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Maternal corticosterone is transferred into the egg yolk

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ABSTRACT

Hormones deposited in the avian egg are considered in many studies to influence or to adjust offspring phenotype to prevailing conditions in an adaptive way. Several studies demonstrated an effect of corticosterone, the main glucocorticoid in birds, injected into the egg on the developing chick, but the injection of steroids in the egg is far from mimicking the natural distribution of the hormone in the egg. Other studies applied a stressor or corticosterone to the mother. However it is still debated whether an increase of circulating corticosterone in the mother translates into higher concentrations of corticosterone in the egg. Therefore, we investigated in captive barn owls *Tyto alba* whether circulating corticosterone in egg-laying females elevated within a physiological range, resulted in the deposition of corticosterone in eggs. We found that an increase in circulating corticosterone in the mother within the naturally occurring range translated into elevated concentrations of corticosterone in the yolk of subsequently laid eggs, indicating a specific time frame and yolk layer of corticosterone deposition. We conclude that increasing maternal plasma corticosterone within a naturally occurring range is an efficient tool to increase corticosterone concentration in the egg and to manipulate conditions for the developing embryo.

1. Introduction

Hormones deposited in the avian egg are considered in many studies to influence or adjust offspring phenotype to prevailing conditions in an adaptive way [23,6,16]. In particular, yolk steroids are thought to represent one of the epigenetic factors that can modify gene expression in response to the maternal environment [12]. While there is substantial evidence of a differential deposition of androgens in eggs and an (adaptive) effect of pre-natal androgen exposure on offspring phenotype [6], the effect of corticosterone (the major glucocorticoid in birds) remains more elusive and the mechanism of corticosterone deposition and its regulation as well as the uptake and metabolism by the embryo are poorly known (reviewed in Groothuis and Schwabl [5]). In contrast to androgens that are produced in the ovary, corticosterone is produced by the adrenal gland and has to reach the oocyte (for deposition in yolk) or the ovulated egg (for deposition in albumen) via the circulation. This poses additional problems regarding how females may regulate the deposition of this hormone independently of its concentration in the maternal circulation, a prerequisite for the development of maternal regulation mechanisms and evolutionary trade-offs.

Several studies demonstrated an effect of corticosterone injected into the egg on the developing chick [35,36,4,17,9,13]. However, the injection of steroids dissolved in oil into the egg is far from mimicking the natural distribution of the hormone in the egg and thus a naturally increased concentration in the egg [38]. In addition, the quantification of corticosterone in egg yolk is difficult, because concentrations are low and gestagens (progesterone, pregnenolone and others) which are present in the yolk in high concentrations can give a signal in a corticosterone immunoassay through cross-reactivity if they are not eliminated through appropriate extractions and antibodies of low cross-reactivity are used [33,32]. This led to the application of unphysiologically high amounts of corticosterone in studies which manipulated egg corticosterone levels for example by injections (reviewed by Henriksen et al. [10]). Experimental administration of corticosterone to the mother [8,2,19], or applying a stressor to the mother [36,2,30,31,28], increased yolk corticosterone concentration (except in Janczak et al. [14]). However, in some of these studies part of the measured corticosterone might in reality be gestagens (see Rettenbacher et al. [33]).

Effects on offspring attributed to increased corticosterone in mothers may in fact be due to unphysiologically high concentrations administered, secondary effects or other hormones in eggs, rather than to elevated corticosterone (e.g. [14]. Two studies failed to detect corticosterone in yolk of chicken eggs from unstressed mothers [33,32] while two other studies detected corticosterone in very low amounts [27,20]. Also, single ACTH injections in mothers failed to induce a detectable deposition of corticosterone in

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chicken eggs; only when high (pharmacological) doses of corticosterone were given orally, corticosterone has been unequivocally found in yolk [32]. The question therefore remains whether increases in circulating maternal corticosterone (by administering corticosterone in physiological doses or by imposing a stressor) actually translates into an increase in the concentration of corticosterone in the egg.

In this study we investigated in captive barn owls *Tyto alba* whether circulating corticosterone in egg-laying females elevated within a physiological, rather than pharmacological, range results in the deposition of measurable corticosterone in eggs. We also determined which eggs laid after corticosterone administration were affected and which yolk layer, since it is known that hormones are not homogeneously distributed in the avian egg and radioactivity from the plasma is deposited in layers [15,21,7]. We implanted captive breeding females either with a corticosterone-releasing implant or with a placebo-implant after having laid the second egg of a clutch. The increase in maternal plasma corticosterone was measured 2 days after implantation. We collected eggs and determined the concentration of corticosterone in different yolk layers. With this study we aimed at testing whether exogenous corticosterone reaches the avian egg.

2. Methods

2.1. Experiment

All experiments were carried out under a permit from the 'Landespräsidium für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (Recklinghausen, Germany)'. We used 6 paired captive American barn owl females, *Tyto alba pratincola*, from the Rheinisch-Westfälische Technische Hochschule (RWTH) University of Aachen, Germany, between 2006 and 2008. Whenever a female started a clutch, we randomly allocated the female to one of three treatment groups, except for the broods following a corticosterone-treatment which were always assigned to placebo. One group received a corticosterone-releasing implant the day after the female laid its second egg (hereafter corticosterone female, $n = 11$). The second group received a placebo-implant the day after the female laid its second egg (hereafter placebo female, $n = 7$). The third group received the same handling treatment without receiving an implant the day after the female laid its second egg (hereafter handled female, $n = 4$). All 6 females were used as their own control, which means that all females received at least one corticosterone-implant and at least one placebo implant. All 6 females were used more than once per year but an individual received not more than one corticosterone implant per year. There was a minimum of 30 days (range 30–301 days, mean 142 days ± 27 (SD)) between two consecutive treatments. The first egg was left to the breeding female, all other eggs were collected on the day of egg-laying and immediately frozen until analysis; hence, the second egg of a clutch was used to determine the yolk-corticosterone concentration before the treatment (hereafter egg of day 0 of the experiment).

The implants (diameter 5 mm) are made up of a biodegradable carrier-binder containing either 15 or 10 mg corticosterone or, for placebo, only of the biodegradable carrier-binder (Innovative Research of America, Sarasota, FL). We implanted the pellet under the skin of the flank above the knee through a small incision, which was closed with tissue adhesive (Histoacryl, Braun, Germany). The implants were specified to have a given constant release rate of 7 days in rats, but in the barn owl it appeared that implants increased circulating corticosterone above normal baseline level 2–3 days following implantation [24].

On the day of implantation of the females (day 0 of the experiment) and 2–3 days later we took a blood sample from the brachial vein within 3 min of capturing the females to measure total corticosterone concentration in plasma. Blood samples taken within 3 min after disturbing a bird can be taken as a measure of baseline corticosterone without being affected by capture and handling stress ([34], own unpubl. data).

2.2. Plasma corticosterone measurement

The blood was collected with heparinised capillary tubes, immediately centrifuged and stored at −20 °C until analysis in the laboratory of the Swiss Ornithological Institute. Plasma corticosterone concentration was determined using an enzyme immunoassay [25,26]. Corticosterone was extracted from plasma with 4 ml dichloromethane (5 µl plasma diluted with 195 µl water). All samples were run in triplicate. The dilution of the corticosterone antibody (Chemicon; cross-reactivity: 11-dehydrocorticosterone 0.35%, progesterone 0.004%, 18-OH-DOC 0.01%, cortisol 0.12%, 18-OH-B 0.02% and aldosterone 0.06%) was 1:8000. HRP (1:400,000) linked to corticosterone served as enzyme label and ABTS as substrate. The concentration of corticosterone in plasma samples was calculated by using a standard curve run in duplicate on each plate. The detection limit of the assay is 1 ng/ml. Plasma pools from chicken with a low and a high corticosterone concentration were included as internal controls on each plate. Intra-assay variation was 9% and 11%, and inter-assay variation was 12% and 21%, for the low and high concentration of the internal control, respectively. For details on the assay see Müller et al. [22].

2.3. Quantification of corticosterone in the yolk

Corticosterone in egg yolk was analyzed in the laboratory of the Veterinary University of Vienna. Each yolk sphere was divided into three concentric layers (see [32,15,7]). From each layer, 0.15 g of yolk was diluted in 600 µl of distilled water, vortexed for 30 s and frozen overnight. On the next day, 3 ml of 100% methanol was added. Samples were shaken for 30 min and frozen overnight. After centrifugation, 1 ml of the supernatant was evaporated under a stream of nitrogen and then resuspended in 500 µl of assay buffer. Corticosterone concentrations were determined in 50 µl duplicates by a corticosterone enzyme immunoassay described by Palme and Möstl ([29]; cross-reactivity: progesterone 1.4%, deoxycorticosterone 5.4%, 17-alpha-hydroxyprogesterone 1.3%, 11-dehydrocorticosterone 1.04%). The sensitivity of the assay was 3.5 pg per tube. The inter- and intra-assay variation was calculated by running a yolk-extract sample in duplicates from a pool of barn owl yolk on each plate. The intra- and inter-assay coefficients of variation were 11% and 16%, respectively. Recovery from the extraction was 90 ± 4% for corticosterone. Detection limits for corticosterone was 0.3 ng/g yolk.

2.4. High-performance liquid-chromatographic separations (HPLC) of yolk extracts

From two eggs laid on day 3 after implantation (i.e. with high corticosterone concentrations), the entire outermost yolk layer (minus the 0.15 g used for the analysis described above) was carefully homogenized by swirling with a mini spatula. About 3.8 g of the outermost yolk were prepared to run in two separate HPLC runs as described by Rettenbacher et al. [33]. Briefly, the samples 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. 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® C18 cartridge (Fa WATERS; Part No. WAT051910) 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 μl was used to determine recoveries of radioactivity, while the rest was injected onto an HPLC column. 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/min 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 analyzed for corticosterone (EIA described above) and for radioactivity to determine the elution position of pregnenolone by liquid scintillation counting (Packard Tri-Carb 2100TR, Meriden, CT, USA).

2.5. Data analysis

Statistical analyses were done with the software package R version 2.12.0 [1]. To test for the effect of corticosterone administration on plasma-corticosterone in breeding females, we used mixed-effect models with total corticosterone concentration in plasma as the dependent variable and female identity as random intercept to correct for the repeated measures on females. Total corticosterone was log-transformed for normality of residuals. Since our sample size per treatment of females was quite small, we tested in a first step whether there was a difference between handled- and placebo-females in corticosterone concentration on day 0 of the experiment and 2–3 days after the start of the experiment. We included treatment (handled vs. placebo) as fixed factor. Since there was no difference in total corticosterone between the two control groups we merged the handled and placebo-group (called control-females) for all subsequent analyses. In a second step we analyzed the effect of the corticosterone-implants on plasma corticosterone concentration in females. Log-transformed corticosterone was the dependent variable and female identity the random intercept. We included treatment (control vs. corticosterone females) and day of the experiment (day 0 and day 2–3) as factors and their interaction.

To test for the effect of female treatment on yolk-corticosterone concentration we performed a mixed-effect model with yolk-corticosterone as the dependent variable, clutch number nested in female identity as random intercept, and treatment (control vs. corticosterone), egg category and their interaction as fixed factors. In the barn owl the period of rapid yolk deposition lasts about 11 days and is completed 2.4 days before oviposition [3]. We therefore used three categories of eggs: (a) eggs laid 1–0 days before the start of the experiment (egg-category 0); these are the second eggs of a clutch; (b) eggs laid 3–5 days after the start of the experiment (egg-category 1) and (c) eggs laid between day 6 and 18 after the start of the experiment (egg-category 2). We did not analyze eggs laid one and 2 days after the start of the experiment since during this time period the rapid-yolk deposition was already completed and most likely no corticosterone could have been transferred to the yolk anymore. Note that the results do not change if we restrict the analysis to the first application per treatment for each individual.

3. Results

3.1. Total corticosterone in the plasma of females

Total corticosterone concentration in plasma was similar in the placebo and handled females (mixed-effect model with female identity as random factor, day 0: $F_{1,4} = 0.01$, $p = 0.9$, day 2: $F_{1,4} = 0.6$, $p = 0.5$). Therefore, for simplicity we merged the placebo and handled females and call them control-females.

Total corticosterone concentration in the plasma of females significantly changed two days after the start of the experiment depending on the treatment (Fig. 1; repeated mixed-effect model with female identity as random factor, interaction treatment × day: $F_{1,30} = 8.3$, $p = 0.007$). Post-hoc tests revealed that on day 0 there was no difference in total corticosterone between corticosterone-females and control-females (mixed-effect model with female identity as random variable for day 0 of the experiment, treatment: $F_{1,14} = 0.2$, $p = 0.6$, mean corticosterone concentration of all groups combined: 4.9 ng/ml ± 1, range: 0.9–16 ng/ml, $n = 21$). Two days after implantation there was a significant increase in corticosterone level in the corticosterone-females compared to the day of implantation (mixed-effect model with female identity as random variable for cort-females, day: $F_{1,12} = 9.5$, $p = 0.009$), but not in the control-females (mixed-effect model with female identity as random variable for control-females, day: $F_{1,14} = 0.4$, $p = 0.5$). There was also a significant difference in corticosterone level between corticosterone-females and control-females 2 days after the start of the experiment (mixed-effect model with female identity as random variable, treatment: $F_{1,11} = 5.6$, $p = 0.03$, mean corticosterone concentration of corticosterone-females: 15.1 ng/ml ± 6.1 (range 4–54, $n = 8$); control-females: 4 ng/ml ± 1.1 (0.9–11, $n = 10$)).

3.2. Corticosterone in egg yolk

Since there was no detectable difference in yolk corticosterone concentration between handled- and placebo-females (mixed-effect model with clutch number nested in female identity as random effect and treatment (handled vs. placebo), egg category and their interaction as covariate, all $p > 0.3$), we merged the eggs of placebo- and handled-females (subsequently called eggs from control-females) for all analyses of yolk corticosterone. In the outer and middle layer of yolk, corticosterone concentration differed significantly between treatment and egg category (Fig. 2, Table 1). In general, highest concentrations were detected in the outer layer which decreased towards the inner layer. Post-hoc tests showed that there was no difference in corticosterone concentration in the outer layer between eggs of corticosterone- and control-females before the

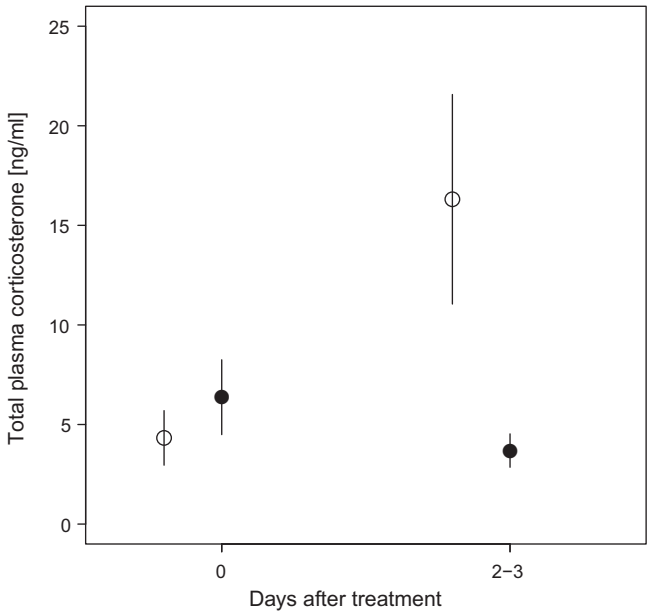

Fig. 1. Total corticosterone concentration (mean ± SE) in the plasma of breeding females on the day of implantation (day 0) and 2–3 days later. Open circles represent corticosterone-treated females ($n = 11$), and closed circles control-females ($n = 11$).

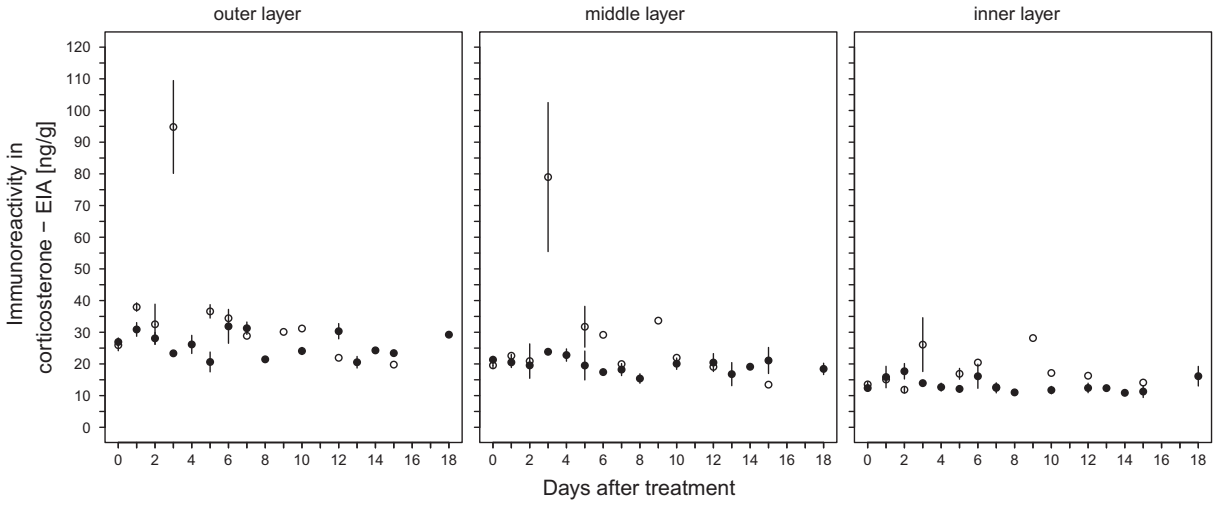

Fig. 2. Mean corticosterone concentration [ng/g] ± SE in three yolk layers of eggs laid before (day 0), and after (day 1–18) female implantation. Open circles represent eggs from females implanted with a corticosterone pellet, closed circles eggs from control-females.

Table 1
Three mixed-effect models with the log-transformed concentration of corticosterone in the outer, middle or inner yolk layer as the dependent variable, treatment (eggs from corticosterone-implanted or control-females) and egg category (0 = before the start of the experiment, 1 = eggs laid 3–5 days after the start of the experiment, 2 = eggs laid 6–18 days after the start of the experiment) as independent variables and clutch number nested in female identity as random intercept. Analyses are based on 60 eggs of 22 clutches of 6 females.

	Outer layer			Middle layer			Inner layer		
	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*
Intercept	1,40	4184	<0.001	1,40	3864	<0.001	1,42	6993	<0.001
Treatment	1,8	20	0.002	1,8	12	0.009	1,8	19	0.002
Egg category	2,40	6	0.004	2,40	10	<0.001	2,42	2	0.1
Egg category × treatment	2,40	15	<0.001	2,40	8	0.001			n.s.

start of the experiment (day 0, outer layer: $F_{1,7} = 0.4$, $p = 0.5$, $n = 16$; middle layer: $F_{1,7} = 1.4$, $p = 0.3$, $n = 16$, Table 2). In eggs laid 3–5 days after female treatment, corticosterone concentration in the outer and middle layer of yolk was generally higher in eggs from corticosterone-females than in eggs from control-females (day 3–5, outer layer: $F_{1,7} = 14$, $p = 0.007$, $n = 15$; middle layer: $F_{1,7} = 7$, $p = 0.03$, $n = 15$, Table 2). In eggs laid more than 5 days after the start of the experiment there was no difference in yolk corticosterone between eggs from corticosterone- and control-females (day 6–18, outer layer: $F_{1,4} = 0.5$, $p = 0.6$, $n = 29$; middle layer: $F_{1,4} = 3$, $p = 0.2$, $n = 29$; Table 2). The difference in egg yolk between eggs from corticosterone- and control-females was most conspicuous on day 3 after implantation with almost a 4-fold increase in the outer and middle layer (Fig. 2).

Table 2
Mean corticosterone concentration in the 3 different yolk layers. Shown are means ± SE for the three different egg categories (0 = eggs laid 1–0 days before the start of the experiment, 3–5 = eggs laid 3–5 days after the start of the experiment, 6–18 = eggs laid 6–18 days after the start of the experiment).

		Egg categories		
		0 d (ng/g) ± SE	3–5 d (ng/g) ± SE	6–18 d (ng/g) ± SE
Cort-females	Outer layer	25 ± 2	61 ± 13	27 ± 2
	Middle layer	20 ± 1	52 ± 13	23 ± 3
	Inner layer	12 ± 1	21 ± 4	18 ± 2
Control-females	Outer layer	27 ± 1	24 ± 2	26 ± 1
	Middle layer	21 ± 1	22 ± 2	19 ± 1
	Inner layer	14 ± 1	13 ± 1	13 ± 1

Corticosterone concentration in the inner yolk layer did not differ significantly between treatment-groups and egg category in the repeated mixed effect model (Table 1). However there was an overall significant effect of treatment (Table 1). Post-hoc analyses revealed that there was no significant difference between yolk-corticosterone concentrations of corticosterone-female and control-female eggs before the treatment (day 0, inner layer: $F_{1,7} = 2$, $p = 0.2$, $n = 16$; Table 2), but a small significant difference between eggs of corticosterone- and control-females laid 3–5 and >5 days after the start of the experiment (day 3–5, inner layer: $F_{1,7} = 7$, $p = 0.03$, $n = 16$; day 6–18, inner layer: $F_{1,4} = 11$, $p = 0.003$, $n = 29$; Table 2, Fig. 2).

In the high-performance liquid chromatographic (HPLC) separations of the outermost yolk layers of two eggs of corticosterone-implanted females laid on day 3 after implantation (i.e. with high corticosterone concentrations), the corticosterone antibody detected several immunoreactive substances (Fig. 3). The first major peak eluted at around fraction 8/9 and the second peak around the previously established elution position of pregnenolone (fraction 16). The third and most pronounced peak of about 5 ng eluted at the previously established elution position of corticosterone (fraction 27–33, Fig. 3). Corticosterone (fractions 27–33) was 44% of the measured immunoreactive corticosterone, whereas 39% was gestagens.

4. Discussion

This study demonstrated that an increase in circulating corticosterone in the mother within the naturally occurring range translated into higher concentrations of corticosterone in the yolk of

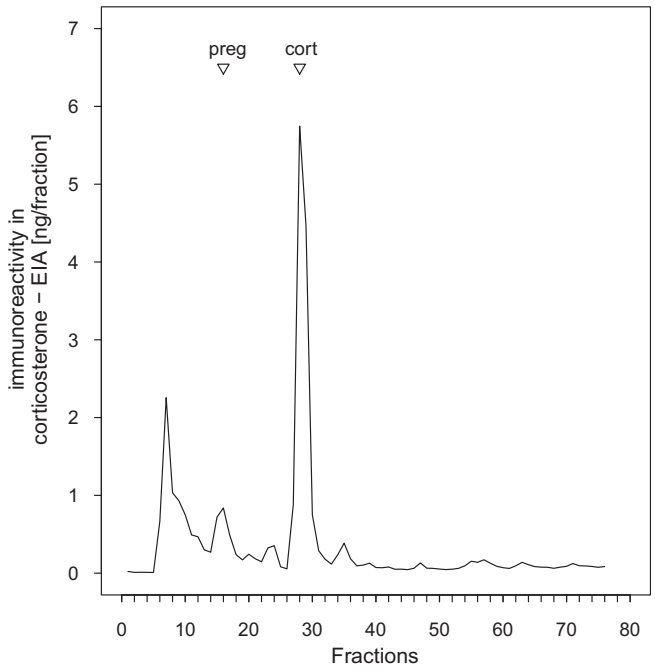

Fig. 3. Immunoreactivity (measured with a corticosterone enzyme immunoassay) of the fractions obtained by high-performance liquid chromatographic (HPLC) separation of extracts of the outer layer of yolk of two eggs from two corticosterone-implanted females. Presented are the mean hormone concentrations per fraction of the two HPLC runs. The triangles mark the approximate elution positions of pregnenolone (preg) and corticosterone (cort), respectively.

subsequently laid eggs. The measured yolk corticosterone values indicated a specific time frame and yolk layer of corticosterone deposition.

The corticosterone pellets used in this study were designed to release corticosterone in rodents at a constant rate. In nestlings of the European subspecies of the barn owl *Tyto alba alba* and *Tyto alba guttata* the release from 15 mg pellets showed a peak in plasma 2 days after implantation which was well within the natural physiological range reached by handling [24]. The American subspecies *T. a. pratincola* used in this study is 42% larger in size than adults of the European subspecies (470 vs. 330 g) and 56% larger than the implanted nestlings of the smaller subspecies (470 vs. 300 g). The 10 and 15 mg corticosterone pellets used in this study caused an increase of circulating corticosterone 2–3 days after implantation which was only about half that in the nestlings of our earlier study (17 vs. 39 ng/ml). Further, female adult barn owls of the European subspecies have a corticosterone response to an acute handling stress of 88 ng/ml ±8 (mean ± SE, $n = 21$, own unpublished data) compared to baseline levels of 15 ng/ml ± 1 ($n = 100$), showing that the circulating levels attained in this study were indeed well within the physiological range of this species. This is confirmed by the fact that in most cases females continued to lay eggs after implantation and thus egg production was not hampered as is the case when corticosterone levels reach high values [18,37].

Eggs laid by females after implantation had a higher yolk concentration of corticosterone than eggs laid before implantation or eggs laid by control-females (placebo-implanted or handled but not implanted). In general, corticosterone concentrations were highest in the outer layer of the yolk and decreased towards the centre of the yolk sphere, similar to what has been found in the yolk of chickens [32]. So far, only part of the studies found an increase of corticosterone in eggs [e.g. 36,2,27], while others did not despite an increase in circulating or excreted corticosterone of the mother [14]. In our study, analysis with HPLC demonstrated that corticosterone was indeed found in eggs of females whose plasma corticosterone levels were artificially elevated. Still, in this study we administered exogenous corticosterone which could have side-effects, for example affecting the negative feedback mechanism [24]. We now need more studies applying a natural stressor to the mother (e.g. predators, human disturbance), rather than exogenous corticosterone, and analysing the occurrence of corticosterone in subsequently laid eggs [11].

The application of corticosterone via self-degradable pellets increased circulating corticosterone during 3–4 days with a peak 2 days after implantation [24]. In this study we found that corticosterone concentration in the outermost yolk layer was elevated in eggs laid 1–5 days after implantation. Very conspicuous, however, was a peak of corticosterone in the outer layer of eggs laid 3 days after implantation. The peak was still visible, but progressively less pronounced, in the middle and inner yolk layer of eggs laid 3 days after implantation, suggesting that circulating corticosterone is mostly deposited in barn owl eggs laid 3 days after implantation, i.e. during the peak in maternal circulating corticosterone. This is in contrast to an implantation study in Japanese quail *Coturnix japonica* which found elevated corticosterone in egg yolk laid 7 days after implantation, but not in those laid 5 days after implantation, while plasma concentration in mothers was back to normal within 4 days after implantation [8]. In barn swallow *Hirundo rustica*, eggs laid the day after the start of applying a stressor showed higher corticosterone concentration in albumen than eggs from control clutches [36]. Barn owl eggs normally take about 2.4 days between ovulation and egg-laying [3]. Hence, from our study it seems that circulating corticosterone is mainly deposited during a short time when the outer yolk layer is formed, shortly before ovulation. Because the middle and inner yolk layers have been formed before implantation (rapid yolk deposition in the barn owl takes about 11 days, Durant et al. [3]), the corticosterone peaks observed in the middle and inner yolk layers of eggs laid 3 days after implantation could be explained by passive diffusion from the outer yolk layers towards the centre. In chicken eggs from untreated mothers a low corticosterone peak was found only in the inner, but not outer yolk by HPLC, which may reflect the steroidogenic activity of the follicle cells during oocyte growth [33]. These authors speculated that corticosterone is only transferred to the egg during early yolk formation, while we have indications of the opposite. Interestingly, quail subjected to restraint stress produced eggs with elevated concentrations of corticosterone in all follicular stages, while in control birds corticosterone concentration was highest in the smallest follicular stages [28]. Taking these studies together, it appears that under non-stressful conditions, corticosterone transfer is highest into the inner yolk layers, but may also accumulate in the middle and especially the outer yolk regions when the mother has increased concentrations of circulating corticosterone. The outer layers are formed last, just before ovulation and might thus reflect plasma increases most accurately, as was demonstrated for deposition of radiolabelled hormones from plasma to yolk [32,7]. However, the mechanism of corticosterone incorporation into yolk remains to be elucidated further, as well as possible diffusion into albumen or other yolk layers and further metabolization in the yolk [e.g. 2,5,38,28].

In conclusion, this study validated the widely used method of administering corticosterone to mothers in order to induce an increase in corticosterone concentration in egg yolk. This results in a natural deposition of corticosterone in eggs and therefore circumvents problems encountered when injecting corticosterone in eggs which results in an unnatural distribution of corticosterone within the egg [38]. Most corticosterone transferred to eggs is deposited in only one egg after inducing a 1–2 day peak of circulating corticosterone in females and is distributed unequally within

the yolk, which may affect how and when corticosterone is available for the developing embryo.

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