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Hegyi, Gergely; Herenyi, Marton; Szoellosi, Eszter; Rosivall, Balazs; Toeroek, Janos; Groothuis, Ton G. G.; Szöllősi, Eszter

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Original Article

Yolk androstenedione, but not testosterone, predicts offspring fate and reflects parental quality

Gergely Hegyi,[a] Márton Herényi,[a] Eszter Szöllősi,[a] Balázs Rosivall,[a] János Török,[a] and Ton G.G. Groothuis[b]
[a]Behavioural Ecology Group, Department of Systematic Zoology and Ecology, Eötvös Loránd University, Pázmány Péter sétány 1/C, H-1117 Budapest, Hungary and [b]Department of Behavioural Biology, University of Groningen, PO Box 14, 9750 AA Haren, The Netherlands

Yolk androgen deposition is a widely investigated maternal effect in birds, but its adaptive value is at present unclear. The offspring fitness correlates of natural yolk androgen levels are virtually unknown, whereas manipulations largely focused on testosterone and neglected other androgens. We determined yolk concentrations of the 2 dominant androgens, androstenedione and testosterone, from all eggs in collared flycatcher clutches and followed the fate of individual offspring from these eggs in a crossfostering experiment. Yolk concentration of androstenedione was much higher than that of testosterone. Offspring from eggs with relatively higher androstenedione concentrations within a clutch were relatively large after hatching, grew slower thereafter, and had a higher recruitment rate in subsequent years. The increase of androstenedione with laying order and its within-clutch variance were negatively correlated with a condition-dependent female ornament, perhaps indicating compensatory hormone deposition into later hatching eggs by females in low condition. Yolk testosterone variation within or among clutches was not related to any measured aspect of offspring or parental quality. Our results suggest that in some species, especially those with much more androstenedione than testosterone in the yolk, androstenedione and not testosterone may be the yolk androgen with a long-term function and adaptive deposition pattern. *Key words:* androgen, female ornament, growth, maternal effect, recruitment. *[Behav Ecol 22:29–38 (2011)]*

In oviparous vertebrates, maternal effects through the egg are now being recognized as potential means of adjusting offspring phenotype to parental or environmental quality (Mousseau and Fox 1998). This view has gradually developed to include more refined scenarios. For example, some authors discussed maternal effects in the framework of individual optimization, whereby every brood or individual offspring has its own optimal level of maternal substances (Groothuis et al. 2005). Others noted that maternal effects are a potential source of conflict, whereby the opposing interests of mother and offspring may lead to an arms race between maternal manipulation and offspring response (Müller, Lessells, et al. 2007). In addition, it has been repeatedly emphasized that maternal effects may slow down or accelerate the evolution of phenotypic traits (Wolf et al. 1998; Qvarnström and Price 2001). Maternal effects through the egg include macronutrient content (Sinervo et al. 1992, Styrsky et al. 2000) and other substances, such as steroids (Hayward and Wingfield 2004; Groothuis et al. 2005), carotenoids and vitamins (Royle et al. 2001; Saino et al. 2003), and immunoglobulins (Saino et al. 2002; Hargitai et al. 2006). The research of bioactive molecules in wild bird eggs started to flourish with the discovery of maternal androgens in the yolk of canaries and zebra finches (Schwabl 1993), and these hormones have remained a central topic ever since then.

The adaptive hypothesis that yolk androgens are a maternal tool to adjust the offspring to environmental conditions was inspired by variation in androgen levels with determinants of offspring prospects, such as laying order (Royle et al. 2001), offspring sex (Gilbert et al. 2005), parental attractiveness (Safran et al. 2008), or various environmental conditions (e.g., Verboven et al. 2003; Tschirren et al. 2004). However, the function of these hormones will remain obscure until their effects on the offspring are clarified. Some experimental yolk hormone injections yielded clear positive effects on offspring (e.g., Eising et al. 2001; Navara et al. 2006b; Müller, Deptuch, et al. 2007, Partecke and Schwabl 2008), but many others had negative or context-dependent effects (e.g., von Engelhardt et al. 2006; Rubolini et al. 2006; Sockman et al. 2008; Sandell et al. 2009). Proposed explanations for this ambiguity include parent–offspring conflict (Müller, Lessells, et al. 2007) and poor experimental design due to lack of mechanistic knowledge (Carere and Balthazart 2007; Groothuis and Schwabl 2008; Navara and Mendonca 2008) or methodological and technical issues (von Engelhardt and Groothuis 2005; Groothuis and von Engelhardt 2005; von Engelhardt et al. 2009).

There are 2 potential main reasons for the controversial results. The first is the general focus on testosterone (T) and the neglect of other yolk androgens, which are all part of the same metabolic pathway for sterogenesis as T. Androgen metabolism in the yolk has been suggested to modulate effects on the offspring (Elf and Fivizzani 2002; Paitz and Bowden 2008; von Engelhardt et al. 2009, but see Eising et al. 2003), so other yolk androgens might be at least as important to measure and manipulate as T itself. These

Address correspondence to G. Hegyi. E-mail:everest@ludens.elte.hu.

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androgens (see Schwabl 1993; Groothuis and Schwabl 2002) include 5-alpha-dihydrotestosterone (DHT), which is generally more potent than T, and androstenedione (A4), which has a lower receptor affinity than T (Sonneveld et al. 2006). A4 can be an important precursor of T in the yolk (Bruggeman et al. 2002), and its concentration is often higher than that of T (Gil et al. 2007; Schwabl et al. 2007). The only study that manipulated all 3 androgens separately in the yolk showed positive effects of elevated A4 and DHT but negative effects of increased T on hatchling Japanese quails (Hegyi and Schwabl 2010). An important question is therefore what role each androgen plays in the yolk and whether assaying or manipulating only T or just the combination of T and A4 together, as done so far, is sufficient.

The second factor that hampers progress in yolk androgen research is the lack of field studies correlating yolk hormone levels with offspring fitness. When investigating the adaptiveness of variation in a behavioral, morphological, or physiological trait, the 2 vital steps are to examine correlations of natural trait expression with proxies of fitness and to manipulate trait expression to detect its causal effects on fitness. These 2 steps are clearly seen in the studies of clutch size (Daan et al. 1990) or egg size (Christians 2002) but not in studies of yolk androgens. Many studies have manipulated yolk androgen levels, whereas others examined the adjustment of androgen levels to various intrinsic and extrinsic factors (reviewed in Gil 2003, 2008; Groothuis et al. 2005). However, these should have acted as follow-up studies that are designed based on the naturally observed relationship between trait expression (i.e., yolk androgen level) and offspring fitness and should aim to explain this relationship. Paradoxically, however, the yolk androgen–offspring fitness relationship remains almost completely unknown. Since the pioneering studies of Schwabl (1993, 1996), to the best of our knowledge, only a single study has sampled individual eggs for androgens and followed the fate of the young from these eggs, but the study only analyzed the degree of hatching asynchrony in the brood (Ellis et al. 2001). Some studies examined androgen patterns and nestling fate in different eggs of the same clutch (e.g., Gil et al. 2006) or in different clutches (e.g., Verboven et al. 2003), but this is clearly a less sensitive approach than following the future of offspring from the same eggs from which the hormones are measured. An especially serious lack of knowledge concerns the proposed adaptive adjustment of within-clutch yolk androgen allocation to individual or environmental quality (Groothuis et al. 2005). Studies have so far shown context-dependent within-clutch adjustments (Gilbert et al. 2005; Rutstein et al. 2005) and laying order dependence in the effect of injections (Müller et al. 2010), but there is no information from natural populations on how yolk androgen levels are related to offspring fitness depending on the position of the egg in the laying order.

Here, we describe a crossfostering experiment to examine the patterns and potential adaptive value of natural yolk androgen concentrations in a Hungarian population of collared flycatchers (*Ficedula albicollis*). In this species, A4 is present in much higher concentrations in the yolk than T (Swedish population; Gil et al. 2007; Tschirren et al. 2009; also see Tobler et al. 2007 for data from the sister species), and although T levels seemed to be strongly heritable, A4 levels were apparently more plastic and reactive to environmental conditions than T (Sweden; Tschirren et al. 2009). Yolk T levels were related to male age, although not to male forehead patch size (FPS; Hungary; Michl et al. 2005), and they also reacted to female social environment (Hungary; Hargitai et al. 2009). However, experimental addition of A4 + T to the yolk had very little effect on nestlings (Sweden; Pitala et al. 2009). This incoherent picture requires clarification, so an investigation of the relation between different androgens and nestling fate in this species is highly desirable.

We focused on the following questions. First, which of the androgens shows a clear relation between its concentration in the yolk and 1) the fitness estimates of the bird hatching from that egg and 2) parental or environmental traits? Difference in these patterns between T and other androgens would potentially question the prevailing correlative and experimental practice in this field. Second, do the correlations of androgens with parental traits, environmental factors, and offspring fitness appear within clutches or among clutches? This has important practical implications for the design of experimental studies.

We took yolk biopsies from all eggs in a clutch before incubation, followed hatching in an incubator, individually marked the chicks, and transferred the whole brood to a different nest. In this way, we could follow the offspring fitness correlates of natural within-brood androgen patterns while randomizing posthatching parental effects. We measured mass growth up to fledging and monitored the return rate of nestlings to the population in the next 2 years. The latter is important because it is a much more direct estimate of offspring fitness than reported in most of the literature so far. In addition, we also assessed the patterns of androgens in relation to laying order, offspring sex, and the body size and sexual ornamentation of male and female parents. In all analyses, we clearly distinguished within-clutch from among-clutch variation.

MATERIALS AND METHODS

Study species, study site, and nest selection

The collared flycatcher is a small (typically less than 15 g), long-distance migratory, insectivorous hole-nesting passerine. Early in the breeding season, most clutches contain 6 or 7 eggs. Nestlings typically hatch 12–14 days after clutch completion. They are reared by both parents and are considered structurally fully grown at the age of 13 days. The present experiment was done in the breeding season of 2007 in a nest-box–breeding population in the Pilis Mountains, Hungary (lat 47°43′N, long 19°01′E; for details of the study area, see Török and Tóth 1988). We selected 24 relatively synchronous nests where the male had been identified as after second year. This was necessary because broods of yearling males had been shown to differ from those of older males in yolk T content (Michl et al. 2005) and nestling growth (Hegyi, Rosivall, et al. 2006).

Yolk sampling

Nests were visited daily, and eggs were numbered using a waterproof marker. In 6- and 7-egg clutches, there is no measurable incubation before laying the fifth egg (Hegyi G, Herényi M, Szöllősi E, Rosivall B, Török J, unpublished data, also see the hatching time patterns presented below), so hormone decline due to embryonic development is unlikely before this time. Therefore, to ensure that our data not only reflect the hormone concentrations deposited by females but also minimize disturbance, we sampled the first 5 eggs of each clutch on the day of laying the fifth egg and the sixth and occasional seventh eggs on the morning when they were laid. Eggs were replaced with dummy eggs and taken to the nearby field station. Sampling was done in a dark room, using a purpose-built apparatus with 25G butterfly infusion sets, a small syringe, and illumination from below the egg to ensure penetration of the yolk. The egg was weighed to the nearest milligrams before taking the biopsy, and the amount of yolk removed was also noted

to the nearest 0.1 mg. The mean mass of biopsies was 7.4 mg (standard deviation = 12.1), which is approximately 2% of total yolk weight. Sampling was successful from 152 of the 159 eggs. The penetration site was cleaned with ethanol before sampling, and the hole on the shell was closed using a tiny piece of transparent wound dressing. Eggs were returned to the nest within an hour of removal. Hatching success of the sampled eggs was high (84.9%). All samples were taken by the same person. Systematic biases in hormone concentrations due to layered yolk composition (Lipar et al. 1999) are unlikely because flycatcher yolks are small and highly mobile so that penetration depth could not be standardized. However, because hormone concentrations were significantly repeatable within clutches (see RESULTS), potential measurement error did not remove the biological information from the data.

Hatching and crossfostering

A few days before expected hatching, eggs were candled daily. When imminent hatching was noticed, the whole clutch was replaced with dummy eggs and put into an incubator while separating individual eggs with paper dividers. Eggs were incubated at 37.2 °C and at 70–80% humidity. The incubator was checked every 20 min from 05:00–21:00 h. Newly hatched nestlings were weighed to the nearest milligrams, individually marked by removing tufts of down from the head, and brought to a different experimental nest that hatched on the same day. In this way, whole broods were reciprocally crossfostered between pairs of nests so that all nestlings were raised in a foreign nest, but within-brood competitive relationships remained unchanged. One experimental clutch had been abandoned earlier during incubation, so we had to foster one brood to a nonexperimental nest that hatched on the same day. From the eggs that died before putting into the incubator, failed to hatch in the incubator, or died during hatching, the embryos were sampled for molecular sexing.

The nestling growth period

Once in the foster nest, the mass of nestlings was measured every second day up to the age of 14 days to the nearest 0.1 g. Measurement ages were determined based on the oldest nestling of the brood, so late-hatching young were measured at younger ages. Nestlings that died during growth were tissue sampled for molecular sexing. Nestlings were ringed using numbered aluminum rings at the age of 6 days and blood sampled between 8 and 10 days of age. Parents were caught at the nest at 8 days of nestling age. They were ringed if necessary, and tarsus length and plumage ornaments were measured to the nearest 0.1 mm using calipers. We measured the maximum height and width of the forehead patch in males (Hegyi, Török, et al. 2006) and the visible lengths of white on the outer vanes of primaries 4–8 on both sexes (Török et al. 2003; Hegyi et al. 2008). All males were of the same age category (see above), whereas for females, newly ringed individuals or yearling recruits were assigned as yearlings and other females as older (see Hegyi et al. 2008 for validation). Finally, the whole nest-box plot system of approximately 800 boxes was thoroughly monitored in the 2 years after the experiment, and any recruit from the experimental broods was noted. All nestlings and available embryos were sexed using polymerase chain reaction techniques (see Rosivall et al. 2004 for details).

Determination of yolk hormone concentrations

In this study, our initial aim was to determine 3 androgens (T, DHT, and A4) from the biopsies. However, when calibrating the assays using $N = 8$ pooled yolk samples of 200 mg each, we found that the concentration of DHT was between 0.4 and 0.8 pg/mg and too low to measure reliably in the small yolk samples. Therefore, DHT was not determined from the small biopsies of the present experiment. Although its concentrations as estimated from the pool yolks were 2 and 3 magnitudes lower than those reported here for T and A4, respectively (see RESULTS), the higher affinity of DHT to the androgen receptor than T or A4 (reviewed in Groothuis and Schwabl 2008) implies that the functional importance of yolk DHT cannot be discounted in this species, and this hormone needs further study.

We analyzed androgens by radioimmunoassay (RIA). The yolk samples were thawed and homogenized with distilled water. Aliquots of this yolk/water emulsion were mixed with 50 µl of 3H tracer T (ca. 2000 counts per min) to assess extraction efficiency. The samples were extracted twice with 2.5 ml of 70% diethyl ether/30% petroleum ether (vol/vol) and dried under a stream of nitrogen. Extracts were then redissolved in 1 ml of 70% methanol, centrifuged, and decanted. The supernatant was dried under a stream of nitrogen and reconstituted in phosphate-buffered saline.

For measuring DHT, we used a highly specific double antibody RIA from Diagnostic System Laboratories, Webster, TX, having a sensitivity of 4 pg/ml. The highest crossreactivity of the antibody was with androstandiol glucuronide (3.3%), and it was only 0.6% with T. For T, we used a kit from the same company (DSL-4000 Coated Tube RIA) having a sensitivity of 0.08 ng/ml and crossreactivities of 5.8% with DHT and 2.3% with A4. For A4, we used the kit DSL-3800 Coated Tube RIA, with a sensitivity of 0.03 ng/ml and crossreactivity with androsterone of only 0.33%. The kits had been validated using column chromatography.

Average recovery rate was 84%. We corrected the measured androgen concentrations (picograms per milligram yolk) for extraction efficiency. Dilution curves showed good parallelism with the standard curve, confirming the reliability of extraction and assay protocols and suggesting the absence of other confounding steroids in the extracts. Yolks were randomly distributed between 2 assays each for T and A4. We included duplicates of pooled collared flycatcher yolk samples in each assay to calculate intra- and inter-assay variation. Intra-assay variation was 3.1% for A4 and 2.6% for T. Inter-assay variation was 7.6% for A4 and 8.7% for T.

Eleven samples had to be omitted from the data set due to failure of the digital scale when taking the biopsy, failure of the hormone determination, or estimated hormone concentrations falling outside the standard curve, leaving $n = 141$ data points for the present analyses.

Statistical analyses

Yolk androgen concentrations were log transformed before analyses. Male FPS was quantified as the product of maximum height and width and male and female wing patch sizes (WPS) as the sum of the measured white segments. Females generally lay at dawn, so the total hatching time of an egg (incubation time until hatching) was estimated to the nearest hour by taking 05:00 on the day of laying the sixth egg as the onset of incubation for eggs 1–6 and moving this one day forward for the seventh egg. The analyzed clutch size of the experimental clutches was either 6 or 7 (see above), so raw laying order effects would have been confounded with clutch size (van de Pol and Verhulst 2006). Therefore, laying order was recoded as a continuous variable with 3 values, 1 for the first 2 eggs, 3 for the last 2 eggs, and 2 for the 2 or 3 intermediate eggs. Chicks from the same brood often hatched in groups, and the incubator was not checked regularly during the night, so we categorized hatching order as early versus late so that

chicks hatched at the same time were put into the same category, and whenever possible, at least 2 nestlings belonged to both categories in every brood.

We summarized nestling growth using principal components analysis (PCA). Classic growth curves are rarely adequate for natural nestling growth trajectories (Brown et al. 2007) and also produce correlated parameters, necessitating further dimension reduction. Using raw multiple measurements or more complex curve fitting methods would have hindered interpretation by producing many parameter estimates. PCA produces a small number of orthogonal growth axes that can be interpreted independently (Stevens 1986). Nestlings that died before 12 days of age were omitted from these analyses because they did not have all the data we needed. This concerned one whole brood (most likely predation of a parent) and 18 individual nestlings of which 14 were runts coming from late-hatched eggs. Therefore, the growth trajectory of these young would not have been informative even if we had chosen to analyze stage-specific growth data. We initially entered 6 input variables in the PCA: total hatching time (a possible determinant of posthatching growth), mass at hatching (the start point of posthatching growth), mass at the 2-day measurement of the brood (i.e., initial mass incorporating rank differences due to hatching order), 12-day mass (an approximate of fledging mass because several nestlings had already disappeared by the age of 14 days), maximum mass increment (the maximum increase of mass during any 2-day growth period, which indicates resource acquisition or assimilation capacity), and maximum relative mass increment (the maximum increase of mass if divided by initial mass during any 2-day growth period, which indicates compensatory growth capacity). Two- and 12-day masses were squared, and growth increments were log transformed before analysis to normalize their distributions. The first 2 principal components (PCs) had eigenvalues larger than 1. All input variables except hatching time had a strong loading ($r > 0.5$) in at least one PC, so we ran another analysis with the exclusion of hatching time. The results of this second analysis are shown in Table 1. PC1 (explaining half of the variance) correlated negatively with hatching mass and 2-day mass and positively with maximum relative mass increment but only weakly with the remaining 2 variables. PC2 (explaining a quarter of the variance) correlated positively with 12-day mass and maximum mass increment but only weakly with the rest of the variables. Therefore, nestlings with large PC1 values were small at hatching but showed large relative growth rates, whereas those with large PC2 values had large absolute growth rates and were large before fledging. Hatching time was analyzed as a separate trait.

We assessed the repeatability of egg size and yolk androgen concentrations by 1-way analysis of variance as described in Lessells and Boag (1987) and Becker (1984), using Statistica 5.5 (Stat Soft, Inc.). We analyzed the determinants of egg mass and yolk androgen concentrations in general linear mixed models using the MIXED module of SAS 9.1, with brood as a random factor, embryo sex, and female binary age as fixed factors and laying order (see above), female tarsus length, age-standardized female WPS, male tarsus length, male FPS, and male WPS as continuous predictors. Nests with one or more parental traits missing were omitted. To see whether the pattern of egg quality over the laying order depended on parental attributes, we also entered the interactions of laying order with all parental traits. We did not test sex interactions due to the apparent lack of functional value for sex-dependent allocation (see next paragraph). Finally, we used Pearson correlation to see whether within-clutch variance in egg attributes depended on parental body size or ornamentation.

Nestling development data were analyzed in general linear mixed models. For hatching failure, nestling mortality, and nestling recruitment, we used generalized linear mixed models with binomial error and logit link (GLIMMIX macro of SAS 9.1). The random factor was brood in all cases. In mixed model analyses, effects of covariates that vary both within and between random factor levels (clutch in our case) are difficult to interpret (van de Pol and Verhulst 2006). Therefore, we used within-subject centering to separate the within-clutch and among-clutch effects of egg mass and yolk androgens (van de Pol and Wright 2009). This means that we entered 2 independent variables for all 3 egg attributes: the clutch mean and the deviation from the clutch mean. These variables were calculated anew for each data set, that is, all analyzed eggs (hatching), all hatched nestlings (nestling mortality), and all young that survived to 12 days of age (growth and recruitment). We also entered offspring order and offspring sex into each model. The offspring order variable differed among the models. For hatching time and egg mortality, egg order was coded as 6 and 7 versus earlier because it was these 2 eggs that differed from the rest of the clutch in hatching time in a preliminary analysis. For nestling growth, nestling mortality, and recruitment, nestling order was coded as early hatched or late hatched as described previously. An experimental study in the Swedish population suggested sex-dependent effect of androgens on growth (Pitala et al. 2009), so we also entered the interactions of sex with egg attributes, but we did not detect any significant sex-dependent hormone effect (results not shown here). Therefore, we present the main effect models here. We used backward removal of nonsignificant terms in all linear models, with reintroduction of the removed terms to the final model one by one.

Table 1

Factor loadings (Pearson *r*) and explained variances in the PCA of nestling growth trajectories

Term	PC1	PC2
Hatching mass	**−0.813**	0.218
2-day mass	**−0.860**	−0.135
12-day mass	−0.365	**0.737**
MMI	0.170	**0.821**
MRMI	**0.860**	0.223
Variance explained	46.1%	26.7%

Large effects ($r > 0.5$) are marked with bold. PC, principal component; MMI, maximum mass increment; MRMI, maximum relative mass increment.

RESULTS

Repeatability and determinants of egg size and yolk androgens

Mean (± standard error [SE]) egg mass was 1796 ± 11 mg. The mean concentrations of androgens were 28.75 ± 1.04 pg/mg for T and 206.06 ± 1.02 pg/mg for A4. The proportion of among-clutch variation was very large in egg mass (repeatability [intraclass correlation, r_I] ± SE: 0.782 ± 0.058; $F_{23,117} = 22.10$, $P < 0.001$), relatively large in A4 concentration ($r_I = 0.472 \pm 0.096$; $F_{23,117} = 6.25$, $P < 0.001$), and smaller but still significant in T concentration ($r_I = 0.207 \pm 0.088$; $F_{23,117} = 2.53$, $P < 0.001$). Egg mass was negatively related to both hormones at the among-clutch level (with T, $r = -0.441$, $P = 0.031$, $N = 24$ and with A4, $r = -0.431$, $P = 0.036$, $N = 24$) but not so within clutches (with T,

Table 2
Egg mass and yolk androgen concentrations in relation to embryo sex, laying order, and parental phenotype

	Egg mass		Yolk T		Yolk A4	
Terms	*F*	df	*F*	df	*F*	df
Sex	1.50	1, 56.1	0.12	1, 74.3	2.48	1, 63
Laying order	9.54**	1, 56.3	0.22	1, 66.3	5.99*	1, 60.5
Female binary age	0.01	1, 12	0.10	1, 15.2	0.09	1, 11.5
Female tarsus length	0.23	1, 12	0.01	1, 14.3	4.84*	1, 60.3
Female WPS[a]	0.09	1, 17.4	0.07	1, 14.8	1.19	1, 59.9
Male tarsus length	0.03	1, 12	0.00	1, 14.5	1.11	1, 11
Male FPS	0.15	1, 12	0.32	1, 14.9	0.10	1, 11.6
Male WPS	0.35	1, 11.9	2.47	1, 16.2	2.10	1, 10.4
Laying order × female binary age	0.27	1, 55.2	0.09	1, 65	0.26	1, 57.5
Laying order × female tarsus length	3.69	1, 55.4	1.70	1, 65.2	5.95*	1, 60.5
Laying order × female WPS	5.78*	1, 56.2	0.54	1, 60.3	7.73**	1, 59.4
Laying order × male tarsus length	0.27	1, 55.1	0.54	1, 63.1	0.00	1, 56.6
Laying order × male FPS	1.80	1, 55.2	0.02	1, 63.1	0.00	1, 57
Laying order × male WPS	1.14	1, 55.2	1.06	1, 67.5	0.14	1, 56.4

Results are from general linear mixed models. Backward model simplification was used; results for terms not in the final model were calculated by reintroduction. df, degrees of freedom.

[a] Age standardized.

*$P < 0.05$, **$P < 0.01$.

$r = 0.045$, $P = 0.594$, $N = 141$ and with A4, $r = -0.102$, $P = 0.230$, $N = 141$). The 2 androgens were positively correlated with each other with an approximately medium effect size at both levels, but the relationship was not significant at the among-clutch level, probably due to the low sample size (among clutches, $r = 0.324$, $P = 0.122$, $N = 24$ and within clutches, $r = 0.253$, $P = 0.002$, $N = 141$).

Among the potential predictors of egg mass we considered, there was a significant interaction between laying order and female WPS (see all details in Table 2). Egg mass increased with laying order in small-patched females but changed very little in large-patched females (Figure 1A). Sex, parental traits, and other interactions with laying order were nonsignificant. We did not find any significant main effect or interaction for yolk T concentration (Table 2). Yolk A4 concentration, however, showed significant interaction between laying order and female WPS in the same way as egg mass (Table 2). These A4 levels were also significantly influenced by an interaction between laying order and tarsus length: (Table 2). Yolk A4 level increased with laying order in females with small body size and small WPS but changed little in females with large phenotypic values (Figure 1B,C). Finally,

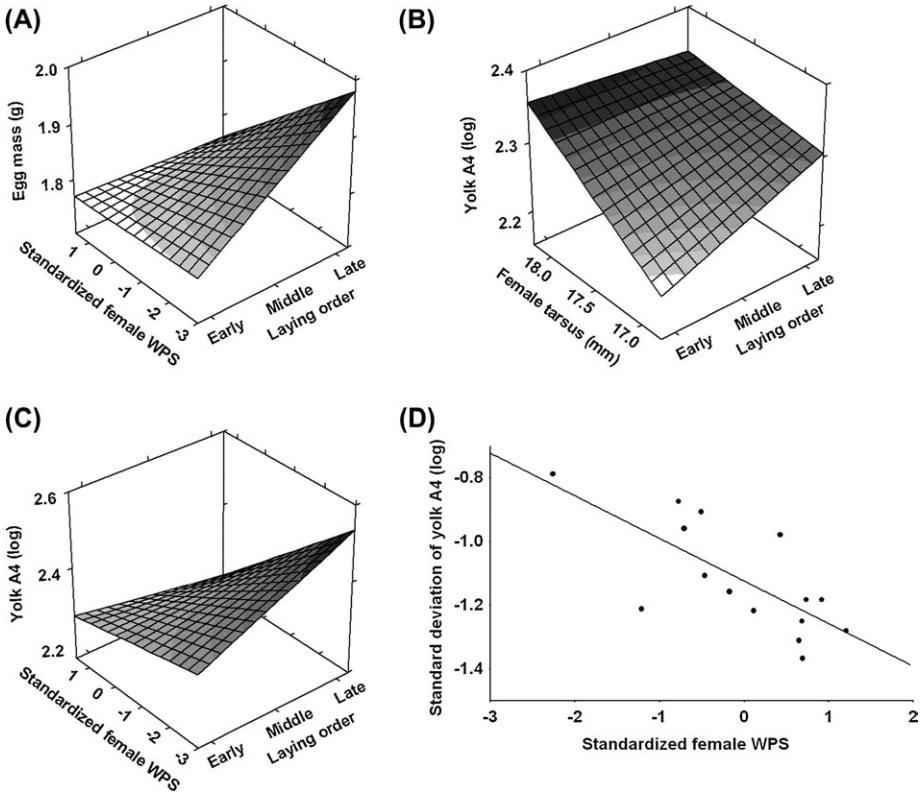

Figure 1
Within-brood patterns of egg quality in relation to female quality; (A) egg mass, laying order, and female wing patch size; (B) yolk androstenedione level, laying order, and female tarsus length; (C) yolk androstenedione level, laying order, and female wing patch size; (D) within-clutch variance of yolk androstenedione level and female wing patch size. The surfaces in (A)–(C) show the linear model estimates and were fit in Statistica 5.5.

Table 3
Offspring development and recruitment in relation to sex, offspring order, egg mass, and yolk androgen concentrations

	Egg mortality		Hatching time		Nestling mortality		Growth PC1		Growth PC2		Recruitment	
Terms	*F*	df	*F*	df	*F*	df	*F*	df	*F*	df	*F*	df
Sex	6.58*	1, 123	0.30	1, 74.1	1.57	1, 105	4.39*	1, 79.9	0.03	1, 78.9	0.15	1, 88.9
Offspring order[a]	0.47	1, 117	18.89***	1, 73.6	4.18*	1, 104	19.87***	1, 71.7	0.31	1, 73.2	0.19	1, 82.9
Among-clutch egg mass	2.79	1, 20.1	0.00	1, 19	0.73	1, 18.9	37.43***	1, 18	0.12	1, 18.7	0.20	1, 16.2
Among-clutch yolk T	4.12†	1, 18.8	2.03	1, 18.9	0.07	1, 14.5	0.04	1, 16.7	0.58	1, 18.5	0.32	1, 13.8
Among-clutch yolk A4	0.64	1, 20.6	0.06	1, 18.7	1.21	1, 16.9	0.03	1, 15.9	0.83	1, 18	2.68	1, 19.9
Within-clutch egg mass	15.36***	1, 112	1.30	1, 71.1	0.68	1, 92.7	9.10**	1, 68.1	4.39*	1, 71.7	0.21	1, 79.7
Within-clutch yolk T	1.23	1, 113	0.04	1, 71	1.21	1, 93	0.11	1, 67.2	0.10	1, 70.7	3.51	1, 91
Within-clutch yolk A4	0.51	1, 109	3.13	1, 71	0.06	1, 91.5	4.14*	1, 68.5	0.15	1, 70.7	6.39*	1, 88

Results are from general linear mixed models (hatching time and growth) or generalized linear mixed models with binomial error and logit link (mortality and recruitment). Backward model simplification was used; results for terms not in the final model were calculated by reintroduction.
[a] Offspring order is laying order for egg mortality and hatching time but hatching order for nestling mortality, growth, and recruitment.
†$P < 0.06$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

the within-clutch variance of yolk A4 concentration was very strongly negatively related to female WPS (Figure 1D; $r = -0.724$, $P = 0.002$, $N = 15$), but there was no significant relationship for other parental traits or egg attributes (female WPS and egg mass, $r = 0.510$, $P = 0.052$ and all other correlations abs(r) < 0.375, $P > 0.169$).

Offspring mortality, development, and recruitment

Within the same clutch, eggs that did not hatch were smaller and more likely to be male than those that hatched (see all statistical details in Table 3). Moreover, eggs from clutches with higher mean yolk T concentrations were marginally less likely to hatch ($P = 0.057$; Figure 2A). Neither the within-clutch nor the among-clutch components of variation in egg mass or yolk androgens predicted the hatching time of individual eggs, and the only significant predictor for this was laying order (see all details in Table 3), with eggs 6 and 7 hatching later than the earlier laid eggs. Nestling mortality after hatching was predicted only by hatching order (Table 3), with late-hatched chicks being twice as likely to die as early-hatched ones (21.3% and 10.3% mortality, respectively). The PC1 of nestling growth showed a significant negative effect of within-clutch relative yolk A4 level (Figure 2B). This implies that chicks from eggs with a high A4 concentration relative to the clutch mean hatched with larger weights and subsequently grew slower than their nest mates. In addition, male chicks and late-hatched young showed larger PC1 values than females or early-hatched young, and both the within- and among-clutch components of egg mass had very strong negative effects on growth PC1. In the case of growth PC2, the only significant effect was that of within-clutch relative egg mass (positive). Finally, the recruitment of experimental nestlings in the next 2 years was significantly positively related to within-clutch relative A4 concentration (Figure 2C) but not significantly related to any other predictor we considered (see details in Table 3).

DISCUSSION

Here, we used a comprehensive experimental design not employed previously in any wild species by following the fate of chicks from known hormone concentrations in a crossfoster design to identify the predictors and offspring fitness correlates of different yolk androgens both within and among clutches. From our small samples, we could determine the 2 dominant yolk androgens (T and A4), whereas the much less concentrated DHT will require further studies. For T and A4, we can now answer the 2 main questions posed in the INTRODUCTION. First, there was a clear difference between the 2 hormones in deposition pattern and consequences for the offspring. Contrary to expectations based on the traditional view, A4 showed indication for both functional importance and differential deposition whereas T did not. Second, the apparent adaptive variation of A4 appeared at the within-clutch level and not among clutches. As we discuss below, these results have broad implications for yolk androgen research in birds, both for understanding the underlying mechanisms and for the design and interpretation of correlative and experimental studies.

Yolk androgen levels and offspring fate

We observed significant but moderate within-clutch repeatability for T and A4, so there was scope to expect plasticity in hormone levels both among and within clutches (also see Tschirren et al. 2009). The 2 androgens were positively correlated with each other both among and within clutches, but about 90% of variation in one hormone was independent of variation in the other, leaving ample room for different adjustment patterns. Egg mass was negatively correlated with androgen concentrations at the among-clutch level but not within clutches. The absence of a negative correlation within clutches implies that the among-clutch correlation was not due to dilution of the same hormone amount in a larger egg but rather an individual-level allocation decision or constraint (Groothuis and Schwabl 2002).

Within-clutch differences in yolk androgen levels did not predict hatching success, but eggs in clutches with relatively high mean T levels were marginally less likely to hatch. In our population, experimental territorial intrusions during egg laying elevated the mean T level of the clutch (Hargitai et al. 2009), so the low hatching success of high T clutches we found here may be due to socially stressed females incubating less efficiently (Criscuolo et al. 2005). Interestingly, concentrations of yolk A4 but not T predicted nestling fitness components after hatching. The first PC of nestling growth contrasted chicks with small initial mass and subsequently fast relative growth and chicks with initially large mass but subsequently slower relative growth. In addition to nestling sex, hatching order, and egg size, growth PC1 was predicted by within-clutch A4 differences. This suggests that high A4 eggs

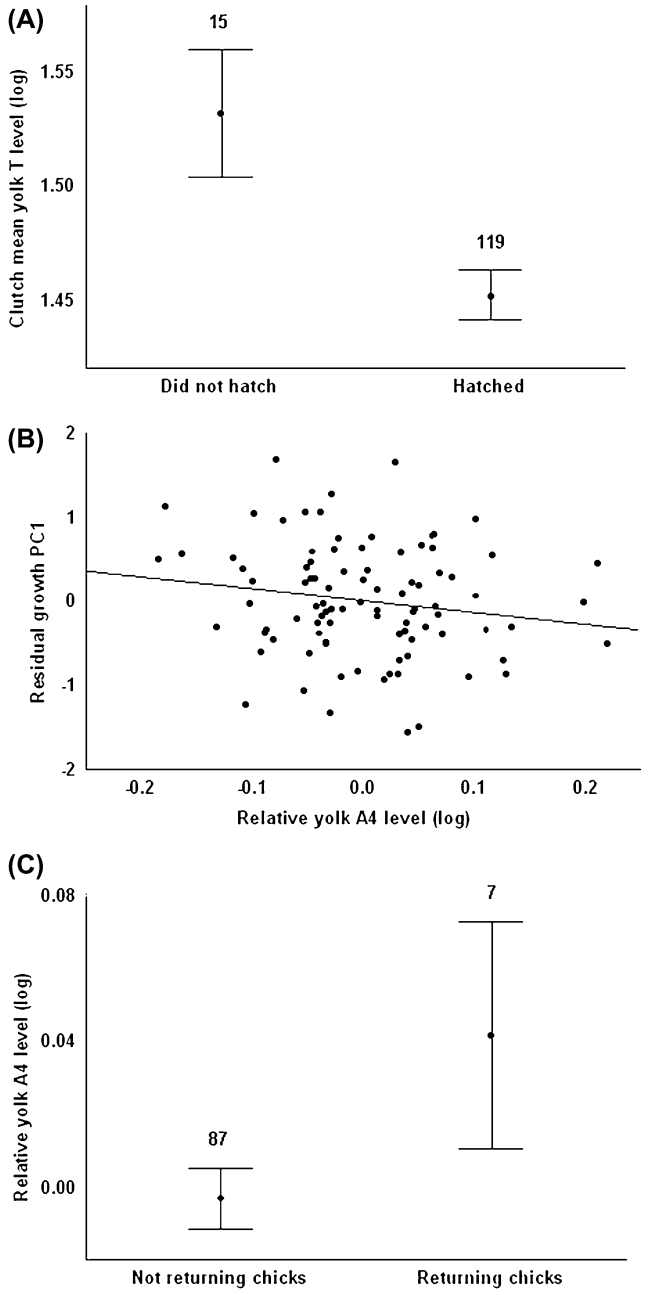

Figure 2
Correlation of yolk androgens with the fate of young when the nestlings are raised in a foreign nest from hatching; (A) clutch mean testosterone level and individual egg mortality; (B) within-clutch relative androstenedione level and nestling growth; (C) within-clutch relative androstenedione level and nestling recruitment. Means are shown with SE; numbers above boxes denote sample size. Panel B shows residual growth principal component values corrected for nestling sex, hatching order, and the within- and among-clutch variation of egg mass.

gave rise to larger hatchlings, presumably due to enhanced embryonic development, but these nestlings subsequently grew relatively slower to reach normal fledging weight. This could be beneficial during the postnatal phase, for example, in terms of lower short-term food demands or lower oxidative stress (Metcalfe and Monaghan 2001).

We also examined, to the best of our knowledge for the first time, the recruitment of individual nestlings in relation to natural levels of yolk androgens. The positive effect of within-clutch relative A4 level on recruitment did not seem to be due to natal dispersal (see Tschirren et al. 2007) because the dispersal distance of the recruited nestlings was very weakly related to yolk androgens, although the power of these tests was low (results not shown). Our within-clutch results and the large-scale among-clutch hormone manipulation data of Tschirren et al. (2007) suggest that yolk androgens may determine the fate of young well into adulthood in natural populations (also see Müller, Dijkstra, et al. 2009 for a different long-term effect in the wild and Eising et al. 2006 and Strasser and Schwabl 2004 for results on wild birds in seminatural or captive conditions).

In our study, the developmental and recruitment benefits of high yolk A4 appeared exclusively within clutches, which might be due to the masking effect of other stronger determinants of the very large among-clutch variation of nestling fitness in our population (Török et al. 2004). In case of tight optimization at the among-clutch level, crossfostering may confuse the natural relationship between maternal effects and offspring fitness among clutches (see Hinde et al. 2010), but this is unlikely in our population where the environment is fluctuating and even clutch size is not individually optimized (Török et al. 2004). The within-clutch pattern was likely not due to androgens mediating direct sibling competition for parentally delivered food (e.g., Schwabl 1993; Schwabl et al. 1997; Eising et al. 2001) because the growth PC phenotype associated with high A4 implied larger initial mass and slower relative growth. An advantage in indirect nestling competition is still feasible if these initially large chicks experienced more rapid development despite their slower mass growth and therefore fledged earlier or benefited in the postfledging care period, a critical time for passerine nestlings (Naef-Daenzer et al. 2001). There are also explanations for the recruitment pattern that do not assume nestling competition. For example, as mentioned above, slower relative growth in high A4 nestlings may lead to lower oxidative stress and thereby higher survival (Metcalfe and Monaghan 2001). Alternatively, the A4 effect may act independently of growth, for example, by producing different personalities with different mortality patterns (Daisley et al. 2005; Eising et al. 2006).

Parental traits and yolk androgen levels

Yolk androgen levels often reflect sexual ornamentation of the male partner (positive: Gil et al. 2004 and negative: Navara et al. 2006a), but female ornamentation (Kraaijeveld et al. 2007) has rarely been examined in this respect (but see Safran et al. 2008). Moreover, although yolk hormone manipulation experiments focused on within-clutch variation (but see Müller, Deptuch, et al. 2007), studies of differential allocation in relation to sexual ornaments mostly investigated among-clutch differences in hormone levels (e.g., Gil et al. 1999; Bolund et al. 2009, but see Dentressangle et al. 2008; Kingma et al. 2009). If male or female ornamental traits indicate parental quality and food supply to offspring (Møller 1993; Møller and Jennions 2001; Siefferman and Hill 2005) and yolk steroids facilitate brood survival or brood reduction under poor food availability (Royle et al. 2001; Eising and Groothuis 2003), we might expect females to adjust the lay-order pattern of yolk androgens in response to ornamental indicators of parental abilities.

Here, we found that no aspect of yolk androgen deposition correlated with the plumage ornaments of males (also see Michl et al. 2005). However, A4 patterns with laying order were related to female tarsus length and also female WPS, a condition-dependent ornament (Hegyi et al. 2008). Moreover, the interactive effect of laying order and female WPS was

similar on egg mass and yolk A4, although A4 and egg mass are not correlated within clutches and negatively correlated among clutches. This may indicate the parallel adjustment of several egg components to female quality.

Late-laid eggs seemed to be preferentially provided with A4 by low-quality females (small size or small WPS) but not by high-quality females. Moreover, the within-clutch variance of A4 in females with small WPS also increased compared with large-patched females, which may further exaggerate the difference between early- and late-laid eggs. This pattern is precisely what we expected based on the apparent consequences of high yolk A4, which seems to improve chick quality and recruitment within but not among clutches. These results may not be due to correlations of A4 with other egg constituents because there was no within-clutch relationship between yolk hormones and macronutrients (egg mass), and other egg components known to correlate with laying order (immunoglobulins and beta carotene; Hargitai et al. 2006; Török et al. 2007) or with yolk T (beta carotene; Török et al. 2007) are not known to have clear growth effects. Therefore, the coherent correlations of yolk A4 with parental quality, nestling growth, and especially nestling recruitment may reflect a tightly regulated adaptive yolk androgen allocation system, although experiments are necessary to confirm this. More studies of female ornamentation and egg composition are needed to determine whether correlations between female ornaments and offspring quality (e.g., Roulin et al. 2000) are due to genetic or rather maternal effects (also see Fitzpatrick et al. 1995). Studies of differential allocation into eggs in relation to parental ornamentation also need to pay more attention to the within-clutch pattern of egg quality (Dentressangle et al. 2008; Kingma et al. 2009).

The functional significance of yolk A4

In our study, both nestling fate and parental traits were related to yolk A4 but unrelated to yolk T. All the dozens of yolk hormone manipulation experiments conducted so far (with the exception of Hegyi and Schwabl 2010) added either T or a mixture of T and A4 to the yolk and brought contradictory results (see INTRODUCTION). The early suggestion that the generally large amount of yolk A4 could act as a precursor of T and other steroids for the embryo (see, e.g., Groothuis and Schwabl 2002) has been practically neglected. Embryos may supply themselves with T from the A4 pool throughout development to avoid the possible toxic effects of high exposure to T early in development and instead expose themselves to the hormone at their optimal time and optimal dosage. If so, initial T concentrations in the yolk would likely serve a short-term purpose, and manipulating early T would not represent a favorable experimental technique to examine adaptive androgen-mediated maternal effects (Hegyi and Schwabl 2010). Moreover, if multiple yolk androgens are weakly interrelated and their deposition patterns are different, then even manipulating them together in the same direction (e.g., T + A4) will yield misleading results. Two species in which the apparent allocation of yolk T seemed consistent with the experimental effects of the hormone were the great tit (Tschirren et al. 2007) and the canary (Schwabl 1996, but see Müller, Vergauwen, et al. 2009). In both of these species, T is the most concentrated androgen in the egg in contrast to most other avian species (see data in the Appendix 1 of Gil et al. 2007).

In the collared flycatcher, the concentrations of yolk androgens we found show a dominance of A4 (consistent with Gil et al. 2007, also see Tobler et al. 2007 for data from the sister species). The highly coherent correlation structure between parental quality, yolk hormone level, and nestling fitness traits we found for A4 was completely absent for T. In other studies of this species, yolk A4 seemed more sensitive to large-scale environmental effects than yolk T (Tschirren et al. 2009). High yolk T rather seemed to reflect bad current environmental conditions or stress to the female (Michl et al. 2005; Hargitai et al. 2009, also see our hatching success data), and experimental manipulations of yolk T and A4 together had very weak and significantly sex-dependent effects on nestlings (Pitala et al. 2009). Although yolk T is positively correlated with yolk A4, most of their variation seems independent, so their regulation and function may also differ. It is therefore possible that collared flycatcher females use A4 but not T to pervasively adjust the phenotype of young to environmental conditions. The small amount of yolk T deposited may nevertheless have a function, most likely in early embryonic development. Separate manipulations of A4 and T in the yolk will be the next step to directly test these suggestions. Future experimental studies of yolk androgens may need to redirect their focus from T to multiple individual androgens, especially in species where T is not the most concentrated androgen in the yolk (Groothuis and Schwabl 2002, 2008; Hegyi and Schwabl 2010).

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