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Corticosterone: effects on feather quality and deposition into feathers

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Summary

1. The concentration of the glucocorticoid hormone corticosterone (CORT) is increasingly used in ecology and conservation biology as an integrated measure of the historical record of an individual's hypothalamo–pituitary–adrenal (HPA) activity during feather growth. However, where and how CORT is incorporated in feathers is incompletely known.

2. We therefore examined whether CORT is reliably measured with an enzyme immunoassay, where CORT is incorporated in the feather and where it affects feather quality, and whether CORT incorporation is related to plasma CORT levels, feather growth rate and melanin pigmentation.

3. During the regrowth of plucked tail feathers, we injected pigeons with tritium-labelled CORT, and implanted a CORT-releasing pellet to increase plasma CORT concentration for about 3 days. In feather segments, we measured labelled CORT (DPM_{3H}) and we quantified CORT with an enzyme immunoassay EIA ($CORT_{EIA}$) and double-checked the results with ultra-high performance liquid chromatography–tandem mass spectrometry (LCMS) ($CORT_{MS}$).

4. Administered CORT affected feather structure and colour at the very base of the feather (epidermal collar, ramogenic zone) and reduced growth rate. In contrast, incorporation of CORT into the feather happened mainly in the blood quill, as shown with all three methods (DPM_{3H}, $CORT_{EIA}$ and $CORT_{MS}$).

5. Incorporation of CORT into feathers was only roughly proportional to plasma concentration, proportional to feather growth rate and increased with melanin pigmentation.

6. Measuring CORT in feather is a way to reveal past events of increased stress during feather growth in birds.

Key-words: corticosterone, corticosterone incorporation, fault bar, feather, feather growth rate, melanin

Introduction

The concentration of the glucocorticoid hormone corticosterone (CORT) is a widely used measure to assess a bird's condition. At baseline levels, CORT plays an important role in energy regulation and the maintenance of homoeostatic energy balance (Sapolsky, Romero & Munck 2000). In situations of stress, CORT is released in high amounts and coordinates physiological and behavioural responses to unpredictable environmental challenges (Wingfield *et al.* 1998). Repeatedly or chronically high CORT concentrations may have negative consequences for an individual's health and reproduction (Sapolsky, Romero & Munck 2000).

Usually, CORT is measured in plasma which provides a point in time measure informing about the current physiological state. CORT can also be monitored using metabolites present in faeces which integrate CORT release over the last hours (Goymann 2005). Finally, CORT is deposited in feathers during their growth. Because feathers are inert when full-grown, feather CORT provides a historical record of an individual's CORT release during the period of feather growth. CORT analysis in feathers is advantageous when an integrative measure of CORT is of interest, as in studies correlating individual traits or environmental conditions during feather growth with the birds' physiology (e.g. Koren *et al.* 2012; Legagneux *et al.* 2013). Hence, there is an increasing demand to use feather CORT as a retrospective measure of the hypothalamo–pituitary–adrenal (HPA) axis activity.

In mammals including humans, hair analysis is an established and well-validated method to track steroid hormones (Thieme *et al.* 2003; Skoluda *et al.* 2012). However, only few validation studies of hormone analysis in feathers have been published (Bortolotti *et al.* 2008, 2009; Lattin *et al.* 2011; Hõrak *et al.* 2013). Despite good evidence that feather CORT is measurable, open questions remained. Firstly, findings from CORT-implanted birds suggested an additional way of CORT incorporation other than via the circulation, but application via preen oil was rejected (Lattin *et al.* 2011). Hence, the

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question where and when CORT is incorporated into the feather remains open. Secondly, CORT antibodies from different companies gave different results suggesting cross-reactivity with other steroid hormones (Lattin *et al.* 2011) or CORT metabolites. Lastly, it is unknown whether glucocorticoids are incorporated into feathers proportionally to the concentration of hormone in the blood.

The first aim of our study was to find out whether CORT in feathers can be reliably measured with the antibody used in our enzyme immunoassay (EIA). We therefore measured CORT both with an EIA ($CORT_{EIA}$) and with ultra-high performance liquid chromatography–tandem mass spectrometry (LCMS) ($CORT_{MS}$).

Our second aim was to determine where and when CORT is incorporated into the feather. For that, we injected pigeons during feather growth with a low concentration of tritium-labelled CORT to trace the pulse of labelled CORT (DPM_{3H}) in the feather. Additionally, in a subsample of birds, we implanted a CORT-releasing pellet during feather growth to increase plasma CORT concentration to stress response levels for about 3 days. We expected firstly that CORT could be incorporated at the epidermal collar and ramogenic zone (Lucas & Stettenheim 1972), that is at the very base of the growing feather where cell proliferation takes place. Secondly, CORT incorporation could take place during cell differentiation and keratinization, that is when the growing feather contains the vascularized feather pulp and is wrapped up in a feather sheath, a stage called blood quill. Thirdly, CORT could be applied onto the growing or full-grown feather via preen gland secretions or, in pigeons and a few other taxa, the powder downs. If a pulse of CORT is incorporated at the epidermal collar, added CORT should appear in the full-grown feather more proximally than if incorporated via diffusion from the pulp which protrudes from the skin (Fig. 1). If CORT is added onto the feather through preening, CORT should be found all over the feather grown at the time of CORT pulse or over the entire feather if spread out later. To test this last possibility, we cut each feather longitudinally and washed one half with diluted soap. We then measured $CORT_{EIA}$, $CORT_{MS}$ and DPM_{3H} in segments of the regrown feathers and compared it with CORT of the original feathers which allowed us to track CORT incorporation by three different methods and on a fine time-scale.

Bortolotti *et al.* (2008) suggested that feather CORT is incorporated proportional to growth rate and not feather mass. However, they had to assume a constant growth rate and a test with variable growth rates is lacking. The application of exogenous CORT slows down feather growth and impairs feather quality (DesRochers *et al.* 2009; Müller, Jenni-Eiermann & Jenni 2009; Almasi *et al.* 2012). By applying exogenous CORT, we obtained an increased variability of growth rate in regrown feathers. This allowed disentangling a time-dependent from a mass-dependent CORT incorporation, the third aim of this study.

The fourth aim was to test whether circulating CORT is incorporated into feathers proportional to plasma CORT level, or whether additional factors determine CORT incorporation. For hair, pigmentation is a major factor determining drug incorporation (Nakahara, Takahashi & Kikura 1995). Melanized hair takes up more steroids than blond hair (Höld *et al.* 1996), because melanin functions as a major binding site (Joseph, Su & Cone 1996). In birds, the binding affinity of CORT to feather pigments has not been studied. Correlations between eumelanin or pheomelanin coloration and feather CORT were found (Bortolotti *et al.* 2008). However, such studies cannot disentangle whether feather CORT depends on

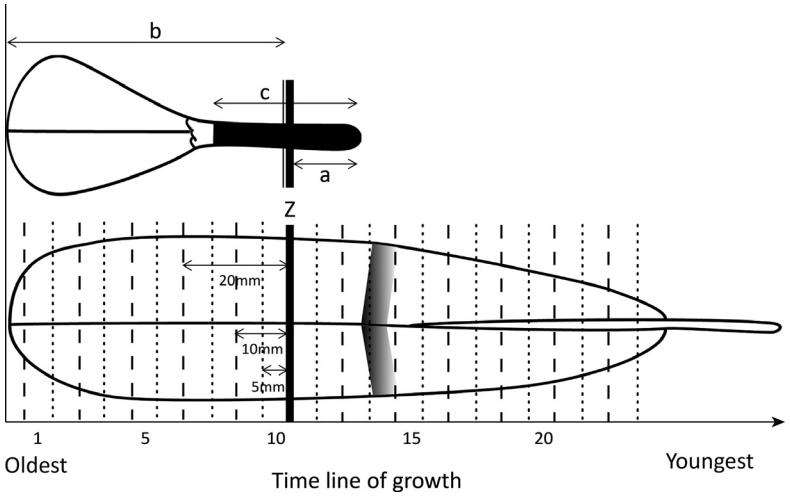

Fig. 1. Diagrams of a growing tail feather at the time of CORT implantation or tritium injection and of the same feather full-grown. The double solid line indicates the skin (Z). Feather length from skin to tip is distance b. The part of the feather quill in the skin (distance a) is 14·5 mm ± 2·4 (mean ± SD) long. The blood quill (distance c, 30·4 mm ± 3·1) is indicated in black. The bold line (Z) indicates the imaginary line at distance b from the feather tip (the length the feather had reached from the skin at tritium injection or at CORT pellet implantation). From this, line segments were cut above and below: 5-mm segments (dotted and broken lines) for tritium counting, 10-mm segments (broken lines) for enzyme immunoassay (EIA), 20-mm segments for mass spectrometry. Segment numbers are indicated below the *x*-axis. The feather was first cut longitudinally (not indicated) to separate inner and outer vane. The shaded area indicates the part of feather whose structure and colour was changed by the CORT released from the pellet, starting at 13·9 mm ± 1·9 (mean ± SD; $n = 19$) proximal of line Z and being of variable length.

the physicochemical and CORT-binding properties of pigments or on plasma CORT during feather growth which may at the same time be correlated with coloration. Therefore, we investigated whether feather CORT depended on melanin coloration, plasma CORT concentration or both.

Materials and methods

EXPERIMENTAL PROTOCOL

Fifteen feral pigeons *Columba livia domestica* were caught in Basel (Switzerland) on 9 February 2012 and transported to the Swiss Ornithological Institute, Sempach. The pigeons were individually marked and kept in groups of five individuals in three aviaries (2 m × 0·8 m × 0·8 m) in three different rooms under natural daylight with water and food *ad libitum* (standard mixture for pigeons, Rust-Rain, Switzerland). After 18 days of acclimatization, a first blood sample was taken (27 February) within less than 3 min from entering the room until the end of sampling. Blood was centrifuged within an hour, the plasma transferred into tubes and frozen at −20°C until analysis. The same day three tail feathers were plucked, measured and kept until analysis (original feathers). Two weeks later (12 March), when the feathers were half-way regrown, a second blood sample was taken. The day after (13 March) 50 μCi ^{3}H-Corticosterone (NET-399 Corticosterone, [1,2,6,7-^{3}H(N)], specific activity 70 Ci mmol^{-1}; Perkin Elmer®, Waltham, MA, USA) dissolved in 0·2 mL 0·9% NaCl (a dose of 247 ng CORT) was injected intraperitoneally in all 15 pigeons. On 14 March, a 15-mg self-degradable CORT-releasing pellet (G-111, Innovative Research of America, Sarasota, FL, USA) was implanted in a subsample of eight individuals (Müller, Jenni-Eiermann & Jenni 2009). Two and eight days after implantation a third and fourth blood sample was taken. When the feathers were full-grown, a fifth blood sample was taken and the regrown feathers were plucked on 10 April (regrown feathers).

The amount of tritium-labelled CORT injected peritoneally was too small to noticeably raise plasma CORT concentration, while the pellets raised plasma CORT substantially (Appendix S1).

At each handling, the pigeons were weighed and the growing feathers measured (from skin to tip). One CORT pellet-implanted individual died on 25 March before the feathers were fully grown and before the fifth blood sampling occasion.

MEASUREMENTS ON WHOLE FEATHERS AND PIGEONS

Plucked tail feathers, original and regrown, were measured (total length), weighed and photographed on mm-paper. From the photographs, we determined whether a change in structure or colour occurred. We also determined the colour values of the different feather segments (5, 10 or 20 mm wide) as used in subsequent analyses separately for the inner and outer vane. The mean colour value of the red–green–blue colour space (RGB), as given by Adobe Photoshop CS3 extended 10.0.1, of a rectangle put onto the inner or outer vane of a segment was taken. Feather's colours were different shades of grey and varied between black (RGB colour value 0) and white (RGB colour value 255) and showed no visible other colour. The colour pigments of 'spread' (blackish) feral pigeons consist mainly (97·5%) of eumelanins (Haase *et al*. 1992).

From three additional feral pigeons, a total of seven growing tail feathers were measured *in situ* (length from the skin to the tip) and plucked (total length). From this, we determined that 14·5 mm ± 2·4 (mean ± SD) of the 30·4 mm ± 3·1 feather quill was in the skin and 15·9 mm protruded out of the skin (Fig. 1).

FEATHER AND PLASMA EXTRACTION

Each feather was cut longitudinal in the middle of the rachis. One half was washed with diluted soap to determine whether CORT is applied through preening, while the second half was not. After drying, the two halves of the feather were taped on a cardboard and cut horizontally at the length the feather had reached at tritium injection or CORT pellet implantation, respectively (line Z in Fig. 1). The four feather pieces were further cut into 5-, 10- or 20-mm segments depending on the assay (5-mm segments for tritium counting, 10-mm segments for EIA, 20-mm segments for LCMS). Each segment was weighed to the nearest 0·01 mg (Sartorius Supermicro S4; Electronic Ultramicro Balance, Göttingen, Germany).

For EIA feather extraction, we followed Bortolotti *et al*. (2009), while for LCMS, several cleaning steps were added (Appendix S2). For the analysis of plasma corticosterone, see Appendix S2.

QUANTIFICATION OF CORTICOSTERONE WITH LCMS AND EIA

CORT was quantified by LCMS at the University in Neuchâtel using an Ultimate 3000 RS system (Dionex, Thermo Fisher Scientific, Waltham, MA, USA) coupled to a 4000 QTrap (ABSciex, Framingham, MA, USA) equipped with a Turbo VTM ion source. For details, see Appendix S2. Plasma and feather CORT concentration was measured with an EIA in the laboratory of the Swiss Ornithological Institute in Sempach (Appendix S2).

$CORT_{EIA}$ measured on one tail feather and $CORT_{MS}$ determined on another tail feather of the same individual provided very similar values (Appendix S3).

MEASUREMENT OF TRITIUM-LABELLED CORTICOSTERONE

Feather segments were placed into a glass vial with 1 mL of Soluene®-350 (PerkinElmer, Cat Nr. 6003038) and heated at 60°C for 1 h in an oven. The mixture was vortexed rigorously, incubated for 36–48 h at room temperature in the dark and placed into an ultrasonic bath for 15 s 10 mL of Hionic-Fluor (PerkinElmer, Cat. Nr. 6013319) was added to each vial before counting for 10 min.

DATA ANALYSIS AND SAMPLE SIZES

From the parameters measured, the following variables were calculated: $CORT_{MS}$ and $CORT_{EIA}$ were expressed as the concentration per ng feather material in each half of the 20-mm or 10-mm feather segment or per mm feather length of the segment. Similarly, DPM_{3H} was expressed per ng or per mm feather length of the 5-mm segment.

There were three types of feathers: (a) original feathers grown in the wild (15 birds); (b) regrown feathers of birds injected only with tritium-labelled CORT (seven birds); (c) regrown feathers of birds injected with tritium-labelled CORT and having received a CORT implant (eight birds).

$CORT_{MS}$ was measured in one original and the corresponding regrown feather of seven individuals implanted with a CORT pellet. $CORT_{EIA}$ was measured in one original and the corresponding regrown feather of all eight individuals implanted with a CORT pellet

and additionally in one regrown feather of five birds injected only with tritium-labelled CORT. DPM_{3H} was measured in one original and the corresponding regrown feather of three birds implanted with a CORT pellet and seven birds injected only with tritium-labelled CORT. The remaining feathers were used for preliminary tests or were broken during growth. Total feather length and weight was only determined in intact feathers (n = 38 pairs of original and regrown feathers).

In all regrown feathers, growth rates (mm day^{-1}) were calculated from the feather-length measures taken from the skin to the feather tip. Because we started measuring the growing feathers only 1 day before injection (2 days before implantation), we do not have growth rates before this. The growth rate measured at the skin actually reflects the growth taking place at the base of the feather follicle 10–15 mm further down into the skin.

The difference in growth rate (mm day^{-1}) of feathers from CORT-implanted and non-implanted individuals was analysed with a linear mixed model (SPSS 18, Hong Kong) including date, implanted/non-implanted and the interaction of date × implanted/non-implanted as fixed factors and individual identity as random factor. The length and mass of original and regrown tail feathers were compared with a linear mixed model including implanted/non-implanted as fixed factor, and feather position nested in individual identity as random factors.

The dependence of the concentration of $CORT_{MS}$ (ln-transformed) or $CORT_{EIA}$ or DPM_{3H} per mm feather length on various factors was analysed with linear mixed models (SPSS 18) with individual identity (equals feather identity) as random factor. The fixed factors were feather generation (original, regrown feathers of CORT-implanted individuals, regrown feathers of tritium-injected individuals), segment, washed or non-washed, vane (inner, outer) and interactions between feather generation, segment and washed/non-washed as fixed factors. Non-significant interactions were excluded from the model. Three of the 10 individuals analysed for DPM_{3H} obtained a corticosterone pellet, but this did not affect DPM_{3H} in the model described above ($P > 0{\cdot}2$) and was therefore omitted.

Results

EFFECT OF CORTICOSTERONE ON FEATHER QUALITY AND FEATHER GROWTH RATE

The implantation of a CORT pellet had a strong effect on feather growth rate ($P < 0{\cdot}001$ for the fixed factors date, implanted/non-implanted and interaction of date × implanted/non-implanted in a mixed model analysis with individual identity as random factor, three feathers each of eight CORT-implanted and seven non-implanted birds). The day after implantation, growth rate decreased to about 50% of that of non-implanted birds and continued to be low for at least 8 days (Fig. 2). Thirteen days after implantation, growth rate of implanted birds approached that of non-implanted birds. Afterwards, non-implanted birds reduced growth rate, because their feathers approached full length, while implanted birds showed somewhat higher growth rates and thus compensated. Tail feathers regrew to similar length and mass as the original feather in implanted and non-implanted birds (for feather length and mass: effect of pellet $P > 0{\cdot}5$ in a mixed model analysis with feather position nested in individual identity as random factors; $P > 0{\cdot}05$ for the overall difference between original and regrown feathers: $-0{\cdot}30$ mm ± 5·83 ($0{\cdot}23$% of mean feather length) and $-0{\cdot}011$ g ± $0{\cdot}027$ (7·24% of mean feather mass), n = 38 pairs of intact feathers).

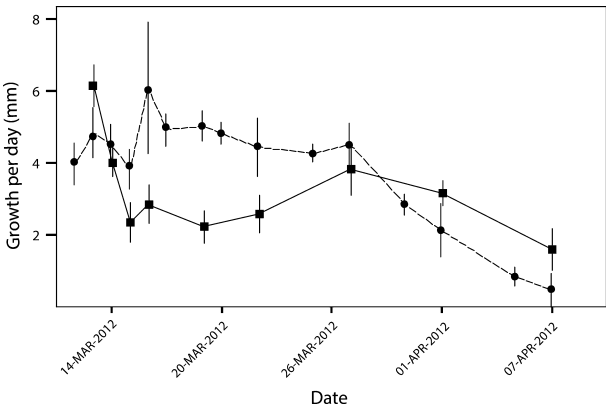

Fig. 2. Mean feather growth rate per day (±SE) of birds with (squares, solid line) and without (dots, broken line) a CORT pellet implanted on 14 March. Sample size is three feathers each of eight CORT-implanted and seven non-implanted birds.

All feathers growing in CORT-implanted birds showed a clear and abrupt change in structure and colour, while all regrown feathers of non-implanted birds showed no such pattern (Fig. 3). In some of the feathers, structure and colour was again normal further proximally, while in others, the entire proximal part was altered. The change in structure and colour occurred 13·9 mm ± 1·9 (mean ± SD; n = 19) further proximal than the length of the feather protruding from the skin 1 day after CORT implantation. As a consequence, segment 13 + 14, which was growing just after implantation, had a lower mass than in original feathers (Fig. 4). Therefore, the abrupt change in structure and colour due to CORT administration occurred at the very base of the feather.

INCORPORATION OF CORTICOSTERONE INTO THE FEATHER

$CORT_{MS}$ concentration per mm feather length varied significantly between feather generations in interaction with segments and differed between washed and non-washed feather parts (Table 1). The regrown feathers of CORT-implanted birds had higher $CORT_{MS}$, particularly in segments 7–10 and 11–14, than the corresponding original feathers, and the washed feathers had lower $CORT_{MS}$ than the non-washed feathers (Fig. 5a).

$CORT_{EIA}$ concentration per mm feather length varied significantly with feather generation in interaction with segment and washing (Table 1). The regrown feathers of CORT-implanted birds had significantly higher CORT concentrations, especially in segments 5–12, than both the corresponding original feathers and the regrown feathers of non-implanted birds (Fig. 5b). Washing reduced the concentration of CORT in the regrown feathers of implanted birds (Fig. 5b).

DPM_{3H} per mm feather length varied significantly with segment depending on feather generation, and with vane, but not with washing (Table 1). The middle segments of tail feathers of

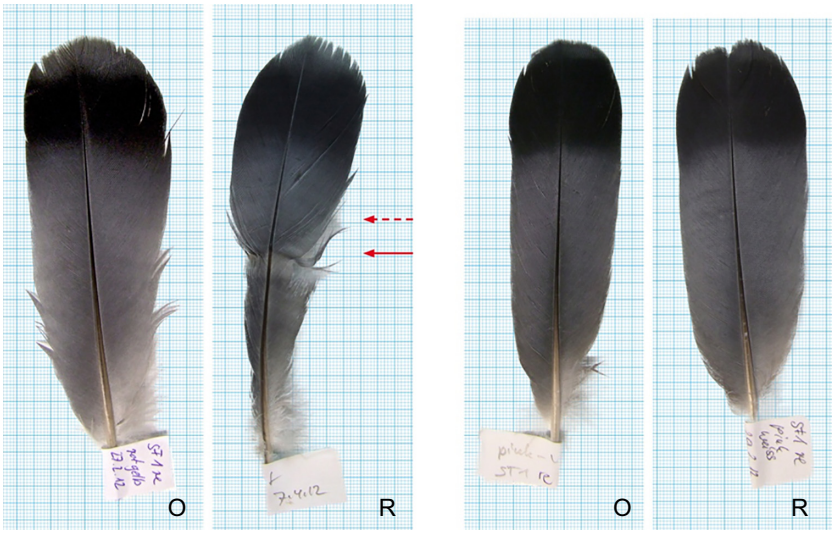

Fig. 3. Original (O) and regrown (R) innermost right tail feather of two feral pigeons. The individual on the right did not receive a corticosterone pellet, and both feathers are similar. The individual on the left received a corticosterone pellet, and the solid arrow indicates the position (on the rachis) of the sudden change in colour and structure. The length of the regrowing feather (measured from the skin, distance b in Fig. 1) at the time of corticosterone pellet implantation is indicated with the dotted arrow.

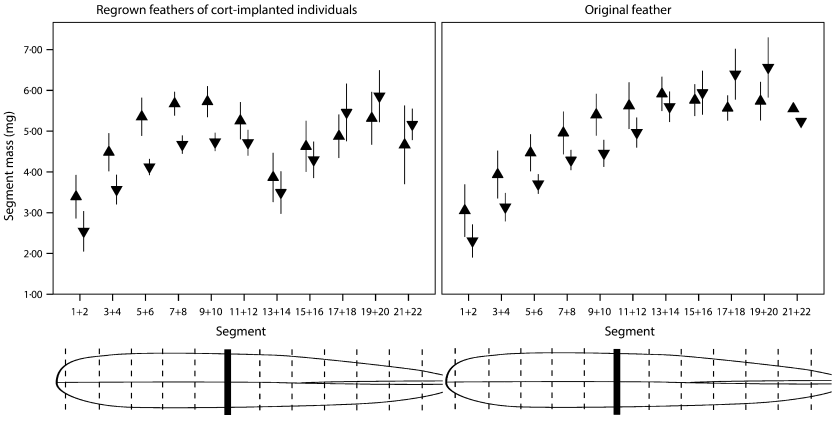

Fig. 4. Mean feather mass (±SE) of segments of the inner (pointing-up triangles) and outer vane (pointing-down triangles) of regrown feathers of CORT-implanted birds ($n = 8$) and their corresponding original feathers. Feather diagrams indicate the segments relative to the length of the feather from the skin at the time of CORT implantation (bold line, cf. Fig. 1).

Table 1. Separate mixed models with the concentration of corticosterone per mm feather length measured with mass spectrometry ($CORT_{MS}$, ln-transformed) or with enzyme immunoassay ($CORT_{EIA}$) or the concentration of tritium (DPM_{3H}) per mm feather length as the dependent variable and the fixed factors feather generation (original, regrown feathers of CORT-implanted individuals, regrown feathers of tritium-injected individuals), segment, washed or non-washed, vane (inner, outer) and interactions between feather generation, segment and washed/non-washed. Individual identity was included as random factor. Non-significant interactions (ns) were excluded

	$\ln(CORT_{MS})$			$CORT_{EIA}$			DPM_{3H}		
	d.f.	*F*	*P*	d.f.	*F*	*P*	d.f.	*F*	*P*
Feather generation	1	68·044	0·000	2	14·310	0·000	1	169·348	0·000
Segment	3	2·552	0·063	10	0·989	0·453	21	16·395	0·000
Washed/non-washed	1	11·283	0·001	1	7·874	0·005	1	0·249	0·618
Vane	1	0·020	0·889	1	0·272	0·602	1	5·377	0·021
Feather generation × Segment	3	4·559	0·006	19	1·787	0·023	19	16·274	0·000
Feather generation × Washed			ns	2	18·606	0·000			ns
Feather generation × Segment × Washed			ns			ns			ns

tritium-injected birds (especially segments 6–14) had a much higher DPM_{3H} than the most distal and the proximal segments (Fig. 5c). The inner vane had higher DPM_{3H} per mm feather length than the outer vane (effect size 1·535 ± 0·662 SE).

DEPENDENCE OF FEATHER CORTICOSTERONE ON FEATHER GROWTH RATE

We investigated whether CORT is incorporated in feathers in a time-dependent fashion in two ways. Following Bortolotti *et al.* (2009), we first examined whether CORT concentration in original feathers was constant over the entire feather length when expressing per mm or per mg feather. Along a feather, the mass per segment increases from distal to proximal. The assumption, however, is that exposure to circulating CORT does not vary in a systematic manner during feather growth (although it may vary stochastically), and hence, CORT concentration should not vary systematically along a feather.

Contrary to the assumption, $CORT_{EIA}$ per mg feather decreased with segment number, that is from distal to proximal

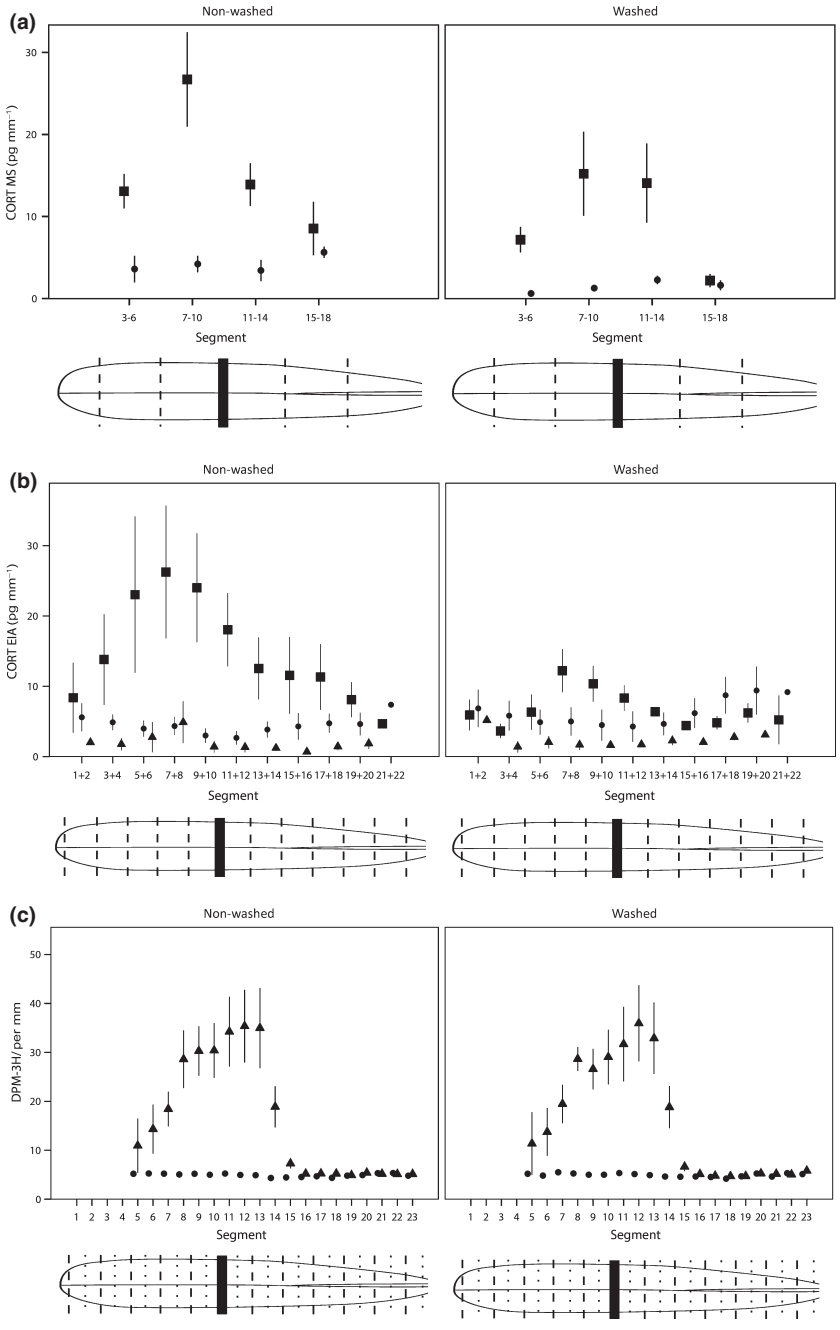

Fig. 5. Mean concentration of corticosterone per mm feather length (±SE) as determined with (a) mass spectrometry ($CORT_{MS}$), (b) enzyme immunoassay ($CORT_{EIA}$) and (c) mean concentration of tritium per mm feather length (DPM_{3H}) in different segments. Left panel: non-washed, right panel: washed vane. Original feathers are indicated with small dots, regrown feathers of CORT-implanted individuals with squares and regrown feathers of tritium-injected individuals with triangles. Note that in (b) the regrown feathers of tritium-injected individuals are from different individuals than the original feathers. In all other panels, the corresponding original and regrown feathers were analysed. Symbols may mask small SE. The diagrams below the panels indicate the segments relative to the length of the feather from the skin at the time of CORT implantation or labelled CORT injection (bold line, cf. Fig. 1).

(Mixed model with individual as random factor, $n = 8$ original non-washed feather, vanes $F = 24{\cdot}2$, $P < 0{\cdot}001$) and was also negatively related to the feather mass of the segment ($F = 46{\cdot}7$, $P < 0{\cdot}001$, slope $= -3{\cdot}26 \pm 0{\cdot}48$ SE), because feather mass of a segment increased from distal to proximal ($F = 140{\cdot}2$, $P < 0{\cdot}001$). Feather colour or vane did not affect this relationship (feather colour $F = 1{\cdot}03$, $P = 0{\cdot}32$, vane $F = 2{\cdot}31$, $P = 0{\cdot}18$, when included into the model above). In accordance with the above assumption, $CORT_{EIA}$ expressed per mm feather length was not significantly related to segment number or feather mass of the segment ($F = 0{\cdot}02$, $P = 0{\cdot}90$; $F = 0{\cdot}09$, $P = 0{\cdot}76$), that is was constant along the feather. Again, feather colour or vane did not affect this relationship (feather colour $F = 0{\cdot}72$, $P = 0{\cdot}40$, vane $F = 2{\cdot}22$, $P = 0{\cdot}19$, when included into the model above). A similar analysis with $CORT_{MS}$ was not possible, because the feather segments of 20 mm each had a more similar mass.

If CORT is incorporated in feather in a time-dependent fashion, expressing CORT per mm feather length assumes that feather growth rate is constant. In a second step, we therefore examined whether growth rate *per se* affects CORT incorporation into feathers. We could do so because we measured growth rate directly in our birds exhibiting variable growth rates due to CORT pellets. We looked only at DPM_{3H} because the CORT pellets increased $CORT_{EIA}$ and $CORT_{MS}$.

In feather segment 13 of tritium-injected birds ($n = 9$, because growth rate could not reliably be determined in one

individual), the variation of growth rate was increased because two of the nine individuals also received a CORT pellet which reduced growth rate. For the segments 5–12, we do not have data on growth rate and from segment 14 onwards, DPM_{3H} was already at low values (Fig. 5c). DPM_{3H} per mm and per mg feather mass of segment 13 were both negatively related to growth rate ($F = 22{\cdot}7$, $P = 0{\cdot}002$, slope $-14{\cdot}73 \pm 3{\cdot}09$ and $F = 83{\cdot}92$, $P < 0{\cdot}001$, slope $-33{\cdot}73 \pm 3{\cdot}68$; Mixed models with individual as random factor and washing as fixed factor; washing was not significant and removed). Hence, more tritium was incorporated per feather length and per feather mass if this part of the feather was exposed for a longer time in the blood quill (Fig. 6).

DEPENDENCE OF FEATHER CORTICOSTERONE ON PLASMA LEVELS AND FEATHER MELANISM

Plasma CORT varied widely between 0·9 and 171·5 ng mL^{-1} 2 days after implantation of the CORT pellet (eight pellet-implanted birds and five birds only injected with tritium-labelled CORT). $CORT_{EIA}$ measured in the unwashed halves of segments 9 + 10 and 11 + 12, which were in the blood quill stage at the time of plasma sampling, depended on the concentration of plasma CORT and on the coloration of the feather segment (Table 2). $CORT_{EIA}$ increased with increasing plasma CORT (Fig. 7) and with increasing colour intensity. Considering only the eight pellet-implanted birds, these relationships were not significant. In the other segments, not in the blood quill stage at the time of plasma sampling, relationships between $CORT_{EIA}$ and plasma CORT or feather colour were less strong or not significant. A similar analysis with $CORT_{MS}$ did not yield significant relationships, probably because the segments were wider and the sample size lower ($n = 7$).

A dependence of feather CORT on coloration was evident in tritium-injected birds. DPM_{3H} per mm feather length in regrown feathers varied with feather segment colour in interaction with segment (Table 3). In segments 8–13, where tritium-labelled CORT was incorporated (Fig. 5c), significantly more tritium was incorporated in darker segments (as an example, see segment 12 in Fig. 8a). Also, the sum of tritium in segments 8–13 was related to the mean coloration of these segments (Fig. 8b) (linear regression: $n = 10$, $r = 0{\cdot}72$, $P = 0{\cdot}018$, slope $= -3{\cdot}31 \pm 1{\cdot}11$). Because we did not determine tritium in plasma, we cannot test for a dependence of DPM_{3H} on plasma concentration.

Discussion

This study demonstrated that tritium-labelled CORT and experimentally increased circulating CORT showed up in feathers and can be measured with an EIA. Surprisingly, the effect of CORT on feather structure and the incorporation of CORT into feathers happened at different sites. While CORT affected feather structure at the epidermal collar and ramogenic zone, its incorporation into the feather happened mainly in the blood quill. Incorporation of CORT into feathers was roughly proportional to plasma concentration and depended on growth rate and melanism.

Table 2. Dependence of $CORT_{EIA}$ of segment 9 + 10 or 11 + 12 (ln-transformed) on the concentration of plasma CORT (sampled 2 days after CORT pellet implantation, ln-transformed) and the coloration of the corresponding feather segment (linear model). $n = 13$ feathers (eight pellet-implanted birds, five birds only injected with tritium-labelled CORT)

	Effect size ± SE	d.f.	*F*	*P*
Segment 9 + 10				
ln(plasma CORT)	0·950 ± 0·152	1	39·3	<0·001
Colour	−0·025 ± 0·007	1	12·8	0·005
Segment 11 + 12				
ln(plasma CORT)	0·731 ± 0·163	1	20·0	0·001
Colour	−0·022 ± 0·007	1	9·5	0·012

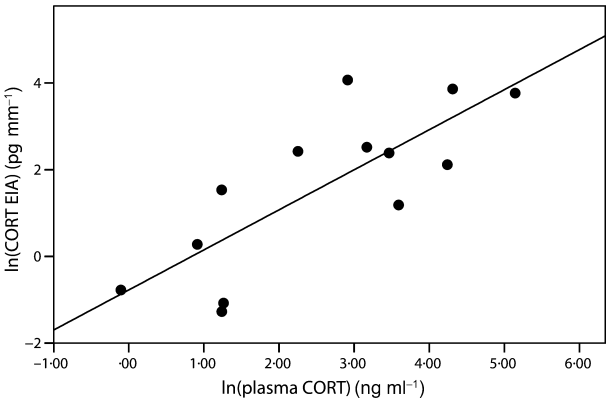

Fig. 7. Relationship between the concentration of CORT in plasma 2 days after CORT pellet implantation and $CORT_{EIA}$ in segment 9 + 10. For statistics, see text. $n = 13$ regrown feathers.

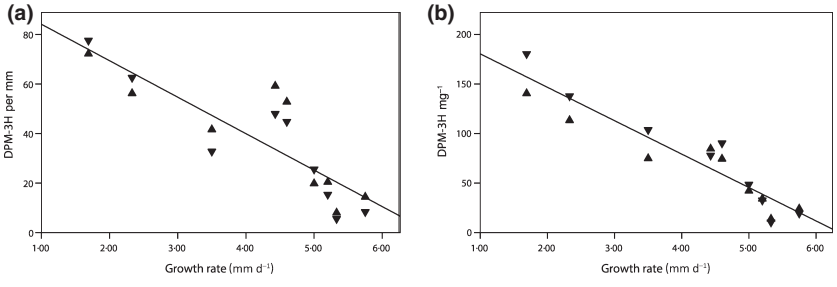

Fig. 6. Relationship between growth rate and the concentration of tritium (DPM_{3H}) in segment 13 (see Fig. 5c) expressed per mm feather length (a) and per mg feather material (b) in regrown feathers ($n = 9$). For each bird, the value for the inner (pointing-up triangles) and outer vane (pointing-down triangles) is given.

Table 3. Mixed model for regrown feathers of tritium-injected birds with the concentration of tritium (DPM_{3H}) per mm feather length as the dependent variable, the fixed factors colour, segment, vane (inner, outer) and the interaction segment × colour, and individual identity as random factor. The effect size was 3·16 ± 1·18 for the inner vane. The interaction vane × colour was not significant ($F = 0{\cdot}68$, $P = 0{\cdot}41$) and removed from the model

	d.f.	*F*	*P*
Colour	1	0·241	0·62
Segment	19	11·687	<0·001
Vane	1	7·175	0·008
Segment × Colour	19	3·428	<0·001

CORTICOSTERONE IN FEATHER CAN BE MEASURED WITH AN ENZYME IMMUNOASSAY

Doubts were raised as to whether CORT antibodies actually measure CORT in feathers or whether they might cross-react with other substances (Lattin *et al.* 2011). The antibody used in our laboratory has a low cross-reactivity with other steroids (Appendix S1) and did detect CORT. $CORT_{EIA}$ correlated highly significantly with $CORT_{MS}$ in the corresponding segment of another tail feather of the same individual. Also, the absolute values determined by the two methods were very similar. Therefore, we are confident that our EIA actually measured CORT in feathers and not cross-reactive substances.

CORTICOSTERONE AFFECTS FEATHER QUALITY AT THE EPIDERMAL COLLAR AND RAMOGENIC ZONE

All CORT-implanted birds showed an abrupt change in feather structure and colour and a lighter weight of these parts, confirming earlier studies which showed that CORT administration as well as increased endogenous CORT during feather growth impair feather quality (Roulin *et al.* 2008; DesRochers *et al.* 2009). The structural impairment of the feathers started on average 13·9 mm more proximally than the point where the feather protruded from the skin 1 day after CORT implantation (Fig. 3) which corresponds to the feather length in the skin of about 14·5 mm. Therefore, the administration of exogenous CORT must have affected feather structure and colour at the base of the follicle, where cell division during feather growth takes place (Lucas & Stettenheim 1972).

CORT pellets also halved growth rate after implantation for at least 8 days. Thereafter, growth rate increased again and the tail feathers finally regrew to similar length and mass as the original feathers. This agrees with findings in growing feathers of adults and nestlings with experimentally elevated circulating CORT (Romero, Strochlic & Wingfield 2005; Müller, Jenni-Eiermann & Jenni 2009; Almasi *et al.* 2012).

CORTICOSTERONE IS INCORPORATED INTO FEATHER IN THE BLOOD QUILL

We experimentally applied two kinds of CORT pulses during feather growth: labelled CORT which did not increase circulating CORT and CORT from an implant which raised circulating CORT substantially. Both CORT pulses resulted in a very similar pattern of CORT (DPM_{3H}, $CORT_{EIA}$, $CORT_{MS}$) along the feather, which allows distinguishing between the three hypotheses of CORT incorporation put forward in the introduction.

Surprisingly, CORT was not incorporated at the base of the growing feather where CORT affects feather structure, the first hypothesis put forward, but – as stated by the second hypothesis – in those feather segments containing the blood quill at the time, the CORT pulse was applied (similar to Lattin *et al.* 2011). The third hypothesis, that CORT is applied onto feather via preen gland secretions or powder downs, is not likely, because precisely those feather segments containing labelled or increased CORT are protected by feather sheaths at the time of the CORT pulse. Lattin *et al.* (2011) could not find CORT in preen gland extract and also rejected this hypothesis.

Our results show for the first time that CORT is incorporated into the feather not at cell proliferation (epidermal collar and ramogenic zone), but at cell differentiation (blood quill). At this stage, feather cells incorporate pigments and keratinize while building the fine structures (Lucas & Stettenheim 1972; Yu *et al.* 2004). Nutrients and carotenoid pigments are supplied to the feather from the pulp, the highly vascularized centre of the blood quill around which the developing feather is wrapped (Lucas & Stettenheim 1972; Yu *et al.* 2004). It is therefore likely that CORT is absorbed by the feather via diffusion from the blood vessels of the pulp. Because CORT is lipophilic, as carotenoids, a similar way of incorporation can be imagined. Diffusion of steroids from exogenous sources into hair after keratinization was shown for mammals (Thieme *et al.* 2003).

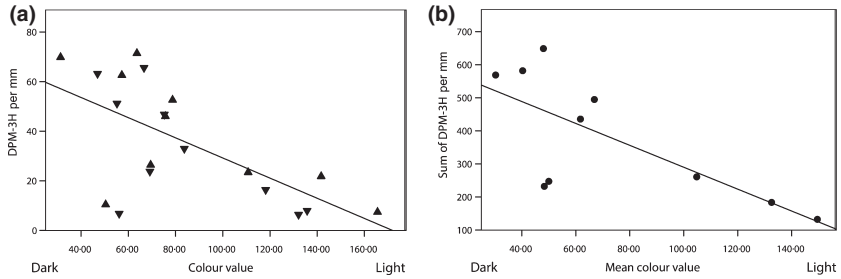

Fig. 8. Dependence of tritium concentration (DPM_{3H}) per mm feather length on segment colour of regrown feathers of birds injected only with labelled corticosterone ($n = 10$ pigeons). (a) As an example shown for segment 12 and for inner (pointing-up triangles) and outer vane (pointing-down triangles). (b) Sum of DPM_{3H} per mm feather length over segments 8–13 vs. mean colour of these segments (both vanes taken together).

We found that CORT is not elevated in feather segments impaired in structure and colour by CORT. Such fault bars are supposed to result from nutritional or physiological stress (Machmer *et al*. 1992; Jovani, Montalvo & Sabaté 2014). One explanation might be that CORT binding is impeded because less binding sites are available due to the reduced structures and the reduced melanin concentration in fault bars. Another explanation could be that at least part of CORT is metabolized in the epidermal collar and ramogenic zone and only metabolites are incorporated. Bortolotti *et al*. (2008) showed that the skin of birds is capable of metabolizing steroid hormones. Up to now, no study measured CORT specifically in fault bars, but only in feather segments containing fault bars including the adjacent parts (Bortolotti *et al*. 2009) which, according to our findings, might contain high CORT. Hence, further studies are needed which should take into account the effect of growth rate and melanism on CORT in feathers.

There is evidence that CORT is not only incorporated biochemically into the feather but can also bind to the surface of the feather. In our study, CORT concentration in regrown feathers of birds with CORT pellets was lowered by washing. Excluding CORT administration to feathers via preen gland excretions, this could indicate that stress levels of circulating CORT over days result in increased feather CORT not only through incorporation but also through surface attachment. Accordingly, no effect of washing was detected for DPM_{3H}, that is when circulating CORT was at normal (low) concentrations. Also, Bortolotti *et al*. (2008) did not find an effect of washing in birds without exogenous CORT administration. Alternatively, tritium at the α-position could be exchanged in aqueous solution, or labelled CORT could be metabolized and tritium be incorporated separately or with some catabolites. This could explain that we found more tritium in the broader inner, than outer vane of a segment, thus a dependence on feather mass which was absent in $CORT_{EIA}$ and $CORT_{MS}$. It therefore remains unclear whether CORT attached to the surface of feathers occurs only at high concentrations.

FEATHER CORTICOSTERONE CONCENTRATION DEPENDS ON FEATHER GROWTH RATE

By assuming a constant growth rate, Bortolotti *et al*. (2009) concluded that feather CORT is incorporated in a time-dependent and not mass-dependent fashion. With similar analyses, we support this. CORT of original feathers expressed per mm length did not vary systematically along the feather, but did so when expressed per mg, in parallel with feather mass. We also did not find a difference in CORT per mm between the narrow outer and the broader inner vane, but for CORT per mg.

When examining feather segments formed under various growth rate regimes, we found an increase of DPM_{3H} with decreasing growth rate whether DPM_{3H} was expressed per mm feather length or per mg feather material. This indicates that a longer exposure of the feather segment in the blood quill to circulating CORT increased CORT incorporation irrespective of the mass of the exposed feather material. Hence, for the first time, we could support the time-dependent uptake of CORT into feather by examining its dependence on actual growth rates.

FEATHER CORTICOSTERONE DEPENDENCE ON PLASMA CORTICOSTERONE AND FEATHER MELANISM

For the first time, we could investigate simultaneously whether feather CORT reflects plasma CORT and/or depends on pigmentation, as found in hair (Nakahara, Takahashi & Kikura 1995). We found evidence that feather CORT depended both on plasma concentration during feather growth and on feather eumelanin coloration.

$CORT_{EIA}$ increased with increasing plasma CORT which may indicate that circulating CORT is indeed reflected quantitatively in feather CORT. However, the correlation is relatively weak considering that it is based on ln-transformed values and that there was no significant correlation when considering only the eight CORT-implanted birds. It is noteworthy that we worked with a high individual variation of plasma CORT concentrations which is probably not usually found in wild birds. Bortolotti *et al*. (2008) also found that feather CORT was unrelated to baseline plasma CORT, but was related to stress-induced plasma CORT levels. They argued that CORT reactivity to stressful events is reflected in feather CORT.

The weak relationship we found between plasma and feather CORT may be caused by the skin being a CORT secreting and absorbing tissue itself. A cutaneous equivalent of the HPA axis has been found in mammalian skin (Slominski 2005) and in hair follicles (Ito *et al*. 2005). In house sparrow skin, glucocorticoid receptors have been found (Lattin *et al*. 2012), but there is yet no evidence of a cutaneous equivalent of the HPA axis is in avian skin or feather follicles.

$CORT_{EIA}$ increased with increasing colour intensity indicating that eumelanin pigments affect CORT binding into feathers. We did not find studies investigating the specific binding of cortisol or CORT to melanin, but there are indications that pigments positively affect binding properties of steroids and that melanin is a likely candidate as a specific binding site (Cone 1996). Also, melanin may indirectly favour CORT uptake by changing the pH-gradient between blood and hair matrix. pH in hair is more acidic with melanin and favours drug uptake (Nakahara, Takahashi & Kikura 1995). However, whether this is also the case in feathers remains to be shown, because pH in feathers (unlike in hair) becomes basophilic during keratinization (Lucas & Stettenheim 1972).

Conclusions and perspectives

We showed that CORT in feathers can be measured with an EIA, and washing is not necessary. Furthermore, we showed that melanin enhances CORT uptake into feather and has to be taken into account. Care is also needed when feather CORT is used as a proxy of the quality or stress sensitivity of an individual because feather CORT might simply reflect the physicochemical properties of the pigments when differences in coloration between individuals are involved. We also confirmed that the deposition of CORT is time-dependent.

Finally, we demonstrate that elevated circulating CORT affects feather structure and colour at the epidermal collar and ramogenic zone, whereas it is incorporated into the feather in the blood quill. This is of importance when the aim is to relate feather CORT to specific events in time during feather growth.

Many open questions remain, for example: Is feather CORT a measure of circulating CORT integrated over the time of growth or do other processes interfere (e.g. effect of melanin; CORT production in the skin)? What consequences have the low circulating baseline and stress levels of CORT during natural moult (Romero, Strochlic & Wingfield 2005) for feather CORT to reflect periods of stress? Nevertheless, feather CORT will undoubtedly be used increasingly as a convenient way in birds to reveal past events of increased stress during feather growth.

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Data accessibility

Data available from the Dryad Digital Repository: http://doi.org/10.5061/dryad.7tm53.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Appendix S1. Effect of tritium-labelled CORT injection and CORT pellet implantation on plasma concentrations.

Appendix S2. Feather extraction and CORT determination with EIA and LCMS.

Appendix S3. Comparison of corticosterone concentrations obtained by EIA and LCMS quantification.

Supporting Information

Appendix S1: Effect of tritium-labelled CORT injection and CORT-pellet implantation on plasma concentrations

The amount of tritium-labelled CORT injected peritoneally (247 ng) was probably too small to noticeably raise plasma CORT concentration. An instantaneous release into the blood stream would have increased blood concentration by about 8.2 ng/ml (assuming 30 ml blood in a pigeon), but because uptake from the body cavity takes some time and breakdown occurs (half-time of CORT in pigeons is 23 min; Chan, Bradley & Holmes 1972), the expected rise in plasma CORT concentration was insignificant. We did not see any significantly increased plasma concentration three days after injection (mean 2.65 ng/ml ± 1.14 SD, n = 7 birds without CORT implants) compared with the two concentrations measured before injection (1.46 ng/ml ± 1.72, 1.85 ng/ml ± 1.41) and the two measures nine and 28 days after injection (2.04 ng/ml ± 1.66, 2.09 ng/ml ± 1.56; P = 0.65 Mixed Model with individual identity as random factor).

The pellets substantially raised plasma CORT in the 8 treated birds from 0.88 ng/ml ± 1.13 (SD) on 27 February and 1.39 ng/ml ± 1.92 two days before pellet implantation to 54.44 ng/ml ± 52.66 (range 9.51 – 171.45) two days after pellet implantation. Plasma CORT decreased to 14.57 ng/ml ± 12.26 eight days after implantation and came back to pre-treatment levels of 1.68 ng/ml ± 1.56 on 10 April. In the 7 non-implanted birds, plasma CORT levels remained low (see above).

Appendix S2: Feather extraction and CORT determination with EIA and LCMS

Feathers were extracted for CORT determination with EIA following Bortolotti et al. (2009). The minced feather segments were mixed with 5 ml of methanol (HPLC grade), placed in a sonication water bath for 30 min, and incubated overnight in a 50°C water bath. The sample was filtered through Whatman filter paper (Grade 4, 47 mm) into a new test tube. The minced feather segments, the sample vial, and the filter paper were washed with 2 ml methanol and added to the methanol extract. Extracts were evaporated under nitrogen gas at 50°C in a SBHCONC/1 Sample Concentrator. For the EIA extracts were reconstituted (final concentration 1 mg feather per well) in assay buffer (phosphate buffer 0.1M pH 7.0, with 0.1% BSA).

For LCMS the extracts were reconstituted in 50 µl methanol, 945 µl H_2O (milliQ) and 5 µl of d8-corticosterone (d8-CORT) as internal standard (1µg/ml; final concentration 50ng/ml). The reconstituted samples were placed in a sonication water bath for 15 sec. Thereafter a solid phase extraction was run using Phenomenex® 8B-S100-AAK columns (polymeric reversed phase, 2 ml, 10 mg/ml) including the following steps: (1) Conditioning with 1 ml methanol; (2) equilibration with 1 ml 5% methanol in milliQ; (3) sample loading; (4) washing with 1 ml 5% methanol in H_2O_{bidest} followed by 1 ml hexane; (5) elution with 1 ml ethyl-acetate into a 1.5 ml tube. The extracts were dried in a vacuum centrifuge at 40°C for 1 hr, reconstituted in 100 µl 50% methanol in H_2O (milliQ), placed in an ultrasonic bath for 30 sec and centrifuged for 90 s at 14000 rpm. The supernatant was transferred into a vial for LMCS.

For the analysis of plasma corticosterone 20 µl plasma and 180 µl water (H_2O_{bidest}) was extracted with 4 ml dichloromethane, dried in a 48°C water bath and reconstituted in phosphate buffer.

CORT was quantified by LMCS at the University in Neuchâtel using an Ultimate 3000 RS system (Dionex, Thermo Fisher Scientific) coupled to a 4000 QTrap (ABSciex) equipped with a Turbo VTM ion source. Chromatographic separation was carried out on an Acquity UPLC® BEH C18 column (1.7 µm particle size, 50x2.1 i.d. mm, Waters). Using milli-Q H_2O with 0.05% formic acid as mobile phase A and acetonitrile with 0.05% formic acid as mobile phase B, 10 µl of the reconstituted steroid fraction was injected onto the column and eluted with the following gradient conditions: 20-70 % B for 5 min, 70-100 % B for 1 min, 100 % B for 2 min, and re-equilibration at 20 % B for 3 min. The flow rate was set to 0.4 ml/min. CORT and d8-corticosterone (d8-CORT) were monitored in positive ionization using the multiple reaction monitoring (MRM) mode. Optimized parameters specific for CORT and d8-CORT respectively were as follows: transitions 347.1/121.1 and 355.1/125.0, collision energy 57 eV and 31 eV, declustering potential 76 V and 91 V, entrance potential 10 V for both, and collision cell exit potential 8 V and 10 V. Other source and collision cell parameters were as follows: ion spray voltage (IS) +5500V, temperature (TEM) 600°C, ion source gas 1 (GS1) 30 psi, ion source gas 2 (GS2) 30 psi, curtain gas (CUR) 15 psi, collision gas (CAD) 5 psi. Quantification was performed using standard curves calculated from standard solutions of CORT at 10, 50, 100, 250 and 500 ng/ml, each containing d8-CORT as internal standard at a constant concentration of 50 ng/ml.

Plasma and feather CORT concentration was measured in triplicates using an enzyme-immunoassay (EIA, Munro & Lasley, 1988) in the laboratory of the Swiss Ornithological Institute in Sempach. The dilution of the CORT 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 1:8’000. HRP (horseradish peroxidase, 1:400’000) linked to CORT served as enzyme label and 2,2’Azino-bis(3-ethylbenzo-thiazoline-6-sulfonicacid)diammonium salt (ABTS) as substrate. The concentration of CORT in plasma samples was calculated by using the standard curve run in

duplicate on each plate. A plasma pool from chicken was included as internal control on each plate. Inter-assay variation was 10.43 % and 4.91%, intra-assay variation 2.32 % and 3.35% for feather and plasma CORT analysis, respectively. For validation 5 series of diluted feather samples were run against 5 standard curves. The dilution curves were parallel to the standard curve.

Feather sample mass varied between 0.86 and 10.25 mg (mean 4.81 ± 1.61 SD). We did not find a dependence of CORT concentration, expressed per mm feather length, on feather sample mass (Mixed Model analysis of original feathers and re-grown feathers of only tritium-injected birds with individual as random factor and with washing and feather sample mass as fixed factors; $P = 0.164$ for feather sample mass, slope +0.166 ± 0.119 SE, $n = 248$). As in Lattin et al. (2011), CORT concentration expressed per mg feather material was significantly dependent on feather sample mass ($P < 0.001$ for feather sample mass, slope -1.900 ± 0.288 SE, $n = 248$). However, as explained in Bortolotti et al. (2009) and in the section 'Dependence of feather corticosterone on feather growth rate' of the Results, expressing CORT per feather length is the biologically meaningful way. Hence, the negative relationship of CORT (per mg feather material) on feather sample mass simply reflects the fact that CORT is 'more diluted' when feather sections of similar length are more heavy.

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Appendix S3: Comparison of corticosterone concentrations obtained by EIA and LCMS-quantification

We compared the concentration of CORT determined by EIA on one tail feather with that determined by LCMS on another tail feather of the same individual as follows. The values of CORT measured by EIA (expressed as the concentration per mm segment length) of two adjacent 10 mm feather segments were averaged to provide a value to be compared with the value determined by LCMS of the corresponding 20 mm segment of the other feather. We matched the washed segments from the EIA analysis with the washed segment from the LCMS analysis and the non-washed with the non-washed, because washing had a major influence on the concentration of CORT in both analyses. Because the data set included many low values and a few high values, we ln-transformed the EIA and the LCMS values to obtain normally distributed residuals. In a linear mixed model with the individual as random intercept, the concentration of CORT determined by EIA was highly significantly related to that determined by LCMS on the corresponding segment of another tail feather of the same individual, while washing or vane (inner or outer) had no significant influence and were removed ($F = 25.62$, $P < 0.001$, $n = 80$). The absolute values determined by the two methods were similar (e.g. the mean on the ln-scale was 1.83 ± 0.15 SE for LCMS data and 1.94 ± 0.11 for EIA data, $n = 80$) and there was no significant difference between paired values (paired t-test $P = 0.45$). Thus, despite the fact that we measured CORT in two different tail feathers, the values obtained by the two methods were very comparable.