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Coloration signals the ability to cope with elevated stress hormones: effects of corticosterone on growth of barn owls are associated with melanism

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Abstract

Stressful situations during development can shape the phenotype for life by provoking a trade-off between development and survival. Stress hormones, mainly glucocorticoids, play an important orchestrating role in this trade-off. Hence, how stress sensitive an animal is critically determines the phenotype and ultimately fitness. In several species, darker eumelanic individuals are less sensitive to stressful conditions than less eumelanic conspecifics, which may be due to the pleiotropic effects of genes affecting both coloration and physiological traits. We experimentally tested whether the degree of melanin-based coloration is associated with the sensitivity to an endocrine response to stressful situations in the barn owl. We artificially administered the mediator of a hormonal stress response, corticosterone, to nestlings to examine the prediction that corticosterone-induced reduction in growth rate is more pronounced in light eumelanic nestlings than in darker nest mates. To examine whether such an effect may be genetically determined, we swapped hatchlings between randomly chosen pairs of nests. We first showed that corticosterone affects growth and, thus, shapes the phenotype. Second, we found that under corticosterone administration, nestlings with large black spots grew better than nestlings with small black spots. As in the barn owl the expression of eumelanin-based coloration is heritable and not sensitive to environmental conditions, it is therefore a reliable, genetically based sign of the ability to cope with an increase in blood corticosterone level.

Introduction

During development, an animal is particularly sensitive to environmental influences which can shape the phenotype for life and upon which selection acts (Lindström, 1999; Stearns & Hoekstra, 2005). How an animal reacts to the particular environmental conditions depends on its genetically inherited reaction norms; that is, genotypes regulate differently their phenotype along an environmental gradient. Most natural environments are heterogeneous in space and time, which allows the persistence of different genetic reaction norms and the evolutionary stability of genetic polymorphisms (Kawecki & Ebert, 2004; Hedrick, 2006). During the developmental phase, energetic restrictions or other environmental perturbations may provoke a trade-off between development and survival (see Metcalfe & Monaghan, 2001). The resulting phenotype critically depends on how an animal deals with this trade-off, and hence what its reaction norm is.

Glucocorticoids play an important orchestrating role in the trade-off between maintenance and development (e.g. Charmandari *et al.*, 2005). Across all vertebrate taxa, the activation of the hypothalamo-pituitary-adrenal (HPA) axis leading to a rise in glucocorticoids helps an animal to redirect energy and behaviour from current activities and processes into a survival mode, to cope with a critical situation (Wingfield *et al.*, 1998). Elevated

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glucocorticoids inhibit anabolic processes including growth (Müller *et al.*, 2009b) and affect many phenotypic traits. Therefore, glucocorticoids as mediators of environmental conditions are a mechanistic factor causing developmental plasticity (Dufty *et al.*, 2002). Hence, how sensitive an animal's HPA axis reacts to stressors and how sensitive it is to glucocorticoids (what we collectively term stress sensitivity) critically determines the phenotype under given environmental conditions and ultimately fitness. Indeed, inter-individual variation in sensitivity to stressful situations can be due to heritable variation in the glucocorticoid response to an acute stressor (Satterlee & Johnson, 1988; Carere *et al.*, 2001; Evans *et al.*, 2006; Roberts *et al.*, 2007; Hazard *et al.*, 2008). Therefore, stressful environments, induced for instance by parasites or food scarcity, may select for phenotypes that are able to cope with stressful situations (Roulin *et al.*, 2008).

In many species, physiological, morphological, behavioural and reproductive traits are associated with colour such as melanin-based coloration which is the most common type of colour traits in vertebrates (Roulin, 2004). Of particular interest is the finding that in several species, darker eumelanic individuals are less sensitive to stressful conditions or to corticosterone administration than less eumelanic conspecifics (Johnston & Janiga, 1995; Senar *et al.*, 2000; Almasi *et al.*, 2008, 2010; Roulin *et al.*, 2008; Dreiss *et al.*, 2010). This association suggests that dark- and light coloured individuals may be adapted to different environmental conditions (Jawor & Breitwisch, 2003; Roulin, 2004), which may be due to the pleiotropic effects of genes affecting both coloration and physiological traits. Indeed, based on a literature review of genetic and pharmacological studies, Ducrest *et al.* (2008) proposed a genetic mechanism to explain how eumelanin-based coloration could be associated with the HPA axis and the sensitivity to stressful situations in vertebrates.

In the present study, we experimentally tested whether the degree of eumelanin-based coloration is associated with the ability to cope with elevated circulating corticosterone [as mimicking a stressful situation inducing a rise in glucocorticoids (Love & Williams, 2008)]. We used the barn owl *Tyto alba* as a model species because it exhibits a pronounced variation in melanin-based coloration, and we already showed that darker eumelanic individuals show a reduced corticosterone response to an acute stressor compared to less eumelanic individuals (Almasi *et al.*, 2010). The expression of eumelanin-based coloration (size of black spots on the body underside) is under strong sex-linked genetic control and only weakly sensitive to environmental conditions (Roulin *et al.*, 2010). Still, variation between genetic siblings is high (similar in order to that between nestlings from different parents; Roulin & Dijkstra, 2003), which allows us to study relationships between the degree of eumelanin coloration and stress sensitivity at the within-nest level. Extra-pair paternity is very rare in the barn owl (only one young out of 211 was fathered by another male than the one that was feeding it, Roulin *et al.*, 2004).

We tested whether corticosterone, the main glucocorticoid in birds, affects growth and thus may shape the phenotype. To this end, we artificially administered corticosterone, the main hormonal mediator of a stress response in birds, to barn owl nestlings. We implanted a corticosterone-releasing pellet in half of the nestlings, and in the other half, we implanted a placebo pellet. The rationale of experimentally elevating the corticosterone level in our experiment was to simulate an endocrine stress response without the possible confounding direct effects of applying a stressor (e.g. energy restrictions in food restriction experiments, as is done frequently). Up to now, there are only a few studies investigating the effect of corticosterone alone on growth in altricial birds under natural conditions in the wild (Hull *et al.*, 2007; Müller *et al.*, 2009b). We artificially elevated corticosterone levels within the naturally occurring range and for only a few days to allow for compensatory growth after corticosterone levels returned to preexperimental baseline levels (Müller *et al.*, 2009a).

As we implanted nestlings after they produced black spots, we tested the hypothesis that variation in the ability to cope with elevated corticosterone is linked to melanin-based coloration. We examined the prediction that corticosterone-induced reduction in growth rate is less pronounced in darker eumelanic owlets than in pale-coloured nest mates. The specific prediction to our experimental design is that eumelanic nestlings grow better than less eumelanic conspecifics when implanted with a corticosterone-releasing pellet, but not necessarily when implanted with a placebo pellet. To examine whether the association between coloration and the ability to withstand an experimental rise in corticosterone could be under genetic control or environmentally mediated, we allocated genotypes randomly among the different rearing environments. To this end, we swapped hatchlings between randomly chosen pairs of nest, that is, on average 26 days before implanting them with a corticosterone-releasing pellet or a placebo pellet.

Methods

Study site and study species

The study was carried out in a 190-km^2 area in western Switzerland (46°49′N, 06°56′E) in 2004. Barn owls lay 2–11 eggs between March and July, and the eggs hatch asynchronously on average every 2–3 days generating a pronounced within-brood age hierarchy. Maximal growth rate starts when nestlings are around 17 days old and around 40 days of age nestlings lose weight before fledging. Nestlings take their first flight at about 55 days of age. Variation in plumage traits is already

visible in nestlings when they acquire the juvenile plumage (from day 50), with females typically displaying on average a darker reddish-brown plumage than males (a pheomelanin-based trait) and more and larger black spots (a eumelanin-based trait) than males, although individuals of both sexes can display any phenotype and phenotypes of siblings can vary strongly. Cross-fostering experiments carried out with a large number of nests revealed a strong effect of the nest of origin but no significant effect of environmental rearing conditions on plumage traits, suggesting a strong genetic control (Roulin *et al.*, 1998, 2010; Roulin & Dijkstra, 2003).

Experimental design

To partition the response to an experimental elevation of corticosterone level into the origin-related and environmental components, we performed a partial cross-fostering experiment using 26 broods. At hatching, broods were matched in 13 nest-box pairs (hereafter 'pair') with the criterion that nestlings were similarly aged. For each brood used in cross-fostering experiments, two of the four oldest hatchlings (mean ± SD age in days, 2.7 ± 2) from one nest were randomly chosen and swapped with the same number of similarly aged hatchlings from another nest of the same 'pair'. In this way, each experimental nest contained nestlings of two origins. Nestlings were thus raised by their biological parents either in their nest of origin or in the nest of foster parents referred to as 'nest of rearing'. Thus, for individuals raised by biological parents, the nests of origin and rearing were the same, whereas for individuals raised by foster parents, the nests of rearing and of origin were different.

To investigate the effect of increased plasma levels of corticosterone on nestling growth, we implanted the four oldest nestlings (mean age ± SD at implantation, 29 ± 4 days; range, 21–35 days) with either a corticosterone or a placebo implant. The implants (diameter, 5 mm) are 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. One of the cross-fostered individuals and one of the non-cross-fostered nest mates were implanted with a corticosterone pellet (hereafter cort-nestlings), and the other cross-fostered individual and non-cross-fostered nest mate with a placebo pellet (hereafter placebo-nestlings). Brood size ranged between 2 and 9. In nine broods, only two nestlings survived till the day of implantation, and we implanted one nestling with a corticosterone implant and the other individual with a placebo implant. In total 43 nestlings received a corticosterone implant and 43 other nestlings a placebo implant (31 further nestlings raised in the 26 experimental nests were not implanted). At the day of implantation, cort- and placebo-nestlings did not differ with respect to age [cort-nestlings, 29 ± 3 days (SD); placebo-nestlings, 29 ± 5 days; Student's *t*-test, $t_{74} = 0.71$, $P = 0.48$], body mass (cort-nestlings, 330 ± 38 g; placebo-nestlings, 323 ± 55 g; $t_{74} = 0.62$, $P = 0.54$), and wing length (cort-nestlings, 133 ± 20 mm; placebo-nestlings, 129 ± 30 mm; $t_{73} = 0.69$, $P = 0.49$). We allocated as many female as male nestlings in the two treatments (19 cort-males and 24 cort-females, 17 placebo-males and 26 placebo-females; χ^2-test, $\chi^2 = 0.19$, $P = 0.66$).

Plasma corticosterone

To monitor the effect of the implants on circulating total corticosterone, we collected blood samples just before implantation, 2, 6 and 20 days after implantation, by puncturing the brachial vein and collecting the blood with heparinized capillary tubes. Samples were immediately centrifuged and the plasma stored in liquid nitrogen. Since nestlings start to increase plasma corticosterone levels 3 min after having their nest-box opened (B.A., personal observation), we used only blood samples taken within 3 min of first opening the nest-box as baseline corticosterone level for analysis. Plasma corticosterone concentration was determined using an enzyme immunoassay (Munro & Stabenfeldt, 1984; Munro & Lasley, 1988). Corticosterone was extracted from plasma with 4 mL dichloromethane (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. Horseradish peroxidase (HRP, 1 : 400 000) linked to corticosterone served as enzyme label and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) as substrate. The concentration of corticosterone in plasma samples was calculated 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 10% and inter-assay variation from 12% to 15%, depending on the concentration of the internal controls. The detection threshold of this assay is 1 ng mL^{-1}.

Effectiveness of corticosterone implantation

In this study (a subset of the data reported in Müller *et al.*, 2009a), corticosterone implantation significantly increased corticosterone concentration above baseline level (mixed-effect model with 'nestling identity' nested in 'nest of rearing' and 'nestling identity' nested in 'nest of origin' as random factor for the four corticosterone

measurements (days 0, 2, 6 and 20); implant × days after implantation: LR = 4.14, $P = 0.04$). Post hoc tests showed that there was no difference in corticosterone levels between cort- and placebo-nestlings at the day of implantation (day 0: overall mean, 11.5 ng mL^{-1} ± 0.9 SE and $n = 31$; post hoc mixed-effect model with 'nest of rearing' and 'nest of origin' as random factor, LR = 0.9 and $P = 0.30$). This demonstrates that we allocated nestlings to the two treatments randomly with respect to baseline corticosterone level. Two days after corticosterone implantation, cort-nestlings had a significantly elevated corticosterone level compared to placebo-nestlings (29.1 ng mL^{-1} ± 2.5, $n = 20$ vs. 9.0 ng mL^{-1} ± 1.2, $n = 13$; LR = 25.5, $P < 0.001$). Six (LR = 0.05, $P = 0.80$) and 20 (LR = 0.06, $P = 0.80$) days after implantation, circulating corticosterone was at baseline levels, and there was no difference between the treatments anymore (overall mean: 6 days, 12.5 ng mL^{-1} ± 1.3, $n = 41$ nestlings; 20 days, 10.6 ng mL^{-1} ± 0.9, $n = 41$).

The increase in corticosterone due to the corticosterone implant was well below the increase in corticosterone as a response to handling (60 ng mL^{-1}, Müller *et al.*, 2009a) and comparable with 32 h of food deprivation (20 ng mL^{-1} ± 17 starved individuals, 7 ng mL^{-1} ± 10 fed individuals; A. Dreiss, personal communication). Food deprivation over several days easily occurs during bad weather under natural situations. Hence, the circulating levels attained by the pellets in this study were indeed within the naturally occurring range and duration of this species.

Assessment of nestling growth, plumage traits and gender

To investigate the effect of corticosterone administration on growth, we weighed all 86 nestlings to the nearest 0.1 g and measured maximum wing length to the nearest mm with a ruler with a zero stop on the day of implantation, as well as 2, 6, 14 and 20 days after implantation. A single person recorded plumage traits in all nestlings a few days before fledging. We placed a 60 × 40 mm frame on four body parts (breast, belly, one flank and one underside of the wings) where we measured the diameters of a large number of representative spots to the nearest 0.1 mm with a slide calliper. For each body part, we calculated a mean spot diameter. For each bird, we then calculated a mean spot diameter using the means taken on the four body parts (for details of the method see Roulin & Dijkstra, 2003; Roulin, 2004). Note that the measurement of spot diameter in nestlings is repeatable within individuals (see Roulin, 1999). The sex of all nestlings was determined using the CHD gene (see Py *et al.*, 2006). All measurements were taken blind to the experimental treatment.

Since we implanted nestlings after black spots had been produced, we checked whether we randomly allocated the two treatments with respect to this plumage trait. Indeed, nestlings implanted with a corticosterone-releasing pellet or a placebo pellet did not differ in spot diameter (mixed-effect model with 'nest of origin' as random factor, implant: LR = 1.65, $P = 0.20$; mean spot diameter of cort-nestlings: 1.4 ± 0.07 mm; placebo-nestling 1.4 ± 0.07 mm).

Statistical procedure

The growth curves of nestling body mass and wing length were analysed using mixed-effect models with the two random factors 'nest of origin' and 'nest of rearing' both nested in 'pair' plus their interactions with the fixed factor 'implantation' (i.e. corticosterone vs. placebo). To keep the random model as simple as possible, the term 'pair' was removed when it did not explain a measurable part of the variance (< 0.01% of the explained variance). Since implantation of corticosterone pellets caused a decline in body mass in the first 2 days after implantation but not thereafter (see Results) and because wing-length growth was more strongly reduced during the first 2 days, we first calculated the change in body mass or wing length between day 0 and day 2 of the experiment (hereafter 'initial body mass change' and 'initial wing-length change') and performed a mixed-effect model analysis with 'initial body mass change' or 'initial wing-length change' as response variable. 'Implantation' (corticosterone vs. placebo) and 'sex' were introduced as categorical variables, and nestling age at the start of the experiment ('age'), and brood size as covariates. We built the model with all possible interactions and compared it with a one-term simpler model. Models were compared with the log-likelihood ratio test (LR), and the more complicated model was kept when it was significantly better than the simpler model, otherwise the simpler model was kept. We always included 'nestling age' into the model to correct for the pronounced age hierarchy in barn owl broods, which results in body mass and size differences between nest mates. We then made a separate analysis with the best model including 'nestling spot diameter' plus its interaction with 'implant'. Using a similar model selection procedure, we reduced the model so that it contained only significant variables and variables involved in significant interactions. All random and fixed effects of the final models were tested using a 'Monte Carlo simulation' approach after Faraway (2006). Thereby, the distribution of the likelihood ratio for comparing an alternative model (containing term X) with a null model (model without term X) is approximated using Monte Carlo simulation. We simulated 500 times a set of response values from the null model and calculated the likelihood ratio between the alternative and the null model for each set of simulated response values. From these 500 likelihood ratios, an approximation of the distribution of the likelihood ratio was obtained and used instead of a chi-square distribution to obtain the *P*-value (Faraway, 2006).

To describe body mass gain and wing-length growth from day 2 to day 20 after the start of the experiment, we fitted for each individual a linear regression to the measurements taken at day 2, 6, 14 and 20, with day and the square of day as predictors. We thus obtained the linear effect β_1 as a measurement for the 'linear secondary mass gain' and 'linear secondary wing-length growth', respectively, and the quadratic effect β_2 as a measurement for the 'quadratic secondary mass gain' and 'quadratic secondary wing-length growth', respectively. We performed four separate mixed-effect model analyses with linear secondary mass gain, quadratic secondary mass gain, linear secondary wing-length growth or quadratic secondary wing-length growth of each nestling as the response variable. We used a similar random model and fixed effect structure (i.e. including nest of origin, nest of rearing, and pair as random variables and implantation, sex, nestling age and brood size as fixed effects), and model selection procedure as described above. Finally, to see whether corticosterone implantation had a long-term effect on nestling body size and on wing length shortly before fledging, we analysed body mass and wing length measured on day 20, that is, a few days before fledging, using the same mixed-effect model analysis as described above.

All statistical tests were carried out using the software package R version 2.10.1 (R Development Core Team, 2009). Means are quoted ± SE if not indicated otherwise. *P*-values ≤ 0.05 were considered as significant.

Ethical note

Nestling survival was not affected by corticosterone administration. In the 26 broods, a total number of 117 nestlings hatched and 108 survived until fledging (92%), a value comparable with a previous nonexperimental study in 2005 when 159 of 179 nestlings survived until fledging (88.2%). Of these 117 nestlings, 86 obtained either a corticosterone or a placebo implant and 81 of them survived until fledging (three cort- and two placebo-nestlings died before fledging). The study was carried out under legal authorization of the 'Service vétérinaire du canton de Vaud', permit no. 1736.

Results

Body mass

During the first 2 days, post-implantation corticosterone implantation significantly reduced body mass compared with placebo-nestlings (Table 1, Fig. 1). The age of the nestlings also had a small but significant effect on the initial body mass change (Table 1) but not in interaction with 'implant'. The random effect 'nest of rearing' explained a significant proportion of the variance in initial mass change. 'Nest of origin', and the interactions of 'nest of rearing' and 'nest of origin' with 'implant' as well as brood size, spot diameter and spot diameter in interaction with implant did not explain a significant proportion of the variance in initial mass change.

Body mass growth between day 2 and day 20 was analysed with the 'linear' and 'quadratic secondary mass gain' of the growth curve. 'Implant' and 'nestling spot diameter' had a significant effect neither on the 'linear', nor on the 'quadratic secondary mass gain' of the nestlings (Table 1). The interaction 'age × sex' was significant in the linear and quadratic secondary mass gain analysis, whereas the main effect 'age' was only significant in the linear secondary mass gain analysis (Fig. 1).

Table 1 Results of the mixed-effect model analyses with body mass growth ['initial mass change' (mass change from day 0 to day 2 of the experiment), 'linear secondary mass gain' (slope of the growth curve from day 2 to day 20 of the experiment) and 'quadratic secondary mass gain' (quadratic effect of the growth curve from day 2 to day 20)] as dependent variables. Shown are degree of freedom (d.f.), log-likelihood ratio (LR) and the *P*-values obtained with the bootstrap method (Faraway, 2006). Measurements were taken on 43 cort- and 43 placebo-nestlings on five different days during a period of 20 days. Variables without a significant contribution were excluded from the models, except for nest of origin, nest of rearing, age, implant and nestling spot diameter which were always kept in the model. Significant *P*-values (≤ 0.05) are given in bold.

	Initial mass change				Linear secondary mass gain				Quadratic secondary mass gain			
	d.f.	LR	P_{boot}	Estimate ± SE	d.f.	LR	P_{boot}	Estimate ± SE	d.f.	LR	P_{boot}	Estimate ± SE
Random effects												
Nest of origin	1	0.0	1		1	0.0	0.7		1	0.0	0.7	
Nest of rearing	1	7.3	**< 0.002**		1	27.9	**< 0.002**		1	19.9	**< 0.002**	
Fixed effects												
Intercept				19.4 ± 19.1				0.4 ± 7.9				0.7 ± 0.4
Sex, female					1	1.0	0.3	28.9 ± 9.4	1	0.5	0.6	−1.4 ± 0.5
Age	1	4.4	0.05	−1.3 ± 0.6	1	8.5	**< 0.002**	0.1 ± 0.3	1	0.5	0.5	−0.02 ± 0.01
Brood size												
Implant, placebo	1	18.4	**0.002**	21.5 ± 4.6	1	1.7	0.2	−1.4 ± 1.1	1	0.1	0.7	0.02 ± 0.06
Spot diameter (Dia)	1	0.0	0.9	−0.05 ± 0.6	1	1.4	0.3	0.3 ± 0.2	1	0.6	0.4	−0.01 ± 0.01
Age × sex					1	8.1	**< 0.002**	−1.0 ± 0.3	1	7.7	**0.002**	0.05 ± 0.01

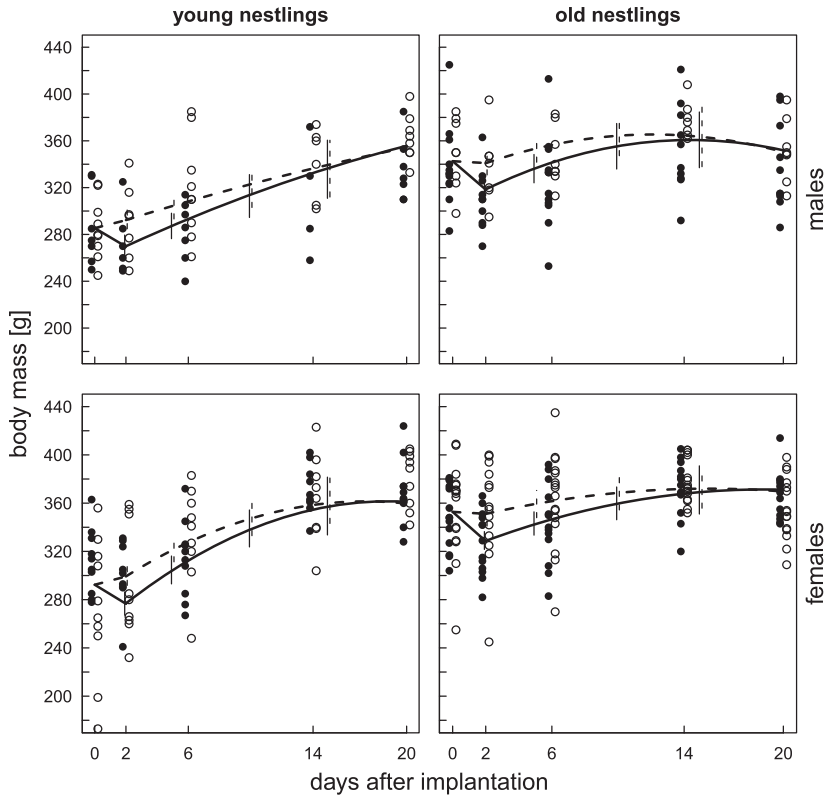

Fig. 1 Body mass growth from day 0 to day 20 of the experiment of young (left panel) and old nestlings (right panel) and males (upper panel) and females (lower panel) separately. Individuals implanted at an age below the median (28.8 days) were denoted 'young nestlings' and those implanted above the median 'old nestlings'. Closed symbols represent cort-nestlings, open symbols placebo-nestlings. Lines represent the predicted mass and the vertical bars confidence intervals of the full model (see Table 1) of cort- (continuous line) and placebo-nestlings (dashed line).

The random effect 'nest of rearing' explained a significant proportion of the variance in the 'linear' and 'quadratic secondary mass gain'. Brood size, 'nest of origin' and the interactions of 'implant' with 'nest of origin' or 'nest of rearing' had no significant influence on the 'linear' and 'quadratic secondary mass gain'.

Body mass shortly before fledging (20 days after corticosterone implantation) was not affected by the treatment anymore (term 'implant' in Table 2 not significant). However, fledging body mass was differentially affected by corticosterone implants depending on nestling spot diameter as shown by the significant interaction between 'implant × nestling spot diameter' (Table 2). Cort-nestlings with small spots were lighter in body mass than cort-nestlings with large spots, whereas body mass was not significantly associated with spot diameter in placebo-nestlings (Fig. 2). The interaction 'nest of origin × implant' had a small but significant effect on nestling fledging body mass (Table 2), indicating that nestlings of different origins reacted differently to the implant. Brood size, 'nest of origin', 'nest of rearing' alone and 'nest of rearing × implant' had no significant influence on fledging body mass.

Table 2 Results of the mixed-effect model analysis with fledging body mass as dependent variable. Shown are degree of freedom (d.f.), log-likelihood ratio (LR) and the *P*-values obtained with the bootstrap method (Faraway, 2006). Measurements were taken on 43 cort- and 43 placebo-nestlings. Variables without a significant contribution were excluded from the models, except for nest of origin, nest of rearing, age, implant and nestling spot diameter which were always kept in the model. Significant *P*-values (≤ 0.05) are given in bold.

	Fledging body mass			
	d.f.	LR	P_{boot}	Estimate ± SE
Random effects				
Pair	1	0.3	0.2	
Pair × nest of origin	1	0.8	0.2	
Pair × nest of rearing	1	0.0	0.7	
Nest of origin × implant	1	2.6	**0.05**	
Fixed effects				
Intercept				335.6 ± 23.3
Sex, female	1	7.9	**< 0.002**	16.9 ± 5.8
Age	1	1.7	0.2	−1.0 ± 0.7
Brood size				
Implant, placebo	1	0.8	0.4	70.9 ± 17.9
Spot diameter (Dia)	1	1.2	0.3	2.8 ± 0.9
Implant × Dia	1	13.0	**< 0.002**	−4.8 ± 1.1

Wing length

At the peak of corticosterone release (the first 2 days after implantation), corticosterone implants reduced wing-length growth significantly (Table 3). Age at implantation also significantly influenced the 'initial wing-length change' but not interaction with 'implant'. No other

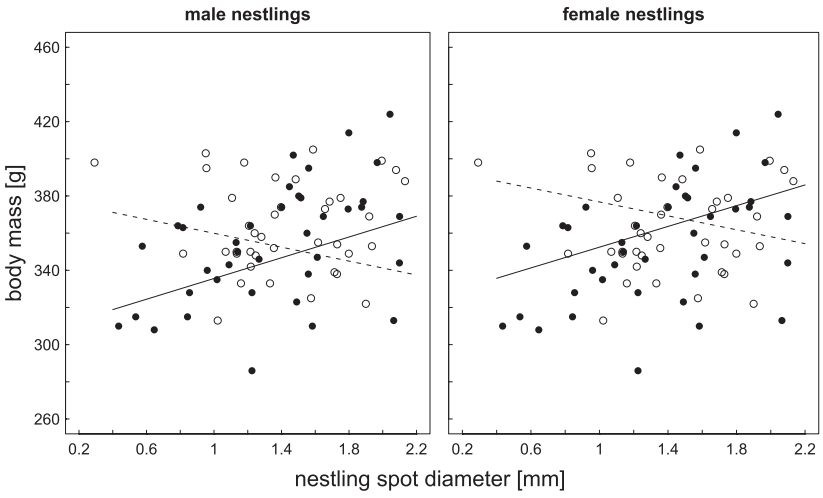

Fig. 2 Body mass shortly before fledging of male nestlings and female nestlings. Closed symbols represent cort-nestlings, open symbols placebo-nestlings. Lines represent the predicted mass of the full model (see Table 2) of cort- (continuous line) and placebo-nestlings (dashed line). *Post hoc* test showed that cort-nestlings with large spots were significantly heavier than cort-nestlings with smaller spots (mixed-effect model with 'nest of rearing' and 'nest of origin' as random factor, LRT = 6.81, $P = 0.009$), whereas in placebo-nestlings, body mass was not associated with spot diameter (mixed-effect model with 'nest of rearing' and 'nest of origin' as random factor, LRT = 1.43, $P = 0.232$).

Table 3 Results of the mixed-effect model analysis with wing-length growth ['initial wing-length change' (wing-length change from day 0 to day 2 of the experiment), 'linear secondary wing-length gain' (slope of the growth curve from day 2 to day 20 of the experiment), and 'quadratic secondary wing-length gain' (quadratic effect of the wing-length growth curve)] as dependent variables. Shown are degree of freedom (d.f.), log-likelihood ratio (LR) and the *P*-values obtained with the bootstrap method (Faraway, 2006). Measurements were taken on 43 cort- and 43 placebo-nestlings on five different days during a period of 20 days. Where indicated with an asterisk, the random effects are: 'pair × nest of origin' instead of only 'nest of origin' and 'pair × nest of rearing', respectively. Variables without a significant contribution were excluded from the models, except for nest of origin, nest of rearing, age, implant and nestling spot diameter which were always kept in the model. Significant *P*-values (≤ 0.05) are given in bold.

	Initial wing-length change*				Linear secondary wing-length growth				Quadratic secondary wing-length growth			
	d.f.	LR	P_{boot}	Estimate ± SE	d.f.	LR	P_{boot}	Estimate ± SE	d.f.	LR	P_{boot}	Estimate ± SE
Random effects												
Pair	1	4.8	**0.006**									
Nest of origin	1	0.0	1		1	0.0	1		1	0.0	1	
Nest of rearing	1	0.0	1		1	39.2	**< 0.002**		1	41.5	**< 0.002**	
Fixed effects												
Intercept				13.6 ± 3.6				5.9 ± 0.7				0.1 ± 0.02
Sex, female												
Age	1	4.0	**0.05**	−0.2 ± 0.1	1	19.6	**< 0.002**	−0.1 ± 0.02				
Brood size												
Implant, placebo	1	14.3	**< 0.002**	3.2 ± 0.8	1	33.1	**< 0.002**	2.4 ± 0.5	1	25.5	**< 0.002**	−0.1 ± 0.02
Spot diameter (Dia)	1	0.3	0.6	−0.1 ± 0.1	1	5.9	**0.03**	0.1 ± 0.03	1	2.2	0.2	−0.004 ± 0.001
Implant × Dia					1	7.2	**0.01**	−0.1 ± 0.03	1	5.4	**0.03**	0.04 ± 0.001

fixed effect had a significant effect on 'initial wing-length change'.

After the peak of corticosterone release, between day 2 and day 20 after implantation, corticosterone treatment differentially affected wing-length growth depending on spot diameter of the nestlings. This effect was significant in the linear and quadratic term of secondary wing-length growth (Table 3). Cort-nestlings had a reduced linear secondary wing-length growth but a stronger quadratic secondary wing-length growth which resulted in a more upward curved growth curve. Nestlings with large spots were less negatively affected by the cort-implants than nestlings with smaller spots (Fig. 3). Age at implantation had a significant negative effect on linear secondary wing-length growth but none on the quadratic secondary wing-length growth. 'Nest of rearing' explained a significant proportion of the variance of the linear and quadratic secondary and the quadratic wing-length growth. 'Nest of origin', and the interactions 'nest of rearing' and 'nest of origin' with 'implant' had no significant effect on wing-length growth.

Wing length at fledging was significantly shorter in cort-nestlings (mean wing length, 222 ± 3 mm) than in placebo-nestlings (226 ± 3 mm). Age at implantation had a significant positive influence on fledging wing length, and female fledglings had significantly longer wings than male fledglings (Table 4). The interaction 'implant × brood size' had a significant negative effect for

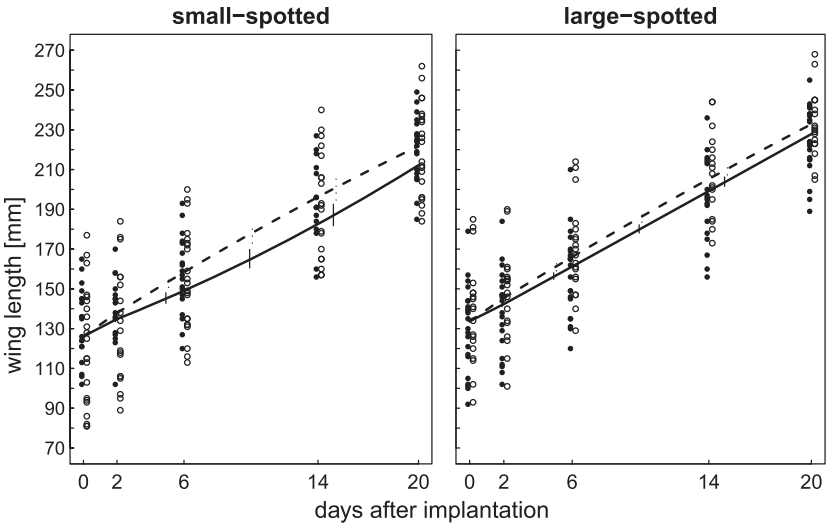

Fig. 3 Wing-length growth from day 0 to day 20 of the experiment of nestlings with small spots and nestlings with larger spots. Individuals with spot diameter below the median (1.44 mm) were denoted 'small spotted' and those nestlings with spot diameter above the median 'large spotted'. Closed symbols represent cort-nestlings and open symbols placebo-nestlings. Lines represent the predicted wing length and vertical bars confidence intervals of the full model (see Table 3) of cort- (continuous line) and placebo-nestlings (dashed line). *Post hoc* tests showed that wings of large-spotted cort-nestlings grew more rapidly than wings of small-spotted cort-nestlings.

Table 4 Results of the mixed-effect model analysis with fledging wing length as dependent variable. Shown are degree of freedom (d.f.), log-likelihood ratio (LR), and the *P*-values obtained with the bootstrap method (Faraway, 2006). Measurements were taken on 43 cort- and 43 placebo-nestlings. Significant *P*-values (≤ 0.05) are given in bold.

	Fledging wing length			
	d.f.	LR	P_{boot}	Estimate ± SE
Random effects				
Pair	1	2.9	**0.04**	
Pair × nest of origin	1	0.0	0.6	
Pair × nest of rearing	1	9.4	**< 0.002**	
Fixed effects				
Intercept				92.1 ± 6.2
Sex, female	1	11.2	**0.002**	4.2 ± 0.1
Age	1	183.8	**< 0.002**	4.4 ± 1.2
Brood size	1	0.1	0.7	−0.8 ± 0.9
Implant, placebo	1	19.4	**< 0.002**	−7.2 ± 3.4
Spot diameter (Dia)	1	3.2	0.1	0.3 ± 0.1
Implant × brood size	1	12.0	**< 0.002**	2.5 ± 0.6

corticosterone nestlings. The interaction 'implant × spot diameter' was not significant (LR = 0.1, d.f. = 1, $P = 0.77$). 'Nest of rearing' explained a significant proportion of the variance of fledging wing length. Neither 'nest of origin' alone, nor the interaction 'implant' with 'nest of rearing' or 'nest of origin' explained a significant proportion of the variance of fledging wing length.

Discussion

In this study, we showed that an elevation of circulating plasma corticosterone during a few days within the naturally occurring range reduces growth differentially depending on the genotype of the barn owl nestlings. The individual sensitivity to corticosterone administration measured in terms of wing-length growth and fledging body mass varied with nestling spot diameter, a heritable eumelanin-based trait. After corticosterone administration, nestlings with larger black spots were heavier before fledging and their wing-length growth was less reduced than in nestlings with smaller black spots.

Effect of corticosterone administration on growth

Glucocorticoids directly interfere with the growth hormone-IGF-1 axis. High glucocorticoid levels reduce growth hormone secretion, decrease bone formation and inhibit IGF-1 signalling, which leads to catabolic and anti-anabolic effects on muscle proteins (Hochberg, 2002). As a consequence, an artificial increase in corticosterone level leads to a decrease in growth rate as already observed in similar experiments carried out in birds and mammals (Huang *et al.*, 2000; Sapolsky *et al.*, 2000; Hull *et al.*, 2007; Müller *et al.*, 2009b). In accordance, corticosterone implantation in nestling barn owls reduced growth in both the parameters measured, body mass and wing length. By elevating corticosterone without food restriction, we could add further evidence that growth under stressful conditions is under differential regulation of corticosterone and not only a direct consequence of nutrient shortage. Therefore, corticosterone is likely involved in the control of developmental plasticity and hence phenotypic variation.

The effect of elevated corticosterone levels on growth differed slightly between body mass and wing length. The effect on body mass was very pronounced during the first 2 days when the elevation of corticosterone was at its maximum. After the period of high plasma corticosterone levels, we could not detect a statistically significant difference between the body mass growth curves of the two groups. However, when looking at the model-predicted growth curves (Fig. 1), we see a small

compensatory growth of body mass growth in cort-nestlings compared with placebo-nestlings and no difference in fledging body mass between the two groups. Hence, there is support for a compensatory growth of body mass in cort-nestlings, as has already been observed in other species after a period of stress (Bize *et al.*, 2006; Müller *et al.*, 2009b). The growth-reducing effect of corticosterone administration on wing length lasted longer than the actual period of elevated plasma corticosterone levels due to the corticosterone implants. This is shown by the significantly smaller slope of the linear secondary wing-length growth. In contrast, the quadratic term of wing-length growth was stronger in cort- than in placebo-nestlings, suggesting an acceleration of wing-length growth, which began only several days after corticosterone levels, returned to baseline. However, the acceleration of wing-length growth was not so strong that the cort-nestlings could fully catch up in wing length until 20 days after cort-implantation. A similar differential pattern of compensatory growth of various growth parameters has been found in the Alpine swift (*Apus melba*) after a temporary period of food shortage (Bize *et al.*, 2006) and in European kestrels *Falco tinnunculus* after corticosterone administration (Müller *et al.*, 2009b).

After fledging (with the birds inaccessible to us), the body mass may still change, and wing feathers may still grow and are subsequently moulted each year, so that cort-nestlings might still catch up fully in body mass and wing length. Even if compensated, a temporarily reduced growth may induce fitness costs (Metcalfe & Monaghan, 2001). Hence, the individual variation in how birds react to elevated levels of corticosterone during growth is of great importance for their success in life, and this will be discussed in the following section.

Corticosterone-mediated variation in growth rate in relation to the degree of eumelanin-based coloration

Variation in growth rates *per se* could be attributed to the nest of rearing (i.e. the local conditions or the rearing parents) and nestling age because of age-specific growth. No variation in growth rates was found with brood size, nest of origin and spot size. In contrast to general growth, individual variation in corticosterone-mediated reduction in growth rate (i.e. interactions with the factor implant in Tables 1–4) was significantly explained only by spot diameter (for fledging body mass and secondary wing-length growth) and nest of origin (fledging body mass), and not by factors such as nest of rearing, age, sex or brood size (interactions of implant with these factors were not significant, Tables 1–4), with the exception of fledging wing length which was more reduced in cort-nestlings when in large broods.

Therefore, although growth was mainly affected by local factors acting in the nest of rearing, variation in corticosterone-mediated reductions in growth rate was more explained by intrinsic properties of the nestlings (spot diameter and nest of origin). Nestlings with large eumelanic spots showed higher body mass and accelerated wing-length growth after a short period of experimentally elevated corticosterone compared to small-spotted and placebo-nestlings. More eumelanic nestlings might have some intrinsic factors which make them better able to cope with elevated corticosterone levels. The link between eumelanin-based coloration (a heritable trait in barn owls; Roulin & Dijkstra, 2003) and the ability to cope with elevated corticosterone could have a genetic basis. Possibly more or less eumelanic individuals differ behaviourally; for example, more eumelanic nestlings beg more and hence receive more food which in turn enables them to deal better with elevated corticosterone levels (Almasi *et al.*, 2009). Another possibility is that more eumelanic birds are better able to regulate exogenous corticosterone and hence are less affected by the effects of elevated corticosterone on growth. We showed earlier that both the release and the regulation of corticosterone are associated with eumelanin-based coloration (Almasi *et al.*, 2010), an association which is not only restricted to barn owls but also confirmed in other bird species and vertebrates (e.g. Schwabl, 1995; Kittilsen *et al.*, 2009).

The physiological differences associated with the size of black spots probably explain why large- and small-spotted barn owl nestlings respond behaviourally in a different way in front of a predator with larger-spotted individuals being less reactive than smaller-spotted conspecifics (Van den Brink *et al.*, in press). Thus, darker eumelanic individuals may have an advantage in terms of growth potentially indicating that the degree of eumelanin-based coloration may be directionally selected in stressful environments as suggested in another study (Roulin *et al.*, 2008). In contrast, small-spotted individuals are more aggressive and reactive in front of a predator which may give them an advantage under predation risk (Van den Brink *et al.*, in press). Differently coloured individuals may therefore be adapted to different environments which may explain the evolutionary stability of colour variation in a heterogeneous environment. Indeed, a previous study suggested that melanic individuals are better adapted to stressful environmental conditions than light melanic conspecifics that better perform in relaxed environments (Roulin *et al.*, 2008).

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