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RESEARCH ARTICLE

Characterizing the timing of yolk testosterone metabolism and the effects of etiocholanolone on development in avian eggs

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ABSTRACT

Maternal transfer of steroids to eggs can elicit permanent effects on offspring phenotype. Although testosterone was thought to be a key mediator of maternal effects in birds, we now know that vertebrate embryos actively regulate their exposure to maternal testosterone through steroid metabolism, suggesting testosterone metabolites, not testosterone, may elicit the observed phenotypic effects. To address the role steroid metabolism plays in mediating yolk testosterone effects, we used European starling (*Sturnus vulgaris*) eggs to characterize the timing of testosterone metabolism and determine whether etiocholanolone, a prominent metabolite of testosterone in avian embryos, is capable of affecting early embryonic development. Tritiated testosterone was injected into freshly laid eggs to characterize steroid movement and metabolism during early development. Varying levels of etiocholanolone were also injected into eggs, with incubation for either 3 or 5 days, to test whether etiocholanolone influences the early growth of embryonic tissues. The conversion of testosterone to etiocholanolone was initiated within 12 h of injection, but the increase in etiocholanolone was transient, indicating that etiocholanolone is also subject to metabolism, and that exposure to maternal etiocholanolone is limited to a short period during early development. Exogenous etiocholanolone manipulation had no significant effect on the growth rate of the embryos or extra-embryonic membranes early in development. Thus, the conversion of testosterone to etiocholanolone may be an inactivation pathway that buffers the embryo from maternal steroids, with any effects of yolk testosterone resulting from testosterone that escapes metabolism; alternatively, etiocholanolone may influence processes other than growth or take additional time to manifest.

KEY WORDS: Maternal effects, Phenotypic variation, Steroid metabolism, Embryonic development, *Sturnus vulgaris*

INTRODUCTION

Successful offspring development requires that the mother provides a variety of resources (Starck and Ricklefs, 1998; Deeming and Reynolds, 2015), but the amount of these resources can vary between offspring. Such non-genetic maternal contributions that alter offspring fitness are known as maternal effects. When maternal resource contributions underlying maternal effects vary, phenotypic variation in offspring can arise (Mousseau and Fox, 1998). Steroid hormones are a type of maternal resource that mediate maternal effects (Eising et al., 2001; Groothuis et al., 2005; von Engelhardt et al., 2009; Riedstra et al., 2013; Williams and Groothuis, 2015), and have been extensively investigated, in part because they have long been known to influence developing tissues and produce long-lasting or permanent organizational effects (Phoenix et al., 1959; Goy and McEwen, 1980; Arnold and Breedlove, 1985). From this work, we recognize that steroids can coordinate sex-specific development of target tissues including the brain and genitalia (Goy and McEwen, 1980; Jost, 1970).

Given that avian eggs contain testosterone at oviposition (Schwabl, 1993; Groothuis et al., 2005; Merrill et al., 2017, 2018), it has been hypothesized that females have the ability to alter testosterone levels in their eggs, thereby influencing offspring development and phenotype (reviewed in Williams and Groothuis, 2015; Groothuis et al., 2019). Embryonic growth rates have been shown to be sensitive to levels of yolk androgens (reviewed in Schwabl et al., 2007; Gil et al., 2007). Androgen-treated eggs tend to hatch sooner than untreated eggs (Eising et al., 2001; Muller and Eens, 2009; Muriel et al., 2015a; but see Muriel et al., 2013) and, similarly, species with higher levels of yolk androgens tend to have relatively shorter incubation periods (Gorman and Williams, 2005; Schwabl et al., 2007; Gil et al., 2007). In addition to shortening the duration of embryonic development, yolk androgens can affect the development of muscles associated with hatching (Lipar and Ketterson, 2000; Navara and Mendonça, 2008) as well as humoral (Groothuis et al., 2005; Clairardin et al., 2011) and cellular (Navara and Mendonça, 2008) immunity. In response to the wide array of phenotypic effects that have been linked to yolk androgens, there has been an increased effort to characterize the proximate mechanisms underlying these maternal effects (Carere and Balthazart, 2007; Groothuis and Schwabl, 2008; Groothuis et al., 2019).

Evidence is now accumulating to suggest that embryos of oviparous vertebrates modulate their exposure to maternal steroids (reviewed in Paitz and Bowden, 2013). While dilution by albumen during development, or the movement of steroids from the yolk to the embryo, could explain a decline in yolk steroid levels during development (Groothuis and Schwabl, 2008), many studies demonstrate that this decline in yolk steroids is due to steroid metabolism (Paitz and Bowden, 2008; von Engelhardt et al., 2009; Paitz and Casto, 2012; Paitz and Cagney, 2019; Paitz et al., 2019). Birds such as chickens (*Gallus gallus*) (von Engelhardt et al., 2009), Japanese quail (*Coturnix japonica*) (Vassallo et al., 2014, 2018), and European starlings (*Sturnus vulgaris*) (Paitz et al., 2011; Paitz and Casto, 2012) all metabolize yolk steroids early in development. In European starling eggs, yolk testosterone levels rapidly decline during the first 5 days of development (Paitz et al., 2011). In rock pigeons (*Columba livia*), a similar decrease in yolk progesterone, 17-hydroxyprogesterone, androstenedione and testosterone occurs after 4.5 days of development (Kumar et al., 2018). Further, evidence of steroid metabolism in fertilized, but not unfertilized, eggs indicates that the embryo likely plays a role in steroid metabolism (Kumar et al., 2018). Given the apparent ubiquity with which maternal steroids are metabolized during early

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development, one critical remaining question is: are the respective metabolites capable of affecting subsequent embryonic development?

A major metabolite of testosterone is etiocholanolone; several enzymatic steps are necessary to convert testosterone into etiocholanolone, including 5β-reduction by 5β-reductase, C-17 oxidation by 17β-hydroxysteroid dehydrogenase, and C-3 hydroxylation by 3α-hydroxysteroid dehydrogenase (Paitz et al., 2011; Kumar et al., 2018). Early work in chick blastoderms identified 5β-reduction as the prevalent pathway for testosterone metabolism during embryonic development (Parsons, 1970), and further work demonstrated that 5β-reductase activity is high in recently hatched quail chicks (Balthazart and Ottinger, 1984). In fact, major sites of 5β-reduction include the brain and liver of both young and adult birds (Steimer and Hutchison, 1981), with 5β-reductase activity being two to three orders of magnitude greater in the brain than either 5α-reductase or aromatase activity (Vockel et al., 1990). However, unlike testosterone metabolites produced by 5α-reductase and aromatase, which are more biologically active than testosterone, 5β-reduction of testosterone has been implicated in biological inactivation of testosterone (Balthazart et al., 1990). Given these results, the authors proposed that the high levels of 5β-reductase in the embryonic brain serve as an inactivation shunt to protect specific brain regions from organizational effects of testosterone or its biologically active metabolites (Balthazart et al., 1990).

In both the European starling (Paitz et al., 2011) and the rock pigeon (Kumar et al., 2018), yolk testosterone is converted to the 5β-reduced metabolite etiocholanolone by day 5 of embryonic development. In starlings, almost 80% of the injected testosterone was converted to the conjugated form of etiocholanolone while no other metabolites could be detected with the techniques employed (Paitz et al., 2011). Previous work demonstrates that 5β-reduced androgens can increase erythropoiesis during embryonic development in chicks; specifically, etiocholanolone stimulates the formation of heme and hemoglobin directly in erythroid cells (i.e. not by erythropoietin) (Levere et al., 1967). Additional evidence shows that 5β-reduced compounds influence the production of enzymes in the developing liver (Granick, 1966; Levere et al., 1967; Anderson, et al., 1982; Aragonés et al., 1991). Therefore, it is possible that etiocholanolone may elicit similar effects in starling embryos. However, most of these studies occurred *in vitro* where exogenous etiocholanolone administration was not temporally linked to its endogenous embryonic production. To our knowledge, no studies have investigated the effects of etiocholanolone *in vivo* during the period of development when yolk testosterone is converted to etiocholanolone. A more complete understanding of how and when steroid metabolism occurs in the egg, and what metabolites are produced, would help clarify the potential mechanisms through which yolk testosterone can affect embryonic development.

Given that maternal testosterone is metabolized to etiocholanolone by embryonic day 5 (Paitz et al., 2011; Kumar et al., 2018), we hypothesized that the phenotypic effects of testosterone during early avian development are mediated by the response of embryos and their extra-embryonic membranes to etiocholanolone. The objectives of this study were to determine when and where in the egg testosterone is metabolized, and whether its major metabolite, etiocholanolone, affects the growth of the embryo and extra-embryonic membranes. We used tritiated testosterone to characterize when testosterone is metabolized in the egg, and then used *in vitro* assays to determine which tissues within the egg were capable of metabolizing testosterone. Levels of endogenous yolk etiocholanolone were quantified across early embryonic development to characterize concentrations prior to, and following the onset of, incubation. These studies demonstrated that yolk testosterone is rapidly converted to etiocholanolone, which was subsequently conjugated shortly thereafter, resulting in a transient rise in yolk etiocholanolone levels. Based on this pattern of endogenous etiocholanolone exposure, we then tested whether exogenous etiocholanolone could influence embryonic growth during this early period of development before it is metabolized.

MATERIALS AND METHODS

Egg collection

Eggs were collected from starling (*Sturnus vulgaris* Linnaeus 1758) nests found in four nest box colonies in McLean County, IL, USA, located on properties owned by Illinois State University (see Pryor and Casto, 2015, for a detailed description). As starlings are an introduced agricultural pest species, permits are not required to collect their eggs, but monitoring of nests, collecting of freshly laid eggs and use of painted wooden decoy eggs to encourage normal patterns of egg laying was reviewed and approved by the Illinois State University Institutional Animal Care and Use Committee.

In ovo metabolism of ^{3}H-testosterone

Given that ^{3}H-testosterone injected into starling eggs is completely metabolized by day 5 of incubation (Paitz et al., 2011), our initial goal was to more precisely determine when this metabolism occurs. In 2017, 38 starling eggs from 36 different clutches were collected, taken to the laboratory, and injections into the albumen were performed with 150,000 cpm of ^{3}H-testosterone (specific activity 100 Ci mmol^{-1}; Perkin-Elmer, Waltham, MA, USA) in 5 µl of sesame oil using a Hamilton microliter syringe. Super glue (Henkel Corporation, Rocky Hill, CT, USA) was used to seal the hole in each egg shell and allowed to dry. To avoid any confounding effects of laying order, eggs were randomly assigned to treatments. The eggs were then placed in a rotating incubator (Model 1202, 280 W circulated air incubator, GQF MFG Co. Inc., Savannah, GA, USA) at a constant 37.5°C and 60% humidity. Eggs were removed at designated sampling times (n=6 per sampling time): 12, 24, 48, 72, 96 or 120 h of incubation. Following removal from the incubator, all eggs were frozen at −20°C until analysis of steroids occurred. Frozen eggs were thawed and the yolk was separated from the albumen and each component was weighed. Once thawed and opened, all eggs appeared to be fertilized, as evidenced by embryonic development, with some eggs incubated for more than 72 h containing distinct blood pools from early stage embryos. When present, this tissue was included in the yolk mass recordings as it was not feasible to separate it from the rest of the sample in eggs that had been frozen.

To extract testosterone and its metabolites, each yolk was then homogenized and 0.5 g of the homogenate was placed in 4 ml methanol and vortexed for 10–15 s. Samples were then frozen at −20°C for at least 24 h to precipitate proteins and neutral lipids, then removed from the freezer and centrifuged at 1000 ***g*** for 15 min. The supernatant was collected and solid phase extraction was used to separate free and conjugated steroids (Paitz et al., 2011). Solid phase extraction was performed using Sep-Pak® cartridges (Sep-Pak® Plus single use C18 cartridges, Waters, Dublin, Ireland). Yolk extracts were diluted with 45 ml of water and run through cartridges under vacuum pressure. Free steroids were then eluted with 5 ml of diethyl ether, and subsequently conjugated steroids were eluted with 5 ml methanol. The conjugated steroids from each cartridge were collected and later characterized and quantified for radioactivity. In

order to further separate free steroids, the free steroid fraction was subjected to celite chromatography (Wingfield and Farner, 1975; Paitz et al., 2011). Following this separation protocol, increasing concentrations of an ethyl acetate:isooctane solution were added to each column; androstenedione elutes in the 0% fraction, etiocholanolone in the 10% fraction and testosterone in the 20% fraction (Paitz et al., 2011). However, we do not know the fractions in which other potential intermediate metabolites (i.e. etiocholanedione, 5β-dihydrotestosterone) elute so the radioactivity levels within each fraction may represent multiple metabolites. Each fraction was then dried and resuspended in scintillation fluid for quantification of radioactivity.

Because the results of the 2017 study suggested testosterone was already completely metabolized prior to the start of sampling, 12 h after ^{3}H-testosterone injection, we conducted a follow-up study in 2018 using higher concentrations of exogenous testosterone and earlier sampling points. In the summer of 2018, 25 freshly laid starling eggs were injected into the albumen with 2,000,000 cpm of ^{3}H-testosterone in 5 µl of sesame oil using a Hamilton microliter syringe and sealed with super glue, as above. To prevent any steroid metabolism, the five eggs randomly selected to be sampled at hour zero were snap frozen on dry ice immediately following the injection. The remaining 20 eggs were then placed in a rotating incubator at a constant 37.5°C and 60% humidity to be randomly sampled at 4, 8 or 12 h of incubation. Eggs were removed from incubation at designated sampling times (n=5 per sampling time). Following removal, all eggs were frozen at −20°C until analysis of steroids occurred. Frozen eggs were thawed but, unlike in 2017, we homogenized the yolk with the albumen and extracted steroids from 0.5 g of this homogenate, as we wanted to recover as much of our injected ^{3}H-testosterone as possible in order to determine the onset of steroid metabolism.

In vitro metabolism of ^{3}H-testosterone

Given that the metabolism of ^{3}H-testosterone occurs relatively early in embryonic development, in 2018 we wanted to examine which, if any, tissue types of freshly laid eggs were capable of metabolizing testosterone. To do this, yolk, albumen or a 1:1 mixture of the two was used as the tissue source. We mixed 0.5 g tissue sample from freshly laid eggs with 100,000 cpm of ^{3}H-testosterone in 200 µl of homogenization buffer (250 mmol l^{-1} sucrose, 5 mmol l^{-1} $MgCl_2$, 100 mmol l^{-1} Tris-HCl), and ran metabolism assays for 120 min. These assays were then quenched by the addition of 4 ml of ice-cold methanol. Samples were then subjected to solid phase extraction and celite chromatography as described above. To further verify the ability of yolks from freshly laid eggs to metabolize ^{3}H-testosterone, an additional five eggs were used in a time course experiment during which the assays described above were terminated at 0, 30, 60 and 120 min with subsamples being removed from each egg homogenate at each time point prior to extraction and chromatography.

Endogenous etiocholanolone levels

In 2017, in addition to characterizing the timing of testosterone metabolism, we quantified the endogenous levels of etiocholanolone (presumably of maternal origin) and how those levels changed during development in freshly laid eggs (n=10) and eggs that had been incubated for 5 days (n=10). Upon sampling, eggs were frozen at −20°C until steroid analyses were performed. Eggs were thawed, the shells removed and the mass of the yolk and albumen recorded and used to calculate the total amount of etiocholanolone in the various egg tissues. Yolk fractions from all incubated eggs contained early stage embryos that were not separated out, but included in the yolk mass. To extract etiocholanolone, the yolk fractions were homogenized and 0.5 g of the homogenate was mixed with 4 ml methanol and subjected to the same solid phase steroid extraction technique as described previously.

Etiocholanolone levels were quantified using LC/MS/MS at the Metabolomics Center at the University of Illinois (Urbana-Champaign, IL, USA) using a 5500 QTRAP mass spectrometer (AB Sciex, Foster City, CA, USA). This technique has been used to quantify a variety of steroids in the yolk of passerine eggs (Merrill et al., 2017, 2018). Briefly, the LC separation was performed on a Phenomenex C6 Phenyl column (2.0×100 mm, 3 m) with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). The flow rate was 0.25 ml min^{-1}. The linear gradient was as follows: 0–1 min, 80% A; 10 min, 65% A; 15 min, 50% A; 20 min, 40% A; 25 min, 30% A; 30 min, 20% A; 30.5–38 min, 80% A. The autosampler was set at 5°C. The injection volume was 5 µl. Etiocholanolone had a retention time of 20.2 min and was quantified on a 5500 QTRAP mass spectrometer (AB Sciex, Foster City, CA, USA) under positive electrospray ionization (ESI) with an ion spray voltage of 5500 V. The source temperature was 500°C. The curtain gas, ion source gas 1 and ion source gas 2 were 36 psi, 50 psi and 65 psi, respectively. Multiple reaction monitoring (MRM) was used to measure etiocholanolone with a Q1–Q3 transition of 291.0–255.0 (m/z).

Effects of etiocholanolone on embryonic growth

In the spring of 2018, 133 starling eggs were collected on the day they were laid and brought back to the lab. Eggs were divided among four different etiocholanolone dose treatments designed to represent one, two or four standard deviations based upon the mean concentration found in freshly laid eggs in the 2017 study (4.6± 1.8 ng, mean±s.d.). This resulted in treatment of 0 ng (control), 2.5 ng (low), 5.0 ng (medium) or 10.0 ng (high) of etiocholanolone dissolved in sesame oil. To avoid any confounding effects of laying order, eggs were randomly assigned to treatments. In addition to the injected eggs, nine uninjected eggs were immediately frozen at −20°C after weighing to serve as controls for baseline steroid levels. Following injections, eggs were incubated for either 3 or 5 days (±1 h). At the appropriate sampling times, all eggs were frozen and stored at −20°C until analysis of steroids occurred.

Following incubation, embryos and their extra-embryonic membranes were collected and weighed to determine their combined wet mass, and the yolk and albumen were separated and stored frozen for subsequent steroid analysis. Embryos and their membranes were then placed in a drying oven at 37°C and dried to a constant mass. The tissue was then used for molecular sexing of the embryo (Nettle et al., 2013). Briefly, 50 µl of 0.2 mol l^{-1} NaOH was added to each dried embryo, and placed in a 75°C heat block for 20 min to promote cell lysis (Haunshi et al., 2008). Samples were removed from the heat block and 300 µl of 0.04 mol l^{-1} Tris-HCl was added to neutralize the solution. Sex was determined by amplifying the sexually dimorphic chromodomain-helicase-DNA binding (CHD) genes using CHD forward primer (5′-GTTACTGATTCGTCTACGAGA-3′) and CHD reverse primer (5′-AATTCCCCTTTTATTGATCCATC-3′) (Nettle et al., 2013). Each PCR reaction contained: 6.7 µl nanopure water, 4.0 µl of 5× GoTaq Flexi buffer (Promega, Madison, WI, USA), 1.6 µl of 25 mmol l^{-1} $MgCl_2$, 1.0 µl of 20 mmol l^{-1} CHD forward primer, 1.0 µl of 20 mmol l^{-1} CHD reverse primer, 0.5 µl dNTPs, 0.2 µl Taq and 5 µl (100 ng) DNA from each embryo sample for a final reaction concentration of 20 µl. Samples were separated by gel

electrophoresis (1.5% agarose gel, 90 min at 95 V) and visualized under UV light. Using this protocol, males exhibit a single band while females exhibit two bands.

We used a subset of the eggs collected in 2018 to quantify etiocholanolone and confirm that our manipulations were within the physiological range. The subset contained n=9 freshly laid eggs, n=3 oil-injected eggs that were incubated for 3 days, n=6 oil-injected eggs that were incubated for 5 days, n=6 high-dose etiocholanolone-injected eggs that were incubated for 3 days and n=5 high-dose etiocholanolone-injected eggs that were incubated for 5 days. Because it was not feasible to separate yolk and albumen while also sampling embryos during development, for all eggs, yolk–albumen homogenates were used for steroid quantification. Etiocholanolone was extracted from 0.5 g of homogenate and quantified using LC/MS/MS as described above.

Statistical analyses

All analyses were run using SAS statistical software (v. 9.4, SAS Institute, Cary, NC, USA). Multivariate analyses of variance (MANOVA) were used to examine how tritiated metabolite levels changed across development in both the 2017 and 2018 *in ovo* metabolism studies using sampling time as a fixed effect. To test how the concentration of each metabolite changed over time, separate analyses of variance (ANOVA) and *post hoc* comparisons were performed. Concentrations were log transformed prior to analysis to normalize the data and homogenize variances. Sampling time was included as a fixed effect. *Post hoc* comparisons (Tukey's HSD) were performed to test for differences between sampling times.

An ANOVA and *post hoc* comparisons were used to compare the concentrations of etiocholanolone produced from each tissue type in our *in vitro* metabolism study. Tissue type was included as a fixed effect in this analysis. We also used a mixed-model ANOVA to examine the change in etiocholanolone produced over the course of 2 h *in vitro*. This analysis included egg as a random effect to account for the fact that samples at one time point were not independent from those at previous time points because they came from the same egg. *Post hoc* comparisons were performed for each 30 min time point from 0 to 120 min. The concentration of etiocholanolone was log transformed prior to analysis to normalize the data and homogenize variances. Assay duration was included as a fixed effect. An ANOVA that included incubation duration as a fixed effect was used to determine whether endogenous etiocholanolone levels changed over the first 5 days of development.

ANOVA were used to assess the effect of etiocholanolone dose on the combined mass of the embryo and extra-embryonic membranes. Incubation duration, treatment and sex of the embryo were included as fixed effects, while clutch and within-clutch position in the laying order were included as random effects. Embryonic tissue mass was log transformed prior to analysis to normalize the data and homogenize variances, as the untransformed data were not normally distributed. All data reported in this manuscript are available in Dataset 1.

RESULTS

In ovo metabolism of ^{3}H-testosterone

Our average recovery of radioactivity in the yolk of each egg was 68% (102,125 cpm per egg), which is consistent with similar studies (Paitz and Bowden, 2008; von Engelhardt et al., 2009), and levels of testosterone recovered at the initial 12 h sampling period (1535±178 cpm, mean±s.e.m.) represented only 1% of the total radioactivity recovered, suggesting a very rapid metabolism of the exogenously applied testosterone. Results from the MANOVA show that there was a significant change in metabolite levels over time ($F_{5,29}$=4.63, P=0.003). Despite the low concentrations of ^{3}H-testosterone at the 12 h initial sampling, we found that testosterone levels decreased over the first 5 days of development ($F_{5,29}$=5.27, P=0.0015; Fig. 1A). We also observed a transient rise then fall in both ^{3}H-androstenedione ($F_{5,29}$=26.35, P<0.0001; Fig. 1B) and ^{3}H-etiocholanolone levels over the first 5 days of development ($F_{5,29}$=5.80, P=0.0008; Fig. 1C). Levels of the conjugated steroid ^{3}H-etiocholanolone glucuronide increased by day 5 of development ($F_{5,29}$=30.31, P<0.0001; Fig. 1D).

In our follow-up study in 2018, with increased ^{3}H-testosterone, earlier sampling periods and an overall recovery of 79% of the total radioactivity, we found that ^{3}H-testosterone concentrations decreased during the first 12 h of development ($F_{3,15}$=5.56, P=0.0091; Fig. 2A). We further observed increases in ^{3}H-androstenedione ($F_{3,15}$=23.05, P<0.0001; Fig. 2B) and ^{3}H-etiocholanolone ($F_{3,15}$=5.49, P=0.0095; Fig. 2C) levels over the first 4 h.

In vitro metabolism of ^{3}H-testosterone

Through *in vitro* testing, we sought to examine in which tissues of the egg metabolism takes place. We determined that, relative to the albumen, the yolk is the most capable of converting ^{3}H-testosterone to ^{3}H-etiocholanolone. While there was a small amount of conversion occurring in the albumen, the yolk and mixed tissues produced significantly more ^{3}H-etiocholanolone ($F_{2,12}$=97.42, P<0.0001). *Post hoc* comparisons showed that both the yolk (8827±910 cpm g^{-1}; mean±s.d.) and mixed tissues (9561±1507 cpm g^{-1}) produced significantly higher amounts of ^{3}H-etiocholanolone than the albumen (1343±287 cpm g^{-1}) (both P<0.001); however, the yolk did not differ from the mixed tissues in the amount of ^{3}H-etiocholanolone produced (P=0.5169).

The ability of the yolk to convert ^{3}H-testosterone to ^{3}H-etiocholanolone was further verified with the time course experiment (Fig. 3). We determined that there was a significant amount of ^{3}H-etiocholanolone produced in the yolk after 120 min *in vitro* ($F_{3,12}$=51.68, P<0.0001) and that egg of origin also had a significant effect on the rate of metabolism ($F_{4,12}$=4.34, P=0.02). *Post hoc* comparisons show that there was a significant increase in ^{3}H-etiocholanolone levels after just 30 min (P=0.005) and again after 120 min (P<0.0004) of mixing ^{3}H-testosterone with yolk.

Endogenous etiocholanolone levels

Yolks from freshly laid eggs contained an average etiocholanolone concentration of 0.91±0.17 ng g^{-1} (mean±s.d.), which resulted in an absolute amount of 4.6±1.8 ng of etiocholanolone per yolk (Fig. 4). There was a significant increase in the amount of endogenous etiocholanolone present in eggs after 5 days of incubation ($F_{1,18}$=45.99, P<0.001; Fig. 4).

Effects of etiocholanolone on embryonic growth

In eggs injected with etiocholanolone, there was a significant effect of incubation duration ($F_{1,10}$=18.16, P=0.0017) and injection dosage ($F_{1,10}$=22.28, P=0.0008) on etiocholanolone level (Fig. 5), but the sex of the embryo did not affect etiocholanolone level (P=0.9411). We also evaluated the effect of incubation duration on etiocholanolone concentration in unincubated/uninjected eggs and 3 and 5 day incubated/oil-injected eggs and found that levels of etiocholanolone changed across development ($F_{2,15}$=15.30, P=0.0002; Fig. 5). Our *post hoc* comparisons showed that levels on day 3 of incubation were higher than those on days 0 and 5 (both P<0.0004), while levels on day 0 did not differ from those on day 5 of incubation (P=0.9602).

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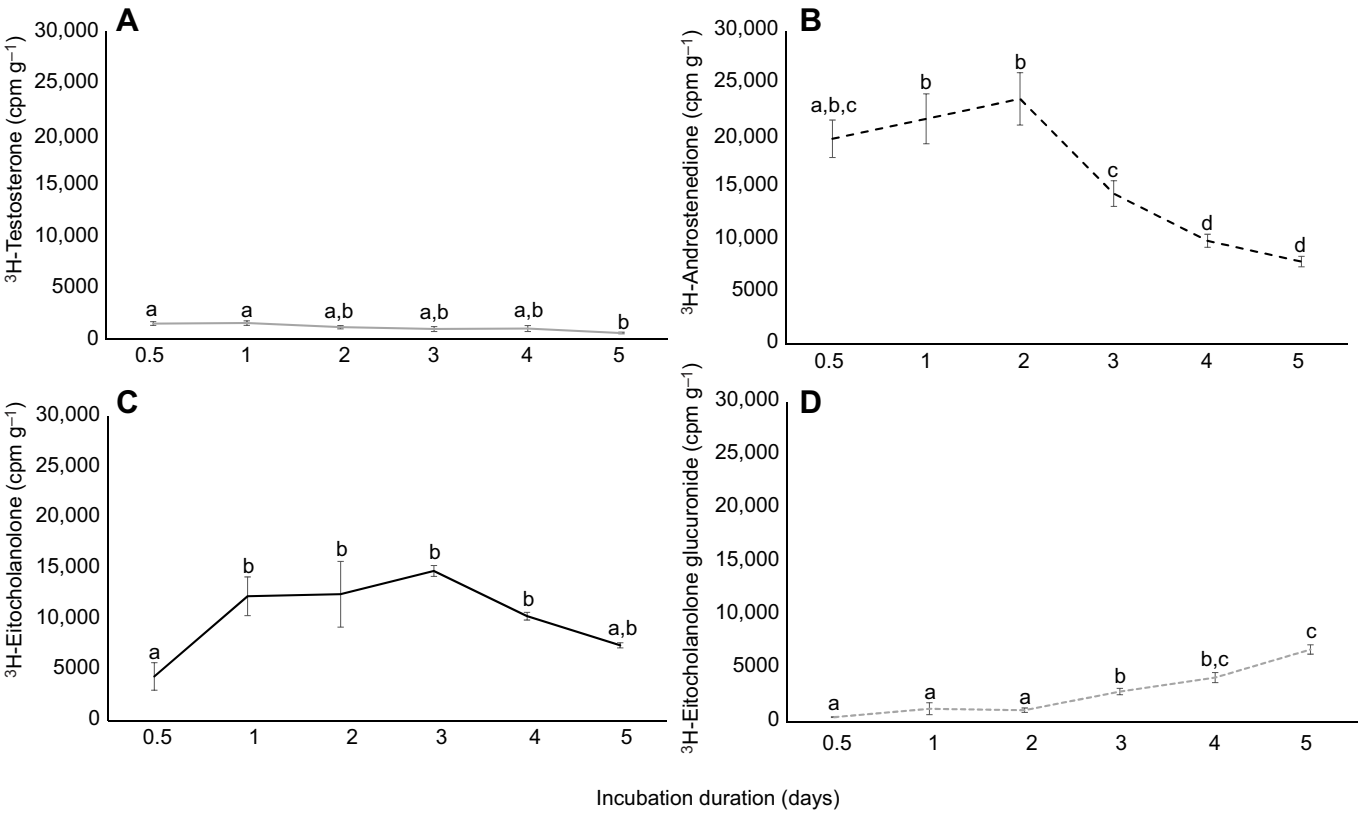

Fig. 1. Concentration of ^{3}H-testosterone, its metabolites (androstenedione and etiocholanolone) and conjugates (etiocholanolone glucuronide) over the first 5 days of incubation. (A) Testosterone; (B) androstenedione; (C) etiocholanolone; (D) etiocholanolone glucuronide. Values with different letters significantly differ from each other. Error bars denote s.e.m. for each incubation duration. *n*=5–6 eggs for each sampling period. Data from 2017.

We used tissue mass as a proxy for growth and found no significant effect of our treatments on the combined mass of the embryo and extra-embryonic membranes ($F_{3,12}$=0.73, P=0.5347; Fig. 6). As expected, there was a significant effect of incubation duration on growth ($F_{1,12}$=25.77, P<0.0001), but the sex of the embryo did not affect mass ($F_{1,12}$=0.22, P=0.6405). Overall, the sex ratio did not deviate from 50:50 male:female with 49.5% males produced.

DISCUSSION

Previous studies have provided evidence for the metabolism of testosterone in bird eggs, but a detailed understanding of when and where this metabolism takes place was lacking. In the present study, we determined that testosterone metabolism occurs rapidly, within hours of the initiation of incubation, confirmed our previous finding (Paitz et al., 2011) that metabolism results in the production of the metabolite etiocholanolone, and demonstrated that yolk possesses the enzymes necessary to carry out this metabolism. Our investigations of ^{3}H-testosterone metabolism *in ovo*, as well as endogenous etiocholanolone levels, show an early rise and subsequent fall in etiocholanolone levels over the first 5 days of development. We hypothesize the decline in etiocholanolone is due to conversion to a conjugated form as our data illustrate an increase in conjugate concentration that corresponds with the reduction in etiocholanolone levels. One potential outcome of the metabolism of maternal testosterone is that embryos are exposed to potentially active metabolites, such as etiocholanolone. Although a transient peak in etiocholanolone was found during early development, we determined that experimental transient exposure to any of a variety of etiocholanolone concentrations does not affect gross measures of growth in early embryos or extra-embryonic membranes over the first 5 days of development. Whether the effects of early exposure to etiocholanolone arise later in development or whether the metabolism of testosterone is an inactivation pathway that buffers the developing embryo from yolk testosterone effects remains to be determined.

Although prior studies demonstrated that testosterone is metabolized *in ovo* very early in development (von Engelhardt et al., 2009) and that etiocholanolone levels rise during this same developmental time period (Kumar et al., 2018), many questions about the origin of etiocholanolone and the metabolism of testosterone were not addressed. Through the use of tritiated testosterone, we were able to clearly demonstrate that the early metabolism of testosterone results in the production of etiocholanolone, as opposed to etiocholanolone being produced from other potential precursors. Our findings also support the idea that maternally derived enzymes are present in the yolk when eggs are laid, and ultimately contribute to the metabolism of testosterone. We showed that testosterone metabolism is occurring during the first 12 h of development, suggesting that metabolism can occur at the onset of incubation, and we determined that the yolk is the primary site for testosterone metabolism, presumably through enzymes found in the yolk. These enzymes are likely to originate from the mother, as ovarian follicles produce 5β-reductase (Wiebe et al., 1990); however, we cannot ignore studies which report that this metabolism does not occur without a viable embryo (Paitz and Casto, 2012; Kumar et al., 2018). Therefore, it is possible that maternal 5β-reductase enzymes are transferred to the yolk during oogenesis, but the developing

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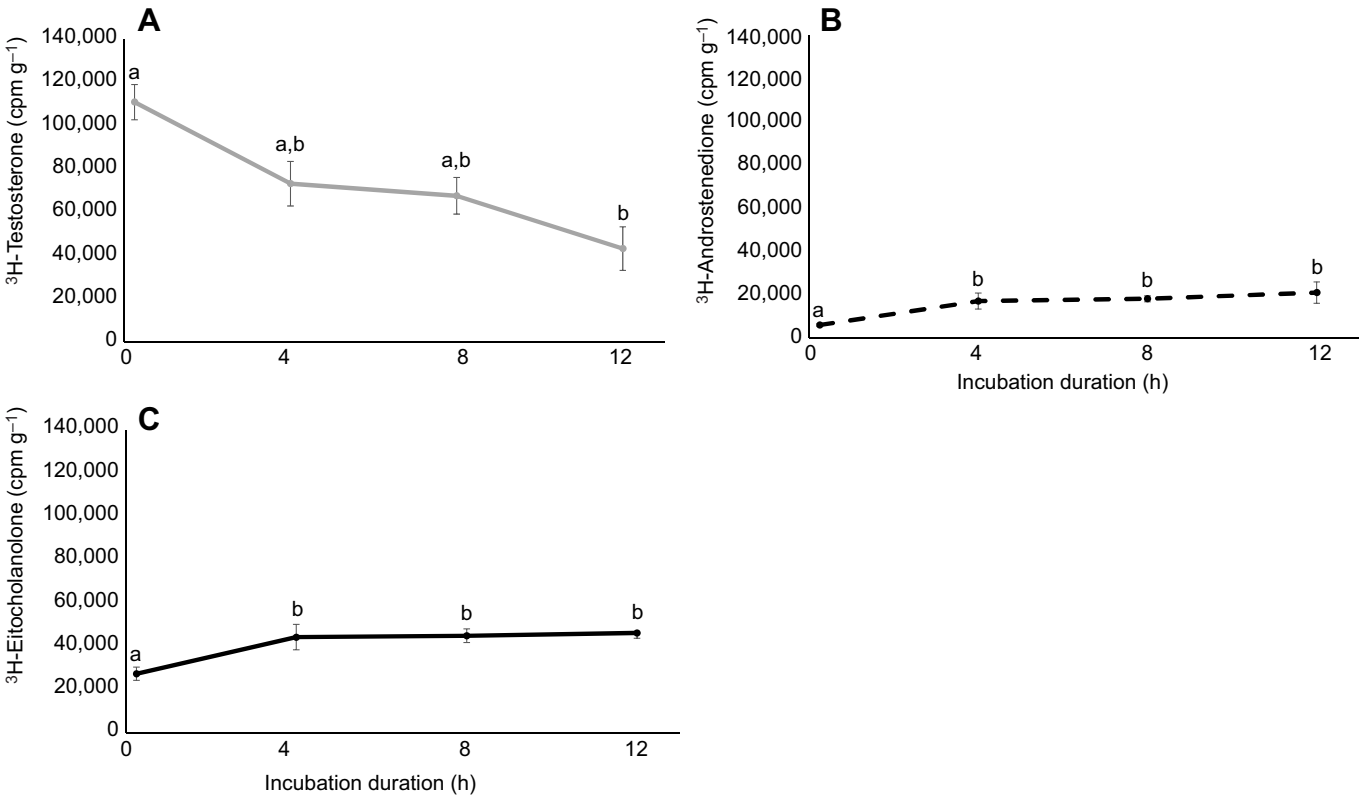

Fig. 2. Concentration of ^{3}H-testosterone and its metabolites over the first 12 h of incubation. (A) Testosterone; (B) androstenedione; (C) etiocholanolone. Values with different letters significantly differ from each other. Error bars denote the s.e.m. for each incubation duration. *n*=4–5 eggs for each sampling period. Data from 2018.

embryo may play a role in either activating these enzymes or creating an environment conducive to metabolism as unfertilized eggs do not exhibit testosterone metabolism.

Although the metabolism of testosterone results in an increase in etiocholanolone, we observed that this increase was transient over the first 5 days of development. As testosterone metabolism begins, there is an increase in the amount of etiocholanolone in the yolk; however, these levels quickly decline by day 5 as a result of conjugation (Paitz et al., 2011). Our results indicate that etiocholanolone is present not only at day 5 of development but also at day 0, suggesting the embryo is likely exposed to these transient etiocholanolone levels during the early stages of development. However, using mass as a proxy for growth, we found no effect of etiocholanolone on the development of the embryo or extra-embryonic membranes, throughout the first 5 days of development.

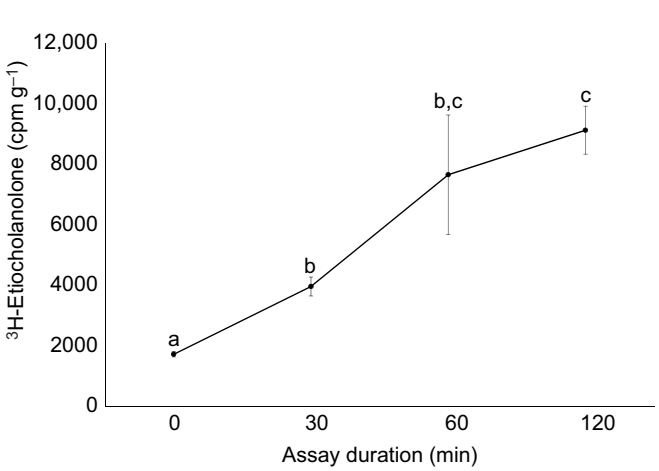

Fig. 3. Time course of etiocholanolone production *in vitro*. Values with different letters significantly differ from each other. Error bars denote the s.e.m. for each assay duration. *n*=5 samples for each sampling period. Data from 2018.

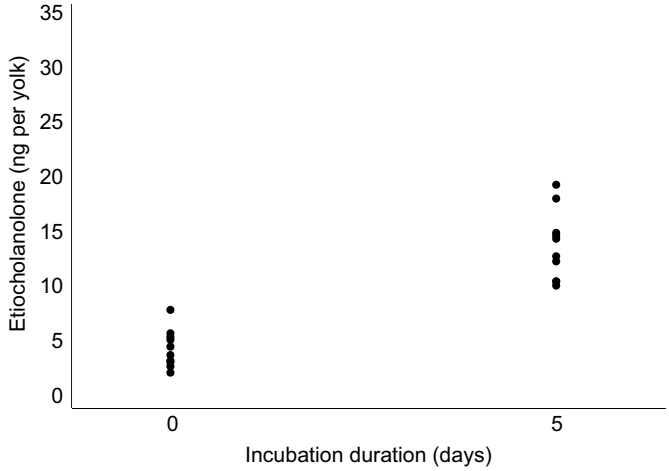

Fig. 4. Yolk etiocholanolone levels in eggs incubated for 5 days. Endogenous etiocholanolone levels were significantly different on day 5 versus those on day 0 of development (P<0.005). *n*=10 eggs per sampling period. Data from 2017.

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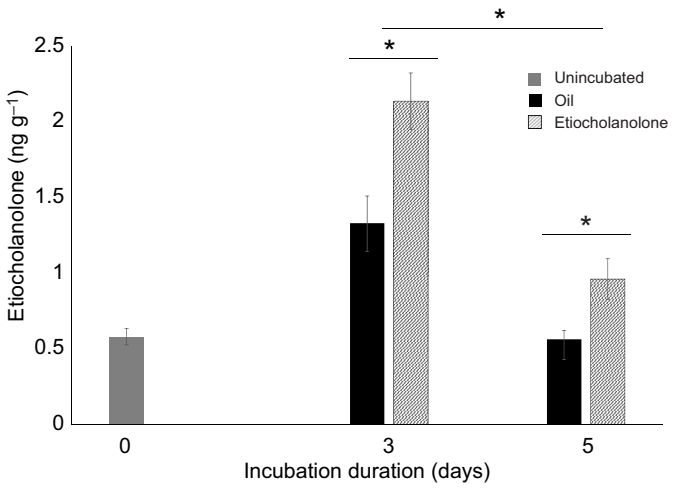

Fig. 5. Quantification of endogenous etiocholanolone in injected eggs over the first 5 days of development. Eggs were unincubated or injected with oil or etiocholanolone and incubated for 5 days. Asterisks indicate significant differences in either etiocholanolone dosage or incubation duration. Error bars denote the s.e.m. for each treatment. *n*=9 for unincubated eggs and *n*=3–6 for the other treatments. Data from 2018.

One question still remaining is how do any effects of testosterone occur when it is metabolized so early in development? We show that testosterone is metabolized to etiocholanolone at the onset of incubation, but effects on tarsus length (Navara et al., 2005, 2006; Navara and Mendonça, 2008; Muriel et al., 2013) and the development of the musculus complexus (Navara and Mendonça, 2008; Chin et al., 2009) have all been documented in nestlings following *in ovo* testosterone manipulation. However, recent studies suggest these effects are tissue and context dependent (Muriel et al., 2015a,b), which may be attributed to an escape of testosterone from metabolism, possibly explaining why some yolk testosterone effects may not arise until later stages of development (Muriel et al., 2015a).

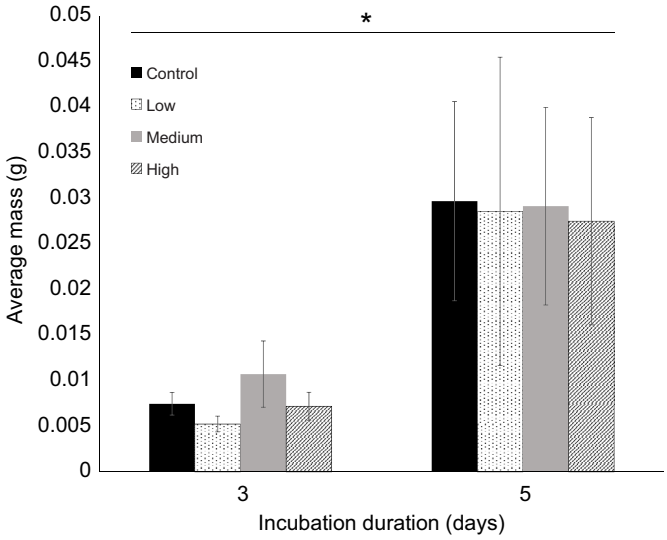

Fig. 6. Average mass of embryo and extraembryonic membrane tissues from each treatment on days 3 and 5 of development. Treatments were control (0 ng) and low (2.5 ng), medium (5 ng) and high (10 ng) etiocholanolone. The asterisk indicates a significant difference between incubation durations. Error bars denote the s.e.m. for each treatment on each sampling day. *n*=9–18 for each sampling period. Data from 2018.

This escape could be due to an overabundance of testosterone in the egg, or perhaps reduced activity of testosterone-metabolizing enzymes in the yolk.

Another possible reason for the observed developmental effects could be that etiocholanolone effects are independent of early growth. In the blastoderm stage of chicken development, 5β-reduced steroids, similar to etiocholanolone, induced erythropoiesis directly in cells (Levere et al., 1967) and perhaps etiocholanolone is responsible for a similar process in the European starling (Parsons, 1970). It is also possible that etiocholanolone alters early gene expression or endocrine set points, which, in turn, could lead to the development of phenotypic effects, such as impaired or enhanced immune function and muscle development, later in development. Further work is necessary to decipher the effects, if any, that etiocholanolone has on offspring phenotype.

There may also be different critical windows of development when maternal steroids or their metabolites have a heightened propensity to elicit phenotypic effects in offspring. A prior study, performed in Japanese quail eggs, suggests that injections of testosterone are most effective at altering offspring phenotypes related to sexual differentiation between days 10 and 15 of embryonic development (Adkins, 1979). Steroids are known to act during a critical period to irreversibly organize tissues early in development and can act later in development and elicit activational effects (Carere and Balthazart, 2007). Therefore, it is possible that the potential for phenotypic effects mediated by early embryonic exposure to etiocholanolone was established in embryonic starlings, but would not appear until later in development, after our sampling occurred, perhaps even after hatching. In future studies, following etiocholanolone-treated embryos throughout their embryonic and post-hatching development, when more detailed phenotypic analyses could be undertaken, could help evaluate this possibility.

Overall, we show that testosterone is metabolized within the first 12 h of embryonic development. Additionally, the metabolism of testosterone is associated with the accumulation of etiocholanolone, a putatively inactive metabolite of the 5β-reduction pathway. The clearance of this secondary metabolite is associated with increased levels of the steroid conjugate etiocholanolone glucuronide, which is also found in the yolk. Recent work suggests this metabolism pathway occurs in multiple species of birds (von Engelhardt et al., 2009; Kumar et al., 2018, 2019; Paitz et al., 2011; Vassallo et al., 2018), suggesting that steroid metabolism is conserved. Finally, the role of 5β-reduction as an inactivation pathway (Balthazart et al., 1990) is further supported by our finding that etiocholanolone had no effect on the development of the embryo and extra-embryonic membrane mass. Therefore, the *in ovo* metabolism of testosterone to etiocholanolone may serve as a buffer mechanism to protect the developing embryo from maternal steroids, but further work is needed to determine whether early exposures affect later stages of offspring development.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: N.C., R.B., J.M.C., R.T.P.; Methodology: N.C., J.M.C., R.T.P.; Formal analysis: N.C., R.T.P.; Investigation: N.C., R.A., J.M.C., R.T.P.;

Resources: N.C., R.B., J.M.C., R.T.P.; Data curation: N.C., J.M.C., R.T.P.; Writing - original draft: N.C., R.B., J.M.C., R.T.P.; Writing - review & editing: N.C., R.A., R.B., J.M.C., R.T.P.; Supervision: R.T.P.

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