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1 **Characterisation of melanosomes involved in the production of non-iridescent structural feather**

2 **colours and their detection in the fossil record**

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12 **Abstract**

13 Non-iridescent structural colour in avian feathers is produced by coherent light scattering through quasi-

14 ordered nanocavities in the keratin cortex of the barbs. To absorb unscattered light, melanosomes form a

15 basal layer underneath the nanocavities. It has been shown that throughout Aves, melanosome morphology

16 reflects broad categories of melanin-based coloration, as well as iridescence, allowing identification of

17 palaeocolours in exceptionally preserved fossils. However, no studies have yet investigated the morphology

18 of melanosomes in non-iridescent structural colour. Here, we analyse a wide sample of melanosomes from

19 feathers that express noniridescent structural colour from a phylogenetically broad range of extant avians

20 to describe their morphology and compare them with other avian melanosome categories. We find that

21 investigated melanosomes are typically wide (approx. 300 nm) and long (approx. 1400 nm), distinct from

22 melanosomes found in black, brown and iridescent feathers, but overlapping significantly with

23 melanosomes from grey feathers. This may suggest a developmental, and perhaps evolutionary, relationship

24 between grey coloration and non-iridescent structural colours. We show that through analyses of fossil

25 melanosomes, melanosomes indicative of non-iridescent structural colour can be predicted in an Eocene

26 stem group roller (Eocoracias: Coraciiformes) and with phylogenetic comparative methods the likely hue

27 can be surmised. The overlap between melanosomes from grey and non-iridescent structurally coloured

28 feathers complicates their distinction in fossil samples

29 where keratin does not preserve. However, the abundance of grey coloration relative to non-iridescent

30 structural coloration makes the former a more likely occurrence except in phylogenetically bracketed

31 specimens like the specimen of Eocoracias studied here.

32

33 **1. Introduction**

34   Melanosomes produce colours in avian feathers through the selective absorption and reflection of

35 specific wavelengths of light by the melanin pigment molecules they contain [1]. Structural colours, on the

36 other hand, are produced by light scattering through ordered nanostructural arrangements of keratin,

37 melanosomes and/or air within the feather [2]. The differences in refractive index between these contrasting

38 phases generate some of the most vivid colours known in nature. Iridescent structural colours are produced

39 through angle-dependent, coherent light scattering by layers of melanosomes and keratin in the feather

40 barbules [2]. Another, distinct mechanism of structural colour production is generated by coherent

41 scattering of light by a medullary or spongy layer underneath a keratin cortex, and above a basal

42 melanosome layer [2–4] which serves to absorb unscattered light. Unlike iridescence, this type of structural

43 colour is consistent from different viewing angles and is located in feather barbs. It is generally referred to

44 as non-iridescent structural colour [5] and produces a range of colours that is perceived by human observers

45 in the spectrum between blue, violet and turquoise [4]. When combined with yellow pigments (for example

46 carotenoids), it can also produce green hues [4]. The basal melanosome layer is important in the production

47 of non-iridescent structural colour as it absorbs incoherently scattered white light, preventing it from being

48 reflected back to an observer. In the case of amelanism, a genetic disorder causing a lack of melanin

49 biosynthesis in birds, the resulting colour of birds that would normally exhibit non-iridescent structural

50 colours is pale, or 'washed-out' [5].

51   Melanin has been shown to have high preservation potential in fossils, both at a chemical and

52 macrostructural level, often preserving the three-dimensional morphology of the lysosome-derived

53 organelles containing the melanin (melanosomes) as well as the spherical granules of cephalopod ink [6–

54 11]. Melanosomes are responsible for the exceptional fossilization of many vertebrate soft tissues, such as

55 skin, hair and feathers as well as eyes and some internal organs, such as the liver [9]. Work investigating

56 the morphologies of melanosomes from extant avian taxa has shown a clear link between their shape,

57 colours produced and the mechanism of their production [11]. Reddish-brown phaeomelanin is contained

58 within ovoid melanosomes that are on average approximately 500 nm long in birds and mammals [11,12],

59 while black eumelanin is contained within a more oblong melanosome, averaging 800–1000 nm in length

60 [11,12]. Melanosomes may have a mixed eumelanin/phaeomelanin composition and their morphology

61 seems to form a spectrum between these end members. Eumelanin-rich melanosomes can vary further in

size and shape which correspond to a further spectrum of colours and coloration mechanisms, such as iridescent and grey [11,12]. Furthermore, penguins have been observed to display melanosomes of a unique morphology which requires further investigation [10].

Inferences of colour in extinct avian and non-avian taxa have been done in the past using melanosome morphology and statistical methods, such as quadratic discriminant analysis [7,9–11,13]. These analyses have shown that plumage colours can be predicted through melanosome morphology alone with 82% accuracy. These predictions are currently based on broad colour categories and mechanisms of colour production involving melanosomes (black, brown, grey and iridescent—after the colours of feathers from which melanosomes were extracted) [11] as well as a category for extant penguin melanosomes [10]. Predicting the coloration of fossil birds and dinosaurs has allowed aspects of their ecology and lifestyle (such as signalling strategies, behaviour and habitat preference) to be inferred [10,11,13,14]. To date, non-iridescent structural colour has not been investigated in the fossil record. It has been shown that keratin degrades during the process of fossilization [15,16]. Hence, the reliability of reconstructing non-iridescent structural colours, which are generated by keratin nanocavities in feather barbs would be impossible. However, since melanosomes associated with non-iridescent structural colours will preserve, these could potentially be conflated with other melanosomes involved in other colour production mechanisms.

Here we explore the morphology of melanosomes from feathers that produce non-iridescent structural colour to determine whether they are distinct from previously identified melanosome categories used to predict palaeocolour in quadratic discriminant analyses. We also investigate the early Eocene stem group coraciiform Eocoracias brachyptera with preserved feathering from Messel Formation in Germany [17]. The modern relatives of this taxon ostensibly display non-iridescent structural colours. Furthermore, to corroborate the phylogenetic bracketing of Eocoracias and its likelihood of displaying non-iridescent structural colour, we conducted an ancestral state reconstruction and evaluated the likely ancestral hues of non-iridescent structural colour.

2. Materials and Methods

2.1. Sampling of feathers and melanosome extraction

A total of 72 feather samples expressing non-iridescent structural colour were collected at the Zoological Museum, Natural History Museum of Denmark, University of Copenhagen (see electronic supplementary material for details of samples). The presence of non-iridescent structural colour in each feather sample was confirmed based on the phylogenetic sample used in Saranathan et al. [18].

92 Feathers were cleaned with ethanol, and the parts of the feather expressing non-iridescent structural

93 colour were cut for sampling. These coloured sections were then subjected to the enzymatic extraction

94 method described in Colleary et al. [8] to remove the keratin and extract melanosomes. The resulting pellets

95 of melanin were mounted on scanning electron microscopy (SEM) stubs and gold coated prior to

96 investigation with a SEM instrument.

97 **2.2. Fossil specimen**

98 A new specimen of the stem group roller E. brachyptera (figure 1) from the Early Eocene Messel Formation

99 in Germany [17,19], housed in the collections of the Senckenberg Research Institute, Frankfurt (SMF) was

100 investigated in this study (collection number: SMF-ME 1450A). This fossil was chosen due to its position

101 as sister to the clade containing the extant Coraciidae (true rollers), a clade in which non-iridescent

102 structural colour is common, and the Brachypteraciidae (ground rollers), which show a more restricted

103 occurrence [20]. This allowed for determination of whether non-iridescent structural colour was present in

104 the stem group representative of this colourful clade. It also provides an ideal test case for ascertaining

105 whether these colours can be detected in the fossil record. A total of 12 samples were removed from the

106 plumage of the fossil by gently scraping small sections of organic material (generally under 2 mm2) off the

107 surface of the preserved feathers using a clean scalpel (figure 1). These samples were mounted on SEM

108 stubs and sputter coated with gold for SEM imaging.

109 **2.3. Investigation of melanosome morphology**

110 The gold coated fossil feather samples and melanosome extracts were investigated using a Zeiss

111 Sigma HD VP field emission SEM under high vacuum mode at an accelerating voltage of 12–20 keV and

112 a working distance of 10 mm. Enough images were produced to be able to measure 100 fully exposed (non-

113 overlapping) melanosomes per sample. To assess the morphology of melanosomes, the width (nm), length

114 (nm) and aspect ratio of 100 melanosomes were measured for each sample using ImageJ [21]. Additional

115 calculations were carried out for each sample: mean, standard deviation, coefficient of variance (CV) and

116 skew for length, width and aspect ratio of each sample. Results were added to a previously constructed

117 dataset from Li et al. [11].

118 One-way ANOVA and Tukey's post hoc test were carried out on data in R [22,23] to determine

119 whether melanosomes extracted from feather samples exhibiting non-iridescent structural colour differed

120 significantly in their morphology from other defined melanosome morphologies (from black, brown, grey,

121 iridescent and penguin feathers). Quadratic discriminant analyses (QDA) were used to investigate the

122 accuracy of colour predictions when non-iridescent structural colour was added as a separate colour

123   category [11]. This method uses extant feather colour categories as the independent variable, and
124   melanosome morphology measurements as the dependent variable. The model fitted to a training subset
125   was applied to the test subset to determine its accuracy. This process was performed 100 times, to account
126   for random sample selection of training and test subsets. Forward stepwise regression was performed to test
127   which of the eight dependent variables (length, length CV, length skew, width, width CV, width skew,
128   aspect ratio and aspect ratio skew) explained most of the variance in the model. Finally, the values of the
129   first two variables (those that explained the variation best) obtained by forward stepwise regression were
130   plotted to provide a visual assessment of melanosome categories.

131   **2.4 Ancestral state reconstruction in the Coraciidae**

132   Phylogenetic comparative methods were used to investigate the likely colour of E. brachyptera. A database
133   of bird plumage coloration for Coraciiformes was constructed based on visual assessment and plumage
134   descriptions from the Handbook of the Birds of the World [20]. Colour states were coded based on the
135   presence and absence of black colour, grey colour, non-iridescent structural colour, and carotenoids across
136   the plumage (electronic supplementary material). We chose grey and non-iridescent structural colour as
137   categories because the melanosomes that produce these colours are similar in morphology (result of this
138   study). Carotenoids were chosen as a colour category due to their combined effect with non-iridescent
139   structural colour on the overall coloration of bird plumage. Presence of carotenoids that produce specific
140   colours was confirmed based on Thomas et al. for selected bird species [24]. The black colour category
141   was chosen as several samples from E. brachyptera feathers were predicted as black in the QDA (see
142   below). Each colour was treated as a discrete character state with absence coded as 0 and presence as 1.
143   The same process was repeated for both males and females. The posterior probability of the tip state for E.
144   brachyptera was estimated, given an equal prior probability of 0.5 for each state. A stochastic character
145   mapping approach was implemented using the function make.simmap in the R package phytools [25], while
146   the phylogenetic backbone was taken from 1000 randomly sampled trees from Jetz et al. [26]. The fossil
147   taxon E. brachyptera was added to each of the 1000 trees from Jetz et al. as outgroup to crown
148   Coraciiformes; the age of the tip for E. brachyptera was set at 48 Ma and the branching time that E.
149   brachyptera diverged from the Coraciiformes was randomly sampled from a uniform distribution bounded
150   by the total-group Coraciiformes age for that tree and 48 Ma using motmot [27]. Using these trees, a
151   consensus tree was built using maxCladeCred in the R package phangorn [28].

152   **3. Results**

153   **3.1. Melanosome morphology in feathers with non-iridescent structural colour**

154   Melanosomes extracted from feathers that exhibit noniridescent structural colour are all ellipsoidal
155   in morphology. Their mean length, width and aspect ratio are 1249.2 nm+ 230.6 nm, 408.5 nm+186 nm,
156   and 3.4+0.9, respectively. The most significant variables that determine the shape of melanosomes (length,
157   width and aspect ratio), were compared among groups divided by colour categories. Results of the one-way
158   ANOVA indicated that there are groups that are not significantly different. The Tukey's post hoc test
159   revealed that the melanosomes extracted from feathers exhibiting non-iridescent structural colours were
160   significantly different ($p < 0.05$) in their morphology from all other colour categories except grey (see
161   electronic supplementary material for details). No significant difference was found in any variable, i.e.
162   length, width and aspect ratio, with melanosomes from grey feathers ($p = 1.0$, $p = 0.99$, $p = 0.99$
163   respectively). There was also no significant difference between the length of melanosomes from feathers
164   expressing non-iridescent structural colours and melanosomes from iridescent feathers ($p = 0.35$), the width
165   of melanosomes from non-iridescent structural colour feathers and 'penguin-type' melanosomes ($p = 0.99$)
166   and the aspect ratio of melanosomes from feathers that express non-iridescent structural colour and
167   melanosomes from black feathers ($p = 0.53$) (figure 2a–c), highlighting the importance of including
168   multiple shape variables in statistical analyses of melanosome morphology–colour relationships.

169   **3.2. Accuracy of melanosome prediction**

170   As the two variables that explain most of the variation in the data based on the forward stepwise
171   addition, length and width were plotted separately for each colour category with 95% confidence intervals
172   (electronic supplementary material). The 100 simulations demonstrate the accuracy of the QDA. Samples
173   were predicted accurately in 61.8% of the cases, which is a drop from 82% before the introduction of the
174   non-iridescent structural colour category. Percentages of accurate prediction within each colour category
175   are outlined in figure 3. Melanosome morphology (length and width) is the strongest predictor for category
176   of non-iridescent structural colour (75.5%), followed by decreasing percentage of accurate prediction:
177   brown (71.8%), iridescent (63.6%), 'penguin-type' (62.2%), grey (54.7%) and black (45.4%). The accuracy
178   of melanosome categorization in the Li et al. database was reported as 82% [11]. The same methodology
179   of accurate prediction was also performed on just the Li et al. database [11]. The 'penguin-type'
180   melanosome category showed the highest percentage of accurate prediction (81.2%) followed by iridescent
181   (72.2%), brown (73%), grey (63.5%) and black (55.6%). When melanosomes from feathers expressing non-
182   iridescent structural colours were included in the Li et al. database and treated as 'unknown' in terms of
183   colour category, 29 samples were predicted as grey, 28 as black, seven as 'penguin-type', six as iridescent,
184   and two as brown.

185   **3.3. Reconstruction of the plumage colour of E. brachyptera**

186   Of the 12 samples taken from E. brachyptera 10 contained melanosomes visible using SEM. These
187   melanosomes were all notably large, conforming to the ones found in non-iridescent structural colours and
188   those characteristic for grey colour (with average length: 1462.02 nm, width: 483.09 nm and aspect ratio:
189   3.1). From the QDA, seven samples were predicted as belonging to grey colour category (abdomen, ventral
190   side of the neck, dorsal side of the neck, caudal region of the skull, lower part of the neck, sternum, dorsal
191   region in front of synsacrum), while three samples were predicted as black colour category (the dorsal side
192   of the neck, rump and the tail).

193   The ancestral state reconstruction of the plumage coloration in E. brachyptera predicted a high
194   probability of non-iridescent structural colour being present (0.99 posterior probability for males and
195   females). Black colour was also likely to have been present (posterior probability 0.75 for males and 0.77
196   females). However, both grey (posterior probability 0.19 for males, and 0.17 for females) and carotenoids
197   (0.04 for males, and 0.02 for females) were not likely to have been present (figure 4).

198   Figure 5 shows a reconstruction of E. brachyptera with a hypothetical plumage coloration based
199   on the results of our study. We did not recover melanosomes in the primary feathers. This is likely due to
200   the sampling method rather than a lack of pigmentation, as these feathers are preserved as dark organics in
201   the same way as the areas with melanosomes present. Due to the preparation methods used for Messel
202   fossils, using resin and often coating them with lacquer, removing organics suitable for imaging under the
203   SEM is difficult because microstructural details are obscured, including melanosomes. Therefore,
204   melanosomes will only likely be exposed if the organics from between the resin and lacquer are removed
205   and imaged. Parts of the neck, tail and rump of E. brachyptera were reconstructed as black due to the
206   support from results of the study of melanosome morphology and ancestral state reconstruction. Based on
207   the same combination of methods we reconstructed the rest of the plumage as blue. The wing feathers were
208   reconstructed as black and blue, following the ancestral state reconstruction and the melanosome
209   morphology detected in the other parts of the bird's plumage. Our results show, however, that melanosome
210   shape alone does not allow for a distinction between grey and non-iridescent structural colour for E.
211   brachyptera.

212   **4. Discussion**

213   **4.1. Reconstruction of non-iridescent structural colour in the fossil record**

214   Distinct melanosome shapes have been determined for black, brown, grey, and iridescent colours,
215   and for special 'penguintype' melanosomes [11]. The introduction of a melanosome type associated with
216   non-iridescent structural colour reduced the accuracy of melanosome categorization from 82% to 61.9%.

Investigated melanosomes are generally distinct to most other melanosome categories but overlap significantly with those significant for grey feather coloration. Given the clear correlation between broad colour categories however, melanosome morphology is currently the best way to determine palaeocolour, particularly for non-iridescent structural colour, grey and iridescent, which can only be determined based on melanosome shape. In this study, the QDA have high accuracy when determining non-iridescent structural colour, iridescence and brown colour while black and grey dropped in accuracy (figure 2). Some of these issues could be surmised to be alleviated by coupling melanosome analysis with chemical analyses, such as time-of-flight secondary ion mass spectrometry, which can distinguish between relative amounts of eumelanin and phaeomelanin in fossil and extant melanosomes [8], and through further probing of the relatively broad colour categories, which in reality form a spectrum of hues. However, chemical analyses will likely not be able to distinguish melanin from melanosomes involved in the production of grey and non-iridescent structural colour and black and iridescent.

The colours of the studied avian taxa included in the ancestral state reconstruction revealed a high probability for the existence of non-iridescent structural colour, and black, while there was a low probability of the occurrence of carotenoid-based and grey colours in the plumage of E. brachyptera. By contrast, the morphology of most melanosomes obtained from E. brachyptera predicted grey rather than non-iridescent structural colour in the QDA. In the case of the Eocoracias fossil, it therefore appears that incorporating ancestral state reconstructions with the aim of discriminating between overlapping melanosome morphologies (e.g. grey colour and non-iridescent structural colour) is an essential method for reconstructing the likely plumage coloration in fossil taxa.

Due to the poor preservation potential of keratin [15,16], the 'spongy' barb cortex responsible for selective light scattering is lost in fossil feathers, leaving only melanosomes. The lack of significant morphological differences between melanosomes characteristic of grey and those associated with non-iridescent structural colour makes them hard to distinguish from one another in fossils. Overall, non-iridescent structural colour occurs in about 10 extant avian lineages (Anseriformes, Galliformes, Columbiformes, Musophagiformes, Procellariiformes, Gruiformes, Charadriiformes, Coliiformes, Coraciiformes, Piciformes, Psittaciformes and Passeriformes) out of 61 lineages according to Stoddard & Prum [29] and is usually even highly restricted within each. Hence, the most conservative inference would be that an avian fossil outside these clades would likely exhibit grey coloration rather than non-iridescent structural colours.

4.2. Developmental pathways in non-iridescent structural and grey colours – a possible evolutionary link?

248   Our observed overlap between melanosomes involved in the production of grey colour and non-iridescent
249   structural colour is of interest for understanding the role of melanosome morphology in plumage
250   development. Initially, melanosomes are transported into keratinocytes during the early stages of feather
251   development [30]. The melanosome-laden keratinocytes are arranged to form the major feather structures—
252   the calamus, the barb and the barbule [30,31]. After death of the keratinocytes, melanosomes are observed
253   to arrange themselves while the cell hardens by polymerizing keratin. This self-assembly process is best
254   known from studies of iridescent feathers, in which melanosomes are arranged to create photonic
255   nanostructures [31]. Maia et al. [31] have suggested that this self-assembly takes place due to depletion–
256   attraction forces between the melanosomes and the polymerizing keratin. It has been shown that the size,
257   shape and concentration of melanosomes may be important factors in facilitating this process. The
258   developmental processes that involve self-assembly mechanisms have been described in feathers with black
259   [31], iridescent [2,31,32] and non-iridescent structural colours [2,33]. So far however, the process of
260   producing melanin-based colours has received little attention and a better understanding of the relative role
261   of the various potential factors in melanosome arrangement (e.g. shape and concentration) is needed. The
262   overlap in melanosome shape between those found in grey feathers and those in feathers expressing non-
263   iridescent structural colour could be indicative of similar developmental mechanisms during the growth of
264   the feather that uses these two colours. Iridescent and melanized feathers have melanosomes concentrated
265   toward the outer region of the feather cortex [31,32,34]. Feathers expressing non-iridescent structural
266   colours differ from this arrangement as melanosomes are concentrated towards the barb core [2,33]. Grey
267   coloration is generated by a more diffuse arrangement of melanosomes and a greater concentration of
268   melanosomes in the core than the cortex. We hypothesize that a transition from grey colour to non-iridescent
269   structural colours would be a potential pathway for the evolution of non-iridescent structural colour. The
270   larger melanosomes would be driven to concentrate in the feather core, leaving a space for keratin
271   nanocavities to form. We surmise that the generally larger and wider melanosomes facilitate this process,
272   while narrower melanosomes, those involved in black and iridescent colours, are pulled to the cortex of the
273   feather [31,34]. Corroborating the notion that there could be an evolutionary relationship between grey and
274   non-iridescent structural colours, some birds are intermediate in coloration between these two colour
275   categories, such as the Victoria crowned pigeon (Goura victoria). Future studies should focus on the link
276   between grey and non-iridescent structural coloration during feather development and how melanosome
277   morphology influences nanostructural feather assembly outside of well-studied iridescent feathers.

278   **Conclusions**

279   Melanosomes in barbs arranged near the keratin cortex producing non-iridescent structural colour are wider
280   and longer than melanosomes in most other feather colour categories. However, melanosomes isolated from

grey feathers overlap significantly with the shape of melanosomes from feathers expressing non-iridescent structural colour. Introducing melanosomes from non-iridescent structural colour as a colour category in the QDA reduced the accuracy of melanosome colour categorization from 82% to 61.9%. This increases in particular the uncertainty when distinguishing grey from non-iridescent structural colour in palaeocolour inference. As non-iridescent structural colours are relatively phylogenetically constrained in modern birds, their prevalence in extinct taxa would have been similarly limited. Therefore, grey is a more likely occurrence unless phylogenetic or ecological data suggest otherwise. It has been argued that melanosome shape is important in facilitating their position inside the keratin matrix during feather ontogeny and the resultant colour. The similarity of melanosome shape involved in the production of grey and non-iridescent structural colours highlights a possible common dependence on melanosome shape during the development of feather coloration and is potentially linked evolutionary.

Ethics. All feathers were picked from museum skin samples (in accordance with museum staff).

Data accessibility. All data are available in electronic supplementary material: (1) Melanosome measurements—1.1. Measurements of melanosomes isolated from fresh feathers that express non-iridescent structural colour are included as a separate category in the previously existing database from Li. et al. (sheet number 7 in our electronic supplementary material). 1.2. Measurements of melanosomes from the surface of the fossil are under sheet number 2 in the electronic supplementary material. (2) Other important data that we have collected for our research are colour presence/absence matrix that is included in sheet number 3 of the electronic supplementary material.

Authors' contributions. F.B. and J.V. designed the experiment. J.V. and G.M. sampled the fossil. F.B. extracted melanosomes and performed the electron microscopy with assistance from E.-J.G., E.L. and F.M.S. E.-J.G and E.L. analysed a preliminary dataset for their undergraduate practical project, supervised by F.B. and J.V. F.B. collated the database for ancestral state reconstruction and statistical analysis. M.N.P. performed the discrete character reconstruction. M.Z., G.M. and F.B. performed the anatomical description of the fossil. M.Z. made the artistic reconstruction of Eocoracias. F.B. and M.Z. produced figures. All authors contributed to the manuscript.

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410 **Figures.**

411
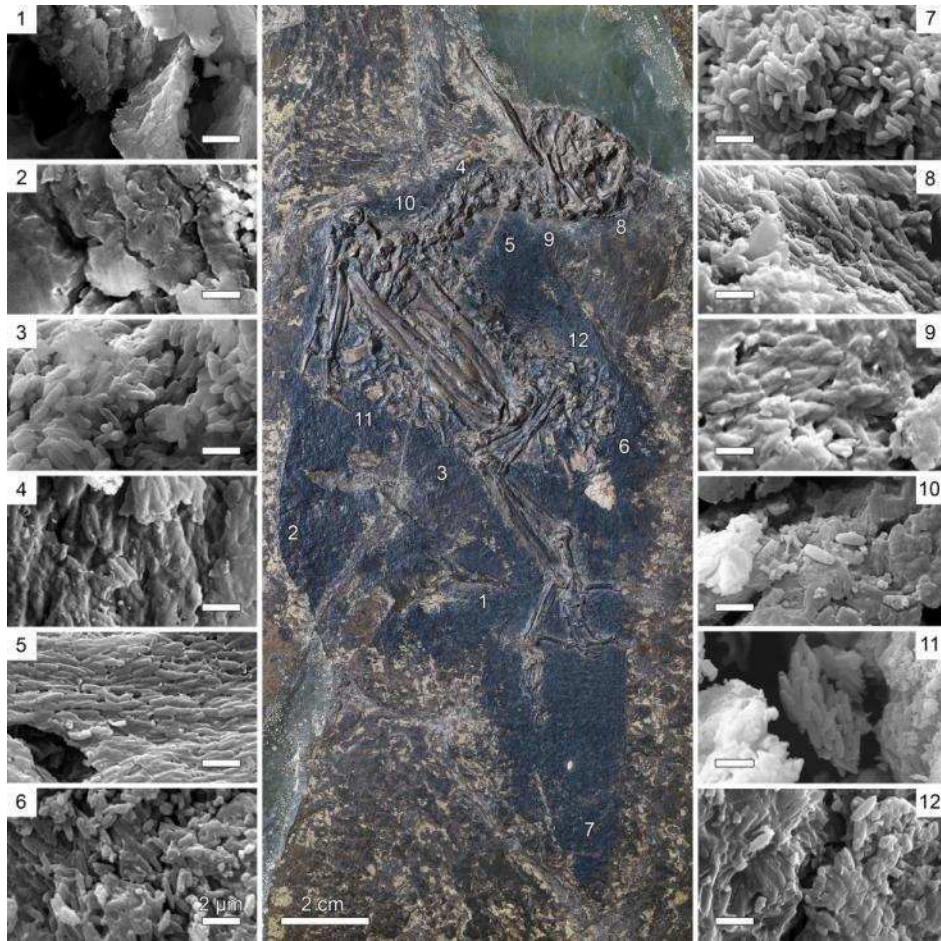
Figure 1. The sampled fossil of Eocoracias brachyptera (SMF-ME 1450A) with SEM images of preserved melanosomes. Numbers indicate where sampling was done and correspond to SEM images. All scale bars for SEM images, 2 mm. (Online version in colour.)
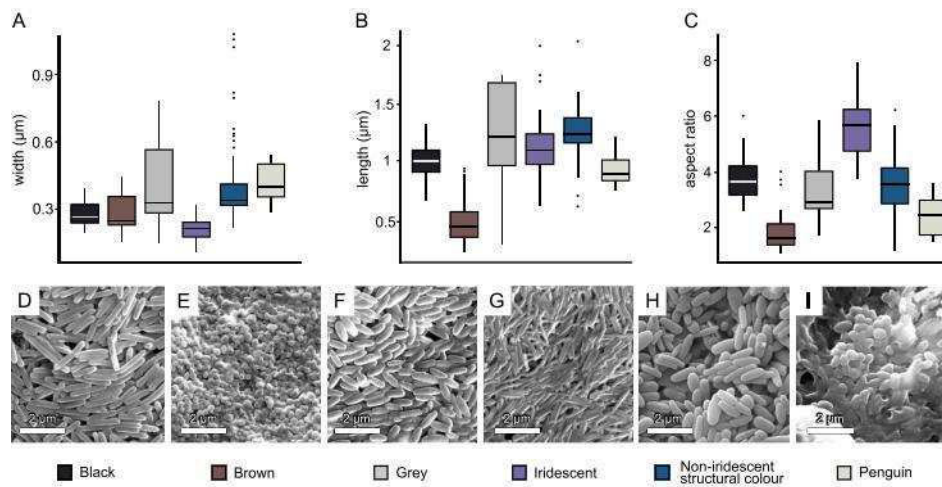

415

416   **Figure 2.** Comparison of melanosome shape (in extant bird feathers) for different colour categories. Box
417   plots of mean width (a), length (b), and aspect ratio (c) of measured melanosomes from 232 birds of various
418   feather types, based on the dataset of Li et al. [11] with non-iridescent structural colour added as a new
419   colour category. SEM images of melanosomes from six colour categories in feathers of the extant taxa: (d
420   ) black feathers of wrinkled hornbill (Rhabdotorrhinus corrugatus), (e) brown feathers of the great jacamar
421   (Jacamerops aureus), (f) grey feather of the toucan barbet (Semnornis ramphastinus), (g) iridescent feather
422   of the great jacamar (Jacamerops aureus), (h) feathers expressing non-iridescent structural colour of the
423   blue paradise flycatcher (Terpsiphone cyanescens), and (i) black feather of an African penguin (Spheniscus
424   demersus). (Online version in colour.)
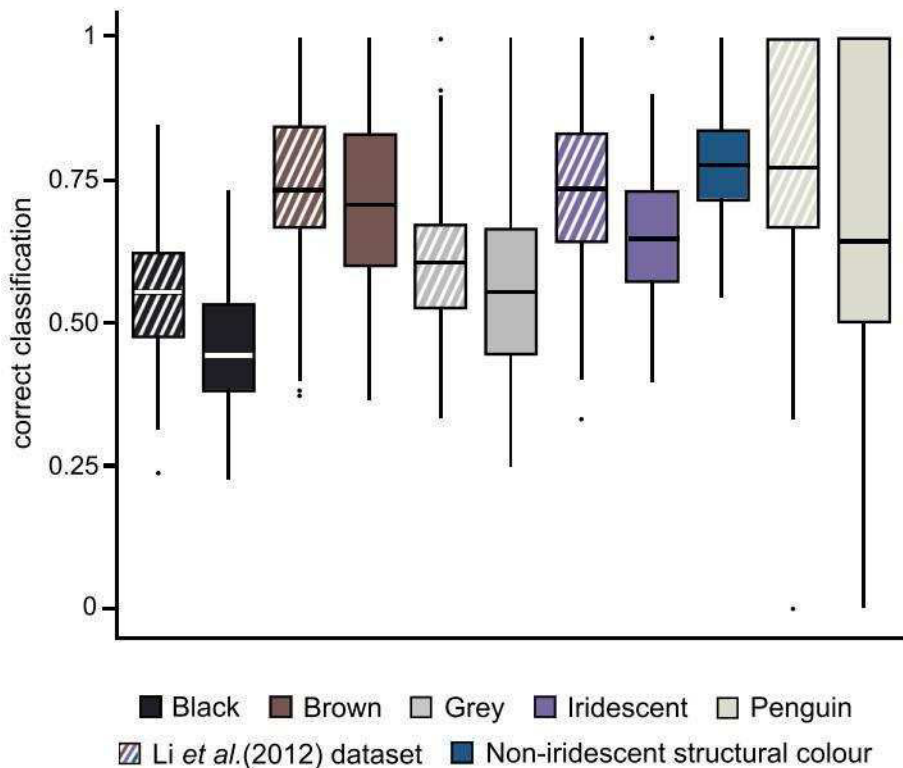
425

426   **Figure 3.** Percentage of correctly classified cases for each colour category based on melanosome
427   morphology in a simulation within a quadratic discriminant analysis (100 repeats). The horizontal line on
428   each box plot indicates the mean value. Cross-hatched boxes indicate correct classifications based on just
429   the Li et al. dataset [11], while solid boxes indicate accurate prediction when non-iridescent structural
430   colour was introduced into the same database. (Online version in colour.)

431
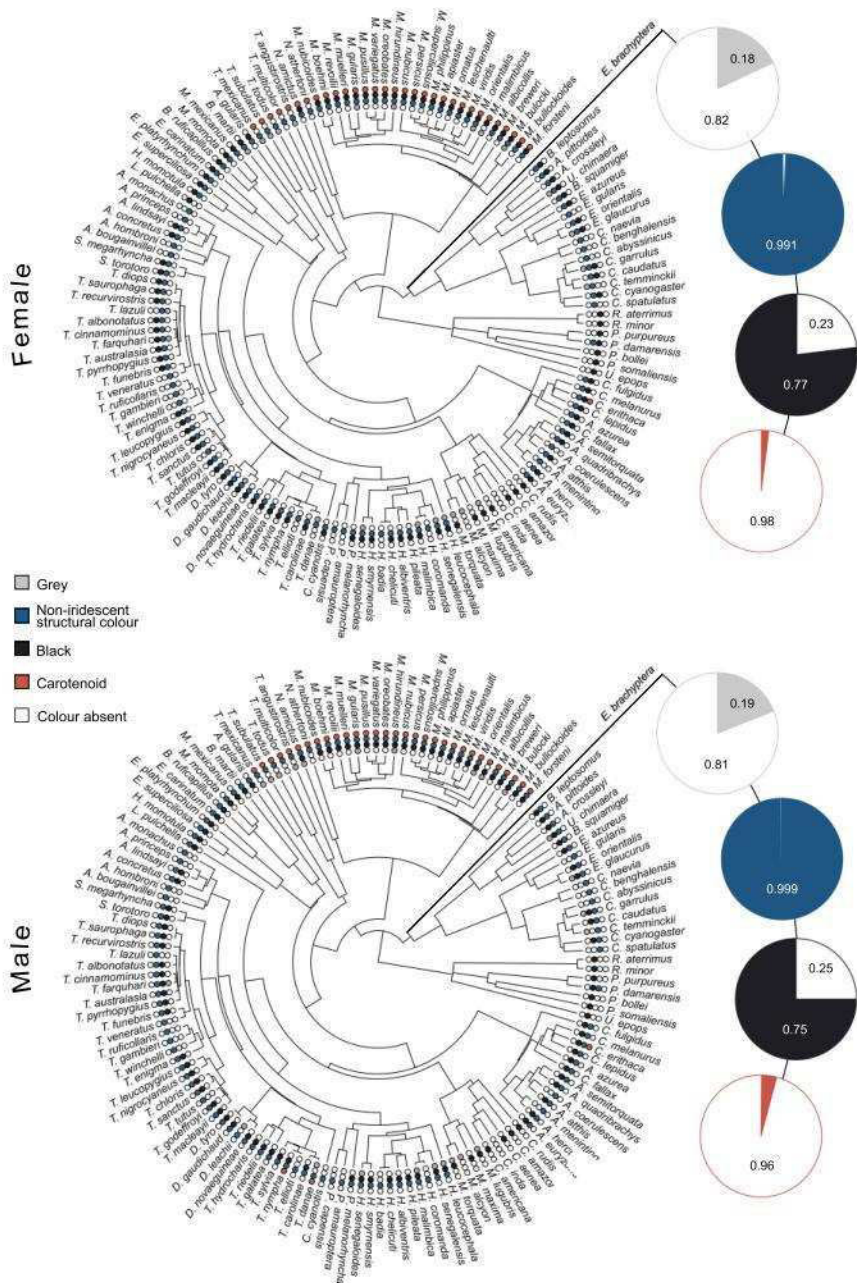
Figure 4. Ancestral state reconstructions of colour presence or absence for the Eocoracias brachyptera fossil for both female and male. Small circles indicate presence or absence of each colour category as indicated in the colour legend. Posterior probabilities of colour presence from stochastic character mapping are represented by large pie charts. (Online version in colour.)

436

Figure 5. Reconstruction of Eocoracias brachyptera with hypothesized plumage coloration. Our reconstruction of the external appearance of the species is based on Mayr and Mourer-Chauviré's description of the fossils [19]. (Online version in colour.)