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*Published in:*  
General and Comparative Endocrinology

*DOI:*  
[10.1016/j.ygcen.2009.05.019](https://doi.org/10.1016/j.ygcen.2009.05.019)

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*Document Version*  
Publisher's PDF, also known as Version of record

*Publication date:*  
2009

[Link to publication in University of Groningen/UMCG research database](#)

*Citation for published version (APA):*  
Rettenbacher, S., Moestl, E., & Groothuis, T. G. G. (2009). Gestagens and glucocorticoids in chicken eggs. *General and Comparative Endocrinology*, 164(2-3), 125-129. <https://doi.org/10.1016/j.ygcen.2009.05.019>

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## Gestagens and glucocorticoids in chicken eggs

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### ARTICLE INFO

#### Article history:

Received 11 February 2009

Revised 23 April 2009

Accepted 27 May 2009

Available online 6 June 2009

#### Keywords:

Birds

EIA

Epigenetic

Gestagens

Glucocorticoids

Maternal effects

Progestins

### ABSTRACT

Avian eggs contain a variety of steroid hormones, which have been attributed as a tool for maternal phenotypic engineering. The majority of studies focuses on androgens, but also significant amounts of progesterone as well as other steroid hormones have been measured. The question if corticosterone is also present in eggs of chickens is currently under debate. The only analytical validation performed so far has failed to demonstrate corticosterone in the yolk of chickens, suggesting that antibodies for corticosterone measurement cross-react with other steroids present in the yolk. In order to investigate this assumption and to characterise potential cross-reacting hormones in more detail, we performed high-performance liquid chromatographic (HPLC) analyses of chicken yolk extracts and determined the concentration of immunoreactive corticosterone, progesterone and cortisol.

The progesterone antibody revealed several immunoreactive substances, including progesterone, pregnenolone and two substances with lower polarity. The corticosterone enzyme immunoassay detected immunoreactive substances at exactly the same elution positions as the progesterone assay and a very small peak at the elution position of corticosterone. Immunoreactive cortisol was not found. In addition, inner and outer regions of the yolk sphere were analysed separately via HPLC. We found different concentrations of immunoreactive substances between the inner and outer yolk regions, probably reflecting the steroidogenic activity of the follicle cells during oocyte growth. We conclude that in homogenised yolk extracts without previous clean-up, the measured corticosterone concentrations may actually reflect those of progesterone and its precursors, most probably being 5 $\alpha$ - and 5 $\beta$ -pregnanones and pregnenolone.

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### 1. Introduction

Avian eggs contain a variety of hormones, which have recently been attributed as a tool for maternal phenotypic engineering (Groothuis et al., 2005; Gil, 2008). The majority of studies investigating these maternal effects focuses on androgens, but also other gonadal steroid hormones have been measured in eggs of various species (e.g. Lipar et al., 1999; Williams et al., 2005; Bertin et al., 2008). Indeed, hormone mediated epigenetic effects have attracted a lot of attention especially of behavioural ecologists over the last decade, as it has been shown that yolk composition depends on environmental factors and maternal status, affecting the offspring both in the short and long-term. Maternal hormones are therefore regarded as a tool to adjust the offspring to current or future conditions in order to maximise fitness (Groothuis et al., 2005; Gil, 2008).

However, when quantifying a certain hormone, one important aspect is to prove the presence of the measured hormone in the egg (von Engelhardt and Groothuis, 2005). To our knowledge, this

has only been carried out for androgens (Schwabl, 1993) and gestagens (Möstl et al., 2001). Validation of the method has become increasingly relevant now that research shifts attention to a wider array of steroid hormones such as glucocorticoids, which are produced in the adrenals and can reach the egg only via the blood, in contrast to gonadal steroids which are produced by ovarian cells, at the interface between female circulation and the oocytes (Groothuis and Schwabl, 2008).

In female birds, the ovary is the main source of sexual steroids, and the three cell theory suggests that the granulosa cells are the predominant source of gestagens, which are used by the theca interna to produce androgens, which are subsequently metabolised to estrogens by the theca externa (Porter et al., 1989). During the course of follicular maturation the steroidogenic capacity of the cells changes, which results in the production of different amounts of hormones during egg development. At later stages, aromatase activity decreases and oestrogens are no longer produced (Kato et al., 1995). In the large preovulatory follicle cells, the enzymatic activity shuts down completely, resulting in an accumulation of progesterone and the preovulatory progesterone peak in the plasma (Etches and Duke, 1984). Besides from entering the bloodstream, from where they signal the current reproductive status

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to the rest of the body, a share of the ovarian hormones also ends up in the yolk, by either passive diffusion or a still unknown mechanism (Groothuis and Schwabl, 2008).

The distribution and proportion of steroid hormones found in the yolk of freshly laid, non-incubated eggs strongly reflect the metabolic activity of the ovarian cells: If the yolk is analysed in concentric layers, gestagens are found mostly in the outer layers, androgens in the middle layers and estrogens in the centre of the yolk (Lipar et al., 1999; Möstl et al., 2001; Hackl et al., 2003). Overall concentrations in the yolk are highest for gestagens and lowest for estrogens, whereas androgens are found in intermediate concentrations. This can be explained by the lipophilic characteristics of the hormones but also by the different diffusion distances from the respective cell layers to the yolk. All these facts support the assumption that the steroid hormones found in the yolk are most likely produced from their own surrounding follicle cells, rather than being transported to the egg via the blood. It therefore remains an open question, if and how steroid hormones from other endocrine organs are deposited into the yolk. Maternal glucocorticoids have been under current investigation, as it has been shown that artificial administration of corticosterone into eggs triggers a suite of changes in the developing embryo (Saino et al., 2005; Hayward et al., 2006; Janczak et al., 2007; Chin et al., 2009). However, it is questionable if glucocorticoids are present in eggs, as the only analytical validation performed so far has failed to demonstrate the presence of corticosterone in the yolk of domestic chickens (Rettenbacher et al., 2005), and the authors assume potential cross-reactions of the corticosterone antibody with progesterone or its precursors. To investigate possible cross-reacting gestagens, we performed high-performance liquid chromatographic (HPLC) analyses of yolk extracts and measured the fractions with a progesterone enzyme immunoassay (EIA) as well as with a corticosterone EIA. In addition, cortisol levels were determined, as it has been described as the predominant glucocorticoid in the early developing embryo (Nakamura et al., 1978). In a second step, we analysed inner and outer regions of the yolk separately, to assess different patterns and/or proportions in gestagen metabolites and quantified gestagen metabolites with two different progesterone antibodies.

## 2. Methods

We analysed eggs obtained from a same-aged group of female Lohmann brown laying hens, obtained from a commercial breeder (R. Schropfer, plc) that were kept in a free-range husbandry system. Eggs were randomly collected and frozen at  $-20^{\circ}\text{C}$  until analysis. The yolks of six eggs were pooled and six subsamples of each 5 g were mixed with 10 ml of double distilled water and tritiated progesterone (Perkin Elmer, MA, USA) to calculate recoveries as well as to have a standard elution pattern for the HPLC. Biological activity of the spike was 1.4 ng. After stirring the mixture for 30 min, 30 ml of 100% MeOH were added dropwise with stirring. After centrifugation, 30 ml of the supernatant were diluted with 45 ml double distilled water and loaded onto a primed Sep-Pak<sup>®</sup> C<sub>18</sub> cartridge via airflow. Cartridges were washed with double distilled water and 30% MeOH and then left to dry overnight. On the next day, elution was performed with 5 ml of 100% MeOH. After evaporation of the solvent, the eluate was resuspended in 1 ml MeOH. An aliquot of 50  $\mu\text{l}$  was used to determine recoveries of radioactivity, while the rest was injected onto an HPLC column. In total, six HPLC runs (one for each subsample) were performed.

We performed straight-phase HPLC with a 70:30 (*n*-hexane:chloroform) eluate and a gradient from 0 to 6% MeOH. Flow was 2 ml per minute and 76 fractions were collected in 30 s intervals. The solvent of the eluting fractions was evaporated and the samples were reconstituted in assay buffer. Aliquots of the fractions

were analysed for progesterone, corticosterone and cortisol (EIAs described below) and radioactivity was determined by liquid scintillation counting (Packard Tri-Carb 2100TR, Meriden, CT, USA).

For the separate investigation of the outer and the inner yolk regions, the outer 2 mm and the central parts of six frozen yolks were pooled and the intermediate parts discarded. For both regions, four subsamples of 5 g each were extracted. Tritiated pregnenolone and corticosterone (Perkin Elmer, MA, USA) were added as tracers. Biological activities of the spikes were 0.87 and 0.5 ng, respectively. Extraction and HPLC separations (eight runs in total) were performed as described previously. Concentrations of immunoreactive steroids were determined with antibodies against Cortisol, Corticosterone (Palme and Möstl, 1997) and  $5\beta$ -pregnane- $3\alpha$ -ol-20-one ( $5\beta$ -20-one EIA; Schwarzenberger et al., 1996). In addition, the HPLC fractions of the separated inner and outer regions were analysed with an antibody against  $5\alpha$ -pregnane- $3\beta$ -ol-20-one ( $5\alpha$ -20-one EIA; Schwarzenberger et al., 1996), to cover different gestagen metabolites. Recovery from the extraction was  $87 \pm 4\%$  for progesterone,  $83 \pm 3\%$  for pregnenolone and  $90 \pm 4\%$  for corticosterone, respectively. Losses during the HPLC procedure were  $23 \pm 15\%$  for progesterone,  $28 \pm 16\%$  for corticosterone and  $39 \pm 12\%$  for pregnenolone. Detection limits were 0.3 ng/g yolk for corticosterone, 0.2 ng/g for progesterone and 10 pg/g for cortisol.

## 3. Results

The elution positions (peaks) of the radiolabelled steroids assessed for the HPLC protocols in this study were fraction 14 for progesterone, fractions 16–18 for pregnenolone, fractions 34–35 for corticosterone and (not assessed in the present study) fraction 49 for cortisol. The elution patterns of the radiolabelled standards from the three presented yolk extracts (see below) are shown in Fig. 1. In our HPLC system, radioinert and radiolabelled steroid hormone standards show the same chromatographic characteristics. In the HPLC fractions of the homogenised yolk extracts, the cortisol antibody detected one main peak of immunoreactivity around fraction 22. In all other fractions, concentrations were below 0.7 ng per fraction. The progesterone EIA found three major immunoreactive peaks eluting around fractions 9, 13/14 and 16/17, respectively, with the second peak showing the same chromatographic mobility as the progesterone standard. In all HPLC runs performed, the first peak was higher than the two others and overall concentrations of immunoreactive metabolites were much higher in the progesterone EIA compared to the cortisol and the

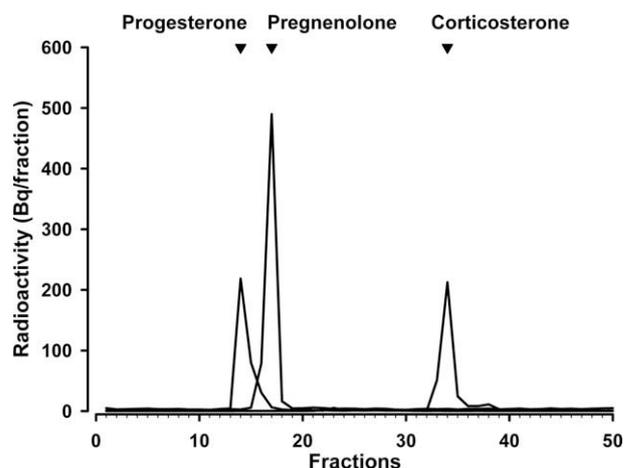
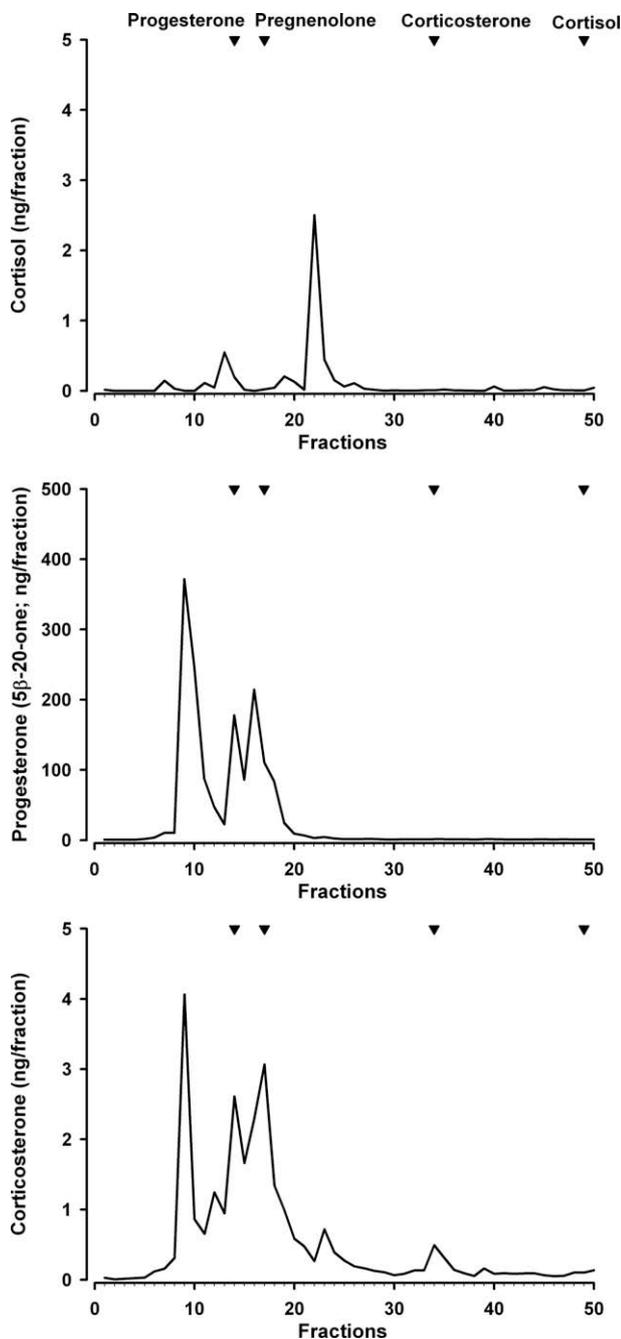


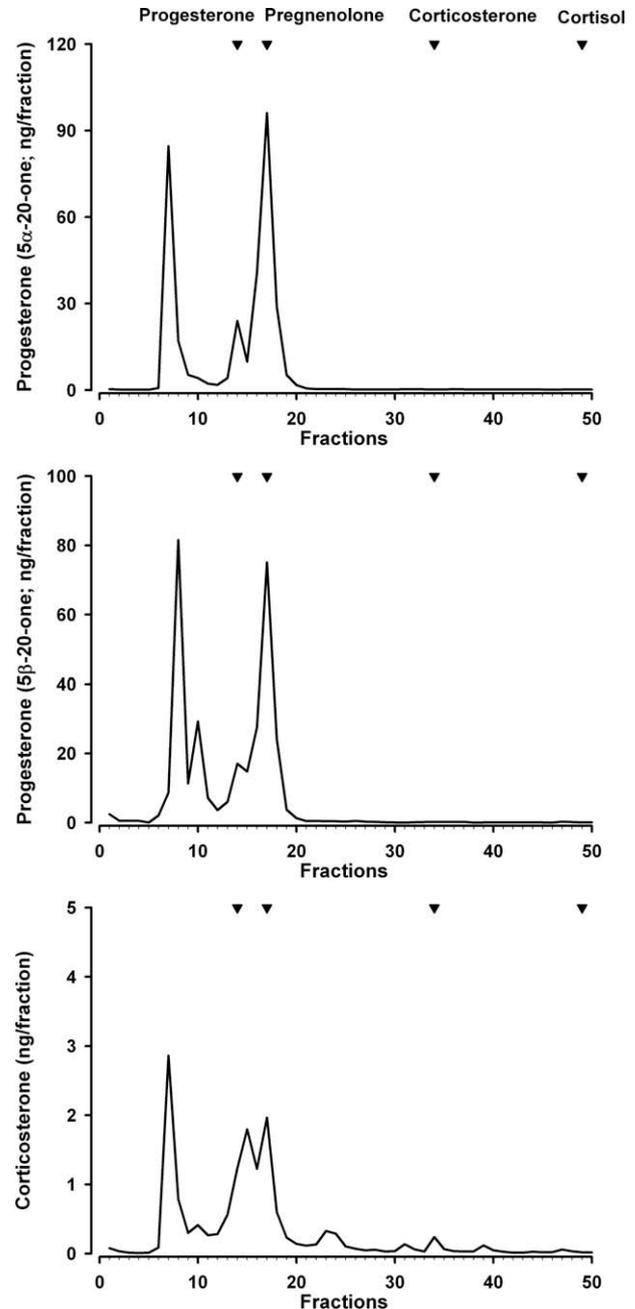
Fig. 1. Radioactivity measured in three separately injected yolk extracts each spiked with one radiolabelled hormone (progesterone, pregnenolone and corticosterone).

corticosterone EIA. The corticosterone antibody detected six immunoreactive peaks in total, three of them at the same elution positions as the progesterone EIA and three small peaks eluting around fractions 12, 22 and 35. In all other fractions, concentrations were below 0.7 ng per fraction. Concentrations of immunoreactive cortisol, progesterone and corticosterone concentrations in the HPLC fractions of one homogenised yolk sample are presented in Fig. 2.

In the HPLC separations of the outer and inner regions, the two progesterone antibodies again detected several immunoreactive substances in both the outer and the inner regions (Figs. 3 and 4). The first two peaks eluted around fractions 8/9 and 10–12.

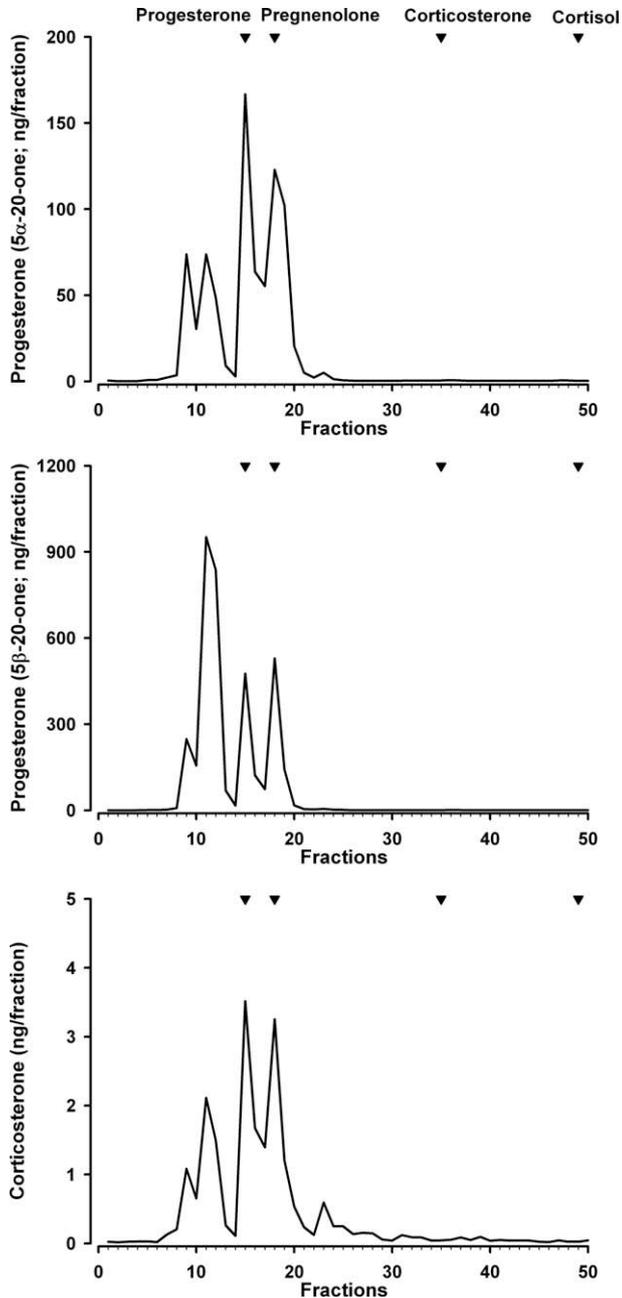


**Fig. 2.** High-performance liquid chromatographic separations of a homogenised yolk extract. Immunoreactivity was measured with a cortisol, a progesterone and a corticosterone enzyme immunoassay. Upside down triangles (▼) mark the approximate elution positions of respective standards.



**Fig. 3.** High-performance liquid chromatographic separations of the inner regions of a yolk extract. Immunoreactivity was measured with two progesterone and a corticosterone enzyme immunoassay. Upside down triangles (▼) mark the approximate elution positions of respective standards.

The third peak eluted at the previously established elution position of progesterone and the fourth peak showed the same chromatographic mobility as the pregnenolone standard. In the inner regions, in both assays the pregnenolone peak was more prominent than the progesterone peak and the first peak was very pronounced (Fig. 3). In the outer regions, this pattern was reversed and higher progesterone concentrations were observed in the 5 $\alpha$ -20-one EIA and high concentrations of the second peak in the 5 $\beta$ -20-one EIA, respectively (Fig. 4). Overall, the 5 $\beta$ -20-one EIA detected higher concentrations of immunoreactive metabolites compared to the 5 $\alpha$ -20-one EIA in the outer regions (paired *t*-test:  $p < 0.003$ ). The corticosterone antibody detected four major peaks at the previously described elution positions in both inner and out-



**Fig. 4.** High-performance liquid chromatographic separations of the outer regions of a yolk extract. Immunoreactivity was measured with two progesterone and a corticosterone enzyme immunoassay. Upside down triangles (▼) mark the approximate elution positions of respective standards.

er regions. In addition, a more polar metabolite in fraction 23 was found. A small peak around fraction 35, the elution position of corticosterone, was observed in the inner regions. In all other fractions, mean concentrations of immunoreactive corticosterone were below 0.7 ng per fraction. As in the progesterone EIAs, the first peak was higher in the inner regions compared to the outer regions and this was reversed for the second peak.

#### 4. Discussion

Corticosterone mediated prenatal maternal effects in birds are recently receiving increasing attention. However, the presence of the hormone in avian eggs has never been proven nor is there sufficient information about cross-reacting substances. The present

study aimed at characterising gestagen metabolites in the yolk of chickens and assessing potential cross-reacting compounds that might interfere with quantification of glucocorticoids or other steroid hormones. We performed HPLC separations of whole yolk extracts as well as of the inner and outer regions of the yolk sphere and determined concentrations of immunoreactive cortisol, progesterone and corticosterone via EIA.

As we did not detect immunoreactive metabolites at the suspected elution position of cortisol, we conclude that there is no cortisol of maternal origin present in the egg at the time of lay and that the cortisol found in the course of embryonic development (Jenkins and Porter, 2004) is probably a product of the embryo's own steroidogenesis.

Analysis of homogenised yolk with a progesterone antibody against 5β-pregnane-3α-ol-20-one revealed the presence of three major peaks, representing at least three or more immunoreactive steroids, with the second peak co-eluting with the progesterone standard and the third one showing the same chromatographic mobility as pregnenolone. This is partly in accordance with earlier findings by Möstl et al. (2001), who also described three major peaks. However, in their study, it was the third peak that co-eluted with progesterone. We assume that due to slight differences in the extraction method we extracted more polar substances (e.g. pregnenolone) better than it was the case in the study by Möstl et al. (2001). The separate analysis of the yolk regions revealed that concentrations of immunoreactive substances between the inner and outer yolk regions differ, and in the outer regions measured with the 5β-20-one EIA, highest concentrations were found. In the inner regions, which are produced earlier during yolk formation, the pregnenolone peak was more prominent, whereas in the later formed outer regions there was more progesterone present. This finding corresponds with the steroidogenic activity and the enzymatic capacities of the follicle cells: In small follicles, which have not entered the follicular hierarchy, the theca cells convert pregnenolone to dehydroepiandrosterone, to further synthesise androstenedione and estradiol via the Δ5-pathway (Lee et al., 1998). As the follicle matures, the enzymatic capacity of the granulosa cells increases rapidly and the steroid production shifts towards the Δ4-pathway, now using progesterone and 17α-hydroxyprogesterone as substrates for androstenedione production (Robinson and Etches, 1986).

In addition to progesterone and pregnenolone, the two progesterone antibodies detected two gestagen metabolites with a lower polarity than progesterone, most likely 5α- and 5β-pregnane-3,20-diones, which were detected by the two antibodies with different affinities. Gomez et al. (1998) postulate the activity of the enzyme 5β-reductase in the follicle, and 5α- and 5β-reduced metabolites have also been found in the chicken brain but their function is yet unclear (Sharp and Massa, 1980). Further characterisation of the gestagens in yolk could be done with a more specific HPLC protocol (Palme et al., 1997). If there is a 5α- and 5β-reductase enzymatic activity in the yolk follicle, the presence of 5α- and 5β-dihydrotestosterone, metabolised from testosterone is also conceivable. 5α-Dihydrotestosterone has already been measured (e.g. Gil et al., 1999; Tschirren et al., 2004), however, validation of these steroid hormone metabolites was not performed yet and should therefore be addressed in future studies.

In both, the homogenised yolk extracts as well as in the separated inner and outer regions, the corticosterone antibody detected immunoreactive substances at exactly the same elution positions as the progesterone antibodies. This supports suggestions by Rettenbacher et al. (2005) that the gestagens in the yolk cross-react with our corticosterone antibody and the measured concentrations in homogenised yolk samples is in fact a signal caused by gestagen metabolites. Cross-reactions, assessed at 50% binding for our assay were only 1.4% for progesterone and pregnenolone and 1.3% for

17 $\alpha$ -hydroxyprogesterone. However, given the high concentrations of gestagens relative to other hormones (von Engelhardt and Groothuis, 2005), even such low cross-reactions can substantially confound measurements.

When yolk regions were analysed separately, we found a small peak in the fractions at the elution position of corticosterone in the inner layers, but in none of the outer layers. This finding raises the question, if corticosterone is transferred to the egg only during early yolk formation, due to a possible alteration in cell permeability or changes in enzymatic activity (enzymes are saturated with other metabolites early in follicular development) as well as the biological relevance of such minor concentrations. In conclusion, our data strongly imply that the measured concentrations of corticosterone may actually reflect those of gestagens. The present study however describes findings in unmanipulated chickens. It remains an open question, if elevation of circulating plasma corticosterone levels in the female within the physiological range will affect yolk hormone composition. A short-term increase in plasma corticosterone concentrations after a single injection of ACTH could not be traced in the eggs (Rettenbacher et al., 2005). In the same study, feeding hens with 0.1 g of crystalline corticosterone resulted in a detectable increase in the yolk. However, this feeding manipulation did not mimic physiological conditions. If plasma corticosterone concentrations are manipulated within the physiological range, a rigorous elimination of the cross-reacting gestagens from the yolk via a clean-up procedure should be performed, before applying a corticosterone immunoassay.

As the present study was conducted in a domesticated species, the situation in wild birds remains elusive. In a number of different avian species, higher yolk corticosterone concentrations were measured after experimental elevation of plasma corticosterone concentrations to high physiological levels (see Groothuis and Schwabl (2008) for a review). Whether this increase is caused by authentic corticosterone and/or yolk gestagens remains to be investigated. Also, the epigenetic potential of gestagens has not been addressed in birds yet.

## Acknowledgments

The authors want to thank Amanda Aichinger for her excellent technical assistance and Alexandra Kuchar for performing the HPLC separations. The support of the Austrian Science Foundation (FWF Project No. 19169-B17) is gratefully acknowledged.

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