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3 **The use of inconsistent terminology for reptile eggshell traits affects the**  
4 **outcome of evolutionary analyses**

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15

16 **Abstract**

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

41

42 **Keywords:** reptile, eggshell, evolution, hard-shelled, soft-shelled, microstructure, Dinosauria,

43 phylogenetic comparative methods

44

## 45 **1) Introduction**

46 The amniotic egg, a major innovation in the evolutionary history of vertebrates, varies in size  
47 and structure associated with specific life history and incubation strategies, which have been  
48 extensively studied in reptiles (Deeming & Ferguson, 1991; Laurin & Reisz, 1997; Reisz, 1997;  
49 Stewart, 1997; Blackburn, 2000a; Shine, 2005; Sander, 2012; Deeming & Ruta, 2014, Deeming  
50 & Reynolds, 2015; Stoddard et al., 2017; Blackburn & Stewart, 2021). Many of these studies  
51 have focused on describing the internal egg organization and embryological development of  
52 extant egg-laying amniote clades – i.e. Monotremata, Lepidosauria, Testudines, Crocodylia,  
53 and Aves (reviews in Blackburn and Stewart, 2021; Starck et al., 2021). The reproductive  
54 strategies of these groups have been studied in the context of their respective radiations, with  
55 some traits identified as major factors of their evolutionary success – e.g. egg shape and size in  
56 birds (Deeming, 2007, 2018a; Dyke & Kaiser, 2010; Deeming & Ruta, 2014; Birchard &  
57 Deeming, 2015; Stoddard et al., 2017; Mytiai, Shatkovska & Ghazali, 2017, 2021; Duursma et  
58 al., 2018; Birkhead et al., 2019; Nagy et al., 2019), or the high flexibility of reproductive traits  
59 in squamates, which facilitated their many independent acquisitions of viviparity (Blackburn,  
60 1993, 2000b, 2006; Shine, 1995, 2005; Andrews & Mathies, 2000; Stewart & Thompson, 2000;  
61 Sites, Reeder & Wiens, 2011; Stewart, 2013, 2015; Pyron & Burbrink, 2014; Griffith et al.,  
62 2015; Blackburn & Stewart, 2021; Whittington et al., 2022).

63 Evolving with diversification of amniote reproductive strategies is the eggshell. The  
64 shells of amniotic eggs all share a similar three-layered structure (Schleich & Kästle, 1988;  
65 Packard & DeMarco, 1991; Mikhailov, 1991a, 1997a, 1997b): the innermost, proteinaceous,  
66 boundary layer; the membrana testacea, also proteinaceous; and the outermost calcareous layer  
67 (CL). However, the inner structure and relative thickness of these layers are highly variable  
68 among extant reptiles (including birds – *sensu* Gauthier, Kluge & Rowe, 1988; Modesto &  
69 Anderson, 2004), but are often classified in two main categories: ‘hard-shelled’ eggs (e.g. all

70 birds and crocodylians, most turtles, most geckos) present a CL organized in discrete calcareous  
71 entities called shell units, which comprise most of the eggshell thickness, while ‘soft-shelled’  
72 eggs (e.g. most squamates, some turtles) have a thin, amorphous CL and a much thicker  
73 membrana testacea (Schleich & Kästle, 1988; Packard & DeMarco, 1991; Mikhailov, 1991a,  
74 1997b). Such eggshell microstructural traits have long been known to be constrained by specific  
75 reproductive strategies, which were originally mostly studied in birds (Simkiss, 1961a; Ar et  
76 al., 1974; Rahn & Ar, 1974).

77         During incubation, the eggshell provides calcium to the embryo and controls water and  
78 gas exchange with its surrounding environment (Paganelli, 1980; Ackerman, Dmi’el & Ar,  
79 1985; Ackerman et al., 1985; Ar & Rahn, 1985; Ar, 1991; Deeming & Thompson, 1991) –  
80 generally through a system of pores, which in birds can present a highly complex structure  
81 (Tyler & Simkiss, 1959; Board, 1982; Grellet-Tinner, Lindsay & Thompson, 2017; Mikhailov  
82 & Zelenkov, 2020). In extant birds and crocodylians, the egg-laying site is a nest or a mound  
83 made from vegetation (Ackerman & Lott, 2004; Mainwaring et al., 2014; Murray, Crother &  
84 Doody, 2020; Hall et al., 2021). Conversely, lepidosaur and turtle eggs are laid either in a tunnel  
85 dug into the ground or above ground with substrate covering (e.g. vegetation, rocks), the latter  
86 being minimal in species that lay ‘hard-shelled’ eggs (Ackerman & Lott, 2004) – although at  
87 least one tortoise species is known to build a nest mound (Kuchling, 1999). Depending on their  
88 location (e.g. on/off the ground) and structure (e.g. open/closed), egg-laying sites present  
89 different conditions of temperature and humidity (Ackerman et al., 1985; Deeming & Ferguson,  
90 1991b; Heenan, 2013). These parameters, along with other evolutionary constraints (e.g.  
91 calcium intake, clutch size – Simkiss, 1961a; Jetz, Sekercioglu & Böhning-Gaese, 2008; Nagy  
92 et al., 2019), have resulted in many specific types of pore and eggshell microstructure among  
93 reptiles (Tyler, 1955; Board, Tullett & Perrott, 1977; Tyler & Fowler, 1978; Board & Scott,  
94 1980; Board, 1982; Packard & DeMarco, 1991; Tanaka, Zelenitsky & Therrien, 2015; Choi et

95 al., 2018), and been the subject of paleobiological hypotheses (Montanari, 2018) – e.g.  
96 regarding nesting strategies in non-avian dinosaurs (Deeming & Unwin, 2004; Varricchio &  
97 Jackson, 2016). For this reason, descriptive traits of eggshell microstructure have been  
98 formalized into a parataxonomical system, with a standardized terminology to classify both  
99 extant and fossil reptile eggshells, regardless of whether the egg layer is known, and study them  
100 in a morphofunctional context (Mikhailov, 1991a, 1997b; Mikhailov, Bray & Hirsch, 1996;  
101 Vianey-Liaud & Zelenitsky, 2003; Khosla & Lucas, 2020).

102         Studies focused on the evolution of such microstructural traits in a phylogenetic context  
103 for all of Reptilia have been lacking. Preservational biases and a neontological/paleontological  
104 decoupling may partially explain the lack of this kind of synoptic study. Soft-shelled eggs are  
105 mostly proteinaceous and extremely rare in the fossil record (Stewart, 1997; Legendre et al.,  
106 2020a; Norell et al., 2020); almost all fossil eggshells consist only in a CL with shell units  
107 (Mikhailov, 1997b). Eggshell parataxonomy, primarily developed to identify isolated fossil  
108 eggs of unknown egg layer, is based on the morphology of these shell units, and cannot be used  
109 to describe eggs that lack them (Schleich & Kästle, 1988; Packard & DeMarco, 1991).  
110 Furthermore, the eggshells of monotremes, the only extant egg-laying amniotes outside reptiles,  
111 have been poorly studied (see next section), preventing inferences of the ancestral eggshell  
112 microstructure in both reptiles and amniotes.

113         The first comparative studies to discuss functional aspects of extant eggshell  
114 microstructure (including comparison between fossil and extant species; Van Straelen, 1928)  
115 were conducted before the formal inclusion of birds in the class Reptilia in the late 1980s  
116 (Gauthier, Kluge & Rowe, 1988; Modesto & Anderson, 2004), resulting in studies that focused  
117 on either birds (Simkiss, 1961a; Ar et al., 1974; Tyler & Fowler, 1978; Paganelli, 1980; Board,  
118 1982; Ar & Rahn, 1985) or non-avian reptiles (Simkiss, 1961b; Packard, Tracy & Roth, 1977;  
119 Packard et al., 1979; Packard & Packard, 1980; Packard, Packard & Boardman, 1982;

120 Ackerman, Dmi'el & Ar, 1985; Ackerman et al., 1985), but never on both groups. In the 1990s  
121 and 2000s, studies of reptile eggshell microstructure focused mostly on taxonomical  
122 descriptions of avian and non-avian dinosaur eggs (e.g. Hirsch & Quinn, 1990; Mikhailov,  
123 1991b, 1997b; Mikhailov, Sabath & Kurzanov, 1994; Khosla & Sahni, 1995; Dauphin, Pickford  
124 & Senut, 1996; Mateus et al., 1997; Vianey-Liaud et al., 1997; Chiappe et al., 1998; López-  
125 Martínez, Canudo & Cuenca-Bescós, 1999; Codrea et al., 2002; Varricchio, Horner & Jackson,  
126 2002; Jackson, Schweitzer & Schmitt, 2002; Jackson & Varricchio, 2003; Vianey-Liaud &  
127 Garcia, 2003; Vianey-Liaud, Khosla & Garcia, 2003; Varricchio & Jackson, 2004; Zelenitsky,  
128 Modesto & Currie, 2002; Zelenitsky & Modesto, 2003; Grellet-Tinner, 2006; Grellet-Tinner et  
129 al., 2006; Jackson et al., 2008; Liang et al., 2009). Apart from a handful of studies on other  
130 reptile species (Hirsch, 1996; Qualls, 1996; Mathies & Andrews, 2000; Heulin et al., 2002;  
131 Osborne & Thompson, 2005; Phillott & Parmenter, 2006), the study of non-dinosaurian reptile  
132 eggshell microstructure was relatively quiescent for two decades, and focused on descriptions  
133 with a small sample size that rarely involved a dialogue between functional study and  
134 comparative work.

135         Phylogenetic comparative approaches have more recently allowed researchers to  
136 perform statistical analyses of quantitative eggshell traits and test specific hypotheses about  
137 their evolution (Mikhailov, 1991b; Varricchio & Jackson, 2004; Grellet-Tinner, 2006; Grellet-  
138 Tinner et al., 2006; Winkler, 2006; Zelenitsky & Therrien, 2008; Tanaka et al., 2011a, 2020;  
139 Araújo et al., 2013; Varricchio & Barta, 2015; Vila, Sellés & Beetschen, 2017; Choi & Lee,  
140 2019). Studies have tested correlations of continuous traits (e.g. eggshell thickness, porosity,  
141 calcium content, water vapor conductance) with life history traits in birds (Birchard &  
142 Deeming, 2009, 2015; Portugal et al., 2014; Attard & Portugal, 2021; McClelland et al., 2021),  
143 dinosaurs including birds (Tanaka, Zelenitsky & Therrien, 2015; Legendre & Clarke, 2021),  
144 archosaurs (Tanaka & Zelenitsky, 2014), squamates (Hallmann & Griebeler, 2015), non-avian

145 reptiles (D’Alba et al., 2021), or across amniotes, albeit with relatively small samples (Stein et  
146 al., 2019; Legendre et al., 2020a). Some of these studies have also reconstructed ancestral states  
147 for these traits – discretized in some cases – and identified correlates that influenced these  
148 evolutionary patterns (Portugal et al., 2014; Tanaka, Zelenitsky & Therrien, 2015; Stein et al.,  
149 2019; Legendre et al., 2020a; Norell et al., 2020; Attard & Portugal, 2021; D’Alba et al., 2021;  
150 Legendre & Clarke, 2021; McClelland et al., 2021). These studies have initiated what is perhaps  
151 the most interesting and controversial debate in this new field of quantitative eggshell research  
152 (Lindgren & Kear, 2020): what was the structure of the ancestral eggshell in dinosaurs,  
153 archosaurs, and reptiles as a whole? How do we assess potential biases in the preservation of  
154 fossil eggs and extant microstructure and function?

155         Recent descriptions of exceptionally-preserved fossil soft-shelled eggs assigned to early  
156 diapsids (Hou et al., 2010), pterosaurs (Unwin & Deeming, 2008; Grellet-Tinner et al., 2014;  
157 Wang et al., 2014, 2017), non-avian dinosaurs (Stein et al., 2019; Norell et al., 2020) and,  
158 possibly, marine reptiles (Legendre et al., 2020a), have led studies to investigate via ancestral  
159 state reconstructions (ASR) the binary trait ‘soft-shelled/hard-shelled’ (sometimes with a third  
160 intermediate state, referred to as a semi-rigid eggshell) on the phylogenetic tree of Reptilia. One  
161 study recovered the first dinosaur and archosaur egg as soft-shelled (Norell et al., 2020) and  
162 another with both as hard-shelled (Legendre et al., 2020a), which was until then the scientific  
163 consensus. These conflicting conclusions were proposed to be due to differences in taxonomic  
164 sampling and ASR methods (Legendre et al., 2020b), but also due to differences in their  
165 respective definitions of what makes an eggshell soft or hard. Indeed, while this dichotomy has  
166 been used to describe eggshells for over a century (Cunningham, 1922), many definitions have  
167 been used by various authors over the past five decades to refer to ‘hard’ and ‘soft’ eggshells  
168 (or synonyms of those) without ever reaching a formal consensus for any of them.

169 In this review, we describe the criteria used in the literature to define the terms ‘soft-  
170 shelled’ and ‘hard-shelled’ for amniote eggs (including monotremes) and the range of eggshell  
171 phenotypes in extant taxa. Then, using a dataset combining those of the two recent studies that  
172 recovered the ancestral archosaur egg as soft-shelled or hard-shelled, respectively (Norell et al.,  
173 2020; Legendre et al., 2020a), we reconstruct evolutionary patterns for a ‘soft/semi-rigid/hard’  
174 discrete trait and an eggshell thickness continuous trait on an updated calibrated phylogeny.  
175 Finally, we discuss how sampling, definitions of eggshell type, and tree calibration influence  
176 the reconstruction of evolutionary patterns for reptilian eggshells, and propose new guidelines  
177 to clarify future analyses and discussion on this topic in a phylogenetic context.

178

## 179 **2) Definitions of hard and soft eggshells among extant amniotes**

### 180 A) Criteria used to define eggshell type

181 The first attempt to formalize distinct categories for reptile eggshells using a sample of several  
182 reptile groups and advanced microscopy techniques was made in a series of papers by Mary  
183 and Gary Packard and colleagues in the late 1970s and early 1980s (Packard, Tracy & Roth,  
184 1977; Packard et al., 1979; Packard & Packard, 1980; Packard, Packard & Boardman, 1982).  
185 Three eggshell types in non-avian reptiles, best summarized in Packard, Packard & Boardman  
186 (1982, p. 138–142), were defined:

187

188 “In summary, reptilian eggs can be divided roughly into three groups based on the  
189 structure of their shells: flexible-shelled eggs with little or no calcareous layer (most  
190 squamates), flexible-shelled eggs with a thick, well-developed calcareous layer  
191 (some chelonians), and rigid-shelled eggs with a well-developed calcareous layer  
192 (crocodilians, some chelonians, and a few squamates). We recognize that grouping  
193 of eggs on the basis of similarities in structure of eggshells is somewhat artificial.

194           Nonetheless, structure of eggshells places certain constraints on the capacity of eggs  
195           to respond to variation in the hydric environment, and these constraints transcend  
196           taxonomic boundaries.”

197

198           These categories were, as explicitly stated here, defined purely in the context of functional  
199           constraints, namely water conductance of the eggshell during incubation (Packard et al., 1979;  
200           Packard & Packard, 1980; Packard, Packard & Boardman, 1982; Ackerman, Dmi’el & Ar,  
201           1985; Ackerman et al., 1985). Eggs in the first category, found in most squamates, have the  
202           most porous shell out of the three, also referred to as parchment-like, and more commonly as  
203           soft-shelled later on (Ackerman, Dmi’el & Ar, 1985; Schleich & Kästle, 1988; Packard &  
204           DeMarco, 1991). These squamate eggs have a very low mineral content (15–30%, versus over  
205           90% in other reptile eggs – Packard, Packard & Boardman, 1982; Schleich & Kästle, 1988;  
206           Andrews, 2017), and thus a high water conductance (about one order of magnitude higher than  
207           in other non-avian reptile eggs, and two orders of magnitude higher than in avian eggs –  
208           Ackerman, Dmi’el & Ar, 1985). As a result, incubation of soft-shelled squamate eggs (i.e., in  
209           oviparous taxa) requires them to be at least partially buried in a dense substrate with high  
210           humidity to allow continuous water intake, while avian eggs, which have the thickest and most  
211           mineralized (i.e. ‘hard’) shells among reptiles, are incubated in much lower temperature and  
212           humidity conditions, and lose water in the process (Ar & Rahn, 1985; Deeming & Thompson,  
213           1991; Thompson & Speake, 2004; Deeming, 2006; Tanaka, Zelenitsky & Therrien, 2015).  
214           Note: the term ‘soft-shelled’ is sometimes also used to refer to avian eggs with poorly calcified  
215           shells due to developmental anomalies (e.g. Simkiss, 1961a).

216           Eggs of other reptile groups have been categorized in this functional context as either  
217           hard-shelled or soft-shelled based on their similarity to either ‘soft’ squamate eggs or avian  
218           eggs, respectively (Figures 1, 2; Belinsky et al., 2004). However, since these studies did not

219 discuss evolutionary hypotheses regarding the emergence of any of these eggshell types, no  
220 explicit hypothesis of primary homology (*sensu* de Pinna, 1991) for ‘soft-shelled’ or ‘hard-  
221 shelled’ as character states was ever defined in a phylogenetic context, thus preventing any  
222 secondary homology for either character state to be clearly supported (or unsupported) in the  
223 Reptilia tree. Similarly, the third, intermediate category defined by Packard, Packard &  
224 Boardman (1982) for some turtle eggs, often referred to as ‘semi-rigid’, or ‘flexible’/‘pliable’  
225 with a mention of shell units being present (Packard et al., 1979; Ackerman, 1980; Packard,  
226 Packard & Boardman, 1982; Ackerman, Dmi’el & Ar, 1985; Ackerman et al., 1985; Deeming  
227 & Thompson, 1991; Packard, 1999; Booth, 2002; Lawver & Jackson, 2014), was never  
228 formally defined as a structural category, and is never discussed as such in recent comparative  
229 studies (e.g. Legendre et al., 2020a; Norell et al., 2020). This is likely due to the fact that most  
230 studies of reptile eggshells did not sample turtle species with semi-rigid eggs, instead focusing  
231 primarily on either birds or squamates – by far the two most species-rich reptile clades  
232 (Barrowclough et al., 2016; Uetz et al., 2021). Thus, despite the explicit admission by Packard,  
233 Packard & Boardman (1982, p. 142) that these three categories were “somewhat artificial”  
234 beyond a difference in water exchange, subsequent literature has often presented these two, or  
235 three categories, as well-defined character states based solely on structural differences, without  
236 investigating their biological relevance or potential homology.

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

244 on top of it (Nys et al., 1999, 2004; Hincke, 2012; Gautron et al., 2021). On the outer surface  
245 of the membrana testacea, calcite (or aragonite in turtle eggshells) crystals deposit around  
246 nucleation centers and form the mammillary layer, i.e. the lowermost, cone-shaped part of shell  
247 units, called mammillae (Mikhailov, 1991a, 1997b; Hincke, 2012). On top of that layer grows  
248 the column-shaped continuous layer – also referred to as the palisade layer in poultry literature  
249 (Gautron et al., 2021), or prismatic layer by paleontologists (Montanari, 2018). This layer,  
250 which can itself comprise several sublayers, constitutes the prismatic-shaped part of shell units,  
251 and represents most of the shell thickness in all hard-shelled eggs (Packard & DeMarco, 1991;  
252 Mikhailov, 1997b; Grellet-Tinner, 2006). In most birds, some turtles, and some lepidosaurs, the  
253 eggshell presents a cuticle, i.e. a thin outer layer on top of shell units, composed of highly  
254 variable relative proportions of calcite, phosphates, proteins, lipids, and polysaccharides  
255 (Mikhailov, 1997a; Kusuda et al., 2013; Samiullah & Roberts, 2014; D’Alba et al., 2017;  
256 review in Kulshreshtha et al., 2022); the cuticle of bird eggs has been proposed as non-  
257 homologous to that of turtles and lepidosaurs, which remains to be tested (Kulshreshtha et al.,  
258 2022). While the inner organization and structure of crystals in a shell unit are highly variable  
259 and used to define major groups in eggshell parataxonomy (Mikhailov, 1991a, 1997b), this  
260 model of outward crystal growth generally follows the same pattern in the eggs of birds,  
261 crocodylians, and turtles (Silyn-Roberts & Sharp, 1986; Packard & DeMarco, 1991; Moreno-  
262 Azanza et al., 2014; Marzola, Russo & Mateus, 2015; Choi et al., 2022b), seemingly supporting  
263 their grouping as hard-shelled. Conversely, in soft-shelled squamate eggs, the ontogenetic  
264 sequence of eggshell formation has been poorly studied, but a general model, distinct from that  
265 of other reptile eggs, has been proposed. After proteinaceous membranes are formed,  
266 crystallization of the thin CL follows the intricate pattern of the outer fibers of the membrana  
267 testacea, suggested to be an additional proteinaceous layer unique to squamates (Packard &  
268 DeMarco, 1991); that model, however, remains to be tested.

269 In general, for both hard-shelled and soft-shelled eggs, the sequence of eggshell  
270 formation has only been described in a limited number of species, especially among non-avian  
271 reptiles, for which only a small proportion of extant species in each major clade has been studied  
272 (Crocodylia: 10 species out of 26 [Marzola, Russo & Mateus, 2015; Uetz et al., 2021];  
273 Lepidosauria: <100 out of >10000 [Packard & DeMarco, 1991; Osborne & Thompson, 2005;  
274 Choi et al., 2018; Uetz et al., 2021]; Testudines: <50 out of 356 [Packard & DeMarco, 1991;  
275 Phillott & Parmenter, 2006; Turtle Taxonomy Working Group, 2017]). Furthermore, many  
276 aspects of shell formation, such as the deposition of a CL without nucleation centers in soft-  
277 shelled eggs (Packard & DeMarco, 1991), remain undescribed even in many of the studied taxa,  
278 and have not been reassessed since the early 1990s.

279 Similarly, the genes involved in the formation of proteins that form the membrana  
280 testacea, which could also help define homologous categories of reptile eggshells, have not  
281 been well characterized. A few proteins have been identified in the eggshell membranes of the  
282 turtle *Pelodiscus sinensis* (Lakshminarayanan et al., 2005) and squamates *Thamnophis sirtalis*  
283 (Hoffman, 1970) and *Salvator merianae* (Campos-Casal et al., 2020). The shell membranes of  
284 two squamates (*Naja atra* and *Salvator merianae*) have been described as intricate random  
285 arrangements of keratin and collagen fibers, conferring them exceptional deformability and  
286 resistance compared to that of the turtle *Mauremys sinensis*, which shows parallel keratin fibers  
287 (Chang & Chen, 2016; Álvarez et al., 2022). Soft lepidosaur eggshells have been shown to  
288 exhibit significant higher amounts of proline than the hard eggshells of birds and geckos, which  
289 might increase their water retention (Cox, Mecham & Sexton, 1982; Sexton et al., 2005). The  
290 potential role of such proteins, however, has only been studied in birds – mostly domestic  
291 chickens in the context of poultry research (Gautron et al., 2021). Over 900 proteins of the  
292 chicken eggshell membrane and over 100 genes encoding for or regulating their expression in  
293 the oviduct have been identified (Du et al., 2015), and some of them have been shown to

294 regulate the size and orientation of calcite crystals in the CL (Dunn et al., 2012; Gautron et al.,  
295 2021). However, their respective roles in eggshell formation and structure, as well as variations  
296 of these among reptile groups, remain poorly known (Hincke, Nys & Gautron, 2010; Hincke,  
297 2012; Du et al., 2015; Gautron et al., 2021).

298 Other morphological traits could also be relevant in this context, such as the structure  
299 of the oviduct, highly variable and well-studied among reptile clades (Blackburn, 1998; Girling,  
300 2002). The specialized oviduct of birds and crocodylians, each region of which produces a  
301 different part of the eggshell sequentially during ontogeny, has been proposed as a  
302 synapomorphy of archosaurs (Palmer & Guillette, 1992). Similarly, the development of  
303 proteinaceous eggshell layers has been described in a few turtle and squamate species  
304 (Hoffman, 1970; Arrieta et al., 2021), sometimes in relation with the structure and function of  
305 the uterine shell glands that produce them (Aitken & Solomon, 1976; Palmer & Guillette, 1988;  
306 Guillette, Fox & Palmer, 1989; Palmer, DeMarco & Guillette, 1993; Corso, Delitala &  
307 Carcupino, 2000; Stewart et al., 2010). In all cases, however, no specific traits of oviductal  
308 morphology have been clearly linked to a given eggshell type. Overall, much more work is thus  
309 needed to identify ontogenetic, morphological, and molecular characters that could potentially  
310 help deciphering the biological reality – or lack thereof – of soft, semi-rigid, and hard eggshells.

311

#### 312 B) Structural variations and inconsistencies in the definitions of hard and soft eggshells

313 In addition to the lack of information on potential ontogenetic and/or genetic differences among  
314 eggshells in reptile taxa, structural differences among eggshells described as hard or soft  
315 prevent these categories from being defined as primary homologies. Here we provide a brief  
316 description of such inconsistencies in four major amniote groups: Crocodylia, Testudines,  
317 Lepidosauria, and Monotremata. The structural characteristics of avian eggshells are not  
318 reviewed here, as they have been extensively described in large-scale comparative studies,

319 some of which in the context of nesting ecology and/or dinosaurian evolution (Tyler & Geake,  
320 1953; Tyler, 1955; Tyler & Simkiss, 1958; Rahn, Ar & Paganelli, 1979; Board, 1982; Ar &  
321 Rahn, 1985; Board & Sparks, 1991; Mikhailov, 1991b, 1997a, 2014; Grellet-Tinner & Chiappe,  
322 2004; Deeming, 2006; Grellet-Tinner et al., 2006; Zelenitsky & Therrien, 2008; Hechenleitner,  
323 Grellet-Tinner & Fiorelli, 2015; D’Alba et al., 2016).

324

325 *i) Crocodylia*

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

343 Crocodilian shell units present a highly stratified continuous layer with peculiar  
344 ornamentation, previously referred to as lacunae, ‘book-like’ structures, or intracascara  
345 (Schleich & Kästle, 1988; Moreno-Azanza et al., 2014; Piazza et al., 2021). In Alligatoridae,  
346 these lacunae have been shown to facilitate gas exchange and hatching (Ferguson, 1982), and  
347 increase embryo survival during nest inundation (Cedillo-Leal et al., 2017). In alligators,  
348 lacunae are formed through outer erosion of the eggshell by acidic secretions from the decaying  
349 vegetation of the nest (Ferguson, 1982). Other authors have hypothesized that erosion might  
350 also take place in the lower oviduct (Schleich & Kästle, 1988), or that such structures can also  
351 form through local inhibition of calcium deposition (Piazza et al., 2021), but neither of the latter  
352 two hypotheses have been tested experimentally, nor on a larger sample of crocodilians.  
353 Differences from avian eggshell and variation within crocodilians in microstructure and  
354 function problematize the use of one composite discrete character state ‘hard-shelled’ for all  
355 extant archosaurs.

356

357 *ii) Testudines*

358 Turtle eggs, the diversity of which prompted the definition of a semi-rigid eggshell type, differ  
359 markedly from avian and crocodilian eggs (see Figure 1). All turtle eggshells have a CL made  
360 of aragonite instead of calcite (Packard et al., 1979; Packard, Packard & Boardman, 1982;  
361 Schleich & Kästle, 1988; Packard & DeMarco, 1991) – although some turtles species can  
362 occasionally produce calcite shells and some squamates aragonitic shells (Baird & Solomon,  
363 1979; Al-Bahry et al., 2009; Guo et al., 2021), which might be due to hitherto unaccounted  
364 factors associated with captivity (Packard & DeMarco, 1991).

365 The CL consists in ‘fan-shaped’ shell units with a spherulitic arrangement of acicular  
366 aragonite crystals (Figure 1; Hirsch, 1983; Silyn-Roberts & Sharp, 1985; Lawver & Jackson,  
367 2016), resulting in large spaces between their bases, distinct from those found in archosaurs

368 (Schleich & Kästle, 1988). This shell unit morphology, unique to turtle eggs, has allowed  
369 paleontologists to assign many fossil eggshells to Testudines (Hirsch, 1983; Schleich & Kästle,  
370 1988; Mikhailov, 1997b; Lawver & Jackson, 2014; Moreno-Azanza et al., 2021; Xu et al.,  
371 2022; but see Ke et al., 2021, for a fossil turtle egg with an unusual shell microstructure), with  
372 an earliest record in the Late Jurassic (Kohring, 1990) and an origin of aragonitic shells in the  
373 Late Cretaceous (Lawver & Jackson, 2017; Choi et al., 2022b; Xu et al., 2022). The relative  
374 shape and size of turtle egg shell units compared to the thickness of the underlying membrana  
375 testacea varies significantly (Lawver & Jackson, 2014; Cadena et al., 2019), from thick and  
376 columnar in most terrestrial turtles to reduced and as thin as the membrane in some freshwater  
377 turtles (Hirsch, 1983; Packard et al., 1979; Packard & Packard, 1988, 1980; Schleich & Kästle,  
378 1988).

379         The eggs of sea turtles (Chelonioidea) present an extreme reduction of the CL, much  
380 thinner than the underlying membrane and so poorly crystallized that shell units cannot be  
381 distinguished from each other (Hirsch, 1983; Schleich & Kästle, 1988; Sahoo et al., 1996;  
382 Phillott & Parmenter, 2006; Sahoo, Mohapatra & Dutta, 2009), similar to soft squamate  
383 eggshells (although the eggs of a fossil sea turtle were recently described as ‘rigid’ – Cadena et  
384 al., 2019). This has resulted in turtle eggshells being discretized as soft (sea turtles), semi-rigid  
385 (some freshwater turtles), or hard (all other species), based on visual assessment of relative  
386 thickness of shell units compared to that of the membrane (Tracy, Packard & Packard, 1978;  
387 Packard et al., 1979; Ackerman, 1980; Packard & Packard, 1980; Packard, Packard &  
388 Boardman, 1982; Hirsch, 1983; Ackerman, Dmi’el & Ar, 1985; Ackerman et al., 1985;  
389 Deeming & Ferguson, 1991b).

390         More recently, however, Kusuda et al. (2013) proposed a classification of turtle  
391 eggshells into six different types, based on the number of sublayers within their shell units  
392 (except type I, found only in species *Pelusios sinuatus*, defined as a complete lack of shell

393 units). The authors argue that their classification matches that of Packard et al., with type I  
394 corresponding to soft shells, type II and III to semi-rigid shells, and type IV to VI to hard shells  
395 (Kusuda et al., 2013). This shows that the initial definition of turtle eggshell type as a three-  
396 state character ignored part of the variation present within eggshell layers, and was overly  
397 simplistic. Furthermore, five out of these six new eggshell types (the sixth one being  
398 autapomorphic) have appeared multiple times in the phylogeny of Testudines and do not  
399 constitute homologous character states (Kusuda et al., 2013), as previously described for other  
400 turtle egg traits (Winkler, 2006), which calls for a more cautious interpretation of turtle eggshell  
401 classifications. Several criteria used to define the new eggshell types might be better  
402 characterized as continuous traits (i.e. type IV is described as identical to type III, but with  
403 “shell units [...] tightly packed with each other” – Kusuda et al., 2013, p. 372), as inferred from  
404 the influence of eggshell thickness and calcification on their variation (Deeming, 2018b).  
405 Future examination of turtle eggshells using more advanced microscopy techniques, e.g.  
406 electron backscatter diffraction (EBSD; Moreno-Azanza et al., 2013; Choi, Han & Lee, 2019),  
407 will likely identify more subcategories inside each of these eggshell types.

408

### 409 *iii) Lepidosauria*

410 Most lepidosaur species were defined by Packard, Packard & Boardman (1982) as soft-shelled  
411 egg layers. However, significant variation of their shell structure problematizes assignment of  
412 eggs in this clade to only one or two functional categories. The best known lepidosaur clade  
413 with a highly distinct eggshell structure is geckos (order Gekkota). Families Gekkonidae,  
414 Phyllodactylidae, and Sphaerodactylidae, representing ~90% of the ~2000 species of geckos  
415 (Uetz et al., 2021), lay eggs that have long been characterized as hard-shelled based on their  
416 CL formed of thick prismatic structures, referred to as ‘shell units’ (Figure 1; Schleich & Kästle,  
417 1988; Packard & DeMarco, 1991; Mikhailov, 1991a, 1997b; Andrews, 2012, 2017; Pike,

418 Andrews & Du, 2012; Choi et al., 2018). Other gecko families (Carphodactylidae,  
419 Diplodactylidae, Eublepharidae, and Pygopodidae) lay eggs with thinner ‘nail-shaped’  
420 structures, described as soft-shelled or semi-rigid depending on the authors (Figure 2; Pike,  
421 Andrews & Du, 2012; Choi et al., 2018). However, among Diplodactylidae, species in the genus  
422 *Eurydactylodes* lay eggs with shell units (Kratochvíl & Frynta, 2006), meaning eggshells with  
423 shell units must have evolved independently at least twice in geckos.

424         Although most gecko eggs are described as hard-shelled based on presence of shell  
425 units, these shell units are not homologous to those found in Archelosauria (*sensu* Crawford et  
426 al., 2015) and differ from them in structure (Figure 1; Choi et al., 2018). EBSD mapping of  
427 archelosaur eggshells shows that their biomineralization starts from the external margin of the  
428 membrana testacea and expands outward (Grellet-Tinner et al., 2011; Moreno-Azanza et al.,  
429 2013; Choi, Han & Lee, 2019; Choi et al., 2022b). Conversely, gecko eggshells with shell units  
430 show an opposite crystallographic configuration – shell units likely grows inward from their  
431 outer surface (see inversed color pattern between eggshells of geckos and other reptiles in  
432 Figure 1; Choi et al., 2018). The CL of several geckos eggs with no shell units (e.g.  
433 Diplodactylidae, Eublepharidae) also shares this growth pattern (Choi et al., 2018), as does the  
434 eggshell of a Cretaceous fossil anguimorph lizard (Fernandez et al., 2015), suggesting this  
435 inward mineralization pattern is a synapomorphy of squamates.

436         Interestingly, the eggshell of the tuatara (*Sphenodon punctatus*), sister taxon to all other  
437 extant lepidosaurs, presents a CL composed of stem-like structures embedded in the  
438 proteinaceous shell membrane, each covered by a cap-like structure on its outer surface  
439 (Packard, Hirsch & Meyer-Rochow, 1982; Packard et al., 1988; Cree, Guillette & Reader,  
440 1996). Such a structure, often described as ‘semi-rigid’ (Packard et al., 1988), is highly similar  
441 to the ‘nail-shaped’ structures found in gecko eggs with no shell units (Choi et al., 2018). This  
442 suggests that these geckos and the tuatara may share a homologous CL organization with ‘nail-

443 shaped' elements and inward mineralization. This organization, which may have given rise to  
444 the shell units seen in other gecko eggs, is distinct from that of Archelosauria and potentially  
445 ancestral to lepidosaurs (Choi et al., 2018). However, confirming that hypothesis would require  
446 studying the eggshells of Dibamidae, a poorly known clade of legless lizards recovered by  
447 several studies as sister group to all other squamates (Pyron, Burbrink & Wiens, 2013; Tonini  
448 et al., 2016). The first description of an egg from the dibamid *Dibamus novaeguineae*  
449 mentioned its "brittle and highly calcareous shell" (Boulenger, 1912, p. 100), without providing  
450 any illustrations or further details. This short description was cited as evidence for the whole  
451 group to likely be classified as 'hard-shelled' (Packard, Packard & Boardman, 1982). Other  
452 eggs from the same dibamid species were later described as having a "thin, flexible shell", the  
453 difference from the shell of the first described egg being attributed to either polymorphism or  
454 specimen degradation (Greer, 1985, p. 140). Since none of these studies investigated dibamid  
455 eggshells using microscopy, their eggshell microstructure remains unknown. Thus, despite the  
456 vast majority of squamate eggs being traditionally described as 'soft-shelled', the potential  
457 homology of eggshell microstructural traits within and outside Lepidosauria remains elusive.

458         Additionally, even in squamate eggs with no shell units, variation has been recorded in  
459 all layers of the eggshell, but has rarely been the subject of classifications analogous to those  
460 of shell units in other reptile eggs. This includes the outer ornamentation of the CL, which can  
461 include grooves and cracks, calcite granules or rosettes, etc., the size and shape of which appear  
462 to be unique to specific clades (Packard et al., 1982; Packard, Packard & Boardman, 1982;  
463 Schleich & Kästle, 1988; Packard et al., 1991; Packard & DeMarco, 1991; Osborne &  
464 Thompson, 2005; Arrieta et al., 2021); bundles of protein fibers in the membrana testacea, often  
465 arranged in highly specific and complex patterns (Packard et al., 1982; Schleich & Kästle, 1988;  
466 Osborne & Thompson, 2005; Legendre et al., 2020a); and mineralized nodules in the membrana  
467 testacea, highly variable in shape and size but almost never reported in formal descriptions

468 (Schleich & Kästle, 1988). Such structures, as well as their location on or within the shell, have  
469 been categorized and discussed in the context of calcification patterns and shell porosity  
470 (Schleich & Kästle, 1988; Kohring, 1995; D’Alba et al., 2021), although such classifications  
471 remain to be tested in a phylogenetic context. Many species also present pore-like structures,  
472 the structure and function of which has never been assessed due to their ‘simple’ aspect  
473 compared to the well-defined pores of hard-shelled eggs (Schleich & Kästle, 1988; Packard &  
474 DeMarco, 1991; Osborne & Thompson, 2005; Arrieta et al., 2021). The CL in some species  
475 has also been reported to be completely absent, or made of hydroxyapatite instead of calcite  
476 (Schleich & Kästle, 1988). These anomalies have been attributed to either human error or  
477 abnormalities in captive specimens, but never subjected to detailed study (Packard & DeMarco,  
478 1991). However, the recent description of hydroxyapatite as the only mineral present in  
479 eggshells of a large sample of wild black and white tegus (*Salvator merianae*) suggests that the  
480 outer mineralized layer of reptile eggshells is indeed not always calcareous (Campos-Casal et  
481 al., 2020).

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

490

491 *iv) Monotremata*

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

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

512

### 513 **3) Ancestral reconstructions of eggshell type and calcareous layer thickness in reptiles**

#### 514 A) Material and methods

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

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

526 A) Shell unit scoring: based on presence ('hard') or absence ('soft') of prismatic shell  
527 units in the CL of the eggshell (Schleich & Kästle, 1988; Packard & DeMarco, 1991; Legendre  
528 et al., 2020a). The 'nail-shaped' structures in the eggshells of the tuatara and some geckos,  
529 distinct from prismatic shell units, are coded as semi-rigid;

530 B) Ratio scoring: a discretization of the continuous ratio 'CL thickness/total eggshell  
531 thickness':  $r \leq 0.5$ : 'soft';  $0.5 < r \leq 0.67$ : 'semi-rigid';  $r > 0.67$ : 'hard' (Hirsch, 1983; Norell et  
532 al., 2020; alluded to in Packard, Packard & Boardman, 1982, with no threshold values  
533 provided). This discretization is often scored through simple visual assessment rather than  
534 compiling the actual ratio, since for most reptile eggshells the CL is either much thicker or  
535 much thinner than the underlying membranes (Packard, Packard & Boardman, 1982; Hirsch,  
536 1983). Furthermore, compilation of the ratio requires proteinaceous shell membranes to be  
537 present in the sampled eggshell and included in thickness measurements. This is not the case

538 for most eggshell studies – either because the specimen is a fossil egg in which the shell  
539 membrane was not preserved, or because the study focused only on eggs for which the CL  
540 represents most of the total thickness and is considered equivalent to it (e.g. in birds), so that  
541 only one measurement is included and the eggs are considered hard-shelled. For this study, we  
542 only measured the ratio to score eggs for which it was visually ambiguous – i.e. the value of  $r$   
543 was not conspicuously inferior to 0.5 or superior to 0.67 (Supporting Information).

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

554 Only 9 species (two non-avian dinosaurs and seven turtles) out of 208 were scored  
555 differently depending on scoring procedure, seemingly showing a general congruence between  
556 the three main ways to define eggshell type – general description (new scoring), discretized  
557 ratio (ratio scoring), and presence/absence of shell units (shell unit scoring). This, however, is  
558 due to the fact that most reptile eggshells present either very thick shell units and a very thin  
559 membrana testacea, or no shell units and a very thick membrana testacea (Schleich & Kästle,  
560 1988; Packard & DeMarco, 1991). These two opposite ends of the spectrum for all three scoring  
561 systems result in similar character states between them, which does not imply that the  
562 underlying definitions of these states are equivalent between scoring systems.

563 All three scoring procedures present potential issues linked with how much of the  
564 original eggshell structural variation they can consider. None of them, for example, includes  
565 information relative to the different types of shell units, which in the case of geckos are known  
566 not to be homologous to those of other reptiles. The new scoring and shell unit scoring neglect  
567 the shell membrane entirely (its thickness and its structure), meaning both eggs without a CL  
568 and eggs with a CL that does not have shell units are coded as ‘soft-shelled’, resulting in  
569 information loss. The ratio scoring also does not considering inner structure of the shell  
570 membrane, but it does consider its thickness in a discretized form. However, this scoring, as  
571 described by Norell et al. (2020), despite being based on previous literature, does not  
572 incorporate any empirical data that would justify the threshold values used to discretize it (see  
573 Legendre et al., 2020b). Additionally, for all scorings, structures that are demonstrably not  
574 homologous to each other are both coded as ‘semi-rigid’ (see previous section), meaning that  
575 the recovery of any clade as ancestrally ‘semi-rigid’ would provide no clear indication of which  
576 eggshell traits were present at that node. For all these reasons, the three scoring systems are  
577 here put to a test of secondary homology through ASR, in order to decipher what biological  
578 information can potentially be inferred from them in a phylogenetic context.

579 We also compiled continuous trait data for CL thickness in  $\mu\text{m}$ . For the continuous  
580 measurement of eggshell thickness, we used CL thickness as a proxy due to the impossibility  
581 of measuring total eggshell thickness in ootaxa that lack a shell membrane. This allowed us to  
582 include total thickness as an equivalent measurement for eggs traditionally considered hard-  
583 shelled, in which only CL thickness is usually reported (i.e. crocodylians, most dinosaurs  
584 including all birds), but not for those traditionally considered semi-rigid or soft-shelled (turtles,  
585 lepidosaurs, pterosaurs, choristoderes). For this reason, we only included taxa in the latter  
586 groups if a measurement of CL thickness was available in its original description. Similarly,  
587 since the significant correlation of eggshell thickness with egg mass influences results of ASR

588 for this trait (Stein et al., 2019; Legendre et al., 2020a; Legendre & Clarke, 2021), we performed  
589 ASR of CL thickness both as an absolute (i.e. as itself, in  $\mu\text{m}$ ) and relative (i.e. as a ratio ‘CL  
590 thickness/egg mass’, in  $\mu\text{m g}^{-1}$ ) trait. For this reason, we only sampled complete eggs for which  
591 egg mass could be estimated from egg length and width, so that all ASR in this study were  
592 performed using the same sample and topology. Original measurements were primarily  
593 collected from the Norell et al. (2020) and Legendre et al. (2020a) datasets ( $n = 92$  and  $n = 148$ ,  
594 respectively; see detail in Supporting Information). The calibrated phylogeny used in all  
595 analyses was based on the tree from Legendre & Clarke (2021), with additional references for  
596 crocodylians, pterosaurs, turtles, and lepidosaurs listed in the Supporting Information.

597 All analyses were performed in R v 4.1.2 (R Core Team, 2022). Continuous traits were  
598 log-transformed prior to analysis (Sokal & Rohlf, 1995). ASR for eggshell type (discrete trait)  
599 were performed in phytools (Revell, 2012, 2013) using Bayesian stochastic character mapping  
600 (SIMMAP – Huelsenbeck, Nielsen & Bollback, 2003; Bollback, 2006). SIMMAP is based on  
601 Bayesian posterior sampling of stochastic character maps using Monte Carlo Markov Chain  
602 (MCMC), which can account for branch length information, rate heterogeneity, and  
603 phylogenetic uncertainty (Huelsenbeck, Nielsen & Bollback, 2003; Bollback, 2006), unlike  
604 maximum parsimony and maximum likelihood methods for discrete traits (Cunningham,  
605 Omland & Oakley, 1998; Huelsenbeck, Nielsen & Bollback, 2003; O’Meara, 2012; Joy et al.,  
606 2016). For each ASR using SIMMAP, we estimated Akaike weights (Burnham & Anderson,  
607 2002) for each of three candidate models for transition rates (equal, symmetrical, and all  
608 different rates), and generated stochastic maps in proportion of the weight of each model out of  
609 1000 simulations (Supporting Code). In order to account for the potentially strong influence of  
610 branch length information on ancestral states (Cunningham, Omland & Oakley, 1998; Joy et  
611 al., 2016), we replicated all analyses using maximum parsimony, which does not consider  
612 branch length information (Cunningham, 1999), in castor (Louca & Doebeli, 2018).

613 For each scoring procedure, we found that the eggshell type recovered for most major  
614 clades using SIMMAP (Table 1; see Results) was the same as that scored for the two  
615 sauropodomorphs *Lufengosaurus* and *Massospondylus* – the only two species in the sample to  
616 be scored differently for each of the three scorings (new scoring: semi-rigid; ratio scoring: soft;  
617 shell unit scoring: hard). They are also, along with the other sauropodomorph *Mussaurus*  
618 (always scored as soft-shelled), the oldest taxa in the tree, i.e. closer in branch length to more  
619 inclusive nodes in the tree than any other species in the sample, which might explain why their  
620 eggshell type strongly influences the one recovered as ancestral for most major clades (e.g. Li,  
621 Steel & Zhang, 2008). To test that prediction, we removed from the sample all other taxa (n =  
622 7, all turtles) that were scored differently for at least one of the three scorings, resulting in a  
623 sample for which all taxa except *Lufengosaurus* and *Massospondylus* are scored identically.  
624 We then replicated all ASR with SIMMAP and maximum parsimony.

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

630

## 631 B) Results

632 For analyses performed on the whole sample (n = 208; Figure 3a,c,e), SIMMAP ASR of  
633 eggshell type recover a high discrepancy in ancestral states of most major reptile clades among  
634 all three scoring procedures (Table 1). For all analyses of eggshell type, Pterosauria are  
635 recovered as ancestrally soft-shelled, since all but one of them are coded as soft-shelled;  
636 similarly, Crocodylia and Aves are always recovered as ancestrally hard-shelled, since all  
637 members of both clades are coded as hard-shelled. The new scoring (Figure 3a) results in a

638 semi-rigid eggshell being the ancestral condition for all major clades (i.e. Reptilia,  
639 Lepidosauria, Archelosauria, Testudines, Archosauria, Ornithodira, Dinosauria, Ornithischia,  
640 and Saurischia) – a surprising result considering only 6 taxa in the whole sample, among which  
641 no ornithischians, were scored as semi-rigid. With the ratio scoring (Figure 3c), all  
642 aforementioned clades are recovered as ancestrally soft-shelled (albeit with low posterior  
643 probabilities – Table 1), with the exception of Testudines and Ornithischia, recovered as  
644 ancestrally semi-rigid. Conversely, with the shell unit scoring (Figure 3e), Reptilia and  
645 Lepidosauria are recovered as ancestrally soft-shelled, but Archelosauria, Archosauria,  
646 Dinosauria and less inclusive clades as ancestrally hard-shelled.

647 SIMMAP ASR performed on the reduced sample (i.e. with identical scoring for all taxa  
648 except *Lufengosaurus* and *Massospondylus*; n = 201) give different results from those  
649 performed on the whole sample (Figure 3b,d,f; Table 1). For all three scorings, Reptilia and  
650 Lepidosauria are recovered as ancestrally soft-shelled, and Testudines and Ornithischia as  
651 ancestrally hard-shelled (the former due to all turtles with semi-rigid eggshells being removed  
652 from the sample). Archelosauria, Archosauria, Ornithodira, Dinosauria, and Saurischia are  
653 recovered as ancestrally soft-shelled if *Lufengosaurus* and *Massospondylus* were scored as soft-  
654 shelled (Figure 3d), but as ancestrally hard-shelled if *Lufengosaurus* and *Massospondylus* were  
655 scored as either semi-rigid (Figure 3b; albeit with low posterior probabilities – see Table 1) or  
656 hard-shelled (Figure 3f). This confirms that *Lufengosaurus* and *Massospondylus* have a  
657 disproportionate effect on the results of SIMMAP ASR, despite representing ~1% of the  
658 sample. Furthermore, for the new scoring, the removal of seven turtle species – only two of  
659 which scored as semi-rigid – changes the ancestral states for all major clades from semi-rigid  
660 to hard (or soft for Reptilia and Lepidosauria), which suggests that these two turtle species also  
661 have a strong influence on those results.

662 ASR performed with maximum parsimony are more congruent between scoring  
663 procedures than those performed using SIMMAP (Figure S1; Table 1). For all three scoring  
664 procedures, an identical probability is recovered for all three character states for the Reptilia  
665 node, resulting in an ambiguous ancestral condition. Lepidosauria are always recovered as  
666 ancestrally semi-rigid, while Archelosauria, Testudines, Archosauria, Ornithodira, Dinosauria,  
667 Ornithischia, and Saurischia are always recovered as ancestrally hard-shelled (Figure S1).  
668 These differences between SIMMAP and parsimony-based ASR for an identical topology show  
669 that branch length information is indeed a strong influence on the eggshell type recovered in  
670 major reptile clades. This is especially true of the ratio scoring, for which all major clades within  
671 Archelosauria are recovered as either ancestrally soft-shelled or semi-rigid by SIMMAP, but as  
672 hard-shelled using parsimony (Figure 3c; Figure S1b). Similarly, for the new scoring, all but  
673 two major clades recovered by SIMMAP as ancestrally semi-rigid are recovered as hard-shelled  
674 by maximum parsimony (Table 1), showing that the abnormal influence of the six taxa scored  
675 as semi-rigid in this scoring is conditional on branch length information. However, unlike  
676 SIMMAP, maximum parsimony ASR performed on the reduced sample are identical to those  
677 performed on the whole sample (Table 1), suggesting that the strong influence of  
678 *Lufengosaurus* and *Massospondylus* on ASR results is independent of branch length  
679 information.

680 ASR for absolute CL thickness as a continuous trait show relatively low values (~30-40  
681  $\mu\text{m}$ ) for Reptilia, Lepidosauria, Archelosauria, Archosauria and Ornithodira. Gekkota,  
682 Testudines and Crocodylia all acquire a thicker CL independently (Figure 4a). Among  
683 Ornithodira, Pterosauria show a strong decrease in CL thickness, as expected from the lack of  
684 CL in most species. Dinosauria also present a low value (41.9  $\mu\text{m}$ ), since they include species  
685 with either thick ( $> 250 \mu\text{m}$ ; Ornithopoda, Sauropoda, most Theropoda) or thin CL  
686 (*Protoceratops* and *Mussaurus*: no CL; *Massospondylus*: 100  $\mu\text{m}$ ; *Lufengosaurus*: 85  $\mu\text{m}$ ) –

687 the latter being much less numerous, but closer in branch length to the Dinosauria node than  
688 the former. *Lufengosaurus* and *Massospondylus* fall in the middle of the spectrum for absolute  
689 CL, much higher than e.g. non-gekkotan lepidosaurs, likely due to the comparatively large size  
690 of their eggs.

691 For relative CL thickness (in  $\mu\text{m g}^{-1}$ ), the pattern is different (Figure 4b): the value at  
692 the node Reptilia remain relatively low (3.46), and nodes Archelosauria and Archosauria show  
693 a decrease (2.66 and 2.19, respectively). Values in Lepidosauria are highly variable, with a  
694 conspicuous increase in Gekkota (20.4) and decrease in Serpentes (1.65) – congruent with a  
695 recent study on non-avian reptiles that identified a similar ASR pattern for shell calcification in  
696 Gekkota and Serpentes (D’Alba et al., 2021). Values increase in Testudines (6.65) and  
697 Crocodylia (4.76), but strongly decrease in Dinosauria (1.32). Among Dinosauria, most  
698 Ornithischia and all Sauropodomorpha show a very low relative CL thickness, while Theropoda  
699 show a gradual increase, with a subsequent decrease in Palaeognathae and further increase in  
700 Eufalconimorphae – a result already described by Legendre & Clarke (2021) using an almost  
701 identical sample of Dinosauria.

702

#### 703 **4) Discussion**

##### 704 A) Influence of branch length information and topology on inference of eggshell type

705 In all ASR of eggshell type performed in this study, the ancestral states recovered for many  
706 major reptile clades is largely conditional on the phylogenetic position and branch length of a  
707 few key taxa in the tree, as was already the case in the two main references used for our sample  
708 (Norell et al., 2020; Legendre et al., 2020a, 2020b). This is due to the fact that, for both Bayesian  
709 and maximum likelihood ASR, the probability of a change of state along a branch increases  
710 with its length (Cunningham, Omland & Oakley, 1998; Huelsenbeck, Nielsen & Bollback,  
711 2003; Joy et al., 2016), which implies that the ancestral state recovered at any given node is

712 more likely to be that of the descendant taxon with the shortest branch length from that node  
713 than the one with the longest branch length (Huelsenbeck & Bollback, 2001; Li, Steel & Zhang,  
714 2008; Hanson-Smith, Kolaczowski & Thornton, 2010; Wright et al., 2015). This results in a  
715 strong bias in the case of nodes with a high difference in length between its two descendant  
716 branches – e.g. Sauropodomorpha, to which the three non-sauropod species in our sample  
717 (*Mussaurus*, *Massospondylus*, and *Lufengosaurus*) are much closer than Sauropoda. Since  
718 these three non-sauropod sauropodomorphs are closer to all most inclusive nodes in the tree  
719 than any other terminal taxa, their eggshell type is more likely to be that recovered for these  
720 clades, giving them a disproportionate influence on all ASR.

721         In our calibrated phylogeny, this effect of proximity of some fossil species to nodes of  
722 interest on ASR is reinforced by the presence of many large clades for which the earliest  
723 diverging tip taxon (i.e. sister taxon to all other parts of this clade) has a character state different  
724 from those of most other members of that clade, which is likely a source of further bias in ASR.  
725 For example, in SIMMAP ASR on our full sample, when *Lufengosaurus* and *Massospondylus*  
726 are coded as semi-rigid, they are not only close to internal nodes of interest, but also share their  
727 semi-rigid state with two deep branches within Lepidosauria: the tuatara (sister taxon of all  
728 other lepidosaurs) and *Eublepharis macularius* (sister taxon of all other geckos). This results in  
729 all major clades, as well as Gekkota, recovered as ancestrally semi-rigid (Figure 3a).  
730 Conversely, when *Lufengosaurus* and *Massospondylus* are coded as soft-shelled, they share this  
731 state with five pterosaurs, which are also closer in time to the Ornithodira and Archosauria  
732 nodes than all but two members of these two clades coded as hard-shelled. This results in  
733 Saurischia, Dinosauria, Ornithodira, Archosauria, and Archelosauria being recovered as  
734 ancestrally soft-shelled, despite the vast majority of species in those clades being coded as hard-  
735 shelled (Figure 3c). Accordingly, when removing branch length information in parsimony-  
736 based ASR, all these clades are recovered as ancestrally hard-shelled (Figure S1). This shows

737 the influence of a few key taxa on ASR results given their temporal proximity to nodes of  
738 interest.

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

758         Similarly, the disproportionate influence of earliest-diverging tip taxa identified in our  
759 ASR has not, to our knowledge, been well-documented. A previous study on the evolution of  
760 squamate viviparity described the tuatara as “relatively uninformative about the root state of  
761 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 10 taxa coded as hard-shelled and one (*Protoceratops*) coded as soft-shelled, but is recovered as ancestrally semi-rigid for both new scoring and ratio scoring. When removing either branch length information or turtles coded as semi-rigid, 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 (Huelsenbeck & Bollback, 2001; Duchêne & Lanfear, 2015; 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 ootaxa, the phylogenetic position of which is highly difficult to infer through phylogenetic reconstruction (Grellet-Tinner et al., 2006; Zelenitsky & Therrien, 2008; Varricchio & Barta, 2015); second, accounting for phylogenetic uncertainty in ASR (especially when using Bayesian methods) only produces a different result when that uncertainty is low (Hanson-Smith, Kolaczkowski & Thornton, 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

787 how such analyses tend to be overly sensitive to a very small number of taxa (~1–4%) due to  
788 their phylogenetic position and calibration. In general, the field of ASR in paleontology is  
789 relatively new, and most biases associated with the inclusion of fossils for e.g. model fitting or  
790 sampling error have only been identified and characterized recently (Hunt & Carrano, 2010;  
791 Bapst, 2014; Soul & Wright, 2021). In this context, our results provide a clear example of  
792 inherent issues of uncertainty in tree topology and calibration, taxon sampling, and character  
793 coding (see next section), which are generally not considered, nor discussed, in recent studies  
794 on reptile eggshell evolution (e.g. Stein et al., 2019; Norell et al., 2020; Legendre et al., 2020a;  
795 D’Alba et al., 2021). We therefore advocate for future studies performing ASR of eggshell type  
796 – or any other discrete or continuous egg trait – to prioritize, when possible, methods that can  
797 account for such biases most efficiently (e.g. hierarchical, split, or hidden rate Bayesian models;  
798 sensitivity analyses using joint estimation from a posterior distribution of trees – King & Lee,  
799 2015; Joy et al., 2016; Wright, Wagner & Wright, 2021; Soul & Wright, 2021), and explicitly  
800 discuss potential biases associated with these methods, sampled taxa, and data used for both  
801 traits and phylogeny tree used in such studies. These extra steps will help facilitate replication  
802 of previous results and improve future discussions on discrepancies between methods and  
803 datasets in this context.

804

805 B) Scoring eggshell type: can we provide biological definitions for hard/soft/semi-rigid  
806 eggshells?

807 ASR performed on absolute and relative CL thickness illustrate the difficulty of assigning a  
808 discrete character state to a specific value, or range of values, for a continuous trait. For absolute  
809 CL thickness, many large clades are recovered with ancestral values that fall near the middle  
810 of the spectrum for that trait, due to the wide range of values taken by terminal taxa in each of  
811 these clades. Following the traditional classification, these intermediate values would be

812 considered equivalent to a semi-rigid eggshell; however, as described in previous sections, the  
813 semi-rigid eggshell type can also be defined as the very specific ‘nail-shaped’ morphology  
814 found in the eggshells of the tuatara and several geckos (Figure 2), which is highly unlikely to  
815 be the ancestral eggshell of Reptilia, Archosauria, or Dinosauria.

816 Similarly, the independent acquisitions of a thick CL (whether absolute or relative) are  
817 clearly associated with the highly distinctive shell unit morphologies documented in each of  
818 these groups (Figure 1; see previous section). Even if the basic structure of a shell unit likely  
819 shows deep homology (*sensu* Shubin, Tabin & Carroll, 1997) among all Archelosauria  
820 (Mikhailov, 1991a, 1997b; Mikhailov, Bray & Hirsch, 1996), the morphological differences  
821 between them constitute the respective synapomorphies of distinct clades among archelosaurs.  
822 Hence the diversity of these shell unit types cannot be fully encompassed by one single ‘hard-  
823 shelled’ character state, which explains why it has rarely, if ever, been the subject of  
824 evolutionary hypotheses in a phylogenetic context. Indeed, the problem of discretizing  
825 continuous traits has been the subject of a four-decade-long debate in phylogenetic systematics,  
826 and none of the many discretization methods available (e.g. divergence weighting, step-matrix  
827 gap-weighting, implied weighting – Gift & Stevens, 1997; Wiens, 2001; Bardin et al., 2014)  
828 have been shown to accurately represent the original distribution of continuous traits (Bardin  
829 et al., 2014; Raven & Maidment, 2017; Worthington, 2017). Additionally, the uneven temporal  
830 distribution of terrestrial Konservat-Lagerstätten (Eliason et al., 2017) may differentially  
831 impact the record of amniote eggs with a thin or absent CL, and statistical approaches that take  
832 into account probability of preservation could be used to put confidence bounds on estimated  
833 ancestral states (Marjanović & Laurin, 2008; Wang et al., 2016; Eliason et al., 2017; Marshall,  
834 2019).

835 An important motivation behind the original definition of eggshell types is that they also  
836 correspond to varying degrees of biomineralization, i.e. calcium content – an important

837 physiological constraint on incubation that has been extensively studied in reptiles (Simkiss,  
838 1961a, 1961b; Packard & Packard, 1984; Silyn-Roberts & Sharp, 1985; Packard, 1994; Matos,  
839 2008; Karlsson & Lilja, 2008; Stewart & Eca, 2010; Österström, Holm & Lilja, 2013;  
840 Deeming, 2018a, 2018b). In that regard, differentiating ‘hard’ and ‘soft’ eggshells could be  
841 considered a functional assessment based on consistency of the CL, roughly indicating the  
842 amount of mineralized calcium in the shell and providing indirect information on reproductive  
843 metabolism (Packard, 1994). However, such a definition is still highly imprecise. Indeed,  
844 despite most of the calcium in the shell being concentrated in the CL, crystalline calcified  
845 elements can be found in other layers as well, their distribution and concentration being highly  
846 variable among reptiles (relative proportions of sulfur and calcium shown in Figure 1; Schleich  
847 & Kästle, 1988; Kohring, 1995; D’Alba et al., 2021). This is especially relevant in eggs with a  
848 thin, poorly calcified CL (i.e. described as ‘soft-shelled’ in lepidosaurs and turtles), which does  
849 not accurately reflect the calcium content of the whole shell. Furthermore, the lack of preserved  
850 proteinaceous layers in most fossil eggshells (Silyn-Roberts & Sharp, 1989; Stewart, 1997;  
851 Kohring, 1999; Grellet-Tinner, 2005; Grellet-Tinner et al., 2011; Norell et al., 2020; Legendre  
852 et al., 2020a) prevents an accurate estimation of their total mineral content. Recent comparative  
853 studies have described the diversity of calcium distribution within extant reptile eggshells,  
854 supporting relative calcium proportion and mapping as key proxies to understand their  
855 evolution (Choi et al., 2018; Campos-Casal et al., 2020; D’Alba et al., 2021; McClelland et al.,  
856 2021). The use of such traits in future studies is likely to improve our understanding of reptile  
857 eggshell microstructure beyond the ‘hard/soft’ dichotomy, and shift the focus of descriptions  
858 from the sole CL to all eggshell layers.

859         In light of the lack of precision in definitions of eggshell type, we conclude that none of  
860 the three eggshell types traditionally used to characterize reptile eggs can be considered  
861 biologically accurate descriptors of their microstructural diversity. The ‘hard/soft’ dichotomy,

862 even with the addition of a third semi-rigid state, is an oversimplification of a much greater  
863 variety of eggshell morphologies, and may “reduce a grayscale continuum into black-and-white  
864 while ignoring important morphological and, potentially, functional information” (D’Alba et  
865 al., 2021, p. 1074). In fact, our ASR of relative CL thickness hints at potential homologous  
866 eggshell structures hitherto undescribed among reptiles: the apomorphic thickness increase in  
867 eufalconimorphs has been proposed to be linked with specific flight strategies among extant  
868 birds, but remains unexplained (Legendre & Clarke, 2021), while the thickness decrease  
869 observed in snakes has, to our knowledge, never been previously reported. This undocumented  
870 diversity would thus be more adequately described through the use of other traits, preferably  
871 not discretized if continuous, and used in combinations that reflect the different eggshell layers  
872 and individual structures inside them, including – but not limited to – shell units.

873         Classic eggshell parataxonomy, while useful in this context to provide preliminary  
874 descriptions based on such traits, has been repeatedly shown to define ootaxa on the basis of  
875 highly homoplastic features (Vianey-Liaud & Zelenitsky, 2003; Zelenitsky & Therrien, 2008;  
876 Mikhailov, 2014), which has resulted in the misidentification of many fossil eggs (Varricchio,  
877 Balanoff & Norell, 2015; Grigorescu, 2017; Choi et al., 2020, 2022a). Therefore, even traits  
878 traditionally used to describe the structure of shell units need to be redefined in this context.  
879 Many of such traits would likely still be defined as discrete categories associated with a  
880 particular reptile clade – e.g. shell units with acicular aragonite crystals, long known to be a  
881 defining feature of turtle eggshells, identifiable even in diagenetic fossil specimens in which  
882 aragonite is replaced with calcite (Hirsch, 1983, 1996; Schleich & Kästle, 1988; Mikhailov,  
883 1997b; Moreno-Azanza et al., 2021), were recently proposed to be a synapomorphy of  
884 Testudines from direct paleontological evidence (Choi et al., 2022b). To avoid potentially  
885 confusing categories based on broad functional characteristics such as traditional eggshell  
886 types, the definition of such discrete traits should be based on explicit structural similarities,

887 which should be argued by authors of future studies as hypotheses of primary homology to  
888 allow their subsequent testing in a phylogenetic framework. Similarly, the lack of a particular  
889 structure should not necessarily be considered by default as one single character state, but  
890 investigated further – including assessment of potential preservational bias for this structure  
891 (e.g. fossil eggs with a thin, non-prismatic CL, proposed to be preserved mostly in oxidative or  
892 euxinic conditions in terrestrial or shallow water environments – Wiemann et al., 2018; Norell  
893 et al., 2020; Legendre et al., 2020a).

894         Several recent studies have shown the value of continuous eggshell traits (e.g. thickness,  
895 calcium content in the shell, crystalline structure of the cuticle, porosity) in studying the  
896 correlation of eggshell microstructure with lifestyle parameters (e.g. body mass, clutch size and  
897 mass, nest structure, water vapor conductance, geographical distribution, ambient temperature  
898 and humidity), and/or reconstruct its evolution in both avian and non-avian reptiles (D’Alba et  
899 al., 2016, 2017, 2021; Legendre & Clarke, 2021; Attard & Portugal, 2021; McClelland et al.,  
900 2021; Kulshreshtha et al., 2022). The characterization of additional traits (e.g. pore structure  
901 and density, calcium concentration, crystalline structure of shell units, thickness and  
902 arrangement of protein fibers in the membrane) will be greatly improved by the systematic use  
903 of advanced microscopy and geochemistry techniques to visualize structures of interest with a  
904 much greater precision (e.g. X-ray or Raman spectroscopy, EBSD, time-of-flight or inductively  
905 coupled mass spectrometry – Moreno-Azanza et al., 2013; Wiemann et al., 2017; Choi, Han &  
906 Lee, 2019; Campos-Casal et al., 2020; Loewy et al., 2020; Alleon et al., 2021; Wiemann &  
907 Briggs, 2022). Future use of such atomized single traits in a phylogenetic comparative context  
908 is thus expected to provide a much clearer picture of the evolution of eggshell microstructure  
909 in reptiles than that of a two- or three-state character (D’Alba et al., 2021; Legendre & Clarke,  
910 2021).

911

## 912 **5) Conclusions**

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

924

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

927 2) Describe both macro- and microanatomy of each specimen, and focus on all parts of  
928 the eggshell (boundary layer, membrana testacea, CL, and cuticle if present);

929 3) Always combine several methods of visualization (e.g. scanning electron  
930 microscopy, EBSD) and chemical analysis (e.g. Raman spectroscopy, energy-dispersive X-ray  
931 spectroscopy, and mass spectrometry) to describe eggshell microstructure and composition and  
932 measure quantitative traits;

933 4) If analyzing a sample of eggs from different species, always correct for phylogenetic  
934 non-independence (Paradis, 2012; Soul & Wright, 2021) and include eggs from every major  
935 clade in your group of interest, including fossils (e.g. a study on Reptilia should always sample

936 avian eggs) – and, if possible, from a wide range of body sizes (e.g. Birchard & Deeming, 2015;  
937 Legendre & Clarke, 2021);

938         5) Review all phylogenetic reconstructions for species in your sample – including fossil  
939 egg parataxonomy – as well as tree calibrations; if possible, include several topologies and  
940 calibrations to account for phylogenetic and temporal uncertainty (Li, Steel & Zhang, 2008;  
941 Cusimano & Renner, 2014; Wright et al., 2015);

942         6) Measure continuous characters (e.g. eggshell thickness, calcium concentration) as  
943 such, and include several of them in multivariate quantitative analyses to account for all parts  
944 of each structure of interest; for a large number of potentially redundant traits, dimensionality  
945 reduction – e.g. principal component analysis – can be performed (Stoddard et al., 2017);

946         7) If performing ASR, test which evolutionary model fits your trait of interest best  
947 (Litsios & Salamin, 2012; King & Lee, 2015; Joy et al., 2016), and use different reconstruction  
948 methods to test the replicability and sensitivity to branch length information of each analysis;

949         8) When defining and analyzing discrete traits, always justify each character state as an  
950 explicit hypothesis of homology, using specific terms referring to clearly identified structures;  
951 if the use of ‘soft’ and ‘hard’ as descriptive terms is necessary when discussing the results of  
952 these analyses, always define precisely what is meant and use those definitions consistently  
953 through the discussion;

954         9) If an evolutionary trend is identified as a result of an ASR, the potential acquisition  
955 of a new trait value or state should always be the subject of an explicit hypothesis of homology,  
956 which must be carefully discussed in the context of phylogenetic uncertainty and sampling  
957 error.

958

959         This list of best practices is not exhaustive, as future definitions of eggshell  
960 microstructural traits are likely to result in new hypothesized synapomorphies for major reptile

961 clades, which might require additional practical and terminological guidelines beyond the scope  
962 of the present study. However, reference to these traits will enable phylogenetic assessment of  
963 variables relevant to a synoptic look at the evolution of reproductive biology in deep time.

964

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976

#### 977 **Author contributions**

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

980

#### 981 **Conflict of interest**

982 The authors declare no conflict of interest.

983

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- 1834

1835 **Supporting Information**

1836 Supporting Information may be found online in the corresponding section of this paper.

1837

1838 **Data Availability Statement**

1839 The data that support the findings of this study are openly available on Github at

1840 [https://github.com/LucasLegendre/eggshell\\_review](https://github.com/LucasLegendre/eggshell_review).

1841

1842 **Table 1.** Ancestral eggshell type with the highest associated probability (indicated in  
1843 parentheses) recovered for each major clade in the tree, for each sample size (whole or reduced),  
1844 reconstruction method (SIMMAP or maximum parsimony), and scoring procedure (new  
1845 scoring, ratio scoring, or shell unit scoring).

<b>For the whole sample (n = 208), using SIMMAP</b>			
<b>Clade</b>	<b>New scoring</b>	<b>Ratio scoring</b>	<b>Shell unit scoring</b>
Reptilia	semi-rigid (0.975)	soft (0.671)	soft (0.672)
Lepidosauria	semi-rigid (0.921)	soft (0.795)	soft (0.704)
Archelosauria	semi-rigid (0.997)	soft (0.535)	hard (0.935)
Testudines	semi-rigid (0.997)	semi-rigid (0.863)	hard (0.997)
Archosauria	semi-rigid (0.997)	soft (0.541)	hard (0.943)
Ornithodira	semi-rigid (0.995)	soft (0.571)	hard (0.942)
Dinosauria	semi-rigid (0.997)	soft (0.558)	hard (0.994)
Ornithischia	semi-rigid (0.997)	semi-rigid (0.781)	hard (0.998)
Saurischia	semi-rigid (0.997)	soft (0.65)	hard (0.996)
<b>For the reduced sample (n = 201), using SIMMAP</b>			
<b>Clade</b>	<b>New scoring</b>	<b>Ratio scoring</b>	<b>Shell unit scoring</b>
Reptilia	soft (0.826)	soft (0.943)	soft (0.654)
Lepidosauria	soft (0.849)	soft (0.959)	soft (0.709)
Archelosauria	hard (0.522)	soft (0.727)	hard (0.926)
Testudines	hard (0.948)	hard (0.931)	hard (0.997)
Archosauria	hard (0.524)	soft (0.726)	hard (0.938)
Ornithodira	hard (0.522)	soft (0.729)	hard (0.941)
Dinosauria	hard (0.536)	soft (0.708)	hard (0.992)
Ornithischia	hard (0.776)	hard (0.714)	hard (0.998)
Saurischia	hard (0.527)	soft (0.723)	hard (0.997)
<b>For the whole sample (n = 208), using maximum parsimony</b>			
<b>Clade</b>	<b>New scoring</b>	<b>Ratio scoring</b>	<b>Shell unit scoring</b>
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)
<b>For the reduced sample (n = 201), using maximum parsimony</b>			
<b>Clade</b>	<b>New scoring</b>	<b>Ratio scoring</b>	<b>Shell unit scoring</b>
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)

1846  
1847

1848 **Figure captions**

1849

1850 **Figure 1.** Microstructural and compositional images of reptile eggshells obtained from EBSD  
1851 (first row) and electron probe microanalyzer (EPMA – second and third rows). The EBSD map  
1852 of the turtle eggshell is an IPF X map, while other EBSD maps are IPF Y maps (IPF: inverse  
1853 pole figure). In the IPF Y map, red indicates calcite growing perpendicular to the eggshell  
1854 surface, while green and blue indicate it growing parallel to the surface. In IPF X map, red and  
1855 green indicate aragonite growing perpendicular to the eggshell surface, while blue indicates it  
1856 growing parallel to the surface. EPMA images show relative concentration of sulphur (S) and  
1857 calcium (Ca), with low concentration coloured blue and high concentration red. The innermost  
1858 part of ‘semi-rigid’ eggshells of geckos (two leftmost columns) are composed of thick  
1859 proteinaceous layers (high concentration of S), while the ‘hard’ eggshells of geckos and  
1860 archelosaurs are mostly composed of CaCO<sub>3</sub> (high concentration of Ca). ‘Semi-rigid’ and  
1861 ‘hard’ gecko eggshells share a similar crystallographic arrangement, reflecting transformational  
1862 homology (Choi et al., 2018). Conversely, the ‘hard’ eggshells of gecko and archelosaurs are  
1863 homoplastic, as inferred from to their opposite crystallographic arrangement (Choi et al., 2018).  
1864 ‘Hard’ eggshells of Aves, Crocodylia, and Testudines are also likely homoplastic (see Text).  
1865 Select taxon silhouettes from PhyloPic (full credits in Supporting Information).

1866

1867 **Figure 2.** Schematic representation of the eggshell microstructures of major amniote groups  
1868 and their phylogenetic relationships. Red, blue, and black colours used in the schematic  
1869 drawings correspond to boundary layer, membrana testacea, and calcareous layer (CL),  
1870 respectively. The microstructure of monotreme eggshells is poorly known (see Text), and is  
1871 therefore not depicted. The tuatara and some gecko (Gekkota) eggshells have ‘nail-shaped’  
1872 structure (=‘semi-rigid’ in traditional terms), but eggshells of other squamates (i.e. outside

1873 Gekkota) just have a thin overlying calcite layer. Testudine eggshells were traditionally  
1874 classified into ‘soft’, ‘semi-rigid’, and ‘hard’, as can be seen in the three schematic images.  
1875 Similar to the polyphyletic ‘hard eggshells’ of amniotes (Figure 1), ‘semi-rigid eggshells’ of  
1876 lepidosaur and testudine eggshells are not homologous with each other. Select taxon silhouettes  
1877 from PhyloPic (full credits in Supporting Information).

1878

1879 **Figure 3.** Ancestral state reconstructions of eggshell type scored as a three-state character –  
1880 ‘soft/semi-rigid/hard’ – on our calibrated phylogeny using SIMMAP (see Text), with labels for  
1881 all clades of interest. (a, b) New scoring. (c, d) Ratio scoring. (e, f) Shell unit scoring. Panels  
1882 on the left (a, c, e) correspond to reconstructions performed on the whole sample (n = 208),  
1883 while panels on the right (b, d, f) correspond to reconstructions performed on the reduced  
1884 sample (n = 201; see Text). Legend for eggshell type (upper left insert in panel a) applies to all  
1885 panels. Non-sauropod sauropodomorphs are labelled ‘S1’, ‘S2’, and ‘S3’ in panels (a), (c), and  
1886 (e), respectively, with a corresponding upper right insert for each panel indicating the topology  
1887 and scoring of these taxa to show their strong influence on each reconstruction (see Text). Select  
1888 taxon silhouettes from PhyloPic (full credits in Supporting Information).

1889

1890 **Figure 4.** Ancestral state reconstruction of (a) absolute and (b) relative calcareous layer (CL)  
1891 thickness, treated as a continuous trait, performed on our calibrated phylogeny (n = 208) using  
1892 maximum likelihood (see Text). All values (x) are log-converted using  $\log_{1p}(x) = \log(1+x)$ ,  
1893 due to the presence of null values in species that do not have a CL. Clades of interest for each  
1894 reconstruction are labelled. Select taxon silhouettes from PhyloPic (full credits in Supporting  
1895 Information).

1896

1897 **Figure S1.** Ancestral state reconstructions of eggshell type scored as a three-state character –  
1898 ‘soft/semi-rigid/hard’ – on our calibrated phylogeny (n = 208) using maximum parsimony (see  
1899 Main Text), with labels for all clades of interest. (a) New scoring. (b) Ratio scoring. (c) Shell  
1900 unit scoring. Legend for eggshell type (upper left insert in panel a) applies to all panels. Similar  
1901 reconstructions on the reduced sample (n = 201; see Main Text) were also performed, but are  
1902 not depicted in this figure due to their results being identical to their respective equivalents on  
1903 the full sample for each scoring procedure (see Main Text). Select taxon silhouettes from  
1904 PhyloPic (full credits in Supporting Information).