Genetic and environmental components of variation in eumelanin and phaeomelanin sex-traits in the barn owl

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Knowledge of the mechanism underlying the expression of melanin-based sex-traits may help us to understand their signalling function. Potential sources of inter-individual variation are the total amount of melanins produced but also how biochemical precursors are allocated into the eumelanin and phaeomelanin pigments responsible for black and reddish-brown colours, respectively. In the barn owl (*Tyto alba*), a eumelanin trait (referred to as 'plumage spottiness') signals immunocompetence towards an artificially administered antigen and parasite resistance in females, whereas a phaeomelanin trait ('plumage coloration') signals investment in reproduction in males. This raises the question whether plumage coloration and spottiness are expressed independent of each other. To investigate this question, we have studied the genetics of these two plumage traits. Crossfostering experiments showed that, for each trait, phenotypic variation has a strong genetic component, whereas no environmental component could be detected. Plumage coloration is autosomally inherited, as suggested

by the similar paternal-to-maternal contribution to offspring coloration. In contrast, plumage spottiness may be sex-linked inherited (in birds, females are heterogametic). That proposition arises from the observation that sons resembled their mother more than their father and that daughters resembled only their father. Despite plumage coloration and spottiness signalling different qualities, these two traits are not inherited independent of each other, darker birds being spottier. This suggests that the extent to which coloration and spottiness are expressed depends on the total amount of melanin produced (with more melanin leading to a both darker and spottier plumage) rather than on differential allocation of melanin into plumage coloration and spottiness (in such a case, darker birds should have been less spotted). A gene controlling the production of melanin pigments may be located on sex-chromosomes, since the phenotypic correlation between coloration and spottiness was stronger in males than in females.

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Introduction

Melanins belong to the major pigments used in the elaboration of reddish-brown, brown, grey and black coloration (Fox, 1976). Melanin production is under genetic control and does not appear to be constrained environmentally (Buckley, 1987; Hearing and Tsukamoto, 1991; Majerus, 1998), suggesting that melanin-based traits may not signal phenotypic quality (Hill and Brawner, 1998, but see Veiga and Puerta, 1996). Consistent with this hypothesis is the finding that in birds, a large proportion of inter-individual variation in melanin-traits is explained by genetic factors (Johnston and Janiga, 1995; Roulin *et al.*, 1998) and condition-dependent effects were usually not detected (Hill and Brawner, 1998; Roulin *et al.*, 1998; McGraw and Hill, 2000; but see, Johnston and Janiga, 1995). However, recent studies shed new light on the possibility that melanin-based traits might signal phenotypic quality. In house sparrows (*Passer domesticus*), the size of the black feather bib on

the throat of males was related to body condition (Veiga and Puerta, 1996; Griffith *et al.*, 1999). In addition, testosterone implants negatively affected bib size in male adults (Gonzalez *et al.*, 2001). In breeding great tits (*Parus major*), the size of the black breast band significantly decreased after ectoparasites had been experimentally added in their nest (Fitze and Richner, 2002).

Individuals may vary in the expression of a melanin-based trait when (1) a different number of feathers are melanised, (2) the proportion of the melanised part of each single feather varies or (3) a different number of feathers are abraded (Bonser, 1995; Gonzalez *et al.*, 2001). Alternatively, individuals may deposit different amounts of melanins in feathers (Johnston and Janiga, 1995) or differentially invest energy into the production of eumelanins (black-brown coloration) and phaeomelanins (reddish-brown). Eu- and phaeomelanin pigments consist of the same biochemical precursors (Hearing and Tsukamoto, 1991), and hence individuals may vary in the respective amount of precursors they allocate into black-brown and reddish-brown ornamental traits. Melanin is synthesised by melanocytes within organelles called melanosomes, and this synthesis is influenced by a variety of extracellular factors. In short, the enzyme tyrosinase triggers the production of melanin precursors,

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and their biosynthesis into phaeomelanins depends on the availability of sulphhydryls, whereas the melanocyte-stimulating hormone (MSH) elicits production of eumelanins (Hearing and Tsukamoto, 1991).

In the barn owl (*Tyto alba*), individuals of a single population can vary with respect to coloration from white to dark reddish-brown (a phaeomelanin trait) and in spottiness from immaculate to heavily marked with black spots (a eumelanin trait). Based on the relatively small sample size ($n = 50$ nests, but most analyses were performed with a sub-sample of these 50 nests), interindividual variation in plumage coloration and spottiness appeared to be under genetic control but not sensitive to environmental factors and body condition (Roulin *et al.*, 1998). In Switzerland, darker reddish-brown males were observed to produce more offspring per breeding attempt and to sustain higher feeding rates, suggesting that these males invest more effort per breeding attempt (Roulin *et al.*, 2001a). If we turn to the other trait, plumage spottiness, heavily spotted females produced offspring that were more immunocompetent towards an artificially administered antigen (Roulin *et al.*, 2000), resistant to ectoparasites (Roulin *et al.*, 2001b) and developmentally stable (Roulin *et al.*, 2003). These results suggest that eu- and phaeomelanin plumage traits signal different qualities. This raises the question of whether plumage coloration and spottiness are expressed independent of each other. To answer this question, we studied the genetics of both plumage traits.

We conducted cross-fostering experiments to test whether (1) variation in plumage coloration and spottiness has a genetic and environmental component and (2) whether these two traits are genetically correlated. We also examined whether plumage traits are autosomally or sex-linked inherited. In birds, males are homogametic (ZZ) and females heterogametic (ZW). The hypothesis of autosomal inheritance predicts that mothers and fathers equally contribute to offspring phenotype. The hypothesis of sex-linked inheritance, in contrast, predicts that mothers resemble sons more than fathers (since mothers pass on their single copy of the Z-chromosome, whereas fathers pass on one of their two copies) and that only fathers significantly contribute to the daughter's phenotype (since only fathers pass on a copy of the Z-chromosome). This latter expectation precludes any prehatching maternal effects on the expression of plumage traits.

Material and methods

Assessment of plumage traits

From 1996 to 2001, Roulin quantified plumage traits on breeding adults and their 55-day-old offspring in a wild barn owl population located in Switzerland (46°49' N/06°56' E). Plumage coloration of the breast, belly, flanks and underside of the wings was scored after comparison with eight colour chips, ranging from I for reddish-brown to VIII for white. Mean colour score from the four body parts provided an index of 'plumage coloration'. Plumage spottiness was recorded on the same body regions by counting the number of spots within a 60 × 40 mm frame and measuring their mean diameter to the nearest 0.1 mm. The formula $100\pi \text{ number of dots} / (\text{mean spot diameter}/2)^2 / (60 \times 40)$ gave the percentage

of the plumage surface covered by black spots. Percentages from the four body regions were averaged, and this mean value was then square root transformed to normalise the data set. The resulting value was our measure of 'plumage spottiness'. Sex of the nestlings was determined using the CHD-molecular method (Roulin *et al.*, 1999). Between 1994 and 2001, 190 different breeding males (each individual having been captured on average 2.07 times) and 227 different breeding females (captured 1.91 times each) were measured. This allowed us to determine how plumage traits change from 1 year to the next. In total, 30 males and 112 females were also measured within the same season (on average 30 days apart; range: 3–134 days). This was useful to determine the extent to which plumage traits were accurately measured. Breeding birds for which primary and secondary feathers all belonged to the same new generation were classified as '1-year-old', and those for which wing feathers were not of the same generation were 'older than 1-year-old' (Taylor, 1994).

Genetic and environmental effects on plumage traits

Sib-sib comparison: To test for genetic and environmental effects on the expression of plumage coloration and spottiness, we carried out nested ANCOVAs using partial cross-fostering experiments. In 1996 (50 nests with in total 198 fledglings), 1998 (39 nests with 167 fledglings) and 2001 (43 nests with 191 fledglings), one to three hatchlings were swapped between nests so that each of these 132 nests contained chicks of two origins. For instance, nestlings born in a nest A (referred to as 'nest of origin') were raised either in the nest of origin A or in another nest B called 'nest of rearing'. In the ANCOVA, we nested the nests of origin and rearing in the pair of cross-foster nests (ie 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 as a factor and position of each individual in the within-brood age hierarchy as a covariate. In the barn owl, eggs hatch every other 2–3 days so that an age hierarchy is established among the siblings. As a consequence, first-hatched nestlings are in better condition than their later-hatched nest-mates (Wilson *et al.*, 1987; Roulin, 1998a) and infested by a lower number of ectoparasites (Roulin, 1998b). If the expression of plumage traits is condition-dependent, nestlings at different positions in the within-brood age hierarchy should develop plumage traits to varying degrees.

Parent-offspring comparison: If in sib-sib comparisons only a genetic component of phenotypic variation is detected, cross-fostered offspring should resemble their genetic parents, but not their foster parents. For a test, we used data from partial cross-fostering experiments (1996, 1998 and 2001) and from an experiment where clutches were swapped between nests (1999). In a multiple regression analysis, mean plumage trait value of cross-fostered offspring was entered as the dependent variable, whereas plumage values of genetic and foster mothers and fathers were the four independent variables. In total, we had a sample of 99 broods in which there were cross-fostered male nestlings and for which plumage traits of genetic and foster parents were known. With respect to cross-fostered female nestlings, we had a sample of 111 broods.

To investigate whether plumage coloration and spottiness are autosomally or sex-linked inherited, we regressed trait values of daughters and sons on those of genetic mother and father. We used data from years when cross-fostering had been performed (1996, 1998, 1999 and 2001) and when no cross-fostering had been conducted (1997). Same-sex siblings (raised in their nest of origin or in a foster nest) were not considered as independent data points. We therefore averaged their plumage trait values to avoid pseudoreplication. In multiple regression analyses, mean offspring values were entered as the dependent variable and trait values of genetic father and mother as independent variables. With respect to plumage coloration, only parents for which plumage characteristics had been measured at 1-year-old were considered, since this trait does not change from the nestling stage to the first year of life but from the first to second year (Roulin, 1999a). With respect to plumage spottiness, we considered all breeding birds, since this trait can change from 1 year to the next, but not in such a consistent way as for plumage coloration (Roulin, 1999a).

Phenotypic correlation between plumage coloration and spottiness

Parent-offspring comparison: To determine whether phenotypic correlation between plumage coloration and spottiness has a genetic basis, we regressed mean plumage coloration of offspring raised in foster nests on plumage spottiness of same-sex genetic parents (data collected in 1996, 1998, 1999 and 2001). In this analysis, we also introduced plumage spottiness of the mate, since pairing with respect to this plumage trait is assortative (Roulin, 1999b). We also compared mean plumage spottiness of cross-fostered offspring with plumage coloration of same-sex genetic parents. We considered only parents for which plumage coloration had been measured when they were 1 year old. We did not control for plumage coloration of the mate because pairing with respect to this trait is not assortative (Roulin, 1999b).

Sib-sib comparison: Using data on partial cross-fostering experiments carried out in 1996, 1998 and 2001, we conducted an ANOVA with mean plumage spottiness of siblings raised in the same nest as the dependent variable, mean plumage coloration of their siblings raised in another nest as a covariate and nest of origin as a factor. We statistically controlled for 'origin' because each genotype appeared twice in the analysis (we compared spottiness of siblings raised in nest A with coloration of siblings raised in nest B, and *vice versa*). This analysis was performed for each sex separately.

Within-individual comparison: We correlated plumage coloration and spottiness measured within individuals. For nestlings, we averaged values of same-sex siblings measured in the nest of origin or in a foster nest (data collected in 1996, 1997, 1998, 1999 and 2001); we had a sample of 187 broods for male nestlings and 193 broods for female nestlings. For adults, we had data on plumage coloration and spottiness for 190 different breeding males and 224 breeding females captured from 1994 to 2001. For individuals captured on several occasions, we calculated the mean plumage trait values.

Statistics

All statistical analyses are two-tailed and *P*-values smaller than 0.05. were considered as significant.

Results

Variation in plumage coloration and spottiness

Although males and females become slightly lighter coloured between the first and second year of age (Roulin, 1999a), an individual that was darker coloured than another one at the first year of age was still darker at the second year (Pearson's correlation between plumage coloration measured in the same individuals at the first and second year of age; males: $r = 0.90$, $n = 27$, $P < 0.001$; females: $r = 0.89$, $n = 17$, $P < 0.001$). With respect to plumage spottiness, birds seem to change from 1 year to the next, but not in such a consistent way (Roulin, 1999a). Between-individual variation is, however, much larger than within-individual variation (repeatability analysis based on measurements of same breeding individuals in different years; male: $r = 0.88 \pm 0.01$, $F = 24.58$, $df = 85, 198$, $P < 0.001$; female: $r = 0.83 \pm 0.02$, $F = 16.14$, $df = 91, 204$, $P < 0.001$). Inter annual variation within individuals may be primarily because of measurement errors as repeatability of measurements taken on the same individuals within the same breeding season was broadly similar (males and females are pooled, $r = 0.93 \pm 0.01$, $F = 26.43$, $df = 111, 112$, $P < 0.001$).

Genetic and environmental effects on plumage traits

Sib-sib comparison: A nested-ANCOVA analysis showed that in nestlings variation in plumage coloration is related to the nest of origin, but neither to the nest of rearing nor to place in the within-brood age hierarchy (Table 1). Similar analysis applied to plumage spottiness revealed that this trait is also heritable and not sensitive to rearing environment nor to within-brood age hierarchy (Table 2).

Table 1 Mixed-model nested ANCOVA on the expression of plumage coloration in nestlings. Data are from 1996, 1998 and 2001

Source	d.f.	F-ratio	P-value
Pair of nests	68, 345	1.1	0.29
Nest of rearing (pair of nests)	67, 345	1.2	0.18
Nest of origin (pair of nests)	71, 345	3.7	<0.0001
Sex	1, 345	130.5	<0.0001
Rank in within-brood age hierarchy	1, 345	0.8	0.36

In this model, the term pair of cross-foster nests was the main effect, while the nests of rearing and nests of origin were nested in the main effect as indicated by the parentheses; sex is a factor and rank in the within-brood age hierarchy, a covariate.

Table 2 Mixed-model nested ANCOVA on the expression of plumage spottiness in nestlings (Similar model as the one presented in Table 1)

Source	d.f.	F-ratio	P-value
Pair of nests	68, 340	1.5	0.054
Nest of rearing (pair of nests)	67, 340	0.8	0.76
Nest of origin (pair of nests)	71, 340	2.9	<0.0001
Sex	1, 340	102.1	<0.0001
Rank in within-brood age hierarchy	1, 340	0.8	0.37

Parent-offspring comparison: Plumage characteristics of cross-fostered nestlings were associated with those of genetic parents (Figures 1 and 2) but not with those of foster parents (Table 3). Autosomal inheritance with

Figure 1 Relation between mean offspring plumage coloration and mean plumage coloration of genetic parents. Data are from cross-fostered and noncross-fostered broods. Mean offspring plumage coloration refers to the mean value of mean plumage coloration of male offspring and mean plumage coloration of female offspring. We considered only broods where plumage coloration of parents was measured when they were 1 year old. Regression line is presented ($h^2 = 0.81 \pm 0.09$, $F = 85.08$, $df = 1, 56$, $P < 0.001$).

Figure 2 Relation between mean offspring plumage spottiness and mean plumage spottiness of genetic parents. Data are from cross-fostered and noncross-fostered broods. Mean offspring plumage spottiness refers to the mean value of mean plumage spottiness of male offspring and mean plumage spottiness of female offspring. Regression line is presented ($h^2 = 0.65 \pm 0.05$, $F = 151.06$, $df = 1, 154$, $P < 0.001$).

respect to plumage coloration is suggested by the similar maternal-to-paternal contributions to the phenotypes of both sons and daughters (Table 4). Sex-linked inheritance with respect to plumage spottiness is suggested by the fact that mothers resembled sons more than fathers and that only fathers significantly contributed to the phenotype of daughters (Table 4; see also Table 3).

Genetic effects on phenotypic correlation between plumage coloration and spottiness

Parent-offspring comparison: Evidence for a genetic component of phenotypic correlation is given by the significant relation between mean plumage coloration of cross-fostered male offspring and plumage spottiness of their genetic father (multiple regression analysis controlling for plumage spottiness of genetic mother: $F = 4.04$, $df = 1, 75$, $P = 0.048$; standardised $\beta = -0.22$). In contrast, the comparison of cross-fostered male offspring plumage spottiness with genetic father's plumage coloration yielded a nonsignificant result (regression coefficient = -0.06 ± 0.05 , $F = 1.19$, $df = 1, 54$, $P = 0.28$). In females, we carried out the same comparisons and both were nonsignificant (multiple regression analysis controlling for plumage spottiness of genetic father: $F = 0.94$, $df = 1, 86$, $P = 0.34$; $\beta = -0.10$ and regression coefficient = 0.03 ± 0.06 , $F = 0.37$, $df = 1, 55$, $P = 0.55$).

Sib-sib comparison: In males, mean plumage spottiness of siblings raised in the same nest was significantly associated with mean plumage coloration of their siblings raised in another nest ($F = 4.52$, $df = 1, 45$, $P = 0.039$; $\beta = -0.30$) after controlling for origin ($F = 5.90$, $df = 45, 45$, $P < 0.001$). In females, this relation was not significant (plumage coloration: $F = 0.11$, $df = 1, 65$, $P = 0.11$; $\beta = -0.03$; origin: $F = 3.72$, $df = 66, 65$, $P < 0.001$).

Within-individual comparison: Plumage spottiness was more strongly correlated with plumage coloration within males (nestlings: regression coefficient = -0.17 ± 0.03 , $F = 33.01$, $df = 1, 185$, $P < 0.001$, Figure 3; breeding adults: regression coefficient = -0.16 ± 0.03 , $F = 26.61$, $df = 1, 188$, $P < 0.001$) than within females (nestlings: regression coefficient = 0.01 ± 0.03 , $F = 0.11$, $df = 1, 191$, $P = 0.74$; breeding adults: regression coefficient = -0.05 ± 0.03 , $F = 4.26$, $df = 1, 222$, $P = 0.04$). In an ANCOVA with plumage spottiness as the dependent variable, sex as a factor and coloration as a covariate, interaction between sex and coloration was significant in both nestlings and adults ($P < 0.01$). This indicates that phenotypic correlation between spottiness and coloration is significantly more intense in males than females.

Discussion

The present work confirms a previous study showing that plumage coloration and spottiness are heritable and that environmental factors and body condition have no detectable effects on their expression (Roulin *et al.*, 1998). Since parent-offspring and sib-sib comparisons showed that only a genetic component of phenotypic variation can be detected, we conclude that the expression of plumage traits is heritable and not, or to a low, undetectable extent, influenced by the quality of rearing environment. The absence of detectable environmental effects is consistent with the fact that heritabilities are

Table 3 Multiple regression analyses between plumage characteristics of cross-fostered offspring, genetic and foster parents

Parents	Plumage coloration		Plumage spottiness	
	Female offspring	Male offspring	Female offspring	Male offspring
Genetic mother	F(1,96)=18.2***	F(1,94)=51.1***	F(1,96)=3.1	F(1,94)=46.5***
Genetic father	F(1,96)=11.2***	F(1,94)=28.8***	F(1,96)=27.0***	F(1,94)=13.46***
Foster mother	F(1,96)=0.4	F(1,94)=2.7	F(1,96)=0.5	F(1,94)=0.2
Foster father	F(1,96)=1.0	F(1,94)=0.7	F(1,96)=0.3	F(1,94)=0.7

Data from cross-fostering experiments performed in 1996, 1998, 1999 and 2001 were pooled. When more than one same-sex offspring was raised in a cross-foster nest, their plumage trait values were averaged. The symbol *** indicates that $P < 0.001$ and no symbol $P > 0.05$.

Table 4 Maternal and paternal contribution to plumage coloration and spottiness of daughters and sons

	Plumage coloration		Plumage spottiness	
	Daughter	Son	Daughter	Son
Maternal contribution	$\beta=0.55$ F(1,64)=39.9***	$\beta=0.50$ F(1,61)=27.3***	$\beta=0.11$ F(1,175)=2.9	$\beta=0.53$ F(1,168)=83.47***
Paternal contribution	$\beta=0.51$ F(1,64)=33.5***	$\beta=0.50$ F(1,61)=27.48***	$\beta=0.50$ F(1,175)=57.5***	$\beta=0.33$ F(1,168)=31.4***

Contribution is given by standardised β calculated from a multiple regression where plumage characteristics of offspring were entered as a dependent variable, and plumage characteristics of genetic mother and father as independent variables. The symbol *** indicates that $P < 0.001$, and no symbol that $P = 0.79$.

Figure 3 Phenotypic correlation between plumage spottiness and coloration in nestling males. Each data point represents mean sibling trait value.

close to 1 (sum of maternal and paternal contributions to offspring phenotype in Table 4). This is of major importance to further investigation of the genetics of these traits. Indeed, it ensures that common environment does not inflate heritability estimates so that we can pool

cross-fostered and noncross-fostered offspring to increase sample size. Using a pooled sample, we found results consistent with the hypothesis that plumage coloration is autosomally inherited and that plumage spottiness is sex-linked inherited.

To our knowledge, the present study is the first one that provides experimental tests consistent with the hypothesis that a phenotypic correlation between plumage traits has a genetic basis. The suggestion that plumage coloration and spottiness are genetically correlated is not surprising because both traits rely on the same biochemical precursors (Hearing and Tsukamoto, 1991). Reddish-brown plumage coloration is constructed from melanins (Fox, 1976), leading some barn owls to develop a melanic black plumage (Literak *et al.*, 1999). Spots are black and also composed of melanins (Fox, 1976). Therefore, the correlation may arise through the expression of a gene that nonrandomly allocates melanin precursors to both plumage traits in a way that darker individuals (particularly true for males) are also more heavily spotted. This indicates that the expression of plumage coloration is not traded against the expression of plumage spottiness. In addition, given that the phenotypic correlation is more intense in males, this gene may be located on the Z sex-chromosome so that males have two copies of the gene and females only one copy. Two other mechanisms may explain sex-specific phenotypic correlation.

First, the genetic correlation may arise because genes that create variation in plumage coloration and spottiness are physically linked on the sexual chromosome Z, and hence males have two copies and females only one. Such a physical linkage should have evolved through selection acting on males to not display both plumage

traits independently. This is, however, unlikely because in such a situation plumage coloration should also be sex-linked inherited, whereas results of the present study rather suggest that this trait is autosomally inherited.

Second, a phenotypic correlation may arise even if genes that create variation in the two plumage traits are located on different chromosomes. In the case where males and females do not mate randomly with respect to plumage coloration and spottiness (ie darker males mating preferentially with more heavily spotted females, or *vice versa*), individuals possessing genes coding for a dark plumage would also possess genes coding for a heavily spotted plumage. The hypothesis of such a nonphysical linkage disequilibrium is unlikely because male plumage coloration was not correlated with plumage spottiness of their female mates ($r = -0.07$, $n = 296$ different breeding pairs captured from 1994 to 2001, $P = 0.23$), and because male plumage spottiness and plumage coloration of their females were not correlated ($r = 0.01$, $n = 296$, $P = 0.89$).

From an adaptive point of view, we can ask why selection may have favoured sex-linked inheritance of plumage spottiness. Since males get a copy of genes coding for both plumage traits from their mother and father under both sex-linked and autosomal inheritance, selective processes acting on males may not provide a satisfactory explanation. With respect to females, sex-linkage may be maintained or have evolved through selective processes acting on females to prevent genes that create variation in plumage spottiness from jumping to one of the 91 other chromosomes (barn owls have 92 pairs of chromosomes, Belterman and De Boer, 1984). Perhaps two copies of the genes may be detrimental to females. Similarly, we can wonder why the phenotypic correlation between the two plumage traits is more intense in males than females. Female plumage spottiness has been shown to signal immunocompetence towards an artificially administered antigen (Roulin *et al*, 2000), ectoparasite resistance of offspring (Roulin *et al*, 2001a) and offspring developmental homeostasis (Roulin *et al*, 2003), whereas female plumage coloration was correlated with laying date (lighter coloured females bred earlier, Roulin *et al*, 2001b). Therefore, if immunocompetence/parasite resistance/developmental homeostasis and an early initiation of breeding activities are qualities that rely on different phenotypic or genotypic characteristics, these qualities may be signalled with traits that are not correlated. These speculations emphasise the importance of determining the genetics of sex-ornaments, and calls for future studies on the genetics of plumage traits in the barn owl.

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