Hormones and Behavior 63 (2013) 80–87

Contents lists available at SciVerse ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

Long-lasting and sex-specific consequences of elevated egg yolk testosterone for social behavior in Japanese quail

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ARTICLE INFO

Article history:
Received 25 May 2012
Revised 12 October 2012
Accepted 23 October 2012
Available online 30 October 2012

Keywords:
Maternal effects
Birds
Yolk testosterone
Social behavior

ABSTRACT

In birds, early exposure to steroid hormones deposited in egg yolks is hypothesized to result in long-lasting effects on brain and behavior. However, the long-term effects of maternal androgens on the development of social behavior, and whether these could interfere with the effects of the endogenous gonadal hormones that mediate sexual differentiation, remain poorly known. To answer these questions, we enhanced yolk testosterone by injecting testosterone (T) in oil into Japanese quail (*Coturnix japonica*) eggs prior to incubation. Vehicle-injected (V) eggs served as controls. From age 3 weeks to 8 weeks, sexual development was measured using morphological and physiological traits, and social behavior was measured, including male-typical sexual behavior. In females, treatment with testosterone boosted growth. Males from T-injected eggs developed an affiliative preference for familiar females and differed from V-injected males in the acoustic features of their crows, whereas sexual interest (looking behavior) and copulatory behavior were not affected. These long-lasting and sex-specific yolk testosterone effects on the development of dimorphic traits, but without disrupting sexual differentiation of reproductive behavior suggest potential organizational effects of maternal testosterone, but acting through separate processes than the endocrine mechanisms previously shown to control sexual differentiation. Separate processes could reflect the action of androgens at different times or on multiple targets that are differentially sensitive to steroids or develop at different rates.

Introduction

Maternal effects occur when the phenotype of an individual is affected by the phenotype of its mother independently of the female's genetic contributions to her offspring (Mousseau and Fox, 1998). Since Schwabl (1993) showed that avian eggs contained substantial and variable amounts of maternal androgens that affect offspring development, maternal effects mediated by yolk hormones have been of great interest as an influence on offspring fitness (Gil, 2008; Griffith and Buchanan, 2010; Groothuis et al., 2005; von Engelhardt and Groothuis, 2011). Many studies have documented short-term effects of yolk hormones on the physiological and behavioral traits of offspring, such as growth rate, begging for food (Gil, 2008; Groothuis et al., 2005), stress sensitivity and social motivation (Daisley et al., 2005) or auditory learning (Bertin et al., 2009). Only a few recent studies have extended these effects to adulthood. Early exposure to testosterone promoted adult exploratory behavior, and increased the expression of sexual traits and the frequency of dominance and sexual displays in male house sparrows (*Passer domesticus*), black-headed gulls (*Larus ridibundus*) and pheasants (*Phasianus colchicus*) (Bonisoli-Alquati et al., 2011; Eising et al., 2006; Partecke and Schwabl, 2008; Ruuskanen and Laaksonen, 2010; Strasser and Schwabl, 2004; Uller et al., 2005), whereas detrimental effects on laying and copulatory behavior were found in female Chinese quail (*Coturnix chinensis*) and pheasants (Bonisoli-Alquati et al., 2011; Rubolini et al., 2007; Uller et al., 2005).

An important question that has not yet been sufficiently addressed is the relationship between long-term effects of maternal androgens and hormonally based sexual differentiation (Carere and Balthazart, 2007). It is well established that steroid action during the embryonic period establishes in an irreversible manner brain sex differences (organizational effects). Then, later in life, circulating steroids can stimulate in a reversible manner the expression of behavior, including behavior that was hormonally organized earlier (activational effects). Avian embryos are exposed to both their endogenous steroids and to maternal hormones deposited in the egg yolk. In precocial birds, the critical period for hormonal organization of sex differences occurs well before hatching, during the phase of yolk utilization (Groothuis and Schwabl, 2008). When maternal androgens have long-term effects, are those occurring through modification of or interference with hormonally organized sexual differentiation? Are they occurring through the same mechanisms, and are their consequences what would be expected, based on what is

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0018-506X/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.yhbeh.2012.10.011

known about sexual differentiation, with respect to the sex and kind of behavior is affected? The alternative is that maternal yolk hormones do not alter sexual differentiation, but instead act through other independent mechanisms (e.g. temporal dissociation or the action of hormones on multiple targets that are differentially sensitive to steroids or develop at different rates), so that their consequences do not fit known patterns of sexual differentiation. For example, if maternal androgens cause an increase in a characteristic that exhibits no sex difference and is not affected by hormonal manipulations during the critical period for sexual differentiation, that result would not reasonably be interpreted as an alteration in sexual differentiation.

The Japanese quail is an excellent model organism for determining the relationship between long-term effects of maternal androgens and hormonally based sexual differentiation. The short time to sexual maturity makes it easier to follow the birds until adulthood. The hormonal bases (both organization and activation) of sexually dimorphic behaviors have been well established in this species. Male-typical behavior such as sexual interest in females (looking at females through a window) and copulatory behavior, and the development of a foam gland, are organized during embryonic development, whereas sex differences in crowing and sexual receptivity result largely from hormonal activation in adulthood (see Balthazart et al., 1995, and Adkins-Regan, 2009 and Carere and Balthazart, 2007 for reviews). Females do not look at other females or show male-typical copulatory behavior unless the eggs from which they hatched were injected with an estrogen synthesis inhibitor (organizational manipulation) followed by adult treatment with testosterone to activate the behaviors. In contrast, adult testosterone treatment alone will activate crowing by females and estradiol treatment of adult males will activate receptivity. Females are larger than males, but size dimorphism in this species is not hormonally organized (Koba et al., 2008).

Furthermore, yolk hormone studies have often assumed that exposure to high testosterone levels in yolk should enhance the expression of male traits, as observed in mammals in response to embryonic or neonatal exposure to androgens (Groothuis et al., 2005; Mateo, 2009). On the contrary, with respect to hormonal organization of sexually differentiated behavior, a number of studies have established in Japanese quail that early exposure to testosterone demasculinizes, not masculinizes, males and does not masculinize females (see Adkins-Regan, 2009 and Balthazart and Ball, 1995 for reviews). These previous studies predict that if maternal yolk hormones act through the same mechanisms as hormonally based sexual differentiation, an increase in yolk testosterone levels is likely to demasculinize male quail, whereas females should not be affected. If they act through independent mechanisms, maternal yolk testosterone should not alter sexual differentiation, but could affect behaviors that are not hormonally organized, as previously explained.

Here, we exposed male and female Japanese quail embryos to a physiological increase in testosterone *in ovo*, prior to incubation. We followed the sexual development of the birds by measuring morphological and behavioral dimorphic traits from 3 to 8 weeks (reproductive adulthood) of age. Measurement included a detailed focus on crowing and the acoustic structure of the crows, as in addition to its important role in activating crowing (Adkins-Regan, 2009; Beani et al., 2000; Chiba and Hosokawa, 2006), testosterone can also affect acoustic features of this male-typical vocalization (Beani et al., 2000; Yazaki et al., 1999).

Materials and methods

(a) Egg injection

Quail eggs were obtained from 42 female Japanese quail between 25 and 32 weeks of age raised and housed in the animal facility at Cornell University. A maximum of two eggs per female was collected. One egg was assigned to the testosterone treatment and the other one to the control. Mean ± SE yolk testosterone concentration in Japanese quail has been determined to be 13 ± 8 ng/g yolk (Gil and Faure, 2007; Hackl et al., 2003; Pilz et al., 2005) and yolk mass is 3.26 ± 0.63 g (Bertin et al., 2008). Therefore, to increase the overall yolk concentration about two standard deviations (16 ng/g), we injected 50 ng of testosterone propionate (Sigma-Aldrich®) suspended in 20 μl vehicle (refined olive oil, Sigma-Aldrich®) in treated eggs (Bertin et al., 2009). In total, 36 fertilized eggs were injected with testosterone (T group) and 41 fertilized eggs with vehicle (V group). The dose of testosterone used to elevate yolk levels was well within the natural range encountered (Hackl et al., 2003). Before injection, all eggs were carefully cleaned and disinfected with 70% ethanol and a hole was bored in the eggshell above the air sac using a sterile 25-G needle. The solution was delivered to the yolk using a 50 μl Hamilton syringe. The injection hole was covered with paraffin. The eggs were left unmoved for 30 min and then incubated for 18 days into an incubator (NatureForm's SAFARI, USA) maintained at 37 °C and 50–60% relative humidity. The incubation and hatching procedure made it possible to keep track of egg/chick treatments but not identity of mothers of eggs; thus mother identity was not included in the statistical analysis.

(b) Birds and housing

Newly hatched chicks were identified by numbered plastic leg bands in three different colors (one per treatment). They were housed in groups of 20–25 birds until 3 weeks of age and in individual battery cages afterwards until 9 weeks of age. To ensure the same early social rearing environment for all treatments, chicks from both groups (T-injected and V-injected) were mixed. Additional quail chicks from non-injected eggs, obtained from 34 female Japanese quail distinct from those used to obtain treated and control eggs, were housed in these mixed groups to provide female stimulus birds for the learned social proximity and two-choice tests described thereafter. These mixed groups were housed in brooders (91 cm (length) × 61 cm (width) × 25 cm (height)) maintained at 33 °C under a 14:10 LD cycle. Food and water were available *ad libitum*.

(c) Behavioral procedures and traits measured

Nineteen T-injected quail (8 males and 11 females) and 20 V-injected quail (10 males and 10 females) were tested and measured following the schedule summarized in Fig. 1. We ran the total of 39 birds in two cohorts because of space and time constraints. Data obtained from the two cohorts were combined after being analyzed for replicate effect. The quail were subjected to three learned social proximity tests per week and one two-choice test every 2 weeks over the 6-week experimental period (from 3-week to 8-week old). The two behavioral tests were carried out to measure the development of sexual behavior and affiliative preference, respectively, in quail from each treatment. In addition, body weight and size of the cloacal vent were determined in both sexes, and size of the proctodeal gland and quantity of foam production were determined in males twice a week. Cloacal vent length was measured with a digital caliper to the nearest 0.1 mm. A similar procedure was used to estimate the size of the proctodeal gland (length x width, mm^2) (Sachs, 1969). The behavioral tests were recorded using digital camcorders (SONY DCR-TRV17 and SONY DCR-TR33) and the behavior was quantified using Stopwatch+ (http://www.cbn-atl.org). We also recorded the first day of laying and of foam production as indicators of the day of sexual maturity in females and males, respectively. All procedures were approved by the Cornell University Institutional Animal Care and Use Committee (protocol number: 2002–0117).

(i) The learned social proximity procedure (LSPP)

Social and sexual motivation was measured in quail using the LSPP similar to that described by Balthazart et al. (1995). Briefly, we quantified the time spent by a focal individual in a proximity

Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
LSPP	Morphology Crowing Blood sample	LSPP	Morphology Crowing	LSPP	Two-choice test[a]

LSPP = Learned Social Proximity Procedure
Morphology = body mass, length of cloacal vent, size of proctodeal gland and foam production

Fig. 1. Weekly testing and measurement schedule over the 6-week experimental period (from 3 weeks to 8 weeks of age). Morphology, crowing and blood sampling were performed at approximately the same time of day on each day of measurement. [a]: Test run every 2 weeks.

area, located in front of a door with a window, providing a view of a female that was subsequently released into the cage.

The apparatus was made up of a testing cage (91 cm (length)× 62 cm (width)×31 cm (height)) and a smaller cage for female stimulus birds (31 cm (length)×19 cm (width)×25 cm (height)) which was centered on the left lateral wall of the main cage and separated from it by a horizontally sliding door (31 cm (width)×25 cm (height)). A small window (1 cm (width)×19 cm (height)) was located in the middle of this door and provided the focal bird with the only visual access to the female. This window could be closed by an opaque panel (5 cm (width)×25 cm (height)). Both the sliding door and the panel could be remotely controlled by the experimenter. When the window was open, the focal bird could only see the female located in the smaller cage if it stood in a proximity area (20.5 cm×30 cm) located in front of the window. The head was used as the cue of entering the proximity area. The front wall of each cage was made of Plexiglas to allow behavioral observations.

The time spent in the proximity area by the focal quail was continuously recorded for 5 min during the pretest period (window and door closed) and then during the test period (window open and door closed). The measure of the social and sexual motivation of the focal quail was then analyzed computing the difference in the time spent in the proximity area between the test and the pretest periods. This difference provided a measure of the specific response to the opening of the window and consequently the view of a female. The door separating the two compartments was then opened and the two birds were allowed to interact for 5 min. During that time, the number of the following sexual behaviors was recorded: head grabs, mounts and cloacal contact movements (CCM) as defined by Adkins and Adler (1972). The two quail were then removed from the experimental apparatus and returned to their home cages.

(ii) Two-choice tests

Affiliative preference for a familiar versus an unknown female was measured using a runway apparatus, based on a similar principle as the treadmill test (Mills and Faure, 1990). The runway was a 118 cm-long straight wire-netting tunnel. At both ends, goal boxes (20 cm (length)×36 cm (width)×41 cm (height)) were separated from the tunnel by a wire-mesh screen. One goal box contained the familiar (Fa) female, i.e. the female stimulus presented in the LSPP, whereas the other goal box contained an unknown (U) female, i.e. from a different housing group and presented to a different focal quail during the LSPP. The tunnel was divided into three zones of equal length (39.33 cm): proximity zones in front of each goal box and a neutral zone in the middle of the tunnel. The two stimulus quail were maintained in their boxes during two successive tests. Thus, side of presentation was counterbalanced across subjects. Test order was randomized. Each focal quail began the trial in the middle of the neutral zone. Trials were 5 min in duration. The latency to enter into and the time spent in each proximity zone were recorded. Data were then analyzed by computing the difference in the latency to enter into the proximity zones and the proportion of time spent in the Fa proximity zone.

(e) Testosterone assays

Testosterone measurements were done in order to confirm that the T-injected males had normal testosterone levels during testing. Blood was collected from T-injected and V-injected males each week (Tuesday mornings) between 09h00 and 11h00 from 3 to 8 weeks of age. Blood was centrifuged at 5125*g* for 6 min and the plasma layer was removed and stored at −80 °C. Plasma testosterone concentration was measured using an enzyme immunoassay (EIA) kit (Cayman Chemical; Ann Arbor, MI, USA). The intra- and inter-assay coefficients of variation (CV) were 7.6% and 62.3%, respectively. Any potential bias related to the high inter-assay CV was minimized as plasma samples were randomized across assays according to treatment and age. In addition, the results for serially diluted quail plasma were parallel to the standard curve, and both the average testosterone levels and the pattern over sexual maturation were similar to those of previous studies (Ottinger and Brinkley, 1979; Ramenofsky, 1984). Therefore, even though the inter-assay CV was high, the testosterone assay otherwise performed well and yielded biologically meaningful data.

(f) Acoustic analysis

The Japanese quail crow is composed of three syllables: two short ones followed by a long and frequency-modulated trill (Guyomarc'h and Guyomarc'h, 1996), which is the characteristic element of the crow (Figure S1). To assess crowing, we measured the number of crows emitted by T- and V-injected males during a 5-min period 1 hour after lights on. Crowing was measured twice weekly (Tuesday and Thursday) from 3 to 8 weeks of age (Fig. 1).

Crow acoustics during social interaction were investigated by analyzing crows emitted by T- and V-injected males during the LSPP. This procedure allowed us to record only one male crowing at a time which is necessary for the subsequent analysis of the acoustic features of the crow. Crows were recorded with a Sennheiser ME67 microphone connected to the camcorder and placed above the cage. The sounds were digitized (sampling frequency = 44.1 kHz) and noise was filtered out using a highpass filter at 2000 kHz with the GoldWave® v5.58 software, based on a noise spectral analysis. For the spectral analysis, 5 crows per bird were selected from the two last weeks of the experimental period (i.e. between 7 and 8 weeks of age). We described the crow of each individual bird by a set of acoustic features (see Table S1 in the electronic supplementary material for a complete list and detailed values) using the Sound Analysis Pro (SAP) 2A.04 software (Tchernichovski and Mitra, 2004). We adjusted amplitude and entropy thresholds in SAP so that each crow would be segmented in syllables. Features were calculated in

9.27 ms windows, every 1 ms of crowing. Subsequent analysis was based on spectral features that change during crow development and could show inter-individual differences (Derégnaucourt et al., 2009; Guyomarc'h et al., 1998): frequency modulation (FM), amplitude modulation (AM), mean frequency and entropy, as automatically calculated by SAP (Table S1).

These various individual spectral features may collectively account for acoustic differences in crowing. We reduced the dimensionality by applying principal component analysis (PCA) to the set of spectral features, and we retained the first two principal components (PC1 and PC2) obtained for each syllable. We then estimated the effect of treatment with testosterone on acoustic structure of crows by comparing PC1 and PC2 between V- and T-injected male quail using a Mann–Whitney *U*-test for independent samples. Effect sizes were calculated using the Glass rank-biserial correlational coefficients (r_{rb}) (Glass and Hopkins, 1984). Following Cohen (1988), an effect size of 0.37 was viewed as large, 0.24 as medium and 0.10 as small. Details can be found in the electronic supplementary material, and in Derégnaucourt et al. (2009) and Fehér et al. (2009).

(g) Data analysis

To estimate the effect of treatment with testosterone on sexual development in quail, we examined the values obtained for all our variables relative to the beginning of sexual maturity (day 0), defined as the first day of laying for females and the first day of foam production for males. As the first day of laying was highly delayed for four T-injected females, we did not have enough data after sexual maturity for these birds. Thus, these females were removed from the statistical analysis except for the comparison of the first day of laying between treatments.

The effect of treatment with testosterone on the development of sexual behavior and affiliative preference in quail was analyzed using a linear model of analysis of variance for repeated measures. We used a model with the cohort (two levels) and the treatment (two levels: V-injected versus T-injected eggs) as between-subjects factors. Time effect was the within-subjects factor. The measures of affiliative preference were analyzed by computing the proportion of time spent in the proximity zone of the Fa stimulus (Fa/[Fa + U]) and the difference in the latency to enter into the proximity zones (Fa − U) during the two-choice test. Preference was defined as the focal bird spending significantly more than 50% of its time in the proximity zone of the Fa stimulus or entering significantly earlier into the proximity zone of the Fa stimulus. We also arcsin transformed the proportion of time spent in the Fa proximity zone in order to meet the assumptions of ANOVA. Female behavioral testing results are not reported, because they are not relevant to the question addressed in this paper.

For the variables reported for both males and females, the sex effect was added to the model as a between-subjects factor. Quail weight and the length of the cloacal vent were analyzed using an analysis of covariance similar to the general linear model described above, except that age was the covariate.

A two-sample approximate test against an alternative hypothesis (Meddis, 1984) was used to compare the age at sexual maturity (first day of laying and of foam production in females and males, respectively), between V- and T-injected quail. This test is a variation of the Mann–Whitney *U*-test for independent samples corrected for a large number of ties in data.

Statistical analyses were performed using Statistica 8.0 (Statsoft, Inc., USA). The general linear models were followed by *post hoc* Fisher LSD tests when appropriate. In all cases, alpha was set to 0.05. Effect sizes were calculated and reported when statistical analyses gave a non-significant tendency following Cohen's (1988) and Fritz et al. (2012) methods. Data are given as mean ± SEM except for the age at sexual maturity which is given as medians and interquartile ranges (25–75%).

Results

(a) Testosterone injections and traits indicating sexual development

There was no significant effect of treatment on hatching success (80.5% *vs.* 78.0%, T- and V-injected eggs respectively, Chi-square test, $\chi^2 = 0.072$, $df = 1$, $p = 0.79$). T-injected females showed a non-significant trend to be delayed in the first day of laying compared to V-injected females (51 (50–66) *vs.* 50 (47–53), respectively, $Z = 1.46$, $p \leq 0.07$, Glass rank-biserial correlational: $r_{rb} = 0.37$ (large effect size as recommended by Cohen (1988)). T- and V-injected males did not differ in the first day of foam production (39 (38–40) *vs.* 38 (38–42), respectively, $Z = 0.60$, $p \leq 0.27$). Significant main effects of sex ($F_{1,229} = 405.9$, $p < 0.001$), treatment ($F_{1,229} = 7.84$, $p = 0.005$) and age ($F_{1,229} = 160.8$, $p < 0.001$) were found for body mass (Fig. 2). We also found a significant interaction between sex and treatment ($F_{1,229} = 9.3$, $p = 0.002$). Both sexes showed a similar pattern of growth with a larger weight gain before sexual maturity. However, females were heavier than males and T-injected females were heavier than V-injected females, suggesting that prehatching exposure to testosterone boosted growth in females (Fisher LSD: $p < 0.001$ in all cases, Fig. 2). Although the other traits indicating sexual development increased with sexual maturity ($p < 0.001$ in all cases, Table 1), there was neither a significant effect of the testosterone treatment nor an interaction between treatment and age or sex for these variables ($p > 0.20$ in all cases, Table 1).

(b) Learned social proximity

Males showed an increase in the time they spent at the window after sexual maturity ($F_{8,112} = 4.1$, $p < 0.001$, Fig. 3). The time male quail spent at the window was not affected by the treatment with testosterone ($F_{1,112} = 0.1$, $p = 0.71$) and there was no significant interaction between treatment and time ($F_{8,112} = 0.9$, $p = 0.48$).

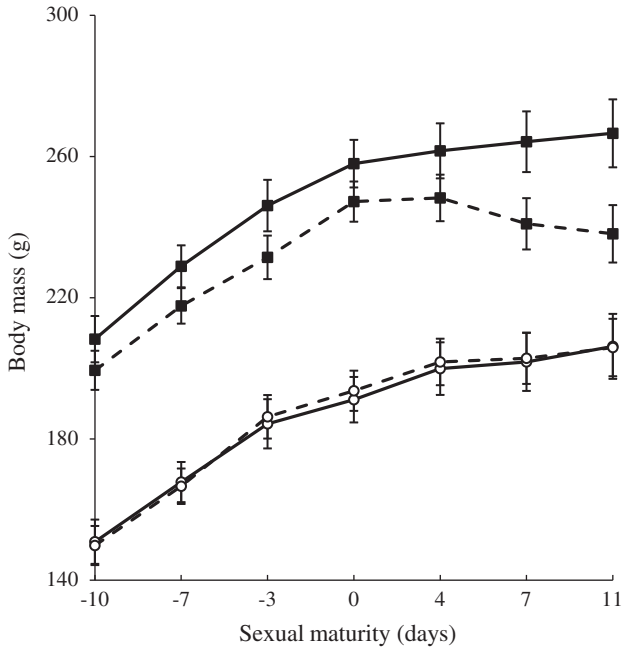

Fig. 2. Mean ± SEM body mass (g) in female (F, filled squares) and male (M, open circles) quail from T-injected ($N_F = 8$, $N_M = 7$, solid lines) and V-injected eggs ($N_F = N_M = 10$, dashed line), measured from 10 days before sexual maturity to 11 days after sexual maturity.

Table 1
Traits indicating sexual development (mean ± SEM) in male (M) and female (F) Japanese quail from T-injected ($N_M = 7$, $N_F = 8$) and V-injected ($N_M = N_F = 10$) eggs measured from 10 days before sexual maturity to 14 days after sexual maturity.

Number of days before (−) and after sexual maturity[a]	Treatment	Length of the cloacal vent (mm)[c]		Size of the proctodeal gland (mm^2)[b]	Foam quantity (ml)[b]	Number of crows per 5 min[b]
		Females	Males	Males	Males	Males
−10	T	7.48 ± 0.58	6.82 ± 0.29			
	V	7.51 ± 057	6.33 ± 0.30			
−7	T	9.13 ± 0.68	6.82 ± 0.29			
	V	8.52 ± 0.49	6.57 ± 0.25			
−3	T	10.04 ± 0.52	7.50 ± 0.41			
	V	9.08 ± 0.43	7.54 ± 0.36			
0	T	10.38 ± 0.51	7.96 ± 0.40	44.91 ± 6.78	0.03 ± 0.01	
	V	9.88 ± 0.36	8.54 ± 0.48	48.26 ± 8.84	0.02 ± 0.01	
4	T	10.39 ± 0.51	8.62 ± 0.47	91.56 ± 8.71	0.03 ± 0.01	3.25 ± 1.74
	V	10.16 ± 0.41	9.16 ± 0.40	87.95 ± 7.85	0.06 ± 0.02	1.75 ± 0.69
7	T	10.78 ± 0.39	9.35 ± 0.35	115.72 ± 6.16	0.07 ± 0.03	3.25 ± 1.60
	V	10.21 ± 0.41	9.41 ± 0.37	124.54 ± 11.30	0.16 ± 0.04	3.90 ± 1.43
11	T	10.98 ± 0.34	9.74 ± 0.45	158.81 ± 11.28	0.12 ± 0.04	2.37 ± 1.27
	V	10.56 ± 0.35	10.05 ± 0.27	165.91 ± 9.63	0.27 ± 0.06	3.80 ± 0.93
14	T			170.01 ± 10.78	0.29 ± 0.10	2.87 ± 1.50
	V			198.10 ± 8.49	0.30 ± 0.03	5.50 ± 1.48

[a] The first day of laying and the first day of foam production indicated the first day of sexual maturity in female and male Japanese quail respectively.
[b] Missing values are due to the fact that the trait was expressed in too few birds to be analyzed.
[c] Because the first day of laying was highly delayed for four T-injected females, there were not enough data to perform the analysis of variance more than 11 days after sexual maturity.

We found no significant effect of treatment or interaction between treatment and the other factors on the number of head grabs, mounts and CCM in males ($F_{5,70} \leq 2.0$, $p \geq 0.10$ in all cases, Table 2).

(c) Two-choice tests

We found a significant effect of the treatment on the proportion of time spent in the proximity zone of the familiar (Fa) stimulus female ($F_{1,14} = 6.5$, $p = 0.024$). The proportion of time was higher in the T- than the V-injected males (Fisher LSD: $p = 0.028$). T-injected males showed an affiliative preference for Fa females, as defined by the focal bird spending at least 50% more time with the Fa than the U females ($p \leq 0.005$), whereas V-injected males showed no preference ($p \leq 0.52$). Data are given in Fig. 4A as the average across all the two-choice tests both before and after sexual maturity because we found neither a significant effect of sexual maturity on the proportion of time spent in the proximity zone of the Fa stimulus female ($F_{1,14} = 0.9$, $p = 0.35$) nor a significant interaction between sexual maturity and treatment ($F_{1,14} = 0.2$, $p = 0.64$)

The treatment with testosterone tended to affect the difference in the latency to enter into the Fa and the U proximity zones but not significantly so ($F_{1,14} = 4.1$, $p = 0.061$, eta squared: $\eta^2 = 0.19$ (large effect size as recommended by Cohen (1988), Fig. 4B). T-injected males entered faster into the proximity zone of Fa females ($p \leq 0.02$ Fig. 4B). Neither a significant effect of sexual maturity ($F_{1,14} = 1.1$, $p = 0.31$) nor a significant interaction between sexual maturity and treatment ($F_{1,14} = 0.2$, $p = 0.69$) was found for this variable.

(d) Plasma testosterone concentrations

Plasma testosterone concentrations in male quail showed a significant increase after sexual maturity ($F_{5,70} = 49.4$, $p < 0.001$, Fig. 5). All males had a testosterone level within the normal range. The analysis revealed neither a significant effect of the pre-hatching testosterone treatment ($F_{1,70} = 0.03$, $p = 0.87$) nor a significant interaction between the treatment and age ($F_{5,70} = 0.5$, $p = 0.74$).

(e) Crowing and acoustic structure

A mean (± SEM) of 7 ± 1 out of the 10 V-injected males and 4 ± 1 out of the 8 T-injected males emitted crows within 1 hour after the light turned on, on each day of observation. The number of males crowing did not differ between the two treatments (Fisher's exact test, $p = 0.63$). Similarly, the number of crows emitted by male quail was not affected by treatment and did not vary following sexual maturity ($F_{1,42} < 0.9$, $p > 0.56$ in all cases, Table 1).

During the LSPP, 6 out of the 10 V-injected males and 8 out of the 8 T-injected males emitted crows. There was no significant difference between the two treatments (Fisher's exact test, $p = 0.09$).

Four T-injected males and one V-injected male were removed from the subsequent acoustic analyses because they did not emit the minimum of 5 crows. In addition, for one of the remaining T-injected males, the first syllable of its crow was removed from the acoustic and statistical analyses due to the poor quality of the spectrogram after digitization. The temporal structure of the crows (total duration, syllable durations and intersyllabic intervals) was not affected by the treatment with testosterone ($U \geq 5.0$, $p \geq 0.45$, in all cases, Table S1). For the analysis of the spectral features of crows, the first three components of the PCA had cumulative Eigenvalues of 6.2, 6.7 and 6.8, and accounted for 78%, 83% and 85% of the total variance for the first, the

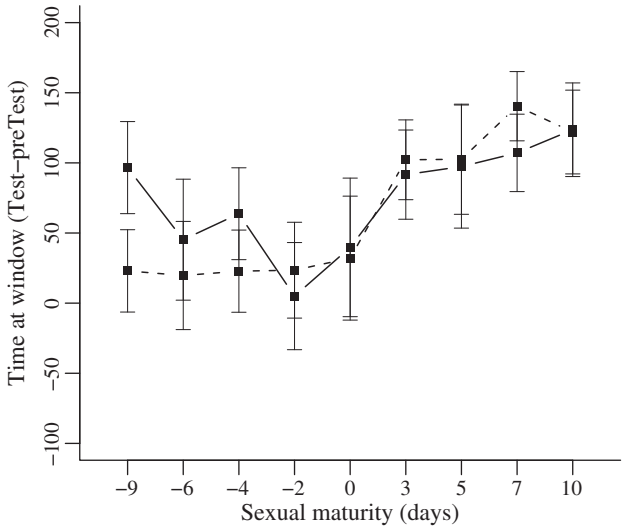

Fig. 3. Mean ± SEM time at window (test–pretest) measured in males from T-injected ($N = 8$, solid line) and V-injected eggs ($N = 10$, dashed line) from 9 days before sexual maturity to 10 days after sexual maturity.

Table 2
Number of head grabs, mounts and cloacal contact movements (CCM) (mean ± SEM) displayed by male Japanese quail from T-injected ($N = 8$) and V-injected ($N = 10$) eggs when given access to the female during the LSPP from 3 days to 14 days after sexual maturity.

Number of days after sexual maturity[a]	Treatment	Grabs	Mounts	CCM
3	T	2.0 ± 2.0	2.0 ± 2.0	0.0 ± 0.0
	V	4.3 ± 2.3	2.6 ± 1.5	0.1 ± 0.1
5	T	21.0 ± 11.8	6.1 ± 3.2	0.5 ± 0.3
	V	9.0 ± 3.9	6.3 ± 2.9	0.2 ± 0.1
7	T	2.6 ± 1.8	1.6 ± 0.8	0.7 ± 0.3
	V	17.1 ± 5.9	6.5 ± 1.8	0.8 ± 0.4
10	T	6.9 ± 3.1	3.7 ± 1.4	1.4 ± 0.5
	V	24.7 ± 10.6	10.2 ± 3.6	1.5 ± 0.7
12	T	12.0 ± 4.4	7.0 ± 2.2	1.7 ± 0.6
	V	18.8 ± 8.8	6.7 ± 1.9	1.7 ± 0.6
14	T	8.0 ± 3.3	5.6 ± 2.3	2.0 ± 0.8
	V	13.8 ± 4.0	6.0 ± 1.2	1.7 ± 0.5

[a] The first day of foam production indicated the first day of sexual maturity for male Japanese quail. The values start at 3 days after sexual maturity because none of the behaviors were displayed prior to that.

second and the third syllables respectively (Table S1). Using the first two principal components of the PCA (PC1 and PC2), we found significant differences in the spectral structure of crows between V- and T-injected male quail (Fig. 6). The first two syllables differed between V- and T-injected males on the basis of PC1 ($U = 0.0$, $r_{rb} = 1.00$, $p = 0.02$ and $U = 1.0$, $r_{rb} = 0.90$, $p = 0.03$ respectively), whereas the third syllable differed on the basis of PC2 ($U = 2.0$, $r_{rb} = 0.80$, $p = 0.05$).

4. Discussion

We found that prehatching exposure to testosterone affected males' affiliative preference and caused acoustic changes in crowing, although motivation to crow was not affected. T-injected males preferred to affiliate with familiar females, whereas V-injected males did not show any preference. Looking behavior and copulatory behavior were not affected by the testosterone treatment. In females, testosterone treatment boosted growth.

What do these results indicate with respect to the relationship between maternal yolk hormone effects and hormonal organization of sexually differentiated behavior in Japanese quail? The fact that the treatment had no demasculinizing effects on male crow frequency, copulation or looking behavior while at the same time having other significant long-term consequences suggests potential organizational effects of maternal yolk testosterone but acting through independent mechanisms from hormonally organized sexual differentiation. The greater body mass of T-injected females also supports independent mechanisms of action, given that size dimorphism in this species is not hormonally organized (Koba et al., 2008).

The affiliative preferences of males were affected by the treatment. It could be argued that this reflects lowered sexual motivation and therefore slight demasculinization. The endocrine mechanism involved in the expression of affiliative preference in the Japanese quail has not yet been determined, but in adult males affiliative preference is correlated with mate choice (White and Galef, 1999) and male-specific interest in female stimuli is known to be hormonally organized (Adkins-Regan, 2011; Balthazart et al., 1997). However, the affiliative preference shown by the males from testosterone treated eggs did not depend on sexual maturity in our study, making it less likely to reflect sexual motivation.

Another explanation for the affiliative preference result is worth considering. In the two-choice test, males have to choose between a female to which they have visual access and then the opportunity for interactions and/or copulation and an unknown female. A preference requires that the male is able to discriminate on the basis of learned familiarity. It is well established that Japanese quail are able to discriminate between familiar and unfamiliar conspecifics (Schweitzer et al., 2009). Yet only the males from testosterone treated eggs preferred the familiar female. Similarly, Bertin et al.(2009) showed that Northern bobwhite quail chicks (*Colinus virginianus*) from T-injected eggs subsequently exposed to a hen's maternal call developed a preference for the familiar call, whereas control chicks did not show any preference. It is possible that our prehatching treatment with testosterone similarly promoted learning of the familiar female characteristics which may explain why T-injected male quail showed a preference for familiar females, independently of sexual motivation, whereas V-injected males

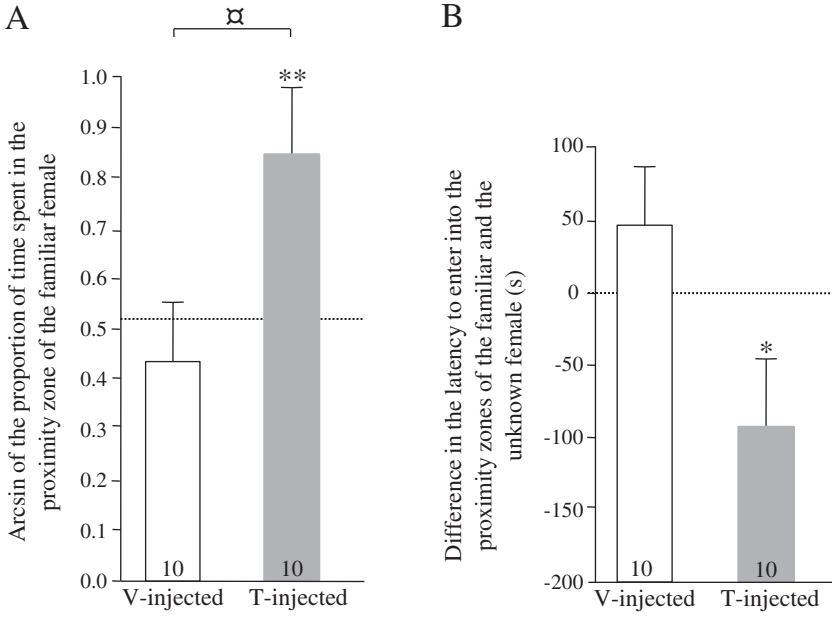

Fig. 4. A) Arcsin of the average proportion of time spent in the proximity zone of the familiar female (mean ± SEM) and B) average difference in the latency to enter into the proximity zones of the familiar and the unfamiliar (U) females measured across all the two-choice tests both before and after sexual maturity, in males from T-injected and V-injected eggs. **, *: significant preference ($p \leq 0.01$; $p \leq 0.05$). ¤: Significant difference between treatments ($p \leq 0.05$). The dotted line indicates the preference threshold: A) the focal bird spending significantly more than 50% of its time in the proximity zone of the Fa stimulus or B) entering significantly earlier into the proximity zone of the Fa stimulus.

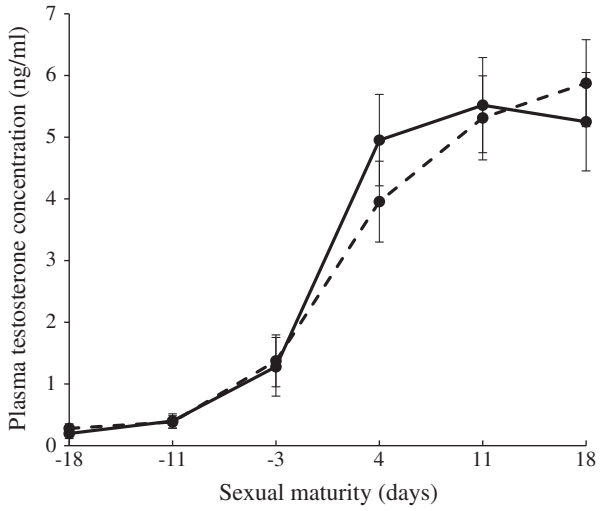

Fig. 5. Mean ± SEM plasma testosterone concentrations (ng/ml) in male quail from T-injected ($N = 8$, solid line) and V-injected eggs ($N = 10$, dashed line), from 18 days before sexual maturity to 18 days after sexual maturity.

did not. This also supports potential organizational effects of yolk maternal testosterone but on other brain regions than those targeted for hormonally organized sexual differentiation.

The acoustics of the males' crows were also affected by the treatment. Again, it could be argued that this reflects lowered sexual motivation and therefore slight demasculinization. The crow is involved in mate attraction (Goodson and Adkins-Regan, 1997; Potash, 1975). The recent finding of Derégnaucourt et al. (2009) suggests that the crow could also be used as a potential cue to discriminate between individuals. We found a global change in the acoustic structure of the crow whose functional consequences are difficult to interpret. Additional work using playback of crows that differ in their acoustic structure would be needed to test a functional hypothesis. Regardless of exactly how the results for affiliation and crow acoustics should be interpreted, both represent long-term and therefore potentially organizational effects of yolk testosterone.

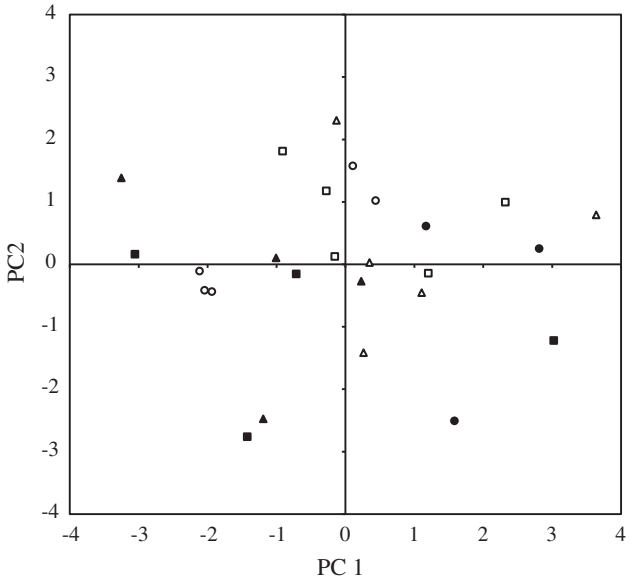

Fig. 6. Changes in acoustic structure of crows in T-injected ($N = 4$, filled symbols) and V-injected ($N = 5$, open symbols) male quail. Principal component analyses calculated from spectral features of the crows, for the three syllables of the vocalization. First syllable: circles, second syllable: triangles, third syllable: squares.

What physiological mechanisms of action can explain how the maternal yolk hormone effects and hormonally organized sexual differentiation are able to coexist? Carere and Balthazart (2007) suggested that there is a temporal dissociation between the two phenomena. Maternally deposited testosterone may no longer be active at the time of sexual differentiation because of high metabolism in the egg or by the embryo (Elf and Fivizzani, 2002; Gilbert et al., 2007; Pilz et al., 2005).

Alternatively, the authors suggested that maternal hormones affect different anatomical targets than the preoptic area and hypothalamus that mediate male-typical sexual behavior under hormonal regulation (Ball and Balthazart, 2004). Indeed, our results and previous studies reported maternal effects on a variety of traits, such as growth rate, begging for food, aggressive and exploratory behavior, immune system competence (Gil, 2008; Griffith and Buchanan, 2010; Groothuis et al., 2005; Ruuskanen and Laaksonen, 2010), stress sensitivity and social motivation (Daisley et al., 2005), and auditory learning (Bertin et al., 2009), which are mediated by the action of androgens on other brain or peripheral (e.g. muscular) targets. It is also possible that these specific targets for the two processes differ in amounts of androgen receptors (AR) in the brain or periphery (Strasser and Schwabl, 2004), or they express AR at different times during incubation (Carere and Balthazart, 2007).

Whatever the mechanisms behind yolk testosterone effects, they may have consequences for the reproductive success of male and female quail. According to the prevailing adaptationist view of maternal effects, mothers alter the phenotype of their offspring to maximize their own lifetime reproductive success and to tailor their offspring to local environmental conditions (Mousseau and Fox, 1998). In our experiment, additional growth in females from treated eggs may in fact result in fitness gains by allowing production of larger and better-quality nestlings in the future (but see Andersson et al., 2004; Müller et al., 2005; Navara et al., 2005; Rubolini et al., 2006, 2007 for detrimental effects of maternal yolk androgens in avian female offspring). As suggested by Andersson et al. (2004), these contrasting effects could result if most energy is allocated to growth and relatively little to immune function or to ovarian development, with negative effects on later ovarian functionality and follicle maturation. In T-injected males, the vocal changes and the affiliative preference towards familiar females are also likely to be social influences on reproduction. As discussed above, they may reflect lower sexual motivation in males, or have consequences for mate choice either by influencing male mating preference or the indicators of male quality used by females to choose between males. Significant evidence exists in birds that females invest in reproductive events in response to male quality (Arnqvist and Kirkpatrick, 2005; Bolund et al., 2009; Forstmeier et al., 2011; Garcia-Fernandez et al., 2010; Horváthová et al., 2012; Kingma et al., 2009; Pryke et al., 2011). Thus, maternal yolk testosterone is likely to have consequences for offspring fitness in both sexes.

In conclusion, elevated yolk testosterone level in Japanese quail eggs has sex-specific and long-term effects over the course of development. The results indicate that yolk testosterone does influence the development of socio-sexual behavior (affiliative preference and crowing), via potential organizational effects, but without disrupting sexual differentiation of male-typical behavior. Thus the two phenomena are able to coexist by acting through independent mechanisms. Additional work, including mechanistic and transgenerational approaches, will help to determine the specific pathway of maternal androgen effects and to assess their potential adaptive value.

We thank Betty S. Hansen for assistance with testosterone assays, Tim Van Deusen and his team for taking care of the animal facility and Rebecca Kurtzman for assistance with behavioral tests. We also thank two anonymous reviewers for their insightful comments and suggestions on a previous version of the manuscript. C.S. is supported by the Fyssen Foundation. Research support was provided by Cornell University.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yhbeh.2012.10.011.

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