〈Original Article〉

Green and purple structural color development by thin film interference and eumelanin distribution of dove feathers

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Summary Depending on the viewing angle, green or purple iridescent colors are reflected from tip of rock dove feathers around the neck. This color development is attributed to thin film interference, and we aimed to clarify the microstructure and pigment distribution that produce the interference color. In this study, Raman micro-spectroscopic and absorption spectrum analyses of dove feathers were performed, and it was confirmed that the pigment eumelanin was most abundant in the feather tips reflecting iridescent colors. By observing the transverse cross-sectioned feathers using optical and transmitting electron microscopes, when the barbules were horizontally arranged, it could be seen that medullae at the feather tips contained a multitude of eumelanin particles. We hypothesized that the distribution of eumelanin particles within the barbule medullae reduced the incoherent scattered light to facilitate structural color reflection from the cortex. When the viewing angle was small relative to the feather surface, green light was reflected from the surface of the horizontally arranged barbules, and when this angle was large, purple light was reflected from barbules. The reflectance spectrum was simulated by changing the incident angle via thin film interference based on the dove feather micro-structure, which proved to be similar to the actual reflectance spectrum of these feathers.

Key words: Dove, Feather, Eumelanin, Thin film interference, Simulation

1. Introduction

In recent years, melanocytes have been found in feathered dinosaur fossils[1], and it is thought that the distribution of melanin is very important in the dermal color expression in the process of evolution from dinosaurs to birds. Melanin is the most common pigment present within animals, which can be further classified as black eumelanin or brown pheomelanin[2]. These pigments can absorb ultraviolet rays and thermal energy, and reduce the bacterial deterioration of keratin fibers in order to protect the body surface[3]. Some bird feathers reflect a variety of

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attractive colors, which indicates differences in bird classes or between individuals, and are effective for influencing mating selection[4, 5]. It is known that bird plumage color originates from a variety of pigments, such as: psittacofulvin in parrots, spheniscin in penguins, porphyrin in the turaco, and carotenoids in the bokmakierie[6, 7]. It has also been reported that nanolevel structures – including the melanin rods of peacock feathers[8-10] as well as the spongy layer of parrot[11, 12] and kingfisher feathers[13] - also reflect a variety of colors. The feathers circulating the neck of the rock dove produce iridescent structural colors that change from green to purple depending on the viewing angle. Nakamura et al.[14] revealed that this coloration was due to thin film interference within the cortex of the barbules. In the current study, the reflectance spectra from rock dove feathers were analyzed using a spectrophotometer, and Raman microspectrometry of the feather pigment was performed to identify eumelanin. The arrangement of barbules attached to the barbs were observed with an optical microscope (OM), and transverse cross sections of the feather segments reflecting iridescent color were confirmed to contain melanin morphology with an transmission electron microscope (TEM). Based on these results, simulation of the reflectance spectra via thin film interference was performed in order to be clarified the three-dimensional arrangement of barbules as well as the effect of melanin distribution on the color reflected from the feathers.

2. Materials and methods

Materials

Rock doves (*Columba livia*) were observed in the field surrounding the Yamazaki University of Animal Health Technology, and iridescent feathers were obtained from carcasses found in the park near the university.

Preparation of thin transverse cross-sections and OM observation

Feathers were embedded in paraffin and sectioned with a Jung microtome to a thickness of 5 μm. The sectioned sample was placed in water on a glass slide, heated at 55°C, and extended for drying. Transverse-sectioned feathers were directly observed using an OM (Olympus Co Ltd., Tokyo, Japan) under transmitted illumination.

TEM observation of transverse cross-sectioned

Samples were prepared as previously described[9] prior to conducting TEM observations of the transverse sections of dove feathers barbules. Observations and imaging of barbules were then performed using a TEM (JEM-1400Plus; JEOL Ltd., Tokyo, Japan) with CCD camera (EM-14830RUBY2; JEOL Ltd., Tokyo, Japan).

Raman micro-spectrometry analysis

Raman micro-spectrometry analysis was performed using a Renishaw inVia™ Raman Microscope with an Ar-laser at emission of 532 nm. A dry feather was deposited on a glass slide and Raman shift was measured at each section.

Extraction and identification of the pigment

Iridescent feather segments were excised. After the measurement of dry weight, sections were immersed in a 5% sodium hydroxide aqueous solution for 12 hours, centrifuged at 3000 rpm for 5 minutes, and the supernatant was collected. Next, an absorption spectrum of this sodium hydroxide extract was measured between 300 - 800 nm using a UVmini-1240 ultraviolet-visible spectrophotometer (Shimadzu Corporation, Tokyo, Japan).

Spectrophotometric analysis

The reflectance spectrum analysis 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). A micro-cellular foamed light reflective sheet (MCPET; Furukawa electric Co. Ltd.) was used as the reference.

3. Results

Iridescent color change of feathers surrounding the neck

The iridescent color of the feathers around the neck of the dove changes between green and purple depending on the viewing angle, with the location of green reflection always occurring on the side of the observer (Fig. 1).

Observation of transverse cross-sectioned feathers

A feather reflecting the iridescent colors was prepared (Fig. 2A); only the tip reflected iridescence, while the root side did not. The feather was embedded in paraffin and transverse cross-sectioned using a microtome. Observing the tip and root sections, respectively, by use of the OM, it was observed that small spherical granules were sparsely distributed in the latter medulla of barb and barbules, but the former medulla of barbules was filled with the black pigment (Fig. 2B). In addition, within the TEM observation of barbules, the medulla at the feather tips contained many spherical granules of approximately 500 nm in diameter, and the cortical thickness of the tip of barbules was uniform between 450 - 500 nm. In the medulla of the root section, melanin granules were only sparsely distributed and the boundary between the cortex and the medulla was unclear. The cortex of the root section had many fine cavities of <0.1 micrometers (Fig. 2C). Confirming the arrangement of barbules on the tip and root locations, the barbules of the former were placed horizontally to be juxtaposed adjacent to each other, and each of the barbules of the latter were vertically arranged with their distance arranged spatially. The change of barbule arrangement and the melanin morphology within the tips and roots of the feather as based on the OM and TEM observations of the transverse cross section are shown as the schematic illustration in Fig. 2D.

Raman micro-spectrometry of pigment

The TEM observation of feathers revealed many black granules in the medulla of barbules (Fig. 2C). When feathers were observed under transmitted illumination, the tips – which normally reflect iridescent color - were instead a deep black color under illumination (Fig. 3A). In order to identify the black pigment, Raman micro-spectroscopy analysis was performed via irradiation with a 532 nm laser to the barbules from α to γ, as shown in Fig. 3A. Consequently, excitation bands were detected at 1380 and 1590 cm^{-1}, similar to the D and G bands of graphene[15] at the α, β, and γ segments, respectively (Fig.3B). These two excitation bands are

Fig. 1 Change in color reflected from feathers around the neck of dove in sunlight depending on the viewing angle.
Dove images were taken in Shizuoka City on April 25, 2019 at 10:00, and were provided by Mr. Tadashi Maruyama from Yamazaki University of Animal Health Technology.

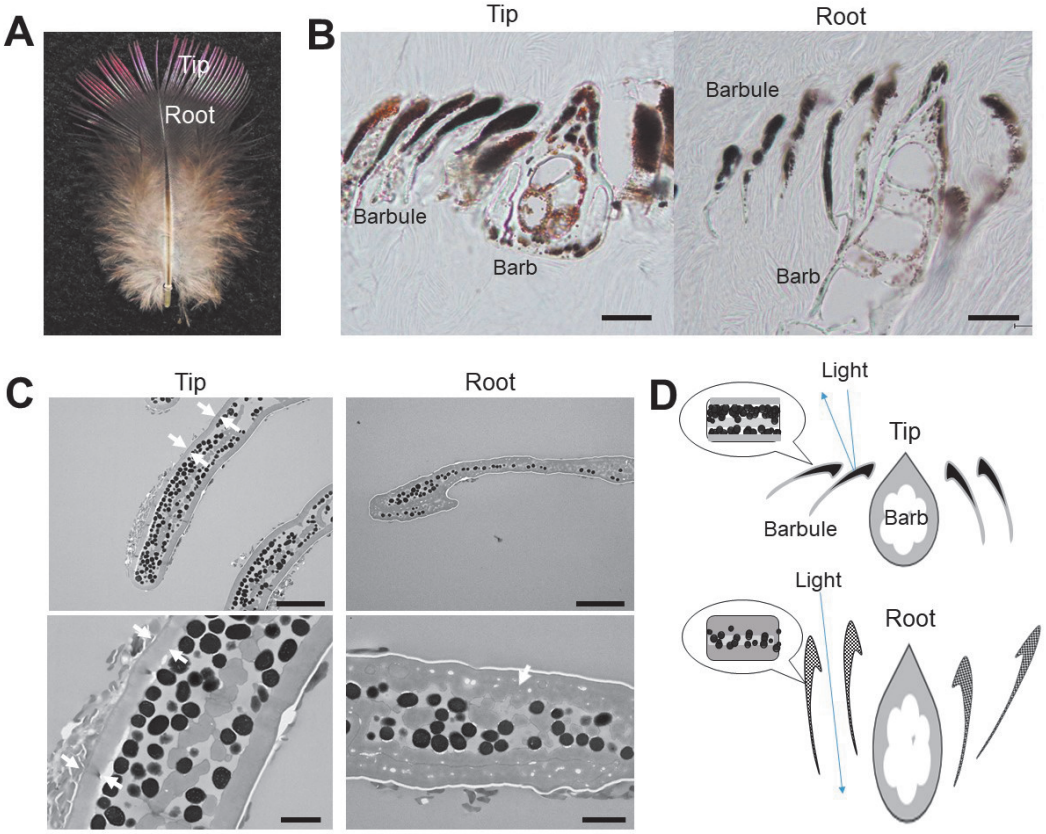

Fig. 2 Observation of the native feathers and transverse cross-sectioned feathers.
A: Under oblique illumination. B: Transverse cross-sectioned tip and root feathers using OM. Scale bar = 10 μm. C: TEM images of transverse cross-sectioned barbules at the tip and the root of the feathers. White arrows indicate the cortex. The thickness of the cortex at the tip of the feather was uniform, indicated by a pair of white arrows facing each other, but the boundary between the cortex and medulla of the root of the feathers was unclear. Upper scale bar = 5 μm, lower scale bar = 1 μm. D: Schematic diagram showing change of melanin morphology (in the balloon) and the arrangement of barbules at the tip and root side of the feather.

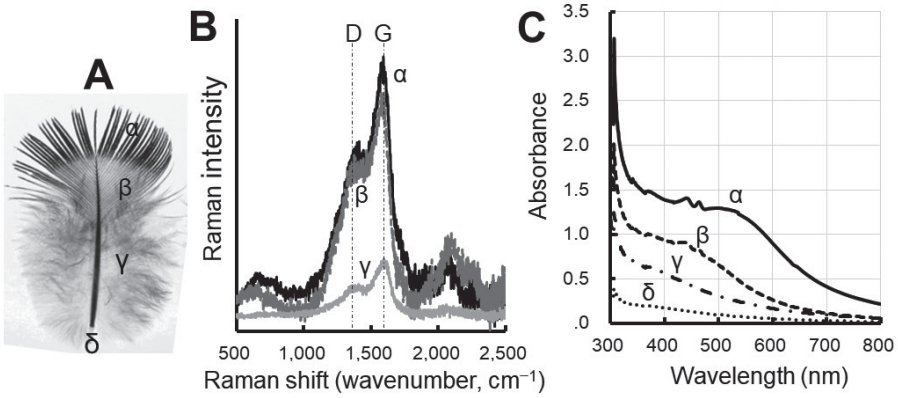

Fig. 3 Observation of the transmittance of rock dove feathers and the identification of pigment.
A: Rock dove feathers under transmitted illumination. Raman microspectrometry and absorption spectrum analysis was performed at the α, β, γ, and δ positions. α: tip of the feathers; β: root of the feathers; γ: downy barbs; δ: rachis. B: Raman spectrogram from α to γ position based on the value of the δ position. The dash-dotted lines, D and G, indicate the Raman spectrum bands observed in disordered graphite that were similar to those observed in eumelanin structure16. C: Spectrum analysis of the pigment extracted from each places from α to δ shown in A with 5% NaOH.

characteristic of eumelanin[16]. The feather was divided and cut into sections from α to δ as shown in Fig. 3A and immersed in a 5% NaOH aqueous solution to dissolve the keratin protein and melanin granules. The absorbance spectra of the respective supernatant fractions after centrifugation of the dissolved solution were measured. A broad peak was observed at approximately 500–600 nm in the NaOH aqueous solution of the α site, but peaks at the β and γ sites were lower, and no peak was observed at the δ site without melanin deposition (Fig. 3C).

Reflectance spectrum and its simulation

The feathers were microscopically observed while changing the incident angle by oblique illumination. When the incident angle relative to the feather surface was small, green light was reflected (Fig. 4A). When the incident angle was large, purple light was reflected from the edge of the curved barbules (Fig. 4B). When the incident angle and the reflection angle (i.e. viewing angle) was set to about 5 degrees and the tip of the feather was pointed to the observer (turning angle = 0), green light was reflected from the feather. Then the reflectance spectrum exhibited two peaks at 500 and 700 nm, of which the wavelengths correspond to green and red, respectively. When the incident angle and the reflection angle (i.e. viewing angle) was set at ~40 degrees and the tip of feather was rotated 90 degrees to the right, purple was reflected. Then the reflectance spectrum exhibited two peaks at 470 and 670 nm, of which the wavelengths correspond to indigo blue and orange, respectively (Fig. 5A). It is well described that reflectance spectrum caused by thin film interference can be estimated by the Fresnel formula. Reflectance spectra from the cortex of these feathers were estimated using simulation software (https://www.filmetricsinc.jp/reflectance-calculator) based on the Fresnel formula[17] provided from Filmetrics Co. Ltd. The formula was applied to the wavelength reflected from the cortex surface. The thickness of the cortex was set at 480 nm based on the TEM images (Fig. 2C), and the refractive indexes of the cortex and eumelanin were set at 1.6 and 1.7 respectively, according to previous research conducted by Leertouwer et al.[18], and Stavenga et al.[19]. When the incident angle was set at 5 degrees, peaks were observed at 500 and 750 nm, of which the reflectance spectrum corresponds to that of the green reflected from the feathers. When the incident

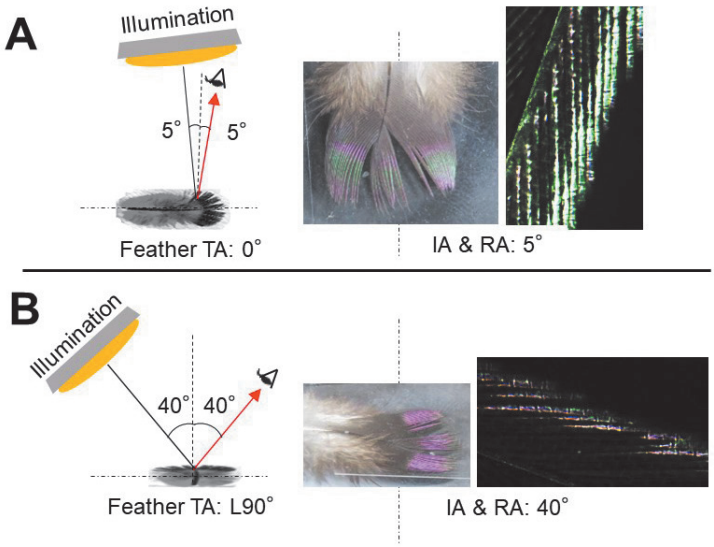

Fig. 4 Schematic diagram outlining the relationship between the incident angle and color reflected from the feather surface.
A: A green color was reflected when the incident angle (IA) was 5 degrees and the turning angle (TA) was 0 degrees. B: A purple color was reflected when the IR was 40 degrees and TA was 90 degrees. A one-dot chain line indicates that TA is 0 degrees.

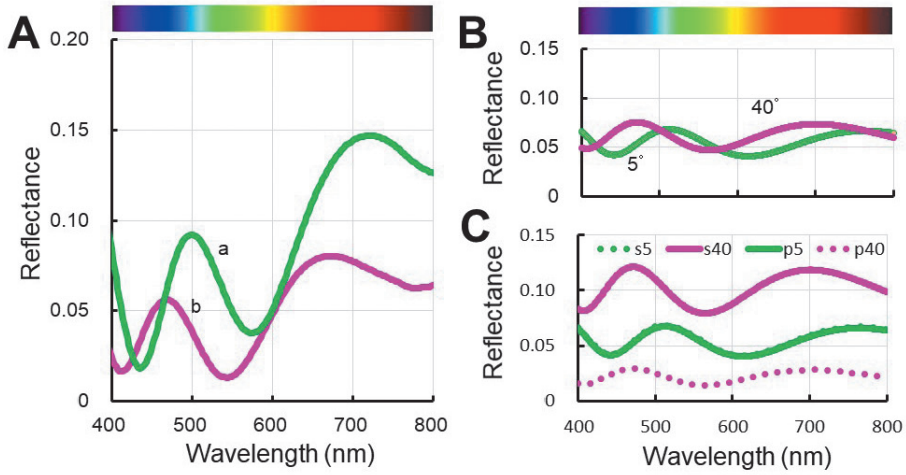

Fig. 5 Reflectance spectrum of dove feathers and simulation of reflectance spectrum from the cortex.

A: green (a) or purple (b) reflectance spectra from the feather surface. B: The simulation graph of mixed polarized reflectance spectra from the feather surface based on the Fresnel formula when the IA was set at 5 and 40 degrees. C: The simulation graph of s- and p-polarized reflectance spectra. The thickness and the refractive index of the cortex of the feather were 480 nm and 1.6 respectively, and the refractive index of eumelanin was set at 1.7.

angle was set at 40 degrees, the reflectance spectrum showed two peaks at 470 and 700 nm, which corresponds to the purple reflectance spectrum of the feathers (Fig. 5B). After dividing the reflection spectrum simulation into p-polarized and s-polarized spectra, s-polarized reflectance is stronger than p-polarized reflectance in the case of purple reflection with a large incident angle (Fig. 5C).

4. Discussion

Attractive bird feather colors are known to originate from unique pigments and nanolevel structures from different bird species[4, 5]. Unique nanolevel structures for structural color development such as a lattice structure comprising rod-shaped melanin particles of peacock tail feathers and a sponge layer comprising keratin layers with cavities of jay feathers are known[10, 20, 21]. The color of the feathers around the neck of the dove varies with the viewing angle in sunlight (Fig. 1), which was also proven to be an iridescent structural color caused by thin film interference of the cortex[14]. In this study, we investigated the role of feather microstructure and melanin distribution in the appearance of the structural color around the neck of doves, which was previously attributed to thin film interference. When observing the tips and roots side of transverse cross-sectioned dove feathers using OM and TEM, the medullae at the feather tips, where iridescent color was reflected (Fig. 2A), contained many dark particles, whereas the medullae at the root side, where iridescent color was not reflected, exhibited sparsely distributed dark particles (Fig. 2B and C). When observing the three-dimensional arrangement of barbules either reflecting or not reflecting iridescent color, these barbules were horizontally or vertically arranged, respectively (Fig. 2B and D). Thus, the structural color development via thin film interference clearly correlated to the three-dimensional arrangement of the barbules. Observing feathers under transmitted illumination, only the tips exhibited a deep black color (Fig.3A). The reason is that the transmitted illumination light is blocked by horizontally arranged barbules of the tips containing many dark particles. When Raman microspectrometric analyses were performed to identify the dark pigment in the tips of the dove feathers referring to the eumelanin-specific two bands[16] (Fig. 3B), the pigment was confirmed to be eumelanin. Further, it was confirmed

using spectrophotometry that eumelanin was most abundant in the tips of the feather reflecting iridescent color (Fig. 3C). Eumelanin particles within the barbules could facilitate the reflection of structural color with a specific wavelength reducing incoherent scattered light. Recent biometric research has revealed that the dark background pigment is important for amorphous nanolevel structural color development, such as within jay feathers[22]. It was proposed that the distribution of eumelanin particles is also important for structural color development of dove feathers via thin film interference.

Dove feathers reflected green light when the IA of illumination light was small and purple when it was large (Fig. 4). To simulate the reflected wavelengths, the thickness of the cortex estimated from TEM and the refractive index of β-keratin and melanin reported in the previous studies[18, 19] were substituted into the Fresnel formula[17] (Fig. 5). These simulation peaks with changing of IA were very similar to the spectra actually reflected from the dove feathers. When the reflection spectrum simulation is divided into p-polarized light and s-polarized light, s-polarized light is stronger than p-polarized light in the case of purple reflection with a large incident angle. It was thought that the direction of illumination was important, for s-polarized light was easily reflected from the surface of semi cylindrical barbules when the light with a large incident angle was irradiated from the side of the feathers.

In conclusion, to reflect iridescent structural color from rock dove feathers by thin film interference, the barbules must be arranged horizontally to achieve proper incident angle for the green or purple color reflection and the thickness of the cortex must be uniform. In addition, it was inferred that eumelanin particles with a suitable refractive index as a substrate of β-keratin are effective only to reflect the light with a specific wavelength reducing scattered light.

Conflicts of interest

The author has no competing interests to declare.

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