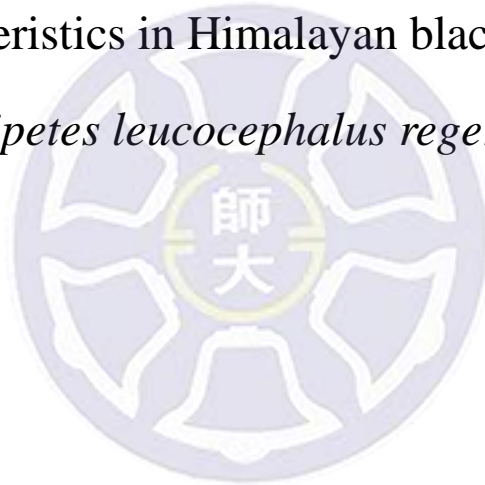


國立台灣師範大學生命科學系 博士論文

類胡蘿蔔素呈色及黑色素呈色的特徵

在紅嘴黑鶉的研究

Studies of carotenoid-based and melanin-based
characteristics in Himalayan black bulbul
(*Hypsipetes leucocephalus regerrimus*)



研究生：洪心怡

Hsin-Yi Hung

指導教授：李壽先 博士

Shou-Hsien Li

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Abstract

Integumentary colorations are essential signals in avian communication. Among all the various color types, carotenoid-based (expressed in yellow, orange or red) and melanin-based (expressed in black, grey or brown) colors are the most common and important traits in birds. Studies have shown that these traits are informative and serve vital functions, such as cues for choosing mates or assessing opponents. Traditionally, in avian communication, carotenoid-based colors are thought to be sexually selected traits and melanin-based colors signal social status. However, the results of various studies indicate these traits serve several functions in different avian species. It suggests that the evolution of both traits is more complex than we used to think. Nevertheless, most relevant studies have focused on the role in sexual dichromatic species, which might be suggested to be under higher pressure of sexual selection. There is a gap of how these traits evolved and what their functions are in less sexually dichromatic species, which might be under different selection regimes from sexually dichromatic ones. In order to understand the imperatives of coloration in avian communication completely, studies to characterize variations and functionality of both traits on less sexually dichromatic species are needed. I chose to study Himalayan black bulbuls (*Hypsipetes leucocephalus nigerrimus*), which are sexually monomorphic to human vision and contain a carotenoid-based bill and tarsi and melanin-based plumage to analyze the possible roles of both types of traits. My results showed that both carotenoid- and several melanin-based parts were sexually dichromatic in avian vision. Furthermore, I found that the

expression of a carotenoid-based bill and melanin-based breast and scapular colorations were correlated with individuals' physical conditions, including immunocompetence and oxidative stress levels. The results of the female-preference test on carotenoid-based traits showed that the red bared parts alone may not be the cues for mate choice for female black bulbuls. My dissertation provides clear evidence that both carotenoid- and melanin-based traits should be informative cues reflecting bearers' physical condition; it suggests that these traits may play a role in the signaling of Himalayan black bulbuls, but the functions of the traits need to be further investigated.

Keywords: carotenoid-based trait, Himalayan black bulbuls (Hypsipetes leucocephalus nigerrimus), melanin-based trait, quality cue, sexual selection



中文摘要

鳥類體表顏色多樣性極大，這些顏色大部分具有功能性，對於鳥類溝通非常重要。在各種體表顏色中，由類胡蘿蔔素呈色（呈現黃、橘或紅色）以及黑色素呈色（黑、灰或棕色）的特徵是最普遍也最重要的特徵。這類特徵通常能傳遞個體的訊息，並且具有重要的功能，例如個體可據此選擇配偶或評估對手。傳統上，類胡蘿蔔素呈色的特徵，被普遍認為是個體選擇配偶的依據，而黑色素呈色的特徵，則被認為與社會互動有關。但是這種觀念已被質疑，因為隨著越來越多的鳥種被研究，發現這兩種特徵在不同種類，可能功能不同，顯示這兩種特徵在鳥類的演化機制，可能比目前所知的更為複雜。除此之外，大部分的相關研究都在探討這兩類特徵在性擇上扮演的角色，所以多是以兩性有明顯型態差異的物種為研究標的（這類物種通常可能受到較高的性擇壓力），較少以在兩性型態差異較小的物種為主題，而這些物種可能受到與兩性型態差異大的物種不同的選汰壓力。為了能更完整地了解顏色在鳥類溝通上的演化機制，需要研究這兩類特徵在兩性型態差異較小的物種上扮演的角色。本研究選擇紅嘴黑鵯 (*Hypsipetes leucocephalus nigerrimus*) 為研究物種，他們具有類胡蘿蔔素呈色的喙部、跗蹠與黑色素呈色的羽毛，對人眼來說是雌雄單型性的鳥種。我的結果顯示，在鳥類視覺上，這兩類特徵在兩性間是有差異的。而類胡蘿

蔔素呈色的喙部與黑色素呈色的胸部與肩部羽毛的顏色表現，也與個體的生理狀況（包括個體的免疫能力與受到的氧化壓力）有關。雌性偏好試驗的結果顯示，單獨類胡蘿蔔素呈色的部位，可能不是雌性黑鵝選擇配偶的依據。我的論文提出清楚的證據，顯示類胡蘿蔔素呈色以及黑色素呈色的特徵，都能顯示紅嘴黑鵝個體的生理狀況，因此對紅嘴黑鵝而言，這些特徵可能具有重要的功能。但是要知道這些特徵在這個物種真正的功能，還需要之後更詳細的研究。

關鍵字：紅嘴黑鵝、類胡蘿蔔素、黑色素、性擇、鳥類通訊



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Chapter One

General introduction

Birds show highly diverse colorations, and even the color variations among species within the same genus can be drastic. How such colorations evolved has always been intriguing to evolutionists. Although some evidence suggests that traits such as white color or plumage patterns (spots, stripes and bars) are selectively neutral and the consequence of developmental constraints (Price and Pavelka 1996, Tickell 2003), most color characteristics in avian species are believed to be adaptive (Hill and McGraw 2006) and serve imperative functions, such as communication signaling. This could include facilitating individual, kin or species recognition (Mayr 1972); enhancing or survivorship through concealing or deceiving to predators (Bortolotti 2006); or promoting appropriate mate choice or social interactions (Bortolotti 2006, Hill 2006). Sometimes, coloration may serve multiple purposes in one single species (Hill and McGraw 2006).

Communication theory

Most research on bird coloration has focused on color as potential signals of quality – cues that communicate information about aspects of the bearer's relative phenotypic and genetic constitution, or other abilities (Dale 2006). According to Stevens' (2013) definition, *signals* are any act or structure that influences the behavior of other organisms, also known

as *receivers*, and which evolved specifically because of that effect. He defines *cue* as an incidental source of information that may influence the behavior of a receiver despite not having evolved under selection for that purpose. Communication theory suggests that *signalers* would use coloration providing information to receivers (Stevens 2013).

Qualities revealed from informative coloration

The broad aspects of “quality” reflected in the color of the bearer include genetic qualities and an individual’s condition. Genetic qualities revealed from the coloration include whether the individuals have good genes and/or better genetic compatibility (Neff and Pitcher 2005). An individual’s condition is revealed from its coloration including physical conditions; social status; and ability to forage, care for young and defend territory (Dale 2006). The information obtained from signalers could be used by receivers to assess the qualities and make choices about potential mates and/ or opponents. In my study, I focused on whether the coloration traits may reflect an individual’s physical condition, namely immunocompetence and oxidative stress levels.

Among all kinds of colorations, carotenoid- and melanin-based traits are the most widely studied and discussed (Hill and McGraw 2006), particularly in birds.

Evolution of carotenoid-based ornaments

Carotenoids, mainly lutein and zeaxanthin, are responsible for red, yellow or orange coloration in vertebrates. Carotenoids also function as natural antioxidants that remove harmful free radicals, which are produced

through normal cellular metabolism or induced by environmental stressors (Chew 1996). Through antioxidative activities, carotenoids can increase the number of lymphocytes or the ability of anti-pathogens and hence, can directly or indirectly regulate multiple somatic processes, such as the functioning of the immune system and molting (Chew and Park 2004, McGraw 2006a). However, an individual's use of carotenoids in the diet is likely limited. Carotenoids cannot be synthesized *de novo* by animals themselves but rather must be ingested from their diet (Brush 1990) and the ability to utilize carotenoids in food is constrained by genotypes and physiological conditions (Alonso-Alvarez et al. 2004, Hill 1991, Latscha 1990, Olson and Owens 1998). The process of different somatic demands competing for limited carotenoids could result in trade-offs of carotenoids allocation among different demands, possibly making the expression of carotenoid-based ornaments correlate with an individual's physical condition. In avian species, males with carotenoid-rich traits usually have higher immunocompetence (e.g., Blount and Matheson 2006, Chew and Park 2004, Faivre et al. 2003), possess higher quality territories (e.g., Bostrom and Ritchison 2006, Casagrande et al. 2006, Reudink et al. 2009), provide better parental care (e.g., Pike et al. 2007, Senar et al. 2002), or have better mobility (e.g., Blount and Matheson 2006) than those with carotenoid-poor traits. Based on the *indicator hypothesis*, which suggests that females would use honestly informative characteristics to choose mates (Andersson and Simmons 2006), carotenoid-based ornaments are suggested as sexually selected (mate-choice) signals in avian species. Studies have shown that

males with carotenoid-richer ornaments are preferred by females (e.g. Hill 1991) and that carotenoid-based plumage is positively correlated with the intensity of sexual selection (Badyaev and Hill 2000, Gray 1996).

However, the concept of carotenoid-based ornaments having mainly evolved through sexual selection has been overemphasized. In some avian species, carotenoid-based ornaments have been found to be functional only as quality cues reflecting an individual's physical condition or parental ability but not as the sexually selected traits (e.g. Pryke et al. 2001), or the main cue of individual identification (Dale 2000). The idea that carotenoid-based ornaments are costly to produce has also been challenged. Some studies suggest that carotenoids are rich in the environment and if over-indigested, may even be toxic to animals (Olson and Owens 1998). Data also show that the variabilities of carotenoid-based ornaments may be under the control of genes (Walsh et al. 2012). Therefore, more studies are required to test how carotenoid-based traits serve as a cue for mate choice.

Evolution of melanin-based traits

Melanins include eumelanin and pheomelanin and cause black, brown, gray, chestnut, or buff coloration in vertebrates (McGraw 2006b). Besides producing pigmentation, the process of melanogenesis reduces the production of free radicals; therefore, melanins are also powerful antioxidants and could affect multiple somatic functions in a way similar to carotenoids (McGraw 2006b). Studies on avian species show that individuals with darker coloration or larger patch sizes of melanin-based

traits are usually more aggressive (e.g. Da Silva et al. 2013, Mennill et al. 2003), possess larger territories (Jawor and Breitwisch 2003), and have better immunocompetence (e.g., Jacquin et al. 2011) than species with lighter coloration or smaller patch size. Unlike carotenoids, melanins can be synthesized within the body from amino acids, which are generally not considered to be limited (Fox 1976, Jawor and Breitwisch 2003). The expression of melanin-based traits is thought to be under the strong control of genes (McGraw 2006b). Hence, melanin-based traits are less costly to be used as signals than carotenoids (Badyaev and Hill 2000). It is conventionally believed that melanin-based ornaments are less likely to be used by females in mate choice and are more likely to be involved in male–male competition (Jawor and Breitwisch 2003, Senar 1999).

However, more studies show that melanin-based ornaments could also serve as sexually selected traits in avian species such as Eurasian penduline tits (*Remiz pendulinus*, Kingma et al. 2008) and yellowthroats (*Geothlypis trichas*, Tarof et al. 2005). In addition, the debate about whether or not the level of melanism is correlated with an individual's condition has been raised; empirical studies on the alpine swifts (*Apus melba*, Bize et al. 2006), house sparrows (*Passer domesticus*) and brown-headed cowbirds (*Molothrus ater*, McGraw et al. 2002) indicate that environmental nutrition and body condition do not significantly influence the expression of melanin-based traits; but studies of barn owls (*Tyto alba*, Roulin et al. 2008) and Eurasian kestrels (*Falco tinnunculus*, Fargallo et al. 2007) showed that melanin-based ornaments were affected by the abundance of nutrition in the habitat. Such controversies indicate the necessity to test if melanin-based ornaments could reflect an

individual's physical quality in a diverse range of species, so that the evolutionary mechanism of such traits can be understood better.

Himalayan black bulbuls

Although there are ample studies about the functions of carotenoid- or melanin-based traits, most of them were conducted in sexually dichromatic species. The studies in less sexually dichromatic species, which may be under less sexual selection compared to sexually dichromatic ones, are relatively rare. In order to understand how impetorative both traits are in avian communication completely, the gap of studying sexually monomorphic species are needed. In this dissertation, I examined the roles of both carotenoid- and melanin-based traits in a sexually monomorphic avian species, Himalayan black bulbuls (*Hypsipetes leucocephalus nigerrimus*). Himalayan black bulbuls are widely distributed in Taiwan's broad-leaf forests at elevations ranging from 100 m to 1500 m. The black bulbul provides both types of pigment-based traits to be measured; it is covered by an entirely black plumage (melanin-based) and its bill and tarsi are both in red (carotenoid-based).

Questions in my dissertation

In my dissertation, I explored four questions:

- (1) Are the carotenoid- and melanin-based characteristics potential cues for sexual selection in black bulbuls? I tested this by examining an indicator of the level of sexual selection, i.e., the degree of sexual dichromatism, from the avian vision (**Chapter Two**)

- (2) Are the carotenoid-based characteristics quality cues that could reflect an individual's physical condition (including immunocompetence and level of oxidative stress) under the stress of molting? Through this experiment, I examined the effects of molting, which is a poorly studied life-history trait, on the allocation of carotenoids between an individual's physical condition and the expression of ornamented traits simultaneously (**Chapter Three**)
- (3) Are the melanin-based plumages quality cues in black bulbuls? If so, a correlation between the coloration of melanin-based plumage and an individual's physical condition (oxidative stress) should be observed (**Chapter Four**)
- (4) Can female black bulbuls use carotenoid-based ornaments (the red bill and tarsi) as sexually selected cues? If so, females black bulbuls would show preferences for redder (carotenoid-richer) males (**Chapter Five**).

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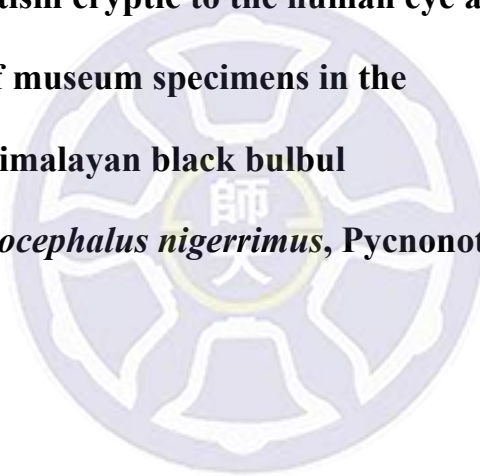
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Chapter Two

**Sexual dichromatism cryptic to the human eye and the
quality of museum specimens in the
Himalayan black bulbul
(*Hypsipetes leucocephalus nigerrimus*, Pycnonotidae)**



Abstract

Sexual dichromatism is an important proxy for the intensity of sexual selection, yet related studies in birds based on museum specimens or conspicuous visual traits in live animals may have led to an underestimation of the intensity and complexity of sexual selection. Using the Himalayan black bulbul (*Hypsipetes leucocephalus nigerrimus*) which is sexually monomorphic to the human eye, I investigated the extent of overall bodily sexual dichromatism. I measured the reflectance both within human visual perceptible range and in the ultra-violet range of two carotenoid-based parts and eight dull, melanin-based parts for each individual live bird or museum skin sampled. I found that males had redder beaks, brighter tarsi and darker plumage than females. These are perceptible to the bird according to a model of color discrimination thresholds, suggesting the existence of multiple cryptic sexually selected traits within the species. I also found significant degradation of the color in skin specimens compared with that in live birds, indicating that sexual dichromatism could be underestimated by using skin specimens alone.

Keywords: carotenoid-based characteristics, cryptic sexual dichromatism, melanin-based plumage, Himalayan black bulbul (Hypsipetes leucocephalus nigerrimus), sexual dichromatism

Introduction

One of the most robust and widely used indexes of the intensity of sexual selection in birds is sexual dichromatism, in which the male is typically brighter or more colorful than the female or has more distinguishing features (e.g., Owens and Hartley 1998, Seddon et al. 2013). Although intersexual differentiation in mating behavior, habitat preference or predator avoidance can also promote the evolution of coloration, sexual dichromatism is considered to be driven mainly by female preference or male-male competition (Andersson 1994) and to function in sexual recognition, individual quality assessment and sexual attraction (Dale 2006). Still, several pitfalls in studies of sexual dichromatism may have led to an overall underestimation of sexual selection in birds. Most significantly, these studies have mainly focused on conspicuous differences (e.g., Bortolotti et al. 1996, Eaton 2005, Gray 1996) in the range 400 to 700nm perceptible to human vision (Cuthill et al. 1999, Neitz and Jacobs 1986). However, birds have a wider visual sensory range (300 to 700nm), and can detect intersexual differences in ultra-violet (UV; 300 to 400nm, Chen et al. 1984). Therefore, UV coloration could be also used as the signal (e.g., Alonso-Alvarez et al. 2004, Siefferman and Hill 2005) or target (Bennett et al. 1997) for mate choice. With the aid of spectrometers, several avian species presumed monochromatic have been found to have dichromatic UV coloration (e.g., Igic et al. 2010, Mays Jr et al. 2004), but more studies are needed to evaluate the prevalence of UV-dichromatism.

Also underrepresented in avian sexual dichromatism studies is the examination of melanin-based coloration, which appears dull to humans

but may still carry signals of individual quality to birds given their superior vision. Melanin-based characteristics have been found to be associated with individual qualities such as social rank, aggressive behavior and immunocompetence and should be no less important targets for sexual selection than carotenoid-based characteristics (reviewed in McGraw 2006b, Kingma et al. 2008, Tarof et al. 2005). Moreover, melanin deposition appears to be controlled by genes and not easily affected by environmental factors such as diet (Fox 1976, Buckley 1989), whereas the expression of carotenoids depends on nutritional status and foraging ability (Hill 1992, Nolan et al. 1998, Thompson et al. 1997). The sexual selection pressures on these traits might differ from those on other types of trait existing in the same organism. However, little attention has been paid to the relative contribution of the two pigment-based colorations within the same species.

The use of museum skin specimens in studies of avian coloration could also lead to the underestimation of sexual dichromatism. The concern that specimens' feather color might fade over time has been noted in several studies: the color degradation might be species dependent and also determined by when the specimen was collected (Doucet and Hill 2009, McNett et al. 2005, Pohland and Mullen 2006). It has been shown that color fading is significant for museum skin specimens collected more than 50 years previously (Pohland and Mullen 2006). But the level of color degradation of newly collected museum specimens has been controversial (Doucet and Hill 2009, McNett et al. 2005, Pohland and Mullen 2006).

In this study, I used a spectrometer to study sexual dichromatism in a

passerine, the Himalayan black bulbul (*H. leucocephalus nigerrimus*), which is sexually monomorphic to the human eye: both sexes are entirely covered by black plumage with a grey patch on their wings (both melanin-based) and a red beak and tarsus (both carotenoid-based (McGraw 2006a). I tested whether intersexual plumage-color differences would be perceptible to the bulbul itself with the Vorobyev-Osorio color discrimination model which is based on the avian tetrahedral color space (Eaton 2005, Stoddard and Prum 2008). I show that sexual dichromatism does exist in the Himalayan black bulbul, providing insights into the potential functional roles of melanin-based and carotenoid-based characteristics in the species. I also found significant fading of museum skin specimens less than five years old. This raises concerns about the use of recently collected skin specimens for study of avian cryptic sexual dichromatism.

Materials and methods

Study Species

The Himalayan black bulbul (*H. leucocephalus nigerrimus*) is a widely distributed species inhabiting broadleaf evergreen and mixed deciduous forests, groves, clearings and edges. A total of 112 live individuals were bought from the pet-shop (San Xing Bird Shop, Taipei, 25.034398,121.504444) during the non-breeding seasons of 2008, 2009 and 2011. The birds were all captured from the southern mountain areas in Taiwan. A blood sample was taken from each bulbul for molecular sex typing before proceeding to color quantification. I also examined 37 specimens from the archives of Taiwan's National Museum of Natural

Science (female: 5, male: 11) and Endemic Species Research Institute (female: 7, male: 14), all of which had been collected within the previous 15 years.

Molecular Sex Typing

Gross DNA was extracted from blood samples with traditional proteinase K digestion followed by LiCl extraction (modified from the procedure of Gemmell and Akiyama 1996). Extracted DNA was resuspended in ddH₂O and stored at -20°C. Less than 100 ng of genomic DNA was added to 12.5 µL of PCR (polymerase chain reaction) mix containing 0.5 mM of each of the dNTPs, 0.3 µM of each PCR primer (2550F / 2718R, Fridolfsson and Ellegren 1999), 10 mM Tris-HCl, 50 mM KCl, 1.5 mM of MgCl₂ and 0.4 U of Taq DNA polymerase (Amersham Biosciences). The PCR profile was 94°C for 3 min, followed by 40 cycles of 95°C for 20 s, 46°C for 30 s and 72°C for 40 s, finished at 72°C for 2 min. The PCR reactions were carried out in iCyclers (Bio-Rad, Hercules, CA, USA). After PCR reactions, I conducted electrophoresis with 1.2% agarose gel to determine the sex. In total, 55 male and 57 female live bulbuls were identified.

Color Measurement

For each individual, the reflectance of ten body regions including two carotenoid-based parts- the beak and tarsus- and eight melanin-based parts- the forehead, nape, back, breast, belly, tail, remige and scapular feathers- were measured by an USB2000 spectrometer (Ocean Optics) with a HL2000 deuterium-halogen light source (Ocean Optics). A

R600-7-UV/125F probe (Ocean Optics) was held perpendicular to the surface of the feathers with a cylindrical cap at the end to standardize measuring distance (5 mm) and to shield ambient light. To calculate relative reflectance, a white standard (Labsphere) was used. To collect the dark reference, the light source was capped by a black plastic plate. Each part was measured three times to calculate repeatability (repeatability > 90% (Lessells and Boag 1987). Due to the obvious fading of carotenoid-based coloration, I did not score the coloration of beak and tarsus in skin specimens. I measured the coloration after checking that there was no obvious stain or abrasion on the surface in order to reduce errors of diminished light reflectance.

Color Quantification

I used a combination of colorimetric variables to quantify coloration. These included hue, total brightness and chroma (Montgomerie 2006). Hue was calculated for beak and tarsus by finding the wavelength of the mean of maximum and minimum reflectance values in the wavelength range 550 to 700nm. Total brightness was calculated for all parts by averaging the reflectance from 300 to 700 nm. Two kinds of chroma were calculated. One was chroma_{RED}, calculated as the proportion of reflectance from 550 to 700 nm in the total brightness for beak and tarsus. The other was chroma_{UV}, calculated as the proportion of reflectance from 300 to 400 nm on the total brightness for all parts.

Color Discrimination

To distinguish between the hue, brightness and chroma of the two sexes, I

used two-way ANOVA to compare the male and female average measurements by considering the cofactor of different examining year. I also used two-way ANOVA to test whether the date of sample collection (in years) had any significant effect on these colorimetric variables. I also compared the same colorimetric variables between skin specimens and the live birds to examine the extent of color by using two-way ANOVA. Because hue, brightness and chroma were compared for each of two carotenoid-based and eight melanin-based body parts, I applied a Bonferroni adjustment for multiple comparisons which reduced the p value from 0.05 to 0.025 in red parts and to 0.00625 in black plumage.

I calculated the variability (standard deviation) of brightness within each sex to test whether the divergence of color differences within the females was different from that within the males at different parts. I also used two-way ANOVA to test whether sexually dichromatic parts had higher variability than non-sexually dichromatic parts.

In addition, considering the different spectral sensitivity of the four avian cone types, I mapped the spectra onto Goldsmith's tetrahedral color space system (Goldsmith 1990) that has recently been recommended for analyzing avian coloration (Eaton 2005, Stoddard and Prum 2008, Stoddard and Prum 2011). I converted the spectrum measured into points within a tetrahedron in which the vertices correspond to exclusive stimulation of the ultraviolet (UV)-, blue (B)-, green (G)- and red (R)-sensitive cones in the avian eye. The quantum catch of each receptor is as follows:

$$Q_i = \int \lambda R_i(\lambda) S(\lambda) I(\lambda) d\lambda,$$

where λ denotes wavelength, $R_i(\lambda)$ is the spectral sensitivity of cone cell

type i (i from 1 to 4 represent the four cone cells, UVS or VS, SWS, MWS and LWS respectively), $S(\lambda)$ is the reflectance spectrum of a given feather patch, $I(\lambda)$ is the irradiance spectrum entering the eye and integration is over the entire avian visual range(300-700 nm). The program Tetracolorspace (Stoddard and Prum 2008) was used for spectrum conversion, and I chose the average spectral sensitivity curves of UVS-type retinas (Endler and Mielke JR 2005) as the candidate avian vision in this study. After calculating the Q_i , I calculated discriminability of color for each pair of average males and females in different body patches using the Vorobyev-Osorio color discrimination model (Vorobyev and Osorio 1998, Vorobyev et al. 1998). The model calculates a distance in avian color space (ΔS) defined by the quantum catch of each receptor type (i.e., cone cell) in the avian retina (Eaton 2005). To calculate ΔS , I used the following formula:

$$(\Delta S)^2 = \frac{[(\omega_1\omega_2)^2(\Delta f_4-\Delta f_3)^2 + (\omega_1\omega_3)^2(\Delta f_4-\Delta f_2)^2 + (\omega_1\omega_4)^2(\Delta f_3-\Delta f_2)^2 + (\omega_2\omega_3)^2(\Delta f_4-\Delta f_1)^2 + (\omega_2\omega_4)^2(\Delta f_3-\Delta f_1)^2 + (\omega_3\omega_4)^2(\Delta f_2-\Delta f_1)^2]}{[(\omega_1\omega_2\omega_3)^2 + (\omega_1\omega_2\omega_4)^2 + (\omega_1\omega_3\omega_4)^2 + (\omega_2\omega_3\omega_4)^2]}$$

where ω_i is the constant noise-to-signal ratio (Weber fraction) for receptor type i , which is based here on empirical estimates from the Pekin robin (*Leiothrix lutea*, $\omega_4=0.05$, following the ratio of the numbers of cones (UV: S: M: L= 1:2:2:4). f_i is proportional to the natural logarithm of the respective receptor quantum catches, which are normalized against an adapting background (equation 2 and 3 of (Vorobyev et al. 1998)). Δf_i is the difference between the signals in receptor i between the stimuli (two colors). When ΔS is below a threshold value 1, colors are assumed to be indistinguishable.

Results

The average spectrums of two sexes were similar in shape but different in total reflectance (Fig. 2.1). In the carotenoid-based beak and tarsus, the spectrums show two peaks at wavelengths ranging from 300 to 400 nm and 600 to 700 nm, which are the reflectance ranges of UV light and carotenoid feathers respectively. Conversely, the spectrums for the melanin-based parts were almost flat but with a slight rise in the UV section. Among the colorimetric variables, the hue and the total brightness were different between sexes in two places. The carotenoid-based beaks of males had higher hues than those of females (male 590.25 ± 0.62 nm, female 587.95 ± 0.71 nm; least square mean \pm SE; Fig. 2.2., two-way ANOVA, $p = 0.016$). At the melanin-based belly, males had lower total brightness than females (male $4.30 \pm 0.21\%$, female: $5.16 \pm 0.20\%$, $p=0.004$, Fig.2.3). As for skin specimens, all parts were the same in the two sexes (S 2.1 Table).

Applying the Vorobyev-Osorio color discrimination model, more parts were found to be significantly dichromatic in live male and female birds, including the carotenoid-based beak and tarsus and the melanin-based belly, remige and tail (Table 2.1). In museum skin specimens, the belly and scapular- in addition to breast- were also found to be sexually dichromatic. As such, different subsets of body parts were found to be sexually dichromatic in live birds and museum skin specimens (Table 2.1).

Color comparisons between live birds and skin specimens showed

coloration fading in several parts. Live birds had higher brightness in breast and scapular but lower brightness in the tail (Table 2.2 a, S2.2 Table); they also had higher chroma_{UV} in every part (Table 2.2 b, S2.2 Table b). Although the sampled skin specimens were all collected less than 20 years previously, an effect of specimens' preserved years was found in the scapular: older specimens showed significantly lower brightness than more recent ones (specimens 15-10 years old $4.52 \pm 0.99\%$, specimens 10-5 years old $5.13 \pm 0.53\%$, specimens less than 5 years old $7.21 \pm 0.69\%$; $F = 3.70$, $p = 0.042$, S2.2 Table), although the difference is not statistically significant after a Bonferroni correction to account for multiple comparisons.

Color variabilities of brightness within females were the same with those within males in all parts (Table 2.3). Additionally, the variabilities of brightness of sexually dichromatic parts (carotenoid-based beak, tarsus and melanin-based belly, remige and tail) were larger than those of sexually monochromatic ones (Table 2.3, variances of sexual dichromatic traits 3.67 ± 0.62 , variances of non-sexual dichromatic traits 1.06 ± 0.50 , Two-way ANOVA with cofactor sex, $F = 10.663$, $p = 0.005$).

Discussion

I have shown significant sexual dichromatism in both carotenoid-based and melanin-based body regions of the Himalayan black bulbul, including in reflectance and spectral shape. Males' redder beak, brighter tarsus and darker plumage were significantly different enough for birds to distinguish between them and females, which could provide an insight

into this species' mating behavior. I also show that color degradation could lead to different results on sexually dichromatism in skin specimens and live birds.

The Himalayan black bulbul and most pycnonotids (part of Pycnonotidae), are dull to humans and listed as monomorphic (Fishpool and Tobias 2005), but my results suggest that the extent of their sexual dichromatism could be underestimated; it is significant but not very large (Table 1; ΔS of Black bird (*Turdus merula*): 5.56-9.21; ΔS of Black cap (*Sylvia atricapilla*): 1.48-16.9; ΔS of Greenfinch (*Carduelis chloris*): 2.26-8.10 (Delhey and Peters 2008), which may be indicative of mild sexual selection. Like most pycnonotids, the Himalayan black bulbul is socially monogamous and provides biparental care (Fishpool and Tobias 2005), personal observation). Dunn et. al. (2010) analyzed more than 1000 species of birds and found lower sexual dimorphism in species with monogamous than with polygynous or lekking mating systems where variance in male mating success is thought to be lower. Nevertheless, other aspects of Himalayan black bulbul and related species' reproductive biology may contribute to sexual dichromatism; these include the genetic mating system and the parental investments of each sex, which need to be investigated further.

The Himalayan black bulbul's sexually dichromatic characteristics could be function-signaling and therefore the objects of sexual selection. In a study of six avian species, Delhey and Peters (2008) found that most function-signaling patches were sexually dichromatic. Sexually dichromatic traits have been proved to function in quality signaling (e.g., Hill 1996, Walker et al. 2013) and agonistic interactions in several avian

species (e.g., Alonso-Alvarez et al. 2004, Bright and Waas 2002, Préault et al. 2002), and are often the object of female choice. Moreover, brightness varied more in the sexually dichromatic parts of the Himalayan black bulbul (beak, tarsus, belly, remige and tail) than in sexually monochromatic ones, consistent with theoretical and empirical expectations (Andersson 1994, Delhey and Peters 2008).

Where males are subject to female mate choice, their sexually selected traits are usually more variable than females' (Andersson 1994, Darwin 1872). The similar variability that I found in both female and black bulbuls' sexually dichromatic traits suggests that mate choice might be mutual in this species. Whereas studies of sexual selection have mostly focused on female choice and male–male competition, data increasingly shows that males can be choosy and benefit from mating females whose reproductive potential is high (reviewed in Edward and Chapman 2011, e.g., Amundsen and Forsgren 2001, Griggio et al. 2005, Jones et al. 2001). Kokko and Johnstone (2002) suggested that high species-specific and high sex-specific mate-encounter rates, high cost of breeding (parental investment), low cost of mate searching and highly variable quality of the opposite sex could promote the evolution of choosiness and that the primary determinant of sex roles in mate choice is parental investment. According to this hypothesis, the sex for which the cost of breeding (mortality during signaling and caring) is the larger should evolve to be choosy. The reproductive biology of Himalayan black bulbuls is unclear, but research on pycnonotids suggests comparable parental care loads between the sexes, and the breeding success is generally low (8.3-15%, Balakrishnan 2010, Fishpool and Tobias 2005) while the rate of predation

is high. As such, high cost of breeding and comparable parental care load between the sexes might promote mutual selection in pycnonotids.

My data also suggest the involvement of multiple Himalayan black bulbul ornaments in sexual selection - as both carotenoid-based and melanin-based characteristics were found to be sexually dichromatic. Studies have shown females choosing mates based on multiple sexual ornaments (Chaine and Lyon 2008, Doucet and Montgomerie 2003); multiple ornaments provide females with different kinds of information in different stages of mate choice (Borgia 1995), or function as redundant signals to improve the accuracy of mate assessment (Johnstone 1994, Moller and Pomiankowski 1993).

Different sets of sexually dichromatic parts were found in live birds and museum skin specimens, and significant degradation of color-whether pigment-based or structural- was found in skin specimens, some of which had been preserved for less than 5 years. These results suggest that the use of skins in avian coloration study may be error-prone, contradicting the previous finding that melanin- and carotenoid-based skins colors remain the same for at least 50 years after preservation (Armenta et al. 2008). Conversely, my results corroborate the conclusion drawn in a study comparing live and skin long-tailed manakins that significant differences in colorimetric variables were attributable to the age of specimens (Doucet and Hill 2009). They also agree with another study that found UV color degradation in preserved skin specimens of some 300 bird species throughout Europe and the USA (Pohland and Mullen 2006). There are many possible reasons for color degradation, including the preservation process, preservation agents, specimen

preparation, contamination or simply age (reviewed in (Doucet and Hill 2009). Given that museum skin specimens are widely used in studies of avian coloration (e.g., Bridge et al. 2008, Kennedy 2010), I suggest that skin specimen coloration should be pre-tested against live birds; measurements obtained from skin samples should be corrected for age and/or condition of preservation, and the results should be interpreted with greater caution.

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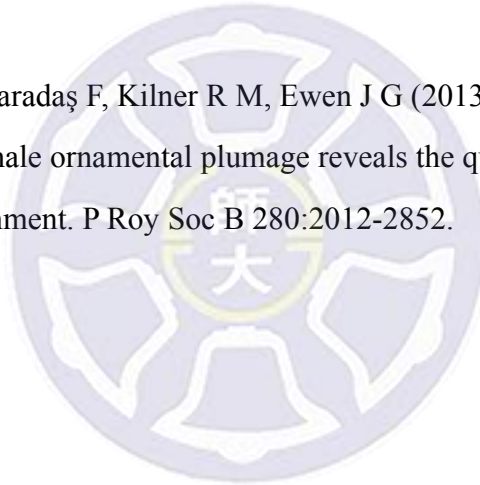


Table 2.1 ΔS between two sexes for live birds and skin specimens in different parts

Parts	Live birds	Skin specimens
Beak	<i>3.15</i>	-
Tarsus	<i>2.28</i>	-
Back	0.28	0.88
Belly	0.64	<i>1.49</i>
Nape	0.08	0.61
Breast	0.09	<i>1.14</i>
Forehead	0.28	0.81
Remige	<i>1.19</i>	0.99
Scapular	0.66	<i>1.12</i>
Tail	<i>3.81</i>	0.52

$\Delta S > 1$ is in bold and italics.

Table 2.2. Post-hoc test (Student's t) of total brightness and chroma_{UV} between live birds and skin specimens after two-way ANOVA test (S2.2 Table)

(a) Total brightness

Parts	Item	Mean* ± SE	Lower CL Difference	Upper CL Difference
Breast	Live	3.49 ± 0.08	0.24	1.00
	Skin	2.87 ± 0.17		
Scapular	Live	6.61 ± 0.19	0.41	2.11
	Skin	5.36 ± 0.38		
Tail	Live	4.23 ± 0.10	-1.44	-0.55
	Skin	5.22 ± 0.20		

*Least Square Mean, unit=%

(b) Chroma_{UV}

Parts	Item	Mean* ± SE	Lower CL Difference	Upper CL Difference
Back	Live	24.13 ± 0.19	1.57	3.29
	Skin	21.70 ± 0.39		
Belly	Live	23.13 ± 0.18	0.60	2.26
	Skin	21.70 ± 0.38		
Nape	Live	24.03 ± 0.23	2.19	4.20
	Skin	20.83 ± 0.45		
Breast	Live	24.10 ± 0.20	2.09	3.93
	Skin	21.09 ± 0.42		
Forehead	Live	23.39 ± 0.24	1.71	3.81
	Skin	20.63 ± 0.48		
Remige	Live	22.99 ± 0.17	0.07	1.64
	Skin	22.13 ± 0.36		
Scapula	Live	23.95 ± 0.16	0.95	2.36
	Skin	22.30 ± 0.32		
Tail	Live	25.79 ± 0.16	0.41	1.87
	Skin	24.65 ± 0.33		

*Least Square Mean, unit=%

Table 2.3. Variabilities (Standard Deviation) of brightness in the same sex among different parts

Parts	Female	Male
Beak	6.59	6.50
Tarsus	5.49	5.52
Back	0.68	0.62
Belly	1.43	1.44
Nape	0.89	0.53
Breast	1.01	0.94
Forehead	0.64	0.60
Remige	1.71	1.45
Scapular feather	1.99	1.98
Tail	0.95	1.14

Chi-square test. *P* < 0.05 is in bold and italics.

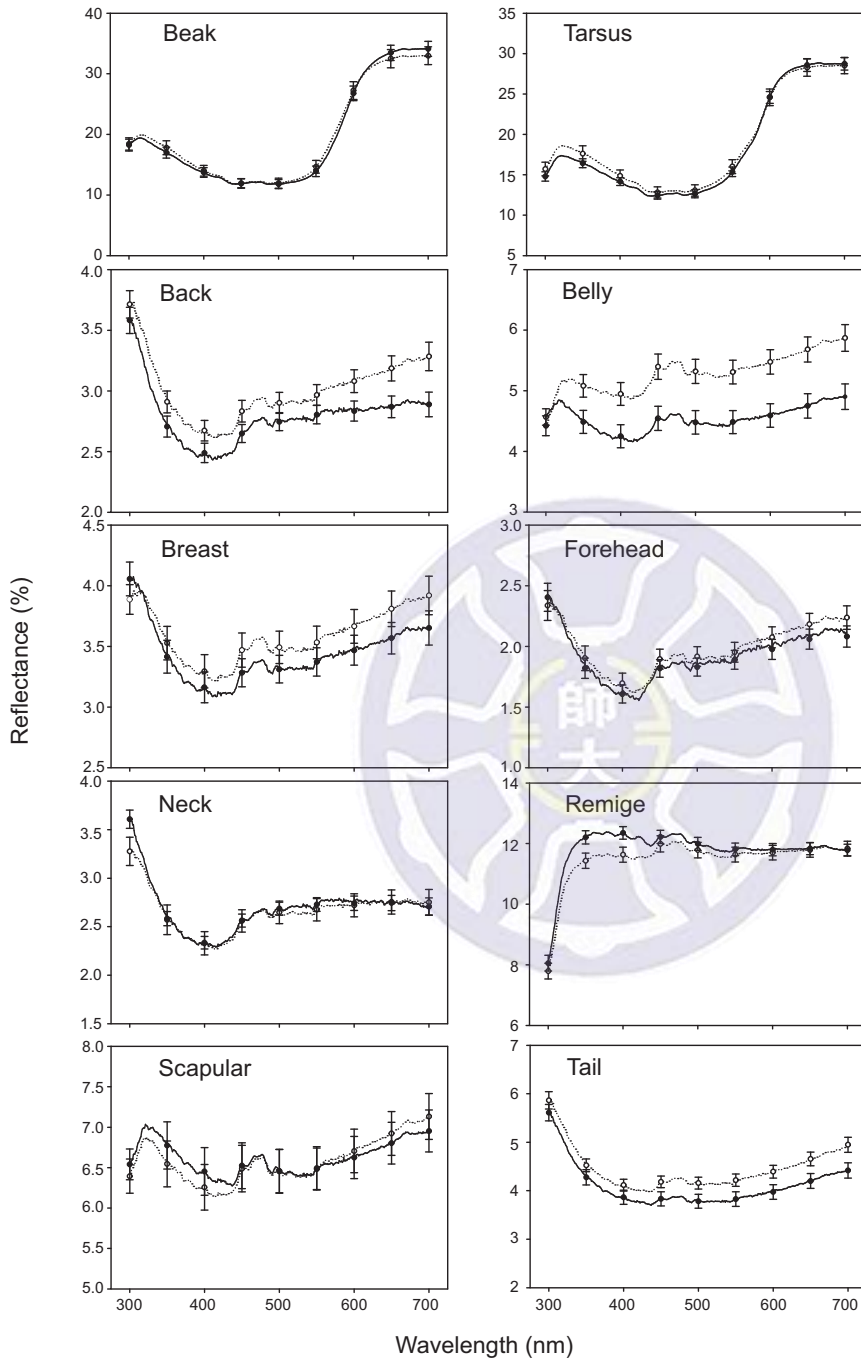


Fig. 2.1. Spectra of ten characteristics in two sexes. Dotted lines indicate spectra of males and solid lines indicate females.

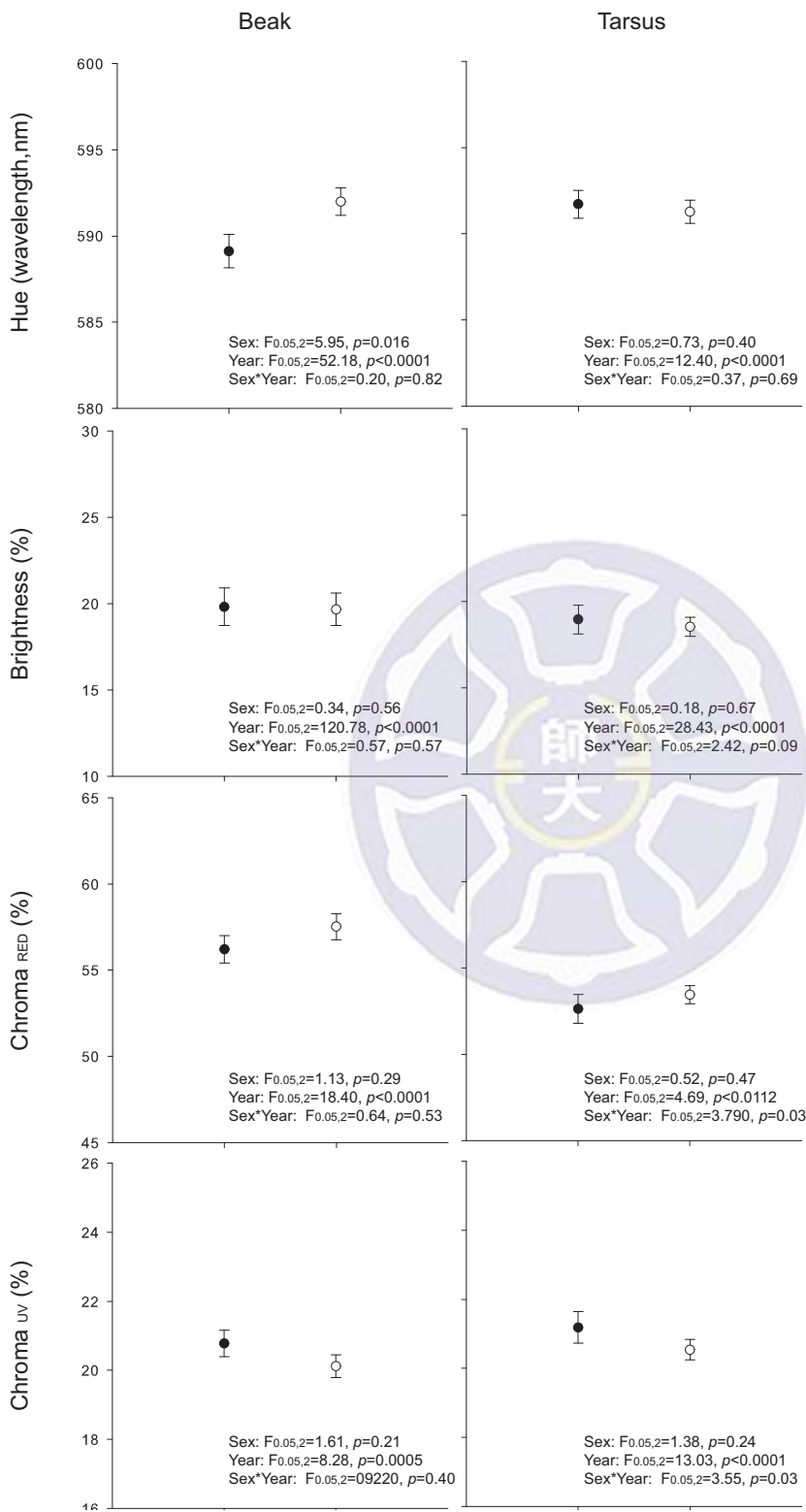


Fig. 2.2. The colorimetric variables in carotenoid-based bill and tarsi between sexes in live birds. The hollow dots indicate males and the solid dots indicate females. Two-way ANOVA test, factor “Year” included three categories: 2008, 2009 and 2011, factor “Sex” included two categories: female and male.



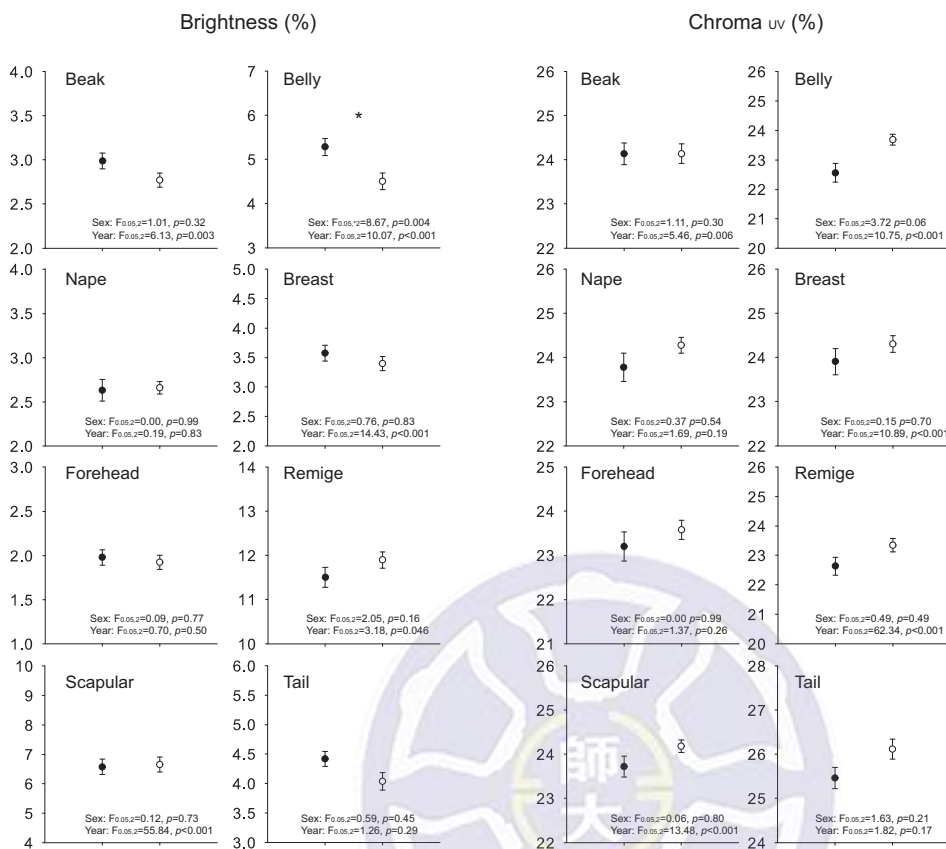
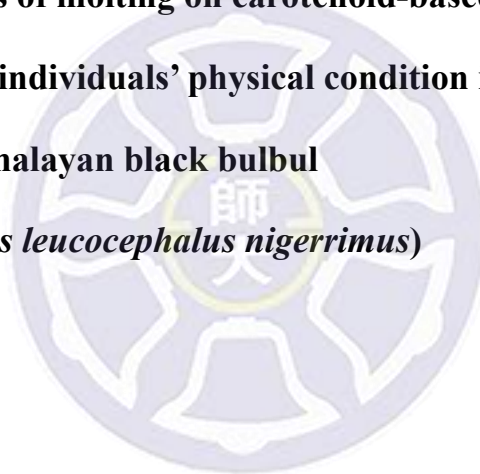


Fig. 2.3. The colorimetric variables in melanin-based characteristics between sexes in live birds. The hollow dots indicate males, and the solid dots indicate females. Two-way ANOVA test factor “Year” included three categories: 2008, 2009 and 2011, factor “Sex” included two categories: female and male. The result of interaction between two factors in each colorimetric variable was not listed because of the insignificant effect of it. The asterisk indicates the significant effect of the factor (adjusted $p < 0.005$).

Chapter Three

**Negative effects of molting on carotenoid-based
characteristics and individuals' physical condition in the
Himalayan black bulbul
(*Hypsipetes leucocephalus nigerrimus*)**



Abstract

Traits that compete limited internal resources against other somatic needs could lead to trade-offs of resource allocations between them; their expression could therefore be correlate. Because the amount of carotenoids available for use is limited and carotenoids can be used in many physical functions, trade-offs arising among carotenoid-based ornaments and other physical demands. Molting is energetically and nutritionally demanding, but the effects of molting on major somatic needs are vague and contentious. In this study, I aimed to investigate whether carotenoid-based ornaments are quality cues that could reflect individuals' physical condition following severe physical stress, namely molting induced by artificial plucking in Himalayan black bulbuls (*Hypsipetes leucocephalus nigerrimus*). I conducted a 2×2 grouping design experiment involving plucking and carotenoid supplementation. My data showed that bill redness was positively correlated with an individual's immunocompetence and negatively correlated with oxidative stress level it experienced, but such correlations were not found in the carotenoid-supplemented group. My results suggest that the bill is a quality cue in black bulbuls, and additional carotenoids would reduce the negative effect of molting on both decorative traits and physical condition.

Keywords: carotenoid-based ornaments, Heterocyte/liphocyte ratio, Himalayan black bulbul (Hypsipetes leucocephalus nigerrimus), molting, quality cues, PHA test.

Introduction

Competition for limited internal resources among different functions could lead to life history trait trade-offs, which may occur between physiological traits expressed either during the same stage or different stages of the life cycle (Zera and Harshman 2001). Signal traits, which could reflect individuals' physical condition or agonistic abilities, are usually costly to their bearers because these traits share resources with other physically demanding ones (e.g., Spencer et al. 2003). Consequently, trade-offs between variable life-history traits and those that could serve as signal traits are to be expected. Among different types of signal traits, carotenoid-based ornaments are the most studied till date.

Besides their ability to producing striking colors (i.e. yellow, orange and red, Fox and Vevers 1960, Latscha 1990), their powerful antioxidant properties also make carotenoids crucial to several life-history traits like breeding (McGraw et al. 2005, Surai 2002), migration (Alan et al. 2013, Metzger and Bairlein 2011) and molting (e.g., McGraw et al. 2006). Nevertheless, animals can only acquire carotenoids from food, and the ability of an animal to utilize the carotenoids in food depends on its genotypes and physiological condition (Alonso-Alvarez et al. 2004, Olson and Owens 1998). It has been suggested that the quantities of carotenoids available to individuals are limited, although carotenoids may be abundant in the natural environments (Olson and Owens 1998). Due to the limited availability of carotenoids, individuals may have to trade-off carotenoids among different life history traits (Alonso-Alvarez et al. 2004). For instance, females may have to balance the benefits of carotenoids with those investing in offspring quality, or of impairing their

own antioxidant damage during reproduction (Bertrand et al. 2006, Biard et al. 2005). In migratory birds, early-arriving males, who usually have a longer reproductive season and greater reproductive success (e.g., Klomp 1970, Perrins 1970), may be strong enough to allocate carotenoids into coloration rather than use them as antioxidants during migration (Ninni et al. 2004). In summary, the appropriate allocation of carotenoids among life traits and physiological functions (i.e., decorative plumage and antioxidation) are necessary in order to maximize individual fitness (e.g. Bertrand et al. 2006, Biard et al. 2005, Faivre et al. 2003, Nordeide et al. 2008).

Molting is energetically and nutritionally demanding (Jenni and Winkler 1994, Klaassen 1995, Kuenzel 2003, Lindstrom et al. 1993) and would decrease the accumulation of circulating plasma carotenoids (Barbosa et al. 2013, Del Val et al. 2014, Del Val et al. 2013). However, to the best of my knowledge, studies that addressing whether molting can affect the expression of carotenoid-based characteristics have been rare. The only relevant study showed that molting speed constrains the expression of yellow throat in the rock sparrows (*Petronia petronia*, (Serra et al. 2007). Meanwhile, the effect of molting on the expression of carotenoids on the bared ornaments like bill, tarsus, or dewlap is also unknown. It is suggested that the expression of carotenoid-based bared parts could reflect an individual's physical condition faster than that of plumage (e.g. Ardia et al. 2010, Faivre et al. 2003). The effects of molting on major somatic functions are vague and contentious. With immunocompetence, an indicator of the individual's immune response, molting has either positive (Sanz et al. 2004) or negative (Martin 2005,

Sanz et al. 2004) correlations, or none at all (Pap et al. 2008). Although it is suggested that oxidative stress is higher during or after molting due to the depletion of plasma carotenoids, the effects of molting on an individual's oxidative stress level has not been tested directly (Del Val et al. 2013). To the best of my knowledge, only one study has found that individuals' oxidative stress levels did not change by natural molting in great tits (*Parus major*, Vaugoyeau et al. 2015). Knowledge of the effect of carotenoid abundance on molting is lacking; therefore, I would like to know whether the effect of molting can be diminished by carotenoid supplementation, because carotenoids are powerful antioxidants.

In this study, I aim to investigate whether carotenoid-based ornaments are the quality cues that reflect individuals' physical condition after a severe physical stress. I used Himalayan black bulbuls (*Hypsipetes leucocephalus nigerrimus*), which have carotenoid-based bills and tarsi, as the study species to examine whether the expression of carotenoid-based characteristics and individuals' physical condition (both immunocompetence and oxidative stress level) could be affected under the stress of molting. Traditionally, the study of molting effects in birds involves the natural induction of molting, but this may be inappropriate under the current study conditions. Because both natural molting and carotenoid allocation can be influenced by hormones, such as testosterone, thyroxine, or corticosterone (Cherel et al. 1988, Rehder et al. 1986), it would be difficult to control the confounding effects of hormones when conducting experiments based on natural molting. I therefore plucked feathers directly from study animals to initiate molting artificially. A 2×2 grouping design was utilized within the carotenoid supplementary group,

tail and secondary feathers were pulled off on half of the birds, while the other half of them served as a control group with their feathers intact. I predicted that molting would have negative effects on the physical condition and cause trade-off of carotenoids allocation in black bulbuls; therefore, I should have observed a significant trade-off between decorative traits and individuals' physical condition in the molting group. I also predicted that the effects of molting could be diminished when the carotenoids were in abundant supply.

Materials and methods

Captive setting and sampling

Thirty-one Himalayan black bulbuls were purchased from a pet-shop in 2010. They were individually housed in cages with opaque covers between them, so that the birds could have acoustic contact but not visual perception of others. They were placed in a room at constant temperature (27°C) and humidity (80%). Blood (150 µL) was collected from each bird using a heparinized capillary tube. Blood samples were stored in a -20°C freezer for molecular sex-typing. In addition, a drop of blood, approximately 5µL, was put on a slide to assay the oxidative stress level.

Molecular sex typing

Genomic DNA was extracted from blood samples with traditional proteinase K digestion followed by LiCl extraction (Gemmell and Akiyama 1996). The polymerase chain reactions (PCRs) program used for molecular sex typing (Fridolfsson and Ellegren 1999) was the same as

that described in Hung et al. (2015 in revision).

Experimental procedures

Experimental design

Thirty-one birds were used in this experiment. After sex typing, individuals of each sex were randomly and equally assigned to four treatment groups: carotenoid-supplemented and plucked [Caro(+), plucked, F = 4, M = 4], carotenoid-supplemented and unplucked [Caro(+), unplucked, F = 2, M = 4], carotenoid-unsupplemented and plucked [Caro(-), plucked, F = 3, M = 4], or control [Caro(-), unplucked, F = 5, M = 5]. In the plucked group, individuals' tails and secondary feathers had been removed. Carotenoid supplementation was initiated on February, 10, 2010, a month prior to the molting experiment. The molting experiment was initiated on the March, 8, 2010, this day also served as the baseline for the immune challenge experiment. During the experimental period, only commercial feed formulated with or without supplementary carotenoids was fed. Oxidative stress testing was conducted on two dates: the baseline day, on which tails and secondary feathers were removed and the testing date, on which the tails and secondary feathers of individuals in the experimental group had grown to half of their average length (approximately three weeks later, around 5 to 5.5 cm for tail feathers and 1.5 to 2.0 cm for secondary feathers). The immune challenge was conducted only on the testing day.

Carotenoid-supplementation experiment

Each individual was fed 40 grams of commercial bird food every day. In the carotenoid-supplemented groups, the additional canthaxanthin (0.9

mg/40 g, Orpharma, Belgium) and lutein (0.32 mg/40g, Orpharma, Belgium) were added to the commercial bird foods.

Physiological conditions

Immune challenge: Phytohaemagglutinin assay (PHA assay)

The PHA assay was conducted on May, 26, 2010. PHA solution (80µl of 2.5ng/µL, Sigma L-1668, Sigma Chemical Co., St. Louis, Mo, USA) was injected into individuals, according to the method of Smits et al. (1999). A pressure-sensitive caliper (TecLock Inc., Japan) was used to measure the thickness of the right wing web before injection to the nearest 0.01 mm. Twenty-four hours after the injection, the level of swelling was measured on the wing web of each individual to determine the degree of immune response (Smits et al. 1999). Individuals with larger swollen wing webs were considered to have stronger immune responses.

Oxidative stress test: Heterocyte to lymphocyte ratio (H/L ratio)

I calculated the ratio of lymphocytes (L) to heterocytes (H) in a total of 100 leukocytes as a measure of oxidative stress for each bulbul individual, according to the method of Vleck et al. (2000). The detailed procedures were the same as that described by Hung et al. (2015 in revision). A higher H/L ratio indicates that the individual were under a higher level of oxidative stress (Gross and Siegel 1983). The assay of oxidative stress was conducted on the baseline and testing dates.

Molting speed measurements

To test whether carotenoid supplementation affected molting speed, I measured the regrowth lengths of tail and secondary feathers on the testing date in the plucked groups, then divided by 21 days to determine each individual's molting speed.

Statistical analysis

Multiple regression analyses were conducted to test whether bill coloration was correlated with the individuals' physical condition, factors, such as sex, plucking treatment and carotenoid supplement treatment, were included in the analysis. In the test of whether molting would affect an individual's condition, due to the interaction effects of plucking and carotenoid supplement treatments that my analysis uncovered, factors including sex, plucking treatment and their interaction were used in Caro(+) and Caro(-) groups respectively.

Results

My data showed that the carotenoid supplementation could affect an individual's physical condition in Himalayan black bulbuls. Coloration comparisons between the Caro(+) and Caro(-) groups before feather-plucking showed that bill coloration in the Caro(+) group was significant redder than that of the Caro(-) group (Hue mean \pm se, Caro(+) 591.16 \pm 1.56 nm, Caro(-) 587.02 \pm 1.11, two-way ANOVA, $t=2.58$, $p=0.01$). My data also showed that the negative correlations between plucking, bill coloration and the individual's physical condition were significant in the group without carotenoid supplementation. In the Caro(-) group, individuals in the plucked group had lower wing web swelling after PHA injection (Table 3.1 a, plucked (mean \pm SE) 1.24 \pm 0.21%, unplucked 1.96 \pm 0.21%, multiple regression, $t = -2.42$, $p = 0.039$) and higher H/L ratios (Table 3.1 b, plucked 0.55 \pm 0.16 %, unplucked 0.08 \pm

0.12 %, multiple regression, $t = 2.26, p = 0.049$); they also had paler bills (Table 3.1c, plucked $581.3 \pm 1.7 \text{ nm}$, unplucked $587.2 \pm 1.7, t = -2.45, p = 0.03$). However, in the Caro(+) group, there were no inter-individual differences within plucked or unplucked groups in any physical indicators (Table 3.1). These data suggest that adding carotenoids buffers the negative effect of molting in Himalayan black bulbuls. I also found that, regardless of plucking, carotenoid supplementation was associated with an increase in the intensity of the red bill coloration (Ls mean \pm SE, Caro(-) 584.3 ± 1.9 , Caro(+) 601.1 ± 1.9 , two-factor ANOVA, $F_{\text{plucking treatment}} = 1.46, p = 0.24, F_{\text{carotenoid treatment}} = 39.31, p < 0.0001, F_{\text{plucking*carotenoid}} = 0.70, p = 0.41$); again, these data suggest that the carotenoid supplementation enhances the red bill coloration. However, I found no effect of carotenoid supplement on molting speed in either secondary or tail feathers (the secondary: Molting Speed_{Caro(+)} = 0.53 ± 0.11 cm/day, Molting Speed_{Caro(-)} = 0.52 ± 0.11 cm/day, $t = -1.07, p = 0.30$; Tail: Molting Speed_{Caro(+)} = 0.20 ± 0.08 cm/day, Molting Speed_{Caro(-)} = 0.26 ± 0.01 com/day, $t = -0.51, p = 0.62$).

I found that regardless of carotenoid supplementation, individuals with redder bill had more severe swelling in the PHA test (Fig. 3.1, multiple regression, estimate_{carotenoid treatment} $-7.45 \pm 1.31, t = -5.66, p < 0.0001$; estimate_{swelling} $5.61 \pm 2.45, t = 2.28, p = 0.03$) and lower H/L ratio (Fig. 3.1, estimate_{carotenoid treatment} $-7.87 \pm 1.27, t = -6.21, p < 0.0001$; estimate_{Log (H/L ratio)} $-6.73 \pm 2.81, t = -2.39, p = 0.02$), indicating that individuals with redder bills had better immunocompetence and were

under lower oxidative stress, relative to those with less intensely colored bills.

Discussion

My data clearly demonstrate that the bill coloration can serve as a quality cue in the Himalayan black bulbuls: the redness of the bill is positively correlated with an individual's immunocompetence and negatively correlated with the oxidative stress level. I also propose that the addition of carotenoids can reduce the effect of molting on both decorative traits and individuals' physical condition in the focal species.

According to the communication theory, informative cues may be perceived and function in animal communication (Dale 2006, Stevens 2013), particularly when such cues could reflect individuals' qualities (Senar 2006). Hence, bill coloration may play essential roles in the communication of Himalayan black bulbuls. Previous data showed that the bill coloration alone may not be a sexually selected cue in the focal species (Chapter Five). A recently published paper suggested that the carotenoid-based bill should be more likely to function as a social signal, as opposed to asexually selected trait (Dey et al. 2015). Unlike feathers, in which carotenoids are deposited during molting, the outer layers of the bill are continuously replaced; therefore bill coloration could reflect a more recent physical condition of its bearer. It is more useful for animals that constantly live in groups to use fast-reacting signals in social interactions, such as the assessment of rivals or the formation of social rankings, than in choosing a mate. Himalayan black bulbuls are socially

monogamous during the breeding season but live in groups during the nonbreeding season. Therefore, it is possible that bill coloration may play an important role in their social interactions, because the presence of social signals could be very beneficial for them to diminish the frequent conflicts that occur in their collective society.

I have clearly demonstrated that molting would have negative effects on individuals' physical conditions and carotenoid-based ornaments. My data suggest that there may be trade-offs of carotenoids occurring between molting and these traits. The trade-offs between the immune response and molting have been found in several avian species (e.g. Kuenzel 2003, Sanz et al. 2004). These trade-offs may occur for various of reasons: first, as noted above, both molting and immune activities impart high resource demands. Martin (2005) found that both activities would increase metabolic rates in house sparrows (*Passer domesticus*). Other lines of evidence indicate that trade-offs between molting and immune activity may also be driven by the availability of protein (e.g. Lochmiller and Deerenberg 2000) or particular hormones, such as prolactin (Kuenzel 2003). My data provide direct evidence that molting would increase individuals' oxidative stress. To the best of my knowledge, this is the first study to provide such evidence. My results also provide evidence that molting decreases the expression of carotenoid-based bare parts. The reasons for the negative effects of molting on these traits have not been tested; however, I suspect that it may be due to competition to the limited resource - carotenoids.

In this study, I did not find the molting effect when the carotenoid supplement was added. A study of molting in gentoo penguins

(*Pygoscelis papua*) showed that plasma carotenoid could be depleted during molting within a week (Barbosa et al. 2013), suggesting that carotenoids could be used rapidly during molting. Therefore, a sufficient supply of carotenoids could decrease the effect of molting on the carotenoid content in our species. Similar results were also found in the study of European starlings (*Sturnus vulgaris*, Casagrande et al. (2015); they discovered that birds receiving extra carotenoids kept singing even during the sickness phase induced by inflammation. Aside from carotenoids, such trade-offs could also be influenced by nutritional quality; reduced nutrient availability can substantially magnify an apparent trade-off, while increased nutrient availability can diminish or obviate it (Zera and Harshman 2001).

In conclusion, my data provide evidence that carotenoid-based bill coloration is informative and may play roles in animal communication in this species. These results also provide direct evidence for a mechanism by which molting would influence several crucial physical traits.

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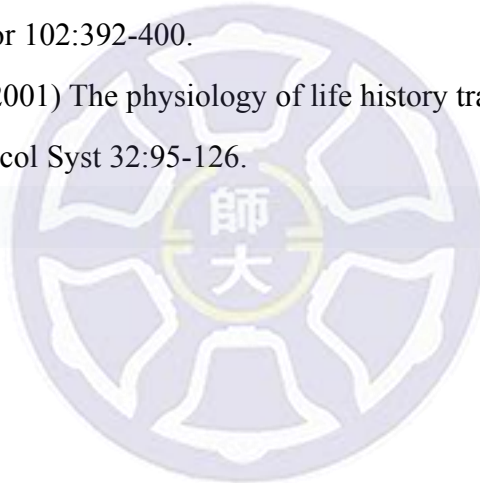


Table 3.1 Multiple regressions of whether molting could affect individuals' physical conditions in Caro(-) group and Caro(+) group respectively.

(a) Swelling

Caro(-) group

Term	Estimate	SE	<i>t</i>	<i>P</i>
Intercept	1.60	0.15	10.79	<.0001
Sex[F]	0.05	0.15	0.36	0.730
Plucking treatment[C]	-0.36	0.15	-2.42	0.039
Sex[F]*Plucking[C]	0.05	0.15	0.31	0.765

Caro(+) group

Term	Estimate	SE	<i>t</i>	<i>P</i>
Intercept	1.85	0.14	13.60	<.0001
Sex[F]	0.13	0.14	0.95	0.364
Plucking treatment[C]	0.16	0.14	1.15	0.278
Sex[F]*Plucking[C]	0.11	0.14	0.82	0.429

(b) Log (H/L ratio)

Caro(-) group

Term	Estimate	SE	<i>t</i>	<i>P</i>
Intercept	0.31	0.10	3.07	0.014
Sex[F]	-0.10	0.10	-0.98	0.353
Plucking treatment[C]	0.23	0.10	2.26	0.049
Sex[F]*Plucking[C]	-0.11	0.10	-1.05	0.323

Caro(+) group

Term	Estimate	SE	<i>t</i>	<i>P</i>
Intercept	0.19	0.16	1.20	0.261
Sex[F]	0.05	0.16	0.31	0.767
Plucking treatment[C]	0.01	0.16	0.08	0.937
Sex[F]*Plucking[C]	-0.08	0.16	-0.52	0.617

(c) Bill coloration (Hue)

Caro(-) group

Term	Estimate	Std Error	t Ratio	Prob> t
Intercept	584.25	1.20	486.61	<.0001
Sex[F]	-1.22	1.20	-1.01	0.332
Tail Ex[C]	-2.94	1.20	-2.45	0.032
Sex[F]*Tail Ex[C]	-0.77	1.20	-0.64	0.535

Caro(+) group

Term	Estimate	Std Error	t Ratio	Prob> t
Intercept	600.76	2.52	238.10	<.0001
Sex[F]	-2.62	2.52	-1.04	0.324
Plucking treatment[C]	-0.83	2.52	-0.33	0.750
Sex[F]*Plucking[C]	-2.32	2.52	-0.92	0.379



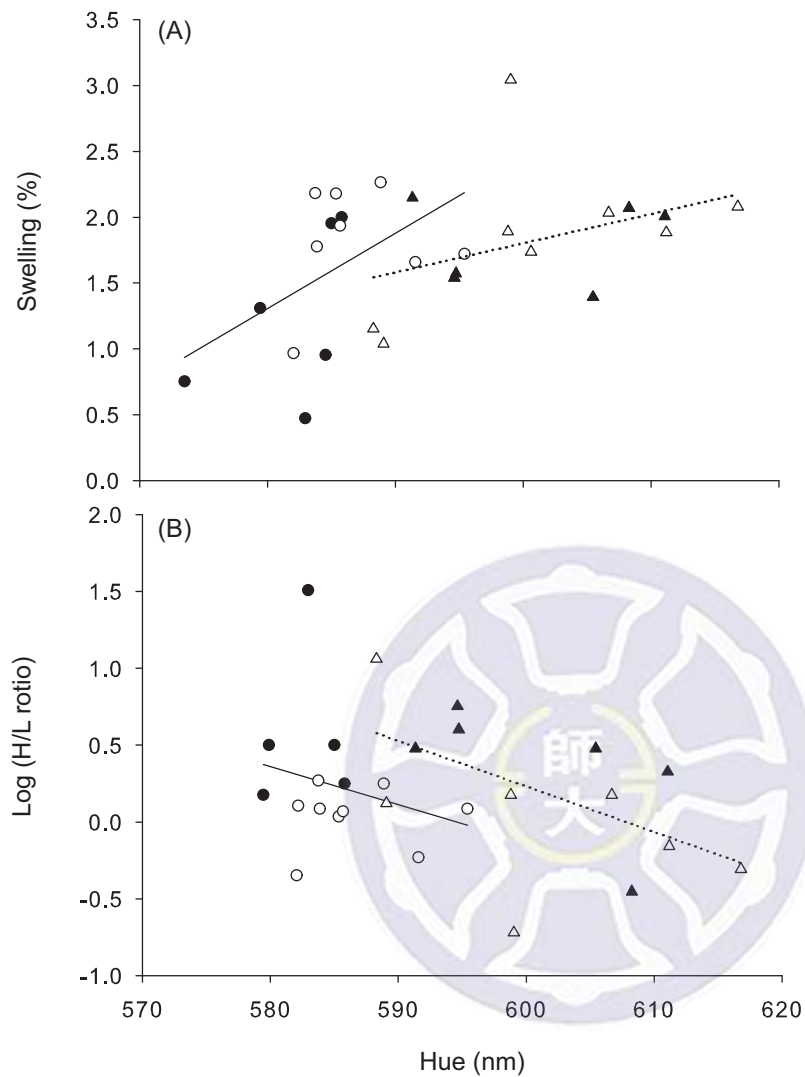


Fig. 3.1. Correlations between beak coloration and individual's physical indicators: (A) swelling (B) Log (H/L ratio). Circles indicate the Caro(-) group while triangles indicate the Caro(+) groups. Hollow symbols represent the unplucking treatment and solid ones represent the plucking ones. Solid lines indicate the regression line in the Caro(+) group and dotted lines indicate the regression lines in the Caro(-) group.

Chapter Four

**Negative correlation between melanin-based plumage
coloration and oxidative stress in**

Himalayan black bulbul

(Hypsipetes leucocephalus nigerrimus)



Abstract

Melanin-based coloration is found to be often associated with variations in physiological and behavioral traits that are typical targets of sexual selection. However, the function of melanin-based traits to their bearers has been investigated less than that of carotenoid-based ones. It is commonly believed that expression of melanin-based pigmentation is genetically controlled. However, whether it could be modulated by physical condition has remained controversial. In this study, I demonstrate that the level of melanin-based plumage pigmentation could be negatively correlated with an individual's oxidative stress level. In this study, I used two groups of black bulbuls (*Hypsipetes leucocephalus nigerrimus*) obtained from a pet shop in 2010 and 2011 to examine the correlation between the melanin-based plumage coloration and the ratio of lymphocytes and heterocytes, which is an indicator of the oxidative stress experienced by an individual. I found a negative correlation between oxidative stress and levels of melanin-based pigmentation on the breast and scapular feathers, but the correlation only appeared in the 2011 group individuals of which had higher oxidative stress levels and brighter plumage (i.e. more melanins) than those of the 2010 group. My data suggest that melanin-based plumage could reflect an individual's physical condition in certain situations. This is consistent with the prediction of the indicator hypothesis. Therefore, melanin-based plumage coloration could be functional in visual communication of Himalayan black bulbuls.

Key words: heterocyte / lymphocyte ratio, Himalayan black bulbuls (Hypsipetes leucocephalus nigerrimus), melanin-based trait, oxidative stress

Introduction

According to the “indicator hypothesis”, females can acquire information about males’ quality by assessing sexually selected traits that reflect their condition (honest signals), such as coloration or other conspicuous characteristics (Andersson and Simmons 2006). Melanins, mainly responsible for black (eumelanins) or reddish and brown (pheomelanin) colors, are considered one of the main pigment classes associated with variations in the physiological and behavioral traits that are typical targets of sexual selection and animal communication. It has been shown that vertebrates with darker coloration are more aggressive, sexually attractive and resistant to stress than those with lighter ones (Ducrest et al. 2008). However, compared with the well-studied carotenoid-based coloration, the function of melanin-based traits to their bearers is less understood.

To human eyes, melanin-based ornaments are usually just dull colored (i.e. black or brown), and are less varied within and between species than carotenoid-based ornaments (Badyaev and Hill 2000). However, the extra cones and oil drops that birds have make them have better color discrimination than humans (Griffith et al. 2006, Jawor and Breitwisch 2003, McGraw 2006). Traditionally, avian melanin-based plumage patterns are characterized by patch size or darkness ranked by human eyes (e.g., Bize et al. 2006), but such methods may highly underestimate variation in darkness and severely hamper our understanding of the functions of melanin-based traits. Fortunately, with the aid of a spectrometer, subtle differences in melanin-based coloration can be quantified, and results indicate that the variation in melanin-based coloration within and between species can be just as much as in

carotenoid-based colorations (e.g., Hill and Brawner 1998).

Animals can synthesize melanins from the aromatic amino acids, phenylalanine and tyrosine. Melanization can be highly heritable (Hill and McGraw 2006, Jawor and Breitwisch 2003). In the past, variations in melanin-based traits were believed mainly to be genetically controlled (Bize et al. 2006). However, more studies suggest that these traits could be condition-dependent: factors such as parasitic infections and diet quality can also influence the level of expression of melanin-based signals (e.g., Fargallo et al. 2007, Jacquin et al. 2011). In particular, oxidative stress could play a critical role in regulating melanization (e.g., Roulin et al. 2008). High oxidative stress, which results from the imbalance between the rate of production of reactive oxygen species (including free radicals) by cell metabolism and the state of repair and antioxidant machinery, can induce ageing and reduce life span (Finkel and Holbrook 2000). The process of melanization could reduce the concentration of free radicals, making melanin an important antioxidant (Ducrest et al. 2008). However, the correlation between oxidative stress levels and the coloration of melanin-based traits are still controversial in birds (e.g., Ducrest et al. 2008, Galván and Alonso-Alvarez 2009). In the current study, I studied the correlation between the brightness of black plumage and individuals' oxidative stress in Himalayan black bulbuls (*Hypsipetes leucocephalus nigerrimus*) to test whether melanin-based plumage could be an indicator of the individuals' physical condition.

The Himalayan black bulbul is widely distributed in Taiwan's broad-leaf forests at elevations from 100 m to 1500 m. Their plumage coloration is entirely melanin-based; black plumage with grey patches on

the scapular feathers and remige. It makes the Himalayan black bulbul a perfect system to investigate the function of melanin-based signaling. In theory, the process of melanin production should reduce the sensibility of stress-regulation processes and result in lower oxidative stress (Ducrest et al. 2008); therefore, I expected a negative correlation between an individual's oxidative stress levels and its black plumage coloration, with brighter individuals (i.e. more melanins) under lower oxidative stress. The results of this study should enhance knowledge about the role of melanin-based coloration in avian communication.

Materials and methods

Study species and captive setting

In total, 18 and 48 Himalayan black bulbuls were bought from a pet shop in Taipei in 2010 and 2011 respectively. Each bird was housed individually in the laboratory. I collected 150 μ L blood from each individual for molecular sex typing. A drop of blood, approximately 5 μ L, of each bird was used as the blood smear for the oxidative stress test.

Molecular sex typing

Genomic DNA was extracted from blood samples with traditional proteinase K digestion followed by LiCl extraction (Gemmell and Akiyama 1996). The detailed programming of polymerase chain reactions (PCRs) for molecular sex typing (Fridolfsson and Ellegren 1999) was the same as in Hung et al. (2015 in revision). I identified 8 females and 10 males in 2010 and 18 females and 30 males in 2011.

Coloration measurements

For each individual, the reflectance of eight regions of melanin-based plumage- including the forehead, nape, back, breast, belly, tail, remige and scapular feathers- was measured using an USB2000 spectrometer (Ocean Optics) with a HL2000 deuterium-halogen light source (Ocean Optics). The measuring procedures were as in Hung et al. (2015 in revision). The brightness of each region was defined as the average of total reflectance within the range of 300-700nm. The data used in this study were extracted and reanalyzed from the data in Hung et al. (2015 in revision). (McGraw et al. 2005) demonstrated that the levels of both eumelanin and phaeomelanin concentration in feathers were significantly and positively correlated with brightness. According to their results, a higher brightness indicates more accumulation of melanin in the feathers

Oxidative stress test

I calculate the ratio of heterocytes (H) to lymphocytes (L) in total 100 leukocytes as a measure of oxidative stress for each bulbul individual following the methods used by Vleck et al. (2000). The blood smear was first dyed with a Wright-Giemsa stain for 3 min and then with 5% PBS for 70 sec. After drying, the numbers of lymphocytes and heterocytes were counted under a microscope at a magnification of 100X with oil immersion and the H/L ratio calculated. The H/L ratio has been proved to be a good indicator of oxidative stress: higher levels of corticosterones can cause higher oxidative stress and also increase the number of heterocytes in the blood (Davis et al. 2008, Gross and Siegel 1983). Because leukocyte numbers change more slowly in response to stress than corticosterone does (Maxwell 1993), H/L ratios provide a more useful and accurate measure of long-term stress than a single measure of

plasma corticosterone (McFarlane and Curtis 1989, Vleck et al. 2000).

Statistical analysis

Multiple regression was used to test whether the brightness of each body part correlated with oxidative stress (H/L ratio) while taking into account the birds' sex and the interaction between sex and oxidative stress. In the tests, the H/L ratio was log transformed to fit the normal distribution.

Two-way ANOVA was conducted to test whether each individual's oxidative stress and the brightness of each its body parts differed significantly between years. In these tests, sex, year and the interaction between sex and year were taken into account.

Results

The average H/L ratio in 2010 was 0.35 ± 0.05 and ranged from 0.1 to 0.4 while the average H/L ratio in 2011 was 1.05 ± 0.13 and ranged from 0.2 to 4.6. Individuals in the 2010 group were under significant lower oxidative stress (higher H/L ratio) than those in the 2011 group (Fig. 4.1, two-way ANOVA, $F= 15.36$, $p=0.0002$). The females' H/L ratio was 0.76 ± 0.15 and the males' was 0.92 ± 0.14 ; there was no significant difference of H/L ratios between the two sexes (Fig. 4.1, two-way ANOVA, $F= 0.73$, $p=0.72$).

The brightness of melanin-based colors differed significantly between Himalayan black bulbuls in the 2010 and 2011 groups (data extracted and reanalyzed from (Hung et al. 2015), in revision). Members of the 2011 group were brighter (i.e. more melanins) than those of the 2010 group, mainly in belly (two-way ANOVA, Table 4.1; L_s mean \pm SE, 2010 group

4.45 ± 0.31%, 2011 group 5.34 ± 0.19%, post-hoc (student's *t*) test, CL: -1.61; -0.1) and breast (Table 4.1; 2010 group 3.22 ± 0.21%, 2011 group 3.82 ± 0.13%, post-hoc (student's *t*) test, CL: -1.10; -0.11). The same tendencies were observed at the remige and scapulars, although there were significant interactions with two factors- sex and year (two-way ANOVA Table 4.1).

The results of multiple regression indicated brighter breast and scapular feathers might be related to lower oxidative stress: there was a significant negative correlation between the brightness of breast and scapulars and oxidative stress (multiple regressions, Table 4.2; linear correlation coefficients in breast, $r_{\text{female}} = -0.23$, $r_{\text{male}} = -0.47$; correlations in scapulars, $r_{\text{female}} = -0.54$, $r_{\text{male}} = -0.23$), but not between oxidative stress and the brightness of the other six body parts. However, this relationship only applied in 2011 and not in 2010: the H/L ratio was not significantly correlated with the brightness of any melanin-based parts (multiple regressions, Table 4.2).

Discussion

My study shows that melanin-based characteristics could be duller in individuals that suffered higher oxidative stress. It consists with the work of Ducrest et al. (2008). They reviewed six studies and discovered that there is significantly negative correlation between the expression of melanin-based characteristics and the level of oxidative stress. It implies that expression of melanin-based traits is condition-dependent and could be the quality cue, which can reflect individual's physical condition (Hill

and McGraw 2006), in the focal species.

However, the significant overall negative relationship between coloration and oxidative stress was not found within any given year. In contrast, individuals in 2011 suffered higher oxidative stress but had brighter plumage (i.e. more melanins). Several recent studies also report similar, positive correlations between oxidative stress and melanin-based coloration (Galván and Alonso-Alvarez 2008, Galván and Alonso-Alvarez 2009, Hõrak et al. 2010), suggesting that an alternative hypothesis about a different interaction between melanin coloration and oxidative stress should be considered in the role of melanin-based signaling.

Glutathione (GSH), another key intracellular antioxidant, has been suggested to inhibit eumelanogenesis and eumelanin-based black ornaments, and might be crucial to the expression of melanin-based traits (Benedetto et al. 1982). GSH is a tripeptide thiol found in virtually all animal cells, and often considered as a vital antioxidant. It functions in the reduction of the disulfide linkages of proteins, in the synthesis of the deoxyribonucleotide precursors of DNA and in the protection of cells against free radicals (Meister 1983). GSH also serves as an agent regulating the process of melanogenesis. Low GSH levels have been associated with the deposition of melanin in bird feathers, whereas high GSH levels inhibit melanogenesis (Galván and Alonso-Alvarez 2008, Galván and Alonso-Alvarez 2009, Hõrak et al. 2010). Hence, GSH might play a role in trade-offs between antioxidants and melanogenesis. I suspect this might be the case in the black bulbuls: individuals in 2011 might had lower GSH than those in 2010, therefore had brighter plumage

but higher oxidative stress.

The different correlation between melanin-based ornaments and oxidative stress in two years- I only discovered a significant relationship in the 2011 group, not in the 2010 group- might also provide support for the GSH hypothesis. A study in rats suggested that corticosterone could decrease glutathione levels (Patel et al. 2002). In my study, individuals in 2011 suffered almost three times the oxidative stress of those in 2010, there it might be expected a relatively lower level of GSH in 2011 than in 2010. With a lower level of GSH, individuals could enhance the darkness or the size of patch of melanin-based ornaments (Galván and Alonso-Alvarez 2008, Galván and Alonso-Alvarez 2009, Hõrak et al. 2010). Therefore the positive correlation between oxidative stress and coloration of melanin-based traits would be expected.

The status of oxidative stress I test here should not reflect the physical condition in real time or during molting, but is rather the long-term condition of an individual. Such long-term oxidative stress could be affected by many factors: genetic, environmental or different life-history stage (Monaghan et al. 2009). Therefore, black bulbuls might be able to use melanin-based coloration to evaluate individual's long-term physical condition. Consequently, the traits might be important in sexual selection or individual assessment in animal aggressive behavior in the Himalayan black bulbul.

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Table 4.1. Two-way ANOVA tests of brightness in eight body parts

Pars	Source	F Ratio	<i>p</i>
Back	Sex	0.50	0.483
	Year ¹	0.47	0.495
	Year*Sex	0.10	0.759
Belly	Sex	0.01	0.921
	Year	5.84	0.019 *
	Year*Sex	3.58	0.063
Breast	Sex	0.89	0.350
	Year	6.05	0.017 *
	Year*Sex	0.11	0.738
Forehead	Sex	1.84	0.181
	Year	0.80	0.375
	Year*Sex	1.71	0.196
Nape	Sex	0.39	0.533
	Year	1.01	0.319
	Year*Sex	0.61	0.439
Remige	Sex	2.61	0.112
	Year	7.95	0.007 *
	Year*Sex	5.27	0.025 *
Scapulars	Sex	3.75	0.057
	Year	13.58	0.001 *
	Year*Sex	4.68	0.035 *
Tail	Sex	1.95	0.168
	Year	2.18	0.145
	Year*Sex	0.70	0.406

¹Factor Year: including 2010 and 2011

²Df=1

**p* < 0.05

Table 4.2 Multiple regressions of brightness in different body parts in 2010 and 2011

Parts	Terms	2010		2011	
		<i>t</i> Ratio	<i>p</i>	<i>t</i> Ratio	<i>p</i>
Back	Intercept	7.25	<.0001	29.49	<.0001
	Sex(F)	0.25	0.808	0.83	0.413
	Log (H/L ratio) ¹	-0.05	0.964	-1.14	0.262
	Sex(F)*Log (H/L ratio)	-1.46	0.166	-1.49	0.144
Belly	Intercept	6.09	<.0001	24.15	<.0001
	Sex(F)	-1.37	0.194	1.53	0.134
	Log (H/L ratio)	0.78	0.447	-1.35	0.183
	Sex(F)*Log (H/L ratio)	-0.85	0.409	0.35	0.728
Breast	Intercept	9.00	<.0001	24.53	<.0001
	Sex(F)	0.65	0.525	0.93	0.358
	Log (H/L ratio)	0.19	0.853	-2.43	0.019 *
	Sex(F)*Log (H/L ratio)	-1.44	0.172	-0.46	0.645
Forehead	Intercept	3.85	0.002	17.16	<.0001
	Sex(F)	1.52	0.150	-0.02	0.984
	Log (H/L ratio)	-0.49	0.635	-0.39	0.696
	Sex(F)*Log (H/L ratio)	-0.43	0.675	0.18	0.858
Nape	Intercept	7.03	<.0001	30.95	<.0001
	Sex(F)	-0.75	0.465	0.01	0.995
	Log (H/L ratio)	0.70	0.497	-0.62	0.539
	Sex(F)*Log (H/L ratio)	0.18	0.858	-1.2	0.238
Scapulars	Intercept	8.07	<.0001	40.47	<.0001
	Sex(F)	-2.22	0.043*	0.03	0.976
	Log (H/L ratio)	-1.01	0.329	-2.37	0.022 *
	Sex(F)*Log (H/L ratio)	-0.76	0.461	-0.93	0.358
Remige	Intercept	5.70	<.0001	49.39	<.0001
	Sex(F)	-2.41	0.032*	0.59	0.555
	Log (H/L ratio)	-0.67	0.515	-0.28	0.782
	Sex(F)*Log (H/L ratio)	-1.60	0.133	-0.78	0.440
Tail	Intercept	6.23	<.0001	27.93	<.0001
	Sex(F)	0.31	0.759	2.1	0.042 *
	Log (H/L ratio)	0.13	0.899	0.34	0.739
	Sex(F)*Log (H/L ratio)	1.01	0.330	-0.36	0.724

¹ Log transformation of heterocytes / lymphocyte ratio due to the non-normal distribution of original H/L data.

**p* < 0.05

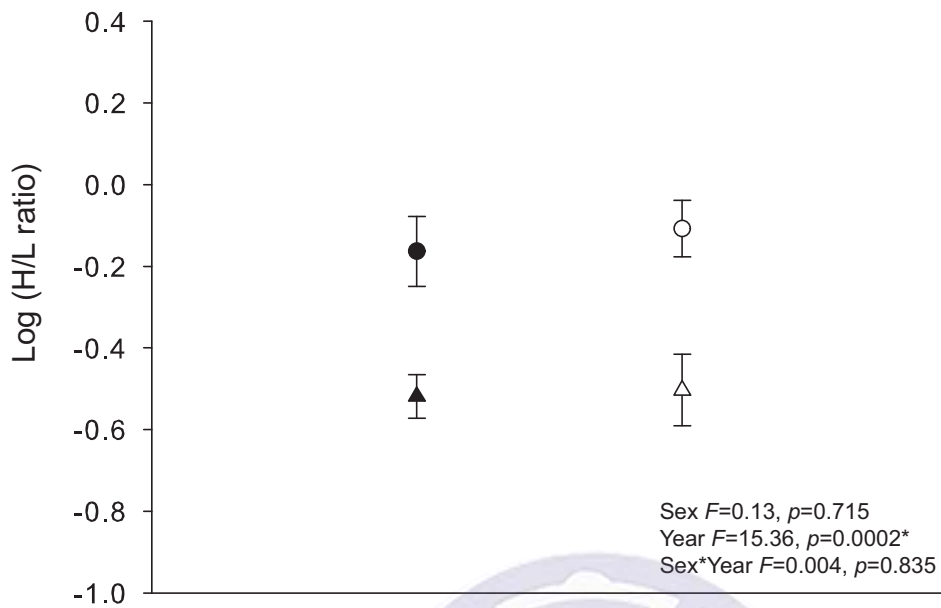


Figure 4.1. The Log (H/L ratio) of two sexes in different years. The circles indicate the average Log (H/L) ratios for 2011 and the triangles indicate those for 2010. The lines indicate the Standard Error. The solid symbols represent the females and the hollow ones represent the males. Two-way ANOVA, factor year includes 2010 and 2011. Df=1, $*p < 0.05$

Chapter Five

**Carotenoid-based traits alone might not be the
sexually selected cues for female
Himalayan black bulbuls
(*Hypsipetes leucocephalus nigerrimus*)**



Abstract

Informative integumentary coloration plays vital roles in bird communication. However, determining whether such ornaments on a bearer can be perceived for a receiver is imperative for understanding avian communication. Carotenoid-based ornaments involving a red, orange, or yellow are considered to reflect the bearers' physical conditions in numerous avian species. According to the "indicator hypothesis", females can use these informative traits as the cue for sexual selection. However, studies have revealed that not every species can use these informative traits as sexually selected traits. In the current study, I tested whether informative carotenoid-based bills and tarsi can act as the target traits of female preference to understand the roles of carotenoid based parts in the Himalayan black bulbul (*Hypsipetes leucocephalus nigerrimus*). The results indicate that females did not have significant preferences for carotenoid-richer (redder) males. I argue that carotenoid-based bills and tarsi alone might not be the sexually selected traits for female Himalayan black bulbuls.

Keywords: carotenoid-based trait, female preference, Himalayan black bulbul (Hypsipetes leucocephalus nigerrimus), sexual selected trait.

Introduction

Birds have a complex and diverse integumentary coloration, which generally indicates information about the bearer; such information can be used to determine the species, age (different appearances among different ages, e.g., Inouye et al. 2001), or an individual bird's quality (e.g., Faivre et al. 2003). Such informative coloration could play essential roles in bird communication, such as being indicators of mate choice (Hill 2006) or assessing opponents (Senar 2006). However, a complete communication involves both a sender and a receiver (Stevens 2013), and determining whether the informative ornament can be perceived by the receiver is imperative to understanding avian communication.

Carotenoid-based ornaments are represented in red, orange, or yellow coloration and are commonly considered to serve as indicators reflecting individual qualities in birds (McGraw 2006). Carotenoids are essential antioxidants and must be ingested by animals (Brush 1990). Through antioxidation, carotenoids can directly or indirectly affect an individual's immune response and affect multiple life history traits such as molting, migration (McGraw 2006). The amount of carotenoids that an animal can use is also limited by the genotypes and physiological conditions of animals themselves (Olson and Owens 1998); therefore, trade-offs must be made in carotenoid allocation between ornamentation and other somatic needs. Such trade-offs should render to correlation between the expression of carotenoid-based ornaments and animals' physical conditions (Svensson and Wong 2011). Studies on birds have revealed that compared with individuals with carotenoid-richer ornaments are typically healthier (e.g., Blount et al. 2003, Faivre et al. 2003, Saks et al.

2003), more agile (e.g., Blount and Matheson 2006, Pryke and Andersson 2003), or more aggressive (e.g., Pryke et al. 2001) compared with those with carotenoid-lesser ornaments; they also provide more favorable resources and better territories for their mates and offspring (e.g., Casagrande et al. 2006, Senar et al. 2002).

Traditionally, according to the “indicator hypotheses” that suggest that females choose mates according to informative traits (Andersson and Simmons 2006), carotenoid-based ornaments are considered to function as sexually selected traits which correlated with male mating success; this has been demonstrated in several avian species (e.g., Blount et al. 2003, Burley and Coopersmith 1987, Faivre et al. 2003). However, other studies have revealed that although carotenoid-based ornaments might reflect an individual’s condition, they might not always be used by females as the targets of mate choice (e.g., Horak et al. 2001, Nolan et al. 2006, Viera et al. 2008). This suggests that the utility of carotenoid-based ornaments in animal communication may be more complicated than conventionally understood.

Himalayan black bulbuls (*Hypsipetes leucocephalus nigerrimus*) have black plumage and carotenoid-based bills and tarsi. The red parts of these birds are conspicuous because of the dull plumage coloration. Previous studies have indicated that the red bill is the indicator of a black bulbul’s physical condition, including its immunocompetence and level of oxidative stress (Chapter Three). According to “indicator hypothesis”, I hypothesize that female black bulbuls choose mates on the basis of the coloration of the red bill and tarsi; specifically, females show higher preferences for males with redder bills and tarsi than for other males. To

test this hypothesis, I artificially reduced the redness of the bill and tarsi to determine whether female bulbuls show preferences to males with redder bills and tarsi (i.e., carotenoid-rich individuals). The results of this study can increase understanding of the roles of carotenoid-based integuments in birds.

Materials and methods

Captive and molecular sex typing

I purchased 47 black bulbuls from a pet-shop in Taiwan in the winter of 2008. Four to five individuals of the same sex were kept in the same cage measuring 40 cm x 40 cm x 60 cm. Different sexes were separated by opaque plastic veils, but acoustic contacts were allowed. All birds were fed with a mixed diet of commercial bird food and fresh fruits daily. The birds were housed in the outdoor and experienced the natural light cycle. I drew 150 μ L of blood from each individual and used for molecular sex-typing. Genomic DNA was extracted from blood samples with traditional proteinase *K* digestion followed by LiCl extraction (Gemmell and Akiyama 1996). The detailed programming of polymerase chain reactions (PCRs) for molecular sex-typing (2550F / 2718R, Fridolfsson and Ellegren 1999) was the same as that of Hung et al. (2015a in revision). A total of 20 males and 27 females were identified.

Color measurements

The reflectance values of the red bill and tarsi were measured using a USB2000 spectrometer (Ocean Optics Inc., Dunedin, U.S.A.) with illumination from an HL2000 halogen light source. The measurement procedures were the same as those of Hung et al. (2015b in revision).

Redness ($\text{Hue}_{550-700\text{nm}}$) was calculated for the bill and tarsi by deriving the wavelength of the mean of maximum and minimum reflectance values in the wavelength range of 550 - 700 nm (Thomas and Gausman 1977). A higher hue indicates higher redness.

Female preferences experiments

Female preference experiments were conducted at the beginning of the reproductive season (March to April) in 2009. For each trial, I randomly selected two males. One as the experimental male, the bill and tarsi of which were artificially painted with orange acrilan, and one as the control male, the bill and tarsi of which were painted with transparent acrilan.

I measured the proximity preference of females that were given a choice of a red-billed (control group) or an orange-billed male (orange group) in a dichotomous choice chamber (Fig. 5.1). I quantified the proximity preference of a female according to the amount of time the female spent in front of a respective male. In each trial, I randomly selected a female bird to be placed in the central cage, and two males to be placed in each of the cages next to the central one. The birds were placed in the apparatus for 15 min to acclimate to the new environment, while water and food were provided ad lib. During the acclimatization period, the birds were visually separated by the opaque plastic veils. After acclimatization, the water, food and veils were removed, and the position of the female was then recorded for 1h with a digital video recorder (Sony DCR-SB62). The amount of time a female spent perched in front of the male's compartment was defined as the preference time. The amount of time a female spent perched in the neutral zone was defined as the neutral time, indicating no preference to either male. I assumed a trial

was successful when the neutral time was less than 30 min. After excluding the neutral time, I calculated the percentages of time females stayed close to the control and orange males, respectively. The female preference toward each male in each trial was arcsine transformed then tested using the paired *t*-test.

I also measured the lengths of the bills, heads, wings and tails and the body weight of tested males to assess the correlation between these traits and female preferences. The precision of the length is to 0.1mm. The statistical analysis was conducted using SAS JMP 7.0.

Results

In the male birds, the average redness (Hue_{550-700nm}) of the bills was 594.5 ± 1.6 nm (mean \pm SE) and that of the tarsi was 589.8 ± 1.9 nm; in the females, the averaged redness of the bills was 593.5 ± 1.4 nm and that of the tarsi was 591.5 ± 1.7 nm. The redness between the control and orange groups was significantly different after treatment (Hue_{orange group} = 579.0 ± 5.2 ; Hue_{control groups} = 601.8 ± 4.4 ; paired *t*-test, $t_{23} = 3.35$, $p = 0.0004$). The experimental coloration ranged within the coloration variability of the males (572.9 - 612.7 nm).

I conducted a total of 24 experimental trials, 16 of the 24 trials which were considered successful. Among the 16 successful trials, the females showed preferences for the red males in seven trials, and showed preferences on orange males in nine trials. Moreover, on average, the females spent 1085.4 ± 184.5 s near control males (red males), 995.3 ± 155.7 s near the experimental males (orange males) and 1451.3 ± 188.2 s

in the neutral zones. I determined that the females did not show higher preferences for the control males (0.51 ± 0.06) than they did for the orange males (0.49 ± 0.06 ; Fig. 5.1, paired t -test, $t_{15} = -0.17$, $p = 0.87$). In addition, the preferences were not correlated with males' morphometric traits (multiple regression, $r^2 = 0.25$, $p = 0.73$, supplementary table 5.1).

Discussion

The results reveal that the females did not significantly prefer the redder males, indicating that the carotenoid-based bared parts might not be the sexually selected trait for female Himalayan black bulbuls. I propose two possible explanations for the failure to detect the preference of females for redder males.

My results are consistent with those of a recent study (Dey et al. 2015), suggesting that carotenoid-based bared parts in birds, like bills, tarsi, eye rings, crests or wattles, may serve as informative cues about physical conditions but not sexually selected cues. Dey et al. (2015) reported a positive correlation between the occurrence of carotenoid bill coloration and two ecological factors associated with high rates of agonistic encounters (colonial breeding and nonbreeding sociality), but not with sexual dichromatism. Previous studies have also suggested that bill coloration can change faster (Ardia et al. 2010, Faivre et al. 2003) than plumage coloration (Ardia et al. 2010) and the bill coloration reflects a bird's real-time quality. Therefore, the carotenoid-based bared parts in

birds are more likely to be selected for social interactions than for mate choice. Because individual birds experience intense competition with numerous unknown birds in nonbreeding flocks and in breeding colonies, the carotenoid-based bared parts of such birds may serve as signals of their physical conditions or fighting ability, enabling them to settle contests quickly without resorting to physical conflicts (West-Eberhard 1983). Therefore, Dey et al. (2015) suggest that carotenoid-based bills might primarily evolve from social interaction, and then subsequently evolve into sexual selection functions through other mechanisms.

Although Himalayan black bulbuls are socially monogamous, they live in groups during nonbreeding seasons (Severinghaus et al. 2012), and they can be extremely aggressive when in groups (personal observation). Therefore, it is imperative for these species to develop honest quality traits reflecting their physical conditions or fighting ability -to avoid physical conflicts. Furthermore, female black bulbuls may not yet be able to discern these traits as a cue for mate choice.

The other potential reason of showing no female preferences on red male is the possibility of using multiple cues in female Himalayan black bulbuls for mate choice. Results from a previous data (Hung et al 2015 in revision) suggested that several black (melanin-based) parts of the plumage are sexually dichromatic and could reflect individuals' oxidative stress, which are the indirect indicators of sexually selected traits (Andersson and Simmons 2006, Andersson 1994). Several avian species, such as Northern cardinals (*Cardinalis cardinalis*, Jawor et al. 2004), have been reported to use multiple cues in mate selections. The benefit of multiple sexual ornaments is that these traits can provide females

different types of information at various stages of the mate choice process (Borgia 1995), or function as redundant signals to improve the accuracy of mate assessment (Johnstone 1994, Moller and Pomiankowski 1993). Females can also use diverse characteristics in different breeding seasons as the current environmental conditions vary (Chaine and Lyon 2008, Qvarnström et al. 2000). Because I manipulated only carotenoid-based bared parts in our experiments, it is possible that females could use other informative characteristics to assess their potential mates.

Studies on the roles of carotenoid-based ornaments in avian species have typically assessed the correlations between the expression of the traits and the bearers' qualities. However, it is necessary to conduct experiments to ensure that the receivers do use these traits. I thus conducted this study to fill the gap regarding the functions of carotenoid-based ornaments in Himalayan black bulbuls' communications. Nevertheless, although my results do not reveal the role of carotenoid-based ornaments in the sexual selection of Himalayan black bulbuls, they can serve as the basis for determining the functions of carotenoid-based traits in other aspects, such as signals of fighting ability (Pryke et al. 2001) or social status (Pryke et al. 2002).

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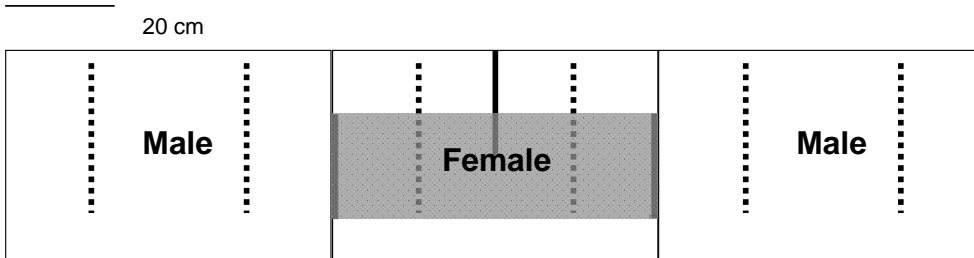
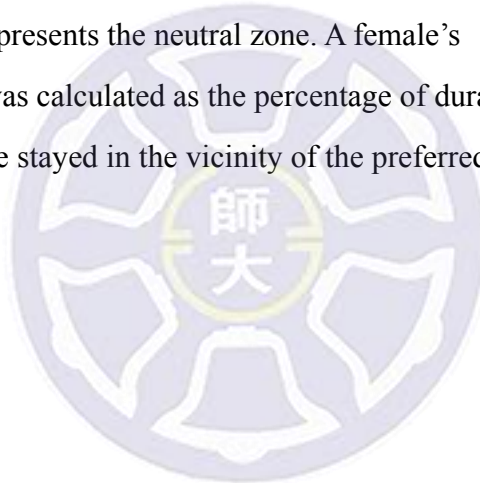


Fig. 5.1 Plain view of the mate choice apparatus (adapted from (Griggio et al. 2007). The dashed lines in the scheme indicate the position of the perches. The bold lines indicate the opaque dividers. Area marked in gray represents the neutral zone. A female's preference for a male was calculated as the percentage of duration of the total time the female stayed in the vicinity of the preferred male (i.e., preference time)



Chapter Six

General conclusions and perspectives

Overviews

My results reveal cryptic sexual dichromatism in both carotenoid-based and melanin-based traits that cannot be detected by the human visual system. The subsequent molting experiments demonstrated that the bill coloration of a bird was highly correlated with its physical conditions: redder bills had higher immunocompetence and incurred lower levels of oxidative stress. A significant correlation was observed between a bird's melanin-based plumage and oxidative stress levels, indicating that the black plumage may be a cue about the physical condition of a bird. However, the insignificant female preference for redder males rejected the hypothesis that the bill coloration served as a sexually selected trait. Although I did not discover the functions of both carotenoid-based and melanin-based traits in the Himalayan black bulbul, according to the communication theory, I suggest that both trait types can play a role in focal species communication.

From the summary of my results (Table 6.1), I found that not all sexually dichromatic characteristics, such as the tarsi, remige and tail, can reflect an individual bird's immunocompetence or oxidative stress level. This may be because I examined only the "quality" of an individual bird in a limited number of aspects. Several types of the quality have been reported to be correlated with the coloration, including parental caring, nesting

sites or territory quality and social ranking or good genes (Dale 2006). In the current study, the sexually dichromatic parts that did not reflect individual bird's physical conditions may serve as indicators of other dimensions of the quality to the birds.

My results suggest the role of carotenoid-based bared parts (such as the bill, eye rings, leg or wattle) in avian species. In contrast to feathers, the color of bared parts may change rapidly and may therefore reflect the short-term physical conditions of the bearer (e.g., Faivre et al. 2003b, Pérez-Rodríguez and Viñuela 2008, Rosenthal et al. 2012). Compared with studies on the plumage of birds, studies exploring the links between birds' physical conditions and coloration of carotenoid-based bared parts are relatively scant. To my knowledge, the roles of the carotenoid-based bill have been studied in only four avian species: European blackbirds (*Turdus merula*, Faivre et al. 2003a, Faivre et al. 2003b), red-legged partridges (*Alectoris rufa*, Pérez-Rodríguez and Viñuela 2008), zebra finches (*Taeniopygia guttata*, Blount et al. 2003), and mallard ducks (*Anas platyrhynchos*, Peters et al. 2004). In these species, the bill coloration has been determined to be positively correlated with immunocompetence (Faivre et al. 2003b, Jawor and Breitwisch 2003, Mougeot et al. 2009, Peters et al. 2004). However, no study has examined the correlation between bill coloration and oxidative stress. My works provide another case that bill coloration was positively correlated with a bird's immunocompetence and negatively correlated with its oxidative stress level. Regarding the functions of the carotenoid-based bill, my results are not conclusive. The carotenoid-based bared parts are the sexually selected traits in the mentioned species. Nevertheless, a recent

comparative study revealed that carotenoid-based bill is highly common in species living in social groups during the nonbreeding season, and in species nesting in colonies; it is not related to either sexual dichromatism or sexual size dimorphism (Dey et al. 2015). The carotenoid-based bill was suggested to primarily evolve as a social signal. Although my results do not provide the function of the carotenoid-based bared parts, they indicated that the red bared parts alone may not be cues for female preference in Himalayan black bulbuls. Himalayan black bulbuls are socially monogamous, but they live in groups during non-breeding seasons; therefore, my results support the hypothesis proposed by Dey et al. (2015).

The concept of sexual selection being the major force producing conspicuous coloration in birds has been subjected to debate recently. Dunn et al. (2015) suggested that natural selection is also attributable to evolution of diverse colorations. Because species with less sexually dichromatic traits may be under lower pressures of sexual selection (Andersson 1994), studying the functions of different pigmentations on less sexually dichromatic species can facilitate understanding the mechanism behind evolution of coloration in birds. To my knowledge, such studies have been conducted on only three less sexually dichromatic species: the great tits (*Parus major*), the blue tits (*Parus caeruleus*) (Delhey and Peters 2008), and the king penguins (*Aptenodytes patagonicus*, Jouventin et al. 2005). Similar to Himalayan black bulbuls, all these species are sexually monomorphic in human visions but sexually dichromatic in avian visions (Delhey and Peters 2008). In all three species, carotenoid-based ornaments serve as only quality signals

(reviewed in Delhey and Peters 2008, Viera et al. 2008), but not sexually selected traits. In great tits, the sexually selected trait is the area of melanin-based plumage on the breast (Delhey and Peters 2008, Norris 1990); whereas in blue tits, it is the UV plumage on the head (Delhey and Peters 2008, Hunt et al. 1999) as for the mutual sexually selected trait. Moreover, in king penguins, it is the UV coloration in the colorful bill as for the mutual selected traits (Nolan et al. 2010). My results are similar to those of aforementioned studies mentioned: carotenoid-based ornaments are quality cues but might not solely serve as sexually selected traits. However, so far I do not have further evidence revealing the mechanism associated with that causes the different functions of carotenoid-based ornaments in less sexual dichromatic avian species.

My results indicate that several carotenoid-based and melanin-based parts were sexually dichromatic or informative. This suggests that multiple ornaments as sexually selected traits in Himalayan black bulbuls. Studies have revealed that females might choose a mate according to multiple sexual ornaments (Chaine and Lyon 2008, Doucet and Montgomerie 2003). The benefit of multiple sexual ornaments is that these traits provide females with different types of information at various stages of the mate choice process (Borgia 1995) or function as redundant signals to improve the accuracy of mate assessment (Johnstone 1994, Moller and Pomiankowski 1993). Furthermore, females can use diverse characteristics in different breeding seasons as the current environmental conditions vary (Chaine and Lyon 2008, Qvarnström et al. 2000). Like in Northern cardinals (*Cardinalis cardinalis*), females use several carotenoid-based parts and the size of melanin-based masks

simultaneously to choose their mates (Jawor and Breitwisch 2004, Jawor et al. 2003).

Future perspectives

Social signal testing

In my dissertation, I did not test whether carotenoid-based or melanin-based traits function as indicators of the social status of an individual or contain information about individual qualities that can be useful in their social interactions, such as hierarchy or aggressiveness. Studies have suggested that both types of traits are functional in social interaction in several avian species (Jawor and Breitwisch 2003, Roulin et al. 2008). Because Himalayan black bulbuls live in groups during nonbreeding seasons and exhibit strong aggressiveness when in captivity with other individuals (personal observation), it is imperative for this species to develop social signals reflecting their physical conditions and fighting ability to reduce conflicts in social interactions. Therefore, I suggest that future studies could examine the correlation between color variations and social ranking (or fighting ability) of both trait types in black bulbuls.

Testing the possibility of other signal traits

In addition to carotenoid-based and melanin-based colorations, other traits can signal an individual's quality or be used for mate choice, such as song (Shutler and Weatherhead 1990) and UV coloration (Prum 2006). I observed UV light reflection from the bill and tarsi in Himalayan black

bulbuls. This can also serve as a target for signaling in black bulbuls.

Apart of UV light, bird songs have also been proved to be quality signals in other species (e.g., Gil and Gahr 2002). The songs of Himalayan black bulbul are simple and can be performed by both sexes (personal observation) in an acoustic range (from 20Hz to 20KHz, Pytte et al. 2004); however, such songs have not been analyzed systematically before. Nevertheless, Himalayan black bulbuls have been reported to sing ultrasonic sound (singing range >20KHz, Li et al. 2011). Several avian species were reported to sing in the ultrasonic sound range, which was determined to be indicator of individual quality in amphibians (e.g., Arch et al. 2008). Testing whether the songs both in acoustic and ultrasonic ranges could be vital functions in the focal species is imperative.

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Table 6.1 The summary of my results

Item	Sexual dichromatism	Quality cues	Sexual selected cues (by females)
Carotenoid-based traits	Bill	□	x
	Tarsus	□	x
Melanin-based traits	Back	x	x
	Belly	x	x
	Breast	x	□
	Forehead	x	x
	Nape	x	x
	Remige	x	x
	Scapula	x	□
Tail	□	x	

“□” indicates “yes” to the question.

“x” indicated “no” to the question

Table S2.1 Two-way ANOVA of different melanin-based parts in skin specimens

Parts	Variables	Total brightness		Chroma _{uv}	
		<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>
Back	Sex	0.79	0.38	1.99	0.17
	Year	1.44	0.26	1.73	0.20
	Sex*Year	1.65	0.22	0.99	0.39
Belly	Sex	1.94	0.18	3.20	0.09
	Year	0.79	0.47	2.52	0.10
	Sex*Year	0.07	0.94	0.11	0.90
Nape	Sex	0.08	0.79	0.71	0.41
	Year	0.38	0.69	0.99	0.39
	Sex*Year	0.51	0.61	1.15	0.34
Breast	Sex	0.05	0.84	4.37	0.05
	Year	0.94	0.41	0.88	0.43
	Sex*Year	0.14	0.87	0.11	0.90
Forehead	Sex	0.00	0.97	1.47	0.24
	Year	0.09	0.91	1.26	0.30
	Sex*Year	2.37	0.12	0.73	0.50
Remige	Sex	0.22	0.65	0.15	0.70
	Year	3.14	0.06	0.11	0.90
	Sex*Year	0.69	0.52	0.05	0.95
Scapular	Sex	0.03	0.86	0.72	0.41
	Year	3.70	0.04	0.34	0.71
	Sex*Year	2.68	0.09	0.11	0.89
Tail	Sex	0.39	0.54	0.96	0.33
	Year	0.39	0.68	0.24	0.79
	Sex*Year	0.35	0.71	0.98	0.39

df: year-2, sex-1, sex*year-2

Year: indicating the years after making into skin specimens, including within 5yrs, 10yrs and 15yrs.

Bold type indicates statistic significant (Bonferroin adjusted $p = 0.005$).

Table S2.2 Two-way ANOVA of different melanin-based parts between live birds and skin specimens

Parts	Variables	Total brightness		Chroma _{uv}	
		<i>F</i>	<i>p</i>	<i>F</i>	<i>p</i>
Back	Item	2.11	0.15	31.24	<.0001
	Sex	2.48	0.12	8.95	0.003
	Item*Sex	2.78	0.10	8.93	0.003
Belly	Item	0.64	0.42	11.63	0.001
	Sex	7.18	<0.001	13.76	<0.001
	Item*Sex	0.02	0.90	1.05	0.31
Nape	Item	4.03	0.05	39.74	<.0001
	Sex	0.05	0.83	9.32	0.003
	Item*Sex	0.01	0.96	4.31	0.04
Breast	Item	10.30	<0.002	41.82	<.0001
	Sex	0.34	0.56	13.10	0.0004
	Item*Sex	0.10	0.75	7.63	0.007
Forehead	Item	0.01	0.94	26.81	<.0001
	Sex	0.17	0.68	9.41	0.003
	Item*Sex	0.72	0.40	5.53	0.02
Remige	Item	0.00	0.97	4.68	0.03
	Sex	2.77	0.10	2.16	0.14
	Item*Sex	0.19	0.66	0.09	0.76
Scapular	Item	8.64	<0.004	21.40	<.0001
	Sex	0.31	0.58	9.77	0.002
	Item*Sex	0.54	0.46	3.35	0.07
Tail	Item	19.52	<.0001	9.42	0.003
	Sex	1.97	0.16	2.30	0.13
	Item*Sex	0.10	0.76	0.06	0.81

Item: live bird and specimen, df= 1

Sex: female and male, df= 1

Item*sex: df=2.

Bold type indicates statistic significant (Bonferroin adjusted $p = 0.005$).

Table S 4.1 Multiple regressions of female preference and male morphometrics.

Term	Estimate	Std Error	t	<i>p</i>
Intercept	-2219.97	1352.23	-1.64	0.13
Bill	425.68	479.23	0.89	0.39
Head	-50.79	301.64	-0.17	0.87
Tarsus	418.40	453.18	0.92	0.38
Wing	30.12	18.58	1.62	0.13
Weight	7.82	7.14	1.09	0.30

Female preference : (the amount of time a female spent on the perch in front of the male* 100) / (total choice time)

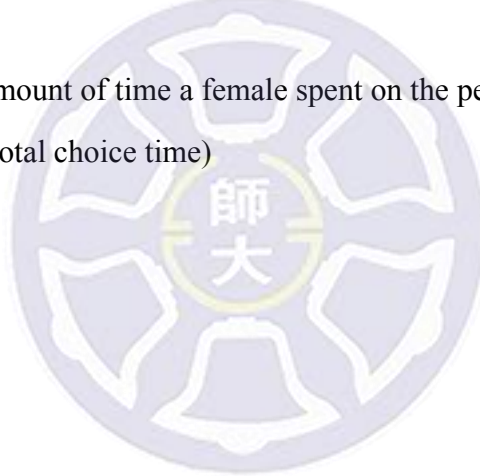


Table S 5.1 Multiple regressions of female preference and male morphometrics.

Term	Estimate	Std Error	t	<i>p</i>
Intercept	-2219.97	1352.23	-1.64	0.13
Bill	425.68	479.23	0.89	0.39
Head	-50.79	301.64	-0.17	0.87
Tarsus	418.40	453.18	0.92	0.38
Wing	30.12	18.58	1.62	0.13
Weight	7.82	7.14	1.09	0.30

Female preference: (the amount of time a female spent on the perch in front of the male* 100) / (total choice time)

