

# Embryonic modulation of maternal steroids in European starlings (*Sturnus vulgaris*)

Ryan T. Paitz\*, Rachel M. Bowden and Joseph M. Casto

*School of Biological Sciences, Illinois State University, Normal, IL 61790, USA*

In birds, maternally derived yolk steroids are a proposed mechanism by which females can adjust individual offspring phenotype to prevailing conditions. However, when interests of mother and offspring differ, parent–offspring conflict will arise and embryonic interests, not those of the mother, should drive offspring response to maternal steroids in eggs. Because of this potential conflict, we investigated the ability of developing bird embryos to process maternally derived yolk steroids. We examined how progesterone, testosterone and oestradiol levels changed in both the yolk/albumen (YA) and the embryo of European starling eggs during the first 10 days of development. Next, we injected tritiated testosterone into eggs at oviposition to characterize potential metabolic pathways during development. Ether extractions separated organic and aqueous metabolites in both the embryo and YA homogenate, after which major steroid metabolites were identified. Results indicate that the concentrations of all three steroids declined during development in the YA homogenate. Exogenous testosterone was primarily metabolized to an aqueous form of etiocholanolone that remained in the YA. These results clearly demonstrate that embryos can modulate their local steroid environment, setting up the potential for parent–offspring conflict. Embryonic regulation must be considered when addressing the evolutionary consequences of maternal steroids in eggs.

**Keywords:** yolk steroids; embryonic development; sexual differentiation; steroid metabolism; etiocholanolone

## 1. INTRODUCTION

Investigations into proximate causes of phenotypic variation have tended to focus on genetic factors, but a larger emphasis is now being placed on how non-genetic factors can shape phenotypic variation. Maternal steroids are one such class of non-genetic factor that influences developing offspring and may allow females to manipulate offspring phenotypes to maximize reproductive success [1,2]. However, when the interest of the mother does not match the interest of the individual offspring, selective pressures on maternal steroid transfer will differ from those on offspring response, resulting in parent–offspring conflict [3]. We must advance our knowledge of whether embryos can regulate such non-genetic factors if we are to understand the potential evolutionary consequences of maternal steroids on offspring phenotype [4].

Most studies investigating the effects of maternal steroids have been conducted in placental mammals, where there is seemingly great potential for direct exposure of the embryo to the maternal environment [5]. The placenta develops from one or more extra-embryonic membranes and forms an interface that facilitates the transfer of substances between mother and offspring [6]. Because of its role in metabolizing steroids [7,8], the placenta is a vital regulator of the endocrinological ‘cross talk’ that occurs between mother and foetus [9]. Disruption of steroid metabolism in the placenta can lead to both foetal [10] and maternal abnormalities [11], supporting the idea that the placenta serves as a buffer between the maternal and foetal endocrine environments. Thus, in

placental mammals, maternal steroid effects are the result of a dynamic interplay between maternal production and embryonic regulation.

In oviparous amniotes, maternal steroid signals are transferred to the yolk during folliculogenesis (reviewed by Paitz & Bowden [12]). At oviposition, the separation of maternal and embryonic endocrine environments produces a discrete initial maternal steroid signal after which there is no further endocrine interaction between mother and embryo. Unfortunately, our understanding of how embryos of oviparous amniotes regulate maternal steroids is minimal, making it difficult to postulate how yolk steroids may affect differentiation of some traits without affecting others (reviewed by Carere & Balthazart [13]).

Our knowledge of how bird embryos may process maternal steroids had been limited to several studies that investigated the ability of early embryonic tissues to metabolize steroids *in vitro*, where it was demonstrated that, by 48 h of incubation, chicken blastoderms are capable of metabolizing several steroids [14–16]. A more recent *in vivo* study in chickens demonstrated that metabolism of exogenous testosterone is initiated during the first 24 h of incubation [17]. In all of these studies, multiple metabolites (both conjugated and unconjugated) were detected, suggesting the presence of numerous metabolic enzymes during this period. The specific tissue of origin for these enzymes remains to be elucidated, but metabolic enzymes are expressed in the chorioallantoic membrane [18] and embryo [19] of the chicken. Additionally, very little is known about the potential biological activity of these metabolites.

Conjugated steroids are typically viewed as metabolites destined for clearance from the body because steroid

\* Author for correspondence (rpaitz@ilstu.edu).

conjugation results in a metabolite that is both biologically inactive and water-soluble [20]. However, during embryonic development in mammals, conjugated steroids are the primary precursors for steroid production by the placenta, where enzymes capable of returning conjugated steroids back to their active form are abundant [21]. The importance of steroid conjugation during embryonic development has recently been addressed in an oviparous amniote, the red-eared slider turtle (*Trachemys scripta*) [22]. This study specifically examined the conjugation of oestradiol by the enzyme oestrogen sulphotransferase and found that the activity of this enzyme significantly increased in the yolk and extra-embryonic membranes during the first 10 days of incubation. During this same period, tritiated oestradiol is converted to a water-soluble metabolite [22] and endogenous oestradiol concentrations in the yolk decline significantly [23]. Together, these studies suggest that the extra-embryonic membranes of oviparous amniotes may serve an important role in metabolizing maternal steroids, just as they do in placental mammals.

Given that embryos of several oviparous amniotes appear to possess the ability to metabolize steroids *in vitro*, we tested the hypothesis that bird embryos are able to modulate maternal steroid signals *in ovo*. Embryonic regulation of the local endocrine milieu may have important implications for the evolutionary consequences of maternally derived yolk steroids. Using the European starling (*Sturnus vulgaris*), we characterized how progesterone, testosterone and oestradiol levels in the egg change during development. We then used injections of tritiated testosterone to examine metabolism of testosterone during development and identify major metabolites, as testosterone is the primary yolk steroid reported to influence offspring development in this species [24,25].

## 2. MATERIAL AND METHODS

### (a) *Egg collection*

Eggs were collected from free-living European starlings breeding in nest boxes at the Illinois State University farm in Normal, IL, USA. Eighteen clutches of eggs, laid between 22 April 2008 and 27 May 2008, were used in the experiments herein. Adults in this newly established colony were unmarked, and individual females may have contributed up to two clutches to these experiments. Based upon the timing of egg-laying and assignment of clutches to each experiment, no female contributed more than one clutch to any experiment.

Laying was monitored and freshly laid eggs were collected daily between 12.00 and 14.00 h. Eggs were replaced with painted wooden decoys that approximated the size, shape, mass and colour of an average starling egg. Decoy eggs were left in first nests until laying ceased and were removed 5–11 days thereafter. Collected eggs were marked with permanent ink on the shell to uniquely identify the clutch and egg number within a clutch. All eggs were weighed to the nearest 0.01 g using an electronic balance and were assigned to study and incubation treatment within approximately 1 h of collection.

### (b) *Experiment 1: steroid levels during development*

In the first experiment, eggs from five clutches were used to characterize steroid concentrations throughout development.

Eggs were either frozen or placed into an incubator (Turn-X Model TX7, Lyon Technologies Inc., Chula Vista, CA, USA) at 37.5°C with 60 per cent humidity. Eggs from each clutch were randomly assigned to sampling groups to minimize any potential laying order effects. One egg from each clutch was then frozen on incubation day 0, 2, 4, 7 and 10. Once all eggs had been frozen ( $n = 25$ ), they were dissected to separate the embryo from the yolk/albumen (YA) fraction. Eggs that did not possess a visible embryo resulted in a homogenate that included the yolk, albumen and embryo. Embryos and the YA fraction were then homogenized independently such that each egg which possessed an embryo resulted in an embryo homogenate and a YA homogenate. The YA homogenate ultimately included yolk, albumen and all extra-embryonic membranes. These components were combined into a single homogenate because it was not feasible to separate them at all stages of development [26]. Egg components were homogenized using a Tissue Tearor homogenizer (BioSpec Products Inc., Bartlesville, OK, USA). A 50 mg sample of homogenate was then collected to quantify progesterone (PROG), testosterone (T) and oestradiol (E<sub>2</sub>) concentrations.

### (c) *Experiment 1: steroid quantification via radioimmunoassay*

Steroid concentrations were quantified via radioimmunoassay (RIA) [23,27,28]. Briefly, samples were diluted in 1 ml of water and 2000 counts per minute (cpm) of steroid tracers were added to each sample. Unconjugated steroids were extracted twice with 3 ml of an ether extraction solvent (a mixture of 30% petroleum ether and 70% diethyl ether). This organic fraction was dried under nitrogen gas, reconstituted in 90 per cent ethanol and stored at -20°C overnight to precipitate neutral lipids. Samples were fractionated via column chromatography; samples were applied directly to the column and eluted using hormone-specific ethyl acetate:isooctane ratios (PROG = 2%, T = 20% and E<sub>2</sub> = 40%). Fractions were then dried and steroid concentrations were measured in competitive binding RIAs with tritiated steroid. Steroid concentrations were calculated based on a standard curve that ranged from 3.91 to 1000 pg for PROG and 1.95 to 500 pg for T and E<sub>2</sub>. Average recovery was 60 per cent for PROG, 56 per cent for T and 58 per cent for E<sub>2</sub>. The intra-assay coefficients of variation were 4 per cent for PROG, 11 per cent for T and 8 per cent for E<sub>2</sub>. Antibodies used were P 1604 (Wien Laboratories, Flanders, NJ, USA) for PROG, T 3003 (Wien Laboratories) for T and 7010 (Biogenesis, Kingston, NH, USA) for E<sub>2</sub>. The tritiated steroids NET 381 (PROG), NET 553 (T) and NET 517 (E<sub>2</sub>) were purchased from Perkin-Elmer (Boston, MA, USA).

### (d) *Experiment 2: radioactive testosterone distribution*

In the second experiment, eggs from 13 clutches were used to characterize the distribution of tritiated testosterone ([<sup>3</sup>H]T) in the egg after 6 days of incubation. Eggs were randomly divided among treatment groups such that each clutch had at least one egg in each of the treatments. Each egg received an injection of 50 000 cpm ([<sup>3</sup>H]T) into the albumen and were then placed into the incubator for 6 days at 37.5°C with 60 per cent humidity; all eggs were frozen at the completion of incubation until analysis. This technique results in incorporation of radioactivity into the yolk, as both the oil bolus and the yolk float to the top of the egg and come in close contact [17]. For the first treatment, eggs were

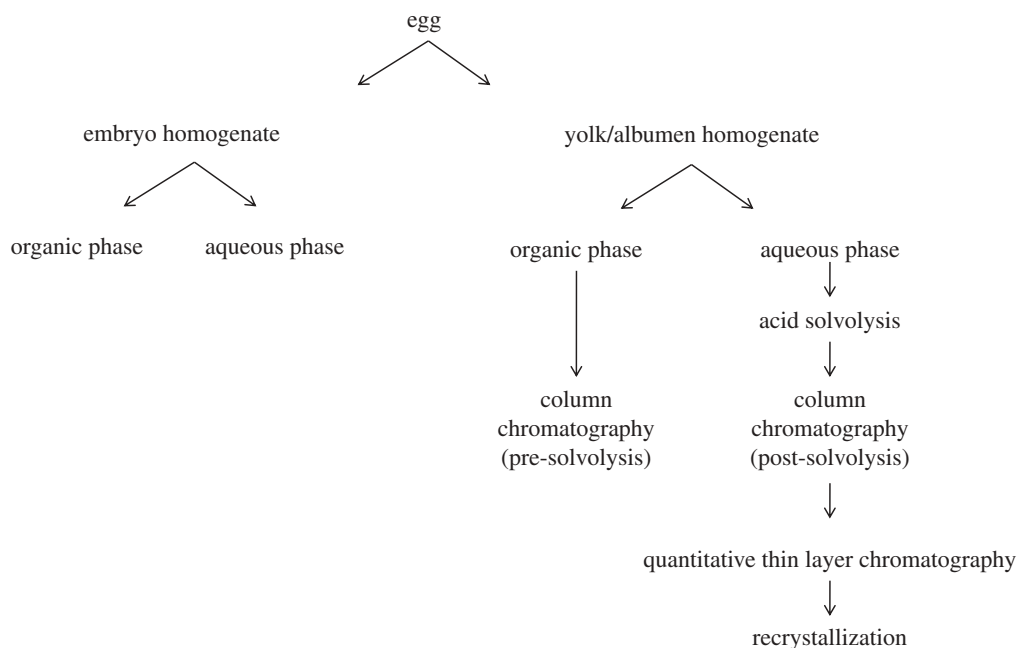


Figure 1. Schematic depicting the experimental techniques used to characterize radioactivity in experiment 2.

immediately injected with 50 000 cpm ( $[^3\text{H}]\text{T}$ ). In the second treatment, eggs were placed at  $4^\circ\text{C}$  for 4 days before injection with ( $[^3\text{H}]\text{T}$ ). This treatment was intended to prevent embryonic development [26] in order to detect if there was any maternal contribution to steroid metabolism. The third treatment was identical to the second treatment except that eggs were placed at  $13^\circ\text{C}$ , the approximate ambient temperature that unincubated eggs would have experienced in the nest, before injection. This treatment was designed to test for any effect of holding eggs prior to injection with ( $[^3\text{H}]\text{T}$ ).

#### (e) *Experiment 2: quantification and identification of radioactive T and metabolites*

Frozen eggs were dissected into embryo and YA fractions and homogenized in the same manner as described above. For this study, only eggs containing embryos were used to determine the distribution of radioactivity within eggs ( $n = 37$ ). Radioactivity levels in 100 mg samples of homogenate dissolved in 1 ml of water were quantified by extracting steroids twice using 3 ml of ether extraction solvent (figure 1). Following a snap-freeze of the aqueous fraction, the organic fraction was decanted, dried and suspended in 1 ml of 90 per cent ethanol while the aqueous fraction thawed. Two 200  $\mu\text{l}$  aliquots of both the organic and aqueous fractions were counted on a Beckman LS 6500 scintillation counter. Total radioactivity of each homogenate was then calculated by multiplying the mean cpm detected in the 100 mg sample by the total component mass.

Fractions containing substantial amounts of radioactivity were subjected to column chromatography to further characterize the radioactivity. To accomplish this, 10 of the original 37 eggs were randomly chosen for chromatography. Two 500 mg aliquots of the YA homogenate from each egg were put through the extraction process as described above and organic phases were combined and dried under nitrogen gas. Aqueous phases were dried separately under nitrogen gas and subjected to acid solvolysis to convert the aqueous conjugated steroids back to their free forms by adding 200  $\mu\text{l}$  of 2.5 M  $\text{H}_2\text{SO}_4$  and 400  $\mu\text{l}$  of saturated NaCl

solution and incubating at  $37^\circ\text{C}$  overnight [29]. These newly freed steroids were then extracted twice in 3 ml ether extraction solvent. The post-solvolysis organic fractions from each of the two aliquots were then combined and dried under nitrogen gas. This process resulted in one pre-solvolysis fraction (containing steroids initially in a free form) and one post-solvolysis fraction (containing steroids initially in a conjugated form) that were each resuspended in 10 per cent ethyl acetate in isooctane, and fractionated via celite chromatography (figure 1). Four ethyl acetate: isooctane ratios (2, 10, 20 and 40%) were passed through the column and collected. These ratios permit the separation of several androgens and  $\text{E}_2$  (androstenedione = 2%, dihydrotestosterone = 10%, T = 20% and  $\text{E}_2$  = 40%) [30]. Each eluate was dried under nitrogen gas, resuspended in 1 ml of ethanol and radioactivity was characterized in each fraction by counting 100  $\mu\text{l}$  of this suspension. Eluates containing large proportions of radioactivity (the 10% fraction: see §3 for more information) were subjected to quantitative thin layer chromatography (TLC) to further characterize the metabolites.

Samples containing radioactivity were spotted onto aluminium-backed silica TLC plates containing a fluorescent indicator (Sigma, St Louis, MO, USA). Unlabelled standards that included androstenedione (Sigma), etiocholanedione, etiocholanol and etiocholanolone (Steraloids, Newport, RI, USA) were over-spotted onto the samples to characterize the co-migration of radioactive metabolites with each standard. Testosterone was not included as a standard because it is not present in the 10 per cent ethyl acetate: isooctane fraction with the column chromatography procedure that we used [30]. Plates were developed in cyclohexane: ethyl acetate (1:1) [17]. Standards were visualized by fluorescence or iodine vapour and were used to identify and sample (excise) 5 mm square inclusive regions of interest from the TLC plate. Initially, excised samples from the plate that included each lane's origin as well as any portions of the lane that did not include a steroid standard were also analysed in order to confirm that radioactivity was not present within these areas of the plates. The excised samples were each added to

3.5 ml of scintillation fluid and counted. Metabolites were verified by repeated recrystallization with known standard to a specific activity [31].

#### (f) Statistical analyses

Mixed model ANOVAs were used to compare steroid levels in the YA and embryo homogenate for each sampling period. For each steroid-homogenate combination, a separate ANOVA was used to compare steroid concentrations and content across development. Day of development was included as a fixed factor and clutch identity and its interaction with day of development were random factors. Total radioactivity levels (cpm) within the YA and embryo homogenate of each egg were compared by a repeated measures-multivariate analysis of variance (RM-MANOVA) with radioactivity levels YA and embryo as response variables, treatment as a fixed factor and clutch of origin included as a random factor. Radioactivity in aqueous versus organic fractions within each tissue was compared by a similar RM-MANOVA with organic radioactivity levels and aqueous radioactivity levels as response variables and clutch of origin included as a random factor. RM-MANOVA was also used to compare radioactivity levels in the different eluates from the column chromatography as well as the radioactivity levels present with each steroid standard on the TLC plates. All statistical tests were performed with SAS software (v. 9.1; SAS Institute, Cary, NC, USA).

### 3. RESULTS

#### (a) Experiment 1: steroid levels during development

Initial concentrations of steroids within the YA homogenate of unincubated eggs (day 0) were (means  $\pm$  s.e.)  $208.9 \pm 25.3$  for PROG,  $0.22 \pm 0.08$  for T, and  $0.1 \pm 0.06$  ng g<sup>-1</sup> for E<sub>2</sub>. PROG ( $F_{4,16} = 9.45$ ,  $p = 0.0004$ ), T ( $F_{4,16} = 7.92$ ,  $p = 0.0012$ ) and E<sub>2</sub> ( $F_{4,16} = 3.52$ ,  $p = 0.031$ ) concentrations in the YA homogenate declined across development. It was only possible to collect embryos starting on day 4 of incubation. All three steroids were detectable in the embryo homogenates, but these concentrations did not change across development (PROG:  $F_{2,5} = 5.21$ ,  $p = 0.059$ ; T:  $F_{2,5} = 0.77$ ,  $p = 0.513$ ; E<sub>2</sub>:  $F_{2,5} = 1.87$ ,  $p = 0.247$ ). Additionally, the absolute amount of all three steroids declined in the YA homogenate (PROG:  $F_{4,16} = 9.77$ ,  $p = 0.0003$ ; T:  $F_{4,15} = 8.34$ ,  $p = 0.0009$ ; E<sub>2</sub>:  $F_{4,16} = 3.68$ ,  $p = 0.0261$ ) (figure 2). The patterns of decline in steroid concentrations and total content were similar. In embryo homogenates, only the amount of PROG changed during development ( $F_{2,5} = 5.96$ ,  $p = 0.048$ ; figure 2). When considering content of the entire eggs (YA content + embryo content), the amount of PROG ( $F_{4,16} = 9.67$ ,  $p = 0.0004$ ) and T ( $F_{4,15} = 3.19$ ,  $p = 0.044$ ), but not E<sub>2</sub> ( $F_{4,16} = 2.31$ ,  $p = 0.102$ ) declined during development. Clutch identity was not a significant source of variation in any analyses.

#### (b) Experiment 2: distribution of radioactivity

Our treatments were designed with the prediction that holding eggs for 4 days at 4°C would prevent embryonic development as was previously reported in zebra finches [26]. However, over 50 per cent of the eggs in each treatment had an embryo present after 6 days of incubation.

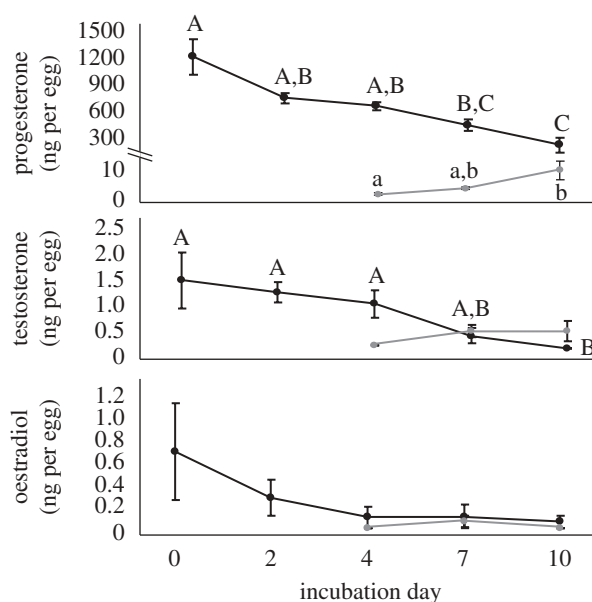


Figure 2. Total steroid content (mean  $\pm$  s.e.) of the yolk/albumen (YA, black) and embryo (grey) fractions over the first 10 days of incubation. Significant differences between groups are indicated for the YA homogenate (capital letters) and the embryo homogenate (lower-case letters). Days not sharing a similar letter are significantly different ( $p < 0.05$ ). Letters were omitted for lines that were statistically similar throughout development.

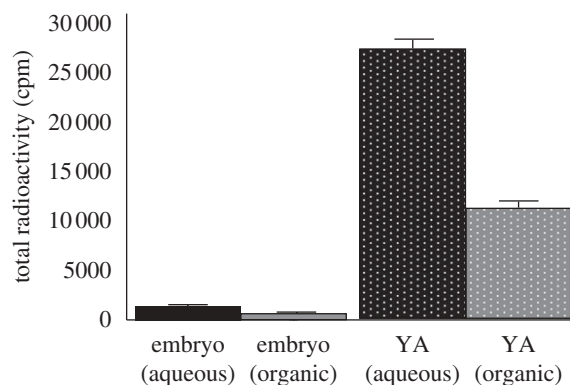


Figure 3. Radioactivity levels in both extraction phases of the embryo and YA homogenate after 6 days of incubation. All groups were significantly different from each other ( $p < 0.001$ ).

Since the three treatments did not differ in survival rates or radioactivity distribution ( $p > 0.1$ ) as predicted, all eggs that contained an embryo were combined into a single analysis of testosterone metabolism. Total radioactivity was higher in the YA homogenate (over 75% of initial application) compared with the embryo homogenate ( $F_{1,22} = 1193$ ,  $p < 0.001$ ). Within the YA homogenate, there was significantly more radioactivity in the organic fraction than in the aqueous fraction ( $F_{1,22} = 182$ ,  $p < 0.001$ ; figure 3). Clutch identity was not a significant source of variation in any analyses. The total radioactivity detected within the embryo was only 0.5 per cent of the initial 50 000 cpm. The remaining 24 per cent of the radioactivity was unaccounted for and was presumably lost during the extraction process.

Because most of the radioactivity was present in the organic and aqueous phase of the YA homogenate

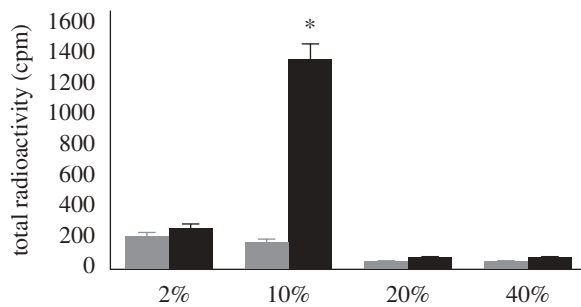


Figure 4. Radioactivity levels in the eluates from column chromatography. Pre-solvolysis levels are in grey and post-solvolysis levels are in black. Within the post-solvolysis fraction, the 10% eluate contain significantly more radioactivity than the other eluates (asterisk corresponds to  $p < 0.05$ ).

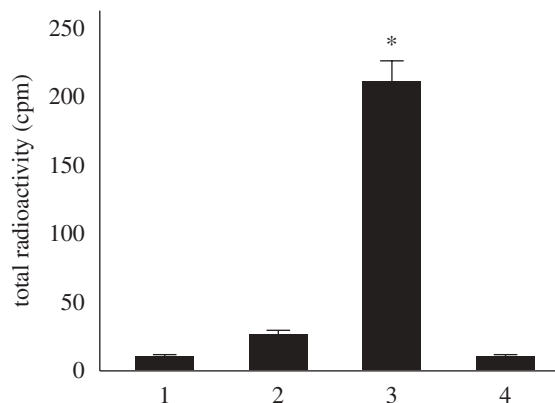


Figure 5. Radioactivity levels detected with each steroid standard used in TLC. (1) Etiocholanedione, (2) androstenedione, (3) etiocholanolone and (4) etiocholanediol were used as standards. More radioactivity co-migrated with etiocholanolone than any other standard (asterisk corresponds to all  $p < 0.001$ ).

(figure 3), these two fractions were subjected to column chromatography for further characterization. Significantly more radioactivity was present in the post-solvolysis fraction than the pre-solvolysis fraction ( $F_{1,2} = 3970$ ,  $p = 0.003$ ), and within the post-solvolysis fraction most radioactivity was present within the 10 per cent eluate (figure 4). The unknown tritiated metabolite(s) from the 10 per cent eluate of the post-solvolysis fraction was subjected to quantitative TLC. More radioactivity co-migrated with etiocholanolone than any other standard (all  $p < 0.001$ ; figure 5). The identity of this tritiated metabolite was confirmed as etiocholanolone by consistent incorporation of radioactivity into crystals formed during sequential recrystallizations with our etiocholanolone standard [31].

#### 4. DISCUSSION

The ability of yolk steroids to influence offspring development has been well documented in avian systems [1], while the lack of a mechanistic understanding of how these effects are mediated has limited the ability of researchers to explain functional relationships [4]. Several studies have shown that yolk steroid levels decrease early in embryonic development [26,32] but the reasons for such a decline remain unidentified. Proposed explanations

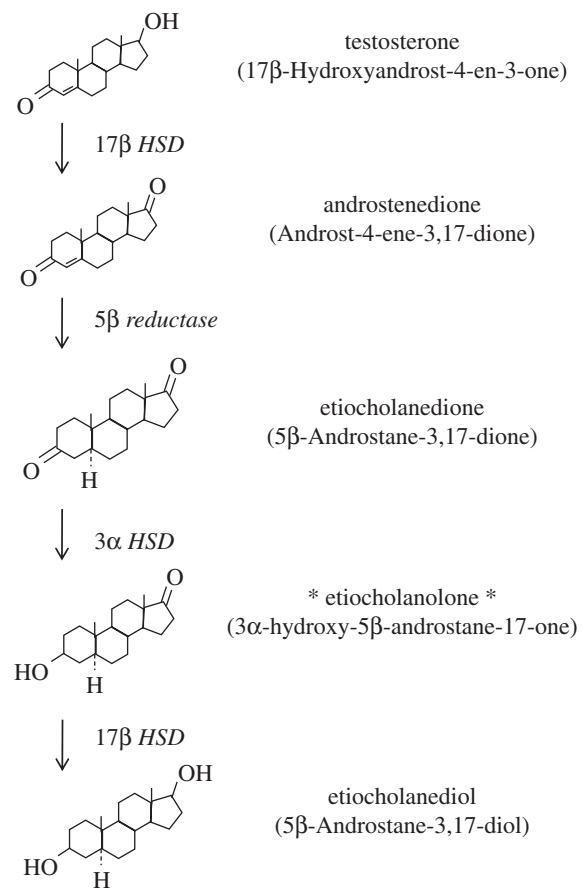


Figure 6. Phase I metabolism of testosterone to etiocholanediol. Asterisks denote major metabolite identified in the present study.

include: dilution of yolk by albumen, leaching of steroids from yolk to albumen and steroid metabolism [4,26]. Our results support the idea that the decline in yolk steroid concentrations is the result of metabolism and not dilution or leaching, since whole egg quantities decline during development. Additional support comes from our finding that exogenous testosterone is metabolized to a conjugated form of etiocholanolone during this period. Together, these data suggest that starling embryos are capable of metabolizing maternal steroids.

*In vitro* studies have demonstrated that early chicken embryos metabolize numerous steroids [14–16], with 5β reduction as the major metabolic pathway. Parsons [14] and Antila *et al.* [16] demonstrated that chicken blastoderms that were less than 48 h old could metabolize testosterone, and that the primary metabolite was etiocholanediol. Importantly, it was demonstrated that unincubated fertile eggs were not capable of metabolizing testosterone [14], indicating that there is probably no maternal contribution of enzymes capable of metabolizing testosterone in the egg. Results from our study are consistent with these studies in that testosterone is reduced to a metabolite in the 5β configuration, but in our *in vivo* study etiocholanolone is conjugated rather than converted to etiocholanediol (figure 6). Our results are also consistent with a recent *in vivo* study demonstrating metabolism of exogenous testosterone over the first 6 days of incubation [17]. While their study did not identify specific metabolites, their TLC results suggest that testosterone is converted to androstenedione and other

metabolites. Unfortunately, the methodology of that study could not differentiate testosterone from etiocholanolone, so it was not possible to determine precisely an end product [33]. The use of column chromatography in the present study allows us to separate these two steroids prior to TLC, as etiocholanolone elutes in the 10 per cent ethyl acetate : isooctane fraction, whereas testosterone does not elute until the 20 per cent ethyl acetate : isooctane fraction. The small amounts of radioactivity present in this 20 per cent eluate in both the pre-solvolysis and post-solvolysis fraction (figure 4) highlights just how little of the radioactivity from the initial testosterone injection is still present in the form of testosterone after 6 days of incubation.

Identification of etiocholanolone as the primary metabolite of testosterone has important implications for understanding the potential embryonic effects of yolk testosterone. Etiocholanolone has been shown to be a potent stimulator of erythropoiesis in mammals [34] and birds [35,36]. The potency of etiocholanolone is closely related to its  $5\beta$  configuration, as several  $5\beta$  androgens and  $5\beta$  pregnanes stimulate erythropoiesis, while their respective  $5\alpha$  epimers are much less potent [35,37]. The conversion of yolk testosterone, and presumably androstenedione, to etiocholanolone may function to stimulate the production of erythrocytes early in development. Chick blastoderms increase erythropoiesis in response to exogenous etiocholanolone [36], and data from the present study suggest that yolk testosterone may serve as an endogenous precursor for etiocholanolone production. The idea that erythropoiesis may be increased in response to increased yolk androgens is consistent with existing data, which suggest that higher yolk androgen levels are associated with increased growth [38–41] and decreased immune function (reviewed by Navara & Mendonca [42]). Because all blood cells originate from haematopoietic stem cells [43], an increase in erythrocyte production to facilitate the demands of increased growth may come at a cost of leucocyte production that might be manifested as decreased immune function [44,45]. In fact, the stimulation of erythropoiesis by etiocholanolone is attributed to effects on early blood cell progenitors and is independent of erythropoietin [34]. Additionally, the effects of etiocholanolone are independent of the androgen receptor and are thought to be modulated through ‘xenobiotic-sensing’ nuclear receptors, such as the constitutive androstane receptor and pregnane X receptor that can bind  $5\beta$  steroids [46] and induce a suite of enzymes related to erythropoiesis [47] and steroid metabolism [12]. Future studies are required to decipher how the effects of etiocholanolone may be elicited, but several lines of research now suggest that the effects attributed to yolk androgens may involve a suite of enzymes, steroids and nuclear receptors.

In addition to characterizing the phase I metabolism of testosterone to etiocholanolone, this study demonstrates the prevalence of phase II conjugating enzymes during embryonic development. The conjugation of yolk steroids by the embryo is intriguing because it potentially addresses several long-standing issues associated with the actions of yolk steroids. By conjugating yolk steroids, embryos create a metabolite that is both water-soluble and inactive. The increased water solubility may facilitate movement from the lipid-rich yolk to the aqueous embryonic environment and address the issue of how an

initially highly lipophilic steroid could move from yolk into the embryo [48]. Previous *in vivo* [17] and *in vitro* [14,16] studies have also demonstrated that embryos are capable of conjugating steroids. The specific mode of conjugation remains to be determined, but the two primary pathways for conjugation in vertebrates, glucuronidation or sulphonation, create metabolites that could be reactivated to the original unconjugated steroid. In mammals, sulphonation is the primary means of conjugation during embryonic development [7]. Steroid sulphatase, the enzyme responsible for deconjugating sulphonated steroids, has been characterized in several vertebrate groups [21] and functions by removing a sulphonyl group, thereby returning the steroid to an active form. The importance of reactivation of sulphonated steroids during embryonic development is highlighted by the role of dehydroepiandrosterone sulphate (DHEA-S) in placental steroid production. In humans, DHEA-S is the chief product of the foetal adrenal gland and serves as the primary precursor for oestrogen production in the placenta [49]. Steroid sulphonation has also been demonstrated in turtles, where  $E_2$  can be converted to oestradiol sulphate by enzymes present in the yolk, extra-embryonic membranes and embryo [22]. Since yolk steroids are conjugated by the developing embryo in birds, it suggests that at least some of the yolk steroid effects reported for this group could ultimately be the result of steroid reactivation via enzymatic conversion.

Despite providing evidence for the embryonic regulation of yolk steroids we cannot, at present, postulate when yolk steroids are influencing development. After 6 days of incubation, the majority of the radioactivity remained in the YA homogenate of starling eggs suggesting that it had yet to reach the embryo, but we could not identify the specific pattern of distribution of the radioactivity because extra-embryonic membranes were homogenized with the yolk and albumen. It is possible that some of the radioactivity was contained within the allantois of the egg as steroid metabolites can be detected in the allantoic waste of birds after hatching [29]. However, we feel it is unlikely that the allantoic waste contained much radioactivity in our eggs because at incubation day 6 when we sampled our embryos, the allantois was extremely small, if visible at all. Further, any radioactivity contained within the allantois would presumably be the result of embryonic clearance, still providing support for the idea that embryos modulate maternal steroid signals early in development. Interestingly, the initial sites of erythropoiesis within the egg are the blood islands within the yolk sac membrane [43]. Thus, it may be possible for yolk androgens to elicit their effects without ever reaching the embryo proper. At this point more study is needed to identify the timing and location of yolk steroid effects, as well as steroid/metabolite(s) actually responsible for producing the effects.

Results from the present study illustrate just how complex the embryonic modulation of maternal steroids can be. Most explanations of the potential adaptive significance of maternal steroids are based on the idea that females can use steroids to confer environmentally relevant information to developing offspring to increase her reproductive success. Embryonic modulation of maternal steroids creates a situation where any effects of

maternal steroids on offspring phenotype result from an interaction between maternal and offspring physiology. The evolution of these maternal effects would then proceed under selective pressures on both the mother and her offspring, and any differences in selective pressures between the two may result in parent–offspring conflict [50]. Our results demonstrate that developing embryos are capable of modulating maternal steroid signals and highlight the importance of understanding embryonic responses to maternal steroids when investigating the evolutionary consequences of such a maternal effect.

This research was conducted according to all applicable federal, state and university regulations. Egg collection procedures were approved by the Illinois State University Institutional Animal Care and Use Committee (protocol no. 08-2008).

We are grateful to Steve Juliano, Sandrine Clairardin and Amy Flowers for their assistance with the project. We would also like to thank Illinois State University for granting permission to place nest boxes on the university farm, Ellen Ketterson for the use of the bird egg incubator and Craig Gatto for assistance with metabolite identification. Financial support for this research was provided by the Beta Lambda Chapter of Phi Sigma to R.T.P. and from the Illinois State University School of Biological Sciences to R.M.B. and J.M.C.

## REFERENCES

- Groothuis, T. G. G., Müller, W., von Engelhardt, N., Carere, C. & Eising, C. 2005 Maternal hormones as a tool to adjust offspring phenotype in avian species. *Neurosci. Biobehav. Rev.* **29**, 329–352. (doi:10.1016/j.neubiorev.2004.12.002)
- Gil, D. 2008 Hormones in avian eggs: physiology, ecology, and behavior. In *Advances in the study of behavior* (eds H. J. Brockmann, T. J. Roper & M. Naguib), pp. 337–395. San Diego, CA: Academic Press.
- Müller, W., Lessells, C. M., Korsten, P. & von Engelhardt, N. 2007 Manipulative signals in family conflict? On the function of maternal yolk hormones in birds. *Am. Nat.* **169**, E84–E96. (doi:10.1086/511962)
- Groothuis, T. G. G. & Schwabl, H. 2008 Hormone-mediated maternal effects in birds: mechanisms matter but what do we know of them? *Phil. Trans. R. Soc. B* **363**, 1647–1661. (doi:10.1098/rstb.2007.0007)
- Glickman, S. E., Cunha, G. R., Drea, C. M., Conley, A. J. & Place, N. J. 2006 Mammalian sexual differentiation: lessons from the spotted hyena. *Trends Endocrinol. Metab.* **17**, 349–356. (doi:10.1016/j.tem.2006.09.005)
- Rothchild, I. 2003 The yolkless egg and the evolution of eutherian viviparity. *Biol. Reprod.* **68**, 337–357. (doi:10.1095/biolreprod.102.004531)
- Levitz, M. 1966 Conjugation and transfer of fetal-placental steroid hormones. *J. Clin. Endocrinol. Metab.* **26**, 773–777. (doi:10.1210/jcem-26-7-773)
- Painter, D. L. & Moore, M. C. 2005 Steroid hormone metabolism by the chorioallantoic placenta of the mountain spiny lizard *Sceloporus jarrovi* as a possible mechanism for buffering maternal-fetal hormone exchange. *Physiol. Biochem. Zool.* **78**, 364–372. (doi:10.1086/430222)
- Pepe, G. J. & Albrecht, E. D. 1995 Actions of placental and fetal adrenal steroid hormones in primate pregnancy. *Endocr. Rev.* **16**, 608–648.
- Shozu, M., Akasofu, K., Harada, T. & Kubota, Y. 1991 A new cause of female pseudohermaphroditism: placental aromatase deficiency. *J. Clin. Endocrinol. Metab.* **72**, 560–566. (doi:10.1210/jcem-72-3-560)
- Harada, N., Ogawa, H., Shozu, M., Yamada, K., Suhara, K., Nishida, E. & Takagi, Y. 1992 Biochemical and molecular genetic analyses on placental aromatase (P-450arom) deficiency. *J. Biol. Chem.* **267**, 4781–4785.
- Paitz, R. T. & Bowden, R. M. 2010 Progesterone metabolites, ‘xenobiotic-sensing’ nuclear receptors, and the metabolism of maternal steroids. *Gen. Comp. Endocrinol.* **166**, 217–221. (doi:10.1016/j.ygcen.2009.11.011)
- Carere, C. & Balthazart, J. 2007 Sexual versus individual differentiation: the controversial role of avian maternal hormones. *Trends Endocrinol. Metab.* **18**, 73–80.
- Parsons, I. C. 1970 Metabolism of testosterone by early chick embryonic blastoderm. *Steroids* **16**, 59–65. (doi:10.1016/S0039-128X(70)80095-2)
- Gonzalez, C. B., Cozza, E. N., Debedners, M. E. O., Lantos, C. P. & Aragonés, A. 1983 Progesterone and its reductive metabolism in steroidogenic tissues of the developing hen embryo. *Gen. Comp. Endocrinol.* **51**, 384–393. (doi:10.1016/0016-6480(83)90054-0)
- Antila, E., Leikola, A. & Tahka, S. 1984 Early steroid metabolism by chick blastoderm *in vitro*. *Steroids* **43**, 315–323. (doi:10.1016/0039-128X(84)90049-7)
- von Engelhardt, N., Henriksen, R. & Groothuis, T. G. G. 2009 Steroids in chicken egg yolk: metabolism and uptake during early embryonic development. *Gen. Comp. Endocrinol.* **163**, 175–183. (doi:10.1016/j.ygcen.2009.04.004)
- Albergotti, L. C., Hamlin, H. J., McCoy, M. W. & Guillette, L. J. 2009 Endocrine activity of extraembryonic membranes extends beyond placental amniotes. *PLoS ONE* **4**, e5452. (doi:10.1371/journal.pone.0005452)
- Bruggeman, V., Van As, P. & Decuypere, E. 2002 Developmental endocrinology of the reproductive axis in the chicken embryo. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **131**, 839–846. (doi:10.1016/S1095-6433(02)00022-3)
- Bernstein, S. & Solomon, S. 1970 *Chemical and biological aspects of steroid conjugation*. New York, NY: Springer.
- Reed, M. J., Purohit, A., Woo, L. W. L., Newman, S. P. & Potter, B. V. L. 2005 Steroid sulfatase: molecular biology, regulation, and inhibition. *Endocr. Rev.* **26**, 171–202. (doi:10.1210/er.2004-0003)
- Paitz, R. T. & Bowden, R. M. 2008 A proposed role of the sulfotransferase/sulfatase pathway in modulating yolk steroid effects. *Integr. Comp. Biol.* **48**, 419–427. (doi:10.1093/icb/icn034)
- Paitz, R. T. & Bowden, R. M. 2009 Rapid decline in the concentrations of three yolk steroids during development: is it embryonic regulation? *Gen. Comp. Endocrinol.* **161**, 246–251. (doi:10.1016/j.ygcen.2009.01.018)
- Pilz, K. M., Quiroga, M., Schwabl, H. & Adkins-Regan, E. 2004 European starling chicks benefit from high yolk testosterone levels during a drought year. *Horm. Behav.* **46**, 179–192. (doi:10.1016/j.yhbeh.2004.03.004)
- Müller, W. & Eens, M. 2009 Elevated yolk androgen levels and the expression of multiple sexually selected male characters. *Horm. Behav.* **55**, 175–181. (doi:10.1016/j.yhbeh.2008.09.012)
- Gilbert, L., Bulmer, E., Arnold, K. E. & Graves, J. A. 2007 Yolk androgens and embryo sex: maternal effects or confounding factors? *Horm. Behav.* **51**, 231–238. (doi:10.1016/j.yhbeh.2006.10.005)
- Wingfield, J. C. & Farner, D. S. 1975 Determination of 5 steroids in avian plasma by radioimmunoassay and competitive-protein-binding. *Steroids* **26**, 311–327. (doi:10.1016/0039-128X(75)90077-X)
- Schwabl, H. 1993 Yolk is a source of maternal testosterone for developing birds. *Proc. Natl Acad. Sci. USA* **90**, 11 446–11 450. (doi:10.1073/pnas.90.24.11446)

- 29 Benowitz-Fredericks, Z. M., Kitaysky, A. & Wingfield, J. 2005 Steroids in allantoic waste: an integrated measure of steroid exposure *in ovo*. *Ann. NY Acad. Sci.* **1046**, 204–213. (doi:10.1196/annals.1343.017)
- 30 Abraham, G. E. 1975 The use of diatomite microcolumns for chromatographic separation of steroids prior to radioimmunoassay. *Path. Biol.* **23**, 889–893.
- 31 Axelrod, L. R., Matthijs, C., Goldzieh, J. W. & Pulliam, J. E. 1965 Definitive identification of microquantities of radioactive steroids by recrystallization to constant specific activity. *Acta Endocrinol.* **49**, 7–77.
- 32 Elf, P. K. & Fivizzani, A. J. 2002 Changes in sex steroid levels in yolks of the leghorn chicken, *Gallus domesticus*, during embryonic development. *J. Exp. Zool.* **293**, 594–600. (doi:10.1002/jez.10169)
- 33 Mattox, V. R., Litwiller, R. D. & Carpenter, P. C. 1979 Comparison of the resolving properties of a group of chromatography systems for a collection of compounds. *J. Chromatogr.* **175**, 243–260.
- 34 Gordon, A. S., Zanjani, E. D., Levere, R. D. & Kappas, A. 1970 Stimulation of mammalian erythropoiesis by 5 beta-H steroid metabolites. *Proc. Natl Acad. Sci. USA* **65**, 919–924. (doi:10.1073/pnas.65.4.919)
- 35 Levere, R. D., Kappas, A. & Granick, S. 1967 Stimulation of hemoglobin synthesis in chick blastoderms by certain 5 beta androstane and 5 beta pregnane steroids. *Proc. Natl Acad. Sci. USA* **58**, 985–990. (doi:10.1073/pnas.58.3.985)
- 36 Irving, R. A., Mainwaring, W. I. P. & Spooner, P. M. 1976 Regulation of hemoglobin synthesis in cultured chick blastoderms by steroids related to 5-beta-androstane. *Biochem. J.* **154**, 81–93.
- 37 Urabe, A., Sassa, S. & Kappas, A. 1979 Influence of steroid hormone metabolites on the *in vitro* development of erythroid colonies derived from human bone marrow. *J. Exp. Med.* **149**, 1314–1325. (doi:10.1084/jem.149.6.1314)
- 38 Schwabl, H. 1996 Maternal testosterone in the avian egg enhances postnatal growth. *Comp. Biochem. Physiol. A* **114**, 271–276. (doi:10.1016/0300-9629(96)00009-6)
- 39 Eising, C. M., Eikenaar, C., Schwabl, H. & Groothuis, T. G. G. 2001 Maternal androgens in black-headed gull (*Larus ridibundus*) eggs: consequences for chick development. *Proc. R. Soc. Lond. B* **268**, 839–846. (doi:10.1098/rspb.2001.1594)
- 40 Gorman, K. B. & Williams, T. D. 2005 Correlated evolution of maternally derived yolk testosterone and early developmental traits in passerine birds. *Biol. Lett.* **1**, 461–464. (doi:10.1098/rsbl.2005.0346)
- 41 Schwabl, H., Palacios, M. G. & Martin, T. E. 2007 Selection for rapid embryo development correlates with embryo exposure to maternal androgens among passerine birds. *Am. Nat.* **170**, 196–206. (doi:10.1086/519397)
- 42 Navara, K. J. & Mendonca, M. T. 2008 Yolk androgens as pleiotropic mediators of physiological processes: a mechanistic review. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **150**, 378–386. (doi:10.1016/j.cbpa.2008.05.002)
- 43 Cumano, A. & Godin, I. 2007 Ontogeny of the hematopoietic system. *Annu. Rev. Immunol.* **25**, 745–785. (doi:10.1146/annurev.immunol.25.022106.141538)
- 44 Kincade, P. W., Medina, K. L. & Smithson, G. 1994 Sex hormones as negative regulators of lymphopoiesis. *Immunol. Rev.* **137**, 119–134. (doi:10.1111/j.1600-065X.1994.tb00661.x)
- 45 Olsen, N. J. & Kovacs, W. J. 2001 Effects of androgens on T and B lymphocyte development. *Immunol. Res.* **23**, 281–288. (doi:10.1385/IR:23:2-3:281)
- 46 Moore, L. B. *et al.* 2000 Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J. Biol. Chem.* **275**, 15 122–15 127. (doi:10.1074/jbc.M001215200)
- 47 Meyer, U. A. 2007 Endo-xenobiotic crosstalk and the regulation of cytochromes P450. *Drug Metab. Rev.* **39**, 639–646. (doi:10.1080/03602530701498737)
- 48 Moore, M. C. & Johnston, G. I. H. 2008 Toward a dynamic model of deposition and utilization of yolk steroids. *Integr. Comp. Biol.* **48**, 411–418. (doi:10.1093/icb/icn079)
- 49 Goodman, H. M. 2003 *Basic medical endocrinology*. Boston, MA: Academic Press.
- 50 Uller, T. 2008 Developmental plasticity and the evolution of parental effects. *Trends Ecol. Evol.* **23**, 432–438. (doi:10.1016/j.tree.2008.04.005)