〈Original Article〉

Distribution of protoporphyrin IX in bird feathers

Toshio Okazaki* and Shinichiro Imamura

Summary When oriental turtle dove plumage was irradiated with ultraviolet A light, all of the feather rachises emitted salmon pink fluorescence. Spectral analysis of salmon pink fluorescence from the rachises and thin-layer chromatography (TLC) and absorption spectrum analysis of samples extracted from feathers were performed to confirm that the rachis pigments were protoporphyrin IX (PpIX) derivatives. This study represents the first demonstration of PpIX in dove plumage. At the same time, salmon pink florescence was also detected in the rachises of the rock dove and brown-eared bulbul as well as the white basal parts of the dorsal feathers of the Japanese tit. As it is known that PpIX has antioxidative activity, it may inhibit the intramolecular radical cyclization of dihydroxyphenylalanine (DOPA)-chrome and facilitate the supply of melanin precursors such as DOPA-quinone to the barbs and barbules. It was believed that PpIX in rachises inhibited melanin deposition.

Key words: bird, feather, UVA, porphyrin, fluorescence

Introduction

The color of bird plumage is generally vivid, beautiful, and diverse compared with that of the coats of mammals. The plumage color indicates differences in bird class and sex, and it influences selection for mating[1,2], antibacterial activity[3], and other characteristics. Although the most common pigment in bird feathers is melanin, other pigments are present in the plumage of several species, such as carotenoids in flamingos[4], psittacofulvin in parakeets[5], spheniscine in penguins[6], and porphyrin (Pp) in owls and bustards[7,8]. Plumage color is determined by the pigment color, and the pigments are mainly distributed on the front side of the plumage, making them easily recognized from the outside by partners. However, it is known that part of Pp is distributed in the backside feathers, and it emits red fluorescence under ultraviolet A (UVA, 320-400 nm) irradiation[7,8]. Recently, we noticed that the rachises of oriental turtle dove plumage emitted salmon pink fluorescence under UVA irradiation similarly as those of owls and bustards. In this study, the pigment emitting salmon pink fluorescence was analyzed using reflection and absorption spectrophotometry,

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Received for publication: July 9, 2019
Accepted for publication: Aug 8, 2019

Raman microspectroscopy, and thin-layer chromatography (TLC). At the same time, the emission of salmon pink fluorescence was confirmed in the plumage of the oriental turtle dove as well as the rock dove, bulbul, and Japanese tit. Our findings that Pp is distributed in the back feathers of various birds may be extremely important for understanding its functions in feather development.

Materials and methods

1) Materials

Oriental turtle dove (*Streptopelia orientalis*), rock dove (*Columba livia domestica*), brown-eared bulbul (*Hypsipetes amaurotis*), Japanese tit (*Parus minor*), white-cheeked starling (*Sturnus cineraceus*), Daurian redstart (*Phoenicurus auroreus*), black-faced bunting (*Emberiza spodocephala*), and budgerigar (*Melopsittacus undulatus*) carcasses were found in parks around Yamazaki University, and their feathers were collected.

2) Observation of plumage under the UVA illumination

The backside plumage of bird wings was irradiated with UVA light (365 nm) from a UVGL-58 handheld UV lamp (Funakoshi, Tokyo, Japan), and red fluorescence was observed.

3) Preparation of thin transverse cross-sections of oriental turtle dove feathers

Oriental turtle dove feathers were buried in paraffin and sectioned using a Jung type microtome to a thickness of 7.5 μm. The sectioned samples were placed in water on slide glass, heated at 55°C, and allowed to dry. The thin transverse cross-section was observed using an optical microscope under UVA illumination.

4) Fluorescence emission spectrum analysis of oriental turtle dove feathers

Fluorescence emission spectrum analysis of oriental turtle dove plumage was performed using a C11009MA mini-spectrometer (Hamamatsu Photonics, Hamamatsu, Japan) under UVA light (365 nm) from a UVGL-58 handheld UV lamp. Protoporphyrin IX (PpIX)/2Na salt (Sigma-Aldrich, Tokyo, Japan) and coproporphyrin III (CpIII) tetramethyl ester (Sigma-Aldrich) dissolved in hydrochloric acid/methanol (3:4) were simultaneously dropped onto a silica TLC plate (Merck KGaA, Darmstadt, Germany) and subjected to fluorescence spectrum analysis. A silica TLC plate was used as a reference material, and the A/D count ratio of the spectrum emitted from the rachis of oriental turtle dove feathers was calculated as the strength ratio of the light emitted from a silica TLC plate.

5) Raman microspectroscopy of oriental turtle dove feathers

Raman microspectrometry of dove feathers and chicken brown eggshells was performed using a Renishaw inVia™ Raman microscope with the 532-nm emission line of an Ar laser. A dry feather was deposited on a glass slide, and the Raman shift was measured.

6) TLC and spectrum analysis of pigments emitting salmon pink fluorescence following UVA irradiation

A mixture of hydrochloric acid and methanol (3:4) was added to the feathers, which were finely pulverized in a mortar. Chloroform was added to the mixture, and after sufficient stirring, centrifugation was performed at 3000 rpm for 5 min. The chloroform/methanol layer was collected and the absorption spectrum was measured between 300 and 800 nm using a UVmini-1240 ultraviolet–visible spectrophotometer. At the same time, TLC was performed on these extracts. The aforementioned TLC plate was used and developed with chloroform/methanol/water (15: 10: 2), and the Rf value was determined under irradiation with UVA at 365 nm. As a control, chloroform/methanol solution of PpIX disodium salt (TIC, Tokyo, Japan), CpIII tetramethyl ester, and biliverdin (Wako, Tokyo, Japan) were used, and the results were compared with sample data.

Results

1) Observation of fluorescence emission and spectrum analysis of the rachises of oriental turtle dove plumage

When UVA was applied to the back of the open wings of the oriental turtle dove, strong salmon pink fluorescence was emitted from the rachises of plumage, which were almost completely covered with barbs and barbules (Fig. 1A). When UVA was applied to feathers taken from each part of the dove plumage, it was confirmed that the rachises of all feathers emitted salmon pink fluorescence. As shown in the left corner of Fig. 1B, many air domes were observed in the rachis medulla, and all rachis cross-sections emitted red fluorescence under UVA irradiation.

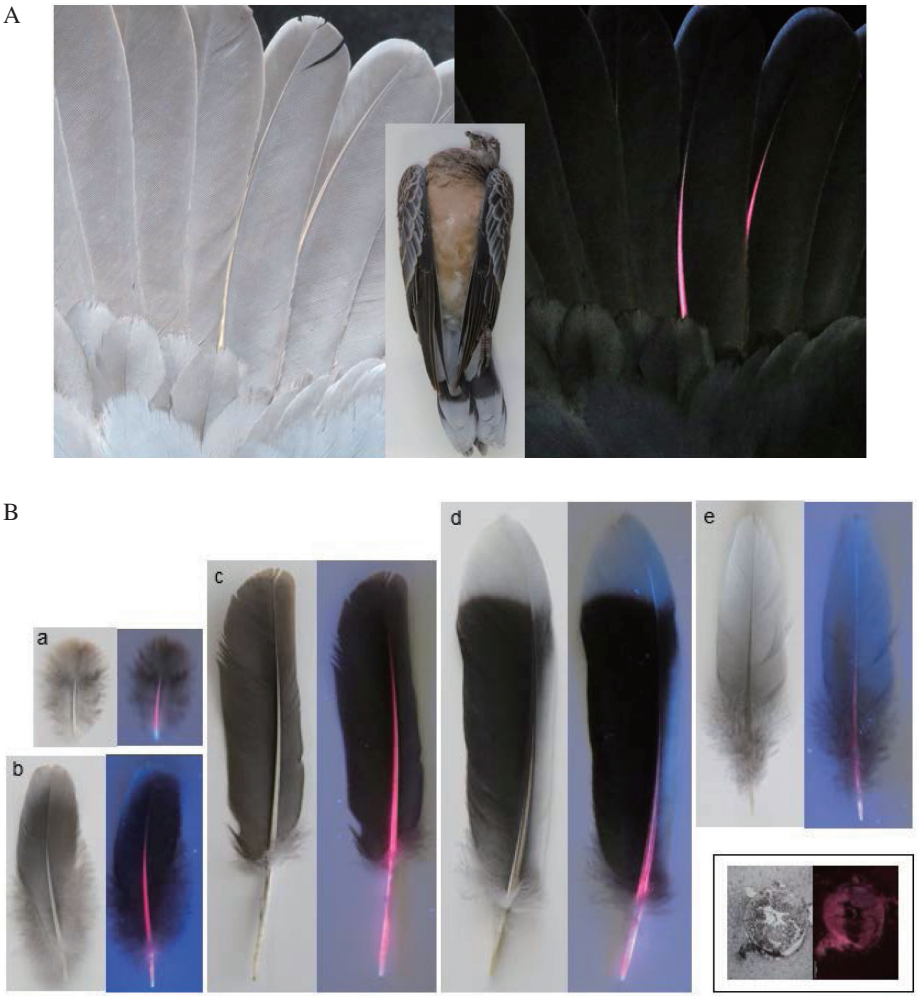

Fig. 1 Observation of salmon pink fluorescence emitted from dove feathers under ultraviolet A (UVA) irradiation

(A) Observation of salmon pink fluorescence emitted from the open wing of an oriental turtle dove under UVA irradiation. Left: under white light; right: under UVA irradiation. (B) Observation of salmon pink fluorescence emitted from oriental turtle dove feathers. Left: under white light; right: under UVA light. The feathers (a–e) were obtained from various parts of the dove body. a: chest; b and c: wing; d and e: tail. Optical microscopic images of transverse cross-sections of oriental turtle dove feather rachises were inserted in the right side corner. Left: transmitted illumination; right: oblique UVA irradiation.

When oriental turtle dove feathers were irradiated under UVA light, the rachises of all feathers, which were white under natural light, emitted strong salmon pink fluorescence. When commercial products of PpIX and CpIII, which were the pigments confirmed within the owl feathers emitting the salmon pink fluorescence under UVA irradiation, were dissolved in chloroform/methanol and added drop wise to a silica plate, the same salmon pink fluorescence was emitted from their spots. The fluorescence spectrum emitted from the rachises of UVA-irradiated dove feathers was compared with those of PpIX and CpIII spots dropped on the TLC plate. As a result, a peak was observed at approximately 670 nm for both the rachis extract and PpIX solution (Fig. 2A).

Raman microspectroscopy was performed on the rachises, and the results were compared with the characteristic Raman peaks of PpIX in brown chicken egg shells[9,10]. The two primary bands from the rachises at 1671 and 1251 cm^{-1} corresponded to amide I and amide III of the keratin protein, respectively[11,12], and the small bands at 1350 and 1580 cm^{-1} were consistent with C=C and lactam stretching of PpIX, respectively[13] (Fig. 2B).

Next, the rachis extract was developed via TLC, and the Rf value obtained under UVA irradiation was compared with data of the PpIX specimen dissolved in chloroform/methanol. In the PpIX specimen, two major salmon pink spots with Rf values of 0.19 and 0.31, respectively, and two minor salmon pink spots with Rf values of 0.22 and 0.36, respectively, were observed. In the rachis extract, two salmon pink spots were also observed, and their Rf values were consistent with the values of the two minor spots of the PpIX specimen (Fig. 3A).

When the absorption spectrum of the chloroform/methanol extract of rachises was analyzed, a peak at 426 nm corresponding to the Soret band of the PpIX specimen and a second peak at 570 nm corresponding to the Q band[14] were detected. Although the characteristic peaks of PpIX were not

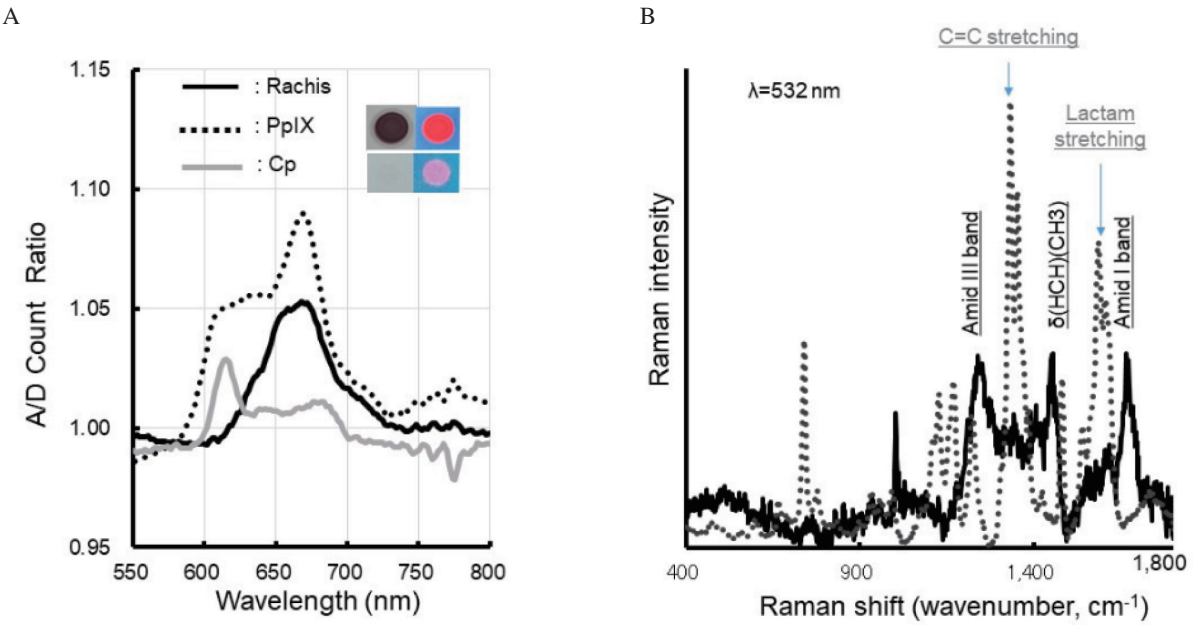

Fig. 2 Fluorescence spectral and Raman spectroscopy analysis of dove feathers
(A) Spectral analysis of fluorescence emitted from the rachises of oriental turtle dove feathers, protoporphyrin IX (PpIX), and coproporphyrin III (CpIII) under UVA irradiation. The inserted images are spots of PpIX and CpIII. Left: under natural light irradiation; right: under UVA irradiation. (B) Raman spectrograms of dove feather rachis and brown chicken egg shell, which included an adequate amount of PpIX[9,10]. Solid line: dove feather rachis; dotted line: brown chicken egg shell. Blue arrows denote the characteristic peaks of PpIX, C=C stretching, and lactam stretching[13].

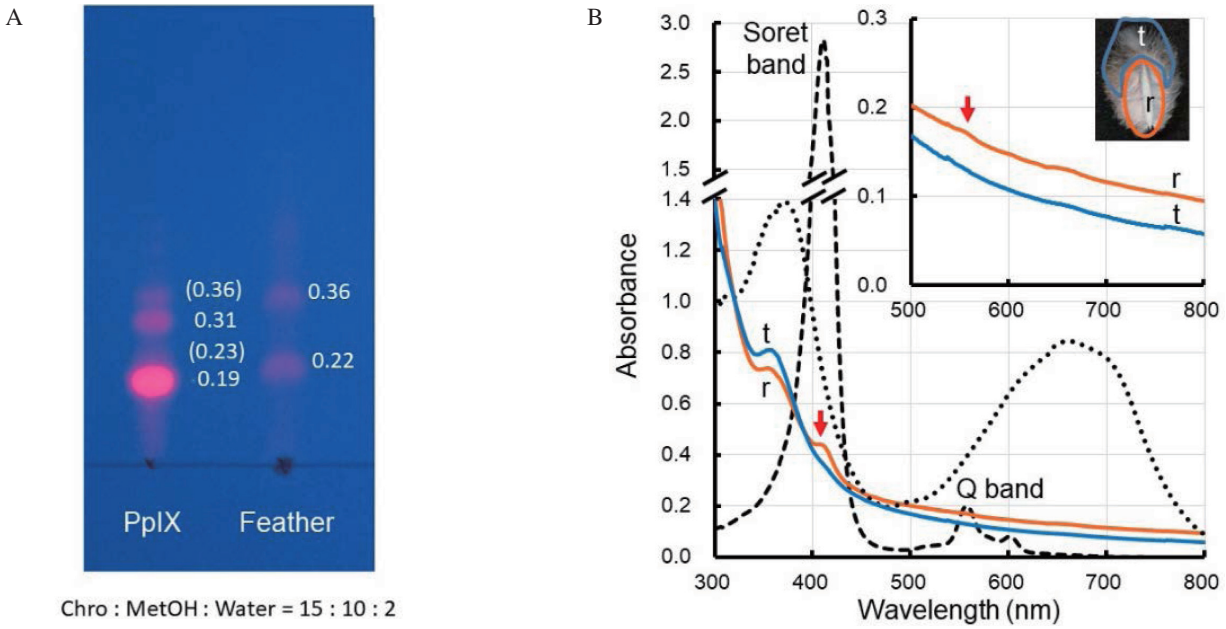

Fig. 3 Identification of dove feather pigments

(A) Thin-layer chromatography of the rachis extract. The spots detected in response to UVA irradiation were compared with those of PpIX. The inserted number indicates the Rf value. Minor peak Rf values are enclosed in parentheses. (B) Absorbance spectrum analysis of dove feathers, which were separated into the tip and rachis. t: feather tip; r: feather rachis; broken line: PpIX specimen; dotted line: biliverdin specimen. Red arrows indicate the peaks of Soret and Q bands of feather extracts.

observed, a clearer peak at 370 nm was observed in the feather tip extract than in rachises. In the rachis and feather tip extracts, a clearer peak at 370 nm, which is consistent with that of biliverdin, was confirmed (Fig. 3B).

2) Observation of salmon pink fluorescence emission from the plumage of birds other than oriental turtle dove under UVA irradiation

Following UVA irradiation of the plumage of various bird species, salmon pink fluorescence was emitted from the rachises of rock doves and brown-eared bulbuls and the white basal parts of the dorsal feathers of Japanese tits (Fig. 4). However, the rachises and back feathers of white-cheeked starlings, Daurian redstarts, black-faced buntings, and budgerigars did not emit salmon pink fluorescence (data not shown).

Discussion

Parts of the white back feathers of owls and white basal parts of the dorsal feathers of bustards emit salmon pink fluorescence under UVA irradiation, and the causative pigments were revealed to be PpIX and CpIII[8]. In this study, we observed similar fluorescence in the rachises of oriental turtle dove, rock dove, and bulbul feathers and the white basal parts of the dorsal feathers of the Japanese tit under UVA irradiation. The TLC data clarified that the pigments in oriental turtle dove feathers were PpIX derivatives. Pp is a square planar macrocycle composed of four pyrroles connected by methane bridges to form aromatic rings, and this structure makes it prone to photodestruction when exposed to visible light. In fact, the salmon pink fluorescence emitted from bustard feathers is attenuated with aging, and the salmon pink color of feathers was bleached by exposure to visible light[8]. The juvenile plumage of black-shouldered kites contains Pp[15], and the area of owl back feathers emitting salmon pink fluorescence under UVA irradiation became narrower with growth[16]. From these observations, Galván et al.[8] hypothesized that the color of Pp

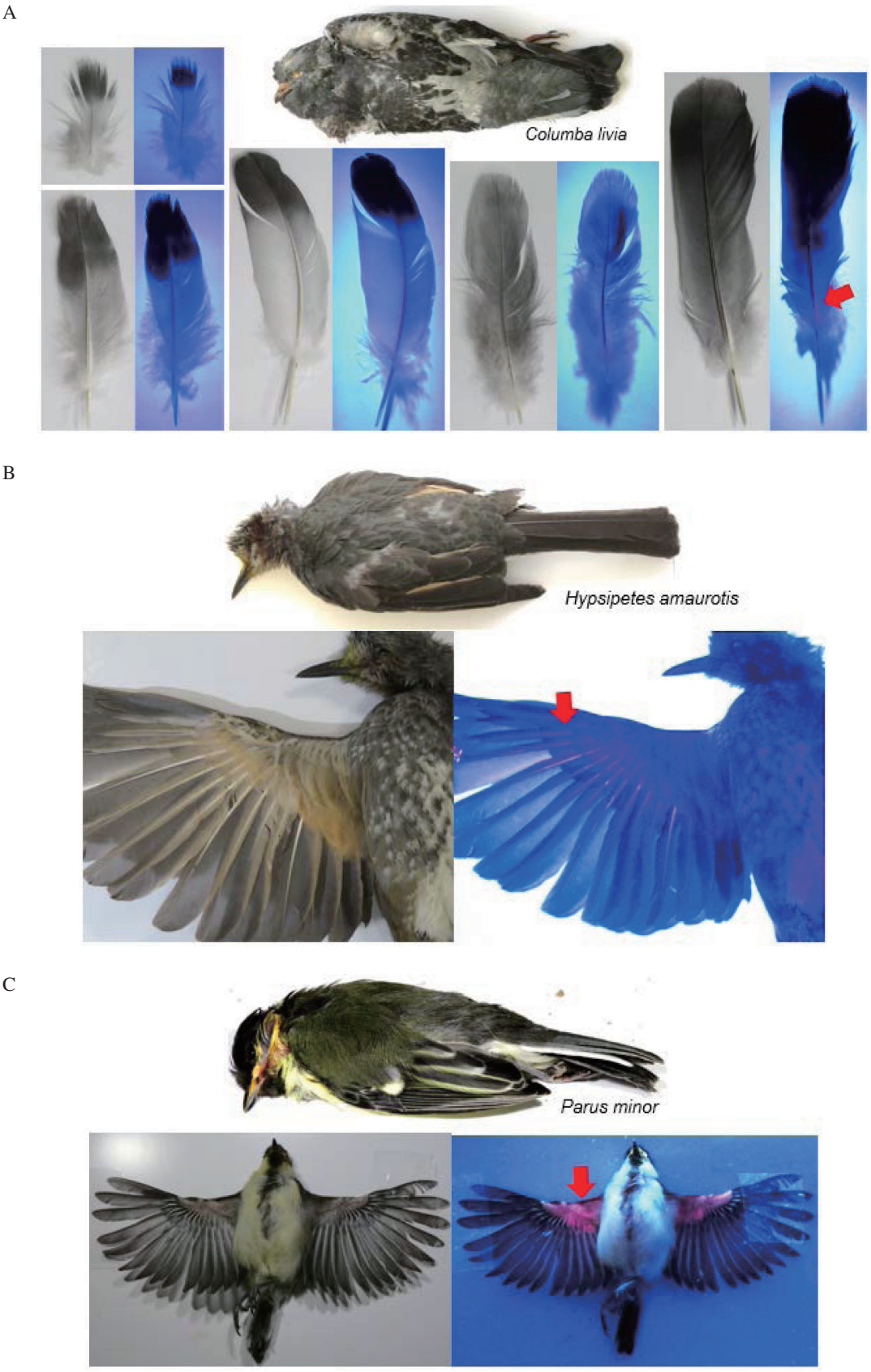

Fig. 4 Observation of salmon pink fluorescence emitted from the plumage of birds other than oriental turtle doves under ultraviolet A (UVA) irradiation

(A) Rock dove (*Columba livia domestica*). (B) Bulbul (*Hypsipetes amaurotis*). (C) Chicken great tit (*Parus minor*). Left: under natural light; right: under UVA irradiation. Red arrows denote the locations of salmon pink fluorescence.

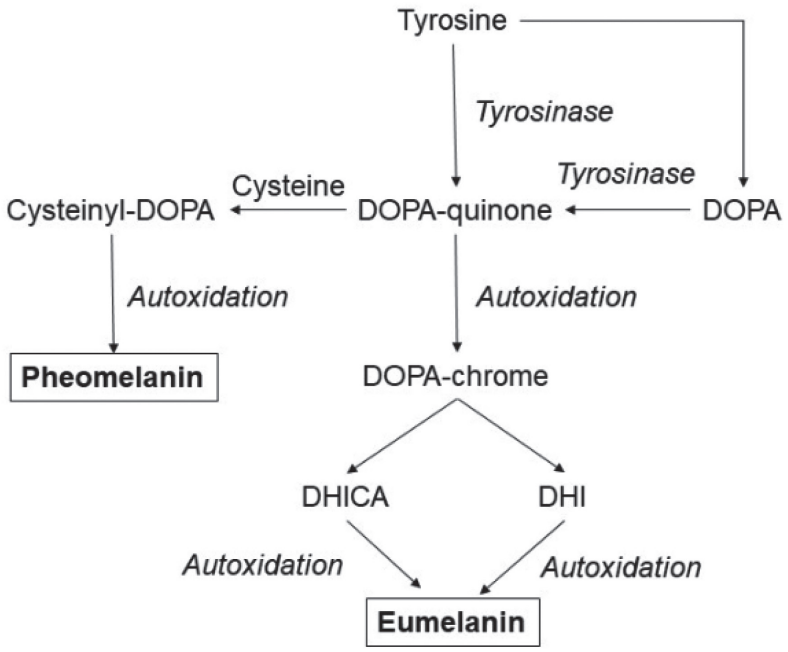

Fig. 5 Melanogenesis pathway
This figure is a simplified representation with reference to the Galván and Solano's paper[18]. DOPA: dihydroxyphenylalanine; DHICA: 5,6-dihydroxyindole-2-carboxylic acid; DHI: 5,6-dihydroxyindole.

served as a signal of virginity and influenced mating. However, the color of the rachises of the oriental turtle dove feathers observed in this study can hardly be seen from outside because the rachises are hidden by barbs and barbules even when the wings are opened. It was believed that PpIX distributed in the rachises might play another important role. Namely, PpIX might inhibit melanization because melanin was not deposited on the rachises of bird feathers or the dorsal feathers. Melanin is derived from tyrosine and more directly from dihydroxyphenylalanine (DOPA) (Fig. 5). After the formation of DOPA from tyrosine, the further conversion from DOPA to DOPA-quinone follows. DOPA-quinone undergoes intramolecular cyclization and oxidation to form DOPA-chrome, which is then converted to 5,6-dihydroxyindole (DHI) or 5,6-dihydroxyindole-2-carboxylic acid (DHICA). Eumelanin is formed by polymerization of DHI and DHICA and their quinones[17,18]. It is known that PpIX exerts antioxidative effects on lipid peroxidation[19,20]. It was assumed that PpIX inhibited the intramolecular radical cyclization of DOPA-quinone to DOPA-chrome via the antioxidant effect of PpIX in the rachises, facilitating the supply of melanin precursors such as DOPA-quinone to the barbs and barbules at the end of the rachises during feather development. As PpIX is metabolized to biliverdin in the tip of barbs and barbules in turtle neck dove feathers, melanization easily proceeds.

In this study, PpIX distribution was confirmed in some bird feathers, and the salmon pink fluorescence level and distribution of PpIX differed among different bird species. The PpIX level may be affected by the rates of PpIX metabolism and photo degradation, and the change in PpIX metabolism in the feathers with the maturation of each bird is unknown.

Acknowledgments

We would like to thank Enago (www.enago.jp) for the English language editing.

Competing interests

The authors have no conflicts of interest.

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