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1	Reconstructing	carotenoid-based a	and structural	coloration in	fossil skin
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- 19
- 20 Running title: fossil skin colour

22 Summary

23

Evidence of original coloration in fossils provides insights into the visual communication 24 25 strategies used by ancient animals and the functional evolution of coloration over time [1-7]. Hitherto, all reconstructions of the colours of the plumage of fossil birds and feathered 26 dinosaurs and reptile integument have been of melanin-based coloration [1-6]. Extant 27 animals also use other mechanisms for producing colour [8] but these have not been 28 identified in fossils. Here we report the first examples of carotenoid-based coloration in the 29 30 fossil record, and of structural coloration in fossil integument. The fossil skin, from a 10 Ma colubrid snake from the Late Miocene Libros Lagerstätte (Teruel, Spain) [9, 10], 31 preserves dermal pigment cells (chromatophores) – xanthophores, iridophores and 32 33 melanophores - in calcium phosphate. Comparison with chromatophore abundance and position in extant reptiles [11-15] indicates that the fossil snake was pale-coloured in 34 ventral regions; dorsal and lateral regions were green with brown-black and yellow-green 35 transverse blotches. Such coloration most likely functioned in substrate matching and 36 intraspecific signalling. Skin replicated in authigenic minerals is not uncommon in 37 exceptionally preserved fossils [16, 17] and dermal pigment cells generate coloration in 38 numerous reptile, amphibian and fish taxa today [18]. Our discovery thus represents a new 39 means by which to reconstruct the original coloration of exceptionally preserved fossil 40 41 vertebrates.

42

43 **Results**

The integument of vertebrates is a complex system with important functions in homeostasis, 44 sensory reception and, via its coloration, visual signaling [18]. Recent studies have reconstructed 45 the melanin-based [2-6] plumage colours of feathered dinosaurs and birds on the basis of 46 preserved melanosomes [2-5] and feather chemistry as revealed by X-ray mapping [6]. Melanin-47 based pigmentation, however, is only one of several pigment-based mechanisms for producing 48 49 colour [18]; evidence of other pigments has not been reported in fossil vertebrates. Examples of fossilized vertebrate skin are not uncommon and have yielded insights into the biology [19-23] 50 of non-feathered dinosaurs and other fossil reptiles, but evidence of original coloration and 51 patterning in fossil skin has, until now, been limited to rare instances of subtle monotonal 52 patterning [5, 19]. Here we report the discovery of intact dermal chromatophores, the pigment 53 cells responsible for coloration and patterning, in a 10 million year old colubrid snake. We use 54 scanning electron microscopy to analyze the relative abundance and vertical position of the 55 chromatophores from different body regions. By comparing these data to those from extant 56 57 snakes, we reconstruct the original integumentary colour patterns of the fossil snake and reveal their ecological functions. 58

59

The fossil snake (Museo Nacional de Ciencias Naturales (CSIC) MNCN 66503) occurs within Vallesian (11.2–8.7 Ma) oil shales of the Libros Gypsum lacustrine sequence [9, 24], which outcrops 25 km SE of Teruel city, NE Spain (40°07'38"N 1°12'1"W). The specimen was recovered during mining operations in the early 20th century; stratigraphic data are not available. It is in lateral aspect and lacks a cranium (Figure 1A), and is assigned to the Colubridae. A more precise taxonomic determination is not possible in the absence of a cranium. The specimen is on permanent display at Dinópolis palaeontological museum in Teruel, Spain.

68 Ultrastructure and chemistry of the fossil snake skin

The fossil skin extends from the vertebrae to the ventral termini of the ribs (Figures 1A, 1B); 69 overlapping scales are evident (Figure 1B). Scanning electron microscopy reveals that the fossil 70 skin, as with many fossilized decay-prone tissues [25], is replicated in calcium phosphate. It 71 72 exhibits a tripartite division into a thin (6-9 µm thick) outer layer that is structureless and nanocrystalline, a thicker (15-25 µm thick) central layer that contains mineralized fibres and 73 74 oblate to spheroidal bodies, and a thick $(100-180 \ \mu m)$ lowermost layer that comprises a plywood-like array of fibres (Figures 1C, 1D). These fossil skin layers correspond to the main 75 layers of the skin in extant reptiles [8, 18], i.e. the epidermis (comprised of keratinized cells), 76 upper dermal stratum spongiosum (loosely packed collagen fibres and chromatophores (pigment 77 cells)) and lower dermal stratum compactum (a dense orthogonal array of collagen fibres). The 78 79 stratum compactum in the fossil snake is locally underlain by a thin (8–13 μ m thick) structureless 80 layer (Figure 1C) that represents the remains of the basement membrane which in extant reptiles separates the skin from the underlying hypodermis [8, 18]. 81

82

The most striking features of the stratum spongiosum in the fossil snake skin are abundant oblate to spheroidal bodies, consistently located immediately below the epidermal-dermal boundary (Figures 1D–K; Figure 2). These bodies fall into three types that are differentiated on their location, size, morphology and internal fill.

87

Type 1 bodies occur at the top of the array; they are small $(1-5 \ \mu m \ x \ 0.4-2 \ \mu m)$ cryptocrystalline discs (Figures 1E, 1F) that can be organized into a layer up to four discs thick (Figures 2C, 2H,

90	2I, Figures S1E–H). As with other features of the skin, the discs are frequently separated from
91	the surrounding matrix by a void (Figure 1F).
92	
93	These discs are underlain by Type 2 bodies, which are larger (3–8 μ m long) irregular spheroids
94	to ovoids that comprise granules of two types: small (0.15–0.4 μ m) subspherical granules with
95	irregular to rounded outlines, and larger (0.8–1.2 μ m) rounded granules with smooth outlines
96	(Figures 1G, 1J). The relative proportions of the two granule types are similar and consistent
97	among the Type 2 bodies (smaller vesicles: $44.2 \pm 4.7\%$; n = 38).
98	
99	The Type 2 bodies are underlain by larger (8–20 μ m long) ovoid features with smooth outlines,
100	and prominent lateral processes. These Type 3 bodies contain densely packed granules with a
101	narrow size distribution (0.18–0.3 µm) (Figures 1H, 1I, 1K).
102	
103	Elemental mapping of the fossil snake skin reveals that the bodies in the stratum spongiosum and
104	dermal collagen fibres contain elevated concentrations of S, and lower concentrations of C and P,
105	relative to other ultrastructures in the skin (Figure 3). No other elements show spatial partitioning
106	among the various structures in the skin.
107	
108	Discussion
109	Interpretation of the bodies as fossil chromatophores
110	The bodies preserved in the stratum spongiosum of the fossil snake are unlikely to be skin
111	glands: in extant snakes, skin glands are restricted to a pair of anal scent glands [18]. Similarly,
112	there is no evidence that the bodies (or their internal granular fill) represent fossilized decay

bacteria [see 26]. The disc-like morphology of the Type 1 bodies is not consistent with that of 113 bacteria. The Type 2 and 3 bodies are too large to represent bacteria, which are usually $0.5-2 \,\mu m$ 114 115 long [27]. Fossil bacteria would be expected to infest the entire tissue during decay (including the dermis), not just specific features such as the interior of the chromatophores. Bacteria could 116 also generate a characteristic texture whereby they pseudomorph the gross geometry of the 117 118 original tissue; if replicated in calcium phosphate this is termed a microbial microfabric [28]. Further, preserved bacteria are not associated with other fossils from Libros: recent geochemical 119 analyses reveal that microbe-like microstructures associated with fossil amphibians from Libros 120 can be convincingly identified as preserved melanosomes [29]. The bodies preserved within the 121 uppermost stratum spongiosum of the fossil snake skin are therefore interpreted as fossil 122 chromatophores, which are common components of the upper stratum spongiosum in extant 123 snakes [18]; the three types of body are interpreted as three different chromatophore types. The 124 skin of the Libros snake is thus preserved as a substrate microfabric [28] whereby 125 126 nanocrystalline calcium phosphate has faithfully replicated the ultrastructure of the tissue. 127 Certain pigments, including melanins, pteridines and carotenoids are known to have an affinity 128 129 for metal cations [30-32]. Elevated levels of sulfur in the dermal chromatophores and collagen fibres may reflect the presence of sulfur-bearing moieties in the original tissue structures [33, 34] 130 131 or the incorporation of sulfur (in the form of sulfate) into the replacement phosphate during 132 mineralization [35]. There is no evidence, however, for partitioning of trace elements among the various chromatophores in the fossil snake skin (Figure 3). This may reflect concentrations 133

below detection limits (< 100 ppm) or overprinting of the original trace element chemistry during

the mineralization process. The fossil chromatophores are therefore interpreted on the basis of

their size, geometry and, in some examples, internal structure compared with those in extant
reptiles [8, 11-15] (Figures 1E-K, Figures S1-S5). Some of the chromatophores in the fossil skin
are present as external moulds; their affinities are resolved by their shape and study (at high
magnification) of the surface texture of the mould (see insets in Figure S1D).

140

141 In extant reptiles, dermal melanophores are readily identified by their position at the base of the chromatophore array, their large size (10–30 μ m wide), prominent lateral processes, and infill of 142 small granules of melanin (melanosomes) with a narrow size distribution [8, 12]. Dermal 143 melanophores typically exhibit ovoid geometries when in the contracted state (whereby 144 melanosomes are restricted to the main body of the melanophore [36]) and have few lateral 145 processes [36] and a low packing density (Figure 1 in [12], Figure 7 in [36], Figure 8 in [37]). 146 The melanosomes vary in size among modern taxa ($0.15-0.8 \,\mu m \log x \, 0.25-0.5 \,\mu m$ wide) but 147 for a given taxon have a small size range (Figure 2a in [11], Figure 1 in [12], Figure 3 in [13], 148 149 Figure 7 in [14], Figure 1 in [15], Figure 1 in [35], Figure 5 in [37]). The Type 3 bodies in the fossil snake skin share all the main characteristics of, and are thus best interpreted as, dermal 150 melanophores. 151

152

Iridophores are small chromatophores (usually 5–10 μ m wide [12, 14, 18]) that have irregular to flattened or disc-like morphologies. They can form vertical stacks up to four cells thick [12] and can occur at the top of the chromatophore array [11, 12] or below an upper layer of xanthophores [12, 14, 15]. The Type 1 bodies in the fossil snake also have a flattened geometry and occur in stacks in some body regions; these features are consistent with an interpretation as iridophores but not as any other ultrastructural feature of the skin. The small size of the fossil iridophores (1– 159 $5 \mu m$) may reflect taxonomic factors (as with melanophores, above) or degradation during the 160 fossilization process. In extant reptiles, iridophores contain angular crystalline platelets of the 161 purines guanine, hypoxanthine or adenine [18]. These platelets are not preserved in the fossil 162 snake, but this is not unexpected: guanine is soluble in dilute acids [38], which are typical 163 products of decay [25].

164

Xanthophores in extant snakes are typically 3–10 µm long and have irregular to spheroidal or 165 ovoid geometries [12, 18]. They have been defined as chromatophores that contain abundant 166 granules of carotenoids and pteridines [12, 18]; others differentiate between primarily 167 carotenoid-bearing xanthophores, and primarily pteridine-bearing erythrophores [12, 18]. The 168 former definition is used herein. Granules of pteridines – pterinosomes – are vesicles $(0.3-1 \,\mu m)$ 169 with a smooth rounded surface, spherical to elongate geometry and internal concentric laminae 170 [13] (Figure 2A in [11], Figure 10 in [12], Figure 10 in [14], Figure 5 in [37], Figure 4 in [39]). 171 172 Carotenoid granules are smaller $(0.15-0.45 \,\mu\text{m})$ and have smooth (Figure 1 in [36]) or irregular (Figure 2 in [12]) outlines, i.e. subrounded to angular geometries [12]. The Type 2 bodies in the 173 fossil snake occur below the iridophores and above the melanophores, and have irregular 174 175 spheroidal to ovoid outlines. The internal granules fall into two discrete types: small subspherical granules with irregular outlines, and larger rounded granules with smooth outlines; these most 176 177 likely correspond to fossil carotenoid and pterinosome vesicles, respectively. The similar 178 proportions of the two granule types in the Type 2 bodies is not consistent with an interpretation 179 as erythrophores [12]. The most parsimonious interpretation is therefore that the irregular spheroidal to ovoid chromatophores in the fossil snake represent xanthophores filled with a 180 181 combination of large pterinosomes and smaller carotenoid granules.

183 **Relating chromatophores to visible hue**

In extant reptiles, the visible hue of the integument is produced by a combination of dermal 184 chromatophores, epidermal melanocytes and epidermal diffraction gratings. In the fossil snake, 185 the epidermis is poorly preserved and thus the former presence of epidermal melanocytes and 186 187 surficial diffraction gratings cannot be determined. The contribution of these features to visible hue and patterning, however, would have been minimal [12, 18, 36, 40]. Epidermal melanocytes 188 are not involved in creating colour patterning [12, 18]; they typically occur only in skin regions 189 of dark brown to black hue, enhancing the effect of a thick dense layer of dermal melanophores 190 [41]. Epidermal diffraction gratings generate weak spectral iridescence that is superimposed on 191 colour patterns generated by dermal chromatophores, which are the primary contributors to 192 visible hue [40]. 193

194

195 Our interpretation of the original colour of the fossil snake is therefore based entirely on the dermal chromatophores. Samples of skin from different body regions of the fossil snake exhibit 196 systematic differences in the type, and relative abundance, of chromatophores (Figures 1C, 2, 197 Figures S1-S5; Table S1); these differences are statistically significant ($\chi^2 = 42.6$; df = 3, 5; $\chi^2_{8} =$ 198 20.09, p < 0.01). There is no evidence that this variation reflects taphonomic factors. The fidelity 199 200 of preservation of the chromatophores does not vary with chromatophore abundance, i.e. the 201 chromatophores are equally well preserved (in terms of definition of external margins and nature of internal fill) where rare and abundant (compare Figures 2A and 2F). Further, the overall 202 fidelity of preservation of the skin does not vary among different body regions, e.g. collagen 203 204 fibres are preserved with equal fidelity throughout. There is thus no evidence that certain regions of the skin were subjected to more extensive decay than others and that the preserved abundance
 of melanosomes is a taphonomic artefact.

207

Synthesis of published literature on reptile chromatophores (Table S2) and primary observations 208 (Figure S4) reveal that in extant reptiles, specific combinations of chromatophores correspond to 209 210 different hues (Table S2). The colours of the fossil snake can thus be reconstructed based on the relative abundance and stratigraphy of the chromatophores. In extant reptiles, iridophores scatter 211 light from crystals of guanine and other purines through thin film interference [8]. Xanthophores 212 are capable of producing a range of yellow, orange and red hues, depending on the relative 213 proportions of carotenoid granules and pterinosomes present [12, 18]. Xanthophores with equal 214 amounts of both granule types – as in the fossils – produce yellowish hues [12]. Melanophores 215 produce brown to black hues as their melanosomes absorb most, or all, wavelengths of light [12]. 216 217

218 Samples 1, 2, 3, 5 and 7 are from lateral body regions; 4 is dorsal, and 6, ventral. Patterning in snakes is typically repeated along the length of the body [8] and thus our colour reconstruction, 219 based on comprehensive sampling of one body region, can be extrapolated to the remainder. 220 221 There is no evidence that the fossil snake skin exhibited white, red, blue, or grey hues. All skin regions studied preserve chromatophores, eliminating the possibility of white hues [12]. There is 222 223 no evidence that any xanthophores comprised primarily pterinosomes, eliminating the possibility 224 of red hues [12, 18, 41]. No skin regions exhibited only iridophores and melanophores, eliminating the possibility of structural blue [11], structural green (Figure S4) and grey hues. 225 Iridophores can reflect specific, or all, visible wavelengths depending on the thickness and 226

organization of the internal purine platelets [8]. Given that the latter are not preserved in thefossils, we cannot comment on their potential contribution to the original colour.

229

Iridophores and xanthophores are abundant and melanophores common in two samples from 230 lateral body regions (samples 5 (Figure 1E) and 7 (Figures 2A, S2)). In extant reptiles, similar 231 232 chromatophore architectures (in particular, the presence of carotenoid-bearing xanthophores and the position of iridophores at the top of the chromatophore array) are associated with green hues 233 [11]. In other lateral body regions (sample 2) melanophores are more abundant and iridophores 234 and xanthophores, less abundant (Figures 2B, S2), suggesting darker, less saturated green hues. 235 Skin samples from other lateral body regions (sample 1) exhibit stacks of iridophores up to four 236 cells thick (Figure 2C), indicating brighter green hues: layering of iridophores markedly 237 increases integument albedo [42]. Conversely, other lateral regions (sample 3) exhibit abundant 238 melanophores; xanthophores are common and iridophores, rare to absent (Figure 2D, S3), 239 240 characteristic of dark brown/black tones [11]. In dorsal regions (sample 4) xanthophores are abundant, iridophores, common and melanophores, rare (Figures 2E, S3), indicating yellowish to 241 pale brown hues [18]. In ventral regions (sample 6), iridophores and xanthophores are abundant, 242 243 and melanophores, rare to absent (Figures 2F, S1), corresponding to cream-coloured hues [40]. 244

The fossil snake can therefore be reconstructed as green with brown/black blotches on its dorsal and lateral surfaces, and pale ventrally (Figure 4). Similar coloration characterizes some extant Colubrid snakes, e.g. *Nerodia floridana* and *Dispholidus typus*.

248

249 **Broader implications**

Green coloration is an effective adaptation for substrate matching in foliage [43]. This cryptic 250 visual signal was enhanced by two pattern elements. The superimposition of brown/black tones 251 252 on the green background formed a disruptive pattern to conceal the body contours [43]. Countershading via dark and light colours on dorsal and ventral surfaces, respectively, decreases 253 apparent relief [44]. Complex patterning indicates a diurnal lifestyle and strong selection for 254 255 substrate matching to reduce visibility to visual predators [45]. Patterning in extant reptiles often comprises a mosaic of elements reflecting antagonistic selective pressures relating to 256 homeostasis and signalling [11]. Bright hues may impact negatively on survival but are 257 implicated in social interactions [46]. Thus the patterning in the fossil snake probably served 258 dual functions in camouflage and intraspecific signalling. 259

260

Until now, reconstructions of the original coloration of fossil vertebrates have been of melanin-261 based mechanisms and from soft tissues preserved as carbonaceous remains. Reconstructions of 262 263 the original colours of vertebrates preserved via this pathway have not been able to incorporate contributions from non-melanin-based coloration mechanisms [3]. Maturation experiments 264 simulating aspects of the organic preservation process have shown that non-melanin-based 265 266 coloration mechanisms have a lower preservation potential than those that are based on melanin [47]. Our discovery confirms that direct evidence for diverse coloration mechanisms can be 267 268 preserved in fossils preserved via an alternative preservation pathway, namely replication of 269 tissues in authigenic minerals, and that the high fidelity of preservation allows original coloration 270 to be reconstructed. The various factors that control phosphatization of soft tissues are known 271 [25] and fossil examples of phosphatized skin are not uncommon; importantly, they have been 272 reported from various taxa and fossil localities [16, 17], suggesting that our discovery has broad

applications in the fossil record. Our discovery should prompt a search for other examples, and is
likely to be the first example of a recurrent phenomenon. Integuments replicated in calcium
phosphate are obvious targets for further attempts to reconstruct colour patterns derived from
melanin and, critically, other pigments and structural coloration mechanisms, across diverse
vertebrate groups.

280 Electron microscopy

281 Samples of fossilised skin were prepared for scanning and transmission electron microscopy as

in [7]. Samples of skin from the extant snake Ahaetulla prasina were frozen with liquid N₂ and

fractured with a scalpel. Samples were examined using a FEI XL-30 ESEM-FEG SEM, a FEI

284 Quanta 650 FEG SEM and a Hitachi S-3500N variable pressure SEM at accelerating voltages of

²⁸⁵ 5–15kV and a JEOL 2100 TEM at an accelerating voltage of 200 kV.

286

287 Electron probe microanalysis

288 Samples of fossilised skin were embedded in resin, polished, and examined using a JEOL JXA

- 289 8530F Electron Microprobe. All maps were produced in wavelength dispersive X-ray
- spectroscopy mode at an accelerating voltage of 15 kV, current of 10 nA and dwell time of 500

ms per pixel.

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293

294 Histology

Samples of skin from the extant snakes *Ahaetulla prasina*, *Crotalus scutulatus* and *Thamnophis sirtalis* were fixed and dehydrated as in [7] and embedded in paraffin wax. 30 µm thick sections
were stained using haematoxylin and eosin.

298

299 Author contributions

MMN designed the study and wrote the manuscript with input from all other authors. SK carried
 out EPMA analyses, and MMN, all other analyses.

302

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309

310 Data Accessibility

311 Data are available at the Cork Open Research Archive (CORA) (<u>http://cora.ucc.ie</u>). Requests for

specimens and samples should be addressed to MMN.

313

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427 Figure legends

428	Figure 1. Preserved skin in a fossil colubrid snake (MNCN 66503). (A) Entire specimen; inset,
429	anterior. Cream-coloured material is fossil skin. Numerals 1–7 indicate sample locations. (B)
430	Overlapping scales. (C–E) Scanning electron micrographs (SEMs) of fractured vertical sections
431	through the skin, showing epidermis (Epi), dermis (De), basement membrane (B),
432	chromatophores (iridophores (I), melanophores (M), xanthophores (X)), stratum spongiosum
433	(Sp), stratum compactum (Sc), and collagen fibres (C). (F–I) Details of iridophore (F),
434	xanthophore (G), melanophores (H, I). (J, K) Transmission electron micrographs of xanthophore
435	(J), melanophore (K). The voids in SEM images typically represent structures that have
436	separated into the counterpart of the sample during preparation.
437	
438	Figure 2. SEMs of vertical sections through the fossil skin showing variation in the relative
439	abundance of different chromatophores (A–C, G–I) with interpretative drawings (D–F, J–L).
440	Encircled numerals correspond to sample numbers in Figure 1(A). (A) Abundant xanthophores,
441	common iridophores and melanophores. Epi., epidermis. (B) Common iridophores and
442	xanthophores, occasional melanophores. (C) Abundant iridophores, common melanophores and
443	xanthophores. (G) Abundant melanophores, common xanthophores, rare iridophores. (H)
444	Abundant xanthophores, occasional iridophores, rare melanophores. (I) Abundant xanthophores
445	and iridophores, rare melanophores. See also Figures S1–S4.

Figure 3. Electron probe microanalysis X-ray maps of a polished vertical section through the
skin of the fossil snake MNCN 66503. Areas mapped in (A) and (B) show the uppermost stratum
spongiosum; the upper surface of the skin is to the left in (A) and associated elemental maps for
C, Mg, Al, P, S, Cl, K, Ca, Mn, Fe and Cs, and to the top of (B) and associated maps for Co, Cu

and Zn. C, collagen fibre; M, melanophore; X, xanthophore. Limited variation in tone in maps
for Cu, Co and Zn indicate consistently low concentrations of these elements over the area
analyzed; colour scale for all other images ranges from blue (low values) to red (high values).
Scale bars: 10 µm.

455

Figure 4. Colour reconstruction of the fossil snake MNCN 66503. (A) Schematic representation
of the relative abundance and position of chromatophores in samples of skin from different body
regions. Numbers denote samples discussed in the text. See also Tables S1, S2. (B) Colour plate
is by Jim Robbins.

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McNamara et al. Figure 3