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Iridescent structural coloration in a crested Cretaceous enantiornithine bird from Jehol Biota

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eLife Assessment

This study presents a potentially **fundamental** analysis of a fossil feather from a 125-million-year-old enantiornithine bird. Using sophisticated 3D microscopic and numerical methods, the authors conclude that the feather was iridescent and brightly colored, possibly indicating that this was a male bird that used its crest in sexual displays. At present, the strength of evidence supporting the conclusions is considered **incomplete** based on methodological shortcomings and questions about taphonomy.

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Abstract

A combination of sectioning and microscopy techniques, along with the application of finite-difference-time-domain modeling on a fossil feather, results in the novel estimation of the range of iridescent colors from the fossilized melanosome type and organization preserved in the elongate head crest feathers of a new Cretaceous enantiornithine bird. The densely packed rod-like melanosomes are estimated to have yielded from red to deep blue iridescent coloration of the head feathers. The shape and density of these melanosomes also may have further increased the feather's structural strength. This occurrence on a likely male individual is highly suggestive of both a signaling function of the iridescent crest, and a potential behavioral role in adjusting the angle of light incidence to control the display of this iridescent structural coloration.

Impact statement

Three-dimensional reconstruction of melanosome organization in an enantiornithine feather crest reveals convergent iridescent coloration.

Introduction

The recognition and study of melanosomes in fossil feathers of non-avialan dinosaurs and birds has added greater complexity to the pattern of their diversity and macroevolution, demonstrating the intertwined evolution of coloration with feather functions across display, thermoregulation, and other uses (Li, Gao et al. 2012, Li, Clarke et al. 2014, Terrill and Shultz 2023). This linkage among colors, structures, and functions early in feather evolution extends even to convergence in iridescent feathers among non-avialan theropods, and it is highly suggestive of commonalities shared among stem and crown birds in terms of their use of colored pigments for camouflage, structural strength, and display (Hu, Clarke et al. 2018, Foth and Rauhut 2020). In a comparative setting with the melanosome feather coloration of living birds, workers have endeavored to reconstruct the colors of fossil feathers using quantitative means focused predominantly on melanosome geometry and density, and the results point to the fossilized melanosomes having produced patterns of black, brown, rufous, grey, and other colors (Vinther, Briggs et al. 2008, Li, Gao et al. 2010, Zhang, Kearns et al. 2010, Li, Gao et al. 2012, Peteya, Clarke et al. 2017, Benton, Dhouailly et al. 2019). Furthermore, the specific shape and arrangement of some fossilized feather melanosomes derived from both non-avialan theropod dinosaurs and birds are similar to those required for producing structurally-based iridescent feather coloration in living birds, a case of independent evolution (Stoddard and Prum 2011, Hu, Clarke et al. 2018, Pan, Li et al. 2022).

Feathers serve multiple functions in birds from comprising an insulatory epidermal layer to forming aerodynamic flight surfaces, and as structures for the containment and display of various pigments. Living birds exhibit diverse feather morphologies and colors, and the significant diversity of known fossil feathers, along with their preserved pigments, points to their past use for camouflage, as well as display and signaling (Prum 2006, Foth and Rauhut 2020, Terrill and Shultz 2023). While the display of most colored feathers are passive and available to any viewer, birds can behaviorally control their signaling through: the erection of feathered ornaments such as the head crest in hoopoes (*Upupa*) or Peacocks (*Pavo*) tails; the exposure of normally hidden brightly colored crown feathers as in Kinglets (*Regulus*); and even the refined control of the black and iridescent coloration in birds such as hummingbirds (Trochilidae) and birds of paradise (Paradisaeidae) (Zi, Yu et al. 2003, Wilts, Michielsen et al. 2014, Stoddard, Eyster et al. 2020). This great variation in structural or iridescent colors results from differences in pigments, their organization, and other structural modifications. For example, the variety of mixed structural colors present in a peacock feather are achieved through changes in the spacing in the keratinous matrix and melanosome organization (Zi, Yu et al. 2003). In addition to the normal range of spectral coloration, hummingbirds can see non-spectral colors as well, and their utility is supported by vision perception experiments (Stoddard, Eyster et al. 2020). The interplay of bird of paradise feather coloration and their vision similarly indicates coordination of their mating displays with spectral properties through the avian visual system (Wilts, Michielsen et al. 2014). Individual birds actively control the signaling functions of their feathers with feathers or ornaments hidden/displayed or even the color presented (as dark/black vs. vibrant/iridescent) being consciously restricted or directed to particular viewers such as potential mates or rivals (Wilts, Michielsen et al. 2014).

Fossilized feathers typically have been evaluated within two-dimensional surface views, limiting the understanding of their overall ultrastructure and perhaps their true coloration. As a step forward in our reconstruction of the structure and function (color display) of early bird feathers, we examined the unique head-crest feathers of a well-preserved specimen (Institute of Vertebrate Paleontology and Paleoanthropology, IVPP V26899) of the Early Cretaceous (~120 Ma) enantiornithine *Shangyang* sp. from the Jiufotang Formation of northeastern China. This fossil preserves a diverse feather assemblage across the body with dense contour feathers, flight remiges, and two rachis-dominated tail feathers (**Figure 1** and figure S1). Among its diverse feather morphologies, this specimen also displays a strikingly enlarged feathered crest on the head that uniquely extends from the caudal end of the skull to a position rostral to the nasal bone (**Figure 1**). For our study, we sampled a long isolated mature feather that is displaced from the caudal part of the crest, as well as three feather fragments extracted from the *in-situ* position of the head crest.

In this study, we utilized serial ultrathin histological sectioning, scanning/transmission electron microscopy (S/TEM) imaging, and finite-difference time-domain (FDTD) modeling to investigate the potential iridescent coloration mechanisms in the elongated crest feathers from this newly discovered enantiornithine specimen of the Jehol Biota. Differing from previous statistical reconstructions of fossil color that measured melanosome aspect ratios, our new approach takes the three-dimensional shape and packing patterns of melanosomes, as well as the keratinous matrix, into consideration. With these novel modeling approaches, a more accurate coloration estimation was produced for its crest feather.

Results

In the new fossil, head crest feathers were selected from the caudal side of the head and sectioned using ultrathin serial sectioning. The overall shape of the sampled crest feather is well-preserved and comprised of elongate and relatively wide barbs. The dominant barb length is proportionally longer than the feather barb examined from the neck and head region of *Confuciusornis* (Foth 2012). The feather is different from the bilaterally symmetric plumulaceous feathers of living birds, and we define it as a barb-dominant feather, since most of the exposed feather tissue is composed of long barbs with a very short and thick calamus. It is estimated to correlate with evolutionary stage II and IIIa of (Prum and Brush 2002), similar to the intermediate feather type proposed as found in an amber fossil (Perrichot, Marion et al. 2008). The new feather described here is distinct from the amber feather in showing an asymmetric shape, radically diverging toward one side (caudal side), and is much larger in size (Perrichot, Marion et al. 2008). There are dense clusters inside the barbs as seen from images of both SEM and TEM.

Ultrathin histological sectioning and application of SEM and S/TEM imaging of the fossil feather samples (see Supplementary materials) resulted in the identification of partially interlinked melanosomes with a derived asymmetric packing pattern (**Figures 2** and **3**). The long axial crack in **Figure 2** may represent the surface of the barbs, resembling a crack formed in maturation experiments (Zhao, Hu et al. 2020). Those melanosome clusters are present and concentrated mostly within the barbs rather than in the barbules of extant bird feathers based on similar size (**Figure 2**). The long axis of the densely packed melanosomes is aligned overall parallel with the barb's long axis (**Figure 2**), which has been confirmed by detailed histological data as well. The melanosomes are arranged in an asymmetric hexagonal conformation, clearly visible in the different sections (see **Figure 3**).

SEM imaging of the cross section and surface view of the sampled feather indicate the presence of a few major barbs without barbules (figures S1 and S3-4). The thickness of the complete feather barb with filled melanosomes (preserved) is around 10 μm, which is much greater than the typical diameter of known barbules, but similar to barbs in extant birds (Freyer, Wilts et al. 2021). The

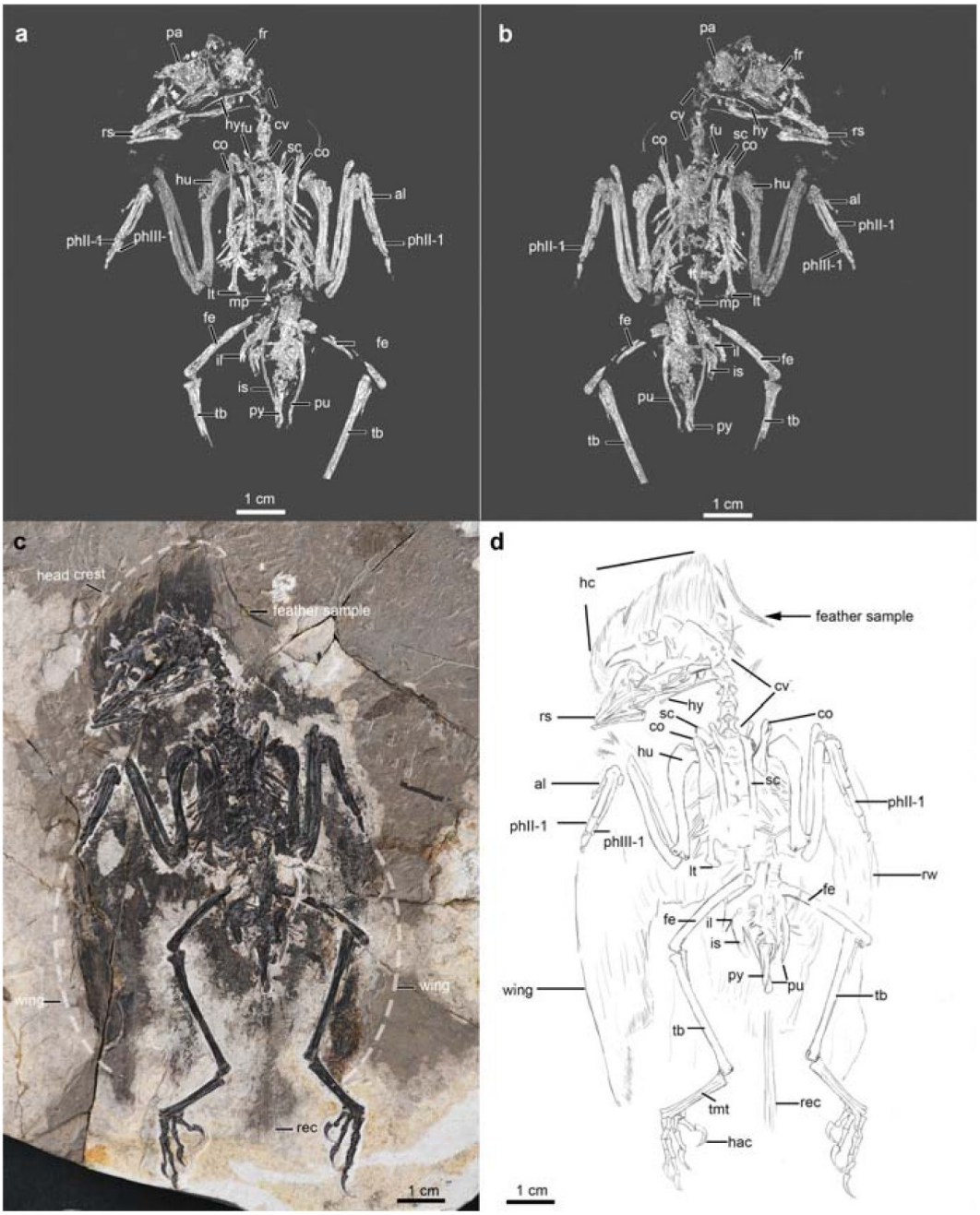

Figure 1

Digital rendering, photography, and line-drawing of the enantiornithine fossil specimen (*Shanyang* sp., IVPP V26899) (a), (c), (d) - dorsal; (b)- ventral view showing the major skeletal elements.

The feather extracted from the crown are labeled as feather sample (**c**). Abbreviations: al, alular metacarpal; co, coracoid; cv, cervical vertebra; fe, femur; fi, fibula; fr frontal; fu, furcula; hac, hallucal ungual; hc, head crest; hu, humerus; hy, hyoid; il, ilium; is, ischium; lt, lateral trabecula; mcf, molted crest feather; mc II, major metacarpal; mp, midline process; ph II-1, first digit of phalanx II; phIII-1, first digit of phalanx III; pa, parietal; pu, pubis; py, pygostyle; r, radius; rec, rectrices; rem, remiges; rs, rostrum; rw, right wing; sc, scapula; sk, skull; st, sternum; tb, tibiotarsus; and tmt, tarsometatarsus.

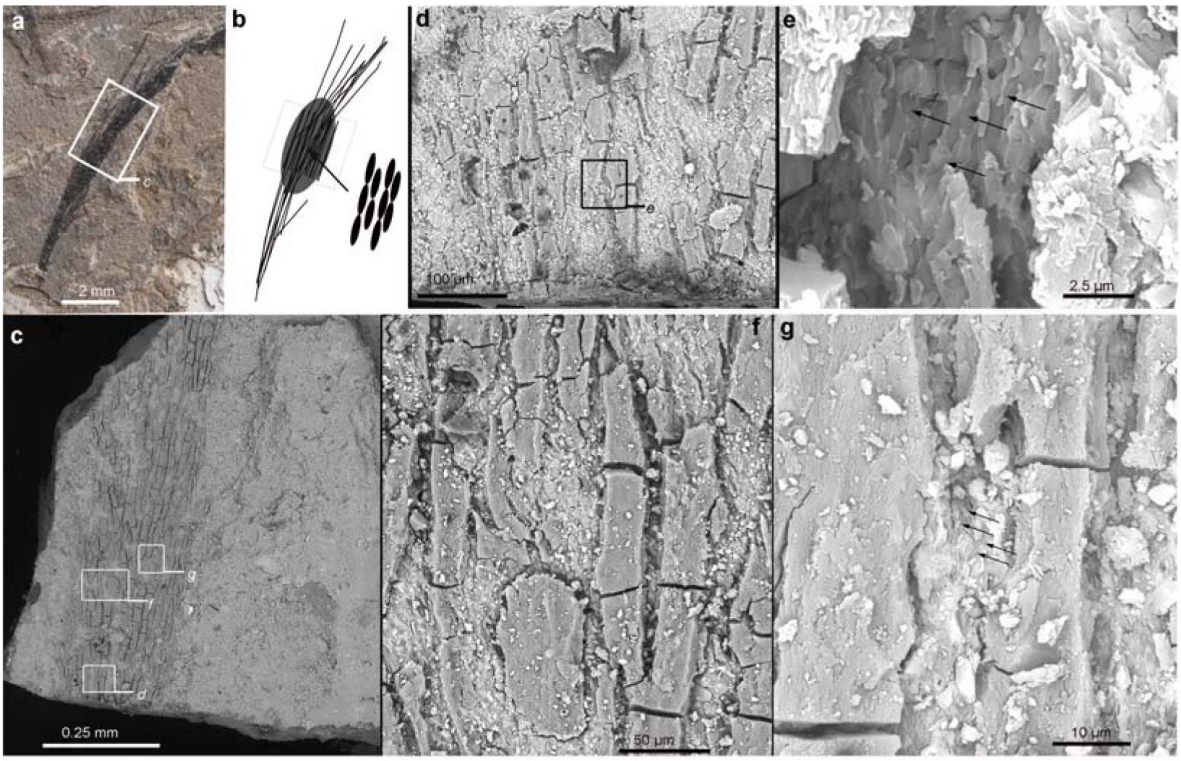

Figure 2

Photograph (a) and simplified line-diagram (b) of the sampled isolated long feather with a focused area from SEM imaging (c-g).

As shown in the SEM images, the long elliptical or oval-shaped melanosomes are tightly aligned with their long axis parallel to the elongated barbs **(e, g)**. The position of enlarged images (**d, e, f, and g**) is indicated as square in (**c, d**); black arrows in (**e**) and (**g**) indicate the melanosome clusters.

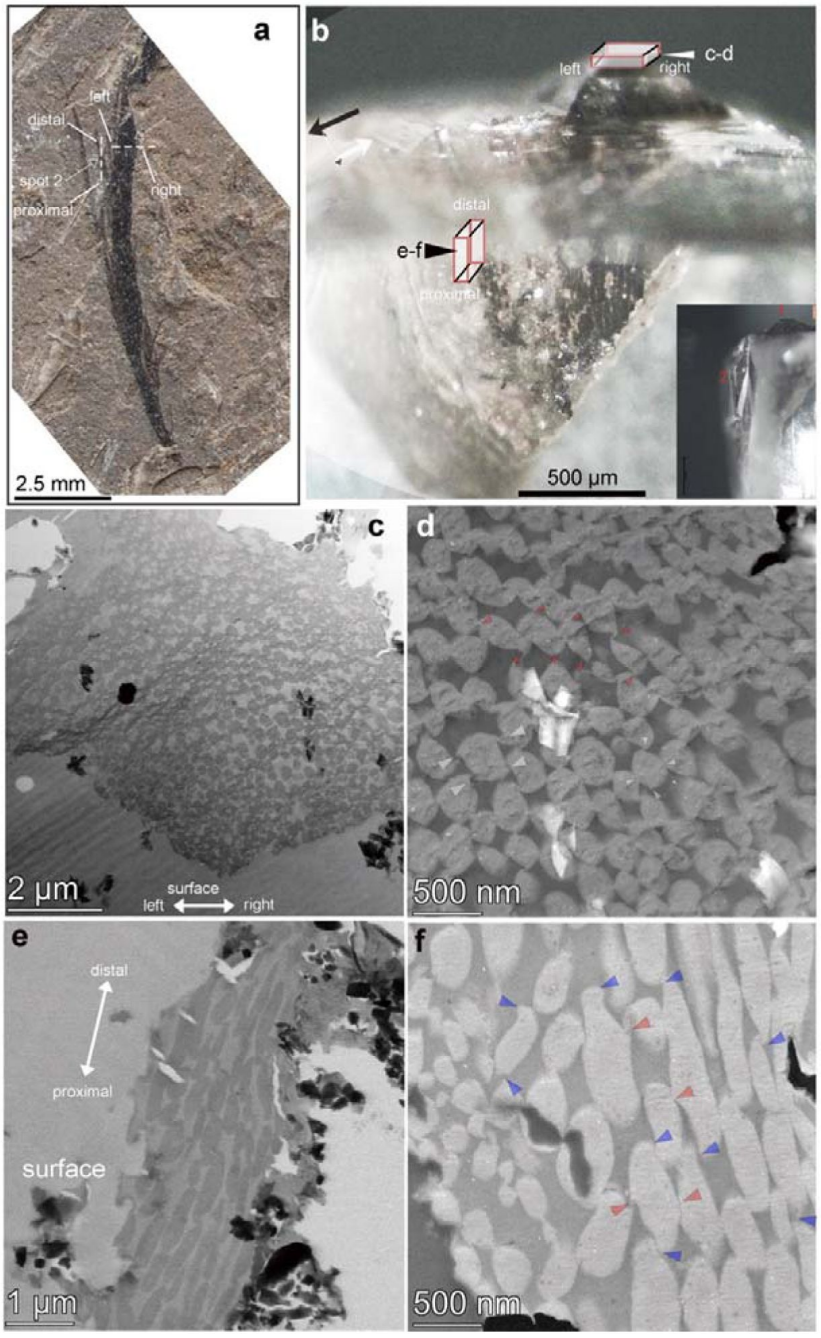

Figure 3.

Photograph of the crest feather (a) and prepared blocks of the feather barb (b) for thin sectioning.

The sampled spots for the sliced feather are indicated as long red-colored cubes with their respective positions in (**b**). (**c**) to (**f**) are (STEM) images obtained from individual slices as indicated in (**b**). The shape and arrangement of the melanosome packing (ACHP, asymmetric compact hexagonal packing) is clearly revealed in (**d**); (**c**) and (**d**) are derived from the cross-section; (**e**) and (**f**) are derived from the nearly longitudinal section. As indicated by the red arrows in (d) and (f), the dorsal and ventral hooks are dominant in the cross-sectional images. On the other hand, hooks connecting the proximal and distal arrays of melanosomes are better visualized in the longitudinal section, as labeled with blue arrows in (f). Orientation of the slices were labeled (left, right, proximal, distal) with their respective petitioning in the fossil before and during sectioning.

three-dimensionally packed melanosomes were studied in both cross and nearly longitudinal sections within the dark colored barbs (one in the middle of the barbs and another in the lateral side of the feather block (**Figure 3a**, b).

Hooks are commonly observed on the oval-shaped melanosomes in cross-sectional views, with two dominant types identified on the dorsal and ventral sides (**Figure 3c-d**, red arrows). These hooks are deflected in opposing directions, linking melanosomes from different arrays (dorsal-ventral) together. The major axis(y) of the oval-shaped melanosomes (mean = 283 nm) is oriented toward the left side in cross-section, while the shorter axis(x) measures approximately 186 nm (Table S2). In oblique or near-longitudinal sections (**Figure 3e-f**), the hooked structures' connections to the distal and proximal sides of neighboring melanosomes are clearly visible (blue arrows, **Figure 3f**). The mean long axis (z) of the melanosomes is approximately 1774 nm (Table S2). A similar pattern occurs in two additional regions of interest within the same feather (figure S5). Although the smaller proximal hooks in these sections are less distinct, this may reflect developmental variation during melanosome formation along the feather barb. Significantly smaller hooks were also observed in cross-sections of *in-situ*feather barbs from the anterior side of the feather crest (figure S6). Based on these observations, we propose that the hooked structures—particularly those on the dorsal, ventral, proximal, and distal sides of the melanosomes—enhance the structural integrity of the barb (figure S7). However, these features may be teratological and unique to this individual, as no similar structures have been reported in other species. These hooks may stabilize the stacked melanosome rods and contribute to increased barb dimensions, such as diameter and length. The sections exhibit modified (or asymmetric) hexagonally packed melanosomes with presence of extra hooked linkages (**Figure 3c-d** and e-f). The long rod-like melanosomes are different from all other known feather melanosomes from both extant and extinct taxa in having some extra hooks and an oblique ellipse shape in cross and longitudinal sections of individual melanosomes (Durrer 1986, Zhang, Kearns et al. 2010). The asymmetric packing of the melanosomes (the major axis leans leftward in cross section) played a major role in the reduction of fossilized keratinous matrix within the barbs, which may correspond to a novel structural coloration in this extinct bird. The close packed hexagonal melanosome pattern found in extant avian feathers yield rounded melanosome outlines in contrast to the oval-shaped melanosomes (see figure S8, x<y) in the perpendicular section here. The asymmetric compact hexagonal packing (ACHP) of the melanosomes is different from the known pattern of melanosomes formed in the structure of barbules among extant birds (Eliason and Shawkey 2012), which has been seen as a regular hexagonal organization. The packing of the melanosomes in an asymmetric pattern, on the microscopic level, might be related to the asymmetrical path of the barb extension direction observed at the macroscopic level (figure S5).

In the computational (FDTD) simulation, when light is incident at a small angle (<30°, both s- and p-polarized) in the X-Y plane, no evident reflection peak was found in the visible spectrum, as shown in **Figure 4c** (e.g., when a viewer observes at this angle, the light is almost completely absorbed, and the crest feather would have a darkened appearance). As the incident angle varies from 40° to 70°, the wavelength corresponding to the reflection peak shifts from 687 nm to 475 nm, and the color of the reflection peak gradually changes from red to deep blue (inset in **Figure 4c** clearly shows how the resulting color varies with the angle of incidence). From the perspective of optical modeling, the generation of iridescent color (reflection peaks in the visible spectrum) is caused by the constructive interference between scattered light produced by these uniquely shaped melanosomes that are compactly arranged in the barbs (Vinther, Briggs et al. 2010). Between blue and red, reflection peaks of green and yellow also are present, corresponding to the wavelengths of 512 nm and 578 nm respectively. It is worth noting that in the case of both p- and s-polarized incident light, the positions of the reflection peaks are nearly identical, with only a slight difference in peak reflectance. Therefore, the three-dimensional multilayer nanostructure model is somehow equivalent to one-dimensional multilayer biphotonic crystals (Saranathan, Forster et al. 2012). In summary, the crest feather of the Cretaceous enantiornithine shows a strikingly angle-dependent and iridescent coloration pattern. When we consider the shrinkage effect on

melanosomes demonstrated experimentally (McNamara, Briggs et al. 2013) and various distances between melanosomes resulting from compression effects (Pan et al. 2019; Zhao et al. 2020), our additional FDTD modeling shows a similar reflection spectrum with a narrower bandwidth of the color peaks (Supplemental Materials).

Discussion

This study of a fossilized feather with a combination of microscopic imaging, ultrathin sectioning, and three-dimensional modeling has provided a more accurate examination of ancient structural coloration and uncovered unusual melanosome structures. Using previous conventional approaches alone, we would have classified this feather crest simply as black or close to iridescent based on its melanosome aspect ratio (Nordén, Eliason et al. 2021). The recognition of overall iridescence links to a commonly found extant bird color range only known previously from the platelet-shaped dinosaurian melanosomes (Hu, Clarke et al. 2018) and a few fossil feathers (Pan, Li et al. 2022) (Nordén, Faber et al. 2019, Nordén, Eliason et al. 2021). The independent evolution of overall iridescence, similar to that found in living birds, was reconstructed here based on a novel array of melanosomes in a feather barb in an extinct non-crown group bird (Nordén, Faber et al. 2019, Yoshioka and Akiyama 2021). The occurrence of iridescent structural coloration in an enlarged head crest on this ancient bird is similar to structures present across many living birds that employ iridescence for intimidation, signaling, and particularly for mate attraction or intraspecific territorial defense (Terrill and Shultz 2023). Given that this individual bird likely was a male based on the occurrence of paired rachis dominant tail feathers (figure S1), the vibrant coloration on a prominent feathered crest suggests that sexual selection may have long played an important role in avialan (feather) macroevolution (Foth 2020, Wang, O'Connor et al. 2021). The vibrant color and its presence on an enlarged feather crest likely are interlinked, and probably indicate an evolutionary and selective relationship among color, feather position, and feather morphology/size.

Our discovery of new melanosome shapes and networks suggests that birds evolved previously unrecognized patterns of color production outside of the limits present among crown group birds. Additionally, these pigments and their dense organization may have made the foundation of the feather darker and allowed for a more intense or vibrant iridescent coloration, a potential relationship that is understudied among living birds. While the interrelationship between melanin content in feathers and increases in structural strength have long been known (Burtt 1981, Burtt Jr and Ichida 2004), perhaps the increased density of melanosomes in this fossil feather barbs added even more structural strength to the feathered crest of this bird in compromise for its lack of a dominant rachis. Additionally, the interlocking hooks among the melanosomes might have aided individual feather barb in resisting shear forces. The modified closely packed melanosome arrays reconstructed here also are potentially advantageous in increasing barb structural integrity and stability in the long barb ramus, and likely affected the visualized spectrum during the display of iridescent coloration. We cannot completely rule out the increased melanosome density in the fossil feather is the result of taphonomy. Size reduction in melanosomes was found previously in a simulation experiment (McNamara, Briggs et al. 2013), but such a reduction is very unlikely to result in the highly organized and consistent melanosome packing present here (McNamara, Briggs et al. 2013, Zhao, Hu et al. 2020). To test the potential impact of melanosome shrinkage and potential taphonomic changes to their spacing, we conducted an additional analysis (See Supplementary Materials), and the results yield similar iridescent spectral peaks as the original setting.

Since the iridescent coloration of living birds is produced by the accumulation of melanosomes mostly positioned in barbules, the identified fossil barbs filled with melanosomes represents a unique phenotype related to an early feather evolutionary and developmental stage. Given the lack of barbules in most early primitive feather forms (or filamentary structures) in non-avialan

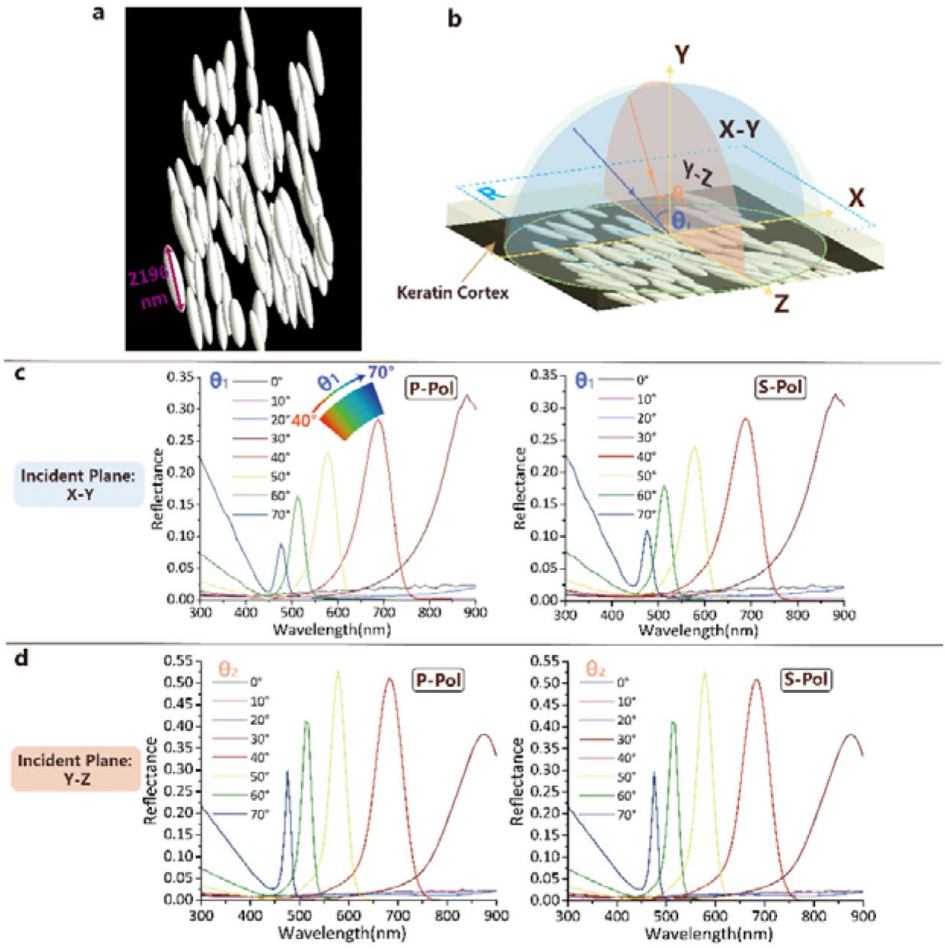

Figure 4.

FDTD modeling results.

(**a**) Representative multilayer melanosome model; (**b**) Schematic drawing of the reflectance calculation setup of the feather barb. X-Y incident plane (blue) is perpendicular, and the Y-Z incident plane (orange) is parallel to the special melanosome longitudinal axis. θ_1 and θ_2 are incident angles in X-Y and Y-Z incident planes, respectively. The blue dashed box (R) indicates the area where the reflection spectra are simulated and calculated. (**c**) Angle-dependent reflectance spectra calculated for p- and s-polarized light by FDTD modeling with X-Y incident plane. (**d**) Angle-dependent reflectance spectra calculated for p- and s-polarized light by FDTD modeling with Y-Z incident plane. Keratin cortex layer was considered in the modeling.

dinosaurs and pterosaurs, the positioning of melanosomes in barbs might be a plesiomorphic feature for ornithodirans broadly (Xu 2019, Cincotta, Nicolaï et al. 2022). With different destinations of melanosome transport during feather formation, the pattern recovered here suggests fundamental differences in feather pigmentation among Cretaceous stem taxa as compared to crown birds. The melanosome-filled barbs evolved earlier in feather evolution before the later appearance of innovations such as the presence of a stiff rachis and colorful barbules observed across living feathers (Yu, Wu et al. 2002).

A focus on the two-dimensional examination of fossil feathers has uncovered a large number of black or dark melanosomes across many early birds and feathered theropods (Li, Clarke et al. 2014, Hu, Clarke et al. 2018). However, our three-dimensional study of a fossilized Jehol feather specimen demonstrates that ultra-histological detail and spatial relationships can be preserved at the microscopic level. Importantly, our discovery suggests that reexamination of previously published 'black' pigmented fossil feathers might uncover a wider occurrence of 'surprising' iridescence and structural coloration among these early birds and non-avialan taxa. Such discoveries will aid in documenting both independent and convergent evolutionary pathways in the history of birds. This research further demonstrates that Jehol fossil feather tissue can be treated as (microscopically) three dimensional specimens allowing for the collection of valuable comparative data and application of comparative analytical techniques that facilitate a better understanding of early feather evolution and function.

Here, we recovered iridescent coloration in fossil bird feathers that was produced by three-dimensional melanosome packing patterns. This discovery dramatically expands the range of fossil bird coloration space, and this diversity in feather coloration in an early bird fits well with its forested environment, as proposed by the coeval fossil plant community (Wu, Li et al. 2023). Tropical forest environments are accompanied by colorful flowing plants and green leaves, and the structural color of this feathered crest is advantageous for bird signaling as well. Forested environments (flowers, leaves, light, and shadow) can be advantageous for birds and their signaling as more colorful feathers are found today in tropical regions as demonstrated among extant vibrant coloration of passeriform birds in particular (Cooney, He et al. 2022).

Materials and Methods

Fossil material

The referred specimen of the enantiornithine bird *Shangyang* sp. (IVPP V 26899) was recovered near La-Ma-Dong Village in Jian-Chang County, within the Jiufotang Formation (dated ~120 Ma). It can be referred to *Shangyang* by the presence of derived features in the premaxillae, tibiotarsus, and sternum (**Figure 1** and Supplementary Materials).

The feather fragment (labeled sample **Figure 1**) extracted from the specimen were mainly analyzed using SEM (Scanning Electron Microscopy) and S/TEM (Scanning/Transmission Electron Microscopy). The extracted feather sample is one of the longest crest feathers, was displaced far from the caudal position of the skull (sample in **Figure 1**), and belongs to a fully mature asymmetric barb dominant feather. In addition to the singular feather, three fragments of other feathers were extracted from the *in-situ* position of the head crest and sampled to validate the revealed structure (Supplementary Materials, figure S6). A small fragment of the right femur was extracted to produce a ground-section to evaluate osteo-histological features in relation to its ontogenetic stage (figures S1 and S2).

Scanning electron microscopy (SEM)

Feather samples coated with gold were examined with a FEI ESEM Quanta 450, which is operated at the Tectonic Laboratory in the China Geological Survey in Beijing, China. The SEM was operated with about 10 mm working distance of the sample under a high voltage of 20 KV. Not only the surface of feather sample was examined, but the raw material of the cross-section exposed in the ultrathin sliced sections also were imaged to characterize an overall distribution and thickness of soft tissue in comparison with the adjacent sediment or matrix (**Figure 2** and figures S3). Additionally, a FIB-SEM (Heliox 5CX Due Beam system) also was further applied to validate the melanosome packing in an extracted volume, and the imaged block is about 11 x 13 x 9μm (figure S7).

Scanning/Transmission Electron Microscopy

The crest feather fragments were embedded in epoxy resin using the SPI-PON 812 Embedding Kit (MNA, EPOK, DDSA, DMP-30)(Luft 1961) at the Key Laboratory of Vertebrate Evolution and Human Origins of the Chinese Academy of Sciences in Beijing in 2022. After a step-wised polymerization at 37°C for 12 hours, 45°C for 12 hours, and 60°C for 48 hours, the epoxy-embedded block was prepared and cut using an ultrathin slicing machine (Lecia EM UC7), equipped with a diamond knife (ultra 45°, 3 mm) for further TEM observations. The ultrathin slices were placed on copper grids with carbon film (200 meshes in ~3 mm diameter copper gird). The thickness of a prepared ultrathin slice is approximately ~70 nm, and the slices were consecutively placed on ~3-5 copper grids. The removed thickness of the sample for making those ~3-5 copper grids for one run of sectioning (serial numbered grids were made) took about ~20 μm of the prepared pyramidal-cubic shaped block (**Figures 3** and 5). The direction of slicing was either perpendicular to the long axis of the barbs, or nearly parallel with the long axis of the barb, for the two spots selected for cutting.

S/TEM imaging was conducted on a Thermo Scientific (FEI) Talos F200X G2 TEM at the IVPP, Beijing during 2021-2022. The Talos F200X was operated at 200 KV with an analytical scanning transmission electron microscope mode (S/TEM). TEM slices were made from the isolated feather adjacent to the head and rostral portion of the skull. The two specific micron-sized spots of the feather were targeted for examination of their microscopic features by ultrathin slicing and examination as shown in **Figure 3**. For the long contour feather, one spot close to the middle of the feather was made as a cross section of the barb, and for the other spot, the slice was made close to the left side of the fragment with a longitudinal slice (**Figure 3**). The strategic consideration of the two directions (and spots) of cutting was to examine the melanosomes and other features in different views in order to visualize these different features and obtain three-dimensional data (see Supplementary Materials).

X-ray computed tomography

The fossil slab was scanned using a GE |phenix| micro-scanner with a resolution of 24.3 μm at Key Laboratory of Vertebrate Evolution and Human Origins, IVPP, Chinese Academy of Sciences, Beijing. For the skeletal anatomy of the bird, μ-CT scans and digital rendering were applied to aid in visualizing the skeletal features of the new specimen (**Figure 1**). The digital rendering of the specimen was completed using Avizo 9.0. (**Figure 1**). Measurements were made using digital calipers, as well as validated in the CT data digitally (Table S1). To achieve optimized image resolution, the field of view was adjusted, resulting in part of the feet being excluded from the CT scan.

Technical protocol for ultrathin slicing

Prior to the work presented here, we obtained numerous practice sections from a few samples of fossil feathers. In our prior samples, the fragmentary feather tissues were used to improve the technician's capability and precision in sectioning exercises by comparison with the desired direction and sectioned direction for given a fossil. There are several key steps elaborated below for the successful repetition of practices of sectioning in the correct alignment. First, we examined the fossil feathers with light microscopy and SEM prior to the embedding process to make sure the barbs were aligned correctly in the epoxy resin. Meanwhile, the small part of the extracted feather sample was aligned, using the microscope (Lecia UC 7), into a proper position during the emending and stage trimming process. The attention to feather direction was performed to assure that the barb major extension direction was aligned with the long axis of the bullet mold (embedding capsule). We also checked the position of the feather fragment in the SPI-Pon 812 during embedding and polymerization. The sliced section should be precise enough to acquire the desired images and information after planning and optimization of each step described above, and before the sectioning. Of course, the ultrathin slicing practices can improve the technician's skill and increase cutting precision which require a gradual learning process.

FDTD modeling of the feather's reflectance

To explore the coloration of the three-dimensionally packed melanosomes, Lumerical finite-difference-time-domain (FDTD) software (Ansys Canada Ltd.) (Inan and Marshall 2011, Sullivan 2013) was used to simulate the visualization of light propagation on the crest feather with regular arrangements of melanosomes (**Figure 1**). The FDTD method allows explicit simulation of light interaction with particular-shaped melanosomes by numerically solving Maxwell's equation in the time domain. The construction of a three-dimensional nanostructure model is based on our characterized data (**Figure 4** and Table S2), and detailed modeling steps are included in Supplementary Materials. The dispersion of the complex refractive index that was applied in our simulation is described and shown in figure S9. In order to mimic real visual conditions, several variables were considered during the simulation, including angle of light incidence in two orthogonal incident planes (X-Y and Y-Z plane) (**Figure 4b**) and polarization state (s- and p-polarized). Usually, p-polarized light is understood to have an electric field direction parallel to the plane of incidence, and s-polarized light has the electric field oriented perpendicular to the incident plane. Reflectance spectra are presented in **Figure 4c-d**.

Caveats about potential taphonomic melanosome changes

The simulated colors here are robust even when taking potential taphonomic changes into consideration. The chance that the highly organized and anatomically oriented nanoscale pattern formed here is the result of geological compression or pressure during fossilization and diagenesis is quite low, and such structures are absent or unseen among known taphonomic artifacts (McNamara, Briggs et al. 2013, Zhao, Hu et al. 2020). At present, we cannot exclude the possibility that sheer stress could result in the observed asymmetric packing of melanosomes. However, that pattern has not been reported in any known experiment or geological process of fossilization including those related to Jehol bird or non-avialan dinosaur fossils. Therefore, we suggest the pattern we obtained here represents original biological structures. Given experimental observations of melanosome shrinkage at high pressures and temperatures (~10% shrinkage) (McNamara, Briggs et al. 2013, Zhao, Hu et al. 2020), we also completed additional modeling with the fossil melanosomes enlarged (110%, see Table S3) along with the fossilized keratinous cortex and matrix. This additional modeling resulted in all of the spectral peaks present in the original modeling being recovered. Therefore, our results are robust even when considering known potential taphonomic artifacts, and both approaches produce similar results with the red-to-blue iridescent coloration having been present in the crown feather (figure S10).

Data and materials availability

All data are available in the main text or the supplementary materials

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Author contributions

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Methodology: JH, MY and ZL

Investigation: ZL, TAS, JH, YM, and JAC

Visualization: JH and ZL

Supervision: ZL and ZZ

Writing—original draft: TAS, ZL, YP, MW, JL and TZ

Writing—review & editing: ZL, JH, TAS, MY, MW, YP, TZ, JL, ZZ, and JAC

Additional files

Supplemental file

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Reviewer #1 (Public review):

Summary:

Li et al describe a novel form of melanosome based iridescence in the crest of an Early Cretaceous enantiornithine avialan bird from the Jehol Group.

This is an interesting manuscript that describes never before seen melanosome structures and explores fossilised feathers through new methods. This paper creates an opening for new work to explore coloration in extinct birds.

Strengths:

A novel set of methods applied to the study of fossil melanosomes.

Comments on revised version:

The authors provided a response to the previous 9 issues, for which additional response is provided here:

(1) I respectfully disagree with the authors justification regarding the crest. They show one specimen of Confuciusornis with short feathers (which appears to be a unique feature of this species, possibly related to the fact it is beaked) but what about the more primitive Eoconfuciusornis, a referred specimen of which superficially has an enormous "crest" (Zheng et al 2017), as does Changchengornis (Ji et al 1999). Regardless, it would make more sense compare this new specimen to other enantiornithines. Although limited by the preservation of body feathers, which is not all that common, the following published enantiornithines also exhibit a "crest": bohaiornithid indet. (Peteya et al 2017); Brevirostruavis (Li et al 2021); Dapingfangornis (Li et al 2006); Eoenantiornis (Zhou et al 2005); Grabauornis (Dalsatt etal 2014); Junornis (Liu et al 2017); Longirostravis (Hou etal 2004); Monoenantiornis (Hu & O'Connor 2016); Neobohaiornis (Shen etal 2024); Orienantiornis (Liu etal 2019); Parabohaironis (Wang 2023); Parapengornis (Hu etal 2015); Paraprotopteryx (Zheng et al 2007); and every specimen of Protopteryx. In fact, every single published enantiornithine that preserves any feathering on the head has the feathers preserved perpendicular to the bone (in fact, the body feathers on all parts of the bed are splayed at a right angle to the bone due to compression), as shown in the confuciuornis specimen image provided by the authors. Since it is highly improbable they all had crests, the authors have no justification for the interpretation that this new specimen was crested. This does not mean that the feathers were not iridescent or take away from the novel methods these authors have used to explore preserved feathers.

(2) Yes, this is possible, but see above for the very strong argument against interpretation of these feathers as forming a crest.

(3) This just further makes the point that the isolated feather is not likely from the head. Since the neck feathers are missing, it is more likely that it is these feathers that have been

disarticulated (and sampled) from the neck region rather than from the very complete looking head feathers; this has significant implications with regards to the birds colour pattern.

(4) Thank you for acknowledging taphonomy.

(5) An interesting hypothesis and one I look forward to seeing explored in the future.

(6) Since the compression is in a single direction, in fact it is not reasonable to assume that distortion would be random. One might predict similar distortion, as with the feathers (spread out from the bone at a 90˚angle) and bone (crushed), which are all compressed in a single direction. However, I agree that such a consistent discovery suggests it is not an artifact of preservation, and only further studies will elucidate this

(7) I still fail to detect this hexagonal pattern - could machine learning be used to quantify this pattern? The random arrangement of white arrows does little to clarify the authors interpretations.

(8) Great to see additional sampling

(9) Thank you for the explanation.

https://doi.org/10.7554/eLife.103628.2.sa2

Reviewer #3 (Public review):

Summary:

The paper presents an in-depth analysis of the original colour of a fossil feather from the crest of a 125-million-year-old enantiornithine bird. From its shape and location, it would be predicted that such a feather might well have shown some striking colour and pattern. The authors apply sophisticated microscopic and numerical methods to determine that the feather was iridescent and brightly coloured, and possibly indicates this was a male bird that used its crest in sexual displays.

Strengths:

The 3D micro-thin-sectioning techniques and the numerical analyses of light transmission are novel and state of the art. The example chosen is a good one, as a crest feather likely to have carried complex and vivid colours as a warning or for use in sexual display. The authors correctly warn that without such 3D study feather colours might be given simply as black from regular 2D analysis, and the alignment evidence for iridescence could be missed.

Weaknesses: Trivial

https://doi.org/10.7554/eLife.103628.2.sa1

Author response:

The following is the authors' response to the original reviews

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *Summary:*

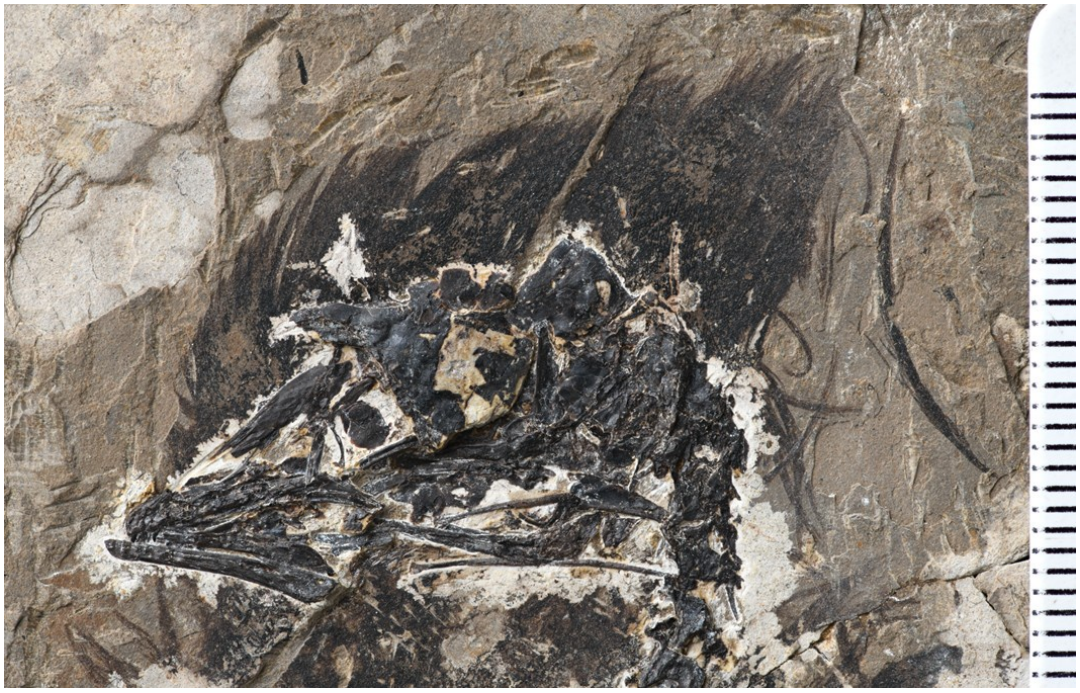
> *Li et al describe a novel form of melanosome based iridescence in the crest of an Early Cretaceous enantiornithine avialan bird from the Jehol Group.*
>
> *Strengths:*
>
> *Novel set of methods applied to the study of fossil melanosomes.*
>
> *Weaknesses:*
>
> *(1) Firstly, several studies have argued that these structures are in fact not a crest, but rather the result of compression. Otherwise, it would seem that a large number of Jehol birds have crests that extend not only along the head but the neck and hindlimb. It is more parsimonious to interpret this as compression as has been demonstrated using actuopaleontology (Foth 2011).*

Firstly, we respectfully acknowledge the reviewer's interpretation.

However, the new specimen we report here is distinct as preserved from *Confuciusornis* (Foth 2011), which belongs to a different clade and exhibits a differently preserved feather crest of a different shape compared to the species described in this study. Figure 3a Foth 2011, Paläontologische Zeitschrift；the cervical feather is much longer than feather from head region in the specimen the referee talked about; It is quite incompletely preserved and much shorter in proportional length (relative to the skull) than the specimen we sampled (see picture below).

Author response image 1.

Our new specimen with well-preserved and the feather crest were interpretated as the originally shaped；the cervical feather is largely absent or very short



In the new specimen there is a large feather crest that gradually extends from the cranial region of the fossil bird, rather than the cervical region, as observed in the previously proposed *Confuciusornis* crest. The feather crest extends in a consistent direction

(caudodistally), and the feathers in the head region of the bird are exceptionally well-preserved, retaining their original shape. The feathers are measured about 1- 2cm at their longest barb. Feathers in the neck are much shorter (see *Confuciusornis* picture above).

> *(2) The primitive morphology of the feather with their long and possibly not interlocking barbs also questions the ability of such feathers to be erected without geologic compression.*

We acknowledge that the specimen must have undergone some degree of compression during diagenesis and fossilization. Given that the rachis itself is already sufficiently thick (that the ligaments everting a crest would attach to), we conclude that it had the structural integrity to remain erect on the skull.

> *(3) The feather is not in situ and therefore there is no way to demonstrate unequivocally that it is indeed from the head (it could just as easily be a neck feather)*

We conclude that it belongs to the head based on the similar suture, overall length, and its close position to the caudal part of the head. There are no similar types of feathers nearby, such as those found on the neck or other areas, which is why we reason that it is a head crest feather. Besides, the shape of the feather we sampled is dramatically different from the much softer and shorter ones detected on the neck.

In addition, we further sampled the crest feather barb from *in situ* preserved feather crest. We also detected a similar pattern to what we originally found regarding the packing of melanosomes. This is now added to the text.

> *(4) Melanosome density may be taphonomic; in fact, in an important paper that is notably not cited here (Pan et al. 2019) the authors note dense melanosome packing and attribute it to taphonomy. This paper describes densely packed (taphonomic) melanosomes in non-avian avialans, specifically stating, "Notably, we propose that the very dense arrangement of melanosomes in the fossil feathers (Fig. 2 B, C, and G-I, yellow arrows) does not reflect in-life distribution, but is, rather, a taphonomic response to postmortem or postburial compression" and if this paper was taken into account it seems the conclusions would have to change drastically. If in this case the density is not taphonomic, this needs to be justified explicitly (although clearly these Jehol and Yanliao fossils are heavily compressed).*

We have added a line acknowledging this possibility. We have accounted for the shrinkage effects caused by heat and compression, as detailed in our Supplementary Information (SI) file. Even when these changes are considered, they do not alter the main conclusions of our study. Besides given most melanosomes we used for simulation are mostly complete and well preserved，we consider the distortion is rather limited or at least minor compared to changes seen in taxonomic experiment shown.

> *(5) Color in modern birds is affected by the outer keratin cortex thickness which is not preserved but the authors note the barbs are much thicker (10um) than extant birds; this surely would have affected color so how can the authors be sure about the color in this feather?*

In extant birds, feather barbs of similar size are primarily composed of air spaces and quasi-ordered keratin structures, largely lacking dense melanosomes. The color-producing barb we have described here does not directly correspond to a feather type in modern birds for comparison. Since there is no direct extant analog to inform the keratin thickness and similar melanosome density, we utilize advanced 3-D FDTD modeling approach to the question of

coloration reconstruction, rather than relying on statistical DFA approaches. In additional to packed melanosomes, the external thin keratin cortex layer is also considered for the simulation.

Additionally, even in the thinner melanosome-packed layers of barbules in living birds, iridescent coloration often is observed (e.g., Rafael Maia J. R. Soc. Interface 2009). This further supports the plausibility of our modeling approach and its relevance to understanding coloration in both extinct and extant species.

> *(6) Authors describe very strange shapes that are not present in extant birds: "...different from all other known feather melanosomes from both extant and extinct taxa in having some extra hooks and an oblique ellipse shape in cross and longitudinal sections of individual melanosome" but again, how can it be determined that this is not the result of taphonomic distortion?*

We consistently observed similar hook-like structures not only in this feather but also in feathers from different positions of the crest. We do not believe that distortion would produce such a regular and consistent pattern; instead, distortion likely would result in random alterations, as demonstrated by prior taphonomic experiments.

> *(7) The authors describe the melanosomes as hexagonally packed but this does not appear to be in fact the case, rather appearing quasi-periodic at best, or random. If the authors could provide some figures to justify this hexagonal interpretation?*

To further validate the regional hexagonal pattern, we expanded our sampling to additional sites. We observed similar patterns not only in various regions of the same barb but also across different feathers (see added SI Figures below). This extensive sampling supports the validity of the melanosome patterns identified in our original analysis.

> *(8) One way to address these concerns would be to sample some additional fossil feathers to see if this is unique or rather due to taphonomy*

We sampled additional areas from the same feather as well as feathers from other regions of the head crest. The packing patterns are generally similar with slight variations in size (figure S6).

> *(9) On a side, why are the feet absent in the CT scan image? "*

To achieve better image resolution, the field of view was adjusted, resulting in part of the feet being excluded from the CT scan.

> ***Reviewer #2 (Public review):***
>
> *Summary:*
>
> *The authors reconstructed the three-dimensional organization of melanosomes in fossilized feathers belonging to a spectacular specimen of a stem avialan from China. The authors then proceed to infer the original coloration and related ecological implications.*
>
> *Strengths:*
>
> *I believe the study is well executed and well explained. The methods are appropriate to support the main conclusions. I particularly appreciate how the authors went beyond the simple morphological inference and interrogated the structural implications of melanosome organization in three dimensions. I also appreciate how the authors were*

> *upfront with the reliability of their methods, results, and limitations of their study. I believe this will be a landmark study for the inference of coloration in extinct species and how to interrogate its significance in the future.*

We thank the referee for these positive comments.

> *Weaknesses:*
>
> *I have a few minor comments.*
>
> *Introduction: I would suggest the authors move the paragraph on coloration in modern birds (lines 75-97) before line 64, as this is part of the reasoning behind the study. I believe this change would improve the flow of the introduction for the general reader.*

We thank the referee for the suggestion, and we made changes accordingly to improve the flow of introduction.

> *Melanosome organization: I was surprised to find little information in the main text regarding this topic. As this is one of the major findings of the study, I would suggest the authors include more information regarding the general geometry/morphology of the single melanosomes and their arrangement in three dimensions.*

We thank the referee for this suggestion. We elaborated on the details of the melanosomes in the results as follows:

Hooks are commonly observed on the oval-shaped melanosomes in cross-sectional views, with two dominant types identified on the dorsal and ventral sides (Figure 3c-d, red arrows). These hooks are deflected in opposing directions, linking melanosomes from different arrays (dorsal-ventral) together. The major axis(y) of the oval-shaped melanosomes (mean = 283 nm) is oriented toward the left side in cross-section, while the shorter axis(x) measures approximately 186 nm (Table S2). In oblique or near-longitudinal sections (Figure 3e-f), the hooked structures' connections to the distal and proximal sides of neighboring melanosomes are clearly visible (blue arrows, Figure 3f). A similar pattern occurs in two additional regions of interest within the same feather (figure S5). Although the smaller proximal hooks in these sections are less distinct, this may reflect developmental variation during melanosome formation along the feather barb. Significantly smaller hooks were also observed in cross-sections of *in-situ* feather barbs from the anterior side of the feather crest (figure S6). The mean long axis (z) of the melanosomes is approximately 1774 nm (Table S2). Based on these observations, we propose that the hooked structures—particularly those on the dorsal, ventral, proximal, and distal sides of the melanosomes—enhance the structural integrity of the barb (figure S7). However, these features may be teratological and unique to this individual, as no similar structures have been reported in other sampled feathers. These hooks may stabilize the stacked melanosome rods and contribute to increased barb dimensions, such as diameter and length. The sections exhibit modified (or asymmetric) hexagonally packed melanosomes with presence of extra hooked linkages (Figure 3c-d and e-f). The long rod-like melanosomes are different from all other known feather melanosomes from both extant and extinct taxa in having some extra hooks and an oblique ellipse shape in cross and longitudinal sections of individual melanosomes (Durrer 1986, Zhang, Kearns et al. 2010). The asymmetric packing of the melanosomes (the major axis leans leftward) played a major role in the reduction of fossilized keratinous matrix within the barbs, which may correspond to a novel structural coloration in this extinct bird. The close packed hexagonal melanosome pattern found in extant avian feathers yield rounded melanosome outlines in contrast to the oval-shaped melanosomes (see figure S8, x<y) in the perpendicular section here. The asymmetric compact hexagonal packing (ACHP) of the melanosomes is different from the known pattern of melanosomes formed in the structure of barbules among extant

birds (Eliason and Shawkey 2012), which has been seen as a regular hexagonal organization. The packing of the melanosomes in an asymmetric pattern, on the microscopic level, might be related to the asymmetrical path of the barb extension direction observed at the macroscopic level (figure S5).

Added Supplemental figure S5. STEM images of cross-sections taken from three different positions (indicated by white dashed lines in a) demonstrate similar melanosome packing styles. Dashed-lines labeled in (a) indicate where the corresponding position of these sections were taken, black arrows indicate the individual barbs that accumulated together in this long crest father. One distinct feature of these sections is the hooked-link structure that aligns the melanosomes into a modified hexagonal, packed arrangement. White arrows (in c, e, g) indicate the hooked structures observed in the selected melanosomes.

Added Supplemental figure S6. STEM images showing melanosome structure from three fragments of the feather crest (indicated by dashed lines and white box in a) reveal the hooked linkages between melanosomes and their surrounding melanosomes structures in (b), (c) and (d). Due to the shorter length of these feather barbs, the hook structures are not as well-defined as those in the longer feather samples shown in the main text.

> *Keratin: the authors use such a term pretty often in the text, but how is this inference justified in the fossil? Can the authors extend on this? Previous studies suggested the presence of degradation products deriving from keratin, rather than immaculated keratin per se.*

We changed to keratinous matrix and material instead. We observed matrix/material in between these melanosomes were filled by organic rich tissue that is proposed to possibly be taphonomically altered keratin.

> *Ontogenetic assessment: the authors infer a sub-adult stage for the specimen, but no evidence or discussion is reported in the SI. Can the authors describe and discuss their interpretations?*

Thanks for the suggestion. We made an osteo-histological section and add our evaluation of the histology of the femoral bone tissue sampled from the specimen to justify assessment of its ontogenetic stage.

See Supplemental figure S2 for Femur Osteo-Histology

SI file Femur Osteo-Histology

Ground sections were acquired from the right side of the femur to assess the osteo-histological features of the bone and its ontogenetic stage. As shown in figure S2, long, flat-shaped lacunae are widely present and densely packed throughout the major part of the bone section. Very few secondary osteocytes are present, and parallel-fibered bone tissue is underdeveloped. The flattened osteocyte lacunae dominate the cellular shape, with observable vascular canals connecting different lacunae. Overall, the osteo-histology indicates that the bird was still in an active growth stage at the time of death, suggesting it was in its sub-adult growth phase.

> *CT scan data: these data should be made freely available upon publication of the study.*

We will release our CT scanning on an open server (https://osf.io/kw7sd/) along with the final version of the manuscript.

> ***Reviewer #3 (Public review):***
>
> *Summary:*
>
> *The paper presents an in-depth analysis of the original colour of a fossil feather from the crest of a 125-million-year-old enantiornithine bird. From its shape and location, it would be predicted that such a feather might well have shown some striking colour and pattern. The authors apply sophisticated microscopic and numerical methods to determine that the feather was iridescent and brightly coloured and possibly indicates this was a male bird that used its crest in sexual displays.*
>
> *Strengths:*
>
> *The 3D micro-thin-sectioning techniques and the numerical analyses of light transmission are novel and state-of-the-art. The example chosen is a good one, as a crest feather is likely to have carried complex and vivid colours as a warning or for use in sexual display. The authors correctly warn that without such 3D study feather colours might be given simply as black from regular 2D analysis, and the alignment evidence for iridescence could be missed.*
>
> *Weaknesses: Trivial.*
>
> ***Recommendations for the authors:***
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> ***Reviewer #3 (Recommendations for the authors):***
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> *In a few places, the paper can be strengthened:*
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> *Dimensionality of study method: In the first paragraph, you set things up (lines 60-62) to say that studies hitherto have been of melanosomes and packing in two dimensions... and I then expect you to say soon after, in the next paragraph, 'Here, we investigate a fossil feather in three dimensions...' or some such, but you don't.*
>
> *You come back to Methods at the end of the Introduction (lines 97-101), but again do not say whether you model the feather in three dimensions or not. Yes, you did - I finally learned at line 104 - you did micro serial sectioning. This needs to shift a long forward into the Introduction.*

Thanks for the suggestions, we utilize serial sectioning to get a different view of the microbodies that are proposed to be melanosomes and reconstructed the three-dimensional volume of the melanosomes, as well as the intercalated keratin.

We restructured the introduction and make clear that the three-dimensional data obtained in this study also was used for modeling and in a more anterior position in the text.

> *In the Results, there are not enough references to images. It's not enough to refer generally to 'Figures 3c-f' [line 133] and then go on to rapidly step through some amazing imagery (text lines 133-146) - you need to add an image citation to each observation so readers can know exactly which image is being described each time.*

We elaborated our description of imaging to better describe the melanosomes in our results section. We add the description of the stack of melanosomes as IN Above (reply of Reviewer #2).

> *The 3D data in Figures 3 and 4 is great and based on huge technical wizardry. The sketch model in Figure 4a is excellent, but could you not attempt an actual 3D block diagram showing the hexagonal arrangement of clusters of aligned melanosomes?*

We have also tried FIB -SEM in an additional place for validation of our ultrathin sections data. See the SI file.

Added figure S7. Targeted feather barb block prepared in FIB-SEM, with volume rendering reconstruction based on the acquired sequential cross-sectional images; the volume reconstruction is visualized in the x-y plane (c-cross section view) and in x-z plane (d-sagittal section view).

Modified Figure S8d shows the 3D model of aligned melanosomes. To show the arrangement more clearly, the schematic XY cross-section of the melanosomes 3D model is shown below (also shown in Supplementary Figure S8d).

> *35: delete 'yield'*

Changed

> *73: 'feather fell' ? = 'feather that has fallen'*

Changed

> *305: excises ?= exercises*

Changed

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