Manipulation of male attractiveness induces rapid changes in avian maternal yolk androgen deposition

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Avian eggs contain maternal androgens that may adjust offspring development to environmental conditions. We review evidence and functional explanations for the relationship between androgen concentrations in avian eggs and male attractiveness. Experimental studies in captive birds show generally positive relationships, but results from correlational and experimental field studies are less consistent, perhaps because they lack a within-female design to control for confounding between-female variation. We analyzed the effect of male attractiveness on yolk levels of maternal androgens in a wild bird, using a correlational and experimental approach with a within-female design. We manipulated the sexually selected UV coloration of the crown feathers of male blue tits (Cyanistes caeruleus) after their female had laid the second egg and measured the subsequent effect on androgen concentrations (testosterone and androstenedione) in the fifth, seventh, and ninth eggs relative to that in the second egg. Levels of testosterone, but not androstenedione, in eggs 5 and 7 were higher for control (attractive) than for UV-reduced (unattractive) males. This effect disappeared in the ninth egg, coinciding with the recovery of UV coloration after manipulation. This suggests that females are capable of rapid adjustments of testosterone deposition in response to changes in their mate's ornamental plumage. However, androgen concentrations in the second egg and pretreatment male crown coloration were not correlated.

Possibly, the combination of relatively small variation in UV coloration before treatment and the influence of unknown confounding variables in the correlational approach resulted in insufficient statistical power to detect such a correlation. Key words: blue tit Cyanistes (formerly Parus) caeruleus, differential allocation, male attractiveness, maternal effects, testosterone, UV coloration, yolk hormones. [Behav Ecol 20:172–179 (2009)]

Maternal effects may represent adaptive transgenerational phenotypic plasticity allowing organisms to optimally adjust offspring phenotype to the environment they are encountering (Mousseau and Fox 1998). Over the last decade, maternally derived hormones in the yolk of avian eggs—in particular androgens—have started to attract much attention as potential mediators of such adaptive maternal effects. Avian mothers deposit variable amounts of androgens in the yolk of their eggs that can have important effects on offspring development (reviewed in Groothuis, Müller, et al. 2005), including begging behavior, the size of the neck muscle, immune function, hatching time, growth, and survival (e.g., Schwabl 1996; Sockman and Schwabl 2000; Eising et al. 2001, 2003; Eising and Groothuis 2003; Pilz et al. 2003; Groothuis, Eising, et al. 2005; Müller et al. 2005).

Although most studies have concentrated on explaining within-clutch variation in yolk androgen concentrations, which may be related to female strategies of brood reduction or compensation of hatching asynchrony, between-clutch variation is often even larger (Reed and Vleck 2001; Groothuis, Müller, et al. 2005). One of the factors that have been indicated to induce this variation is the sexual attractiveness of a female’s mate (Gil et al. 1999). This has been interpreted as a form of increased maternal investment in offspring of which the mother will receive higher fitness returns, as predicted by the differential allocation hypothesis (Burley 1988; Sheldon 2000). Alternatively, females may, by differential hormone allocation, manipulate paternal investment in their offspring (Groothuis, Müller, et al. 2005; Moreno-Rueda 2007; Müller et al. 2007).

Laboratory-based studies have demonstrated that females increase the deposition of androgens to the yolk of their eggs when paired with a more attractive male (see Table 1). In captive zebra finches (Taeniopygia guttata), females paired with males wearing “attractive” red leg rings deposited higher concentrations of yolk androgens than females with males wearing “unattractive” green rings (Gil et al. 1999; but see Rutstein et al. 2004). Captive zebra finch females also deposited higher levels of yolk androgens when paired to a mate that was found to be preferred in a preceding mate choice trial, an effect that was limited to eggs later in the laying sequence (von Engelhardt 2004). Furthermore, in captive canaries (Serinus canaria), females deposited higher levels of androgens in their eggs when exposed to more attractive male song (Gil et al. 2004; Tanvez et al. 2004; but see Marshall et al. 2005). Also in captive peafowl (Pavo cristatus), eggs laid by females assigned to more attractive male mates, with a higher tail eyespot density, contained higher levels of androgens (Loyau et al. 2007).

In contrast to the largely consistent results of laboratory-based studies, in wild bird populations, the overall direction of the relationship between yolk androgen levels and male attractiveness appears less straightforward (Table 1). Experimentally manipulated length of the sexually selected tail streamers of barn swallows had a positive effect on maternal...
Table 1
Overview of studies on the effect of mate attractiveness on yolk androgen deposition, including the type of androgens investigated (testosterone [T], androstenedione [A4], dihydrotestosterone [DHT], and oestradiol [E]) and the relationships found between androgen levels and male attractiveness

<table>
<thead>
<tr>
<th>Species</th>
<th>Male characteristic</th>
<th>Effect</th>
<th>Androgens</th>
<th>Corr/Exp</th>
<th>Captive/wild</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canary Serinus canaria</td>
<td>Song quality</td>
<td>Positive</td>
<td>T and DHT (total)</td>
<td>Exp</td>
<td>Captive</td>
<td>Gil et al. (2004)</td>
</tr>
<tr>
<td>Zebra finch</td>
<td>Ring color</td>
<td>Positive</td>
<td>T</td>
<td>Exp</td>
<td>Captive</td>
<td>Loyau et al. (2007)</td>
</tr>
<tr>
<td>Zebra finch</td>
<td>Ring color</td>
<td>Zero</td>
<td>T</td>
<td>Exp</td>
<td>Captive</td>
<td>Gil et al. (1999)</td>
</tr>
<tr>
<td>Barn swallow Hirundo rustica</td>
<td>Tail length</td>
<td>Positive</td>
<td>A4</td>
<td>Exp</td>
<td>Wild</td>
<td>Saino et al. (2006)</td>
</tr>
<tr>
<td>Barn swallow</td>
<td>Tail length</td>
<td>Zero</td>
<td>A4</td>
<td>Exp</td>
<td>Wild</td>
<td>Safran et al. (2008)</td>
</tr>
<tr>
<td>Barn swallow</td>
<td>Tail length</td>
<td>Zero</td>
<td>A4</td>
<td>Exp</td>
<td>Wild</td>
<td>Michl et al. (2005)</td>
</tr>
<tr>
<td>Collared flycatcher Ficedula albicilla</td>
<td>Plume coloration</td>
<td>Positive</td>
<td>A4</td>
<td>Exp</td>
<td>Wild</td>
<td>Navara et al. (2006)</td>
</tr>
<tr>
<td>House finch</td>
<td>Plume coloration</td>
<td>Negative</td>
<td>T, A4, and DHT (total)</td>
<td>Exp</td>
<td>Wild</td>
<td>Navara et al. (2006)</td>
</tr>
<tr>
<td>House sparrow Passer domesticus</td>
<td>Testosterone level</td>
<td>Zero</td>
<td>T</td>
<td>Exp</td>
<td>Wild</td>
<td>Mazuc et al. (2003)</td>
</tr>
</tbody>
</table>

It is indicated whether studies were correlational (Corr) or experimental (Exp) and whether studies were conducted in wild or captive birds.

* There was no overall effect of male mating status (monogamous vs. polygynous) on mean yolk androgen (T, A4, DHT, and E) concentrations in European starlings, but the within-clutch pattern of A4 depended on mating status.
able to quickly adjust yolk androgen levels to male attractiveness, we expected an increase in these levels from the second egg, laid before treatment, to the fifth and seventh eggs. This is because the rapid yolkling phase during which hormones accumulate in the yolk takes in songbirds between 2.5 and 4 days (Badyaev et al. 2005), after which ovulation and egg laying takes about 1 day. Therefore, eggs laid in a shorter interval than 3 days after the start of treatment are not expected to show an increase in yolk androgen concentrations. Due to the waning of the effect of treatment (Korsten, Limbourg, et al. 2007), later laid eggs might be less affected in their hormone levels. Androgen concentrations in the second egg were used to obtain a baseline measurement for the 2 treatment groups and were correlated to premanipulation of natural crown coloration of males.

**MATERIALS AND METHODS**

**Study area, bird handling, and sample sizes**

The experiment was carried out in the breeding season of 2005 (7 April to 5 June) in a population of blue tits breeding in nest-boxes at “De Vosbergen” estate (ca., 50 ha; 53°08′ N, 06°35′ E), near Groningen, The Netherlands. This population has been intensively studied since 2001. The study area consists of patches of mixed deciduous and coniferous forest interspersed by open grassland. Nest-boxes were checked daily for presence of the first-laid egg.

Males (n = 36) were caught in front of occupied nest-boxes on the day the second egg had been laid, using a mist net and a decoy (a mounted male blue tit) with song playback. Males were subsequently transported in a dark bird bag to the measuring probe was held at a right

**Crown reflectance measurements**

Before the manipulation of the crown UV reflectance, the spectral reflectance of the crown feathers was measured with an USB-2000 spectrophotometer with illumination by a DH-2000 deuterium–halogen light source (both Avantes, Eerbeek, The Netherlands). The measuring probe was held at a right angle against the plumage, that is, both illumination and recording were at 90° to the feathers. During each crown reflectance measurement, we took 5 replicate readings of the same spot and smoothed each of these reflectance spectra by calculating the running mean over 10-nm intervals. Following previous studies of UV color signaling in blue tits (Andersson et al. 1998; Sheldon et al. 1999; Delhey et al. 2003; Griffith et al. 2003; Korsten et al. 2006), we calculated 3 indices describing the variation in crown coloration—“brightness,” “hue,” and “UV chroma”—from each reflectance spectrum and averaged these across the 5 replicate spectra. Brightness was the sum of reflectance between 320 and 700 nm (R_{320–700}), which corresponds to the spectral range visible to blue tits (Hart et al. 2000). Hue was the wavelength of maximum reflectance (R_{max}). UV chroma was the sum of reflectance between 320 and 400 nm divided by the sum of reflectance between 320 and 700 nm (R_{320–400}/R_{320–700}). Both the hue and the UV chroma indices have previously been identified as important predictors of male attractiveness and viability in blue tits (Andersson et al. 1998; Sheldon et al. 1999; Delhey et al. 2003; Griffith et al. 2003). For repeatabilities (varying between 0.50 and 0.75), see Korsten, Vedder, et al. 2007. In one of the 33 males included, the measurement of natural crown reflectance before the manipulation failed, leading to a sample of 32 males of which natural UV reflectance was measured.

The capturing of males and females for crown reflectance measurements took place within relatively short periods (10–21 April and 13 May to 23 June for males and females, respectively) leading to little variation in crown feather wear (see Ørnborg et al. 2002; Delhey et al. 2006), and consequently, crown coloration was not significantly related to the date of

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Pretreatment characteristics of UV-reduced and control-treated male blue tits</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>UV reduced (n = 17)</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Capture date (April, days)</td>
<td>16.35</td>
</tr>
<tr>
<td>Body mass (g)</td>
<td>11.28</td>
</tr>
<tr>
<td>Tarsus length (mm)</td>
<td>16.99</td>
</tr>
<tr>
<td>Brightness</td>
<td>75.86</td>
</tr>
<tr>
<td>Hue (nm)</td>
<td>386.2</td>
</tr>
<tr>
<td>UV chroma</td>
<td>0.290</td>
</tr>
</tbody>
</table>

*a* Pretreatment crown coloration was measured for 15 males only in the control-treated group.
capture in either males (brightness: $r = -0.253$, $P = 0.16$; hue: $r = 0.319$, $P = 0.08$; UV chroma: $r = -0.250$, $P = 0.17$; all $n = 32$) or females (brightness: $r = 0.004$, $P = 0.98$; hue: $r = 0.193$, $P = 0.31$; UV chroma: $r = -0.221$, $P = 0.24$; all $n = 30$).

**Crown UV manipulation**

UV reflectance was reduced with a mixture of duck preen gland fat and UV blocking chemicals (50% Parsol 1789 and 50% Parsol MCX [by volume]; Roche, Basel, Switzerland) as used successfully in previous studies of wild blue tits (e.g., Sheldon et al. 1999; Limbourg et al. 2004; Korsten et al. 2006). Control males were treated with the duck preen gland fat only. This treatment was smeared on the crown feathers and to measure its effect, 3 replicate crown reflectance measures were taken directly after the manipulation following the protocol described above. Males were assigned sequentially to either the UV-reduced or the control treatment.

**Egg collection**

Nest-boxes were visited daily, and newly laid eggs were marked with nontoxic markers until the last egg was laid and clutch size was determined. We collected eggs 2, 5, 7, and 9 from each brood, on the day they were laid. Collected eggs were replaced was determined. We collected eggs 2, 5, 7, and 9 from each clutch. This treatment was smeared on the crown feathers and to measure its effect, 3 replicate crown reflectance measures were taken directly after the manipulation following the protocol described above. Males were assigned sequentially to either the UV-reduced or the control treatment.

However, for unknown reasons, incubation failed and eggs contained no embryos, so that eggs could not be sexed. After incubation, eggs were stored at $-20^\circ C$ until androgen analyses were conducted.

The UV-reduced and control groups did not differ in egg mass of egg 2, laid before treatment (egg mass, mean ± standard error [SE]: 1.08 ± 0.02 and 1.09 ± 0.02 g, respectively, $P = 0.6$), or in yolk mass of egg 2 (mean ± SE: 187.6 ± 17.9 and 178.7 ± 13.3 mg, respectively, $P = 0.7$).

**Androgen quantification**

Androgens (testosterone [T] and androstenedione [A4]) were measured by radioimmunoassay (RIA) after extracting them from the yolk first with ether and then with ethylacetate and isooctane on celite columns (Wingfield and Farner 1975; Schwabl 1993). The whole yolk was removed from the eggs when still frozen and weighed to the nearest 0.001 g using an analytical balance. We then homogenized the yolk in 200 mL of Tris buffer. T and A4 levels were measured in duplicate in 200 mL of Tris buffer. T and A4 levels were measured in duplicates of 50 mL of sample using (Diagnostic System Laboratories) RIA kits.

Due to a laboratory accident (affecting samples randomly), we could not quantify T concentrations for 1 ninth egg in the UV-reduced group, and in 1 control clutch, we could not collect the ninth egg. This makes a total sample size of 130 eggs for T analyses. Due to the same accident, we could not determine A4 levels in 4 second eggs, 2 in each experimental group, as well as in eggs 5, 7, and 9 of 1 clutch of the UV-reduced group. Because we used hormone concentrations in second eggs to correct for basal levels in the eggs, laid after treatment, we had to disregard all these 5 clutches from further data analyses. This left us with 112 eggs of 28 clutches (14 clutches in each group). Due to the same accident and incomplete clutch, we could not determine A4 levels in 1 seventh and 1 ninth egg in each group (all of different clutches), leaving a total of 108 eggs for the analyses of A4 concentrations.

The intra- and interassay coefficients of variation for the 7 T assays were 9.7% and 11.9%, respectively. Those for the 4 A4 assays were 2.0% and 6.6%, respectively.

**Statistical analyses**

To test the effect of UV treatment on yolk testosterone levels, we used a multilevel model that included a random effect for female identity to account for the nonindependence of the eggs produced by a single female. Data on hormone concentrations were log transformed to achieve normality. Because we were interested in the effect of treatment on the change in yolk androgen concentrations between egg 2 (not affected by treatment) and the subsequent eggs (laid after start of treatment), we used hormone concentrations of egg 2 as a covariate in the analyses of the effect of treatment on hormone levels in eggs 5, 7, and 9. This method is the best among several possible approaches for detecting unbiased changes from baseline (Senn 2006). The covariate did not significantly interact with treatment in any of the models.

We expected that the effect of treatment may increase with a longer time interval after the start of treatment (from egg 5 to egg 7), but may decrease thereafter, due to the potentially diminishing treatment effect due to recovery of the UV reflectance with time after application of the UV-reduction treatment (Korsten, Limbourg, et al. 2007). Therefore, we also tested the effects of laying sequence (eggs 5, 7, and 9) and of the interaction of UV treatment and laying sequence on the relative change of yolk androgen concentration. Significance was assessed using the increase in deviance ($\Delta$ deviance, which follows a $\chi^2$ distribution) when a parameter was removed from the model.

Because egg 2 could not be affected by male UV manipulation, we correlated androgen concentrations of egg 2 to natural male crown coloration as measured before manipulation to investigate the natural relationship between yolk androgen concentrations and male crown coloration. To account for potentially confounding factors, we additionally ran 2 separate multiple regression analyses using a stepwise backward selection procedure to explain the variation in yolk concentrations of T and A4. In the first set of models, we entered the 3 male crown color indices (brightness, hue, and UV chroma) together with male age, body mass, and tarsus length as predictors of either T ($n = 32$) or A4 ($n = 28$). In the second set of models, we entered the 3 male crown color indices as well as female characteristics—female crown color indices (brightness, hue, and UV chroma), age, body mass,
As expected, there was a significant effect of the interaction of UV treatment × position in laying sequence on T concentrations (Δ deviance = 4.6, df = 1, P = 0.03; Figure 2a). After treatment, yolk testosterone concentrations were higher in the control group than in the UV-reduced group, and this difference subsequently decreased over the laying sequence. After removing the interaction effect, the overall effects of treatment and egg position were not significant (Δ deviance = 3.1 and 0.1, respectively, P = 0.08 and 0.8, respectively). Post hoc tests revealed that the effect of treatment was significant for egg 7 (Δ deviance = 4.4, P = 0.04) but not for egg 5 (Δ deviance = 2.4, P = 0.1) or egg 9 (Δ deviance = 1.4, P = 0.2).

For A4, the pattern was less clear, and the interaction effect of treatment and egg number did not reach statistical significance (Δ deviance = 3.0, P = 0.08) and neither did egg number nor treatment alone (Δ deviance < 2.3, P > 0.1; Figure 2b).

The same model was used to test the effect of treatment, egg position, and their interaction effects on egg mass. Except for the covariate (egg mass egg 2: [Δ deviance = 5.0, P = 0.03]), none of the predictors yielded significant results (Δ deviance < 2.8, P > 0.1).

Yolk concentrations of T and A4 measured in the second egg before application of treatment did not correlate with natural male crown reflectance before manipulation (T: brightness: r = 0.103, P = 0.58; hue: r = −0.036, P = 0.84; UV chroma: 0.03).
DISCUSSION

One of the most frequently cited factors that may explain variation in androgen concentrations among clutches is male attractiveness. However, results of field studies are much more inconsistent than those of laboratory-based studies (Table 1). Therefore, we performed a field study using a new and more sensitive within-female design, in which we manipulated male attractiveness after the second egg was laid, and determined the relative change in androgen concentrations of subsequently laid eggs. We found that wild female blue tits quickly changed the deposition of testosterone in the yolk of their eggs in response to manipulation of male crown UV coloration, a sexually selected trait. This was not the case for androstenedione, the precursor of testosterone that has a much lower affinity to the androgen receptor than testosterone itself or its metabolite dihydrotestosterone (Sonneveld et al. 2005, 2006).

Relative to the second egg, concentrations of testosterone were lower in the subsequent eggs in clutches of UV-reduced—unattractive—males compared with males that received a control treatment. This effect diminished over the laying sequence and had disappeared in the ninth egg (Figure 2). Most likely, the diminishing effect of treatment was due to a rapid female response to recovery of male crown coloration after a few days in UV-reduced males (Korsten, Limbourg, et al. 2007). Alternatively, in the days after treatment of their mates, females learned that important aspects of male quality other than UV reflectance (those that would normally correlate with UV reflectance) were not affected. The functional explanation for the existence of cues that signal mate quality hinges on the assumption that it is easier/less costly for females to assess quality by such a cue than by trying to determine all separate aspects of a mate’s quality. Therefore, it may well be that when the covariance between signal and mate quality is disrupted due to experimental manipulation of the cue, a female only gradually discovers the lack of correlation between both and will discard the information from the cue.

Several adaptive explanations have been postulated for the finding that avian females produce eggs with elevated androgen concentrations when mated with attractive males. The differential allocation hypothesis (e.g., Gil et al. 1999, 2004, 2006, see in the Introduction) remains speculative because its underlying assumption that androgen allocation is costly to the female remains as yet unsupported (Groothuis, Müller, et al. 2005; Navara et al. 2006; Groothuis and Schwabl 2008). Alternatively, eggs of females with attractive mates may contain more androgens because such females overproduce sons, and eggs bearing male embryos contain relatively high levels of maternal androgens. We view this explanation as unlikely because an overall effect of crown UV manipulation on sex ratio in the blue tit could not be demonstrated (Sheldon et al. 1999; Korsten et al. 2006; Delhez et al. 2007b).

A third explanation for elevated concentrations of maternal androgens in eggs of females with attractive males is that only young sired by high-quality fathers can benefit from these elevated levels because only they are able to withstand the costs of elevated exposure to testosterone (Gil et al. 1999), such as on immune function (Groothuis, Eising, et al. 2005; Groothuis, Müller, et al. 2005; Müller et al. 2005). However, the negative correlation between mate attractiveness and yolk levels of androgens in the house finch (Navara et al. 2006) does not support this hypothesis either.

Finally, females may change yolk hormone deposition in relation to male attractiveness in order to adjust offspring begging behavior to the parental care they expect from their mate (Groothuis, Müller, et al. 2005; Navara et al. 2006; Moreno-Rueda 2007; Müller et al. 2007) if male food provisioning is correlated with his attractiveness (either positively or negatively). Consistent with this idea, female house finches (C. mexicanus) deposit higher levels of androgens in their eggs when paired to unattractive males (Navara et al. 2006), which show reduced nestling feeding (Hill 1991). Unfortunately, the evidence for the relationship between male attractiveness, feeding rate, yolk androgen concentration, and begging behavior of the offspring is not yet analyzed in one and the same species and remains therefore indirect at best. Furthermore, the positive effect of male attractiveness on yolk androgen levels in peafowl, a species without paternal care, does not support this hypothesis. Clearly, the functional explanation for the relationship between male attractiveness and yolk androgen concentrations is not as clear as has been suggested in the literature. Perhaps, the relationship between both factors in different species requires different functional explanations.

Although we found a significant effect of the male UV manipulation on yolk testosterone levels, there was no significant correlation between androgen levels in the second baseline egg and male pretreatment coloration. The inclusion of additional male characteristics (age, body mass, and tarsus) as explanatory variables in a multiple regression analysis, which accounted for the possibility that female androgen deposition was dependent on multiple male cues, did not reveal any significant effect either; nor did the inclusion of female characteristics (age, body mass, tarsus length, and laying date). There are several possible explanations for this discrepancy. First, taking only the second egg as an indicator of yolk hormone levels of the complete clutch may be inaccurate. Indeed, several studies found an effect of male quality on yolk hormone deposition only in interaction with the position of the egg in the laying sequence (von Engelhardt 2004, Gilbert et al. 2005; Gwinner and Schwabl 2005). Second, in the natural situation, blue tit females may only modify yolk androgen deposition if the UV reflectance of the crown plumage of their mate shows a sudden and dramatic decrease. Because UV reflectance is due to structural properties of the feathers that are subject to abrasion and tear and wear (e.g., Delhey et al. 2006), a decrease in UV reflectance can perhaps be caused by disease or damage due to severe fighting. Third, the UV reduction caused by our treatment was very large (UV chroma, mean ± SD, UV reduced: 0.180 ± 0.016; control: 0.288 ± 0.015) compared with the natural variation in male UV reflectance (UV chroma, mean ± SD: 0.290 ± 0.017) that potentially affected androgen levels of egg 2. The relatively small variation in natural male UV reflectance compared with the experimentally induced UV reduction probably leads to reduced statistical power to detect an effect of natural male UV coloration. In addition, unknown confounding variables, which we did not control for in our analyses, may have further decreased the likelihood of detecting an effect of natural variation in male attractiveness on female hormone deposition. This third scenario may be a general issue in field studies on maternal yolk androgens.
leading to the less consistent overall pattern as well as the higher incidence of negative results compared with laboratory studies (see Table 1). Correlative studies with larger sample sizes under standardized laboratory conditions, taking eggs of different laying order into account, and experimental studies analyzing dose-dependent effects of the manipulation of male traits may help to solve the discrepancy between the experimental and correlative studies. In any case, the results of our experimental field study clearly indicate that concentrations of maternal androgens in avian eggs can change surprisingly quickly in reaction to a change in male characteristics.

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