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Deciphering The Consequences Of Yolk Testosterone Metabolism In Birds

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DECIPHERING THE CONSEQUENCES OF YOLK TESTOSTERONE METABOLISM IN BIRDS

NICOLE A. CAMPBELL

38 Pages

Maternal steroids transferred to eggs can elicit permanent effects on various aspects of 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 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 testosterone metabolite in avian embryos, is capable of influencing early embryonic development. Tritiated testosterone (3H-T) was injected into freshly laid eggs to characterize the movement and metabolism during early development. Varying levels of etiocholanolone were also injected into starling eggs and incubated for either three or five days to test whether etiocholanolone influences the early growth of embryonic tissues. Interestingly, the conversion of testosterone to etiocholanolone is initiated within the first 12 hours of embryonic development, but the increase in etiocholanolone is transient; etiocholanolone is also subject to metabolism, suggesting embryos are exposed to elevated levels of etiocholanolone for a short period of time in early development. We found exogenous etiocholanolone manipulation had no significant effect on the growth rate of the embryos or extra-embryonic membranes early in development. These findings suggest the conversion of yolk testosterone to

etiocholanolone may be an inactivation pathway that buffers the embryo from the effects of maternal steroids and the observed effects of yolk testosterone may be modulated by the fraction of testosterone that escapes metabolism.

KEYWORDS: testosterone, steroid metabolism, etiocholanolone, embryology, *Sturnus vulgaris*

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NICOLE A. CAMPBELL

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Fulfillment of the Requirements
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DECIPHERING THE CONSEQUENCES OF YOLK TESTOSTERONE METABOLISM IN BIRDS

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CHAPTER I: TESTOSTERONE METABOLISM AND ETIOCHOLANOLONE EFFECTS

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. When maternal resource variation occurs, maternal effects, manifesting in phenotypic variation, can arise (Mousseau and Fox, 1998). One maternal resource that has been implicated in mediating maternal effects is steroids (Eising et al., 2001; Groothuis et al., 2005; von Engelhardt et al., 2009; Reidstra et al., 2013; Williams and Groothuis, 2015). Much of the early interest in investigating maternal effects focused on steroids, in part because steroids have long been known to influence developing tissues and produce organizational effects that are permanent (Phoenix et al., 1959; Goy and McEwen, 1980). 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 laying (or oviposition) (Schwabl, 1993; Groothuis et al., 2005), it has been hypothesized females have the ability to alter testosterone levels in their eggs, thereby influencing offspring development and phenotype. For example, manipulations of maternal testosterone can affect the development of muscles associated with hatching (Lipar and Ketterson, 2000; Lipar, 2001; Navara and Mendonça, 2008) and flight (Navara and Mendonça, 2008; Chin et al., 2009). Skeletal development is also affected by testosterone; nestlings from eggs injected with testosterone had larger tarsi (Navara et al., 2005, 2006; Navara and Mendonça, 2008), although this increase has recently been found to only occur in male offspring during the first half of embryonic development (Muriel et al.,

2013). Increased exposure to testosterone during development can lead to altered development and functioning of the immune system. For example, both humoral (Groothuis et al., 2005; Clairardin et al., 2011) and cellular (Navara and Mendonça, 2008) immunity are suppressed by androgen exposure. With these findings, it is apparent that testosterone exposure during early development does affect offspring phenotype; however, not all studies report effects from early androgen exposure (Rubolini et al. 2006a), indicating these effects may be context-or species-dependent. Although, one aspect of development in which all studies report similar findings is the inability of yolk testosterone to affect the sexual differentiation of tissues, such as the brain (Carere and Balthazart, 2007).

After studies on the effects of yolk testosterone began to accumulate, the question of how effects of maternal testosterone in the yolk could arise without any apparent effects on sexual differentiation, which was known to be modulated by testosterone (Carere and Balthazart, 2007), quickly arose. A study performed in ring-necked pheasants found that injections of testosterone into egg yolks prior to incubation did not affect sexual ornamentation of the hatchlings (Rubolini et al., 2006b). However, in studies of Japanese quail, estradiol benzoate (EB) injected into eggs prior to day 12 of incubation affects the sexual differentiation of both male and female offspring (Adkins, 1979; Schumacher et al., 1989). Males hatched from eggs injected before day 12 of incubation, showed a demasculinization of their sexual behavior in adulthood, whereas males hatched from eggs injected after day 12 of incubation showed no effect of EB treatment on behavior (Adkins, 1979). These findings suggest there is a critical development window in the process of sexual differentiation by which steroid manipulations can elicit activational effects later in life (Carere and Balthazart, 2007).

While a number of hypotheses have been put forth to address this issue (e.g. “temporal dissociation” and “dose differences”), one question receiving increased attention is whether or not developing embryos modulate the effects of maternal steroids in the yolk via steroid metabolism (Paitz and Bowden, 2008, 2013; Paitz et al., 2011). For example, placental vertebrates also develop in an environment rich in maternal steroids, yet steroids produced by embryonic gonads, not from maternal circulation, direct sexual differentiation (Fuchs and Klopper, 1971). In these taxa, the metabolism of maternal steroids by the placenta is critical to maintaining an endocrine environment that allows developing gonads to properly direct sexual differentiation (Pepe and Albrecht, 1995). In egg-laying species, the chorioallantoic membrane (CAM), which is derived from the chorion and allantois like most placentas (Mossman, 1987), is also capable of steroid metabolism (Paitz and Bowden, 2008; Albergotti et al., 2009).

Evidence is now accumulating to suggest embryos of egg-laying vertebrates modulate their exposure to maternal steroids via steroid metabolism (reviewed in Paitz and Bowden, 2013). While dilution by albumen during development, or simply steroid movement from yolk to the embryo, could explain a decline in yolk steroid levels during development (Groothuis and Schwabl, 2008), many studies demonstrate this decline in yolk steroids is due to steroid metabolism (Paitz and Bowden, 2008; von Engelhardt et al., 2009; Paitz and Casto, 2012). Birds such as chickens (*Gallus gallus*) (von Engelhardt et al., 2009), Japanese quail (*Coturnix japonica*) (Vassallo et al., 2014; Vassallo et al., 2018), and the starling (Paitz et al., 2011; Paitz and Casto, 2012) all metabolize yolk steroids early in development. In European starling eggs, yolk testosterone levels decline rapidly during the first five days of development (Paitz et al., 2011). In rock pigeons, a similar decrease in yolk progesterone, 17-hydroxyprogesterone,

androstenedione, and testosterone occurs after 4.5 days of development (Kumar et al., 2018). Evidence of steroid metabolism in fertilized eggs, but not unfertilized eggs, of both starlings (Paitz and Casto, 2012) and rock pigeons (Kumar et al., 2018), suggests the embryo plays a role in steroid metabolism. Given the evidence that maternal steroids are metabolized during development, one critical remaining question is whether or not the respective metabolites are capable of influencing subsequent embryonic development.

Metabolic Fate of Yolk Steroids

The testosterone metabolites, dihydrotestosterone (DHT) and estradiol (E_2), influence development through their role in sexual differentiation (Siiteri and Wilson, 1974; McEwen et al., 1977). The conversion of testosterone to DHT or E_2 is crucial to the development of the brain and gonads of developing offspring in many taxa (Callard et al., 1978; Ball et al., 2014). The enzyme 5α -reductase is responsible for converting testosterone to DHT while aromatase converts testosterone into E_2 (Andersson et al., 1991; Thigpen et al., 1992), and the localized distribution of these two enzymes has a large effect on how the developmental effects of testosterone are mediated. For example, androgen receptors have a much higher affinity for DHT than testosterone (Siiteri and Wilson, 1974). So in a tissue that contains androgen receptors, it is important that 5α -reductase is also present in order to convert testosterone to its more potent form if needed. On the other hand, E_2 and other estrogens bind to estrogen receptors to produce distinct biological effects, such as activating the neural pathway for the elicitation of lordosis behavior in rats (McEwen et al., 1977). Therefore, it is crucial that

aromatase is localized to tissues which respond best to estrogens, otherwise the steroids will not be able to bind the proper receptor nor elicit their effects.

The metabolic fate of testosterone is not just limited to DHT or E₂. Another metabolite of testosterone is etiocholanolone, which is created through the 5 β -reduction pathway, and 5 β -reductase is one of the enzymes responsible for converting testosterone to etiocholanolone (Steimer and Hutchison, 1981). 5 β -reduction is the major pathway for testosterone metabolism in chick blastoderms (Parsons, 1970), and 5 β -reductase activity is high in recently hatched quail chicks (Balthazart and Ottinger, 1984). In fact, major sites of 5 β -reduction include the brain and liver of both young and adult birds (Steimer and Hutchison, 1981), with 5 β -reductase activity being two to three orders of magnitude greater in the brain than either 5 α -reductase or aromatase activity (Vockel et al., 1990). The 5 β -reduced metabolite, 5 β -DHT, injected in eggs at day nine of incubation elicited no effects on male sexual behavior after hatching (Schumacher et al., 1989), suggesting that this metabolite is inactive with respect to the process of sexual differentiation. Etiocholanolone has been identified as a major metabolite of yolk testosterone in the starling (Paitz et al., 2011) and the rock pigeon (Kumar et al., 2018), where yolk testosterone is converted to etiocholanolone by day five of embryonic development. Prior research suggests that the 5 β -reduction metabolism pathway may be crucial for early development of multiple avian species (Aragonés et al., 1991). Therefore, understanding how and when steroid metabolism and the conversion of steroids to other metabolites occurs in the egg, we can enhance our current understanding of the mechanisms through which yolk testosterone influences embryonic development.

Biological Effects of Etiocholanolone

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). Research demonstrating implants of 5β -reduced androgens appear to be inactive with respect to sexual differentiation in bird embryos (Schumacher et al., 1989; Balthazart et al., 1990) supports this proposed role of inactivation. One such study proposed that the high levels of 5β -reductase in the embryonic brain serve as an inactivation shunt to protect specific areas of the brain from the organizational effects of testosterone or its reduced metabolites (Balthazart et al., 1990).

While 5β -reduced androgens might not affect sexual differentiation, these metabolites were shown to increase erythropoiesis (red blood cell production) during embryonic development in birds (Levere et al., 1967). Previous work demonstrates that etiocholanolone stimulates the formation of heme and hemoglobin directly in erythroid cells (i.e. not by erythropoietin) (Levere et al., 1967). Additional evidence shows 5β -reduced compounds influence the production of enzymes in the liver (Granick, 1966; Levere et al., 1967; Anderson, et al., 1982; Aragonés et al., 1991) that increase cytochrome P450 activity in chick embryos (Anderson et al., 1982). Therefore, it is possible that the 5β -reduced metabolite, etiocholanolone, may elicit similar effects and influence offspring phenotype in birds.

Given the observation that maternal testosterone is metabolized to etiocholanolone in several bird species (Paitz et al., 2011; Kumar et al., 2018), we hypothesized the phenotypic effects of testosterone during early avian development are mediated by the response of the embryos and their extra-embryonic membranes to the 5β -reduced metabolite,

etiocholanolone. The objectives of this experiment were to determine when and where testosterone is metabolized, and whether its major metabolite, etiocholanolone, affects the growth of the embryo and extra-embryonic membranes.

Experimental Methods

Egg Collection

Eggs of European starlings (*Sturnus vulgaris*) were used in all experiments in this study, as previous evidence demonstrates that starling mothers transfer testosterone to the yolks of their eggs (Lipar 2001; Pilz et al., 2003), and that etiocholanolone is a metabolite of yolk testosterone metabolism in starling eggs (Paitz et al., 2011). Starlings are abundant across the Midwestern United States and readily occupy artificial nest boxes during the breeding season. Eggs were collected from nests found in four nest box colonies in McLean County, Illinois, located on properties owned by Illinois State University (see Eisner 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 3H-Testosterone

Since previous work demonstrated that 3H-testosterone injected into starling eggs was completely metabolized by day five 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 and taken to the laboratory. Eggs were then injected with 150,000 CPM of 3H-Testosterone (Specific activity = 100) (Perkin-Elmer, Waltham, MA, USA) in 5 μ L of sesame oil with 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. The eggs were then placed in a rotating incubator (G.Q.F. Manufacturing Co., Savannah, GA. Model "1202" 280W Circulated Air Incubator) at a constant 37.5 °C and 60% humidity. Eggs were removed at designated sampling times: 12, 24, 48, 72, 96, or 120 hours 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. Some of the eggs incubated for more than 72 hours contained distinct blood pools from early stage embryos and this tissue was included in the yolk mass recordings as it was not feasible to separate it.

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 seconds on a Vortex (Fischer Scientific Model G-560). Samples were then frozen at -20°C for at least 24 hours to precipitate proteins and neutral lipids then removed from the freezer and centrifuged (Thermo Scientific Sorvall R1 Centrifuge) at 2000 rpm for 15 minutes. 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 (Waters, Ireland, Sep-Pak® Plus single use C18 cartridges). Each Sep-Pak® cartridge was numbered to match a yolk/methanol solution from the eggs sampled. 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 followed by conjugated steroids being eluted with 5 mL methanol. The conjugated steroids from each cartridge were collected in separate numbered test tubes 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, 5 mL of increasing concentrations of an ethyl acetate:isooctane solution (0%, 10%, and 20%) were added to each column. Androstenedione elutes in the 0% fraction, etiocholanolone in 10% fraction, and testosterone in the 20% fraction (Paitz et al., 2011). Each fraction was then dried and quantified for radioactivity.

Because the results of the 2017 study suggested testosterone was already completely metabolized prior to the 12 hour sampling period, 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 with 2×10^6 CPM of 3H-testosterone in 5 μ L of sesame oil with a Hamilton microliter syringe and sealed as mentioned previously. The five eggs randomly selected to be sampled at hour zero were snap frozen immediately following the injection on dry ice to prevent any steroid metabolism. 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 either four, eight, or 12 hours of incubation. Eggs were frozen upon sampling, 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 3H-testosterone as possible.

In vitro Metabolism of 3H-Testosterone

Given that metabolism of 3H-testosterone occurred so early in development, we wanted to examine which, if any, components of freshly laid eggs were capable of metabolizing testosterone. To do this yolk, albumen, or a 1:1 mixture of the two were used as tissue sources. We mixed 0.5 g tissue samples from freshly laid eggs and mixed with 100,000 CPM of 3H-testosterone in 200 μ l of homogenization buffer (250mM sucrose, 5mM MgCl₂, 100mM Tris-HCl), and ran 120-minute metabolism assays that were quenched by the addition of 4 mL of ice cold methanol. These samples were then subjected to solid phase extraction and celite chromatography as described above. To further verify that 3H-testosterone metabolism occurs in the yolk of freshly laid eggs, a time course experiment was conducted in which the assays described above were terminated at 0, 30, 60, 120 minutes respectively and samples from five different eggs were tested at each time point.

Endogenous Etiocholanolone Levels

In addition to characterizing when testosterone is metabolized, we quantified the endogenous levels of etiocholanolone in freshly laid eggs (presumably of maternal origin) and examined how those levels change during development. To quantify endogenous etiocholanolone levels present in starling eggs, 10 freshly laid starling eggs were frozen at -20°C until steroid analysis was performed and 10 eggs were incubated for five days and then frozen. To extract etiocholanolone, eggs were thawed and de-shelled as mentioned previously, and yolk and albumen mass was recorded in order to back correct sample concentrations to the total amount in the egg. Yolk fractions from all incubated eggs contained early stage embryos

that were not separated out, but included in the yolk mass. The yolks were homogenized and 0.5 g of the homogenate was mixed with 4 mL methanol as mentioned previously and was 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 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; Merrill et al., 2018). Briefly, the LC separation was performed on a Phenomenex C6 Phenyl column (2.0 × 100mm, 3m.) 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. 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 minutes and was quantified on a 5500 QTRAP mass spectrometer (AB Sciex, Foster City, CA, USA) under positive electrospray ionization (ESI) with the ion spray voltage of 5500V. 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 the 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. Clutches were divided among four different injection treatments: sesame oil vehicle (control), 2.5 ng (low), 5.0 ng (medium), or 10.0 ng (high) injections of

etiocholanolone dissolved in sesame oil. Etiocholanolone doses were chosen based on the data on etiocholanolone levels in freshly laid starling eggs that were collected in 2017, where eggs contained on average 4.6 ng of etiocholanolone \pm 1.8 (mean \pm SD). We chose these doses as they represented an approximate increase in etiocholanolone of one, two, and four standard deviations. In addition to the injected eggs, nine eggs were immediately frozen at -20°C after weighing to serve as controls for baseline steroid levels. Following injections, eggs were incubated for either three or five days.

Following incubation, embryos and their extra-embryonic membranes were collected and weighed to collect 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. After noting dried mass, the tissue was then used for molecular sexing of the embryo (Nettle et al., 2013). Briefly, 50 μL of 0.2M Sodium Hydroxide (NaOH) was added to each dried embryo, and placed in a 75°C heat block for 20 minutes to promote cell lysis (Haunshi et al., 2008). Samples were removed from the heat block and 300 μL of 0.04M 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'-AATCCCCTTTTATTGATCCATC-3') (Nettle et al., 2013). Each PCR reaction contained: 6.7 μL nanopure water, 4.0 μL 5X GoTaq Flexi buffer (Promega, Madison, WI, USA), 1.6 μL 25mM MgCl_2 , 1.0 μL 20mM CHD forward primer, 1.0 μL 20mM 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 visualized using gel electrophoresis (1.5% agarose gel) under UV light. The

gels ran for 90 minutes at 95 V. Using this protocol, male individuals show a single band while females produced two bands.

We used a subset of eggs to confirm that our etiocholanolone manipulations were within the physiological range. The subset contained nine freshly laid eggs, three oil-injected eggs that were incubated for 3 days, six oil-injected eggs that were incubated for 5 days, six high-dose etiocholanolone-injected eggs that were incubated for 3 days, and five 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). Analyses of variance were used to examine how tritiated metabolite levels changed across development in both the 2017 and 2018 *in ovo* metabolism studies. Separate analyses of variance and post-hoc comparisons were performed for each metabolite. Concentrations were log transformed prior to analysis to normalize the data as well as homogenize variances. Sampling period was included as a fixed effect. Post-hoc comparisons (Tukey's HSD) were performed to test for differences between sampling periods.

An analysis of variance 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 an analysis of

variance to examine the change in etiocholanolone produced over the course of two hours *in vitro*. Post-hoc comparisons were performed for each 30 minute time point from zero to 120 minutes. The concentration of etiocholanolone was log transformed prior to analysis to normalize the data and homogenize variances. Time point was included as a fixed effect. To determine the change in endogenous etiocholanolone levels over the first five days of development, an analysis of variance was used including the day of development as a fixed effect.

Analyses of variance were used to analyze the treatment effects on mass of the embryo and extra-embryonic membranes. Day of development, treatment, and sex of the embryo were included as fixed effects, while clutch was included as a random effect. Masses of the embryonic tissues were log transformed prior to analysis to normalize the data and homogenize variances, as the raw data did not produce a normalized distribution.

Results

In ovo Metabolism of 3H-Testosterone

Our average recovery of radioactivity per egg was 68% (102,125 CPM per egg), which is consistent with similar studies (Paitz and Bowden, 2008; von Engelhardt et al., 2009). We found the concentration of the injected 3H-testosterone dropped over the course of development *in ovo* ($F_{5,29} = 5.27, p = 0.0015$). Unexpectedly, the levels of testosterone recovered at the initial 12 hour sampling period (1535 ± 178 CPM) (mean \pm SE) only represented 1% of the total radioactivity recovered. We examined testosterone levels changed over the first five days of development ($F_{5,29} = 5.27, p = 0.0015$) (Figure 1a). Other metabolism patterns, such as a change

in androstenedione levels ($F_{5,29} = 26.35, p < 0.0001$) (Figure 1b) and an accumulation of the conjugated steroid, etiocholanolone glucuronide ($F_{5,29} = 30.31, p < 0.0001$) (Figure 2d), were shown to occur during this time period of development. Levels of etiocholanolone changed over the first five days of development ($F_{5,29} = 5.80, p = 0.0008$), and this change resulted in a transient peak occurring between days two and four of development (Figure 1c).

In our follow-up study in 2018, with increased 3H-testosterone and earlier sampling periods, we were able to demonstrate that testosterone metabolism is occurring in the first 12 hours of development. We observed testosterone levels change over the first 12 hours of development ($F_{3,15} = 5.56, p = 0.0091$) (Figure 2a) and again, observed changes in androstenedione ($F_{3,15} = 23.05, p < 0.0001$) (Figure 2b) and etiocholanolone ($F_{3,15} = 5.49, p = 0.0095$) levels (Figure 2c) during this time.

In vitro Metabolism of 3H-Testosterone

We also sought to examine where in the eggs this metabolism takes place; either in the yolk, the albumen, or both through *in vitro* testing. We determined the yolk is most capable of producing etiocholanolone from testosterone. While there is a small amount of etiocholanolone produced in the albumen, the yolk and mixed tissues produced significantly more etiocholanolone ($F_{2,12} = 97.42, p < 0.0001$). Post-hoc comparisons show that both yolk and mixed tissues produced significantly higher amounts of etiocholanolone than albumen (both $p < 0.001$); however, yolk did not differ from the mixed tissues in the amount of etiocholanolone produced ($p = 0.5169$).

The ability of yolk to convert testosterone to etiocholanolone was further verified with the time course data (Figure 3). We determined there was significantly more etiocholanolone produced in the yolk after two hours in vitro ($F_{3,16} = 28.15, p < 0.0001$). Post-hoc comparisons show that there is a significant change in etiocholanolone levels after just 30 minutes ($p = 0.0032$) and in the amount of etiocholanolone produced after 120 minutes of mixing with yolk ($p < 0.0001$).

Endogenous and Manipulated Etiocholanolone Levels

Yolks from freshly laid eggs contained an average of $4.6 \text{ ng} \pm 1.8$ of etiocholanolone (mean \pm SD). There was a significant increase in the amount of etiocholanolone present in eggs after five days of incubation ($F_{1,18} = 45.99, p < 0.001$) (Figure 4).

We then tested to see if etiocholanolone injections produced detectable increases in etiocholanolone levels within various components of the egg. We measured the levels of etiocholanolone between the freshly laid, control, and high etiocholanolone treatments. There was a significant effect of day ($F_{1,10} = 18.16, p = 0.0017$) and treatment ($F_{1,10} = 22.28, p = 0.0008$) on etiocholanolone levels (Figure 5). Sex of the embryo did not affect etiocholanolone levels ($p = 0.9411$). We also evaluated the difference in etiocholanolone concentrations by incubation duration between our frozen and control treatments and found that levels of etiocholanolone changed across development in unmanipulated eggs ($F_{2,15} = 15.30, p = 0.0002$) (Figure 5). Our post-hoc comparisons show that levels on day three of incubation are higher than on days zero and five (both $p < 0.0004$), while levels on day zero did not differ from day five of incubation ($p = 0.9602$).

Effects of Etiocholanolone on Embryonic Growth

We used tissue mass as a proxy for growth and found no significant effect of our treatments on mass of the embryo and extra-embryonic membranes ($F_{3,12} = 0.73, p = 0.5347$) (Figure 6). There was a significant effect of day on growth ($F_{1,12} = 25.77, p < 0.0001$), although sex of the embryo did not affect mass ($F_{1,12} = 0.22, p = 0.6405$). Overall, the sex ratio was approximately 50:50 with 49.5% males produced.

Discussion

Previous studies provide evidence for the metabolism of testosterone in bird eggs, but a detailed understanding of when and where this metabolism takes place was lacking. We determined testosterone metabolism occurs rapidly, essentially as soon as incubation begins, and this metabolism results in the production of the metabolite, etiocholanolone. *In vitro* assays, using freshly laid eggs, demonstrate the yolk possesses the enzymes necessary to carry out this metabolism. Our investigations of 3H-testosterone metabolism *in ovo*, as well as endogenous etiocholanolone levels, show an early rise and subsequent fall in etiocholanolone levels over the first five days of development. We hypothesize the fall 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 (Paitz et al., 2011). The metabolism of maternal testosterone creates a scenario whereby embryos are exposed to potentially active, metabolites, such as etiocholanolone. A transient peak in etiocholanolone was found during early development; however, we determined through our study that this transient exposure does not affect the growth of the embryo or the extra-embryonic

membranes. With this evidence, we propose testosterone metabolism to etiocholanolone is an inactivation pathway which may serve to buffer the process of sexual differentiation in the embryo from the effects of yolk testosterone.

Our findings are some of the first to provide a detailed characterization of what happens to yolk testosterone during development. Studies have demonstrated 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). A recent study also determined that this metabolism pathway occurs in chicken eggs during the first five days of development (Kumar et al. 2019). Through the use of tritiated testosterone, we were able to demonstrate this early metabolism of testosterone results in the production of etiocholanolone as opposed to etiocholanolone produced from other potential precursors. Several of our findings support the idea that maternally derived enzymes are present in the yolk when eggs are laid and contribute to this metabolism. We showed testosterone metabolism occurs by hour 12 of development, suggesting that this metabolism starts at the onset of incubation. We also determined that the yolk of starling eggs is capable of metabolizing testosterone, presumably through enzymes found in the yolk. Thus, these enzymes are likely to originate from the mother, as ovarian follicles produce 5 β -reductase (Wiebe et al., 1990); however, we cannot ignore studies which report this metabolism does not occur without a viable embryo (Paitz and Casto, 2012; Kumar et al., 2018). Therefore, it is possible maternal 5 β -reductase enzymes are transferred to the yolk during oogenesis, but the developing embryo may either play a role in activating these enzymes or by creating an environment conducive to metabolism since unfertilized eggs do not exhibit testosterone metabolism.

Although testosterone metabolism results in an increase of etiocholanolone, we observed that this increase was transient over the first five days of development. As testosterone metabolism begins, we saw an increase in the amount of etiocholanolone accumulating in the yolk; however, these levels quickly decline by day five due to conjugation (Paitz et al., 2011). Our results indicate etiocholanolone is present not only at day five of development, but at day zero as well, suggesting the embryo is likely exposed to these transient etiocholanolone levels during the critical 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.

Etiocholanolone production has been attributed to a mechanism of inactivation of testosterone (Balthazart and Ottinger, 1984). Evidence relating the activity of 5β -reduced metabolites in young and adult birds may provide some insight into the importance of this metabolic pathway. In 20 day old Zebra finches, 5β -reductase activity is two to three orders of magnitude more active than both aromatase and 5α -reductase in selected brain regions of the telencephalon, an area of the brain which regulates learning and birdsong (Vockel et al., 1990). Developing quail also have high levels of 5β -reductase activity in their brains which is often 10 times more active than in adults (Balthazart and Schumacher, 1984) and these levels remain elevated for the duration of the embryonic stage (Balthazart and Ottinger, 1984). The 5β -reduced metabolite, 5β -DHT, had no effect on male sexual behavior post hatching (Schumacher et al., 1989), confirming that 5β -reduced metabolites are inactive compounds with regards to the process of sexual differentiation of the brain. Together, these findings regarding the activity of 5β -reductase enzymes, reflect our own discovery of transient etiocholanolone levels found in

the yolk of starling eggs during early embryonic development. The 5β -reduction pathway is considered to be an inactivation pathway for testosterone (Balthazart and Ottinger, 1984), and therefore it is possible that 5β -reductase “buffers” maternal steroids by rendering them as inactive compounds which cannot influence sexual differentiation of the developing embryo. Therefore, embryos may not be passive agents of maternal effects and seem to have the ability to circumvent any early developmental effects of maternal testosterone through the conversion of testosterone to etiocholanolone.

One question still remaining is how do effects of testosterone exist when it is metabolized so early in development? We show 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 birds following 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, leading to the possibility that some yolk testosterone effects may not arise until later stages of development (Muriel et al., 2015a). 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 possibility for these developmental effects could be due to etiocholanolone effects that 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. It is also possible that etiocholanolone may alter gene activation and expression during early development which could lead to the development of phenotypic effects, such as impaired or enhanced immune function and muscle development, post-hatch.

There may also be a critical window of development where maternal steroids and their metabolites have the highest propensity to elicit phenotypic effects in offspring. A prior study performed in Japanese quail (*Coturnix japonica*) eggs, between days 10 and 15 of embryonic development, suggest injections of testosterone are most effective at altering offspring phenotypes related to sexual differentiation (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 phenotypic effects mediated by etiocholanolone are present in the starling, but may not arise until later in development after our sampling occurred and perhaps may not be visible until after hatching.

Overall, with these findings, we show that testosterone is metabolized within the first four hours of embryonic development. Additionally, the metabolism of testosterone is associated with the accumulation of etiocholanolone, an inactive metabolite of the 5 β -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; Kumar et al., 2019; Paitz et al., 2011; Paitz and Casto, 2012; Vassallo et al., 2014; Vassallo et al., 2018), suggesting this steroid metabolism is conserved. Finally, the role of the 5 β -reduction as an inactivation pathway (Balthazart et al.,

1990) is further supported with 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.

Figure 1 Timing of *In Ovo* Testosterone Metabolism During the First Five Days of

Development: Concentration of 3H-Testosterone and its metabolites over the first five days of incubation. Time points with the same letter do not significantly differ from each other. Error bars denote the standard error for each time point.

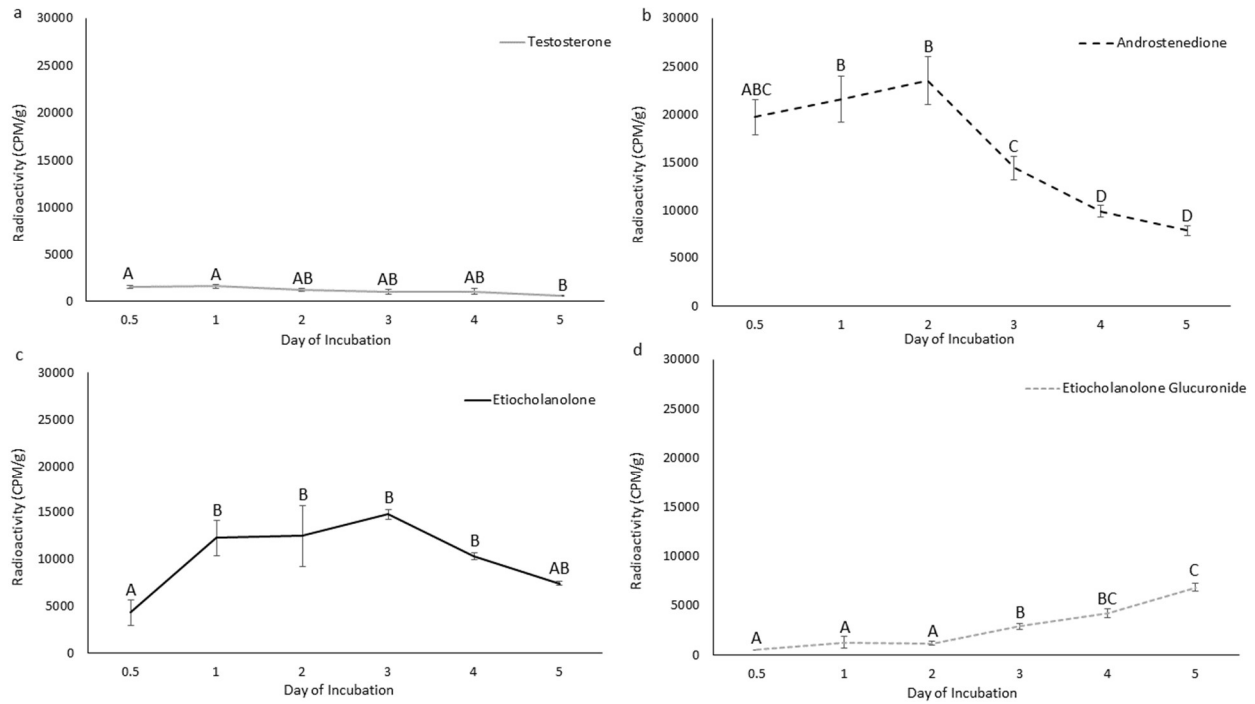


Figure 2 Timing of *In Ovo* Testosterone Metabolism During the First 12 Hours of

Development: Concentration of 3H-Testosterone and its metabolites over the first 12 hours of incubation. Time points with the same letter do not significantly differ from each other. Error bars denote the standard error for each time point.

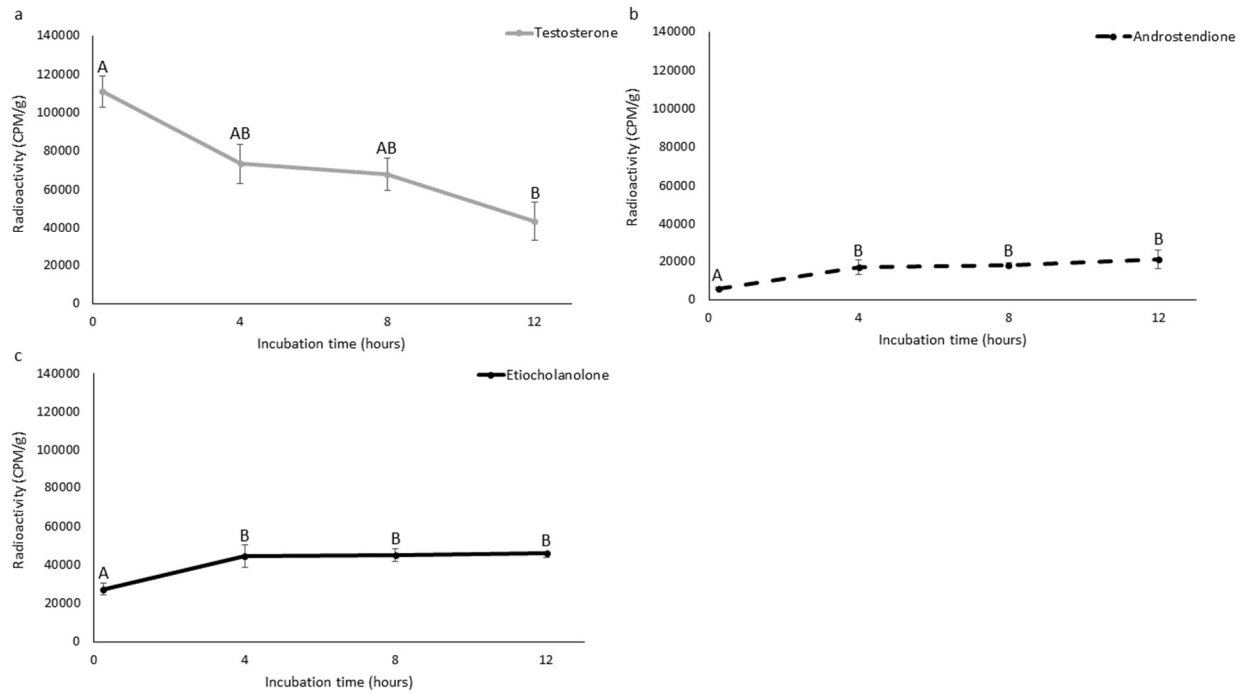


Figure 3 *In Vitro* Etiocholanolone Production: Production of etiocholanolone *in vitro* in 0.5 g yolk over the course of two hours. Time points with the same letter are not significantly different from each other. Error bars denote the standard error for each time point.

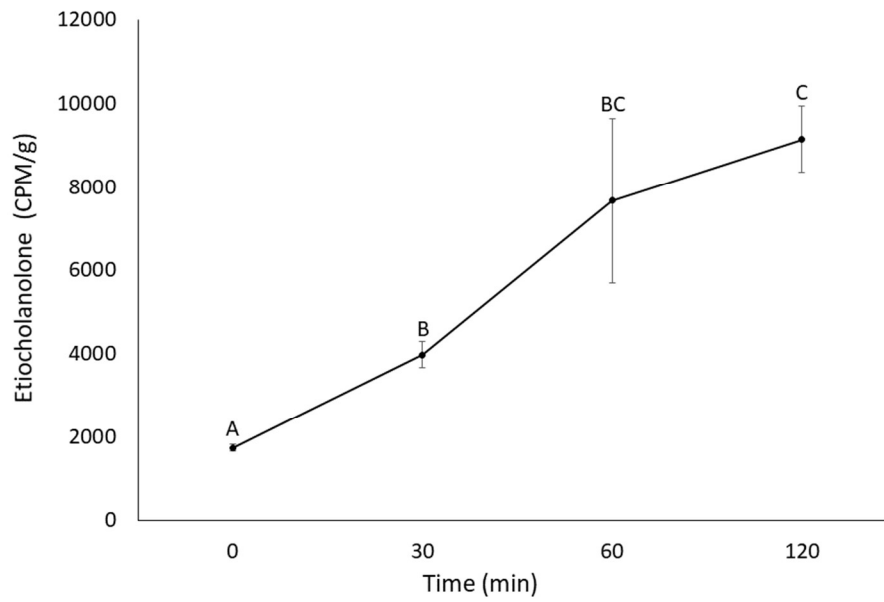


Figure 4 Endogenous Etiocholanolone: Etiocholanolone levels within the yolks of unincubated eggs and eggs that had been incubated for five days. Endogenous etiocholanolone levels are significantly different on day five than on day zero of development ($p < 0.005$).

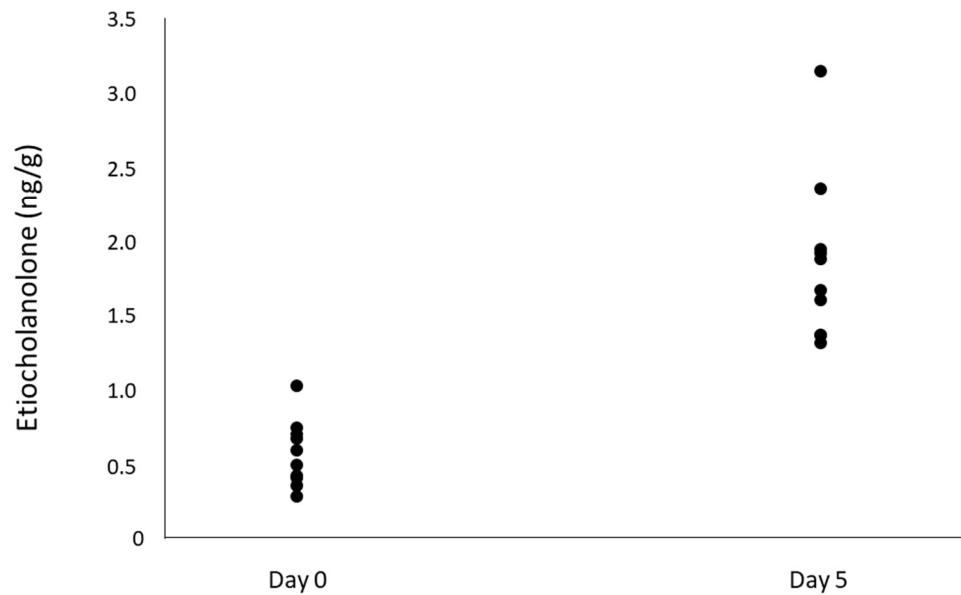


Figure 5 Etiocholanolone Injections Introduced Higher Levels of Etiocholanolone to Eggs:

Treated verses untreated eggs and the amount of etiocholanolone present over the first five days of development. Bars with the same letter are not significantly different from each other. Day 3 eggs significantly differ from day 5 eggs as shown by the asterisk and high etiocholanolone (High Etio) eggs significantly differ from control eggs on both day 3 and day 5 of development as indicated by the asterisk. Error bars denote the standard error for each treatment.

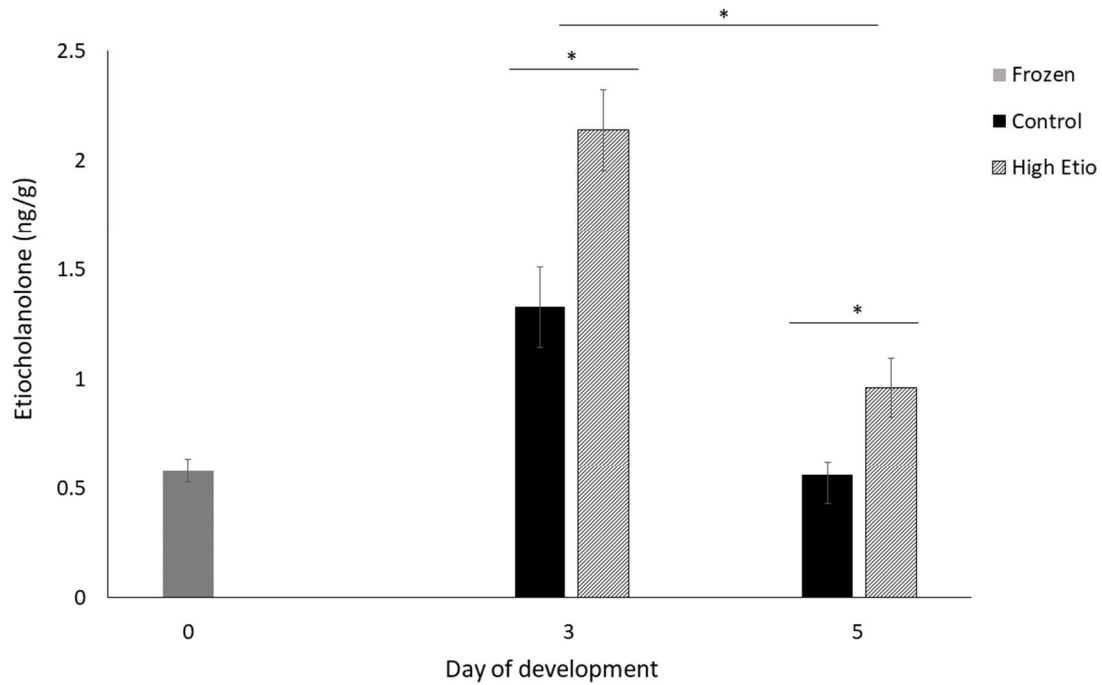
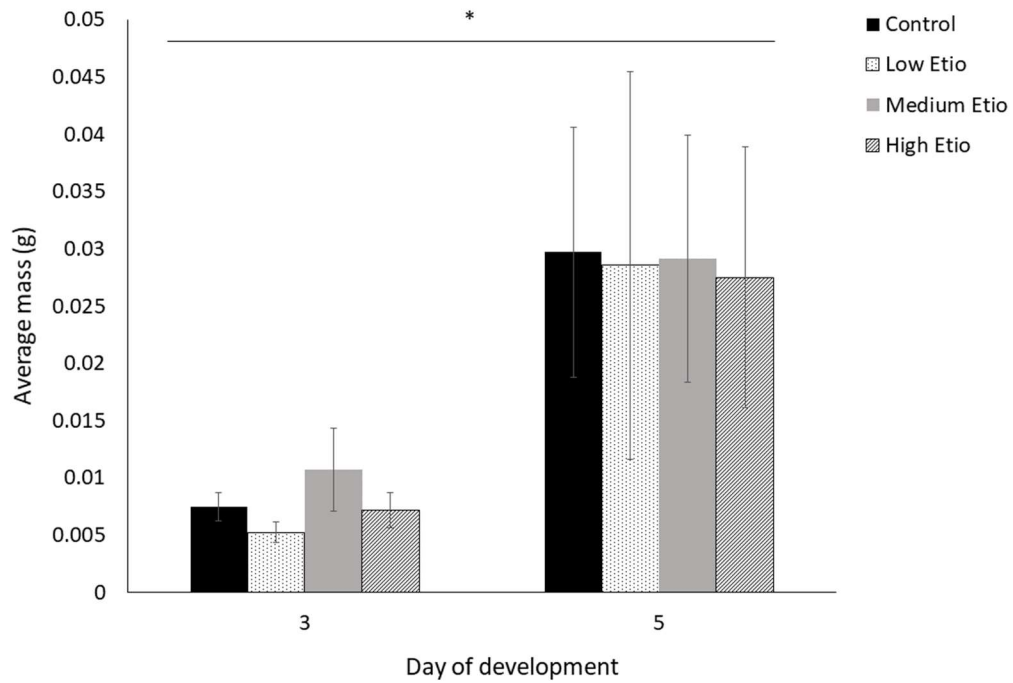


Figure 6 Etiocholanolone Effects on Embryo and Extra-embryonic Membrane Masses: Average mass (in grams) of extracted embryo and extraembryonic membrane tissues from each treatment on days three and five of development. There was a significant effect of day on mass as denoted by the asterisk. Error bars denote the standard error for each treatment on each sampling day.



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