Hormones and Behavior 56 (2009) 416–422

Contents lists available at ScienceDirect

Hormones and Behavior

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

# Increased yolk testosterone facilitates prenatal perceptual learning in Northern bobwhite quail (*Colinus virginianus*)

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ARTICLE INFO

*Article history:*
Received 7 April 2009
Revised 16 July 2009
Accepted 16 July 2009
Available online 30 July 2009

*Keywords:*
Maternal effects
Yolk testosterone
Auditory stimulation
Perceptual learning
Arousal
Behavioral development
*Colinus virginianus*

ABSTRACT

Prenatal learning plays an important role in the ontogeny of behavior and birds provide a useful model to explore whether and how prenatal exposure to hormones of maternal origin can influence prenatal learning and the development of behavior. In this study we assessed if prenatal exposure to yolk testosterone can influence auditory learning in embryos of Northern bobwhite quail (*Colinus virginianus*). We experimentally enhanced testosterone concentrations in bobwhite quail eggs prior to incubation. The embryos from these T-treated eggs as well as control embryos that had received the vehicle-only or were non-treated were exposed to an individual bobwhite hen's maternal call for 120 min over the course of the day prior to hatching. All chicks were tested at 24 h following hatching for their auditory preference between the familiar bobwhite maternal call versus an unfamiliar bobwhite maternal call. T-treated chicks spent significantly more time in proximity to the familiar call compared to the unfamiliar call and also showed shorter latencies to approach the familiar call than control birds. Increased emotional reactivity, i.e. propensity to express fear responses, was also found in T-treated chicks. Baseline heart rates recorded in a second group of T-treated embryos and control embryos did not differ, which suggests no effect of yolk testosterone on baseline arousal level. To our knowledge this is the first demonstration of the influence of prenatal exposure to testosterone on auditory learning.



## Introduction

Avian embryos are more accessible to various types of experiential manipulations than are mammalian species and have thus provided insight into how prenatal experience can guide and constrain attentional selectivity, learning, and memory during early development (see Lickliter, 2005 for a review). A variety of precocial avian species respond selectively to the maternal assembly call of their own species following hatching and, like mammals, show attentional biases that lead them to orient and seek proximity to their mothers in the days following hatching (e.g. Allen, 1977; Gottlieb, 1971; Heaton et al. 1978). A number of studies have demonstrated that the type, amount, as well as the timing of prenatal sensory stimulation can influence the development of this filial behavior (Lickliter, 2000 for a review). For example, bobwhite quail embryos (*Colinus virginianus*) incubated in isolation and denied tactile contact with clutch mates prior to hatching fail to prefer species-specific maternal auditory and visual cues at ages when socially reared chicks reliably demonstrate such filial preferences (Lickliter and Lewkowicz, 1995).

Several studies have shown that bobwhite quail embryos and hatchlings require an average of 240 min exposure to an individual variant of the bobwhite maternal call to show a preference for the familiarized call over a novel variant of the maternal call (e.g., Foushée and Lickliter, 2002; Lickliter et al., 2002; Lickliter and Hellewell, 1992). However, temporally synchronizing a maternal call and a flashing light (thereby providing intersensory redundancy) facilitated prenatal learning of the maternal call. In this bimodal condition quail embryos were found to learn the call four times faster (following 60 min of exposure, Lickliter et al., 2002, 2004) than in unimodal exposure. These and other studies of precocial avian species suggest that there is a range of sensory stimulation that is optimal for species-typical perceptual development. Deviations above or below this optimal range of stimulation appear to modify embryos' arousal level and interfere with the young organisms' ability to selectively attend and learn in the period prior to hatching (Lickliter and Lewkowicz, 1995; Radell and Gottlieb, 1992; Reynolds and Lickliter, 2002, 2004).

In addition to sensory stimulation, avian and mammalian embryos are also exposed to hormones of maternal origin present in their prenatal environment. However, unlike mammals, birds can provide hormones to their offspring only prior to laying their eggs. This constraint suggests that avian embryos could be exposed to maternal hormones from the first day of prenatal development (Nordgreen

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doi:10.1016/j.yhbeh.2009.07.008

et al., 2006). Although the mechanisms remain unclear (Groothuis and Schwabl, 2008), subtle differences in the hormonal environment of avian embryos have been shown to result in numerous phenotypic effects following hatching (e.g., Kaiser and Sachser, 2005; Schwabl, 1996; Daisley et al., 2005). In altricial bird species, correlational as well as experimental studies indicate that prenatal exposure to androgens can influence growth, increase overall activity, increase begging behavior, increase the frequency of aggressive and sexual displays (e.g. Schwabl, 1993, 1996; Eising et al., 2001, 2006; Eising and Groothuis, 2003; Pilz and Smith, 2004; Rubolini et al., 2006), influence social dominance among nest-mates (Schwabl, 1996), and modulate neophobic responses (Tobler and Sandell, 2007). Studies of precocial birds have also highlighted the prenatal influence of androgens on various postnatal phenotypic outcomes (Bertin et al., 2008; Okuliarova et al., 2006, 2007; Daisley et al., 2005). For example, Daisley et al. (2005) reported that young Japanese quail (*Coturnix coturnix japonica*) hatched from eggs injected with testosterone have shorter durations of tonic immobility (i.e. a lower level of emotional reactivity), are less dependent socially, and perform a detour task faster than controls.

Taken together, these studies on avian embryos and chicks indicate that prenatal exposure to enhanced testosterone can modify behavioral development. In light of these findings, we wondered if yolk androgens could be involved in the modulation of embryos' arousal levels and learning capacities during prenatal development, as well as their behavioral profile in the period following hatching. To begin to explore this possibility, we experimentally elevated yolk testosterone levels in a group of bobwhite quail eggs and compared their baseline arousal (as indexed by heart rate), prenatal auditory learning, and postnatal mass gain and emotional reactivity (i.e., propensity to express fear responses) to those of controls chicks. Considering the higher level of activity generally observed in altricial birds prenatally exposed to higher levels of yolk testosterone (e.g. Eising et al., 2001, 2006; Eising and Groothuis, 2003), we predicted that higher levels of androgens during early development would moderately increase the baseline arousal level of embryos and in turn influence their selective attention and perceptual learning in the period prior to hatching.

## Materials and methods

### *Hormonal assays*

To determine the relevant quantity of testosterone to inject into Northern bobwhite quail eggs, we first determined the average endogenous testosterone concentrations and the standard deviation in 25 fertile, unincubated eggs received from a commercial supplier (Strickland, Pooler, GA). Eggs were weighed on electronic scales and assayed with a protocol described in Lipar et al., (1999), Mostl et al., (2001), Hackl et al. (2003) or Bertin et al. (2008). Briefly, the frozen yolk was separated from the albumin and weighed. To extract steroids, after thawing, each mixed yolk was suspended in 10 ml of water and vortexed twice for 30 s. Samples were then stored overnight at 4 °C. Samples were then vortexed and 1 ml of the suspension was transferred into a new vial. The suspension was then diluted with 4 ml methanol, vortexed for 30 min and stored at −20 °C overnight to precipitate apolar lipids. After centrifugation (−15 °C, 2500 *g*, 10 min) 10 µl of the supernatant was used for enzyme immunoassays (EIAs). EIAs were performed on microtitre plates with a double antibody technique described by Palme and Mostl, (1994). The antisera were raised in rabbits against testosterone-3-CMO-bovine-serum-albumin or 5α-androstane-3α-ol-17-oneHS:BSA (for full descriptions of antibodies and validation see Palme and Mostl, 1994; Hirschenhauser et al., 1999; Mostl et al., 2001). The test sensitivity of the assay was 1 fmol/well. All samples were assayed in one plate with an intra-assay variation of 4.7%.

### *Egg injections*

Average yolk testosterone concentration in Northern bobwhite quail was determined to be $4.25 \pm 2.04$ ng/g yolk and the average yolk mass $3.83 \pm 0.49$ g. Therefore, to increase the overall yolk concentration to two standard deviations (4.08 ng/g) we injected 15.6 ng ($4.08 \times 3.83$) of testosterone suspended in 20 µl vehicle (corn oil) in treated eggs. This injection allowed the targeted concentration of 8.33 ng/g to remain within the range of the natural variation observed (minimum: 1.7 ng/g, maximum: 10.49 ng/g). 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 27-G needle. The solution was delivered to the yolk using a 100 µl Hamilton syringe mounting 27-G sterile needle. As described in Rubolini et al. (2006), the injection hole was then sealed by gluing a tiny piece of cleaned and disinfected eggshell to the egg immediately after injection.

For this study, three groups of embryos were tested: a testosterone injected group (T), a vehicle injected group (V-controls) and an untreated group to control for any possible vehicle effect or injection procedure effect (Controls). Fertile unincubated eggs were received weekly from the commercial supplier. Egg mass was balanced between groups and was within the range of variation of the 25 eggs previously used for hormonal assays; eggs were drawn from 6 or more weekly batches to minimize the influence of any inter-batch variability on the study. After injection, eggs were left unmoved for 30 min and then incubated for 23 days. Eggs from the three conditions were incubated together on the same shelf of a Grumbach BSS 160 incubator (Munich, Germany), maintained at 37 °C and 70% relative humidity.

### *Prenatal auditory stimuli*

All the embryos received the same auditory stimulation procedure prenatally. A protocol previously used in studies of bobwhite quail embryos (e.g. Reynolds and Lickliter, 2004; Honeycutt and Lickliter, 2001, 2002; Lickliter et al., 2002) was utilized. On the second half of the 21st day of incubation, embryos were transferred to a portable Grumbach S84 hatcher, maintained at 37.5 °C and 80% relative humidity. On the morning of Day 22 the stimulation procedure started. The auditory stimulus used for prenatal stimulation was a recording of an individual bobwhite maternal call (Call A; for discussion see Auditory preference testing section). The recording was composed of a loop of Call A broadcasted at a rate of 13 calls/minute. The call was broadcast for 5 min each hour during the 24 h period prior to hatching, resulting in a total of 120 min of exposure. The sound level of the hen's call was maintained at a maximum peak intensity of 65 dB, as measured by a Bruel and Kjaer 2232 sound-level meter (B&K Instruments, Marlborough, MA). The recording of the maternal call was broadcast through a speaker connected to an amplifier that received input from a computer. To control for the possible effects of developmental age, only birds that hatched on the 23rd day of incubation were used in our analyses. We obtained 46 T chicks, 34 V-controls chicks, and 40 Control chicks.

### *Heart rate measures*

To assess the potential influence of testosterone on embryos' baseline arousal, we recorded baseline instantaneous heart rate at Day 22 of incubation for another group of 45 embryos (15 T, 15 V-controls and 15 Controls) that were not exposed to any prenatal stimulation. The protocol described by Markham et al. (2006) was followed. Data acquisition was conducted in a Hova-Bator S7501 portable incubator Hova-Bator, Savannah, GA set at 37 °C. To approximate Einthoven's triangle for maximum signal strength, two holes were made on opposing sides in the lower portion of the egg, with the airspace facing

up. The holes were made with a 22-gauge needle and were deep enough to penetrate the egg shell membrane into the amnion, without disturbing the embryo (Moriya et al., 2000). Once the holes were made, the egg was placed on the foam support inside a metal enclosure. The two copper electrodes were bent at a 90° angle 2 mm from the ends, and these ends were placed into the egg holes. Once the shielded metal enclosure and hatcher were closed, the embryo was allowed a 1-min settling period to adjust to the ambient temperature prior to recording. A Coulbourn S73-22 impedance pneumograph coupler (Coulbourn Instruments, Allentown, PA) sent a 50-khz sine wave through the egg to detect instantaneous heart rate. Changes in the sine wave were then amplified via a Coulbourn S7501 bioamplifier at 40,000 × gain and further amplified via a BIOPAC (BIOPAC Systems, Goleta, CA) MP100A amplifier at 2000 × gain. After amplification, the BIOPAC system converted the signal from analog to digital, which permitted the use of Acknowledge 3.5.7 software (BIOPAC Systems, 2005) to digitally apply a 3–10 kHz bandpass filter to the signal, eliminating noise and normalizing the size of the signal. All embryos' baseline instantaneous heart rates (beats-per-minute, BPM) were recorded for 10 min.

*Chick rearing*

Newly hatched chicks (Day 1) were identified by a colored and numbered leg band and weighed. Their mass was recorded daily until Day 4. Chicks were transferred to a sound-proof rearing room and placed in groups of 12–15 same-aged chicks to mimic typical brood conditions for bobwhite quail (Stokes, 1967). To ensure the same early social rearing environment for all treatments, chicks from the three prenatal groups (T-treated, vehicle-treated, no treatment) were mixed. These mixed groups were housed in large plastic tubs (25 cm wide × 15 cm high × 45 cm long) placed on shelves in a Nuaire Model NU-605-500 Animal Isolator (Plymouth, MN). Ambient air temperature was maintained at approximately 35.5 °C in the rearing room. Food and water were available *ad libitum*.

*Auditory preference testing*

The auditory preference of each chick was tested at 24 h after hatching. Each chick was carried in a transport wooden box (10 cm × 10 cm × 10 cm) to a sound-attenuated room. All testing sessions consisted of a 5-min simultaneous choice tests between two variants of the bobwhite maternal assembly call and took place in a circular arena (diameter = 130 cm, height = 24 cm). The surface of the arena was constructed of plywood and painted black. The sides of the arena were constructed out of sheet metal formed into a circle, covered by a layer of sound-attenuating foam and opaque black cloth. Loudspeakers were hidden on opposite sides of the arena. The speakers were connected to independent RCA SA-155 amplifiers (Fort Worth, TX), each connected both to a Sony CDP-XE370 CD player (Tokyo, Japan). Prior to all testing sessions, sound pressure levels at the start location for chicks (a point equidistant from both speakers on the periphery of the arena) were calibrated to a maximum of 65 dB for both speakers using a Brüel and Kjaer Model 2232 sound-level meter. At the start of the session, each chick was placed in a plastic box at the start location and left for a period of 60 s prior to the onset of stimulation. A video camera, mounted on the ceiling above the arena, and a microphone, placed beneath the arena, allowed continuous visual and auditory monitoring of the testing sessions. During testing, two variants of the bobwhite maternal assembly call, Call A (familiar) and Call B (unfamiliar), cleaned of background noise by the Borror Laboratory of Bioacoustics (Columbus, OH), were broadcasted simultaneously. The calls were played at equal repetition rates (13 calls/minute) from opposite sides of the arena and were counterbalanced between the two sides across testing trials. Both maternal calls are similar in phrasing, repetition rate, and frequency modulation and vary primarily in minor peaks of dominant frequency and pitch (see Harshaw and Lickliter 2007; Heaton et al., 1978). A number of studies have demonstrated that bobwhite chicks have no naïve preference for either of these two maternal calls (e.g., Honeycutt and Lickliter, 2001, 2002; Lickliter et al., 2002; Lickliter and Hellewell, 1992). A semicircular approach area, corresponding to 5% of the total surface area of the arena, was demarcated around each speaker area on the monitor used for observing sessions. Upon entry of a chick into one of these areas, the experimenter clicked one of the two buttons in a Visual Basic/Excel program. The button was held down until the chick exited the area. The primary data of interest were cumulative scores for duration of time spent in each approach area and scores for latency of first approach to each area. The latency of first step, the latency of first distress calling, and the number of jumps against the speakers during the test trial were also recorded.

*Emotional and social reactivity testing*

*Tonic-immobility tests*

To test for emotional reactivity (used in its restrictive sense i.e., propensity to express fear responses, Bouissou et al., 1994; Jones, 1996) we followed the protocol described by Jones (1986). On Day 4 (approximately 72 h of age), chicks from each of the three groups were placed on their back and restrained for 10 s prior to release. If more than 10 s lapsed between the release of the quail and their escape, duration of tonic immobility was noted. If not, the experimenter immediately reattempted to induce tonic immobility. If tonic immobility was not induced after five induction attempts, birds scored 0 s for tonic immobility duration. If chicks did not attempt to stand after 5 min, the test was stopped and a maximum of 300 s was scored. The duration of the tonic immobility reaction is considered to be a standard and robust measure of fearfulness (Gallup, 1977; Jones, 1986, 1987). This manipulation induces a reversible catatonic state, the duration of which is positively correlated with general underlying fearfulness (Mills et al., 1994; Jones et al., 1991).

*Separation tests*

To test for reaction to social separation, we used a protocol similar to that described by Launay (1993) and Bertin and Richard-Yris (2004). On Day 5 (approximately 96 h of age) each bird was transported in a wooden box to the sound-attenuated testing room and placed alone in a plastic tub similar to its rearing tub for 3 min. With the video system described above, the experimenter recorded latency of first move, latency of first distress call, number of distress calls, and number of jumps against the walls of the tub. In this test, commonly used in poultry, the number of calls and the number of jumps against the wall are considered to be positively correlated to the motivation of animals to join conspecifics (i.e. social motivation, see Launay, 1993).

All work was carried out in accordance with the recommended institutional guidelines for animal care and research (Florida International University IACUC approval #08-010).

*Data analysis*

Data on the mass of chicks were analyzed using a one-way repeated measures ANOVAR (treatment × time), and subsequent post-hoc test HSD Tukey tests were utilized. The requirements for parametric statistics were not obtained for data on latencies, tonic immobility scores, and frequency of behaviors. Therefore Wilcoxon matched-pairs signed-rank tests were used for within groups comparisons. Kruskall–Wallis and post-hoc Mann–Whitney *U*-tests were used for between group comparisons. Due to the non-independence of the duration scores for the two approach areas, duration scores were converted into proportion of total duration scores. Between-group comparisons were performed on differences

between these proportions (familiar minus unfamiliar) using Kruskall–Wallis and post-hoc Mann–Whitney *U*-tests. Because the usefulness of using corrections for multiple comparisons in cases of low sample size is highly debated and results in a loss of power (e.g. Nakagawa, 2004; Garcia, 2004, Garamszegi, 2006), we present the original *p*-values. Effect sizes were calculated using the Glass rank biserial correlational coefficients ($r_g$). Following Cohen (1988), an effect size of 0.3 was viewed as moderate and 0.1 as small. Data are presented as mean ± SEM. All analyses were performed using Statview software (SAS, Cary, NC), with significance accepted at $p<0.05$.

## Results

### *Heart rates*

The mean BPM did not differ significantly between the three groups (T-treated: 173.71 ± 6.57 BPM, V-controls: 171.43 ± 9.41 BPM, Controls: 167.31 ± 6.96 BPM, Kruskall–Wallis, $H=0.79$, $p=0.67$).

### *Chick growth*

We found no evidence of an effect of the testosterone treatment on the hatching success between our three groups. Out of 220 eggs incubated, we obtained 48 T chicks, 34 V-controls and 42 Controls chicks with a hatching success of respectively 61.5%, 53.76% and 46.53% (Chi-2 test, $p=0.21$). We found a significant effect of the treatment on chick growth (ANOVAR, $F_{2,228}=7.74$, $p=0.0009$). There was an effect of time (ANOVAR, $F_{3,228}=65.83$, $p<0.0001$) and a significant interaction between time and treatment (ANOVAR, $F_{6,228}=14.01$, $p<0.0001$). The mass of chicks at hatching did not differ significantly, but an effect of the treatment was found at Days 3 and 4 (Table 1). T-treated chicks were found to be heavier than V-controls at Day 3 (post-hoc HSD Tukey test, $p=0.02$). At Day 4 they were heavier than both V-controls and Controls chicks (post-hoc HSD Tukey tests, $p<0.001$). V-controls and Controls chicks did not differ significantly from each other on any day following hatching (post-hoc HSD Tukey tests, $p>0.05$).

### *Auditory preferences*

Within group comparisons revealed that neither V-controls nor Controls chicks showed significant difference in duration (Fig. 1) or latency scores (Fig. 2) between the familiar call and the unfamiliar call (Wilcoxon, $p>0.05$ for all comparisons). T-treated chicks, in contrast, spent significantly more time in proximity to the familiar call than in the unfamiliar call (Wilcoxon, $Z=5.11$, $p<0.001$) (Fig. 1) and also approached the familiar call sooner than the unfamiliar call (Wilcoxon, $Z=5.11$, $p<0.001$) (Fig. 2).

T-treated chicks also expressed significantly more jumps against the speaker broadcasting the familiar call than against the speaker broadcasting the unfamiliar call during the test trial (1.08 ± 0.37 versus 0.04 ± 0.04, Wilcoxon, $Z=3.57$, $p<0.0004$), whereas a tendency was observed in V-controls (0.46 ± 0.16 versus 0.11 ± 0.06, Wilcoxon, $Z=1.9$, $p=0.054$) and no difference was found in Controls chicks (0.07 ± 0.05 versus 0.12 ± 0.07, Wilcoxon, $Z=0.82$, $p=0.41$).

**Table 1**
Mean ± SEM mass of T-treated, V-controls and Controls chicks from Day 1 to Day 4.

| Mass | Young | | |
|---|---|---|---|
| | T-treated | V-controls | Controls |
| Day 1 | $7.4 \pm 0.07_a$ | $7.49 \pm 0.08_a$ | $7.46 \pm 0.08_a$ |
| Day 2 | $7.39 \pm 0.08_a$ | $6.96 \pm 0.08_a$ | $7.06 \pm 0.08_a$ |
| Day 3 | $7.85 \pm 0.09_a$ | $7.19 \pm 0.13_b$ | $7.45 \pm 0.14_{ab}$ |
| Day 4 | $8.58 \pm 0.11_a$ | $7.57 \pm 0.15_b$ | $7.7 \pm 0.23_b$ |

Different letters indicate significant differences in post-hoc HSD Tukey tests.

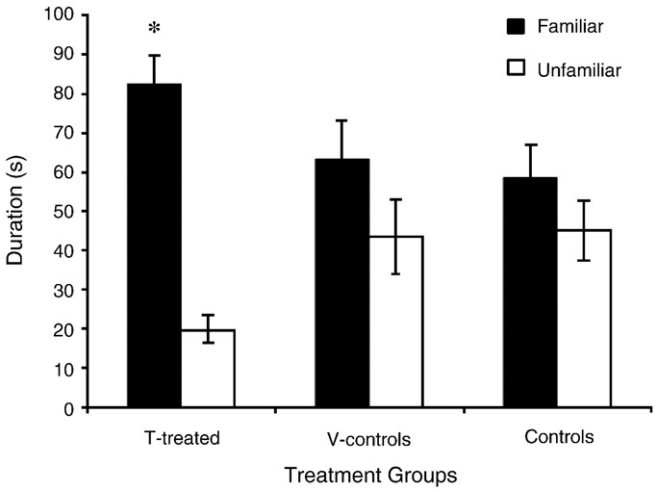


**Fig. 1.** Mean ± SEM duration scores for the familiar and unfamiliar maternal calls. Significance indicated for Wilcoxon matched-pairs signed-rank tests. *$p<0.05$.

Between-groups comparisons revealed significant differences in proportion of time spent in approach areas (Kruskall–Wallis, $H=11.71$, $p=0.0028$). Specifically, T-treated chicks showed a significantly larger proportion of time in the familiar call approach area than V-controls (0.58 ± 0.47 versus 0.21 ± 0.66, Mann–Whitney *U*-test, $U=481$, $r_g=0.33$, $p=0.01$) or Controls chicks (0.58 ± 0.47 versus 0.13 ± 0.67, Mann–Whitney *U*-test, $U=548.5$, $r_g=0.40$, $p=0.001$), whereas V-controls and Controls chicks showed no difference (Mann–Whitney *U*-test, $U=565.5$, $r_g=0.09$, $p=0.53$).

The latency of first step in the arena also differed significantly between treatment groups (Kruskall–Wallis, $H=10.45$, $p=0.005$). T-treated chicks showed a longer latency of first step than V-controls (39.46 ± 6.58 s versus 17.71 ± 4.25 s, Mann–Whitney *U*-test, $U=436$, $r_g=0.32$, $p=0.02$) and Controls chicks (39.46 ± 6.58 s versus 17.1 ± 3.7 s, Mann–Whitney *U*-test, $U=577.5$, $r_g=0.37$, $p=0.003$) whereas V-controls and Controls chicks showed no difference in their latency of first step (Mann–Whitney *U*-test, $U=510.5$, $r_g=0.09$, $p=0.53$).

### *Emotional and social reactivity tests*

Tests revealed significant differences between the three groups of chicks in tonic immobility duration (Kruskall–Wallis, $H=8.16$, $p=0.017$). T-treated chicks showed a significantly longer duration

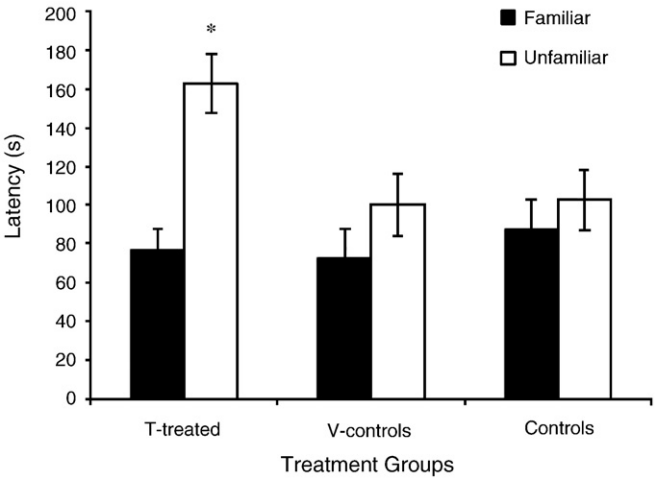


**Fig. 2.** Mean ± SEM latency scores for the familiar and unfamiliar maternal calls. Significance indicated for Wilcoxon matched-pairs signed-rank tests. *$p<0.05$.

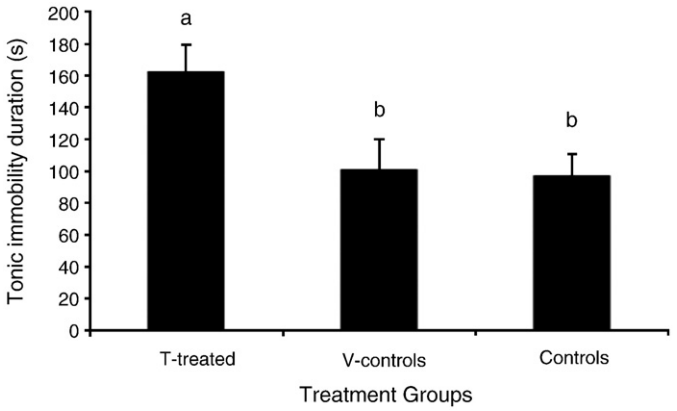


**Fig. 3.** Mean ± SEM durations of tonic immobility. Different letters indicate significant differences in post-hoc Mann–Whitney *U*-tests.

of immobility than both V-controls (Mann–Whitney *U*-test, $U = 445.5$, $r_g = 0.30$, $p = 0.03$) and Controls chicks (Mann–Whitney *U*-test, $U = 616$, $r_g = 0.33$, $p = 0.009$), whereas V-controls and Controls chicks did not differ (Mann–Whitney *U*-test, $U = 525$, $r_g = 0.01$, $p = 0.98$) (Fig. 3). The number of inductions did not differ significantly between the three groups (Kruskall–Wallis, $H = 5.12$, $p = 0.07$).

None of the parameters recorded in the tests of social separation (latency of first move, latency of first distress call, number of distress calls, and number of jumps against the walls of the tub) was found to differ significantly between the three groups (Kruskall–Wallis, $p > 0.05$ for all comparisons).

## Discussion

The results of this study contribute to the growing appreciation of non-genetic maternal influences on offspring's phenotypic outcomes. Mothers transfer to offspring a variety of non-genetic factors in reproduction, including DNA methylation patterns, chromatin marking systems, cytoplasmic chemical gradients, hormones, and a range of sensory stimulation necessary for normal development (see Harper, 2005; Jablonka and Lamb, 2005; Lickliter, 2005; Mameli, 2004 for reviews). Understanding how such developmental resources contribute to the emergence, maintenance, or modification of phenotypic traits has received increasing research attention in the last decade, particularly in the area of neuroendocrinology (see Crews, 2008 for a review). The known organizational and activational effects of hormones during early development provide a potent pathway by which mothers can modify their offspring's development (Gil, 2003). In the present study we found that elevated levels of yolk testosterone in bobwhite quail eggs can influence postnatal growth rates, increase chicks' emotional reactivity, and facilitate embryos' auditory learning. To our knowledge, this is the first demonstration of an effect of testosterone exposure on prenatal perceptual learning.

T-treated, vehicle-treated, and control embryos were all exposed to an individual variant of the maternal call of their own species for 120 min in the day prior to hatching. We found evidence of learning only in the T-treated chicks. T-treated chicks preferred the familiar call over the unfamiliar call in postnatal choice tests and expressed more jumps against the speaker broadcasting the familiar call than the speaker broadcasting the unfamiliar call. The absence of a preference for the familiar call we observed in V-controls and Controls chicks replicates previous studies of bobwhite quail that found that 120 min of prenatal exposure is not sufficient to engender a significant preference for an individual maternal call. Studies of bobwhite quail have consistently shown that embryos and chicks typically require at least 240 min of exposure to an individual maternal call to subsequently show a preference for that call over an unfamiliar call (Foushée and Lickliter, 2002; Honeycutt and Lickliter, 2001, 2002; Lickliter et al., 2002; Lickliter and Hellewell, 1992). In the present study, we showed that T-treated embryos required only half the exposure time usually needed to develop a significant postnatal auditory preference.

Previous studies have suggested a link between arousal level and prenatal learning in bobwhite embryos (Markham et al., 2006; Reynolds and Lickliter, 2002, 2004). Enhancing arousal level of embryos may facilitate learning but a too high level of arousal can also be detrimental. For example, bobwhite quail chicks prenatally exposed to a high dose of norepinephrine (hormone enhancing the activity of the parasympathetic nervous system) were found to be unable to learn a maternal call even with 240 min of exposure (Reynolds and Lickliter, 2002; Markham et al., 2006). Contrary to our prediction we found no difference in baseline heart rates between the T-treated, vehicle-treated, or control groups. The treatment thus appeared to have no effect on baseline autonomic nervous system activity. Given that only T-treated chicks showed enhanced auditory learning, we found no support for the hypothesis that testosterone influences prenatal learning via modulation of embryos' arousal levels.

Whereas no difference in mass was observed across groups at hatching, a higher mass gain was found in T-treated chicks compared to Controls chicks by 24 h of age, suggesting that T-treated chicks have a faster growth rate over the course of early development. Whether the higher mass gain observed in T-treated chicks was the result of a difference in feeding behavior or a difference in metabolic rate could not be determined in the current study, but the profile we obtained is in accordance with previous studies with young birds. For example, higher mass gain has been observed in Japanese quail chicks exposed to higher levels of prenatal androgens (Bertin et al., 2008). Similar findings have also been reported in birds from other orders (canaries, Schwabl 1996; gulls, Eising et al., 2001; Eising and Groothuis, 2003; blue birds, Navara et al. 2005, starlings, Pilz et al., 2004 but see Sockman and Schwabl, 2000, Andersson et al., 2004).

Higher durations of tonic immobility and longer latency of first step in the auditory choice tests were observed in T-treated chicks compared to control birds. Responsiveness induced by physical restraint in tests of tonic immobility is thought to be a fear-related anti-predator response; these responses are commonly used to assess the general underlying fearfulness of individuals, with higher fearfulness correlated with longer duration of tonic immobility (Jones and Waddington, 1992). In poultry, latency of first step in a novel environment is thought to reflect the initial phase of disinhibition following freezing. Freezing, characterized by the absence of both movements and vocalizations (Jones, 1980) is a common neophobic response in a novel environment, and the longer this phase, the higher the presumed level of emotional reactivity. Taken together, our results suggest increased emotional reactivity and fearfulness in bobwhite chicks exposed to elevated testosterone in egg yolk. Our results are in accordance with Okuliarova et al., (2007), who also reported higher tonic immobility duration in 2-day-old Japanese quail from testosterone treated eggs. Higher emotional reactivity has also been reported for young Japanese quail exposed to higher levels of androgens (Bertin et al., 2008). However, Daisley et al. (2005) and Okuliarova et al. (2006) reported lower emotional reactivity in testosterone treated Japanese quail chicks. These contradictory findings could be due to the age when birds were tested, a dose-dependent effect, or the interaction of testosterone with other non-controlled factors such as egg size or the presence of other hormones (e.g. androstenedione, progesterone). Additional research is needed to explore these possibilities.

How did increased yolk testosterone serve to facilitate prenatal perceptual learning in the present study? While we do not yet know the answer to this question, it is interesting to note that circulating testosterone appears to exert an effect on working memory in

rodents. For example, when trained on a T-maze alternation task, castrated males exhibit slower acquisition than intact males (Kritzer et al., 2001). Furthermore, androgen receptors have been found in the hippocampus (Brown et al., 1995; Kerr et al., 1995; Milner et al., 2001, 2005; Simerly et al., 1990; Tabori et al., 2005), suggesting that testosterone or its aromatized metabolite could act directly on the hippocampus to alter memory retention. In birds, circulating steroids are thought to influence song memory and production in songbirds (Ball et al., 2004) and in the great tit it was recently found that the hippocampus, as in mammals, expresses androgen receptors (Hodgson et al., 2008). Only a few studies have investigated the influence of yolk testosterone on song phenotype in oscine birds and results suggest small (Muller and Eens, 2009; Muller et al., 2008) or even detrimental effects of yolk testosterone on song development and song phenotypes (Garamszegi et al., 2007). To our knowledge, the action of testosterone on early memory in non-oscine birds remains unknown but recent findings suggest that the development of the hippocampus is influenced by pre-hatch experience and steroid hormones. Indeed, prenatal auditory stimulation was found to increase the mean synaptic density and height in day-old chick (*Gallus domesticus*) hippocampus (Chaudhury et al., 2009). Injections of corticosterone 24 h or 48 h before hatching were also found to improve retention for a weak passive avoidance task in day-old chicks (Sui et al., 1997).

Given that auditory recognition memory is known to involve homologous brain structures in birds and mammals (particularly the hippocampus, see Bolhuis and Gahr, 2006), we hypothesize that the enhancement in prenatal learning we observed in our T-treated embryos may be the result of improved memory retention. However, further research is needed to explore this and other hypotheses regarding how prenatal exposure to hormones of maternal origin can guide and constrain perceptual development, learning, and memory.

## Acknowledgments

The work reported here was funded by the Foundation Fyssen and NICHD grant HD RO1 048423. We are grateful to Alexandra Kuchar-Schulz for her help with the yolk steroid analyses. We thank Elizabeth Kiely and Barbara Pham-Escobar for their help in data collection. We are also grateful to Christopher Harshaw for his useful advice, Philip Stoddard for the preparation of the hormonal solution and Gregory Loucougaray for his help with statistical analysis.

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