Ibis (2007), **149**, 264–270

Cryptic sexual dichromatism occurs across multiple types of plumage in the Green-backed Tit *Parus monticolus*

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Several recent studies have found instances of cryptic sexual dichromatism within avian taxa. Although this dichromatism has been found in plumage produced through a variety of proximate mechanisms, little is known about how dichromatism varies across these types of plumage within a single species. We used a reflectance spectrometer to measure colour within the Green-backed Tit *Parus monticolus*, a species which displays multiple types of pigment and structural colours. We found significant differences in spectral measurements corresponding to hue, chroma, and brightness between male and female carotenoid, melanin, structural white, grey and structural blue plumage. The only plumage that did not appear to show sexual dichromatism was the olive plumage of the back. These findings suggest that the mechanism(s) producing cryptic dichromatism in the Green-backed Tit are non-specific and act across multiple types of plumage, rather than within a single type, such as carotenoid-based or structurally produced.

Sexual dichromatism, or colour differences between males and females, has been studied by evolutionary biologists and ornithologists for over a century (Darwin 1871; Owens & Hartley 1998). Birds, with their well-developed visual systems and elaborate, colourful displays, exhibit a wide variety of examples of sexual dichromatism across numerous taxa. These cases of dichromatism range from obvious or blatant dichromatism where males and females appear so different that they were initially thought to be separate species, such as the Mallard *Anas platyrhynchos* (Andersson 1994), to subtle or cryptic differences in taxa that were originally thought to be monomorphic, such as the Black-capped Chickadee *Parus atricapillus* (Mennill *et al*. 2003).

The widespread use of reflectance spectrometers over the past decade has contributed greatly to the study of dichromatism. Reflectance spectrometers are quantitative and they are not subject to the biases of the human visual system (Bennett *et al.* 1994). Recently, many cases of dichromatism in the ultraviolet region of the spectrum have been discovered (e.g. Hunt *et al*. 1998, Eaton & Lanyon 2003). Additionally, subtle differences between males and females that are not apparent to the human eye (hereafter referred to as cryptic dichromatism, e.g. Mennill *et al*. 2003) are better quantified, further adding to the list of dichromatic taxa. Dichromatism has now been found across a wide range of avian taxa in many types of plumage, including structural blues (Hunt *et al*. 1998) and whites (Mennill *et al*. 2003), as well as pigment-based colours (e.g. carotenoids, Mays *et al*. 2004; melanins, Mennill *et al*. 2003).

Although many cases of cryptic dichromatism have been described, less is known about how dichromatism varies across plumage types within a single taxon. The Green-backed Tit *Parus monticolus* is an ideal species for studying this variation. Green-backed Tits have colour plumage produced through several different proximate mechanisms (see Discussion), including a bright-yellow breast (with a matt-black stripe running down the centre), a black throat, an iridescent black head (black with some blue visible depending on the viewing angle), an olive back, a blue-grey rump, white cheeks, and a blue wing-patch

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and tail (see illustration in MacKinnon & Phillipps 2000). Both males and females are monochromatic and appear indistinguishable in colour to the human eye, although they differ slightly in pattern, with males having a more pronounced stripe down the centre of their breast (Wang *et al.* 1991). The purpose of this study was to determine whether there were significant colour differences between male and female Green-backed Tits. If dichromatism was present we wanted to investigate whether it occurred within a single type of plumage (e.g. pigment-based or structurally produced, see Discussion) and/or a single body region (e.g. the prominently visible breast or rump), which would suggest a potential signalling role.

METHODS

Measurements

In order to investigate cryptic dichromatism in the Green-backed Tit, we used a reflectance spectrometer to measure specimens from the Taiwan Museum of Natural History and the Taiwan Endemic Species Research Institute. In all, 57 birds were measured: 34 males and 23 females. All were collected within Taiwan, and had been in the collection for 20 years or less at the time of measuring. There was no significant difference between males and females in specimen age (Mann–Whitney *U*-test, $n = 44$, $P = 0.13$) or month of collection (Mann–Whitney *U*-test, $n = 34$, $P = 0.14$). The year and month of collection were not available for all samples.

Measurements were taken from eight different body regions: crown (iridescent black), throat (black), cheek (white), back (olive), rump (blue-grey), lateral tail (blue), wing (folded, blue) and belly (yellow). In all cases, body regions were visually inspected before measurement and any region that did not appear to be in good condition was not measured (e.g. feathers were missing or damaged during the specimen preparation process). Measurements from each specimen were made in triplicate, whenever possible from non-overlapping spots within each body region. Reflectance was measured from 300 to 700 nm using an Ocean Optics (Dunedin, FL, USA) USB2000 spectrometer and a PX-2 full-spectrum light source. A bifurcated fibre-optic cable was used, which allowed for illumination and detection to occur through the same probe. An anodized aluminium cylinder with a 1.27-cm aperture prevented ambient light from entering. All measurements were taken perpendicular to the feather surface and were relative to a Spectralon diffuse white standard (Labsphere, North Sutton, NH, USA) and the dark. After measurement, raw spectral data were binned into 1-nm intervals using custom software, and replicates were averaged.

Colour quantification

Measurements from different body regions produced reflectance spectra with qualitatively different shapes (Fig. 1). Thus, we used a combination of colorimetric variables to quantify colour attributes (reviewed in Andersson & Prager 2006, Montgomerie 2006). Total brightness was calculated for all types of plumage by averaging the reflectance from 300 to 700 nm. In addition, for the breast and back (yellow and olive, respectively) we calculated the wavelength of 50% reflectance (λ_{R50}) by finding the wavelength of the mean between the maximum and minimum reflectance values. This method has been previously used to quantify spectral location, corresponding to hue or colour (Pryke *et al.* 2001) for carotenoid-based colours that typically have a shape resembling a step function (Lythgoe 1979). For the blue wing and tail we found the wavelength of maximum reflectance (λ_{max}) that defines the location of the spectrum and thus the perceived hue or colour for structural colours, which have reflectance peaks rather than plateaus (Sheldon *et al.* 1999). We also calculated saturation for the yellow and olive breast and back by calculating the difference between the maximum and minimum reflectance values (within the range 300–700 nm), divided by the sum of the maximum and minimum reflectance values for these two body regions. For the crown (iridescent black), rump (blue-grey), tail and wing, we calculated the UV chroma (perceptually similar to the saturation, but for consensus with previous studies we refer to it as UV chroma) by calculating the sum of reflectance from 300 to 400 nm divided by the sum of the reflectance from 300 to 700 nm (Siefferman & Hill 2003). We used a Kolmogorov–Smirnov test to confirm that no sets of measurements had distributions that were significantly different from the normal distribution. We then compared male values from each body region with female values from the same region using a *t*-test. Because we were performing multiple comparisons across colours thought to be produced by 4–7 different proximate mechanisms, we only considered differences less than or equal to 0.01 as statistically significant. Finally, we calculated the coefficient of determination for sex (R^2) at all

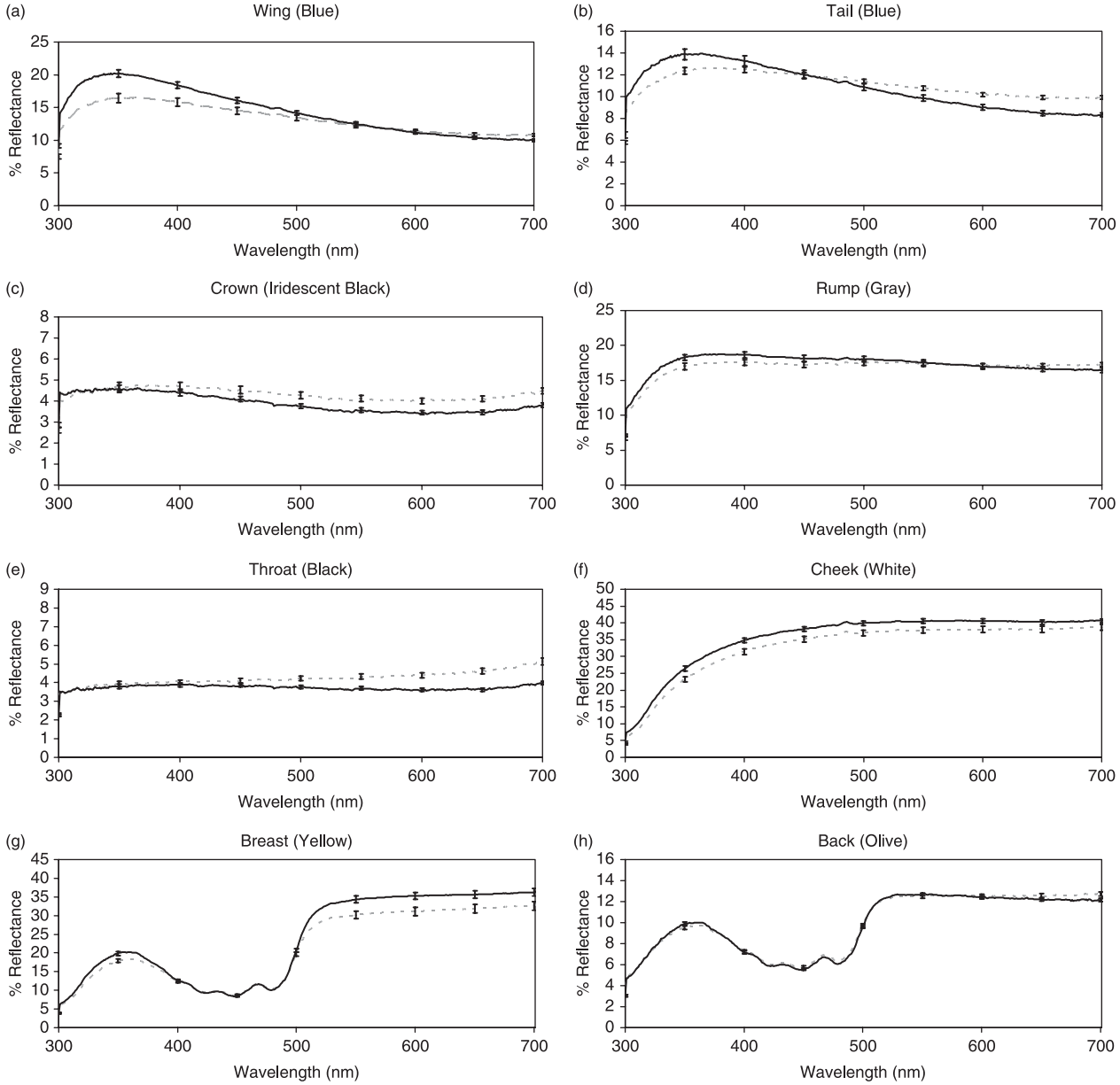

Figure 1. Graphs illustrating reflectance spectra from (a) wing, (b) tail, (c) crown, (d) rump, (e) throat, (f) cheek, (g) breast and (h) back. The solid black line corresponds to male plumage, the dotted grey line to female plumage. Bars represent standard errors.

regions which had significant differences between males and females. All statistics were performed using SPSS version 12.

Molecular sexing

Using molecular methods, we sexed three specimens for which the sex was unknown. Because our results are dependent on specimens being correctly sexed, we also molecularly sexed ten additional specimens (for which tissue samples were readily available) in order to confirm the sex independently. DNA was isolated from tissue samples stored frozen in ethanol using an LiCl extraction method (Gemmell & Akiyama 1996). Previously published sexing primers 2550F and 2718R (Fridolfsson & Ellegren 1999) were used to amplify a fragment of the *CHD* gene using the polymerase chain reaction (PCR). These primers

produce one (Z) band in males and two (Z + W) bands in most females (Fridolfsson & Ellegren 1999). PCR reactions were performed using standard reaction procedures (10 μL reaction volume, 50 ng DNA, 0.2 μM primer, 0.625 mM dNTP, 1× PCR buffer, 0.4 U *Taq* DNA polymerase, and 1.5–2.5 mM $MgCl_2$), denatured (94 °C for 3 min), then amplified for 30 cycles (30 s at 95 °C, 30 s at 46 °C, 90 s at 72 °C with a final 3 min of extension at 72 °C). Finally, PCR products were electrophoresed in 1× TBE buffer for 30 min. Sex was determined based on the number of bands present.

RESULTS

Colour measurements

The maximum reflectance of both the wing and the tail was in the UV region of the spectrum (Fig. 1a & 1b). In addition, we found that the crown, which appeared iridescent black to the human eye (Fig. 1c), and the blue-grey rump (Fig. 1d) also had UV reflectance peaks (particularly in males, see below). The black throat had low reflectance across all wavelengths (Fig. 1e). The white cheek (Fig. 1f) reflectance was constant over the human-visible (400–700 nm) portion of the spectrum but decreased sharply in the UV. The yellow breast had a reflectance spectrum characteristic of carotenoids, with a sharp increase in reflectance at middle wavelengths and a plateau at longer wavelengths (Fig. 1g). The olive colour of the back (Fig. 1h) had the same overall shape as that of the yellow breast, but with a much lower percentage reflectance at longer wavelengths, resulting in a less saturated colour (Table 1). Both male and female measurements within each body region were normally distributed (data not shown).

Dichromatism

We found significant differences ($P \leq 0.01$) between male and female colour measurements across all body regions except for the olive back (Fig. 1g, Table 1). The wing and the tail differed significantly in UV chroma and in λ_{max}, with males having greater UV chroma and a shorter-wavelength λ_{max} in both cases (Fig. 1a & 1b, Table 1). Both the black crown and the grey rump differed significantly in UV chroma, with males having greater UV chroma (Fig. 1c & 1d, Table 1). Both the black throat and the white cheek differed

Table 1. Comparison of quantitative colour values derived from reflectance spectra. A dash indicates regions for which measurement did not apply (see Methods for description of measurements).

			Wavelength λ_{max} or λ_{R50}			Brightness				Saturation or chroma	
	Sex	*n*	(nm)	sd	*P**	(%)	sd	*P**	(%)	sd	*P**
Breast	M	31	502	1.9	0.30	23.8	3.0	**0.01**	62.2	6.8	0.05
	F	22	501	1.6		21.6	2.8		58.1	8.2	
Crown	M	32	–	–		3.9	0.7	0.04	28.8	1.8	**0.00**
	F	22	–	–		4.3	0.8		26.4	1.5	
Cheek	M	26	–	–		35.7	3.1	**0.01**	–	–	
	F	20	–	–		33.0	3.4		–	–	
Back	M	30	498	1.0	0.78	9.8	0.7	0.71	40.0	4.1	0.56
	F	21	498	1.0		9.9	0.7		39.2	4.7	
Rump	M	31	–	–		17.4	2.2	0.42	24.7	1.2	**0.00**
	F	22	–	–		16.9	1.7		23.5	1.4	
Throat	M	31	–	–		3.7	0.5	**0.00**	–	–	
	F	22	–	–		4.2	0.6		–	–	
Tail	M	31	353	15	**0.00**	10.9	1.8	0.63	30.1	1.7	**0.00**
	F	23	373	17		11.1	1.0		26.6	1.3	
Wing	M	34	348	11	**0.00**	14.5	2.0	0.05	32.7	1.7	**0.00**
	F	23	359	11		13.3	2.1		29.2	1.7	

**t*-test, values of 0.01 or less are highlighted in bold.

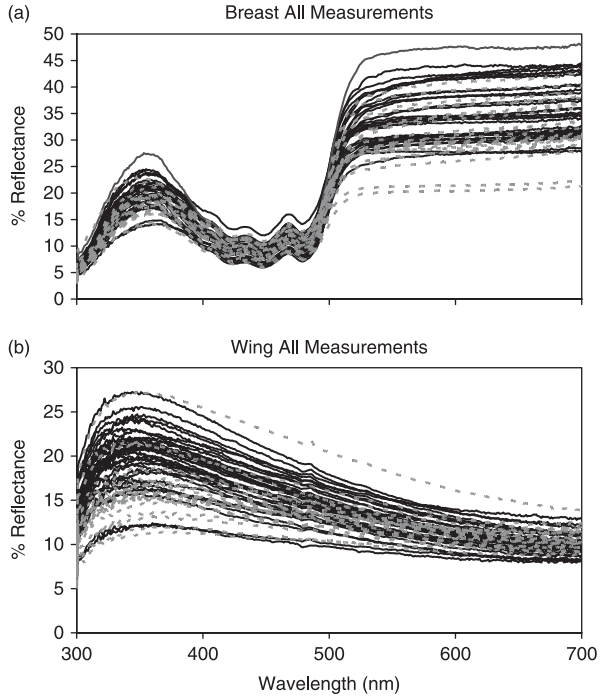

Figure 2. Graph illustrating the amount of male and female overlap at (a) the breast and (b) the wing. Sex explained 13% of the observed variation in breast brightness, 19% of wavelength variation in the wing and 52% of chroma variation in the wing. Each solid black line is a single male, each dotted grey line a female.

significantly in brightness, with males having darker throats and brighter cheeks (Fig. 1e & 1f, Table 1). Finally, the yellow breast differed significantly in brightness between males and females, with males having a greater brightness (Fig. 1h, Table 1).

We found considerable overlap between male and female colour measurements for all body regions measured (e.g. breast and wing, Fig. 2).

Molecular sexing

We were able to unambiguously confirm the sex of the three unknown specimens, all of which were male. The molecular sexing results from the other ten specimens agreed with the specimen's assigned sex in all cases.

DISCUSSION

Through the combined use of quantitative measures of colour, a large sample size and parametric statistics, we were able to detect subtle plumage differences between male and female Green-backed Tits. Although we found statistically significant colour differences between male and female Green-backed Tits across seven different body regions, whether these colour differences are biologically meaningful remains to be determined. Statistically significant differences do not directly translate into differences that are meaningful in a signalling context. On average, sex only explained about one-quarter of the observed variation across body regions (average 27%, range 9–56%). Furthermore, male and female Green-backed Tits also differ in the width of their breast stripe, so colour is not the only cue of sexual identity in this taxon. However, previous behavioural studies have suggested that very subtle plumage differences can have behavioural implications (e.g. Mennill *et al.* 2003, Doucet *et al.* 2005). When combined with the findings of several other recent studies (Eaton & Lanyon 2003, Eaton 2005), our results do suggest that cryptic dichromatism might be common and that true monochromatism could be rarer than previously thought.

Several recent studies have documented that changes in the skin colour of museum specimens may occur over time and that colour may also vary due to the season during which the specimen was collected (e.g. McNett & Marchetti 2005). Although we grouped samples of different ages (time in collection) and seasons in order to have a large sample size, we do not think these groupings are likely to change our finding of significant differences between males and females. If all the males we sampled had been collected recently (or during one season) and all the female specimens were older (or collected during a different season), then changes due to age or season might have been mistaken for sexual differences. However, there were no significant differences between male and female specimens in age or month of collection. Therefore, unless changes in specimen wear occur differentially between male and female specimens, grouping sexes and seasons might increase the error within each sex, but not the comparisons between the sexes. Furthermore, the two females with the lowest breast reflectance and the male with the highest breast reflectance (Fig. 2a) had similar ages and dates of collection (females: March 1989 and March 1991, male: January 1990). The grouping of ages and sexes could be part of the reason why sex explained such a small percentage of the variation.

We used the shape of the reflectance spectrum to determine the likely proximate mechanism that was producing plumage colour for each body region. Although biochemical or microstructural analyses

of colours are ideal, we feel that when carefully interpreted, general information can be derived from spectral shape in some instances. For example, even though they appear quite similar to the human eye (e.g. McGraw *et al*. 2004), reflectance spectra can distinguish between carotenoid-based colours and those produced by melanins because carotenoids characteristically have a secondary peak in the UV, and plateau at longer wavelengths, whereas melanin colours do not (e.g. Hofmann *et al*. in press). Furthermore, our designation of carotenoid, melanin and structural colours in the Green-backed Tit agrees with that of Blue Tits (*Cyanistes caeruleus*) and Great Tits (*Parus major*), for which the proximate mechanisms have been studied in considerable detail (reviewed in Andersson & Prager 2006, McGraw 2006a, 2006b, Prum 2006). These groupings are very general: carotenoid, melanin, and white, grey and blue/UV structural colours. We are not trying to infer that a particular carotenoid or concentration of pigment is present, only that this colour is probably produced by carotenoids.

Finally, the cryptic differences that we found in the Green-backed Tit occur across multiple types of plumage produced by very different proximate mechanisms. Therefore, our results suggest that cryptic dichromatism is not limited to plumage produced by a single proximate mechanism (e.g. pigment or structural colours). These findings differ from those of previous comparative studies of non-cryptic or obvious dichromatism in which different types of plumage (e.g. carotenoids) were found to correlate with (Gray 1996) or have a greater contribution to dichromatism (Badyaev & Hill 2000). Although our methods differed from the comparative approach used in these studies, as we focused on a single monochromatic taxon, our findings suggest that comparing the contribution of different pigments in cryptic and blatantly dichromatic taxa might be an interesting avenue for future research. It would also be interesting to investigate whether there is a continuum between cryptic and blatantly sexual dichromatism. Such studies may provide a better understanding of the ultimate mechanisms driving sexual dichromatism.

C.H. was supported by a National Science Foundation and Taiwan National Science Council East Asia and Pacific Summer Institutes Grant. C.H. would like to thank, in particular, Cheng-Te Yao of the Endemic Species Research Institute for his assistance in accessing collections, logistical support and sharing of expertise. Special thanks are also due to all the members of the Li laboratory for their help and support in Taiwan. Finally, Jeff Leips, Kevin Omland and Tom Cronin provided thoughtful comments on the manuscript.

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Received 24 March 2006; revision accepted 3 August 2006.