〈Research Article〉

Role of spongy layer and melanin granule arrangement on the development of blue structural color of bird feathers

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Summary The blue color of parrot, jay, and kingfisher feathers originate from the spongy layer beneath the cortex, as confirmed using microscopy of feather barb cross sections. The green color reflected from parrot feathers was thought to be caused by a combination of blue structural color derived from the spongy layer and yellow color derived from the yellow pigment psittacofulvin in the cortex. Using optical and scanning electron microscopy, melanin granules in the medulla of blue and green parrot feathers were found to be oval in shape and arranged in a palisade-like structure. This structure was assumed to enhance the blue color reflection based on a simulation using Bragg's formula. The striped, repetitive blue gradation in jay wing feathers was found to be produced by changes in the number and distribution depth of melanin granules. The melanin granules of blue kingfisher feathers were smeared with medullary β-keratin under the spongy layer to form a smooth lump. It was thought that the lump comprising β-keratin and melanin reflected the light that passed through the spongy layer to give iridescence to the blue structural color.

Key words: Feather, Spongy layer, Melanin, Microscopy, Reflectance spectrum

1. Introduction

The feathers of some birds display diverse and beautiful colors originating from various pigments, such as carotenoids, protoporphyrins, and psittacofulvins, distributed in the cortex of their feathers[1,2], which color depth will be changed depending on environmental conditions[3]. However, some of the rich colors displayed originate from nanoscale structures, such as the lattice structure comprising melanin rods observed in the barbules of peacock feathers[4-6] and the spongy layer between the cortex and medulla in the barbs of parrot feathers[7-9]. The color of visible light reflected from nanoscale structures is termed structural color. The spongy layer is known to reflect blue light, and parrots, jays and kingfishers have this layer in the barbs of their feathers[10,11]. The green feathers of parrots reveal a unique color development whereby yellow pigments (psittacofulvins) in the cortex act in combination with structural color to produce various other

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colors[12]. Recently, biometric experiments using artificial amorphous spatial structures have revealed that the blue color reflection from the spongy layer requires a black pigment like melanin to be distributed under the spongy layer[13].

The aim of the present study is to clarify the role of melanin granules in the creation of blue color reflection from the spongy layer. Reflectance spectra from parrot, jay and kingfisher feathers, and their melanin content, were analyzed. In parallel, transverse sections of parrot, jay and kingfisher feathers were prepared and the melanin distribution was observed under an optical microscope (OM) and a scanning electron microscope (SEM). It became clear that the morphology and arrangement of melanin granules beneath the spongy layer observed in the barbs of parrot, jay and kingfisher feathers played an important role in the expression of the characteristic structural color derived from the spongy layer in each species.

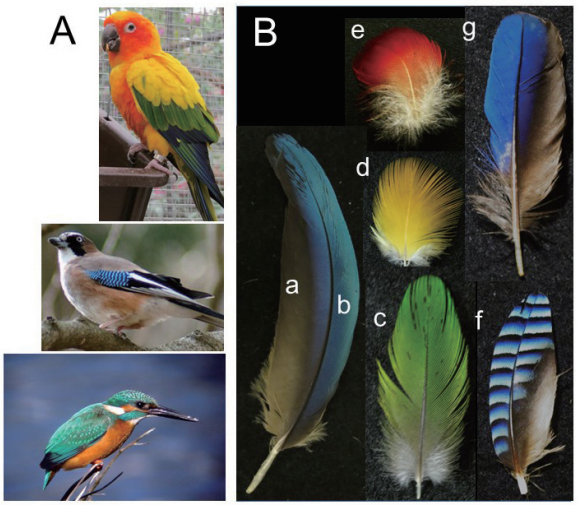

Fig. 1 Photographs of researched birds and their feathers.
(A) From top to bottom, images of sun conure parrot (*Aratinga solstitialis*), jay (*Garrulus glandarius*) and kingfisher (*Alcedo atthis*). Kingfisher photo provided by Mr. Manabu Ohyama. (B) a and b: black and blue; c: green; d: yellow; e: red feathers of the sun conure parrot, *A. solstitialis*; f: blue wing feathers of the jay, *G. glandarius*; g: blue tail feathers of the kingfisher, *A. atthis*.

2. Materials and Methods

Materials

Yellow, red, blue, and green feathers of the sun conure parrot *Aratinga solstitialis*, the wing feathers of the jay *Garrulus glandarius* displaying a striped pattern with a repetitive blue gradation, and the blue tail feathers of the kingfisher *Alcedo atthis* were prepared for this study. Each of these feathers was obtained from one individual. Since each feather has a different color distribution, this study analyzed a single feather that emits the characteristic color of the bird (Fig. 1A and B).

Reflectance spectrum analysis of bird feathers

The reflectance spectrum analysis of the bird feathers was performed using a mini-spectrometer C11009MA (Hamamatsu Photonics, Hamamatsu, Japan) under visible light illumination from an incandescent lamp (JOR 110V 75W E11X1; Tokyo Metaru Kogyo, Tokyo, Japan). Carbon black (Holbein Works Ltd, Tokyo, Japan) and a white reference board (Neewer, Shenzhen, China) were used as the blank and reference materials, respectively. The A/D count ratio of the reflectance spectrum was calculated taking the strength of reflected light from carbon black as 0 and the strength of reflected light from the white reference board as 100.

Extraction of melanin pigment and absorbance spectral analyses

After the measurement of dry weight, bird feathers were immersed in a 5% sodium hydroxide aqueous solution for 48 h, centrifuged at 1620 g for 5 minutes, and the supernatant collected. Next, an absorption spectrum of this sodium hydroxide extract was measured from 300 to 800 nm using a UVmini-1240 ultraviolet-visible spectrophotometer (Shimadzu Corporation, Tokyo, Japan).

Preparation of thin transverse sections of parrot feathers

The barbs of parrot feathers were buried in paraffin and sectioned with a Jung-type microtome to a thickness of 5.0 or 7.5 μm. The sectioned sample

was placed in water on a slide glass, heated at 55 °C, and extended for drying.

Observation of transverse-sectioned barbs

First, transverse-sectioned barbs of parrot feathers of several colors were directly observed using an OM (Olympus Co Ltd., Tokyo, Japan) under transmitted illumination. Next, the encapsulating agent malinol (Muto Pure Chemicals Co Ltd, Tokyo, Japan) was applied by dropper onto the transverse-sectioned barbs and the barbs re-observed. Images of both were taken using a microscopic digital color camera (DP21-SAL; Olympus Co Ltd, Tokyo Japan) for comparison.

Microspectrophotometric analysis of transverse-sectioned barbs

The reflectance spectra from the transverse-sectioned barbs were determined using a microscopic spectrophotometer (MSV-5000; JASCO Co, Tokyo, Japan) with the diameter of the spotlight set at 10 μm.

SEM observations of transverse-sectioned barbs

Gold was deposited onto the transverse-sectioned barbs on a slide glass, and they were then observed using a table top SEM (TM3000; Hitachi Technologies, Tokyo, Japan).

3. Results

Reflectance spectrum analysis of bird feathers

The reflectance spectra of black, blue, green, yellow and red feathers of the parrot and blue feathers of the jay and kingfisher were analyzed using a mini-spectrometer (Fig. 2A). The reflectance spectra of blue parrot and jay feathers showed a very slight peak at 500 nm, while the green parrot feathers showed a very slight peak at 530 nm, a slightly longer wavelength than the blue feathers. In the blue kingfisher feathers, the reflectance spectrum showed a peak, clearer than for the parrot and jay, at 500 nm. In the yellow feathers of the parrot, the reflection intensity rose to and maintained a high level at wavelengths >500 nm, and in the red feathers intensity rose to and maintained a high level at wavelengths >600 nm. The absorption spectra of the bird feather sodium hydroxide solutions for yellow and red parrot feathers showed almost no peak around 400–500 nm derived from melanin. In blue and green parrot feathers, a melanin peak was observed, but the melanin absorption spectrum of jay and kingfisher feathers was higher than that of parrot feathers (Fig. 2B). As described above, the visible reflection intensity of the blue and green parrot feathers and the jay and kingfisher feathers with a high melanin concentration was low, and the visible reflection intensity of the yellow and red parrot feathers with a low melanin concentration was high.

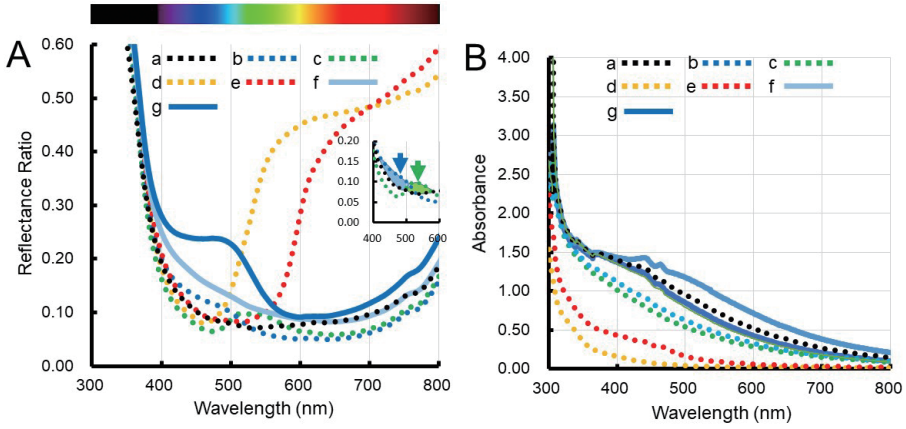

Fig. 2 Reflectance and absorbance spectra of bird feathers.
(A) Feather reflectance ratios. (B) Feather absorbance.

OM and SEM observations of transverse-sectioned barbs

The sun conure parrot, *Aratinga solstitialis*:

Black, blue and green parrot feathers (Fig. 1) sectioned to a thickness of 7.5 μm were observed using OM (Fig. 3A, left side color photos). A melanin pigment was observed in the medulla of the blue and green feathers. As reported in a previous study[12], yellow pigment can be observed in the cortex of yellow, red and green feathers but not in that of black and blue feathers. Direct microscopic observations of native transverse-sectioned barbs of blue and green feathers under transmitted illumination showed orange spongy layers between the cortex and the medulla (Fig. 3A, left side color photos, middle row). A blue color was reflected from transverse-sectioned barbs under oblique illumination (Fig. 3A, left side color photos, bottom row).

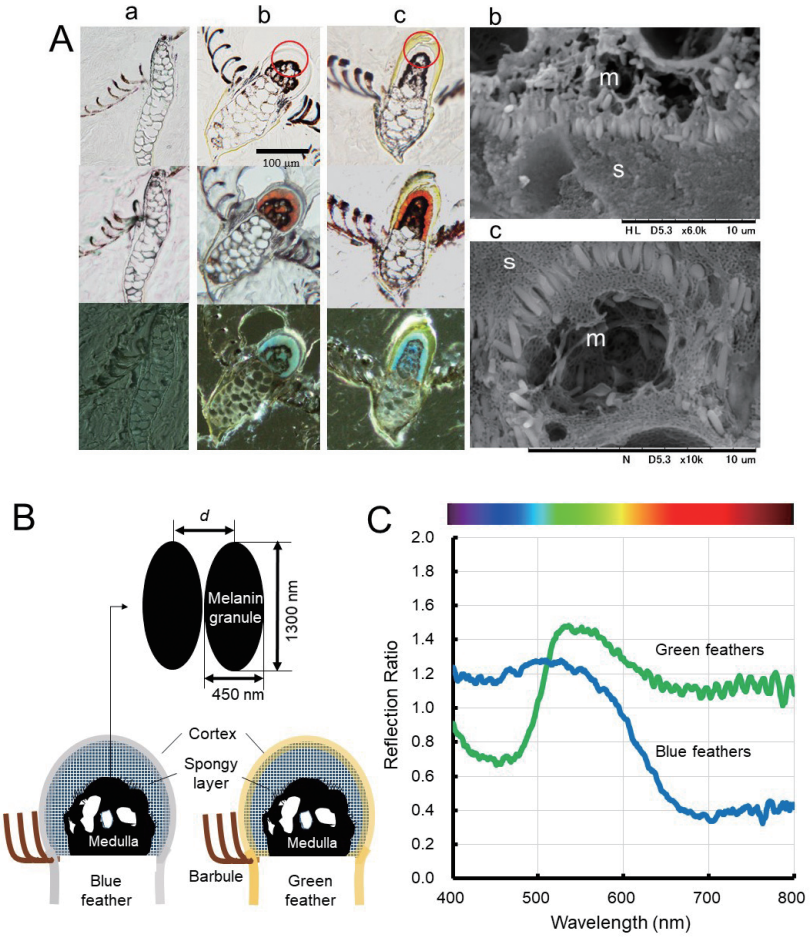

Fig. 3 Microstructure and microspectrophotometric analysis of parrot barb.
(A) Optical and scanning electron micrographs, and microspectrophotometric analysis of transverse-sectioned parrot feathers. The lowercase letters "a, b, c" correspond to the same letters identifying the parrot feathers before slicing shown in Fig. 1B. Left panel (a–c): Color optical micrographs of transverse-sectioned feathers. a–c top row: after encapsulation with malinol; a–c middle and bottom rows: before encapsulation with malinol (top and middle rows: transmitted illumination; bottom row: oblique illumination). Right panel: monochrome SEM observation. s: spongy layer; m: medulla. (B) A schematic of palisade-like structure of melanin granules on the medullary surface. (C) Microspectrophotometric analysis. The spectra were measured at the part of the transverse section of blue and green feathers surrounded by the red circles in part (A).

The orange color that passes through the spongy layer is the complementary color of the blue that is reflected from the spongy layer. When transverse-sectioned barbs were treated with malinol, the color of the spongy layer disappeared and the layer became transparent (Fig. 3A, left side color photos, top row).

The melanin granules in the medulla of blue and green feathers were observed using SEM. This confirmed that the melanin granules were oval in shape and were arranged in a palisade-like structure with their long-axis oriented outward (Fig. 3A, right side, monochrome photograph). The long-axis length of the melanin granules was ~1300 nm, and the short-axis length was ~450 nm. A schematic of palisade-like structure of melanin granules on the medullary surface was provided to Fig. 3B.

Microspectrophotometric analysis was performed at the part of the transverse-sectioned barbs indicated by the red circles in Fig. 3A, locations which included the cortex and the spongy layer of transverse-sectioned barbs. The blue feathers showed a higher reflection intensity at wavelengths shorter than ~500 nm. The green feathers also showed a broad peak around 500 nm, and reflection intensity was high at wavelengths longer than 600 nm (Fig. 3C).

Thus, the spongy layer both of the blue feathers and green feathers reflected the blue structural color. The yellow pigment was only observed in the green feather cortex, and the pigment color also affected the reflection spectrum of the green feather section.

The jay, *Garrulus glandarius*:

Some of the wing feathers of the jay display a striped pattern with a repetitive blue gradation (Fig. 1B-f). Serial transverse sections of these feathers were prepared. Native transverse-sectioned barbs were observed using OM under transmission illumination (Fig. 4 left panel, middle row). Sections were also observed after encapsulation in malinol (Fig. 4 left panel, top row). The spongy layer in the unencapsulated section was orange, becoming colorless and transparent following encapsulation under transmission illumination. Observation of serial sections revealed obvious changes in medullary melanin and its distribution in the spongy layer; in the transverse section of the white portion of the barb, melanin granules were absent in the medulla and numerous cavities were observed (Fig. 4 left panel, left

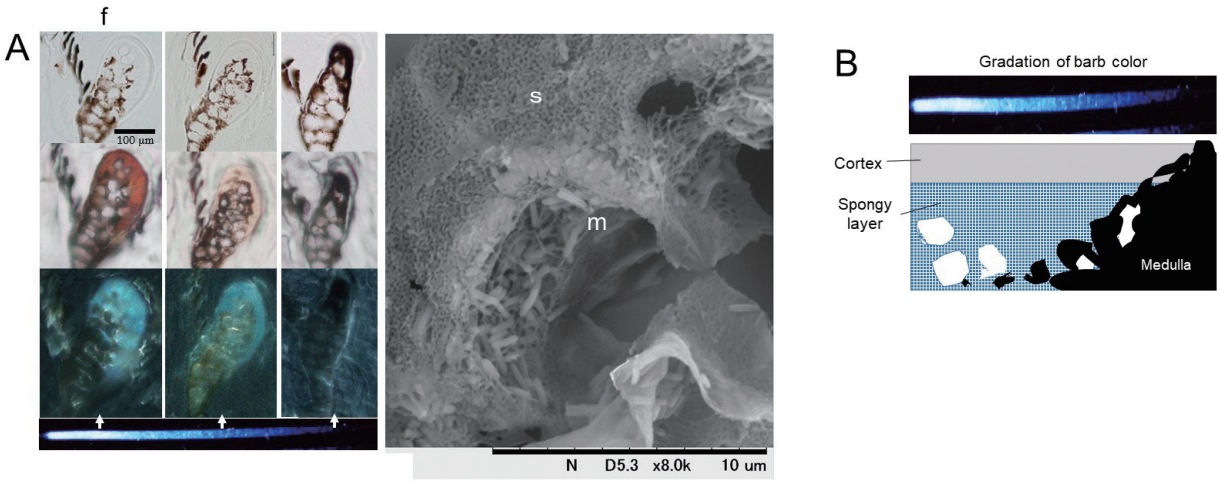

Fig. 4 Microstructure of jay barb.
(A) Optical and scanning electron micrographs of transverse-sectioned jay feathers. Left panel color images: optical microscopy. The letter "f" corresponds to the same letter identifying the jay feather before slicing in Fig. 1B. Left panel top row: after encapsulation with malinol. Left panel middle and bottom rows: before encapsulation with malinol (middle row, transmitted illumination; bottom row: oblique illumination). Right panel monochrome photograph: SEM. s: spongy layer; m: medulla. (B) A schematic of longitudinal section of jay barb.

column). Melanin granules were present in the medulla where the barb color was blue. In addition, the location of the melanin was now on the upper surface of the barb, and the thickness of the spongy layer decreased with an increase in the intensity of the blue color (Fig. 4 left panel, center column). In the black portion of the barb, the spongy layer was absent, and medullary melanin granules adhered to the cortex. The melanin layer thickness increased and now extended to the surface of the barb (Fig. 4 left panel, right column). Under oblique illumination of the transverse-sectioned barbs (Fig. 4 left panel, bottom row), blue light was reflected from the spongy layer, contrasting with the orange color seen under transmitted illumination (Fig. 4 left panel, middle row). Using OM under transmitted illumination the medullary melanin granules were observed to be distributed as lumps on the surface of the medullary foam below the spongy layer. Further observation under SEM revealed that the melanin granules had an elongated oval shape and were randomly attached on the inside of the foamy medulla (Fig. 4 right panel, monochrome photograph). A schematic of melanin granules randomly arranged on the inner surface of the medulla was provided to Fig. 4B.

The kingfisher, *Alcedo atthis*:

As in parrot and jay feathers, transverse-sectioned barbs of blue kingfisher feathers were observed using OM under transmission illumination, then encapsulated in malinol and re-observed (Fig. 5 left panel, middle and top images, respectively). As with the parrot and jay barbs, in the unencapsulated section the spongy layer was orange, and melanin granules were visible on the medulla surface. Under oblique illumination, blue light was reflected from the spongy layer, contrasting with the orange color seen under transmitted illumination (Fig. 5 left panel, bottom image). The melanin of kingfisher feathers was observed to be distributed as a smooth layer on the surface of the medullary foam below the spongy layer. Further observation under SEM revealed that the melanin granules seemed to be smeared with β-keratin on the medullary surface, while the inner wall of the medullary dome was

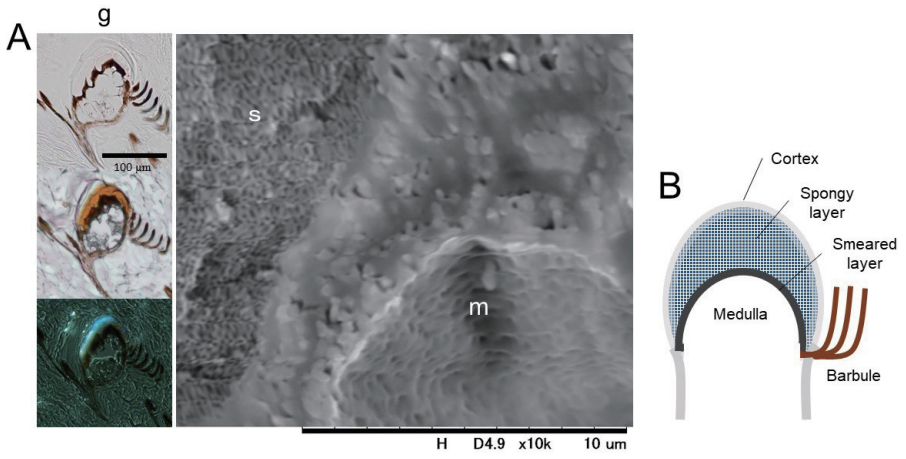

Fig. 5 Microstructure of kingfisher barb
(A) Optical and scanning electron micrographs of transverse-sectioned kingfisher feathers. Left panel color images; optical microscopy. The letter "g" corresponds to the same letter identifying the kingfisher feather before slicing in Fig. 1B. Left panel top row: after encapsulation with malinol. Left panel middle and bottom rows: before encapsulation with malinol (middle row: transmitted illumination; bottom row: oblique illumination). Right panel monochrome image; SEM. s: spongy layer; m: medulla. (B) A schematic of melanin distribution smeared on the surface of kingfisher medulla.

homogeneous with few melanin granules observed on the surface (Fig. 5 right panel, monochrome image). A schematic of melanin distribution smeared on the surface of kingfisher medulla was provided to Fig. 5B.

4. Discussion

The beautiful colors exhibited in parrot feathers arise through a combination of psittacofulvin, a specific yellow pigment in the cortex, and the blue structural color generated by the spongy layer, which consists of nanoscale air cavities and β-keratin between the cortex and the medulla[1,9,14]. Jay and kingfisher feathers have also been shown to reflect the blue structural color derived from the spongy layer[10,11]. The aim of this study was to clarify the role of melanin distribution in the development of the blue color from the spongy layer.

When the reflectance spectra from black, blue, green, yellow, and red parrot feathers, and blue feathers from jay and kingfisher were analyzed, the reflection intensity of yellow and red parrot feathers was seen to be higher than that of blue and green feathers (Fig. 2A). Yellow feathers reflected light at 500 nm or above, and red feathers reflected light at 600 nm or above. Blue feathers weakly reflected light between 400 and 500 nm, and green feathers weakly reflected light between 500 and 600 nm. It was hypothesized that the distribution of melanin might be necessary to emphasize this weak light. Absorbance analysis of each type of feather dissolved in sodium hydroxide revealed that the melanin content of yellow and red parrot feathers was extremely low, while the melanin content of blue and green parrot feathers, and blue jay and kingfisher feathers was high (Fig. 2B). Although the cortex of green parrot feathers includes psittacofulvin pigment[12], like the yellow feathers, the reflection intensity of green feathers was very low. This phenomenon was thought to be due to absorption by melanin. To clarify the relationship between color reflection and melanin distribution in bird feathers, we observed the spongy layer and melanin distribution of parrot, jay, and kingfisher feathers with OM and SEM, and considered the relationship with structural color expression (Figs 3–5).

OM observations of transverse sections of blue and green parrot feathers, and blue feathers of jay and kingfisher revealed that the spongy layer between the cortex and medulla exhibited an orange color under transmitted illumination and reflected blue light under oblique illumination. Moreover, the spongy layer became transparent after treatment with an encapsulating reagent. Based on these phenomena, it was inferred that the spongy layer had an amorphous cavity structure, and that the blue color reflected from the spongy layer was a complementary color of the orange observed under transmitted illumination. When the cross sectioned barb structures of parrot, jay and kingfisher feathers were imaged using SEM it was observed that, in parrot feathers, the melanin granules were an elongated oval shape and distributed in a palisade arrangement on the medullary surface. It has recently been reported that the nanoscale lattice structure of melanin rods in peacock feather barbules reflects the specific beautiful colors[4-6]. Kawamura et al.[15] constructed artificial nanoscale polystyrene particles coated with polydopa, and experimentally proved that the reflectance spectrum from polydopamine (DP)-coated particles changed according to the particle size and the DP refraction index based on Bragg's law. From these studies, it was thought that the palisade-like structure of parrot melanin granules also regulates the reflectance spectra from the medulla, narrowing the spectral range for a sharper color reflection. The long-axis length of the melanin granules of parrot feathers was found to be ~1300 nm, and the short-axis length ~450 nm (Fig. 3B). Although the parrot melanin granule size was significantly larger than the polystyrene granule size used in the simulation by Kawamura et al.[15], unlike in their experiment, the reflectance spectrum from the parrot feathers was measured in this study as via incident light passing from the spongy layer toward the melanin granules. The refractive index of β-keratin in parrot feathers as measured using the Jamin–Lebedeff interference microscopy method[16] is ~1.6, and the refractive index of medullary melanin

is ~1.7[17]. Assuming that the ratio of the air cavity area of the spongy layer to β-keratin is 1:4 based on the scanning data of the spongy layer by ImageJ software (data omitted), the relative refractive index between the spongy layer and the melanin was calculated to be very small, about 1.17. In this study, the center-to-center distance between the melanin particles, taken as the short-axis length, was 450 nm. Bragg's law employs the following formula, in which m is the order of diffraction, λ is the wavelength of light, d is the center-to-center between the nearest particles, n is the refractive index of colloidal particles, and θ is the angle between the incident light and the diffraction crystal planes:

$$m\lambda = \sqrt{\frac{8}{3}d^2(n^2 - \sin^2\theta)}$$

When the appropriate data values are substituted into this formula, the wavelength (λ) of the reflection spectrum at an incident angle (θ) of 90° is 446 nm. This result suggests that the palisade arrangement of the elongated oval melanin granules on the medullary surface of parrot feathers selectively reflects the blue light from the light transmitted through the spongy layer, i.e., the shape and distribution of melanin granules appears to enhance the reflectance of blue light from parrot feathers.

In jay feathers, which bear the blue gradation stripe pattern, few melanin granules were observed under the spongy layer in the white portion of the barbs. Moving toward the blue end of the barb, melanin granules begin to appear and their location changes from the depth of the barb to its surface, the spongy layer becomes narrower and the blue color reflected from the barb becomes darker (Fig. 4). Therefore, the blue color gradation may be caused by a change in broadband absorption based on increases and decreases in the number of melanin granules and their depth. The necessity of melanin in the expression of blue structural color due to the amorphous air cavity structure was proven by the work of Iwata et al.[13] using silica particles on glass and black quartz plates; when the thickness of silica particles smeared on a black quartz plate was thin, the reflection color was blue, and when the layer was thick, the reflection color was white.

Compared with parrot and jay feathers, kingfisher feathers were relatively clear and had a high reflection spectrum near 500 nm, despite having a high melanin content. Referring again to the work of Kawamura et al.[15], when dopamine (DA) was coated on polystyrene particles thinly, light that could not be absorbed by the DA was reflected from the polystyrene surface to produce an iridescent color. In this study, the melanin in kingfisher feathers appeared to be smeared with β-keratin on the medullary surface (Fig. 5). This smeared β-keratin layer containing melanin may reflect the light passing through the spongy layer to produce an iridescent blue color reflected from the spongy layer, in the same manner as the polystyrene particles coated a thin DA layer reported by Kawamura et al.[15]

In conclusion, the intensity of the blue color reflected from the parrot feathers is strengthened by the palisade structure of melanin granules on the medulla. The graded blue striped pattern of jay feathers arises through a change in the number of melanin granules and their location along the feather barb. The iridescence of the blue color of kingfisher feathers originates from the smeared melanin layers on the medulla. It is clear that the distribution of melanin plays a fundamental role in structural color development in the feathers of various birds.

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Conflicts of interests

The author has no conflicts of interest.

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