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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 correlative 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

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Table 1

Overview of studies on the effect of mate attractiveness on yolk androgen deposition, including the type of androgens investigated (testosterone [T], androstenedione [A_4], dihydrotestosterone [DHT], and oestradiol [E]) and the relationships found between androgen levels and male attractiveness

Species	Male characteristic	Effect	Androgens	Corr/Exp	Captive/wild	Reference
Canary *Serinus canaria*	Song quality	Positive	T	Exp	Captive	Gil et al. (2004)
Canary	Song quality	Positive	T and DHT (total)	Exp	Captive	Tanvez et al. (2004)
Canary	Song quality	Zero	T, A_4, DHT, and E	Exp	Captive	Marshall et al. (2005)
Peafowl *Pavo cristatus*	Tail eyespot density	Positive	T	Exp	Captive	Loyau et al. (2007)
Zebra finch *Taeniopygia guttata*	Ring color	Positive	T	Exp	Captive	Gil et al. (1999)
Zebra finch	Ring color	Zero	T	Exp	Captive	Rutstein et al. (2004)
Zebra finch	Preferred as mate by females	Positive	Total androgen level	Exp	Captive	von Engelhardt (2004)
Barn swallow *Hirundo rustica*	Tail length	Positive	A_4	Exp	Wild	Gil et al. (2006)
Barn swallow	Tail length	Zero	A_4	Exp	Wild	Saino et al. (2006)
Barn swallow	Tail length	Zero	A_4	Corr	Wild	Safran et al. (2008)
	Plumage coloration	Positive	A_4	Corr	Wild	
Collared flycatcher *Ficedula albicollis*	Forehead patch	Zero	T	Corr	Wild	Michl et al. (2005)
European starling *Sturnus vulgaris*	Mating status	Zero[a]	T, A_4, DHT, and E	Corr	Wild	Gwinner and Schwabl (2005)
House finch *Carpodacus mexicanus*	Plumage coloration	Negative	T, A_4, and DHT (total)	Corr	Wild	Navara et al. (2006)
House sparrow *Passer domesticus*	Testosterone level	Zero	T	Exp	Wild	Mazuc et al. (2003)

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

[a] There was no overall effect of male mating status (monogamous vs. polygynous) on mean yolk androgen (T, A_4, DHT, and E) concentrations in European starlings, but the within-clutch pattern of A_4 depended on mating status.

androgen deposition in 1 population (*Hirundo rustica*; Gil et al. 2006), but not in another (Saino et al. 2006). In a correlative study in another population, male throat color but not streamer length was correlated with yolk androgen levels (Safran et al. 2008). In a study on free-living house sparrows (*Passer domesticus*), no effect of male testosterone implantation (which was assumed to increase male attractiveness) was found on yolk androgen levels (Mazuc et al. 2003). Likewise, a correlational study on collared flycatchers (*Ficedula albicollis*) found no correlation between yolk androgen levels and male attractiveness (size of the forehead patch; Michl et al. 2005). Furthermore, in European starlings (*Sturnus vulgaris*), no overall effect of male mating status (monogamous vs. polygynous) on yolk androgen concentrations was found (Gwinner and Schwabl 2005). Instead, the within-clutch pattern of the steroid androstenedione appeared to be dependent on male mating status, which was possibly a female response to the expected contribution of the male to nestling care (Gwinner and Schwabl 2005). Finally, in a population of house finches (*Carpodacus mexicanus*), a negative correlation between yolk androgen levels and male ornamental plumage was found (Navara et al. 2006).

The greater inconsistency in the results of field studies compared with laboratory studies may be caused by the fact that in addition to the singly tested aspect of male attractiveness, several other factors may affect yolk androgen levels in the field. This may also explain why correlational studies, taking only a single aspect of male quality into account, may not yield clear results. In addition, the majority of laboratory studies used a sensitive within-female design while such control for between-female variation was not achieved in any of the field studies. Furthermore, differences between studies could be due to differences in timing of the experimental treatments relative to egg laying. Therefore, we conducted a field study that had the following aims: 1) analyzing the effect of an experimental manipulation of mate attractiveness on egg androgen concentrations using a within-female design, which controlled for the confounding effects of between-female variation; 2) comparing correlational and experimental data in the same population; and 3) investigating on what timescale in the egg-laying process females can adjust their hormone deposition in response to male attractiveness.

The blue tit (*Cyanistes* [formerly *Parus*] *caeruleus*) is an excellent model species to experimentally test the effect of male attractiveness on patterns of female yolk androgen deposition. Blue tits have sexually dimorphic UV-reflecting crown feathers (Andersson et al. 1998; Hunt et al. 1998), and the UV coloration of the crown plumage plays a role in female mate choice (Andersson et al. 1998; Hunt et al. 1998; Delhey et al. 2003, 2007a) and may serve as male viability indicator (Sheldon et al. 1999; Griffith et al. 2003; but see Delhey and Kempenaers 2006). It has recently been found that female blue tits decrease their reproductive investment (in terms of nestling food provisioning) in response to experimentally reduced crown UV reflectance of males, and consequently, these females fledge significantly smaller chicks (Limbourg et al. 2004; see also Johnsen et al. 2005).

We manipulated UV coloration of the crown feathers of male blue tits and subsequently measured the effect on the androgen concentrations in the yolk of their females' eggs. UV reflectance of males was first measured and thereafter manipulated on the day their female laid the second egg of the clutch. The UV-reduction treatment was nonpermanent, and UV reflectance is known to recover within days after treatment (Limbourg et al. 2004; Korsten, Limbourg, et al. 2007). Therefore, we collected the second-, fifth-, seventh-, and ninth-laid eggs (mean clutch size in our population: 10.9 ± 1.7 standard deviation [SD], $n = 249$; see Korsten et al. 2006), enabling us to test whether and on what timescale females adjusted yolk androgen levels to changes in male attractiveness. If females are

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 yolking 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 nearby field station, where their age, body mass (to the nearest 0.1 g using a 30-g spring balance), and tarsus (to the nearest 0.1 mm using calipers) were measured. We determined age (1 year or >1 year) based on the color of the primary coverts following Svensson (1992). The natural reflectance of the crown plumage was measured, and thereafter, we manipulated the males' crown UV reflectance (see below). Birds were released in their own territory after treatment. Age did neither differ between UV-reduced and control-treated males did not differ in age ($\chi^2 = 0.01$, degrees of freedom [df] = 1, $P = 0.92$), date of capture, body size (mass and tarsus), and crown coloration before manipulation (for statistics, see Table 2).

During chick feeding (6–10 days after hatching), we caught the male parents of all broods in the study area inside their nest-box using a spring trap to confirm the initial assignment of the experimental males to specific broods. Three males included in our experiment were also caught at another nest-box, indicating these males to be polygynous. All other males in the experiment were recaptured at only one nest-box in the same territory as where they were initially caught, confirming that in all territories the resident male was manipulated. Male polygyny may affect maternal androgen deposition (Gwinner and Schwabl 2005) and thus could potentially confound patterns of yolk hormone deposition in our experiment. Therefore, the 3 polygynous males were excluded from further analyses, yielding a final sample size of 33 experimental clutches (16 controls and 17 UV reduced). After the same catching procedure, we also caught the females of experimental pairs during chick feeding in their nest-boxes ($n = 30$, 3 females were not caught), and we determined their age and measured their body mass, tarsus length, and crown reflectance following the protocol described above.

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 Örnborg et al. 2002; Delhey et al. 2006), and consequently, crown coloration was not significantly related to the date of

Table 2
Pretreatment characteristics of UV-reduced and control-treated male blue tits

	UV reduced ($n = 17$)		Control ($n = 16$)		Test	
	Mean	SE	Mean	SE	t	P
Capture date (April, days)	16.35	0.69	16.25	0.81	0.10	0.92
Body mass (g)	11.28	0.13	11.08	0.14	1.04	0.31
Tarsus length (mm)	16.99	0.08	17.03	0.12	0.33	0.74
Brightness[a]	75.86	3.38	79.06	2.82	0.72	0.48
Hue (nm)[a]	386.2	2.12	387.4	2.59	0.35	0.73
UV chroma[a]	0.290	0.0042	0.291	0.0045	0.20	0.85

[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 with plastic dummy eggs. Collected eggs were incubated for 72 h in an incubator at 35 °C to induce embryonic development for extraction of DNA to be used for molecular sexing. 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 °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 [A_4]) 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 μL of distilled water by vigorous mixing on a vortex facilitated by the addition of a few glass beads. We used a weighed sample of the homogenized yolk for further analyses. A known amount of radioactive T and A_4 (ca., 2000 counts per minute) was added to a weighed subsample (150–280 mg) of the homogenate to assess extraction efficiency, and samples were kept for 1 h at 37 °C for equilibration. Extraction was performed in 3 batches: batch 1 was extracted 3 times with 3 mL of petroleumether:diethylether, 30:70 (vol:vol), batches 2 and 3 were 3 times extracted with 3 mL of diethylether (both methods extract T and A_4 from yolk and yielded similar recoveries, which were on average 56% and 50% for T and A_4, respectively). The 3 ether fractions were decanted from the snap-frozen egg yolk/water phase, combined, and dried under a stream of nitrogen. The dried extract was redissolved in 1 mL of 90% ethanol, stored overnight at −20 °C, and then centrifuged. The supernatant was dried under nitrogen, redissolved in 1 mL of 2% ethylacetate in isooctane, and transferred to diatomaceous earth chromatographic columns (Kieselgur, pro-analysi, Merck, Darmstadt, Germany). Steroids were eluted with 4 mL of pure isooctane (discarded), 4.0 mL of 2% ethylacetate in isooctane (eluate containing A_4), 4.5 mL of 10% ethylacetate in isooctane (discarded), and 4.5 mL of 20% ethylacetate in isooctane (eluate containing T). The eluates were dried and redissolved in 200 μL of Tris buffer. T and A_4 levels were measured in duplicates of 50 μL 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 A_4 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 1 incomplete clutch, we could not determine A_4 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 A_4 concentrations.

The intra- and interassay coefficients of variation for the 7 T assays were 9.7% and 11.9%, respectively. Those for the 4 A_4 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 (Δ deviance, which follows a χ^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 A_4. 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 A_4 concentrations ($n = 28$). 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,

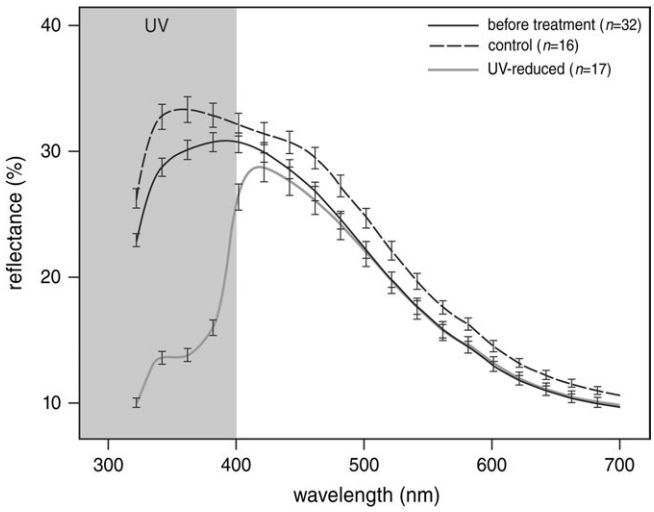

Figure 1
Mean reflectance spectra (±SE at 20-nm intervals) of crown plumage of male blue tits before treatment and after UV-reduction or control treatment (the pretreatment measurement of 1 control male failed).

tarsus length, and lay date—as predictors of either T ($n = 29$) or A_4 concentrations ($n = 25$). Full models were reduced by excluding variables in order of decreasing significance until only variables with $P < 0.05$ would remain in the model. Excluded variables were entered one by one in the final model to confirm their lack of significance. We chose not to run a single analysis including all male and female predictor variables at the same time to avoid the risk of overparameterization of our models given the limited sample size. For the same reason, we did not include interaction effects in the models. Analyses were conducted using MLwiN 2.02 for multilevel models and SPSS 14.0 for all other statistical tests.

RESULTS

The UV-reduction treatment caused a large decrease in the UV reflectance of the crown plumage directly after manipulation (Figure 1). All 3 indices of crown coloration were significantly different between the 2 treatment groups directly after manipulation (Table 3).

Clutch size (which includes collected eggs) did not differ between UV-reduced and control-treated pairs (mean ± SE, UV reduced: 11.8 ± 0.15, control treatment: 12.1 ± 0.25; $t = 0.826$, df = 31, $P = 0.42$).

T and A_4 concentrations of eggs were positively correlated ($r = 0.387$, $P < 0.001$, $n = 109$).

Table 3
Indices of male crown coloration after UV-reduced and control treatment

	UV reduced ($n = 17$)		Control ($n = 16$)			
	Mean	SE	Mean	SE	Test t/U	*P*
Brightness	65.07	2.82	85.27	2.46	5.37[a]	<0.001
Hue (nm)	416.1	0.67	363.7	3.58	0.00[b]	<0.001
UV chroma	0.180	0.0040	0.288	0.0038	19.60[a]	<0.001

[a] *t*-test.
[b] Mann–Whitney *U* test.

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 A_4, 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 A_4 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:

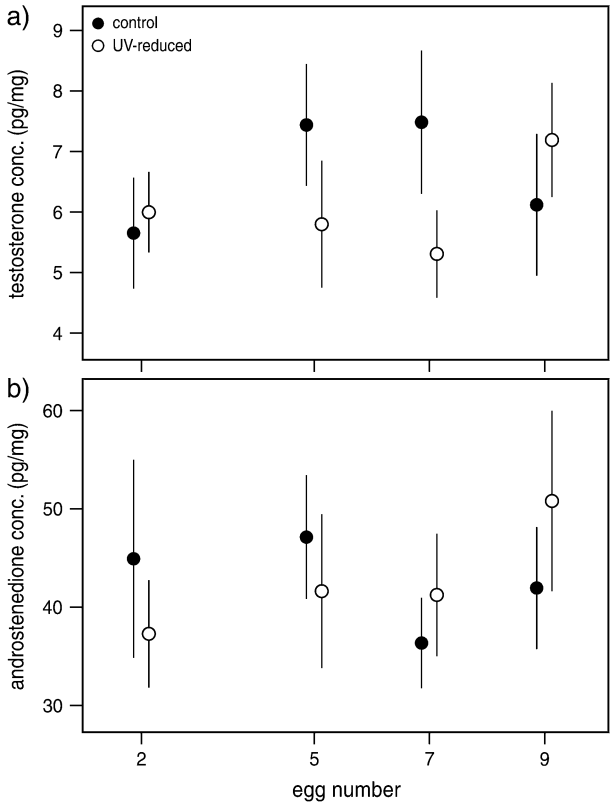

Figure 2
Mean (±SE) yolk testosterone and androstenedione concentration in eggs of UV-reduced (T: $n = 17$ pairs, $n = 66$ eggs, 17 eggs per egg position except for egg 9 were $N = 15$; A_4: $n = 14$ pairs, $n = 54$ eggs, 14 eggs per egg position except for eggs 7 and 9 were $N = 13$) and control pairs (T: $n = 16$ pairs, $n = 64$ eggs, 16 eggs per egg position; A_4: $n = 14$ pairs, $n = 54$ eggs, 14 eggs per egg position except for eggs 7 and 9 were $N = 13$), plotted against position of the egg in the laying sequence (egg number).

$r = 0.140$, $P = 0.44$; all $n = 32$; A_4: brightness: $r = 0.164$, $P = 0.40$; hue: $r = -0.079$, $P = 0.69$; UV chroma: $r = 0.108$, $P = 0.58$; all $n = 28$). Likewise, none of the multiple regression analyses in which we used male or female characteristics in addition to male crown color indices (see Materials and methods) to explain the variation in either yolk T or A_4 concentrations in the second egg yielded a significant model (all P values > 0.15).

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; Delhey et al. 2007b). In addition, consistently higher concentrations of maternal yolk androgen concentrations in avian eggs containing male embryos relative to those containing female embryos have so far not convincingly been demonstrated (Groothuis, Müller, et al. 2005).

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 those 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 relationships among 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 male 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 $\pm$ SD, UV reduced: 0.180 ± 0.016; control: 0.288 ± 0.015) compared with the natural variation in male UV reflectance (UV chroma, mean $\pm$ 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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