RESEARCH ARTICLES

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Extra-pair paternity, testes size and testosterone level in relation to colour polymorphism in the barn owl *Tyto alba*

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In many bird populations, individuals display one of several genetically inherited colour morphs. Colour polymorphism can be maintained by several mechanisms one of which being frequency-dependent selection with colour morphs signalling alternative mating strategies. One morph may be dominant and territorial, and another one adopt a sneaky behaviour to gain access to fertile females. We tested this hypothesis in the barn owl *Tyto alba* in which coloration varies from reddish-brown to white. This trait is heritable and neither sensitive to the environment in which individuals live nor to body condition. In Switzerland, reddish-brown males were observed to feed their brood at a higher rate and to produce more offspring than white males. This observation lead us to hypothesize that white males may equalise fitness by investing more effort in extra-pair copulations. This hypothesis predicts that lighter coloured males produce more extra-pair young, have larger testes and higher levels of circulating testosterone. However, our results are not consistent with these three predictions. First, paternity analyses of 54 broods with a total of 211 offspring revealed that only one young was not sired by the male that was feeding it. Second, testes size was not correlated with male plumage coloration suggesting that white males are not sexually more active. Finally, in nestlings at the time of feather growth testosterone level was not related to plumage coloration suggesting that this androgen is not required for the expression of this plumage trait. Our study therefore indicates that in the barn owl colour polymorphism plays no role in the probability of producing extra-pair young.

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In many birds including raptors, owls, geese, skuas and herons, individual variation in coloration has a strong genetic basis. For example, in the Eleonora's falcon *Falco eleonora* plumage coloration is coded by two alleles at a single locus (Wink et al. 1978), whereas in the white-throated sparrow *Zonotrichia albicollis* colour variation

is due to a chromosomal inversion (Thorneycroft 1966, 1975). Once a genetic polymorphism has evolved in a population, colour morphs can be maintained by the four following mechanisms. First, disruptive selection may favour morphs to be specialised in the exploitation of different ecological niches (Skúlason and Smith 1995). Second, genetic diversity at loci coding for colour polymorphism may be maintained when heterozygous individuals have a selective advantage over homozygous ones. Heterozygous individuals may indeed be less inbred, express deleterious recessive alleles less frequently and display a greater protein diversity allowing them to cope with a greater diversity of stressful factors than homozygous individuals (Brown 1997, Hansson and Westerberg 2002). Third, although a morph may be selected against, polymorphism may be maintained via immigration of the counter-selected morph or via a high mutation rate at genes coding for it. Finally, frequency-dependent selection is believed to be one of the most important mechanism accounting for the evolutionary stability of polymorphism (Maynard-Smith 1982).

Under frequency-dependent selection, a morph provides selective advantages to its bearer, but also some disadvantages, in a way that the net benefit accrued by each morph is equal at an equilibrium frequency. An increase in the frequency of one morph constitutes a disadvantage for all individuals, whereas a decrease provides an advantage, and hence morphs should rapidly return to the equilibrium frequency. Even if this mechanism can play a significant role in the evolutionary stability of colour polymorphism, little evidence has been accumulated for it. In birds, the only potential case is restricted to the ruff *Philomachus pugnax*. In this lekking species, colour plumage polymorphism signals mating strategies. To get access to fertile females, dark and white males adopt a territorial and sneaky courtship behaviour, respectively (Hogan-Warburg 1966, Lank et al. 1995). Both strategies are stable because the mating success of dark aggressive males is greater when white males attend leks at a high than low frequency (Hill 1991). Another example of morph-dependent mating or courtship strategies has been reported in the white-throated sparrow in which white-striped males sing more frequently, are more dominant and sexually more active than tan-striped males (Ficken et al. 1978, Tuttle 2003), behaviours that are physiologically determined (DeVoogd et al. 1995). Finally, in feral pigeons *Columba livia* testes of melanic birds regress less rapidly in winter months than those of blue bar males (Murton et al. 1973) explaining why melanics are gametogenetically more active in winter months (Johnston and Janiga 1995). These three examples suggest that colour morphs may signal alternative mating strategies.

In the present study, we investigated whether plumage coloration of male barn owls signals alternative mating strategies. This bird varies from white (subspecies *T. a. alba* mainly located in southern Europe) to reddish-brown (*T. a. guttata* mainly located in northern Europe; Roulin 2003), a trait for which the expression is under genetic control, but neither sensitive to body condition nor to the environment in which individuals live (Roulin et al. 1998, Roulin and Dijkstra 2003). Although males and females can express any coloration, males are on average lighter coloured (Roulin 1999a). In central Europe, the non-assortative interbreeding of these two subspecies (Baudvin 1975, Roulin 1999b, Matics et al. 2002) generates a pronounced variation in colour. Plumage coloration appears to be non-neutral with respect to life-history components because in a Swiss population reddish-brown males fed their brood at a higher rate and produced more offspring per breeding attempt than light coloured males (Roulin et al. 2001) putting into question how the polymorphism can be stable. A possibility is that white males equalise fitness by copulating with more females allowing them to produce more extra-pair young (EPY) than reddish-brown males.

To test the hypothesis that white males are sexually more active than reddish-brown ones, we determined paternity of offspring in two years using molecular genetic markers. Furthermore, we examined two other predictions of this hypothesis. First, since within-species the sexually more attractive or active males can have larger sexual organs (Møller 1994), we predicted that lighter coloured individuals have larger testes. We investigated this prediction by measuring testes in individuals found dead along highways. Second, if in the barn owl plumage coloration signals alternative mating strategies, its expression may rely on testosterone, a key hormone in male sexual behaviour (individuals with experimentally enhanced levels of testosterone invest less effort in parental care, but increase their mating effort and gain more extra-pair fertilisations, Raouf et al. 1997, but see Peters 2002, van Duyse et al. 2002) and in the expression of plumage traits in some species (Hillgarth and Wingfield 1997). To test this prediction, we assayed testosterone level in nestlings at the time of feather production, and predicted that the level of plumage whiteness is positively correlated with testosterone concentration measured in blood samples.

Methods

Assessment of plumage coloration

A. Roulin determined plumage coloration of barn owls after comparing coloration of the breast, belly, one flank and underside of one wing with eight colour chips ranging from I for reddish-brown to VIII for white.

Mean value of the four colour scores provided an index of overall plumage coloration. The assessment of this plumage trait is reliable (Roulin et al. 1998, Roulin 1999a, b).

Assessment of extra-pair young

We monitored the frequency of extra-pair young in 1997 and 1999 in a 190 km^2 study area located in western Switzerland (46°49′ N, 06°56′ E). A blood sample was collected on the brachial vein of 122 fledglings from 31 broods in 1997 and from 89 fledglings from 23 broods in 1999. In all instances, it was possible to take a blood sample from the two breeding parents. Blood (c. 100 µl) was stored in an EDTA buffer until further analyses. The terminology 'social parents' refers to parents that are feeding related or unrelated offspring, whereas 'genetic parents' refers to the biological parents.

Frequency of EPY was determined in two laboratories, in 1997 by W. Müller and T. Lubjuhn, and in 1999 by M. Wink. In 1997, DNA was isolated according to a standard procedure (Miller et al. 1988) that was slightly modified (see Lubjuhn and Sauer 1999). Five µg DNA of each sample were digested with 50 U of *Alu* I under the conditions given by the manufacturer. The digests were separated on agarose gels (20 × 40 cm; 0.8% agarose; 1–2 V/cm) in 1 × TBE for 60 h. After drying gels, fragments were detected by in-gel hybridization with the ^{32}P-labelled oligonucleotide $(GGAT)_4$ (for further information see Epplen 1992). Bands were scored within the range of 2 to 30 kb. The evaluation was done considering some fundamental rules outlined by Westneat (1990) by trying to assign all bands of the nestling to one of the putative parents. Band-sharing coefficients (BSC) were calculated as $BSC = C \times 2/(A + B)$, with C being the number of shared bands, and A and B the number of bands in the profiles of individual A and B, respectively (Jeffreys et al. 1985, Wetton et al. 1987).

For the samples from 1999, DNA isolation, digestion by restriction enzymes (*Hae* III), agarose electrophoresis, and capillary transfer to a nylon membrane (Biodyne B) followed standard protocols established in the M. Wink's laboratory (Swatschek et al. 1994). Nylon membranes were pre-hybridised in hybridisation mixture (5 × SSPE, 0.1% SDS, 1% powdered milk, 5 × Denhardt's solution; Sambrook et al. 1989) for two hours at 39°C. Hybridization was carried out with a hybridisation mixture containing 10 pmol/mL of the digoxigenated oligonucleotide probe $(GGAT)_4$ (Fresenius) at 39°C overnight. Membranes were washed three times with 6 × SSC for 30 minutes each. DNA/DNA-hybrids were detected by an antibody which was raised against digoxigenin (Boehringer). This antibody was coupled to a phosphatase which in turn produced a coloured precipitate at the sites of hybridisation. After colour reactions were completed, nylon membranes were documented and processed by the Bioprofil system (Fröbel, Lindau). Band-sharing coefficients (BSC) were calculated as explained above for the study performed in 1997.

Measurement of testes size

Between 27.11.96 and 18.3.01, 178 male barn owls have been collected dead along French highways in the regions Lorraine and Champagne. Owls stayed less than a day along the roads before being collected and put in a −20°C freezer until dissection. A.-L. Ducrest measured testes length and width to the nearest 0.1 mm. For each individual, we calculated the mean volume of the left and right testes, extracted from the formula $\pi \times (\text{width}/2)^2 \times \text{length}$, a value that was box-Cox transformed to normalise the data set, and hence to be used in parametric statistical analyses. Birds (n = 125) in which we found a bursa of Fabricius were considered to be 'juveniles'. Indeed, this immune organ is present only in the first year of life (Glick 1983), as shown with data from the Natural History Museum of Basel where this organ was measured in 74 barn owls ringed as nestlings and found dead in Switzerland between 1948 and 1996. Birds with a bursa of Fabricius were aged 20 to 271 days and those without it 143 to 2200 days. We estimated their age by considering number of days separating first of June (arbitrary birth date) and date of cadaver collection. Dead birds in which we did not find any bursa of Fabricius were said to be 'adults' (n = 53). Since testes are larger during the reproductive period, we statistically controlled for the number of days separating date of cadaver collection from the first of August, an arbitrary date for the end of the mating season (in our population egg laying dates span from 5 March to 30 July).

Assessment of circulating testosterone

In 1998, L. Sasvári measured testosterone concentration in birds from the same Swiss population as the one in which paternity analyses were performed. In 39 broods, we took a blood sample in 77 male and 81 female nestlings with a mean age of 48 days (range is 33 to 59 days), an age when colour pigments are deposited in the feathers. Nestling plumage coloration was assessed when they were 55 days, an age when plumage coloration is fully expressed with males being already lighter coloured than females (birds of both sexes become slightly lighter coloured at the first moult taking place one year later, and an individual that was darker coloured than another one at the first year of age is still darker at the second year; Roulin and Dijkstra 2003). After centrifugation, the plasma was immediately stored in a deep-freezer at −20°C, whereas red cells were preserved to determine

the sex of these birds using the molecular techniques gene method (analyses done by C. Dijkstra; for detailed information see Roulin et al. 1999). Plasma samples (100 μl) were extracted with 10 volumes of diethylether, three times (30 min extraction time). Testosterone concentrations were analysed, without chromatography, by radioimmunoassay (RIA). The detection limit was 5 pg/assay tubes. Intra- and inter-assay differences were 8% and 11%, respectively. The antibody (BSA-testosterone-3-hemisuccinate) cross-reacts with 5-α-dihydrotestosterone at a level of 40%, but the known concentration of this androgen in the avian plasma is generally less than 10% testosterone (Adkins-Regan et al. 1990, Galli et al. 1973, De Santo et al. 1983, Rissman and Wingfield 1984), so it probably only slightly modifies the apparent testosterone concentration. The existence of other androgens (androstenedione, dehydroandrosterone and dehydroandrosterone sulphata) in owls is not excluded, but their cross-reaction with the used antibody is less than 1% (Péczely et al. 1980).

Statistics

The relationship between blood concentration in testosterone and nestling plumage coloration was investigated with an ANOVA. Testosterone concentration was the dependent variable, nest a random categorical factor, sex a fixed factor, and rank in the within-brood age hierarchy and age at the time of blood sampling two covariates. We analysed the relationship between testes size and plumage coloration for juveniles and adults separately. In juveniles, testes size was the dependent variable in a multiple regression and both plumage coloration and estimated age two independent variables. In a similar model for adults, plumage coloration and number of days separating date of cadaver collection from the first of August were the two independent variables. All statistical analyses are two-tailed and P-values smaller than 0.05 considered to be significant. Means are followed ±1 S.D.

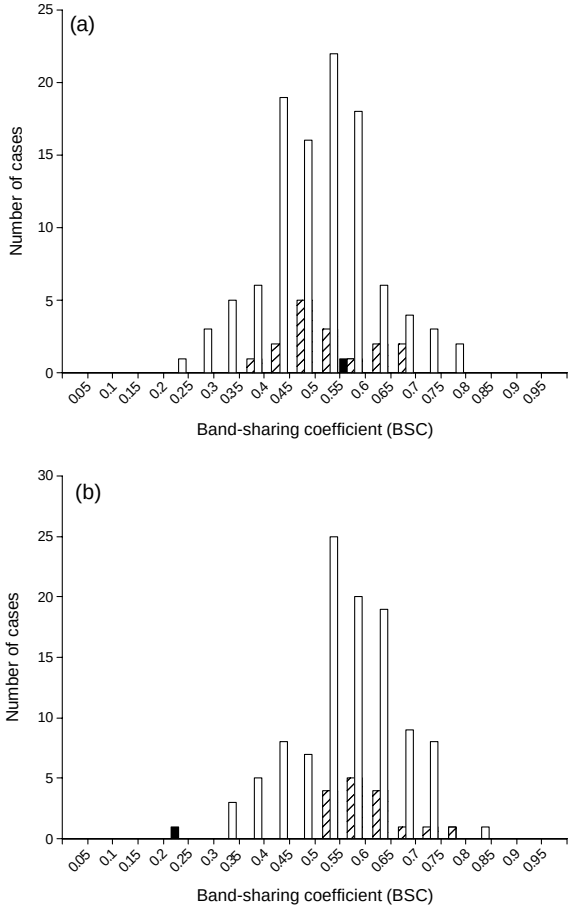


Fig. 1. Histogram of band-sharing coefficients for the study on extra-pair paternity performed for the sample from 1997. Values correspond to the comparison between (a) nestlings and their social mother and (b) nestlings and their social father. Different columns represent values for nestlings with different numbers of mismatches in their banding patterns, i.e. different numbers of fragments that were not present in the banding pattern of either social parent. White columns: nestlings without any mismatch (n = 105), striped columns: nestlings with a single mismatch (n = 16), black columns: nestling with six mismatches (n = 1).

Results

Assessment of extra-pair young

Using the restriction enzyme *Alu* I, we counted on average 26.8 ± 4.4 bands per individual (n = 62 adults) for the sample from 1997. Background BSC for breeding pairs averaged 0.22 ± 0.11 (n = 31), indicating a low genetic relatedness. The banding patterns of most of the nestlings (n = 105) completely matched the banding patterns of their social parents with an average BSC of 0.50 ± 0.11 with their social mother and 0.56 ± 0.10 with their social father (n = 105 each, see also Fig. 1). For 16 nestlings, a single fragment was detected that was not present in the banding patterns of either social parent. Since BSC values for these nestlings did not differ from those of nestlings with no mismatch in their banding patterns (BSC with the social mother = 0.52 ± 0.09, n = 16; BSC with the social father = 0.60 ± 0.07, n = 16; see also Fig. 1), we conclude that the novel fragments were due to mutational events. A single nestling, however, showed six novel fragments in its banding pattern. The BSC of this nestling with its social mother was 0.59, i.e. within the range found for within-pair young (see above and Fig. 1). In contrast, the BSC with its social father was only 0.21, i.e. outside the range found for within-pair young (see above and Fig. 1). Thus, we concluded that this single nestling results from an extra-pair fertilisation. The extra-pair male was reddish-brown

(mean colour score = 4.0) and raised a brood of five chicks two km away from his extra-pair young.

Using the restriction enzyme *Hae* III in 1999, an average of 8.35 ± 0.25 bands were counted (n = 46 adults) with a background BSC of 0.25 ± 0.19 (n = 23). The banding patterns of all 89 nestlings from the 1999 sample completely matched the banding patterns of their social parents with an average BSC of 0.63 ± 0.11 with their social mother and 0.62 ± 0.13 with their social father (n = 89 each). Thus, there was no evidence for the occurrence of extra-pair paternity in the sample from 1999.

Relationship between testes size and plumage coloration

In French birds found dead along highways, mean testes volume was 32.2 mm^3 (range = 4.0–253.2 mm^3). Testes size was not significantly related to plumage coloration in both juveniles (ANOVA: colour: $F = 0.94$, $df = 1,122$, $P = 0.34$, Fig. 2a; estimated age: $F = 19.93$, $df = 1,122$, $P < 0.0001$, Fig. 3a) and adults (colour: $F = 0.04$, $df = 1,50$, $P = 0.84$, Fig. 2b; number of days separating date of cadaver collection from the first of August: $F = 23.82$, $df = 1,50$, $P < 0.0001$, Fig. 3b). These results are robust, since plumage coloration was not significantly associated with testes size in an analysis including month of cadaver collection as a categorical variable (three-way ANOVA, testes size as dependent variable, colour: $F = 0.79$, $df = 1,164$, $P = 0.38$; age (juvenile vs adult): $F = 1193.55$, $df = 1,164$, $P < 0.0001$; month: $F = 8.35$, $df = 1,164$, $P < 0.0001$).

Relationship between testosterone level and plumage coloration

In Swiss nestlings, mean testosterone level was 274.6 ± 65.30 pg/ml (range = 132-464 pg/ml). We found no relationship between testosterone level and plumage coloration (5.17 ± 1.38) in nestlings (ANOVA: $F = 0.13$, $df = 1,115$, $P = 0.72$, Fig. 4; nest: $F = 1.89$, $df = 38,115$, $P = 0.005$; age: $F = 2.02$, $df = 1,115$, $P = 0.16$; sex: $F = 0.16$, $df = 1,115$, $P = 0.69$; rank in within-brood age hierarchy: $F = 4.84$, $df = 1,115$, $P = 0.03$, testosterone concentration was higher in earlier hatched chicks).

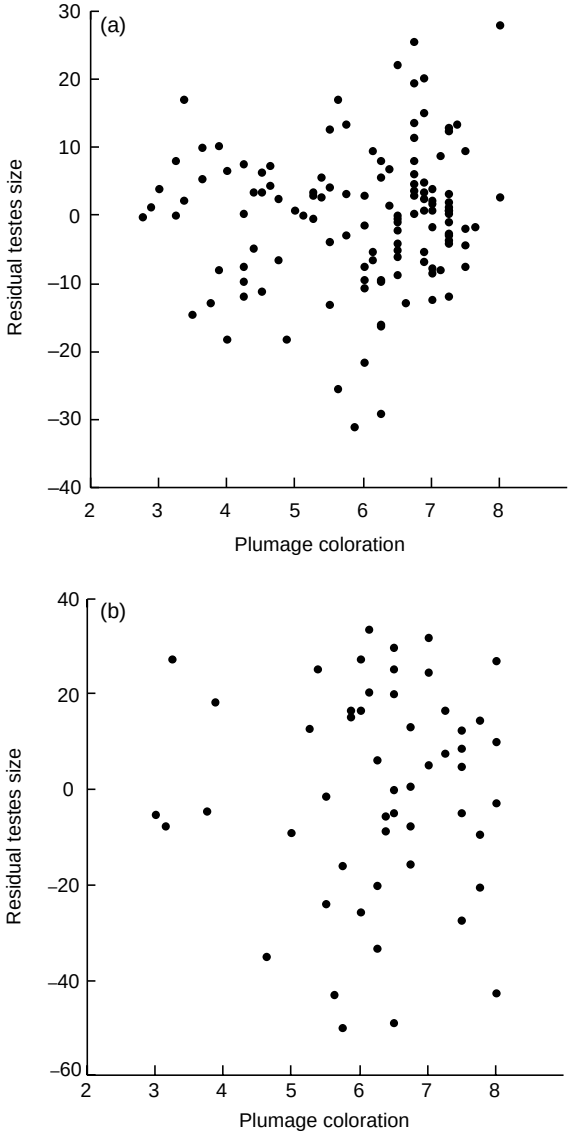
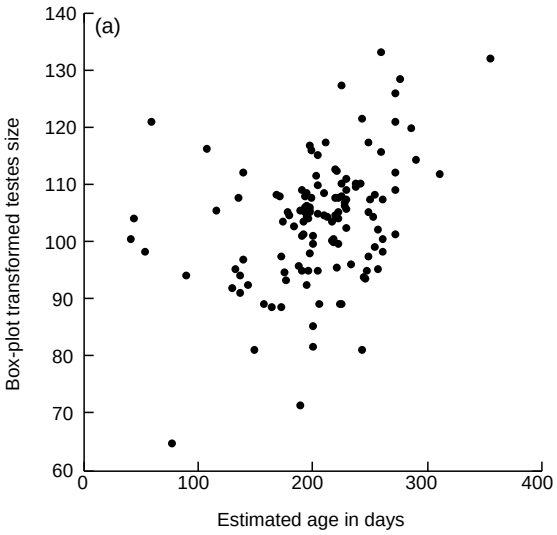


Fig. 2. Relationship between residual testes size and plumage coloration in juvenile (a) and adult barn owls (b). Residuals were extracted from a regression of Box-Cox transformed testes size on estimated age in juveniles (Fig. 2a) or number of days separating date of cadaver collection from the first of August in adults (Fig. 2b).

Discussion

In the present study, we found no evidence for the hypothesis that plumage coloration reflects alternative mating strategies in the barn owl. The frequency of EPY was extremely low with only one extra-pair young out of 211 analysed nestlings. Furthermore, in growing nestlings plumage coloration was not associated with testosterone level suggesting that the expression of this trait does not rely on this androgen. Finally, testes size was not correlated with plumage coloration indicating that white males may not be sexually more active than reddish-brown ones. Therefore, if in our Swiss population reddish-brown males invested more effort in reproduction and produced more offspring (Roulin et al. 2001), white males did not equalise fitness by investing more effort in sexual activities.



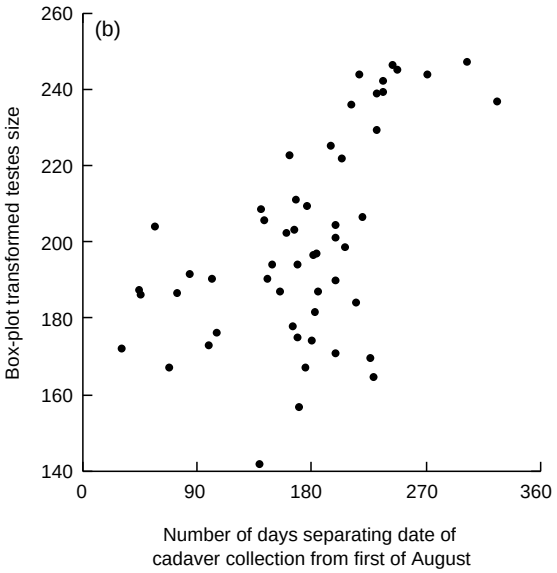
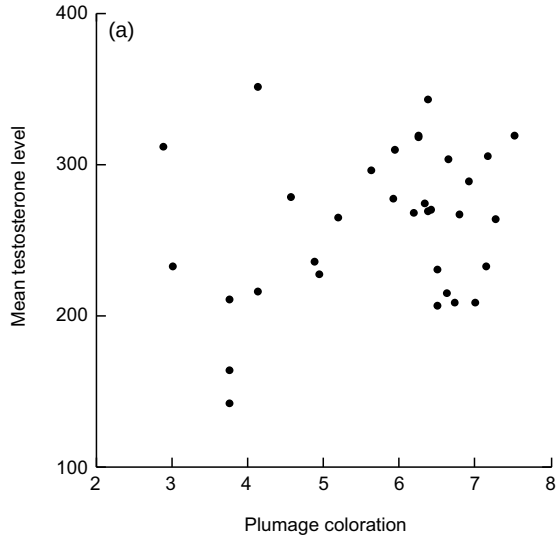


Fig. 3. Relationship between testes size (box-plot transformed) and estimated age in juveniles (a) and number of days separating date of cadaver collection from the first of August in adults (b).



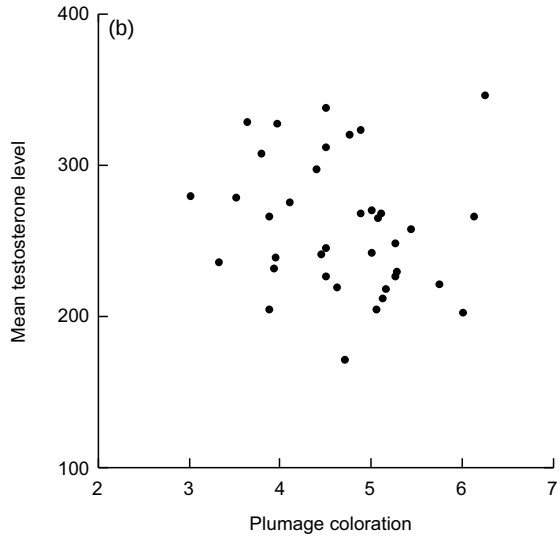


Fig. 4. Relationship between testosterone level and plumage coloration in male (a) and female nestlings (b). Each circle represents mean sibling values.

Although it could be adaptive for white males to enhance their reproductive success through EPY, they apparently fail to do so. This might be caused by the high fitness cost of reduced paternity in species like raptors and owls in which males invest substantial effort in parental care probably leading to the evolution of a high copulation frequency. In the barn owl, copulation frequency is very high with birds copulating almost all year long (January until November). In captivity, copulations commence on average 32 days before egg laying with a frequency of more than one copulation per hour and a maximal number of 70 copulations in 24 hours (Epple 1985). Such a high copulation frequency by the social mate strongly reduces the probability for an extra-pair male to fertilize his female (Birkhead and Møller 1992). The 14 studies in Falconiformes and Strigiformes showed rare or no EPY (Table 1).

Knowledge of the proximate mechanisms of the development of secondary sexual traits is of interest to understand the signaling function of colourful ornaments. In a number of bird species, the expression of plumage traits is testosterone-dependent, a hormone that has a plethora of side effects including a role in regulating aggression, parental care, courtship behaviour and immunocompetence (review in Hillgarth and Wingfield 1997). Testosterone is therefore a candidate for explaining why ornaments frequently signal male quality.

Table 1. Review of studies measuring the frequency of extra-pair young in raptors and owls using molecular techniques.

Species	No. of broods	No. of nestlings	Broods with EPY (%)	No. of EPY (%)	Authors
Black vulture *Coragyps atratus*	16	36	0	0	A
Galapagos hawk *Buteo galapagoensis*	10	66	0	0	B
European sparrowhawk *Accipiter nisius*	33	?	?	? (5.4)	C
Eleonora's falcon *Falco eleonorae*	17	60	0	0	D
Merlin *Falco columbarius*	9	31	0	0	E
Lesser kestrel *Falco naumanni*	26	87	1 (3.8)	3 (3.4)	F
Kestrel *Falco tinnunculus*	75	319	2 (2.7)	6 (1.9)	G
American kestrel *Falco sparverius*	21	89	2 (9.5)	10 (11.2)	H
Barn owl *Tyto alba*	54	211	1 (1.9)	1 (0.5)	I
Little owl Athene noctua	16	53	0	0	J
Screech-owl *Otus asio*	23	80	0	0	K
Flammulated owl *Otus flammeolus*	17	37	0	0	L
Long-eared owl *Asio otus*	12	59	0	0	M
Burrowing owl *Speotyto cunicularia*	18	31	?	2 (6.5)	N

(A) Decker et al. (1993), (B) Faaborg et al. (1995), (C) Parkin in Birkhead and Møller (1992), (D) Swatschek et al. (1993), (E) Warkentin et al. (1994), (F) Negro et al. (1996), (G) Korpimäki et al. (1996), (H) Villarroel et al. (1998), (I) present study, (J) Müller et al. (2001), (K) Lawless et al. (1997), (L) Arsenault et al. (2002), (M) Marks et al. (1999), (N) Johnson (1997).

This hormone has been shown experimentally to reduce parental care (Raouf et al. 1997), and hence we hypothesized that reddish-brown male barn owls invest more effort in reproduction because they have lower levels of circulating testosterone than white males. However, results of the present study show that at the time when pigments are stored in feathers, the level of circulating testosterone does not covary with plumage coloration. This suggests that the expression of plumage coloration does not rely on testosterone. Although we measured this hormone in nestlings instead in breeding adults, testosterone may not explain the observation of colour-dependent investment in reproduction (Roulin et al. 2001). This statement requires further investigation by assaying testosterone in relation to plumage coloration in adults. Whereas male and female nestlings did not differ in testosterone levels, at the time of breeding it is unlikely that adult males and females show similar levels. Even if plumage coloration is not testosterone-dependent, we cannot rule out the possibility that lighter coloured adult males produce more testosterone during the mating season.

The present study shows that white males do not invest more effort in sexual activities than reddish-brown ones. Therefore, the question remains open on how can the colour polymorphism be evolutionary stable in Switzerland if in some years reddish-brown males produce more offspring than white ones (Roulin et al. 2001). Two alternative scenarios to the one proposing that white males produce more EPY have still to be investigated. First, white males may live longer than reddish-brown ones because of their lower investment in reproduction allowing them to produce a similar number of offspring during their lifetime. Long-term data are being currently accumulated to test this hypothesis in the near future. Second, reddish-brown males may have produced more offspring in years that proved to be particularly favorable to this phenotype. Following this hypothesis, white males may achieve a higher reproductive success in other years when environmental conditions favour their phenotype. Under this scenario, colour polymorphism would be maintained under disruptive selection with reddish-brown and white males being adapted to alternative environmental factors. This hypothesis is consistent with the maintenance of the European cline variation in coloration (Roulin 2003), with reddish-brown birds being adapted to environmental factors prevailing in northern Europe and white birds to factors found in southern Europe. Furthermore, in Switzerland reddish-brown birds were found to eat primarily common voles *Microtus arvalis* and white ones wood mice *Apodemus* spp. suggesting that differently coloured birds exploit alternative habitats or that hunting success on alternative prey is colour-dependent (Roulin 2004). Experimental studies are now required to investigate these two alternative scenarios.

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