Neglected aspects of hormone mediated maternal effects

Kumar, Neeraj

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Chapter 5

Early embryonic modification of maternal hormones differs systematically among embryos of different laying order: A study in birds

Neeraj Kumar, Martijn van Faassen, Ido Kema, Manfred Gahr, Ton G.G. Groothuis

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ABSTRACT

Vertebrate embryos are exposed to maternal hormones that can profoundly affect their later phenotype. Although it is known that the embryo can metabolize these maternal hormones, the metabolic outcomes, their quantitative dynamics and timing are poorly understood. Moreover, it is unknown whether embryos can adjust their metabolic activity to, for example, hormones or other maternal signals. We studied the dynamics of maternal steroids in fertilized and unfertilized rock pigeon eggs during early incubation. Embryos of this species are naturally exposed to different amounts of maternal steroids in the egg according to their laying position, which provides a natural context to study differential embryonic regulation of the maternal signals. We used mass spectrometric analyses to map changes in the androgen and estrogen pathways of conversion. We show that the active hormones are heavily metabolized only in fertilized eggs, with a corresponding increase in supposedly less potent metabolites already within one-fourth of total incubation period. Interestingly, the rate of androgen metabolism was different between embryos in different laying positions. The results also warrant a re-interpretation of the timing of hormone mediated maternal effects and the role of the supposedly biologically inactive metabolites. Furthermore, the results also provide a potential solution as to how the embryo can prevent maternal steroids in the egg from interfering with its sexual differentiation processes as we show that the embryo can metabolize most of the maternal steroids before sexual differentiation starts.

1 INTRODUCTION

Over the last decades there is a growing interest in the exposure of the vertebrate embryo to maternal hormones as a potential pathway for adaptive maternal effects. Egg laying species, especially birds (Gil 2008; Groothuis et al. 2005b; von Engelhardt & Groothuis 2011), but also fish (Brown et al. 1988) and reptiles (Paitz & Bowden 2008, 2011; Radder 2007) have been used extensively to study the effects of maternal hormones, especially steroids, in the egg yolk since in oviparous species the embryo develops outside the body of the mother facilitating such manipulations. This has revealed a wide array of effects on the offspring phenotype, ranging from morphology to physiology and behaviour (Groothuis et al., 2005; Schwabl, 1993; von Engelhardt and Groothuis, 2011). Furthermore, systematic variation is found in egg steroid levels associated with the laying order as well as environmental variation surrounding the mother (Eising et al. 2001; Schwabl 1993, 1997a; von Engelhardt & Groothuis 2011), including biotic and abiotic factors (Gil 2008; Hahn 2011; Müller et al. 2002; Welty et al. 2012). However, how, when and which hormones reach the embryo is as yet unclear.

In the course of egg incubation the hormone concentrations in the yolk decline rapidly (birds (Eising et al., 2003; Elf and Fivizzani, 2002; Wilson and McNabb, 1997), reptiles (Bowden et al., 2002; Paitz and Bowden, 2009), fish (Feist & Schreck 1996)). One study showed that hormone levels decline in yolk-albumen homogenates (Paitz et al. 2011) suggesting that the decrease in yolk hormone concentrations is not entirely due to yolk dilution by mixing and/or water influx with albumen, and a few pioneering studies indicate metabolism of maternal yolk steroids by the embryo by conjugation (Paitz et al., 2011; Paitz and Casto, 2012; Vassallo et al., 2014; von Engelhardt et al., 2009). As suggested by Paitz and Bowden (2008, 2013) and von Engelhardt (2009), this opens the possibility that embryos of oviparous species have in fact active control over their endocrine environment like in mammalian species (Braun et al. 2013; Cottrell & Seckl 2009; Del Giudice 2012) which would be favoured by natural selection (Del Giudice, 2012; Mock and Forbes, 1994; Müller et al., 2007; Wilson et al., 2005; Winkler, 1993). This is because of potential parent-offspring conflicts in which the endocrine environment created by the mother might be primarily in the interest of the mother but not always be in the best interest of the offspring as they share only half of their genes. For example, by distributing maternal androgens over the laying order mothers may favour certain offspring over others, creating a conflict with the latter. However, the detailed scope for such role of the embryo in translating maternal hormones is not well understood, especially in bird species, the most widely used species in this field. This includes the timing and quantitative dynamics of embryonic metabolism, metabolic differences based on embryo's laying order in the clutch, the overall metabolic outcomes concerning detailed steroid metabolic pathway such as conversion of less potent metabolites to more potent ones or vice-versa, and their uptake and utilization by the embryo.

The aims of this study were (i) To verify which of the steroids of the androgenic and estrogenic pathway differ in maternal deposition between first and second eggs including the conjugated forms; (ii) To investigate to what extent the decline in yolk hormone levels during the first days of incubation is due to hormone conversion by analysing the decline in hormone amounts of the entire egg between oviposition and 4.5 days of incubation in the unfertilized eggs; (iii) To compare the metabolic profile of incubated fertilized and unfertilized eggs to discern the maternal and embryonic contribution to the steroid metabolism; (iv) To compare the metabolic outcomes of maternal steroid hormones between fertilized first and second eggs that would indicate scope for differential embryonic activity. To this end, we used rock pigeon species (*Columba livia*) because it provides an appropriate natural context to test whether the embryos of different laying order can utilize or metabolize maternal hormonal signals differently as the first and second embryos of a clutch are exposed to different levels of maternal androgens (Hsu et al. 2016). We analysed a wide spectrum of hormone profiles and their metabolites (Fig. 1) over the first 4.5 days of incubation to identify patterns of conversion to biologically active or inactive compounds, including conjugated forms.

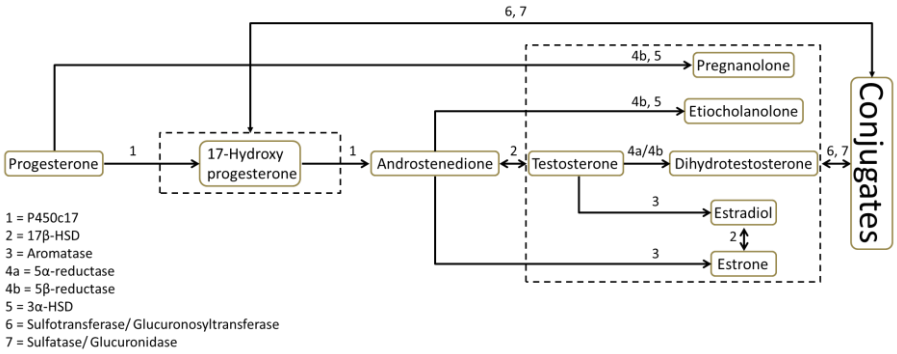

Figure 1. The analysed compounds of the steroid metabolic pathway. All the compounds shown within the dashed boxes can be conjugated. The numbers represent the enzymes involved in the pathway.

2 MATERIALS AND METHODS

2.1 Animal housing

All animal procedures were approved by the animal welfare committee of the University of Groningen under license 6835B. The procedures were carried out at the animal facility of the University of Groningen according to the guidelines and regulations of the committee. Rock pigeons (80 pairs) were housed in outdoor aviaries (45m long x 9.6m wide x 3.75m high) under natural light and temperature conditions, and *ad libitum* access to food and fresh water. All eggs (whether unfertilized or fertilized) were collected under exactly the same housing conditions. Some clutches consisted of unfertilized eggs although all female birds had access to male birds. The food consisted of a mixture of commercial pigeon seeds (Kasper 6721 and Kasper 6712), P40 vitamin supplement (Kasper P40), and small stones or grit. Each bird had a unique combination of coloured leg bands for identification. Nest boxes (60cm x 50cm x 36cm) were provided along with the breeding bowls and nesting material. Daily observations were made for food and water availability, nest building, and egg laying.

2.2 Egg collection and Sample preparation

Eggs were collected during months of August-September. Nests were checked twice daily to monitor egg laying. For hormone measurements at oviposition, yolk and albumen of freshly laid eggs (n=8 for both positions in the laying order) were homogenized and the homogenates were weighed and stored at -20°C until hormone extractions took place. For incubated fertilized and unfertilized groups, eggs were collected from separate birds and were artificially incubated at 37.8°C with 60% relative humidity for four and a half days. The eggs were monitored for the development of the embryo. In case of fertilized eggs, yolk, albumen, and embryo were homogenized (n= 8 for both positions in the laying order) and the homogenates were weighed and stored at -20°C until hormone extractions took place. We expected the embryonic endogenous hormone production to be minimum by 4.5 days of incubation in comparison to larger maternal amounts in the egg as the gonadal differentiation begins only around the sixth day of incubation after which embryonic endogenous hormone production takes place (Andrews et al. 1997; Woods et al. 1975; Yoshida et al. 1996). In case of unfertilized eggs yolk and albumen were homogenized (n = 8 for both positions in the laying order) and the homogenates were weighed and stored at -20°C until hormone extractions took place.

2.3 Extraction and mass spectrometric analyses of steroids

Out of the targeted compounds of the steroidogenic pathway (Fig. 1), etiocholanolone and pregnanolone were analysed by gas chromatography (GC) and the other compounds were analysed by liquid chromatography (LC), combined with tandem mass spectrometry (MS/MS). For LC-MS/MS steroids were extracted from 300 mg homogenates and for GC-MS/MS from 600 mg, using methanol as organic solvent. Details on extraction and mass spectrometry procedures can be found in the electronic supplementary material (ESM).

2.4 Statistical analyses

Statistical analyses were performed using general linear model in IBM-SPSS software (version 23). To test for differences in maternal deposition of hormones between eggs of different position in the laying order, data were analysed taking egg laying order as a fixed factor. To examine the role of maternal factors in the egg in steroid dynamics during early incubation, independent of embryonic activity, we compared steroid levels at oviposition before incubation with levels after four and a half days of incubation in the unfertilized eggs. These data were analysed taking egg laying order (first or second), incubation (day 0 or day 4.5 unfertilized), and their interaction as fixed factors. To examine the role of the embryo itself in steroid dynamics during early incubation, levels of steroids were compared between unfertilized and fertilized eggs, both incubated for 4.5 days. These data were analysed taking egg laying order (first or second), fertilization (unfertilized or fertilized), and their interaction as fixed factors. We re-run both models without interactions in case interaction effects were not significant (otherwise main effects are not meaningful) to estimate the main effects more reliably, and only in case the p-values were qualitatively different these are explicitly mentioned. The weighted least square method was used that does not assume equal variances across groups. Out of the total 480 data points, 17 data points (3.5%) with extreme values (more than three times of the interquartile range (Hoaglin & Iglewicz 1987)) were excluded as outliers. Conjugated etiocholanolone, conjugated pregnanolone, and conjugated estradiol were detectable only in some of the fertilized incubated eggs, and thus were excluded from the statistical analyses due to too few data points.

3. RESULTS

3.1 Differential maternal deposition: day 0 eggs

At the time of oviposition (Fig. 2: day 0, table 1), second eggs had higher levels of 17-hydroxyprogesterone ($p = 0.032$), androstenedione ($p < 0.001$), and testosterone ($p < 0.001$), confirming earlier studies on androgens (Hsu et al. 2016). The levels did not differ between first and second eggs for progesterone, estradiol, and estrone. There was some conjugated estrone and conjugated testosterone already present at oviposition which also did not differ between the egg laying order. Dihydrotestosterone (5α or 5β), etiocholanolone, pregnanolone, and their conjugated forms, as well as conjugated estradiol were all undetectable at oviposition, although with high limit of detection for etiocholanolone (1.0 nmol/L) and pregnanolone (10 nmol/L).

3.2 The role of maternal factors in the egg in steroid dynamics during early incubation: incubation effect on unfertilized eggs

After four and a half days of incubation, hormone amounts in the unfertilized eggs did not significantly differ from eggs at oviposition for any of the analysed steroids (Fig. 2, table 2: column 3). Only the incubation effect for progesterone ($p = 0.052$) became just significant when the interaction effect was removed from the model ($p = 0.043$). There was no significant interaction between egg laying order and incubation except for androstenedione (table 2: column 4), where in first eggs its amount slightly increased ($p = 0.032$) and in second eggs slightly decreased but not significantly ($p = 0.259$) (Fig. 2c).

3.3 The role of the embryo in steroid dynamics during early incubation: different effect of incubation in fertilized and unfertilized eggs

To examine the role of the embryo in steroid dynamics, levels of steroids were compared between unfertilized and fertilized eggs, both incubated for four and a half days (Fig. 2; table 3: column 3). As compared to the unfertilized eggs, in the fertilized eggs there was a highly significant decrease in progesterone, 17-hydroxyprogesterone, androstenedione, and testosterone; and a highly significant increase in etiocholanolone, pregnanolone, and estradiol. The decline in estrone just did not reach statistical significance, but became significant when the interaction was removed from the model ($p < 0.01$). Among conjugated forms, there was no change in conjugated estrone but an increase in conjugated testosterone ($p = 0.01$). Conjugated etiocholanolone, conjugated pregnanolone, and conjugated estradiol were not detectable in the unfertilized eggs but only in some of the

fertilized eggs. Together the results indicate that only in fertilized but not in unfertilized eggs hormone amounts change during early incubation mostly in such a way that unconjugated hormones are metabolized to hormones that are supposed to be biologically inactive.

3.4 Differences between first and second eggs in metabolic activity

There was a significant interaction between egg laying order and fertilization for the decrease of androstenedione ($p = 0.003$, table 3: column 4). It was almost significant for testosterone too ($p = 0.053$, table 3: column 4). The increase in etiocholanolone was also highly significant depending on laying order ($p < 0.001$, table 3: column 4). This indicates differential hormone conversion rate between first and second eggs with a stronger decline in the unconjugated primary androgens and a stronger increase in etiocholanolone in second eggs.

4 DISCUSSION

We analysed for the androgenic pathway the role of the embryo in the decline of androgens during the first days of incubation and to what extent this may be different between embryos of eggs that differ in laying position. As the decline was measured in the entire fertilized egg, including the embryo, the changes in amounts of steroids over incubation cannot be attributed to mixing of yolk-albumen or water influx into yolk or embryonic uptake over the course of incubation, as suggested in the literature (Groothuis & Schwabl 2008) but rather indicates metabolism as has been demonstrated for European starling (Paitz et al. 2011). Because the steroid levels do not change in the unfertilized eggs (Fig. 2, table 2) but only in the fertilized eggs (Fig. 2, table 3), this indicates that the steroid metabolism is due to very early embryonic activity confirming the suggestions made by earlier studies in chicken (von Engelhardt et al. 2009), European starling (Paitz & Casto 2012; Paitz et al. 2011), Japanese quail (Vassallo et al. 2014), and red-eared slider turtle (Paitz & Bowden 2008, 2009, 2013). However, as mixing of yolk, albumen and water occurs in fertilized but not in unfertilized eggs this difference may potentially also explain the decrease in hormone levels occurring only in fertilized eggs in case the metabolic enzymes are deposited by the mother in the albumen. However, this mixing is only very minor at this early stage in the incubation process and may therefore only contribute to a minor extent. Alternatively, the embryonic activity could have been responsible for activating maternal enzymes in the yolk. However, in another study on rock pigeon we did not find any evidence for presence of maternal enzymes in the yolk (Kumar et al. 2018a).

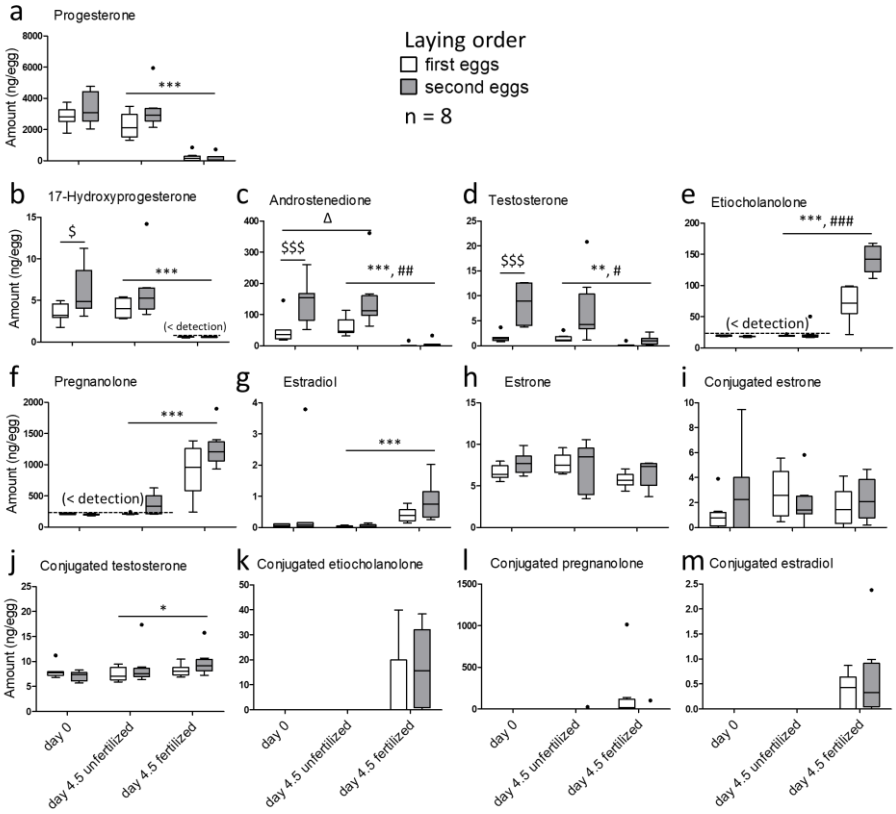

Figure 2. Metabolism of steroids in rock pigeon eggs. The amounts (nanogram per egg) of analysed steroids in entire egg homogenates (except eggshell) at oviposition (day 0) and after 4.5 days of incubation in unfertilized and fertilized eggs of rock pigeon, which produce two-egg clutch (first eggs- white box, second eggs- grey). Panels k, l, and m were not analysed statistically due to too few data points (sample size of detectable and non-zero levels for first eggs was 3, 4, and 5; and for second eggs was 6, 7, and 6 respectively for k, l, and m panels). Significant differences are shown by $ between first and second eggs at oviposition, and by * between unfertilized and fertilized eggs both incubated for 4.5 days. The interaction effects are shown by Δ for the interaction between the egg laying order and incubation in unfertilized eggs, and by # for the interaction between the egg laying order and fertilization. $^{\#}p = 0.053$, $^{\$, *, \Delta}p < 0.05$, $^{**, \#\#}p < 0.01$, $^{\$\$\$, ***, \#\#\#}p < 0.001$.

Table 1. p-values tested for the effect of laying order for the differences in maternal steroids in the egg at oviposition. Significant effects are indicated in bold. NA= not available (not detected).

Steroid	Laying order
Progesterone	0.218
17-Hydroxyprogesterone	**0.032**
Androstenedione	**< 0.001**
Testosterone	**< 0.001**
Etiocholanolone	NA
Pregnanolone	NA
Estradiol	0.791
Estrone	0.053
Conjugated estrone	0.202
Conjugated testosterone	0.233

Table 2 Statistical results of comparisons of steroid amounts in rock pigeon eggs at oviposition (day 0) with unfertilized eggs incubated for 4.5 days: p-values for the effect of laying order, incubation, and their interaction were tested. Significant effects are indicated in bold. NA= not available (not detected).

Steroid	Laying order	Incubation	Interaction (laying order x incubation)
Progesterone	**0.046**	0.052	0.931
17-Hydroxyprogesterone	**0.009**	0.710	0.180
Androstenedione	**< 0.001**	0.871	**0.045**
Testosterone	**< 0.001**	0.608	0.524
Etiocholanolone	NA	NA	NA
Pregnanolone	NA	NA	NA
Estradiol	0.231	0.154	0.443
Estrone	0.622	0.689	0.227
Conjugated estrone	0.545	0.543	0.121
Conjugated testosterone	0.511	0.627	0.488

Table 3. Comparisons of steroids in unfertilized and fertilized eggs after 4.5 days of incubation. p-values are presented for the effect of laying order, fertilization, and their interaction. Significant effects are indicated in bold.

Steroid	Laying order	Fertilization	Interaction (laying order x fertilization)
Progesterone	0.163	**< 0.001**	0.123
17-Hydroxyprogesterone	0.233	**< 0.001**	0.221
Androstenedione	**0.002**	**< 0.001**	**0.003**
Testosterone	**0.007**	**0.003**	0.053
Etiocholanolone	**< 0.001**	**< 0.001**	**< 0.001**
Pregnanolone	**0.011**	**< 0.001**	0.310
Estradiol	**0.047**	**< 0.001**	0.086
Estrone	0.845	0.052	0.375
Conjugated estrone	0.903	0.542	0.260
Conjugated testosterone	0.161	**0.010**	0.168

In addition, we could show for the first time that the hormone dynamics over the first 4.5 days of incubation differed between fertilized eggs of the first and last laying order position. Whether this is a function of initial differences in hormone levels or due to differences in enzymatic activity is as yet unclear. This discovery is important since parent-offspring conflict theory (Godfray, 1995; Trivers, 1974; Wilson et al., 2005; Wolf and Wade, 2001) predicts that embryos that actively respond to or modulate maternal signals in a context-dependent manner are favoured by natural selection (Del Giudice, 2012; Müller et al., 2007; Winkler, 1993). Indeed, the high level of conjugation we demonstrated for the pigeon embryo has also been proposed as a mechanism by which the embryo can regulate action of maternal steroids as conjugated steroids might not bind to the steroid receptors (Paitz & Bowden 2013).

The levels of progesterone, and its downstream metabolites- 17-hydroxyprogesterone, androstenedione, and testosterone, declined over early incubation in fertilized eggs. As there was no increase in the levels of estrone or its conjugate, and dihydrotestosterone or its conjugate and conjugated 17-hydroxyprogesterone were undetectable, none of these downstream metabolites can explain the decline in their precursors. However, the levels of etiocholanolone, pregnanolone and their conjugated forms, none of which were even detectable at oviposition, were very high in the fertilized eggs. Indeed, the decrease in androstenedione and testosterone is quantitatively reflected by the increase in etiocholanolone and its conjugate (table 4). The decrease in progesterone is quantitatively only partly reflected by the increase in pregnanolone and its conjugate (table 4), suggesting formation of other metabolites of progesterone that we did not measure. There was a slight increase in the levels of estradiol and its conjugate. Finally, the laying order difference in the decrease of androstenedione and testosterone levels is reflected by the laying order difference in the formation of etiocholanolone but not in estradiol levels in the fertilized incubated eggs.

It is noteworthy that the absolute levels of active hormones such as testosterone and progesterone decline substantially and extremely early during the incubation. Therefore, if the differential maternal hormonal allocation were indeed responsible for differential offspring phenotype development, such effects are likely to take place on the embryonic tissues within this early incubation period. This highlights that the early incubation period is extremely important to investigate further the mechanisms of action of maternal hormones. Whether the active hormones could already induce receptor-mediated changes in the epigenome and/or transcriptome profile in the embryonic tissues is as yet unknown. For instance, it is unclear whether the embryo already expresses steroid receptors at earlier stages of development than 4.5 days. These receptors are known to be present in the chicken embryonic body tissues only after 4.5 days of incubation for androgens (Endo et al. 2007), 3.5 days for estrogens (Andrews et al. 1997; Smith et al. 1997), and 4 days for progesterone (Guennoun et al. 1987). Although in both species chicks hatch around 21 days

after start of incubation, the pigeon is an altricial species whereas the chicken is a precocial species so that receptor development in the pigeon is expected to be even later in development than in the chicken. It might be possible that the hormones act on the embryo via non-genomic receptors, as has been extensively described for adult birds (Balthazart et al. 2009).

Table 4. Median values (ng/egg) of steroids corresponding to Fig. 2.

	day 0		day 4.5, unfertilized		day 4.5, fertilized	
egg laying order	**first**	**second**	**first**	**second**	**first**	**second**
Progesterone	2803.31	3077.12	2107.39	2887.76	141.17	56.38
17-hydroxyprogesterone	3.22	4.90	4.00	4.26	< 0.61	< 0.57
Androstenedione	28.21	153.91	46.58	108.32	0.77	1.14
Testosterone	1.26	8.97	1.13	4.28	0.07	0.98
Etiocholanolone	< 19.83	<18.03	<19.23	<18.72	71.68	142.10
Pregnanolone	< 217.39	< 197.69	< 210.84	333.36	959.32	1208.98
Estradiol	0.06	0.06	0.03	0.07	0.39	0.75
Estrone	6.36	7.68	7.47	8.47	5.69	7.34
Conjugated estrone	0.76	2.24	2.58	1.38	1.45	2.07
Conjugated testosterone	7.67	7.35	7.06	7.29	8.06	9.13
Conjugated etiocholanolone	0	0	0	0	0	15.67
Conjugated pregnanolone	0	0	0	0	0	0
Conjugated estradiol	0	0	0	0	0.43	0.33

Alternatively, since the levels of the metabolites etiocholanolone and pregnanolone increase rapidly during this early incubation period, these metabolites might be responsible for mediating the maternal effects, but that remains to be studied. Pregnanolone, for instance, could play a role as a potential neurodevelopmental regulator (Matsunaga et al., 2004; Pignataro et al., 1998; Viapiano and De Plazas, 1998). Etiocholanolone and its conjugate were already suggested as androgen metabolites based on metabolism of injected radiolabelled testosterone in the eggs of European starling upon incubation for six days (Paitz et al. 2011). Etiocholanolone has extremely low binding affinity for androgen receptors in mammals (Fang 2003), but nevertheless it has been suggested to influence erythropoiesis in mammals and birds (Gordon et al. 1970; Irving et al., 1976; Levere et al. 1967; Paitz et al., 2011), which could be mediated via non-classical receptors (Losel and Wehling, 2003; Meyer, 2007; Moore et al., 2000; Paitz and Bowden, 2010). Such effects may likely not be limited to any particular target tissue such as brain or gonads and may therefore be responsible for the wide array of effects on the chick phenotype. Finally, there is the possibility that the embryo can convert the conjugated metabolites back to their unconjugated forms at the time and amount needed for the embryo itself. This has been

suggested by Paitz and Bowden, who found that in ovo injection of estradiol sulphate did affect the sex ratio in a turtle species (Paitz & Bowden 2011).

It remains a puzzle why the maternal gonadal steroids in the egg do not interfere with the sexual differentiation of the developing embryo (Carere & Balthazart 2007). This study shows that the embryo is capable of metabolizing most of the active steroids of maternal origin even prior to beginning of sexual differentiation processes, as hypothesised earlier via embryonic 5β-reduction pathways (Paitz & Bowden 2010), and thus provides a potential solution to this problem.

Finally, the difference in maternal hormone levels found between first and second eggs at oviposition is assumed to provide the mother an upperhand in parent-offspring conflict contexts. For example, the higher levels in second eggs have been demonstrated to boost competitive ability of the last hatched chick in the sibling rivalry, but would go at the cost of the older sibling (e.g. (Eising et al., 2001; Hsu et al., 2016; Schwabl, 1993)). This might induce selection on the embryo to enhance or counteract the maternal signal depending on the position in the laying and hatching order. Embryos may detect this position by the differences in egg composition between eggs of different laying order, either in hormone concentration or in the many other compounds in the yolk. Our results show that incubated fertilized eggs of first and second laying position do differ in hormone dynamics. Initial differences between the eggs in maternal hormone concentrations of 17-hydroxyprogesterone and androstenedione tend to diminish during very early incubation of fertilized eggs. However, after 4.5 days of incubation of fertile eggs there are still differences in testosterone with higher levels in second eggs ($p = 0.006$) while large differences in etiocholanolone develop with significantly higher levels in second eggs too ($p < 0.001$).

5 CONCLUSION

In conclusion, we experimentally demonstrate that the absolute levels as well as the relative differences in maternal hormones at oviposition tend to diminish very early during incubation due to embryonic metabolism, with the rate of androgen metabolism being higher in latter laid eggs. This creates a paradox as it is well known that initial differences in these hormones can have substantial effects on the chick, whereas we show that at 4.5 days of incubation these differences are hardly present anymore. This paradox can be solved in three ways: first, the active hormones can already induce receptor-mediated genomic and/or non-genomic changes in embryonic tissues before their depletion; second, steroids may intercalate with DNA and thereby have non-receptor mediated genomic effects; third,

the embryo can convert the inactive metabolites in the course of development back to their biologically active forms. These possibilities are promising avenues for further research.

ETHICS

All the animal research was conducted according to the established guidelines and regulations of the animal welfare committee of the University of Groningen, and all relevant procedures were approved by the committee under the license 6835B.

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SUPPLEMENTARY MATERIAL

Extraction of gonadal steroids from the egg

To each sample (300 mg homogenates for LC-MS/MS and 600 mg for GC-MS/MS) either 50 µl (mixture of $^{13}C_3$ labelled progesterone, 17-hydroxyprogesterone, androstenedione, and testosterone in 50% methanol, each 30 nmol/L, IsoSciences); or 200 µl ($^{2}H_5$ labelled etiocholanolone in pure methanol, 6.7 µmol/L, IsoSciences) of an internal standard was added and left for one hour at room temperature for equilibration. Since for each individual sample the ratio of the added internal standard to the target compound automatically corrected for any potential losses during extraction procedures as well as signal suppression due to differences in ionization efficiency in the mass spectrometer, hence the data were corrected for recovery losses. Internal standards for estrone and estradiol (25 µl of $^{13}C_3$ labelled estrone and estradiol, each 1.0 nmol/L, IsoSciences) were added after the extractions. Each sample was extracted twice in 1 ml methanol by vortexing, followed by centrifugation at 12000xg for 10 minutes at room temperature. The supernatant was transferred to tubes containing 200 mg of solid $ZnCl_2$ for lipid precipitation (Wang et al. 2010). The total volume of the combined supernatants was made to 4 ml by adding 2 ml methanol, and centrifuged at 12000xg for 10 minutes at 4°C. The supernatant was dried under nitrogen gas in a waterbath at 50°C, re-suspended in 1 ml methanol, centrifuged at 12000xg for 10 minutes at room temperature, followed by addition of 1.8 ml water to the supernatant. This mixture was centrifuged at 12000xg for 10 minutes at 4°C. The supernatant was loaded on C18 SPE columns (3 ml, 500 mg, Grace Inc.) pre-equilibrated with 3 ml of methanol, followed by 3 ml of water. After collecting flow through, columns were washed with 3 ml water, and then eluted with 2 ml methanol. The eluent was divided in two equal parts, one part was analyzed without hydrolysis and the other part after hydrolysis, the difference between the two representing the conjugated steroids as hydrolysis converts conjugated compounds to their free forms (e.g. (Mi et al. 2014)).

For sample preparation without hydrolysis 1 ml eluent was dried under vacuum, re-suspended in 150 µl methanol, followed by addition of 350 µl water to make a final concentration of 30% methanol. For sample preparation with hydrolysis 1 ml eluent was dried under vacuum, and re-suspended in 2 ml acetate buffer (0.5 M sodium acetate with 15 g/l sodium ascorbate, pH 4.8). 100 µl of Helix Pomatia (Brunschwig Chemie) was added, vortexed, and incubated at 46°C for 2 hours. The hydrolyzed samples were cooled at room temperature and purified on HLB SPE columns (3ml, Waters Inc) pre-equilibrated with 2 ml of methanol, followed by 2 ml of water. After collecting flow through, columns were washed with 2 ml water, and then eluted with 2 ml methanol. The eluent was dried under vacuum, re-suspended in 150 µl methanol, followed by addition of 350 µl water to make a final concentration of 30% methanol.

Mass Spectrometry

(a) LC-MS/MS

The extracts were analyzed with a XEVO TQ-S tandem mass spectrometer (Waters Corp.), equipped with an Online SPE Manager and ACQUITY UPLC system (Waters Corp.). The UPLC flow rate was set at 0.4 ml/min using 10 mM ammonium acetate, 0.1% formic acid in water and methanol (containing 0.1% formic acid) as mobile phases A and B respectively. The analysis of estradiol and estrone consisted of 0.2 mM ammonium fluoride in 10 % methanol in water and 0.2 mM ammonium fluoride in methanol as mobile phase A and B respectively. For each extract, 40 µl sample was injected for extraction on a XBridge C8 cartridge and chromatographic separation was performed on a Kinetex C18 column (2.1 x 100 mm, 2.6 µm). The mass spectrometer was operated under electrospray ionization mode with following operating conditions: cone voltage of 30 V, desolvation temperature of 600°C and source temperature of 150°C, collision energy between 15-40 eV optimized for different analytes. Quantitative calibration was performed by using a calibration curve using the internal standards for each of the analyte. The analysis was performed by monitoring two mass transitions for each analyte. The monitored multiple reaction monitoring (MRM) transitions (m/z) are shown in the supplementary table S1. The quantification limits were 0.01-0.05 nmol/L, except for dihydrotestosterone (0.1 nmol/L).

(b) GC-MS/MS

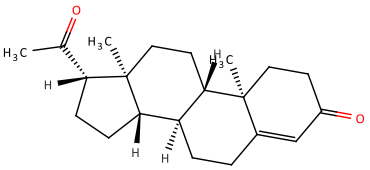
The steroids were extracted using the same procedure as for LC-MS/MS except that HLB cartridges (3 ml, 60 mg) were used for cleanup before hydrolysis, instead of C-18. Steroids were derivatized using 150 µl of methoxyamine (stock solution of 1 g methoxyamine hydrochloride in 50 ml pyridine) by incubating samples at 80°C for 1 hour. After evaporating the solvent at room temperature under nitrogen gas, samples were incubated with 200 µl N-trimethyl silyl imidazole overnight. Derivatized samples were washed with 4 ml n-heptane in 3 ml of 0.1 M HCl by vortexing. The upper heptane layer was collected, first by centrifuging for 4 minutes at 1200xg, and then cryo-phase separation by incubating samples for 1 minute at -45°C. This upper n-heptane layer was washed with 3 ml water by vortexing, centrifuging for 4 minutes at 1200xg, and followed by cryo-phase separation by incubating samples for 1 minute at -45ºC. The upper n-heptane layer was transferred to a fresh tube, evaporated and then re-suspended in 200 µl n-heptane.

Steroids were chromatographically separated on a J&W CP-Sil 5 CB column (15 m x 250 µm x 0.25 µm). A 7890A GC with 7000 Triple Quadrupole Detector (Agilent) was used for separation and detection using electron impact and multiple reaction monitoring. Nitrogen was used as collision gas (flow 1.5 mL/min), Helium as quench gas (flow 2.25 mL/min) and carrier gas (2 mL/min). For each sample 5 µl was injected at 65°C, with the MS source at

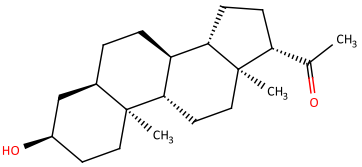
270°C and both quadrupoles at 150°C. Chromatography was performed using a temperature program for optimal separation: 1 min 50°C, ramp 50°C/min until 200°C, and finally ramp 2.5°C/min until 230°C. Electron impact was performed at 70 eV. The monitored MRM transitions (m/z) are shown in the supplementary table S1. The quantification limit for etiocholanolone was 1.0 nmol/L, and for pregnanolone 10.0 nmol/L. The higher limit of detection is a technical challenge associated with measuring these steroids in the gas phase in GC procedure.

Supplementary Table S1. Multiple Reaction Monitoring (MRM) transitions.

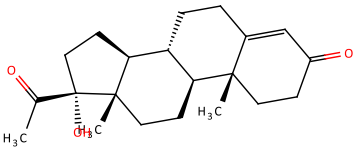
Compound	MRM I	MRM II
Progesterone	315 > 97	315 > 109
17-Hydroxyprogesterone	331 > 97	331 > 109
Dehydroepiandrosterone	253 > 197	271 > 213
Androstenedione	287 > 97	287 > 109
Testosterone	289 > 97	289 > 109
Pregnanolone	388 > 298	-
Etiocholanolone	360 > 270	360 > 213
Dihydrotestosterone	291 > 159	291 > 255
Estrone	269 > 145	269 > 159
Estradiol	271 > 145	271 > 183



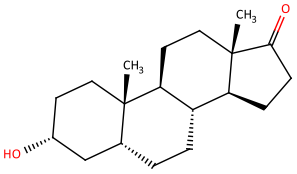
Progesterone



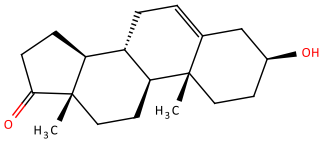
Pregnanolone



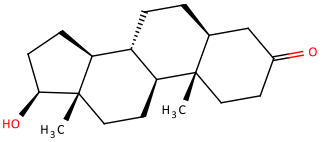
17-Hydroxyprogesterone



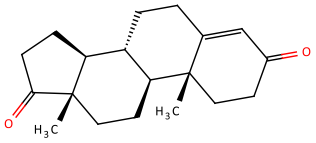
Etiocholanolone



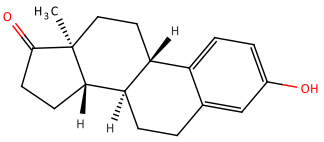
Dehydroepiandrosterone



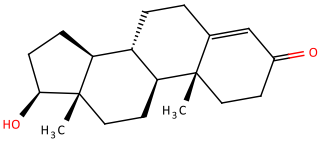
5α-Dihydrotestosterone



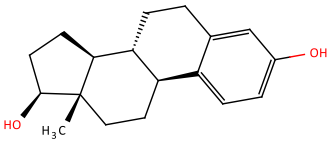
Androstenedione



Estrone



Testosterone



Estradiol

Supplementary Figure 1. Chemical structures of the compounds listed in supplementary table S1.