Melanosomes or Microbes: Testing an Alternative Hypothesis for the Origin of Microbodies in Fossil Feathers

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Microbodies associated with fossil feathers, originally attributed to microbial biofilm, have been reinterpreted as melanosomes: pigment-containing, eukaryotic organelles. This interpretation generated hypotheses regarding coloration in non-avian and avian dinosaurs. Because melanosomes and microbes overlap in size, distribution and morphology, we re-evaluate both hypotheses. We compare melanosomes within feathers of extant chickens with patterns induced by microbial overgrowth on the same feathers, using scanning (SEM), field emission (FESEM) and transmission (TEM) electron microscopy. Melanosomes are always internal, embedded in a morphologically distinct keratinous matrix. Conversely, microbes grow across the surface of feathers in continuous layers, more consistent with published images from fossil feathers. We compare our results to both published literature and new data from a fossil feather ascribed to Gansus yumenensis (ANSP 23403). ‘Mouldic impressions’ were observed in association with both the feather and sediment grains, supporting a microbial origin. We propose criteria for distinguishing between these two microbodies.

In 1995, approximately 1μm long elongate microbodies were observed on the surface of fossilized feathers from the Eocene of Messel (Germany), Oligocene of Cereste (France) and Jurassic Solnhofen Limestone (Germany)1 using scanning electron microscopy (SEM). These microstructures were proposed to represent mineralized microorganisms and the gycocalyx they secrete, and, based upon this interpretation, a new mode of fossilization was proposed1. This was further supported by the observation of similar microbodies associated with fish, mammals and other material in the same deposit, and a microbial origin for these has not been disputed2,3. However, a decade after their discovery, the microbodies associated with fossil feathers were reinterpreted as eukaryotic melanosomes4; intracellular, membrane-bound organelles where melanin pigment is synthesized and stored5. Because melanosomes vary in morphology in extant feathers6, it was additionally proposed that color could be inferred in fossil feathers solely on the shape of these structures; round indicating red and brown hues and oblate indicating black and/or grey7–13. Finally, aspects of behavior, physiology and ecology were posited for avian and non-avian dinosaurs7–12 based solely upon these morphological data.

Bacteria and the extracellular polymeric substances (EPS) they secrete are known to fossilize14,15. Because bacterial cells contain a cell wall composed of resistant, cross-linked peptidoglycan polymers16, they are hypothesized to have greater preservation potential than eukaryotic intracellular organelles17, protected only by a lipid bilayer. The fossil record contains many examples of fossilized bacteria and biofilms2,15,10,19, and some processes leading to their preservation have been elucidated in the lab20. Fossilization of intracellular organelles is extremely rare, even when cell-like microstructures retaining transparency and flexibility persist21–23, but morphologies consistent with organelles have been noted24. Whether these represent molecule for molecule replacement in mineral or original components cannot be determined without chemical data. Because melanosomes and microbes overlap in shape and size, differentiating between the two is critical for supporting hypotheses of behavior7, evolutionary significance9,10, and/or ecology11 in extinct organisms.

Because intracellular organelles are definitive evidence for a eukaryotic source, it is imperative to differentiate between melanin and melanosomes before proposing far-reaching interpretations of color, habitat, niche and
Melanin is an organic pigment derived from tyrosine residues that are highly cross-linked and resistant to degradation. Both eukaryotes and prokaryotes produce melanin pigments, the basic structural unit of which is unknown. Melanosomes are membrane-bound intracellular organelles within specialized cells called melanocytes where melanin pigments are polymerized by enzymes and stored. Melanosomes have also been referred to as melain “granules”, a term more appropriate to describe irregular clusters of the melanin pigment regardless of source. During maturation of keratinous pigmented tissues, including skin, hair, and feathers, melanocytes are transferred from melanocytes to keratin-producing cells, where they become embedded within the keratinous matrix. While melanin chemistry confers high preservation potential to the pigment, this resistance to degradation has not been shown to extend to the membrane-bound organelles containing pigment grains. In fact, melanosomes are related to lysosomes, organelles producing enzymes involved in autolytic degradation, therefore indicating a predisposition for rapid degradation. Finally, bacteria are prokaryotic organisms that may or may not secrete an enzyme-rich exopolymeric substance that facilitates bacterial adherence to a substrate and that may assist in degradative processes. Melanosomes, as membrane-bound organelles, are not produced by bacteria, although many do produce and utilize the pigment melanin.

Data presented in previously published works describing fossil material have not eliminated either a eukaryotic melanosome or prokaryotic biofilm source for the microbodies associated with fossil feathers; thus, both hypotheses remain valid. We conducted actualistic experiments on extant feathers to test the hypothesis that microbodies observed in fossil feathers are more consistent with melanosomes than degrading bacteria colonizing the surface. We incubated extant feathers with pond inoculum (Fig. 2a and b), show microbial growth on the surface of the feather. Microbial bodies are dense and overlapping, form a virtually continuous mat across the feather surface and follow feather contours (Supplementary Fig. S2), in contrast to the relatively less abundant and non-overlapping distribution of melanosomes depicted in Fig. 1. In some regions, microbial bodies demonstrate a high degree of alignment (Fig. 2a, arrows), similar to patterns ascribed to fossil melanosomes (Figure 2d and e, reprinted with permission). Also see Fig. 1b in Vinther et al. (2008) and Fig. 1c and e in Vinther et al. (2010).

It was previously noted that purported melanosomes are easier to observe in degraded fossil feathers, but we show that microbial cells can also be removed from their EPS, leaving ‘mouldic impressions’ (Fig. 2b) similar to those noted in fossil feathers (Fig. 2c, reprinted with permission). We propose that because microbes and their EPS are participants in degradation, it is more parsimonious to attribute these bodies to microbial overgrowth than to published data for fossils. We also examined a Chinese fossil feather using SEM-EDX to visualize microbodies or ‘mouldic impressions’ similar to those observed in previous studies of fossil feathers.

**Results**

Figure 1 shows that electron-dense melanosomes are visible using transmission electron microscopy (TEM) and are embedded within the keratinous matrix of both pigmented feathers (Fig. 1b, c), but are lacking in white feathers (Fig. 1a). These distinct bodies appear either elongate or round, depending on cutting aspect, but rarely overlap, are often separated, and are always completely surrounded by the keratinous matrix of the feather. Conversely, cells from cultured, endospore-forming B. cereus are external to the feather (Fig. 1d) and are more similar in size, shape, distribution and location to previously published work on fossil melanosomes in side by side comparisons (Fig. 1f, reprinted with permission).
Figure 2 | FE-SEM micrographs of biofilm overgrowth of extant feathers compared with published images of fossil feathers. (a) Guineafowl feathers exposed to naturally occurring culture show strong alignment of microbial cells (arrows). (b) Higher magnification of biofilm edge in (a) showing where bacteria cells have been eliminated from the surrounding matrix (arrow), leaving voids similar to those figured in (c), which were identified as “…eumelanosomes preserved as moulds inside small areas that are separated from each other by anastomosing ridges of degraded feather (at arrows in c)” (scale bar: 5 μm). Reprinted by permission from Macmillan Publishers Ltd: [NATURE]13, copyright (2010). (d) “Strongly aligned, closely spaced, eumelanosomes preserved as solid bodies,” in Confuciusornis feathers (scale bar: 2 μm). Reprinted by permission from Macmillan Publishers Ltd: [NATURE]13, copyright (2010). (e) “Melanosomes (arrows)” figured in Carney et al. 2012 (scale bar: 1 μm). Reprinted by permission from Macmillan Publishers Ltd: [NATURE COMMUNICATIONS]7, copyright (2012).

Figure 3 | SEM images of melanosomes from extant Gallus gallus feathers. Black (a,b) and brown (c,d) chicken feathers sectioned longitudinally. (b) Although the feather is visually black, both ovate eumelanosomes (arrow) and round phaeomelanosomes (arrowhead) are present. (c) Phaeomelanosomes (arrowhead) are also observed in the brown feather. (d) Melanosomes exhibit unsmooth, granular surfaces. Using this method, like those observed in TEM, melanosomes appear randomly oriented, rather than in dense mats as reported for fossils (see text). Considerable size variation (e.g., ~0.5 μm–~2 μm for the eumelanosomes) is observed between all melanosomes.
melanosomes embedded in a keratinous matrix without chemical data.

We have examined many modern pigmented feathers (at least 20) prepared by fresh fracture, cryofracture, and/or sectioning in multiple planes, but definitively identifying melanosomes under SEM is not trivial. Consistent with other observations, we found that, in most cases, modern feathers must be treated to reduce or remove keratin before melanosomes embedded within the matrix are visible; alternatively, feathers embedded in resin and longitudinally sectioned allow visualization under SEM. Without treatment, melanosomes were seen in only one feather after fresh fracturing (Supplementary Fig. S3). Melanosomes in extant chicken feathers are more sparse and have a non-overlapping distribution (Fig. 1b and c) compared to bacteria (Fig. 1d and e) when both are viewed in TEM. Furthermore, under our experimental conditions, microbes grew across the surfaces of the feathers in densely packed layers, more similar to what have been presented as fossil melanosomes than to in situ melanosomes in TEM sections of modern feathers. Never were melanosomes observed in whole mounts of extant feathers, as has been reported for visualization of fossil feather melanosomes, nor were they observed without extensive manipulation.

Following the protocol in Li et al. 2010, we were able to observe melanosome exposure, and that these cannot be differentiated from melanosomes embedded in a keratinous matrix without chemical data.

Melanosomes (dashed arrow) are always internal to the feather surface, sparsely distributed and non-overlapping. (b) Higher magnification of boxed area in (a) shows interaction (arrowhead) of bacteria (arrow) with barb surface (white line). White dashed arrow depicts internal, electron-opaque melanosome. Without staining, (c) bacteria are not visible on the external surface, as they are not normally electron-opaque, in contrast with easily visualized, internal, electron-opaque melanosomes (dashed arrow). The melanin pigment is inherently electron dense; no staining is necessary. (d) Enlarged image of unstained section in (c) shows vacuoles associated with internal melanosomes, as has been noted previously. Keratin matrix completely surrounds melanosomes, making them difficult to image in SEM without additional treatment.

Microbial bodies are easily distinguished from melanosomes in extant feathers, because they are not electron-dense and because melanosomes are always internal and embedded in the keratinous matrix while microbes are present as surface overgrowth (Fig. 2a, b and Supplementary Fig. S1). Additionally, their electron-dense nature makes melanosomes visible in both stained and unstained sections (Fig. 4), while microbes are only clearly visible after heavy metal staining (Fig. 4a and b).

We also examined an isolated fossil feather (Fig. 5) (ANSP 23403) collected from the Xiagou Formation (Lower Cretaceous) in northwestern Gansu Province, China, and ascribed to the bird *Gansus yumenensis*. As reported in other work, 'mouldic impressions' (Fig. 6) were observed associated with the fossil feather. Although the feather exhibited regions of different color (black apically (Fig. 5a), brown more basally (Fig. 5b)) the 'mouldic impressions' did not differ in type or distribution between the black and brown regions. In addition, these impressions were also observed on sediment grains (the identity of which is confirmed by EDS (Fig. 7))
superficially associated with the feather. Because the sediment grains are clearly not part of the feather structure, yet retain ‘mouldic impressions’, a microbial origin for these impression structures is favored. SEM-EDS analysis revealed that the fossil feather (Fig. 7 and 8) is composed primarily of C and Fe, although quantitative data from the brown portion (Fig. 8) indicates a reduced amount of C compared to the black region (Fig. 7) (see Table 1 for quantitative elemental data). The sedimentary matrix surrounding the feather, as well as the sediment grains observed associated with the fossil material, are composed primarily of O, Si and Al.

Discussion

We evaluate statements used by others to support a melanosome origin for microbodies in fossil feathers, and put forth alternative interpretations equally supported by the same data. (1.) Localization of microbodies to ‘dark’ and absence from ‘light’ regions of one fossil feather support a melanosome origin. Three hypotheses exist to explain the striped patterning of the fossil feather reported in Vinther et al. 2008. First, the original feather, in life, was also striped, with melanosomes distributed in the colored regions of the feather and no melanosomes in the uncolored parts, and the fossils preserve this original pattern. Second, microbial overgrowth on the surface of the feather is distributed according to the relatively more nutrient-rich pigmented feather regions over unpigmented areas7,13. Third, microbes preferentially colonized and completely degraded those feather regions most easily broken down (e.g. unpigmented)7,14,15, thus were no longer present in these regions during fossilization, but continued to act on the more resistant, melanin-containing regions. (2.) Densely packed and aligned/organized layers support a melanosome origin (Fig. 2c–e)7,13. Recently published research indicates original melanosome geometry and distribution are altered with heat and pressure16, diagenetic processes affecting fossils, and that have not been taken into account in previous research claiming melanosome morphology in fossil feathers reflects their original color.
Data presented here show microbial overgrowth is dense and can be aligned, whereas internal melanosomes are more sparsely distributed and relatively random in orientation as observed in both SEM and TEM. Our data show this description is more consistent with biofilm overgrowth (Fig. 2a, b) than melanosomes (Fig. 1b and c). In addition, bacteria can align (Fig. 1e, 2a, and Supplementary Fig. S1c) and follow the contours of a feather (Supplementary Fig. S2) in layers. Unlike most bacteria, which usually exhibit more uniform and smooth cell surfaces59,60, melanosomes, as observed in a previous study26, are not smooth, but rather have bumpy and non-uniform surfaces (Fig. 3b and d). Because bacteria have a tough cell wall external to the plasma membrane59, this granular topography26 may be more difficult to observe in melanin-containing bacteria than in eukaryotic melanosomes. This remains to be tested.

Elements consistent with melanin, including Ca2+, Cu2+ and Zn2+43–44, are associated with feathers and thus support a melanosome origin. However, these biomarkers are also used and/or sequestered by bacteria45,46, including common soil bacteria and other microorganisms, and are also part of the sedimentary environment. These microorganisms are also capable of synthesizing melanin59, thus elemental data alone cannot be used to discriminate microbes from melanosomes.

Our examination of the fossil feather ascribed to Gansus yumenensis showed no microbodies of any type, but did reveal ‘mouldic impressions’ (Fig. 6) similar to those described in previous studies. However, these impressions did not vary between black and brown regions of the feather, and were also observed on sediment grains (Fig. 6) (confirmed with EDS data) associated with the fossil. Therefore, because sediment grains do not contain melanosomes, it more parsimonious to propose these ‘mouldic impressions’ represent a microbial origin (remnant EPS) than intracellular structures derived from the original feather. A very similar image was presented in Barden et al. 2010 (Fig. 1H) but elemental data were not mapped, so identification as a sediment grain cannot be confirmed in their paper. Visual, textural (Fig. 5 and Fig. S4), and elemental data (Fig. 7 and 8 and Table 1) from the fossil feather suggest differential diagenetic processes acting on the different regions of the feather. The reason for these differences is beyond the scope of this paper and requires additional studies.

Pending the identification of definitive molecular or chemical signals unique to either melanosomes or microbes in extant feathers that are likely to persist across geological time, distinguishing microbes from melanosomes in fossils may be difficult. Until new data are presented, we propose the following criteria to support a melanosome origin for microbodies associated with fossil feathers: 1) a taphonomic mechanism must be demonstrated for removing resistant keratin while leaving the intracellular organelles intact; 2) electron-dense material should be localized to the microbodies using TEM-EELS (TEM coupled with energy loss spectroscopy) or TEM-EDX; 3) melanosome-specific (e.g. cargo proteins5) or bacteria-specific (e.g., peptidoglycans16) biomolecules should be localized to the structures to eliminate the alternative, using in situ surface techniques (e.g., time of flight secondary ion mass spectrometry (TOF-SIMS42)), or other softer mass spectrometry imaging methods.

The ‘mouldic impressions’ described in fossil feathers imply that the microbodies were once present, and subsequently degraded from an amorphous material that retained the impression through fossilization. Therefore, if the structures are melanosomes, this material

Figure 7 | SEM-EDS data derived from the black fossil feather region. The six dominant elements (~95% of detected electrons) are presented in order of decreasing abundance by weight percent. Elemental map and quantitative data (Table 1) suggest the fossil feather is composed primarily of C although Fe is also abundant. In contrast, Al is localized to the sediments and not in the feather. The surrounding sediment is composed primarily of O and Si which is consistent with previous analyses53. The data demonstrate that the ~8 μm-sized spheres shown in Fig. S4 are composed of O and Si, consistent with sediment grains and not part of the original feather structure.
should resemble β-keratin, because the β-keratin matrix of feathers is highly resistant to degradation. β-keratin is a rigid structural protein comprising ~80% of the organic matrix of mature feathers. Its multiple cross-links, twisted-pleated-sheet tertiary structure and hydrophobic amino acid composition confer high preservation potential to structures comprised of this protein. Mammals do not produce β-keratin, thus common contamination by human keratins can be easily recognized. If microbial, the material should retain microbial-specific biomarkers in the environment immediately surrounding these microbodies.

Some papers state the keratin matrix is completely degraded in fossil feathers and no feather structure remains, but fail to state what material, then, retains 'mouldic impressions'. If feather structure is not preserved, how is the object identifiable as a fossil feather, and how has a biofilm source been eliminated? The presence or absence of keratin has not been tested in any fossil feather purporting to contain melanosomes. Yet, the melanosome hypothesis posits that melanosomes are preserved in 'life position'. Modern feather melanosomes ('in life') are always embedded in a keratinous matrix, thus the 'mouldic impressions' cited by many in support of the melanosome hypothesis are assumed to be made in the original keratinous matrix of the feather, an assumption that has never been tested.

It should be noted that handling history and full depositional description are often not included in studies purporting to recover fossil melanosomes. Excavation of a fossil feather as part and counterpart could be interpreted differently than a feather collected as a whole specimen. This information is critical for determining where these microbodies are localized (ie. inside versus on the surface of the feather).

More importantly, even if irrefutable data support a melanosome origin for microbodies in a given fossil, imparting color to the entire organism, or even the entire feather, based upon their presence cannot be inferred. All melanized feathers in extant birds contain both eumelanin and phaeomelanin; it is the relative abundances of these two melamins that determine the expressed color of a pigmented feather. Claiming a 'red-orange' or 'black-grey' color for entire fossil organisms based upon identification of round or elongate morphologies is overly simplistic, because in living birds, pigment molecules of multiple types are employed to confer hues of brown, red, orange, etc. Coloration of feathers is complex, the result of expression of more than one type of pigment (e.g. porphyrins, carotenoids), which may be more labile, with lower preservation potential, than the relatively resistant melamins. Without preservation of all pigments originally employed, original organismal color cannot be interpreted with accuracy.

The initially proposed hypothesis of a microbial origin for these microstructures observed in multiple fossilized feathers, as well as other fossil material from the Messel deposits, has not been refuted.

Table 1 | SEM-EDS quantitative data for the fossil feather. Both black (Fig. 7) and brown (Fig. 8) regions of the feather are presented as weight (ms%) and molar (mol%) percentages in decreasing abundance. * indicates element not auto-detected by instrument.

|       | Black ms% | mol% | Brown ms% | mol% |
|-------|-----------|------|-----------|------|
| O     | 37.92     | 41.36| 45.29     | 57.03|
| C     | 28.06     | 34.76| 19.12     | 13.72|
| Si    | 10.99     | 6.83 | 5.93      | 7.11 |
| Fe    | 9.90      | 3.09 | 8.39      | 3.03 |
| Al    | 5.75      | 3.72 | 7.30      | 12.24|
| Ca    | 2.20      | 0.96 | 2.59      | 2.27 |
or indeed addressed, with data presented in previous studies, and is supported by the data we present herein. The present data do not support the melanosome hypothesis for these fossilized microstructures. Morphology alone is insufficient to distinguish between a melanosome and/or microbial origin, but data that capitalize on distinct chemical differences between melanosomes and microbes are needed to support one hypothesis over the other. With the exception of one fossil feather study where the chemical data are not of high resolution\textsuperscript{15}, the only in-depth chemical data presented for microbodies in the fossil record that seem to support a melanosome origin are derived from marine\textsuperscript{12,13,14} rather than terrestrial environments, where preservational conditions are very different than the lacustrine environments from which most feathers have been recovered\textsuperscript{1}. Additionally, geochemical data from fossil feather ‘melanosomes’ are compared only with that derived from extant melanosomes; microbodies are not included in the comparative data\textsuperscript{2}.

Furthermore, because the shape of melanosomes has been used to interpret color\textsuperscript{4,7–11} and behavior\textsuperscript{7,11} in extinct animals, distinguishing melanosomes from microbes is critical to acceptance or rejection of these hypotheses. As McGraw warned ‘’[…] it is wise to withhold these hypotheses. As McGraw warned ‘’[…] it is wise to withhold

### Methods

**Fossil feather specimen.** For more information on the geologic context of the fossil feather specimen (ANSP 23403) ascribed to Gansus yumenensis see the Supplementary Methods section online.

**Scanning electron microscopy (SEM) and SEM with energy dispersive x-ray spectroscopy (SEM-EDX).** For whole mount/surface analysis, G. yumenensis feathers were gently washed in E-pure water and air dried.

To view melanosomes, approximately 1 mm sections were taken from G. yumenensis feathers, and washed in phosphate buffered saline, dehydrated in two changes of 70% ethanol for 30 minutes with rocking followed by a one hour incubation of 2:1 LR white: 70% ethanol. Samples were then incubated for one hour each in two changes of 100% LR white, followed by a third incubation overnight. Feathers were placed in gelatin capsules with the long axis parallel to the length of the capsule, filled with LR white and covered to exclude oxygen. Resin was polymerized for 24 hours at 60 °C. A Leica EMUC6 ultra-microtome with a Diatome 45° knife was used to cut 5 and 10 µm longitudinal (parallel to long axis of the barb) sections.

Samples were mounted on double-sided carbon tape and imaged using a JEOL JSM-6010LA analytical SEM controlled by JEOL InTouchScope version 1.05A software. Some images (Fig. 3 and Supplementary Fig. S1b, S1c, S2a and S3c) were captured after applying a 3–6 nm gold/palladium coating. All EDS data of the uncoated fossil feather specimen (ANSP 23403) ascribed to Numida meleagris were collected from the Poultry Teaching Unit at North Carolina State University. (All methods described above for visualizing melanosomes in SEM. One sample of feathers, and washed in phosphate buffered saline, dehydrated in two changes of 70% ethanol. Samples were then incubated for one hour each in two changes of 70% alcohol, followed by a third incubation overnight. Feathers were placed in gelatin capsules with the long axis parallel to the length of the capsule, filled with LR white and covered to exclude oxygen. Resin was polymerized for 24 hours at 60 °C. A Leica EMUC6 ultra-microtome with a Diatome 45° knife was used to cut 5 and 10 µm longitudinal (parallel to long axis of the barb) sections.

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**Author contributions**

A.E.M., E.A.J. and W.Z. performed data collection and research. D.L. and M.C.L. collected and described specimens of *Gansus yumenensis*. K.I.L. provided the geologic description of the *Gansus* site and performed initial analyses on the fossil specimen. A.E.M. and M.H.S. wrote the manuscript.

**Additional information**

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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