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In ovo metabolism of progesterone to 5β-pregnanedione in chicken eggs: Implications for how yolk progesterone influences embryonic development

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ABSTRACT

Progesterone has received substantial attention for the essential role it plays in establishing and maintaining pregnancy in placental vertebrates. Despite the prevalence of progesterone during development, relatively little is known about how embryos respond to progesterone. This is true of placental vertebrates as well as egg-laying vertebrates where levels of progesterone in the yolk tend to be higher than most other steroids in the yolk. Bird eggs provide an opportunity to investigate the effects of progesterone on embryonic development because progesterone can be easily manipulated without any confounding effects on maternal physiology. To understand how progesterone might influence embryonic development, it is important to characterize the metabolic fate of progesterone given its potential to be converted to a wide range of steroids. We investigated the metabolic fate of tritiated progesterone over the first four days of development using chicken eggs (*Gallus gallus*) and identified 5β-pregnanedione as the primary metabolite during this period. After only one day of development, 5β-pregnanedione could be detected within the yolk. Levels of 5β-pregnanedione in both the yolk and albumen tended to rise early in development but conjugated metabolites began to accumulate towards the end of our sampling period. Additionally, *in vitro* assays using embryo homogenates collected after 72 h of development demonstrated that embryos were capable of carrying out the conversion of progesterone to 5β-pregnanedione. Overall these results have important implications for deciphering the mechanisms through which yolk progesterone might influence embryonic development. Effects could arise via progesterone receptors or receptors capable of binding 5β-pregnanedione but we found no evidence that progesterone is serving as a precursor for androgen or estrogen production.

1. Introduction

Almost all aspects of vertebrate reproduction are coordinated, at least in part, by steroid cues produced by the endocrine system (reviewed in Norris and Carr, 2013). Progesterone is one of the most critical of these steroids as it is a pleiotropic regulator of reproductive physiology in females (Lydon et al., 1996; Conneely and Jericevic, 2002) that is "unequivocally required in all mammals for support of conceptus (embryo/fetus and associated membranes) survival and development" (Spencer and Bazer, 2002). As such, levels of progesterone are elevated at the time of reproduction to elicit uterine changes, such as increased vascularization and decidualization, which prepare the uterus for pregnancy (Byrns, 2014). After implantation, progesterone levels continue to rise until parturition (Mesiano, 2001). A large portion of this progesterone is produced by the transient corpus luteum (Stocco et al., 2007) and placenta (Pasqualini and Chetrite, 2016). At the time of ovulation, the corpus luteum is the primary tissue responsible for progesterone production while the placenta takes over this role during pregnancy (Niswender et al., 2000; Mesiano, 2001). The importance of this progesterone for successful pregnancies is demonstrated by the use of antiprogesterones like RU486 to interrupt pregnancies (Baulieu, 1991) and synthetic progestogens to prevent preterm birth (Byrns, 2014). While it is widely accepted that progesterone plays a central role in the establishment and maintenance of pregnancy, the vast majority of this work has focused on the role progesterone plays in regulating maternal physiology in placental mammals. Relatively little is known about the direct effects this elevated progesterone has on embryonic development (Clemente et al., 2009).

This lack of knowledge is largely due to the difficulty in manipulating embryonic progesterone signaling during development without also eliciting changes in the maternal environment. Thus effects on the developing embryos may be direct responses to progesterone manipulations or indirect responses to changes in maternal physiology. One solution to this problem is to study the effects of progesterone on

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embryonic development using egg-laying amniotes such as birds. Similar to mammals, progesterone plays an important role in coordinating reproduction. Progesterone levels are elevated around the time of ovulation (Johnson and van Tienhoven, 1980) primarily as a result of progesterone production by the preovulatory follicle (Johnson, 2015). One consequence of this is that bird eggs then contain high levels of progesterone when they are laid (Lipar et al., 1999; Rettenbacher et al., 2009; Paitz et al., 2011; Della Costa et al., 2017). The effects of maternal steroids on embryonic development in birds has been the subject of research for decades (Schwabl, 1993; Groothuis et al., 2005; Williams and Groothuis, 2015). However, most of these studies have tended to focus on androgens (Schwabl, 1993; Clairardin et al., 2011; Merrill et al., 2017, reviewed in Groothuis et al., 2019) and glucocorticoids (Hayward et al., 2006; Love and Williams, 2008; Vassallo et al., 2014; Vassallo et al., 2019) while relatively few studies have been done on progestogens (Renden and Benoff, 1980; Bertin et al., 2008; Bertin et al., 2013; Iqbal et al., 2014; Herrington et al., 2015; Herrington et al., 2016). The reason for this paucity of studies on progesterone is not clear as concentrations of progesterone in bird eggs tend to be far higher than those of androgens and glucocorticoids (Lipar et al., 1999; Rettenbacher et al., 2009; Paitz et al., 2011; Poisbleau et al., 2011; Bertin et al., 2013; Merrill et al., 2018).

Understanding how maternal progesterone may influence embryonic development is interesting because progesterone not only has the potential to elicit direct effects via progesterone receptors (Conneely and Jericevic, 2002), but it can also be converted to a variety of progestogens, androgens, estrogens, or glucocorticoids with relatively few enzymatic steps (Payne and Hales, 2004). Not surprisingly, the few documented effects of progesterone manipulations on bird embryos are quite variable. Progesterone has been shown to slow developmental rates (Renden and Benoff, 1980, Iqbal et al., 2014), increase embryonic heart rate (Herrington et al., 2016), and moderate auditory learning (Herrington et al., 2015; Herrington et al., 2016). None of these studies investigated whether the observed effects were elicited via direct activation of progesterone receptors or elicited by a metabolite of progesterone. Given that progesterone can serve as a precursor for a relatively large number of steroids, it is important to know if or how maternal progesterone is metabolized by bird embryos. Accumulating evidence suggests that bird embryos are capable of metabolizing maternal steroids during development (von Engelhardt et al., 2009; Paitz and Bowden, 2010; Paitz et al., 2011; Paitz and Casto, 2012; Vassallo et al., 2014; Kumar et al., 2018; Kumar et al., 2019; Vassallo et al., 2019). Only one of these studies identified specific metabolites (Paitz et al., 2011); where testosterone was converted to a conjugated form of etiocholanolone (5β-ANDROSTAN-3α-OL-17-ONE) in European starling (*Sturnus vulgaris*) eggs. This result suggests that several enzymes can be present in early development (5β reductase, 17β hydroxysteroid dehydrogenase, 3α hydroxysteroid dehydrogenase). Early *in vitro* work demonstrated these enzymes can be detected in 48 h old chick blastoderms (Parsons, 1970) and metabolize testosterone. Importantly, progesterone is also subject to metabolism by many of these same enzymes.

To explore how maternal progesterone might be metabolized by developing bird embryos, we characterized the movement and metabolism of tritiated progesterone during the first four days of development in chicken (*Gallus gallus domesticus*) eggs. By determining the metabolic fate of maternal progesterone, we can begin to identify the pathways through which the high levels of maternal progesterone might impact embryonic development.

2. Methods

2.1. Egg treatments and sampling

For this study, 30 fertile Leghorn chicken eggs were purchased from the University of Illinois poultry farm. All eggs received an injection of 250,000 CPM of (1,2,6,7-^{3}H; Specific Activity = 96.6 Ci/mmole) progesterone (Perkin-Elmer, Waltham, MA, USA) dissolved in 10 µl of sesame oil into the albumen and were incubated at 37.8 °C and 55% humidity. Five eggs were then removed from the incubator after 2, 12, 24, 48, 72 and 96 h respectively, and frozen at −20 °C.

Steroids were injected into the albumen, as opposed to the yolk, because injections into the yolk tend to decrease survival rates compared to injections into the albumen. While steroids are primarily located in the yolk at the time of oviposition, they can also be detected in the albumen (De Baere et al., 2015). Additionally, comparisons of yolk versus albumen injections have found that these different approaches do affect the distribution of the steroid in the egg, but they do not affect how quickly the steroid/steroid metabolites are taken up into the embryo or the metabolic fate of the injected steroid (Vassallo et al., 2019). Both approaches likely create a situation where the embryo is developing near the injected oil since both the embryo and the injected oil float to the top of the egg (von Engelhardt et al., 2009). Additionally, the physical separation of yolk and albumen is transient since the vitelline membrane surrounding the yolk degrades and ruptures in the first few days of development (Jensen, 1969). Injections into the yolk (von Engelhardt et al., 2009; Kumar et al., 2018; Kumar et al., 2019) and albumen (Paitz et al., 2011; Paitz et al., 2012) have been used successfully to investigate yolk steroid metabolism with generally similar outcomes.

2.2. Distribution of free steroids and conjugated metabolites

To initially characterize the distribution of progesterone and potential metabolites, eggs were removed from the freezer and separated into “albumen” and “yolk” components. The albumen component consisted of thick and thin albumen outside of the vitelline membrane (Greenwood and Bolton, 1956) while the yolk component consisted of the contents within the vitelline membrane including yolk, sub-embryonic fluid, and the embryo/membranes. The presence of an embryo was confirmed in all eggs sampled after 12 h of development and was assumed to be present at all earlier sampling points where it was difficult to differentiate between fertile and infertile eggs after the freeze/thaw process. Both the yolk and albumen were homogenized and 1 g of each was used for solid-phase extraction (SPE) to separate free steroids from steroid conjugates (Newman et al., 2008).

For SPE, 4 ml of methanol was added to each homogenate, vortexed, and placed at −20 °C overnight to precipitate proteins and neutral lipids (Kozlowski et al. 2009). Samples were centrifuged at 2000 rpm for 20 min and the resulting supernatant was transferred to a 50 ml conical vial. Water was then added to each vial to bring the total volume up to 50 ml of extract thereby diluting the methanol extract to a concentration less than 10% methanol in water. This 50 ml of extract was then drawn through a Sep-Pak Plus C18 cartridge (Waters, Ltd., Miliford, MA, USA) under vacuum. Free steroids were eluted from the cartridge with 5 ml of diethyl ether and the conjugated steroids were then eluted with 5 ml of methanol. Ether and methanol fractions were then dried under a stream of nitrogen gas and radioactivity was quantified in each fraction. The amount of radioactivity in the free and conjugated fraction of each 1 g of tissue was used to calculate total amounts of free and conjugated steroids in the yolk and albumen respectively. To test for significant changes in the amount of radioactivity in each of these four fractions, separate ANOVAs using sampling point as a fixed factor were conducted. Data were log transformed prior to analysis and *post hoc* (Tukey’s HSD) comparisons were conducted to test for differences between sampling points. All statistical tests were conducted using SAS v. 9.4 (SAS Institute, Cary, NC, USA).

2.3. TLC characterization of metabolites

Upon determining which tissues within the egg contained radioactivity, we used thin-layer chromatography (TLC) to identify the

primary metabolites of progesterone in the free steroid fraction following SPE (Paitz and Bowden, 2008; von Engelhardt et al., 2009; Paitz et al., 2012; Paitz and Bowden, 2013). Here, free steroids were extracted from 5 g of yolk and albumen using the SPE technique described above. Levels of conjugated steroids were too low to conduct TLC. The dried ether extracts from yolk and albumen were dissolved in 25 µl of methanol containing 1 µg of progesterone, 5α-pregnanedione, and 5β-pregnanedione (Steraloids, Newport, RI) as standards and applied to 20 × 5 cm aluminum plates coated with silica G that contained a UV indicator (Sorbtech, Norcross, GA). Plates were then developed to the 12 cm mark in a solvent system of ethyl acetate:cyclohexane (1:1) (Mattox et al., 1979). This system was chosen specifically to separate progesterone, 5α-pregnanedione, and 5β-pregnanedione after preliminary plates indicated the primary metabolite (only metabolite comprising more than 10% of the total radioactivity) was more mobile than progesterone while the standards for testosterone, androstenedione, estradiol, and corticosterone were all less mobile. After plates were developed, standards were visualized under iodine vapor and UV light. To determine the distribution of radioactivity on the plate, each plate was initially cut into 22 individual 1 cm × 0.5 cm sections (the 1 cm below the origin was not analyzed) and the amount of radioactivity in each section was quantified (Paitz et al., 2011; Paitz et al., 2012) (Fig. 1). 5β-pregnanedione was verified as the primary metabolite repeated recrystallization to a specific activity (Axelrod et al., 1965; Paitz et al., 2011). To compare how 5β-pregnanedione and progesterone levels changed over time in both the yolk and albumen, ANOVAs using sampling point as a fixed factor were conducted. Data were arcsine-square root transformed prior to analysis and *post hoc* (Tukey's HSD) comparisons were conducted to test for differences between sampling points.

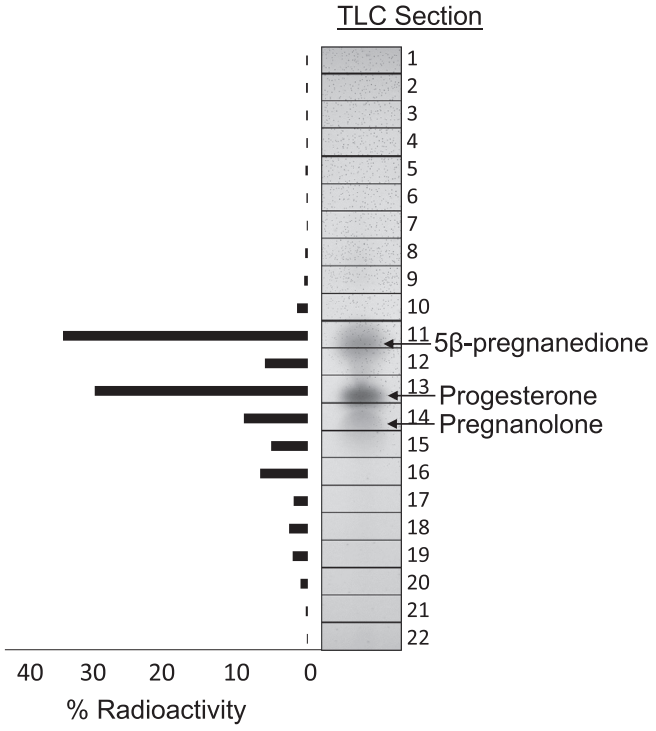

Fig. 1. Example of TLC separation of tritiated metabolites from the free fraction following solid phase extraction. Radioactivity levels were quantified in each 0.5 × 1 cm section and reported as a percentage of the total radioactivity present on the TLC plate. Each plate contained standards for 5β-pregnanedione, progesterone, and pregnanolone.

2.4. Progesterone metabolism in embryo homogenates

To examine whether embryos possessed the enzymes necessary to metabolize progesterone, we conducted an *in vitro* metabolism assay using homogenates of 72 h old embryos. Briefly, 4 embryos (including the developing chorioallantoic membrane) were harvested from eggs that had been incubated 72 h. Each embryo was homogenized in 2 ml of homogenization buffer (250 mM sucrose, 5 mM $MgCl_2$, 100 mM Tris-HCl) using a Potter-Elvehjem homogenizer. Homogenates were divided into four 500 ml aliquots and 100,000 CPM of ^{3}H-progeseterone (dissolved in 100 µl of homogenization buffer) was added to each aliquot. One aliquot from each embryo was immediately frozen while the other three aliquots were incubated at 37.8 °C for 1, 2, and 4 h respectively. To quantify progesterone metabolism, free steroids were extracted and subjected to TLC as described above. Time dependent changes in 5β-pregnanedione levels were analyzed with an ANOVA similar to what was used to analyze 5β-pregnanedione levels in the yolk and albumen.

3. Results

3.1. Distribution of free steroids and conjugated metabolites

Injected progesterone was metabolized over the first four days of incubation in chicken eggs. Two hours after the injection into the albumen, most radioactivity was detected as free steroids within the albumen (Fig. 2). As development proceeded, free steroids in the albumen dropped ($F_{5,24}$ = 2.36, P = 0.041) and began to accumulate in the yolk within 24 h of incubation ($F_{5,24}$ = 10.89, P < 0.0001). After 96 h of incubation, steroid conjugates could be detected in both the albumen ($F_{5,24}$ = 8.85, P < 0.0001) and yolk ($F_{5,24}$ = 16.36, P < 0.0001) (Fig. 2). We recovered 64% of the original 250,000 CPM on average; ranging from 57% at the 2 h sampling point to 72% at the 96 h sampling point.

3.2. TLC characterization of free steroids

When we examined the metabolic fate of progesterone within the yolk and albumen, we found that 5β-pregnanedione was the primary metabolite within the free steroids for both tissues. 5β-pregnanedione was first detected within the yolk after 24 h ($F_{4,11}$ = 22.60, P < 0.0001) and could then be detected within the albumen after 48 h ($F_{4,11}$ =, P < 0.0001) (Fig. 3). Presumably this 5β-pregnanedione is further metabolized and subsequently conjugated around 96 h of

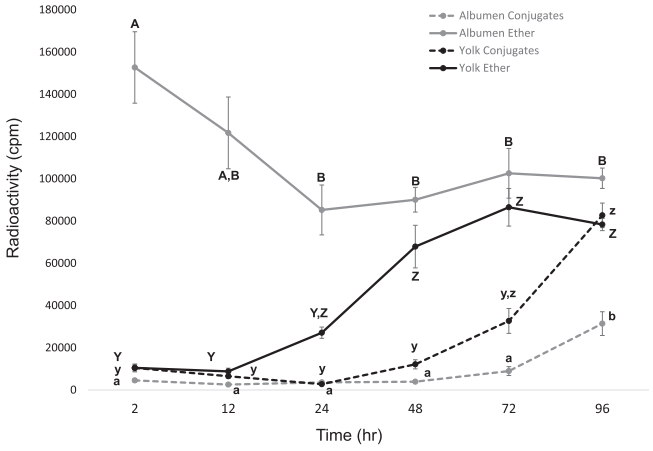

Fig. 2. Distribution of radioactivity (mean ± SE) in the yolk and albumen across the first four days of development. Free and conjugated steroids were separated using solid phase extraction. For each tissue (yolk and albumen) and fraction (free and conjugated) combination, days not sharing a letter are significantly different (p < 0.05).

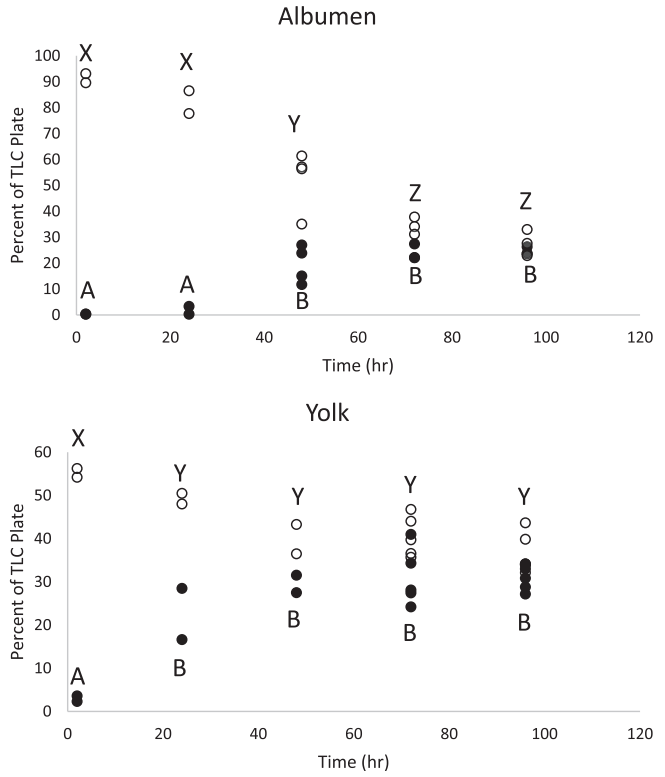

Fig. 3. Levels of 5β-pregnanedione (Solid Circles) and progesterone (Open Circles) in the free fraction of the yolk and albumen as determined by TLC. Values represent the percentage of the radioactivity on each plate that was found co-migrating with the 5β-pregnanedione standard. Days not sharing a letter for each respective steroid are significantly different ($p < 0.05$).

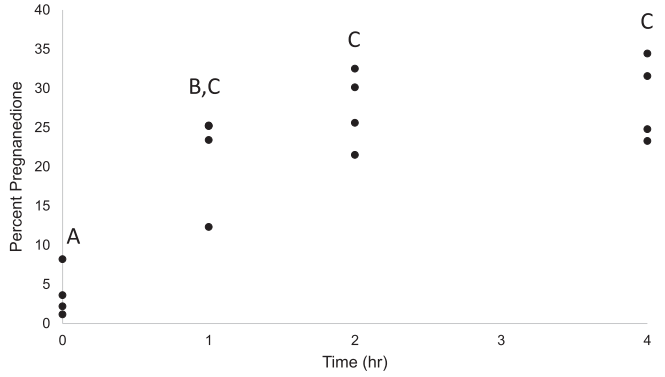

Fig. 4. Time course of 5β-pregnanedione produced by homogenates of 72 h old embryos *in vitro*. Values represent the percentage of the radioactivity on each TLC plate that was found co-migrating with the 5β-pregnanedione standard. Times not sharing a letter are significantly different ($p < 0.05$).

development. However, we did not characterize the fate of the steroid conjugates. Finally, we found that 72 h old embryos were capable of converting progesterone to 5β-pregnanedione *in vitro* ($F_{3,12} = 25.97$, $P < 0.0001$) (Fig. 4).

4. Discussion

We found that progesterone is metabolized early in development *in ovo* and that most of this metabolism occurs via 5β-reduction of progesterone to 5β-pregnanedione. Developing embryos were shown to have the capacity to convert progesterone to 5β-pregnanedione suggesting they are at least partially responsible for the observed *in ovo* metabolism. Given the potential metabolic fates of progesterone, such as androgens, estrogens, and glucocorticoids, our findings have important implications for understanding how progesterone might impact development. By having 5β-reduction of its primary metabolic fate, maternal progesterone does not appear to be serving as a precursor for the production of active androgens, estrogens, or glucocorticoids. Consistent with this idea, levels of androgens in the yolk of bird eggs drop early in development suggesting they are not being produced from abundant progestogen precursors (Elf and Fivizzani, 2002; Paitz et al., 2011; Kumar et al., 2018; Kumar et al., 2019). With regards to progesterone itself, some of the injected progesterone remained present after 96 h of development and could presumably elicit effects via progesterone receptors once they are present. In chicken embryos, progesterone receptors have been detected in the brain as early as day four of incubation (Guennoun et al., 1987) and in the oviduct as early as day six of incubation (Camacho-Arroyo et al., 2007). The effects of maternal progesterone may therefore be mediated by progesterone receptors but early conversion of progesterone to 5β-pregnanedione opens up additional mechanisms by which progesterone might impact development.

Relatively few studies have investigated the specific pathways avian embryos use to metabolize steroids during the first few days of development, but those that have also report 5β-reduction as the primary route of metabolism (Parsons, 1970; Paitz et al., 2011; Kumar et al., 2018). These studies focused on the metabolism of testosterone, but the enzyme responsible for this conversion, 5β reductase (AKR1D1), is also responsible for the 5β-reduction of progesterone (Chen and Penning, 2014). In the European starling (*Sturnus vulgaris*), AKR1D1 was shown to be present in the first five days of embryonic development and capable of metabolizing yolk testosterone during this period (Paitz et al., 2011). More recently, work in the rock pigeon (*Columbia livia*) has shown that 5β-reduced metabolites of both testosterone (i.e. ethiocholanolone) and progesterone (i.e. pregnanolone) accumulate in the egg during the first 4.5 days of development (Kumar et al., 2018). These results indicate that the 5β-reduction of yolk steroids occurs very early in development for several bird species. It is worth noting that in both of these studies as well as the current study, embryonic exposure to 5β-reduced steroids may be limited to early development since metabolites are further metabolized via conjugation as development proceeds (Paitz et al., 2011; Kumar et al., 2018). The conjugation of maternal steroids occurs in a wide number of egg-laying vertebrates (Paitz and Bowden, 2008; Paitz and Bowden, 2013). With accumulating evidence suggesting the 5β-reduction of maternal steroids is the most prevalent metabolic pathway in avian embryos, it is necessary to understand how 5β-reduced steroids may or may not impact development.

The effects of 5β-reduced steroids have been studied in several contexts in avian embryos. 5β-reduced steroids, such as 5β-pregnanedione and etiocholanolone, are potent inducers of δ-aminolevulinate synthase (ALAS), the rate limiting enzyme for heme biosynthesis (Anderson et al., 1982; Aragonés et al., 1991). This heme is subsequently used in the developing liver and extraembryonic membranes (Granick and Kappas, 1967; Levere et al., 1967) for the production of hemeproteins such as hemoglobins (Irving et al., 1976) and cytochrome P450s (Rifkind et al., 1973). Thus, 5β-reduced metabolites have been proposed to be endogenous regulators of heme dependent processes like erythropoiesis and steroid/drug metabolism (Aragonés et al., 1991). However, the 5β-reduction of androgens, which is prolific in the brain of avian embryos appears to be an inactivation pathway for testosterone in that 5β-reduced androgens have little to no impact on the differentiation or activation of reproductive behavior (Adkins, 1977; Davies et al., 1980; Steimer and Hutchison, 1981; Delville et al., 1984; Balthazart et al., 1983). Taken together, these data indicate that 5β-reduced steroids may be able influence growth related processes in the embryo while not influencing sexual differentiation of the brain (Paitz et al., 2011; Kumar et al., 2018). This has important implications for understanding how maternal steroids can elicit phenotypic effects while allowing steroid cues from the embryonic gonads to direct sexual differentiation (Carere and Balthazart, 2007).

As with all steroids, the physiological response of the organism is largely dependent on the specificity and distribution of hormone receptors. A major breakthrough in characterizing the mechanisms underlying the potential effects of 5β-reduced steroids came with the discovery of several "orphan nuclear receptors" that were capable of binding 5β-reduced pregnanes and androstanes. Both the pregnane X receptor (PXR) (Kliewer et al., 1998) and constitutive androstane receptor (CAR) (Baes et al., 1994) bind 5β-reduced steroids (Kliewer et al., 1998; Moore et al., 2000). Importantly, both of these receptors also activate an enhancer of *ALAS* (Fraser et al., 2002) which is consistent with earlier findings that 5β-reduced steroids induce δ-aminolevulinic acid and heme production (Granick and Kappas, 1967; Levere et al., 1967). Birds possess a single nuclear receptor related to both PXR and CXR, the chicken xenobiotic receptor (CXR) (Handschin et al., 2000), which is also capable of binding 5β-reduced steroids and inducing ALAS production (Handschin et al., 2001). This creates a situation where maternal steroids, upon 5β-reduction, could bind CXR in embryonic tissues to elicit effects on hemoglobin or cytochrome P450 production without binding androgen or estrogen receptors responsible for coordinating sexual differentiation. In order for this to happen, the metabolites must actually reach the target tissues and recent studies suggest this might not always be the case (Vassallo et al., 2019; Kumar et al., 2019). Only a small fraction (approximately 2%) of yolk corticosterone (Vassallo et al., 2019) reaches developing quail embryos without first being metabolized, while yolk testosterone (Kumar et al., 2019), and androstenedione (Kumar et al., 2019) do not reach pigeon embryos at all. Metabolites of yolk steroids do appear to reach the embryo at higher concentrations (von Engelhardt et al., 2009; Vassallo et al., 2014; Vassallo et al., 2019).

More studies are necessary to characterize the role 5β-pregnanedione might play in regulating development of avian embryos and how variation in maternal progesterone, or even maternal 5β-pregnanedione, could lead to maternal effects. Circumstantial evidence indicates 5β-pregnanedione may have very important effects early developmental processes that require heme production. Both erythropoiesis (Levere et al., 1967) and steroid metabolism (Parsons, 1970, Paitz et al., 2011) occur very in development. Our observed metabolism of progesterone to 5β-pregnanedione provides a mechanism by which the high levels of maternal progesterone in the yolk can affect these early processes. Variation in maternal progesterone could alter the intensity or duration of these processes and ultimately impact offspring fitness. Whether or not this happens still needs to be tested but the results from our study highlight the need to advance our understanding of 5β-pregnanedione and other 5β-reduced steroids with *in vivo* and *in ovo* studies.

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Declaration of Competing Interest

None.

Data Statement

The data supporting this publication are available as a supplemental file.

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