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Effects of prenatal yolk androgens on armaments and ornaments of the ring-necked pheasant

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Abstract Mothers may profoundly affect offspring phenotype and performance by adjusting egg components, including steroid hormones. We studied the effects of elevated prenatal testosterone (T) exposure in the ring-necked pheasant on the expression of a suite of male and female traits, including perinatal response to stress, immune response, growth, and secondary sexual traits. Prenatal T levels were increased by injecting the yolk of unincubated eggs with physiological doses of the hormone. Yolk T injection resulted in a reduced length of male tarsal spurs, a trait which positively predicts male success in intra- and intersexual selection and viability, whereas no direct effect on male wattle characteristics or plumage traits of either sex was observed. Female spur length was also negatively affected by T, but to a lesser extent than in males. In addition, the covariation between male secondary sexual traits, which are reliable quality indicators, differed between T and control males, suggesting that the manipulation may have altered the assessment of overall male quality by other males and females. In conclusion, the negative effects of elevated yolk T on spur length, a trait which positively predicts male fitness, coupled with the lack of effects on growth or other traits in both sexes, provided limited evidence for mothers being subjected to a trade-off between positive and negative consequences of yolk T deposition on offspring traits and suggest that directional selection for reduced yolk T levels may occur in the ring-necked pheasant.

Keywords Maternal effects · Multiple ornaments · *Phasianus colchicus* · Secondary sexual traits · Sex-specific effects · Testosterone

Introduction

Variation in egg composition can have profound consequences on the phenotype of the offspring (Williams 1994; Mousseau and Fox 1998; Price 1998; Dufty et al. 2002; Gil 2003; Groothuis et al. 2005a). By adjusting egg quality, individual females may thus influence offspring performance, under the limitations imposed by extrinsic conditions and maternal physiological constraints (Mousseau and Fox 1998; Price 1998; Groothuis et al. 2005a). The expression of such maternal effects mediated by egg quality may depend on maternal conditions and maternal environment or may be affected by complex interactions between maternal and offspring genes, and extrinsic factors (Dufty et al. 2002). Since maternal effects can enhance phenotypic variation, for example through epistatic effects, which have the potential to affect the rate and direction of evolutionary changes (Wade 1998; Wolf et al. 1998), they have become the focus of increasing research interest.

Cleidoic eggs of birds are provisioned by the mother with quantitatively minor components, including sex hormones (Schwabl 1993; reviews in Gil 2003; Groothuis et al. 2005a). Sex hormones are likely candidates as mediators of early maternal effects because maternal hormonal profiles and hormone transfer to the eggs may vary according to contingent maternal state and environmental conditions (Dufty et al. 2002; reviews in Gil 2003; Groothuis et al. 2005a). In addition, variable concentrations of egg hormones can have marked effects on offspring phenotype, and these effects may depend on the offspring genetic makeup, as simply exemplified by sex-related variation in susceptibility to sex hormones (Adkins-Regan et al. 1995; Williams 1999; von Engelhardt et al. 2004; Rubolini et al. *in press*). In fact, in vertebrates, sex hormones are known to exert pervasive effects on the development of several organs and apparatus, including for example the brain, and variation in the

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hormonal milieu during early life stages therefore has the potential to affect several traits related to individual performance and fitness (Dufty et al. 2002; reviews in Gil 2003; Groothuis et al. 2005a).

In birds, the function of the transmission of variable amounts of androgens has been addressed by a number of experimental studies that have adopted a manipulative approach by increasing the concentration of hormones in the eggs (e.g., Schwabl 1996; Eising et al. 2001; Eising and Groothuis 2003; Andersson et al. 2004; Groothuis et al. 2005b; Rubolini et al. *in press*; Saino et al. *in press*). An increase in yolk androgens results in shorter time to hatching (Eising et al. 2001; Eising and Groothuis 2003) and enhances the development of specific embryonic muscles (e.g., the hatching muscle; Lipar and Ketterson 2000) or postnatal growth (Schwabl 1996; Eising et al. 2001). However, androgens can also postpone hatching, and negative effects or no effects of yolk androgens on phenotype or viability have been reported (Sockman and Schwabl 2000; Navara et al. 2005; Henry and Burke 1999; Sockman and Schwabl 2000; Andersson et al. 2004; Uller et al. 2005; Rubolini et al. *in press*). In addition, development and functioning of the vertebrate immune system are believed to be generally negatively influenced by androgens (e.g., Folstad and Karter 1992; Duffy et al. 2000; Belliure et al. 2004; Groothuis et al. 2005b; see review in Grossman 1984). For example, in chickens, exposure to elevated androgen levels early in life can accelerate the regression of a major primary immune organ, the bursa of Fabricius, with potential negative consequences for humoral acquired immunity (e.g., Hirota et al. 1976; Glick 1984).

Therefore, at present, no general conclusion can be drawn concerning the consequences of maternal egg androgens for the offspring in avian species. Variation in egg hormone concentration may depend on transgenerational trade-offs, whereby high androgen levels in the mother may enhance offspring quality while having detrimental effects on the mother itself (Staub and de Beer 1997; Groothuis et al. 2005a). However, variation in the sign of the effect of androgens depending on the phenotypic value of the specific offspring trait under scrutiny strongly suggests that maternal decisions on transfer of androgens to the eggs may also result from complex trade-offs between antagonistic effects on different traits (reviews in Gil 2003; Groothuis et al. 2005a). This scenario may be further complicated if the effects of androgens vary with the sex of the offspring because of differential susceptibility of males and females to androgenizing factors or of different metabolic processing of egg androgens, which may be aromatized to estrogens at different rates in the two sexes. However, only few studies analyzed sex-related effects of egg androgens in birds, finding either negative effects of elevated egg androgens on female phenotype (e.g., Henry and Burke 1999; Rubolini et al. *in press*; Saino et al. *in press*) or no differential effects in relation to sex (Schwabl 1996; Strasser and Schwabl 2004; Uller et al. 2005).

Despite the fact that sex hormones are among the main determinants of morphological and functional differenti-

ation of the two sexes, there is relatively little information on the organizational effects of androgens on male sexual traits. Experimental manipulation of testosterone (T) in house sparrow (*Passer domesticus*) eggs resulted in males having a large badge, which is a sexually selected trait (Strasser and Schwabl 2004). In addition, individuals of both sexes originating from testosterone-treated eggs were more likely to prevail in dominance relationships at a food source compared to individuals of the same sex originating from control eggs (Strasser and Schwabl 2004). In the Chinese quail (*Coturnix chinensis*), yolk T inoculation resulted in reduced testes mass, but no effects on sexual plumage ornamentation were detected (Uller et al. 2005).

The aim of this study was to analyze the organizational effects of an increase in T levels within the physiological limits in unincubated eggs of the ring-necked pheasant (*Phasianus colchicus*) on a diverse suite of traits in the two sexes. At hatching and at later ages (until completion of skeletal development), we measured body mass and size (as reflected by tarsus length) (hereafter referred to as “ordinary” morphological traits), *in vivo* T-cell-mediated immune response, and innate fearful response to stressful conditions resulting from handling (“tonic immobility” response; Gallup 1977; Jones 1986, 1989). In addition, we measured a number of sexually dimorphic ornamental traits, including length of the tail and spurs in both sexes and length of head feather ornaments (ear tufts) and wattle characteristics (size and intensity of the red coloration) of males (hereafter referred to as “sexual” traits). Finally, we analyzed the pattern of covariation between male secondary sexual characters in individuals originating from T-injected and sham-injected eggs.

Materials and methods

Study organism

The ring-necked pheasant is a large (≈ 1 kg), polygynous galliform with uniparental maternal care of the progeny (Cramp 1998). Males vigorously defend mating territories, and those that are not successful in defending a territory usually do not acquire mates (Hill and Robertson 1988; Biadi and Mayot 1990; Cramp 1998). Sexual dimorphism is marked, both in terms of size and coloration: males are larger and have a colorful plumage, whereas females are cryptic (Cramp 1998). Males have ear tufts and red wattles, which are absent in females. Both sexes possess a long tail and spurs, which are considerably longer in males than in females (Cramp 1998). Different male ornaments seem to have partly different roles in sexual selection (see review in Mateos 1998). Head ornaments, including wattle size and ear tufts, and plumage brightness appear to be mainly relevant to male-male interactions both during the breeding season and after breeding, when males may gather in unisexual groups, where aggressions frequently occur (Hill and Robertson 1988; Mateos and Carranza 1997; Cramp 1998). However, ear tufts and tail length are also known to

influence female mate choice, larger and showy ornaments being preferred to smaller and drab ones (Mateos and Carranza 1995). The role of spurs in sexual selection may also be dual: von Schantz et al. (1989) showed experimentally that spur length affects harem size, whereas other studies revealed that spur length did not directly affect female choice but is rather important in predicting the outcome of male-male competition, dominant males showing longer spurs than subordinates (Mateos and Carranza 1996). In this study, sexual traits were measured during January, at 7 months of age (see below), when male and female plumage traits are fully developed and males begin to defend breeding territories in southern Europe (Mateos 1998; Papeschi et al. 2000). Previous studies showed that male traits measured at this time of the year are reliable predictors of male social rank during the breeding season (Papeschi et al. 2003). In addition, male wattle size in January is positively correlated with circulating androgen levels (Papeschi et al. 2000), which are known to enhance aggressiveness and dominance in intrasexual interactions (Briganti et al. 1999).

General procedures, subjects, and housing conditions

The study was carried out at a game farm during 2004–2005. In spring, we randomly selected 800 eggs from a set of eggs laid over 1 week by approximately 1,000 females kept in 155 polygynous groups under natural photoperiodic conditions. Natural clutch size ranges between 8 and 15 eggs (Cramp 1998), but under intensive farming conditions, females lay eggs continuously between the end of March and the end of June. Eggs were collected daily and maintained at 16°C until the day of treatment, which occurred within a maximum of 7 days after laying. To assign the eggs to either experimental group (T injection or inoculation with the vehicle only), the eggs were first arranged in random sequence and then split in two groups of 400 eggs. The eggs of each group were then randomly assigned to subgroups of five eggs (pentads). The eggs of the first pentad were injected with T, those of the second pentad were injected with the vehicle (sesame oil) and served as controls, and so forth, alternating the treatments between consecutive pentads. Each of the two groups of 400 eggs was treated by a different experimenter on a single day. This procedure implies that each injector treated the same random sample of eggs for each experimental group. Prior to injection, eggs were left with the acute pole upwards for approximately 30 min. The eggshell at the acute pole was carefully cleaned and disinfected, and a hole was opened with a heat-sterilized pin of the same diameter as the needle used for injection. Injection was made using a 250- μ l Hamilton syringe mounting a 25-gauge, 16-mm-long needle. The hole was sealed by glueing a small piece of eggshell immediately after injection. In order to check whether injection occurred in the yolk, we performed preliminary trials on pheasant eggs, where we injected a solution of a red food dye in sesame oil by completely

inserting needles of different lengths in the egg. These eggs were deep-frozen immediately after injection and subsequently dissected while still frozen to check for the location of the red food dye in the egg. Injections using the 16-mm-long needle were well within the yolk in all of ten trial eggs.

The amount of T injected was decided based on the variation recorded in a sample of 73 pheasant eggs (data kindly provided by F. Dessì-Fulgheri, Università di Firenze). We injected 40 ng T (4-androsten-17- β -ol-3-one; Sigma, Germany) dissolved in 20 μ l sterile sesame oil, corresponding to two standard deviations of the total amount estimated for the yolk in the sample of 73 eggs. However, we also checked for T concentration in a random sample of 13 eggs belonging to the same set from which those included in the experiment were extracted. Mean T concentration (see below for assay) was 6.95 ng/g (2.28 SD), yielding an estimated mean amount of 73.4 ng (24.0 SD) for an average yolk of 10.6 g. Thus, the amount of T we injected increased the concentration of the hormone in the egg by 1.67 SDs, as estimated in the same population of females at the same time of the season (see also Romano et al. 2005). Control eggs were injected with 20 μ l sterile sesame oil using the same procedure as for T injection. Although maternal yolk androgen concentration has been shown to vary with laying sequence in several species (see review in Gil 2003), no information is available for pheasants, and the laying order of individual eggs could not be assessed in this study due to constraints imposed by game-farming practices. However, eggs used in the present study were assigned to treatments at random, so that variation of yolk hormones with the laying sequence should not confound our experimental manipulation. All the eggs were incubated together in the same incubator on six different layers (three layers for each treatment). Eggs from the two treatments were not mixed in order to distinguish chicks from the two groups at hatching, and treatments were assigned randomly to layers. Chicks could thus be assigned to their original treatment but could not be assigned to their original egg because newly hatched chicks cannot be individually separated in layers of large commercial incubators. Paternity or maternity of individual chicks could not be assigned because of practical limitations imposed by intensive game farming and the lack of available highly polymorphic microsatellite markers for this species (Baratti et al. 2001). Newly hatched chicks were marked with a numbered plastic band that was replaced with bands of a larger size at later ages. Out of 400 eggs for each group, 178 (44.5%) T-injected and 171 (42.8%) control eggs hatched, a nonsignificant difference ($\chi^2=0.25$, $df=1$, $P=0.61$). Egg failures in artificially incubated eggs at the game farm where the study has been conducted usually account for approximately 15–20%. Injection in the yolk therefore caused an increase in hatching failure by approximately 35–40%. All birds were kept in a single group in a large room under controlled thermal conditions until 35 days of age and later transferred to a 10 \times 40 \times 3-m outdoor aviary until day 210 (at 7 months of age), under natural photoperiodic and atmospheric conditions. Water and food were provided ad libitum.

Measurement of ordinary traits, tonic immobility, and immunity

At hatching (day 0, i.e., within 24 h after the chicks were extracted from the incubator), and on days 15 and 90 after hatching, we measured the length of the tarsus that did not wear the band using a digital caliper (≈ 0.01 mm) and the body mass using an electronic balance (≈ 0.01 g at hatching and day 15) or a spring balance (≈ 1 g at day 90). Tarsus measurements at day 90 are likely to reflect tarsus length of adult birds, as shown by comparisons of mean values of our experimental birds with those reported by Meriggi (1992) for adult pheasants (*t* test, males: $t_{197}=0.87$, $P=0.39$; females: $t_{218}=0.62$, $P=0.54$). At hatching, before taking morphological measurements, we also measured tonic immobility, which is a catatonic-like, fear-potentiated state of reduced responsiveness induced by physical restraint, commonly observed in galliforms and other avian taxa (Gallup 1977; Erhard et al. 1999). Tonic immobility response was induced by applying a modified version of the protocol devised by Jones (1989), by placing each chick on its back in a U-shaped container and restraining it with the hand for approximately 10 s. When released, the chicks usually right themselves within minutes. However, considerable variation exists among chicks (e.g., Erhard et al. 1999; Rubolini et al. 2005), and variable duration of the tonic immobility posture is considered as a measure of innate fearfulness in birds. Trials lasted a maximum of 2 min. If the chick had not uprighted itself within the time of the trial, we scored 120 s. Only 0.7% of the chicks had not uprighted themselves at the end of the trial.

At day 15, we measured the intensity of the T-cell-mediated immune response according to a standard *in vivo* cutaneous hypersensitivity test (Lochmiller et al. 1993; Saino et al. 1997). We first measured the thickness of both wing webs using a pressure-sensitive micrometer (≈ 0.01 mm). The web of the right wing was then injected with 0.2 mg phytohemagglutinin (PHA) dissolved in 0.05 ml phosphate buffered saline (PBS), and the same amount of PBS was injected in the left wing web to serve as a control. Twenty-four hours later, the thickness of both wing webs was measured again. The change in thickness of the wing web injected with PHA minus the change in thickness of the wing web injected with PBS was used as an index of T-cell-mediated immune response, according to several previous studies (Lochmiller et al. 1993; Saino et al. 1997).

Measurement of sexual traits

At day 210 (at 7 months of age), we measured the length of the longest tail feather (both sexes) using a ruler (≈ 1 mm), the length of both ear tufts (males) (with a ruler, ≈ 1 mm), and the length of the spur (including tarsus width, e.g., von Schantz et al. 1989; Göransson et al. 1990) of the leg that did not wear the band (both sexes) using a digital caliper (≈ 0.01 mm). The mean value of controlateral ear tufts was used in the analyses. Feather measurements were not taken on individuals with broken feather tips (see Table 2 for sam-

ple sizes). A standard picture of the head of males was taken with a digital camera (Nikon Coolpix 5700) under constant light conditions [1,000-W halogen bulb mounted on a Kaiser 3085 portable photographic lamp; the light was filtered by an azure transparent conversion filter (Spotfilter Blu 201) from artificial to natural daylight (5,600 K)] in a dark room. Pictures were taken against a gray baseboard, with a thin 5-cm plastic ruler (divisions 1 mm) held just above the comb. Two standard reference color chips were placed on the baseboard and included in each picture. For each picture, we obtained the wattle area (area of the red fleshy wattle, wattle size hereafter) by measuring the number of pixels (“Lasso tool” in Adobe Photoshop 7.0) and appropriately transforming it to a true surface measurement (square centimeter) by means of the reference ruler placed on the head. Wattle size was highly repeatable, as estimated on a random sample of 12 males that were photographed twice ($F_{11,12}=30.86$, $P<0.001$; repeatability=0.94, according to Sokal and Rohlf 1995). We also obtained an estimate of the intensity of the red color component of the wattle on the red-green-blue (RGB) scale, given that wattle redness could be a sexually selected trait, as it is a carotenoid-dependent trait which reliably reflects male nutritional conditions (Ohlsson et al. 2002, 2003). Red intensity (R) was estimated as the mean red intensity of each pixel of a standard elliptical surface (60×30 pixels, totalling 1420 pixels), centered on the wattle at 1.2 cm below the eye. The ellipse was always fully comprised within the wattle in all pictures. In order to control for slight among-pictures variation in illumination, the R color component was corrected for each picture using the two reference color chips, following the procedures outlined in Villafuerte and Negro (1998). The corrected red intensity value was then divided by its luminosity, which is a function of all three corrected primary color components (red, green, and blue) (see Villafuerte and Negro 1998 and <http://www.ebd.csic.es/rv/index.html> for details of the procedure). This standardized red intensity (wattle color hereafter) was significantly repeatable, as estimated on a random sample of 12 males that were photographed twice ($F_{11,12}=6.34$, $P=0.002$; repeatability=0.72). Wattle size or color measurements could not be obtained for a few individuals because the head resulted in an incorrect position in the picture. This explains the discrepancies in the size of the samples reported in Table 2. In all statistical analyses, we used always the maximum available sample size.

Two people injected the eggs, performed the tonic immobility and immunity tests, and measured all the morphological variables, always blindly of the treatment of individual eggs and chicks. In all analyses, wattle size (square centimeter) was square-root-transformed to account for allometric relationships with linear measurements (such as tail, ear tufts, and spur length).

Testosterone assay

To assay testosterone in the 13 unincubated reference eggs (see above), the yolk was separated from the albumen and

stored at -20°C . Before assay, whole yolks were accurately mixed. One hundred mg of yolk was suspended in 1 ml water, and 500 μl of the suspension was extracted in 4 ml diethyl ether. The suspension was shaken for 2 min and centrifuged for 10 min. Diethyl ether was separated from water by immersion in a bath of liquid nitrogen and evaporated under a nitrogen flow. The pellet was resuspended in iso-octane saturated with ethylene glycol. T was separated from the other ether-soluble components on diatomaceous earth

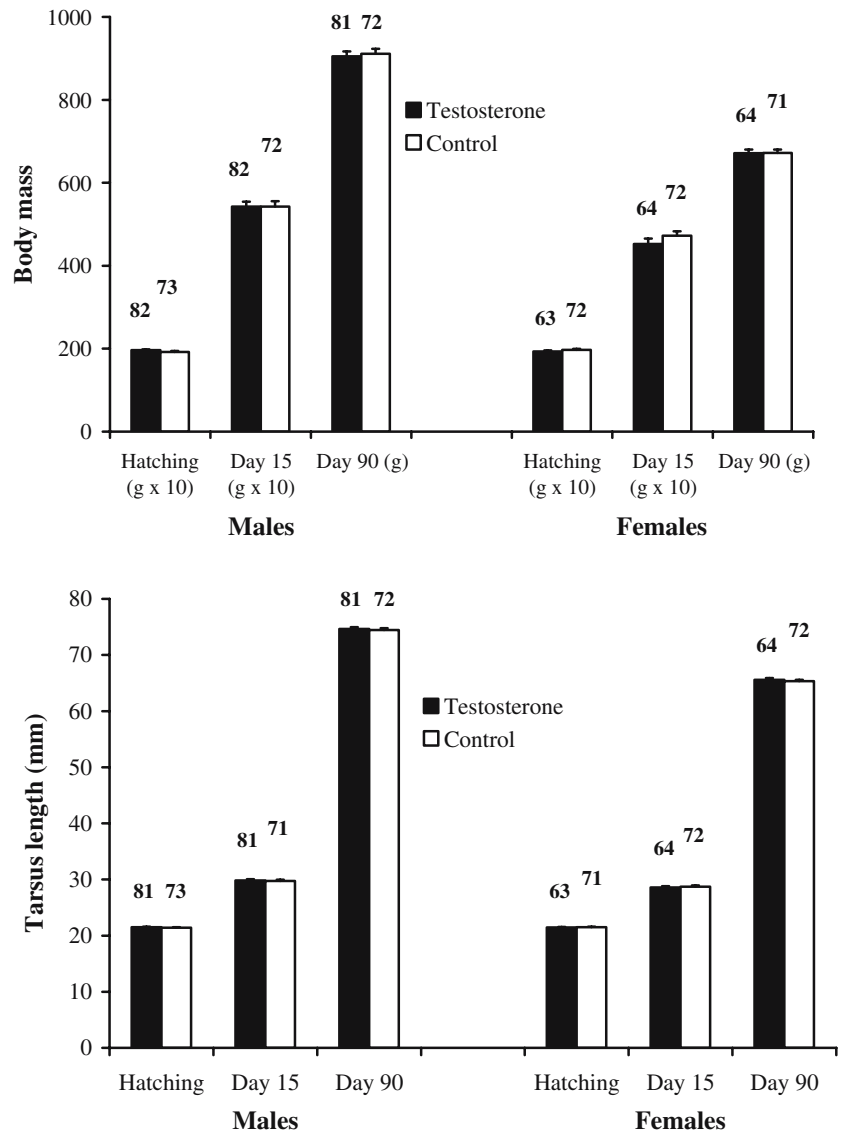
chromatography columns. T was assayed using ^{125}I radioimmunoassay kit purchased from ORION Diagnostica (Espoo, Finland). All eggs were analyzed in a single assay. Intra-assay coefficient of variation, as determined on a single sample assayed in triplicate, was 13.0%. Based on these analyses, the dose of T that was injected increased the concentration of the hormone by 1.67 SDs of the mean recorded in the 13 reference eggs (see above and Romano et al. 2005).

Table 1 Analyses of variance of phenotype of ring-necked pheasants in relation to sex and egg treatment (T or control)

	Model with interaction			Model without interaction		
	<i>df</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>F</i>	<i>P</i>
Hatching (day 0)						
Body mass						
Treatment	1	0.03	0.86	1	0.09	0.77
Sex	1	0.15	0.70	1	0.16	0.69
Treatment \times sex	1	2.99	0.09			
Error	286			287		
Tarsus length						
Treatment	1	0.02	0.88	1	0.04	0.84
Sex	1	0.17	0.68	1	0.17	0.68
Treatment \times sex	1	0.60	0.44			
Error	284			285		
Tonic immobility						
Treatment	1	2.20	0.14	1	2.50	0.12
Sex	1	1.74	0.19	1	1.77	0.18
Treatment \times sex	1	2.09	0.15			
Error	285			286		
Day 15						
Body mass						
Treatment	1	0.64	0.43	1	0.57	0.45
Sex	1	42.52	<0.001	1	42.56	<0.001
Treatment \times sex	1	0.66	0.42			
Error	286			287		
Tarsus length						
Treatment	1	0.01	0.92	1	0.004	0.95
Sex	1	22.78	<0.001	1	22.84	<0.001
Treatment \times sex	1	0.32	0.57			
Error	284			285		
Immune response						
Treatment	1	0.72	0.40	1	0.63	0.43
Sex	1	0.04	0.84	1	0.04	0.85
Treatment \times sex	1	0.47	0.50			
Error	280			281		
Day 90						
Body mass						
Treatment	1	0.11	0.74	1	0.13	0.72
Sex	1	498.17	<0.001	1	499.79	<0.001
Treatment \times sex	1	0.08	0.78			
Error	284			285		
Tarsus length						
Treatment	1	0.47	0.49	1	0.47	0.49
Sex	1	882.02	<0.001	1	885.12	<0.001
Treatment \times sex	1	0.01	0.94			
Error	285			286		

See Fig. 1 for sample sizes of each treatment by sex group

Fig. 1 Mean (SE) body mass and tarsus length of male and female ring-necked pheasants originating from testosterone-injected or sham-injected control eggs recorded at hatching and 15 or 90 days after hatching. Sample sizes are reported above bars. For representation on the same scale, body mass at hatching and at day 15 are multiplied by 10



Statistical analyses

The effects of elevated yolk T on the trait of interest were investigated by means of analyses of variance. Independent factors were treatment of the egg of origin (T-injected or control) and sex (when relevant). Interaction terms were removed from the model when not significant. The association between pairs of sexual traits was investigated by means of analyses of covariance, where one trait was entered as the dependent variable and the other trait as a covariate, whereas treatment was included as a two-level factor. In these analyses, which were run separately for each sex, the treatment \times covariate interaction was included to test for a difference in the slopes of the relationship between traits in the two experimental groups. However, analyses of covariance imply the definition of a dependent and an independent variable (the covariate) (Sokal and Rohlf 1995). In the ring-necked pheasant, we could not establish causal relationships between traits. We therefore investigated whether the co-

Table 2 Mean (SE; *n*) for sexual traits of the ring-necked pheasant according to sex and experimental group (T or control)

Character	Males		Females	
	T	Control	T	Control
Spur length (mm)	20.0 (0.21; 61)	21.2 (0.20; 56)	8.5 (0.08; 59)	8.7 (0.06; 68)
Tail length (cm)	46.6 (0.41; 62)	46.4 (0.49; 55)	24.8 (0.21; 60)	25.0 (0.21; 68)
Ear tuft length (mm)	18.2 (0.23; 62)	18.3 (0.24; 57)	–	–
Wattle size (cm ²)	2.48 (0.05; 53)	2.43 (0.05; 51)	–	–
Wattle color	2.25 (0.04; 52)	2.19 (0.05; 50)	–	–

Sample size varies between traits because of missing data (see “Materials and methods”). See Table 3 for statistical analyses

Table 3 Analyses of variance of sexual traits in relation to egg treatment and sex (when relevant) of ring-necked pheasant

	<i>df</i>	<i>F</i>	<i>P</i>
Spur length			
Treatment	1	25.70	<0.001
Sex	1	6540.65	<0.001
Treatment × sex	1	11.61	0.001
Error	240		
Tail length			
Treatment	1	0.001	0.98
Sex	1	4060.22	<0.001
Error	242		
Ear tuft length			
Treatment	1	0.02	0.89
Error	117		
Wattle size			
Treatment	1	0.57	0.45
Error	102		
Wattle color			
Treatment	1	0.75	0.39
Error	100		

See Table 2 for sample sizes

variation between pairs of traits differed in slope among treatments by running, for each pair of variables, two separate analyses in which the dependent variable and the covariate were swapped (see Table 4). In fact, results of statistical tests on factor × covariate interaction terms are sensitive to the structure of the model, i.e., whether the model is built using either variable as a covariate or as a dependent variable. All statistical analyses were performed by means of the SPSS 11.5 software.

Results

Effects of yolk T on ordinary traits, tonic immobility, and immune response

Yolk T inoculation had no effect on egg hatchability (see “Materials and methods”), survival, and offspring sex ratio at day 90, both T-treated and control eggs originating 145

individuals (male to female ratio; T eggs 81:64, control eggs 73:72). For T eggs, there was a tendency to originate more males than females (binomial $P=0.18$), although the difference was far from significance on a two-way contingency table ($\chi^2=0.89$, $df=1$, $P=0.34$). The effect of hormone treatment on body size and mass of hatchlings and the young at later ages did not differ between the sexes (Table 1). After removal of the interaction term, T treatment had no effects on body mass or size at all ages (Table 1, Fig. 1). There was no sexual dimorphism in mass or size at hatching, whereas males were larger and heavier than females at days 15 and 90 (Table 1). Although we could not control for the effect of egg size (see “Materials and methods”), we performed an analysis of variance of mass and tarsus increase between day 0 and day 15 (i.e., over the linear part of the growth curve) in relation to treatment and sex in order to account for initial size differences between chicks. This analysis confirmed that mass and tarsus growth rates were unaffected by T treatment in both sexes [treatment × sex interaction: body mass, $F_{1,284}=0.42$, $P=0.52$; tarsus length, $F_{1,280}=0.11$, $P=0.75$; effect of treatment (interaction removed): body mass, $F_{1,285}=0.49$, $P=0.48$; tarsus length, $F_{1,281}<0.01$, $P=0.97$].

At hatching, the behavioral response to acute stress arising from handling (“tonic immobility”) did not differ between sexes or treatments [mean for T males: 6.86 s (1.46 SE), $n=81$; control males: 13.18 s (3.24 SE), $n=73$; T females: 7.14 s (1.64 SE) $n=63$; control females: 7.22 s (1.73 SE), $n=72$; Table 1]. Egg T treatment did not differentially affect the intensity of the T-cell-mediated immune response in the two sexes [mean wing web swelling, T males: 1.24 mm (0.06 SE), $n=83$; control males: 1.23 mm (0.06), $n=71$; T females: 1.27 mm (0.07), $n=62$; control females: 1.18 mm (0.08), $n=68$; Table 1]. In the simplified model, the intensity of the immune response was unaffected by treatment or sex (Table 1).

Effects of yolk T on sexual traits

Two homologous sexually dimorphic traits were measured in both sexes, i.e., spur and tail length. In both sexes, individuals originating from T eggs had shorter spurs (Tables 2, 3). However, the effect of T treatment on spur

Table 4 *F*-values for the treatment × covariate interaction term in analyses of covariance (including egg treatment as a factor and each of the other traits as a covariate) of each secondary sexual trait of ring-necked pheasant males

	Spur length	Tail length	Ear tuft length	Wattle size	Wattle color
Spur length	–	NS	NS	$F_{1,97}=4.62^*$	NS
Tail length	NS	–	$F_{1,113}=10.63^{***}$	$F_{1,97}=8.97^{**}$	NS
Ear tuft length	NS	$F_{1,113}=9.88^{***}$	–	NS	NS
Wattle size	$F_{1,97}=5.67^*$	$F_{1,97}=8.75^{**}$	NS	–	NS
Wattle color	NS	NS	NS	NS	–

The upper triangle of the matrix includes the effects of the interaction term when column variables were considered as dependent variables and row variables as covariates, whereas the lower triangle shows the same effects when variables were swapped (see “Statistical analyses” and “Results” for explanation)

NS Not significant

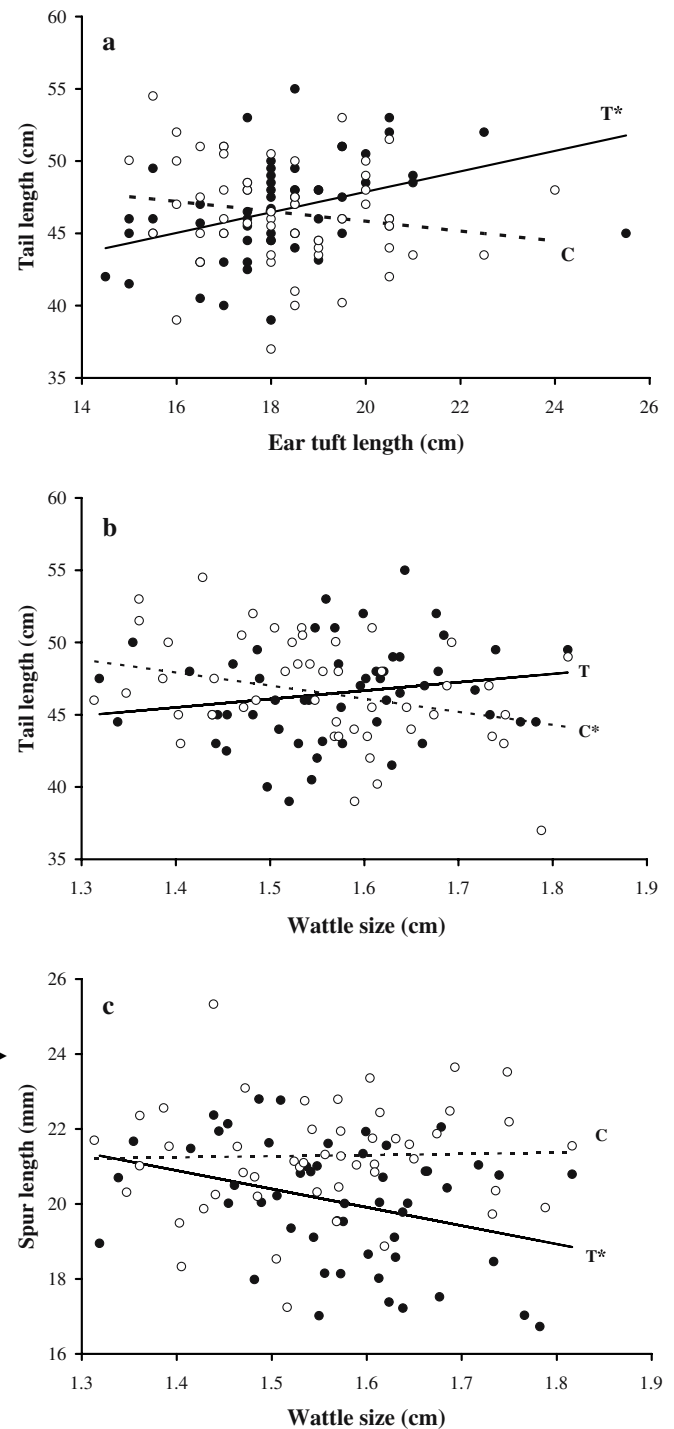
* $0.01<P<0.05$; ** $0.001<P<0.005$; *** $P<0.001$

length was larger in males than in females, both in absolute and relative terms (Tables 2, 3). In males, the difference in mean spur length between treatments corresponded to 0.80 SDs of the mean spur length observed among controls, whereas in females, the difference between treatments corresponded to 0.40 SDs of the mean estimated for controls (Table 2). A within-sex comparison further confirmed that the spurs of both males and females originating from T eggs were shorter than those of individuals originating from control eggs (*t* test, males: $t_{115}=4.34$, $P<0.001$; females: $t_{125}=2.50$, $P=0.014$). The significant effect of treatment on spur length was unaffected by including tarsus length in analyses of covariance both on males (effect of tarsus length: $F_{1,112}=17.29$, $P<0.001$; effect of treatment: $F_{1,112}=19.30$, $P<0.001$) and females (effect of tarsus length: $F_{1,124}=13.20$, $P<0.001$; effect of treatment: $F_{1,124}=7.38$, $P=0.008$). Tail length was not differentially affected by T treatment in the two sexes (Table 2; $F_{1,241}=0.30$, $P=0.58$). When the interaction was removed from the model, tail length was unaffected by egg treatment (Table 3). Finally, none of the sexual traits that were measured only on males (ear tufts, wattle size, or color) was influenced by egg hormone treatment (Tables 2, 3).

Effects of yolk T on the covariation among sexual traits

The slope of the relationships between wattle size and tail or spur length, respectively, and between ear tuft and tail length differed between T and control males (Table 4; Fig. 2). None of the analyses on other pairs of traits showed a significant effect of the treatment \times covariate interaction (Table 4). All results were unaffected by swapping dependent and independent variables (Table 4). Linear regression analyses carried out for each experimental group revealed a complex pattern of variation in the association between traits among T or control males. Ear tuft and tail length were positively related in T males, whereas this relationship was nonsignificant among controls (Fig. 2a). However, tail length did not covary with wattle size among T males, but significantly decreased with increasing wattle size among controls (Fig. 2b). Finally, spur length significantly declined with wattle size in T males, whereas it did not change among controls (Fig. 2c). Removal of the non-

significant interactions from the models revealed no significant covariation between pairs of male traits, with the exception of a positive covariation between wattle red coloration and wattle size [$F_{1,99}=7.38$, $P=0.008$, coefficient=0.713 (0.262 SE)]. Among females, the slope of the relationship between spur and tail length did not differ between treatments ($F_{1,123}=0.91$, $P=0.34$; results unchanged when the dependent and the independent variables were swapped). Removal of the interaction term showed no significant covariation between the two traits ($F_{1,124}<0.01$,



$P=0.99$). Analyses of covariance of spur length of the two sexes combined showed no significant effect of the two- or three-way interactions between treatment, sex, and tail length (details not shown).

Discussion

The main aim of this study of the ring-necked pheasant was to investigate the effect of an experimental increase in yolk T levels on the expression of male secondary sexual characters relevant to male-male competition and female mate choice. T treatment was found to negatively affect spur length in both sexes, although male spurs were more affected than female ones, whereas it did not affect the expression of plumage ornaments or wattle characteristics. In addition, T treatment modified the natural pattern of covariation between male secondary sexual traits. In fact, the negative correlation between tail length and wattle size observed among control birds was not found among T males. Conversely, T treatment resulted in a positive correlation between tail length and ear tuft length, and in a negative correlation between spur length and wattle size, whereas these correlations were not observed among males originating from sham-inoculated eggs.

Effects of elevated prenatal T on phenotype

In general, epigenetic variation in morphological, behavioral, or other traits due to early exposure to steroid hormones is thought to arise via two mechanisms of phenotypic plasticity (Badyaev 2002; Dufty et al. 2002). First, early exposure to variable steroid hormone levels can have a long-term priming effect on synthesis and secretion of hormones later in life. Second, variation in steroid levels may affect sensitivity of target tissues to the activational effects of steroids during growth or adult life. For example, in vertebrates, secretion of the growth hormone (GH, a major factor in somatic growth modulation) by the pituitary ultimately depends on gonadal hormones via their effect on the release of hypothalamic factors (see discussion in Badyaev 2002). Gonadal hormones can modulate the expression of pituitary receptors for hypothalamic stimulators of GH release, resulting in variation in GH profiles (Kamegai et al. 1999). Prenatal exposure to variable steroid hormone levels can therefore imprint the sensitivity of the pituitary to the mediators controlling GH release (Gatford et al. 1998) and can influence the generation of GH secretory patterns (Chowen et al. 1996; Veldhuis and Iranmanesh 1996). Finally, sexual differences in the steroid milieu during early development can affect the expression of hormone receptors and hormone-secreting cells, with persistent long-term consequences on the sensitivity to hormones in the two sexes (Brandstetter et al. 2000; Lopez et al. 1995). In this context, sex-specific patterns of hormone secretion and/or sensitivity of target tissues may lead to sex differences in the phenotypic response to an altered prenatal hormonal

environment (e.g., Strasser and Schwabl 2004, Rubolini et al. *in press*, Saino et al. *in press*).

The sex-specific negative effects of *in ovo* T treatment on spur length observed in this study could result from any of these mechanisms, whereby, for example, increased exposure to T during early developmental stages may have differentially affected the pattern of secretion of hormones or the sensitivity of the mesodermal cells originating the spur to testosterone in the two sexes. Previous studies showed that T administration to juvenile male pheasants had no effect on spur length but resulted in increased wattle size (Briganti et al. 1999), suggesting that the effect of elevated T on spur length varies during ontogeny and that long-term organizational effects of prenatal T exposure differ from short-term effects of elevated circulating T on phenotypically plastic traits such as wattle size, which was unaffected by elevated prenatal T in our study.

The function of spurs in female ring-necked pheasants is unclear, as all the studies we are aware of concerning this trait in pheasants focus on males, where long spurs have been experimentally shown to affect female choice and to predict social dominance rank, success in intrasexual competition, and viability (review in Mateos 1998). The negative effects of physiological T levels on a secondary sexual trait of males, which positively predicts success in sexual selection (von Schantz et al. 1989), obviously suggest that directional selection exists for a reduction of T levels in the eggs, unless these negative effects of T on males are balanced by positive effects on other traits important either in natural or sexual selection contexts (e.g., behavioral traits). In addition, among T males, a negative relationship between spur length and the size of the wattle was observed. This indicates that in T (but not in control) males, an increase in wattle size was accompanied by a further reduction in spur length (see Fig. 2c). Thus, it appears that exposure to increased prenatal T levels would result in a trade-off between the expression of spurs and wattles.

Interestingly, an earlier study showed that spur length positively predicted T-cell-mediated immune response of fully grown male pheasants in captivity (Ohlsson et al. 2002). Thus, spur length is negatively affected by experimentally elevated *in ovo* testosterone levels and positively covaries with T-cell-mediated immunity. This pattern is consistent with the idea that elevated T levels have negative effects on immunity because long spurs may result from low T levels and reflect high T-cell-mediated immune response. The relationship between spur length and T-cell-mediated immunity was not observed in the present study in any of the sex \times treatment experimental groups (data not shown). However, it must be stressed that we measured T-cell-mediated immune response at day 15 because we gave priority to the investigation of the effect of T on immunity soon after birth, whereas spur length was measured 7 months later.

As it seems unlikely that females benefit from having shorter spurs, T levels in the eggs are unlikely to be the outcome of a trade-off between contrasting T effects on

spurs in the two sexes. We analyzed a number of “ordinary” characters of individuals of both sexes, including body size and mass at different ages and perinatal behavioral response to stress, which may be related to antipredator behavior and a major component of the acquired immunity early in life, but we failed to find any effect of T on these traits. Of course, high T levels could enhance the expression of other traits that we did not measure, although we considered most of the sexually selected male morphological characters (see Mateos and Carranza 1995, 1997 and Mateos 1998 for a review) and other important fitness traits such as body size, immunity, and behavior.

Previous studies of the effect of exposure to experimentally elevated T levels *in ovo* on post-hatching growth and behavior of birds have provided mixed evidence (e.g., Schwabl 1993, 1996; Eising et al. 2001; Eising and Groothuis 2003; Sockman and Schwabl 2000; Andersson et al. 2004; see “Introduction”). The few studies where the effect of elevated T levels on male secondary sexual characters has been investigated also revealed contrasting patterns: in the house sparrow, a positive effect on feather ornamental coloration of males and social dominance behavior of both sexes has been documented (Strasser and Schwabl 2004), whereas in the Chinese quail, the expression of male plumage traits was unaffected (Uller et al. 2005). Thus, present results suggest that the possible trade-offs between contrasting effects of T on different offspring traits are elusive and require the scrutiny of an even larger number of traits in order to be identified.

Alternatively, the level of androgens in the eggs may be the result of an intergenerational trade-off between the costs and benefits of high T levels in the mother and the eggs rather than the outcome of a trade-off between the expression of different traits in the offspring. This is however unlikely in our study case, as the negative, rather than positive, effects of T on a trait which is related to fitness in males should select for lower transfer of T to the eggs, with potentially beneficial effects also for the mother since high, rather than low, T levels are known to be costly in females (e.g., Searcy 1988; Rutkowska et al. 2005 for studies on birds; review in Staub and de Beer 1997).

Finally, the negative effect of T administration on spur length may have resulted from an increased *in ovo* level of estrogens because T can be converted into 17- β -estradiol by aromatase (Becker et al. 1992), and, in birds, the expression of male plumage ornamentation in females is inhibited by estrogens (Owens and Short 1995). We do not know whether germinative tissues that originate the spurs are sensitive to androgens, estrogens, or both. However, spurs and feathers share a common embryological origin, both being mesodermic derivatives (Romanoff 1960). Shorter spurs in females may therefore have resulted from inhibition of the male character by estrogens in females, and a reduction of spur length in both sexes following T administration in the eggs may have resulted from increased aromatization of T into estradiol. Estradiol in avian eggs appears to be much less abundant than testosterone (e.g., Caldwell Hahn et al. 2005 and our un-

published data on pheasants). Thus, the aromatization of even a small proportion of the injected T into estradiol could have resulted in a marked proportional effect on estradiol concentration in the egg, which may also differentially affect spur length of males and females.

Covariation between multiple sexual traits

Previous studies of pheasants have shown positive correlations between the expression of male secondary sexual characters (e.g., Papeschi et al. 2000; review in Mateos 1998). However, among our control subjects, we found only a single positive correlation (color and size of the wattle), whereas the correlation between wattle size and tail length was negative. None of the other eight pair-wise correlations between the five secondary sexual characters we measured attained statistical significance. Our tests were based on relatively large samples, similar to those involved in previous studies, where generalized positive covariations have been found between some of the characters we measured (Papeschi et al. 2000). Thus, the power of the statistical tests should not have generated the observed discrepancies between studies. Moreover, age of the focal subjects was also similar between the present and previous studies (Papeschi et al. 2000).

Several of the male traits we investigated (i.e., spur, tail, and ear tuft length) have been shown to influence female mate preference and should therefore be regarded as ornaments (*sensu* Berglund et al. 1996). The current theory on the evolution of multiple male ornaments posits that different male ornamental characters have evolved to serve the function of revealing to choosy females different aspects of male quality (the “multiple message” hypothesis) or to provide the female with multiple ways of assessing the quality of a potential mate (the “redundant signal” hypothesis) (Møller and Pomiankowski 1993). Both hypotheses do not lead to explicit predictions about the extent of the covariation between male characters (Møller and Pomiankowski 1993). However, it could be speculated that if each male trait is correlated with condition in a different way, as envisaged by the redundant signal hypothesis, no covariation between traits should be expected. Conversely, according to the multiple message hypothesis, the pattern of covariation between male ornaments will depend on the relationship between the different components of male quality that are advertised by these traits. Present results concerning the covariation among ornamental traits observed in control birds are therefore compatible with both hypotheses for the evolution of male ornaments in the pheasant (see Mateos 1998). In addition, the above-mentioned ornaments appear to have a dual function, being also relevant to intrasexual competition, according to the armament-ornament model (Berglund et al. 1996). However, to our knowledge, the evolutionary consequences of the combined effects of intra- and intersexual selection on the covariation between male traits have not been explored from a theoretical perspective. At present, we are therefore unable to

fully assess the implications of our results concerning character covariation in the context of the evolution of male armaments and ornaments in the pheasant. Interestingly, however, the negative correlation between wattle size and tail length in control males, which is inconsistent with previous findings (e.g., Papeschi et al. 2000), may suggest the existence of a trade-off between the expression of multiple signals (see also Andersson et al. 2002).

In any case, the effects of elevated yolk T exposure on the covariation between male traits indicate that prenatal T may constrain or otherwise influence the simultaneous expression of armaments and ornamental characters, with potential consequences on the overall assessment of individual male quality both by females and other males.

In conclusion, our study showed that elevated prenatal T exposure had negative effects on an important sexually selected trait in males and highlighted that variable yolk androgen levels may affect the simultaneous expression of multiple secondary sexual traits, which are reliable quality indicators. These results, together with the lack of positive effects of prenatal T on any of the diverse ordinary and sexually selected traits we measured, suggest that either we missed the traits that were positively affected by androgens or that maternal androgen transfer to the eggs may involve long-term costs for male offspring in the ring-necked pheasant.

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