

WITHIN-CLUTCH VARIATION IN YOLK ANDROGENS IN RELATION TO FEMALE EXPRESSION OF A MALE ORNAMENT IN PIED FLYCATCHERS *FICEDULA HYPOLEUCA*

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SUMMARY.—*Within-clutch variation in yolk androgens in relation to female expression of a male ornament in pied flycatchers Ficedula hypoleuca.*

Aims: Female birds deposit variable amounts of androgens in their eggs, and research shows that these hormones can influence several aspects of offspring development and phenotype. Some evidence suggests that yolk androgen deposition may be a costly investment for the female bird. We studied the relationship between female expression of a male ornament and yolk androgen deposition in the pied flycatcher *Ficedula hypoleuca*. In this species, some females present a white forehead patch, which is an ornament typically restricted to males. Female expression of this trait has previously been found to be associated with older age and low levels of blood parasites. We predicted that, if yolk androgen deposition is costly, eggs from females displaying a white forehead patch should contain higher concentrations of yolk androgens than those laid by females lacking that ornament.

Location: La Hiruela, central Spain.

Methods: We measured testosterone (T) and androstenedione (A4) levels in 10 pied flycatcher broods, after an ether extraction, following a radioimmunoassay method.

Results and Conclusions: We found that yolk T and A4 concentrations increased with laying order. However, the increase in A4 with laying order was steeper for females without a forehead patch than for those expressing it. Also, and contrary to expectations, we found a marginally significant trend for females with white foreheads to have lower yolk-T levels. We discuss these results with reference to the adaptive function of hatching asynchrony.

Key words: yolk androgens, testosterone, pied flycatcher, hatching asynchrony, female ornamentation.

RESUMEN.—*Variación dentro de la puesta en el contenido de andrógenos en relación a la expresión de un ornamento masculino en las hembras del papamoscas cerrojillo Ficedula hypoleuca.*

Objetivos: Los huevos de las aves contienen andrógenos de origen maternal que influyen en el desarrollo y fenotipo de su descendencia. Dentro de una misma especie, existe una gran variación entre hembras en los niveles de hormonas de sus huevos, lo sugiere un complejo equilibrio entre costes y beneficios. En este artículo estudiamos la relación entre la expresión de un ornamento masculino en las hembras del papamoscas cerrojillo *Ficedula hypoleuca* y el contenido de andrógenos en los huevos. En esta especie, algunas hembras presentan una mancha blanca en la frente, que es un carácter sexual (ornamento) típico de los machos. Estudios previos muestran que la expresión de este rasgo en hembras se encuentra

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asociado a su edad y a niveles bajos de parásitos sanguíneos. Predecimos que, si la inversión de andrógenos en la yema es costosa, los huevos de las hembras que presenten una frente blanca deberían tener niveles más altos de andrógenos que los de las hembras sin la frente blanca.

Localidad: La Hiruela, España central.

Métodos: Medimos los niveles de testosterona (T) y androstenediona (A4) en los huevos de 10 puestas de papamoscas cerrojillos, tras extraer los esteroides con éter, usando métodos de radioinmunoensayo.

Resultados y Conclusiones: Encontramos que los niveles de T y A4 disminuían con el orden de puesta. Sin embargo, el incremento en A4 era más elevado para las hembras que no presentaban una frente blanca que para las hembras que sí la presentaban. Por otro lado, y en contra de las predicciones, encontramos una diferencia marginalmente significativa en el sentido de menores niveles de T en las yemas de las hembras ornamentadas. Discutimos estos resultados con relación a las posibles funciones adaptativas de la sincronía de eclosión.

Palabras clave: andrógenos de la yema, testosterona, papamoscas cerrojillo, asincronía de eclosión, ornamentos femeninos.

INTRODUCTION

Maternal effects are receiving increasing attention as adaptive mechanisms that parents can use to facultative modify the development of their offspring (Mousseau and Fox, 1998). A particularly interesting example of maternal effect is the deposition of androgens in avian egg yolks (Gil, 2003). Evidence in a number of species shows that increased amounts of androgens in the yolk can result in a faster development in the egg, increased begging rates, bolder behavioural phenotypes and larger ornaments (Schwabl, 1996; Eising and Groothuis, 2003; Pilz *et al.*, 2004; Strasser and Schwabl, 2004; Daisley *et al.*, 2005; Eising *et al.*, 2007).

However, increased androgen levels are not always beneficial for offspring, and there is also evidence for dose-dependent responses, increases in mortality and reduced immunity (Sockman and Schwabl, 2000; Navara *et al.*, 2005). Females have been shown to increase their deposition of yolk androgens when paired to attractive males (Gil *et al.*, 1999; Gil *et al.*, 2006b), suggesting that this investment may constitute an example of differential allocation. In agreement with the differential allocation hypothesis (Burley, 1988), several lines of evidence suggest that yolk androgen deposition is costly both for nestlings (Müller *et al.*,

2005) and females (Gil *et al.*, 2004a; Rutkowska *et al.*, 2005; Saino *et al.*, 2007).

In many sexually dimorphic bird species, a minority of females display morphological traits that are usually restricted to male plumage. These traits are often expressed to a smaller degree than in males, and there is large variation between females in their expression. The presence of these ornaments has traditionally been explained as a consequence of a strong genetic correlation between the sexes, but evidence is now accumulating for a role of these ornaments in sexual selection (Amundsen, 2000; Amundsen and Pärn, 2006). For instance, assortative mating with respect to ornamentation suggests the action of a process of mutual mate choice (Møller, 1993; Potti and Merino, 1996; Komdeur *et al.*, 2005). A recent study in the Eastern bluebird *Sialia sialis* has shown condition-dependent plumage colouration in females (Siefferman and Hill, 2005), suggesting that the same processes of honest signalling that apply to male ornamentation could be extended to females in other species. In the pied flycatcher *Ficedula hypoleuca*, some females exhibit white forehead patches similar to those that are sexually selected in males (Potti, 1993). Female expression of this trait is associated with older age and with low levels of trypanosome infection (Potti, 1993; Potti and Merino, 1996), suggest-

ing its possible role in signalling quality in this species, although no experimental study has been conducted to explore whether this trait actually serves a signalling function in females.

Given the link between yolk androgen and sexual selection, we set out to investigate the differences in yolk androgens in relationship to the presence of a white forehead in female pied flycatchers in a Spanish population, in a largely exploratory manner. We predicted that, if egg yolk androgen is costly to females, female pied flycatchers expressing a white forehead patch should deposit higher amounts of yolk androgens than females not expressing this trait.

We also examined the distribution of yolk androgens with respect to the laying sequence, since different patterns can modify the effects of hatching asynchrony on offspring growth (Schwabl *et al.*, 1997): a pattern of increasing yolk androgen concentration should minimise the effects of hatching asynchrony while a decreasing pattern would have the opposite effect. Given the conflicting predictions of the numerous hypotheses proposed to explain hatching asynchrony (Viñuela, 2000), it is not straightforward to predict the differences that females with and without a white forehead should present in the distribution of yolk androgens within a clutch. If the effects of hatching asynchrony are used by parents to ease brood reduction or to reduce rearing costs at the expense of late-hatched offspring fitness (Lack, 1954; Hussell, 1972), we expect females with a white forehead to present a distribution of androgens that would increase with laying order, whereas those without the white forehead should show a flat or a descending pattern. This is because older females (those more likely to express white foreheads) are less expected to require brood reduction than younger females, and such a pattern should allow late hatching chicks to catch up in growth. However, if producing broods with large size differences among nestlings is advantageous for the offspring but costly for parents

(Slagsvold and Lifjeld, 1989), we expect females presenting a white forehead to present a flat distribution of androgens with respect to laying order, since such a pattern would reinforce the effects of hatching asynchrony. On the contrary, those females without white foreheads should present an increasing pattern of androgens along the laying order.

Finally, if the effects of hatching asynchrony are modulated by female quality, we would expect older, higher quality females to be able to raise a full brood without requiring androgen compensation. In this case we would expect females with a white forehead to present no variation in androgen levels across laying, whereas females without a white forehead should increase androgen levels with laying order. These predictions are based on the assumption of no differences between females in timing of incubation in relation to presence of a white forehead, previously ascertained in the population of study (J. Potti, *unpub. data*).

MATERIAL AND METHODS

The work was made in 2000 in a long-term studied population of pied flycatchers breeding in nestboxes in La Hiruela, central Spain (for details see: Potti and Merino, 1996; Potti, 1998). Nests were visited daily during laying time in order to mark freshly laid eggs. Eggs from 10 clutches (each comprising 6 eggs) were collected one day after the clutch was completed, after capturing, ringing and measuring incubating females. Laying order data was available for 8 of these clutches. The presence or absence of a white forehead patch was recorded (Potti, 1993).

Androgen assays

Yolks were dissected from the eggs and placed in a test tube where they were homogenised by hand with a small spatula. A small sample of

yolk (ca. 50 mg) was taken from this tube and steroids extracted following a standard ether method (Gil *et al.*, 2004b). Extraction recoveries were calculated on a small sample of yolk by adding 1000 CPM of tritiated hormones, and estimated to be > 90 % for both T and A4. Given this high recovery rate, we considered unnecessary to correct for sample-specific recovery rates. Assays were conducted at the Centre d'Etudes Biologiques de Chizé (CBC), using antibodies from P.A.R.I.S. laboratories (France). Briefly, samples were incubated overnight at 4 °C with ca. 6000 CPM of ³H labelled hormone and a specific antibody. Bound and free fractions were separated by dextran-coated charcoal and centrifuged. A Packard 1600 liquid scintillation counter was used to count activity of the bound fractions. Cross-reactivity of T antibody was estimated to be lower than 2 % for all androgens tested, except 5 α -DHT (27 %) and A4 (1.6 %). Cross-reactivity of A4 antibody was lower than 1 % for all androgens tested. All samples were assayed in duplicate. Intra-assay coefficients of variation were 5.2 and 13.6 for T and A4, respectively. Inter-assay coefficients of variation were 6.9 and 17.3 for T and A4.

Statistical analyses

Androgen concentrations were log transformed to approximate normal distributions. Data were analysed using a mixed model procedure in SAS (Proc Mixed). Since there are large differences between nests in yolk androgen levels, nest identity was fitted as a random factor in all models. Degrees of freedom were calculated using the Satterthwaite correction (Littell *et al.*, 1996). Residuals from the models were checked for normality throughout.

RESULTS

Yolks contained an average (SE) concentration of 6.04 (0.25) pg/mg of T and 17.05 (0.98)

pg/mg of A4. The concentrations of the two androgens were highly correlated with each other (Pearson correlation: $r = 0.829$, $n = 59$, $P < 0.001$). There were large differences between nests in androgen concentration (T: $F_{9,49} = 8.09$, $P < 0.001$; A4: $F_{9,49} = 13.18$, $P < 0.001$).

We ran random models for each androgen to test whether females with and without a white forehead differed in the levels of yolk androgen in their eggs, including nest identity as a random factor to account for within-nest similarity and avoid pseudoreplication. Since yolk androgen is often related to egg laying order, we included laying order as a covariate as well as its interaction with presence of a white forehead. T concentration was found to increase with laying order ($F_{1,37.8} = 8.26$, $P = 0.006$; estimate (SE) = 0.35 (0.12); Fig. 1), and there was a marginally significant trend for females expressing white forehead patches to have smaller concentrations than those without ($F_{1,5.78} = 4.23$, $P = 0.08$; least squares means (SE) of females without white forehead: 7.27 (0.65) vs. females with white foreheads: 5.57 (0.50)). A4 similarly increased with laying order ($F_{1,37} = 12.71$, $P = 0.001$; estimate (SE) = 1.47 (0.41)) and, although there were no differences between females with and without white foreheads ($F_{1,11} = 0.17$, $P = 0.68$), the interaction term between female ornamentation and laying order was significant ($F_{1,37} = 5.66$, $P = 0.026$). The nature of the interaction can be appreciated in Figure 2: the increase of A4 with laying order is steeper for females without a white forehead than with females with it.

DISCUSSION

Several studies have shown that avian nestlings can acquire growing and developmental benefits from high yolk androgen concentration (Schwabl, 1996; Lipar and Ketterson, 2000; Eising *et al.*, 2001), and that these higher concentrations may induce higher im-

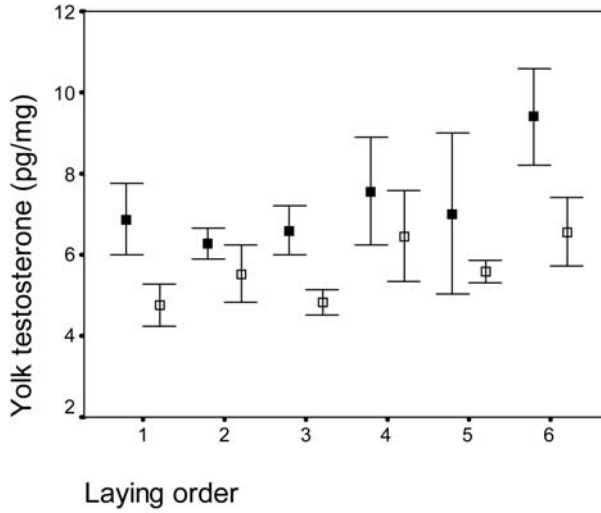


FIG. 1.—Distribution of testosterone (mean \pm SE) along the laying order for female pied flycatchers with (white squares) and without (black squares) a white forehead patch.

[Distribución de la cantidad de testosterona (media \pm SE) en relación al orden de puesta en hembras de papamoscas cerrojillo con (cuadrados blancos) y sin (cuadrados negros) la marcha blanca frontal.]

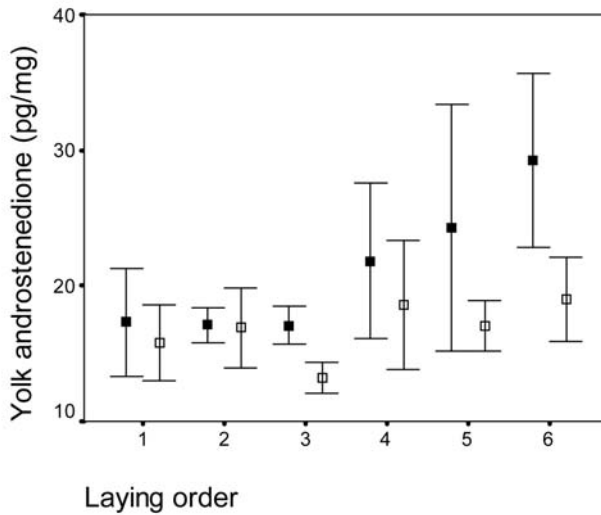


FIG. 2.—Distribution of androstenedione (mean \pm 1 SE) along the laying order for females with (white squares) and without (black squares) a white forehead patch.

[Distribución de la cantidad de androstenediona (media \pm SE) en relación al orden de puesta en hembras de papamoscas cerrojillo con (cuadrados blancos) y sin (cuadrados negros) la marcha blanca frontal.]

mune costs in nestlings (Müller *et al.*, 2005). Additional evidence for costs of high yolk androgen levels for nestlings or females, is suggested by decreases in yolk androgen in experimentally infested great tit *Parus major* nests (Tschirren *et al.*, 2004), in female zebra finches *Taeniopygia guttata* raised in large brood sizes (Gil *et al.*, 2004a), and in female house martins *Delichon urbicum* exposed to an immune challenge (Gil *et al.*, 2006a). Furthermore, older females have been shown to deposit higher levels of androgens in the European starling *Sturnus vulgaris* (Pilz *et al.*, 2003).

Since pied flycatcher females that show a white forehead are older and suffer less from parasites than non ornamented females (Potti and Merino, 1996), we expected a positive covariance between yolk androgen levels and female ornamentation. Contrary to expectations, androgen yolk concentrations in eggs of ornamented female pied flycatchers were not higher than in those of unornamented females. Rather on the contrary, we found a non significant trend for T in the opposite direction.

Although our findings are in contradiction to the mainstream prediction as outlined above, they provide additional evidence suggesting that benefits of high androgens may not be as general as originally thought. For instance, a study in the lesser black backed gull *Larus fuscus* has shown that females fed with food supplements did not lay eggs with higher amounts of androgens (Verboven *et al.*, 2003), as it would be expected if this was a costly resource. In the same line, the effects of increased yolk androgen levels do not always result in enhanced growth. Evidence of detrimental effects (Sockman and Schwabl, 2000), dose dependent responses (Navara *et al.*, 2005), sex-specific differences in androgen optima (Saino *et al.*, 2006; von Engelhardt *et al.*, 2006) and benefits limited to periods of food restriction (Pilz *et al.*, 2004) strongly suggest that yolk androgens are complex modifiers of phenotypes, and that the balance of benefits and costs may depend on parental and offspring phenotypes. Future re-

search should explore this field, analysing the long term effects of yolk androgens as a function of individual quality.

We also found that females that expressed a white forehead presented a flatter, more egalitarian distribution of A4 along the laying order than females without that trait. Since nestlings from eggs of the last positions of the laying order typically hatch later than their siblings in this population (Potti, 1998), such a distribution of androgens would imply that offspring of females with a white forehead hatching from these late laying positions would be at a greater disadvantage than that of females without the white forehead. In short, this would enhance size differences among nestlings created by an early start of incubation. The fact that females with a white forehead presented this flat distribution of androgens in this population is in agreement with a hypothesis of hatching asynchrony that posits that nestlings from asynchronous broods develop better, and that feeding asynchronous broods is more costly to parents (Slagsvold and Lifjeld, 1989). Alternatively, if the negative effect of hatching asynchrony for late nestlings is stronger for low quality females, it could be argued that females without a white forehead need to compensate these effects by means of yolk androgens, whereas ornamented females would be able to raise a full clutch without the need of compensation. However, previous evidence in the same population of the present study has failed to identify any benefits of hatching asynchrony despite a large sample size (Potti, 1998) hence further discussion is unwarranted until more data are available.

An alternative explanation for differences in androgen distribution along the laying order is that ornamented females may differ from non ornamented females in patterns of hatching asynchrony and, thus, differences in yolk androgen levels may be confounded by differences in incubation schedule. However, unpublished data for this population show that female expression of a white forehead patch is unrelated to hatching asynchrony (J. Potti,

unpub. data), suggesting that this hypothesis does not hold.

An additional possibility is that differences in yolk androgens levels in relation to female ornamentation might be an epiphenomenon of proximal mechanisms of plumage determination. Although several physiological mechanisms have been found to control sexual colour dimorphism in birds, male coloration in the Passeriformes seems to be generally dependent on high levels of luteinizing hormone (Kimball and Ligon, 1999). It is to be expected that female expression of a male trait would be based on a male-like modification of the typical female endocrine milieu. We believe that future research on the proximate basis of this trait in the pied flycatcher should examine a proximal link with yolk androgen deposition.

Although a good deal of research has accumulated in the last decade on the costs and benefits of yolk androgen in birds (for reviews see Gil, 2003; Groothuis *et al.*, 2005), we are still far from a good understanding of this maternal effect. Paradoxical results such as ours add to this complex picture and call for studies that take individual condition into account (McNamara and Houston, 1996).

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