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# Enhanced yolk testosterone influences behavioral phenotype independent of sex in Japanese quail chicks *Coturnix japonica*

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**Abstract**

Studies have demonstrated an effect of yolk testosterone levels on the physiology and behavior of nestling birds. In order to investigate this phenomenon experimentally in a precocial bird, we enhanced yolk testosterone, but within the physiological range, by injecting 50 ng testosterone in ethanol into Japanese quail *Coturnix japonica* eggs prior to incubation. The chicks hatching from these as well as from control eggs that had received the carrier-only or were left unmanipulated were subject to a number of behavioral tests from hatching to the age of 3 weeks. In addition, fecal samples were taken during a 90-min isolation period to determine a physiological response to a stressor. Experimental chicks performed a detour task faster and approached novel objects sooner than did the controls. Chicks from treated eggs took a longer time to start distress vocalizing and also produced less distress calls during open-field trials, took on average a larger number of trials for them to show tonic immobility and also excreted lower levels of corticosterone metabolites (BM) than did the controls. In response to a stressor, excreted BM was initially higher in the control chicks, as compared to the experimental birds. Induced behavioral effects were independent of sex with no sex treatment interactions found. In sum, experimentally enhanced testosterone levels in the eggs shifted individual behavioral phenotype towards "bold" or "proactive", irrespective of sex. We conclude that testosterone in the yolk influences the coping style of hatchlings and may be a potential means of maternal influence on offspring phenotype.
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*Keywords:* Quail; Coturnix; Steroid; Egg yolk; Epigenetics; Coping style

Phenotypic characters are based on genetic inheritance. In addition, certain epigenetic factors may come into play. Most notably, parental effects may influence offspring phenotype. In birds, for example, females may increase their level of care (De Lope and Moller, 1993), lay more eggs (Petrie and Williams, 1993) or lay larger eggs (Cunningham and Russell, 2000). In addition, it has been shown that egg yolk contains high concentrations of steroids and that the amount deposited may be under maternal influence (Burley and Vadehra, 1989; Schwabl, 1993).

Recent evidence suggests that the amount of steroid deposited in the egg yolk may be influenced by the female's social and physical environments (see Eising et al., 2001; Schwabl, 1996a,b). In this way, yolk steroid levels are influenced by breeding density (Schwabl, 1997), male attractiveness (Gil et al., 1999), social intrusion (Mazuc et al., 2003) and/or female condition (Verboven et al., 2003). These differences in steroid levels may result in epigenetically derived phenotype variation within a clutch, since steroids have strong organizational effects on the developing embryonic nervous system (Sapolsky, 1992) and have morphological, physiological and behavioral effects on offspring traits (Eising, 2004; Ketterson and Nolan, 1999; Ros, 1999).

Most studies have focused on the effects of maternally derived testosterone in the eggs of asynchronously hatch-

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ing birds. Thus, egg testosterone levels in canary *Serinus canaria* (Schwabl, 1993) and black-headed gull *Larus ridibundus* clutches (Eising et al., 2001) are thought to counteract the effects of laying asynchrony. Chicks hatching from late-laid eggs, which have higher testosterone levels, are better able to compete with their earlier-hatched and larger siblings for food, show increased growth, and have an increased hatching muscle size (Lipar and Ketterson, 2000). By contrast, in the American kestrel *Falco sparverius* increased yolk testosterone levels correlated with reduced growth and survival (Sockman and Schwabl, 2000).

Most of the early work did not control for hatchling sex. Recently, it was found that yolk testosterone may be part of the sex-determining process (Petrie et al., 2001) and laying date and sequence can indeed be related to sex allocation (Krebs et al., 2002). Hence, the relationship between yolk testosterone and enhanced chick competitiveness found may have been due to a sex difference in behavioral dispositions.

The bird species described above are more or less nest-bound during the chick phase. We suggest that a potential adaptive function of maternal yolk steroid manipulations may also be present in true precocial birds. Indeed, potential differences in foraging capabilities in chickens, *Gallus domesticus*, have been reported (Riedstra and Groothuis, 2000). However, because competition between precocial siblings is likely to be low and of a different nature to competition in nest-bound birds, we suggest that the effects of elevated egg-yolk testosterone may be expressed in more subtle directions in precocial birds (Persson and Andersson, 1999). For example, long-term effects of in ovo steroid exposure may lead to sustained differences in overall behavioral and physiological responsiveness of the hatchlings, termed "coping-styles" (Koolhaas et al., 1999). "Proactive" individuals would be quick, but superficial in exploring novel objects (Dingemanse et al., 2002), would be less socially dependent and respond with reduced glucocorticoid secretion to challenging situations than more "reactive" individuals. Also, proactive types tend to be bolder towards predators and show increased dispersal and hence may have reduced survival compared to reactive types (Dingemanse, 2003).

Hence, to test whether increased yolk testosterone levels would indeed shift individual behavioral phenotype towards "proactive", we applied a suitable battery of behavioral tests. We examined some behavioral and hormonal correlates of increased testosterone exposure in the egg in the hatchlings of a precocial bird, the Japanese quail. In line with the evidence from nest-bound species, we predicted that additional testosterone in the egg would shift individual behavioral phenotype of the chicks towards a proactive coping style (see Koolhaas et al., 1999). We hypothesized that yolk androgen not only affects nestling competitiveness (see above), but has long-term effects on individual behavioral phenotype and hence will affect life histories (Dingemanse, 2003; Eising, 2004). Finally, we asked whether enhanced testosterone in the egg would produce similar effects on behavioral phenotype in both sexes. Towards these goals, we performed a series of experiments with hatchlings to measure activity levels, motivational and neophobic responses as well as hormone excretion and reactivity. Our results indeed demonstrate the post-hatching effects of elevated egg testosterone levels, which were in the direction we had expected.

## Materials and methods

### *Animals and housing*

Japanese quail *Coturnix japonica* provide a useful working model for examining phenotypic traits (the expression of behaviors and physiology) since the young hatch after 18 days in the egg, demonstrate a wide repertoire of behaviors within days post hatch, show sexual dimorphic plumage after only 3 weeks and are sexually mature within 6 weeks.

Quail eggs were obtained from a commercial source (Winkler GmbH, Salzburg) which used in excess of 50 laying females, reducing the likelihood of there being so many siblings in the experimental batch as to affect the experimental outcome. Following injection (see below) the eggs were transferred to an incubator (37.4°C, 60% relative humidity). Eggs were turned twice per day until day 14 at which time air humidity was increased. Chicks hatched between days 17 and 20 and were left for 24 h in the hatching box of the incubator. They were then taken out and transferred to a home cage: a compartment of 110 × 30 × 35 cm, with sawdust on the floor. All chicks, testosterone-treated and controls mixed, were initially housed in a single home cage (between 15 and 26 chicks in each hatch) allowing a full range of social behaviors to be expressed and to ensure the same early social environment for all treatment groups. Chicks were individually marked using a unique color ring combination. Food (chicken starter mash, initially pulverized) and water were provided ad libitum. The photoperiod ran from 0700 to 2200 h (15:9 h L:D) with warmth provided by dull-emitter heating lamps (250 W). At approximately 10 days of age, the chicks were taken to larger cages 180 × 130 × 110 cm.

### *Testosterone injections*

Prior to incubation, some of the eggs received an injection of 50 ng testosterone (Sigma) dissolved in 20 μl 70% ethanol: This was delivered to the yolk using a 27-G sterile needle after a small hole had been bored in the egg-shell above the air sac and the needle was lowered into the egg yolk. The dose of testosterone used to elevate yolk levels was well within the natural range encountered (Hackl et al., 2003). The injection hole was covered with a paraffin/

vaseline mix (40:60 w/w), which solidifies at a temperature below 50°C. The eggs were left unmoved for 2 h and then placed in an incubator (37.4°C). The chicks hatching from these testosterone-treated eggs were the T group. Two control groups were also used. One group received no injection (C) whilst a second received an injection of the carrier only (70% ethanol; E).

*Behavioral tasks*

At various times after hatching, chicks were taken from the home cage to a testing cage. All experiments were video recorded with a Sony DCR-VX700E digital tape recorder and subsequently analyzed using the Observer video-pro system (Noldus), with individuals scored for latencies, etc. (see below). Not all chicks were used for each test, e.g., those chicks used in the detour task as 'goal' chicks; numbers are outlined under each subheading. When tested, chicks were taken in a random order to eliminate any effects of order.

*Open field test*

At 3 and 7 days of age, chicks were tested individually in a circular open field measuring 50 cm in diameter with a 60-cm-high matte-black wall surrounding a sawdust-covered floor. The chick was taken from its home group and placed in the center of the open field at which time a stopwatch was started. Behaviors of the chick were recorded remotely via a video camera placed directly above the open field. Behaviors recorded included the latency until first distress call; the number of distress calls emitted; the number of attempted escapes (attempts to jump at the walls of the arena). In addition, when playing back the video cassette on a monitor, we placed a grid (6 × 6 squares) over the screen and scored the number of times the chick crossed the grid lines as an index of motor activity during the isolation. After 12 min, the chick was returned to its home group and the open field was cleaned after each test.

*Detour*

Chicks between 5 and 7 days of age were taken individually to a test compartment. In the center of the compartment (150 × 95 cm), we placed a U-shaped barrier to produce an inner corridor 12-cm long and 10-cm wide and two external side corridors 12-cm wide each. On the closed end of the barrier, there was a small grid opening (10 × 7 cm with a grid size of 0.5 cm). On the opposite, external wall of the arena (10 cm from the grid) another compartment (20 × 20 cm) was separated from the test compartment by a grid partition into which we placed a group of five chicks, the 'goal' chicks, and not used as subjects, just before each experimental session. The chicks in this compartment were provided with water and food (chicken starter) and were kept warm by a warming lamp positioned directly overhead.

At the beginning of each test, we positioned a chick inside the U-shaped barrier facing the small grid through which the goal chicks could be seen. In order to approach the group, the isolated chick had to turn around and walk away from the goal chicks, towards the open end of the barrier, and exit by turning either left or right down the side corridors. In doing so, visual contact to the group would be lost until the test chick reached one of the outside corners of the U-shaped barrier. The time taken for each chick to reach this point from the starting position was recorded. In addition, the latency to start emitting distress calls and the number of times the individual left the front area of the barrier and returned were recorded. A cutoff time of 120 s was used. Once the chick had successfully completed the task, i.e., taking the detour under the 2 min, it was taken and placed with the goal chick group for 3 min before being returned to its home cage. Each chick was subsequently retested on five further occasions (after 30 min, 3 h, 24 h, 48 h and 72 h) with the same behavioral parameters recorded. Any chicks not performing the task on the first attempt were not used in subsequent analyses.

*Novel object tests*

A test compartment (85 × 45 cm) was prepared with a video camera and light bulb (40 W) placed directly above the center at a height of 60 cm. Each chick was tested individually between 10 and 15 days of age and was allowed two periods of 5 min before the test to habituate to the compartment. A novel object was introduced into the compartment and the chick repositioned to the opposite end. Three trials per chick and hence three novel objects were used in total: (A) a plastic toy model, (B) a cube (1.8 × 1.8 × 1.8 cm) and (C) a small nonfunctioning toy motor.

The latency to move and the onset of distress calls together with the total number of distress calls emitted were recorded. The time to first contact to the novel object was also measured. In addition, the chick's position was recorded at 10-s intervals over a 2-min period after being placed in the center of the test arena. On playback of the video the compartment was divided into five equal-sized sections on the horizontal axis by a grid placed on the television screen (Jones and Waddington, 1992). The chick scored 1 if it was in the area containing the novel object with the score increasing through 2 to 5 at the far end of the compartment. The total scored by each chick was the sum of 12 scans (minimum score = 12, maximum = 60) with high scores reflecting high avoidance of the object.

*Tonic immobility*

At 18 days of age, the quail were challenged with a restraint task. Birds were removed individually from the home cage and taken in a box to the testing room

approximately 30 m away. There, the quail was removed from the box and restrained on its right side for 15 s by the experimenter. After this time, the experimenter gently lifted both hands off the bird's body. The time taken for the bird to move was recorded: if there was no movement for the 15 s counting from the removal of hands then the bird was recorded as having been immobilized and given a score of 0. If the bird did move in the 15 s, however, then the bird was restrained and the procedure carried out again (Jones and Waddington, 1992). The number of restraint trials required to induce a 15-s immobilization of the quail was recorded up to a maximum of six trials. If no restraint was successful during this time, the bird was scored as a 6.

*Hormone measurements*

Fecal samples were collected for individual quail during and following a 90-min "isolation" period. This was necessary since we considered collecting single fecal samples would induce an irregular and nonspecific stress to the chicks, which could not be controlled for and since at least 0.25 g of fecal material is needed for an adequate determination of hormone with our assay procedure. The experiment consisted of placing 2-week-old quail into individual compartments (10 × 10 cm; water ad libitum, heated from above) from where they could see but not directly interact with other conspecifics. The floor consisted of a removable panel, which was gently slid out from underneath the quail, in order to collect the fecal material. Feces were collected in this way every 30 min up to 90 min, with all material from each individual from each time point collected and frozen (at −20°C) separately. Subsequently, the material was analyzed for steroid (corticosterone and testosterone-metabolites) content.

Steroid analysis from feces has been thoroughly tested in avian models. Most notably, significant correlations between plasma testosterone and its metabolites in feces have been found in geese (Hirschenhauser et al., 2000; Kotrschal et al., 1998). In addition, it has been shown that steroid excretion starts from 15 min after a significant/stressful event and peaks 30 min to 2 h later in geese (Krawany, 1996). We have assumed that a reduced time factor may be applicable to quail due to higher metabolic rate.

Fecal samples (0.25 g) were extracted with 1.25 ml water plus 1.5 ml methanol, hydrolyzed with a mixture of β-glucuronidase/arylsulfatase (Merck 4114), and determined by enzyme immunoassay (EIA; Möstl et al., 1987) using group-specific antibodies as described elsewhere in detail (corticosterone assay: Kotrschal et al., 1998; testosterone assay: Hirschenhauser et al., 1999a,b). The sensitivity of the assay was less than 2 pg/well, and concentration limits for reliable measurement ranged from 0.5 to 45 ng/g feces for testosterone metabolites (TM) and from 0.4 to 98.7 ng/g feces for corticosterone metabolites (BM). The intra-assay coefficient of variation was 15.6% for TM and 17.4% for BM, and the inter-assay coefficient of variation was 12.8% for TM and 13.7% for BM. Such relatively high values are quite normal for analyzing hormones from feces, mainly due to the number of different steps involved which all enhance variability.

*Statistical analyses*

For all statistical analyses, the SPSS (Chicago, IL) package was used. A Kolmogorov–Smirnov (K–S) test was used to determine whether the data sets were normally distributed. Data that were significantly different from the normal distribution were transformed: logarithmically unless otherwise stated. Outliers were tested for by Cook's distance measures and were removed from the analyses only when they had a significant effect on the test. They are included in the figures.

Repeated measures two-way ANOVA was used throughout for data analysis. In all cases, the between-subject factors were sex and treatment. Within-subject variables varied with the experiment and included latencies and counts of behaviors. In all cases, one variable at a time was tested in the ANOVA model. Post hoc tests were carried out where necessary. Data are presented as mean ± standard error of the mean (SEM) where appropriate. Only statistically significant results, including interactions between the dependent variables, are reported in the results section below except where the authors believe it salient to report nonsignificance.

## Results

*Testosterone injection and sex ratio*

In total, 28 testosterone-treated individuals (17 male, 11 female, group T) hatched, together with 16 (8 male, 8 female, group E) carrier-injected and 36 (21 male, 15 female, group C) noninjected controls. There was no effect of group on the sex ratio of the hatch (Chi-square, $\chi^2 = 0.497$, $df = 2$, $P = 0.78$). There was a significant effect of treatment on hatching, however, with the 28 T chicks coming from a total of 102 injected eggs, the 16 E chicks from 40 eggs, and the 36 noninjected controls from 62 eggs (Chi-square, $\chi^2 = 15.174$, $df = 2$, $P = 0.001$). We used a generalized linear model with binomial errors to test for a significant difference in the proportion of hatchlings by treatment. We performed two orthogonal contrasts of (i) control (C) vs. E-injected and T-injected, and (ii) E-injected vs. T-injected. The first contrast was significant ($z = -3.075$, $P = 0.002$) whilst the second showed a tendency but was not significant ($z = 1.351$, $P = 0.102$), suggesting this effect was due to the injection per se rather than to the substance injected.

*Open field tests*

A two-way repeated measures ANOVA was used in the analysis with sex and treatment as the independent factors. The number of calls and the latency to call and to move were used as the repeated measures in separate tests. Differences were found between the groups (egg-injected controls E = 16, 9 male, 7 female; noninjected controls C = 22, 13 male, 9 female; treated T = 26, 14 male, 12 female) for number of distress calls ($F_{2,58}$ = 12.545, $P$ = 0.0001; Fig. 1) and the latency to emit distress calls ($F_{2,58}$ = 3.365, $P$ = 0.036; Fig. 1). Specifically, the treated chicks called less than the other two groups and showed an increased latency to start calling (post hoc Tukey HSD tests all $P < 0.05$). There was also a treatment difference in the latency to move (C-chicks 7.9 ± 2.1 s, E 8.1 ± 1.8 s, T 11.8 ± 2.9 s; $F_{2,58}$ = 3.265, $P$ = 0.04) but not in the amount the chick moved over the 12-min isolation.

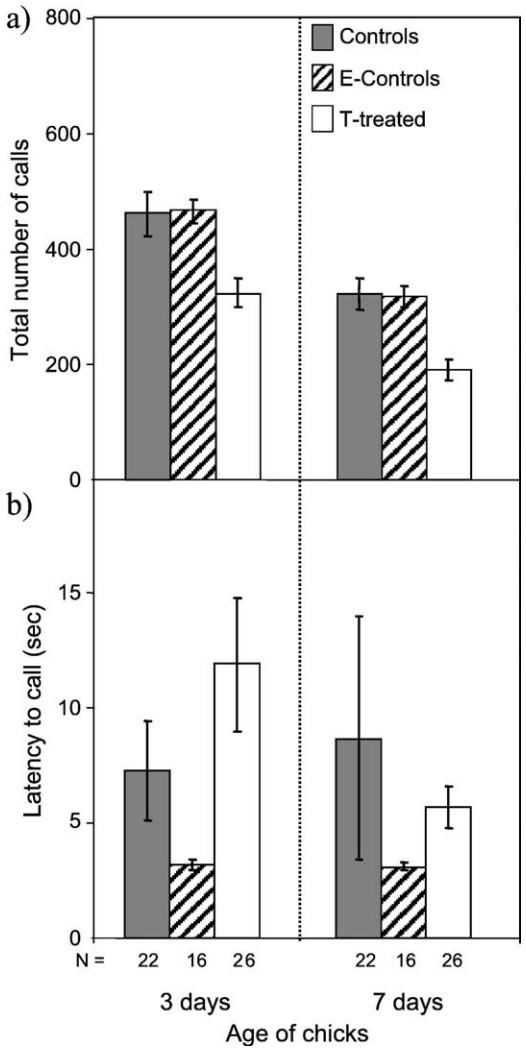


Fig. 1. The total number of distress calls emitted (a) and the latency to start calling (b) during a 12-min isolation period for 3- and 7-day-old chicks from control, ethanol-injected (E controls) and testosterone-injected eggs. Bar charts show means ± SEM.

Younger chicks moved significantly more than the older chicks (3-day chicks 49.56 ± 3.46 units; 7-day chicks 31.61 ± 2.07 units, $F_{1,58}$ = 20.789, $P$ = 0.0001) and called more often (3-day chicks 406.88 ± 19.1; 7-day chicks 267.53 ± 14.52, $F_{1,58}$ = 45.965, $P$ = 0.0001) as determined by tests of within-subject effects in the ANOVA model.

*Detour tests*

Not all chicks performed the detour on first trial but there was no difference according to treatment; 19 T-chicks from 26 (73.8%) completed the task on first attempt, 15 C-chicks from 26 (57.7%) and 6 E-chicks from 10 (60%; Chi-square, $\chi^2$ = 1.45, $df$ = 2, $P$ = 0.484). Only the data from those chicks that did complete the detour at the first attempt were used for subsequent analyses.

A two-way repeated measures ANOVA was carried out on the data following transformation (C = 15, 9 males, 6 females; E = 6, 3 males, 3 females; T = 19, 10 males, 9 females) with time taken to perform the detour as the repeated measure. As expected, there was a significant effect of trial on detour latency as determined by within-subject tests ($F_{3,102}$ = 12.262, $P$ = 0.0001). There was a tendency for the treated chicks to perform the detour faster than the controls (C-chicks 64.71 ± 5.1 s, E 56.7 ± 8.8 s, T 44.17 ± 4,72 s; $F_{2,34}$ = 2.729, $P$ = 0.068). It was apparent that on the first successful detour attempt treated chicks were faster (C-chicks 94.28 ± 14.02 s, E 82.9 ± 11.02 s, T 58.89 ± 9.7 s).

*Novel object*

A two-way repeated measures ANOVA was used in the analysis with proximity to and latency to contact the novel object and the number of pecks at the novel object as the repeated measures with the test performed for each variable separately (T = 18, 9 males, 9 females; C = 17, 11 males, 6 females; E = 9, 6 males, 3 females). There was an effect of treatment on latency to first contact to the different objects ($F_{2,38}$ = 17.238, $P$ = 0.0001; Fig. 2) with post hoc tests (Tukey HSD tests all $P < 0.05$), showing that treated chicks were faster than the controls. No other treatment effects were found.

Males were faster to first contact than females (males 28.7 ± 2.0 s, females 31.8 ± 3.7 s; $F_{1,38}$ = 5.124, $P$ = 0.029). In addition, the chicks reacted differently between the three novel objects according to within-subject tests in the ANOVA in terms of proximity ($F_{2,76}$ = 4.097, $P$ = 0.009 on square-root transformed data: toy 30.2 ± 2.52; die 24.3 ± 2.58 and motor 24.1 ± 2.2 units) and latency to contact ($F_{2,76}$ = 3.292, $P$ = 0.0001: see Fig. 2).

*Tonic immobility*

The number of trials to tonic immobility was significantly different for treatment ($F_{2,34}$ = 26.24, $P$ = 0.0001)

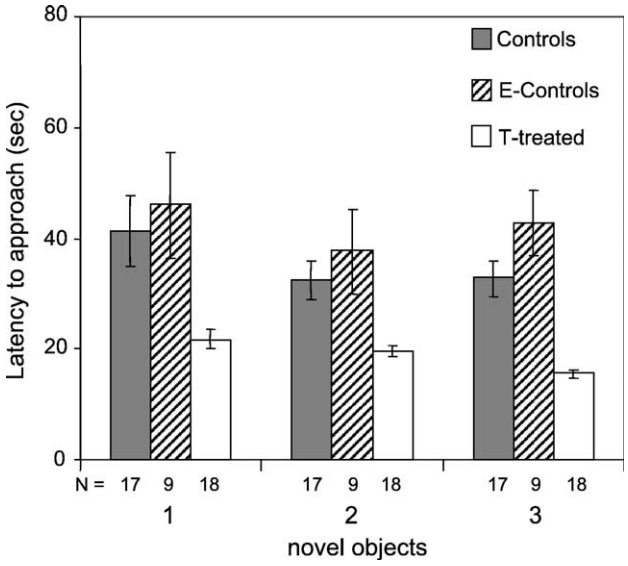


Fig. 2. The time taken to approach novel objects by chicks hatching from control, ethanol-injected (E Controls) and testosterone-injected (T) eggs. The three novel objects used were (1) a plastic toy, (2) a colored cube and (3) a small mechanical motor. Bar charts show means ± SEM.

and sex ($F_{1,34}$ = 13.734, $P$ = 0.001; for 19 C chicks, 11 male, 8 female; 5 E chicks 4 male, 1 female; and 16 T chicks, 8 male, 8 female). Post hoc tests revealed significant differences between the treated group and both the control groups C and E (Tukey HSD tests all $P < 0.05$). No differences were found between the two control groups, however, so these groups were combined in the following descriptive statistics due to the low number of E chicks. On average, it took 3.2 ± 1.1 trials for control birds (C and E) to remain immobilized for the 15 s with 4 birds (16.6%) never attaining immobility. In comparison, the treated birds took 5.4 ± 1.1 trials with 10 birds (62.5%) never being restrained successfully. Female birds were more likely to undergo tonic immobility ($n$ = 11/18) during the trials than the males ($n$ = 3/22; $F_{1,38}$ = 11.785, $P$ = 0.002). However, a one-way ANOVA demonstrated no effect of treatment or sex on the duration of the immobility once induced.

*Hormone data*

The fecal metabolites from a total of 22 (13 male, 9 female) control and 17 (9 male, 8 female) treated quail were analyzed following the 90-min "isolation" period. All control quail were from non-ethanol-injected eggs. All hormone values were transformed to produce a normally distributed data set. Initially, a two-way repeated measures ANOVA was carried out on the data set with sex and treatment as the independent factors and hormone values as the repeated measures. One individual (a female T) was removed from the analyses since the BM levels had a significant effect on the overall analysis (as determined by Cook's distance plot and subsequent removal of these data from the model).

*Testosterone excretion*

There was a difference between males and females ($F_{1,33}$ = 6.102, $P$ = 0.019) with females excreting higher amounts of testosterone metabolites (TM) overall (males 3.474 ± 0.316, females 6.83 ± 1.051 ng/g feces). There was a nonsignificant trend ($F_{1,33}$ = 3.655, $P$ = 0.064) for treated quail to excrete increased TM compared to the controls over the period (see Fig. 3). However, there was no significant interaction between sex and treatment ($F_{1,33}$ = 0.098, $P$ = 0.757).

*Corticosterone excretion*

The controls excreted more corticosterone metabolite (BM) during the 90-min period overall than did the treated birds (controls 30.35 ± 2.25 ng/g; treated, 25.87 ± 1.61 ng/g; $F_{1,35}$ = 3.955, $P$ = 0.046; see Fig. 3). There was no effect of sex and no treatment–sex interaction present.

## Discussion

We have shown that the addition of exogenous testosterone to the egg yolk, mimicking enhanced testosterone of maternal origin, significantly influences the expression of behavioral and physiological phenotype in a precocial bird species, the Japanese quail *C. japonica*. Changes were according to predictions based on the assumption that enhanced androgens early in ontogeny would shift individual behavioral phenotype towards 'proactive' (Koolhaas et al., 1999) or 'fast' (Drent and Marchetti, 1999). Chicks from eggs that had received an additional dose of testosterone (T) into the yolk prior to incubation seemed to be less socially dependent than the controls. T chicks, (i) took a longer time to start calling and called less often during an isolation period, (ii) were able to perform a detour task faster and (iii) excreted a reduced level of BM following a social challenge compared to controls. Also, in agreement with our expectation that 'proactives' deal more actively with challenges, chicks from T-injected eggs (iv) approached and investigated a novel object sooner and (v) were immobilized only after significantly more restraint periods than was observed in the controls. No differences in any of the behavioral or physiological parameters were associated with the injection of ethanol as a carrier, suggesting that the effects seen were specific to testosterone.

Even though chicks from T-treated eggs produced fewer distress calls than controls, this was not accompanied by a decrease in motor activity as would have been expected had the calling been associated with enhanced fearfulness (Faure et al., 1983). In fact, casual observation suggested that the chicks were less disturbed than controls by the trials. In addition, treated birds were also significantly less likely to be induced to tonic immobility than the controls. Such reduced responsiveness induced by physical restraint is held

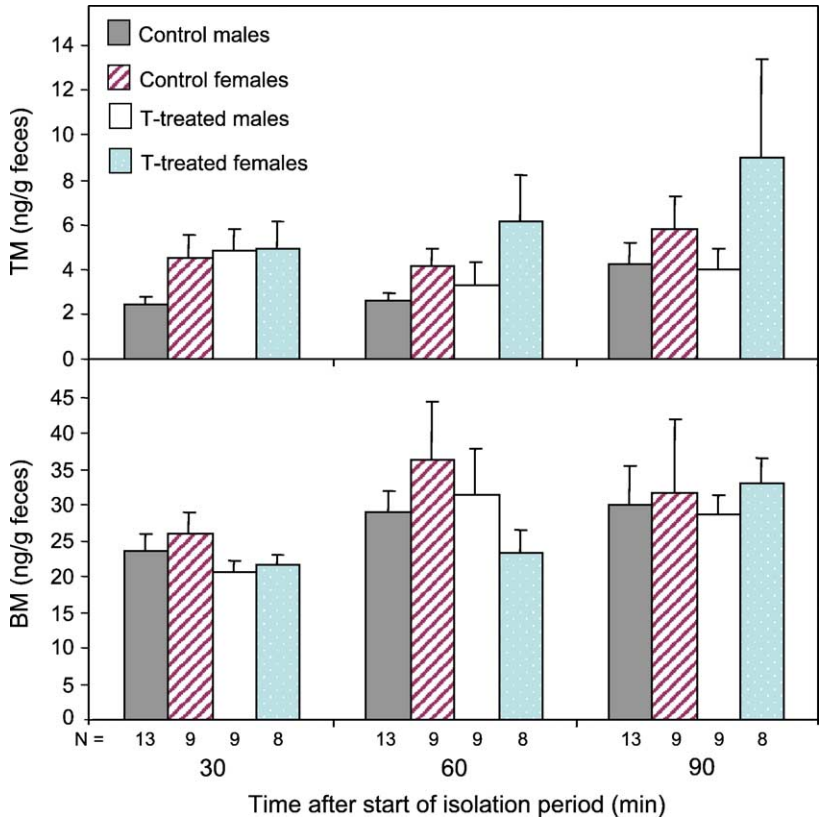


Fig. 3. Graphs showing testosterone metabolite (TM) and corticosterone metabolite (BM) excretion during a 90-min isolation period for quail chicks hatching from 22 (13 male, 9 female) control and 17 (9 male, 8 female) testosterone-treated eggs. Bar charts show means ± SEM.

to be a fear-related anti-predator response (Jones and Waddington, 1992). Reduced fearfulness in treated individuals was also apparent by their approaching novel objects sooner than the controls. However, overall, time spent near the novel object did not differ between treated and controls, indicating that individuals from the two groups did not differ in exploratory behavior per se, but rather in the perception of a fearful event.

Evidently, chicks hatching from eggs containing testosterone-enhanced yolk were less fearful in the open field, tonic-immobility and novel-object situations than were those hatching from control eggs. This became particularly apparent in detour behavior. It has been previously shown that testosterone treatment produces changes in attention and perception during learning (Clifton et al., 1986) and that T-treated individuals are sooner to detach from their mother as an imprinting object (Gvaryahu et al., 1986). Hence, our birds from T-treated eggs were more likely to perform the task at the first time and were also faster to detour, not because they were more but because they were less attracted to their siblings, since it was seemingly easier for them to move away from their social companions before joining them.

That these behavioral differences may have a physiological underpinning is demonstrated by the levels of steroid metabolites excreted following a 90-min social isolation. It has been previously demonstrated that fast and slow-detour chicks also differ in their adrenocortical responses to a stressor (e.g., Marin and Jones, 1999), indirectly suggesting an effect of yolk-testosterone on HPA development. This is supported by our present results, since the treated birds showed a significantly lower BM excretion during the course of the 90-min isolation (Fig. 3). This is in alignment with findings of a general inhibitory effect of testosterone on HPA responses to a stressor in mammals (e.g., Viau and Meaney, 1996). In rats, neonatal gonadectomy results in elevated corticosterone levels in response to a stressor (McCormick et al., 1998). The additional T injected to the yolks of the quail in the present study seemingly initially down-regulated the HPA responses. Differences in terms of basal HPA axis and gonadal axis activity have also been observed between coping styles. Thus, short attack latency ('proactive') mice and wild-type male rats both demonstrate high baseline values of testosterone release (Ruiter et al., 1992; Schuurman, 1981) whilst there is a reduced circadian peak of HPA activity compared to controls (Korte et al., 1996). These data resemble ours, where a reduced BM excretion is found in chicks from T-enhanced eggs following isolation. Our data also show a trend for increased TM in the treated birds following this social isolation.

### *Stress physiology and the brain*

Some underlying mechanisms for egg yolk T actions have been suggested previously. For example, pre-hatch

treatment with T reduced the number of distress vocalizations (DV) during a period of isolation in male quail chicks (Bernroider et al., 1996). This is paralleled by our present findings. The dorso-medial mesencephalic areas, with their opioid peptides under testosterone modulation (Deviche and Güntürkün, 1992; Takeuchi et al., 1996), are known to be involved in the generation of DV (Yazaki et al., 1999). There is a steroid (T)-induced organizational/activational effect on opioid sources in this region, with a decrease in DV associated with testosterone treatment (Bernroider et al., 1996). It is therefore likely that other brain areas associated with fear/anxiety are also influenced, directly or indirectly, by yolk steroids during development leading to altered receptor and enzyme activity and regulation. Such potentially subtle changes in the physiological environment of the embryo may lead to measurable changes in behavior and physiology of the hatchling. The quail embryo has all T-metabolizing pathways functioning by day 10 (Hutchison and Schumacher, 1986), suggesting that yolk steroids can be fully utilized by this stage.

We suggest that some mechanism produces differences in sociality between the two groups with the treated quail faster to perform the detour initially and also subject to increased stress response in the presence of others. Such a potential mechanism may be that of lateralization of brain function. Differences in brain laterality exist between male and female chicks (e.g., Regolin and Vallortigara, 1996; Vallortigara, 1996), which are influenced by exogenous addition of testosterone (Schwarz and Rogers, 1992). Hence, the addition of steroid to the egg yolk may have influenced the degree of brain lateralization and also certain affective behaviors including those relating to social interactions (see Bradshaw and Rogers, 1993; Trevarthen, 1996).

### *A mechanism of maternal influence*

Our results suggest that differential steroid deposition in the yolk may be a potential means of female epigenetic influence on phenotype in a precocial bird. Steroid hormone deposition differs from female to female. In addition, steroid hormones differ in concentration throughout the yolk and between yolks of the same clutch (Lipar et al., 1999; Möstl et al., 2001; Sockman and Schwabl, 2000). The mechanism by which gonadal steroids are transferred to the egg, however, is still not fully understood. The transfer of steroids from the peripheral circulation appears to be limited (Arcos, 1972; Hackl et al., 2003; Möstl et al., 2001). Indeed, Mazuc et al. (2003) showed that yolk testosterone was negatively correlated with systemic maternal testosterone in House sparrows (*Passer domesticus*). It is possible that maternal systemic levels of luteinizing hormone affect steroid production in the theca and granulosa cells (Tilly and Johnson, 1989), which seem to produce the bulk of yolk steroids (Hackl et al., 2003).

By whatever mechanism, the female may be able to influence the phenotypic characteristics of her offspring including sex and behavioral phenotype in response to environmental conditions. Our experiments show that those individuals receiving a higher concentration of testosterone in the egg, are less neophobic and more economical in their response to certain stressors. Thus, via the epigenetic mechanism of steroid input into the yolk, the female may be able to favor certain individuals by modifying certain characteristics and to bring about a fine adjustment of individual differences in behavior in response to environmental variability. However, particularly at high yolk T levels, mothers may trade off potential benefits with considerable costs (Eising, 2004). These include reduced growth, an important factor affecting survivorship at fledging (Schmutz, 1993) and a reduced Bursa of Fabricius size, suggesting a reduced immunocompetence (Fennell et al., 1996). Also, individuals receiving higher doses of early androgens venture further from home (Dingemanse et al., 2003; Vom Saal, 1984). Hence, individuals from high T eggs may be disposed to disperse, but may also be relatively poor survivors. For quail, this aspect of survivorship remains speculative.

Even though hatchlings of injected eggs were only slightly male-biased, we cannot exclude that enhanced yolk testosterone levels affect sex determination (Petrie et al., 2001) or, alternatively, that male embryos are more robust than female embryos with regards to enhanced yolk testosterone levels and, hence, show a higher hatchability; since genetic sex was not determined. Most previous studies on maternal manipulation of offspring behavioral phenotype have not controlled for sex (e.g., Schwabl, 1993). This has recently raised doubts as to whether the effects measured were indeed due to developmental T-modulated behavioral phenotype per se or simply a side effect of T-modulated sex allocation (Petrie et al., 2001). Thus, if males were intrinsically bolder (or more "proactive"; Koolhaas et al., 1999) than females, a sex bias in laying-date and laying sequence (which was indeed found, see Krebs et al., 2002) would suffice to explain the T-related shifts in individual behavioral phenotype. Our present data show that the behavioral phenotype of chicks from T-injected eggs is shifted towards proactive irrespective of sex, since no treatment-sex interactions were found in the data. In addition, Eising et al. (2003) were also able to show that male and female eggs of chickens (*G. domesticus*) contained similar levels of testosterone and androstenedione in their yolks. This leaves the likelihood that maternal manipulation affects behavioral phenotype directly by enhanced yolk androgens rather than by affecting sex allocation and in this way indirectly affecting offspring behavioral phenotype. However, there remains the possibility that some other sex-specific mechanism may affect embryonic yolk steroid use. It will be necessary to investigate whether bird mothers in different species are able to allocate offspring sex and offspring behavioral phenotype independently from each other or whether yolk androgens always affect both. In other words, will there be (species-specific) decoupling of sex allocation from behavior or will enhanced yolk androgen levels,

targeted at producing a more proactive behavioral phenotype (sensu Koolhaas et al. (1999)), also unavoidably affect sex allocation?

In summary, we have shown that there is a shift in phenotype towards proactive associated with an increase in egg yolk testosterone. This shift was irrespective and independent of the sex of the offspring. Thus, via differential steroid deposition, the female may be able to influence the phenotype of her offspring and thus their survivorship and ultimately their fitness.

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## References

Arcos, M., 1972. Steroids in egg yolk. Steroids 19, 25–34.

Bernroider, G., Holztrattner, M., Rottner, K., 1996. Sex steroid–opioid interactions associated with the temporal component of avian calling patterns. Horm. Behav. 30, 583–589.

Bradshaw, J., Rogers, L., 1993. The Evolution of Lateral Asymmetries, Language, Tool Use and Intellect. Academic Press, Inc., San Diego.

Burley, R.W., Vadehra, D.V., 1989. The Avian Egg. Wiley, New York.

Clifton, P.G., Andrew, R.J., Rainey, C.R., 1986. Effects of gonadal steroids on attack and on memory processing in the domestic chick. Physiol. Behav. 37, 701–707.

Cunningham, E.J.A., Russell, A.F., 2000. Egg investment is influenced by male attractiveness in the mallard. Nature 404, 74–76.

De Lope, F., Moller, A.P., 1993. Female reproductive effort depends on the degree of ornamentation of their mates. Evolution 47, 1152–1160.

Deviche, P., Güntürkün, O., 1992. Peptides for calling? An immunohistochemical study of the avian n. intercollicularis. Brain Res. 569, 93–99.

Dingemanse, N.J., 2003. Natural selection and avian personality in a fluctuating environment. University of Utrecht, Netherlands (PhD thesis).

Dingemanse, N.J., Both, C., Drent, P.J., van Oers, K., van Noordwijk, A.J., 2002. Repeatability and heritability of exploratory behaviour in great tits from the wild. Anim. Behav. 64, 929–937.

Dingemanse, N.J., Both, C., van Noordwijk, A.J., Ruten, A.L., Drent, P.J., 2003. Natal dispersal and personalities in great tits (*Parus major*). Proc. R. Soc. Lond., B 270, 741–747.

Drent, P.J., Marchetti, C., 1999. Individuality, exploration and foraging in hand-raised juvenile Great tits. In: Adams, N.J., Slotow, R.H. (Eds.), Proc. 22nd Intl. Ornith. Congress, Durban. BirdLife South Africa, Johannesburg, pp. 896–914.

Eising, C.E., 2004. Mother knows best? Costs and benefits of differential maternal hormone allocation in birds. University of Groningen, The Netherlands (PhD Thesis).

Eising, C.E., Eikenaar, C., Schwabl, H., Groothuis, T.G.G., 2001. Maternal androgens in black-headed gull (*Larus ridibundus*) eggs: consequences for chick development. Proc. R. Soc. Lond., B 268, 839–846.

Eising, C.E., Müller, W., Dijkstra, C., Groothuis, T.G.G., 2003. Maternal androgens in egg yolks: relation with sex, incubation time and embryonic growth. Gen. Comp. Endocrinol. 132, 241–247.

Faure, J.M., Jones, R.B., Bessei, W., 1983. Fear and social motivation as factors in open field behavior of the domestic chick: a theoretical consideration. Biol. Behav. 8, 103–116.

Fennell, M.J., Radecki, S.V., Proudman, J.A., Scanes, C.G., 1996. The suppressive effects of testosterone on growth in young chickens appears to be mediated via a peripheral androgen receptor; studies of the anti-androgen ICI 176,334. Poult. Sci. 75, 763–766.

Gil, D., Graves, J., Hazon, N., Wells, A., 1999. Male attractiveness and differential testosterone investment in zebra finch eggs. Science 286, 126–128.

Gvaryahu, G., Snapir, N., Robinzon, B., Goodman, G., el Halawani, M.E., Grimm, V.E., 1986. The gonadotrophic-axis involvement in the course of the filial following response in the domestic fowl chick. Physiol. Behav. 38, 651–656.

Hackl, R., Bromundt, V., Daisley, J., Kotrschal, K., Möstl, E., 2003. Distribution and origin of gonadal steroids in the yolk of Japanese quail eggs (*Coturnix coturnix japonica*). J. Comp. Physiol. (B) 173, 327–331.

Hirschenhauser, K., Möstl, E., Kotrschal, K., 1999a. Seasonal patterns of sex steroids determined from feces in different social categories of greylag geese (*Anser anser*). Gen. Comp. Endocrinol. 114, 67–79.

Hirschenhauser, K., Möstl, E., Kotrschal, K., 1999b. Within-pair testosterone covariation and reproductive output in Greylag geese *Anser anser*. Ibis 141, 577–586.

Hirschenhauser, K., Möstl, E., Péczely, P., Wallner, B., Dittami, J., Kotrschal, K., 2000. Seasonal relationships between plasma and fecal testosterone in response to GnRH in domestic ganders. Gen. Comp. Endocrinol. 118, 1–11.

Hutchison, J.B., Schumacher, M., 1986. Development of testosterone-metabolizing pathways in the avian brain: enzyme localization and characteristics. Brain Res. 390, 33–42.

Jones, R.B., Waddington, D., 1992. Modification of fear in domestic chicks, *Gallus gallus domesticus*, via regular handling and early environmental enrichment. Anim. Behav. 43, 1021–1033.

Ketterson, E.D., Nolan Jr., V., 1999. Adaptation, exaptation, and constraint: a hormonal perspective. Am. Nat. 154, S4–S25.

Koolhaas, J.M., Korte, S.M., De Boer, S.F., Van der Vegt, B.J., Van Reenen, C.G., Hopster, H., De Jong, I.C., Ruis, M.A.W., Blokhuis, H.J., 1999. Coping styles in animals: current status in behavior and stress physiology. Neurosci. Biobehav. Rev. 23, 925–935.

Korte, S.M., Meijer, O.C., De Kloet, E.R., Buwalda, B., Keijser, J., Sluyter, F., van Oortmerssen, G., Bohus, B., 1996. Enhanced 5-HT1A receptor expression in forebrain regions of aggressive house mice. Brain Res. 736, 338–343.

Kotrschal, K., Hirschenhauser, K., Möstl, E., 1998. Dominance and stress is seasonal in greylag geese. Anim. Behav. 55, 171–176.

Krawany, M., 1996. Die Entwicklung einer nicht invasiven Methode zum Nachweis von Steroid-hormonmetaboliten im Kot von Gänsen. Vet. Univ. of Vienna, Vienna (PhD thesis).

Krebs, E.A., Green, D.J., Double, M.C., Griffiths, R., 2002. Laying date and laying sequence influence the sex ratio of crimson rosella broods. Behav. Ecol. Sociobiol. 51, 447–454.

Lipar, J.L., Ketterson, E.D., 2000. Maternally derived yolk testosterone enhances the development of the hatching muscle in the red-winged blackbird *Agelaius phoeniceus*. Proc. R. Soc. Lond., B 267, 2005–2010.

Lipar, J.L., Ketterson, E.D., Nolan Jr., V., Casto, J.M., 1999. Egg yolk layers in the concentration of steroid hormones in two avian species. Gen. Comp. Endocrinol. 115, 220–227.

Marin, R.H., Jones, R.B., 1999. Latency to traverse a T-maze at 2 days of age and later adrenocortical responses to an acute stressor in chicks. Physiol. Behav. 66, 809–813.

Mazuc, J., Bonneaud, C., Chastel, O., Sorci, G., 2003. Social environment affects female and egg testosterone levels in the house sparrow (*Passer domesticus*). Ecol. Lett. 6, 1084–1090.

McCormick, C.M., Furey, B.F., Child, M., Sawyer, M.J., Donohue, S.M., 1998. Neonatal sex hormones have 'organizational' effects on the

hypothalamic–pituitary–adrenal axis of male rats. Brain Res. Dev. Brain Res. 105, 295–307.
Möstl, E., Meyer, H.H., Bamberg, E., von Hegel, G., 1987. Oestrogen determination in feces of mares by EIA on microtitre plates. Proc. Symp. Analysis of Steroids Sopron, pp. 219–224 (Hungary).
Möstl, E., Spendier, H., Kotrschal, K., 2001. Concentration of immunoreactive progesterone and androgens in the yolk of hens' eggs *Gallus domesticus*. Wien. Tieraerztl. Monatsschr. 88, 62–65.
Persson, I., Andersson, G., 1999. Intraclutch hatch synchronisation in pheasants and mallard ducks. Ethology 105, 1087–1096.
Petrie, M., Williams, A., 1993. Peahens lay more eggs for peacocks with larger trains. Proc. Royal Soc. Lond., B 251, 127–131.
Petrie, M., Schwabl, H., Brande-Lavridsen, N., Burke, T., 2001. Maternal investment. Sex differences in avian yolk hormone levels. Nature 412, 498–499.
Regolin, L., Vallortigara, G., 1996. Lateral asymmetries during responses to novel-colored objects in the domestic chick: a developmental study. Behav. Processes 37, 67–74.
Riedstra, B., Groothuis, T., 2000. The effects of prenatal androgen treatment in domestic chickens. Abstract VII Int. Symp. Avian Endocrin., Varanasi, India.
Ros, A.F.H., 1999. Effects of testosterone on growth, plumage pigmentation and mortality in black-headed gull chicks. Ibis 141, 451–459.
Ruiter, A.J., Koolhaas, J.M., Keijser, J.N., Oortmerssen, G.Av., Bohus, B., 1992. Differential testosterone secretory capacity of the testes of aggressive and non-aggressive house mice during ontogeny. Aggressive Behav. 18, 149–157.
Sapolsky, R.M., 1992. The neuroendocrinology of the stress response. In: Becker, J.B., Breedlove, S.M., Crews, D. (Eds.), Behavioral Endocrinology. MIT Press, Cambridge, MA, pp. 287–324.
Schmutz, J.A., 1993. Survival and pre-fledging body mass in juvenile emperor geese. Condor 95, 222–225.
Schuurman, T., 1981. Hormonal correlates of aggressive behavior in adult male rats. In: McConnel, P.S., Boer, G.J., Romijn, H.J., Van de Pool, N.E., Corner, M.A. (Eds.), Adaptive Capabilities of the Nervous System. Amsterdam, Elsevier, pp. 415–420.
Schwabl, H., 1993. Yolk is a source of maternal testosterone for developing birds. Proc. Natl. Acad. Sci. 90, 11449–11450.
Schwabl, H., 1996a. Maternal testosterone in the avian egg enhances postnatal growth. Comp. Biochem. Physiol. 114A, 271–276.
Schwabl, H., 1996b. Environment modifies the testosterone levels of a female bird and its eggs. J. Exp. Zool. 276, 157–163.
Schwabl, H., 1997. Maternal steroid hormones in the egg. In: Harvey, S., Etches, R.J. (Eds.), Perspectives in Avian Endocrinology. Endocrinology Ltd., Bristol.
Schwarz, I.M., Rogers, L.J., 1992. Testosterone: a role in the development of brain asymmetry in the chick. Neurosci. Lett. 146, 167–170.
Sockman, K.W., Schwabl, H., 2000. Yolk androgens reduce offspring survival. Proc. R. Soc. Lond., B 267, 1451–1456.
Takeuchi, H., Yazaki, Y., Matsushima, T., Aoki, K., 1996. Expression of Fos-like immunoreactivity in the brain of quail chick emitting the isolation-induced distress calls. Neurosci. Lett. 220, 91–94.
Tilly, J.L., Johnson, A.L., 1989. Regulation of androstenedione production by adenosine 3′,5′-monophosphate and phorbol myristate acetate in ovarian thecal cells of the domestic hen. Endocrinology 125, 1691–1699.
Trevarthen, C., 1996. Lateral asymmetries in infancy: implications for the development of the hemispheres. Neurosci. Biobehav. Rev. 20, 571–586.
Vallortigara, G., 1996. Learning of colour and position cues in domestic chicks: males are better at position, females at colour. Behav. Processes 36, 289–296.
Verboven, N., Monaghan, P., Evans, D.M., Schwabl, H., Evans, N., Whitelaw, C., Nager, R.G., 2003. Maternal condition, yolk androgens and offspring performance: a supplemental feeding experiment in the lesser black-backed gull (*Larus fuscus*). Proc. R. Soc. Lond., B 270, 2223–2232.
Viau, V., Meaney, M.J., 1996. The inhibitory effect of testosterone on hypothalamic-pituitary-adrenal responses to stress is mediated by the medial preoptic area. J. Neurosci. 16, 1866–1876.
Vom Saal, F.S., 1984. The intrauterine position phenomenon: effects on physiology, aggressive behavior and population dynamics in house mice. Prog. Clin. Biol. Res. 169, 135–179.
Yazaki, Y., Matsushima, T., Aoki, K., 1999. Testosterone modulates stimulation-induced calling behavior in Japanese quails. J. Comp. Physiol. [A] 184, 13–19.