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

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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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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