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Regulation of stress response is heritable and functionally linked to melanin-based coloration

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Abstract

Sexual selection theory posits that ornaments can signal the genetic quality of an individual. Eumelanin-based coloration is such an ornament and can signal the ability to cope with a physiological stress response because the melanocortin system regulates eumelanogenesis as well as physiological stress responses. In the present article, we experimentally investigated whether the stronger stress sensitivity of light than dark eumelanic individuals stems from differential regulation of stress hormones. Our study shows that darker eumelanic barn owl nestlings have a lower corticosterone release after a stressful event, an association, which was also inherited from the mother (but not the father) to the offspring. Additionally, nestlings sired by darker eumelanic mothers more quickly reduced experimentally elevated corticosterone levels. This provides a solution as to how ornamented individuals can be more resistant to various sources of stress than drab conspecifics. Our study suggests that eumelanin-based coloration can be a sexually selected signal of resistance to stressful events.

Introduction

Sexually selected traits signal many aspects of phenotypic quality such as competitive ability (West & Packer, 2002), resistance to parasite attack (Hamilton & Zuk, 1982) and to oxidative stress (von Schantz *et al.*, 1999), and the ability to cope with stressful environmental factors that lead to glucocorticoid responses (Roulin *et al.*, 2008b). Covariation between secondary sexual characters and other phenotypic traits provides the basis for sexual selection to proceed as females can obtain information on male quality by assessing their coloration or any other conspicuous trait. Sexual selection studies were fruitful at showing that females indeed select males based on colour traits (Andersson & Iwasa, 1996), but the exact genetic and physiological mechanisms underlying a covariation between coloration and other phenotypic traits are still poorly known.

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Investigating the proximate mechanisms that link coloration to other phenotypic traits is not trivial as studies of sexual selection usually refer to the notion of good genes without going further into the identification of which genes are actually implicated. We thus first need to identify candidate genes that are in linkage disequilibrium with those coding for sexually selected traits. Then, we need to describe the cascade from gene expression to the actual action of the gene products that lead bright individuals to perform better than drab conspecifics. Knowledge of these genetic and physiological mechanisms is key to appraise the exact signalling function of sexually selected traits. Recent developments in this field indeed showed that the situation might be more intricate than previously believed. Several researchers showed that colour traits that are directionally selected can be associated with behavioural syndromes, i.e. differently coloured individuals differ consistently in temperament or personality over a variety of contexts (Réale *et al.*, 2007). For example, although in several sparrow species darker males are more aggressive than lighter-coloured individuals (Watt, 1986; Liker & Barta, 2001; Torda *et al.*, 2004), they seem to achieve

similar fitness as lightly coloured individuals (Nakagawa *et al.*, 2007). This potentially implies that differently coloured individuals may represent alternative strategies to derive the same fitness, with dark individuals being more aggressive than lighter-coloured conspecifics that adopt prudent behaviours. The recent proposition that variation in melanin-based coloration may signal behavioural syndromes is based on the finding that products of the proopiomelanocortin (*POMC*) gene bind not only to the melanocortin-1-receptor (Mc1R) that triggers the synthesis of eumelanin pigments but also to four other melanocortin receptors that regulate very different functions including sexual behaviour, aggressiveness, exocrine gland activity, immune function, energy homeostasis, and hypothalamic–pituitary–adrenal (HPA) stress response (Ducrest *et al.*, 2008). Pleiotropic effects of the melanocortin system imply that suites of phenotypic traits become correlated including melanin-based coloration.

The pleiotropic effects of the melanocortin system offer the possibility to investigate the link between sexually selected traits and the signalled qualities. To this end, we consider the HPA stress response, one of the pleiotropic effects of the melanocortin system. Environmental and social stressful factors including for instance food shortage, immune challenges and presence of predators or competitors induce a HPA stress response (Wingfield *et al.*, 1998; Kitaysky *et al.*, 2001; Cockrem & Silverin, 2002; Romero, 2004), resulting in an increase in melanocortins (adrenocorticotropin hormone, ACTH) and circulating glucocorticoids (Simpson & Waterman, 1988; Sapolsky *et al.*, 2000). Glucocorticoids are responsible for an adaptive stress response, which allows individuals to allocate optimally resources among competing physiological functions including for instance immunity, sexual activity, reproductive investment, and growth (Hofer & East, 1998; Charmandari *et al.*, 2005). Interestingly, not only environmental conditions but also other melanocortins including MSH (melanin-stimulating hormones) modulate the HPA stress response. Administration of α-MSH previous to an acute stressful event reduced the glucocorticoid stress response in rats (Racca *et al.*, 2005). As α-MSH induces the production of eumelanic pigments, darker individuals are therefore predicted to be less sensitive to stressful factors (Ducrest *et al.*, 2008). Several empirical studies are consistent with this prediction (Rohwer & Wingfield, 1981; Johnston & Janiga, 1995; Roulin *et al.*, 2001, 2003, 2008b; Almasi *et al.*, 2008). However, the mechanism underlying the differential regulation of the HPA stress response by dark and light eumelanic individuals is not yet known. We propose that the stronger sensitivity to stressful environmental factors of light than dark eumelanic individuals can be explained by two nonmutually exclusive hypotheses. The 'release' hypothesis proposes that a given stressor leads to a weaker immediate release of glucocorticoids in dark than light eumelanic individuals, and the 'regulation' hypothesis proposes that the HPA-axis of darker eumelanic individuals better regulates circulating corticosterone levels and thus faster returns to prestress glucocorticoid levels than of lighter individuals. A tight regulation of corticosterone is essential as elevated levels are disadvantageous in the absence of a stressor (reduced immunity and resistance to oxidative stress (Stier *et al.*, 2009), and impaired growth (Sapolsky *et al.*, 2000)).

In the present article, we report an experimental study where we (i) test predictions derived from the release and regulation hypotheses and (ii) test whether the association between eumelanin-based coloration and stress sensitivity is inherited from the biological parents. We considered the barn owl that displays black eumelanic spots of varying size at the tip of feathers, a heritable and sexually dimorphic trait with females being on average larger spotted than males (Roulin & Dijkstra, 2003). In our Swiss population, spot size is directionally selected in females (Roulin *et al.* submitted) as it is a criterion in mate choice (Roulin, 1999; Roulin & Altwegg, 2007) and signals aspects of female quality including survival (Roulin & Altwegg, 2007) and offspring quality (Roulin, 2004). In a previous study (Almasi *et al.*, 2008), we already showed that darker eumelanic breeding males better cope with an experimental elevation in circulating corticosterone because their feeding rate was less affected than in light coloured conspecifics. Thus, the barn owl is particularly appropriate to examine the release and regulation hypotheses.

We tested the release hypothesis in nestling barn owls by measuring the release of total corticosterone level because of a standardized stressful event induced by handling birds, a standard method used by many researchers (e.g. Wingfield *et al.*, 1995; Clinchy *et al.*, 2004). We predict that darker individuals have a lower corticosterone release as a response to a stressful event than lighter eumelanic individuals. We then tested the regulation hypothesis by manipulating the level of glucocorticoids through implanting nestlings with a corticosterone-releasing pellet (hereafter cort-nestlings) or with a placebo pellet (hereafter placebo-nestlings). We predict that darker individuals are able to keep corticosterone levels lower after a corticosterone challenge than lighter eumelanic individuals, and thus we expect to find a stronger negative relationship between eumelanin-based coloration and corticosterone in cort- than placebo-nestlings. To demonstrate that such covariation is heritable, we cross-fostered eggs or hatchlings to allocate genotypes randomly among the rearing environments. We thus took care that foster and biological parents did not resemble each other with respect to melanin-based coloration. In such a situation, any relationship between nestling corticosterone levels and plumage traits measured in nestlings or in their biological parents may be assigned to prehatching maternal effects or to genetics but not to posthatching environmental effects.

Methods

Study design and cross-fostering experiment

The barn owl is a medium-sized nocturnal bird that breeds mainly in nest boxes and hunts in open agricultural areas. Two to 11 eggs hatch asynchronously on average every second day. The study was carried out on a plain of 190 km^2 at an altitude ranging from 430 to 520 m in western Switzerland (46°49′N, 06°56′E) in 2004 and 2006. The area is dominated by agriculture and holds a barn owl population of 20–80 pairs breeding in 110 nest boxes put in barns. In 2004, 47 pairs bred, of which 39 raised at least one fledgling successfully, and nestlings were in prime body condition. In 2006, only 29 pairs started to breed and 21 raised successfully their brood until fledging, and body mass of the nestlings was lower than in 2004 (Almasi *et al.*, unpublished).

In 2004, 22 broods with similar hatching dates were matched in pairs, and two of the four-first-hatched nestlings, randomly chosen, were swapped with similarly aged nestlings from the matched nest (mean age at cross fostering: 2.6 days ± 1.9 (SE)). One of the two cross-fostered individuals was later implanted with a corticosterone-releasing pellet (corticosterone-nestling) and the other cross-fostered individual with a placebo pellet (placebo-nestling). Additionally, one of the two oldest noncross-fostered nestling received a corticosterone-releasing pellet and the other a placebo pellet. Because we had a maximum of four experimental chicks per brood, the youngest nestlings in broods with more than four nestlings (n = 12 in total) were unmanipulated. In 2006, 26 broods with similar laying dates were matched in pairs, and the four-first-laid eggs were swapped between pairs of nests, and we later implanted two cross-fostered individuals with a corticosterone-releasing pellet and the two other cross-fostered individuals with a placebo pellet. Again, as we manipulated a maximum of four nestlings per brood, some nestlings (n = 7 individuals in total) remained unmanipulated. For the present study, we only considered cross-fostered manipulated nestlings. Thus, in total, we had a sample of 58 cross-fostered cort-nestlings (22 nestlings in 2004 and 36 in 2006) and 54 cross-fostered placebo-nestlings (22 in 2004 and 32 in 2006). All nestlings were colour marked on their clipped nail until being ringed with an aluminium ring at the age of 14 days. Our aim being to correlate nestling corticosterone level with spot diameter measured in nestling themselves or in their biological parents, we verified that there was no relationship between spot diameter of biological and foster parents. This was the case in both 2004 and 2006 (Pearson correlations, all P-values > 0.2). Furthermore, in the 2 years, spot diameter of the biological mother was not correlated with spot diameter of the biological father (Pearson correlation: P-values > 0.8). Finally, both in 2004 and 2006 mother spot diameter was not correlated with laying date and clutch size (Pearson correlations, P-values > 0.2).

The implants (diameter 5 mm) were made up of a biodegradable carrier-binder containing 15 mg corticosterone or, for placebo, only of the biodegradable carrier-binder (Innovative Research of America, Sarasota, FL, USA). We implanted the pellet under the skin of the flank above the knee through a small incision, which was closed with tissue adhesive (Histoacryl, Braun, Germany). The implants were specified to have a given constant release rate of 7 days in rats, but in the barn owl it appeared that implants increased circulating corticosterone above normal baseline level during 2–3 days (Müller *et al.*, 2009). On the day of implantation, cort- and placebo-nestlings did not differ with respect to age (mean ± SD: 25.9 ± 1 days vs. 25.6 ± 1 days; Student's t-test, t = 0.1, d.f. = 110, P = 0.9), body mass (292 ± 6 g vs. 286 ± 10 g; Welch t-test, t = 0.7, d.f. = 91.4, P = 0.58), wing length (111 ± 3.78 mm vs. 108 ± 4.98 mm; Student's t-test, t = 0.6, d.f. = 110, P = 0.5) and sex-ratio (chi-test, χ^2 = 0.1; P = 0.8). The Swiss committee for animal research approved the study (animal experiment permit from the 'service vétérinaire du canton de Vaud' no 1736). Nestling survival was not affected by corticosterone administration. In the 48 broods, a total number of 174 nestlings received either a corticosterone-releasing pellet (92 nestlings) or a placebo pellet (82 nestlings). One hundred and fifty-five (89%) of these nestlings survived until fledging (81 (88%) of corticosterone, 74 (90%) of placebo-nestlings survived), a value comparable to a previous nonexperimental study in 2005 when 159 of 179 nestlings survived until fledging (88%).

Plasma corticosterone level

To monitor the effect of the implants on baseline and stress-induced corticosterone, blood samples were taken from the brachial vein of all experimental nestlings before implantation, 2 and 20 days after implantation. Because a significant increase in circulating corticosterone levels was observed 3 min after capture of the nestlings (own observation and Romero & Reed, 2005), corticosterone levels measured in blood samples collected within 3 min after capturing the nestlings were considered to be baseline levels and used in further analysis. We termed all samples taken within 3 min after capturing the nestling as baseline, even if they were from corticosterone-treated nestlings. We measured handling stress-induced corticosterone in 46 nestlings at the day of implantation. Nestlings were kept in cloth bags after taking a baseline blood sample, and an additional blood sample was taken 26 ± 6 min (mean ± SD) after capturing each nestling. Variation in the time to take the second blood sample was not correlated with handling stress-induced corticosterone level (Person correlation, r = −0.1, P = 0.4, n = 194), and hence we did not take into account this factor in subsequent analyses.

The blood was collected with heparinized capillary tubes, immediately centrifuged and the plasma stored in liquid nitrogen. After transport to the laboratory, the samples were stored at –20 °C until analysis. Plasma corticosterone concentration was determined using an enzyme immunoassay (Munro & Stabenfeldt, 1984; Munro & Lasley, 1988). Corticosterone was extracted from plasma with 4 mL dichlormethane (5 μL plasma diluted with 195 μL water). All samples were run in triplicates. The dilution of the corticosterone antibody (Chemicon; cross-reactivity: 11-dehydrocorticosterone 0.35%, progesterone 0.004%, 18-OH-DOC 0.01%, cortisol 0.12%, 18-OH-B 0.02% and aldosterone 0.06%) was 1 : 8000. HRP (1 : 400 000) linked to corticosterone served as enzyme label and ABTS as substrate. The concentration of corticosterone in plasma samples was calculated by using a standard curve run in duplicates on each plate. Plasma pools from chicken with a low and a high corticosterone concentration were included as internal controls on each plate. Intra-assay variation ranges from 5% to 13% and inter-assay variation from 12% to 21%, depending on the year and concentration of the internal controls. For details on the assay, see Müller *et al.* (2006).

To estimate free corticosterone, we measured corticosterone-binding globulin (CBG) capacity using a radioligand-binding assay with tritiated corticosterone (described in Breuner *et al.*, 2003). Briefly, plasma was stripped of endogenous steroids with dextran-coated charcoal solution for 20 min. Plasma dilution was optimized for barn owls yielding a dilution of 1 : 450 and an incubation period of 2 h and temperature of 4 °C. All samples were run in triplicates. Total binding was determined using 50 μL buffer (50 mM Tris), 50 μL ^{3H}CORT (20 nM ^{3H}CORT) and 50 μL stripped plasma. Nonspecific binding was determined using 50 μL unlabelled corticosterone (1 μM cort) instead of buffer. Glass fibre filters were soaked in 25 mM Tris with 0.3% polyethylenimine for 1 h before vacuum filtration (Brandel Harvester, Shelton, CT, USA). Filters were rapidly rinsed with 9 mL rinse buffer (25 mM Tris; 3 rinses of 3 mL). Following filtration, radioactivity bound to the filters was measured by standard liquid scintillation spectroscopy (scintillation cocktail Ultima Gold™ LLT, Perkin Elmer, Gaithersburg, MD, USA). The equilibrium binding parameters for the specific binding of ^{3H}CORT were determined through equilibrium saturation binding assay of pooled barn owl plasma and ^{3H}CORT concentration between 0.2 and 10.7 nM. Mean (± SE) affinity estimates (dissociation constant K_d) of corticosterone for CBG in barn owls were 4.11 ± 0.34 nM. Individual hormone-binding capacity was estimated using point sample analysis. Percentage of CBG bound in the assay was estimated using concentration of ^{3H}CORT and K_d from the saturation analysis with the following formula: % bound = $[^{3H}CORT]/([^{3H}CORT] + K_d)$ and ranged from 77% to 83%. Before estimating free hormone levels, all point samples were corrected to 100% for analysis. Free hormone levels were estimated according to the equation in Barsano & Baumann (1989). A plasma standard was included in all CBG assays, which yielded intra-assay coefficients of variation of 6% and an inter-assay coefficient of variation of 23%.

Assessment of plumage traits

We assessed the size of the black spots, an eumelanin-based trait in breeding adults and their 55-day-old offspring. We reliably measured variation in size (repeatability is 0.92) of black spots by placing on the breast, belly, flanks and underside of the wings a 60 × 40 mm frame within which a single person counted spots and measured their diameter to the nearest 0.1 mm (Roulin *et al.*, 2004). For each body part, we calculated a mean spot diameter and mean number of spots, and the mean value from the four body parts were used in the statistical analyses. As the body underside of barn owls varies not only with respect to eumelanin-based coloration but also to phaeomelanin-based coloration, we compared coloration of breast, belly, one flank and one underside of the wing with eight colour chips, ranging from 1 for reddish-brown to 8 for white and calculated a mean phaeomelanic coloration per bird. Sex of nestlings was determined using the CHD-gene method (for details see Roulin, 1999), whereas breeding females were recognized by the presence of a brood patch.

Statistical procedure

Statistical analyses were carried out using the statistical software package R version 2.4.1 (R Development Core Team, 2006). To analyse baseline corticosterone levels, we performed analyses only on blood samples collected within 3 min after opening nest-box. Therefore, sample sizes vary between the different visits and analyses. Because the plasma volume of some samples was low, we could not analyse free corticosterone in all samples (for details on sample size see Table 1). All analyses were carried out using only cross-fostered nestlings.

To test the release hypothesis proposing that darker individuals mount a lower stress response after an acute stressor, we performed separate mixed-effect model analyses with baseline total and stress-induced total corticosterone as dependent variable in separate models, nestling spot diameter as continuous variable and nestling sex as fixed factor. Site where individuals were raised was included as random factor to account for the fact that nestlings in the same nest were not statistically independent. In a second step, we tested whether the ability to mount a stress response is inherited. We performed the same model selection procedure as described earlier for spot size of the biological mother and spot size of the biological father in separate models because we were not able to capture two males. We

Table 1 Number of nestling barn owls used to perform statistical analyses. Day 0, 2 and 20 refer to the number of days after having implanted a corticosterone or placebo pellet.

	# of implanted nestlings		# of nestlings (stress-induced total cort)		# of nestlings (baseline total cort)		# of nestlings (baseline free cort)	
	C	*P*	C	*P*	C	*P*	C	*P*
2004	22	22						
Day 0					11	10	13	11
Day 2				15	18	16	17	14
Day 20					9	11	11	12
2006	36	32						
Day 0					36	30	35	30
Day 2				31	23	23	19	22
Day 20					30	23	24	23

then performed similar analysis of phaeomelanin-based coloration of biological and foster parents and nestlings in separate models.

To test the regulation hypothesis proposing that variation in the regulation of artificially elevated corticosterone is associated with the size of black spots, we performed two separate repeated mixed-effect model analyses with either baseline total corticosterone or baseline-free corticosterone as dependent variables. As fixed factors, we included implant (corticosterone vs. placebo), day (0, 2 and 20 days after implantation), nestling sex and year (2004 and 2006), and as three continuous covariates nestling age at day 0, nestling body mass at day 0 and spot diameter of the nestling, as well as all interactions. To control for pseudo-replication, nest site and nestling identity nested in rearing site were included as two random factors. The final model was obtained by removing all nonsignificant interactions and nonsignificant variables not involved in significant interactions with a stepwise backward procedure. In a second step, we replaced nestling spot diameter with spot diameter of the biological parents to test whether variation corticosterone levels is associated with plumage traits of the biological parents and thus is an inherited trait. We performed the same model selection procedure as described earlier for spot size of the biological mother and spot size of the biological father in separate models. In a third step, we performed similar analysis with phaeomelanin-based coloration of the parents and nestlings in separate models. *P*-values < 0.05 (two-tailed) were considered as significant.

Results

Handling stress-induced corticosterone levels and the release hypothesis

To test the hypothesis that dark eumelanic individuals mount a lower stress response than lighter eumelanic individuals, we related handling stress-induced total corticosterone levels of 46 nestlings taken 26 ± 6 (SD) minutes after capturing to nestling spot size. Darker eumelanic nestlings mounted a significantly lower corticosterone response after capture and handling restraint (mixed-effect model with site as random variable, spot diameter of nestlings: $F_{1,10} = 8.5$, $P = 0.02$, $n = 46$, Fig. 1). Sex and the interaction of sex by spot diameter of the biological mother were not significant and hence removed from the final model.

To test whether the association between eumelanin-based coloration and handling stress-induced corticosterone level is an inherited trait, we replaced spot diameter of the nestlings by spot diameter of the biological mother in a similar mixed-effect analysis. Handling stress-induced total corticosterone level was significantly negatively correlated with spot diameter of the biological mother (mixed-effect model with site as random variable, spot diameter of the biological mother: $F_{1,29} = 4.7$, $P = 0.038$, $n = 46$, Fig 1). Sex and the interaction of sex by spot diameter of the biological mother were not significant and hence removed from the final model. Similar analysis with spot diameter of the biological

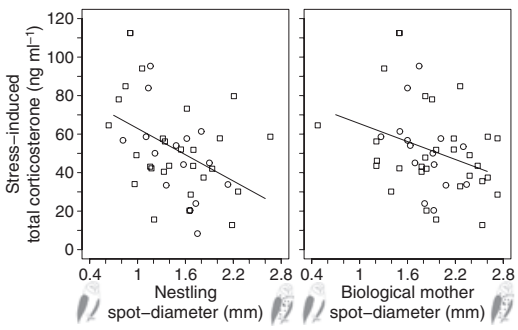

Fig. 1 Total corticosterone level measured on average 26 min after having started to handle cross-fostered nestling barn owls in relation to nestling spot diameter and spot diameter of the biological mother in 2004 (circles) and 2006 (squares).

father, the foster father and foster mother were not significant (all $P > 0.1$), showing that covariation between stress response and eumelanin-based coloration was inherited from the mother but probably not from the father. Phaeomelanin-based coloration of the nestlings, biological and foster parents was not associated with the handling-induced stress response (all $P > 0.1$).

Baseline corticosterone levels taken within 3 min after capturing the nestlings were not associated with spot diameter and phaeomelanin-based coloration measured in nestlings and in biological and foster parents (all $P > 0.3$). Thus, only the stress-induced corticosterone level was associated with spot diameter and not corticosterone level measured prior to the stressful event.

Experimental manipulation of corticosterone levels and the regulation hypothesis

We examined the regulation hypothesis stating that darker offspring and offspring sired by darker mothers reduce faster the level of corticosterone after experimental corticosterone administration. We thus analysed baseline corticosterone levels of nestlings implanted with a corticosterone-releasing pellet or a placebo pellet in relation to melanin-based coloration of the nestlings themselves and of their biological parents. This hypothesis predicts that the negative correlation between baseline corticosterone levels and spot diameter of the nestlings or of the biological mother should be stronger in cort-nestlings than in placebo-nestlings. As expected, baseline total and free corticosterone levels were associated with spot diameter of the biological mother in interaction with implant group (Table 2; Figs 2 and 3). Sex and the interaction of sex by spot diameter of the biological mother were not significant and therefore removed from the final model. Similar analyses with spot diameter and phaeomelanin-based coloration of the nestling, biological father and foster parents proved not significant (all $P > 0.1$).

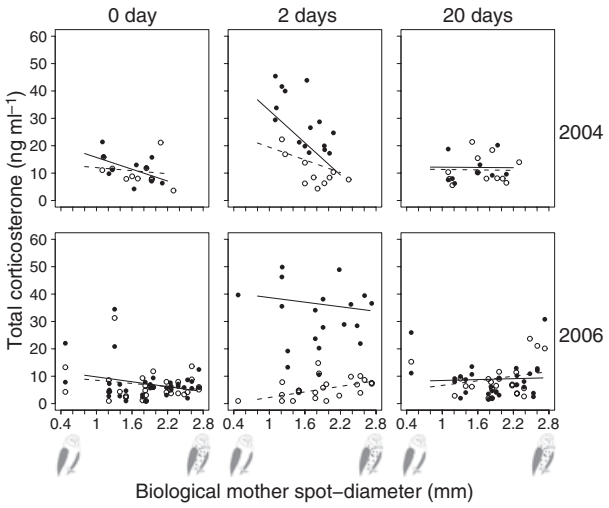

Fig 2 Total corticosterone levels of cross-fostered nestlings in 2004 (upper panels) and 2006 (lower panels) just before implantation (0 day), 2 and 20 days after implantation of a corticosterone-releasing (dots) or placebo pellet (circles) in relation to spot diameter of the biological mother. Lines, extracted from the repeated mixed-effect model, represent the predicted corticosterone levels of cort-nestlings (continuous line) and placebo-nestlings (dashed line).

Post hoc analyses showed that the relationship between baseline total and free corticosterone level and spot size was significant only in cort-nestlings 2 days after implantation (i.e. at the peak of corticosterone release, Müller *et al.*, 2009). Because the interaction between year and

Table 2 Mixed-effect analyses with baseline total and baseline free corticosterone levels as dependent variables in separate models. As random factors we included nestling identity nested in rearing site. Degrees of freedom, *F*-, and *P*-values are reported. The analysis of baseline total corticosterone is based on 289 measurements taken on three different days in 120 nestlings from 48 different broods. The analysis of baseline free corticosterone is based on 270 measurements taken on three different days in 119 nestlings from 48 different broods. The fixed factor 'implant' indicates whether nestlings were implanted with a corticosterone-releasing or placebo pellet. 'Day' indicates whether blood samples were taken on the day of implantation, 2 or 20 days later.

	Baseline total corticosterone			Baseline free corticosterone		
	d.f.	*F*	*P*	d.f.	*F*	*P*
Intercept	1,152	626.16	< 0.001	1,142	229.69	< 0.001
Year	1,42	9.85	< 0.001	1,42	30.17	< 0.001
Implant	1,67	43.62	< 0.001	1,69	11.23	0.001
Day	2,152	34.51	< 0.001	2,142	11.72	< 0.001
Spot-diameter genetic mother	1,42	5.20	0.028	1,42	13.52	< 0.001
Year*implant	2,67	3.63	0.032			N.S.
Year*spot-diameter genetic mother	2,42	5.04	0.011	2,42	7.13	0.002
Implant*day	2,152	39.24	< 0.001	2,142	13.01	< 0.001
Implant*spot-diameter genetic mother	1,67	4.90	0.030	1,69	7.38	0.008
Day*spot-diameter genetic mother			N.S.	2,142	5.64	0.001

implant was significant in the analysis of baseline total corticosterone, we performed separate *post hoc* analyses for each year. Independently of the year, baseline total corticosterone level at the day of implantation was not associated with spot diameter of the biological mother (*post hoc* mixed-effect model with site as random variable; spot diameter of biological mother: $F_{1,13} = 1.5$, $P = 0.2$ in 2004 and $F_{1,17} = 1.3$, $P = 0.3$ in 2006). Two days later, cort-nestlings sired by biological mothers with larger black spots showed a lower baseline total corticosterone levels than cort-nestlings sired by mothers with smaller black spots in 2004 (*post hoc* mixed-effect model; interaction implant × spot diameter of the biological mother: $F_{1,8} = 7.0$, $P = 0.012$, Fig. 2a), but not in 2006 ($F_{1,26} = 1.8$, $P = 0.2$, Fig. 2b). Twenty days after implantation, baseline total corticosterone was no more associated with the spot diameter of the biological mother in cort- and placebo-nestlings (*post hoc* mixed-effect model with site as random variable; spot diameter of biological mother: $F_{1,13} = 0.3$, $P = 0.6$ in 2004, $F_{1,34} = 0.01$, $P = 0.9$ in 2006; Fig. 2).

With respect to baseline-free corticosterone level, we pooled data of 2004 and 2006 in Fig. 3 because the interaction between year and implant was not significant ($P > 0.1$, Table 1). At the day of implantation, baseline-free corticosterone levels tended to be associated with spot diameter of the biological mother (*post hoc* mixed-effect model with site as random variable; spot diameter of biological mother: $F_{1,33} = 3.9$, $P = 0.058$), but not in interaction with the implant (spot diameter of biological mother × implant, $F_{1,50} = 1.7$, $P = 0.2$). Two days after implantation, baseline-free corticosterone levels were higher in cort-nestlings born from small-spotted mothers than in nestlings born from large-spotted mothers and in placebo-nestlings (*post hoc* mixed-effect-model; implant × spot diameter of biological mother: $F_{1,30} = 8.5$, $P = 0.006$). Twenty days after implantation, baseline-free corticosterone levels were no longer associated with spot diameter of the biological mother (*post hoc* mixed-effect model; spot diameter of biological mother: $F_{1,31} = 0.8$, $P = 0.4$; implant: $F_{1,34} = 0.3$, $P = 0.6$; interaction: $F_{1,33} = 0.4$, $P = 0.5$).

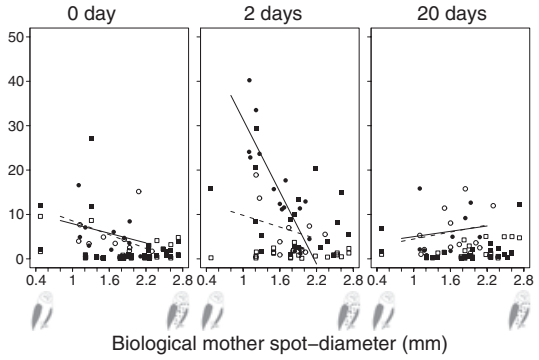

Fig 3 Free corticosterone levels of cross-fostered nestlings in 2004 (circles) and 2006 (squares) just before implantation (0 day), 2 and 20 days after implantation of a corticosterone-releasing (closed symbols) or placebo pellet (open symbols) in relation to spot diameter of the biological mother. Lines, extracted from the repeated mixed-effect model, represent the predicted corticosterone levels of cort (continuous line) and placebo-nestlings (dashed line).

Discussion

The results of the present study are consistent with both the release and regulation hypotheses. First, the release of corticosterone to a standardized stress situation was associated with eumelanin-based coloration. Barn owls with large black spots mounted a lower corticosterone response to capture-induced handling stress than lighter eumelanic individuals. Further, this covariation was inherited from the mother to the offspring as cross-fostered offspring mounted a lower corticosterone stress response when born from dark compared to lightly coloured biological mothers. In agreement with the regulation hypothesis the degree of maternal (but not nestling) eumelanin-based coloration was positively associated with the ability of cross-fostered offspring to quickly return to pre-implantation baseline corticosterone levels. However for total corticosterone this was only true in the year with benign environmental conditions (2004) and not in 2006 when environmental conditions were more severe for barn owls, whereas regulation of free corticosterone was not significantly affected by yearly variation in environmental conditions. The link we report in the present study between eumelanin-based coloration and both the release and regulation of corticosterone is consistent with the proposition that this colour trait can signal resistance to stress (Ducrest *et al.*, 2008; Roulin *et al.*, 2008b).

Differential regulation of corticosterone and fitness consequences

By implanting cross-fostered nestlings with a corticosterone-releasing pellet we induced an artificial rise in baseline corticosterone level, and during the peak period of corticosterone release nestlings showed lower corticosterone levels when their biological mother displayed larger black spots. This suggests that the regulation of corticosterone, either through endogenous corticosterone production or through clearance rate, is more flexible in offspring from dark mothers than in offspring from light mothers. This relationship was detected in 2004 and 2006 with respect to the biologically active fraction of corticosterone (i.e. free corticosterone) that enters tissue to interact with receptors (Mendel, 1989). In contrast, the relationship between total corticosterone and eumelanin-based coloration of the biological mother was less pronounced and detected only in 2004. Total corticosterone consists of free circulating corticosterone

and the fraction bound to CBG that has a reservoir function to carry corticosterone to specific sites where a local increase in free corticosterone is required as for example during inflammation (Pemberton *et al.*, 1988). The stronger association between eumelanin-based coloration of the mother and free corticosterone, rather than total corticosterone, probably stems from the fact that free corticosterone can additionally be regulated through variation in CBG capacity (Breuner *et al.*, 2003; Romero *et al.*, 2006; Almasi *et al.*, 2009), whereas the level of total corticosterone depends on the production of endogenous corticosterone for which the regulation is condition dependent. In 2004 environmental conditions were much better than in 2006 (see Methods). According to the hypothesis that regulation of CBG increases the flexibility for organisms to respond to environmental stressors (Breuner *et al.*, 2003), baseline total corticosterone did not differ between 2004 and 2006, whereas baseline CBG capacity was higher in 2006 than 2004 and thus baseline free corticosterone level lower in 2006 (Almasi *et al.*, 2009). Future studies will have to determine the relative role of the regulation of endogenous corticosterone release vs. CBG capacity in generating a relationship between eumelanin-based coloration and free corticosterone.

The more flexible regulation of circulating corticosterone by offspring sired by mothers displaying large black spots may have important fitness consequences. Two recent studies showed that the effects of artificially elevated corticosterone levels vary with eumelanin-based coloration. In a study performed in 2005, we showed that lighter eumelanic barn owl males provision their brood at a higher rate, but when corticosterone was artificially elevated the reduction in provisioning rates was much more pronounced in light than darker eumelanic males (Almasi *et al.*, 2008). This suggests that the negative impact of a high level of corticosterone during a few days on reproductive activities is more pronounced in light than darker eumelanic individuals. Possibly lighter eumelanic placebo-males invested more in feeding their offspring to compensate for their lower performance in periods of intense stress. Furthermore, in the same sample of nestlings as used in the present study we demonstrated that artificial administration of corticosterone reduced nestling growth rate to a larger extent in light than darker eumelanic birds (Almasi *et al.* unpublished). The present study additionally showed that the colour-specific ability to regulate corticosterone could be inherited from the mother to the offspring. Altogether the three studies performed in the barn owl (present study, Almasi *et al.*, 2008; Almasi *et al.*, unpublished) provide strong evidence for the hypothesis that eumelanin-based coloration is associated with the ability to cope with stressful environments as has also been shown in the Alpine swift *Apus melba* (Roulin *et al.*, 2008b) and European kestrel (*Falco tinnunculus*) (Fargallo *et al.*, 2007).

Potential proximate mechanisms underlying a link between the regulation of the HPA-axis and eumelanin-based coloration

The observation that the ability to return elevated corticosterone levels to normal baseline levels was associated with eumelanin-based coloration measured in biological mothers but not in the nestlings can be explained by three mechanisms. First, darker mothers may pass on genes to the offspring, which improve their resistance to stress. The fact that resistance to stress was correlated with eumelanin-based coloration measured in mothers, but neither in the nestlings themselves nor in biological fathers suggests that only the gene copy passed on by the mother is expressed in the offspring (genomic imprinting). We showed in the same barn owl nestlings that exogenous corticosterone reduced phaeomelanin-based coloration during feather growth and can thus mediate the condition-dependent component of melanin-based coloration (Roulin *et al.*, 2008a; see also Fitze & Richner, 2002; Fargallo *et al.*, 2007). However, as black eumelanic spots were already produced at the time of implanting corticosterone pellets, our results are rather consistent with a linkage disequilibrium between genes coding for melanogenesis and those coding for the regulation of the HPA-axis. Second, darker mothers may be in better condition and produce embryos of better quality (maternal effect). Third, darker mothers may provide better parental care. The third possibility can be ruled out as nestlings were raised by foster mothers for which the size of black spots was not correlated with that of the offsprings' biological mother.

Although we cannot firmly discriminate between genomic imprinting and maternal effects (Hager *et al.*, 2008), we discuss a hypothesis proposed by Ducrest *et al.* (2008), which predicts that the covariation between the degree of melanin-based coloration and other phenotypic traits including stress sensitivity (this study and Almasi *et al.*, 2008), sexual behaviour (West & Packer, 2002), and immune function (Roulin, 2004) comes from pleiotropic effects of genes regulating melanogenesis. The *POMC*-gene codes for four different melanocortins (α-, β-, γ-MSH and ACTH). α- and β-MSH bind to the melanocortin-1 receptor (Mc1R) and trigger eumelanin synthesis (and bind with lower affinity to the four other McRs that regulate energy homeostasis, immune and cardiovascular functions, sexual behaviour, and glucocorticoid stress response). ACTH binds also to Mc1R and to Mc2R, which stimulates the production of glucocorticoids. Experimental studies showed that in the presence of α-MSH, less corticosterone is secreted as a response to a stressor (Milligan *et al.*, 1998; Racca *et al.*, 2005). Additionally, α-MSH also blocks the decrease in CBG as a response to a stressor (Milligan *et al.*, 1998), which suggests that α-MSH interferes with the HPA-axis. Possible mechanisms are that α-MSH directly blocks Mc2R, so that ACTH cannot trigger corticosterone secretion

anymore or α-MSH regulates ACTH production through negative feedback. The hypothesis that melanocortins mediate covariations between melanin production and other phenotypic traits by binding to McRs relies on the assumption that the activity of peptides derived from the *POMC*-gene at the sites where melanin pigments are produced reflects a similar peptide activity in organs where genes coding for the other phenotypic traits are expressed (Ducrest *et al.*, 2008). The above-mentioned hypothesis can explain the negative relationship of total and free corticosterone levels with eumelanin-based coloration of the biological mothers (with the exception of total corticosterone in the year with unfavourable environmental conditions) and gives evidence that the ability to regulate corticosterone is heritable. The association between eumelanin-based coloration of the biological mother (and not of the nestlings) and the ability to regulate corticosterone can indicate imprinting on the mother genes coding for the receptors to regulate the HPA-axis (Chong *et al.*, 2007). The next key step is to demonstrate directly the role of melanocortins to generate covariation between eumelanin-based coloration and other phenotypic traits including resistance to stress.

Conclusion

This study provides evidence for a genetic correlation between melanin-based coloration and the ability to release and regulate stress hormones. Darker eumelanic birds and birds sired by darker eumelanic mothers have a lower corticosterone release to a standardized stressor. Natural stressors, e.g. food restriction induce a corticosterone increase above baseline levels (Kitaysky *et al.*, 2001; Romero & Wikelski, 2001), but whether individuals with a greater increase have stronger fitness consequences still needs to be shown. We induced an increase in corticosterone level by handling nestlings rather than by imposing food restriction, as the latter experimental design will have many side effects apart from an effect on the regulation of corticosterone. Thus, our design is appropriate and used by many researchers.

Further, birds sired by darker eumelanic mothers are better able to regulate elevated corticosterone levels to normal baseline levels. A tight regulation of corticosterone levels to environmental stimuli is crucial to maintain homeostasis and to prevent the animal from deleterious effects of chronically elevated corticosterone levels (Sapolsky *et al.*, 2000; Stier *et al.*, 2009). Regulation of circulating glucocorticoids may be a key mechanism explaining why some individuals are fitter than others, which can be selected by sexual selection through eumelanin-based coloration.

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