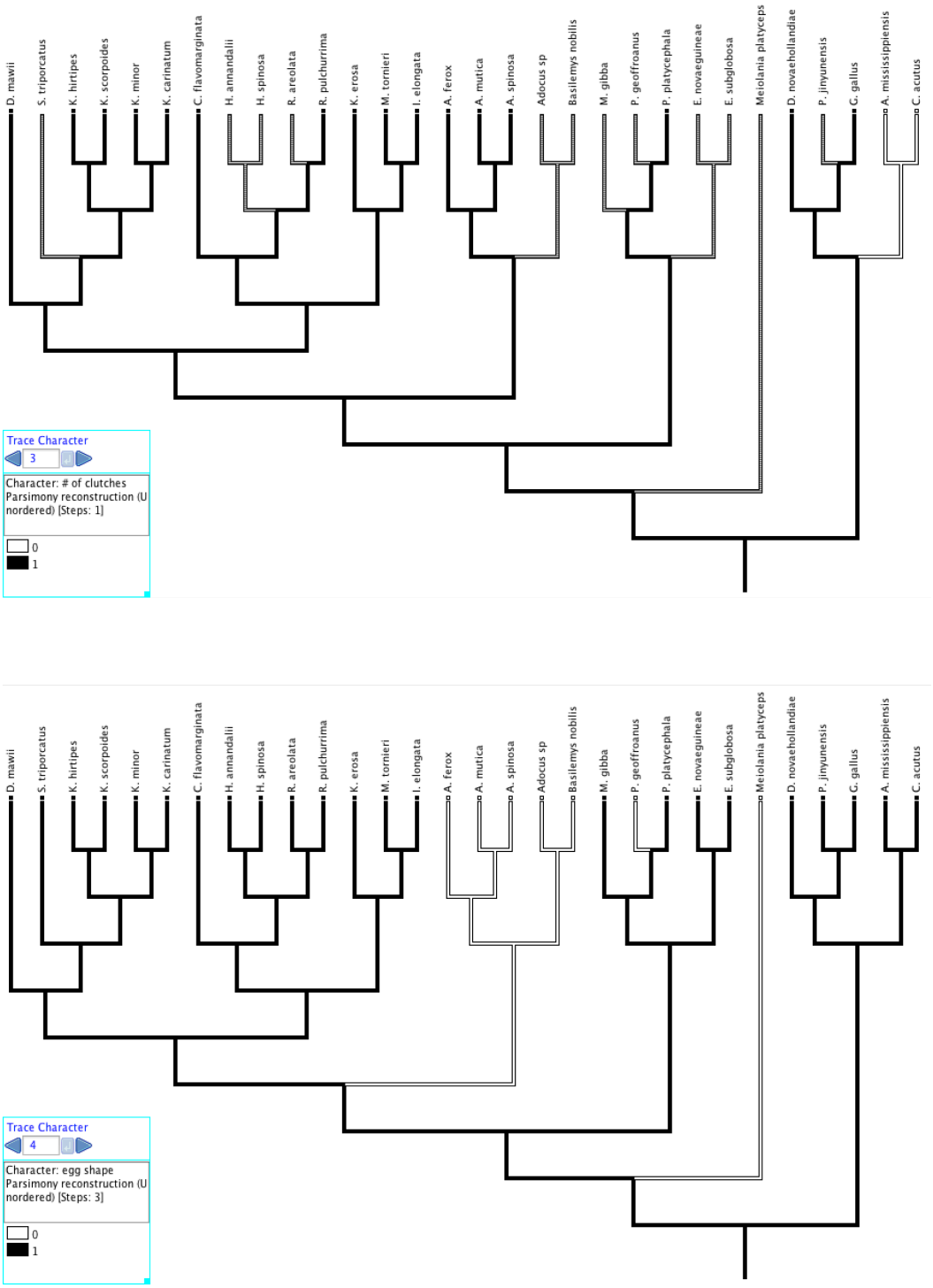
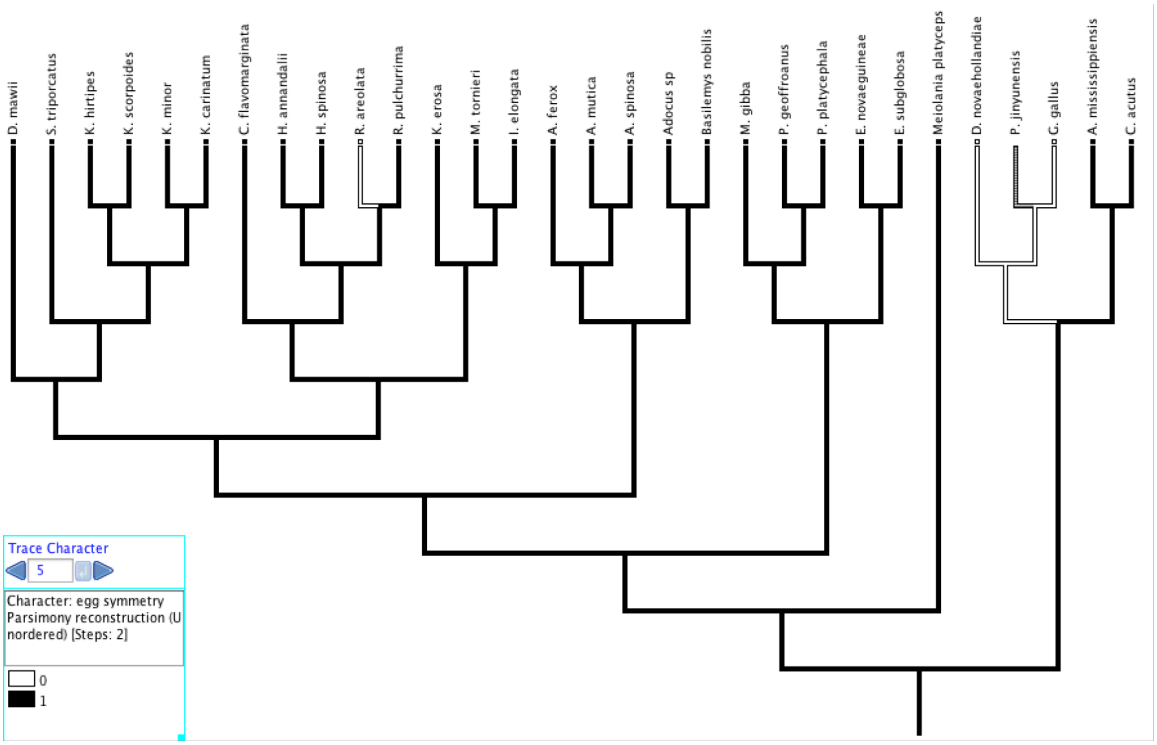


Figure 27. Ancestral state reconstruction of characters 3 and 4.



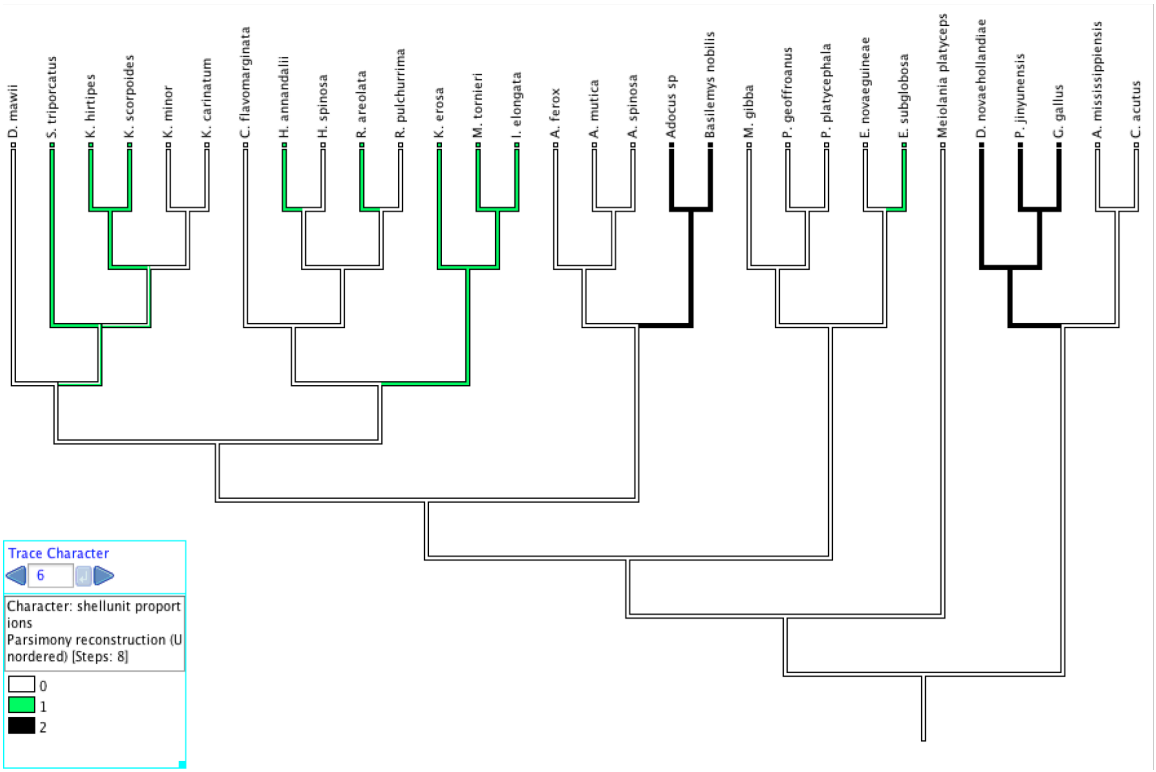


Figure 28. Ancestral state reconstruction of characters 5 and 6.

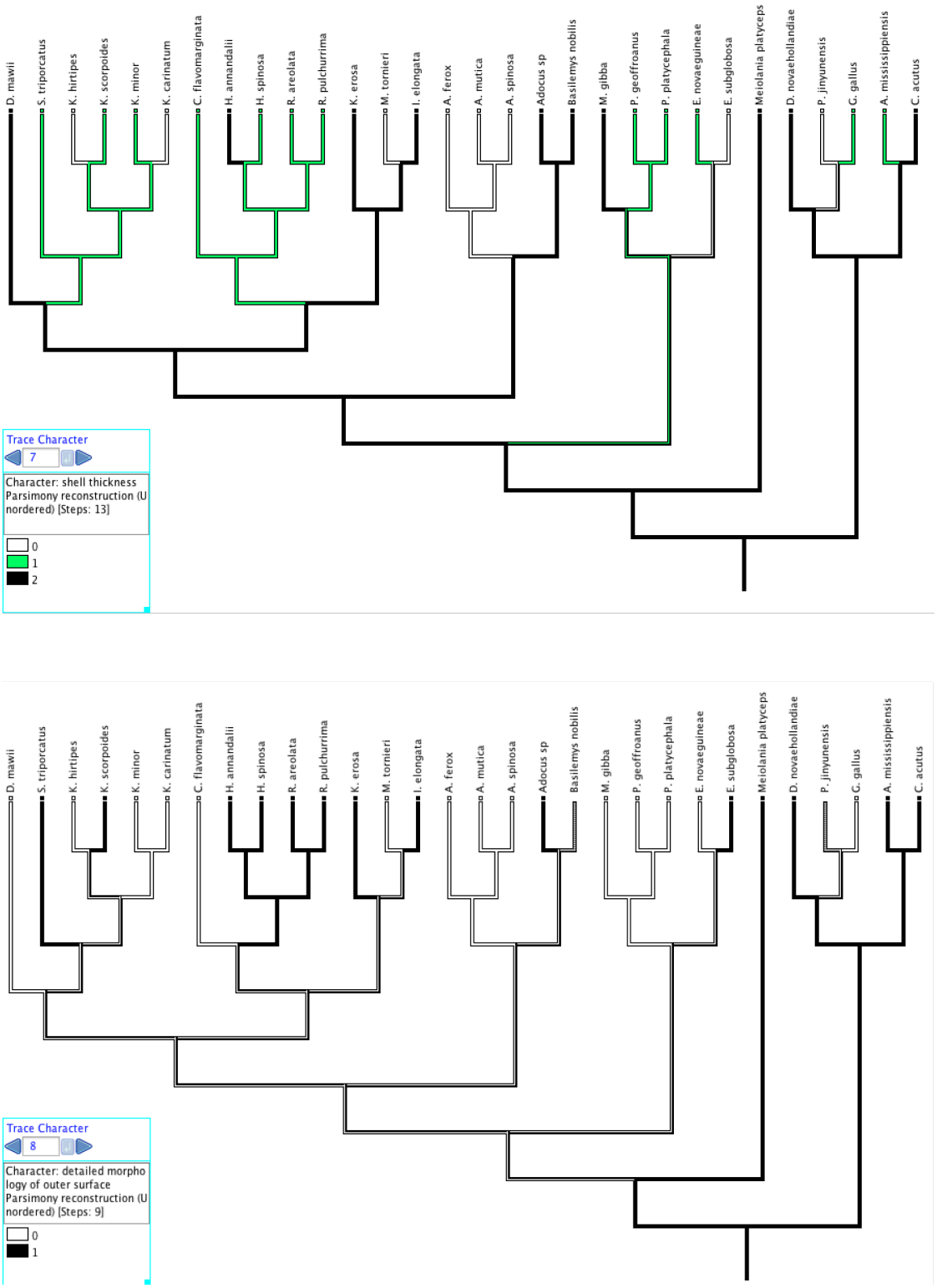
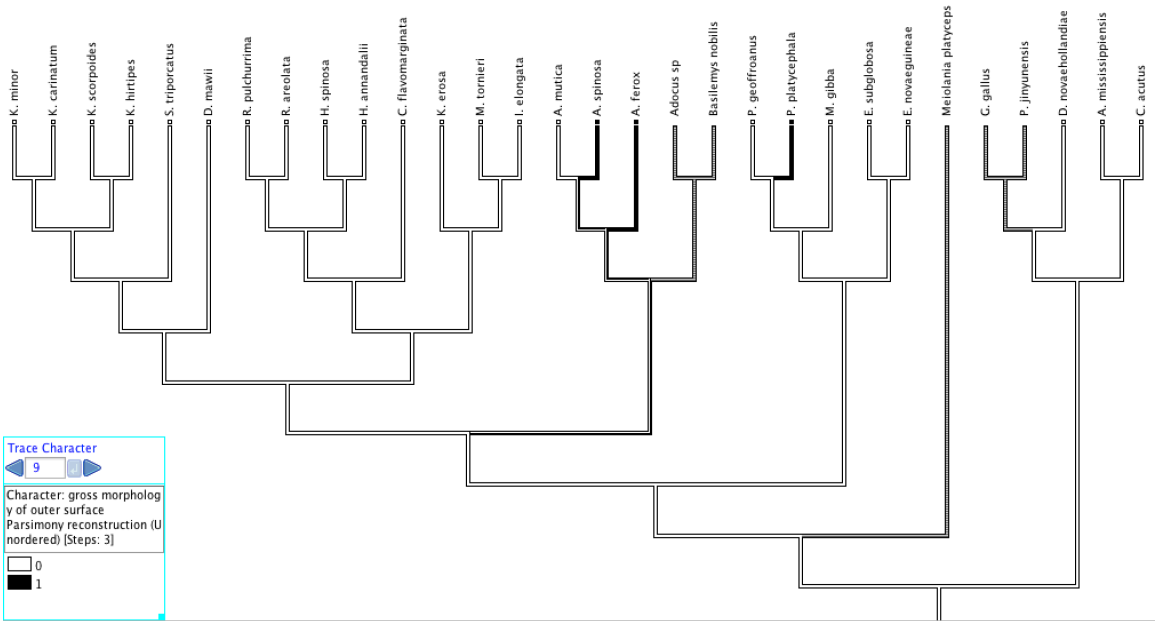


Figure 29. Ancestral state reconstruction of characters 7 and 8.



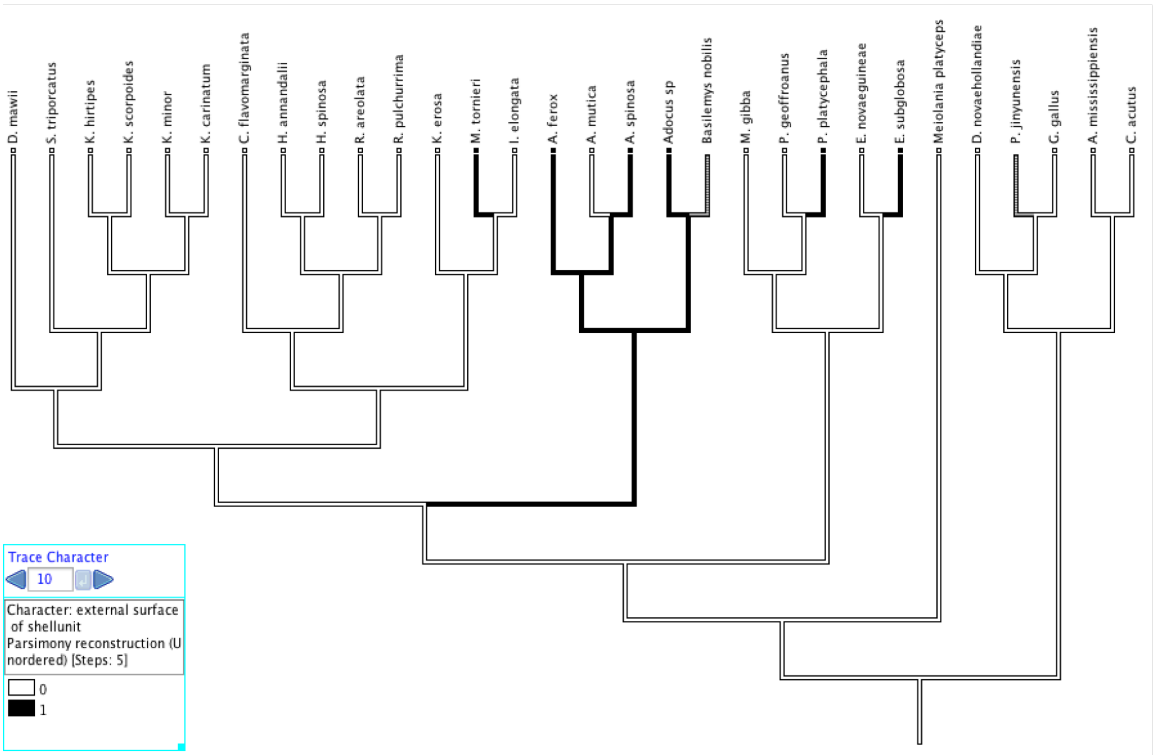


Figure 30. Ancestral state reconstruction of characters 9 and 10.

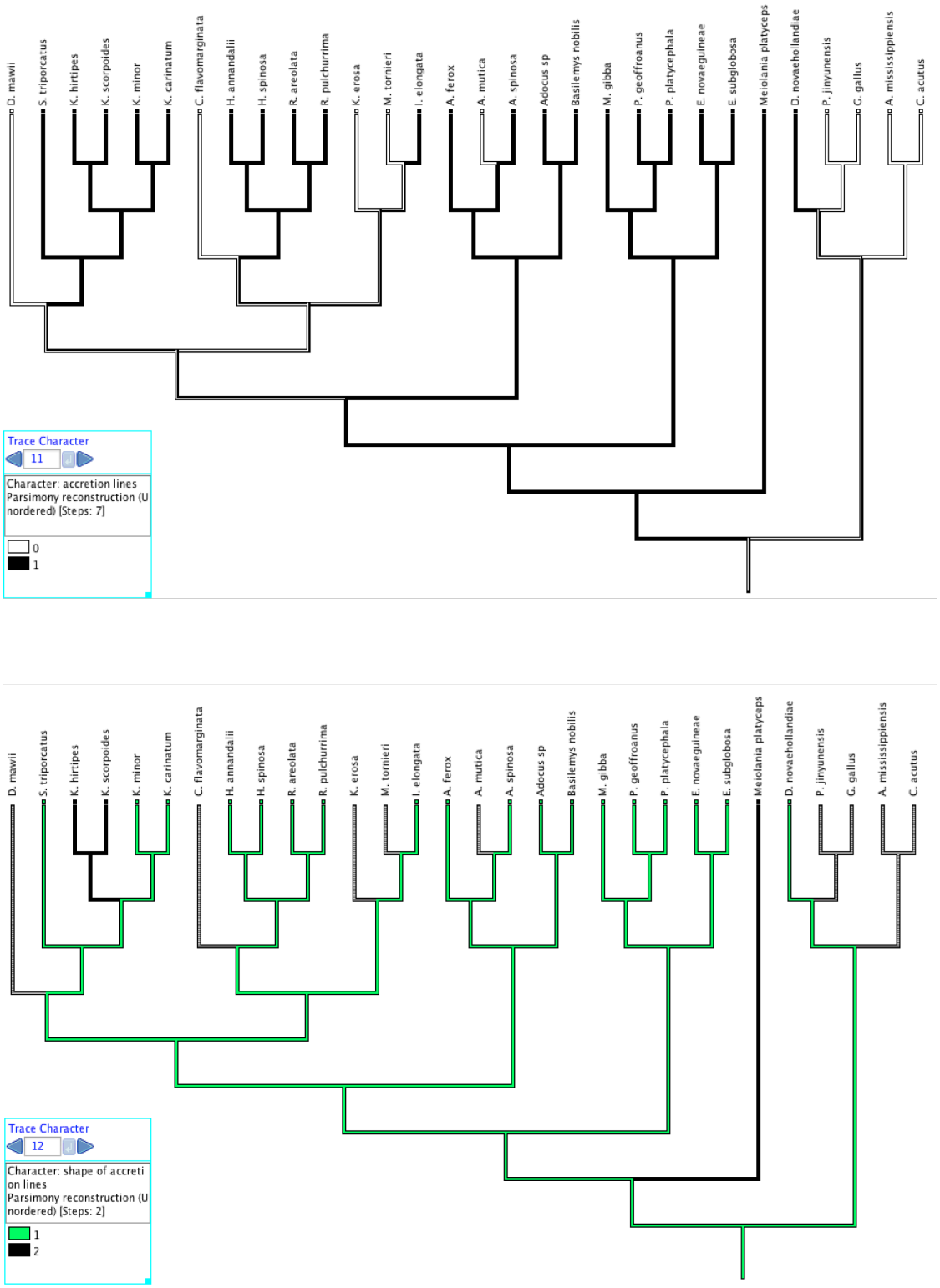


Figure 31. Ancestral state reconstruction of characters 11 and 12.

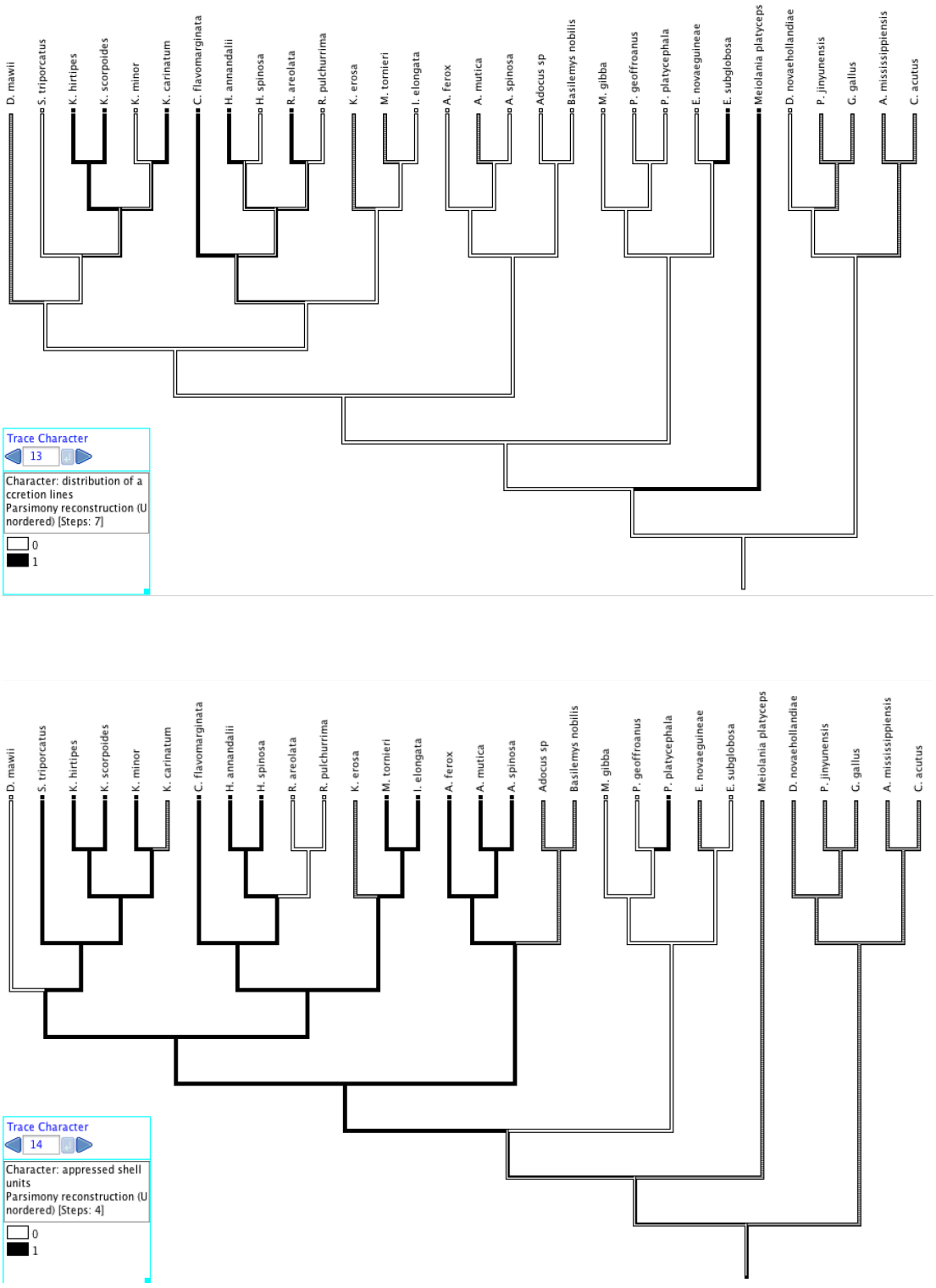


Figure 32. Ancestral state reconstruction of characters 13 and 14.

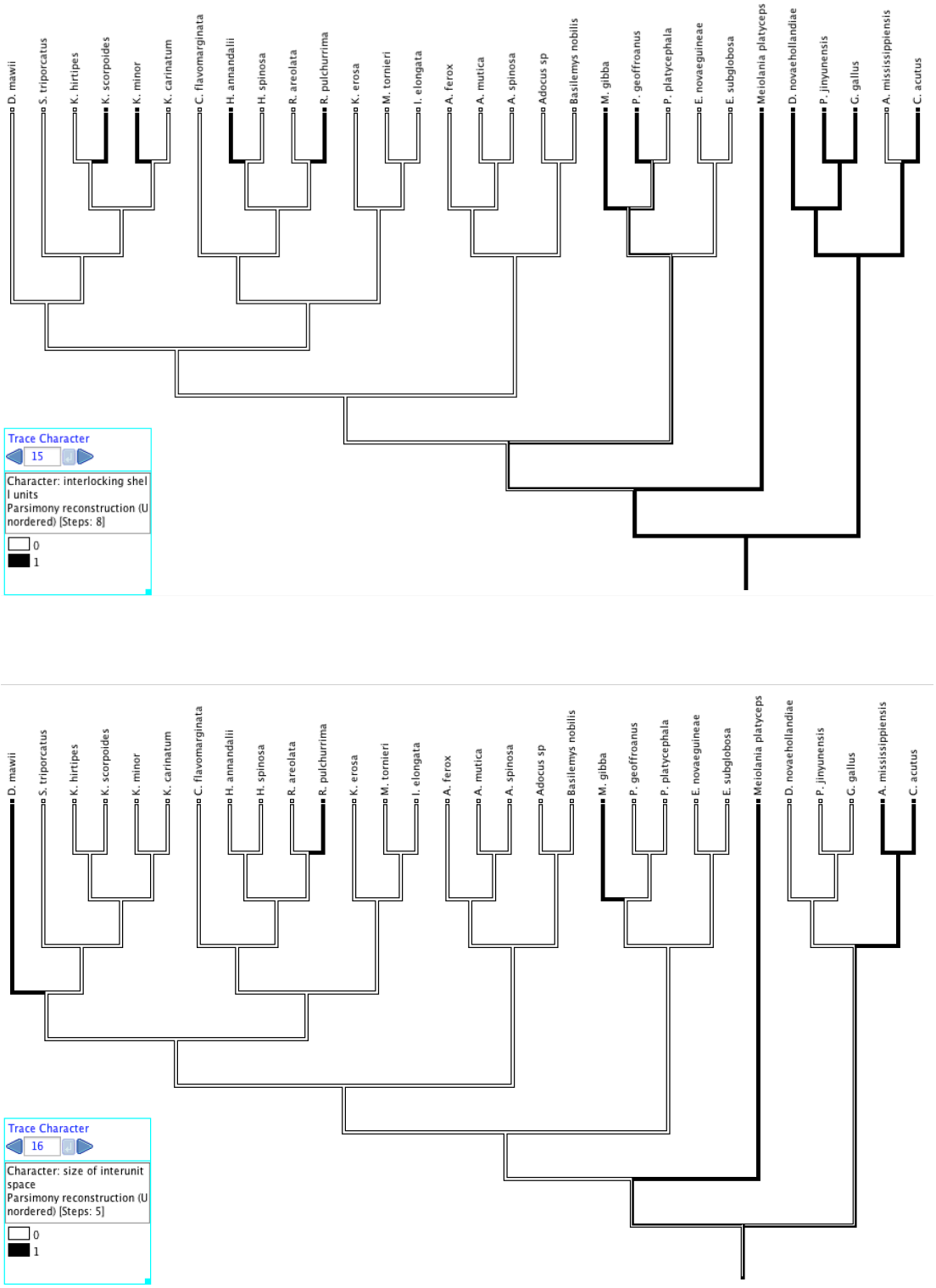
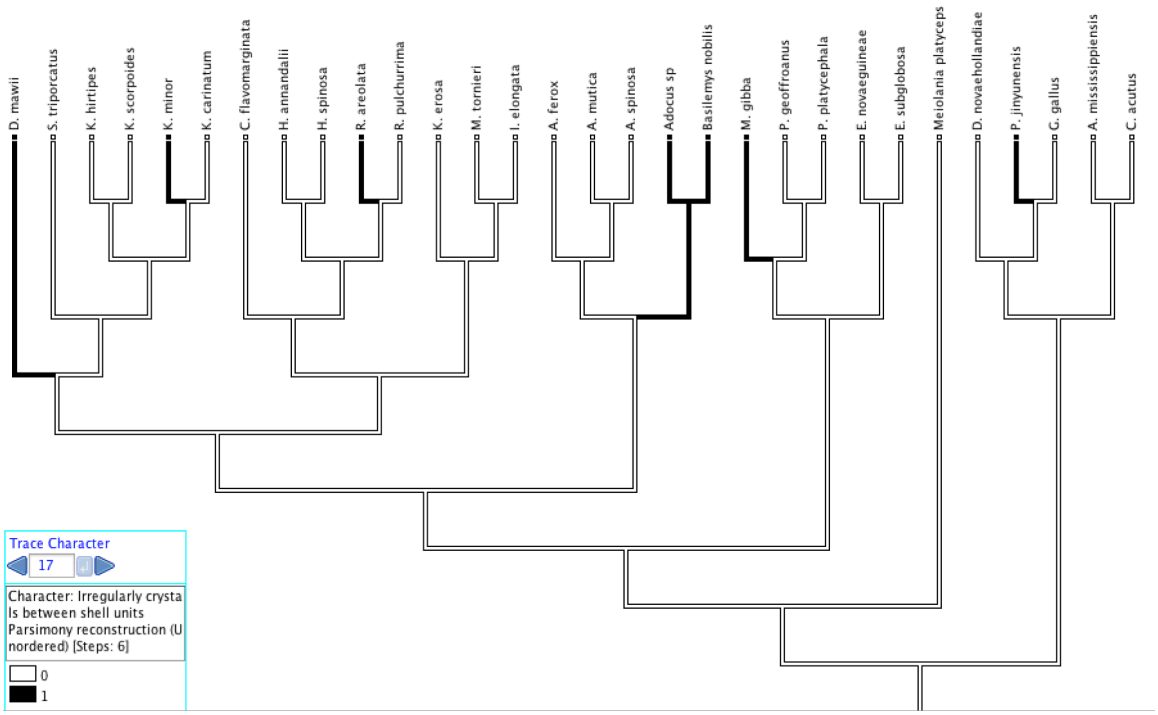


Figure 33. Ancestral state reconstruction of characters 15 and 16.



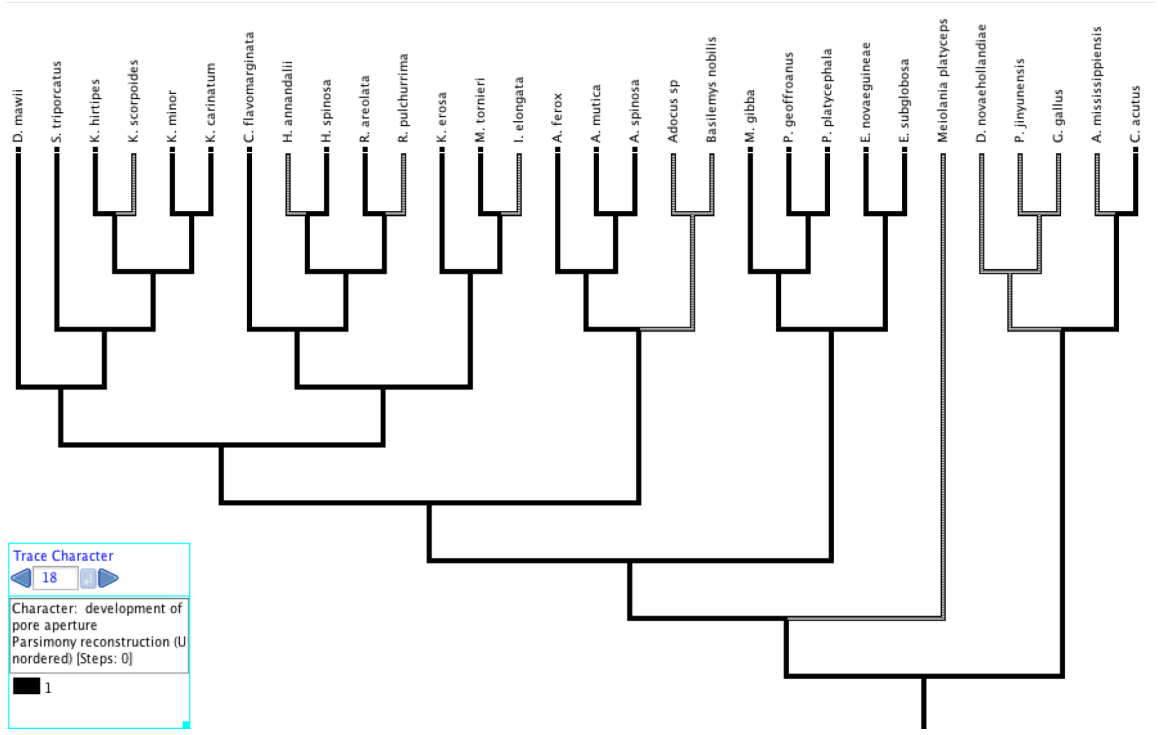
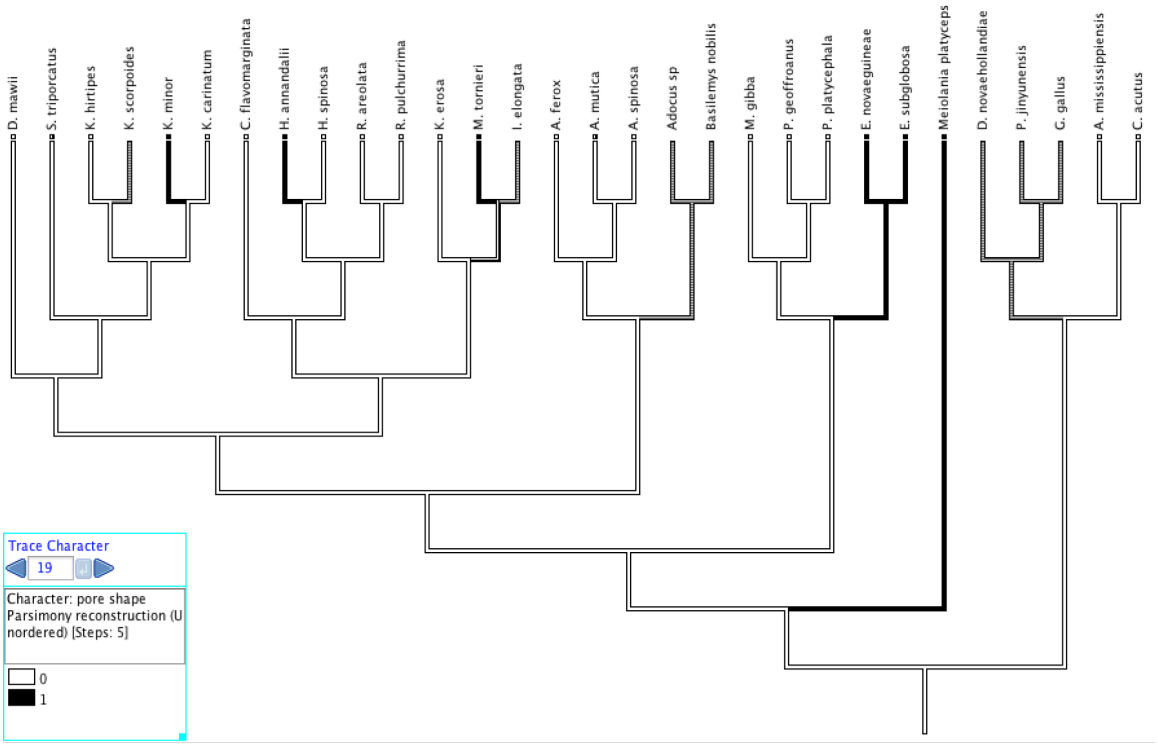


Figure 34. Ancestral state reconstruction of characters 17 and 18.



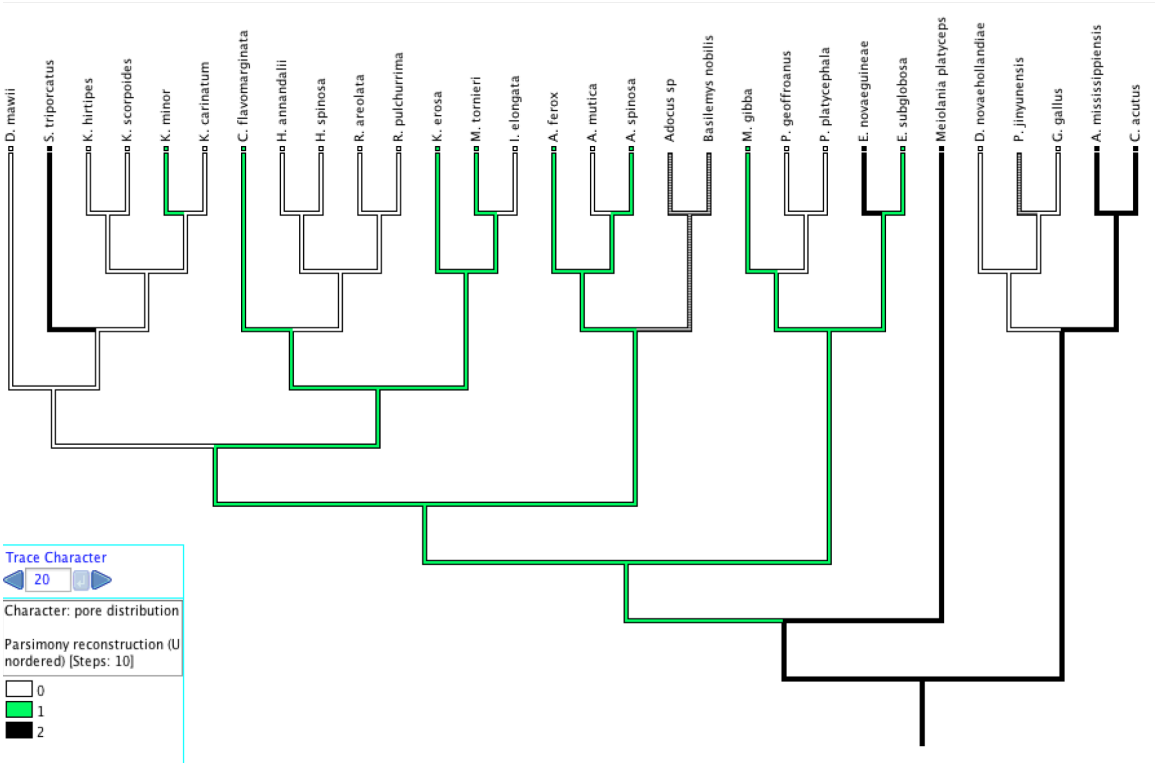


Figure 35. Ancestral state reconstruction of characters 19 and 20.

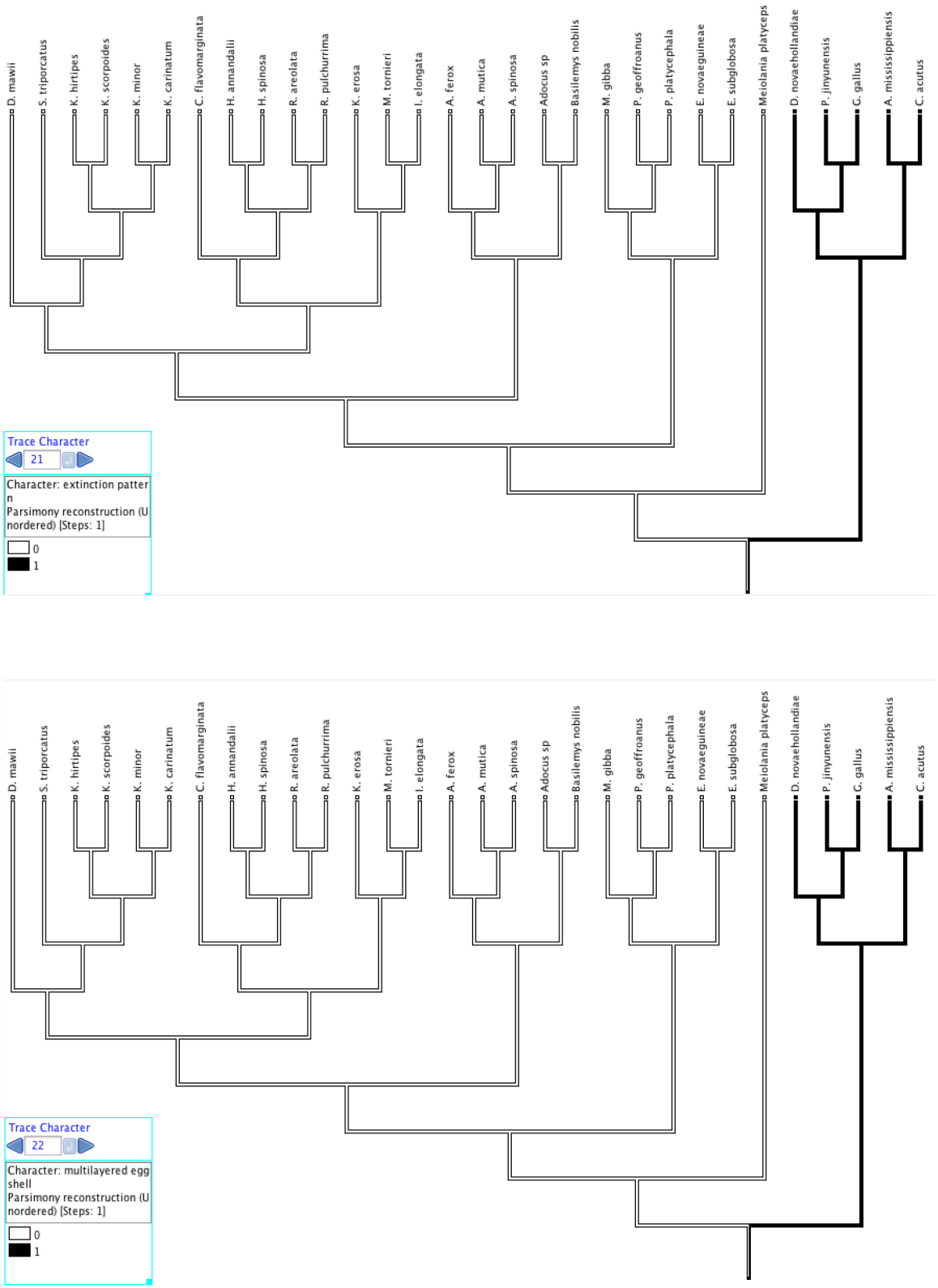
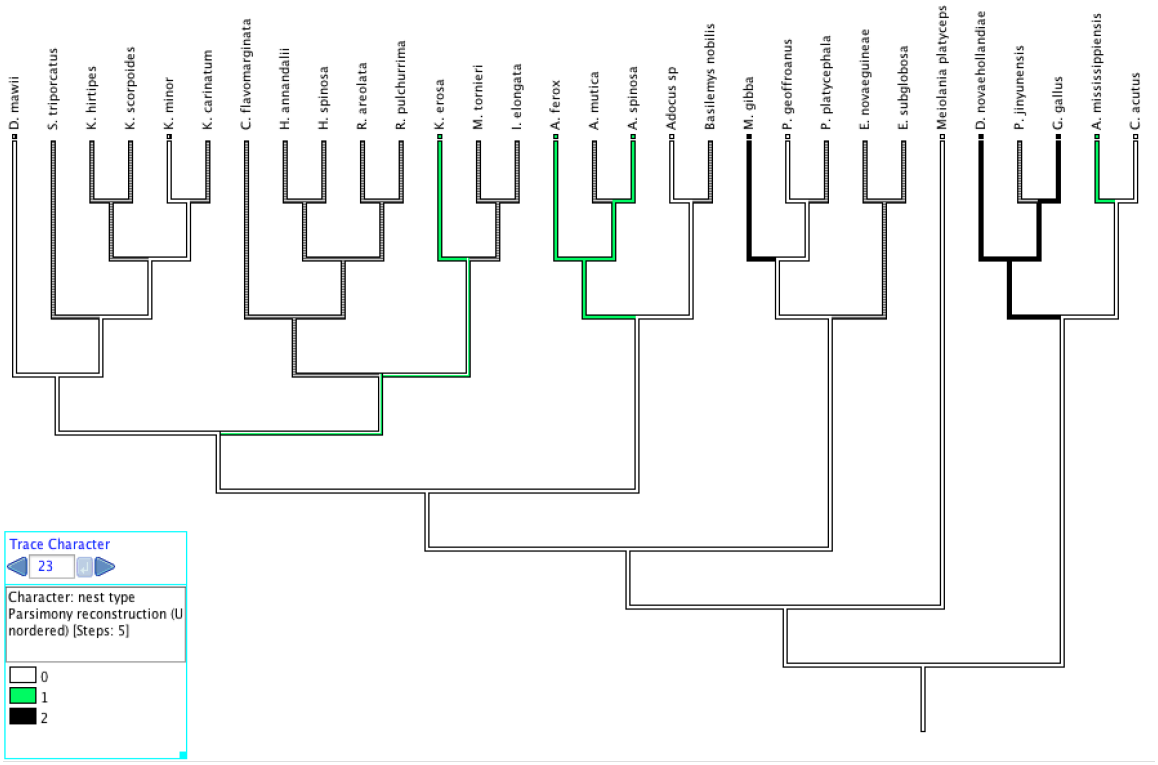


Figure 36. Ancestral state reconstruction of characters 21 and 22.



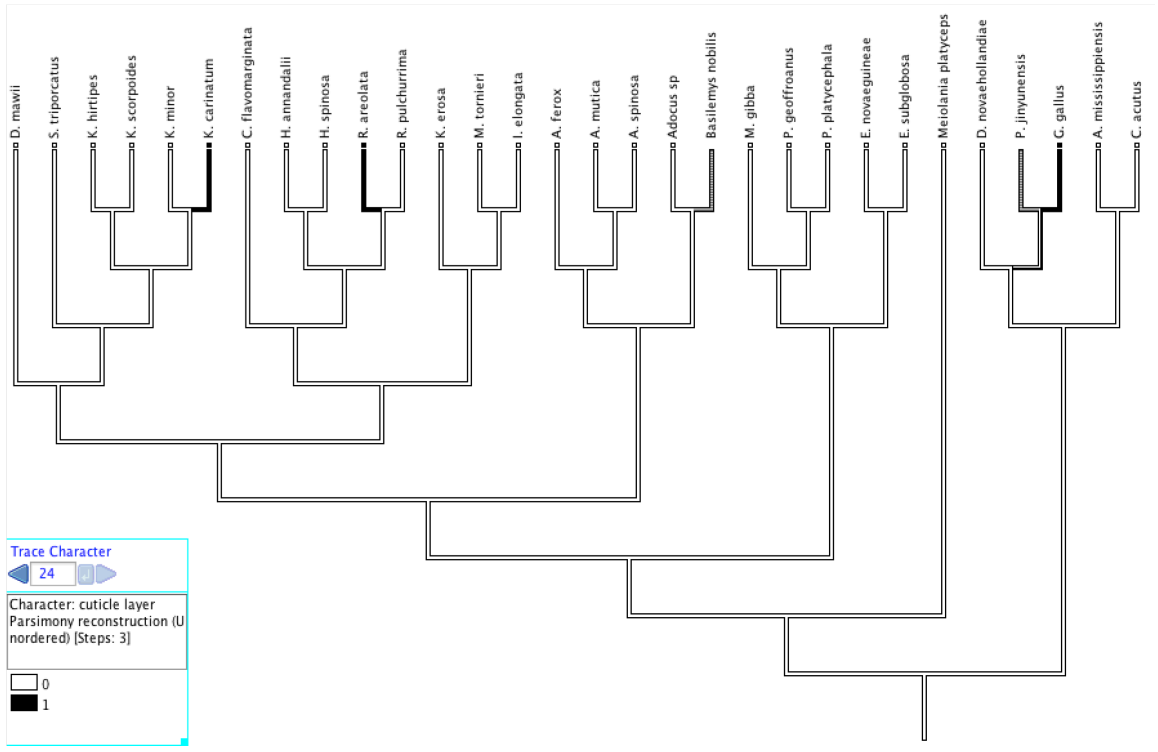


Figure 37. Ancestral state reconstruction of characters 23 and 24.

CHAPTER EIGHT

CONCLUSIONS

1. The fossil record of turtle eggs extends from the Middle Jurassic to the Pleistocene, and specimens are known from every continent except Antarctica.

2. Fossil turtle eggs are relatively abundant in Asia, Europe and North America but are poorly represented in Gondwana. *Testudoolithus oosp.* eggs described here come from the Late Cretaceous of Madagascar and represent the first occurrence of fossil turtle eggs from the island and only the fourth definitive evidence of turtle eggs from the Mesozoic of Gondwana.

3. A weathered clutch of 16 eggs from the Late Cretaceous Judith River Formation of Montana contains embryos are assigned to *Testudoolithus zelenitskyae* oosp. nov. At least one egg exhibits multilayered eggshell, a condition in modern turtles resulting from prolonged egg retention.

4. Eggs and their embryonic remains in MOR 710 are likely referable to *Adocus sp.* based on similarities with specimens from Alberta and Utah and demonstrate a late stage (23 or 25) of development when compared to extant turtle taxa. This suggests that termination of embryonic development likely occurred just prior to hatching.

5. An estimated adult carapace length 35.0–54.5 cm was calculated for the female turtle that laid the *T. zelenitskyae* clutch, using a modified data set and the equation of Elgar and Heaphy (1989). Modification was necessary to correct errors in the original equation.

6. Carapace length estimations are based on egg mass. However, calculating egg mass uses a constant for the density of a birds egg. Since turtle eggs have a slightly higher density, carapace lengths must be treated as a minimum estimate.

7. *Testudoolithus lordhowensis* oosp. nov. represents the first described eggs from the Pleistocene of Lord Howe Island, Australia. Egg mass and comparison to modern taxa suggest a female carapace length of approximately 75 cm.

8. *Testudoolithus lordhowensis* are assigned to the stem turtle *Meiolania platyceps* based on the following evidence: similar estimated body size as adult specimens on Lord Howe Island, *M. platyceps* represents the only turtle reported from the island, and distance from the mainland suggests colonization by turtle species was likely a rare event.

9. The presence of aragonite mineral in *M. platyceps* eggs suggests that the evolution of the aragonite eggshell occurred no later than the Early or Middle Jurassic.

10. The high gas conductance rate (170.27 mg H20 day^{-1} $Torr^{-1}$) of *Meiolania platyceps* eggs, compares favorably with those of modern tortoises and indicates that the eggs were incubated in a humid nesting environment. The presence of at least two superimposed layers of eggs further suggests that *M. platyceps* deposited its clutch in an excavated hole nest. This nesting strategy likely evolved no later than the Early to Middle Jurassic.

11. My study represents the first analysis to compare modern turtle eggshell to fossil specimens under cathodoluminescence. Modern eggshell exhibits dull blue and dark green luminescence. Similar blue and dark green luminescence is observed in the fossil taxa *Meiolania platyceps, Adocus* sp. and *Basilemys nobilis* indicating the presence and minimal alteration of the original aragonite. In contrast, bright orange to red luminescence observed in *Testudoolithus* eggs from Madagascar indicate that diagenetic calcite or dolomite replaced the original eggshell. Cathodoluminescence, therefore, provides an important tool for assessing diagenesis in fossil eggshell and distinguishing characters useful in cladistic analyses.

12. Cladistic analysis utilizing egg and reproductive characters are rarely performed on taxa outside of Dinosauria. My morphological analysis using 24 characters, 25 turtle taxa only resolved the clades Trionychidae and Adocia, with the remaining turtle taxa comprising a large polytomy. This poor resolution indicates that homoplasy (e.g., convergent evolution and/or character reversals) is likely due to species' habitats and incubation environments or the limited number of informative characters.

13. Egg and reproductive characters, when combined with molecular data, have more resolving potential toward the top of trees. Therefore, combined analyses that includes osteologic and/or molectular date are more useful in cladistics analyses until new characters are discovered.

14. Limitations of cladistic analyses of turtle egg and reproductive characters include the small number of informative characters, missing data, few positively identified fossil specimens, intraspecific variation and diagenesis of eggshell.

15. Cladistic analyses of dinosaur eggs demonstrate a greater potential for more informative characters than analyses of turtle eggs. This is likely due to the long and complex history of theropod reproduction and the relatively conservative nature of turtle evolution. For this same reason, the five oospecies currently named within *Testudoolithus,* lack many distinguishing features and several are

diagnosed by unique combinations of characters. Thus, *Testudoolithus* is on the verge of becoming a "wastebasket" taxon.

16. More taxonomically identified fossil turtle eggs are needed in order to hypothesize their phylogenetic relationships. Using data sets that combine osteology with egg and reproductive characters will further enhance resolution.

17. The phylogenetic relationship of Crocodylia, as hypothesized in analyses based on egg and reproductive characters, is strongly dependent upon the number of structural eggshell layers. For example, coding crocodilian eggs as single layered pushes them into Testudines, but they are resolved as archosaurs when coded for multiple eggshell layers.

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