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Corticosterone in bird eggs: The importance of analytical validation

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■ Summary

It was recently found that high concentrations of chicken yolk gestagens and gestagen metabolites hamper corticosterone quantification via immunoassays. However, the situation in chicken albumen is still unresolved. In addition, the ratio of steroid hormone in the yolk of wild birds might differ. To investigate these matters, corticosterone and gestagens were measured in individual fractions of high-performance liquid-chromatographic separations of chicken albumen and yolk of red jungle fowl. Similarly, yolk extracts of hens with corticosterone-releasing implants or placebos were analysed to assess the impact of elevated plasma corticosterone concentrations on authentic yolk corticosterone levels. We also compared the results of a previously used corticosterone enzyme immunoassay (EIA) to those from a commercial radioimmunoassay (RIA) kit. The analytical validations of chicken albumen, bankiva yolk and yolks from hens with or without artificially elevated plasma corticosterone levels indicated that the main share of the immunoreactivity measured via corticosterone immunoassays was caused by substances other than authentic

Schlüsselwörter: Kortisol, Glukokorticoide, maternaler Stress, Enzymimmunoassay, Kreuzreaktionen, Steroid.

■ Zusammenfassung

Kortikosteron im Vogelei: Die Wichtigkeit der analytischen Validierung

Einleitung

Kürzlich wurde festgestellt, dass Eidotter von Hennen hohe Konzentrationen an Gestagenen und Gestagenmetaboliten enthält, welche die Quantifizierung von Kortikosteron mittels Immunoassays behindern. Ob dies auch im Eiklar so ist, war bisher unklar. Weiters könnte Eidotter von Wildvögeln ein anderes Verhältnis von Steroidhormonen aufweisen.

Material und Methoden

Es wurden daher Dotterproben von Bankivahühnern und Eiklar von Haushühnern nach Extraktion mittels Hochleistungs-Flüssigkeitschromatographie getrennt und Kortikosteron sowie Gestagene in den einzelnen Fraktionen quantifiziert. In gleicher Weise wurden Dotterproben von Hennen, die mit einem kortikosteronfreisetzenden Pellet oder einem Placebo implantiert waren, analysiert, um die Auswirkungen von erhöhtem Plasmakortikosteronspiegel auf die Dotterkortikosteronkonzentration zu testen. Zusätzlich verglichen wir den zuvor

verwendeten EIA mit einem kommerziellen RIA Kit.

Ergebnisse

Die analytischen Validierungen von Hühnereiklar, Bankivadotter und Dotter von Hennen mit erhöhten oder nicht erhöhten Plasmakortikosteronspiegeln ergaben, dass der Hauptanteil der von den Kortikosteronimmunoassays gemessenen Immunreaktivität nicht von Kortikosteron selbst, sondern von anderen Substanzen verursacht wurde. Im Eiklar konnte echtes Kortikosteron nicht nachgewiesen werden. Bei der Analyse von Bankivadotter wurden drei Gestagenspitzen mit Konzentrationen von bis zu 2000 ng pro Fraktion entdeckt, während der Kortikosteronspitzenwert bei 0.8 ng pro Fraktion lag. Beide Kortikosteronimmunoassays fanden einen etwas höheren Kortikosteronwert im Dotter der mit dem Kortikosteronpellet implantierten Henne (EIA: 0.7 ng; RIA: 0.5 ng pro Fraktion) im Vergleich zum Kontrolltier (EIA: 0.5 ng; RIA: 0.2 ng pro Fraktion), aber beide Antikörper banden auch etliche andere Substanzen, höchstwahrscheinlich Gestagene.

Schlussfolgerungen

Unsere Ergebnisse zeigen, dass obwohl ein geringer Teil von zirkulierendem Kortikosteron in den Dotter überzutreten scheint, die direkte Messung von Kortikosteron im Vogelei mittels Immunassays nicht anzuraten ist.

corticosterone. In albumen, the concentration of authentic corticosterone was below the detection limit. Analysis of bankiva yolk revealed three major gestagen peaks with concentrations of up to 2000 ng per fraction and a corticosterone peak of about 0.8 ng per fraction. Both corticosterone assays found a slightly higher corticosterone peak in a corticosterone-implanted hen's yolk (EIA: 0.7 ng; RIA: 0.5 ng per fraction) compared to the sham-treated female (EIA: 0.5 ng; RIA: 0.2 ng per fraction) but both antibodies also bound to several other substances, presumably gestagens. Although a certain amount of circulating corticosterone might pass into the yolk, direct quantification of corticosterone in non-homogenized avian egg samples via immunoassays is not advisable.

Abbreviations: EIA = enzyme immunoassay; HPLC = high-performance liquid-chromatography; RIA = radioimmunoassay

■ Introduction

Prior to ovulation, the avian follicle produces a variety of steroid hormones (ETCHES and DUKE, 1994), which partly accumulate in the yolk of the egg. Ample research has shown that these hormones influence both physiology and behaviour of the developing bird embryo. Maternal steroid hormone deposition in eggs is therefore regarded as a mechanism to optimize reproductive success and hormone-mediated maternal effects have recently received considerable attention. Most studies focus on functional approaches, although the validity of the analytical methods applied has not yet been critically assessed. CEKAN (1979) defines a 'valid' assay as the one that yields results identical to the true value within the limits of experimental error. The author also highlights the relevance of both the specificity of the antiserum and the ratio between the steroid of interest and impurities in the sample. If a cross-reacting substance is present in high concentrations, measurements can be confounded, despite a low level of cross-reactivity. The latter point is especially important when a previously uncharacterized matrix is analysed and it is important to assess which substances are actually measured by the method used (MÖSTL et al., 2005). When high-performance liquid-chromatographic (HPLC) separations of chicken yolk extracts were analysed with a corticosterone immunoassay, it was found that the corticosterone antibody detects high amounts of immunoreactive substances that differ from authentic corticosterone (RETTENBACHER et al., 2005). The authors concluded that the signal measured by the corticosterone immunoassay in homogenized yolk is not caused by corticosterone itself. It was subsequently found that these immunoreactive substances show the same chromatographic characteristics as the gestagens progesterone and pregnenolone and the gestagen metabolites 5α- and 5β-pregnane-3,20-diones, all of which cross-react with the corticosterone antibody used (RETTENBACHER et al., 2009).

Compared to other gonadal steroids, gestagens are present at extremely high levels in the yolk, partly due to their lipophilic characteristics, but also because they are produced by the granulosa cells, which are closest to the yolk, whereas androgens and oestrogens are synthesized further away in the theca cell layer. Absolute levels differ slightly between studies due to differences in analytical methods but progesterone concentrations of about 1000 ng/g yolk are generally reported (e.g. LIPAR et al., 1999; MÖSTL et al., 2001; BERTIN et al., 2008) and pregnenolone levels have been reported to be about 10-fold higher than those of progesterone (HARTMANN et al., 1998). The absolute concentrations of corticosterone are much lower than those of gestagens, probably because the glucocorticoid corticosterone is mainly produced in the adrenocortex and has to reach the oocyte via circulation. By means of gas chromatography-mass spectrometry (GC-MS), SAS et al. (2006) found corticosterone levels of between 0.2 ng and 0.9 ng/g yolk. This illustrates that even a low cross-reactivity of about 0.1% of a corticosterone antibody with e.g. progesterone would already result in an overestimation of the actual corticosterone concentrations in yolk. This poses serious limitations to the application of immunoassays for the quantification of corticosterone in yolk without previous clean-up and has frequently led to an overestimation of actual yolk corticosterone levels.

The situation in the albumen remains elusive, as analytical validations have not yet been performed. In contrast to the yolk, which accumulates during the maturation of the steroid-synthesising follicle, albumen is secreted in the oviduct and progesterone concentrations in the albumen are much lower than in yolk (MÖSTL et al., 2001). Quantification of corticosterone in albumen might therefore be less confounded by cross-reacting compounds. To investigate this point we performed HPLC immunograms of chicken albumen samples and compared measurements of a progesterone and a corticosterone enzyme immunoassay (EIA). Similarly, we conducted analytical validations for yolk of bankivas (*Gallus gallus bankiva*). Bankiva or red jungle fowl is the ancestor of the domestic chicken (SAWAI, 2010) and in contrast to domestic chicken strains has not been subjected to artificial selection for reproduction (ROMANOV et al., 1999). Its egg yolk might thus have a different hormone composition. In addition, the impact of experimentally elevated plasma corticosterone concentrations on yolk corticosterone has not yet been addressed with the proper analytical methods in chickens, as egg yolks analysed previously were collected from unstressed chickens (RETTENBACHER et al., 2009). We therefore analysed yolks from hens with or without corticosterone-releasing implants. We also compared measurements made with the previously used in-house corticosterone antibody with those

obtained with a commercially available RIA. The aim was to extend our knowledge about hormones in the yolk of a domestic species and to shed light on the situation in bird eggs in general.

Material and methods

Animals and sample collection

Eggs for the albumen analysis were obtained from a same-aged group of female Lohman brown laying hens that were kept in a free-range husbandry system. Yolks of the same eggs were analysed in a previous study (RETTENBACHER et al., 2009). Eggs from bankiva chickens were obtained from the University of Groningen's laboratory breeding population. Female and male bankivas were housed pair wise in outdoor aviaries. Domestic chickens and jungle fowl were not being used for experiment around the time eggs were collected.

Yolks of chickens with elevated concentrations of plasma corticosterone were obtained from an experiment where chickens were implanted with corticosterone-releasing pellets or placebo pellets (for details see HENRIKSEN et al., 2011a). Following implantation, concentrations of plasma corticosterone were significantly elevated over a period of nine days. We used eggs that were yolked when the females' plasma corticosterone levels were maximally elevated for our analysis: Deposition of radiolabelled hormones or substances such as Sudan dyes into the yolk of chickens occurs during the last eleven days of yolk formation (GRAU, 1976; GILBERT et al., 1983; RETTENBACHER et al., 2005) and an additional 25-hour period is necessary for albumen and shell formation (WARREN and SCOTT, 1934). Therefore, authentic yolk corticosterone concentrations were analysed via HPLC immunograms of eggs from day 14 after implantation: such eggs would have experienced peak concentrations of circulating plasma corticosterone.

Extraction of egg albumen

From each of six eggs, 10 ml of albumen were mixed with 10 ml of double-distilled water and 1 ml of saturated $NaHCO_3$ and stirred for 30 min. Tritiated progesterone and corticosterone (all radiolabelled hormones from Perkin Elmer, MA, USA) were added as standards. 40 ml of methanol (100%) was added dropwise and the mixture was stirred for 30 min. After centrifugation, 30 ml of the supernatant was diluted with 45 ml of double-distilled water and loaded onto a primed Sep-Pak® C_{18} cartridge (Waters) via airflow. Cartridges were washed with 10 ml each of double-distilled water and methanol (30%) and left to dry overnight. Elution was performed the following day with 5 ml methanol (100%). Samples were dried under nitrogen flow, suspended in 0.1 ml of n-hexane:chloroform (70:30) and injected onto the HPLC column (for chromatographic conditions see below).

Extraction of yolk

Yolks of two bankiva eggs were homogenized and 5 g of each yolk sample was mixed with 10 ml of double-distilled water and stirred for 30 min. For the chicken yolks, two eggs laid on day 14 after implantation were analysed. One egg was collected from a female that had been implanted with a corticosterone-releasing pellet (cort chicken), the other from a placebo pellet-implanted chicken (placebo chicken). For recoveries and standards, radiolabelled corticosterone was added to all yolk samples and radiolabelled pregnenolone was added to the bankiva yolk samples. After drop wise addition of 30 ml methanol, samples were stirred for 30 min. After centrifugation, supernatants were processed as described above for the albumen. Straight-phase HPLC separations were performed under room-temperature with a LiChrosorb® Si 60 (10 μm)-filled column (Forschungszentrum Seibersdorf, Austria) using a mixture of n-hexane:chloroform (70:30) as eluate and a linear methanol gradient from 0–6% methanol. The flow was 2 ml per min and 76 fractions were collected at 30 s intervals. The eluate was evaporated and samples were reconstituted in assay buffer. Aliquots of the fractions were used to determine radioactivity via liquid scintillation counting (Packard Tri-Carb 2100TR, Meriden, CT).

Immunoassays

In the chicken albumen and bankiva yolk extracts, concentrations of immunoreactive corticosterone and progesterone were determined using antibodies against corticosterone (PALME and MÖSTL, 1997) and 5β-pregnane-3α-ol-20-one (SCHWARZENBERGER et al., 1996) as described previously (RETTENBACHER et al., 2009). The yolk samples of the pellet-implanted chickens were also analysed with a commercial RIA (MP Biomedicals, Costa Mesa, CA) as described by GOERLICH et al. (2010).

Results

The elution positions of the radiolabelled standards differ between the individual chromatograms, which we attribute to the fact that HPLC separation was performed at room temperature. Nevertheless, a reliable separation of corticosterone from cross-reacting gestagens was achieved in all runs. The elution positions of the radiolabelled standards in the albumen analysis were fraction 16 for progesterone and fraction 35 for corticosterone. In the bankiva yolk analysis, the pregnenolone standard eluted in fraction 15 and the corticosterone standard in fraction 28, while fraction 32 was established for the corticosterone

standard in the chicken yolk analysis. The exact elution positions of gestagens were not assessed in the chicken yolk HPLCs. Mean (±s.d.) recovery of the radio-labelled tracers was 87±13% for progesterone and 87±9% for corticosterone. The recovery of radiolabelled pregnenolone (used only for bankiva yolks) was not assessed. The detection limits of the enzyme immunoassays were 0.4 ng per fraction for progesterone and 0.01 ng per fraction for corticosterone in albumen. In the yolks, the detection limit for corticosterone was 0.01 ng/G both for the EIA and for the RIA. For practical reasons, chicken yolk extracts were analysed several weeks after the analysis of albumen and bankiva yolk extracts, but HPLC separation of corticosterone and placebo pellet-implanted chicken yolk extracts was performed on the same day and under the same conditions.

Albumen

In the albumen, concentrations of immunoreactive metabolites measured by the corticosterone EIA did not exceed 0.8 ng in any fraction. Two large peaks of about 0.8 ng were detected in fractions 12 and 16, with the second peak co-eluting with the progesterone standard. A third smaller peak was found around fraction 20. No peaks were visible at the elution position of corticosterone (fraction 35) and concentrations were below 0.07 ng in all other fractions. In the progesterone EIA, immunoreactive peaks were detected in exactly the same fractions as in the corticosterone EIA but in this case the two peaks in fractions 12 and 16 were not of the same height: the first peak was around 18 ng per fraction and the second peak, co-eluting with the progesterone standard, was only about 4 ng per fraction. Concentrations were below 1 ng in all other fractions (see Fig. 1a).

Yolk of bankiva

In the HPLC separations of the bankiva yolk extracts, the corticosterone EIA detected three peaks around fractions 7, 10 and 15 with concentrations of about 1.2 ng to 2 ng per fraction. The third peak co-eluted with the pregnenolone standard. A much smaller peak of about 0.3 ng eluted in fraction 20. At the elution position of authentic corticosterone, a peak of about 0.8 ng per fraction was detected. In all other fractions, immunoreactivity was below 0.3 ng per fraction. The progesterone EIA detected immunoreactive peaks in the same fractions but concentrations declined from first to second and third peak, ranging from 1700 ng in fraction 7 to 310 ng in fraction 10 and 170 ng in fraction 15, respectively (see Fig. 1b).

Pellet-implanted chicken

In the yolk of the corticosterone pellet-implanted chicken, the corticosterone EIA detected one peak of about 1.7 ng in fraction 7. Between fractions 13 and 17, several immunoreactive substances eluted

as one large second peak with concentrations of between 1 and 2 ng per fraction. An immunoreactive peak of about 0.7 ng per fraction was observed at the elution position of corticosterone. In all other fractions, concentrations were below 0.08 ng. When the same fractions were analysed with the corticosterone RIA, six individual peaks between fractions 7 and 25 could be distinguished, with concentrations ranging from 0.8 ng to 0.2 ng per fraction. A corticosterone peak of about 0.5 ng was found in fraction 32. In addition a very small peak around fraction 38 was visible, which was not observed in the EIA analysis (see Figure 2a). In the placebo chicken yolk, the corticosterone EIA detected three major non-polar peaks in fractions 8, 12 and 17 of about 2.2 ng, 3 ng and 1.6 ng per fraction, respectively. A peak at the elution position of corticosterone of 0.5 ng per fraction was also observed. The corticosterone RIA detected five or more immunoreactive substances in fractions 8 to 23 with concentrations ranging from 0.6 ng to 0.4 ng and a peak of about 0.2 ng per fraction at the elution position of corticosterone. Again, substances with higher polarity that had a longer retention time than corticosterone eluting around fraction 38 were detected by the corticosterone RIA but not by the EIA (see Fig. 2b).

■ Discussion

Quantification of corticosterone in eggs has been used as a non-invasive method to quantify stress as well as to assess the impact of maternal stress on offspring. However, analytical validations should always be performed before embarking on the analysis of a previously uncharacterized matrix. The crucial aspect is a thorough evaluation of what is picked up by the antibody used and whether the resulting measurement truly reflects the substance of interest at biologically relevant concentrations. To test a newly developed antibody, CEKAN (1979) coined the term 'assay validity'. To assess which substance in a sample is actually measured, the term 'analytical validation' has evolved in relation to non-invasive monitoring of hormones in excreta (MÖSTL et al., 2005; TOUMA and PALME, 2005). In this approach, samples are first subjected to chromatographic purification and the collected fractions are measured by means of the immunoassays in question. In this manner, RETTENBACHER et al. (2009) revealed that chicken egg yolk contains high concentrations of gestagens and gestagen metabolites that give rise to a signal in a corticosterone enzyme immunoassay. Due to the vast differences in absolute concentrations between gestagens and corticosterone, quantification of corticosterone via immunoassays in chicken yolk is hampered, even when cross-reactivities with e.g. progesterone seem small.
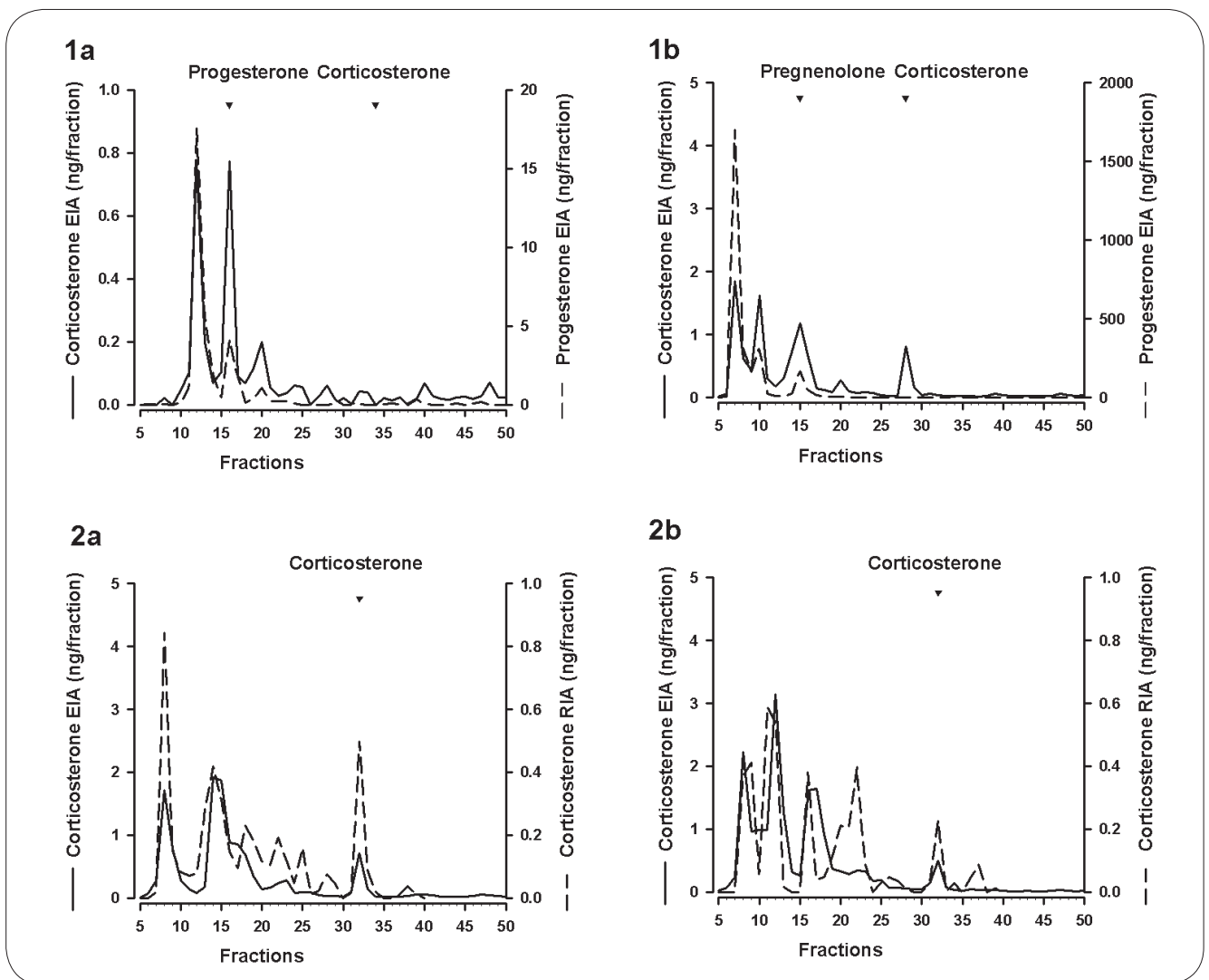
Fig. 1a: High-performance liquid chromatographic separations of chicken albumen. Immunoreactivity was measured with a corticosterone enzyme immunoassay (solid line) and a progesterone enzyme immunoassay (stippled line). Elution positions of the standards are indicated by arrows.

Fig. 1b: High-performance liquid chromatographic separations of bankiva yolk. Immunoreactvity was measured with a corticosterone enzyme immunoassay (solid line) and a progesterone enzyme immunoassay (stippled line). Elution positions of the standards are indicated by arrows.

Fig. 2a: High-performance liquid chromatographic separations of yolk from a chicken implanted with a corticosterone-releasing pellet. Immunoreactivity was measured with a corticosterone enzyme immunoassay (solid line) and a corticosterone radio immunoassay (stippled line). Elution position of the corticosterone standard is indicated by an arrow.

Fig. 2b: High-performance liquid chromatographic separations of yolk from a chicken implanted with a placebo pellet. Immunoreactivity was measured with a corticosterone enzyme immunoassay (solid line) and a corticosterone radio immunoassay (stippled line). Elution position of the corticosterone standard is indicated by an arrow.

This result related to the analysis of yolks of unstressed chickens only, using a single in-house corticosterone antibody (RETTENBACHER et al., 2009). We have now assessed whether corticosterone can be reliably measured in albumen, which contains lower levels of gestagens than yolk. Furthermore, yolk extracts of a hen with experimentally elevated plasma corticosterone levels and a sham-treated female were analysed to assess the impact of elevated plasma corticosterone concentrations on the measurement of authentic yolk corticosterone. Furthermore, the corticosterone EIA previously used was compared to a commercial RIA kit. We found that, substances other than authentic corticosterone caused signals in the corticosterone immunoassays in all samples analysed. This was the case for both yolk and albumen and for artificially stressed chickens and non-domestic birds. In every immunogram performed, at least four individual peaks were visible when individual fractions were measured by immunoassay. Similarly to findings by MÖSTL et al. (2001) and RETTENBACHER et al. (2009), three major peaks were seen in analyses of chicken albumen and bankiva yolk. Absolute concentrations differed greatly between albumen and yolk, in line with previous observations (MÖSTL et al., 2001; JANCZAK et al.,

2009), but both in albumen and in yolk the first peak was predominant and had a different chromatographic mobility from that of the standards. The second peak co-eluted with progesterone and the third peak with pregnenolone. RETTENBACHER et al. (2009) suggested that highly apolar substances such as 5α- and 5β-reduced compounds (5α-and 5β-pregnane-3,20-dione) are present in yolk. Follicle cells are capable of synthesizing a plethora of steroids (WIEBE et al., 1990), so it is conceivable that several unidentified gestagen and their metabolites are present in the yolk. In the HPLC immunograms, each peak represents at least one immunoreactive substance but it is also possible that two or more substances with similar chromatographic characteristics elute as one larger peak. The observation that the first peak showed the highest immunoreactivity does not imply that the corresponding substance is present at the highest concentrations in the yolk: the peak might result from two different compounds that elute in the same fractions.

It is also likely that the progesterone antibody used has a higher affinity for e.g. 5β-pregnanes than for pregnenolone. It should be noted that exhaustive identification of the cross-reacting substances was not the main aim of the present study, which was undertaken to assess whether they bind to corticosterone antibodies. The corticosterone assay and the progesterone assay detected peaks in exactly the same fractions, both in chicken albumen and in bankiva yolk, suggesting that the antibodies in both EIAs bind to the same steroids. Furthermore, we analysed HPLC separations of the pellet and placebo-implanted hens' yolks with two different immunoassays. Both tests indicated the presence of several cross-reacting compounds. Compared to the EIA, the RIA detected a number of additional peaks and also measured immunoreactive substances with a higher polarity than corticosterone. The differences between the patterns found with the RIA and the EIA again suggest that individual fractions contain several metabolites with different affinities to the two antibodies. The two antibodies were raised against the same immunogen (corticosterone-3-CMO) but slight differences were visible in the two immunograms. Previous comparison of another commercial antibody to the in-house antibody gave similar results, although the percentages of cross-reactivities with progesterone differ greatly between the three antibodies (1.4% in the EIA and 0.02% in the RIA used in the present study; 15% in the RIA used by QUILLFELDT et al., 2011). This underlines that in addition to knowing the specificity of an antibody (i.e. its cross-reactions with particular steroids) it is necessary to assess which substances are present in the matrix to be analysed and whether they cross-react with the antibody used.

To date, all corticosterone immunoassays tested have largely measured immunoreactive substances with chromatographic characteristics different from authentic corticosterone (QUILLFELDT et al., 2011; this study). From these results it seems highly likely that all antibodies raised against corticosterone coupled on position three will cross-react with yolk gestagens and are thus not suitable for the quantification of corticosterone in egg yolk. It is therefore not advisable to perform corticosterone quantification in yolk by means of immunoassays, even when cross-reactivities seem small. The present study found corticosterone levels in the albumen to be below the detection limit, which fits with the observation that overall hormone concentrations are much lower in albumen than in yolk (see above). It is still unclear whether steroids diffuse from the lipophilic yolk into the albumen after the egg is produced or are transferred from the plasma into the albumen later in the formation of the egg, probably during albumen secretion in the oviduct. Further studies should use more sensitive methods to evaluate whether plasma corticosterone concentrations are reflected in the albumen. We did not analyse albumen from hens with experimentally elevated plasma corticosterone levels in the present study. Given the small increase of authentic corticosterone in the yolk after pellet implantation, we think it unlikely that there will be detectable concentrations of corticosterone in albumen.

Steroid hormones in the blood reach the oocyte during yolk formation (ARCOS 1972; HACKL et al., 2003; RETTENBACHER et al., 2005) and it is conceivable that a share of circulating corticosterone is transferred from the plasma into the yolk. From the few studies on the topic that rigorously excluded cross-reacting compounds it could be inferred that corticosterone is transferred from plasma into the yolk in a dosage-dependent manner: In the present study, yolks from chickens with plasma corticosterone levels elevated within the physiological range were analysed and a slightly higher corticosterone peak was detected in the yolk of the corticosterone-pellet implanted chicken (EIA: 0.7 ng; RIA: 0.5 ng per fraction) than in the placebo-treated control (EIA: 0.5 ng; RIA: 0.2 ng per fraction). Eggs for the present analysis were collected from laying hens implanted with pellets designed for a continuous release of 30 mg corticosterone over 60 days (HENRIKSEN et al., 2011a). Although the yolks were produced while plasma corticosterone levels were significantly elevated, the observed difference in yolk corticosterone concentrations between pellet and placebo-implanted animals was rather small. Studies where plasma corticosterone concentrations were considerably elevated above physiological levels clearly show that a certain share of corticosterone passes from the circulation into the yolk during egg formation. Transfer of corticosterone into egg yolk was observed after feeding 0.1 g of crystallized corticosterone to laying hens (RETTENBACHER et al., 2005) and, after implantation of barn owls with corticosterone-releasing pellets,

authentic corticosterone concentrations of about 5 ng were found in their yolks (ALMASI et al., 2012), whereas a single injection of ACTH in chickens was not reflected in elevated yolk corticosterone concentrations (RETTENBACHER et al., 2005). These data indicate that transfer of plasma corticosterone into egg yolk depends on the extent of the artificial elevation of plasma corticosterone. Whether a natural stressor also elevates yolk corticosterone levels has not been evaluated with appropriate analytical methods. Nevertheless, the work cited above shows that endogenous corticosterone can pass into the yolk if it is present in the plasma in high concentrations and/or elevated over a prolonged time span.

A peak of 0.8 ng corticosterone was detected in bankiva yolk. As the corticosterone levels in bankiva plasma were not assessed in the present study, we do not know whether the yolks were produced under stressful conditions or whether baseline glucocorticoid levels are higher than in the domestic chickens. Unfortunately, the few studies that used the proper analytical methods to measure yolk corticosterone levels in birds with unmanipulated plasma corticosterone levels do not report plasma corticosterone concentrations. We can thus only speculate on the levels of circulating corticosterone levels in these females. In yolks of caged and free-ranged hens, SAS et al. (2006) found corticosterone concentrations between 0.2 ng and 0.9 ng/g. In bankiva yolk, a distinct corticosterone peak of about 0.8 ng was visible (this study). Our analyses of placebo pellet implanted chickens with two immunoassays revealed the presence of peaks of about 0.5 ng and 0.2 ng in the yolk, whereas previous studies reported authentic corticosterone peaks of about 0.7 ng only in the inner regions of the yolk (RETTENBACHER et al., 2009) or undetectable levels of corticosterone in the yolk (RETTENBACHER et al., 2005). Similar observations were made in southern rockhopper penguins and imperial shags: in both species some yolks were found to contain corticosterone whereas others did not (QUILLFELDT et al., 2011). This indicates that the corticosterone content of individual eggs might differ and enhances the need for further investigations.

Concluding remarks

From the present findings we conclude that the yolks of bird species other than domestic chicken also contain a number of cross-reacting substances that hamper quantification of authentic corticosterone, which is itself present in the yolk only in minimal amounts. The same situation was observed in chicken albumen, where authentic corticosterone was not found in detectable amounts. Although artificial elevation of plasma corticosterone concentrations over a longer period seems to increase concentrations of corticosterone in the yolk, direct quantification in the unhomogenized yolk is not advisable. The literature is littered with studies that have overestimated the actual yolk corticosterone concentrations due to analytical flaws of this kind. As a result, there is a misplaced confidence that amounts of yolk corticosterone accurately reflect changes in circulating concentrations.

It has been postulated that transferred maternal corticosterone acts as a signal for the developing embryo (HENRIKSEN et al., 2011b). However, it is important to consider the impact of local glucocorticoid metabolism. A recent *in vitro* study showed that chicken follicle cells metabolize corticosterone (RETTENBACHER et al., 2013), probably by means of enzymes such as 11β-HSD and 20β-HSD that have been found in the chicken ovary (KLUSONOVA et al., 2008). Whether degradation is performed solely to avoid local glucocorticoid overexposure in the ovarian tissue or might also have a protective function for the chicken embryo remains to be clarified. Furthermore, the biological relevance of the transferred concentrations for the developing embryo might be disputed. Besides ovarian corticosterone metabolism (RETTENBACHER et al., 2013), embryonic tissues also contain enzymes that degrade corticosterone (VON ENGELHARD et al., 2009), suggesting that the developing bird has some control over the amount of maternal corticosterone to which it is exposed. Although the time course of embryonic yolk consumption is not fully elucidated, it is unlikely that the entire amount of corticosterone is incorporated before the onset of endogenous glucocorticoid production around day eight of incubation (JENKINS and PORTER, 2004). It is therefore highly questionable whether the low concentrations of yolk corticosterone have any biological relevance for the developing embryo. The role of corticosterone as a mediator of transgenerational stress in birds should thus be critically evaluated. Alternative pathways by which mothers might signal stressful conditions to their offspring have recently been elucidated. Elevated plasma corticosterone affects egg composition as well as concentrations of reproductive hormones in the yolk (HENRIKSEN et al., 2011a). Parameters such as yolk testosterone levels or egg weight might serve as an indirect but more appropriate measure of maternal stress.

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