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Between-female variation in house sparrow yolk testosterone concentration is negatively associated with *CYP19A1* (aromatase) mRNA expression in ovarian follicles

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ABSTRACT

Maternally-derived yolk androgens influence the development and long-term phenotype of offspring in oviparous species. Between-female variation in the amounts of these yolk androgens has been associated with a number of social and environmental factors, suggesting that the variation is adaptive, but the mechanisms behind it are unknown. Using two different approaches, we tested the hypothesis that variation in yolk androgen levels across individuals is associated with variation in their capacity to synthesize androgens. First, we injected female house sparrows with exogenous gonadotropin-releasing hormone (GnRH) to maximally stimulate ovarian steroidogenesis. Second, we collected pre-ovulatory follicle tissue and quantified the mRNA expression of four key enzymes of the steroid synthesis pathway: steroidogenic acute regulatory protein (*StAR*), cytochrome P450-side chain cleavage enzyme (*CYP11A1*), 17β-hydroxysteroid dehydrogenase (*HSD17B1*), and aromatase (*CYP19A1*). Thirty minutes after GnRH injection, androgen concentrations in both the plasma and in the yolks of pre-ovulatory follicles were significantly elevated compared to controls. However, this measure of steroidogenic capacity did not explain variation in yolk testosterone levels, although physiological differences between house sparrows and more widely studied poultry models were revealed by this approach. Steroidogenic enzyme mRNA levels were detectable in all samples and were significantly lower in the most mature pre-ovulatory follicles. Of the four measured genes, *CYP19A1* expression exhibited a significant negative relationship with yolk testosterone concentrations in laid eggs, revealing a key mechanism for between-female variation in yolk testosterone. Furthermore, this suggests that any factors which alter the expression of *CYP19A1* within an individual female could have dramatic effects on offspring phenotype.

1. Introduction

Avian eggs are produced sequentially, where one ovarian follicle per day is selected from a large follicle pool for rapid maturation and eventual egg formation [21]. Once recruited into this pre-ovulatory pathway, the oocyte accumulates liver-derived lipoprotein yolk precursors via receptor-mediated endocytosis [47]. This process of rapid yolk deposition takes 3–4 days in songbirds [61] and ceases near, or at, the time the oocyte is ovulated. Several pre-ovulatory follicles develop simultaneously and are thought to remain in a size hierarchy where the largest follicle (F1) is the next to be ovulated and smaller follicles (F2, F3, etc.) are each one day behind the other in their stage of maturation (but see [4]). Prior to and during rapid yolk deposition, follicle cells of the granulosa and theca layers that surround the oocyte produce steroids from the precursor cholesterol [21]. These ovarian steroids regulate many physiological processes in the female during breeding [19,21], but they have also been shown to accumulate in the yolks of eggs [48]. Such yolk steroids, especially androgens, have since been a key component of hormone-mediated maternal effects research in birds [11] as well as other oviparous vertebrates [7,27,42].

Through either the measurement or manipulation of endogenous levels, avian yolk androgens have been shown to affect many different aspects of the embryonic and nestling phenotype, including growth and morphology [8,50,57], immunocompetence [1,29], and behavior [6,50,55]. Yolk androgens can also have long-lasting effects on behavioral and sexually-selected morphological traits in adult birds [38,44,45,53]. Thus variation in yolk androgen concentrations has the potential to differentially affect the survival and reproductive success of individual birds.

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Importantly, patterns of variation in yolk androgen concentrations have been found at multiple levels, including differences between the eggs of a single clutch [14,48,56] and in the profiles of various yolk androgens across species [13,25,51]. However, a strikingly large amount of variation is often observed between individual conspecific females (e.g., [41]). Many social and environmental factors experienced at the time of breeding have been shown to either correlate with or influence yolk androgen concentrations across females (for reviews, see [11,16]). These findings have led to the hypothesis that the accumulation of yolk androgens is an adaptive mechanism allowing females to adjust offspring phenotype to the predicted prevailing environment [11,16,49,50]. However, the endogenous mechanisms that actually regulate yolk androgen accumulation and variation remain virtually unknown [15]. Such processes must be identified before a full understanding of the function and evolution of yolk steroids can be achieved [15].

Clues to these mechanisms come from recent studies that found high within-female repeatability [9,32,56] and even heritability [34,58] of yolk androgen concentrations. Such results implicate a genetic component to yolk androgen variation. This is supported by studies involving an injection of exogenous gonadotropin-releasing hormone (GnRH) to stimulate maximal production of ovarian steroids. The magnitude of the response to GnRH, detected as an increase in plasma testosterone concentrations, was positively associated with yolk testosterone levels [18,30,40]. Thus, yolk androgen levels appear to be correlated with a female's capacity to produce androgens, suggesting that there is between-female variation in the expression of genes that regulate androgen production.

In the current study, we used a GnRH injection to determine whether the overall androgen-producing capacity of female house sparrows (*Passer domesticus*) is associated with yolk androgen concentrations, as in other studies [18,30,40]. In addition to measuring the plasma testosterone response to GnRH, we tested whether yolk androgen concentrations within pre-ovulatory follicles were responsive to the GnRH injection. Furthermore, we sought to identify a novel, mechanistic source of yolk androgen variation between females by quantifying the mRNA expression of four key steroidogenic genes in ovarian follicles. Though individual differences likely exist at all levels of the hypothalamus–pituitary–gonadal (HPG) axis (e.g., in the expression of receptors that mediate sensitivity to negative feedback at the hypothalamus or pituitary gland), we focused on the ovarian follicular tissue, as it is the primary site of synthesis of the androgens that accumulate in yolk [15,17]. To our knowledge, this is the first study to identify a gene product responsible for yolk androgen variation between females, thus revealing a crucial part of the regulatory mechanism.

2. Materials and methods

2.1. Animal collection and maintenance

House sparrows were collected at livestock facilities in Pullman, Washington and Moscow, Idaho using mist nets in March, 2009. All trapping and experimental protocols were approved by the WSU IACUC board and performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Each bird was given a unique pair of colored leg bands for identification. A total of 75 birds (36 females and 39 males) were distributed approximately equally into six large ($2.3 \times 4.6 \times 2.3$ m) outdoor aviaries that contained nine wooden nest boxes ($13 \times 15 \times 13$ cm). Grass hay and polyester fibers (pillow batting) were provided for nest material. The birds received *ad libitum* access to fresh water and wild bird seed, and a supplemental mixture of peanut butter, hard-boiled egg, crushed eggshell, dry quick oats, and water was provided fresh daily. Pair identity at each nest box was recorded either during nest building or the day when the first egg was laid. Identities of the birds in active nest boxes were continually assessed throughout the experiment; multiple females were never observed at the same nest box during the same time period.

2.2. Previous tamoxifen treatment

As part of another study earlier in the breeding season prior to the GnRH experiment, most female house sparrows in the current study were subjected to an experiment involving injections of tamoxifen, a selective estrogen receptor modulator [35]. Depending on the tissue, tamoxifen has been shown to act as both an antagonist [59] and agonist [26] of the estrogen receptor in birds. Though tamoxifen is rapidly metabolized [60], it could have induced long-term changes that altered the expression of steroidogenic machinery. The median time from the last injection of tamoxifen to the GnRH experiment for a given bird was 24 days (range: 4–36 days); both the previous treatment (tamoxifen or control) and the interval between experiments were included as initial covariates in all of the current analyses. In order to increase available degrees of freedom, these variables were then removed if no effect was found.

2.3. GnRH injection and follicle collection

A pilot experiment was conducted to confirm the responsiveness of house sparrows to heterologous chicken GnRH (Sigma-Aldrich, St. Louis, MO) and to determine the minimum effective dose (see Supplementary Material). Females were exposed to one of two treatments (GnRH or vehicle-only control) on the day they laid the first egg of a new clutch; all eggs were collected on the morning they were laid and stored at −20 °C until yolk androgen analysis. Females initiating a new clutch were captured in a hand net and approximately 250 μL of blood was collected from the wing vein; plasma was separated by centrifugation at 6000*g* for 10 min and stored at −20 °C until analysis. Immediately after bleeding, the female was injected in the pectoral muscle with either 1.25 μg GnRH in 50 μL of phosphate-buffered saline (PBS) or 50 μL PBS alone. The bird was then placed in a cloth bag in a quiet room for the duration of the experiment. Approximately 30 min after injection (median = 32 min., range = 31–40 min.), a second 250 μL blood sample was taken and the bird was then immediately euthanized by CO_2. All yolky follicles were removed one-by-one, punctured to drain the yolk, and flash-frozen in liquid nitrogen. Follicle tissue was stored at −80 °C until RNA extraction (see Section 2.4). The median time from euthanasia until a given follicular tissue was frozen was 7 min (range = 4–11 min); this time was included as an initial covariate in all RNA analyses, but was removed if there was no effect. In addition to follicle tissue, the oocyte that had been ovulated earlier that day (if present) was removed from the oviduct and stored at −20 °C until yolk steroid analysis. Yolk that was drained from the pre-ovulatory follicles was collected and stored at −20 °C until steroid analysis. Thus, from an individual female, we collected tissue from the largest pre-ovulatory follicle (F1) and the next-largest pre-ovulatory follicle (F2). In addition, we collected yolk samples from F2 and F1 pre-ovulatory follicles, the ovulated oocyte retrieved from the oviduct (Eov), and the first egg of the clutch that was laid earlier that day (E1) (Fig. 1). Note that not every female possessed each type of follicle or egg.

2.4. Steroid hormone measurement

Using established protocols [48,61], plasma steroids were extracted with diethyl ether, separated on diatomaceous earth