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Steroids in chicken egg yolk: Metabolism and uptake during early embryonic development

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ARTICLE INFO

ABSTRACT

Effects of maternal hormones may adaptively adjust offspring development to prevailing conditions. However, Darwinian fitness of parents is maximized by investing in more than one offspring while each individual offspring benefits from receiving maximal investment. The control of mother and offspring over hormone-mediated maternal effects is thought to play a key role in the outcome of parent–offspring conflict, but these control mechanisms have hardly been studied.

We investigated the potential embryonic control by analysing the changes in distribution and metabolism of steroid hormones in the egg during the first 6 days of incubation using injections of radiolabelled testosterone and corticosterone in freshly laid eggs. After 1 day of incubation the highest amount of radioactivity was concentrated in a small area at the top of the yolk. This challenges the use of hormones in oil as mimicking natural exposure. During incubation radioactivity spread within the egg with highest concentrations in yolk and yolk sac and lower concentrations in albumen, embryo, allantois, and amnion. Steroids were metabolised to other unconjugated and conjugated steroids, perhaps facilitating embryonic steroid uptake. Our study shows that the injected radiolabel is metabolised in the egg and taken up by the embryo, giving the embryo potential control over the effects of maternal hormones and thereby limiting maternal control over the outcome of hormone-mediated maternal effects.

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1. Introduction

Vertebrate embryos are exposed to substantial amounts of steroid hormones from their mother. Birds offer excellent models to study the mechanism and function of these hormone-mediated maternal effects since the embryo develops outside the mother's body, facilitating measurement and manipulation of early hormone exposure. Over the last decade there has been a surge in interest in the role of steroid hormones in avian yolk. The levels of these hormones in the egg vary both within- and between clutches in relation to a range of factors, such as position in the laying order, food availability, season, quality of the male or density and social interactions and have both short- and long-lasting effects on offspring development (Gil, 2003, 2008; Groothuis et al., 2005; Groothuis and Schwabl, 2008; Moore and Johnston, 2008; von Engelhardt and Groothuis, 2005). These findings have led to the hypothesis that by the modification of yolk hormone levels offspring development can be adaptively adjusted to prevailing conditions.

If we assume that the effects of hormones in the yolk indeed have an adaptive function and are not just an epiphenomenon of production of hormones for the maternal circulation, this raises the question, whether the effects of yolk hormones of maternal origin are adaptive for the mother or the offspring. While parents often benefit from more equal distribution of resources over more than one offspring, each individual offspring will benefit from obtaining a higher share of parental investment. Although maternal hormones may be signals rather than resources, they affect resource distribution by parental investment via affecting rate of development, begging behaviour, growth and sibling competition. It is often implicitly assumed that the embryo is only a passive receiver of the hormonal signals of the mother, providing the mother the upper hand in the parent–young conflict (Schwabl, 1993), but evolution might have provided the embryo with some degree of control or flexibility in the response mechanisms (Carere and Balthazart, 2007; Groothuis and Schwabl, 2008; Müller et al., 2007). A similar, but even more complex situation is discussed in the context of potential parent–offspring conflict in mammals regarding the effects of hormones produced by mother, placenta and offspring (Haig, 1993, 1996).

First, to exert an effect on offspring, hormones deposited into the egg before onset of embryonic development have to be taken up by the developing embryo. To date it is unclear how the highly lipophilic steroids are extracted from the yolk by the embryo (Moore and Johnston, 2008), In addition, the dynamics of the distri-

bution of the hormone within the yolk is relevant, since at laying different yolk layers contain different concentrations of steroids (Elf and Fivizzani, 2002; Groothuis et al., 2005).

Second, in order to respond to the maternal hormones the embryo needs to express receptors and establish physiological pathways (second messengers, DNA binding sites, etc.) that can result in an effect of the hormones on gene expression or non-genomic modulation of embryonic physiology. Indeed, the presence of steroid hormone receptors by day 4 has been demonstrated through detection of mRNA of androgen and oestrogen receptors (Smith et al., 1997; Andrews et al., 1997; Endo et al., 2007) and immunohistochemically using antibodies against progesterone receptor (Guennon et al., 1987) or oestrogen receptor (Andrews et al., 1997). Since this occurs before embryonic differentiation of the gonads and before endogenous steroids are detected (see Bruggeman et al., 2002) it is possible that receptor expression at this stage is an adaptation to maternal steroids.

Third, the embryo may produce hormone-metabolising enzymes that inactivate or modify the activity of maternal hormones. Enzymes or the mRNA for enzymes that convert steroids have been detected as early as day 2 of incubation (Nomura et al., 1999; Woods and Weeks, 1969). Parsons (1970) found that testosterone is clearly metabolized by day 2 blastoderm to mainly 5-β-reduced androgens. He was interested in these reduced androgens, since it had earlier been found that they control haemoglobin and porphyrin formation (Irving et al., 1976; Levere et al., 1967). Parsons even speculated about maternal steroids as a source for these metabolites. However, this suggestion has not been followed up and it is still unclear whether these metabolites derive from steroid hormones of maternal origin. The metabolism of corticosterone, the main embryonic corticosteroid has not been studied to our knowledge, but cortisol, one of the corticosteroids produced by chicken embryos from about day 9 (Kalliecharan and Hall, 1974) is converted in vitro primarily to 20-β-dihydrocortisol by the chorioallantoic membrane of 6- to 7-day-old embryos (McNatt et al., 1992). It would therefore be interesting to study whether maternal cortisol is present in the egg.

Unfortunately there are so far no studies of these control mechanisms in birds. A recent study very similar to ours using turtle eggs (Paitz and Bowden, 2008) found that radioactive oestradiol administered to the shell at laying was recovered in albumen and yolk, but not in the embryo within a few days of incubation and was converted to more polar compounds by enzymes that could be detected in the yolk, extra-embryonic membranes and the embryo but not the albumen. This study suggested conversion of steroids to water-soluble forms would help the transport of the hormone to the embryo.

In our study, we focused on the distribution, uptake and metabolism of steroid hormones from the yolk by the developing embryo. To this end we injected radiolabelled testosterone and corticosterone into freshly laid chicken eggs, incubated them for 1, 3 or 6 days and looked at the distribution of radioactivity in the different compartments of the egg and used thin layer chromatography for investigating the presence of different metabolites. We used testosterone and corticosterone, because these hormones have received the most interest so far in the field of avian maternal hormones and have clear and strong effects on embryonic development with long-lasting consequences.

2. Material and methods

2.1. Injections and incubation

We used three eggs for each hormone treatment and incubation time and therefore refrained from statistical analysis. Eggs were in-

jected and incubated in an incubator that turned eggs every 6 h by ca. 90 °C. Eggs were placed lying on their side, i.e., with the long axis horizontally, mimicking its natural position in a nest Under these conditions, due to the lower density of the embryo the yolk always turns so that the embryo is on its top. We therefore use this asymmetry to refer to top/up for the side of the egg where the embryo develops and bottom/down as the opposite side, while the blunt and pointed end of the eggs face sideways. For the injection, a small hole was drilled through the shell from the side in the middle of the long axis and about one-third from the top into freshly laid chicken eggs, which were then injected with ca. 64 kBq (2 μCi) of radiolabelled testosterone or 33 kBq (1 μCi) corticosterone in 50 μl sesame oil using a Hamilton syringe. For an average 60 g chicken egg this would result in a concentration of ca 1 Bq/mg (testosterone) or 0.5 Bq/mg (corticosterone) assuming even distribution over the whole egg (or, since maternal hormones are mostly found in the yolk, ca. 3 Bq/mg yolk (testosterone and 1.7 Bq/mg yolk (corticosterone) based on an average 20 g yolk). This is equal to ca. 0.06 pg/mg for both testosterone (specific activity ca. 5.1 TBq/mmol and molecular weight 288.43 g/mol) and corticosterone (specific activity ca. 3.1 TBq/mmol and molecular weight 346.46 g/mol). This equals about one-tenth of average testosterone levels of ca. 0.5 pg/mg measured in chicken yolk (Elf and Fivizzani, 2002) and much less than reported levels of corticosterone ranging between ca. 1.4 (Eriksen et al., 2003) and ca. 20 pg/mg (Rettenbacher et al., 2005), However, it should be noted that very little if any corticosterone may be present in avian eggs because immunoassays may primarily measure other substances that cross-react with the antibodies used (Rettenbacher et al., 2005).

Eggs were incubated for 1, 3 or 6 days at 37 °C and 50–70% relative humidity with their long axis horizontally and then frozen in the same position at −20 °C with the upper part marked for orientation purposes. Unfortunately, no embryo, extraembryonic tissue or blood vessels were visible in 3 day incubated eggs injected with testosterone, which may have been due to early embryonic death or infertility, but most likely not due to the treatment since all corticosterone-treated eggs and 6 day incubated eggs contained embryos.

2.2. Separation of eggs

Eggs were separated in a cold room (ca. 10 °C) by slowly thawing the eggs until they could be cut and separated in different components. If necessary, samples were (temporarily) placed on ice or dry ice to slow or stop the thawing process. Eggs were separated as described below depending upon the number of days of incubation.

2.2.1. One day incubated eggs

With the help of the mark on the top of the egg the eggs were oriented so that the part with the developing embryo would be on top. The shell of the egg was removed and a disk of about 5 mm thickness was cut transversely through the middle of the yolk from top to bottom. This slice was subsequently cut from top to bottom in three about equally wide sections and the middle section again cut horizontally into five approximately equally large parts of yolk plus the albumen at the top and the albumen at the bottom of the egg (see Fig. 1, left drawing).

In all testosterone-treated eggs and two of the three corticosterone-treated eggs a small area of lighter coloured material (ca. 4 mm in diameter) could be identified embedded in the upper part of the yolk at the border between yolk and albumen. This droplet was in the same area where we would also expect the developing embryo, but we could not see the latter in the partially thawed day 1 eggs. We assumed this droplet was the injected oil containing labelled steroid, which was confirmed during analysis, because of
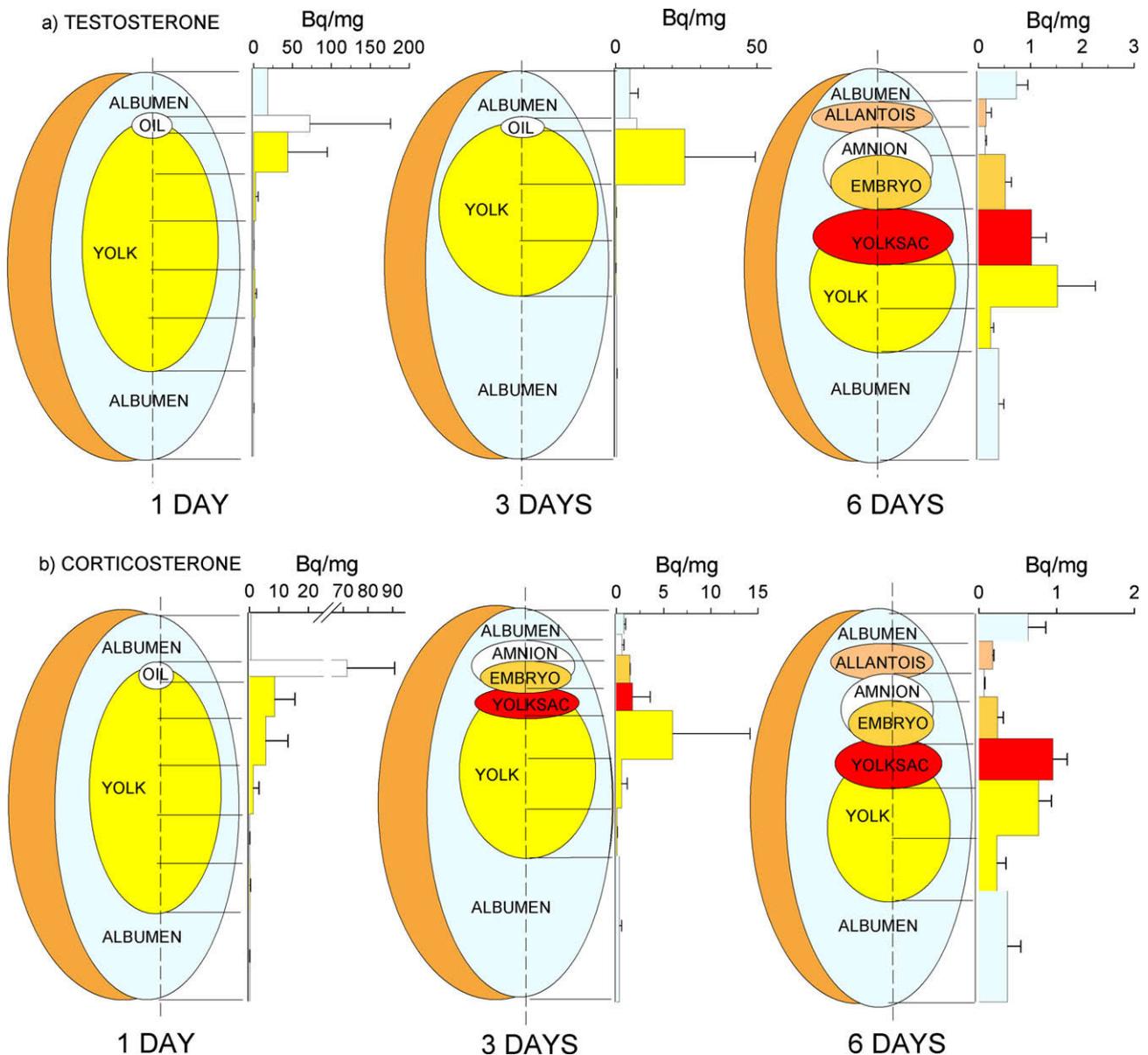
Fig. 1. Distribution of radioactivity in becquerel/mg of sample (mean and standard deviation) in a vertical transect of the egg for (a) testosterone and (b) corticosterone, showing the different egg components schematically, not in relation to their true size. In eggs incubated for 6 days, yolk could be separated in an upper thin yolk and a lower thick yolk corresponding approximately to the upper and lower part of the yolk shown.

the high levels of radioactivity it contained. The droplet was removed and analysed separately, although it could not be clearly separated from the surrounding material and was mixed with some adjacent yolk, sub-embryonic fluid and/or albumen.

2.2.2. Two days incubated eggs

The shell was removed and the embryo on top of the yolk was carefully removed. For all testosterone-treated eggs (which contained no visible embryo) we could also still identify the presumed oil droplet and analysed it separately. Tissue and blood vessels of the yolk sac were removed from the yolk. A disk was then cut as for day 1 eggs, except that the smaller central section was only cut into an upper, middle and lower part (Fig. 1, middle drawing).

2.2.3. Six days incubated eggs

Again the shell was removed and the embryo removed from the top of the egg. The embryo was rinsed with 0.9% NaCl. The amnion

was opened and the amniotic liquid collected. The allantois was separated from the embryo and the yolk sac tissue including blood vessels was removed from the yolk. Again a disk was cut through the middle of the egg, the middle section cut and separated in an upper and a lower part and albumen from above and below the yolk (Fig. 1, right drawing).

For all eggs, the remaining albumen and yolk was separated and collected to be used for extraction and thin layer chromatography. In 6 days incubated eggs, the yolk consists of two parts that can be easily separated: a lower part that resembles the yolk in fresh eggs and where the yolk rings are still visible (thick yolk) and an upper part directly below the embryo and covered by the yolk sac membrane which is more watery, due to uptake of water from the albumen (thin yolk or sub-embryonic fluid) (New, 1956; Romanoff, 1943).

All tissue samples (embryo, yolk sac, amnion, allantois) were weighed (between 3.9 and 1642 mg, depending upon the type of sample) and homogenized using a sonicator while keeping the

tissue on ice. Samples were frozen at −20 °C until further analysis. Yolk and albumen samples were homogenized either also using a sonicator or by the addition of some glass balls and vortexing. Up to 2 ml of water was added for homogenization for either very small or very dense samples and for rinsing of the sonicator to reduce losses of samples.

2.3. Scintillation counting

A small aliquot (between 50 and 500 µl, depending upon the size of the sample) of each homogenized sample was mixed with scintillation fluid (Ultima Gold, Perkin–Elmer) and analysed on a Liquid Scintillation Analyzer (Perkin–Elmer) and counts converted to becquerel using the tSIE method with an external standard and a quench correction curve made with egg yolk. Results are expressed per milligram of sample.

2.4. Extraction

The remainder of the homogenates (ca. 1–4 ml) was extracted 3 times with 2 volumes of diethyl ether to extract the apolar (mostly free) from the polar (mostly conjugated) steroids. An aliquot of the residual polar phase (between 100 and 200 µl) was counted to estimate the proportion of conjugated steroids. Ether extracts were dried, re-dissolved in 70% methanol and frozen over night at −20 °C to precipitate polar lipids that could interfere with the chromatography. Samples were centrifuged, the supernatants dried at 45 °C under a stream of nitrogen and redissolved in 50 µl ethyl acetate. Ten microlitres of this sample was counted to estimate the proportion of free steroids, the remaining 40 µl was used for thin layer chromatography (see below). All radioactivity lost during the extraction in the methanol precipitates, by absorption to the glass, or in other ways is included in the unaccounted fraction. Egg yolk, albumen and tissue are a complex matrix of proteins and lipids, so that, a clean and complete extraction of various metabolites with different physicochemical properties is difficult to obtain so that large losses during the required clean-up procedures for chromatography are usual.

2.5. Thin layer chromatography and autoradiography

Samples in ethyl acetate were spotted onto a thin layer chromatography plate (Sigma, containing a fluorescence indicator) about 1 cm from the bottom of the plate and with about 1 cm distance between successive samples. Unlabeled (ca. 1 µg) and labelled standards (progesterone, androstenedione, testosterone and corticosterone) were spotted as standards at the side and in the middle of the plate. Some test-plates were also run under the same conditions with additional unlabelled standards which moved up in the following order (upper, fastest moving first): 5-α-androstanedione > progesterone > 17-β-estradiol > 5-α-dihydrotestosterone > androstenedione > 5-β-dihydrotestosterone > testosterone > corticosterone > cortisol. The plates were developed by dipping them horizontally for varying amounts of time into solvent at the bottom of a container. The plates were dipped once into 100% methanol until the solvent front had moved up about 1.2 cm to concentrate the samples into a small zone. Then the plates were dipped into ethyl acetate:cyclohexane (1:1) until the solvent front had moved up about 7–12 cm. Separation of the unlabelled steroid standards was ascertained by their absorption of the fluorescence from the plate under UV-light. The plates were exposed for several days to a phosphor screen (Packard: type TR, tritium sensitive) and the latent image was detected using a Cyclone storage phosphor system (Perkin–Elmer).

3. Results

3.1. Distribution of radioactivity in the egg

After 1 day of incubation, highest concentrations of radioactivity were found in both hormone treatments in the upper area of the yolk, where the embryo develops and where the oil droplet moved to after injection (Fig. 1). Lower concentrations were found in the yolk directly below the droplet. Only very low or background levels of radioactivity were detected in the lower half of the yolk and the albumen.

After 3 days of incubation most radioactivity was found in the upper part of the yolk. We only detected embryos in eggs injected with corticosterone and we found some radioactivity in the yolk sac tissue, the embryo, amnion and albumen in the upper part of the egg, indicating hormone uptake by the embryo.

After 6 days of incubation, radioactivity was further distributed in the egg but the highest concentrations were still found in the yolk directly below the developing embryo and in the yolk sac membrane. Lower amounts were measured in the embryo and albumen and the lowest concentrations in lower part of the yolk, amnion and allantois.

3.2. Proportions of free and conjugated steroids

Fig. 2 shows that for all tissues and both hormones the apolar fraction decreased rapidly with days of incubation. The polar fraction containing presumably mostly conjugated steroids increased rapidly with incubation. Relatively large amounts of radioactivity of the original sample homogenates were not recovered in some extracts presumably because parts of the samples stuck to the sample tubes after extraction and drying and could not be re-dissolved and/or because they were contained in the methanol precipitates. Since this unaccounted radioactivity was extracted by ether from the homogenates it was probably mostly part of the apolar fraction.

3.3. Thin layer chromatography of free steroids

The fraction extracted with diethyl ether, containing the free (including potentially unmodified injected) hormones was separated on thin layer chromatography. For testosterone injected eggs we found the following: Already after 1 day of incubation at least one metabolite could be observed in the upper area of the egg, both in the yolk and the albumen, where the highest concentrations of radioactivity were measured and where the developing embryo would be expected (Fig. 3a). Its position indicates this metabolite could be androstenedione, but further verification is required since the resolution of the thin layer chromatography does not exclude the possibility that other testosterone metabolites may be found at the same position. No metabolites were observed in the lower yolk areas. After 3 and 6 days of incubation several metabolites were observed in all egg components analysed while after 6 days there was still only one compound at the expected position of testosterone in the lowest part of the yolk, suggesting no metabolism in this area.

For corticosterone injected eggs (Fig. 3b), no metabolism was observed after 1 day of incubation, except perhaps a more polar compound in the area where the embryo would develop and where highest concentrations of radioactivity were observed. After 3 days all radioactivity was still observed in the position of corticosterone, except for amniotic fluid where no radioactivity was observed at the position of corticosterone but at a lower position, suggesting a very polar metabolite, that moved only very little from the origin (the origin is the location where the sample was applied and can be
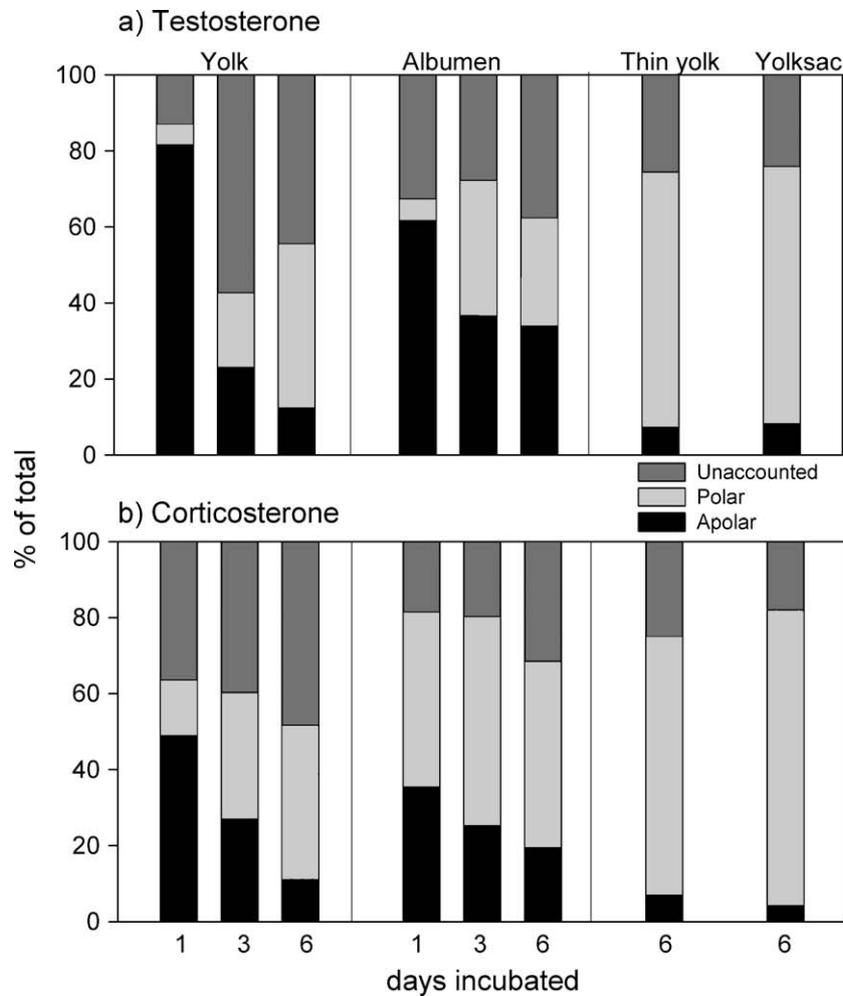
Fig. 2. Proportion of apolar (presumably mostly free) and apolar (presumably mostly conjugated) metabolites for (a) testosterone and (b) corticosterone in proportion of the total radioactivity of homogenates. Relatively large amounts of radioactivity in some fractions remained unaccounted presumably because the samples were sticking to the tubes after drying and could not be completely redissolved.

seen as the lowest dark spot in some standard lanes, an impurity that did not move from the origin). In eggs incubated for 6 days we observed radioactivity both at the position of corticosterone and more polar compounds.

4. Discussion

In the field of hormone-mediated maternal effects it is often assumed that the embryo acts as a passive receiver of the maternal hormones. However, in the light of parent–offspring conflict, the evolutionary optimum for the hormone effects on the offspring may be different for parents and offspring (Groothuis and Schwabl, 2008; Müller et al., 2007). Therefore we studied to what extent the embryo may play an active role in this conflict by investigating embryonic response mechanisms to the maternal hormone exposure. In addition, we analysed the distribution and uptake of in ovo injections of hormones, that may have important repercussions for manipulation and measurement of maternal hormones (Groothuis and von Engelhardt, 2005; von Engelhardt and Groothuis, 2005). We focused on testosterone and corticosterone which have attracted most attention in research on maternal hormones, since they have strong effects on offspring development and phenotype. It has to be noted, however, that only the presence of testosterone and other androgens in yolk has so far been clearly confirmed using mass spectrometry, while so far all studies on corticosterone have

relied on immunoassays that may detect structurally similar substances other than corticosterone (Rettenbacher et al., 2005). The biological relevance of our findings regarding corticosterone depends therefore on its natural endogenous presence in eggs.

4.1. Distribution dynamics and potential consequences

After 1 day of incubation the radioactivity was highly concentrated in the upper area of the yolk and usually the oil droplet was still visible as a lighter coloured droplet within the yellow yolk. Since the label was injected into the middle of the yolk, this shows that the oil droplet with the label moves upwards due to the lower density of sesame oil compared to yolk. This is consistent with earlier studies that injected eggs with a coloured substance dissolved in oil (Walker, 1967). This result suggest that in many studies in which eggs have been injected with hormones the blastoderm, being on top of the yolk, may have been exposed to pharmacological levels of the hormone, despite scaling the injection dose to the total amount of hormone in the yolk of eggs of that species. Moreover, since the yolk layers differ in hormone concentrations and may be taken up at different times in development, such a bolus of hormone may not mimic the natural distribution of the hormone in eggs at all.

However, there are two reasons why hormone injections in ovo may be less artificial than suggested above so that manip-
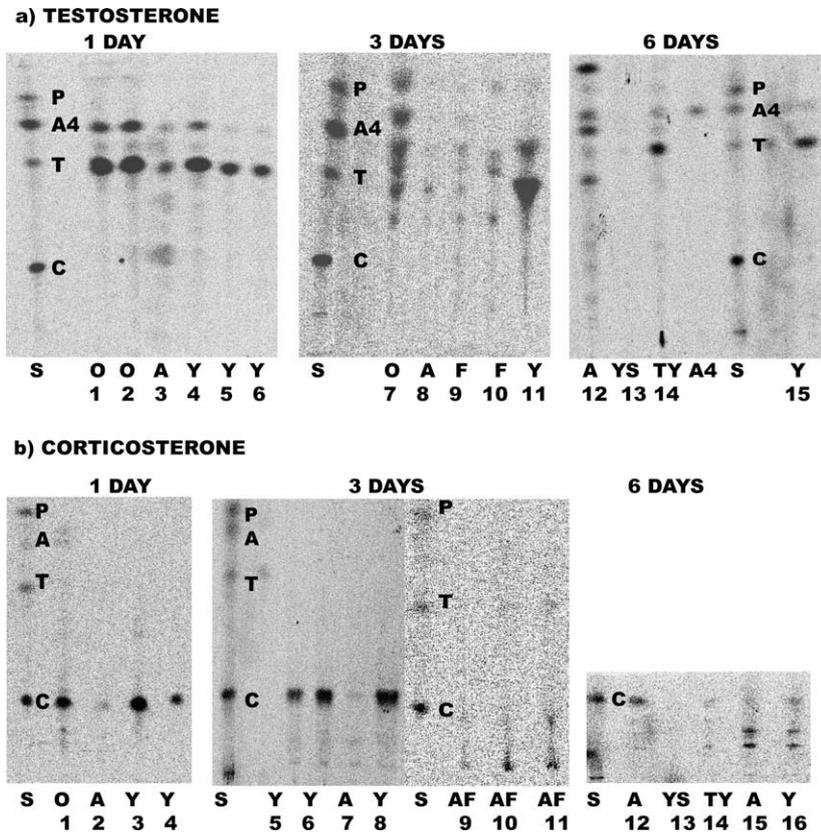
Fig. 3. Examples for separation of the apolar (free) fraction on thin layer chromatography. Samples and standards (S) are applied at the bottom of the plates and eluted from bottom to top. The least polar substances move fastest, the position of labelled standards is indicated to the right of the standards as follows: P (progesterone), A4 (androstenedione), T (testosterone) and C (corticosterone). The lowest spot seen in some standard lanes is an impurity at the location where the samples were applied. Egg components are indicated by letters as follows: O (oil droplet), Y (yolk), A (albumen), F (sub-embryonic fluid), YS (yolk sac), TY (thin yolk), AF (amniotic fluid). Samples: (a) Testosterone: 1 day incubated: S: standard, 1, 2: oil droplet at upper border of yolk and albumen; 3: albumen; 4, 5, 6: yolk with increasing distance from blastoderm. 3 days incubated: S: standard; 7: highly concentrated label at upper border of yolk and albumen; 8: albumen; 9, 10: sub-embryonic fluid; 11: yolk. 6 days incubated: 12: albumen; 13: yolk sac membrane; 14: thin yolk; A4: androstenedione-standard; S: standards; 15: yolk. (b) Corticosterone: 1 day incubated: S: standards; 1: oil droplet at upper border of yolk and albumen; 2: albumen; 3, 4: yolk with increasing distance from blastoderm. 3 days incubated: S: standards; 5, 6: yolk; 7: albumen; 8: yolk; S: standards; 9, 10, 11: amniotic fluid. 6 days incubated: S: standards; 12: albumen; 13: yolk sac membrane; 14: thin yolk; 15: albumen; 16: yolk.

ulations may not result in embryonic exposure to extreme hormone concentrations. First, the blastoderm is not always exactly on top of the yolk so that it may not always come in direct contact with the oil droplet. Second, in the course of early incubation the hormone spreads out within the egg in the upper thin yolk that is formed by mixing of yolk with water from the albumen resulting also in dissolution of the droplet and dilution of the initially highly concentrated hormone. Only the lower thick yolk retains the layered structure so that hormones are probably released successively here at the time of yolk uptake of particular layers. Third, since the developing blastoderm is separated from the yolk by the sub-embryonic fluid (New, 1956; Romanoff, 1943), which consists mainly of water being therefore very polar, it is likely that at least the free steroids will diffuse much more easily into the yolk than in the direction of the embryo.

To overcome the problem of artificial exposure by means of hormone injection, hormones could be injected in different dosages according to the concentrations in the different layers of the yolk and the time that that specific layer is consumed by the embryo. However, the latter is not yet known, while the possibility exists that the hormones are not taken up during the process of yolk consumption but are perhaps taken up only after conversion to a water-soluble form from the watery phase (see also Paitz and Bowden, 2008).

By day 3 the label had clearly mixed with the yolk. Although label was still present mostly in the upper yolk area of the egg, lower concentrations could now be detected in the developing embryonic tissues that were visible only for the eggs injected with corticosterone. This indicates for the first time that the embryo does take up maternal hormones or metabolites derived from these hormones at early stages of embryonic development.

By day 6, the label is still primarily found in the upper area of the yolk but also in relatively high concentrations in the yolk sac tissues, showing that the hormones are continued to be taken up by the developing tissues. Concentrations in allantois and amnion are lower than in the surrounding albumen and in the embryo. Allantois and amnion consist mostly of water so that the apolar original label would not dissolve well, but by day 6 most has been converted to more polar metabolites so that uptake would be expected. But since both allantois and amnion are growing rapidly during early embryonic development, this might result in a dilution of the label entering from the embryo, yolk or albumen. Also for other substances large variation of relative concentrations due to barriers between embryonic circulation, allantois and amnion have been observed (ten Busch et al., 1997).

In conclusion, the radiolabel is spreading during incubation from the yolk into all other compartments and is taken up by the developing embryo, but how far different processes, such as

diffusion, active uptake and active excretion play a role, remains to be studied.

4.2. Metabolism

4.2.1. Polar and apolar metabolites

The label was converted during embryonic development to more polar metabolites. These were presumably mostly conjugated steroids, since conjugation increases solubility in water, but the actual substances were not identified and further biochemical characterization of the polar substances was out of the scope of this study. This conversion may explain the rapid decrease in yolk hormone levels within the first days of incubation reported in previous studies (Bowden et al., 2002; Eising et al., 2003; Elf and Fivizzani, 2002). Steroid metabolism by the embryo and the extraembryonic membranes (yolk sac, allantois, amnion, chorion) has been observed in all species studied so far, in fish (Antila, 1984; Piferrer and Donaldson, 1994), amphibians (Antila, 1977), reptiles (Painter and Moore, 2005; Paitz and Bowden, 2008), birds (Antila et al., 1984; Parsons, 1970) and mammals (Levitz, 1966). Chick blastoderm can metabolise steroid hormones in vitro even within the first 2 days after laying (Antila et al., 1984; Parsons, 1970), producing both conjugated and free steroids. This early conversion of radiolabelled hormone would explain the substantial shift in the fraction of apolar and unaccounted fraction already between day 1 and day 3 under the assumption that the blastoderm in the eggs incubated for 3 days with testosterone had been alive but not (yet) produced red blood cells so that the embryo was not visible.

The proportion of conjugates was highest in the yolk sac tissue and in the thin yolk (sub-embryonic fluid), while there were less metabolites in the yolk further removed from the developing embryo. Since we would expect that the enzymes would be present everywhere in the yolk if they are of maternal origin, this suggests that the enzymes producing the metabolites are produced by embryonic tissues rather than of maternal origin. We therefore think that the metabolites in eggs that were incubated for 3 days with testosterone and contained no visible embryos were produced by embryos that died very early in development rather than by enzymes of maternal origin although we can currently not exclude this possibility.

In turtles, Paitz and Bowden (2008) found enzymatic activity that could convert free steroids into sulfonated conjugates in yolk and embryo but not in the albumen. This suggests that the increase in polar metabolites in the albumen is not due to enzymes in the albumen but due to diffusion or transport from the yolk into the albumen. In both the polar albumen and apolar yolk the proportions of polar and apolar steroids are surprisingly similar, which may relate to the movement of water from the albumen to the thin yolk, which could result in transportation of the polar metabolites from the albumen to the yolk and a decrease in concentration of the free steroids in the yolk.

Non-ether-extractable metabolites may include sulfates (Paitz and Bowden, 2008), glucuronides (Yeoh et al., 1996), fatty acid esters (Hochberg et al., 1977) and other conjugates. Steroid conjugates are often thought to be an inactive form of steroid that increases solubility in water and facilitate excretion (Pang et al., 1994), but conjugates may also be a transport form that can be converted again to active steroids and moreover, may be active compounds themselves (Coughtrie et al., 1998) that are somewhat protected from metabolism by conjugation (Hochberg et al., 1991). As neurosteroids they may modulate neurotransmitter systems (Baulieu and Robel, 1990; Compagnone and Mellon, 2000) and can act as sex pheromones, as studied especially in fish (Colombo et al., 1980; van den Hurk and Lambert, 1983). Interestingly, by converting the free steroids to conjugated forms the embryo may facilitate and regulate hormone uptake via the watery phase in the sub-embryonic fluid.

We did not further analyse metabolites in amnion and allantois, because until day 6 their size was very small and concentrations of label much lower than in the other tissues. Previous studies could not detect in both tissues free or conjugated steroids at any stage before endogenous hormone production starts (Epple et al., 1997; Gill et al., 1983).

4.2.2. Different forms of apolar metabolites

In the ether-extractable fraction, containing mostly the free steroids, we found one to several other metabolites after 1 day of incubation for testosterone and after 3 or more days of incubation also for corticosterone. The number of different metabolites increased during incubation. Metabolites were mostly found in the areas close to the developing embryo, i.e., in the upper area of yolk and albumen, sub-embryonic fluid or thin yolk, amniotic fluid and yolk sac. No or fewer metabolites were observed in the lower yolk that probably had not been in contact with embryonic or extraembryonic tissues, suggesting again that metabolism occurs only in active tissues and that mixing or diffusion especially within the yolk is limited.

We do not know which unconjugated metabolites could be formed from testosterone, but previous in vitro studies show that 5-β-reduced androgens (Antila et al., 1984; Parsons, 1970) can be produced even by 48 h old embryos. Aromatase which converts androgens to estrogens has only been found from day 6 (Haffen and Cedard, 1967) so that a production of estrogens is unlikely. The chorioallantoic membrane of 6-day-old chicken embryos metabolises cortisol (McNatt et al., 1992) to 20-β-dihydrocortisol, but corticosterone metabolism has not been studied to our knowledge. Further characterization and identification of the metabolites is planned.

Again, the most important question is whether the metabolites formed are active or inactive compounds. Usually 5-β-reduction is thought to represent an inactivation pathway, but the same 5-β-reduced androgens and progestins that the blastoderm can produce during early development (Antila et al., 1984; Parsons, 1970) affect various processes during early embryonic development, such as blood formation (Irving et al., 1976; Levere et al., 1967), and act as neurosteroids (Aragones et al., 1991; Viapiano and de Plazas, 1998). Some effects of corticosteroids on embryonic development may also be mediated by metabolites that are usually considered inactive since they have no classical glucocorticoid activity. 20-β-dihydrocortisol is formed by embryonic tissue from cortisol (McNatt et al., 1992) and has anti-angiogenic activity in chicken embryos as also other corticosteroids and their metabolites (Crum and Folkman, 1984; Folkman and Ingber, 1987).

5. Conclusion

A bolus injection of steroid hormone dissolved in sesame oil into the yolk will concentrate near the area where the embryo develops and subsequently diffuses to other parts of the egg, including embryonic tissue. After 6 days of incubation the hormone is still not evenly distributed over the yolk, challenging the widely use of hormone injections in the yolk as mimicking natural levels of exposure. The hormones are metabolized to other so far unidentified compounds but the original compounds appear to remain present as well, especially in the remaining parts of the yolk. In particular, there is a rapid increase in more polar, probably conjugated steroids, possibly to help the uptake of steroids by the developing embryo. Both parent compound and metabolites may play an important role in early embryonic development by regulating blood formation during early embryonic development, by act-

ing as neurosteroids and by affecting offspring development in various other ways.

Acknowledgments

We especially thank Erich Möstl for introducing us to the very useful technique of thin layer chromatography for analysing steroid metabolites. We thank Bonnie de Vries and Maarten Lasthuizen for technical help with the analysis of yolk hormones and Cees Vermeulen, Peter Wiersema and Jan Keijzer for contributing advice and equipment to do thin layer chromatography and autoradiography. Two anonymous reviewers provided very helpful comments on the paper. We gratefully acknowledge financial support from the EU – Marie Curie Intra-European Fellowship MEIF-CT-2006-039362 to Nikolaus von Engelhardt and from the Austrian Science Fund - project number 19169 to Rie Henriksen.

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