

## REVIEW ARTICLE

# The diverse terminology of reptile eggshell microstructure and its effect on phylogenetic comparative analyses

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**Funding information**

Howard Hughes Medical Institute, Grant/  
Award Number: GT10473; National  
Research Foundation of Korea, Grant/  
Award Number: 2020R1A6A3A03038316

**Abstract**

Reptile eggshell ensures water and gas exchange during incubation and plays a key role in reproductive success. The diversity of reptilian incubation and life history strategies has led to many clade-specific structural adaptations of their eggshell, which have been studied in extant taxa (i.e. birds, crocodylians, turtles, and lepidosaurs). Most studies on non-avian eggshells were performed over 30 years ago and categorized reptile eggshells into two main types: “hard” and “soft” – sometimes with a third intermediate category, “semi-rigid.” In recent years, however, debate over the evolution of eggshell structure of major reptile clades has revealed how definitions of hard and soft eggshells influence inferred deep-time evolutionary patterns. Here, we review the diversity of extant and fossil eggshell with a focus on major reptile clades, and the criteria that have been used to define hard, soft, and semi-rigid eggshells. We show that all scoring approaches that retain these categories discretize continuous quantitative traits (e.g. eggshell thickness) and do not consider independent variation of other functionally important microstructural traits (e.g. degree of calcification, shell unit inner structure). We demonstrate the effect of three published approaches to discretizing eggshell type into hard, semi-rigid, and soft on ancestral state reconstructions using 200+ species representing all major extant and extinct reptile clades. These approaches result in different ancestral states for all major clades including Archosauria and Dinosauria, despite a difference in scoring for only 1–4% of the sample. Proposed scenarios of reptile eggshell evolution are highly conditioned by sampling, tree calibration, and lack of congruence between definitions of eggshell type. We conclude that the traditional “soft/hard/semi-rigid” classification of reptilian eggshells should be abandoned and provide guidelines for future descriptions focusing on specific functionally relevant characteristics (e.g. inner structures of shell units, pores, and membrane elements), analyses of these traits in a phylogenetic context, and sampling of previously undescribed taxa, including fossil eggs.

**KEYWORDS**

dinosauria, eggshell, evolution, hard-shelled, microstructure, phylogenetic comparative methods, reptile, soft-shelled

[Corrections added on 14 July 2022, after first online publication: a mistake in the phylogenetic position of one species in the tree was identified after publication. This does not affect the results and discussion and does not change the conclusions of the paper, but it does affect the colour patterns in Fig. 3 and Fig. 4 and values in Table 1, as well as a few sentences and values in the Results section. These errors have been corrected in this version]

## 1 | INTRODUCTION

The amniotic egg, a major innovation in the evolutionary history of vertebrates, varies in size and structure associated with specific life history and incubation strategies, which have been extensively studied in reptiles (Blackburn, 2000a, 2000b; Blackburn & Stewart, 2021; Deeming & Ferguson, 1991a; Deeming & Reynolds, 2015; Deeming & Ruta, 2014; Laurin & Reisz, 1997; Reisz, 1997; Sander, 2012; Shine, 2005; Stewart, 1997; Stoddard et al., 2017). Many of these studies have focused on describing the internal egg organization and embryological development of extant egg-laying amniote clades – i.e. Monotremata, Lepidosauria, Testudines, Crocodylia, and Aves (reviews in Blackburn & Stewart, 2021; Starck et al., 2021). The reproductive strategies of these groups have been studied in the context of their respective radiations, with some traits identified as major factors of their evolutionary success – e.g. egg shape and size in birds (Birchard & Deeming, 2015; Birkhead et al., 2019; Deeming, 2007, 2018a; Deeming & Ruta, 2014; Duursma et al., 2018; Dyke & Kaiser, 2010; Mytiai et al., 2017, 2021; Nagy et al., 2019; Stoddard et al., 2017), or the high flexibility of reproductive traits in squamates, which facilitated their many independent acquisitions of viviparity (Andrews & Mathies, 2000; Blackburn, 1993, 2000a, 2000b, 2006; Blackburn & Stewart, 2021; Griffith et al., 2015; Pyron & Burbrink, 2014; Shine, 1995, 2005; Sites et al., 2011; Stewart, 2013, 2015; Stewart & Thompson, 2000; Whittington et al., 2022).

Evolving with diversification of amniote reproductive strategies is the eggshell. The shells of amniotic eggs all share a similar three-layered structure (Mikhailov, 1991a, 1997a, 1997b; Packard & DeMarco, 1991; Schleich & Kästle, 1988): the innermost, proteinaceous, boundary layer; the membrana testacea, also proteinaceous; and the outermost calcareous layer (CL). However, the inner structure and relative thickness of these layers are highly variable among extant reptiles (including birds – *sensu* Gauthier et al., 1988; Modesto & Anderson, 2004), but are often classified in two main categories: “hard-shelled” eggs (e.g. all birds and crocodylians, most turtles, most geckos) present a CL organized in discrete calcareous entities called shell units, which comprise most of the eggshell thickness, while “soft-shelled” eggs (e.g. most squamates, some turtles) have a thin, amorphous CL and a much thicker membrana testacea (Mikhailov, 1991a, 1997b; Packard & DeMarco, 1991; Schleich & Kästle, 1988). Such eggshell microstructural traits have long been known to be constrained by specific reproductive strategies, which were originally mostly studied in birds (Ar et al., 1974; Rahn & Ar, 1974; Simkiss, 1961a).

During incubation, the eggshell provides calcium to the embryo and controls water and gas exchange with its surrounding environment (Ackerman, Dmi'el, & Ar, 1985; Ackerman, Seagrave, et al., 1985; Ar, 1991; Ar & Rahn, 1985; Deeming & Thompson, 1991; Paganelli, 1980) – generally through a system of pores, which in birds can present a highly complex structure (Board, 1982; Grellet-Tinner et al., 2017; Mikhailov & Zelenkov, 2020; Tyler & Simkiss, 1959). In extant birds and crocodylians, the egg-laying site is a nest or a mound made from vegetation (Ackerman & Lott, 2004; Hall et al., *in press*;

Mainwaring et al., 2014; Murray et al., 2020). Conversely, lepidosaur and turtle eggs are laid either in a tunnel dug into the ground or above ground with substrate covering (e.g. vegetation, rocks), the latter being minimal in species that lay “hard-shelled” eggs (Ackerman & Lott, 2004) – although at least one tortoise species is known to build a nest mound (Kuchling, 1999). Depending on their location (e.g. on/off the ground) and structure (e.g. open/closed), egg-laying sites present different conditions of temperature and humidity (Ackerman, Seagrave, et al., 1985; Deeming & Ferguson, 1991b; Heenan, 2013). These parameters, along with other evolutionary constraints (e.g. calcium intake, clutch size – Jetz et al., 2008; Nagy et al., 2019; Simkiss, 1961a), have resulted in many specific types of pore and eggshell microstructure among reptiles (Board, 1982; Board et al., 1977; Board & Scott, 1980; Choi et al., 2018; Packard & DeMarco, 1991; Tanaka et al., 2015; Tyler, 1955; Tyler & Fowler, 1978), and been the subject of paleobiological hypotheses (Montanari, 2018) – e.g. regarding nesting strategies in non-avian dinosaurs (Deeming & Unwin, 2004; Varricchio & Jackson, 2016). For this reason, descriptive traits of eggshell microstructure have been formalized into a parataxonomical system, with a standardized terminology to classify both extant and fossil reptile eggshells, regardless of whether the egg layer is known, and study them in a morphofunctional context (Khosla & Lucas, 2020; Mikhailov, 1991a, 1997b; Mikhailov et al., 1996; Vianey-Liaud & Zelenitsky, 2003).

Studies focused on the evolution of such microstructural traits in a phylogenetic context for all of Reptilia have been lacking. Preservational biases and a neontological/paleontological decoupling may partially explain the lack of this kind of synoptic study. Soft-shelled eggs are mostly proteinaceous and extremely rare in the fossil record (Legendre, Rubilar-Rogers, Musser, et al., 2020; Norell et al., 2020; Stewart, 1997); almost all fossil eggshells consist only in a CL with shell units (Mikhailov, 1997b). Eggshell parataxonomy, primarily developed to identify isolated fossil eggs of unknown egg layer, is based on the morphology of these shell units and cannot be used to describe eggs that lack them (Packard & DeMarco, 1991; Schleich & Kästle, 1988). Furthermore, the eggshells of monotremes, the only extant egg-laying amniotes outside reptiles, have been poorly studied (see next section), preventing inferences of the ancestral eggshell microstructure in both reptiles and amniotes.

The first comparative studies to discuss functional aspects of extant eggshell microstructure (including comparison between fossil and extant species; Straelen, 1928) were conducted before the formal inclusion of birds in the class Reptilia in the late 1980s (Gauthier et al., 1988; Modesto & Anderson, 2004), resulting in studies that focused on either birds (Ar et al., 1974; Ar & Rahn, 1985; Board, 1982; Paganelli, 1980; Simkiss, 1961a; Tyler & Fowler, 1978) or non-avian reptiles (Ackerman, Dmi'el, & Ar, 1985; Ackerman, Seagrave, et al., 1985; Packard et al., 1977, 1979; Packard & Packard, 1980; Packard, Packard, & Boardman, 1982; Simkiss, 1961b), but never on both groups. In the 1990s and 2000s, studies of reptile eggshell microstructure focused mostly on taxonomical descriptions of avian and non-avian dinosaur eggs (e.g. Chiappe et al., 1998; Codrea et al., 2002; Dauphin

et al., 1996; Grellet-Tinner, 2006; Grellet-Tinner et al., 2006; Hirsch & Quinn, 1990; Jackson et al., 2002, 2008; Jackson & Varricchio, 2003; Khosla & Sahni, 1995; Liang et al., 2009; López-Martínez et al., 1999; Mateus et al., 1997; Mikhailov, 1991b, 1997b; Mikhailov et al., 1994; Varricchio et al., 2002; Varricchio & Jackson, 2004; Vianey-Liaud et al., 1997, 2003; Vianey-Liaud & Garcia, 2003; Zelenitsky et al., 2002; Zelenitsky & Modesto, 2003). Apart from a handful of studies on other reptile species (Heulin et al., 2002; Hirsch, 1996; Mathies & Andrews, 2000; Osborne & Thompson, 2005; Phillott & Parmenter, 2006; Qualls, 1996), the study of non-dinosaurian reptile eggshell microstructure was relatively quiescent for two decades, and focused on descriptions with a small sample size that rarely involved a dialogue between functional study and comparative work.

Phylogenetic comparative approaches have more recently allowed researchers to perform statistical analyses of quantitative eggshell traits and test specific hypotheses about their evolution (Araújo et al., 2013; Choi & Lee, 2019; Grellet-Tinner, 2006; Grellet-Tinner et al., 2006; Mikhailov, 1991b; Tanaka et al., 2020; Tanaka, Lü, et al., 2011; Varricchio & Barta, 2015; Varricchio & Jackson, 2004; Vila et al., 2017; Winkler, 2006; Zelenitsky & Therrien, 2008). Studies have tested correlations of continuous traits (e.g. eggshell thickness, porosity, calcium content, water vapor conductance) with life history traits in birds (Attard & Portugal, 2021; Birchard & Deeming, 2009, 2015; McClelland et al., 2021; Portugal et al., 2014), dinosaurs including birds (Legendre & Clarke, 2021; Tanaka et al., 2015), archosaurs (Tanaka & Zelenitsky, 2014), squamates (Hallmann & Griebeler, 2015), non-avian reptiles (D'Alba et al., 2021), or across amniotes, albeit with relatively small samples (Legendre, Rubilar-Rogers, Musser, et al., 2020; Stein et al., 2019). Some of these studies have also reconstructed ancestral states for these traits – discretized in some cases – and identified correlates that influenced these evolutionary patterns (Attard & Portugal, 2021; D'Alba et al., 2021; Legendre & Clarke, 2021; Legendre, Rubilar-Rogers, Musser, et al., 2020; McClelland et al., 2021; Norell et al., 2020; Portugal et al., 2014; Stein et al., 2019; Tanaka et al., 2015). These studies have initiated what is perhaps the most interesting and controversial debate in this new field of quantitative eggshell research (Lindgren & Kear, 2020): what was the structure of the ancestral eggshell in dinosaurs, archosaurs, and reptiles as a whole? How do we assess potential biases in the preservation of fossil eggs and extant microstructure and function?

Recent descriptions of exceptionally preserved fossil soft-shelled eggs assigned to early diapsids (Hou et al., 2010), pterosaurs (Grellet-Tinner et al., 2014; Unwin & Deeming, 2008; Wang et al., 2014, 2017), non-avian dinosaurs (Norell et al., 2020; Stein et al., 2019) and, possibly, marine reptiles (Legendre, Rubilar-Rogers, Musser, et al., 2020), have led studies to investigate via ancestral state reconstructions (ASR) the binary trait “soft-shelled/hard-shelled” (sometimes with a third intermediate state, referred to as a semi-rigid eggshell) on the phylogenetic tree of Reptilia. One study recovered the first dinosaur and archosaur egg as soft-shelled (Norell et al., 2020) and another with both as hard-shelled (Legendre,

Rubilar-Rogers, Musser, et al., 2020), which was until then the scientific consensus. These conflicting conclusions were proposed to be due to differences in taxonomic sampling and ASR methods (Legendre, Rubilar-Rogers, Vargas, & Clarke, 2020), but also due to differences in their respective definitions of what makes an eggshell soft or hard. Indeed, while this dichotomy has been used to describe eggshells for over a century (Cunningham, 1922), many definitions have been used by various authors over the past five decades to refer to “hard” and “soft” eggshells (or synonyms of those) without ever reaching a formal consensus for any of them.

In this review, we describe the criteria used in the literature to define the terms “soft-shelled” and “hard-shelled” for amniote eggs (including monotremes) and the range of eggshell phenotypes in extant taxa. Then, using a dataset combining those of the two recent studies that recovered the ancestral archosaur egg as soft-shelled or hard-shelled, respectively (Legendre, Rubilar-Rogers, Musser, et al., 2020; Norell et al., 2020), we reconstruct evolutionary patterns for a “soft/semi-rigid/hard” discrete trait and an eggshell thickness continuous trait on an updated calibrated phylogeny. Finally, we discuss how sampling, definitions of eggshell type, and tree calibration influence the reconstruction of evolutionary patterns for reptilian eggshells, and propose new guidelines to clarify future analyses and discussion on this topic in a phylogenetic context.

## 2 | DEFINITIONS OF HARD AND SOFT EGGSHELLS AMONG EXTANT AMNIOTES

### 2.1 | Criteria used to define eggshell type

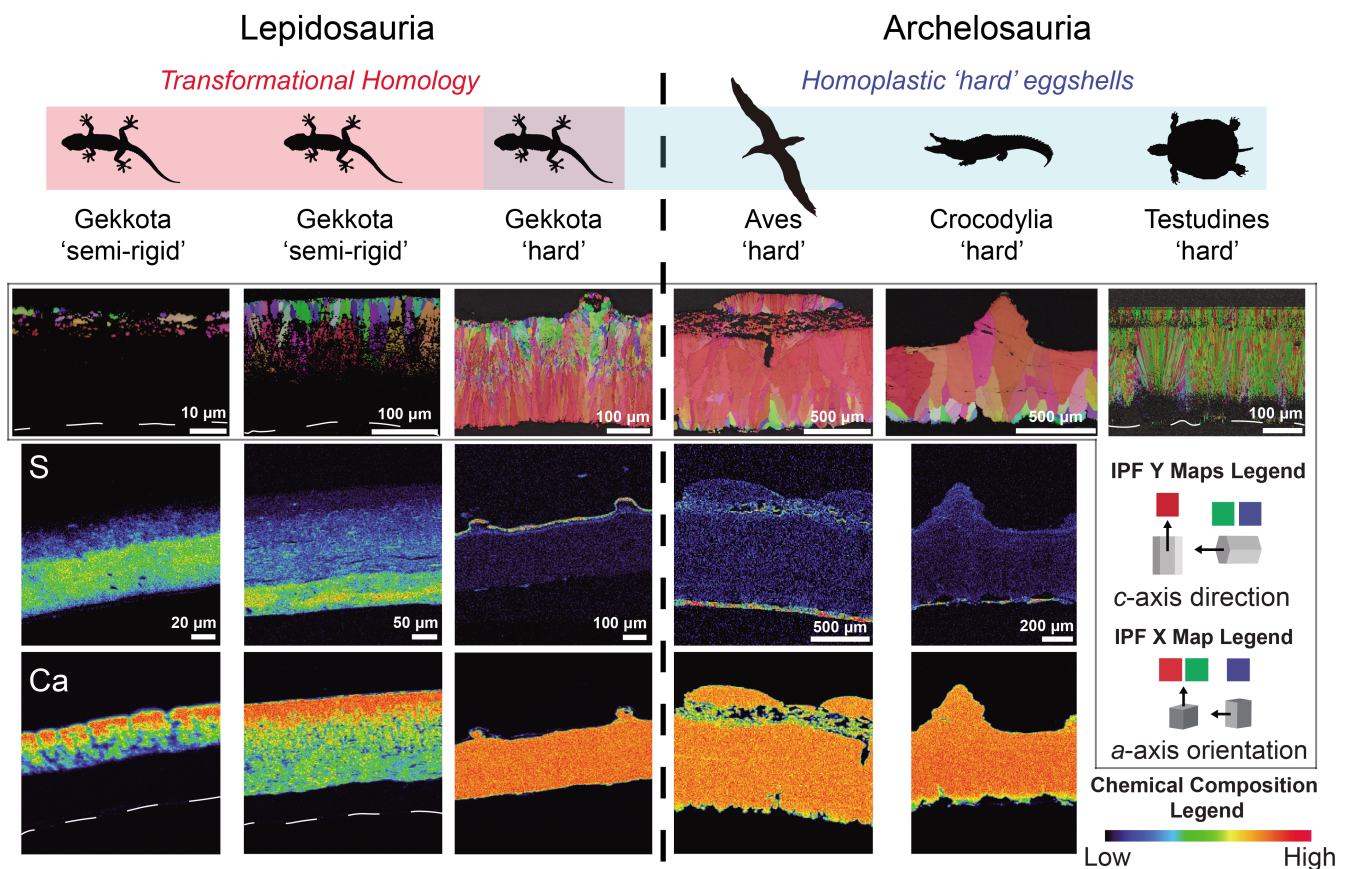
The first attempt to formalize distinct categories for reptile eggshells using a sample of several reptile groups and advanced microscopy techniques was made in a series of papers by Mary and Gary Packard and colleagues in the late 1970s and early 1980s (Packard et al., 1977, 1979; Packard & Packard, 1980; Packard, Packard, & Boardman, 1982). Three eggshell types in non-avian reptiles, best summarized in Packard, Packard and Boardman (1982, p. 138–142), were defined:

“In summary, reptilian eggs can be divided roughly into three groups based on the structure of their shells: flexible-shelled eggs with little or no calcareous layer (most squamates), flexible-shelled eggs with a thick, well-developed calcareous layer (some chelonians), and rigid-shelled eggs with a well-developed calcareous layer (crocodilians, some chelonians, and a few squamates). We recognize that grouping of eggs on the basis of similarities in structure of eggshells is somewhat artificial. Nonetheless, structure of eggshells places certain constraints on the capacity of eggs to respond to variation in the hydric environment, and these constraints transcend taxonomic boundaries.”

These categories were, as explicitly stated here, defined purely in the context of functional constraints, namely water conductance of the eggshell during incubation (Ackerman, Dmi'el, & Ar, 1985; Ackerman, Seagrave, et al., 1985; Packard et al., 1979; Packard & Packard, 1980; Packard, Packard, & Boardman, 1982). Eggs in the first category, found in most squamates, have the most porous shell out of the three, also referred to as parchment-like, and more commonly as soft-shelled later on (Ackerman, Dmi'el, & Ar, 1985; Packard & DeMarco, 1991; Schleich & Kästle, 1988). These squamate eggs have a very low mineral content (15–30%, versus over 90% in other reptile eggs – Andrews, 2017; Packard, Packard, & Boardman, 1982; Schleich & Kästle, 1988), and thus a high water conductance (about one order of magnitude higher than in other non-avian reptile eggs, and two orders of magnitude higher than in avian eggs – Ackerman, Dmi'el, & Ar, 1985). As a result, incubation of soft-shelled squamate eggs (i.e., in oviparous taxa) requires them to be at least partially buried in a dense substrate with high humidity

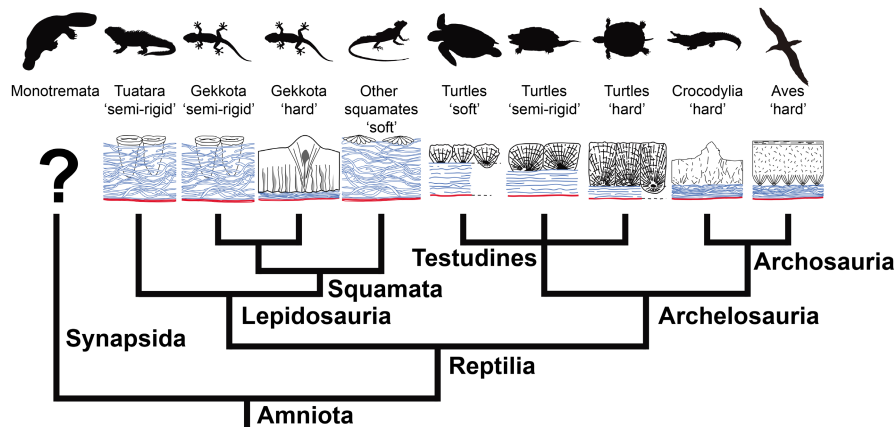
to allow continuous water intake, while avian eggs, which have the thickest and most mineralized (i.e. “hard”) shells among reptiles, are incubated in much lower temperature and humidity conditions, and lose water in the process (Ar & Rahn, 1985; Deeming, 2006; Deeming & Thompson, 1991; Tanaka et al., 2015; Thompson & Speake, 2004). Note: the term “soft-shelled” is sometimes also used to refer to avian eggs with poorly calcified shells due to developmental anomalies (e.g. Simkiss, 1961a).

Eggs of other reptile groups have been categorized in this functional context as either hard-shelled or soft-shelled based on their similarity to either “soft” squamate eggs or avian eggs, respectively (Figures 1, 2; Belinsky et al., 2004). However, since these studies did not discuss evolutionary hypotheses regarding the emergence of any of these eggshell types, no explicit hypothesis of primary homology (*sensu* Pinna, 1991) for “soft-shelled” or “hard-shelled” as character states was ever defined in a phylogenetic context, thus preventing any secondary homology for either character state to



**FIGURE 1** Microstructural and compositional images of reptile eggshells obtained from EBSD (first row) and electron probe microanalyzer (EPMA – second and third rows). The EBSD map of the turtle eggshell is an IPF X map, while other EBSD maps are IPF Y maps (IPF: inverse pole figure). In the IPF Y map, red indicates calcite growing perpendicular to the eggshell surface, while green and blue indicate it growing parallel to the surface. In IPF X map, red and green indicate aragonite growing perpendicular to the eggshell surface, while blue indicates it growing parallel to the surface. EPMA images show relative concentration of sulphur (S) and calcium (Ca), with low concentration colored blue and high concentration red. The innermost part of “semi-rigid” eggshells of geckos (two leftmost columns) are composed of thick proteinaceous layers (high concentration of S), while the “hard” eggshells of geckos and archelosaurs are mostly composed of  $\text{CaCO}_3$  (high concentration of Ca). “Semi-rigid” and “hard” gecko eggshells share a similar crystallographic arrangement, reflecting transformational homology (Choi et al., 2018). Conversely, the “hard” eggshells of gecko and archelosaurs are homoplastic, as inferred from their opposite crystallographic arrangement (Choi et al., 2018). “Hard” eggshells of Aves, Crocodylia, and Testudines are also likely homoplastic (see Text). Select taxon silhouettes from PhyloPic (full credits in Supporting Information).





**FIGURE 2** Schematic representation of the eggshell microstructures of major amniote groups and their phylogenetic relationships. Red, blue, and black colors used in the schematic drawings correspond to boundary layer, membrana testacea, and calcareous layer (CL), respectively. The microstructure of monotreme eggshells is poorly known (see Text), and is therefore not depicted. The tuatara and some gecko (Gekkota) eggshells have “nail-shaped” structure (=“semi-rigid” in traditional terms), but eggshells of other squamates (i.e. outside Gekkota) just have a thin overlying calcite layer. Testudine eggshells were traditionally classified into “soft,” “semi-rigid,” and “hard,” as can be seen in the three schematic images. Similar to the polyphyletic “hard eggshells” of amniotes (Figure 1), “semi-rigid eggshells” of lepidosaur and testudine eggshells are not homologous with each other. Select taxon silhouettes from PhyloPic (full credits in Supporting Information).

be clearly supported (or unsupported) in the Reptilia tree. Similarly, the third, intermediate category defined by Packard, Packard and Boardman (1982) for some turtle eggs, often referred to as “semi-rigid,” or “flexible”/“pliable” with a mention of shell units being present (Ackerman, 1980; Ackerman, Dmi'el, & Ar, 1985; Ackerman, Seagrave, et al., 1985; Booth, 2002; Deeming & Thompson, 1991; Lawver & Jackson, 2014; Packard, 1999; Packard et al., 1979; Packard, Packard, & Boardman, 1982), was never formally defined as a structural category, and is never discussed as such in recent comparative studies (e.g. Legendre, Rubilar-Rogers, Musser, et al., 2020; Norell et al., 2020). This is likely due to the fact that most studies of reptile eggshells did not sample turtle species with semi-rigid eggs, instead focusing primarily on either birds or squamates – by far the two most species-rich reptile clades (Barrowclough et al., 2016; Uetz et al., 2021). Thus, despite the explicit admission by Packard, Packard and Boardman (1982, p. 142) that these three categories were “somewhat artificial” beyond a difference in water exchange, subsequent literature has often presented these two, or three categories, as well-defined character states based solely on structural differences, without investigating their biological relevance or potential homology.

One criterion that could in theory help identify either “hard-shelled” or “soft-shelled” eggs as a homologous character state is their shell formation during ontogeny, the general sequence of which appears to be highly conserved in all reptile eggs studied in this context. That sequence has been primarily described in avian eggs, generally domestic chicken (Board & Sparks, 1991; Gautron et al., 1996, 2021; Hincke, 2012; Nys et al., 1999, 2004). The avian eggshell is formed from the inside out: first in the isthmus region of the oviduct, where the proteinaceous membranes are produced; then in the uterus, where the mineralized shell forms on top of it (Gautron et al., 2021; Hincke, 2012; Nys et al., 1999, 2004). On the outer surface of the membrana testacea, calcite (or aragonite in

turtle eggshells) crystals deposit around nucleation centers and form the mammillary layer, i.e. the lowermost, cone-shaped part of shell units, called mammillae (Hincke, 2012; Mikhailov, 1991a, 1997b). On top of that layer grows the column-shaped continuous layer – also referred to as the palisade layer in poultry literature (Gautron et al., 2021), or prismatic layer by paleontologists (Montanari, 2018). This layer, which can itself comprise several sublayers, constitutes the prismatic-shaped part of shell units and represents most of the shell thickness in all hard-shelled eggs (Grellet-Tinner, 2006; Mikhailov, 1997b; Packard & DeMarco, 1991). In most birds, some turtles, and some lepidosaurs, the eggshell presents a cuticle, i.e. a thin outer layer on top of shell units, composed of highly variable relative proportions of calcite, phosphates, proteins, lipids, and polysaccharides (Figure 1; D'Alba et al., 2017; Kusuda et al., 2013; Mikhailov, 1997a; Samiullah & Roberts, 2014; review in Kulshreshtha et al., 2022); the cuticle of bird eggs has been proposed as non-homologous to that of turtles and lepidosaurs, which remains to be tested (Kulshreshtha et al., 2022). While the inner organization and structure of crystals in a shell unit are highly variable and used to define major groups in eggshell parataxonomy (Mikhailov, 1991a, 1997b), this model of outward crystal growth generally follows the same pattern in the eggs of birds, crocodylians, and turtles (Choi, Kim, et al., 2022; Marzola et al., 2015; Moreno-Azanza et al., 2014; Packard & DeMarco, 1991; Silyn-Roberts & Sharp, 1986), seemingly supporting their grouping as hard-shelled. Conversely, in soft-shelled squamate eggs, the ontogenetic sequence of eggshell formation has been poorly studied, but a general model, distinct from that of other reptile eggs, has been proposed. After proteinaceous membranes are formed, crystallization of the thin CL follows the intricate pattern of the outer fibers of the membrana testacea, suggested to be an additional proteinaceous layer unique to squamates (Packard & DeMarco, 1991); that model, however, remains to be tested.

In general, for both hard-shelled and soft-shelled eggs, the sequence of eggshell formation has only been described in a limited number of species, especially among non-avian reptiles, for which only a small proportion of extant species in each major clade has been studied (Crocodylia: 10 species out of 26 [Marzola et al., 2015; Uetz et al., 2021]; Lepidosauria: <100 out of >10,000 [Choi et al., 2018; Osborne & Thompson, 2005; Packard & DeMarco, 1991; Uetz et al., 2021]; Testudines: <50 out of 356 [Packard & DeMarco, 1991; Phillott & Parmenter, 2006; Turtle Taxonomy Working Group, 2017]). Furthermore, many aspects of shell formation, such as the deposition of a CL without nucleation centers in soft-shelled eggs (Packard & DeMarco, 1991), remain undescribed even in many of the studied taxa, and have not been reassessed since the early 1990s.

Similarly, the genes involved in the formation of proteins that form the membrana testacea, which could also help define homologous categories of reptile eggshells, have not been well characterized. A few proteins have been identified in the eggshell membranes of the turtle *Pelodiscus sinensis* (Lakshminarayanan et al., 2005) and squamates *Thamnophis sirtalis* (Hoffman, 1970), and *Salvator merianae* (Campos-Casal et al., 2020). The shell membranes of two squamates (*Naja atra* and *Salvator merianae*) have been described as intricate random arrangements of keratin and collagen fibers, conferring them exceptional deformability and resistance compared with that of the turtle *Mauremys sinensis*, which shows parallel keratin fibers (Álvarez et al., 2022; Chang & Chen, 2016). Soft lepidosaur eggshells have been shown to exhibit significant higher amounts of proline than the hard eggshells of birds and geckos, which might increase their water retention (Cox et al., 1982; Sexton et al., 2005). The potential role of such proteins, however, has only been studied in birds – mostly domestic chickens in the context of poultry research (Gautron et al., 2021). Over 900 proteins of the chicken eggshell membrane and over 100 genes encoding for or regulating their expression in the oviduct have been identified (Du et al., 2015), and some of them have been shown to regulate the size and orientation of calcite crystals in the CL (Dunn et al., 2012; Gautron et al., 2021). However, their respective roles in eggshell formation and structure, as well as variations of these among reptile groups, remain poorly known (Du et al., 2015; Gautron et al., 2021; Hincke et al., 2010; Hincke, 2012).

Other morphological traits could also be relevant in this context, such as the structure of the oviduct, highly variable and well-studied among reptile clades (Blackburn, 1998; Girling, 2002). The specialized oviduct of birds and crocodylians, each region of which produces a different part of the eggshell sequentially during ontogeny, has been proposed as a synapomorphy of archosaurs (Palmer & Guillelte, 1992). Similarly, the development of proteinaceous eggshell layers has been described in a few turtle and squamate species (Arrieta et al., 2021; Hoffman, 1970), sometimes in relation with the structure and function of the uterine shell glands that produce them (Aitken & Solomon, 1976; Corso et al., 2000; Guillelte et al., 1989; Palmer & Guillelte, 1988; Palmer et al., 1993; Stewart et al., 2010). In all cases, however, no specific traits of oviductal morphology have been clearly linked to a given eggshell type. Overall, much more

work is thus needed to identify ontogenetic, morphological, and molecular characters that could potentially help deciphering the biological reality – or lack thereof – of soft, semi-rigid, and hard eggshells.

## 2.2 | Structural variations and inconsistencies in the definitions of hard and soft eggshells

In addition to the lack of information on potential ontogenetic and/or genetic differences among eggshells in reptile taxa, structural differences among eggshells described as hard or soft prevent these categories from being defined as primary homologies. Here we provide a brief description of such inconsistencies in four major amniote groups: Crocodylia, Testudines, Lepidosauria, and Monotremata. The structural characteristics of avian eggshells are not reviewed here, as they have been extensively described in large-scale comparative studies, some of which in the context of nesting ecology and/or dinosaurian evolution (Ar & Rahn, 1985; Board, 1982; Board & Sparks, 1991; D'Alba et al., 2016; Deeming, 2006; Grellet-Tinner & Chiappe, 2004; Grellet-Tinner et al., 2006; Hechenleitner et al., 2015; Mikhailov, 1991b, 1997a, 2014; Rahn et al., 1979; Tyler, 1955; Tyler & Geake, 1953; Tyler & Simkiss, 1958; Zelenitsky & Therrien, 2008).

### 2.2.1 | Crocodylia

Crocodylian eggs are generally considered to be very similar in structure to avian eggs (i.e. well-defined shell units and a much thicker CL than in lepidosaurs), and commonly referred to as “hard-shelled” (Figure 1; Deeming & Ferguson, 1990; Marzola et al., 2015). However, they present many clear differences from avian eggs: their pore density is much lower (average of 5–22 pores/cm<sup>2</sup> [Marzola et al., 2015] versus 45–316 pores/cm<sup>2</sup> in birds [Rokitka & Rahn, 1987; Tullett, 1975; Tyler, 1955]), and their eggshell is thinner than that of avian eggs of the same size (Legendre, Rubilar-Rogers, Musser, et al., 2020; Marzola et al., 2015), resulting in a much higher water conductance (1.4 to 4 times that of similar-sized birds – Deeming & Thompson, 1991). Furthermore, their shell units are organized in a much looser pattern compared with those of birds due to their trapezoidal shape, resulting in empty spaces at their base compared with the more columnar shape found in birds (Figure 1; Marzola et al., 2015; Mikhailov, 1997b; Moreno-Azanza et al., 2014; Schleich & Kästle, 1988). This peculiar microstructure of crocodylian eggs has been known from spatiotemporally diverse fossil localities – e.g. Eocene (Hirsch & Kohring, 1992), Cretaceous (Jackson & Varricchio, 2016; Moreno-Azanza et al., 2015; Tanaka, Zelenitsky, et al., 2011), with the earliest record dating back to the Late Jurassic (Russo et al., 2017), showing that the microstructure of crocodylian eggshells was acquired comparatively early in their history (Moreno-Azanza et al., 2015).

Crocodylian shell units present a highly stratified continuous layer with peculiar ornamentation, previously referred to as lacunae, “book-like” structures, or intracascarial space (Moreno-Azanza

et al., 2014; Piazza et al., 2021; Schleich & Kästle, 1988). In Alligatoridae, these lacunae have been shown to facilitate gas exchange and hatching (Ferguson, 1982), and increase embryo survival during nest inundation (Cedillo-Leal et al., 2017). In alligators, lacunae are formed through outer erosion of the eggshell by acidic secretions from the decaying vegetation of the nest (Ferguson, 1982). Other authors have hypothesized that erosion might also take place in the lower oviduct (Schleich & Kästle, 1988), or that such structures can also form through local inhibition of calcium deposition (Piazza et al., 2021), but neither of the latter two hypotheses have been tested experimentally, nor on a larger sample of crocodylians. Differences from avian eggshell and variation within crocodylians in microstructure and function problematize the use of one composite discrete character state “hard-shelled” for all extant archosaurs.

### 2.2.2 | Testudines

Turtle eggs, the diversity of which prompted the definition of a semi-rigid eggshell type, differ markedly from avian and crocodylian eggs (see Figure 1). All turtle eggshells have a CL made of aragonite instead of calcite (Packard et al., 1979; Packard & DeMarco, 1991; Packard, Packard, & Boardman, 1982; Schleich & Kästle, 1988) – although some turtles species can occasionally produce calcite shells and some squamates aragonitic shells (Al-Bahry et al., 2009; Baird & Solomon, 1979; Guo et al., 2021), which might be due to hitherto unaccounted factors associated with captivity (Packard & DeMarco, 1991).

The CL consists in “fan-shaped” shell units with a spherulitic arrangement of acicular aragonite crystals (Figure 1; Hirsch, 1983; Lawver & Jackson, 2016; Silyn-Roberts & Sharp, 1985), resulting in large spaces between their bases, distinct from those found in archosaurs (Schleich & Kästle, 1988). This shell unit morphology, unique to turtle eggs, has allowed paleontologists to assign many fossil eggshells to Testudines (Hirsch, 1983; Lawver & Jackson, 2014; Mikhailov, 1997b; Moreno-Azanza et al., 2021; Schleich & Kästle, 1988; but see Ke et al., 2021; Xu et al., 2022, for fossil turtle eggs with an unusual shell microstructure), with an earliest record in the Late Jurassic (Kohring, 1990) and empirical evidence of aragonitic shells in the Late Cretaceous (Choi, Kim, et al., 2022; Ferguson & Tapanila, 2022; Xu et al., 2022). The relative shape and size of turtle egg shell units compared with the thickness of the underlying membrana testacea varies significantly (Cadena et al., 2019; Lawver & Jackson, 2014), from thick and columnar in most terrestrial turtles to reduced and as thin as the membrane in some freshwater turtles (Hirsch, 1983; Packard et al., 1979; Packard & Packard, 1980, 1988; Schleich & Kästle, 1988).

The eggs of sea turtles (Chelonioidea) present an extreme reduction of the CL, much thinner than the underlying membrane and so poorly crystallized that shell units cannot be distinguished from each other (Hirsch, 1983; Phillott & Parmenter, 2006; Sahoo et al., 1996, 2009; Schleich & Kästle, 1988), similar to soft squamate eggshells (although the eggs of a fossil sea turtle were recently described as

“rigid” – Cadena et al., 2019). This has resulted in turtle eggshells being discretized as soft (sea turtles), semi-rigid (some freshwater turtles), or hard (all other species), based on visual assessment of relative thickness of shell units compared with that of the membrane (Ackerman, 1980; Ackerman, Dmi'el, & Ar, 1985; Ackerman, Seagrave, et al., 1985; Deeming & Ferguson, 1991b; Hirsch, 1983; Packard et al., 1979; Packard, Packard, & Boardman, 1982; Packard & Packard, 1980; Tracy et al., 1978).

More recently, however, Kusuda et al. (2013) proposed a classification of turtle eggshells into six different types, based on the number of sublayers within their shell units (except type I, found only in species *Pelusios sinuatus*, defined as a complete lack of shell units). The authors argue that their classification matches that of Packard, Packard and Boardman (1982), with type I corresponding to soft shells, type II and III to semi-rigid shells, and type IV to VI to hard shells (Kusuda et al., 2013). This shows that the initial definition of turtle eggshell type as a three-state character ignored part of the variation present within eggshell layers, and was overly simplistic. Furthermore, five out of these six new eggshell types (the sixth one being autapomorphic) have appeared multiple times in the phylogeny of Testudines and do not constitute homologous character states (Kusuda et al., 2013), as previously described for other turtle egg traits (Winkler, 2006), which calls for a more cautious interpretation of turtle eggshell classifications. Several criteria used to define the new eggshell types might be better characterized as continuous traits (i.e. type IV is described as identical to type III, but with “shell units [...] tightly packed with each other” – Kusuda et al., 2013, p. 372), as inferred from the influence of eggshell thickness and calcification on their variation (Deeming, 2018b). Future examination of turtle eggshells using more advanced microscopy techniques, e.g. electron backscatter diffraction (EBSD; Choi et al., 2019; Moreno-Azanza et al., 2013), will likely identify more subcategories inside each of these eggshell types.

### 2.2.3 | Lepidosauria

Most lepidosaur species were defined by Packard, Packard and Boardman (1982) as soft-shelled egg layers. However, significant variation of their shell structure problematizes assignment of eggs in this clade to only one or two functional categories. The best known lepidosaur clade with a highly distinct eggshell structure is geckos (order Gekkota). Families Gekkonidae, Phyllodactylidae, and Sphaerodactylidae, representing ~90% of the ~2000 species of geckos (Uetz et al., 2021), lay eggs that have long been characterized as hard-shelled based on their CL formed of thick prismatic structures, referred to as “shell units” (Figure 1; Andrews, 2012, 2017; Choi et al., 2018; Mikhailov, 1991a, 1997b; Packard & DeMarco, 1991; Pike et al., 2012; Schleich & Kästle, 1988). Other gecko families (Carphodactylidae, Diplodactylidae, Eublepharidae, and Pygopodidae) lay eggs with thinner “nail-shaped” structures, described as soft-shelled or semi-rigid depending on the authors (Figure 2; Choi et al., 2018; Pike et al., 2012). However, among

Diplodactylidae, species in the genus *Eurydactyloides* lay eggs with shell units (Kratohvil & Frynta, 2006), meaning eggshells with shell units must have evolved independently at least twice in geckos.

Although most gecko eggs are described as hard-shelled based on presence of shell units, these shell units are not homologous to those found in Archelosauria (*sensu* Crawford et al., 2015) and differ from them in structure (Figure 1; Choi et al., 2018). EBSD mapping of archelosaur eggshells shows that their biomineralization starts from the external margin of the membrana testacea and expands outward (Choi et al., 2019; Choi, Kim, et al., 2022; Grellet-Tinner et al., 2011; Moreno-Azanza et al., 2013). Conversely, gecko eggshells with shell units show an opposite crystallographic configuration – shell units likely grow inward from their outer surface (see inversed color pattern between eggshells of geckos and other reptiles in Figure 1; Choi et al., 2018). The CL of several gecko eggs with no shell units (e.g. Diplodactylidae, Eublepharidae) also shares this growth pattern (Choi et al., 2018), as does the eggshell of a Cretaceous fossil anguimorph lizard (Fernandez et al., 2015), suggesting this inward mineralization pattern is a synapomorphy of squamates.

Interestingly, the eggshell of the tuatara (*Sphenodon punctatus*), sister taxon to all other extant lepidosaurs, presents a CL composed of stem-like structures embedded in the proteinaceous shell membrane, each covered by a cap-like structure on its outer surface (Cree et al., 1996; Packard, Hirsch, & Meyer-Rochow, 1982; Packard et al., 1988). Such a structure, often described as “semi-rigid” (Packard et al., 1988), is highly similar to the “nail-shaped” structures found in gecko eggs with no shell units (Choi et al., 2018). This suggests that these geckos and the tuatara may share a homologous CL organization with “nail-shaped” elements and inward mineralization. This organization, which may have given rise to the shell units seen in other gecko eggs, is distinct from that of Archelosauria and potentially ancestral to lepidosaurs (Choi et al., 2018). However, confirming that hypothesis would require studying the eggshells of Dibamidae, a poorly known clade of legless lizards recovered by several studies as sister group to all other squamates (Pyron et al., 2013; Tonini et al., 2016). The first description of an egg from the dibamid *Dibamus novaeguineae* mentioned its “brittle and highly calcareous shell” (Boulenger, 1912, p. 100), without providing any illustrations or further details. This short description was cited as evidence for the whole group to likely be classified as “hard-shelled” (Packard, Packard, & Boardman, 1982). Other eggs from the same dibamid species were later described as having a “thin, flexible shell,” the difference from the shell of the first described egg being attributed to either polymorphism or specimen degradation (Greer, 1985, p. 140). Since none of these studies investigated dibamid eggshells using microscopy, their eggshell microstructure remains unknown. Thus, despite the vast majority of squamate eggs being traditionally described as “soft-shelled,” the potential homology of eggshell microstructural traits within and outside Lepidosauria remains elusive.

Additionally, even in squamate eggs with no shell units, variation has been recorded in all layers of the eggshell, but has rarely been the subject of classifications analogous to those of shell units in other reptile eggs. This includes the outer ornamentation of the

CL, which can include grooves and cracks, calcite granules, or rosettes, etc., the size and shape of which appear to be unique to specific clades (Arrieta et al., 2021; Osborne & Thompson, 2005; Packard & DeMarco, 1991; Packard et al., 1982; Packard, Packard, & Boardman, 1982; Packard et al., 1991; Schleich & Kästle, 1988); bundles of protein fibers in the membrana testacea, often arranged in highly specific and complex patterns (Legendre, Rubilar-Rogers, Musser, et al., 2020; Osborne & Thompson, 2005; Packard et al., 1982; Schleich & Kästle, 1988); and mineralized nodules in the membrana testacea, highly variable in shape and size but almost never reported in formal descriptions (Schleich & Kästle, 1988). Such structures, as well as their location on or within the shell, have been categorized and discussed in the context of calcification patterns and shell porosity (D'Alba et al., 2021; Kohring, 1995; Schleich & Kästle, 1988), although such classifications remain to be tested in a phylogenetic context. Many species also present pore-like structures, the structure and function of which has never been assessed due to their “simple” aspect compared with the well-defined pores of hard-shelled eggs (Arrieta et al., 2021; Osborne & Thompson, 2005; Packard & DeMarco, 1991; Schleich & Kästle, 1988). The CL in some species has also been reported to be completely absent, or made of hydroxyapatite instead of calcite (Schleich & Kästle, 1988). These anomalies have been attributed to either human error or abnormalities in captive specimens, but never subjected to detailed study (Packard & DeMarco, 1991). However, the recent description of hydroxyapatite as the only mineral present in eggshells of a large sample of wild black and white tegus (*Salvator merianae*) suggests that the outer mineralized layer of reptile eggshells is indeed not always calcareous (Campos-Casal et al., 2020).

Packard and DeMarco (1991, p. 65), in their seminal review of eggshell microstructure in non-avian reptiles, concluded: “Clearly, many questions concerning shell formation in oviparous reptiles remain to be answered. [...] Additional studies of shell formation in oviparous squamates undoubtedly will contribute importantly to our understanding of this process in reptiles generally.” As detailed here, this conclusion remains true 30 years later, and the grouping of so many distinct eggshell morphologies under a single “soft-shelled” category has likely prevented the atomization of many discrete, phylogenetically informative traits that would improve our knowledge of the evolution of lepidosaur eggshells.

#### 2.2.4 | Monotremata

The eggshell structure of monotremes, the only extant egg-laying amniotes outside Reptilia, is even less well-known than that of reptiles, likely due to the difficulty of breeding them in captivity (Temple-Smith & Grant, 2001). Although their inner egg structure and embryology have been well described (Blackburn & Stewart, 2021; Griffiths, 1978; Hughes, 1984), their eggshell, usually referred to as soft, has only been described extensively in two publications (Hill & Hill, 1933; Hughes & Carrick, 1978).



Hill and Hill (1933) described complete ontogenetic series for the platypus and short-beaked echidna and reported a three-layered shell in laid eggs. The outmost layer was described as densely mineralized, granular in texture, much thicker than the other two, and with distinct pore canals – i.e. similar to the CL of eggs described as hard-shelled. A recent study of eggshell thickness in amniotes using Hill and Hill's measurements recovered the platypus CL as comparable in thickness to that of similar-sized bird eggs (Legendre, Rubilar-Rogers, Musser, et al., 2020). Hughes and Carrick (1978) described a similar three-layered shell in an intra-uterine platypus egg of unspecified ontogenetic age, with its outer layer much thinner, i.e. closer to that of eggs previously described as soft-shelled (130 to 208  $\mu\text{m}$  [Hill & Hill, 1933] versus 49  $\mu\text{m}$  [Hughes & Carrick, 1978]). Since both studies predate the more standardized terminologies for eggshell microstructure of Packard, Packard and Boardman (1982) and Mikhailov (1991b), a comparison with reptile morphologies requires a reassessment of character states and measurements in an extrauterine (laid) egg before defining potential microstructural states for assessment of secondary homology.

### 3 | ANCESTRAL RECONSTRUCTIONS OF EGG SHELL TYPE AND CALCAREOUS LAYER THICKNESS IN REPTILES

#### 3.1 | Material and methods

Using eggshell microstructure data collected from the literature, we assembled a sample of egg measurements for 208 reptile species (Table S1; Supporting Information; Legendre, 2022). We then compiled eggshell microstructural traits from these measurements, each corresponding to one possible scoring strategy (i.e. hypothesis of primary homology), to be tested for secondary homology (i.e. synapomorphies) using ancestral state reconstruction (ASR) on a phylogeny of Reptilia. The first set of traits includes three distinct scorings of a three-state discrete trait, corresponding to the three traditionally defined eggshell types (“hard”/“semi-rigid”/“soft”). The “semi-rigid” character state applies to shells with either “nail-shaped” structures (e.g. tuatara) or shell units as thick as their underlying membrane (e.g. some sauropodomorphs and turtles).

The three eggshell types were based on distinct criteria, each of them previously used in the literature:

- A Shell unit scoring: based on presence ('hard') or absence ('soft') of prismatic shell units in the CL of the eggshell (Legendre, Rubilar-Rogers, Musser, et al., 2020; Packard & DeMarco, 1991; Schleich & Kästle, 1988). The “nail-shaped” structures in the eggshells of the tuatara and some geckos, distinct from prismatic shell units, are coded as semi-rigid;
- B Ratio scoring: a discretization of the continuous ratio “CL thickness/total eggshell thickness”:  $r \leq 0.5$ : “soft”;  $0.5 < r \leq 0.67$ : “semi-rigid”;  $r > 0.67$ : “hard” (Hirsch, 1983; Norell et al., 2020; alluded to in Packard, Packard, & Boardman, 1982, with no threshold

values provided). This discretization is often scored through simple visual assessment rather than compiling the actual ratio, since for most reptile eggshells the CL is either much thicker or much thinner than the underlying membranes (Hirsch, 1983; Packard, Packard, & Boardman, 1982). Furthermore, compilation of the ratio requires proteinaceous shell membranes to be present in the sampled eggshell and included in thickness measurements. This is not the case for most eggshell studies – either because the specimen is a fossil egg in which the shell membrane was not preserved, or because the study focused only on eggs for which the CL represents most of the total thickness and is considered equivalent to it (e.g. in birds), so that only one measurement is included and the eggs are considered hard-shelled. For this study, we only measured the ratio to score eggs for which it was visually ambiguous – i.e. the value of  $r$  was not conspicuously inferior to 0.5 or superior to 0.67 (Supporting Information).

- C A new scoring procedure: while most species in our sample are scored identically for shell unit and ratio scoring procedures (see below), a few species present a difference between scorings (e.g. turtle eggs traditionally defined as semi-rigid). Since the original definition of such “ambiguous” eggshell types was often based on visual assessment (consistency and general aspect of the CL – e.g. Hirsch, 1983), it can potentially differ from both previous scorings. In order to reflect that original definition and test its relevance against the two scorings defined above in a phylogenetic context, we introduced a third scoring: species for which shell unit scoring and ratio scoring differed were also scored following a general visual assessment, which in some cases resulted in a score distinct from the previous two (see detail for each species in Supporting Information).

Only nine species (two non-avian dinosaurs and seven turtles) out of 208 were scored differently depending on scoring procedure, seemingly showing a general congruence between the three main ways to define eggshell type – general description (new scoring), discretized ratio (ratio scoring), and presence/absence of shell units (shell unit scoring). This, however, is due to the fact that most reptile eggshells present either very thick shell units and a very thin membrana testacea, or no shell units and a very thick membrana testacea (Packard & DeMarco, 1991; Schleich & Kästle, 1988). These two opposite ends of the spectrum for all three scoring systems result in similar character states between them, which does not imply that the underlying definitions of these states are equivalent between scoring systems.

All three scoring procedures present potential issues linked with how much of the original eggshell structural variation they can consider. None of them, for example, includes information relative to the different types of shell units, which in the case of geckos are known not to be homologous to those of other reptiles. The new scoring and shell unit scoring neglect the shell membrane entirely (its thickness and its structure), meaning both eggs without a CL and eggs with a CL that do not have shell units that are coded as “soft-shelled,” resulting in information loss. The ratio scoring also

does not consider inner structure of the shell membrane, but it does consider its thickness in a discretized form. However, this scoring, as described by Norell et al. (2020), despite being based on previous literature, does not incorporate any empirical data that would justify the threshold values used to discretize it (see Legendre, Rubilar-Rogers, Vargas, & Clarke, 2020). Additionally, for all scorings, structures that are demonstrably not homologous to each other are both coded as “semi-rigid” (see previous section), meaning that the recovery of any clade as ancestrally “semi-rigid” would provide no clear indication of which eggshell traits were present at that node. For all these reasons, the three scoring systems are here put to a test of secondary homology through ASR, in order to decipher what biological information can potentially be inferred from them in a phylogenetic context.

We also compiled continuous trait data for CL thickness (in  $\mu\text{m}$ ). For the continuous measurement of eggshell thickness, we used CL thickness as a proxy due to the impossibility of measuring total eggshell thickness in ootaxa that lack a shell membrane. This allowed us to include total thickness as an equivalent measurement for eggs traditionally considered hard-shelled, in which only CL thickness is usually reported (i.e. crocodylians, most dinosaurs including all birds), but not for those traditionally considered semi-rigid or soft-shelled (turtles, lepidosaurs, pterosaurs, choristoderes). For this reason, we only included taxa in the latter groups if a measurement of CL thickness was available in its original description. Similarly, since the significant correlation of eggshell thickness with egg mass influences results of ASR for this trait (Legendre, Rubilar-Rogers, Musser, et al., 2020; Legendre & Clarke, 2021; Stein et al., 2019), we performed ASR of CL thickness both as an absolute (i.e. as itself, in  $\mu\text{m}$ ) and relative (i.e. as a ratio “CL thickness/egg mass,” in  $\mu\text{m g}^{-1}$ ) trait. For this reason, we only sampled complete eggs for which egg mass could be estimated from egg length and width, so that all ASR in this study were performed using the same sample and topology. Original measurements were primarily collected from the Norell et al. (2020) and Legendre, Rubilar-Rogers, Musser, et al. (2020) datasets ( $n = 92$  and  $n = 148$ , respectively; see detail in [Supporting Information](#)). The calibrated phylogeny used in all analyses was based on the tree from Legendre and Clarke (2021), with additional references for crocodylians, pterosaurs, turtles, and lepidosaurs listed in the [Supporting Information](#).

All analyses were performed in R v 4.1.2 (R Core Team, 2022). Continuous traits were log-transformed prior to analysis (Sokal & Rohlf, 1995). ASR for eggshell type (discrete trait) were performed in phytools (Revell, 2012, 2013) using Bayesian stochastic character mapping (SIMMAP – Bollback, 2006; Huelsenbeck et al., 2003). SIMMAP is based on Bayesian posterior sampling of stochastic character maps using Monte-Carlo Markov Chain (MCMC), which can account for branch length information, rate heterogeneity, and phylogenetic uncertainty (Bollback, 2006; Huelsenbeck et al., 2003), unlike maximum parsimony and maximum likelihood methods for discrete traits (Cunningham et al., 1998; Huelsenbeck et al., 2003; Joy et al., 2016; O’Meara, 2012). For each ASR using SIMMAP, we estimated Akaike weights (Burnham & Anderson, 2002) for each

of three candidate models for transition rates (equal, symmetrical, and all different rates) and generated stochastic maps in proportion of the weight of each model out of 1000 simulations (Supporting Code). In order to account for the potentially strong influence of branch length information on ancestral states (Cunningham et al., 1998; Joy et al., 2016), we replicated all analyses using maximum parsimony, which does not consider branch length information (Cunningham, 1999), in castor (Louca & Doebeli, 2018).

For each scoring procedure, we found that the eggshell type recovered for most major clades using SIMMAP (Table 1; see Results) was the same as that scored for the two sauropodomorphs *Lufengosaurus* and *Massospondylus* – the only two species in the sample to be scored differently for each of the three scorings (new scoring: semi-rigid; ratio scoring: soft; shell unit scoring: hard). They are also, along with the other sauropodomorph *Mussaurus* (always scored as soft-shelled), the oldest taxa in the tree, i.e. closer in branch length to more inclusive nodes in the tree than any other species in the sample, which might explain why their eggshell type strongly influences the one recovered as ancestral for most major clades (e.g. Li et al., 2008). To test that prediction, we removed from the sample all other taxa ( $n = 7$ , all turtles) that were scored differently for at least one of the three scorings, resulting in a sample for which all taxa except *Lufengosaurus* and *Massospondylus* are scored identically. We then replicated all ASR with SIMMAP and maximum parsimony.

For CL thickness, we performed ASR using a maximum likelihood Brownian Motion model using “contMap” in phytools (Revell, 2012, 2013); polytomies were resolved using “multi2di” in ape (Paradis, 2012). The use of a Brownian Motion model was justified by the high phylogenetic signal ( $\lambda > 0.999$ ;  $p < 0.001$ ) recovered for both absolute and relative CL thickness, using “phylosig” in phytools.

## 3.2 | Results

For analyses performed on the whole sample ( $n = 208$ ; Figure 3a,c,e), SIMMAP ASR of eggshell type recover a high discrepancy in ancestral states of most major reptile clades among all three scoring procedures (Table 1). For all analyses of eggshell type, Pterosauria are recovered as ancestrally soft-shelled, since all but one of them are coded as soft-shelled; similarly, Crocodylia and Aves are always recovered as ancestrally hard-shelled, since all members of both clades are coded as hard-shelled. The new scoring (Figure 3a) results in a semi-rigid eggshell being the ancestral condition for all major clades (i.e. Reptilia, Lepidosauria, Archelosauria, Testudines, Archosauria, Ornithodira, Dinosauria, Ornithischia, and Saurischia) – a surprising result considering only six taxa in the whole sample, among which no ornithischians, were scored as semi-rigid. With the ratio scoring (Figure 3c), all aforementioned clades are recovered as ancestrally soft-shelled, with the exception of Testudines recovered as ancestrally semi-rigid. Conversely, with the shell unit scoring (Figure 3e), Reptilia and Lepidosauria are recovered as ancestrally soft-shelled,

**TABLE 1** Ancestral eggshell type with the highest associated probability (indicated in parentheses) recovered for each major clade in the tree, for each sample size (whole or reduced), reconstruction method (SIMMAP or maximum parsimony), and scoring procedure (new scoring, ratio scoring, or shell unit scoring)

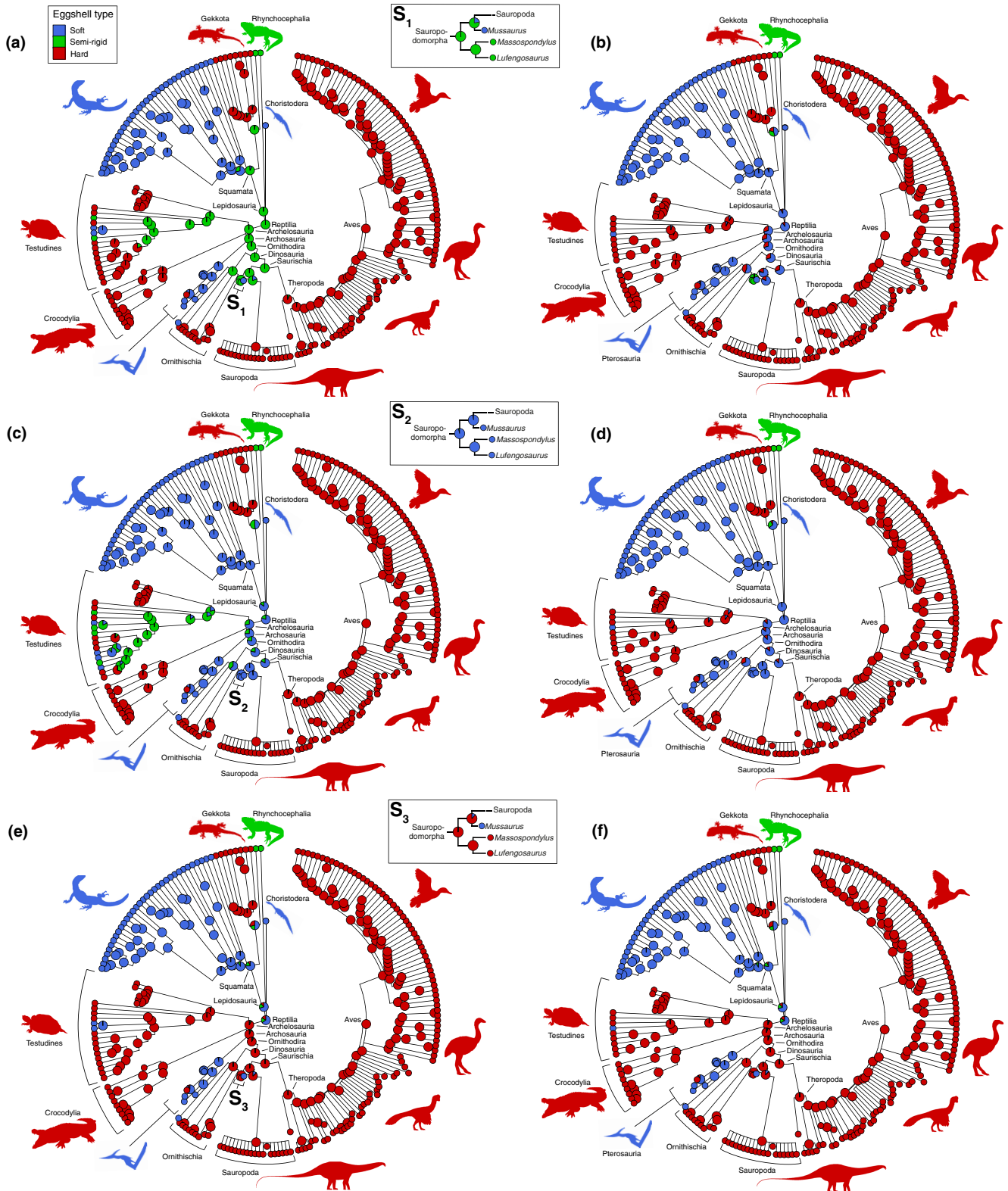
Clade	New scoring	Ratio scoring	Shell unit scoring
For the whole sample ( $n = 208$ ), using SIMMAP			
Reptilia	Semi-rigid (0.998)	Soft (0.784)	Soft (0.673)
Lepidosauria	Semi-rigid (0.995)	Soft (0.838)	Soft (0.711)
Archelosauria	Semi-rigid (0.999)	Soft (0.738)	Hard (0.917)
Testudines	Semi-rigid (0.999)	Semi-rigid (0.776)	Hard (0.993)
Archosauria	Semi-rigid (0.999)	Soft (0.753)	Hard (0.929)
Ornithodira	Semi-rigid (0.999)	Soft (0.794)	Hard (0.931)
Dinosauria	Semi-rigid (0.999)	Soft (0.789)	Hard (0.985)
Ornithischia	Semi-rigid (0.998)	Soft (0.644)	Hard (0.997)
Saurischia	Semi-rigid (0.999)	Soft (0.831)	Hard (0.994)
For the reduced sample ( $n = 201$ ), using SIMMAP			
Reptilia	Soft (0.900)	Soft (0.968)	Soft (0.659)
Lepidosauria	Soft (0.906)	Soft (0.981)	Soft (0.709)
Archelosauria	Soft (0.702)	Soft (0.867)	Hard (0.931)
Testudines	Hard (0.925)	Hard (0.895)	Hard (0.995)
Archosauria	Soft (0.701)	Soft (0.867)	Hard (0.941)
Ornithodira	Soft (0.706)	Soft (0.867)	Hard (0.941)
Dinosauria	Soft (0.694)	Soft (0.858)	Hard (0.991)
Ornithischia	Soft (0.531)	Soft (0.676)	Hard (0.997)
Saurischia	Soft (0.699)	Soft (0.865)	Hard (0.998)
For the whole sample ( $n = 208$ ), using maximum parsimony			
Reptilia	Each state at 0.333	Each state at 0.333	Each state at 0.333
Lepidosauria	Semi-rigid (0.6)	Semi-rigid (0.6)	Semi-rigid (0.6)
Archelosauria	Hard (1)	Hard (1)	Hard (1)
Testudines	Hard (1)	Hard (1)	Hard (1)
Archosauria	Hard (1)	Hard (1)	Hard (1)
Ornithodira	Hard (1)	Hard (1)	Hard (1)
Dinosauria	Hard (1)	Hard (1)	Hard (1)
Ornithischia	Hard (1)	Hard (1)	Hard (1)
Saurischia	Hard (1)	Hard (1)	Hard (1)
For the reduced sample ( $n = 201$ ), using maximum parsimony			
Reptilia	Each state at 0.333	Each state at 0.333	Each state at 0.333
Lepidosauria	Semi-rigid (0.6)	Semi-rigid (0.6)	Semi-rigid (0.6)
Archelosauria	Hard (1)	Hard (1)	Hard (1)
Testudines	Hard (1)	Hard (1)	Hard (1)
Archosauria	Hard (1)	Hard (1)	Hard (1)
Ornithodira	Hard (1)	Hard (1)	Hard (1)
Dinosauria	Hard (1)	Hard (1)	Hard (1)
Ornithischia	Hard (1)	Hard (1)	Hard (1)
Saurischia	Hard (1)	Hard (1)	Hard (1)

[Corrections added on 14 July 2022, after first online publication: In Table 1 values have been updated in this version]

but Archelosauria, Archosauria, Dinosauria, and less inclusive clades as ancestrally hard-shelled.

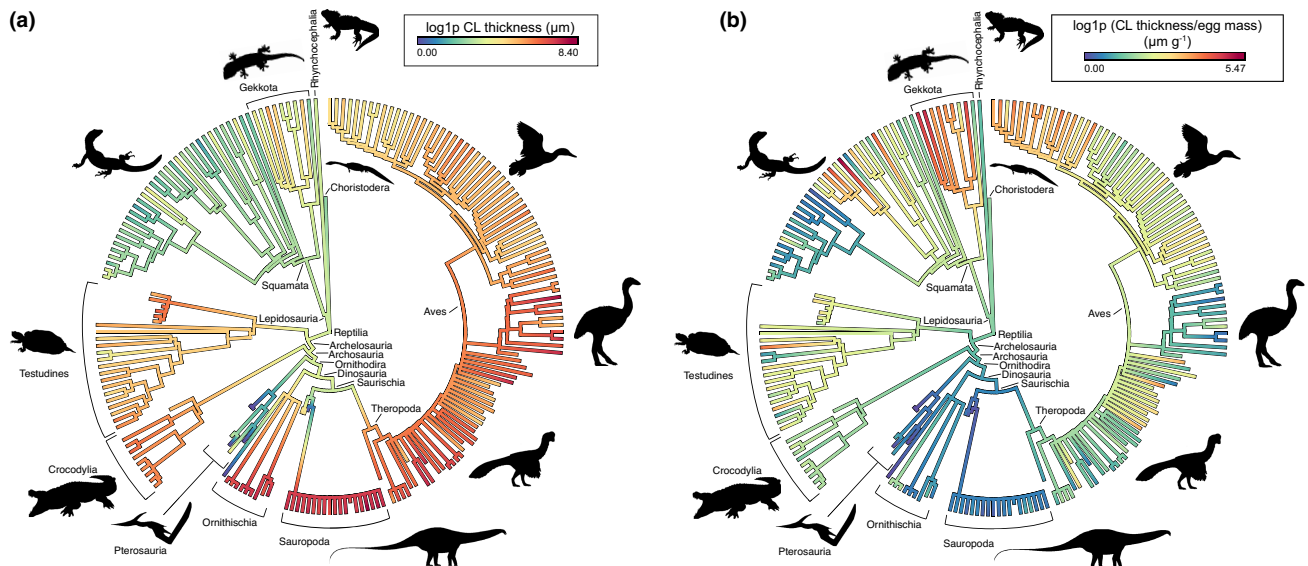
SIMMAP ASR performed on the reduced sample (i.e. with identical scoring for all taxa except *Lufengosaurus* and *Massospondylus*;

$n = 201$ ) give different results from those performed on the whole sample (Figure 3b,d,f; Table 1). For all three scorings, Reptilia and Lepidosauria are recovered as ancestrally soft-shelled, and Testudines as ancestrally hard-shelled (due to all turtles with



**FIGURE 3** Ancestral state reconstructions of eggshell type scored as a three-state character – “soft/semi-rigid/hard” – on our calibrated phylogeny using SIMMAP (see Text), with labels for all clades of interest. (a, b) New scoring. (c, d) Ratio scoring. (e, f) Shell unit scoring. Panels on the left (a, c, e) correspond to reconstructions performed on the whole sample ( $n = 208$ ), while panels on the right (b, d, f) correspond to reconstructions performed on the reduced sample ( $n = 201$ ; see Text). Legend for eggshell type (upper left insert in panel a) applies to all panels. Non-sauropod sauropodomorphs are labeled “S1,” “S2,” and “S3” in panels (a), (c), and (e), respectively, with a corresponding upper right insert for each panel indicating the topology and scoring of these taxa to show their strong influence on each reconstruction (see Text). Select taxon silhouettes from PhyloPic (full credits in Supporting Information). [Correction added on 14 July 2022, after first online publication: Figure 3 has been updated in this version]





**FIGURE 4** Ancestral state reconstruction of (a) absolute and (b) relative calcareous layer (CL) thickness, treated as a continuous trait, performed on our calibrated phylogeny ( $n = 208$ ) using maximum likelihood (see Text). All values ( $x$ ) are log-converted using  $\log_{1p}(x) = \log(1 + x)$ , due to the presence of null values in species that do not have a CL. Clades of interest for each reconstruction are labeled. Select taxon silhouettes from PhyloPic (full credits in Supporting Information). [Correction added on 14 July 2022, after first online publication: Figure 4 has been updated in this version]

semi-rigid eggshells being removed from the sample). Archelosauria, Archosauria, Ornithodira, Dinosauria, Ornithischia, and Saurischia are recovered as ancestrally soft-shelled if *Lufengosaurus* and *Massospondylus* were scored as either semi-rigid (Figure 3b) or soft-shelled (Figure 3d), but as ancestrally hard-shelled if *Lufengosaurus* and *Massospondylus* were scored as or hard-shelled (Figure 3f). This confirms that *Lufengosaurus* and *Massospondylus* have a disproportionate effect on the results of SIMMAP ASR, despite representing ~1% of the sample. Furthermore, for the new scoring, the removal of seven turtle species – only two of which scored as semi-rigid – changes the ancestral states for all major clades from semi-rigid to soft (or hard for Testudines), which suggests that these two turtle species also have a strong influence on those results.

ASR performed with maximum parsimony are more congruent between scoring procedures than those performed using SIMMAP (Table 1; Figure S1). For all three scoring procedures, an identical probability is recovered for all three character states for the Reptilia node, resulting in an ambiguous ancestral condition. Lepidosauria are always recovered as ancestrally semi-rigid, while Archelosauria, Testudines, Archosauria, Ornithodira, Dinosauria, Ornithischia, and Saurischia are always recovered as ancestrally hard-shelled (Figure S1). These differences between SIMMAP and parsimony-based ASR for an identical topology show that branch length information is indeed a strong influence on the eggshell type recovered in major reptile clades. This is especially true of the ratio scoring, for which all major clades within Archelosauria are recovered as either ancestrally soft-shelled or semi-rigid by SIMMAP, but as hard-shelled using parsimony (Figure 3c; Figure S1b). Similarly, for the new scoring, all but two major clades recovered by SIMMAP as ancestrally semi-rigid are recovered as hard-shelled by maximum parsimony (Table 1), showing that the abnormal influence of the six taxa

scored as semi-rigid in this scoring is conditional on branch length information. However, unlike SIMMAP, maximum parsimony ASR performed on the reduced sample are identical to those performed on the whole sample (Table 1), suggesting that the strong influence of *Lufengosaurus* and *Massospondylus* on ASR results is independent of branch length information.

ASR for absolute CL thickness as a continuous trait show relatively low values (~30–40  $\mu\text{m}$ ) for Reptilia, Lepidosauria, Archelosauria, Archosauria, and Ornithodira. Gekkota, Testudines, and Crocodylia all acquire a thicker CL independently (Figure 4a). Among Ornithodira, Pterosauria show a strong decrease in CL thickness, as expected from the lack of CL in most species. Dinosauria also present a low value (38.2  $\mu\text{m}$ ), since they include species with either thick (>250  $\mu\text{m}$ ; Ornithopoda, Sauropoda, most Theropoda) or thin CL (*Protoceratops* and *Mussaurus*: no CL; *Massospondylus*: 100  $\mu\text{m}$ ; *Lufengosaurus*: 85  $\mu\text{m}$ ) – the latter being much less numerous, but closer in branch length to the Dinosauria node than the former. *Lufengosaurus* and *Massospondylus* fall in the middle of the spectrum for absolute CL, much higher than e.g. non-gekkotan lepidosaurs, likely due to the comparatively large size of their eggs.

For relative CL thickness (in  $\mu\text{m g}^{-1}$ ), the pattern is different (Figure 4b): the value at the node Reptilia remains relatively low (3.44), and nodes Archelosauria and Archosauria show a decrease (2.63 and 2.16, respectively). Values in Lepidosauria are highly variable, with a conspicuous increase in Gekkota (20.4) and decrease in Serpentes (1.65) – congruent with a recent study on non-avian reptiles that identified a similar ASR pattern for shell calcification in Gekkota and Serpentes (D'Alba et al., 2021). Values increase in Testudines (6.62) and Crocodylia (4.75), but strongly decrease in Dinosauria (1.28). Among Dinosauria, most Ornithischia and all Sauropodomorpha show a very low relative CL thickness, while

Theropoda show a gradual increase, with a subsequent decrease in Palaeognathae and further increase in Eufalconimorphae – a result already described by Legendre and Clarke (2021) using an almost identical sample of Dinosauria.

## 4 | DISCUSSION

### 4.1 | Influence of branch length information and topology on inference of eggshell type

In all ASR of eggshell type performed in this study, the ancestral states recovered for many major reptile clades is largely conditional on the phylogenetic position and branch length of a few key taxa in the tree, as was already the case in the two main references used for our sample (Legendre, Rubilar-Rogers, Musser, et al., 2020; Legendre, Rubilar-Rogers, Vargas, & Clarke, 2020; Norell et al., 2020). This is due to the fact that, for both Bayesian and maximum likelihood ASR, the probability of a change of state along a branch increases with its length (Cunningham et al., 1998; Huelsenbeck et al., 2003; Joy et al., 2016), which implies that the ancestral state recovered at any given node is more likely to be that of the descendant taxon with the shortest branch length from that node than the one with the longest branch length (Hanson-Smith et al., 2010; Huelsenbeck & Bollback, 2001; Li et al., 2008; Wright et al., 2015). This results in a strong bias in the case of nodes with a high difference in length between its two descendant branches – e.g. Sauropodomorpha, to which the three non-sauropod species in our sample (*Mussaurus*, *Massospondylus*, and *Lufengosaurus*) are much closer than Sauropoda. Since these three non-sauropod sauropodomorphs are closer to all most inclusive nodes in the tree than any other terminal taxa, their eggshell type is more likely to be that recovered for these clades, giving them a disproportionate influence on all ASR.

In our calibrated phylogeny, this effect of proximity of some fossil species to nodes of interest on ASR is reinforced by the presence of many large clades for which the earliest diverging tip taxon (i.e. sister taxon to all other parts of this clade) has a character state different from those of most other members of that clade, which is likely a source of further bias in ASR. For example, in SIMMAP ASR on our full sample, when *Lufengosaurus* and *Massospondylus* are coded as semi-rigid, they are not only close to internal nodes of interest, but also share their semi-rigid state with two deep branches within Lepidosauria: the tuatara (sister taxon of all other lepidosaurs) and *Eublepharis macularius* (sister taxon of all other geckos). This results in all major clades, as well as Gekkota, recovered as ancestrally semi-rigid (Figure 3a). Conversely, when *Lufengosaurus* and *Massospondylus* are coded as soft-shelled, they share this state with five pterosaurs, which are also closer in time to the Ornithodira and Archosauria nodes than all but two members of these two clades coded as hard-shelled. This results in Saurischia, Ornithischia, Dinosauria, Ornithodira, Archosauria, and Archelosauria being recovered as ancestrally

soft-shelled, despite the vast majority of species in those clades being coded as hard-shelled (Figure 3c). Accordingly, when removing branch length information in parsimony-based ASR, all these clades are recovered as ancestrally hard-shelled (Figure S1). This shows the influence of a few key taxa on ASR results given their temporal proximity to nodes of interest.

While such a bias has been described in several studies on both simulated and empirical datasets (Cascini et al., 2019; Cunningham et al., 1998; Cusimano & Renner, 2014; Hanson-Smith et al., 2010; Huelsenbeck & Bollback, 2001; Li et al., 2008; Wright et al., 2015), its effects on ASR that include fossils have rarely been discussed. This is due to the fact that most ASR in the literature (Cusimano & Renner, 2014; McCann et al., 2016) use either (1) molecular phylogenies with branch lengths representing the number of nucleotide substitutions per site, i.e. an evolutionary model not necessarily proportional to time calibration (Drummond et al., 2012; Huelsenbeck & Bollback, 2001; Ishikawa et al., 2019; Matsumoto et al., 2015); or (2) time-calibrated trees of extant species (i.e. ultrametric trees), in which no terminal taxon can have such a disproportionate influence. Including fossils in time-calibrated trees for ASR requires precise evaluation of their phylogenetic position and fossil record sampling bias (Bapst, 2014), as well as their effect on the underlying evolutionary model of the trait of interest (Cascini et al., 2019; King & Lee, 2015; Litsios & Salamin, 2012; Wilson et al., in press) – both of which have been the subject of considerable debate in the paleontological community (Bapst, 2014; Soul & Wright, 2021). While the inclusion of both fossil and extant species generally improves ASR accuracy – especially when using discrete traits, which are less sensitive to sampling bias and model misspecification (Puttick, 2016; Soul & Wright, 2021), the effect of such a high discrepancy in distance between nodes and terminal taxa on ASR with time-calibrated trees remains to be assessed.

Similarly, the disproportionate influence of earliest-diverging tip taxa identified in our ASR has not, to our knowledge, been well-documented. A previous study on the evolution of squamate viviparity described the tuatara as “relatively uninformative about the root state of squamates” due to the long branch separating it from the Lepidosauria node (Wright et al., 2015, p. 513). Our results suggest the opposite, but this is in part also due to the position of *Eublepharis* – the only other lepidosaur with an eggshell coded as semi-rigid like the tuatara, showing that ASR can be biased by very specific combinations of topology and branch lengths, which are likely to be only understood on a case-by-case basis. Another important factor in this context is tree resolution, since our tree comprises many polytomies, including the base of several key clades (i.e. Reptilia, Ornithischia, Sauropoda). Phylogenetic uncertainty has been shown to result in an overestimation of transitions (i.e. independent origins of a character state) in the tree (Duchêne & Lanfear, 2015). This can be seen in our tree for the polytomy at the base of Ornithischia: the clade contains nine taxa coded as hard-shelled and one (*Protoceratops*) coded as soft-shelled, but is recovered as ancestrally semi-rigid for the new scoring and ancestrally soft-shelled for the ratio scoring. When removing branch

length information, the clade is recovered as ancestrally hard-shelled. Such combinations of biases in branch length information, sampling error, and phylogenetic uncertainty impact the results of all ASR and should be accounted for in studies that perform them (Duchêne & Lanfear, 2015; Huelsenbeck & Bollback, 2001; Wright et al., 2015).

In this study, we updated the topology and calibration of the phylogeny used in ASR using several references (Supporting Information), but did not account for all alternative topologies and calibrations. There are two reasons for this: first, our sample includes many outgroups, the phylogenetic position of which is highly difficult to infer through phylogenetic reconstruction (Grellet-Tinner et al., 2006; Varricchio & Barta, 2015; Zelenitsky & Therrien, 2008); second, accounting for phylogenetic uncertainty in ASR (especially when using Bayesian methods) only produces a different result when that uncertainty is low (Hanson-Smith et al., 2010), which is not the case here, since the true tree for many taxa in our sample has never been assessed. The aim of ASR performed in this study, however, is not to provide biologically accurate ASR of our traits of interest, but rather to show how such analyses tend to be overly sensitive to a very small number of taxa (~1–4%) due to their phylogenetic position and calibration. In general, the field of ASR in paleontology is relatively new, and most biases associated with the inclusion of fossils for e.g. model fitting or sampling error have only been identified and characterized recently (Bapst, 2014; Hunt & Carrano, 2010; Soul & Wright, 2021). In this context, our results provide a clear example of inherent issues of uncertainty in tree topology and calibration, taxon sampling, and character coding (see next section), which are generally not considered, nor discussed, in recent studies on reptile eggshell evolution (e.g. D'Alba et al., 2021; Legendre, Rubilar-Rogers, Musser, et al., 2020; Norell et al., 2020; Stein et al., 2019). We therefore advocate for future studies performing ASR of eggshell type – or any other discrete or continuous egg trait – to prioritize, when possible, methods that can account for such biases most efficiently (e.g. hierarchical, split, or hidden rate Bayesian models; sensitivity analyses using joint estimation from a posterior distribution of trees – Joy et al., 2016; King & Lee, 2015; Soul & Wright, 2021; Wilson et al., *in press*; Wright et al., 2021), and explicitly discuss potential biases associated with these methods, sampled taxa, and data used for both traits and phylogeny tree used in such studies. These extra steps will help facilitate replication of previous results and improve future discussions on discrepancies between methods and datasets in this context.

#### 4.2 | Scoring eggshell type: can we provide biological definitions for hard/soft/semi-rigid eggshells?

ASR performed on absolute and relative CL thickness illustrate the difficulty of assigning a discrete character state to a specific value, or range of values, for a continuous trait. For absolute CL thickness,

many large clades are recovered with ancestral values that fall near the middle of the spectrum for that trait, due to the wide range of values taken by terminal taxa in each of these clades. Following the traditional classification, these intermediate values would be considered equivalent to a semi-rigid eggshell; however, as described in previous sections, the semi-rigid eggshell type can also be defined as the very specific “nail-shaped” morphology found in the eggshells of the tuatara and several geckos (Figure 2), which is highly unlikely to be the ancestral eggshell of Reptilia, Archosauria, or Dinosauria.

Similarly, the independent acquisitions of a thick CL (whether absolute or relative) are clearly associated with the highly distinctive shell unit morphologies documented in each of these groups (Figure 1; see previous section). Even if the basic structure of a shell unit likely shows deep homology (*sensu* Shubin et al., 1997) among all Archelosauria (Mikhailov, 1991a, 1997b; Mikhailov et al., 1996), the morphological differences between them constitute the respective synapomorphies of distinct clades among archelosaurs. Hence the diversity of these shell unit types cannot be fully encompassed by one single “hard-shelled” character state, which explains why it has rarely, if ever, been the subject of evolutionary hypotheses in a phylogenetic context. Indeed, the problem of discretizing continuous traits has been the subject of a four-decade-long debate in phylogenetic systematics, and none of the many discretization methods available (e.g. divergence weighting, step-matrix gap-weighting, implied weighting – Bardin et al., 2014; Gift & Stevens, 1997; Wiens, 2001) have been shown to accurately represent the original distribution of continuous traits (Bardin et al., 2014; Raven & Maidment, 2017; Worthington, 2017). Additionally, the uneven temporal distribution of terrestrial Konservat-Lagerstätten (Eliason et al., 2017) may differentially impact the record of amniote eggs with a thin or absent CL, and statistical approaches that consider probability of preservation could be used to put confidence bounds on estimated ancestral states (Eliason et al., 2017; Marjanović & Laurin, 2008; Marshall, 2019; Wang et al., 2016).

An important motivation behind the original definition of eggshell types is that they also correspond to varying degrees of biomineralization, i.e. calcium content – an important physiological constraint on incubation that has been extensively studied in reptiles (Deeming, 2018a, 2018b; Karlsson & Lilja, 2008; Matos, 2008; Österström et al., 2013; Packard, 1994; Packard & Packard, 1984; Silyn-Roberts & Sharp, 1985; Simkiss, 1961a, 1961b; Stewart & Ecaj, 2010). In that regard, differentiating “hard” and “soft” eggshells could be considered a functional assessment based on consistency of the CL, roughly indicating the amount of mineralized calcium in the shell and providing indirect information on reproductive metabolism (Packard, 1994). However, such a definition is still highly imprecise. Indeed, despite most of the calcium in the shell being concentrated in the CL, crystalline calcified elements can be found in other layers as well, their distribution and concentration being highly variable among reptiles (relative proportions of sulfur and calcium shown in Figure 1; D'Alba et al., 2021; Kohring, 1995; Schleich & Kästle, 1988). This is especially relevant in eggs with a thin, poorly calcified CL (i.e. described as “soft-shelled” in lepidosaurs and turtles), which do not accurately reflect the calcium

content of the whole shell. Furthermore, the lack of preserved proteinaceous layers in most fossil eggshells (Grellet-Tinner, 2005; Grellet-Tinner et al., 2011; Kohring, 1999; Legendre, Rubilar-Rogers, Musser, et al., 2020; Norell et al., 2020; Silyn-Roberts & Sharp, 1989; Stewart, 1997) prevents an accurate estimation of their total mineral content. Recent comparative studies have described the diversity of calcium distribution within extant reptile eggshells, supporting relative calcium proportion and mapping as key proxies to understand their evolution (Campos-Casal et al., 2020; Choi et al., 2018; D'Alba et al., 2021; McClelland et al., 2021). The use of such traits in future studies is likely to improve our understanding of reptile eggshell microstructure beyond the "hard/soft" dichotomy, and shift the focus of descriptions from the sole CL to all eggshell layers.

In light of the lack of precision in definitions of eggshell type, we conclude that none of the three eggshell types traditionally used to characterize reptile eggs can be considered biologically accurate descriptors of their microstructural diversity. The "hard/soft" dichotomy, even with the addition of a third semi-rigid state, is an oversimplification of a much greater variety of eggshell morphologies, and may "reduce a grayscale continuum into black-and-white while ignoring important morphological and, potentially, functional information" (D'Alba et al., 2021, p. 1074). In fact, our ASR of relative CL thickness hints at potential homologous eggshell structures hitherto undescribed among reptiles: the apomorphic thickness increase in eufalconimorphs has been proposed to be linked with specific flight strategies among extant birds, but remains unexplained (Legendre & Clarke, 2021), while the thickness decrease observed in snakes has, to our knowledge, never been previously reported. This undocumented diversity would thus be more adequately described through the use of other traits, preferably not discretized if continuous, and used in combinations that reflect the different eggshell layers and individual structures inside them, including – but not limited to – shell units.

Classic eggshell parataxonomy, while useful in this context to provide morphological descriptions based on such traits, has been repeatedly shown to define ootaxa on the basis of highly homoplastic features (Mikhailov, 2014; Vianey-Liaud & Zelenitsky, 2003; Zelenitsky & Therrien, 2008), which has resulted in the misidentification of many fossil eggs (Choi et al., 2020; Choi, Barta, et al., 2022; Grigorescu, 2017; Varricchio et al., 2015). Therefore, even traits traditionally used to describe the structure of shell units need to be redefined in this context. Many of such traits would likely still be defined as discrete categories associated with a particular reptile clade – e.g. shell units with acicular aragonite crystals, long known to be a defining feature of turtle eggshells, identifiable even in diagenetic fossil specimens in which aragonite is replaced with calcite (Hirsch, 1983, 1996; Mikhailov, 1997b; Moreno-Azanza et al., 2021; Schleich & Kästle, 1988), were recently proposed to be a synapomorphy of Testudines from direct paleontological evidence (Choi, Kim, et al., 2022). To avoid potentially confusing categories based on broad functional characteristics such as traditional eggshell types, the definition of such discrete traits should be based on explicit structural similarities, which should be argued by authors of future studies as hypotheses of primary homology to allow their

subsequent testing in a phylogenetic framework. Similarly, the lack of a particular structure should not necessarily be considered by default as one single character state, but investigated further – including assessment of potential preservational bias for this structure (e.g. fossil eggs with a thin, non-prismatic CL, proposed to be preserved mostly in oxidative or euxinic conditions in terrestrial or shallow water environments – Legendre, Rubilar-Rogers, Musser, et al., 2020; Norell et al., 2020; Wiemann et al., 2018).

Several recent studies have shown the value of continuous eggshell traits (e.g. thickness, calcium content in the shell, crystalline structure of the cuticle, porosity) in studying the correlation of eggshell microstructure with lifestyle parameters (e.g. body mass, clutch size and mass, nest structure, water vapor conductance, geographical distribution, ambient temperature, and humidity), and/or reconstruct its evolution in both avian and non-avian reptiles (Attard & Portugal, 2021; D'Alba et al., 2016, 2017, 2021; Kulshreshtha et al., 2022; Legendre & Clarke, 2021; McClelland et al., 2021). The characterization of additional traits (e.g. pore structure and density, calcium concentration, crystalline structure of shell units, thickness and arrangement of protein fibers in the membrane) will be greatly improved by the systematic use of advanced microscopy and geochemistry techniques to visualize structures of interest with a much greater precision (e.g. X-ray or Raman spectroscopy, EBSD, time-of-flight or inductively coupled mass spectrometry – Alleon et al., 2021; Campos-Casal et al., 2020; Choi et al., 2019; Choi, Kim, et al., 2022; Loewy et al., 2020; Moreno-Azanza et al., 2013; Wiemann et al., 2017; Wiemann & Briggs, 2022). Future use of such atomized single traits in a phylogenetic comparative context is thus expected to provide a much clearer picture of the evolution of eggshell microstructure in reptiles than that of a two- or three-state character (D'Alba et al., 2021; Legendre & Clarke, 2021).

## 5 | CONCLUSIONS

In this review, we have shown that the scoring of soft, hard, and semi-rigid eggshell types shows considerable variation depending on authors, and that this variation can lead to completely different ancestral state reconstructions of eggshell microstructure, even with a difference in scoring for only 1% of the sample. New data are needed on well-preserved eggs of known egg layer identity to resolve the states ancestral to key clades – notably on individual structures within each major eggshell layer used to define traditional eggshell types, which have hitherto been critically overlooked. Such microstructural differences between eggshells, potentially crucial to understand the evolution of reptilian eggs, cannot be assessed with a three-state terminology. Therefore, we also recommend a series of guidelines to account for as many aspects of eggshell microstructure as possible when describing and analyzing new egg specimens:

1. If possible, always sample and illustrate eggshell fragments on several parts of the egg to consider intra-specimen variation, including both mineralized and proteinaceous layers;



2. Describe both macro- and microanatomy of each specimen, and focus on all parts of the eggshell (boundary layer, membrana testacea, CL, and cuticle if present);
3. Always combine several methods of visualization (e.g. scanning electron microscopy, EBSD) and chemical analysis (e.g. Raman spectroscopy, energy-dispersive X-ray spectroscopy, mass spectrometry) to describe eggshell microstructure and composition and measure quantitative traits;
4. If analyzing a sample of eggs from different species, always correct for phylogenetic non-independence (Paradis, 2012; Soul & Wright, 2021) and include eggs from every major clade in your group of interest, including fossils (e.g. a study on Reptilia should always sample avian eggs) – and, if possible, from a wide range of body sizes (e.g. Birchard & Deeming, 2015; Legendre & Clarke, 2021);
5. Review all phylogenetic reconstructions for species in your sample – including fossil egg parataxonomy – as well as tree calibrations; if possible, include several topologies and calibrations to account for phylogenetic and temporal uncertainty (Cusimano & Renner, 2014; Li et al., 2008; Wright et al., 2015);
6. Measure continuous characters (e.g. eggshell thickness, calcium concentration) as such, and include several of them in multivariate quantitative analyses to account for all parts of each structure of interest; for a large number of potentially redundant traits, dimensionality reduction – e.g. principal component analysis – can be performed (Stoddard et al., 2017);
7. If performing ASR, test which evolutionary model fits your trait of interest best (King & Lee, 2015; Litsios & Salamin, 2012; Wilson et al., in press), and use different reconstruction methods to test the replicability and sensitivity to branch length information of each analysis;
8. When defining and analyzing discrete traits, always justify each character state as an explicit hypothesis of homology, using specific terms referring to clearly identified structures; if the use of “soft” and “hard” as descriptive terms is necessary when discussing the results of these analyses, always define precisely what is meant and use those definitions consistently through the discussion;
9. If an evolutionary trend is identified as a result of an ASR, the potential acquisition of a new trait value or state should always be the subject of an explicit hypothesis of homology, which must be carefully discussed in the context of phylogenetic uncertainty and sampling error.

This list of best practices is not exhaustive, as future definitions of eggshell microstructural traits are likely to result in new hypothesized synapomorphies for major reptile clades, which might require additional practical and terminological guidelines beyond the scope of the present study. However, reference to these traits will enable phylogenetic assessment of variables relevant to a synoptic look at the evolution of reproductive biology in deep time.

#### AUTHOR CONTRIBUTIONS

LJL and JAC designed the research; LJL acquired and analyzed the data; LJL and SC drafted the manuscript; all authors revised and approved the final draft of the article.

#### ACKNOWLEDGMENTS

We thank S. Davis, G. Musser, D. Rubilar-Rogers, and A. Vargas for discussion; K. Tanaka and S. Zhang for providing the material for EBSD images of crocodylian and turtle eggshells used in Figure 1; N.-H. Kim for providing schematic drawings of lepidosaur eggshells; M. Fabbri, J. Wiemann, and D. Zelenitsky for providing the original dataset and phylogenetic tree used in Norell et al. (2020); and two anonymous reviewers for their constructive remarks and suggestions. Select taxon silhouettes in Figures 1–4 were modified from files under Public Domain licence from [phylopic.org](http://phylopic.org) (see Supporting Information). This work was supported by a grant to the University of Texas at Austin from the Howard Hughes Medical Institute through the Science Education Program (GT10473) to JAC and LJL, and by a Basic Science Program through the National Research Foundation of Korea (2020R1A6A3A03038316) to SC.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

#### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available on Github at [https://github.com/LucasLegendre/eggshell\\_review](https://github.com/LucasLegendre/eggshell_review).

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## SUPPORTING INFORMATION

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**How to cite this article:** Legendre, L.J., Choi, S. & Clarke, J.A. (2022) The diverse terminology of reptile eggshell microstructure and its effect on phylogenetic comparative analyses. *Journal of Anatomy*, 241, 641–666. Available from: <https://doi.org/10.1111/joa.13723>