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Temporal variation in glucocorticoid levels during the resting phase is associated in opposite way with maternal and paternal melanic coloration

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Abstract

Sex-dependent selection can help maintain sexual dimorphism. When the magnitude of selection exerted on a heritable sex trait differs between the sexes, it may prevent each sex to reach its phenotypic optimum. As a consequence, the benefit of expressing a sex trait to a given value may differ between males and females favouring sex-specific adaptations associated with different values of a sex trait. The level of metabolites regulated by genes that are under sex-dependent selection may therefore covary with the degree of ornamentation differently in the two sexes. We investigated this prediction in the barn owl, a species in which females display on average larger black spots on the plumage than males, a heritable ornament. This melanin-based colour trait is strongly selected in females and weakly counter-selected in males indicating sex-dependent selection. In nestling barn owls, we found that daily variation in baseline corticosterone levels, a key hormone that mediates life history trade-offs, covaries with spot diameter displayed by their biological parents. When their mother displayed larger spots, nestlings had lower corticosterone levels in the morning and higher levels in the evening, whereas the opposite pattern was found with the size of paternal spots. Our study suggests a link between daily regulation of glucocorticoids and sex-dependent selection exerted on sexually dimorphic melanin-based ornaments.

Introduction

The maintenance of sexual dimorphism is a central issue in evolutionary biology. Natural and sexual selection promote the evolution of sex differences in the expression of many phenotypic traits. Typically, females invest more effort than males in reproduction leading to the evolution of cryptic traits in females to hide while taking care of the offspring and of showy ornaments in males as a means to enhance the mating success. The situation might be different in species with sex-role reversal (Paczolt & Jones, 2010) in which males invest more effort in reproduction than females, as it is often the case in raptors and owls. The two sexes may thus be differentially selected to express an ornament implying that the benefit of expressing such a trait to a given value may be sex specific. This may select for sex-specific adaptations to maximize the benefit of displaying an ornament. In this case, we predict that the level of metabolites regulated by genes that are under sex-dependent selection covaries with the degree of ornamentation differently in the two sexes.

Identifying the physiological pathway affected by genes that are under sex-dependent selection requires a biological system for which a candidate gene is known to pleiotropically mediate the expression of a secondary sexual character and major physiological traits. An interesting case is the melanocortin system. In vertebrates, the proopiomelanocortin gene (*POMC*) encodes

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for melanocortin hormones that trigger the production of black eumelanin pigments and are essential to regulate the hypothalamic–pituitary–adrenal axis (HPA). The HPA consists of the hypothalamic corticotropin-releasing hormone, which stimulates the pituitaric adrenocorticotropin hormone (ACTH) and further activates the synthesis of glucocorticoids (cortisol and corticosterone) (Charmandari *et al.*, 2005). In the animal kingdom, melanin is the most widespread pigment and participates in the elaboration of many ornaments. Differently coloured individuals may thus differentially regulate the corticosterone levels (Almasi *et al.*, 2010). It would be interesting to tackle this issue in a species in which the benefit of displaying melanin-based coloration is sex specific because the covariation between coloration and corticosterone levels may be sex specific.

Baseline corticosterone mediates trade-offs between life history traits such as self-maintenance, growth and reproduction (Romero, 2004; Landys *et al.*, 2006; Bonier *et al.*, 2009). A change in environmental challenges such as food limitation can lead to an increase in baseline corticosterone levels to reallocate resources to body maintenance by inducing catabolism of body reserves to fuel the metabolism (e.g. Kitaysky *et al.*, 1999; Jenni-Eiermann *et al.*, 2008). Baseline corticosterone levels also regulate seasonal (Romero, 2002; Criscuolo *et al.*, 2005) and daily biological rhythms. For instance, levels of this hormone typically increase with onset of feeding and activity cycles in vertebrates (e.g. Pancak & Taylor, 1983; Jessop *et al.*, 2002). In diurnal animals, the peak in corticosterone levels is shortly before the active period (e.g. Breuner *et al.*, 1999), a pattern that can be perturbed by the duration of days and nights and by circadian rhythm (Steiger, 2002).

In the barn owl, females display on average larger black eumelanic spots on feather tips than males, a strongly selected trait in females and weakly counter-selected in males (Roulin *et al.*, 2010). Spot size is a criterion in male mate choice (Roulin, 1999; Roulin & Altwegg, 2007) and advertises aspects of female genetic quality (Roulin, 2004). To investigate whether the daily variation in corticosterone levels is associated with spot size differently in the two sexes, we monitored variation in daily baseline corticosterone levels in nestlings in relation to the size of black spots displayed by their biological and foster parents; some nestlings were swapped between nests soon after hatching to randomize genotypes among environments. If there is a relationship between offspring corticosterone levels and spot diameters measured in their biological parents, we can suspect that the association between corticosterone levels and melanin-based coloration is heritable rather than environmentally mediated. We expect an association between the daily baseline corticosterone levels in nestlings and the size of black spots displayed by their biological parents because we previously observed that this eumelanic trait is linked to the balance between energy intake and expenditure (Roulin, 2009; Dreiss *et al.*, in press), a property that can be partly under the control of corticosterone (Kitaysky *et al.*, 1999; Jenni-Eiermann *et al.*, 2008). In the evening, but not in the morning, breeding females with large black spots are heavier than conspecifics displaying smaller spots (Roulin, 2009). We monitored baseline corticosterone in nestlings because our aim is not only to link daily variation in this hormone with melanin-based coloration measured in the nestlings themselves but also in their parents. As we will see, this is crucial because there is a link between nestling corticosterone levels and spot diameter measured in their biological parents, not in the nestlings themselves. If we would have measured corticosterone levels in parents, our study would have been purely correlative (a within-individual phenotypic correlation can be mediated not only by genetic factors but also environmental factors), whereas an association between offspring corticosterone levels and spot diameter of biological parents is likely to be caused by genetic factors.

Methods

Baseline corticosterone levels

We carried out the study between 2004 and 2006 in western Switzerland (46°49′N, 06°56′E) in a wild population of barn owls located in an area of 190 km^2. We regularly monitored nests to determine clutch size and hatching date. We performed cross-fostering experiments to allocate genotypes randomly among the environments. In 2004 and 2006, we matched broods in pairs with similar hatching dates, and in 2004 two of the four first-born nestlings were randomly chosen and swapped with similarly aged nestlings from the matched nest [mean age at cross-fostering: 3.0 days ± 0.2 (SE)]. In 2006, four of the first-laid eggs were randomly chosen and swapped with similarly aged eggs from the matched nest. In 2005, no cross-fostering experiment was carried out. Shortly after hatching, all nestlings were colour marked on their clipped nail until being ringed with an aluminium ring at the age of 14 days. Nestling rank in the within-brood age hierarchy was easily determined because of pronounced hatching asynchrony with each egg hatching every 2.5 days. Rank 1 was assigned to the oldest nestling, rank 2 to the second-oldest nestling and so on. Sex of nestlings was determined using molecular markers (Py *et al.*, 2006), and breeding females were recognized by the presence of a brood patch.

One person reliably (repeatability is 0.92) measured the size of black spots in breeding adults and their 55-day-old offspring. A 60 mm × 40 mm frame was placed on the breast within which the diameter of spots was measured to the nearest 0.1 mm. A mean spot diameter was calculated and used in the statistical analyses. Spot diameter of biological and foster parents

was not correlated both in 2004 and 2006, the 2 years when we did cross-fostering experiments (Pearson's correlations, all *P*-values > 0.09). In the 3 years, mother and father mean spot diameters were not correlated with laying date and clutch size (*P*-values > 0.40).

To investigate the change in baseline corticosterone levels during the daylight hours from 8 AM to 7 PM, we collected 641 blood samples in 328 nestlings from 76 nests. Mean age of the nestlings was 33 days ± 12 (SD) (range 5–62, mean age in 2004: 39 days ± 9; 2005: 35 days ± 11; 2006: 21 ± 9 days). Blood samples were taken from the brachial vein and collected with heparinised capillary tubes, immediately centrifuged and the plasma stored at −20 °C until analysis. Because a strong increase in circulating corticosterone levels is observed 3 min after capture of the nestlings (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 analyses. To correct for the small increase of corticosterone within the first 3 min after capture (Pearson's correlation: $r = 0.20$, $n = 641$, $P < 0.0001$; slope is 0.17 ± 0.03 ng mL^{-1} per min handling), we included the time expressed in seconds from start of the capture to blood collection (defined as 'disturbance time') into the model.

Plasma total corticosterone concentration was determined using an enzyme immunoassay (Munro & Stabenfeldt, 1984; Munro & Lasley, 1988). We diluted 5 μL plasma with 195 μL water to extract corticosterone from plasma with 4 mL dichloromethane. 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 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) served as substrate. The concentration of total 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 13% and inter-assay variation from 12% to 21%, depending on the year and concentration of the internal control. Corticosterone values were log-transformed to obtain a normal distribution of the data.

Statistical procedure

Statistical analyses were done using the statistical software package R version 2.10 (R Development Core Team, 2009). To separate the effects of the rearing environment and the genetic background of the nestlings on the variation in baseline corticosterone levels, we considered only the data collected on cross-fostered nestlings in 2004 and 2006; we had 197 measurements of corticosterone levels in 102 individuals from 42 nests. We performed a mixed-effect model analysis with corticosterone levels as the dependent variable and mean spot diameter of the biological mother and father plus their interaction as covariates. Year, sex and rank of the nestlings in the within-brood age hierarchy were included as factors and the amount of time between the moment when we started to disturb the nestlings and collected a blood sample (disturbance time), nestling body mass and age as covariates. To correct for the nonindependence of siblings and account for repeated sampling within the same individuals, nestling identity nested within-brood identity was introduced as random factor. Nonsignificant terms of the full model were stepwise backward eliminated. Using the same sample of individuals, we then carried out similar analyses by considering spot diameter displayed by the foster parents.

Because these preliminary analyses showed that baseline corticosterone levels were associated with plumage traits measured in the biological parents but not in the foster parents, we considered cross-fostered and non-cross-fostered nestlings together; we had 641 measurements of corticosterone levels in 328 nestlings from 76 nests monitored in 2004, 2005 and 2006. To analyse the pattern of baseline corticosterone levels during the resting phase, we performed similar analyses as previously by adding hour of the day as an extra covariate. To confirm that rearing conditions did not inflate the relationship between corticosterone levels and spot diameter, we tested whether the factor 'cross-fostering' (i.e. whether a given nestling was raised by biological or foster parents) was not significant alone or in interaction with hour of the day and spot diameter of the biological parents. As expected, this factor was not significant (results not shown) and thus in the final model, we removed it to simplify the analyses. Final models only contained significant effects and main effects involved in significant interactions. We performed similar models by replacing spot diameter of the parents by spot diameter of the nestlings themselves. Analyses are two-tailed, and significance level is fixed at 0.05.

Results

In 2004 and 2006, baseline corticosterone levels of cross-fostered nestlings were associated with spot diameter of the biological mother in interaction with spot diameter of the biological father but not with spot diameter of the foster parents (Table 1).

To investigate whether nestlings born from small-spotted parents or large-spotted parents regulate baseline corticosterone levels differently during the resting phase, we considered data collected in cross-fostered and non-cross-fostered nestlings from the 3 years of the study (2004, 2005 and 2006). This analysis was possible because pairing with respect to spot diameter was

Table 1 Mixed-model ANCOVA testing the relationship between baseline total corticosterone levels in cross-fostered barn owl nestlings and the size of eumelanic black plumage spots measured in the biological (a) and foster (b) parents. Baseline total corticosterone was the dependent variable and year, nestling body mass, spot-diameter of the biological (a) or foster (b) parents the independent variables. We included nestling identity nested in site as random factor. The analysis is based on 197 measurements of 102 individuals from 42 nests in 2004 and 2006. Significant terms are written in bold.

	df	*F*	*P*
(a)			
Intercept	**1,94**	**2872**	**< 0.001**
Year	**1,37**	**47**	**< 0.001**
Nestling body mass	1,94	0	0.80
Spot diameter of biological mother (M)	1,37	1	0.20
Spot diameter of biological father (F)	1,37	2	0.20
M × F	**1,37**	**6**	**0.02**
(b)			
Intercept	**1,94**	**2336**	**< 0.001**
Year	**1,37**	**41**	**< 0.001**
Nestling body mass	1,94	0	0.80
Spot diameter of foster mother (M)	1,37	0	0.90
Spot diameter of foster father (F)	1,37	2	0.10
M × F	1,37	1	0.50

random in 2004 (Pearson correlation between spot diameter of the biological mother and her male mate: $r = 0.10$, $n = 23$, $P = 0.80$), 2005 ($r = 0.20$, $n = 31$, $P = 0.40$) and 2006 ($r = 0.30$, $n = 14$, $P = 0.30$) implying that we had a wide range of combinations of maternal and paternal spot diameters (Fig. 1). Spot diameter of the biological mother and father was differentially associated with offspring corticosterone levels (interaction 'spot-diameter biological mother × spot-diameter biological father' in Table 2). At the beginning of the resting phase, nestlings had more corticosterone when their mother and father displayed larger and smaller black spots, respectively (left panel of Fig. 2). At the middle of the resting phase, there was no relationship anymore between offspring corticosterone levels and parental spot diameter (panel in the middle of Fig. 2). Shortly before nestlings became active again, they had more corticosterone when their mother and father had smaller and larger black spots, respectively (right panel of Fig. 2). Thus, from the beginning to the end of the resting phase, the relationship between offspring corticosterone levels and mother spot diameter changed from positive to negative (interaction 'hour × spot-diameter biological mother' in Table 2), whereas in males it changed from negative to positive (interaction 'hour × spot-diameter biological father' in Table 2). In an analysis, where we included only spot diameter of the mother but not the father, the interaction 'hour × spot-diameter biological mother' was significant ($F_{1,310} = 9.00$, $P = 0.003$); similarly in an analysis where we included only spot diameter of the father but not of the mother, the interaction 'hour × spot-diameter biological father' was also significant ($F_{1,310} = 4.00$, $P = 0.041$). Because spot diameter is strongly heritable (Roulin *et al.*, 2010) and the relationship between corticosterone levels was of opposite sign with father and mother spot diameter, it is not surprising if similar analyses where we replaced parental spot diameters by nestling spot diameter proved not significant (*P*-values > 0.10).

The significant 'year' effect in Table 2 indicates that nestlings had more corticosterone in 2004 than in 2005 than in 2006. Overall corticosterone levels increased from the morning to the evening (Fig. 3).

Discussion

The present study in the barn owl shows that a melanin-based ornament is associated with circadian regulation of

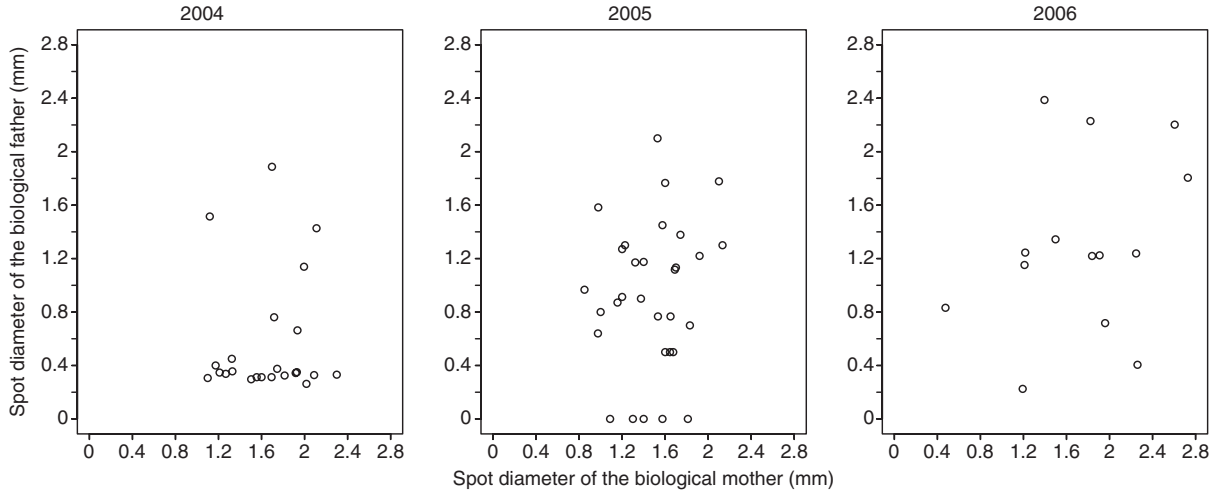

Fig. 1 Spot diameter of the biological mother in relation to spot diameter of her male mate in 2004, 2005 and 2006.

Table 2 Mixed-model ANCOVA testing the pattern in baseline total corticosterone levels during daylight hours from 8 AM to 7 PM in nestling barn owls in relation to the diameter of black spots displayed by their biological mother and biological father. The analysis is based on 641 measurements of 328 individuals from 76 nests from 2004, 2005 and 2006. Significant terms are written in bold.

	df	*F*	*P*
Intercept	**1,309**	**11751**	**< 0.0001**
Year	**2,247**	**43**	**< 0.0001**
Disturbance time	**1,309**	**44**	**< 0.0001**
Hour of the day	**1, 309**	**14**	**< 0.0001**
Spot-diameter biological mother (M)	1,247	0	0.70
Spot-diameter biological father (F)	1,247	0	0.60
Hour of the day × M	**1,309**	**9**	**0.003**
Hour of the day × F	**1,309**	**7**	**0.007**
M × F	**1,247**	**8**	**0.006**

Log-transformed baseline corticosterone levels were the dependent variable, and we included hour of the day, nestling body mass and spot diameter of the biological mother and father as four covariates. Year, sex of nestlings, position in the within-brood age hierarchy and cross fostering status (whether nestlings were raised by biological or foster parents) were included as factors; in first analyses sex, rank and cross-fostering status (i.e. whether nestlings were raised by biological or foster parents) proved not significant alone or in interactions and were therefore removed from the final analysis presented in the table. To control for the nonindependence of siblings and account for repeated sampling within the same individuals, we introduced the nest of origin and nestling identity nested in the nest of origin as two random factors. Nonsignificant terms of the full model were stepwise backward eliminated. The sign 'x' indicates 'interaction'. Even if the time span between the moment when we disturbed nestlings and took a blood sample (i.e. disturbance time) was weakly associated with corticosterone levels, we statistically controlled for this variable.

baseline corticosterone levels but in opposite direction with mother and father spot diameter. In the following, we discuss why corticosterone levels increase during the resting phase (daylight hours) in a nocturnal bird, why corticosterone levels are associated with a eumelanin-based trait and the importance of our results in the context of sex-specific selection.

In line with previous studies showing that daily elevations of glucocorticoids coincides with the onset of feeding and activity cycles (review in Landys *et al.*, 2006), we found that baseline corticosterone levels increased from 8 AM to 7 PM. As can be seen in Fig. 3, there was large variation in the level of corticosterone suggesting that individuals may not all regulate daily levels of corticosterone in a similar way. Accordingly, nestlings showed the typical daily regulation of corticosterone (low levels in the morning and high levels in the evening at the beginning of the active phase) when their biological parents displayed plumage black spots of a size typical of the other sex, i.e. small black spots in mothers and large spots in fathers. In contrast, when parents displayed plumage spots typical of their own sex (i.e. large spots in females and small spots in males), nestlings showed high levels of baseline corticosterone in the morning and lower levels in the evening (Fig. 2). To date it is unclear why offspring regulate corticosterone levels differently from the general pattern when their biological parents show the typical ornament of their own sex. This result is stimulating and will thus deserve more research. The fact that variation in corticosterone levels was associated with melanin-based coloration displayed by the biological parents but not the foster parents indicates that diurnal variation of corticosterone is heritable as already shown in humans (Linkowski *et al.*, 1993). Because baseline corticosterone levels are involved in several key physiological processes including sleep and vigilance (Steiger, 2002), we may expect circadian rhythm and sleeping activity to be associated with the size of parental black spots. No data are yet available to examine these predictions.

From a proximate point of view, the link between a eumelanin-based trait and baseline corticosterone levels (and also stress-induced levels, see Almasi *et al.*, 2010) could be induced by the melanocortin system. The *POMC* gene encodes for melanocortin hormones that trigger eumelanogenesis and are necessary for the biochemical cascade leading to the secretion of glucocorticoids (Charmandari *et al.*, 2005). In several animals including humans (Adan & Natale, 2002; Roenneberg *et al.*, 2004; Lehnkering & Siegmund, 2007) and *Drosophila melanogaster* (Helfrich-Förster, 2000), daily activity rhythm differs between the sexes. Because the *POMC* gene shows daily rhythms of expression (Seres *et al.*, 2004), it would be interesting to investigate whether in the barn owl daily variation in the expression of the *POMC* gene and of genes involved in circadian rhythm is sex- and colour-specific. Whatever the exact underlying mechanism, eumelanin-based coloration may be used by conspecifics as a signal of the way daily activity is regulated. Depending on whether it is adaptive or not to show pronounced daily variation in corticosterone levels in nestlings, selection may favour assortative or disassortative pairing with respect to this colour trait. Based on Fig. 2, we predict that if pairing is assortative the offspring should not show a strong daily pattern of corticosterone regulation. In contrast, if parents pair disassortatively the offspring should show a strong daily pattern of corticosterone regulation. In that case, corticosterone levels should be low in the morning and high in the evening if the father displays large black spots and his mate small spots; the offspring should have high corticosterone levels in the morning and low levels in the evening if the father displays small spots and his mate large spots. Interestingly, in some years, we indeed observed that pairing with respect to spot size is assortative and in other years disassortative or random (Fig. 1). Our study on the regulation of corticosterone may therefore provide some insights into an understanding of the adaptive value of variation in the pattern of

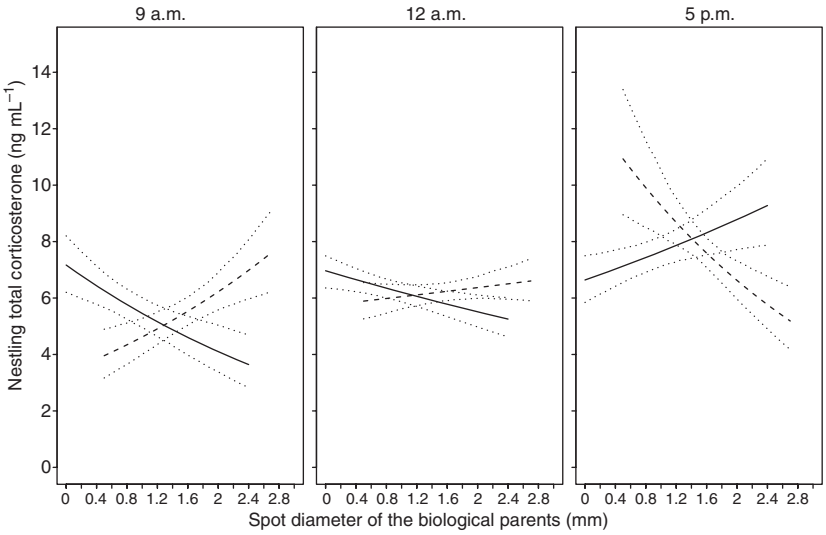

Fig. 2 Predicted baseline corticosterone levels (with 95% confidence intervals) in the barn owl nestlings in relation to spot diameter of the biological father (solid line) and biological mother (dotted line), based on the model presented in Table 2. The panel on the left represents levels at the beginning of the resting phase of the barn owl nestlings (9 AM), the panel in the middle the levels in the middle of the resting phase (12 AM) and the panel on the right the levels shortly before nestlings start to be active again (5 PM). Data were collected in 2004, 2005 and 2006.

pairing. Accordingly, studies in insects and humans demonstrated that stress can affect the mating pattern, with stressed individuals mating disassortatively and nonstressed individuals assortatively (Lopez, 1999; Hingle *et al.*, 2001; Lass-Hannemann *et al.*, 2010). Thus, our observations on the regulation of corticosterone raise new avenues of research on a potential link between mate choice, melanin-based ornaments and glucocorticoids.

Many evolutionary biologists are currently studying sexually antagonistic selection (e.g. Bonduriansky & Chenoweth, 2009). Males and females show different reproductive interests and thus face different selection pressures. This promotes the evolution of intralocus and interlocus sexual conflicts. Males are selected to evolve traits that enhance their reproductive success at the females' expense, and vice-versa. When this sex conflict is exerted on the same trait in the two sexes, this may promote the evolution of sex-specific gene expression to allow each sex to reach its phenotypic optimum as much as possible (Mank, 2009). Although genomic studies have shown that many genes display sex-biased expression (e.g. in 18% of the genes in 18-day chicken embryos) (Mank *et al.*, 2008), we are not aware of any study testing the pattern of covariation between sex-biased gene expression and sexually selected traits. Thus, the identity of the genes and of their products that result from sexually antagonistic selection being exerted on secondary sexual characters are yet unknown. This would require the measurement of gene expression levels or of the levels of gene products in relation to the degree of a sexually selected ornament in the two sexes. Our study provides an attempt to link a sex trait with variation in a key hormone involved in many physiological processes in both sexes. As said earlier, we measured baseline corticosterone levels

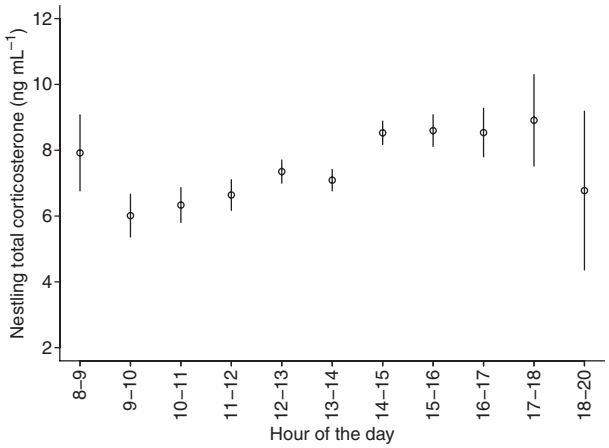

Fig. 3 Baseline corticosterone levels (mean ± SE) in nestling barn owls in relation to hour of the day. Data were collected in 2004, 2005 and 2006.

because of the predicted association between this hormone and melanin-based coloration. The finding that the signs of the covariation between baseline corticosterone levels in nestlings and the size of black spots of the biological mother and of the biological father are different suggest that this colour trait may be sexually antagonistically selected and that corticosterone mediates sex conflict. Accordingly, a recent long-term study showed that selection exerted on the size of black spots is sex dependent with females being strongly positively selected to display large spots, whereas males may be weakly selected to display small spots (Roulin *et al.*, 2010). It would be interesting to test whether sex-dependent selection is partly mediated by corticosterone.

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