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Corticosterone mediates the condition-dependent component of melanin-based coloration

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The handicap principle of sexual selection theory states that colourful phenotypic traits signal aspects of individual quality because only individuals in prime condition can afford to produce and bear conspicuous traits. Melanin-based pigments participate in the elaboration of many secondary sexual characters and, given their role in sexual selection, melanin-based coloration may therefore honestly reflect individual quality. Although the expression of melanism is usually under genetic control, in some species it is condition dependent. However, the underlying physiological mechanism is yet unknown. Based on the negative feedback link between corticosterone and melanogenesis (melanocortins, tyrosinase) in response to stressful environmental factors, we hypothesize that corticosterone mediates the condition-dependent component of melanism. This hypothesis predicts that stressful factors induce a rise in circulating corticosterone which inhibits the secretion of melanocortins and tyrosinase and in turn melanin production. We tested this prediction by manipulating the level of corticosterone at the time of melanin production in nestling barn owls, *Tyto alba*, a species showing heritable variation in the degree of phaeomelanism from reddish-brown to white. The finding that corticosterone-implanted nestlings produced feathers with less phaeomelanic coloration than placebo-implanted nestlings is consistent with the hypothesis that the environment-mediated reduction in the degree of melanism is, at least in part, caused by a rise in corticosterone. In species in which the expression of melanin-based coloration is condition dependent, we now need a test showing that individuals with less corticosterone and more melanin-based signals are individuals in better condition.

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Colourful skin, feathers and cuticles are among the most striking visible phenotypic aspects in many animals. Among the different mechanisms responsible for variation in coloration, deposition of melanin pigments is probably the most widespread. Melanin-based coloration is sensitive to both natural and sexual selection by playing important roles in prey–predator interaction, thermoregulation and social interactions (Majerus 1998; Jawor & Breitwisch 2003; Roulin 2004a). Although the expression of melanin-based coloration is often under genetic control (Majerus 1998; Gantz & Fong 2003; Roulin & Dijkstra

2003), significant environmental components have been detected in several species (e.g. Griffith et al. 1999; Horth 2003; Fargallo et al. 2007), suggesting that the degree of melanism can sometimes honestly signal aspects of individual quality as initially proposed by Zahavi (1975) under the handicap principle of sexual selection. Indeed, in many species of vertebrates melanin-based ornaments are used as criteria in mate choice (Majerus 1998; Jawor & Breitwisch 2003; Roulin 2004a) and the degree of melanism covaries with life history, morphological, behavioural and physiological traits (Roulin 2004a). However, the physiological mechanism that mediates the environmental component of interindividual variation in melanism is not yet known (Griffith et al. 2006). Identifying the molecules that mediate the environmental component of variation is key because these molecules may affect other important phenotypic traits, potentially explaining why less melanic individuals can perform less well than deeply melanic conspecifics (Jensen et al. 2004). To appraise this question, a detailed knowledge of the mechanism underlying the production of melanin pigments is necessary.

Melanin consists principally of two heteropolymers, the brown to black eumelanin and yellow to reddish-brown phaeomelanin that are synthesized in melanocytes in eyes, skin, hair, feathers and cuticle. Melanin synthesis starts with the hydroxylation of L-tyrosine by the rate-limiting enzyme tyrosinase to DOPA and dopaquinone. During eumelanin synthesis the tyrosine-related enzymes TYRP1 and DCT further transform dopaquinone into di-hydroxyindole-derived precursors of eumelanin (DHI and DHICA), whereas incorporation of cysteine or gluta-thione to dopaquinone produces phaeomelanin pigments (Prota 1992). The most important regulators of melano-genesis are the melanocortin 1-receptor (Mc1R), its antag-onist the agouti signalling protein (ASIP) and agonists the melanocortins melanin-stimulating hormones (MSHs) and adrenocorticotropic hormone (ACTH) which are post-translational products of the prohormone encoded by the pro-opiomelanocortin (POMC) gene (Eberle 1988). Binding of ASIP and melanocortins to Mc1R triggers the synthesis of phaeomelanin and eumelanin pigments, respectively (Slominski et al. 2004).

Melanocortins regulate not only the production of melanin (Jimbow et al. 2000; Rees 2003) but also a number of other physiological functions including the stress response principally through ACTH, which is part of the hypothalamic–pituitary–adrenal axis (HPA) (Simpson & Waterman 1988). The HPA consists of the hypothalamic corticotropin-releasing hormone (CRH) that signals to pituitaric ACTH, which further stimulates adrenal gluco-corticoid synthesis including cortisol and corticosterone. The latter two glucocorticoids are responsible for a time-limited and adaptive stress response, and they control glu-coneogenesis, lipolysis, immune function, sexual activity, growth and development to adequately respond to stress (Hofer & East 1998; Charmandari et al. 2005). Glucocorti-coids exert their effects through receptors which are ex-pressed in most tissues in vertebrates including the skin and hair follicle cells where melanin pigments are produced and packed (Ito et al. 2005). During the stress response glucocorticoids have inhibiting effects on melanogenesis (Slominski et al. 2004) by reducing the transcription of POMC, Mc1R and tyrosinase and the activity of DCT (Arnold et al. 1975; Ermak & Slominski 1997) and by induc-ing hair follicle regression (Paus et al. 1994).

Based on the known negative feedback link between corticosterone and melanocortins during the stress re-sponse, we tested experimentally in wild barn owls, *Tyto alba*, whether an elevation in corticosterone level reduces the production of melanin-based coloration. This is an im-portant experiment, because it could reveal that cortico-sterone mediates the observed environmental effects on melanin production found in other species of birds (house sparrow, *Passer domesticus*, Griffith et al. 1999; European kestrel, *Falco tinnunculus*, Fargallo et al. 2007). We there-fore manipulated the level of circulating corticosterone in nestling barn owls which vary from reddish-brown to white, a phaeomelanin-based trait, and from heavily marked with black spots to immaculate, a eumelanin-based trait (Roulin 2004b). We show that interindividual variation in the degree of reddish-brownness is due to the deposition of phaeomelanin pigments and not of por-phyrin and carotenoids, two other reddish pigments. Also, we report an experiment where we implanted subcutane-ously pellets of corticosterone or placebo in 15- to 39-day-old nestlings to test the effect of corticosterone on the production of phaeomelanin-based feather coloration. We could not test the effect of corticosterone on eumelan-ism because black spots, located at the tip of feathers, were already produced at the time of implantation, while phaeomelanic pigments were still deposited in the grow-ing feathers (feathers are entirely reddish-brown).

METHODS

Study Organism

In most worldwide distributed barn owl populations, individuals vary in the degree of phaeomelanism from reddish-brown to white and in the degree of eumelanism from heavily marked with black spots to immaculate. Plumage traits are genetically correlated with darker reddish-brown birds displaying more and larger black spots (Roulin & Dijkstra 2003; Roulin 2004b). Nestlings express the full variation in these two plumage traits, and feathers grown at the nestling stage are not moulted until the second year of age, that is, after the first breeding attempt. Cross-fostering experiments have demonstrated that the resemblance in coloration of related individuals is heritable and not influenced by the environment at least not to a detectable extent (Roulin & Dijkstra 2003). Although the two sexes can express any plumage trait, females are on average more melanic than males, that is, darker reddish-brown and displaying more and larger black spots. These two plumage traits appear to play a role in mate choice with males potentially preferring heavily over lightly spotted females (Roulin 1999; Roulin & Altwegg 2007) and females potentially preferring white over reddish-brown males (Roulin & Altwegg 2007). The size of black spots covaries positively with humoral immu-nocompetence (Roulin et al. 2000), parasite resistance (Roulin et al. 2001a), developmental homeostasis (Roulin

et al. 2003), calcium bone concentration (Roulin et al. 2006), age at maturity and survival prospects (Roulin & Altwegg 2007). Darker reddish-brown males were observed to produce more (Roulin et al. 2001b) and heavier (Roulin et al., in press) offspring. Therefore, the two melanin-based colour traits covary with important physiological and life history traits.

Experimental Design

The study was carried out in 2004, 2005 and 2006 in western Switzerland. To experimentally investigate the effect of corticosterone on the expression of phaeomelanin-based coloration in nestlings, B. Almasi implanted subcutaneously in the flank either a pellet (diameter 0.5 cm, biodegradable carrier-binder containing 15 mg corticosterone; Innovative Research of America, Florida) releasing corticosterone over 4 days which resulted in a circulating corticosterone level of about 25 ng/ml (baseline level before implantation was 7.94 ng/ml; B. Almasi, unpublished data) or a placebo pellet (biodegradable carrier-binder without corticosterone). A small incision was made and the implant was placed under the skin which was then closed with adhesive tissue (Histoacryl; Braun, Switzerland). In 39 broods we implanted two nestlings with corticosterone (hereafter denoted as 'corticosterone-nestlings') and two other individuals with placebo (hereafter denoted as 'placebo-nestlings'), in two broods we had three corticosterone-nestlings and two placebo-nestlings, in eight broods two corticosterone-nestlings and one placebo-nestling, and in 23 broods one corticosterone-nestling and one placebo-nestling. Using molecular methods we determined nestling sex in all but one individual (not enough blood was collected for this individual) (Py et al. 2006).

Because some individuals died or fledged earlier than anticipated, we collected breast feathers to measure phaeomelanin-based coloration in 108 corticosterone-nestlings (58 in 2004, 14 in 2005 and 36 in 2006) and 96 placebo-nestlings (52 in 2004, 13 in 2005 and 31 in 2006) aged 55 days. Similar proportions of female (61 of 113 experimental females; 54.0%) and male (47 of 90 males, 52.2%) nestlings were implanted with corticosterone (chi-square test: $\chi^2_1 = 0.06, P = 0.80$). Mean $\pm$ SD age at the time of injection (28 ± 5 days; range $15-39$ days) did not differ between corticosterone- and placebo-nestlings (mixed-model ANOVA with age as the dependent variable and nest of rearing as a random factor: treatment: $F_{1,144.4} = 2.38, P = 0.13$). At the time of implantation, corticosterone- and placebo-nestlings did not differ in wing length (mixed-model ANCOVA: treatment: $F_{1,147.4} = 2.77$, $P = 0.10$; age: $F_{1,169.7} = 3691.2$, $P < 0.0001$) and body mass (mixed-model ANCOVA: treatment: $F_{1,138.9} = 0.25$, $P = 0.62$; age: $F_{1,188} = 42.60$, $P < 0.0001$; age$^2 = F_{1,186.2} = 18.13$, $P < 0.0001$). Finally, parents of corticosterone- and placebo-nestlings did not differ in plumage coloration, number of spots and spot diameter (Student's t tests on mean nest values: all $P > 0.70$).

Before implanting pellets, we collected a 20-µl blood sample to measure baseline corticosterone level (ng/ml) by puncturing the brachial vein and collecting the blood with heparinized capillaries. Samples were immediately centrifuged and the plasma was stored in liquid nitrogen. After transport to the laboratory, the samples were stored at $-20°$C until analysis in the next autumn. An increase in circulating corticosterone after an initial stress is detected after 3 min (Romero & Reed 2005; B. Almasi, unpublished data). Thus, we measured baseline corticosterone level only if blood samples were collected within 3 min of first opening the nestbox. For this reason, we could measure baseline corticosterone level in 113 of the 204 experimental individuals. Plasma corticosterone concentration was determined using an enzyme immunoassay (Munro & Stabenfeldt 1984; Munro & Lasley 1988) following Müller et al. (2006).

Assessment of Plumage Coloration and Number and Size of Black Spots

A. Roulin measured plumage traits in nestlings and their parents in the field. Plumage coloration was assessed on the breast by comparison with eight colour chips from 1 (dark reddish-brown) to 8 (white) (Roulin 1999); for the human eye, on each feather there is a slight continuous gradation in coloration with the top being darker than the base, and thus coloration was assessed only at the top of feathers. On the same body part, black spots were also counted within a 60×40 mm frame and measured to the nearest 0.1 mm. A mean spot diameter value was calculated and used in the statistical analyses. Assessment of plumage traits was done without being aware of which individual was implanted with a corticosterone or a placebo pellet, since at that time only B. Almasi knew this information.

When nestlings were 55 days of age, we collected three breast feathers of each experimental nestling by cutting fully grown (i.e. metabolically inert) feathers off at their base using a scissor. These feathers were collected at the same place and were representative of all breast feathers because coloration is uniform on each body part. In November 2006, A. Rossi-Pedruzzi superposed the three feathers and stuck them on a black paper in a black box equipped with a fluorescent tube (8w/20-640 bl-super). A picture of the collected feathers was taken with a digital camera (Konika Minolta; Dimage A200) fixed at a distance of 27 cm from the feathers. Pictures were imported into the software Adobe Photoshop CS2 to measure hue (value that allows a colour to be distinguished as red, blue, yellow, and so on), saturation (or chroma; indicates the purity or strength of a colour) and brightness (indicates the relative lightness of a colour, i.e. the proportion of white and black) on three randomly chosen points at the top and three other points in the middle of the feathers; we took care not to measure coloration of the black spots. For each of these two feather regions, we then calculated a mean value for hue, saturation and brightness. Coloration measured in the field using colour chips was more strongly correlated with saturation (Pearson correlation: $r = -0.69, N = 204$, $P < 0.0001$) than with hue ($r = 0.43, N = 204, P < 0.0001$) and brightness ($r = 0.06, N = 204, P = 0.40$). To quantify

the effect of corticosterone on phaeomelanin-based colora-tion, we calculated the differences in saturation, hue and brightness of the mean of the three measurements on the top and the mean of the three measurements at the middle of the feathers. This value is denoted 'contrast in feather saturation', 'hue' and 'brightness'. We used this procedure because in many cases a decoloured band in the middle of feathers was apparent, probably the effect of corticosterone (see Results).

Feather Content of Melanin, Porphyrin and Carotenoid

K. Wakamatsu identified the concentration in phaeo-melanin and eumelanin pigments in one entire feather collected on the flank of 10 adults. As previously de-scribed (Ito & Wakamatsu 1994; Wakamatsu & Ito 2002), melanin pigments were chemically degraded and then using high-performance liquid chromatography (HPLC) we quantified pyrrole-2,3,5-tricarboxylic acid (PTCA), which is a specific degradation product of eumelanin, and 4-amino-3-hydroxyphenylalanine (4-AHP), which is a specific degradation product of phaeomelanin. Briefly, 3.0–9.0 mg of each feather was homogenized with 0.5 ml water by using a Ten-Brocke glass homogenizer. For the HPLC determination of eumelanin, feather homogenates (100 μl) were oxidized with potassium per-manganate ($KMnO_4$) to give PTCA, which was quantified with HPLC using ultraviolet detection. Each determina-tion was performed in duplicate. For the HPLC determi-nation of phaeomelanin, feather homogenates (100 μl) were hydrolyzed with hydriodic acid (HI) to give 4-AHP, which was quantified with electrochemical detection; 1 ng of PTCA corresponds to 50 ng of eumelanin, and 1 ng of 4-AHP corresponds to 9 ng of phaeomelanin.

I. Miksík determined the concentration in porphyrins in one feather weighing 10–25 mg collected on the flank or belly from each of 11 individuals found dead along French highways in 2003. Using a method similar to that to deter-mine porphyrins in eggshells (Miksík et al. 1996), these feathers were esterified in a solution of 10 ml absolute methanol (LiChrosolv; gradient grade for chromatogra-phy; Merck, Darmstadt, Germany) containing 5% concen-trated sulphuric acid. This procedure was done at room temperature in the dark under an atmosphere of N_2. One day later extracts were decanted and mixed with 2.5 ml chloroform (Merck; chloroform GR, ISO) and 5 ml distilled water before being shaken. The lower chloroform phase was collected and the higher water phase was once again extracted with chloroform. The two extractions were pooled together before being evaporated to dryness and re-constituted in 0.5 ml chloroform. Standard for the quanti-fication (protoporphyrin IX; Sigma, St. Louis, MO, U.S.A.) was treated using the same procedure. To extract porphy-rins strongly packed in feathers, the remaining feathers were hydrolyzed under alkaline conditions (0.1 M NaOH, 105°C) for 18 h under N_2, filtered, neutralized with HCl, evaporated and reconstituted in a methanol solution con-taining 5% concentrated sulphuric acid. This reconstituted solution was then esterified and the extracts were collected as described above. In the feather extracts, we determined and quantified protoporphyrin IX in the form of dimethy-lester. We performed reversed-phase high-performance chromatography using an Agilent 1100 LC system (Agi-lent, Palo Alto, CA, U.S.A.) consisting of a degasser, binary pump, autosampler, thermostatted column compartment, and multiwavelength and fluorescence detectors. Chro-matographic separation was carried out in a Zorbax Eclipse XDB C18 column (150 × 2.1 mm I.D.; Agilent). The sam-ple (20 μl) was injected into the column and eluted with a gradient consisting of (A) methanol–water–pyridin 35:65:0.25 v/v and (B) methanol–acetonitrile–pyridine 90:10:0.25 v/v (flow rate 0.25 ml/min, temperature 55°C). The gradient started at A/B 80:20 reaching A/B 10:90 after 15 min. For the next 10 min the elution was isocratic followed by another 10-min isocratic elution at 100% B. Elution was monitored by absorbance at 410 nm and by fluorescence at $405_{ex}/620_{em}$ nm.

J. D. Blount analysed barn owl feathers for carotenoid pigments using standard methods (Hudon & Brush 1992; Stradi et al. 1995). Four or five feathers collected from the flank of 10 individuals were washed in ethanol (30 s) and hexane (30 s) before being blotted dry on filter paper. Sam-ples (3–5 mg) of the coloured barbules were then cut from feathers and ground to a fine powder using a mixer mill (Model MM200 with zirconium oxide jars and balls; Retsch GmbH, Haan, Germany) at 30 Hz for 15 min in the presence of 3 ml methanol. The resultant mixture was centrifuged at 12 000g at 4°C for 5 min. After centrifu-gation, the methanol was collected and then evaporated to dryness in a vacuum at room temperature using a sam-ple concentrator. Samples were then redissolved in 1 ml methanol ready for determination of carotenoids if pres-ent, which was done using two methods. First, to deter-mine the fine spectrum and wavelength of maximum absorbance (λ_{max}) of samples, the absorbance at 1-nm in-tervals between 400 and 500 nm was measured using a bench top spectrophotometer (Nicolet Evolution 500; Thermo Electron Corp., Hemel Hemstead, U.K.). Second, samples (50 μl) were injected into a Dionex HPLC system (Dionex Corp., California, U.S.A.) fitted with a 5-μm ODS guard column and a Spherisorb ODS2, 5 μ C_{18} reverse-phase column (250 × 4.6 mm) (part PSS831915; Waters Corp., Massachusetts, U.S.A.) maintained at 20°C in a ther-mostatted column compartment (TCC-100; Dionex). The mobile phase consisted of a linear gradient starting with 100% solution A (acetonitrile–methanol, 85:15, v/v) and ending with 100% solution B (acetonitrile–dichloro-methane–methanol, 70:20:10, v/v) over 24 min at a flow rate of 2 ml/min^{-1}. Data were collected from 350 to 600 nm using a photodiode array detector (PDA-100; Dionex). For both absorbance spectrophotometry and HPLC, J.D.B. also ran replicates of a positive control (carot-enoids extracted from canary feathers in methanol) to validate the isolation and determination procedures.

Statistical Analysis

Statistical tests were computed with the JMP statistical package version 6 and P values lower than 0.05 are consid-ered significant. Means are quoted ±SD.

Ethical Note

The experiment was under legal authorization of the 'service vétérinaire du canton de Vaud' (no. 1736). The moderate level of the injection of corticosterone had only a transient effect on nestling body mass (mean $\pm$ SE body mass before implantation in 49 corticosterone- and 45 placebo-nestlings in 2004: 331 ± 7 g versus 323 ± 7 g; Student's t test: $t_{92} = 0.79$, $P = 0.43$; 6 days later: 322 ± 6 g versus 344 ± 6 g; $t_{90} = 2.66$, $P = 0.009$), and around fledging (i.e. 50 days of age) there was no difference in body mass in corticosterone- and placebo-individuals (350.4 ± 3.7 versus 356.6 ± 3.5 g; Student's t test: $t_{182} = 1.20$, $P = 0.23$). Before fledging a similar proportion of corticosterone-nestlings (10 of 122; 8.2%) and placebo-nestlings (8 of 114; 7.0%) died (chi-square test: $\chi^2_1 = 0.12$, $P = 0.73$). Because in 2005 we implanted only two nestlings per brood, we could test whether implanting a pellet decreased survival between the time of implantation and fledging. This was not the case because 20 of 179 (11.2%) nonimplanted nestlings died while 3 of 36 (8.3%) implanted nestlings died ($\chi^2_1 = 0.25$, $P = 0.62$).

RESULTS

Feather Content of Melanin, Porphyrin and Carotenoid

Concentrations of eumelanin and phaeomelanin in feathers were 486 ± 49.4 and 3751.47 ± 1333.2 ng/mg, respectively. In a stepwise ANCOVA, feather concentration of phaeomelanin was associated with plumage coloration measured on a scale from 1 to 8 ($F_{1,8} = 26.76$, $P = 0.0008$) but not with number of spots, spot diameter and sex (all $P > 0.47$). Feather concentration of eumelanin tended to be higher in females than in males ($F_{1,8} = 4.88$, $P = 0.058$); plumage coloration, number of spots and spot diameter did not explain any significant variation in eumelanin (all $P > 0.11$). Mean feather concentration of porphyrin was 132 ± 68 µg/g. Porphyrin concentration was not significantly correlated with plumage coloration (Pearson correlation: $r = -0.20$, $N = 11$, $P = 0.55$). No carotenoids were detected.

Baseline Corticosterone Level

Before pellet implantation, mean baseline corticosterone level was 7.94 ± 6.69 ng/ml. This level was not associated with the degree of reddish-brownness (i.e. saturation) measured at the tip of feathers (mixed-model ANOVA with log-transformed baseline corticosterone level as the dependent variable and nest as a random variable: $F_{1,108.3} = 1.61$, $P = 0.21$).

Effect of Corticosterone on Phaeomelanin-based Coloration

In 64 of the 108 (59.3%) corticosterone-nestlings, there was a visible decoloured feather zone located 4–18 mm (8.7 ± 3.6 mm) from the tip (the collected feather was 20–40 mm long). A similar decoloured zone was visible in only five of the 96 (5.2%) placebo-nestlings (chi-square test: $\chi^2_1 = 66.3$, $P < 0.0001$). Decoloured zones were visible on the collected feathers but also on the entire plumage when we had the owls in hand. In corticosterone-nestlings, presence/absence of a decoloured zone was not associated with nestling age at the time of implantation, sex or plumage traits (logistic regression with presence/absence of decoloured zone as dependent variable (0/1): all $P > 0.51$). The distance between the tip of feathers and the decoloured zone was positively correlated with age at implantation ($r = 0.54$, $N = 64$, $P < 0.0001$), which is consistent with the feather part above the decoloured zone being already produced at the time of implantation.

In both placebo- and corticosterone-nestlings, coloration was more saturated (i.e. more reddish-brown; 25.0 ± 11.2 versus 25.11 ± 10.1) at the tip of feathers than in the middle where the feather was decoloured (17.3 ± 7.5 versus 14.3 ± 7.0) (paired t tests: placebo-nestlings: $t_{95} = 8.84$, $P < 0.0001$; corticosterone-nestlings: $t_{107} = 13.18$, $P < 0.0001$). Contrast in saturation was more pronounced in female (10.3 ± 8.1) than in male (4.8 ± 8.0) nestlings (mixed-model two-way ANOVA with contrast as the dependent variable and nest as a random variable: $F_{1,198} = 16.51$, $P < 0.0001$) and in corticosterone- than in placebo-nestlings (Fig. 1; same model: $F_{1,150.9} = 8.82$, $P = 0.0035$); interaction between sex and treatment was not significant ($P = 0.56$) and including year in the model did not modify the results. The effect of our treatment was detected at the middle of feathers where coloration was lighter in corticosterone- than in placebo-nestlings (Student's t test: $t_{202} = 2.97$, $P = 0.0034$), whereas no treatment effect was detected at the tip of feathers which is consistent with this feather part being already produced at the time of implantation ($t_{202} = 0.07$, $P = 0.95$). Within
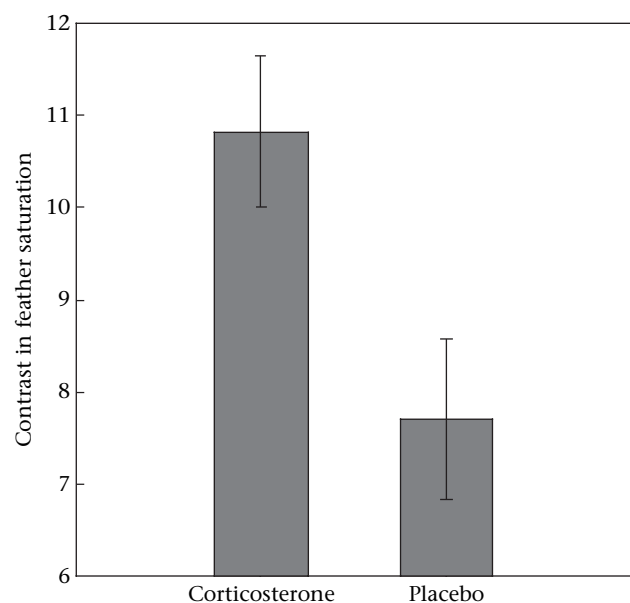
Figure 1. Contrast in feather saturation in nestlings implanted with corticosterone and placebo. Contrast refers to the difference in saturation between the tip and the middle of feathers.

corticosterone-individuals saturation at the tip of feathers was positively correlated with saturation in the middle of feathers (mixed-model ANOVA with saturation in the middle of feathers as the dependent variable and nest as a random factor: saturation at the tip of feathers: $F_{1,100.9} = 22.11$, $P < 0.0001$; sex: $F_{1,102.4} = 7.03$, $P = 0.009$; females being darker than males: number of spots: $F_{1,97.82} = 0.20$, $P = 0.66$; spot diameter: $F_{1,102.1} = 1.67$, $P = 0.20$). This indicates that the reduction in saturation due to corticosterone is proportional to the degree of phaeomelanism in the absence of extra corticosterone but is not proportional to the degree of eumelanism (i.e. number and size of black spots). Corticosterone did not affect feather hue and brightness (results not shown).

DISCUSSION

Phaeomelanism varies gradually from dark reddish-brown at the tip to a lighter coloration at the middle of feathers, a gradient that was more pronounced in corticosterone- than in placebo-nestlings. This indicates that corticosterone reduced the deposition of phaeomelanin pigments in the middle of the feathers; because black spots were already produced at the time of implantation (spots are located at the tip of the feathers), we could not test the possibility that this hormone also regulates the deposition of eumelanin pigments. A decoloured feather band was detected in 59.3% of corticosterone-individuals.

Implantation of corticosterone pellets elevated circulating corticosterone to a level of 25 ng/ml on average over a period of 4 days (baseline level before implantation is 7.94 ng/ml on average), before corticosterone returns to baseline level (B. Almasi, unpublished data), indicating that the effect of our experiment should have been transient. Accordingly, the decoloured zone in the middle of feathers of corticosterone-individuals measured approximately 5–10 mm; a similar decoloured zone was observed in only 5.2% of placebo-nestlings, indicating that in natural conditions decoloration can happen, and thus the production of phaeomelanin pigments can sometimes be condition dependent in the barn owl. This is consistent with the finding that so-called fault bars (deterioration of feather structure and pigmentation) on wing and tail feathers have been associated with stressful events (King & Murphy 1984; Murphy et al. 1988, 1989; Machmer et al. 1992).

Based on the genetic mechanisms underlying melanin biosynthesis, we predicted that an injection of corticosterone should decrease the synthesis or deposition of phaeomelanin pigments and in turn reddish-brown feather coloration. This is consistent with the finding that an injection of corticosterone decreased saturation (a measure of the strength of a colour) but neither hue (a value that indicates coloration) nor brightness (a value that indicates the relative lightness of a colour). Melanin pigments are synthesized in lysosome-like organelles called melanosomes (Slominski et al. 2004) which are then transferred from melanocytes into surrounding keratinocytes that are progressively incorporated from the feather germ follicle to the feather filaments along with

keratin (Lin et al. 2006). Corticosterone may affect feather pigmentation in two major ways. First, glucocorticoids can inhibit the transcription of tyrosinase, Mc1-R and POMC in the feather bud as shown in murine skin (Ermak & Slominski 1997), and thus synthesis of both phaeomelanin and eumelanin should be reduced in the feather bud. Accordingly, corticosterone decreased the production of phaeomelanin pigments as shown by the decoloured feather zone; the effect was transient because pellets released corticosterone during only 4 days, as shown by regular blood sampling (B. Almasi, unpublished data). Second, because the plasma level of corticosterone was elevated for a period of 4 days, nestlings were probably in a state of moderate stress. Therefore, the injection of corticosterone may have reduced the level of ACTH through a negative feedback loop. This may have occurred in the pituitary gland, but also in feather buds, because mammalian hair follicle and dermal fibroblast of the skin display a functional HPA axis equivalent to the hypothalamic–pituitary–adrenal axis (Ito et al. 2005; Slominski et al. 2005). Reduction of POMC-derived peptides reduces melanocyte proliferation and migration and principally eumelanin synthesis (Suzuki et al. 1996). This may have led to a decrease in the production or deposition of phaeomelanin pigments. Corticosterone can therefore mediate the condition-dependent component of melanin-based coloration if there is interindividual variation in corticosterone level or in the sensitivity to the effect of corticosterone, which is the case in several organisms (Koolhaas et al. 1999; Evans et al. 2006). The general applicability of our results in other vertebrates needs testing and whether the effect of corticosterone on the deposition of melanin pigments is stronger in sexually selected than in nonsexually selected traits (Cotton et al. 2004) also should be investigated.

Although the degree of melanin-based coloration can be under strong genetic control, the feedback loop between the production of ACTH (one hormone responsible for melanin production) and that of corticosterone during the stress response may account for the environmental component of interindividual variation in the degree of melanism observed in other bird species (Griffith et al. 1999; Fargallo et al. 2007). Interestingly, corticosterone altered phaeomelanin production only when experimentally elevated, whereas baseline corticosterone level was not correlated with coloration, indicating that corticosterone affects melanin production only if elevated during prolonged periods of stress. In the barn owl, although the expression of phaeomelanism is under strong genetic control (Roulin et al. 1998; Roulin & Dijkstra 2003), we observed a decoloured feather zone in 5.2% of the placebo-implanted nestlings. This suggests that a heritable melanin-based trait can sometimes be sensitive to environmental factors.

Although in the barn owl an experimental elevation in corticosterone level resulted in a reduction in the degree of reddish-brownness, a manipulation of brood size did not alter the expression of this trait (Roulin et al. 1998), although manipulating brood size induced a clear change in offspring body condition (Roulin et al. 1999). Furthermore, the degree of phaeomelanism was not associated with the

place of nestlings in the within-brood age hierarchy (Roulin & Dijkstra 2003), although late hatched individuals have more ectoparasites and lower access to food resources than their early hatched siblings. Because a reduction in food supply can lead to an increase in corticosterone level (e.g. Kitaysky et al. 2001), it appears that in the barn owl a relatively high threshold in corticosterone level has to be reached and maintained during a sufficiently long period of time to result in a significant reduction in melanin-based coloration. Our experimental treatment resulted in a three-fold increase of total corticosterone level during 4 days, values that are naturally observed after a short starvation period of 1 night; a stress response to an acute stressor results in a four- to five-fold increase of total corticosterone level (B. Almasi, unpublished data). If barn owls appear to be relatively unaffected by stressful factors in their expression of melanin-based coloration, a lower threshold in corticosterone level may be sufficient to induce a change in melanin-based coloration in species such as the house sparrow (Griffith et al. 1999) and European kestrel (Fargallo et al. 2007) in which environmental conditions can lead to a clear change in the expression of eumelanin-based colour traits. Apparently, directional sexual selection exerted on the degree of eumelanism in these two bird species may increase the susceptibility to corticosterone, a proposition that deserves further testing.

Acknowledgments

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