Corticosterone in Chicken Eggs

S. RETTENBACHER,[a] E. MÖSTL,[a] R. HACKL,[b] AND R. PALME[a]

[a]*Institute of Biochemistry, Department of Natural Sciences, University of Veterinary Medicine, A-1210 Vienna, Austria*

[b]*Clinic for Avian, Reptile, and Fish Medicine, Department of Farm Animals and Herd Management, University of Veterinary Medicine, A-1210 Vienna, Austria*

ABSTRACT: **Birds are discussed as models for prenatal stress. In this study, several experiments were conducted to gain basic knowledge of if, how, and when maternal adrenocortical activity is reflected by corticosterone concentrations in the egg. Radiolabeled corticosterone was administered to 10 laying hens to investigate the uptake into as well as the distribution within the eggs. The yolk was dissected in concentric layers and analyzed. Less than 1% of the administered radioactivity entered the egg but was, however, not evenly distributed. On the day after injection, highest radioactivity (Bq/g) was detected in the albumen and the outmost layer, whereas concentration peaked 4–7 days later in the inner layers. In two other experiments, increased plasma levels of corticosterone were induced by injection of adrenocorticotropic hormone (ACTH) or feeding of corticosterone. Again, yolk disks were cut in layers and analyzed with a corticosterone enzyme immunoassay. No effect of the ACTH administration was detected, whereas feeding of corticosterone resulted in increased immunoreactive corticosterone concentrations in the yolk. Straight-phase high-performance liquid chromatographic (HPLC) separations were also performed to characterize immunoreactive steroids in the yolk. Two close-eluting peaks at the approximate elution position of corticosterone could be observed after the feeding experiment, whereas in untreated control eggs they were absent. It was concluded that transfer from plasma to egg is low for corticosterone and that further investigations concerning the transport mechanisms and the exact nature of yolk steroids are necessary.**

KEYWORDS: **corticosterone; eggs; poultry; chicken**

INTRODUCTION

Environmental perturbations elevate concentrations of glucocorticoids in the blood to maintain homeostasis and to trigger physiological and behavioral reaction patterns toward survival.[1] Plasma glucocorticoid concentrations are therefore widely used to monitor stress responses in various species.[2–5] If the adverse conditions causing higher hormone levels do not suppress reproduction, they may influence the phenotype of the offspring to maximize success under the constraints of the local en-

Address for correspondence: S. Rettenbacher, Institute of Biochemistry, Department of Natural Sciences, University of Veterinary Medicine, Veterinärplatz 1, A-1210 Vienna, Austria. Voice: +43-1-25077-4114; fax: +43-1-25077-4190.
sophie.rettenbacher@vu-wien.ac.at

Ann. N.Y. Acad. Sci. 1046: 193–203 (2005).
doi: 10.1196/annals.1343.016

vironment. In mammals, stress during gestation has profound deleterious effects on subsequent offspring, and the detrimental consequences of prenatal stress have been broadly shown.[6] In chicken, Lay and Wilson found that administration of corticosterone mimics some, but not all, of the effects of prenatal stress in mammals.[7]

In the blood, corticosterone is reversibly bound to proteins.[8] Basal plasma concentrations of corticosterone in chickens are around 1.3 ng/mL.[9] It is assumed that the amount of corticosterone in the yolk is influenced via passive diffusion by the levels of plasma corticosterone,[10] but it has never been verified that the corticosterone concentration in the yolk correlates with the circulating systemic corticosterone levels. Little is known about how corticosterone gets into the eggs, and basic knowledge about the distribution of stress hormones in the yolk and albumen is needed. Although the influence of maternal sex steroids on the developing embryo has been elucidated broadly,[11] it is not possible to draw analogous conclusions for corticosterone, because it is produced mainly in the adrenal glands, whereas sex steroids are synthesized by the ovary.

The aim of this study was to gain some basic information about the transport of corticosterone from the plasma into the egg and its distribution within the yolk. For this purpose, radiolabeled corticosterone was administered intravenously to mature laying hens. In addition, the concentration of corticosterone in the plasma was increased to elucidate consequences on the corticosterone levels in the eggs. A characterization of the yolk steroids was also performed.

MATERIALS AND METHODS

Animals

The experiment is described in detail by Rettenbacher *et al.*[12] The analyzed eggs came from laying hybrids, ISA brown, obtained from a commercial breeder (R. Schropper PLC, Gloggnitz, Austria). There were weekly intervals between experiments. Permission for performing the animal experiment was obtained from the Federal Ministry of Education, Science, and Culture (GZ 68.205/59-Pr/4/2002).

Radiometabolism Study

Ten birds were administered 1.7 MBq (i.e., 46 μCi) of ^{3}H-labeled corticosterone, dissolved in 1 mL of 0.9% NaCl solution containing 10% (vol/vol) ethanol into the vena cutanea ulnaris. The radiolabeled corticosterone (NET-399; [1,2,6,7-^{3}H(N)]corticosterone; 2830.5 GBq/mmol) was obtained from New England Nuclear (Perkin-Elmer, Boston, MA). During the next 12 days, eggs were collected and stored at −24°C. Previously laid eggs were used to determine background levels.

For analysis, the frozen eggs were incubated at room temperature for a few minutes, until the shells could be removed. During defrosting, the albumen changed its consistency to semifrozen and could be simply scraped off with a spatula. Amounts of albumen were quantified, and 0.5 mL was put directly into scintillation vials to measure radioactivity.

After weighing, the yolk was divided into five concentric layers. Therefore, a disk of approximately 3 mm was cut out, leaving two hemispheres. The radius of the disk

was measured and divided by 5. With a pair of dividers, these five layers, approximately 2 to 3 mm thick, were separated from each other. In the following text, the first layer (layer 1) represents the outermost layer, and the subsequent numbers represent the following layers, with the fifth layer being the central layer. A total of 0.15 g of each layer was extracted with 4 mL of 80% (vol/vol) methanol by shaking for 30 min. After centrifugation, aliquots of the supernatant (0.5 mL in duplicates) were mixed with 6 mL of scintillation fluid (Quicksafe A, No. 100800; Zinsser Analytic, Maidenhead, UK) and measured in a liquid scintillation counter (Packard Tri-Carb 2100TR; Meriden, CT). Radioactivity levels were expressed as becquerels per gram of yolk.

In one animal, we calculated the mean radioactivity of the whole yolk as if it would have been homogenized prior to analysis. Therefore, the weight of each layer was calculated and multiplied with the measured layer concentration (Bq/g) to give the total radioactivity present. The sum of all five layers was divided by the weight of the whole yolk to calculate the mean concentration of the total yolk.

Injection of ACTH and Feeding of Corticosterone

To stimulate adrenocortical activity, we administered 2 mL (0.25 mg) of ACTH (Synacthen; Ciba-Geigy, Basel, Switzerland) to all 10 animals. Eggs were collected during the next four days and stored at −24°C. Previously laid eggs were used to determine background levels.

In a separate experiment, all hens were fed 0.1 g of corticosterone, mixed with a bit of moistened food. The ingestion was completed in about 30 min. Eggs were collected for 6 days.

The preparation of the yolk layers and the extraction were performed in the same way as described for the radiometabolism study. In the feeding experiment, after centrifugation, 10 µL aliquots of the supernatant, diluted with assay buffer (1:1.5), could be directly measured in a corticosterone enzyme immunoassay (EIA).[13] The sensitivity of the assay was 0.8 pg/well; the intra- and interassay coefficients of variation (CVs) were 10% and 13%, respectively. In the ACTH experiment, methanolic yolk extracts were purified with Sep-Pak C_{18} cartridges (1g; Waters, Milford, MA) as described by Möstl *et al.*[14] before analysis with the same EIA.

High-Performance Liquid Chromatography

One sample (extract of 0.15 g of yolk) from the feeding experiment, containing maximum corticosterone concentration, was used for high-performance liquid chromatography (HPLC). The supernatant of the extract was subjected to a cleanup procedure, as described by Rettenbacher *et al.*[12] Straight-phase HPLC separation was performed on a Lichrosorb Si 60 column (10 µm, 25 × 0.4 cm; Forschungszentrum Seibersdorf, Vienna, Austria), as described by Palme and Möstl.[13] Fractions were then analyzed by the corticosterone EIA.[13]

In another experiment, HPLC separations were performed after extraction of control eggs with diethyl ether. Therefore, 16 g of a homogenized yolk was divided into portions of 0.5 g, 0.5 mL of water was added, and the mixture was extracted twice with 5 mL of diethyl ether. The ether phase was transferred into a new vial and evaporated. A total of 2 mL of 100% methanol was added to resuspend the extracted sub-

stances. After addition of 400 μL of water and centrifugation for 10 min at 2500 × *g*, the supernatants were pooled. The cleanup procedure and the HPLC separation were performed as previously described.

RESULTS

Administration of Radioactivity

After injection of ^{3}H-labeled corticosterone, 108 eggs were collected and analyzed. Only 0.42 ± 0.07% (mean ± SD) of the total recovered radioactivity[12] was found in the yolk, and 0.25 ± 0.05% in the albumen. Peak concentrations of radioactivity in the eggs decreased during the sampling period. A stepwise transition of the

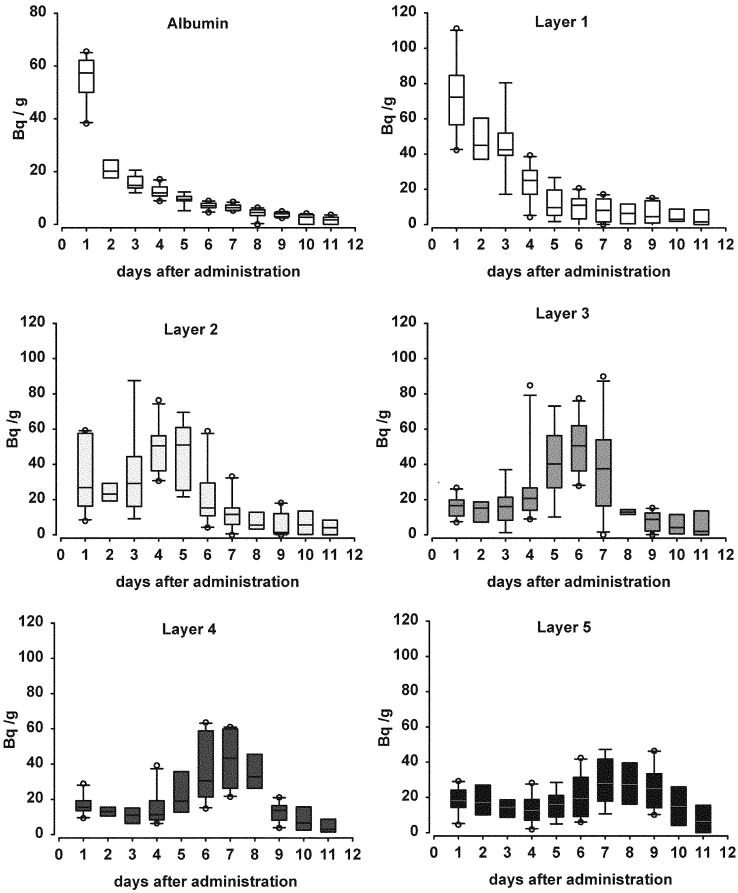

FIGURE 1. Radioactivity (Bq/g) in albumen and different yolk layers after administration of ^{3}H-labeled corticosterone to 10 laying hens. Data are given as box-plot diagrams showing medians (*lines in the boxes*), 25% and 75% quartiles (*boxes*), 10% and 90% ranges (*whiskers*), and outliers (*dots*).

daily maximal concentration of radioactivity from the surface layer of the first laid egg to the center of the tenth egg was observed (FIG. 1). Highest concentrations were found in layer 1 on day 1 after injection (116 Bq/g) and in the albumen (66 Bq/g). Peak concentrations in the second layer were reached on the third day. Between days 4 and 7, the concentration maxima could be found in layer 3. Layer 4 showed the highest concentrations of radiolabeled corticosterone on days 6 and 7, and layer 5 on days 7 and 8 (FIG. 1). Eleven days after administration, background levels were reached in all layers. Differences between layers as well as between days were statistically significant (one-way ANOVA on ranks, $P < .001$, $h = 377$). To isolate the groups that differ from the others, we applied a multiple-comparison procedure (Dunn's method). Due to the large number of groups, details are not given but can be obtained upon request.

The calculated results from the homogenized yolk showed a continuous decrease of radioactivity over the sampling period (FIG. 2).

Administration of ACTH

After administration of ACTH, no significant changes in corticosterone concentrations within the same layers could be monitored during the sampling period. In all the eggs analyzed, the highest concentrations were found in the outermost layers, the values decreased toward the center, and the lowest concentrations were found in the central layer of the analyzed yolks. This pattern remained unchanged throughout the sampling period (TABLE 1).

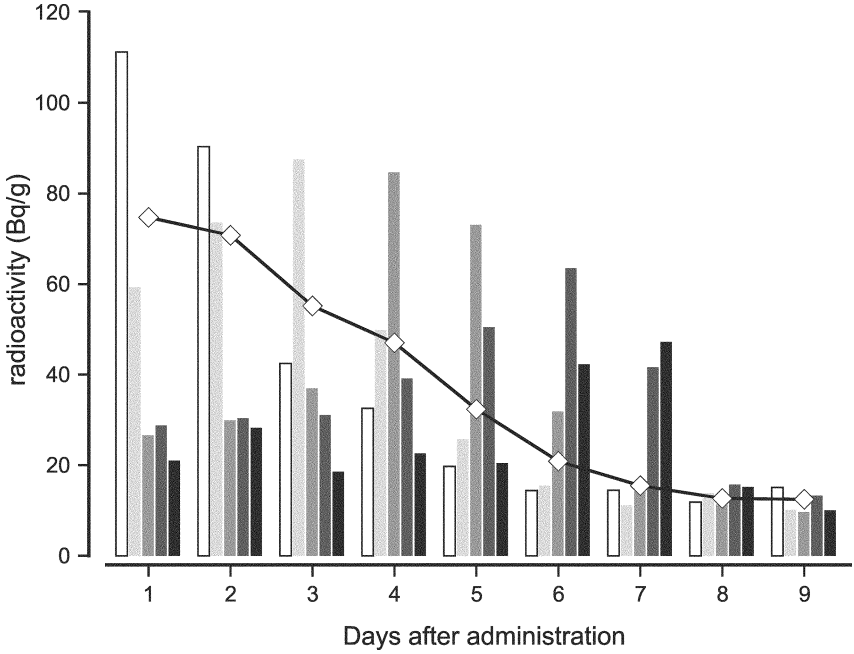

FIGURE 2. Radioactivity in the yolk of one animal. Data resulting from the consecutive layers are given as *bars;* data from the homogenized yolk (calculated) are given as line plot.

TABLE 1. Concentrations of immunoreactive corticosterone (ng/g; mean ± SD) in the different yolk layers after administration of ACTH

	Layer 1	Layer 2	Layer 3	Layer 4	Layer 5
Day 0	43.0 (±5.5)	46.7 (±9.6)	28.9 (±9.8)	16.2 (±2.6)	7.2 (±1.3)
Day 1	36.9 (±3.8)	35.5 (±10.4)	22.4 (±8.7)	16.1 (±10.4)	7.8 (±1.9)
Day 2	37.5 (±12.7)	35.2 (±2.9)	20.7 (±4.5)	9.8 (±4.1)	7.3 (±1.6)
Day 3	32.6 (±13.8)	30.1 (±7.2)	19.2 (±5.3)	9.0 (±3.1)	9.7 (±4.4)
Day 4	37.4 (±11.5)	35.3 (±5.9)	21.4 (±2.2)	11.2 (±4.4)	9.8 (±3.8)

TABLE 2. Concentrations of immunoreactive corticosterone (ng/g) in yolk layers after feeding corticosterone

	Layer 1	Layer 2	Layer 3	Layer 4	Layer 5
Day 0	43.1	43.3	28.3	16.4	7.9
	(37.7 – 49.6)	(37.7 – 61.1)	(18.6 – 44.9)	(11.9 – 18.7)	(5.5 – 15.5)
Day 1	96.1	31.0	12.0	9.5	9.0
	(58.3 – 182.1)	(11.7 – 70.9)	(5.3 – 14.4)	(4.8 –14.6)	(6.7 – 11.7)
Day 3	55.2	65.9	33.5	15.5	9.8
	(17.9 – 91.8)	(59.8 – 119.8)	(20.7 – 52.9)	(7.8 – 18.3)	(5.1 – 14.8)
Day 4	62.8	88.8	104.0	45.3	16.1
	(56. 4 – 178.1)	(55.7 – 132.5)	(20.2 – 122.1)	(14.0 – 49.4)	(15.7 – 19.9)
Day 5	58.3	100.3	93.2	71.6	28.4
	(49.4 – 122.7)	(55.1 – 169.4)	(54.7 – 407.1)	(15.5 – 131.1)	(18.8 – 50.1)
Day 6	72.7	107.6	120.1	88.2	39.7
	(59.2 – 86.1)	(93.5 – 121.7)	(81.1 – 159.1)	(36.6 – 139.8)	(22.3 – 57.2)

NOTE: The median and the range (min–max) is given.

Feeding Corticosterone

The distribution pattern of immunoreactive corticosterone was similar to that of radioactivity, but not so distinctive, because fewer eggs were obtained during the collection period due to a decreased laying performance. On the first day after feeding, the highest concentrations were measured in the outermost layers (58–182 ng/g; median = 96), and concentrations decreased toward the center of the yolk. Two days later, the highest concentration was detected in the second layer. The absolute highest concentration of corticosterone was found on day 5 after feeding in layer 3 (407 ng/g of yolk; TABLE 2).

High-Performance Liquid Chromatography

HPLC separations of one sample's extract of the feeding experiment containing peak concentrations measured by the corticosterone EIA revealed the presence of different immunoreactive substances (FIG. 3a). There were two main, close eluting peaks around the elution position of corticosterone and cortisone (fraction 42).

In the ether extracts of an untreated egg, the corticosterone EIA detected a sharp peak at fraction 6, resembling very apolar substances eluting closely to the solvent front (FIG. 3b), and some smaller apolar peaks between fractions 14 and 20. No peaks at the elution positions of corticosterone and cortisone were visible.

Levels of immunoreactive substances cannot be compared between both HPLC separations because there were different amounts of yolk processed. In the feeding experiment, only one sample (resulting from 0.15 g of yolk) was used, whereas the whole yolk of a normal egg was used for ether extraction, and methodological losses were not evaluated. Therefore, only levels within the HPLCs can be compared.

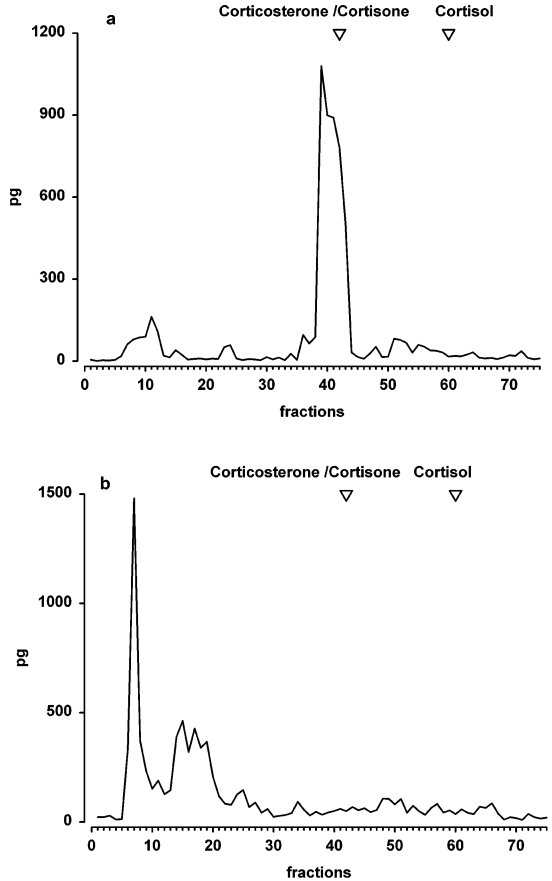

FIGURE 3. High-performance liquid chromatographic separations of yolk extracts obtained (**a**) from the feeding experiment and (**b**) from control eggs . Immunoreactivity was measured in a corticosterone EIA. *Open upside down triangles* mark the approximate elution positions of respective standards. Note that the absolute values cannot be compared between the two immunograms.

DISCUSSION

In this study, several investigations were conducted to gain basic knowledge about corticosterone transport into and distribution within the chicken's egg. We focused mainly on the situation in the yolk, and therefore albumen was not taken into particular consideration. Albumen was measured only in the radioactivity experiment, and the findings resembled the results from the outermost layer. We therefore conclude that for the other experiments, the situation in the albumen is partly reflected by the findings in the outermost layer of the yolk.

As shown by the radiometabolism study, the amount of recovered radioactivity in the eggs was less than 1% of the total recovery. This finding leads to the conclusion that the transfer of circulating corticosterone into the egg is rather low. The recovered radioactivity was distributed in a quite distinctive pattern, which can be explained by the fact that the injected radioactivity reaches follicles at different stages of their development. In follicles that are close to ovulation, radioactivity was found in the outermost layer and in the albumen, whereas follicles in an earlier stage of development accumulated radioactivity closer to the center. Hackl *et al.*[15] performed a similar experiment in quails. They found that 0.12% of radiolabeled testosterone was excreted via the yolk, whereas about 60% of the administered radioactivity was recovered in the feces. They also observed the same characteristic distribution pattern, with the radioactivity being deposited in layers, but the maximum concentrations were reached on the third day in the third layer. This difference could be explained by the slower resorption due to the intramuscular injection done by Hackl *et al.*,[15] whereas in this study, the intravenous administration resulted in an immediate increase of ^{3}H-labeled corticosterone in the blood. The fact that in the feeding experiment the maximum was also reached on day 3 supports this assumption.

Although administration of ACTH increased adrenocortical activity,[12] no changes of the corticosterone concentration could be monitored in the yolk. The corticosterone assay detected a characteristic pattern, with concentrations being higher in the outer layers and lowest in the central layers, but the absolute values remained more or less constant throughout the sampling period and did not differ from those of the control eggs. As Hackl *et al.*[15] found a similar distribution pattern for progesterone, our results could be explained by cross-reactions of the antibody used in the corticosterone EIA with other steroids, probably of gonadal origin. El-lethey *et al.*[16] showed that feeding of corticosterone increased plasma concentrations in chicken. In our study, after an orally administered dosage of 0.1 g of corticosterone, an increase of immunoreactive metabolites in the yolk could be detected by our corticosterone EIA. These results from the corticosterone feeding experiment indicate that high concentrations of circulating corticosterone result in a minor transfer of corticosterone into the yolk, which is detectable by the EIA. These findings are in accordance with the radiometabolism study, which showed that transfer from plasma to yolk is small. Although feeding corticosterone resulted in decreased laying performance, and therefore fewer eggs were available for analysis, a similar layer pattern as was found for radioactivity appeared.

In the HPLC separation of the feeding sample, the large peak detected by the corticosterone assay very likely reflects some of the administered corticosterone. Because we did not check for methodological losses, it was not possible to calculate the concentrations of the metabolites. Also, a comparison between the two HPLC sepa-

rations concerning the amounts of the measured hormones cannot be performed. In the feeding experiment, one sample containing peak concentrations was used for the HPLC separations, whereas a whole yolk of an untreated egg was ether extracted for the other investigation. HPLC separations were done to characterize the hormones immunologically, not for their quantification. In the ether-extracted control eggs, no peak was visible at the elution position of corticosterone. This finding leads to the conclusion that in untreated eggs corticosterone is present only in trace amounts, if at all, and cannot be detected by our EIA.

From these findings, we conclude that unphysiologically high concentrations of plasma corticosterone, as obtained by feeding, are reflected in the yolk and that intravenously administered radioactivity can be recovered to some degree in the egg, whereas a short-term increase of adrenocortical activity, as provoked by ACTH administration, cannot be traced in the eggs.

Monitoring changes that are caused by increased adrenocortical activity is therefore not possible with the corticosterone EIA we used. In the literature, the reported concentrations are very much alike, although different species were investigated by different laboratory methods. Hayward *et al.*[10] found concentrations of 0.92 ng/g of corticosterone in yolk of quails and 2.06 ng/g after implantation of subcutaneous corticosterone deposits. Eriksen *et al.*[17] reported corticosterone concentrations between 1.17 and 1.55 ng/mL in unfertilized eggs. In the albumen, Downing and Bryden[8] measured concentrations between 1 and 1.5 ng/g of corticosterone. None of these authors characterized the measured immunoreactive steroids by means of HPLC immunograms. It is very likely that the reported corticosterone concentrations may, at least in part, be reflections of cross-reacting substances or blank values of the assays used.

Basic knowledge about the correlation between plasma and yolk corticosterone concentration is still lacking and difficult to establish. First, corticosterone concentrations in plasma can change quickly due to handling and manipulation stress and therefore rarely reflect basal values. As a better option, concentration of corticosterone metabolites in fecal samples, which reflect adrenocortical activity,[12] can be used as a reference in such studies where serial bleeding is necessary. Second, as this study points out, it is of major importance to take into account that the steroids are not evenly distributed within the yolk. Lipar *et al.*[18] pointed out that the exact definition of the sampling site is essential for interpreting results concerning gonadal steroids. In most studies, the yolk is homogenized or obtained via puncture, which can contribute to variations in the hormone concentrations. The occurrence of cross-reactions with sex steroids that are also not evenly distributed[15] must be kept in mind.

Another consideration might be that corticosterone is metabolized prior to the deposition in the egg and is therefore not detectable with corticosterone antibodies. Because of the low amount of radioactivity found in the eggs, HPLC separations to characterize these radioactive substances could not be performed.

The low transition of radioactivity from plasma to the egg, the unchanged distribution pattern after the ACTH administration, and the results of the HPLC, in which no corticosterone could be detected, are very strong indicators that a relationship between plasma and egg concerning corticosterone is difficult to establish. Our findings resemble those of Downing and Bryden,[8] who investigated plasma and albumen concentrations and did not find a correlation. There is some evidence that an influ-

ence of maternal stress on the offspring exists,[10] but this is probably more complicated to ascertain than simply measuring corticosterone concentrations in the eggs.

Therefore, further investigations concerning the yolk consumption of the embryo, the effects of steroids on the developing organism, and the transfer mechanism of adrenal steroids into the egg are necessary.

ACKNOWLEDGMENTS

We thank the staff of the Clinic for Avian, Reptile, and Fish Medicine for keeping the animals during the experiments, A. Kuchar-Schulz for performing the HPLC separations, and A. Zechner for excellent laboratory assistance.

REFERENCES

1. SILVERIN, B. 1998. Stress responses in birds. Avian Poult. Biol. Rev. **9:** 153–168.
2. BEUVING, G. 1983. Corticosteroids in welfare research of laying hens. *In* Indicators Relevant to Farm Animal Welfare. D. Smidt, Ed.: 47–53. Martinus Nijhoff Publishers. Boston.
3. MUNCK, A., P.M. GUYRE & N.J. HOLBROOK. 1984. Physiological functions of glucocorticoids in stress and their relation to pharmacological actions. Endocr. Rev. **5:** 25–44.
4. BROOM, D.M. & K.G. JOHNSON. 1993. Stress and Animal Welfare (Animal Behavior Series). Chapman & Hall. London.
5. VON HOLST, D. 1998. The concept of stress and its relevance for animal behavior. Adv. Study Behav. **27:** 1–131.
6. KAISER, S. & N. SACHSER. 2005. The effects of prenatal stress onbehaviour: mechanisms and function. Neurosci. Biobehav. Rev. **29:** 283–294.
7. LAY, D.C. & M.E. WILSON. 2002. Development of the chicken as a model for prenatal stress. J. Anim. Sci. **80:** 1954–1961.
8. DOWNING, J.A. & W.L. BRYDEN. 2002. A non-invasive test of stress in laying hens. RIRDC Publication No. 01/143. Available at: http://www.rirdc.gov.au/reports/EGGS/01-143.pdf (last accessed: December 9, 2004).
9. DEHNHARD, M., A. SCHREER, O. KRONE, *et al*. 2003. Measurement of plasma corticosterone and fecal glucocorticoid metabolites in the chicken *(Gallus domesticus)*, the great cormorant *(Phalacrocorax carbo)*, and the goshawk *(Accipiter gentilis).* Gen. Comp. Endocrinol. **131:** 345–352.
10. HAYWARD, L.S. & J.C. WINGFIELD. 2004. Maternal corticosterone is transferred to avian yolk and may alter offspring growth and adult phenotype. Gen. Comp. Endocrinol. **135:** 365–371.
11. GIL, D. 2003. Golden eggs: maternal manipulation of offspring phenotype by egg androgen in birds. Ardeola **50:** 281–294.
12. RETTENBACHER, S., E. MÖSTL, R. HACKL, *et al.* 2004. Measurement of corticosterone metabolites in chicken droppings. Br. Poult. Sci. **45:** 704–711.
13. PALME, R. & E. MÖSTL. 1997. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. Int. J. Mammal. Biol. **62**(Suppl. II): 192–197.
14. MÖSTL, E., H. SPENDIER & K. KOTRSCHAL. 2001. Concentration of immunoreactive progesterone and androgens in the yolk of hen's eggs *(Gallus domesticus).* Wien. Tieraerztl. Monschr. **88:** 62–65.
15. HACKL, R., V. BROMUNDT, J. DAISLEY, *et al*. 2003. Distribution and origin of steroid hormones in the yolk of Japanese quail eggs *(Coturnix coturnix japonica)*. J. Comp. Physiol. [B] **173:** 327–331.
16. EL-LETHEY, H., T.W. JUNGI & B. HUBER-EICHER. 2001. Effects of feeding corticosterone and housing conditions on feather pecking in laying hens *(Gallus gallus domesticus)*. Physiol. Behav. **73:** 243–251.

17. ERIKSEN, M.S., A. HAUG, P.A. TORJESEN & M. BAKKEN. 2003. Prenatal exposure to corticosterone impairs embryonic development and increases fluctuating asymmetry in chickens *(Gallus gallus domesticus).* Br. Poult. Sci. **44:** 690–697.
18. LIPAR, J.L., E.D. KETTERSON, V. NOLAN, JR. & J.M. CASTO. 1999. Egg yolk layers vary in the concentration of steroid hormones in two avian species. Gen. Comp. Endocrinol. **115:** 220–227.