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Specimen details

•FUM-N-1450 is the holotype of *Eosphargis breineri* Nielsen, 1959, and it includes the articulated remains of a large-sized (estimated total body length ~1.5 m) leatherback turtle. The specimen was collected at the Knudeklint locality on the Island of Fur in northern Jutland, Denmark[S1,S2]. The fossil is preserved in calcareous concretions originating from the stratigraphic level 'ash layer minus 33' of the Fur Formation[S3]. This particular deposit has been associated with the Island of Lundy eruption in the Bristol Chanel[S3], and its age has been estimated to be about 55 Ma (Early Eocene)[S4]. Because the fossil-bearing nodules were found embedded in ash, it is conceivable that the turtle perished from suffocation during the eruption event, a scenario that also may explain the absence of bite marks and scratches inflicted by scavenging bony fish and/or sharks. Presumably, the carcass ended up in the anoxic bottom sediments, tilted somewhat to the left, and subsequently was encased in three lens-shaped nodules comprising the rear of the animal, the anterior left of the trunk, and the skull, respectively. Blackish soft tissue matter was found in the concretion containing the posterior part of the turtle, and appears to originate from the area between the left hind limb and anus. FUM-N-1450 has not been treated with any preservative or consolidant.

•SMU 76532 consists of a semi-articulated skeleton (preserved portion ~11 m in length) assigned to the tylosaurine mosasaur *Tylosaurus nepaeolicus*[S5]. The specimen was found approximately 1.6 km southeast of Terlingua ghost town in the Late Coniacian (~86 Ma) San Vicente Member of the Boquillas Formation. Portions of the skull, including the diagnostic quadrates, were recovered along with a number of trunk vertebrae, part of the anterior tail, pelvic girdle, and hind limb elements associated with a scattering of smaller materials representing stomach contents (including the durophagous shark *Ptychodus mortoni* and another mosasaur referred to the plioplatecarpine genus *Platecarpus*[S5]). An articulated section of rhomboidal 'scales' (~40 × 35 mm) preserved as matt blackish material was discovered during the excavation of SMU 76532. The 'scales' are relatively uniform in size (measuring 1.5–2.3 mm in length and 2.0–2.6 mm in width), and obliquely arrayed into an alternating pattern. The slab preserving the 'scales' was originally treated with very thin polyvinyl butyral dissolved in acetone; however, further preparations in 2012 uncovered an area with untreated 'scales'. Both treated and untreated 'scales' were examined in this study. Data from the latter are presented in Fig. 3 and Extended Data Figs 2 and 3.

•YORYM 1993.338 consists of an essentially articulated section of ichthyosaur caudal vertebrae (overall length of segment ~21 cm) with associated soft tissues preserved as blackish material, found in a calcareous concretion from the Sinemurian (~196–190 Ma) of Black Ven, Dorset, England (see ref. 11 for details). Even though the fossil preserves in exquisite detail the outline of a lunate tail fin in left lateral view, it has not been possible to confidently assign it to a particular genus or species[11]. YORYM 1993.338 was mechanically prepared without application of preservatives.

Fossil melanosome measurements

•FUM-N-1450: mean length = 812.6 nm [number measured (*n*) = 75, standard deviation (SD) = 195.7, min = 450.4, max = 1439.0], mean width = 467.8 nm (*n* = 75, SD = 88.7, min = 278.4, max = 674.3).

•SMU 76532: mean length = 504.9 nm (*n* = 75, SD = 125.8, min = 207.6, max = 808.9), mean width = 323.0 nm (*n* = 75, SD = 66.5, min = 184.4, max = 523.3).

•YORYM 1993.338: mean length = 778.5 nm (*n* = 75, SD = 192.2, min = 409.8, max = 1245.1), mean width = 483.9 nm (*n* = 75, SD = 97.1, min = 318.8, max = 651.3).

Additional material examined

In addition to the fossil specimens described above, the following molecules and structures were examined in this study (a selection of spectra from compounds with a molecular structure similar to that of eumelanin is presented in Extended Data Fig. 5):

Melanins

- Natural eumelanin from the cephalopod *Sepia officinalis* (Sigma-Aldrich).
- Natural melanin (mostly phaeomelanin) from feathers of the Rhode Island Red chicken, *Gallus gallus domesticus* (extracted according to protocol in ref. S6).
- Synthetic eumelanin (Fisher Scientific).

Chromatophore compounds

- Xanthophyll from marigold (Sigma-Aldrich).
- Guanine (Sigma-Aldrich).
- Isoxanthopterin (Sigma-Aldrich).
- Riboflavin (Sigma-Aldrich).

Porphyrins

- Coproporphyrin I dihydrochloride (Sigma-Aldrich).
- Hemin chloride (MP Biomedicals).
- Chlorophyll *a* from *Anacytis nidulans* (Sigma-Aldrich).

Compounds structurally similar to melanin

- Copper (II) phthalocyanine (Sigma-Aldrich).
- Copper (II) acetylacetonate (Sigma-Aldrich).

Microbial mat samples

These were obtained from a methane seep on the NW Black Sea shelf (one sample)[S7] and from rock surfaces in the Tunnel of Äspö (Äspö Hard Rock Laboratory, SE Sweden; two samples from sites located at 2,200 and 3,440 m from the tunnel entrance, respectively). The Black Sea sample is largely comprised of methanotrophic archaea, whereas the Äspö mats represent phototrophic systems with abundant cyanobacteria. The microbial mats were analysed as cryosections, as previously described[S7]. For spectral data, see ref. 16.

Spectral differences between fossil 'skin' samples and melanin standards

Although our ToF-SIMS analyses demonstrate a detailed agreement between the spectra acquired from the three fossil 'skin' samples and the two eumelanin standards, including major peaks at 49, 50, 66, 73, 74, 97, 98, 121, 122, 145, and 146 u, substantial differences also do occur. The latter are mainly of two types (Extended Data Fig. 2):

(i) A substantially increased signal from peaks corresponding to sulphur-containing organic fragments of C_nHS^--type, where n = 2 (57 u), 4 (81 u) and 6 (105 u), in the fossil spectra compared to the natural eumelanin spectrum (the synthetic eumelanin spectrum shows no substantial signal from these peaks).

(ii) A consistently lower signal from peaks corresponding to C_nN^-, where n = 3 (50 u), 5 (74 u), 7 (98 u), 9 (121 u), and 11 (146 u), in relation to the preceding peak; that is, C_nH^-, where n = 4 (49 u), 6 (73 u), 8 (97 u), 10 (120 u), and 12 (145 u), in the fossil spectra compared to the natural eumelanin spectrum.

The natural 'phaeomelanin' spectrum (Extended Data Fig. 5) displays prominent peaks assignable to sulphur-containing organic fragments, including C_nHS^- [where n = 2 (57 u), 4 (81 u) and 6 (105 u)], C_nNS^- [where n = 1 (58 u), 3 (82 u), 5 (106 u), and 6 (118 u)] and C_6NSO^- (134 u), in addition to unidentified peaks with elevated intensities occurring at 99, 100, 149, and 150 u, respectively (peaks at 63 and 79 u belong to phosphate-containing impurities). Substantial signal from characteristic eumelanin peaks is also present in the 'phaeomelanin' spectrum in the mass range < 100 u, and the peaks show a relative intensity distribution similar to that of the natural eumelanin spectrum. This is likely a consequence of

eumelanin impurities in the extracted 'phaeomelanin' sample (almost all extant avian melanosomes contain a mixture of eu- and phaeomelanin[29]).

Spectral differences between our samples were evaluated by principal component analysis (PCA). Extended Data Fig. 6 shows a score plot and a loadings plot from a PCA analysis that includes our three fossil 'skin' samples, different melanins and a selection of other molecular standards (compounds showing few spectral similarities with eumelanin and/or the fossil samples were not included in the analysis). A total of 57 peaks, selected from the synthetic eumelanin (43) and 'phaeomelanin' (14) spectra, were included in the analysis (Extended Data Fig. 6c). The score plot shows distinct separations between the various samples (Extended Data Fig. 6a), indicating substantial spectral differences. Characteristics of these dissimilarities are visualised in the loadings plot (Extended Data Fig. 6b). The position of each peak indicates that it has a relatively high signal intensity in spectra located at a corresponding position in the score plot (and, conversely, that spectra located in other areas have relatively lower intensities of this particular peak). The score plot shows the eumelanin standards associated with the mosasaur and leatherback turtle spectra to the right (positive PC1 scores), whereas 'phaeomelanin' and the other standards are located to the left (negative PC1 score). The ichthyosaur spectra are positioned in the lower central part of the diagram (negative PC2 score). Close inspection of the loadings plot shows that with the exception of the C_nHS^- peaks, the 'phaeomelanin' peaks are all localised to the region of the 'phaeomelanin' spectra in the score plot. The C_nHS^- peaks are, on the other hand, located in the region of the ichthyosaur spectra, indicating that these peaks are particularly strong in that sample. Comparison between the 'phaeomelanin' and ichthyosaur spectra (both directly and in the PCA analysis) thus shows that it is mainly C_nHS^--related peaks occurring in the ichthyosaur spectra, while the other 'characteristic' 'phaeomelanin' peaks show much lower signal intensities in the ichthyosaur spectra than would be expected had they been derived from this biochrome. When comparing the fossil spectra with one another, their relative positions in the score plot indicate an increasing C_nHS^- signal in the order: mosasaur < leatherback turtle < ichthyosaur.

Even though we cannot exclude minor contributions from phaeomelanin pigment in the fossil 'skin' spectra due to the presence of sulphur, the results from our PCA analysis suggest that the sulphur-containing fragment ions originate largely from another source, presumably the diagenetic incorporation of sulphur with the eumelanin molecule[S8]. This hypothesis is corroborated by: (1) the co-localisation of sulphur-containing fragment peaks [C_nHS^-, where n = 2 (57 u), 4 (81 u) and 6 (105 u)] with eumelanin peaks in the ion images (Extended Data Fig. 4); and (2) the fact that these peaks are also present in the natural eumelanin spectrum, albeit at lower relative intensities when compared to the fossil spectra (Extended Data Fig. 2). *Sepia* melanin is generally considered to be pure eumelanin[S9], and although the origin of sulphur atoms in modern eumelanin pigments is not fully understood[S10,S11], sulphur incorporation is nonetheless possible[S10]. Additionally, as stated above, the other 'characteristic' 'phaeomelanin' peaks show much lower signal intensities in the ichthyosaur spectra than would be expected had they instead been derived from phaeomelanin. Hence, the relatively close localisation of the 'phaeomelanin' and ichthyosaur spectra in the score plot (Extended Data Fig. 6a) is by all likelihood a combination of: (1) eumelanin residues in the modern 'phaeomelanin' spectrum; and (2) diagenetically induced sulphur incorporation with the fossil eumelanin structure.

Compared to the eumelanin standard spectra, the fossil spectra are all located in the lower part of the score plot (lower PC2 scores). Examination of the eumelanin region in the loadings plot (positive PC1 scores) thus provides information regarding differences in relative intensity distribution of the eumelanin peaks between the eumelanin standards and fossil samples. For instance, it is readily apparent that peaks assigned to C_nN^-, where n = 3, 5, 7, 9,

11, and 13, are located in the upper part of the loadings plot (positive PC2 loadings), suggesting that these peaks are strong in the eumelanin spectra relative to the fossil spectra. Similarly, peaks assigned to C_nH^-, where n = 4, 6, 8, and 10, are located in the lower part of the loadings plot (negative PC2 loadings), indicating that these peaks are relatively strong in the spectra acquired from the fossil samples. Because these differences appear consistently, they presumably reflect minor modifications of the eumelanin molecular structure during diagenesis.

Rationale for assignment of microstructures to fossilised melanosomes

Although some microbes are able to produce melanin[S12–S14], we find it highly unlikely that the eumelanin-containing structures we identified in three phylogenetically diverse specimens all represent decomposed prokaryote mats[7,13,S15], because the pigment is associated with integument consistent with significant melanisation in living reptiles (Extended Data Fig. 7)[3,4,S16]. The fossil microbodies presented herein are also solid with a seemingly homogenous internal content (Fig. 3c, arrowheads), similar to melanosomes of extant organisms[17]. Conversely, purified bacterial melanins form amorphous deposits[S17], due to the fact that melanin production generally occurs extracellularly in these microbes[S14], and melanin is usually localised to the cell wall in eukaryotic microorganisms, resulting in hollow structures (so-called 'melanin ghosts'[S12–S14]). Furthermore, melanised microbe ghosts frequently display crater-like bud scars[S12,S13], marks that are absent on the surface of the ancient eumelanin-containing microbodies. Hence, we conclude that the microstructures preserved in FUM-N-1450, SMU 76532 and YORYM 1993.338 are the fossilised remains of endogenous pigment organelles.

It has previously been shown that multimillion-year-old melanosomes contain almost exclusively melanin, suggesting that other biomolecules, such as proteins and lipids, are lost during the process of fossilisation[16]. In extant animals, melanosomes develop in specialised pigment cells (melanophores/melanocytes) through four morphologically distinct stages and melanin deposition increases with organelle maturation[S18]. Thus, it is reasonable to assume that fully melanised melanosomes are more likely to be retained in the fossil record, and that the complex, cross-linked polymeric structure of melanin prevents these colour-producing organelles from disintegrating despite an absence of lipids and structural proteins in their fossilised state. However, some slight alterations in size and geometry may have occurred, and are presumably the effects of temperature and pressure during burial[S19].

Finally, it should be pointed out that the taphonomy of skin is different from that of feathers[S20], as is the deposition of melanin within each of these tissues[S21]; it is currently unknown what effects these factors may have on the mechanism(s) and likelihood of melanin preservation in the fossil record.

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