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Proximate basis of the covariation between a melanin-based female ornament and offspring quality

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Abstract In contradiction to sexual selection theory, several studies showed that although the expression of melanin-based ornaments is usually under strong genetic control and weakly sensitive to the environment and body condition, they can signal individual quality. Covariation between a melanin-based ornament and phenotypic quality may result from pleiotropic effects of genes involved in the production of melanin pigments. Two categories of genes responsible for variation in melanin production may be relevant, namely those that trigger melanin production (yes or no response) and those that determine the amount of pigments produced. To investigate which of these two hypotheses is the most likely, I reanalysed data collected from barn owls (*Tyto alba*). The underparts of this bird vary from immaculate to heavily marked with black spots of varying size. Published cross-fostering experiments have shown that the proportion of the plumage surface covered with black spots, a eumelanin composite trait so-called "plumage spottiness", in females positively covaries with offspring humoral immunocompetence, and negatively with offspring parasite resistance (i.e. the ability to reduce fecundity of ectoparasites) and fluctuating asymmetry of wing feathers. However, it is unclear which component of plumage spottiness causes these relationships, namely genes responsible for variation in number of spots or in spot diameter. Number of spots reflects variation in the expression of genes triggering the switch from no eumelanin production to production, whereas spot diameter reflects variation in the expression of genes determining the amount of eumelanin produced per spot. In the present study, multiple regression analyses, performed on the same data sets, showed that humoral immunocompetence, parasite resistance and wing fluctuating asymmetry of cross-fostered offspring covary with spot diameter measured in their genetic mother, but not with number of spots. This suggests that genes responsible for variation in the quantity of eumelanin produced per spot are responsible for covariation between a melanin ornament and individual attributes. In contrast, genes responsible for variation in number of black spots may not play a significant role. Covariation between a eumelanin female trait and offspring quality may therefore be due to an indirect effect of melanin production.

Keywords Female ornament · Fluctuating asymmetry · Genetic variation · Immunocompetence · Melanin

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Introduction

Evolutionary biologists studying the signalling function of colour traits distinguish carotenoid- from melanin-based ornaments (e.g. Owens and Hartley 1998; Badyaev and Hill 2000). This distinction is based on the premise that colour traits can honestly signal quality only if they are costly to produce or to bear (Zahavi 1975; Grafen 1990). The expression of carotenoid-based traits is sensitive to environmental factors such as dietary carotenoids (Hill et al. 1994), body condition and parasites (Fitze and Richner 2001), whereas the expression of melanin pigments is under strong genetic control (Hearing and Tsukamoto 1991; Roulin et al. 1998; Roulin and Dijkstra 2003). For this reason, melanin-based traits were hypothesised to not reveal individual quality in contrast to carotenoid-based traits (Badyaev and Hill 2000). However, several studies contradict this hypothesis, because melanin-based traits were experimentally shown to covary with individual attributes such as gonad size in feral pigeons (*Columba livia*, Murton et al. 1973) and dominance status in sparrows (Rohwer and Rohwer 1978). Furthermore, in barn owls (*Tyto alba*) females for which the proportion of

the plumage surface was covered by a larger amount of black spots (a eumelanin composite trait so-called "plumage spottiness") produced offspring that were better able to raise antibodies specifically directed against an artificially administrated antigen (Roulin et al. 2000), to reduce the fecundity of the ectoparasite *Carnus hemapterus* (Roulin et al. 2001a), and to grow feathers of the right and left wing symmetrically (Roulin et al. 2003a). The proximate basis of such covariations is still unknown.

Distinct genes can determine independently the switch from absence of black eumelanin pigments to its production and the amount that is produced. In birds and mammals, mutations at the melanocortin-1 receptor (Mc1r) are usually responsible for the presence or absence of a eumelanin coloration (Barsh 1996; Theron et al. 2001; Mundy et al. 2003; Nachman et al. 2003), whereas up-regulation of extracellular α-melanin stimulating hormone (α-MSH) causes continuous variation in the amount of eumelanin produced (Jawor and Breitwisch 2003) as shown experimentally in humans (Lerner and McGuire 1961) and mice (Geschwind 1966). Three distinct mechanisms can therefore be responsible for a covariation between a eumelanin-based trait and individual attributes. First, melanin pigments can directly affect physical and biological processes for example by protecting organisms against solar radiation (e.g. Berry and Willmer 1986) or pathogens (Mackintosh 2001; Wilson et al. 2001). Thermal melanism arises because dark surfaces absorb and radiate heat more quickly than pale surfaces (Majerus 1998). Melanin can trap microorganisms within humoral capsules, and biochemicals produced during melanogenesis can have antimicrobial activities (Mackintosh 2001). Second, an indirect effect of melanin production may be due to genes responsible for an abrupt change in coloration (e.g. from black to yellow due to mutations at Mc1r, Theron et al. 2001). However, I am not aware of any study demonstrating that mutations at genes such as Mc1r have pleiotropic effects on physiological processes such as immunocompetence, parasite resistance and developmental homeostasis. Third, another kind of indirect effect may arise if genes responsible for variation in the amount of eumelanin pigments (e.g. α-MSH, Jawor and Breitwisch 2003) have pleiotropic effects on important individual attributes. This mechanism is plausible because α-MSH plays an active role in innate host defence and other physiological processes (e.g. Catania et al. 2000; Ichiyama et al. 2000). The second and third mechanisms are worth considering because individuals may simultaneously vary in the probability that a given body surface entails melanin pigments (e.g. a feather may have a black spot or none) and the amount of melanin deposited on this body surface (e.g. a black spot can be small or large). Therefore, individuals can signal different qualities if they differ in both number and size of black spots. This is a biologically relevant aspect, since in several species mate choice can be exerted on multiple ornaments (Calkins and Burley 2003).

For a move into the direction of determining the proximate mechanism underlying covariation between eumelanin-based ornaments and phenotypic quality, I reanalysed published experimental data collected in the barn owl. This bird displays black spots on its ventral side (a eumelanin-based trait), and it also varies with respect to another polymorphic trait so-called plumage coloration (a phaeomelanin-based trait; continuous variation between reddish-brown and white). Plumage coloration is genetically correlated with plumage spottiness, reddish-brown individuals being spottier than white ones (Roulin 2003; Roulin and Dijkstra 2003). The proportion of the plumage surface covered by spots (plumage spottiness) in genetic mothers is associated with humoral immunocompetence (Roulin et al. 2000), parasite resistance (Roulin et al. 2001a), and fluctuating asymmetry (Roulin et al. 2003a) measured in their cross-fostered offspring. To record female plumage spottiness, an index of the amount of eumelanin produced, black spots were counted and their diameters measured. Determination of which of these two measures (i.e. number of spots or spot diameter) is associated with these three offspring attributes would provide relevant insights into which kind of genes may be responsible for covariation between a eumelanin-based trait and phenotypic qualities. For this purpose, I performed three multiple regression analyses with, in turn, humoral immunocompetence, parasite resistance and fluctuating asymmetry as dependent variables. In these three models, number of black spots and spot diameter were two independent variables (these multiple regression analyses were not published because at that time I did not realise their importance). I also included "plumage coloration" as a third independent variable. A significant contribution of the number of black spots would indicate that genes that trigger the production of black spots in the mother (independently of their size) play a role in offspring phenotypic traits. A significant contribution of spot diameter would reveal that genes responsible for variation in the amount of eumelanin pigments produced per spot in the mother plays such a role. Inter-individual variation in the amount of eumelanin pigments produced per spot could be due to inter-individual variation in the number of melanocytes or in melanocyte expression. This aspect is currently being investigated, and hence will not be further discussed in the present paper.

To better appreciate results from these multiple regression analyses, I provide detailed descriptions of variation and covariation in plumage spottiness, number of spots, spot diameter and plumage coloration. Furthermore, I experimentally test whether the expression of number of spots and spot diameter is under genetic control and sensitive to the rearing environment. Finally, I investigate whether number of spots and spot diameter are sex-linked or autosomally inherited (for similar tests on plumage spottiness and coloration see Roulin and Dijkstra 2003). Under the hypothesis of autosomal inheritance, mothers and fathers should contribute equally to offspring phenotype. Under the hypothesis of sex-linkage, sons should resemble mothers more than fathers, since mothers pass on their single copy of the Z-chromosome, whereas fathers pass on one of their two copies. Only fathers should contribute significantly to the daughter's phenotype, since

fathers but not mothers pass on a copy of the Z chromosome.

Materials and methods

Study site and the assessment of plumage traits

The study was performed in western Switzerland between 1996 and 2003 on a plain covering 190 km^2 at an altitude of 430–520 m. One hundred and ten nest-boxes have been mounted on the external wall of barns since 1987. Each year, breeding adults and their offspring were captured and ringed. Breeding females were recognised by the presence of a brood patch. When breeders had not been ringed as nestlings, I considered individuals as "yearlings" if all primary and secondary wing feathers belonged to the same generation, as '2 years old" if only the sixth primary had already been renewed, and as '3 years old" if more primary or secondary wing feathers were already renewed (Taylor 1993). The sex of the nestlings was determined using the CHD-gene method (for details see Roulin et al. 1999).

To record the trait plumage coloration, I compared coloration of breast, belly, one flank and one underside of the wings with eight colour chips, ranging from I for reddish-brown to VIII for white. This method is reliable (Roulin 1999a). A previous study showed that although males and females can express any coloration, males are on average lighter coloured, and individuals of both sexes become lighter coloured between the first and second year of age (Roulin 1999b). A bird that is darker than another one at the first year is still darker at the second year (Roulin and Dijkstra 2003).

To record variation in number and size of spots, I placed on the same four body parts a 60×40-mm frame within which I counted spots and measured the diameter of one to 26 of the most representative spots to the nearest 0.1 mm. For each body part, I calculated mean spot diameter, a value denoted "spot diameter". The proportion of the plumage surface covered by black spots, referred to as plumage spottiness, was estimated with the formula $100\times\pi\times$number of spots$\times$(mean spot diameter/2)^2/(60×40). Number of spots, spot diameter and plumage spottiness values of the two flanks were averaged, as well as those of the two wings. The method of assessing plumage spottiness has already been shown to be reliable (repeatability is 0.93; Roulin and Dijkstra 2003). To assess the reliability of counting spots and measuring their diameter, I measured 33 breeding males and 116 breeding females twice within the same season. Range in number of days between two successive measurements is 3 and 134 days. The repeatability of measuring number of spots was 0.93 in breeding males (one-way ANOVA, $F_{29,36}$=26.54, P<0.0001) and 0.89 in breeding females ($F_{194,174}$=15.96, P<0.0001), and the repeatability of measuring spot diameter 0.92 in breeding males ($F_{29,36}$=24.88, P<0.0001) and 0.84 in breeding females ($F_{91,140}$=11.45, P<0.0001). Methods of assessing plumage traits are therefore accurate.

Variation in plumage traits

To study variation in coloration, spottiness, spot diameter and number of spots on the breast, belly, flanks and underside of the wings, I measured 195 different breeding males and 253 different breeding females. In 1996, 144 birds were measured, 74 in 1997, 96 in 1998, 80 in 1999, 95 in 2000, 98 in 2001, 149 in 2002 and 92 in 2003. Trait values of individuals measured in more than 1 year were averaged so that each individual appeared only once per analysis. To examine how plumage traits varies on each body part, for each sex I calculated a mean value, SD, coefficient of variation and range.

Covariation among plumage traits

To investigate covariations between plumage traits, I considered the same 195 breeding males and 253 breeding females as above. Values for the breast, belly, flanks and underside of the wings were averaged and used in statistical analyses (these mean values were also used in subsequent analyses). Contributions of number of spots and spot diameter to the plumage surface covered by black spots (i.e. plumage spottiness) were given by standardised β calculated from a multiple regression where plumage spottiness was entered as a dependent variable, and number of spots and spot diameter as two independent variables. To determine whether the covariation between number of spots and spot diameter is sex-specific, I ran an ANCOVA with spot diameter as a dependent variable, sex as a factor and number of spots as a covariate. The hypothesis of a sex-specific covariation is supported if the interaction between sex and number of spots is significant.

Black and reddish-brown coloration are due to the deposition of eumelanin and phaeomelanin pigments, respectively (Hearing and Tsukamoto 1991; Jawor and Breitwisch 2003). Recent studies showed that plumage coloration and spottiness are genetically correlated (Roulin 2003; Roulin and Dijkstra 2003), and hence via a multiple regression I investigated whether coloration (dependent variable) is mainly associated with number of spots or spot diameter (two independent variables).

Genetics of number of spots and spot diameter

The expression of plumage spottiness has already been shown to be under genetic control and not sensitive to rearing environment or body condition (Roulin et al. 1998; Roulin and Dijkstra 2003). To investigate whether the expression of both number of spots and spot diameter is also under strong genetic control, I used data from partial cross-fostering experiments conducted in 1996 (51 nests with 193 nestlings), 1998 (38 nests with 167 nestlings), 2001 (43 nests with 193 nestlings) and 2002 (62 nests with 278 nestlings). At hatching, half of nestlings were swapped between nests so that each nest contained offspring of two origins. This design is useful to partition variation in phenotypic traits into genetic and environmental components. Nestlings born in nest A, denoted the "nest of origin", were raised either in the nest of origin A or in another nest B called "nest of rearing" (nests of origin and rearing are the same when nestlings born in nest A are also raised in nest A). In a mixed-model ANOVA, I nested the nests of origin and rearing in the pair of cross-foster nests (i.e. nests A and B belonged to pair 1, whereas nests C and D to pair 2, and so on). Sex of the nestlings was entered in the model as a factor.

Parent-offspring comparison has already demonstrated that plumage spottiness is sex-linked inherited (Roulin and Dijkstra 2003). Because this observation does not necessarily imply that both number of spots and spot diameter are also sex-linked inherited, I investigated the genetics of both traits. If the expression of these plumage traits is under genetic control and not sensitive to rearing environment, I can use all the data collected between 1996 and 2002, whether or not offspring were cross-fostered between nests. For each breeding pair, I measured trait values of daughters and of sons as explained above, and for each sex calculated mean trait values. Predictions of autosomal vs. sex-linked inheritance, as explained earlier, were tested with multiple regression analyses. Mean trait values of same-sex offspring were the dependent variables, and trait values of genetic father and genetic mother two independent variables.

Covariation between offspring attributes and number of spots versus spot diameter

Humoral immunocompetence was defined as the quantity of antibodies specifically directed towards a subcutaneous injection of sheep red blood cells (SRBC). In 1998, I cross-fostered half of the

hatchlings between pairs of nests. When the oldest chick of each brood was 40 days old, I immunised all nestlings (nest-mates were therefore not injected at the same age because of hatching asynchrony). Blood samples were taken from the brachial vein just before immunisation, and 3, 8, 13 and 18 days after. An indirect haemagglutination assay allowed measurement of antibody concentration in these samples. Antibody concentration was maximal at day 8 and 13 (Roulin et al. 2000), and hence I calculated a mean value to be used in the statistical analyses. Because nestlings raised in the same nest mounted a similar humoral immune response, I removed variation in the amount of antibodies explained by the variable "nest of rearing", "pair of cross-foster nests" and "age of the nestlings at the time of immunisation" (antibody response is greater in older chicks) in a mixed-model nested ANOVA. The residuals extracted reflect origin-related effects of mounting an immune response towards SRBC, denoted "SRBC-response" and were used in statistical analyses. Age of genetic mothers was correlated neither with SRBC-response measured in their cross-fostered offspring (Pearson correlation: r=0.25, n=38 experimental nests, P=0.13), nor with number of spots (r=−0.20, n=38, P=0.23) and spot diameter measured in genetic mothers (r=0.20, n=38, P=0.22). For more information see Roulin et al. (2000).

Resistance to ectoparasites was assessed by measuring fecundity of the blood-sucking ectoparasite *C. hemapterus* (Diptera: Carnidae). This parasite is common in the barn owl with 98% of the nests being infested (Roulin 1998) and chicks harbouring on average 50 flies on their body (range=1–273) (Roulin et al. 2003b). Females are easily recognisable with their white abdomen containing up to 109 eggs (Roulin 1999c). In 1999, I swapped clutches between nests, and once the first-hatched chick was 20-days-old, I collected five gravid females per chick every 5 days up to fledging. In total, 2,087 flies were sampled and put singly in a 1.5-ml Eppendorf tube at 37°C for 24 h. After that time, I counted eggs laid in tubes. For each nest-box visit and for each chick, I calculated mean number of eggs laid by *C. hemapterus*. Then, a mean sibling value was calculated to be used in statistical analyses. Age of genetic mothers was correlated neither with number of eggs laid by *C. hemapterus* collected on their cross-fostered offspring (r=−0.05, n=31 experimental nests, P=0.83), nor with number of spots (r=−0.10, n=31, P=0.58) and spot diameter measured in genetic mothers (r=0.08, n=31, P=0.68). More information on this experiment can be found in Roulin et al. (2001a).

In 2001, I measured fluctuating asymmetry of primary wing feathers in growing nestlings. For this purpose, I cross-fostered half of hatchlings between pairs of nests. When cross-fostered offspring were 50 days of age, two adjacent feathers were laid on top of one another, and the distance D from tip to tip was measured. In total, there were nine such distances, D1 for the distance between the primaries P10 and P9, D2 for the distance between P9 and P8, and so on. For each distance Di, I subtracted the value found in the right wing from the value found in the left wing (Di_{right}–Di_{left}), and took the absolute value. This was done for the nine differences D1–D9, and the sum of these nine unsigned values was $\log_{10}$-transformed to normalise the data distribution. The latter value is the measure of fluctuating asymmetry. The assessment of fluctuating asymmetry is reliable (Roulin et al. 2003a). Age of genetic mothers was correlated neither with fluctuating asymmetry measured in their cross-fostered offspring (r=−0.17, n=43 experimental nests, P=0.26), nor with number of spots (r=−0.16, n=43, P=0.31) and spot diameter measured in genetic mothers (r=0.26, n=43, P=0.09). For more information see Roulin et al. (2003a).

Statistical analyses

All statistical analyses were two-tailed and P-values <0.05 were considered significant.

Results

Variation in plumage traits

Mean, SD, coefficient of variation and range of plumage coloration, number of spots, spot diameter and plumage spottiness measured on the breast, belly, flanks and underside of the wings are presented in Table 1. Plumage coloration was most variable on the breast in males and on the flanks in females. Plumage spottiness, spot diameter and number of spots were most variable on the belly in both sexes.

Covariation among plumage traits

Variation in spot diameter explained more of the variation in plumage spottiness than number of spots in both breeding males (multiple regression with plumage spottiness as dependent variable, spot diameter as first independent variable, $F_{1,192}$=680.58, P<0.0001; β=0.64; number of spots as second independent variable, $F_{1,192}$=256.45, P<0.0001; β=0.39) and females (spot diameter: $F_{1,252}$=1260.74, P<0.0001; β=0.65; number of spots, $F_{1,252}$=623.94, P<0.0001; β=0.45). It was necessary to carry out separate analyses for each sex because the covariation between number of spots and spot diameter was significantly stronger in males than females (ANCOVA with spot diameter as dependent variable, number of spots as covariate and sex as factor; interaction, $F_{1,446}$=5.17, P=0.02). Pearson correlation between number of spots and spot diameter was 0.76 (n=195, P<0.0001) in males and 0.54 (n=253, P<0.0001) in females.

Plumage coloration, an autosomally inherited trait (Roulin and Dijkstra 2003), was mainly associated with number of spots and weakly with spot diameter in breeding males (multiple regression with plumage coloration as dependent variable, number of spots as first independent variable, $F_{1,192}$=51.34, P<0.0001, β=−0.71; spot diameter as second variable, $F_{1,192}$=4.64, P=0.03, β=0.21) and females (number of spots, $F_{1,252}$=4.03, P=0.046, β=−0.15; spot diameter, $F_{1,252}$=0.13, P=0.72).

Genetics of number of spots and spot diameter

Partial cross-fostering experiments showed that the expression of both number of spots and spot diameter is under genetic control and not sensitive to rearing environment (Table 2). Genes responsible for variation in number of spots appear to be autosomally inherited as suggested by the similar paternal-to-maternal contribution to this trait in offspring (Table 3). In contrast, mean spot diameter may be sex-linked inherited because sons resembled their mother more than their father and daughters resembled only their father (Table 3). The significant maternal contribution to spot diameter of daughters presented in Table 3 was in fact explained by annual variation in female spot diameter (daughter spot

Table 1 Plumage coloration (1 for reddish-brown and VIII for white), plumage spottiness, number of spots and spot diameter (0.1 mm). Values are separately given for breast, belly, flank (mean value of left and right flanks) and underside of the wings (mean value of left and right wings). The greatest coefficients of variation (*CV*) are written *in italics*

	Plumage coloration				Plumage spottiness				Spot diameter				Number of spots			
	Breast	Belly	Flank	Wing	Breast	Belly	Flank	Wing	Breast	Belly	Flank	Wing	Breast	Belly	Flank	Wing
195 different breeding males																
Mean	5.58	7.00	5.86	7.35	1.41	1.00	3.32	1.77	8.42	9.86	14.03	11.65	33.33	16.07	41.35	27.18
±SD	±1.63	±1.45	±1.56	±1.10	±1.82	±1.46	±2.68	±1.98	±4.08	±5.07	±4.22	±4.36	±26.66	±13.28	±15.72	±14.28
CV	*29.14*	20.78	26.68	15.02	128.80	*146.65*	80.75	111.50	48.44	*51.47*	30.05	37.40	79.99	*82.67*	38.02	52.54
Range	2–8	2.5–8	2.5–8	3–8	0–8.04	0–8.27	0.01–15.72	0–11.01	0–21.13	0–24.21	2.8–28.97	0–24.05	0–106	0–61	1–77	0–73
253 different breeding females																
Mean	4.10	5.90	4.53	5.93	2.93	1.95	5.71	4.07	12.30	13.99	17.59	15.21	49.53	23.47	51.53	46.85
±SD	±1.28	±1.80	±1.46	±1.67	±2.07	±1.65	±2.96	±2.59	±3.58	±4.30	±3.93	±3.62	±21.94	±12.00	±12.89	±14.54
CV	31.24	30.46	*32.22*	28.12	70.71	*84.57*	51.79	63.64	29.15	*30.75*	22.36	23.78	44.31	*51.12*	25.00	31.03
Range	1–8	2–8	1–8	2–8	0.03–10.52	0–9.12	0.74–17.00	0.37–17.41	2.45–20.80	0–22.92	8.16–30.16	3.47–28.55	2–104	0–58	24–89	17–85

diameter as dependent variable, father spot diameter as independent variable, $F_{1,248}$=85.67, P<0.0001, β=0.48; mother spot diameter as second variable, $F_{1,248}$=1.68, P=0.20, β=0.07; year as a category factor, $F_{6,248}$=4.48, P=0.0002). Annual variation may be due to either measurement errors or a change in the frequency of genes responsible for variation in spot size. All other results presented in Table 3 did not change after inclusion of year as a factor.

Covariation between offspring attributes and number of spots vs. spot diameter

SRBC-response of cross-fostered offspring was greater when the genetic mother displayed larger spots (multiple regression with spot diameter as first independent variable, $F_{1,34}$=9.39, P=0.004, β=0.68; number of spots as second variable, $F_{1,34}$=1.07, P=0.31; plumage coloration as third variable, $F_{1,34}$=0.27, P=0.60). Pearson correlation coefficient between spot diameter measured in genetic mothers and quantity of antibodies specifically directed towards SRBC measured in the cross-fostered offspring is 0.48 (n=38, P=0.0025; Pearson correlation coefficient with plumage spottiness of genetic mothers was 0.36, Roulin et al. 2000).

C. hemapterus collected on cross-fostered offspring laid a lower number of eggs when the genetic mother displayed larger black spots (multiple regression with spot diameter as first independent variable, $F_{1,27}$=8.35, P=0.008, β=−0.63; number of spots as second variable, $F_{1,27}$=0.20, P=0.67; plumage coloration as third variable, $F_{1,27}$=0.09, P=0.77). Pearson correlation coefficient between spot diameter measured in genetic mothers and number of eggs laid by *C. hemapterus* collected on the cross-fostered offspring is −0.59 (n=31, P=0.0005; Pearson correlation coefficient with plumage spottiness of genetic mothers was −0.45, Roulin et al. 2001a).

In a multiple regression analysis, spot diameter of genetic mothers was significantly associated with wing

Table 2 Mixed-model nested ANOVA on number of spots and spot diameter in nestlings. Data come from partial cross-fostering experiments carried out in 1996, 1998, 2001 and 2002 (in this model, the term pair of nests was the main effect, while the nests of rearing and nests of origin were nested in the main effect as indicated in parentheses; sex is a fixed factor)

Source	*df*	*F*-ratio	*P*
Number of spots			
Pair of nests	102, 523	1.44	0.04
Nest of rearing (pair of nests)	97, 523	0.86	0.82
Nest of origin (pair of nests)	105, 523	2.58	<0.0001
Sex	1, 523	175.28	<0.0001
Spot diameter			
Pair of nests	102, 523	1.42	0.05
Nest of rearing (pair of nests)	97, 523	0.74	0.97
Nest of origin (pair of nests)	105, 523	2.89	<0.0001
Sex	1, 523	173.13	<0.0001

Table 3 Maternal and paternal contribution to number of spots and spot diameter of daughters and sons. Contribution is given by standardised β calculated from a multiple regression where plumage characteristics of offspring were in turn entered as a dependent variable, and plumage characteristics of genetic mother and father as two independent variables. Each pair appears only once in each analysis

	Number of spots		Spot diameter	
	Daughter	Son	Daughter	Son
Maternal contribution	β=0.26, $F_{1,218}$=17.40**	β=0.47, $F_{1,208}$=72.90**	β=0.12, $F_{1,218}$=4.74*	β=0.49, $F_{1,208}$=86.65**
Paternal contribution	β=0.27, $F_{1,218}$=19.12**	β=0.36, $F_{1,208}$=43.86**	β=0.52, $F_{1,218}$=81.24**	β=0.38, $F_{1,208}$=50.24**

*P=0.03, **P<0.0001

fluctuating asymmetry measured in their cross-fostered offspring (spot diameter as first independent variable, $F_{1,39}$=3.99, P=0.05; number of spots as second variable, $F_{1,39}$=0.11, P=0.74; plumage coloration as third variable, $F_{1,39}$=0.11, P=0.74). Pearson correlation between spot diameter measured in genetic mothers and feather fluctuating asymmetry measured in the cross-fostered offspring was r=−0.35, n=43, P=0.02; Pearson correlation coefficient with plumage spottiness of genetic mothers was −0.33, Roulin et al. 2003a, b).

Discussion

The re-analysis of previously published results showed that covariations between a eumelanin-based female plumage trait and offspring attributes including humoral immunocompetence, parasite resistance and fluctuating asymmetry is explained by spot diameter, but not by number of spots and plumage coloration. Because these phenotypic attributes were measured in offspring raised by randomly chosen foster parents, the present study suggests that genes responsible for variation in the amount of eumelanin pigments produced per spot have pleiotropic effects on important individual attributes. In contrast, genes responsible for variation in number of spots may not play a significant role. Therefore, gene products involved in the production of eumelanin pigments generate covariation between a eumelanin-based ornament and individual quality.

One of the main messages of the present study is that an ornament is often a composite trait and each component should be measured separately. Three reasons support such a method of measuring ornaments. First, phaeomelanin- and eumelanin-based ornaments can have different signalling functions (Roulin et al. 2001b), and hence analyses that do not distinguish between these two kinds of ornaments may fail to detect any covariation with life history or behavioural traits. This is important because in most studies on genetic colour polymorphism, individuals were classified in a discrete number of morphs although individuals frequently vary continuously with respect to several traits (Roulin, in press). The use of morphs instead of its composite traits may reduce the statistical power to detect significant covariation between colour traits and phenotypic qualities. Second, genes involved in different components of a same ornament (e.g. plumage spottiness with the components "number of spots" and "spot diameter") may be located on autosomes and sex-chromosomes. This is of importance because sex-linked genes can have pleiotropic effects on sex determination mechanisms (Ellegren 2000), sexually selected traits (Reinhold 1998) or on traits that are beneficial to one sex but detrimental to the other (Rice 1984). Knowledge of the mode of inheritance may therefore allow generation of predictions regarding the signalling function of ornaments and their different components. Third, different genes may contribute to various extents to the production of an ornament. In the barn owl, both spot diameter and number of spots contribute to the extent to which plumage is covered with black pigments. Therefore, there is a non-negligible probability of failing to detect the signalling function of a eumelanin-based trait if one considers plumage spottiness instead of its components: number of spots and spot diameter. Spot diameter covaries with offspring phenotypic attributes including a measure of immunocompetence, parasite resistance and fluctuating asymmetry, but it still remains unclear whether inter-individual variation in number of spots has a signalling function. Because number of spots and spot diameter are strongly correlated within males (r=0.76), these two traits may have a redundant signalling function in this sex. In contrast, because covariation between number of spots and their size is less intense within females (r=0.54), these two traits may signal different qualities in this sex. More studies are required to investigate these issues.

The present study is a further step in understanding the genetic architecture of colourful plumage traits. Parent-offspring comparisons showed that both number of spots (a eumelanin trait; present study) and plumage coloration (a pheomelanin trait; Roulin and Dijkstra 2003) are autosomally inherited, whereas spot diameter is sex-linked inherited (present study). These findings are consistent with the observation that plumage coloration is mainly associated with number of spots indicating that these two traits share an autosomally inherited gene. This gene might convert a metabolite (e.g. L-tyrosine) into both eumelanin and phaeomelanin pigments (Jawor and Breitwisch 2003), and hence the greater the expression of this gene the darker coloured and spottier birds are. Even if number of spots is autosomally inherited and spot diameter is sex-linked inherited, these two traits are correlated within

individuals suggesting that the expression of number and size of black spots partly relies on a common gene. Because the covariation between number of spots and spot diameter was significantly greater in males than females, this gene may be located on the Z sex chromosome. Its function may be to trigger spot production (yes or no response), a process that may increase in frequency (i.e. more feathers produce one or several spots) when gene expression is elevated. Assuming that this gene also influences spot size, expression of this gene may simultaneously influence number and size of black spots. Detailed physiological data are required to test this hypothesis.

In conclusion, the eumelanin-based female ornament plumage spottiness is a composite ornament composed of the traits number of spots and spot diameter. However, only the sex-linked inherited trait spot diameter measured in genetic mothers was significantly associated with humoral immunocompetence, parasite resistance and fluctuating asymmetry of wing feathers of cross-fostered offspring. This is an important finding with which to further investigate not only the signalling function of black spots in the barn owl, but also in other bird species displaying a similar trait. Current analyses are mainly done to determine whether spot diameter reflects individual qualities not only in Switzerland where the lightly spotted subspecies *T. alba alba* and heavily spotted subspecies *T. alba guttata* interbreed, but also in other worldwide distributed subspecies. Little can be said here, but preliminary analyses show that spot diameter may have a signalling function in populations other than the Swiss one (e.g. barn owls display larger spots in the southern compared to northern hemisphere). Therefore, the reanalysis of already published data was not a mere trial to republish the same data. Previous papers on the signalling function of plumage spottiness in the barn owl were in the context of the evolution of female ornaments. This focus disregarded the fact that black spots are due to eumelanin pigments. At that time, interest in melanin-based traits was not as pronounced as it is today. Consideration of black spots as a melanin-based ornament raises new fundamental question, and the present paper is a crucial move into this direction by providing a foundation for future studies.

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References

Badyaev AV, Hill GE (2000) Evolution of sexual dichromatism: contribution of carotenoid versus melanin-based coloration. Biol J Linn Soc 69:153–172

Barsh GS (1996) The genetics of pigmentation: from fancy genes to complex traits. TIGS 12:299–305

Berry AJ, Willmer PG (1986) Temperature and the colour polymorphism of *Philaenus spumarius* (Homoptera: Aphrophoridae). Ecol Entomol 11:251–259

Calkins JD, Burley NT (2003) Mate choice for multiple ornaments in the California quail (*Callipepla californica*). Anim Behav 65:69–81

Catania A, Cutuli M, Garofalo L, Carlin A, Airaghi L, Barcellini W, Lipton JM (2000) The neuropeptide α-MSH in host defense. Ann NY Acad Sci 917:227–231

Ellegren H (2000) Evolution of the avian sex chromosomes and their role in sex determination. Trends Ecol Evol 15:188–192

Fitze PS, Richner H (2001) Differential effects of parasite on ornamental structures based on melanins and carotenoids. Behav Ecol 13:401–407

Geschwind II (1966) Change in hair color in mice induced by injection of α-MSH. Endocrinology 79:1165–1167

Grafen A (1990) Biological signals as handicaps. J Theor Biol 144:517–546

Hearing VJ, Tsukamoto K (1991) Enzymatic control of pigmentation in mammals. FASEB 5:2902–2909

Hill GE, Montgomerie R, Inouye CY, Dales J (1994) Influence of dietary carotenoids on plasma and plumage colour in house finch: intra- and intersexual variation. Funct Ecol 8:343–350

Ichiyama T, Sato S, Okada K, Catania A, Lipton JM (2000) The neuroimmunomodulatory peptide α-MSH. Ann N Y Acad Sci 917:221–226

Jawor JM, Breitwisch R (2003) Melanin ornaments, honesty, and sexual selection. Auk 120:249–265

Lerner AB, McGuire JS (1961) Effect of alpha- and beta-melanocyte stimulating hormones on the skin colour of man. Nature 189:176–179

Mackintosh JA (2001) The antimicrobial properties of melanocytes, melanosomes and melanin and the evolution of black skin. J Theor Biol 211:101–113

Majerus MEN (1998) Melanism, evolution in action. Oxford University Press, Oxford

Mundy NI, Kelly J, Theron E, Hawkins K (2003) Evolutionary genetics of the melanocortin-1 receptor in vertebrates. Ann N Y Acad Sci 994:307–312

Murton RK, Westwood NJ, Thearle RJP (1973) Polymorphism and the evolution of continuous breeding season in the pigeon *Columba livia*. J Reprod Fertil Suppl 19:561–575

Nachman MW, Hoekstra HE, D'Agostino SL (2003) The genetic basis of adaptive melanism in pocket mice. Proc Natl Acad Sci U S A 100:5268–5273

Owens IPF, Hartley IR (1998) Sexual dimorphism in birds: why are there so many different forms of dimorphism? Proc R Soc Lond B 265:397–407

Reinhold K (1998) Sex linkage among genes controlling sexually selected traits. Behav Ecol Sociobiol 44:1–7

Rice WR (1984) Sex chromosome and the evolution of sexual dimorphism. Evolution 38:735–742

Rohwer S, Rohwer FC (1978) Status signalling in harris sparrows: experimental deceptions achieved. Anim Behav 26:1012–1022

Roulin A (1998) Cycle de reproduction et abondance du diptère parasite *Carnus hemapterus* dans les nichées de chouettes effraies *Tyto alba*. Alauda 66:265–272

Roulin A (1999a) Nonrandom pairing by male barn owls *Tyto alba* with respect to a female plumage trait. Behav Ecol 10:688–695

Roulin A (1999b) Delayed maturation of plumage coloration and plumage spottiness in the barn owl *Tyto alba*. J Ornithol 140:193–197

Roulin A (1999c) Fécondité de la mouche *Carnus hemapterus*, parasite des jeunes chouettes effraies (*Tyto alba*). Alauda 67:205–212

Roulin A (2003) Geographic variation in sexually selected traits: a role for direct selection or genetic correlation? J Avian Biol 34:251–258

Roulin A (in press) The evolution, maintenance and adaptive function of genetic colour polymorphism in birds. Biol Rev

Roulin A, Dijkstra C (2003) Genetic and environmental components of variation in eumelanin and phaeomelanin sex-traits in the barn owl. Heredity 90:359–364

Roulin A, Richner H, Ducrest A-L (1998) Genetic, environmental and condition-dependent effects on female and male ornamentation in the barn owl *Tyto alba*. Evolution 52:1451–1460

Roulin A, Ducrest A-L, Dijkstra C (1999) Effect of brood size manipulations on parents and offspring in the barn owl *Tyto alba*. Ardea 87:91–100

Roulin A, Jungi TW, Pfister H, Dijkstra C (2000) Female barn owls (*Tyto alba*) advertise good genes. Proc R Soc Lond B 267:937–941

Roulin A, Riols C, Dijkstra C, Ducrest A-L (2001a) Female plumage spottiness and parasite resistance in the barn owl (*Tyto alba*). Behav Ecol 12:103–110

Roulin A, Riols C, Dijkstra C, Ducrest A-L (2001b) Female- and male-specific signals of quality in the barn owl. J Evol Biol 14:255–267

Roulin A, Ducrest A-L, Balloux F, Dijkstra C, Riols C (2003a) A female melanin-ornament signals offspring fluctuating asymmetry in the barn owl. Proc R Soc Lond B 270:167–171

Roulin A, Brinkhof MWG, Bize P, Richner H, Jungi TW, Bavoux C, Boileau N, Burneleau G (2003b) Which chick is tasty to parasites? The importance of host immunology versus parasite life history. J Anim Ecol 72:75–81

Taylor IR (1993) Age and sex determination of Barn Owls *Tyto alba alba*. Ring Migr 14:94–102

Theron E, Hawkins K, Bermingham E, Ricklefs RE, Mundy NI (2001) The molecular basis of avian plumage polymorphism in the wild: a melanocortin-1-receptor point mutation is perfectly associated with the melanic plumage morph of the bananaquit, *Coereba flaveola*. Curr Biol 11:550–557

Wilson K, Cotter SC, Reeson AF, Pell JK (2001) Melanism and disease resistance in insects. Ecol Lett 4:637–649

Zahavi A (1975) Mate selection: a selection for a handicap. J Theor Biol 53:205–214