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# Biological activity of oestradiol sulphate in an oviparous amniote: implications for maternal steroid effects

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Understanding the many factors that underlie phenotypic variation is of profound importance to evolutionary biologists. The embryonic endocrine environment is one such factor that has received much attention. In placental amniotes, the dynamic interaction of maternal and embryonic steroid production and metabolism is critical to regulating the endocrine environment. Less is known about how embryos of oviparous amniotes regulate their endocrine environment because most studies have focused on relating initial steroid levels in the yolk at oviposition to offspring phenotype. We tested the hypothesis that embryos of oviparous amniotes regulate their endocrine environment by conjugating maternal steroids and subsequently using the metabolites as precursors for steroid production later in development. Using the red-eared slider turtle (*Trachemys scripta*), we first characterized the conjugation of exogenous oestradiol to either oestradiol glucuronide or oestradiol sulphate (E<sub>2</sub>-S) *in ovo* during the first 15 days of development. Results show that oestradiol is primarily conjugated to E<sub>2</sub>-S. We then examined whether E<sub>2</sub>-S influenced sex determination and report that E<sub>2</sub>-S increases the production of female offspring. These data demonstrate that oviparous amniotes can both sulphonate steroids and respond to sulphonated steroids during embryonic development in a manner similar to placental amniotes.

**Keywords:** yolk steroids; steroid metabolism; maternal effect; oestradiol sulphate

## 1. INTRODUCTION

One primary goal of evolutionary biology is to understand how phenotypic variation is created and lost in response to selective pressures. The phenotype of an individual will ultimately be the result of an interaction between its own genotype and the environment it encounters. Phenotypic variation resulting from differences in the endocrine environment experienced during embryonic development has received ample attention over the years [1,2]. This attention is derived from the fact that early endocrine signals, specifically steroid hormones, have the ability to produce effects that persist throughout an organism's lifetime and influence vital processes such as reproduction [3,4]. Most of our understanding of how steroids influence development comes from experimental manipulations or maternal conditions where steroid signalling is disrupted. Examples of this include exogenous steroid application [5], enzyme inhibitor application [6], enzyme deficiencies [7,8] and gene knockouts [9]. These studies illustrate how variation in steroid signals can lead to variation in offspring phenotypes.

In light of data demonstrating that early steroid signals can produce long-term phenotypic effects, research in placental mammals has examined how embryos undergo normal development in the endocrinologically active maternal environment present during pregnancy. These studies report that the mammalian placenta, which develops from one or more extra-embryonic membranes [10], possesses an abundance of steroidogenic enzymes [1]. In addition to enzymes, such as cytochrome P450s, that are

responsible for the biotransformation of steroids [1], there are also enzymes responsible for conjugation of steroids present in the placenta [11]. Steroid conjugation is mostly carried out by two families of enzymes: UDP-glucuronosyl transferases or sulphotransferases, with sulphotransferases being the most prevalent during embryonic development [12]. The conjugated steroids are more hydrophilic than their parent steroid and are typically considered inactive owing to their inability to bind steroid receptors [13,14]. Importantly, these conjugated steroids, both sulphonated and glucuronidated, are potential precursors for steroid production as they can be enzymatically converted back to their original, active form. Thus, it appears that placental enzymes function to modulate maternal steroid signals prior to their passing into foetal circulation, and also to modulate foetal signals prior to their passing into maternal circulation [1,15].

While work in placental mammals has primarily focused on how offspring are protected from maternal steroid signals, most work done in oviparous amniotes has focused on maternal steroid signals as a means to influence offspring phenotypes in an adaptive manner [2,16]. In oviparous amniotes such as reptiles [17] and birds [18], maternal steroids accumulate in the yolk during folliculogenesis, and this results in a discrete contribution of maternal steroids being present at oviposition. In bird eggs, concentrations of androgens in the yolk can vary systematically [18] and have been shown to influence a suite of offspring traits [2,19,20]. Interestingly, not all steroid-sensitive traits are affected by yolk steroids and the effects can be context-dependent, but the reason for this phenomenon remains unresolved [21]. Recent research in birds has demonstrated that

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exogenous steroids are metabolized *in vivo* during the early part of embryonic development [22,23], concurrent with a decline in endogenous yolk steroid levels [23]. One commonality between both *in vivo* studies and several earlier *in vitro* studies [24,25] is that steroid conjugation is prevalent during the early part of embryonic development. And while steroid conjugation is known to provide precursors for steroid production in placental mammals [1], very little is known about the role of steroid conjugates in oviparous amniotes.

Steroid conjugation has been proposed as a crucial process in the regulation of maternal steroid signals in oviparous amniotes [26], with most of the empirical support for this hypothesis coming from work done in the red-eared slider turtle (*Trachemys scripta*). Although not necessarily interpreted within the context of maternal steroid effects, the effects of early steroid manipulations, specifically 17 $\beta$ -oestradiol ( $E_2$ ), are well characterized in this species. *Trachemys scripta* possesses temperature-dependent sex determination where embryos incubated at cooler temperatures (less than 28°C) develop as males. The percentage of female embryos starts to increase as temperatures exceed 28°C, with temperatures above 30°C resulting in all embryos developing as females [5]. In this system, exogenous  $E_2$  has consistently and repeatedly been used to override the temperature cue to experimentally produce females at temperatures that would normally produce males [5], providing the initial evidence that early oestradiol levels play a role in sex determination. Embryos are most sensitive to exogenous manipulation during the middle third of incubation when gonadal differentiation is occurring [27]. The application of 1.0  $\mu\text{g}$  of  $E_2$  to *T. scripta* eggs incubating at a male-producing temperature (26°C) did not produce any females when applied at embryonic stage 12, but produced over 80 per cent females when applied at embryonic stage 18 [27]. However, application of higher doses of  $E_2$  (10  $\mu\text{g}$ ) at both of these stages produced 100 per cent females [27]. Natural variation in endogenous yolk  $E_2$  levels is also associated with an increased production of female offspring in the closely related painted turtle (*Chrysemys picta*) [17]. Concentrations of  $E_2$  in the yolk decline significantly by day 10 of development in *T. scripta* [28]. During this same period, sulphotransferase enzyme activity increases significantly in the yolk and extra-embryonic membranes, and exogenous  $E_2$  is conjugated *in ovo* [26]. Overall, the data from turtles suggest that yolk  $E_2$  is conjugated to oestradiol sulphate ( $E_2\text{-S}$ ) during the early part of development. Combining the turtle data with those from placental mammals demonstrating that embryos use sulphonated steroids as precursors for steroid production, we hypothesized that yolk  $E_2$  effects in turtles result from the sulphonation of  $E_2$  to  $E_2\text{-S}$  early in development and the subsequent reactivation to  $E_2$  later in development. To test this hypothesis, we first characterized how exogenous  $E_2$  is conjugated to confirm that sulphonation is the primary conjugation pathway in *T. scripta* eggs. We also examined the potential for  $E_2$  to be glucuronidated as an alternative route of conjugation. We then examined whether *T. scripta* embryos were capable of responding to exogenous  $E_2\text{-S}$ . These studies provide data that allow important comparisons to be made between placental and oviparous amniotes with regard to how they

regulate maternal steroid signals during embryonic development.

## 2. MATERIAL AND METHODS

### (a) *Experiment 1: characterization of oestradiol conjugation*

To characterize the mode of oestradiol conjugation, we used 15 frozen yolk samples from five clutches that were previously used to quantify  $E_2$  levels in egg following exogenous  $E_2$  application [28]. Briefly, eggs were treated within 24 h of oviposition with 10  $\mu\text{g}$  of  $E_2$  dissolved in 10  $\mu\text{l}$  of ethanol, except for one egg from each clutch that was immediately frozen (day 0) to allow characterization of initial endogenous levels of conjugated  $E_2$ . Eggs were then incubated at 31°C, and one egg from each clutch was sampled on day 5 and day 15 of development. A single female-producing temperature was chosen for this experiment because the rate of yolk oestradiol metabolism is not influenced by incubation temperature [5]. At the time of sampling, yolks were isolated, homogenized and then frozen at -20°C.

Oestradiol glucuronide ( $E_2\text{-G}$ ) and  $E_2\text{-S}$  were quantified using a modified procedure of Geisler *et al.* [29]. From each yolk, two separate 100 mg aliquots were dissolved in 200  $\mu\text{l}$  of phosphate-buffered saline (pH = 7.4) in a 1.5 ml micro-centrifuge tube. Aliquots ( $n = 6$ ) containing 250 pg of  $E_2\text{-3-G}$  (Steraloids, Newport, RI, USA) or  $E_2\text{-3-S}$  (Sigma-Aldrich, St Louis, MO, USA) were included in the assay as standards to quantify percentage recovery and calculate intra-assay variation. Then, 800  $\mu\text{l}$  of 80 per cent methanol was added to each aliquot, vortexed and transferred to a 12  $\times$  75 mm glass test tube. Each micro-centrifuge tube was then rinsed with 200  $\mu\text{l}$  of methanol, vortexed and added to the test tube. Tubes were centrifuged at 3000 r.p.m. for 15 min at 0°C, followed by a decanting of the supernatant into a fresh test tube. The tube containing the yolk sample was then rinsed with an additional 1 ml of methanol, vortexed and spun again. The supernatants were combined and stored at 0°C to allow sedimentation of the neutral lipids. The following day, samples were spun at 2000 r.p.m. for 10 min at 0°C to pellet neutral lipids. The supernatants, containing conjugated and unconjugated steroids, were decanted into a fresh test tube and dried under nitrogen gas.

Conjugated steroids were separated from unconjugated steroids using solid-phase extraction. Sep Pak  $C_{18}$  cartridges (Waters Inc., Milford, MA, USA) were conditioned by washing with 5 ml of methanol, then 5 ml of Nanopure water. Samples were reconstituted in 1 ml of water and added to the cartridges. Once the samples were loaded, cartridges were washed with 5 ml of water, and unconjugated steroids were eluted with 5 ml of diethyl ether. Finally, conjugated steroids were eluted with 5 ml of methanol. Conjugated steroids were converted back to their free form by enzymatic hydrolysis. One aliquot was hydrolysed with  $\beta$ -glucuronidase purified from bovine liver to convert  $E_2\text{-G}$  back to  $E_2$  (Sigma-Aldrich) [30] while the other was hydrolysed with steroid sulphatase (STS) purified from abalone entrails (Sigma-Aldrich) to convert  $E_2\text{-S}$  back to  $E_2$  [29]. Purified enzymes were dissolved in sodium acetate buffer (pH = 5.0) at a concentration of 5  $\text{mg ml}^{-1}$  for  $\beta$ -glucuronidase and 1  $\text{mg ml}^{-1}$  for sulphatase. Hydrolysis was carried out by adding 2 ml of the enzyme solution to the conjugated steroids and incubating for 24 h at 37°C. The newly freed steroids were extracted with 3 ml diethyl ether (2 $\times$ ), dried

under nitrogen gas and reconstituted in 1 ml of 10 per cent ethyl acetate in isoctane.

$E_2$  was then quantified in each sample by radioimmunoassay (RIA) [26,31]. Tritiated oestradiol (2000 cpm) was added to each sample to calculate percentage recovery during the RIA process, and steroids were fractionated using column chromatography to minimize any effects of antibody cross-reactivity.  $E_2$  was quantified in its respective fraction with a competitive binding assay using an antibody specific to  $E_2$  (reported cross-reactivities are 14% with oestrone, 5% with oestriol and less than 0.01% with all other reported steroids; Antibody 7010, Biogenesis, Kingston, NH, USA). Samples were run in duplicate and compared with a standard curve that ranged from 5 to 500  $\text{pg g}^{-1}$ .  $E_2$ -G levels are quantified as the amount of  $E_2$  within the fraction hydrolysed with  $\beta$ -glucuronidase, while  $E_2$ -S levels are quantified as the amount of  $E_2$  within the fraction hydrolysed with sulphatase.

### (b) Experiment 2: biological activity of oestradiol sulphate

The second experiment characterized the biological activity of  $E_2$ -S by examining its ability to influence sex determination in *T. scripta*. To do this, 24 clutches of eggs were obtained from females collected at Banner Marsh Fish and Wildlife Area in central Illinois. Clutches were either treated within 24 h of oviposition ( $n = 12$ ) or after 20 days of incubation ( $n = 12$ ) as this is the end of the first third of embryonic development when gonadal differentiation begins in this species [5]. At the time of treatment, eggs from each clutch were randomized and received one of four doses of  $E_2$ -S (0, 0.1, 1.0 or 10  $\mu\text{g}$ ) dissolved in 70 per cent ethanol and applied topically in a 5  $\mu\text{l}$  bolus. Eggs were partially buried in moist vermiculite and incubated at 28°C for the entirety of development. Incubating eggs at this male-biased temperature allowed us to detect any increase in the number of female hatchlings following treatment. The sex of each hatchling was then characterized by macroscopic inspection of the gonads and Müllerian ducts a minimum of 60 days post-hatch [32]. Procedures were approved by the Illinois State University Institutional Animal Care and Use Committee, and eggs collected under permission of the Illinois Department of Natural Resources.

### (c) Statistical analyses

To test for differences in conjugated steroid levels, we performed a repeated-measures MANOVA with steroid levels (corrected for percentage recovery) as dependent variables, day of development as a fixed factor and clutch of origin as a random factor. Steroid levels were log-transformed prior to analysis. To compare sex ratios between the different doses of  $E_2$ -S, Fisher's exact tests were used. All statistical tests were conducted in SAS v. 9.1 (SAS Institute, Cary, NC, USA).

## 3. RESULTS

Endogenous levels of both  $E_2$ -G and  $E_2$ -S were low to undetectable within the yolk at the time of oviposition. Following  $E_2$  treatment,  $E_2$ -G and  $E_2$ -S were detectable within the yolk, with  $E_2$ -S concentrations being significantly higher than those of  $E_2$ -G ( $F_{1,3} = 188$ ,  $p = 0.001$ ; figure 1). Concentrations of  $E_2$ -G and  $E_2$ -S did not change from day 5 to day 15 ( $F_{1,3} = 1.41$ ,  $p = 0.32$ ; figure 1). Mean corrected values ( $\text{ng g}^{-1}$ ) for  $E_2$ -G were 34.6 on day 5 and 32.9 on day 15, while values for

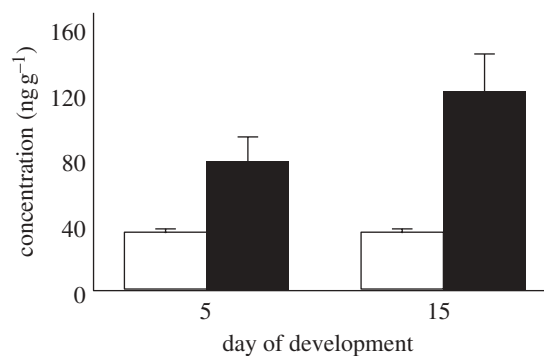


Figure 1. Mean concentrations (corrected values) of  $E_2$ -G (white) and  $E_2$ -S (black) in the yolk.  $E_2$ -S concentrations were significantly higher than  $E_2$ -G ( $p = 0.001$ ), with concentrations remaining stable during development ( $p = 0.32$ ).

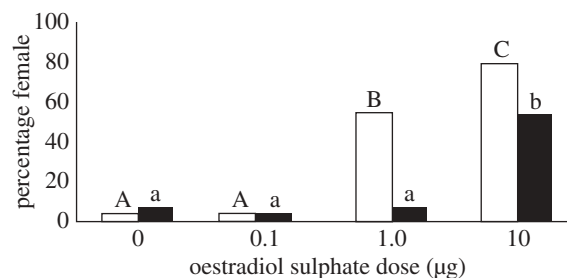


Figure 2. Sex ratios of hatchlings treated with  $E_2$ -S at day 0 (white) or at day 20 (black). Significant differences between groups are indicated for eggs treated at day 0 (capital letters) and those treated on day 20 (lower-case letters). Groups not sharing a similar letter are significantly different ( $p < 0.05$ ).

$E_2$ -S were 81.2 and 134.8, respectively. Percentage recovery for  $E_2$ -G and  $E_2$ -S was 37 and 51 per cent, respectively. Intra-assay variation was 15 per cent for  $E_2$ -G and 13 per cent for  $E_2$ -S.

Exogenous  $E_2$ -S had a significant effect on hatchling sex ratios. For eggs treated at oviposition (day 0), both the 1 and 10  $\mu\text{g}$  dose led to an increased production of female hatchlings compared with the 0 and 0.1  $\mu\text{g}$  dose (figure 2;  $p < 0.001$ ). For eggs treated on day 20, only the 10.0  $\mu\text{g}$  dose led to an increased production of female hatchlings (figure 2;  $p < 0.001$ ). All treatment groups contained 26–30 hatchlings.

## 4. DISCUSSION

We hypothesized that embryos of oviparous amniotes primarily sulphonate maternal steroids and use the resulting sulphonated steroids as precursors for steroid production later in development, similar to embryos of placental mammals. Data from the first experiment demonstrate that (i) levels of conjugated steroids are very low at oviposition, (ii)  $E_2$  is conjugated during the first 5 days of development and (iii) sulphonation is the primary conjugation pathway (figure 1). Results from the second experiment suggest that *T. scripta* embryos are capable of responding to  $E_2$ -S, as evidenced by the increased production of female hatchlings (figure 2). These results also provide support for the idea that the sulphotransferase/sulphatase pathway plays an important role in the modulation of maternal steroid signals in oviparous amniotes just as it does in placental amniotes. Demonstrating that

embryos can sulphonate steroids, and that sulphonated steroids can influence offspring development, has important implications for how we interpret the effects of maternally derived yolk steroids by advancing our knowledge of how embryos regulate their endocrine environment.

Maternally derived yolk steroids have been extensively studied since they were first proposed as a means by which females could influence offspring phenotype [2,18,19]. Numerous studies have shown that concentrations of various steroids in the yolk decline during development [23,28,33–36]. In *T. scripta*, concentrations of yolk  $E_2$  are significantly higher in the female's second clutch of the season, but, despite this initial difference, concentrations by day 10 of development are low/undetectable in both groups, and this decline is not influenced by incubation temperature [5]. In fact,  $E_2$  concentrations in the same yolks used in this current study were shown to significantly decline from day 5 to day 15 [26]. When calculating the total amount of  $E_2$  measured within these eggs (yolk + albumen + embryo), amounts declined from 0.217  $\mu\text{g}$  on day 5 to 0.024  $\mu\text{g}$  on day 15, which only represents 2 and 0.02 per cent, respectively, of the initial 10  $\mu\text{g}$  that was initially applied on day 0 [26]. The current study demonstrated that some of this  $E_2$  was conjugated to  $E_2\text{-G}$  and  $E_2\text{-S}$ . The total amount of  $E_2\text{-G}$  in just the yolk was 0.178  $\mu\text{g}$  on day 5 and 0.161  $\mu\text{g}$  on day 15, while the total amount of  $E_2\text{-S}$  was 0.419  $\mu\text{g}$  on day 5 and 0.661  $\mu\text{g}$  on day 15. These levels still only account for less than 10 per cent of the initial  $E_2$  that was applied. Potential explanations for the unaccounted-for  $E_2$  include, but are not limited to, incomplete transfer of steroid through the eggshell, presence of  $E_2\text{-G}$  and  $E_2\text{-S}$  in the albumen or embryo (only yolk was examined in the present study) and metabolism of  $E_2$  to different oestrogens. While it is possible that some of the initial  $E_2$  remained on the eggshell, a previous study using tritiated  $E_2$  demonstrated that this topical route of administration resulted in over 70 per cent transfer of tritiated  $E_2$  into the egg [26]. This study also demonstrated that water-soluble metabolites of  $E_2$  were present in the yolk and albumen in approximately equal amounts, suggesting that  $E_2\text{-G}$  and  $E_2\text{-S}$  would be present in the albumen in addition to the yolk [26]. At this point, we have no measure of the conversion of  $E_2$  to other oestrogens such as oestrone, but work in the European starling (*Sturnus vulgaris*) has shown that exogenous testosterone is converted to other androgen metabolites, with less than 5 per cent of the initial testosterone detectable after 6 days of incubation [23]. More work is needed to characterize  $E_2$  metabolites throughout the egg during embryonic development in *T. scripta*. Regardless of the ultimate fate of the steroid, the data from this study demonstrate that even a supraphysiological dose of  $E_2$  (10  $\mu\text{g}$ ) can be almost completely metabolized during the first third of development and result in levels of  $E_2\text{-S}$  in the yolk that are 85 times higher than  $E_2$  levels in the entire egg.

Sulphonation has been established as the primary steroid conjugation pathway during early embryonic development in mammals for some time [12]. With regard to the sulphonation of  $E_2$ , the enzyme primarily responsible for this conversion is oestrogen sulphotransferase (SULT1E1) (EC 2.8.2.4). The resulting

metabolite,  $E_2\text{-S}$ , has increased water solubility and is considered inactive [13,14]. However,  $E_2\text{-S}$  (along with other sulphonated steroids) can be reactivated by the enzyme STS (EC 3.1.6.2), highlighting the importance of STS as a producer of active steroids. The ability of  $E_2\text{-S}$  to influence sex determination in *T. scripta* suggests the presence of STS activity during embryonic development, and STS expression has been reported in embryonic [37] and foetal [38] tissues. With evidence mounting that maternal steroids are sulphonated during the early part of embryonic development in oviparous amniotes [23,26,28], embryonic STS activity becomes a critical part of understanding when and where maternal steroid effects can arise. The sulphonation of maternal steroids by developing embryos results in the conversion of lipophilic, active steroids to water-soluble, inactive forms. The temporary inactivation of steroids via sulphonation may help reconcile the apparent contradiction that maternal steroids can influence individual differentiation without influencing other processes (such as sexual differentiation [21]) by allowing for tissue-specific reactivation of sulphonated steroids by STS.

In *T. scripta*, this specific contradiction is not present, as gonadal differentiation is sensitive to  $E_2$  [5]. But this system does provide a steroid-sensitive trait that is dichotomous (male versus female), allowing us to clearly demonstrate that sulphonated steroids can influence development in an oviparous amniote. And despite the difference between turtles and birds in the relative influence of steroids on gonadal differentiation, other aspects of yolk steroid signalling are similar. For example, both taxa exhibit a decline in yolk steroid concentrations across development as well as *in ovo* steroid metabolism early in development [39]. Because of their sensitivity to  $E_2$ , it may be even more important for turtles to convert maternal steroids to inactive metabolites to prevent a potential skewing of sex ratios. Our finding that the lowest dose of  $E_2\text{-S}$  did not influence sex ratios suggests that embryos can buffer themselves from a maternal effect on gonadal differentiation when steroid levels are low. Consistent with this finding, endogenous steroids primarily influence offspring sex ratios late in the nesting season [17], when yolks from second clutches contain almost 10 times the amount of  $E_2$  as first clutches [28,40]. Embryos appear more sensitive to  $E_2\text{-S}$  when applied immediately at oviposition versus day 20 of development, but the reasons for this remain unknown. Numerous factors that influence  $E_2\text{-S}$  movement and reactivation are likely to differ between these two points of development. Interestingly, we did not detect any  $E_2\text{-G}$  or  $E_2\text{-S}$  in the yolks at the time of oviposition, suggesting that these substances are not transferred to the yolk during folliculogenesis. At this point, data suggest that both turtles and birds, and probably oviparous vertebrates more generally, are able to modulate maternal steroid signals, thus demonstrating an important embryonic contribution to the manifestation of maternal steroid effects.

By demonstrating that embryos of *T. scripta* can both sulphonate steroids and respond to sulphonated steroids, this study illustrates the complex and dynamic nature of yolk steroid effects. The interaction between maternal signal and embryonic response that we report is very similar to what is found in placental amniotes and suggests that a common pathway is used in the modulation of

maternal steroids. Since embryonic steroid milieus during sexual differentiation can produce long-term phenotypic effects and disruption of these milieus often leads to abnormal reproductive behaviour in adults [21], sulphonation may allow embryos to use maternal steroids as precursors for steroid production without the risk associated with transporting active steroid signals. The presence of steroid sulphonation during embryonic development in an oviparous amniote may also help explain how viviparity evolved in vertebrates [41]. One potential issue with the transition from oviparity to viviparity is that the embryo would be subjected to the maternal environment, including steroid signalling, throughout development. Sulphonation is the mechanism by which placental amniotes are thought to protect themselves from the potentially detrimental effects of maternal steroids [11], and thus our finding of a similar mechanism in an oviparous system suggests that one major barrier to viviparity is gone. Overall, emerging data indicate that oviparous and placental amniotes modulate maternal steroid signals in a similar manner. More studies are needed to characterize the role of sulphonated steroids during embryonic development if we are to fully understand how maternal steroid signals create phenotypic variation in offspring, but it now appears that oviparous amniotes provide a powerful system in which to study the regulation of maternal steroid signals. The discrete nature of the egg facilitates investigation into the mechanisms of how embryos regulate their endocrine environment because there is a separation from the maternal endocrine environment. By advancing our knowledge of how embryos regulate their endocrine environment, we will develop a more complete understanding as to how phenotypic variation is created.

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