A guide to the field of palaeo colour

Melanin and other pigments can fossilise: Reconstructing colour patterns from ancient organisms can give new insights to ecology and behaviour

Jakob Vinther

Melanin, and other pigments have recently been shown to preserve over geologic time scales, and are found in several different organisms. This opens up the possibility of inferring colours and colour patterns ranging from invertebrates to feathered dinosaurs and mammals. An emerging discipline is palaeo colour: colour plays an important role in display and camouflage as well as in integumental strengthening and protection, which makes possible the hitherto difficult task of doing inferences about past ecologies, behaviours, and organismal appearance. Several studies and techniques have been presented in the last couple of years that have described ways to characterize pigment patterns. Here, I will review the available methods and the likely applications to understand past ecologies. A golden age of colourized dinosaurs and other animals is now dawning upon us, which may elucidate the nature of ancient predator prey interactions and display structures.

Keywords:
camouflage; dinosaur; display; melanin; melanosome; pigment

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Introduction

Colour is an integral aspect of organisms and their behaviour. Colour patterns allow animals to camouflage themselves from predators in their surroundings. Conspicuous patterns serve in species recognition, sexual display or advertising poisonous organisms (aposematism) or mimicking the latter. Other conspicuous colour patterns may serve in dazzle camouflage, affecting predators' perception of prey trajectory and speed [1], yet others can startle predators and disruptive colour markings serve to obliterate the outline of an organism [2, 3].

Pigments also serve other roles than imparting colour. Melanin acts as an antimicrobial agent [4] in vertebrate organs such as liver, spleen and the peritoneum, as well as delicate external integuments such as the scrotum and anus. Melanin can be an integral part of invertebrate immune systems, and act as a strengthening agent in keratinous tissues such as beaks [5], scales and feathers [6]. Melanins in nervous systems (neuromelanin) and eye retinas are chemically distinct from other integumental melanins, and might serve in modulating nervous impulses [7] or scavenging toxic compounds and metals [8]. Their absence, as in some cases of albinism, is correlated with conditions such as deafness [7], as Charles Darwin observed in his blue eyed, white cat [9].

The key function of pigments and colour patterns and their relationship to organismal behaviour and interactions are therefore important to understand their ethology. However, the study of fossil pigments has until recently been largely neglected. Some fossils are soft-bodied or lightly sclerotized in nature and preserve as carbonaceous residues such as fossil leaves, algae, feathers, insects and less familiar organisms such as eurypterids and graptolites. Palaeontologists and geochemists have sought to understand which organic compounds might be preserved in such fossils and how to identify them [10–12]. It has been surmised that organic pigments indeed might be preserved in fossil material [13]. Fossil insects are frequently found with clear light and dark-colour patterns (Fig. 1F–H), and even iridescence (Fig. 2E) [14]. Fossil mollusc shells are occasionally found with colour patterns [15] as old as the Ordovician (488–460 Ma) (Fig. 1D), and trilobites with dark stripes are known from the Ordovician

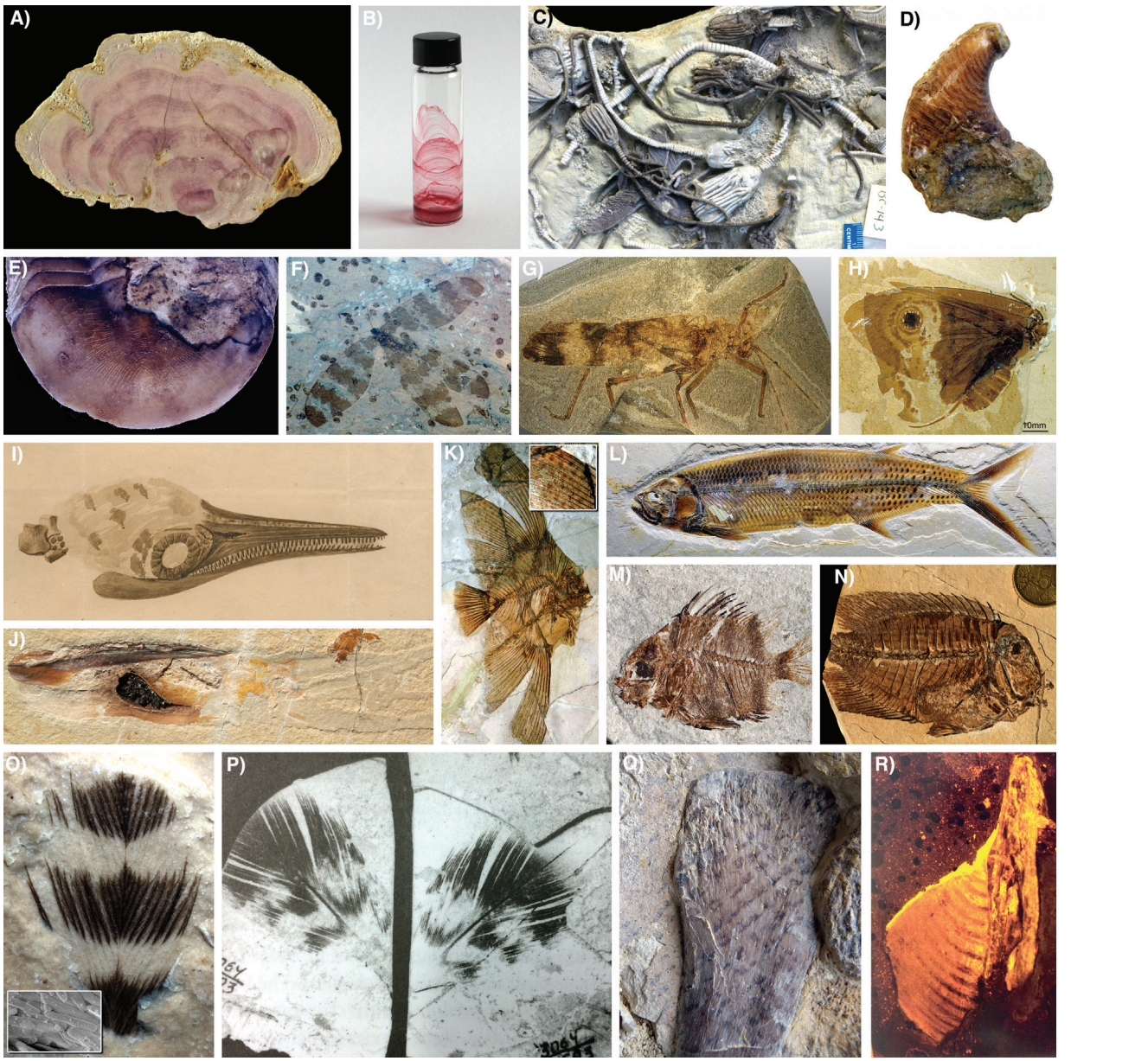


Figure 1. Examples of fossil pigments and colour patterns in the fossil record. **A**: the Jurassic red alga, *Solenopora*, preserving pink-colored borolithochrome [20]. **B**: Extracted borolithochrome pigment. **C**: The crinoids *Elegantocrinus*, *Cribanocrinus*, *Cusacrinus* and *Strimplecrinus* (Carboniferous, Indiana, USA) preserving different amounts of organic remains, likely derived from fringelites [21]. **D**: The Silurian (~425 Ma) cephalopod *Phragmoceras imbricatum* preserving colour banding [15]. **E**: The trilobite, *Ilaenus* (Kunda, Ordovician, St. Petersburg, Russia) preserving dark radial stripes on its pygidium. **F**: A Jurassic lacewing, from the Daohuguo beds (late Jurassic, inner Mongolia, China) preserving transverse stripes on its wings (photographed in museum exhibit, Liaoning). **G**: The grasshopper *Pseudotettigonia* amoena (early Eocene, fur formation, Denmark), preserving colour bands. **H**: A kalligrammatid lacewing (Daohuguo), with a wing eye spot. **I**: Painting of ichthyosaur fossil, by Elisabeth Philpot (1780–1857, a British fossil collector), using ink from a squid fossil preserving the ink sac in coeval layers as the ichthyosaur fossil (Lyme Regis, early Jurassic) to paint the picture. **J**: Fossil stem octopus, *Glyphiteuthis* (late Cretaceous, Lebanon), preserving soft tissues, including the ink sac. **K**: The fossil angelfish, *Eoplatax* (Eocene, Monte Bolca), preserving spots on the anal and dorsal fin (detailed). **L**: The fish *Thrissops* from the late Jurassic Solnhofen Plattenkalks, preserving countershading anteriorly and a speckled pattern on its scales posteriorly. **M**: A juvenile specimen of *Archaephippus asper* (Monte Bolca, Italy, Eocene) preserving vertical stripes. **N**: The holotype of *Frigosorbinia baldwinae* (Monte Bolca, Italy, Eocene), preserving horizontal stripes. **O**: isolated body contour feather preserving transverse colour bands (Crato Formation, Cretaceous, Brazil), inset show melanosomes preserved in the dark region of the specimen under electron microscope [22]. **P**: Isolated body contour feather (Mongolia, Early Cretaceous), preserving irregular banding [23, 24]. **Q**: Skin impressions superimposed on associated bones and sedimentary matrix of a mosasaur skeleton, *Plateosaurus* [25], with pigment patterns associated with the scales. **R**: A head crest from the pterosaur, *Pterorhynchus*, preserving arcuate, transverse colour bands [26]. **A**, **B**, courtesy of Klaus Wolkenstein; **C**, courtesy of William I. Ausich, Ohio State University; **D**, Vojtêch Turek, Prague; **E**, Andrei Ivantsov, Palaeontological Institute, Moscow; **E**, Henrik Madsen, Molermuseet, Mors, Denmark; H, Dong Ren, Beijing; **I**, Kathleen Santry, Oxford Natural History Museum; **P**, Alexander Kellner, Brazil; **Q**, Johan Lindgren, Lund University and Mike Caldwell, U. Alberta; **R**, Sylvia Czerkas.

(Fig. 1E) and Middle Cambrian (~509 Ma) [16], which is likely indirect evidence for visual predation then.

When fossil collectors in the early 19th century explored the lower Jurassic (~190 Ma) near Lyme Regis on the Dorset coast of South England they often encountered fossil cephalopods with a dark 'splotch', shaped like an ink sac (Fig. 1J). Emily Philpot, who was the mentor of celebrated fellow fossil collector Mary Anning, demonstrated that the fossil 'ink' could be dissolved in water and used to write and paint (Fig. 1I). In 1973, German researchers demonstrated that fossil ink from lower Jurassic specimens yielded almost similar infrared spectra as modern ink [17]. In addition, studies in the middle of the 20th century indicated the preservation of melanocytes, the pigment bearing cells in the skin of exceptionally preserved middle Eocene (48–41 Ma) frogs [18] and lower Jurassic ichthyosaurs (~190 Ma) [19]. These studies were limited to light microscopic observations. Little progress was made in the decades after these discoveries; part of the reason was a misinterpretation of fossil melanins as bacterial remains.

When fossil bacteria became melanosomes

The 1960s experienced a revolution in biology with the introduction of electron microscopy. This allowed the study of micron to nanometre scale structures and thus detailed histological characterisation of tissues and organelles. Electron microscopy really took off in palaeontology in the 1970s and 1980s, and ushered in renewed interest in soft tissue preservation. When researchers magnified fossil feathers and mammal hairs from the famous middle Eocene Messel locality they encountered large patches with sausage shaped to spherical objects approximately half a micron to one micron in length [27]. They looked remarkably similar to bacteria, which had – just a little earlier – also been imaged with SEM for the first time. They were even aligned in swaths similar to growing bacterial colonies and were therefore suggested to be autolithified bacterial mats on the surface of the carcass [27].

Several studies followed, which characterised the preservation of skin and feathers, and attributed microbodies to bacterial remains [28–31]. Incidentally this research provided crucial evidence for an emerging paradigm highlighting the importance of microbial mats and bacterial presence during fossilisation [30, 32, 33]. Scientists have on occasion noted the presence of colour patterns in fossil feathers (Fig. 1O and P) [24, 29, 34, 35]. Some in particular found it puzzling that colour patterns were preserved in feathers, while, in the same time, should have been replaced by bacteria [34].

This paradigm shifted considerably when the same structures were reinterpreted as melanin-bearing melanosomes [22]. The insight came from studying the peculiar case of three-dimensionally preserved ink sacs in Jurassic and Cretaceous cephalopods. Electron microscopy analyses show that the fossil ink melanin is preserved as small spherical granules, identical to modern ink samples from cephalopods [36]. As cephalopod melanin is chemically identical to vertebrate melanin, it led to the question as to whether melanin would be preserved in such fossils in a similar fashion. Organic residues in fossil vertebrates are usually melanin-bearing structures, such as integument, eyes and certain internal organs. In order to explore, the possibility that these organic residues might be melanin remains fossil feathers were chosen as an initial target. Fossil feathers are relatively common, and, in light of eventually being able to study feathered dinosaurs, they seemed appropriate [22].

Melanin is contained within melanosomes (lysosome-derived organelles) and is transported, intact, into keratinous tissues, such as feathers, scales and hair or retained within the melanocyte cell in the lower epidermis. These organelles vary from oblong to spherical objects about one micron in length.

When an isolated early Eocene (54 Ma) bird skull with a halo of feathers and a well preserved eye were taken under the electron microscope, swaths of oblong objects were observed in the feathers, identical to black-coloured melanosomes in modern ones [22]. Furthermore, the melanosome-like microbodies in the feathers were similarly aligned along the length of the barbs and barbules. The fossil eye contained a mixture of oblong and sub-spherical objects like melanosomes observed in modern bird retinas. Another isolated Cretaceous feather [29] with conspicuous transverse banding (Fig. 1O) preserved melanosomes with a similar alignment along the barbules in addition to the clear presence of oblong microbodies only in the dark regions and not in the lighter regions.

The observed arrangement of these fossil microbodies in addition to the robust microscopical evidence for melanin granules in cephalopod ink sacs was considered compelling evidence that these structures should be interpreted as melanin-containing melanosomes rather than bacteria [22]. The structures were clearly solid organic masses with several physical similarities to melanin-containing melanosomes, including the characteristic arrangement. The resulting publication [22] laid out the argument that different colours can be inferred in fossil feathers and even feathered dinosaurs: this is due to the fact that there are two different types of melanin in integuments and most organs – eumelanin and phaeomelanin, which, respectively, give black to dark brown colour and rufous red to buff yellow colours. These are shaped as either elongate 'sausages' (eumelanosomes) or as tiny sub-micrometric 'meatballs' (phaeomelanosomes) [37, 38].

Iridescence (metallic sheen) is formed in feathers by regular arrangement of melanosomes within the barbules, which, due to their relative refractive index to the keratin matrix, can create coherent scattering of certain wavelengths of light [39]. These arrangements can be preserved intact in exceptional circumstances, as is seen in an isolated 47-million-year-old feather from Messel (Fig. 2A–D) [40, 41], which would have exhibited thin film iridescence. A silvery sheen in the iridescent parts of the fossil (Fig. 2B) is due to a complex interaction between the iridescent barbules and wedges of sediment, covering parts of the distal, iridescent barbules [41] and a secondary artefact. Melanosomes in iridescent feathers are also in some cases highly modified, and can be hollow, flattened or disc shaped [42] – physical features that would be a good indication of original iridescence if observed in a fossil feather.

The discovery that structures formerly identified as bacteria (see Box 1) are melanosomes paved the way for investigations of melanin-based colouration and iridescence in whole-bodied fossils such as feathered dinosaurs [38, 43–45], birds [46, 47]

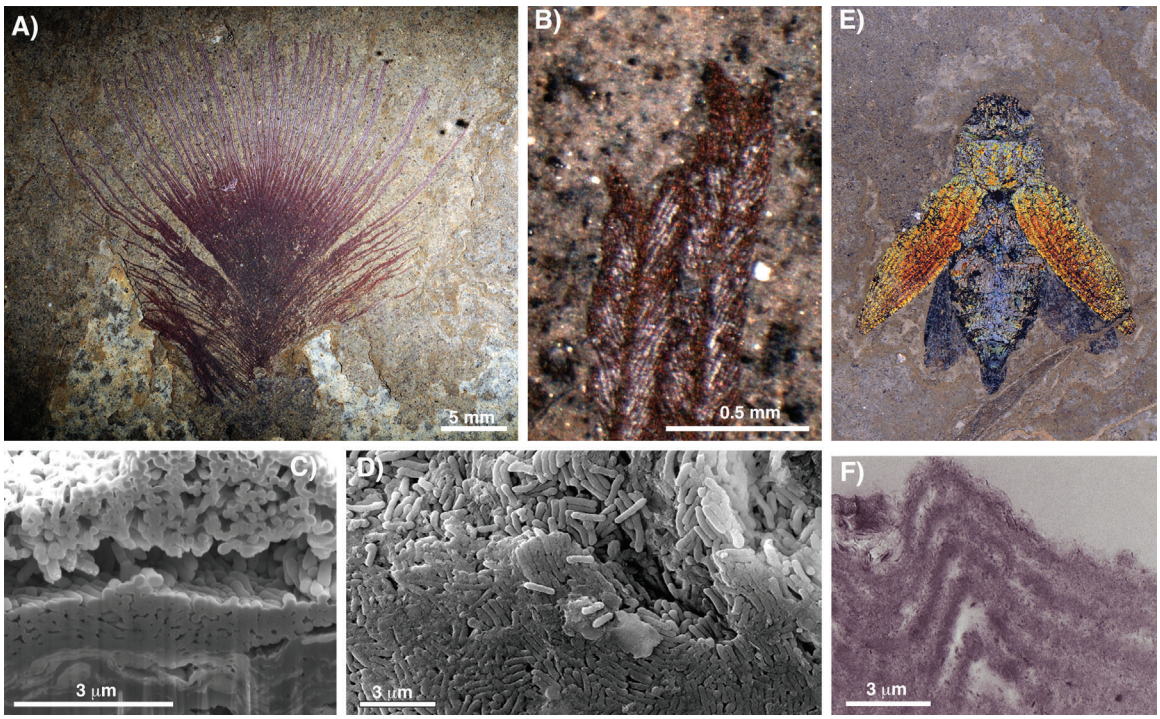


Figure 2. Iridescence and structural colours in fossils. **A–D**: Fossil iridescent feather [40, 41] from the Eocene Messel locality (47 million years old). **A**: The complete feather. **B**: Detail of iridescent feather in **A**, showing the silvery sheen observed in distal barbules. **C**: Focused Ion Beam SEM cross section of iridescent barbules, showing densely packed melanosomes on the dorsal surface, facing down. This demonstrates that the feather was only iridescent on the dorsal surface. **D**: Dorsal surface of feather from the counterpart, showing a smooth layer of melanosomes broken up in the top part of the view. **E**: Fossil Jewel beetle from Messel, preserving visible iridescence, length about 22 mm. **F**: Transmission Electron Microscopy image of a different beetle elytron from the same locality, showing the multilayer nanostructure responsible for the observed iridescence [14]. **A**, **B** and **D**: images of the author, **C**: image by James D. Schiffbauer; **E**, Courtesy of Dr. Stefan Schaal and Anika Vogel; **F**, Courtesy of Andrew Parker.

and other organisms [48] at a microscopic as well as chemical level.

Melanosome morphology is a reliable predictor of colour in mammals and birds

It is observed that there are two distinct chemical forms of melanin. Eumelanin (black to dark brown in colour), which is a complex molecule consisting of di-hydroxyindole and di-hydroxyindole carboxylic acid monomers complexly polymerised and cross linked [56, 57] to a macromolecule of yet unappreciated dimensions [58]. Phaeomelanin (reddish brown to buff yellow in colour) is derived from monomer units of sulphur-containing benzothiazine [59, 60]. The organelles synthesising these appear to be very conserved in morphology: eumelanosomes are oblong, sausage-shaped structures with a length of about a micrometer, while phaeomelanosomes are shaped like little subspherical 'meatballs' about 500 nm in diameter (Fig. 3). These morphological distinctions are conserved across mammals and birds [22], while detailed knowledge of the cause for this correlation between melanin chemistry and melanosome morphology still need clarification. It is unclear how phaeomelanin is distributed across other amniotes and metazoans and how their morphology is expressed. It was long thought that perhaps phaeomelanin was unique to mammals and birds, having perhaps evolved convergently [61]. However, recent studies have found phaeomelanin in tortoises [62], frogs [63] and chiton molluscs [64] and suggest a more widespread and earlier origin.

Modern birds are now well studied, and for the purpose of correlating fossil melanosomes in feathered non-avian dinosaurs a survey of feathers of distinct colour was assembled and their melanosomes were measured [38]. This dataset showed a good correlation between distinct colour categories and morphological variables, enabling the distinction between red/brown, black and grey colour in a canonical discriminant analysis with an overall accuracy of about 90%. A subsequent study produced a larger dataset, including melanosomes from iridescent feathers [44] as well as black penguin feathers, because their melanosomes are distinctly wider than other typical melanosomes [46]. With the added categories the accuracy is 82% [44]. The original analysis also included melanosome density as a variable [38], but this has subsequently been omitted [44]. It is difficult to correlate the density of melanosomes within a feather fossil as the keratin has degraded away and the melanosomes are condensed into a thinner layer.

Brightness gradients are often observed in unweathered fossil feathers, which resemble patterns observed in modern feathers. A simple type is a lighter pigmented base of the

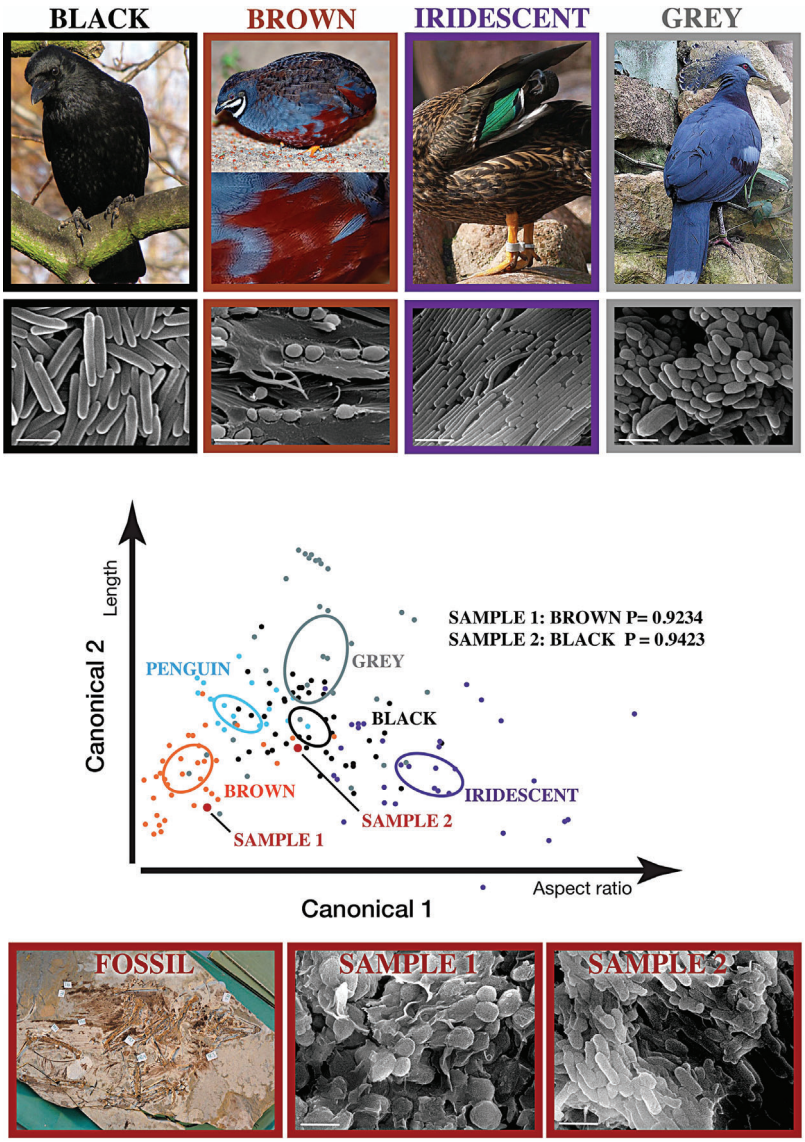


Figure 3. Inferring colour patterns from fossil melanosomes. Feathers from modern birds of distinct colours have their melanosomes imaged under a scanning electron microscope and are measured for their length, width, aspect ratio and the skew and coefficient of variation of each. Fossil samples (in this case a specimen of *Confuciusornis*, early Cretaceous, China) and their melanosomes are measured. By stepwise selection of a subset of the above variables based on their significance, colour can be predicted from the unknown fossil samples in a quadratic discriminant analysis with a probability. Bird images (black carrion crow, *Corvus corone*; King Quail, *Coturnix chinensis*; Mellers Duck, *Anas melleri*; Victoria Crowned Pigeon, *Goura victoria*) all from wikipedia, licenced under the Creative Commons attribution 2.0, attribution share alike 2.5, 3.0 and public domain, respectively. SEM images, courtesy of Natasha Vitek, Samuel Manning and Tamatea Nathan and the author.

feather, which is common in water birds [65]. It is observed that the density of melanosomes in these fossil feathers correlates with the relative observed brightness. Similarly, relative density of melanosomes in modern birds gives different relative brightness in colour [65]. So, while fossil and modern melanosome assemblages might not be directly comparable, it is possible to infer relative colour brightness within a fossil [65].

An important aspect in determining colour from melanosome morphology is the conserved nature and correlation with different pigmentary, and structural colours. However, it was observed in fossils that when both impressions of melanosomes and three dimensionally intact melanosomes are preserved in the same specimen, the voids are often larger than the melanosome itself. When analysed separately, these can result in significant [46] to minor [66] variations in predictions of original colour. What is likely to be occurring is shrinkage due to loss of CO_2, CH_4, H_2O and N_2 during burial and subsequent diagenetic maturation of the material [11]. When fossil squid ink sacs were analysed, about 10–15% intact melanin was found in Jurassic specimens from southern England [67], while none was recovered from similarly aged samples from Germany [68]. The only difference between the specimens is that the German formations have a deeper burial history and thus experienced a higher temperature/pressure regime; morphologically, they are identical. The alteration of the chemical structure clearly does not always affect the integrity of melanosomes, but loss of material likely condenses their structure. Artificial maturation experiments confirm this notion [69, 70]. However, as the most important variables in the aforementioned melanosome quadratic discriminant analysis are the aspect ratio, near-isometric shrinkage should have little effect. Other variables used are the skew and coefficient of variation of the length, width and aspect ratio, which should also be buffered from minor shrinkage, which is observed to be around (10–20%). However, length of the melanosomes was considered an important variable when the iridescent category was introduced [44]. One way to mitigate this effect is either to measure the imprints, which should have formed early and thus reflect the original dimensions closer; or, alternatively, to scale up the three-dimensionally preserved

Box 1

Bacteria or melanosomes?

It has been maintained by some that bacteria may account for the observed organic microbodies in fossil vertebrate tissues even in light of the recent evidence for these being melanosomes [31, 49–51]. It has similarly been argued, based on experimental studies [52, 53], that naturally occurring bacteria on decaying feathers are indistinguishable in size and shape from aligned melanosomes. However, these studies [53] did not account for the marked size difference between the bacteria observed and melanosomes. The bacteria observed in one particular experiment [53] were about 4–5 μm in length and more than a micron wide. This is more than double the size of any known melanosome, fossil or extant, and five times the size of a typical eumelanosome. There is currently no evidence that bacteria fossilise as isolated three-dimensional bodies. Bacteria *are* known as fossils, but as inclusions in cherts and organic biomarkers. Also, whilst even melanin-synthesizing bacteria [54] and single-celled fungi [55] exist, their chemistry is different and their size and distribution does not overlap with the narrow range of morphologies seen in melanosomes. These microbial melanin bodies are present as thin sheets in fungi [55] or secreted extracellularly [54]. Melanosomes are generally solid bodies of melanin, while few iridescent birds have hollow melanosomes [42]. These will not be conflated with melanin-synthesizing microbes given their structural, morphological and chemical differences.

melanosomes by 10–20% based on knowledge of the degree of thermal alteration and the correlated shrinkage.

Currently, evidence exists that melanosome morphology can be used to differentiate brown and black in mammals; and black, iridescent, grey and brown in birds. However, the extent to which this is applicable in other vertebrates and along the dinosaurian stem remains to be assessed [43]. A similar comparative dataset for mammals has yet to be published, although a comparative trend in morphology between phaeomelanosomes and eumelanosomes exists [37, 43]. Non-iridescent structural colours are generated by nanostructural arrangements in the keratin matrix, such as air bubbles and other air cavities [39]. As keratin does not preserve, non-iridescent structural colour will be moot from the fossil record of birds, which is a major limitation as many birds exhibit this such as jays, coraciiforms and parrots.

New mass spectroscopic techniques can be used to characterize fossil and fresh melanin

Since the infrared absorbance study in 1973 [17] of fossil cephalopod melanin, little had been done to characterise melanin in fossils. However, with the renewed interest in fossil melanin in recent years, several studies have taken on the chemical characterisation of fossil melanin. The most comprehensive study is by Glass et al. [67], which characterised melanin in fossil ink sacs using a suite of chemical techniques, including mass spectroscopy of oxidative products, analysis of pyrolysates and spectral absorption. In this study, the mass spectroscopy of oxidates demonstrated that about 10–15% of the fossil ink from British Jurassic ink sacs was intact eumelanin, whilst the remainder had decomposed. Pyrolysis GCMS was able to demonstrate a suite of melanin-derived pyrolysates in addition to a suite of diagenetic components, some of which had become secondarily sulfurised [67]. It also takes small quantities of material. A subsequent study [68] looked at fossil ink from German cephalopods of roughly the same age, but which had experienced deeper burial and thus organic maturation. These, however, did not contain significant amounts of intact melanin (0.01% of modern samples), but did contain similar pyrolysate compounds to the British ink sacs, which suggests the wisdom of using PY-GCMS for diagnosing fingerprints of fossil melanin even when chemically altered.

Another approach that has recently been implemented is synchrotron-based photoionisation mass spectroscopy [71], which was used to characterise black, brown and grey feathers. This method is interesting, as it analyses minute sample surfaces and obtains very specific high molecular weight ions to diagnose by, but has not been applied to fossils yet (This method should not be mistaken for x-ray syncrotron fluorescence methods, see Box 2).

A similar method is Time of Flight Secondary Ion Mass Spectroscopy (ToF SIMS), which has been used to characterise fossil melanin from fish eyes [72] and reptile skin [48]. This method relies on bombarding the surface with ions, fragmenting the substrate molecules, some of which will become ionised in the process. By collecting either the negatively or positively charged ions, a spectrum of secondary ions are collected and separated by the mass-dependent time-of-flight to the detector. The method is not optimal for characterising high molecular weight ions, but is particularly good in characterising spectra of secondary ions, which are of lower molecular weight. Thus a spectrum of molecular ion peaks is generated, which by themselves are not diagnostic, but their relative frequency might be. Lindgren et al. demonstrated a remarkable similarity between low molecular mass spectra derived from melanin and from fossil melanosomes, which could be discriminated from bacterial cultures [72] and chemically similar porphyrins [48].

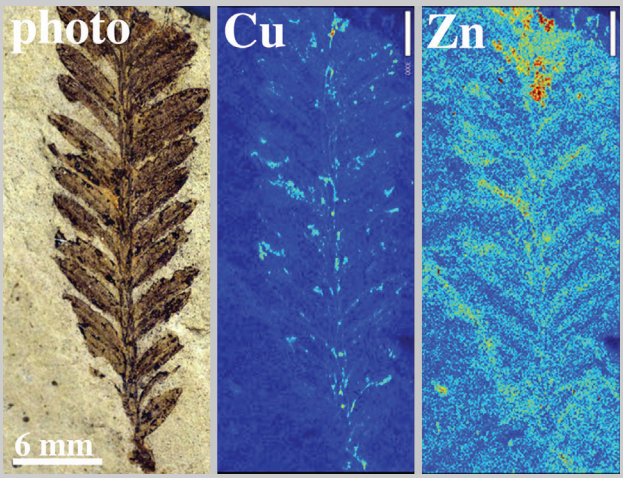
A great deal has been achieved in the last couple of years in confirming the presence of melanin in melanosome microbodies, which puts the old bacterial paradigm to rest (but see Box 1). The next step will be to characterise melanin from more diverse organisms and tissues, and characterise fossil phaeomelanin.

Other pigments to be added to the palaeo-palette

In birds, other important pigments utilised are, predominantly, lipid based carotenoids and psittacofulvins – e.g. in parrots [78] – which are derived from the diet. Some groups, such as

Box 2

Trace metals as biomarkers?



X-ray synchrotron fluorescence imaging of a fossil leaf.

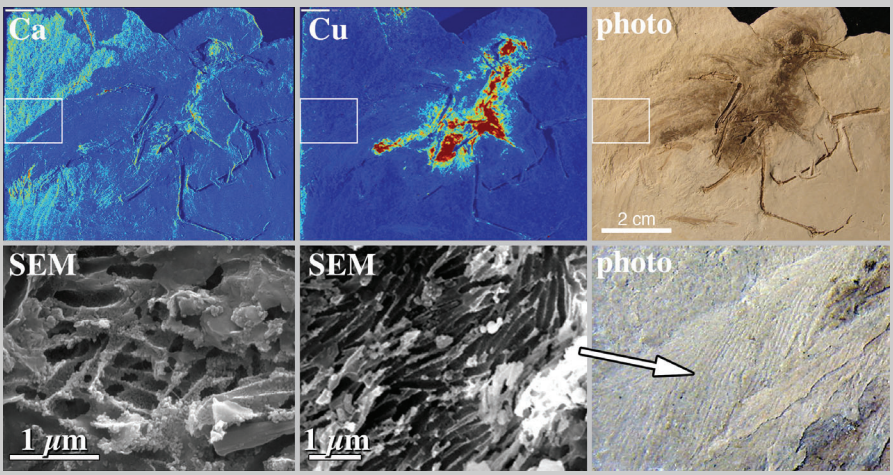
For decades, palaeontologists have characterised fossils under the SEM using energy dispersive X-ray spectroscopy, which can characterize the spatial distribution of elements across a surface. More recently, synchrotron X-ray fluorescence and X-ray absorption techniques has been introduced for characterising the elemental composition of fossils [73] and other living tissues. One study inspected fossil feathers [74], and proposed that metals absorbed by melanin through organic chelation could be diagnostic of melanin. More specifically it was argued that:

1: Organically chelated copper is a biomarker for eumelanin [74].

2: Trace metal distributions may be preserved after dissolution of the melanosome and allow for characterisation of original colour patterns [74].

This method would prove useful if the above statements were true, because it allows the scanning of large surfaces, whereas SEM is complicated when dealing with samples larger than a centimetre. However, there are problems with the biomarker method.

A biomarker is defined as '*an organic compound in natural waters, sediments, soils, fossils, crude oils or coal that can be unambiguously linked to specific precursor molecules made by living organisms*' [12]. This should mean that the observed organically chelated copper should not be detected in organic material of different origin than melanin. This statement is unfortunately not addressed in the paper [74] using appropriate controls such as negative samples. Several organic compounds chelate copper, e.g. the widespread porphyrins, which would look very similar to melanin in its mode of chelation and thus also coordination chemistry; humic acids derived from decomposed organic material [75] poses a similar challenge for disambiguation. Indeed, copper and zinc are observed in the organic residues of an Eocene (48 Ma), Metasequoia leaf shown here using synchrotron X-ray fluorescence and as have been subsequently shown elsewhere [76]. This leaf does not contain melanin, hence rejecting the notion [74] that organically chelated trace metals can be used as biomarkers.



X-ray synchrotron fluorescence imaging of a fossil bird.

Furthermore, the metals are tightly associated to the organic molecules that chelate them, which also mean that they will disappear if the organic material leeches away. Depicted is an undescribed Oligocene zygodactylid bird from the Ruby Paper Shale of Montana (Yale Peabody Museum). Feathers with organic preservation are present in the body contour region while the distal primary feathers (zoomed in region) are white and devoid of any organic material. A copper map shows a clear relationship to the distribution of organic material, which could lead to the interpretation that the body was darkly pigmented with melanin, whereas the primary feathers were white, similar to some recent reconstructions using this method [74]. However, electron microscopy reveals melanosomes in both the light and dark feathers as impressions: thus both copper and organic material was lost here. In order to reliably interpret colour patterns in fossils, microscopic studies are necessary to investigate the distribution and type of melanin present by studying the melanosomes present. A recent study of the complete specimens of Archaeopteryx [77], which has no preserved organic material, is therefore most likely misinterpreting weathering patterns, secondary precipitates and remains from human handling, rather than colour patterns.

owls and turacos, use porphyrins [79]. Spheniscins (putative pterin like pigment) are unique to penguins [80]. Mammals do not utilise pigments other than melanin in their pelage. In other amniotes, chromatophores are utilised, which consist of melanophores, xanthophores (pteridine and carotene containing pigment cells) and iridophores (discussed later).

While much has been done to characterise melanin from fossils, characterising other pigments has seen less attention. Nonetheless, pigments have been characterised from fossil red algae (borolithochromes) [20] (Fig. 1A and B) as well as crinoids (hypericinoids and brominated anthraquinone pigments) [81–84] (Fig. 1C). Both carotenoids and porphyrins are well known as constituents in bitumen and crude oil [85], indicating that it could also be found directly in fossil material. Heme-derived compounds (geoporphyrins) were recently discovered in the blood-filled stomach of a fossil Eocene mosquito [86], hence demonstrating that porphyrins can remain in situ in sedimentary rocks (although often remobilized). Fossil leaves have been found to contain several organic compounds in situ, such as the flavonoid pigments [87], which can give yellow, red or blue pigment, and are also known to colour certain flowers and berries. Leaves are known to be visibly green and orange in certain localities, such as the Miocene Clarkia beds of Idaho.

It thus seems possible to find other pigments in fossilised animal skins and feathers. Aromatic-, polycyclic- and lipid-derived pigments are indeed highly likely to fossilise in anoxic to euxinic conditions because of their robust cross linked and often polymerised molecular structure. However, detection of carotenoids in feathers has been attempted with little luck [88]. In birds, carotenoids are limited (albeit convergent) in distribution (40% of perching birds and 13% of non-perching birds [89]), which makes carotenoids less likely to be found in fossils outside of passerine birds, as well as non-avian theropods. It is observed that carotenoids, as well as porphyrins, are masked by melanin if co-occurring in feathers [90], which means that melanised feathers can be confidently assigned to possible colour by their morphology and melanin chemistry. It is also of concern that these pigments can remobilise during burial, and that porphyrins and carotenoids are ubiquitous derivatives of decaying algae and leaves in these deposits, which might contaminate fossils.

Thus, other pigments are increasingly likely to be found in animals (Supplementary Information Table S1). Caution should however be exercised in attributing them to the fossil, by carefully comparing fossil material with that of adjacent sediments; it should also be appreciated that other pigments likely have a lower preservation potential than melanin.

Melanin-based colours elucidate the origin and types of sexual display in feather-bearing dinosaurs

The study of melanin-based colours of feathered dinosaurs has opened an avenue for understanding the role of display and pigmentation in the evolution of the feather. An intriguing hypothesis emerged from the study of *Anchiornis* (Fig. 4A and B) [38] based on the observation that there is a remarkable coincidence in the appearance of complex pinnate feathers in maniraptorans and elaborate colour patterns within the feather. Simple colour patterns are seen in basal theropods, such as *Sinosauropteryx* (Fig. 4G), which exhibit filamentous integumentary appendages. As pinnate feathers evolved, they became elaborate structures, displaying more ornate colour patterns as is witnessed in the striped tail feathers of *Caudipteryx* [35](Fig. 4C and D), and the spangled feathers of *Anchiornis*. *Caudipteryx* is assumed to be a ground-dwelling dinosaur and more basal than presumed arboreal forms, such as *Anchiornis* and *Microraptor* (Fig. 4C). Thus the coincidence of pinnate feathers and within-feather colour patterns seems potentially causally linked, and this suggests that the evolution of pinnate feathers was for display. By extension, this suggests that feathers for aerial locomotion were exapted from feathers that originally evolved for display purposes [38]. The red head crest suggests a signalling function in *Anchiornis*, while the spangled pattern on the fore- and hindlimbs could be camouflage, well suited for an arboreal species in dappled lighting environment. Congruent with the hypothesis that pinnate feathers evolved for display is the observation that the fine divisions of a pinnate feather, the barbules, harbour melanosome-based iridescent nanostructures. *Microraptor* is a four-winged deinonychosaurian theropod dinosaur [91], which exhibits iridescent melanosome morphologies [44] (Fig. 4C). This demonstrates first and foremost the antiquity of iridescence, but also the rapid elaboration of the colour repertoire permitted by the pinnate feather anatomy. The striped tail of *Sinosauropteryx* resembles the putative camouflage-based banding seen in, for example iguanian lizards and some nocturnal mammals [92]. Thus, it seems feasible that, via the pinnate feather, avian-type intraspecific communication had evolved in maniraptoran/paravian dinosaurs, but this theory needs further testing.

A recent extensive survey of melanosomes in stem birds, mammals and other tetrapods showed a distinct shift in melanosome morphological diversity in mammals and birds, extending down to paravian/maniraptoran dinosaurs [43]. It was proposed that this could reflect a shift in physiology of some sort. However, an alternative scenario is suggested by the fact that both derived maniraptoran dinosaurs and mammals are covered by dense appendicular integument (hair and feathers). This resulted in the loss of the chromatophore, an integumentary complex that allows for diverse and dynamic colour patterns (as detailed below). It is therefore more feasible that the diverse range of melanosomes in warm-blooded animals is a reflection of the necessity to evolve new approaches to making colours and colour patterns. These novel approaches came through the elaboration of more radical variants of phaeomelanin- and eumelanin-based colours than seen in chromatophore bearing amniotes where melanin mainly serves to provide dark colouration and to absorp unscattered light by iridophores. The elaboration of melanosomes into photonic nanostructures as well as grey colours in birds could thus be due to the evolution of keratinous insulatory structures, and resulting loss of chromatophores rather than the physiological shift to warm bloodedness or similar. A comparable elaboration of pure eumelanin- and phaeomelanin-based colour patterns is observed in the keratinous shield of tortoises [62].

Figure 4. Dinosaurs with inferred and reconstructed colour patterns. **A**: *Anchiornis huxleyi* (late middle Jurassic, ~160 Ma, Liaoning, China, presumed paravian theropod) from the Shandong Tianyu museum. Notice the spangled colour pattern preserved on forelimb and hind-limb feathers. **B**: Reconstruction of *Anchiornis* based on examination of melanosome morphology across a well-preserved specimen [38]. **C**: The four-winged dinosaur, *Microraptor* (Early Cretaceous, ~120 Ma, Liaoning, China, paravian theropod), preserving long, asymmetric pinnate feathers on forelimbs and hindlimbs. **D**: Reconstruction of *Microraptor*, based on statistical comparison of melanosomes to modern birds inferring it to be iridescent [44]. **E**: *Caudipteryx* (early Cretaceous, ~120 Ma, Liaoning, China, presumed basal maniraptoran theropod), preserving pinnate feathering along forelimbs and tail. The tail preserves [35] dense transverse light and dark banding (**F**). **G**: The basal theropod, *Sinosauropteryx prima* (early Cretaceous, ~120 Ma, Liaoning, China) [93], preserving light and dark bands along the tail, including melanin remains in the eye (iris) and stomach (presumably liver, peritoneum and potentially lungs). **A**, Courtesy of Robert Clark; **B**, courtesy of Carl Buell (Illustrator) and Carl Zimmer (Commissioner), from the Book 'Evolution – Making sense of Life' (Zimmer and Emlen, Roberts and Co. Publishers); **C–F**, Courtesy of Mick Ellison; **G**, Authors' own photo, with permission from Yunbai Zhang, Nanjing.

With the ability to detect melanin in fossil feathers, several emerging questions about the evolution of such structures for display and camouflage are opened up. Very few taxa have been studied so far, and detailed comparative studies could reveal sexual dimorphism and cryptic species in the fossil record as well as detailing the evolution of the avian integument as a colour bearing structures. The study of the transition from a chromatophore-bearing colourful integument to the similarly colourful feathery integument is now possible with the vast number of feathered dinosaurs and other stem birds from China and other localities.

Colours in mammals can give clues to their positional behaviour and the environment that they inhabited

Mammals generally have a limited colour vision compared to most other animals, being effectively dichromatic. Trichromacy has however re-evolved in primates and marsupials [94], and some rodents exhibit some sensitivity to UV light [95]. In effect, colouration in mammals is mainly restricted to camouflage, with some inter- and intra-specific signalling and sexual signalling in some primates and a few other mammals [92]. Colours derive entirely from elaboration of phaeomelanin and eumelanin, and structural colouration is rarely present [96, 97]. A variety of camouflage patterns is observed, such as background matching/pattern blending with spotted patterns, resembling patterns of dappled light in arboreal species in closed environments. Stripes are generally seen in grassland-dwelling species [92]. Disruptive colouration is not conclusively confirmed, but some contrasting patterns seems to be correlated to aposematism [98–100]. The most common type of colour pattern is countershading, which serves to enhance background matching by reducing contrasting shadow created in well-lit environments. The top–down component in lighting means that countershading is closely tied to positional behaviour, as is seen in primates where countershading is negatively correlated to vertical posture [101]. The selective pressure for countershading is often lost at greater size, as is observed in cetaceans [102]. The correlation between countershading, environment and other factors are well studied; in ruminants there is a distinct correlation in sharpness of countershading to the openness of the environment inhabited, latitudinal solar radiation and body size [103].

In whales, distinct, colour markings are correlated to communal behaviour, fast swimming and ostentatious behaviour at the water surface, which seem connected to intraspecific communication [102]. White markings and asymmetric colouration are correlated to diet (fish, krill and squid) and might serve to disorient prey and allow for visible coordination among hunting individuals [102]. Many mammals, especially ungulates, exhibit contrasting white and black markings, which are seen to be correlated to gregarious species in open environments and thus seem to serve intra- and inter-specific communication [92].

Widespread investigation of countershading in fossil mammals is permitted by the general preservation of specimens in lateral aspect, which is due to their anatomy (bats are an exception). For example, the Eocene primate *Darwinius massilae* from Messel [104] exhibits hair impressions on both the belly and back which have similar brightness, and thus suggest a vertical life posture. This notion will need confirmation from electron microscopical investigation.

Mammals might serve as modern analogues to many fossil groups whose modern closest descendants have a different life-style today. An example is the ground-dwelling ornithischian and sauropodan dinosaurs, which in many ways resemble mammals more than birds in ecology. Mesozoic marine reptiles might also see analogues to modern whales rather than terrestrial reptiles. Discrepancies from mammal analogues will also be of interest for understanding unique extinct ecologies.

Caution should be exercised when interpreting fossil colour in other amniotes

The amniotes inherited integumentary chromatophores from its fish ancestors. Chromatophores are colour-producing organs that combine different pigments and structural colouration to produce a variety of colours. For example, the common colour, green, is produced by yellow pigments (xanthophores) in combination with iridiophores, which scatter blue light (Fig. 5). In order for the scattered light waves to be filtered from the unscattered light, a basal layer of melanosomes serves for absorption. Iridiophores consist of platelets with guanine crystals inside; these cellular structures are unlikely to fossilise in most localities, even those with exceptional preservation. Similarly, xanthophores and red-coloured erythrophores contain pteridines and carotenoids, which are likely to remobilise during fossilisation and not readily be identified or discriminated from contamination by the surrounding sediment. The only likely constituent of a chromatophore complex to be readily fossilised is the melanin-bearing melanosomes of the melanophores. It is observed that even white and yellow-white patches, such as white dorsal stripes in lizards [105], or the belly [106], will have a basal layer of melanin. It is also observed in a juvenile species of crocodile that melanophores migrate vertically in the epidermis of the belly to shift from a counter shaded state to a fully melanised brown colour in a diurnal cycle [107]. Thus, the recent interpretation of ichthyosaurs as lacking countershading and being completely externally melanised [48] is inconsistent due to the lack of knowledge about the distribution of iridophores and xanthophores, inherent in reptile skins. Thus, a completely melanised fossil reptile could have been countershaded or green, or another colour entirely (Fig. 5).

Nonetheless, distinct and original melanised colour patterns are sometimes observed, especially when incorporated into keratinised integument as is seen in a Palaeocene turtle [108], the scales of a mosasaur [25] (Fig. 1Q) or the head crest of a pterosaur [26] (Fig. 1R). The incorporation of melanin into a dead, keratinised integument ensures the loss of chromatophores. Thus, unless distinct colour patterns are observed in amniote skins, any inference of colour patterns such as countershading or lack thereof should not be made.

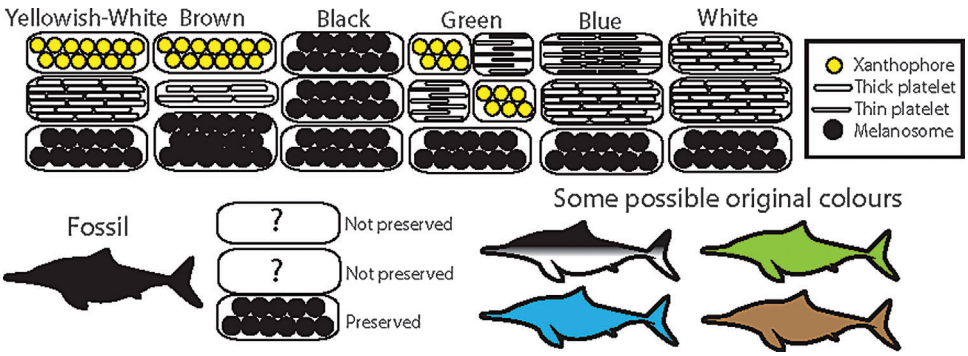


Figure 5. Chromatophores, colour production and uncertainty in reconstructing colouration in fossil (non-endothermic) vertebrates. Above, the arrangement of pigment bodies, xanthophores and iridocytes over a melanophore result in a spectrum of colours (modified from [105]). The iridocytes do not preserve readily in fossils, xanthophores and the pigments (pterine or carotenoids) within have not been identified in soft tissue fossils. Thus characterizing a fossil ichthyosaur (bottom left) based only on chemical and morphological characterization of melanosomes will need to take the presence of chromatophores into account, as the resulting colours could be multifarious (bottom right) given that hitherto only the contribution due to melanin has been characterised.

Melanin-based colour patterns in fishes are good indicators of the environment inhabited

The aquatic environment offers a whole different range of conditions for signalling and camouflage compared with air. Visibility is generally low to non-existent and light is filtered through the water column, making it spectrally and spatially varied [109]. In open waters, the day-time light levels from above are on average four orders of magnitude higher than from below and one or two orders of magnitude lower than from the side [110, 111]. This results in organisms exhibiting marked counter shading in well-lit open water environments. In fresh water environments, it is observed that more turbid lakes are inhabited by fish with less conspicuous countershading and other colour traits, as is seen in Boreal perch [112]. This is probably a result of relaxed selection. Fishes also communicate with colour patterns: shallow water fishes in particular often exhibit colourful and contrasting patterns in addition to sexual dimorphism [113]. Sexual selection and distinct colour traits have been observed to correlate with increased speciation and diversity in cichlids [114]. It has been argued that colourful coral reef fishes can exhibit both camouflage and communication [115], as the colour patterns blur at a distance, matching background light, but at close range they exhibit noticeable colour marks. Many of these fishes also live in close proximity to colourful substrates, such as sponges and corals that they resemble. Another important point is that fishes lack long-wave photoreceptors, and are more tuned towards UV sensitivity, which means that fishes that appear colourful to the human eye are likely to be well camouflaged to a fish eye [111, 115].

Fishes utilise several pigments as well as iridocytes to create diverse colour patterns and mirrored camouflage [111]. These will not necessarily be available to study in the fossil record. Melanin is known to be preserved in fishes [22, 72], and provides some important colour pattern information. Melanin-based colour patterns are regularly observed in fossil fishes. A particularly diverse assemblage is the Eocene Monte Bolca assemblage (Fig. 1K, M and N) as well as the Jurassic aged Ettling in Bavaria, Germany (Fig. 1L). Colour patterns are also documented in the diverse and disparate chondricthyan fauna from the Carboniferous Bear Gulch [116], which preserves countershading and transverse pigmented stripes as well as internal organs [117]. This is also seen in a Permian actinopterygian fish [118].

The study of colour patterns in fossil fishes is likely to provide evidence of the inhabited environment. Assemblages with conspicuous countershading are likely to reflect open water fishes (Fig. 1L), while forms with conspicuous vertical (Fig. 1M) and horizontal (Fig. 1N) colour bandings as well as spots (Fig. 1K) are likely inhabitants of well-lit shallow and benthic environments in which dazzle camouflage and background matching are advantageous.

Insect colour patterns can reveal ancient predator–prey interactions

Insects mainly camouflage themselves by countershading, as is seen in caterpillars [119], by disruptive colouration that breaks up the shape of the insect body, and by background matching. Poisonous or unpalatable insects might exhibit conspicuous colours to highlight their unsuitability as food (aposematism). Many insects might mimic these aposematic markings, as is seen in wasp-mimicking hoverflies, beetles and mantidflies. In some diurnal insects (dragon flies and butterflies in particular), intra-specific recognition plays a role by signalling dominance. Eyespots evolved in response to attacking birds. Many moths, butterflies, grasshoppers, stick insects and mantids exhibit wings that in relaxed position are concealed, but a pair of eyespots can be suddenly revealed when spread and thus create a startling effect upon the attacking predator. Smaller eyespots are present in some butterfly species on the underside of wings. These are deflection marks.

Fossil insects are very commonly found with preserved colour patterns as light and dark patches. Sometimes, the

cuticle preserves its three-dimensional layering, and, hence photonic nanostructures thus resulting in the survival of iridescence from the living creature (Fig. 2E and F) [14]. The diagenetic alterations are very likely to alter these iridescent colours, but it is possible to reconstruct the original hues by physical modelling [120]. In contrast to birds, which widely use iridescence in intraspecific signalling, insects generally utilise structural colours in camouflage or aposematism.

By comparing fossil colour patterns with those in modern counterparts and analogues, their function can be hypothesised. Therefore certain behaviours might be traced back through time, such as intra-specific communication in ancient dragonflies; but perhaps more interesting is to trace the nature of ancient predator–prey relationships. In some cases, ancient colour patterns can show that lineages lived in different niches compared with today: examples are the neuropterans. The extinct lineage of neuropterans called the Kalligrammatidae (165–125 Ma), exhibit a remarkable array of eye spots on their wings (Fig. 1H) [121] (also known as the butterflies of the Mesozoic, due to their morphology and exorbitantly large size). These eyespots demonstrate a startling or deflection strategy, which suggest diurnal activity. Modern neuropterans are usually nocturnal and much smaller. Eyespots in modern butterflies are generally a deterrence pattern against avian predators approaching with speed from a distance. This suggests that these ancient kalligrammatids were predated upon by airborne carnivores with good visual systems. Could these have been the paravian dinosaurs that took to the skies at this time? *Anchiornis* (Fig. 4A and B) is among those early flighted (more likely gliding) dinosaurs known from coeval beds [121], and is a potential predator of these lace wings. This period saw an apparent arms race between paravian/avialan dinosaurs and pterosaurs [122], and it cannot be excluded that pterosaurs were also predators that provided some of the selection pressure for the evolution of this strategy although pterosaurs saw an earlier appearance in the Triassic. In the same time period, remarkable cases of leaf mimesis as camouflage is known in insects [123, 124], which is almost certainly a response to the emergence of predators with complex visual systems – seemingly the early flighted dinosaurs and pterosaurs.

Conclusions and outlook

Detecting and analysing fossil pigments provides an avenue to understanding new aspects of behavioural evolution and the interaction of fossil organisms with their palaeo environments. The most ubiquitous pigment is melanin, which is the only pigment that can be reliably detected in the fossil record both morphologically under an electron microscope (particularly in vertebrates) and chemically. Other pigments can be preserved in fossils, but they are more limited in distribution, have a different preservation potential, and are more difficult to detect. Certain limitations are inherent in inferring all aspects of pigmentary and structural colours, but recent studies have opened up a tool kit that allow for reconstructing a greater palette than ever before. Palaeo colour has gone from a world in 50 shades of grey to one painted in Technicolor, and the coming years will certainly see a great number of innovative studies looking at colours of the past.

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