Dinosaur origin of egg color: oviraptors laid blue-green eggs

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Protoporphyrin (PP) and biliverdin (BV), give rise to the enormous diversity in avian egg coloration. Egg color serves several ecological purposes, including post-mating signaling and camouflage. Egg camouflage represents a major character of open-nesting birds which accomplish protection of their unhatched offspring against visually oriented predators by cryptic egg coloration. Cryptic coloration evolved to match the predominant shades of color found in the nesting environment. Such a selection pressure for the evolution of colored or cryptic eggs should be present in all open nesting birds and relatives. Many birds are open-nesting, but protect their eggs by continuous brooding, and thus exhibit no or minimal eggshell pigmentation. Their closest extant relatives, crocodiles, protect their eggs by burial and have unpigmented eggs. This phylogenetic pattern led to the assumption that colored eggs evolved within crown birds. The mosaic evolution of supposedly avian traits in non-avian theropod dinosaurs, however, such as the supposed evolution of partially open nesting behavior in oviraptorids, argues against this long-established theory. Using a double-checking liquid chromatography ESI-Q-TOF mass spectrometry routine, we traced the origin of colored eggs to their non-avian dinosaur ancestors by providing the first record of the avian eggshell pigments protoporphyrin and biliverdin in the eggshells of Late Cretaceous oviraptorid dinosaurs. The eggshell parataxon *Macroolithus yaotunensis* can be assigned to the oviraptor *Heyuannia huangi* based on exceptionally preserved, late developmental stage embryo remains. The analyzed eggshells are from three Late Cretaceous fluvial deposits ranging from eastern to southernmost China. Reevaluation of these taphonomic settings, and a consideration of patterns in the porosity of completely preserved eggs support an at least partially open nesting behavior for oviraptorosaurs. Such a nest arrangement corresponds with our reconstruction of blue-green eggs for oviraptors. According to the sexual signaling

hypothesis, the reconstructed blue-green eggs support the origin of previously hypothesized avian paternal care in oviraptorid dinosaurs. Preserved dinosaur egg color not only pushes the current limits of the vertebrate molecular and associated soft tissue fossil record, but also provides a perspective on the potential application of this unexplored paleontological resource.

1   **Dinosaur Origin of Egg Color: Oviraptors Laid Blue-Green Eggs**

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27 **ABSTRACT**

28 Protoporphyrin (PP) and biliverdin (BV), give rise to the enormous diversity in avian egg

29 coloration. Egg color serves several ecological purposes, including post-mating signaling and

30 camouflage. Egg camouflage represents a major character of open-nesting birds which

31 accomplish protection of their unhatched offspring against visually oriented predators by cryptic

32 egg coloration. Cryptic coloration evolved to match the predominant shades of color found in the

33 nesting environment. Such a selection pressure for the evolution of colored or cryptic eggs

34 should be present in all open nesting birds and relatives. Many birds are open-nesting, but protect

35 their eggs by continuous brooding, and thus exhibit no or minimal eggshell pigmentation. Their

36 closest extant relatives, crocodiles, protect their eggs by burial and have unpigmented eggs. This

37 phylogenetic pattern led to the assumption that colored eggs evolved within crown birds. The

38 mosaic evolution of supposedly avian traits in non-avian theropod dinosaurs, however, such as

39 the supposed evolution of partially open nesting behavior in oviraptorids, argues against this

40 long-established theory. Using a double-checking liquid chromatography ESI-Q-TOF mass

41 spectrometry routine, we traced the origin of colored eggs to their non-avian dinosaur ancestors

42 by providing the first record of the avian eggshell pigments protoporphyrin and biliverdin in the

43 eggshells of Late Cretaceous oviraptorid dinosaurs. The eggshell parataxon *Macroolithus*

44 *yaotunensis* can be reliably assigned to the oviraptor *Heyuannia huangi* based on exceptionally

45 preserved, late developmental stage embryo remains. The analyzed eggshells are from three Late

46 Cretaceous fluvial deposits ranging from eastern to southernmost China. Reevaluation of these

47 taphonomic settings, and a consideration of patterns in the porosity of completely preserved eggs

48 support an at least partially open nesting behavior for oviraptorosaurs. Such a nest arrangement

49 corresponds with our reconstruction of blue-green eggs for oviraptors. According to the sexual

50 signaling hypothesis, the reconstructed blue-green eggs support the origin of previously

51 hypothesized avian paternal care in oviraptorid dinosaurs. Preserved dinosaur egg color not only

52 pushes the current limits of the vertebrate molecular and associated soft tissue fossil record, but

53 also provides a perspective on the potential application of this unexplored paleontological

54 resource.

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56

57   **INTRODUCTION**

58   Birds offer the most diverse displays of color and shape among modern vertebrates

59   (Stoddard & Prum, 2011). In contrast to their closest modern relatives, the crocodiles, avian eggs

60   range widely in size and shape, and the avian key innovation seems to be variation in color

61   (Cassey et al., 2012). Nature`s repertoire ranges from immaculate (homogeneously colored)

62   reddish brown and white eggs in chicken (*Gallus domesticus*), to light beige with dark brown

63   maculation (speckling) in the oystercatcher (*Haematopus ostralegus*), to light blue in the

64   American robin (*Turdus migratorius*), to the intensive bluish-green of emu eggs (*Dromaius*

65   *novaehollandiae*) at the end of the spectrum (Cassey et al., 2012).

66   All other amniotes, including non-avian reptiles and monotreme mammals, lack eggshell

67   color (Packard & Seymour, 1997). Non-avian amniotes can protect their eggs by burying them

68   (Leighton et al., 2009), or by continuously guarding the nest (Komdeur et al., 1999). Colored

69   eggs are present in most modern birds which build open nests, as the eggs are vulnerable due to

70   periods without parental guarding (Komdeur et al., 1999; Gillis et al., 2012). Birds with

71   biparental brooding behavior minimize the periods during which a clutch is unattended and

72   vulnerable, and sometimes reduce egg coloration (Komdeur et al., 1999). Complete reduction of

73   eggshell coloration is observed in many cavity nesting and cave-breeding birds (Hewitson, 1864)

74   confirming the visual signaling component of egg color function (Castilla et al., 2007). Visual

75   signaling resulting in camouflage of an egg clutch is largely dependent on eggshell coloration

76   relative to the color shade of the nesting background (Wallace, 1890; Stoddard et al., 2016).

77   Such negative signaling also offers protection against brood parasitism because of, e.g., elaborate

78   egg color innovations that allow recognition of parasite eggs among the clutch (Newton, 1896).

79   In addition to signaling, numerous other functions of egg color pigments have been proposed,

80   such as antimicrobial effects (Ishikawa, 2010), protection from solar radiation (Lahti, 2008), and

81   eggshell mechanical reinforcement (Gosler, Higham & Reynolds, 2005). In a phylogenetic

82   analysis of egg shell coloration and color patterning, Kilner (2006) concluded that the ancestral

83   egg color of avian of extant birds was white, and that egg coloration evolved multiple times

84   within crown birds (see Kilner, 2006).

85   This phylogenetic inference ignores the fact that many birds with white eggs, including

86   ostrich (*Struthio*), rhea (*Rhea*) and elephant bird (*Aepyornis*) contain minor amounts of eggshell

87 pigment, and that their reduced pigment most likely represents an evolutionary reaction to the
88 brooding-based reduced selection pressure on coloring their eggs (Kennedy & Vevers, 1975).

89 We hypothesize egg coloration evolved after the switch from burying eggs to building an
90 open and exposed nest (consistent with Gillis et al., 2012). Selection for egg color would only
91 have occurred after the eggs themselves became visible to parents, conspecifics, predators, or
92 parasites (Kilner, 2006).

93 While most dinosaurs buried their eggs (reviewed by Varricchio & Jackson, 2016), there
94 is ample evidence that bird-like, non-avian eumaniraptoran dinosaurs, i.e., Oviraptorosauria,
95 Dromaeosauridae, and Troodontidae, built open ground nests with at least partially exposed eggs
96 (Varricchio & Jackson, 2016, Norell et al., 1995). Partial exposure accounts for the arrangement
97 of their strongly elongated eggs stacked and partially buried almost vertically in the nest material
98 in circular layers either with (seemingly primitive) or without (derived) an empty space in the
99 center of the nest (Norell et al., 1995). Late Cretaceous oviraptorosaurid eggs from China and
100 Mongolia (Norell et al., 1995; reviewed in Varricchio & Jackson 2016) are frequently preserved,
101 and because they are laid in exposed, partially open nests (Norell et al., 1995), they may have
102 been pigmented like many bird eggs.

103 Only two chemical compounds act as avian eggshell pigments and give rise to virtually
104 all observed bird egg colors and patterns–the tetrapyrrols protoporphyrin (PP) and biliverdin
105 (BV) (Kennedy & Vevers, 1975). Both are participants in the vertebrate heme cycle:
106 protoporphyrin is a reddish-brown heme precursor, while biliverdin is a blue-greenish heme
107 catabolite (Ryter & Tyrrell, 2000). In contrast to the linear BV, the cyclic PP exhibits a stronger
108 resonance stabilization (Falk, 1964). Due to their different metabolic functions, PP and BV have
109 distinctively different chemical properties: while PP is lipophilic and rather unreactive, BV is
110 much more reactive due to its hydrophilic, oxidated, linear arrangement (Gorchein et al., 2009).
111 BV is distributed throughout the entire thickness of the prismatic layer in eggshells, most
112 probably linked to matrix proteins, while PP is currently thought to be present in high
113 concentrations only in the outermost eggshell cuticle layer (Wang et al., 2009). Related avian
114 pigments are incorporated into integumentary structures, such as, for example, the PP relative
115 uroporphyrin III which occurs as red turacine pigment in the feathers of herbivorous,
116 musophagid birds (Rimington, 1939). Structurally, the two avian eggshell pigments show a
117 similarity to the most common vertebrate pigments eu- and pheomelanin, in being N-

118 heterocycles (Watt et al., 2009) although their origins are very different in vertebrate secondary

119 metabolism (Kubo & Furusawa, 1991).

120   In terms of pigment taphonomy, the oldest confirmed record of eggshell PP and BV

121 traces is in subfossil moa eggs from New Zealand (Igic et al. 2009) which demonstrates their

122 preservation potential on a time scale of 10^3 years, but also the loss of the more labile BV

123 through time due to degradation processes, dissolution and transport via percolating aqueous

124 fluids. Other related biomolecules with a reported fossil record are hemes (Greenwalt et al.,

125 2013), and chlorophylls (Leavitt, 1993). Both have been identified with minimal or no diagenetic

126 alteration from Mesozoic and Cenozoic fossil deposits, supporting the possibility of eggshell

127 pigment preservation in fluvial or alluvial oxidative deposits such as those from eastern and

128 southern China.

129   We identified unmodified, preserved PP and BV eggshell pigments in all three

130 oviraptorid samples and proved that these eggshells were the sole source of pigments by

131 demonstrating the absence of BV and PP in the investigated sample of adjacent sediment. We

132 also provide support for the preservation of dinosaur eggshell cuticle (previously suggested by

133 Mikhailov, 1991; Schweitzer et al. 2002; Varricchio and Jackson 2004) based on cuticular PP

134 storage. These observations phylogenetically extend the presence of an avian-like eggshell

135 pigmentation back to oviraptorids. Using the known concentrations of our commercial pigment

136 standards and PP and BV concentrations in emu eggshell as a sensitivity control and quality

137 marker for our analytic system, we reconstructed a visually evident blue-green egg color for

138 *Heyuannia* incorporating experimental and taphonomic corrections. Our eggshell zonal porosity

139 reevaluation reconstructed an at least partially open nest for the oviraptorid *Heyuannia huangi*

140 (based on Varricchio et al. 2008; Deeming, 2006; see Supplemental Information).

141

142 **MATERIALS AND METHODS**

143   Institutional abbreviations

144 NMNS: National Museum of Natural Sciences, Taichung, Taiwan

145 PFMM: Paleowonders Fossils and Mineral Museum, Taipei, Taiwan

146 STIPB: Steinmann Institute of Geology, Mineralogy, and Paleontology, Division of

147   Paleontology, University of Bonn, Bonn, Germany

148 ZFMK: Zoologisches Forschungsinstitut und Museum Alexander Koenig, Bonn,

149   Germany

150

Extant eggshell material

152   The emu eggshells (*Dromaius novaehollandiae*) were produced by captive birds and are

153   stored in the ZFMK collections (ZFMK uncat.). Emu eggshell is reported to contain minimal

154   amounts of PP in addition to some of the highest naturally occurring amounts of BV (Gorchein et

155   al., 2012).

156

Fossil eggshell material

158   We sampled three oviraptorid *Macroolithus yaotunensis* eggs from the collections of the

159   NMNS and STIPB covering three geographically and taphonomically distinct Chinese deposits.

160   Investigated specimens were collected in the Liguanqiao Basin near Nanyang in the province of

161   Henan (STIPB E54/1), from the Hongcheng Basin in the province of Jiangxi (NMNS CYN-

162   2004-DINO-05/I), and the Nanxiong Basin in province of Guangdong (STIPB E54/3). Detailed

163   descriptions of the localities in context of geological and taphonomic settings are included in the

164   Supplemental Information. Macroscopically, all oviraptorid eggshell samples had a blackish to

165   blackish-brownish (after cleaning them from adherent sediment) color, revealing a very subtle

166   shimmer of blue-green at angled light conditions.

167   Historically, samples derived from the Liguanqiao Basin are Late Cretaceous in age and

168   derived from the fluvial/alluvial deposits (red sandstones) of the Hugang Formation. They have

169   been housed in STIPB since 1983 and were previously described by Erben (1995). Preserved

170   oviraptorid eggs from the Hongcheng Basin in the province of Jiangxi were obtained from the

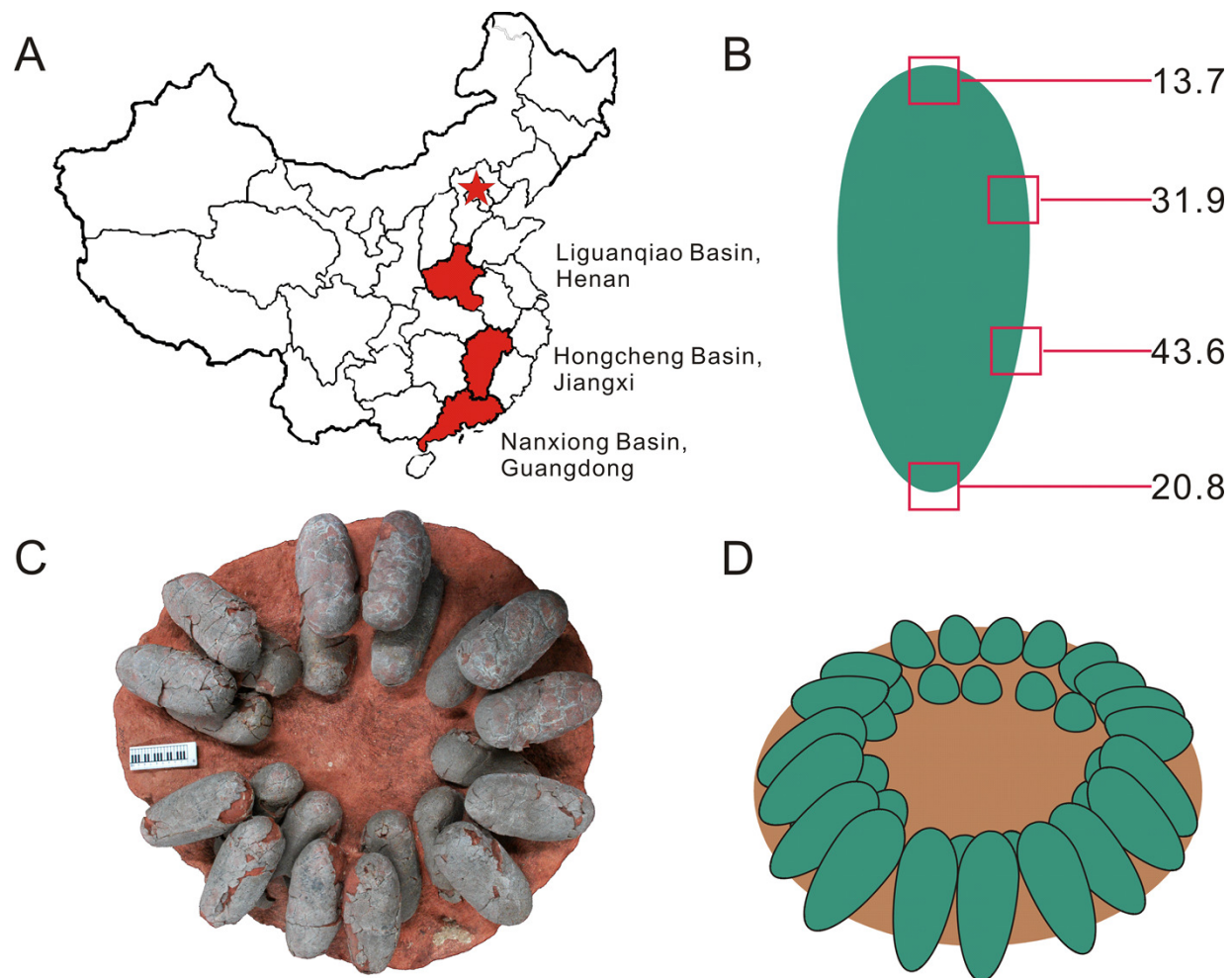
171   Late Cretaceous Tangbian Formation which comprises fluvial red sandstones. The Hongcheng

172   Basin and the Nanxiong Basin may belong to the same extended basin complex (Liu, 1999). The

173   Late Cretaceous strata of the Nanxiong Basin are divided into the Yuanpu Formation and the

174   overlying Pingling Formation. The Yuanpu Formation, which might be correlated with the fossil-

175   rich Mongolian Djadochta Formation, yielded our eggshell samples from alluvial sediments (red

176   silt-sandstones), and is dated as Maastrichtian in age (Zhao et al., 1991).

177
178   **Figure 1** (A) Pair of oviraptorid *Heyuannia* eggs (NMNS CYN-2004-DINO-05) from the Chinese
179   province of Jiangxi before sampling. Porosity measurements and calculations of water vapor conductance
180   are based on these eggs. Pieces of eggshell from each of the four zones depicted in (B) were used in
181   porosity measurements. (B) Egg model separated into four zones used for zonal porosity measurements.
182   Therefore, double half-prolate spheroids and cone models of the idealized egg were used to estimate the
183   zonal surface areas to eventually approximate water vapor conductance. Zone 1 represents the blunt end
184   of the egg, zones 2 and 3 the mid portions, zone 4 represents the pointed end of the egg.
185

186   One of two preserved complete eggs (Fig. 1A) from the Chinese province of Jiangxi

187   (NMNS CYN-2004-DINO-05) which were previously assigned to the oviraptorid egg parataxon

188   *Macroolithus yaotunensis* was sampled over four zones of the egg (Fig. 1B, Fig. 2), prepared for

189   histology, and then used for porosity measurements (see Supplemental Information). These four

190   zones represent the blunt, middle, and acute parts of the egg, and were separated to approach

191   zonal differences in porosity values which were tested for maximum porosity at the mid portions

192   to indicate egg storage in an open nest (based on Varricchio et al. 2008). Measured porosity

193   values were compared to published dinosaur and avian porosity patterns and used to calculate the

194   eggshell water vapor conductivity. Samples for chemical analyses were taken separately.

195   Sediment adhering to the complete eggs (NMNS CYN-2004-DINO-05/I) was sampled

196   additionally to confirm that we are not dealing with wholesale sample contamination. A single

197   sediment sample (red silty sandstone) was available to test against wholesale contamination with

198   BV and PP of the sample since only the complete oviraptorid eggs from the province of Jiangxi

199   provided original, attached matrix sediment. The two-remaining fossil oviraptor eggshell

200   samples represent isolated fragments freed of original matrix.

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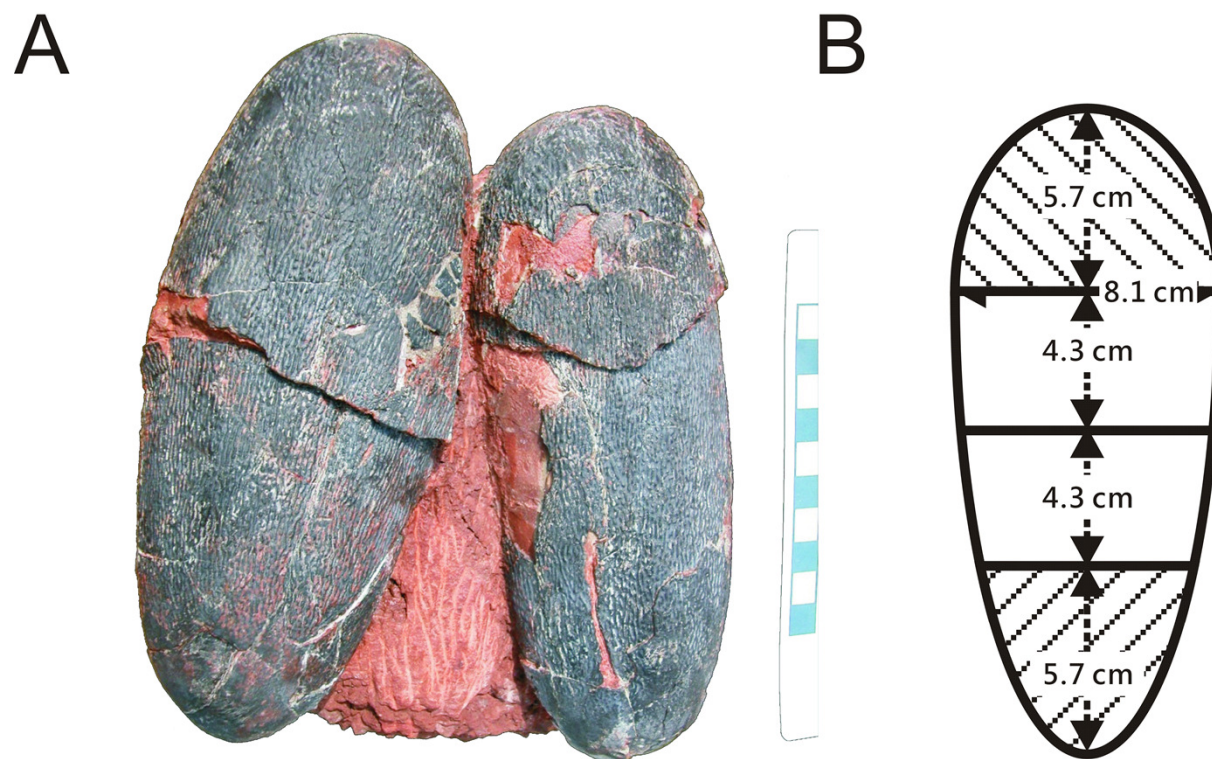
203   **Figure 2** Provenance of *Heyuannia* eggshell, reconstructed zonal egg water vapor conductance, oviraptor
204   clutch structure, and corrected, reconstructed egg color. (A) Geographical map of China. The capital city,
205   Beijing, is indicated by the red star. Red shaded provinces indicate the three different localities where the
206   specimens were collected: the Liguanqiao Basin in Henan, the Hongcheng Basin in Jiangxi, and the
207   Nanxiong Basin in Guangdong (see SI). (B) The reconstructed color and average zonal water vapor
208   conductance of the left Jiangxi *Heyuannia huangi* egg (NMNS CYN-2004-DINO-05/I) calculated from
209   BV and PP concentrations and porosity measurements (see Supplemental Information). (C) Top view of

210 an oviraptor clutch (PFMM 0010403018). This clutch illustrates how eggs are arranged in pairs with their
211 blunt ends pointing to the clutch center. The eggs are arranged in layers separated by sediment. (D)
212 Reconstruction of a partially open oviraptorid nest. Note that the original inclination of the eggs would
213 have been steeper than their preserved attitude (C) due to sediment compaction.

214

215

216

Methods

218   We used two commercial standards (biliverdin dihydrochloride and protoporphyrin IX,

219 purchased from Sigma Aldrich), one extant bird eggshell sample (emu), the three fossil

220 *Heyuannia huangi* eggshell samples, and one sediment sample (reddish sandstone) for High

221 Performance Liquid Chromatography coupled to Electrospray Ionization Quadrupole Time-of-

222 Flight Mass Spectrometry (HPLC ESI Q-ToF MS).

223   The basis for this sample selection was (1) to demonstrate reproducibility in three fossil

224 eggshell samples, (2) to exclude the possibility of wholesale contamination due to sample or

225 system exogenous PP or BV input by analyzing sediment adhering to a sample of eggshell, (3) to

226 provide a sensitivity control and quality marker of the analytical routine based on precise

227 detection and quantification of emu eggshell (ranging from the upper (BV) to the lower (PP)

228 detection limit in terms of pigment concentrations), and (4) to generate a calibrated concentration

229 signal for quantification based on the known concentrations of the commercial standards.

230   Adhering sediment and other superficial contaminations were chemically removed from

231 all eggshell samples by a boosted decalcification of the outermost sample surfaces. 500 μL of

232 disodium EDTA solution (100 mg/mL), adjusted to a pH of 7.2, were added to the 180-562 mg

233 eggshell samples and sediment control sample, each of which was stored in 1 mL Eppendorf

234 tube. Samples were incubated for 5 min and then transferred to fresh Eppendorf tubes. The

235 decalcification residue was discarded.

236   Pigment decalcification was performed immediately after preliminary cleaning of the

237 samples. The three oviraptorid eggshell samples, the emu eggshell, and the sediment control

238 sample were incubated again in the EDTA solution which was already used for initial cleaning,

239 this time for 5 min. During this 5 min, the sample tubes were vortexed three times for 1 min.

240 Vortexing was performed with uncapped tubes to allow outgassing of the carbon dioxide

241 generated. After 5 min of incubation in the EDTA decalcification solution, all sample tubes were

242 centrifuged at 15,000g for 1 min. After centrifuging, the supernatant solutions were collected in

243   separate tubes, while the decalcified sample precipitates were filled up with fresh EDTA solution

244   from the stock. Incubation of 5 min followed, including vortexing 3x in uncapped tubes, as in the

245   previous step. The samples were centrifuged again at 15,000g for 1 min, supernatants were

246   collected, and the precipitates filled up with fresh EDTA solution. As in the previous step,

247   incubation of 5 min including vortexing of the uncapped sample tubes followed. After a final

248   round of centrifuging for 1 min, supernatant solutions were collected, and the partially

249   decalcified precipitates were used for the final pigment extraction. 1 mL of acetonitrile/acetic

250   acid (4:1, *v/v*) was added to the decalcified sample pellets for 10 min of incubation, including 2

251   min of vortex-mixing. Afterwards the sample tubes were centrifuged at 15,000g for 2 min, and

252   the supernatant solution holding the pigment extract were transferred into fresh Eppendorf tubes,

253   and stored in a dark environment at 4°C. The commercial standards were dissolved in the same

254   acetonitrile-acetic acid solution (4:1, *v/v*) and stored with the sample pigment extracts.

255   The filtered extracts and commercial standard solutions were stored less than 24 h before

256   they were injected into an HPLC Dionex Ultimate 3000 (Thermo Scientific) separating sample

257   compounds by using a EC50/2 Nucleodur C18 Gravity 3 μm column (Macherey-Nagel).

258   Reverse-phase HPLC was run at a flow rate of 0.3 mL/min. HPLC was started at 90% H_2O

259   containing 0.1% acetic acid. The gradient started after 1 min and reached 100% acetonitrile after

260   14 min. For additional 7 min, the column was flushed with 100% acetonitrile (containing 0.1%

261   acetic acid). For the biliverdin analysis, 15 μL sample solution were injected, and 20 μL for the

262   protoporphyrin detection. 2 min of washing runs between each sample extract cleaned the entire

263   system. The liquid chromatography system was coupled to a micrOTOF-Q mass spectrometer

264   (Bruker) with an electrospray ionization (ESI) source inducing positive ionization.

265   Data were collected in positive full scan MS mode over the range of 50 – 1000 m/z, using

266   a capillary voltage of 4.5 kV and an end plate offset of -500 V. The dry heater of the ESI source

267   was set at 200°C. Nitrogen desolution and nebulizer gas flow was 10.0 L/min; the nebulizer was

268   run at 2.2 bar. Time-of-Flight (TOF) detection allowed the determination of the accurate masses

269   of biliverdin and protoporphyrin.

270

271   **RESULTS**

272   We reliably identified both BV, as $[M+H]^+$ with 583.2520 m/z (calculated mass:

273   583.2551 g/mol) after 8 min retention time, and PP, as $[M+H]^+$ complex with 563.2623 m/z

274   (calculated mass: 563.2653 g/mol) after 14 min retention time (Fig. 3A) in the commercial

275   standard solutions, the emu eggshell, and the three fossil oviraptorid eggshell samples. The

276   commercial standard solutions of known concentrations were used to identify the chemotrait-

277   specific, diagnostic retention times for BV and PP on the chromatographic column. Elution of

278   BV was consistent after 8 min, while elution of PP delayed consistently until 14 min of the run

279   mobile phase gradient (consistent with Igic et al., 2009). A second compound-diagnostic trait

280   was provided by ionization after elution from the chromatographic column, followed by exact

281   mass determination of the $[M+H]^+$ ion complexes. Differing behavior of BV and PP native to

282   eggshells compared to commercially purified BV and PP was ruled out by identical retention

283   times, numbers of isoforms/tautomers, and exact mass peaks of emu eggshell BV and PP and the

284   commercial standard solutions. Furthermore, maximum sensitivity of the HPLC ESI MS system

285   was demonstrated by precise detection of pigment concentrations in the emu eggshell solution

286   which approached the upper (BV) and the lower (PP) detection limits. The extracted ion

287   chromatograms (EICs) for BV and PP obtained from the sediment sample whole ion mass

288   spectrum yielded signals within broader tolerances of the BV and PP exact masses, but no peaks

289   corresponding to the commercial standard calibrated retention times were identified. Absence of

290   a diagnostic mass peak after 8 min retention time on the column for BV, and after 14 min

291   retention time on the column for PP, is a significant demonstration of absence of trace amounts

292   BV and PP in the sediment sample. Thereby, contamination of the samples or the detection

293   system was excluded and originality of the detected pigments in the oviraptorid eggshell samples

294   is guaranteed.

295   Quantification of the detected pigment concentrations based on commercial standard

296   calibration was determined by application of an experiment-empirical correction for the

297   extraction loss of BV due to its increased hydrophily of the fossil oviraptor eggs.

298   We found the highest preserved concentrations of BV in the eggshells from Henan (6 nmol/g),

299   followed by the eggshells from Jiangxi (2 nmol/g) and those from Guangdong (1 nmol/g). The

300   preserved PP concentrations ranged from 2 nmol/g in both the Henan and Jiangxi eggshells to 1

301   nmol/g in the Guangdong eggshells. Our empirical correction applied to the fossil eggshell

302   samples yielded very realistic pigment concentration estimates for the emu eggshell of 2 nmol/g

303   PP, and 266 nmol/g BV which fall into the reported range of emu eggshell pigment

304   concentrations in the scientific literature (Table S1). The bluish shimmer of the fossil oviraptorid

305 eggshells suggests generally higher BV concentrations than those we detected, as also found in a

306 similar study on pigment preservation in subfossil moa eggshells using the same methodology.

307 The color of the fossil eggs suggested higher BV concentrations than the authors managed to

308 detect. Taken together, our study and the study by Igic et al. (2009) imply that bluish or greenish

309 coloring degradation products of BV remain which therefore shows slightly different chemical

310 properties and different exact masses, and is thus not detected by an LC MS system targeting

311 unmodified compounds measurable against commercial standards.

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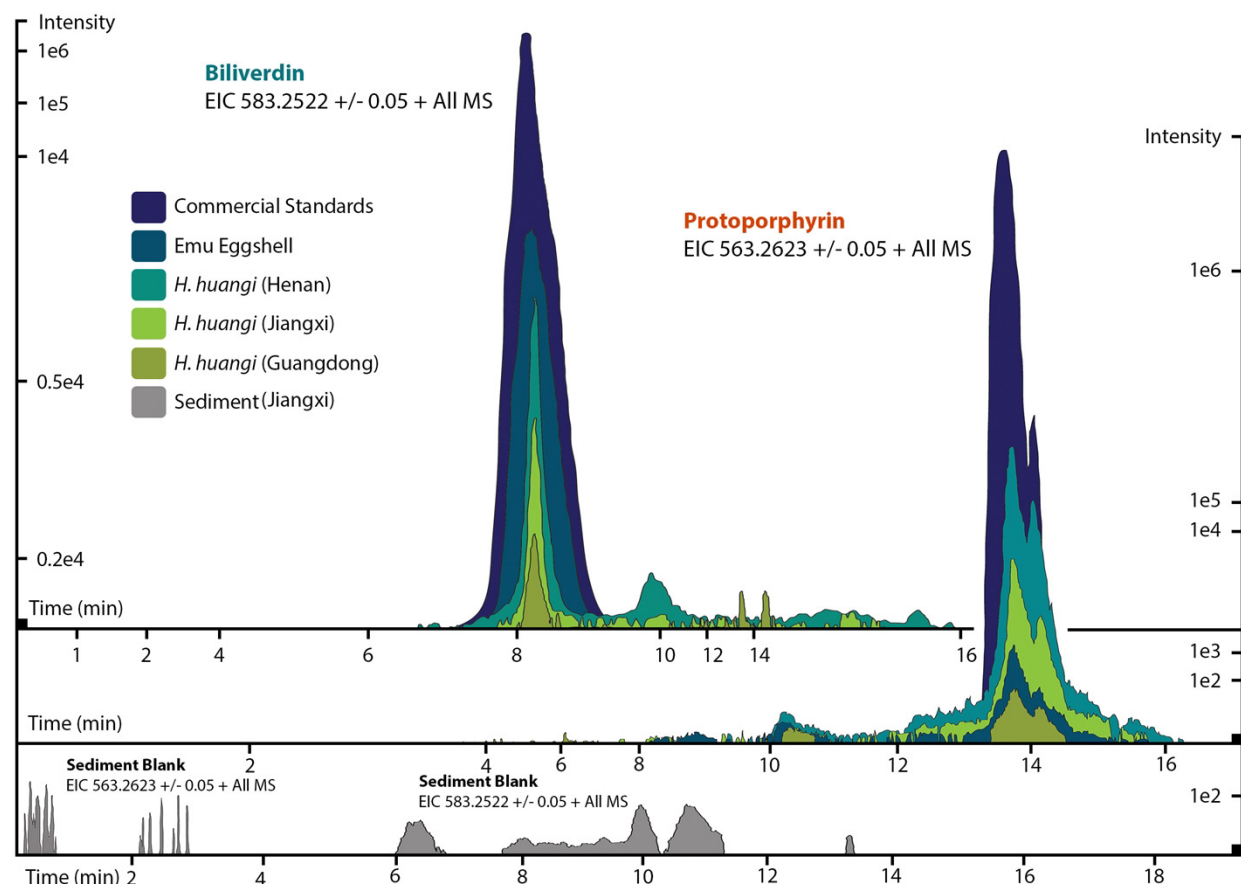

313 **Figure 3** ESI (+) MS extracted ion chromatograms (EICs) for mass 583.2520 ± 0.01/0.05 m/z, indicative

314 of BV, and mass 563.2653 ± 0.01/0.05 m/z, indicative of PP. We identified BV and PP by retention time,

315 exact mass and isoform/tautomer separation. EICs for 583.2520 ± 0.01/0.05 m/z are depicted for the

316 commercial BV standard, emu eggshell, extracts of *Heyuannia huangi* eggshells derived from the Chinese

317 provinces Henan, Jiangxi, and Guangdong and the sediment control extract from Jiangxi. Unmodified BV

318 elutes after 8 min retention time, and was proven present for the biliverdin commercial standard, the emu

319 eggshell, and the three oviraptorid eggshells. The sediment sample was used as control for contamination,

320 and its EIC does not show a peak after 8 min retention time, proving the absence of biliverdin in the

321 sediment sample and the originality of biliverdin detected for the eggshell samples. (B) EICs for 563.2653

322 ± 0.01/0.05 m/z are depicted for the commercial PP standard, emu eggshell, *Heyuannia huangi* eggshell

323 derived from the Chinese provinces Henan, Jiangxi, and Guangdong, and the sediment sample from
324 Jiangxi. Unmodified PP elutes after 14 min and was proven present for the PP commercial standard, the
325 emu eggshell, and the fossil oviraptorid eggshells. There is no peak in the PP EIC for the sediment sample
326 after 14 min, what proves the absence of PP in the sediment control sample, and the originality of PP in
327 the eggshell samples. Peak intensity correlates with pigment concentrations in the extracts.
328

329   To demonstrate the perceivability of a visual color signal based on the detected pigment

330 concentrations in the oviraptorid eggshells, we plotted our pigment concentrations into the

331 comprehensive pigment concentration-color matrix of Cassey et al. (2012). Our three dinosaur

332 egg color data points fall in the visibly olive-green color range between *Haliaetus albicilla* and

333 *Circus aeruginosus* (Cassey et al. 2012). They plot in the cluster of unspotted eggs, suggesting

334 an immaculate, homogenous coloration. No patterns were visible in the fossil eggs (Fig. 1).

335   Since the preserved fossil oviraptor eggshell color suggests originally higher BV

336 concentrations, taphonomy needs to be considered to generate a realistic, native oviraptor egg

337 color reconstruction. Because BV is more reactive and more hydrophilic, and thus soluble in

338 sediment-percolating aqueous fluids, the concentrations of unmodified, preserved pigments after

339 at least 66 million years of sedimentary burial are much more likely to be significantly lowered

340 than those of the more stable, hydrophobic PP (Falk, 1964). Therefore, the taphonomic

341 projections of our preserved pigment concentrations in the avian egg color space (Cassey et al.

342 2012) realistically lift the investigated oviraptor egg colors significantly towards much higher

343 BV values, while the shift towards increased PP values would be only minimal. However, our

344 fossil oviraptor eggs would remain deeply nested within the area of unspotted eggs (based on

345 Cassey et al. 2012). Such an additional taphonomic correction of the reconstructed egg color

346 approximates an intensively blue-greenish oviraptorid egg color. Whether the differences in

347 preserved pigment concentrations between the three fossil oviraptor egg samples from different

348 localities reflect intraspecific variation in egg color or different taphonomic conditions in the

349 deposits cannot be reliably assessed at this point and requires future investigations. Since these

350 differences in preserved pigment concentrations in the oviraptorid eggshells affect the BV values

351 much stronger (range 6 – 1 nmol/g) than they affect the PP values (range 2 – 1 nmol/g), we

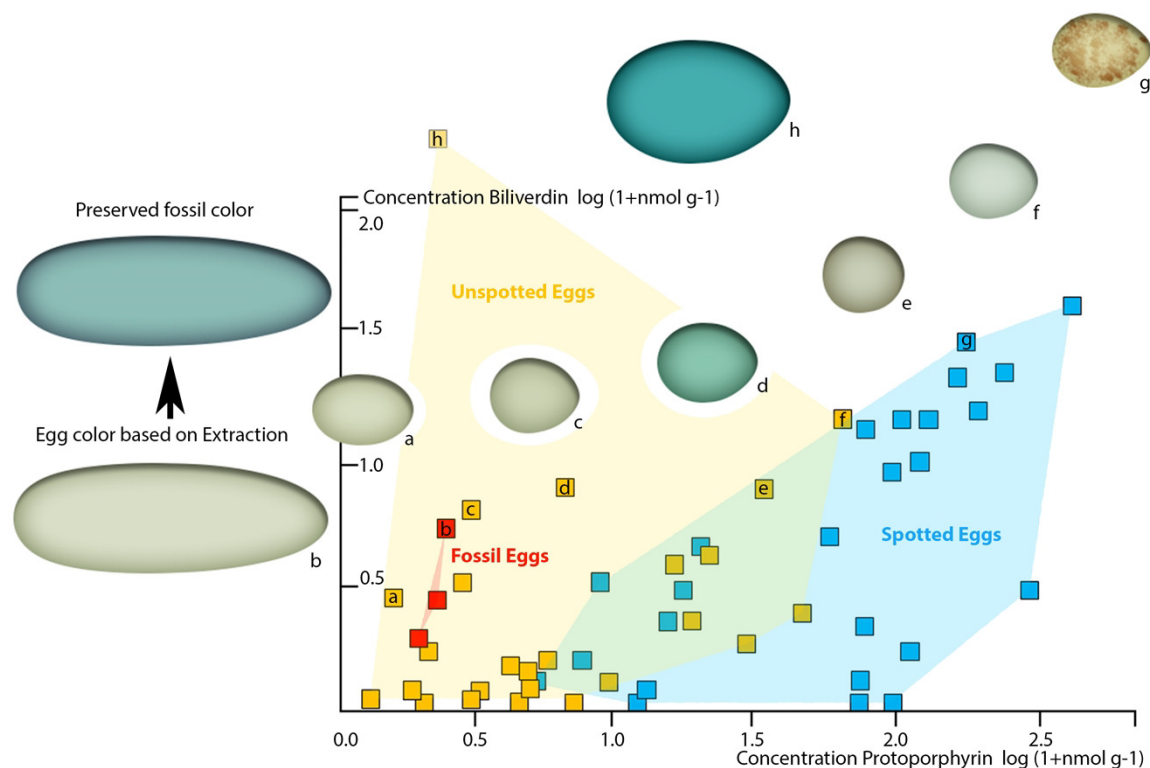
352 assume that differences in color are more likely to be taphonomic. *In vivo* intraspecific variation

353 of egg color would most likely affect BV and PP concentrations equally, while taphonomic

354 effects affect BV concentrations much stronger than PP concentrations (Falk et al., 1964). Also,

355 the original egg color is overprinted by a generally blackish-brownish hue (Fig. 1). This

356   brownish discoloration traces back to preserved, oxidatively crosslinked eggshell organic matrix

357   proteins of the AGE/ALE-type (Wiemann et al. 2016).

358

359   **Figure 4** Plot of oviraptor egg and different avian egg biliverdin concentrations against their

360   protoporphyrin concentrations with example for the overall color impression based on the dataset

361   published by Cassey et al. 2012. Avian eggs cluster together moderately separated into unspotted (yellow

362   cluster, yellow squares) and spotted (blue cluster, blue squares) eggs. Fossil oviraptorid eggs span a color

363   space (red cluster) and are represented by red squares, all nested within definitely unspotted eggs. The

364   color examples extracted from Cassey et al. 2012 indicated for the preserved oviraptor egg pigments

365   concentrations a visibly olive-green color (framed by a: *Haliaetus albicilla*; c: *Circus aeruginosus*). This

366   color estimate lies still below the preserved bluish shimmer of the eggshells. Egg colors at cluster edges

367   are extracted from Cassey et al. 2012 and labelled from a – h.

368

369   Our reconstruction of colored eggs for oviraptors is consistent with our reevaluation of

370   the oviraptorid nesting mode: we consider oviraptor eggs as lying at least partially open in the

371   nest (consistent with Norell et al., 1995). This reconstruction is based on the estimated water

372   vapor conductance of 108.66 mg H_2O day^{-1} Torr^{-1} for the *Heyuannia* egg NMNS CYN-2004-

373   DINO-05/I from the province of Jiangxi (Fig. 1A). This value is calculated from the four zonal

374   conductances deduced from zonal porosity counts (Supplemental Information). The highest

375 values for shell porosity were found in the middle portion of the oviraptor egg (zones 2 and 3),

376 and especially pronounced in zone 3 with a resultant conductance of 43.6 mg H_2O day^{-1} Torr^{-1}

377 (Table S5). The pointed end (zone 4) which is stuck in the nest material, has a calculated

378 conductance of 22.88 mg H_2O day^{-1} Torr^{-1}, while the exposed blunt end (zone 1) has a calculated

379 conductance of 13.77 mg H_2O day^{-1} Torr^{-1} (Table S5).

380

381 **DISCUSSION**

382   Some aerobic microorganisms are known to produce protoporphyrin as an intermediate of

383 their cytochrome c biosynthesis, and they also use biliverdin which represents a highly-

384 conserved metabolite. Thus, a microbial origin of the pigments that we detected from our

385 oviraptorid egg samples needs to be excluded. We use the double-checking LC MS approach

386 (based on Gorchein et al. 2009) applied to sediment adherent to the oviraptorid eggshells from

387 Jiangxi to reliably exclude any wholesale sample contamination, contamination of the LC MS

388 system, of used laboratory equipment or chemicals. Absence of any traces of BV or PP in our

389 sediment control sample proves the cleanliness of our analytical system and supplies, as it proves

390 absence of whole sample contamination based on potentially abundant microbes in the deposits.

391 The only alternative source of the detected oviraptorid eggshell pigments would be eggshell-only

392 contamination with microbially derived BV and PP (Woodward & Dailey, 1995). The preserved

393 bluish-greenish shimmer of the investigated eggshells strongly argues against this. Also, the

394 preserved pigment concentrations and differing BV and PP ratios eliminate the possibility of

395 eggshell-only microbial contamination. The preserved concentrations fall within the empirical

396 correction for pigment loss during the extraction routine (see Figure 4). Thus, the most

397 parsimonious conclusion is that we have documented oviraptorid eggshell pigments.

398   Our results push the origin of pigmented eggshells phylogenetically back to oviraptorid

399 dinosaurs (see Figure 5) (Kilner, 2006). To test for convergent evolution or homology of egg

400 color between oviraptorids and crown birds, future investigations of pigmentation in other

401 eumaniraptoran eggshells are required. Oviraptorid dinosaurs incorporated the same pigments

402 and isoforms into their eggshells, out of a pool of dozens of possible staining secondary

403 metabolites (McGraw et al., 2006; Hubbard et al., 2010; Stoddard & Prum, 2011). If dinosaur

404 egg color was a convergent character relative to the colored eggs of crown birds, the same color

405 effect could have been achieved by use of different metabolites. Non-avian dinosaurs and

406   modern birds apparently use the same molecules to create eggshell coloration. There are no
407   studies available testing for pigment constraints during the shell formation based on
408   simultaneous shell crystallite growth and pigment incorporation into the eggshell organic matrix.
409   Also, potential constraints of metabolite transport mechanisms or *de novo* synthesis sites in the
410   archosaur shell gland are yet unknown, as is metabolite permeability through the archosaur shell
411   gland wall. In any case, we can infer based on our results that oviraptor shell glands generally
412   worked in a similar way avian shell glands do: due to its hydrophilic behavior biliverdin is
413   preferentially incorporated into the organic matrix of the eggshell prismatic zone which holds the
414   calcite crystallites (Falk, 1964). Biomaterial studies suggested thioether linkage of BV to
415   cysteine-rich proteins, allowing an energy-efficient storage close to the ion lattice of calcite
416   crystals (Lamparter et al. 2004; Salewski et al., 2013). Based on this presumably general storage
417   mechanism of BV in biocomposite materials, we infer a similar storage in oviraptor eggshells.
418   PP is secreted during the final steps of eggshell formation, while the storage mechanism is yet
419   unclear. Due to its hydrophobic properties, PP is currently thought to occur in highest
420   concentrations in the waxy eggshell cuticle (Baird et al., 1975). Thus, evidence for PP in fossil
421   oviraptorid eggshells strengthens the support for hypothesized preservation of eggshell cuticle
422   through deep time (Mikhailov, 1991; Schweitzer et al. 2002; Varricchio and Jackson 2004). Both
423   pigments are supposed to be synthesized *de novo* in the shell gland tissue (Wang et al., 2009).
424   Combining our pigment analyses with the outcomes of studies on oviraptorid clutch
425   arrangements (Huh et al. 2014, Pu et al. 2017), it can be summed up that oviraptorid eggs were
426   most probably laid in overlapping circles, partially stuck in the nesting material, with their blunt
427   ends exposed, pointing upwards in an almost vertical orientation. Partially exposed, blue-green
428   eggs, stacked in circular layers, allow the inference of a similarly colored nesting material. In
429   modern birds, blue-green eggs stored in ground nests are found in Casuariformes, including
430   emus and cassowaries (Coombs, 1989). Emus and cassowaries lay their eggs in soil mounds
431   covered with leaf litter and vegetation, so that egg color and nesting background match in tone
432   (Coombs et al., 1989). Considering arid to subtropical paleoclimate reconstructions for Late
433   Cretaceous China (Hsu, 1983), and the fluvial/alluvial red silt-sandstone sedimentology of the
434   fossil laegerstaetten from Henan, Jiangxi and Guangdong provinces, vegetation coverage of the
435   nest base comparable to emu and cassowary nests, adjacent to permanent or rather ephemeral
436   river systems would offer an environment requiring blue-greenish egg colors for nest crypsis. An

437   alternative explanation to only cryptic functions of blue-green eggs has been described for

438   tinamous (*Tinamus major*): blue-green eggs in tinamous (*Tinamus major*) have been

439   demonstrated to be non-cryptic and to be laid in environments where the egg color significantly

440   contrasts the nesting background color (Brennan, 2010). In tinamous, nest predation depends not

441   on egg color cues, but mainly on either visual or chemical parental cues during clutch incubation,

442   and egg color is supposed to have evolved for intraspecific signaling (Brennan, 2010).

443   Intraspecifically, blue-green egg color in modern birds has been associated with paternal care,

444   communal nesting, and postmating sexual signaling (Moreno & Osorno, 2003; Handfort &

445   Mares, 1985). Paternal care was previously hypothesized to have dinosaur origin, tracing back to

446   oviraptorid dinosaurs, and thus, poses a good fit our finding of oviraptorid blue-green egg color

447   (Varricchio et al. 2008, but see Birchard et al. 2013). Communal nesting, as seen in polyandrous

448   birds like emus and cassowaries, represents a reproductive strategy that might apply for non-

449   avian dinosaurs, but has never been tested for (Handfort & Mares, 1985). Preliminary studies

450   based on eggshell chemical signatures identifying multiple maternal individuals contributing

451   eggs to one clutch hint at communal nesting in oviraptorid dinosaurs (Yang et al., 2016). Finally,

452   postmating sexual signaling according to the Sexual Signaling Hypothesis appears controversial

453   in the ornithological literature (Moreno & Osorno, 2003; Krist & Grim, 2007). This hypothesis

454   suggests intensive blue-green egg color as a quality marker for maternal health and

455   immunocapability (Moreno & Osorno, 2003). Post-mating sexual signaling then occurs due to

456   BV being incorporated in high concentrations into the eggshell because it is dispensible to the

457   maternal metabolism (Moreno & Osorno, 2003). The general idea goes back to the handicap

458   hypothesis (Roberts et al., 2004), and assumes that the male is motivated by the confirmation of

459   the female health status and thus, the offspring quality (Moreno & Osorno, 2003).

460   The similarity in reproductive strategies between crown birds and derived non-avian

461   maniraptorans may reflect shared selective pressures: the recent discovery of the anseriform

462   *Vegavis* in Late Cretaceous deposits from Antarctica provides evidence that crown birds

463   coexisted with non-avian dinosaurs (Clarke et al., 2016). The presence of neognath birds in the

464   Late Cretaceous implies that paleognath birds already diverged at this point (based on avian

465   phylogeny by Prum et al., 2015). We would like to suggest new avenues of research based on the

466   perspective of the potential coexistence, shared selection pressures, and niche competition of

467   secondarily flightless paleognath birds and oviraptorid, dromeosaurid, and troodontid dinosaurs.

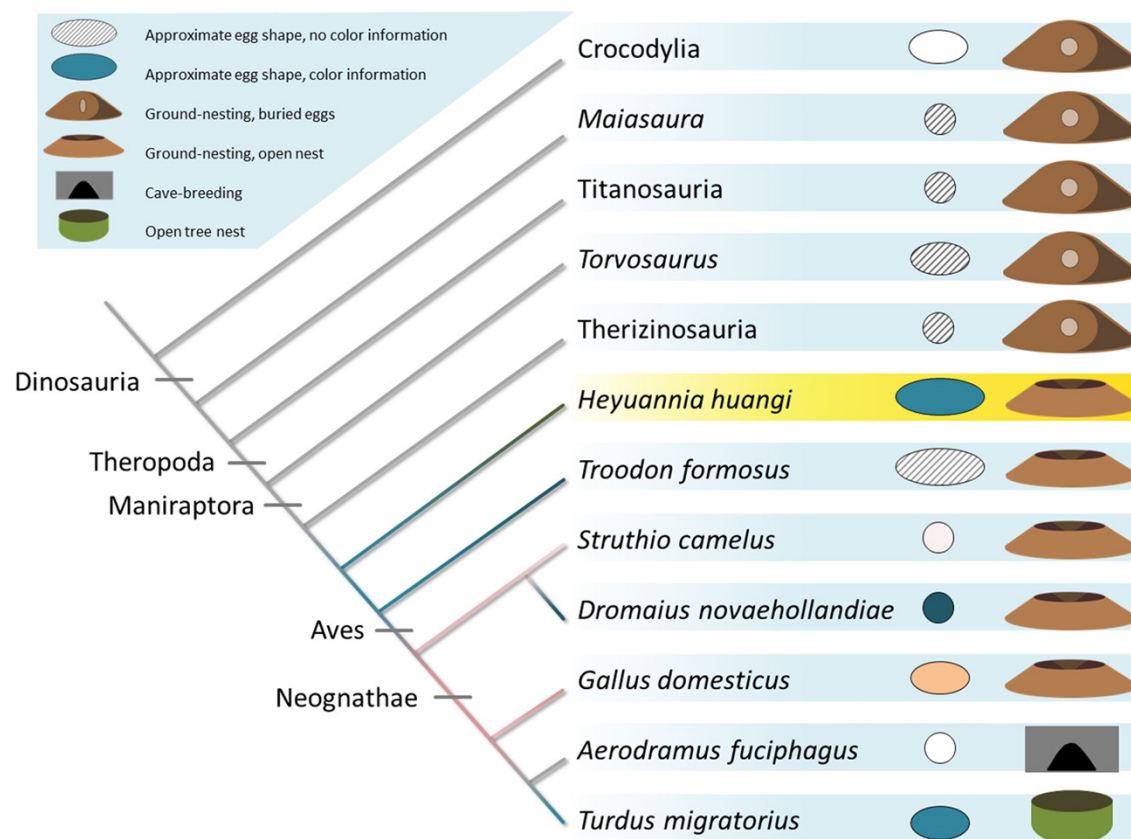



Figure 5 Evolution of egg coloration (egg item), egg shape (egg item), and nesting type (nest item) in archosaurs. Listed (as successive outgroups) are ornithischian dinosaurs, such as *Maiasaura*, for sauropods, such as titanosaurs, and theropod dinosaurs, including *Torvosaurus*, therizinosaurs, oviraptors, troodontids, and crown birds (Varricchio & Jackson 2016). The topology of the tree is based on Nesbitt, 2011(Archosauria), Sereno, 1999 (Dinosauria), Carrano et al., 2012 (Tetanurae), Turner et al., 2012 (Paraves), Prum et al., 2015 (Aves). Porosity data and clutch structure indicate a fully buried nesting type for most dinosaur taxa (Varricchio & Jackson 2016). Buried eggs which are indicated by the nesting item next to the egg item for each terminal taxon appear to be plesiomorphic. Oviraptorid dinosaurs, here represented by *Heyuannia huangi*, built at least partially open nests, concurrent with the phylogenetically most basal appearance of PP and BV in dinosaur eggshell (egg item). In modern birds, eggshell pigmentation varies with nesting microenvironment. Presence and kind of the (more abundant) eggshell pigment is represented by the color of the cladogram branch, and the egg item next to each terminal taxon. Beige egg colors indicate PP being more abundant, whereas bluish egg colors indicate BV being more abundant.

CONCLUSIONS

Our study extends the origin of colored eggs from crown birds to oviraptorid dinosaurs. The result has important implication both for the origin of avian biology and the reproductive

488   biology of theropods dinosaurs. This work also broadens the scope of paleontological research

489   on molecular preservation and ecology to hard vertebrate tissues. Our study ties together

490   previous hypotheses on the eumaniraptoran origin of partially open nesting, and paternal care.

491   Also, potential future avenues for investigation are posed by the potential linkage between blue-

492   green egg color and communal nesting, as well as polyandry, which represent yet unaddressed

493   topics in extinct archosaurs.

494   The second aspect of our work focuses on its implications for molecular and soft tissue

495   preservation through deep time. Chemically stable, relatively small biological molecules such as

496   PP and BV appear to be protected from complete degradation over millions of years in carbonate

497   biomineral matrices, in an oxidative sediment milieu. Similar biomolecule preservation may also

498   be present in enamel, dentine and bone mineral. Ancient biomolecules and the soft tissues which

499   they construct pave the way to trace life and its behaviors through time and, thus, invite further

500   studies since they are easily detectable, more abundant than expected, and revolutionary in their

501   ecological implications.

502

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517

518   Supplemental Information

519   Supplemental information for this article is available (PeerJ).

520

521

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