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Published in:
American Naturalist

DOI:
10.1086/659996

Publication date:
2011

Experimental Evidence for Genetic Heritability of Maternal Hormone Transfer to Offspring

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Submitted July 23, 2010; Accepted March 1, 2011; Electronically published May 4, 2011

Dryad data: http://dx.doi.org/10.5061/dryad.8698.

abstract: In many animal species, embryos are exposed to maternal hormones that affect their development. Maternal hormone transfer varies with environmental conditions of the mother and is often interpreted as being shaped by natural selection to adjust the offspring to prevailing environmental conditions. Such hormone transfer requires genetic variability, which has not yet been experimentally demonstrated. Our study reports direct evidence for additive genetic variance of maternal androgens through a bidirectional selection on yolk testosterone (T) levels in Japanese quail. Lines selected for high egg T (HET) and low egg T (LET) concentration differed in yolk levels of this androgen, resulting in high realized heritability ($h^2 = 0.42$). Correlated responses to selection on other gonadal hormones indicated that selection specifically targeted biologically active androgens. Eggs of HET quail contained higher androstenedione and lower estradiol concentrations than did those of LET quail, with no line differences in yolk progesterone concentration. Plasma T concentrations in adult females were not affected by selection, seriously challenging the hypothesis that transfer of maternal hormones to offspring is constrained by hormone levels in a mother's circulation. Our results suggest that transfer of maternal T represents an indirect genetic effect that has important consequences for the evolution of traits in offspring.

Keywords: yolk testosterone, sex steroids, quail, maternal effects, indirect genetic effects.

Introduction

Maternal effects occur throughout the animal kingdom via multiple and often subtle pathways and are now being recognized as an important source of adaptive phenotypic plasticity that has significant implications for understanding both proximate and ultimate analyses of behavior (Mousseau and Fox 1998; Groothuis and Schwabl 2008). Because prenatal hormone exposure can have long-term effects on brain and behavior and because maternal hormone production is strongly influenced by environmental factors, transfer of maternal hormones to the embryo—a widespread phenomenon among vertebrates—offers the mother an excellent tool to adjust offspring development to current environmental circumstances (Groothuis et al. 2005). Most progress in this field has been made using bird species, for three reasons. First, transfer of maternal substances to the egg takes place within a well-defined time window and can easily be measured and manipulated after the egg is laid. Second, birds are a well-known study model in behavioral ecology, facilitating ultimate approaches. Third, all avian eggs studied to date contain substantial amounts of maternal androgens, and their yolk concentrations vary systematically with environmental and social factors, such as ectoparasitic load (Tschirren et al. 2004), level of social competition (Tanvez et al. 2008), aggressive intrusions in the territory (Navara et al. 2006), and food abundance (Gasparini et al. 2007). Moreover, experimental manipulation of yolk androgen levels has profound effects on behavior, physiology, and morphology during both the chick and the adult phase (for reviews, see Groothuis et al. 2005; Gil 2008). Maternal hormones in avian egg yolk have therefore been widely studied as potential transgenerational mediators of epigenetic effects between maternal and offspring phenotypes.

Although maternal effects can be determined by both the genotype and the environment experienced by the mother, the genetic component of hormone concentration in egg yolk has hardly been examined. Potential genetic variability is important because most authors assume that the transfer of maternal hormones to the egg reflects an adaptive maternal effect shaped by Darwinian selection (Groothuis et al. 2005; Müller et al. 2007), for which additive genetic variance is required (Groothuis et al. 2008). These maternal influences, which have both an environmental and a genetic component, cause indirect genetic

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Am. Nat. 2011. Vol. 177, pp. 824–834.
DOI: 10.1086/659996

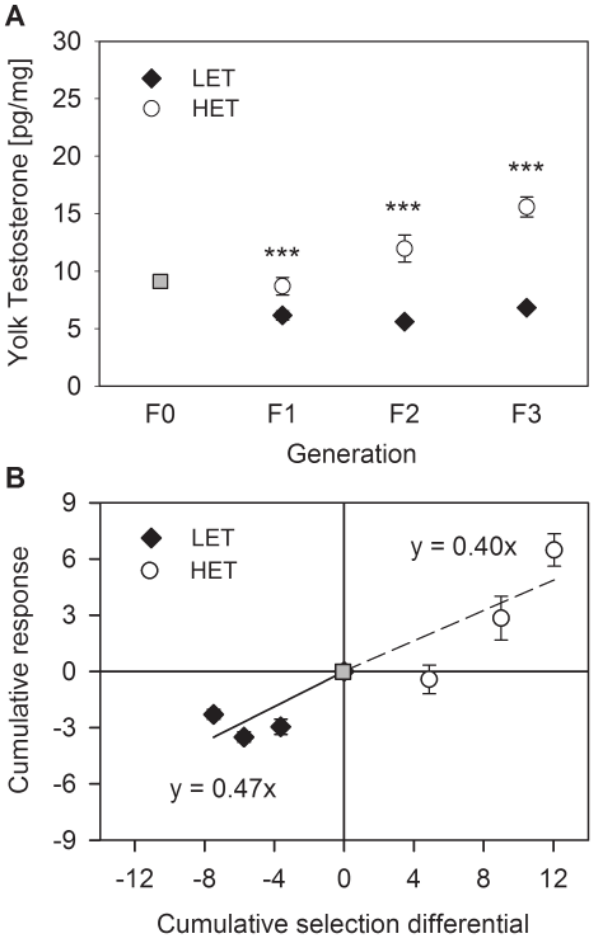

Figure 1: Response to the bidirectional selection on low egg testosterone (LET) and high egg testosterone (HET) yolk concentration over three generations, expressed as (*A*) generation means ± SE (*three asterisks*, $P < .001$) and (*B*) the linear regression of the cumulative response (±SE) on the cumulative selection differential forced through zero. The slope of the regression is plotted separately for each line. Gray squares represent the randomly bred stock population of Japanese quail (F_0 generation).

effects that may profoundly contribute to an evolutionary response of such controlled offspring traits (Wolf et al. 1998).

There is only indirect and multi-interpretable evidence for such genetic effects. Consistent individual differences have been reported in androgen concentrations in the eggs of female pied flycatchers (*Ficedula hypoleuca*) across the breeding season (Tobler et al. 2007) and in eggs of European starlings (*Sturnus vulgaris*) across 2 years (Eising et al. 2008). In addition, significant differences in yolk androgen concentrations have been found between behaviorally selected lines of Japanese quail (*Coturnix japonica*; Gil and Faure 2007; Bertin et al. 2009) and great tits (*Parus major*; Groothuis et al. 2008). However, it is still unclear whether this covariation is due to direct coselection on yolk hormone accumulation, to an indirect effect of selection on behavior that is under the control of steroid hormones, or to an effect of selection on phenotypic traits of males that subsequently influence yolk hormone deposition in their female partners. A recent study of a wild population of collared flycatchers (*Ficedula albicollis*) reported a significant heritability for yolk testosterone (T) concentrations on the basis of resemblance between egg hormones of mothers and daughters (Tschirren et al. 2009). Nevertheless, a direct test of heritable genetic variance of maternal hormone accumulation in the egg is as yet still missing. The aim of our study was to fill this gap by means of artificial selection of Japanese quail for this trait.

In a previous study, we found high interfemale differences in yolk T deposition combined with high repeatability of this trait within individual female Japanese quail, both among three eggs of the same laying sequence and over the reproductive cycle (Okuliarova et al. 2009). These results suggest that yolk T deposition has a genetic component and motivated us to start a bidirectional selection of Japanese quail for yolk T concentrations. The experiment had three aims. First, we evaluated the extent to which artificial selection can alter maternal hormone deposition under stable environmental and social conditions, providing an estimation of the heritability of yolk T concentrations. Second, to further understand where selection would act, we investigated the effect of selection on other steroid hormones of the main steroidogenic pathway—namely, the two precursors of T, progesterone (P_4) and androstenedione (A_4), and one metabolite of T, estradiol (E_2). Third, we investigated the extent to which selection for yolk T levels is accompanied by changes in the concentrations of this androgen in the maternal circulation. This is important because in the case of such a linkage changes in yolk hormone deposition have direct consequences for the mother, possibly constraining evolution and inducing a trade-off in the maternal system—currently a heavily debated issue (see Groothuis and Schwabl 2008; Moore and Johnston 2008).

Material and Methods

Animals and Housing

The initial population used for our selection experiment originated from the laying strain of a randomly bred population of Japanese quail maintained at the Institute of Animal Biochemistry and Genetics, Slovak Academy of Sciences, Ivanka pri Dunaji, Slovak Republic. From the age of 5 weeks onward, two females were housed with

Table 1: Results of a mixed general linear model demonstrating differences in yolk testosterone (T) concentrations between the low egg T and high egg T lines within the first, second, and third filial generations

		F_1 generation			F_2 generation			F_3 generation		
Yolk T	Effect	df	*F*	*P*	df	*F*	*P*	df	*F*	*P*
Intercept	Fixed	1	2,058.21	.000	1	1,829.29	.000	1	6,895.46	.000
Line	Fixed	1	15.10	.000	1	54.42	.000	1	206.54	.000
Cage effect	Random	28	.50	.963	34	1.70	.068	28	.41	.988
Female effect	Random	27	10.23	.000	32	7.10	.000	25	9.09	.000
Error		108			129			105		

one male in group cages (0.26 m × 0.48 m × 0.26 m [length × width × height]) under a long-day photoperiod (16L : 8D). For breeding to the next generation, eggs were incubated under standard conditions (temperature of 37° ± 0.2°C and 50%–60% relative humidity) in a forced-draught incubator (Bios Midi) with automatic turning of eggs. Before hatching, eggs were allocated to individual compartments to ensure individual identification. After hatching, chicks were reared in four separate boxes (1.2 m × 0.6 m × 0.3 m; two for each line, containing 30–40 animals) under continuous light until sexual maturity (age of 5 weeks). Temperature was gradually reduced from 37°C to 21°C during a 3-week period. Commercial starter mash was replaced by complete feed mixture at the age of 4 weeks. Food and water were provided ad lib. Procedures for the care and use of animals were in accordance with the laws and regulations of the Slovak Republic and were approved by the Ethics Committee of the Institute of Animal Biochemistry and Genetics (license SK PC 22004).

Selection Procedure

The initial population (the F_0 generation) consisted of 80 females and 40 males randomly selected from the stock population. At the age of 19–20 weeks, two or three eggs were collected per female and used for an analysis of T concentrations in egg yolk. During this period, we kept only one female with the male to enable egg identification, and the other was transferred to female group cages. Females were switched 5 days later, on average, and the eggs of the second female were collected. Females ($N = 6$) that did not lay eggs or that laid only one egg during the sampling period were excluded from the experiment. We selected 18 breeding pairs (9 females with the highest and 9 females with the lowest concentrations of yolk T and 18 males) as parents of the F_1 generation. At the age of 32–33 weeks, 10 eggs on average from each of the 18 selected females were collected and incubated until hatching. The progeny of females with the lowest and highest egg T concentrations were used to create the low egg T (LET) and the high egg T (HET) lines, respectively. The same selection criteria and experimental procedure were used for the F_1, F_2, and F_3 generations. In the F_1 generation, 19 breeding pairs (10 LET and 9 HET) were selected as parents of the F_2 generation; in the F_2 generation, 17 breeding pairs (8 LET and 9 HET) were selected as parents of the F_3 generation. Females were always mated with males from their same line and their same generation. No selection criteria were applied for males, and they were assigned to females only with regard to their sibling relationship, to avoid inbreeding.

The initial randomly bred population was reproduced simultaneously with the selected lines, and after three generations of selection, yolk T concentrations in the eggs of randomly bred females ($N = 20$) was measured.

Blood Sample Collection

We obtained blood samples for plasma T analysis from females of the F_2 generation ($N = 70$). Quails were sampled at the age of 11 weeks, 1 day before egg collection for yolk hormone analysis. Blood was collected from the wing vein by means of a heparinized syringe within 3 min after capture between 9:00 a.m. and noon.

Yolk and Plasma Steroid Extraction and Assays

Eggs were collected on the day of laying, and the following external egg quality traits were measured: egg mass, yolk mass, and eggshell mass. The eggshell was cleaned and weighed after being dried at 80°C. Yolks were thoroughly homogenized and stored at −20°C until steroid extraction. For the yolk T assay, subsamples of 40–45 mg of yolk were used. First, they were diluted in 500 μL of deionized water and vortexed with the addition of two glass beads for 3 min. Approximately 1,500–2,000 dpm of [^{3}H]-testosterone was added to each sample for individual recovery calculation, and samples were equilibrated overnight at 4°C. Thereafter, they were applied on solid-phase columns filled with Extrelut NT (Merck, Darmstadt) and extracted three times: twice with 2 mL and once with 1 mL of a mixture

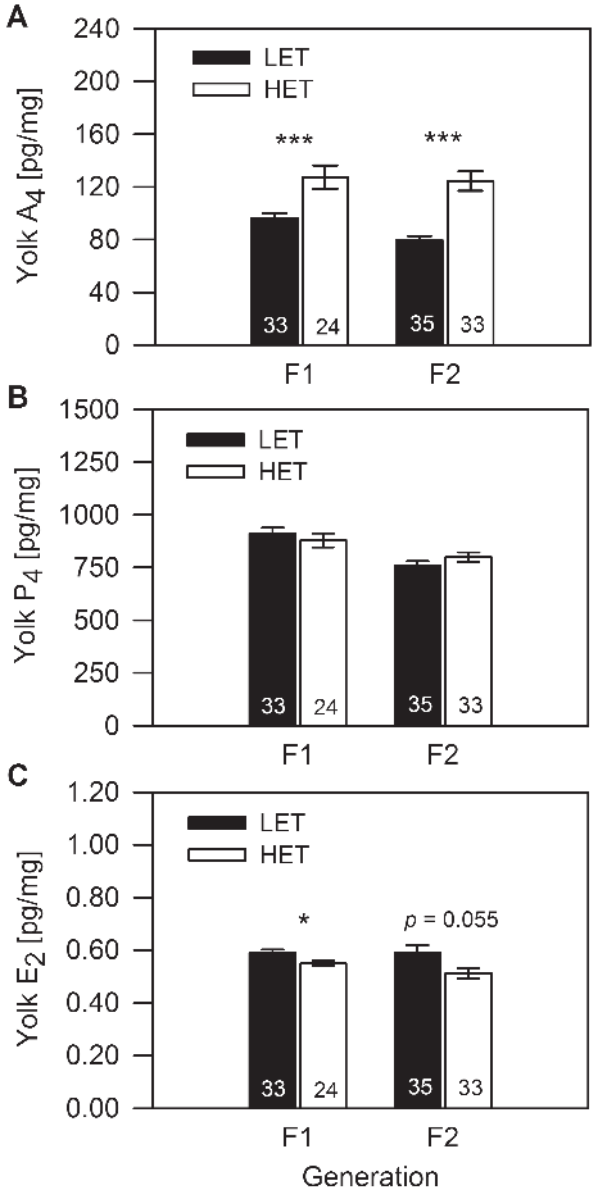

Figure 2: Correlated response of other yolk steroids (mean ± SE) to the bidirectional selection on low egg testosterone (LET) and high egg testosterone (HET) yolk concentration over two filial generations: (*A*) androstenedione (A_4), (*B*) progesterone (P_4), and (*C*) estradiol (E_2). Sample sizes (*N* females) are depicted in the bars. *One asterisk*, $P < .05$; *three asterisks*, $P < .001$.

of diethyl ether and petroleum ether (7 : 3). After being evaporated under a stream of nitrogen, dried extracts were reconstituted in 300 μL of phosphate buffer (pH 7.5) and frozen until measurement of T by a radioimmunoassay validated in our laboratory (Okuliarova et al. 2010). Average recovery for yolk T was 65.3% ± 0.3%. Yolk T concentrations were measured in 20-μL aliquots of the extract using [1,2,6,7-^{3}H]-testosterone (Amersham Biosciences; specific activity = 3.52 TBq/mmol) and a specific antibody generated in rabbits against testosterone-3-(carboxymethyl)oxime–bovine serum albumin conjugate. Cross-reactivity of antiserum for the yolk T assay was 9.6% with 5α-dihydrotestosterone (5α-DHT), 0.1% with androstenediol, and <0.1% with other steroids (Zeman et al. 1986). Bound and free T fractions were separated by dextran-coated charcoal and centrifuged at 3,000 rpm for 10 min at −20°C. All samples of the F_0 ($N = 218$), F_1 ($N = 165$), F_2 ($N = 197$), and F_3 ($N = 160$) generations were measured in 9 assays, with an intra-assay variation coefficient ranging from 0.3% to 11.6% and an interassay variation coefficient of 14.7%.

Yolk A_4, P_4, and E_2 concentrations were analyzed from F_1- and F_2-generation eggs in the laboratory of T. G. G. Groothuis. A part of thawed yolk was thoroughly homogenized in deionized water (1 : 2), and a subsample of this emulsion (~200 mg of pure yolk) was used for steroid extraction. Tritiated T (4,500 cpm) was added to each sample and allowed to equilibrate overnight at 4°C, to estimate recoveries. The samples were extracted three times with 2 mL of diethyl ether/petroleum ether (7 : 3). After centrifugation and snap-freezing, the ether phases were decanted, combined, and dried under a stream of nitrogen. The extracts were redissolved in 2 mL of 70% methanol and left to precipitate for 2 days at −20°C. Thereafter, samples were centrifuged, decanted, and dried under a stream of nitrogen. Dried extracts were reconstituted in 200 μL of phosphate-buffered saline. Extraction of plasma samples was performed in the same way as for the yolk samples in the laboratory of T. G. G. Groothuis; 100 μL of plasma was used, and dried extracts were reconstituted in 100 μL of phosphate-buffered saline. The extraction recovery was 67.8% ± 0.3% for yolk samples and 75.4% ± 0.7% for plasma samples. Yolk A_4, P_4, and E_2 concentrations and plasma T concentrations were determined by radioimmunoassay with DSL kits (Diagnostic System Laboratories). Cross-reactivity of antibody specific for T in the plasma assay was 2.3% with A_4. The cross-reactivity of the A_4 antibody with other androgens was <0.02%. The intra-assay variation coefficients ranged from 2.5% to 9.1% for A_4, from 0.4% to 5.2% for P_4, and from 1.9% to 9.2% for E_2, and the coefficient was 8.3% for plasma T. The inter-assay variation coefficients for determination of yolk A_4, P_4, and E_2 were 9.3%, 5.3%, and 4.7%, respectively (plasma T was determined in one assay).

Statistical Analysis

Concentrations of yolk hormones (T, A_4, P_4, and E_2) between the LET and HET line within each generation were

Table 2: Results of a mixed general linear model demonstrating differences in yolk androstenedione (A_4), progesterone (P_4), and estradiol (E_2) concentrations separately between the low egg testosterone and high egg testosterone lines within the first and second filial generations

		F_1 generation			F_2 generation		
	Effect	df	*F*	*P*	df	*F*	*P*
Yolk A_4:							
Intercept	Fixed	1	1,000.95	.000	1	599.59	.000
Line	Fixed	1	20.49	.000	1	28.83	.000
Cage effect	Random	28	.42	.986	34	1.31	.225
Female effect	Random	27	12.82	.000	32	6.34	.000
Error		108			127		
Yolk P_4:							
Intercept	Fixed	1	2,041.94	.000	1	3,381.54	.000
Line	Fixed	1	.78	.385	1	2.21	.144
Cage effect	Random	28	.77	.754	34	.52	.969
Female effect	Random	27	6.83	.000	32	6.47	.000
Error		108			127		
Yolk E_2:							
Intercept	Fixed	1	6,749.02	.000	1	1,366.80	.000
Line	Fixed	1	5.12	.031	1	3.96	.055
Cage effect	Random	28	1.12	.384	34	1.78	.052
Female effect	Random	27	2.58	.000	32	2.52	.000
Error		108			127		

compared using a mixed general linear model. Line was tested as a fixed factor, and the effect of cage nested within line and the effect of female nested within cage were included as random factors. All data were examined to fit the normal distribution, and log transformation was used for yolk T and E_2 concentrations. Plasma T concentrations of adult females were also log transformed and compared between the selected lines with the independent *t*-test. Egg mass, yolk mass, and eggshell mass were statistically analyzed in the same way as yolk hormones.

The realized heritability (h^2) of yolk T concentration was estimated as the slope of the linear regression of the cumulative response to selection (R_C) on the cumulative selection differential (S_C) forced through zero (Falconer 1981). The R_C was calculated as the difference between the mean yolk T concentration for female offspring and for the initial (randomly bred) population. The selection differential (S) was calculated as the difference between the mean yolk T concentration for selected mothers and for the whole population from which they were chosen. The sum of selection differentials resulted in the S_C. These calculations of heritability were made separately for each line and for the entire population, using the divergence between the HET and the LET line.

The heritability of A_4, P_4, and E_2 in egg yolk was estimated on the basis of mother-daughter regression, using twice the slope of the linear regression of the mean value for daughters on the value for mothers (37 pairs; Lynch and Walsh 1998). SEs of regression coefficients were used to estimate SEs of heritability. Grouped data from both the F_1 and F_2 generations of the HET and LET lines were used in the mother-daughter analysis.

The relationships among concentrations of yolk hormones (T, A_4, P_4, and E_2) and between plasma and yolk T levels were evaluated by means of Pearson correlation coefficients. The correlation between plasma and yolk T levels was performed with egg yolks that were in the second and third hierarchical follicle stages (17 LET and 12 HET) when blood samples were obtained from birds. During these follicular stages, maximum T deposition in yolk occurs (Okuliarova et al. 2010).

Results

Response of Yolk T Concentrations to Selection

In our randomly bred population (F_0 generation), yolk T concentrations ranged from 4.5 to 21.1 pg/mg of yolk, with a mean ($\pm$SE) of 9.1 $\pm$ 0.4 pg/mg. After three generations of selection, the mean yolk T concentrations were 6.8 $\pm$ 0.3 and 15.6 $\pm$ 0.9 pg/mg in the LET and HET lines, respectively, whereas in eggs of the randomly bred females (control) the concentrations were found to be in the middle of this range (10.7 $\pm$ 0.7 pg/mg; $N = 20$ females).

Already in the F_1 and subsequently in the F_2 and F_3 generations, yolk T concentrations differed significantly

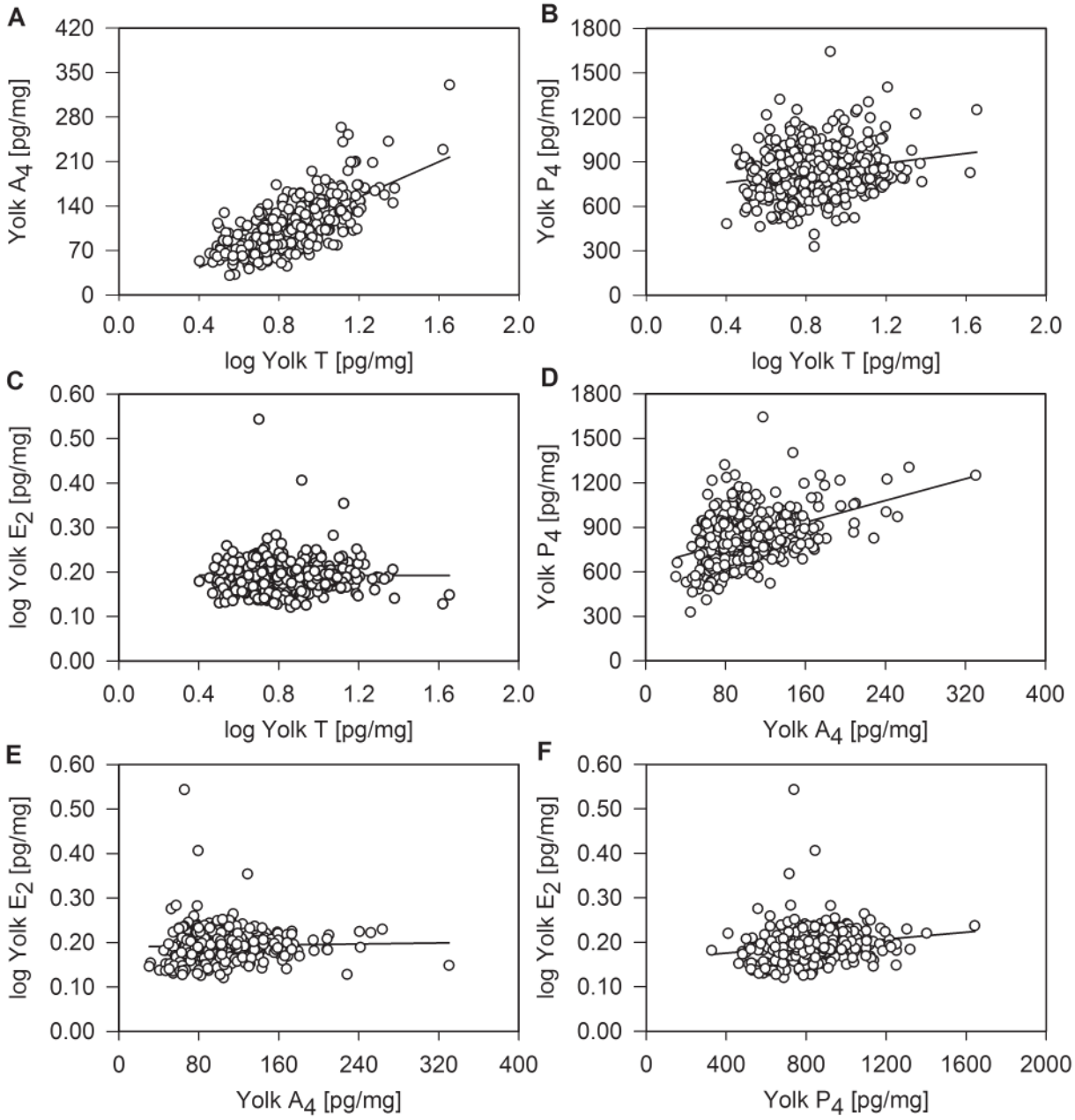

Figure 3: Phenotypic correlations among yolk testosterone (T), androstenedione (A_4), progesterone (P_4), and estradiol (E_2) in eggs of Japanese quail. The sample size ($N = 360$) includes all eggs of each female of the low and high egg testosterone lines of the first and second filial generations.

between the two selected lines in the expected direction, with higher levels in the HET line and lower levels in the LET line (fig. 1*A*; table 1). In addition, females differed consistently from each other within lines, whereas a cage effect was not apparent (table 1).

Realized Heritability of Yolk T Concentrations

The realized heritability of yolk T concentrations based on both selected lines was 0.42 ± 0.03 and differed significantly from zero ($r^2 = 0.97$, $F = 253.48$, df = 1, 3,

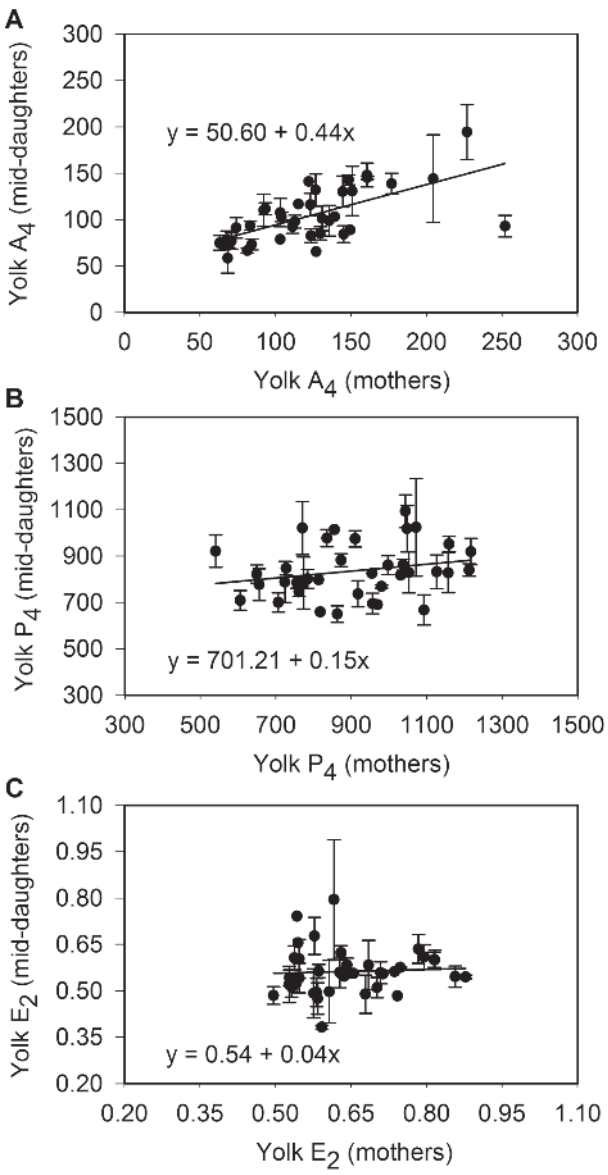

Figure 4: Mother-daughter regressions for yolk (*A*) androstenedione (A_4), (*B*) progesterone (P_4), and (*C*) estradiol (E_2), based on averages ($\pm$SE) for all daughters of the same mother (125 individuals from 37 families).

$P < .01$). The realized heritability was estimated to be 0.47 ± 0.11 in the LET line and 0.40 ± 0.11 in the HET line. In both cases, heritabilities significantly differed from zero (LET: $r^2 = 0.48$, $F = 18.36$, df $= 1, 3$, $P < .05$; HET: $r^2 = 0.70$, $F = 13.76$, df $= 1, 3$, $P < .05$; fig. 1*B*), and the difference in heritability between the lines was not significant ($t = -0.155$, $P = .883$). In addition, mean selection differentials did not differ between lines (-2.49 in the LET line and 4.04 in the HET line; $t = -1.920$, df $= 4$, $P = .127$).

Correlated Response of Unselected Yolk Hormones to Selection

Compared with eggs of the LET line, eggs of the HET line contained significantly higher concentrations of A_4, the androgen precursor of T, in both the F_1 and the F_2 generation (fig. 2*A*; table 2). Such differences were not found in yolk P_4 levels (fig. 2*B*; table 2). Yolk E_2 concentrations showed the opposite pattern compared with both androgens (fig. 2*C*; table 2). As with T, females differed consistently within lines for all three hormones, whereas there was no effect of cage (table 2).

Phenotypic correlations among yolk hormone levels were calculated on the basis of all eggs ($N = 360$) of both lines of the F_1 and F_2 generations (fig. 3). A strong positive correlation was found between yolk T and A_4 concentrations ($r = 0.719$, $P < .001$; fig. 3*A*). Both T and A_4 concentrations were positively related to P_4 concentrations in egg yolk (T vs. P_4: $r = 0.196$, $P < .001$; A_4 vs. P_4: $r = 0.424$, $P < .001$; fig. 3*B*, 3*D*), and a weak positive correlation was found between yolk P_4 and E_2 concentrations ($r = 0.183$, $P < .001$; fig. 3*F*). The correlations between T and E_2 levels ($r = -0.003$, $P = .953$; fig. 3*C*) and A_4 and E_2 levels ($r = 0.031$, $P = .563$; fig. 3*E*) were not significant.

Twice the slope of the linear regression ($2b \pm 2$ SE) of the mean yolk hormone concentration in the eggs of daughters (125 individuals from 37 families of the F_1 and F_2 generations) on the mean value for mother eggs showed a significant heritability estimate for yolk A_4 concentrations ($h^2 = 0.87 \pm 0.18$, $r^2 = 0.41$, $F = 24.508$, df $= 1, 35$, $P < .001$; fig. 4*A*), whereas no significant resemblance between mother and daughters was found for yolk P_4 concentrations ($h^2 = 0.30 \pm 0.22$, $r^2 = 0.05$, $F = 1.917$, df $= 1, 35$, $P = .175$; fig. 4*B*) or for E_2 concentrations ($h^2 = 0.08 \pm 0.25$, $r^2 = 0.003$, $F = 0.108$, df $= 1, 35$, $P = .744$; fig. 4*C*).

Correlated Response of Plasma T Levels to Selection

No differences in circulating T levels between the LET and HET lines were revealed ($t = 0.115$, df $= 68$, $P = .909$; fig. 5). Plasma T concentrations did not correlate with yolk T levels in either the LET line ($r = 0.238$, $P = .358$, $N = 17$) or the HET line ($r = 0.301$, $P = .340$, $N = 12$; fig. 6).

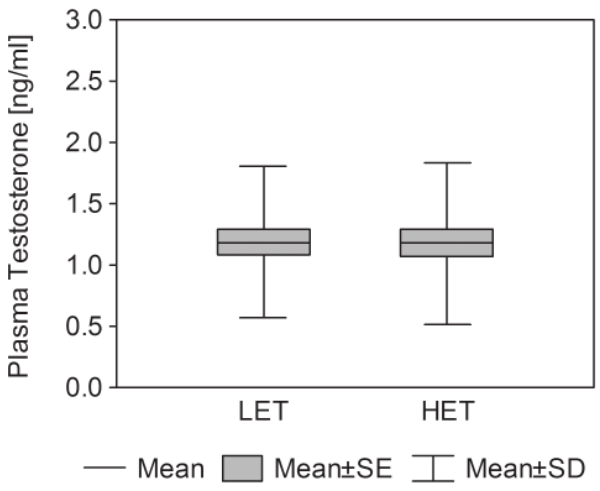

Figure 5: Plasma testosterone concentration in adult female Japanese quail (age of 11 weeks) compared between the low egg testosterone (LET; $N = 35$) and high egg testosterone (HET; $N = 35$) lines in the second filial generation.

Response of External Egg Quality Traits to Selection

External egg quality traits did not differ between the LET and HET lines over three filial generations (all $P > .1$). Mean egg mass, yolk mass, and eggshell mass in the LET and HET lines are summarized in table 3.

Discussion

The aim of this study was to test additive genetic variation for hormone transfer from mother to offspring, its potential underlying pathways, and its relation to circulating hormone levels in the mother. We used a bird model because maternal androgens in avian egg yolk have been demonstrated to profoundly modulate offspring development. These maternal effects have been interpreted as an adaptive phenomenon requiring additive genetic variance, which has not yet been proved.

Evidence for Additive Genetic Effects

Our results show for the first time that maternal T concentrations in egg yolk respond significantly to bidirectional selection, demonstrating a direct contribution of additive genetic variance to the phenotypic variability of this trait. The realized heritability of yolk T concentrations was $h^2 = 0.42$ over both lines and was $h^2 = 0.47$ and 0.40 in the LET and HET lines, respectively. We did not find an asymmetrical response to selection over three generations, whereas heritability estimates and mean selection differentials did not differ between lines. Despite the lack of significance, the selection differential was larger in the HET line than in the LET line, suggesting that the response to selection tended to be stronger in an upward rather than downward direction. This pattern is consistent with other findings indicating that low lines may have more limited potential for selection than high lines (Satterlee and Johnson 1988; Evans et al. 2006).

We are aware of only one published study showing heritability of yolk T levels ($h^2 = 0.75$), calculated as the parent-offspring regression in a wild population of collared flycatcher (Tschirren et al. 2009). This heritability estimate was higher than our results. This is intriguing because that field study could obviously not keep rearing conditions constant, as in our study, and it may suggest that apart from additive genetic variance maternal effects, indirect environmental effects are involved too. Hormones deposited in egg yolk are considered to be well-suited candidates to mediate epigenetic maternal effects on offspring phenotype (Mousseau and Fox 1998). Yolk T has been demonstrated to influence growth (Schwabl 1996; Eising et al. 2001), immune response (Müller et al. 2005; Sandell et al. 2009), and several behaviors of chicks (Eising and Groothuis 2003; Daisley et al. 2005; Okuliarova et al. 2007). Thus, because of the presence of genetic variance for yolk T levels, this trait can possess a maternal indirect genetic effect (Wolf 2003). Such indirect genetic effects are environmental influences on the offspring phenotype that are due to the expression of maternal genes that provide this environment (Wolf et al. 1998). Such effects can strengthen or weaken the direct genetic effects and significantly alter

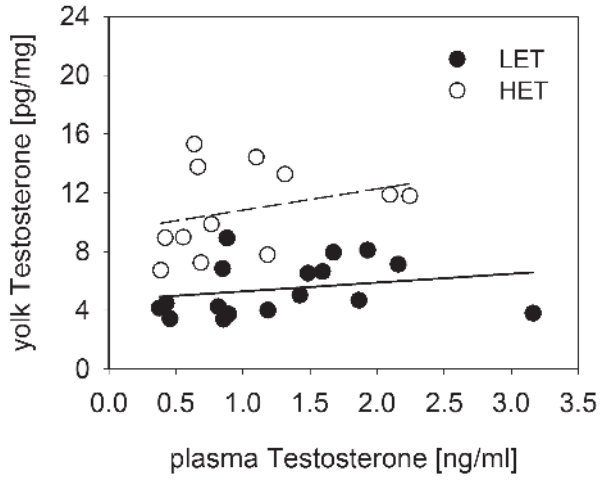

Figure 6: Relationship between plasma and yolk testosterone concentrations in the low egg testosterone (LET; $r = 0.238$, NS, $N = 17$) and high egg testosterone (HET; $r = 0.312$, NS, $N = 12$) lines of Japanese quail. Blood was collected when analyzed eggs were in the second and third hierarchical follicle stages.

Table 3: External egg quality traits (mean ± SE) in the low egg testosterone (LET) and high egg testosterone (HET) lines over three filial generations

	F_1 generation		F_2 generation		F_3 generation	
	LET line ($N = 95$)	HET line ($N = 70$)	LET line ($N = 103$)	HET line ($N = 94$)	LET line ($N = 75$)	HET line ($N = 85$)
Egg mass (g)	9.60 ± .12	9.61 ± .18	8.89 ± .12	8.79 ± .13	8.78 ± .15	8.76 ± .15
Yolk mass (g)	2.83 ± .05	2.86 ± .07	2.59 ± .05	2.57 ± .07	2.71 ± .07	2.69 ± .06
Eggshell mass (g)	.861 ± .018	.890 ± .021	.816 ± .011	.833 ± .015	.786 ± .016	.826 ± .013

Note: N = number of measured eggs.

the evolutionary dynamics of phenotypic characteristics under natural selection (Hunt and Simmons 2002; McAdam and Boutin 2004). Our data indicate the presence of additive genetic variance for yolk T transfer, but potentially elevated steroid levels in yolk may also program daughters to produce eggs with either high or low T levels. To our knowledge, there is no evidence for this possibility, because yolk T concentrations were not affected in eggs of female precocial pheasants (*Phasianus colchicus*) that had hatched from eggs with experimentally increased T content (Rubolini et al. 2007). Moreover, in such a case one would expect an effect of selection in the first generation only, which was clearly not the case in our study.

Target of Selection

To understand which pathway of steroidogenesis was affected by selection, we analyzed its effect on other yolk steroid hormones. A_4, the direct precursor of T in the metabolic pathway, was affected along with T itself, and we detected a strong positive correlation between both androgens in yolk. Moreover, we estimated significant heritability of yolk A_4 concentrations on the basis of mother-daughter regression. In the study with collared flycatchers, the investigators did not find heritable variation for yolk A_4 and argued that it may be more sensitive to environmental circumstances than T (Tschirren et al. 2009). In our study, which provided a much more constant environment, the strong correlative response of yolk A_4 to selection, together with its high heritable variation, indicates that the selection did not affect the rate of conversion from A_4 to T by the enzyme 17β-hydroxysteroid dehydrogenase and suggests that the biosynthesis of T further upstream in the pathway was targeted.

Intriguingly, yolk P_4 was not affected by selection. Its concentrations did not differ between the lines, and the mother-daughter regression did not reveal significant heritability. Both findings suggest that the conversion from P_4 to A_4 in the Δ^4 pathway was not altered. Therefore, the selection may preferentially act on the Δ^5 pathway, in which A_4 is formed from pregnenolone through dihydroepiandrosterone (DHEA; Robinson and Etches 1986). Because P_4 is a biologically active hormone—in contrast to DHEA, which has a very low affinity to androgen receptors—the results suggest that there is specific genetic variance for T deposition circumventing possible biological effects mediated via P_4.

Although yolk concentrations of E_2, a biologically active metabolite of T, were slightly lower in the HET line than in the LET line, we did not find a significant heritability for this hormone based on mother-daughter regression. These results indicate that the selection may specifically affect the additive genetic variance of yolk androgens. Reduced levels of yolk E_2 can result from increased conversion of T to 5α-DHT, a biologically active androgen that was not measured in our study. Nonetheless, given that 5α-DHT has been demonstrated to be present in avian egg yolk (Groothuis et al. 2005) and can affect offspring phenotype (Schwabl et al. 2007), further studies should also take this hormone into account.

Mechanism of Hormone Accumulation

The third aim of our study concerned the question of whether mothers are able to regulate yolk hormone levels, influencing offspring development independently of their own circulating hormone levels. If not, mothers are faced with a trade-off between hormone effects on their offspring and on themselves, constraining the evolution of hormone-mediated maternal effects (Groothuis and Schwabl 2008; Moore and Johnston 2008). The selection on yolk T provided a unique opportunity to test these possibilities by correlating plasma hormone levels in the female around the time of egg production with those in her eggs. In contrast to yolk androgen concentrations, plasma T concentrations of the females did not differ between the LET and HET lines in the F_2 generation, and there was no correlative relationship between yolk and plasma T levels sampled in a follicular stage, when maximum T deposition occurs (Okuliarova et al. 2010). Contrary to previous suggestions (reviewed in Groothuis and Schwabl 2008), this finding indicates that mothers may not be faced with the above-mentioned trade-off. Furthermore, the absence of a response of systemic T con-

centrations to selection for egg yolk levels suggests that maternal effects via egg hormones are not just an epiphenomenon of the reproductive system or an exadaptation (Groothuis et al. 2005). Natural selection seems to have specifically shaped the mechanisms of hormone transfer to the egg in order to not disrupt physiological ranges of T levels in the mother, which are essential for her own reproductive processes. This is consistent with the fact that these hormones are produced in the tissue surrounding the follicle (e.g., Bahr et al. 1983; Groothuis and Schwabl 2008), facilitating hormone accumulation in the developing ovum separately from uptake in the maternal circulation. A recent study applying labeled hormone indeed found very low uptake of T from the plasma into developing eggs in a lizard species (Cohen and Wade 2010).

In conclusion, our results demonstrate heritable genetic variation of transgenerational hormone transfer in a bird model, indicating that the process of yolk hormone accumulation can be a target of natural selection and may be a fitness-related trait. In addition, the results indicate that selection for T in egg yolk specifically affected androgen deposition, leaving other biologically active steroids in the yolk and T in the maternal circulation unaffected. This strongly supports the interpretation that yolk hormone deposition has been shaped by natural selection without posing severe constrains on the mother. The selection model provides an alternative approach to the study of hormone-mediated maternal effects, which up to now have been investigated either by direct manipulation of hormonal content in avian eggs or by manipulation of the social and ecological conditions experienced by females during the egg-laying period. Artificial selection would enable testing of the consequences of differences in androgen levels within the physiological range of the species, a requirement not always met by egg-injection studies.

Acknowledgments

This study was supported by the Slovak Research and Development Agency (APVV 0047-10) and the Scientific Grant Agency of the Ministry of Education of the Slovak Republic and of the Slovak Academy of Sciences (VEGA 1/0365/10).

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Associate Editor: Ellen D. Ketterson
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Cyanura macrolopha (long-crested jay): "When I was travelling westward in the spring of 1864, I saw some of these jays in the Raton Mountains, in New Mexico. ... An elegant dashing fellow, of good presence if not good manners. ... A stranger to modesty, and forbearance." From "The Long-Crested Jay" by Elliott Coues (*American Naturalist*, 1871, 5:770–775).