From the Institute of Medical Biochemistry[1], Department of Biomedical Sciences and the Konrad Lorenz Institute of Ethology[2], Department of Integrative Biology and Evolution, University of Veterinary Medicine Vienna, Austria

Patterns of excreted glucocorticoid metabolites change during development – analytical and physiological implications

M. STÖWE[1]*, S. RETTENBACHER[1], J.M. BUSSO[1], A. GRASSE[2], K. MAHR[2], W. VOGL[2], H. WINKLER[2] and E. MÖSTL[1]

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Summary

In view of the consequences of glucocorticoid (GC) production, inter-individual comparisons of the hormonal response to challenges are of interest. GC excretion is widely assessed non-invasively via measurements of immunoreactive glucocorticoid metabolites (CM) in excreta by means of enzyme immunoassays (EIAs). A few methodological issues need to be considered to ensure valid results. We examined whether and how patterns (mixture) of excreted CM change during development (early and late nestling stage, adults) in blue tits and compared two EIAs. Assay 1 had been successfully validated for a number of bird species. Assay 2 used an antibody against the same antigen but raised in a different individual. The difference could affect antibody cross-reactivity and therefore the suitability of assay 2 had to be validated. We compared the results of the two assays in samples of adult blue tits (handling stress experiment), chicken and quail (ACTH challenges). The CM patterns of 3–4 days old blue tit nestlings differed markedly from those of 11–12 days old nestlings and adults. Sex differences in CM patterns were found in all age classes. While assay 2 cannot be recommended for measuring stress responses in adult blue tits, it was suitable for use in chicken and quail. Our results show that a change in antibody charge may have a remarkable impact on antibody cross-reactivities with CM and may affect the biological sensitivity of an assay to measure hormonal stress responses. Furthermore, the suitability of an assay is highly species-dependent.

Abbreviations: CBG = corticosteroid binding globulin; CM = corticosterone metabolites; EIA = enzyme immunoassay; GC = glucocorticoid; HPA = hypothalamo-pituitary-adrenal; RP-HPLC = reversed-phase high performance liquid chromatography

Zusammenfassung

Altersabhängigkeit des Musters ausgeschiedener Glukokortikoidmetaboliten

Einleitung

Zur nicht-invasiven Erfassung von Glukokortikoiden (GC) bietet sich die Messung von Glukokortikoidmetaboliten (CM) in den Exkrementen via Enzymimmunoassays (EIAs) an. Vergleiche zwischen Individuen bezüglich ihrer hormonellen Stressreaktionen sind in der Verhaltensforschung von großem Interesse. Um aussagekräftige Ergebnisse zu erhalten, müssen jedoch einige methodische Punkte beachtet werden. Ein EIA ist dann geeignet, wenn seine Antikörper mit den nach einem stressbedingten GC-Anstieg ausgeschiedenen CM interagieren. Das CM-Muster hängt nicht nur von der Spezies, sondern auch von Alter sowie Geschlecht der Tiere ab. Wir untersuchten hier bei Blaumeisen (*Cyanistes caeruleus*), ob und wie sich die immunreaktiven CM-Muster während der Entwicklung verändern. Weiters verglichen wir zwei verschiedene EIAs: Assay 1 wurde bereits erfolgreich für eine Reihe von Vogelspezies validiert. Für den Antikörper von Assay 2 wurde zwar das gleiche Antigen verwendet, die Antikörper wurden jedoch von einem anderen Tier produziert. Da dies möglicherweise die Kreuzreaktionen beeinflusst, musste Assay 2 erneut validiert werden.

Material und Methode

Kotprobenextrakte von Blaumeisen unterschiedlichen Alters (frühe und späte Nestlingsphase, Adulttiere) wurden mittels Hochleistungs-Flüssigkeitschromatographie getrennt und die CM-Konzentrationen in den einzelnen Fraktionen gemessen. Wir verglichen die beiden EIAs bezüglich ihrer Sensitivität, CM-Anstiege nach einem „Handling“ (Stressexperiment) im Kot von adulten Blaumeisen bzw.

nach ACTH-Stimulationstests bei Wachteln und Haushühnern zu detektieren.

Ergebnisse

CM-Muster von 3–4 Tage alten Blaumeisennestlingen unterschieden sich signifikant von denen 11–12 Tage alter Nestlinge und adulten Tieren. Die CM-Muster variierten in allen Altersklassen zwischen den Geschlechtern. Assay 2 zeigte sich geeignet, stressbedingte CM-Anstiege bei Hühnern und Wachteln zu messen, ist jedoch für Blaumeisen nicht zu empfehlen. Die beiden Assays unterscheiden sich deutlich in ihren Kreuzreaktionen mit Steroidmetaboliten, was den Unterschied in ihrer Sensitivität Änderungen in ausgeschiedenen CM-Mengen zu detektieren erklärt.

Schlussfolgerungen

Ob ein bestimmter EIA zur Messung von GC-Abbauprodukten im Kot eingesetzt werden kann, ist nicht nur speziesspezifisch, sondern kann neben dem Geschlecht auch noch vom Alter der Fokustiere abhängen. Unsere Ergebnisse zeigen eindrucksvoll, dass eine Änderung in der Antikörpercharge die Kreuzreaktionen des Antikörpers beeinflusst, was sich merklich auf die biologische Sensitivität des EIAs auswirken kann.

Introduction

In view of the wide-ranging modulating effects and potential long-term consequences of high glucocorticoid (GC) levels, it is interesting to compare individual secretions of GCs in response to challenges. Both baseline and stress-induced GC secretion show ample inter- and intra-individual variation. Differences in hypothalamo-pituitary-adrenal (HPA) axis reactivity result from differences in genetic background (EVANS et al., 2006; FRAISSE and COCKREM, 2006), from maternal effects (in mammals, e.g. KAISER and SACHSER, 2005, and birds, reviewed in HENRIKSEN et al., 2011), from maternal care (e.g. HENNESSY, 1997; BRANCHI, 2009; BANERJEE et al., 2012), from developmental stage (STARCK and RICKLEFS, 1998; BLAS et al., 2006, and references therein), from body condition (SOCKMAN and SCHWABL, 2001; BREWER et al., 2008; LENDVAI et al., 2009), from coping style/personality (e.g. CARERE et al., 2003; STÖWE et al., 2010), from season (breeding, raising of young: KOTRSCHAL et al., 1998; HIRSCHENHAUSER et al., 2000) and from migration (JENNI et al., 2000). Social context also has a notable impact on GC secretion. Depending on the situation, social contact may be a cause of stress (e.g. conflicts, reviewed in CREEL et al., 2013) or have calming and encouraging effects (social support, e.g. HENNESSY et al., 2006; STÖWE et al., 2006, 2009; FRASER et al., 2008; FRASER and BUGNYAR, 2010). In addition, the concentration of corticosteroid binding globulin CBG) in the blood and the number of GC receptor binding sites modulate GC actions (ROSNER, 1990, see discussion).

There is considerable interest in measuring GCs non-invasively in a wide range of species. Determining the amounts of glucocorticoid metabolites (CM) in urine, faeces or saliva is particularly useful when repeated sampling (especially in small animals) is necessary. Individuals do not need to be handled, which is an enormous advantage when studying free-ranging animals. Moreover, sample collection poses no burden for the animal if it is habituated to the presence of humans and samples are collected after delivery. The time lag between hormone secretion into the bloodstream and excretion of metabolites facilitates data collection without affecting baseline GC levels (from stress caused by approach and sample collection). Furthermore, sample collection is far easier than when corticosterone or cortisol are measured in the blood (TOUMA and PALME, 2005; PALME et al., 2005; SHERIFF et al., 2011, even when blood samples are taken from animals that are approached and caught rather quickly, e.g. nestlings in nest-boxes: GIL et al., 2008).

There are a few methodological issues that need to be considered to ensure meaningful results when CM are determined in excreta. The immunoassay applied has to be validated for each species separately, testing both males and females, as sex differences in CM patterns (mixture of excreted CM) have been observed in several species (e.g. GOYMANN, 2005; TOUMA and PALME, 2005; STÖWE et al., 2010). Differences in metabolite structures may affect the measured CM concentrations, because antibodies vary in their affinity to different metabolites (MÖSTL et al., 2005, see also below). In addition, the time lag from GC secretion to excretion should be considered (PALME, 2005), bearing in mind that the animals' (circadian) activity (TOUMA et al., 2003), changes in diet (e.g. seasonal, GOYMANN, 2005) and rate of food intake (GOYMANN et al., 2006) may affect gut passage times and CM excretion.

Furthermore, the developmental stage could affect the biochemical structure of the GC metabolites (i.e. size and polarity). There are several lines of evidence for this. Even though the major glucocorticoid in birds is corticosterone, the birds' adrenal glands also secrete cortisol during the embryonic phase and around hatching (DE MATOS, 2008, and references therein). In addition, the rate of metabolism, gut flora and gut passage times certainly differ between nestlings and adults. The differences will probably affect not only the concentrations of the metabolites but also the composition of the excreted CM. Nevertheless, we are unaware of any previous

investigations of this topic in birds, despite the interest in the effects of hormone secretion during development.

One of the core steps of an enzyme immunoassay (EIA) is the binding of antibodies to certain groups of metabolites. In the course of the measuring procedure a fixed amount of labelled steroid is added. Depending upon the concentration of the steroid metabolites in the sample in question, more or less labelled steroid will be bound by the antibodies. The amount of labelled steroid bound enables the concentration of steroid metabolites to be determined. If the metabolites involved in the antibody-metabolite interactions change (different metabolites excreted) the CM concentrations measured may vary significantly, depending on the cross-reactivity of the assay used (MÖSTL et al., 2005).

We now report a study to address two issues. The first part was aimed at testing whether and how GC metabolite patterns change over development in a passerine, the blue tit, *Cyanistes caeruleus*. To this end, we used reversed-phase high performance liquid chromatography (RP-HPLC) and EIAs to analyse samples of male and female nestlings at the beginning (3–4 days of age) and towards the end of the nestling phase (11–12 days of age) and of adults. RETTENBACHER et al. (2004) showed that an assay with antibodies binding steroids with a 3,11-dioxo configuration (4-pregnenes, 5α-pregnanes and 5α-androstanes) is well suited to measuring stress responses in chicken, *Gallus gallus domesticus*. In the meantime the assay (subsequently referred to as assay 1 and described in detail by RETTENBACHER et al., 2004) has been successfully validated for several other avian species (e.g. capercaillie, *Tetrao urogallus*: THIEL et al., 2005; black grouse, *Tetrao tetrix*: BALTIC et al., 2005; blue tit, pied flycatcher, *Ficedula hypoleuca*: LOBATO et al., 2008; Japanese quail, *Coturnix japonica*: BUSSO et al., 2013). Assay 1 suffers from a susceptibility to disturbances (e.g. slight temperature changes). For the antibody of assay 2 the same antigen was used to immunize rabbits, although the antibodies of assay 1 and 2 were derived from two separate immunization processes (different individuals).

The second part of the study was aimed at testing whether assay 2 could be a more robust alternative for assay 1. To validate the ability of assay 2 to detect stress-related increases in CM in blue tits, we conducted a handling stress experiment and analysed the droppings collected with assays 1 and 2. In addition, we used assay 2 to re-analyse samples of ACTH challenges previously conducted with female chicken and male quail and compared the results to those obtained with assay 1.

Materials and Methods

Animals and sample collection

Blue tits are hole-nesting, territorial, non-migratory passerines. The clutch size ranges from 4–14 eggs and young fledge at 16–23 days of age and continue to be fed by both parents for another 2–3 weeks (GLUTZ VON BLOTZHEIM et al., 1993).

From 5.5. – 22.5.2010 we monitored first-brood blue tit nestlings in nest-boxes distributed at Buchberg (48° 12.8' N, 16° 12.8' E, 21 ha) in the Viennese Woods (Austria). The nest-boxes are being used in a long-term study by H. W. and W. V.. We regularly visited the nest-boxes to monitor clutch size and hatching dates. When nestlings were 3–5 days old, we marked them individually with coloured cotton bands attached to the tarsi. We also fed them mealworms to induce defaecation, collected droppings and returned the nestlings to their nests. We visited each nest-box every second day at the same time of day to collect droppings until nestlings were 10–12 days old, when we stopped nest-visits to avoid causing premature fledging. We substituted the cotton leg bands with aluminium rings (MPIO-Vogelwarte Radolfzell) when nestlings were 8–10 days old. At this time, we also took a buccal smear from each bird with a cotton swab (Raucotupf sterile cotton tipped applicators, Lohmann and Rauscher) for genetic sex determination. Both droppings and buccal smears were stored on ice while we were in the field and thereafter kept frozen at -20 °C until analysis. Data were collected between 9:20 and 18:20. Ringing and handling of the nestlings was approved by the conservation department (MA22) of the city of Vienna (permit numbers MA22-740/07 and MA22-216/210 to H. W. and W. V.).

In February 2013 we conducted a catching/handling stress experiment with six adult blue tits (almost two years old, four females and two males) housed together in a single aviary at the Konrad Lorenz Institute of Ethology (indoor enclosure - 2.75 x 2.8 x 2.1 m - connected to an outdoor enclosure - 1.7 x 2.8 x 2.1 m) under natural light conditions. Birds were caught with nets, put in cotton bags and carried into an experimental room, where they were released into individual cages (81 x 38 x 38 cm). Birds remained in these cages for the duration of sample collection (11:00 until 14:40). Cages were equipped with wooden perches and drawers at the bottom. Water and food were available *ad libitum* (a mixture of sunflower seeds, peanuts, millet, curd, pieces of carrots and apples, mealworms and food for insectivorous birds, i.e. 2/3 egg food, Witte Molen BV, Netherlands, 1/3 soft-food, Claus GmbH, Germany). The bottom of the drawers was covered with paper to facilitate the collection of droppings. Every 25 min all droppings per bird were collected and stored on ice during sample collection, then at -20 °C until being processed for

hormone analysis. Blue tits were bled at the alar vein for sex determination before being returned to their home aviary. All experimental procedures were approved by the Austrian animal welfare commission (permit number 68.205/0220-II/3b 2012 to H. Hoi).

To determine the sex of the blue tits, we extracted DNA from the nestlings' buccal smear swabs using the QIAamp® DNA Micro Kit (Qiagen, N.V. Hilden, Netherlands). For molecular sexing of nestlings and adults we used primers P2 and P8 (Invitrogen™, for primer sequences see GRIFFITHS et al., 1998). PCR amplifications were performed in a Biometra® T-gradient Thermo-cycler machine. Separation was achieved via gel electrophoresis (3% agarose, GRIFFITHS et al., 1998) giving one band for males and two bands for females.

To evaluate the ability of assay 2 to detect stress responses in two other bird species, we analysed droppings collected after ACTH challenge tests from female chickens (RETTENBACHER, unpublished) and male quails (BUSSO et al., 2013). We analysed samples with both assay 1 and assay 2 (for details see below) to compare absolute CM amounts and relative increases of measured CM concentrations between the assays. For the ACTH challenge tests in chicken, we used a same-aged group of 12 adult female Lohmann brown laying hens (older than 22 weeks) obtained from a commercial breeder (Schropper GmbH, Gloggnitz, Austria) housed in single cages as described by RETTENBACHER et al. (2004). After an adaptation period of five days, birds were injected in the pectorial muscle with 0.2 mg of a synthetic ACTH analogue (Synacthen-Depot® Novartis Pharma GmbH, Vienna, Austria) per kg body weight. Droppings were collected and stored as described previously (RETTENBACHER et al., 2004). The experiment was approved by the Austrian animal welfare commission (permit number 68.205/0196-BrGT/2006 to S. R.).

The procedure for the ACTH challenge in quails was described by BUSSO et al. (2013). We used samples from eight males (from three groups, see BUSSO et al., 2013: short day - responders, n=3; short day - non responders, n=3; long day, n=2).

Sample analysis

To examine whether CM excretion patterns in blue tits change with age, 0.05 g of wet droppings were vortexed in 0.5 ml methanol (60%) for 15 min (PALME et al., 2013), then centrifuged and the supernatants pooled: one pool (1 ml) per sex and age-class (3–4 days old nestlings, 11–12 days old nestlings, adults). We added tritiated cortisol (New England Nuclear, Dreieich, Germany) to the pools of the 3–4 days old nestlings and the adult birds to estimate steroid loss due to clean-up and chromatography procedures. Methanolic supernatants were diluted with double-distilled water (1:10) and cleaned using an activated Sep-Pak C_{18} cartridge (1 g, Waters, Milford, MA, USA). We eluted steroid metabolites with 4 ml 80% methanol. The eluates were dried under a stream of nitrogen and re-dissolved in 100 µl 20% methanol before running a RP-HPLC (linear water/methanol gradient: 20%–100%, column: Novopack C_{18}, 3.9 x 150 mm, Waters, WAT086344, flow rate: 1 ml/min, three fractions per min). Note that the HPLC separation differed substantially from that performed in LOBATO et al. (2008).

To estimate recovery rates of the radiolabelled tracer, 0.05 ml of each of the RP-HPLC fractions 52–62 (elution position of cortisol at fractions 56 and 57) were transferred into scintillation vials, mixed with 8 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, USA). Recovery was 80% for 3–4 days old male and 96% for female nestlings, 74% for adult males and 87% for adult females.

Two different assays were used to measure metabolites in the RP-HPLC fractions. One cortisone EIA (assay 1, RETTENBACHER et al., 2004) has been validated previously for blue tits (LOBATO et al., 2008). The antibodies of assay 1 and of the second cortisone EIA (assay 2) were raised in a rabbit, although from a different immunization process (in a different individual). The same antigen as used for the antibody described by RETTENBACHER et al. (2004) was used. As the antibodies were raised in different individuals, their suitability for detecting CM related to stress responses should be re-tested, even if the same antigen was used for immunization.

For assay 2, the working dilution of the antibody was 1:30,000. 4-pregnene-17α,21-diol-3,11,20-trione, linked at position C-20 to carboxymethyloxime (CMO), was used as biotinylated label (MÖSTL et al., 2005). Microtitre plates were protein A-coated (OUSCHAN et al., 2013). The working dilution of the label was 1:1,000,000. Cortisone (4-pregnene-17α,21-diol-3,11,20-trione) was used as standard (range of the curve from 2 to 500 pg/well); the 50% intercept was about 85 pg. Tab. 2 shows the cross-reactions of assay 2 and compares them to those described for assay 1 by RETTENBACHER et al. (2004).

As bacterial enzymes may hydrolyse conjugated metabolites, thereby changing metabolite patterns in droppings/faeces after defecation (and sample collection, see PALME, 2012 and references therein) and even after thawing (MÖSTL et al., 2005), we also examined the effects of hydrolysis on the amounts of CM detected by the different assays. We evaporated 0.05 ml of the first 56 fractions, containing largely conjugated, polar metabolites (unconjugated metabolites elute in later fractions), then dissolved CM in 0.1 ml sodium acetate buffer (pH=4.8) and 0.2 µl β-glucuronidase/aryl sulfatase (Merck 1.04114.0002) and incubated the solutions at 38 °C for 18 h.

The processing of the chicken and quail samples is described in RETTENBACHER et al. (2004) and BUSSO et al. (2013). In both species sample extracts were analysed with assays 1 and 2.

Data analysis

To test for potential differences in CM patterns during development in blue tits, we added the CM values (ng/fraction) of five fractions each (fraction 1–5, 6–10, 11–15 etc.) and compared the sums between the age classes with a Kruskal-Wallis test followed by Mann-Whitney U tests and Bonferroni corrections. CM increases (we considered only the first peak in excreted CM) in response to the handling (blue tits) and ACTH challenges (chicken, quail) were tested with Wilcoxon tests. To examine whether the results from assay 1 correlated with those from assay 2, we used Spearman rank-order correlations. In quails, we correlated CM values measured with assays 1 and 2 for each individual separately and thereafter calculated the mean±SD of the r_s values of the single correlations. In blue tits and chickens, we did not have sufficient data per individual to proceed in this manner so we correlated individual baseline CM values measured with assay 1 to those measured with assay 2 and did the same with peak CM values. All tests were calculated by hand according to SIEGEL and CASTELLAN (1988) for n<10, as was the case for adult blue tits and for quail (Wilcoxon tests). For n>10 (comparisons of CM excretion patterns during development in blue tits, chicken data and between assay correlations in quail) we used the program SPSS (IBM SPSS Statistics 19). Test results are given two-tailed. Unless otherwise indicated, mean±SD values are given throughout the paper.

Results

CM excretion during development

Excreted CM differed significantly between age classes. Using assay 1, the CM patterns of adult blue tits were significantly different from CM excreted by nestlings (Fig. 1a–c, except for the CM of 3–4 days old male nestlings, which did not differ from those of adult males). There was no significant difference between early and late stages of nestlings ($n_{3–4\ days\ old\ nestlings}=n_{11–12\ days\ old\ nestlings}=n_{adults}=19$ sums of five HPLC fractions each; Kruskal-Wallis test, df=2, males: $\chi^2=8.14$, p=0.017, females: $\chi^2=25.68$, p<0.001; post-hoc comparisons: Mann Whitney U tests, results see Tab. 1). When the samples were analysed with cortisone assay 2, only adult females differed significantly from 3–4 days old females and early stage male nestlings from late stage male nestlings (Fig. 1d–f, $n_{3–4\ days\ old\ nestlings}=n_{11–12\ days\ old\ nestlings}=n_{adults}=19$ sums of five fractions each; Kruskal-Wallis test, df=2, males: $\chi^2=7.67$, p=0.022, females: $\chi^2=7.73$, p=0.021; post-hoc comparisons: Mann Whitney U tests, see Tab. 1).

In both cortisone assays, hydrolysis led to an increase in CM concentrations in the first 55 HPLC fractions in nestling droppings (Figs. 1 and 2, assay 1: 3–4 days old males: 13-fold increase, females: 2.6-fold; 11–12 days old males and females: 3.1-fold; assay 2: 3–4 days old males: 1.7-fold, females: 11.9-fold; 11–12 days old females: 1.3-fold). Only in samples of 11–12 days old males did assay 2 detect significantly (33%) lower amounts of CM after hydrolysis compared to a sample analysis without hydrolysis steps (Figs. 1 and 2). In adults, higher CM concentrations were measured when sample processing did not include hydrolysis (assay 1: males: 1.4 times higher amounts, females: 1.5; assay 2: females: 1.3 times higher). In samples from adult males, hydrolysis led to a twofold increase in CM when measured with assay 2 (Figs. 1 and 2).

Handling stress and ACTH challenges in adult birds: comparison of assay 1 and 2

Even though CM values measured with assay 1 and 2 were positively correlated (Spearman rank-order correlation, n=6, baseline values: $r_s=0.99$, p=0.005; peak values: $r_s=0.99$, p=0.005), assay 1 detected a significant increase in CM 1.5–2 hours after the handling challenge in adult blue tits (Wilcoxon test, n=6, T+=21, p=0.03, Fig. 3), while assay 2 reflected this increase only by trend (Wilcoxon test, n=6, T+=20, p=0.06, Fig. 3). Compared to baseline CM levels, levels of CM measured with assay 1 increased 2.2±1-fold in response to the handling stress, whereas the increase was 1.3±0.3-fold when measured using assay 2.

In chickens, only baseline CM values were correlated between assays (Spearman rank-order correlations, n=12, baseline values: $r_s=0.71$, p=0.009, peak values: $r_s=0.32$, p=0.31). We measured significant increases in CM in response to the ACTH challenge with both assays (Wilcoxon test, n=12, assay 1: z=-3.06, p=0.002; assay 2: z=-2.82, p=0.005). With assay 1, CM values increased 16±10.5-fold and with assay 2 CM values were 5±3.4 times higher 5–7 hours after ACTH administration (Fig. 4).

In all quails, CM values measured with assay 1 highly correlated with those of assay 2 (Spearman rank-order correlation, individual r_s: 0.85±0.084, $p_{max}=0.005$, $p_{min}=<0.001$, $p_{median}=0.001$). Both assays revealed a significant increase in CM in response to the ACTH challenge (Wilcoxon test, n=8, assay 1 and 2: T+=36, p=0.008). With assay 1, CM values increased 4±1.6-fold and with assay 2 the CM values were 3.8±2.2 times higher 2–3 hours after ACTH injection (Fig. 5).

Tab. 1: Comparison of excreted CM between age classes for male and female blue tits. We added the CM values (ng/fraction) of five consecutive fractions (fractions 1–5, 6–10, 11–20 etc.) of each HPLC immunogram for cortisone assays 1 and 2 and tested for differences between age classes using Kruskal-Wallis tests (see results) and Mann-Whitney U tests as post-hoc tests: $n_1=n_2=n_3=19$ sums of five fractions each. $P<0.017$ remain significant after non-sequential Bonferroni correction (indicated in bold).

	11–12 days old males		adult males	
	assay 1	**assay 2**	**assay 1**	**assay 2**
3–4 days old males	z=-0.22, p=0.82	z=-2.67, **p=0.007**	z=-1.63, p=0.11	z=-1.68, p=0.096
11–12 days old males			z=-3.14, **p=0.002**	z=-1.21, p=0.23
	11–12 days old females		**adult females**	
3–4 days old females	z=-0.99, p=0.32	z=-1.85, p=0.065	z=-4.29, **p<0.001**	z=-2.51, **p=0.011**
11–12 days old females			z=-4.33, **p<0.001**	z=-1.39, p=0.16

Tab. 2: Between assay comparison of cross-reactions (in %) with diverse steroids that have a similar structure to that of cortisone.

Steroid	assay 1	assay 2
4-pregnene-17α,21-diol-3,11,20-trione	**100***	**100**
4-androstene-3,11,17-trione	**30***	**< 1**
5α-androstane-3,11,17-trione	**20***	**40**
4-pregnene-11β, 17α,20α,21-tetrol-3-one	**9***	**< 1**
4-pregnene-3,20-dione	**2***	**59**
5β-androstane-3,11,17-trione	**2***	**< 1**
4-pregnene-11β,21-diol-3,20-dione	**2***	**55**
5β-androstane-3α-ol-11,17-dione	**< 1***	**< 1**
5α-pregnane-17α,21-diol-3,11,20-trione	**134**	**78**
5β-pregnane-17α,21-diol-3,11,20-trione	**20**	**< 1**

* as published in RETTENBACHER et al., 2004

Discussion

We aimed at examining changes in excreted CM during the development of blue tits by means of two different assays. As assay 2 was newly developed and not extensively validated, we used handling (blue tits) and ACTH challenge tests (chickens and quails) as a basis for comparing the two assays and for assessing the suitability of assay 2 for measuring CM excretion in the three species.

The patterns of excreted glucocorticoid metabolites did indeed change during development (Fig. 1). Those of 3–4 days old nestlings differed markedly from those of 11–12 days old nestlings and of adults. Several factors (secretion of different steroids, corticosteroid binding globulin, food/gut passage times, gut flora composition, see below) may influence how steroids are metabolized and there are diverse and not mutually exclusive explanations for the differences observed between age-groups. First, during the embryonic stage and around the time of hatching the nestlings' adrenal glands synthesize not only corticosterone in considerable amounts but also cortisol, cortisone and sex steroids (DE MATOS, 2008, and references therein). Secretion of cortisol and cortisone rapidly declines after hatching (DE MATOS, 2008) but it is conceivable that measurable amounts of metabolites of these steroids are present in droppings of 3–4 days old nestlings. Based on our data, we cannot assess whether a potential period of hyposensitivity to stress (WADA et al., 2007) affects CM excretion in the early nestling stage.

Between age-class differences in food composition will not have a notable impact on the levels of CM measured in the present study, because blue tit nestlings are fed insects throughout the nestling period (the type of insect might be larger towards the end of the nestling period but there is no switch between insect feed and seeds) and adults are almost entirely insectivorous during the breeding season (PERRINS, 1991).

With regard to gut flora, a number of studies have investigated the effects of food composition on growth and resistance to pathogens in farmed birds, although little is known about gut flora composition in wild bird populations. Recent data show that the gastrointestinal tract of black-legged kittiwake chicks (*Rissa tridactyla*) is colonized by many transient species. With age, gut bacteria communities gradually

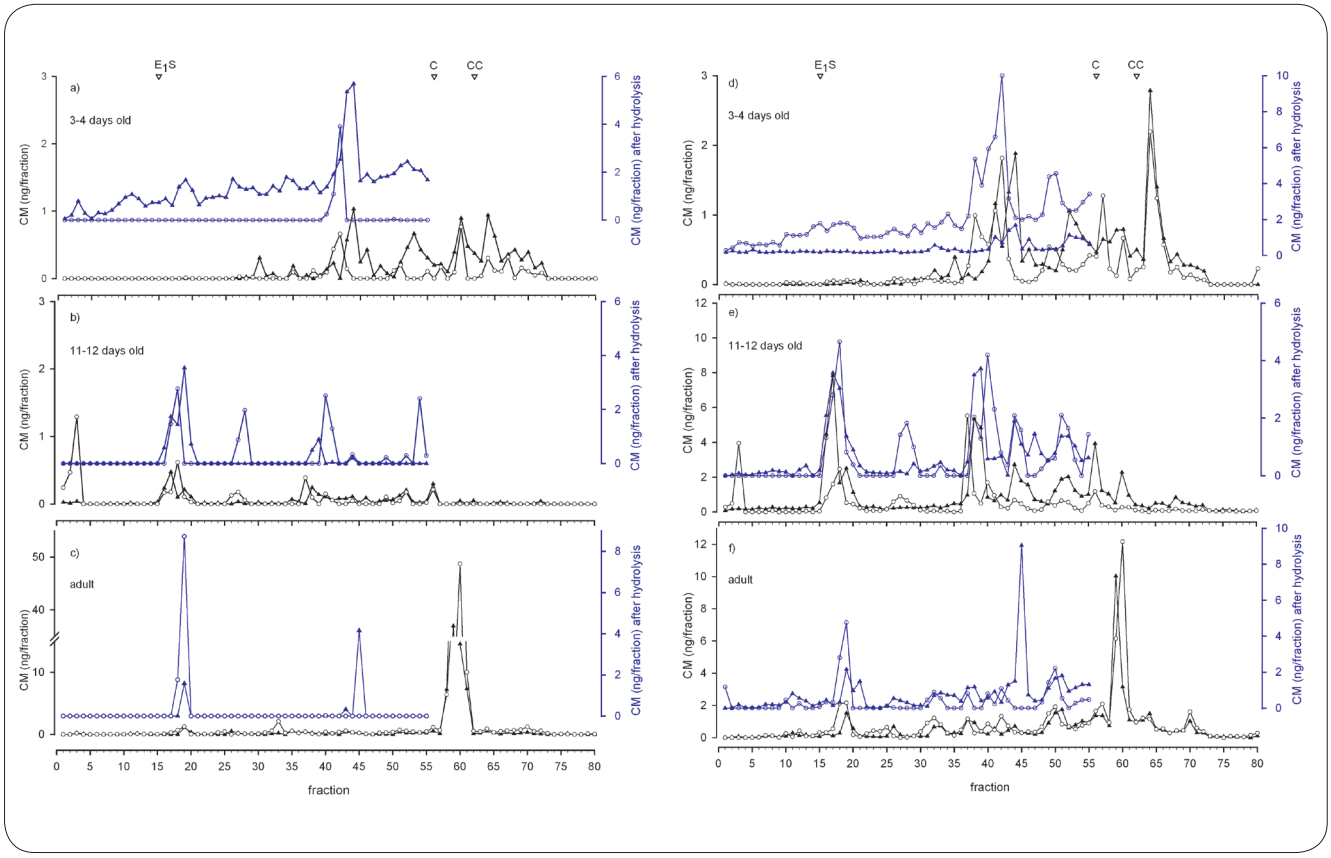

Fig. 1: HPLC immunograms: CM concentrations (ng/fraction) measured with cortisone assay 1 (a, b, c) and cortisone assay 2 (d, e, f) in 3–4 days old nestlings, 11–12 days old nestlings and adults. Blue lines show immunograms with additional hydrolysis before EIA analysis (y-axis on the right), black lines without hydrolysis (y-axis on the left). In both cases, males are represented by filled triangles and females by open circles. The white triangles on the top indicate fractions where standards elute: C = cortisol, CC = corticosterone, E_1S = oestrone sulphate.

change to a more stable adult state. Given that parents regurgitate the food they feed to their chicks and that chicks remain in the nest until fledging (thus avoiding contact with bacteria outside the nest), it is surprising that chicks and adults only share a small fraction of bacterial operational taxonomic units (VAN DONGEN et al., 2013). Changes in gut bacteria load and community composition with age have also been observed in songbirds (MILLS et al., 1999, tree swallows, *Tachycineta bicolor*) and there are indications that nest (differences between nests bigger than between species: cross fostering experiment with blue and great tits, *Parus major*: LUCAS and HEEB, 2005), weather conditions, thermoregulatory capacity and food availability (pied flycatchers, *Ficedula hypoleuca*: GONZÁLEZ-BRAOJOS et al., 2012) also effect bacterial communities in the gut. Individual variation in gut flora appears fairly consistent but sex-related differences have not previously been observed (BENSKIN et al., 2010). Thus, the sex differences in CM we observed in all age classes (with both of the assays used) are probably due to differences in steroid metabolism in the liver and not because of sex-related differences in the composition of gut bacteria.

Irrespective of the causes of sex differences in CM patterns, the fact that they have been detected in a

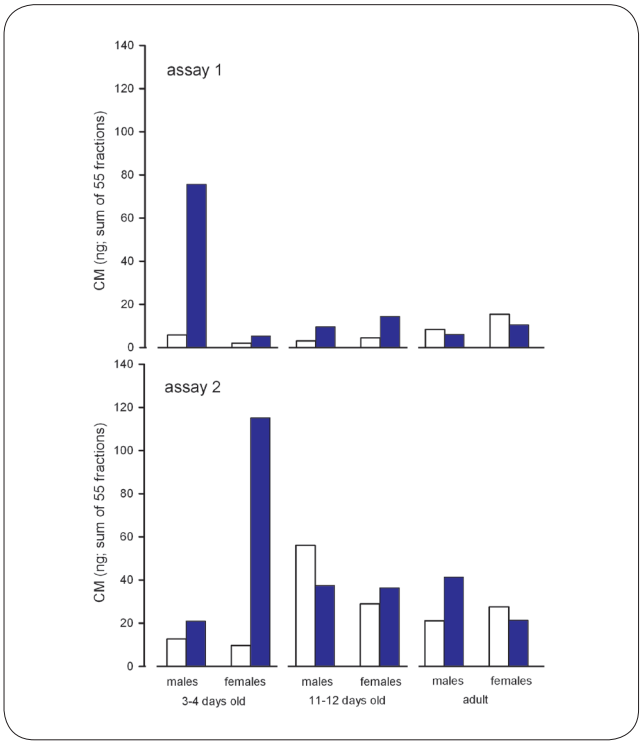

Fig. 2: Amounts (ng) of immunoreactive metabolites measured in RP-HPLC fractions 1–55, before (white bars) and after (blue bars) hydrolysis, using cortisone assays 1 and 2 in all three age classes and both sexes.

variety of species (GOYMANN, 2005; PALME et al., 2005; STÖWE et al., 2010) and in several age classes (present study) implies that it is necessary to examine sex differences carefully before directly comparing stress responses in males and females. Moreover, our results highlight that a direct comparison between age classes (especially involving the early nestling stage in which CM differ most from those in later developmental stages) should be performed with caution if at all.

Finally it is important to consider the impact of corticosteroid binding globulin (CBG), which affects the amount of GC that can be metabolized and excreted. In most animals (BREUNER and ORCHINIK, 2002) the major portion (90–95%) of circulating GCs is bound to CBG. Only the remaining unbound 5–10% triggers responses to acute stress (the free hormone hypothesis, see ROSNER, 1990). Unbound GCs rapidly traverse cell walls and enter cells, where they are metabolized (e.g. CARSIA and HARVEY, 2000). When the resulting metabolites are excreted, they may be detected in urine and faeces (or in birds' droppings). Thus, the CM concentrations measured in excreta presumably correspond to the proportion of unbound GC (5–10%) in the blood (PALME et al., 2005). This is why correlating CM values with plasma corticosterone values only makes sense when free corticosterone values in plasma are considered (and not total = free plus bound corticosterone). Aligning the correct values remains difficult due to the time lag in excretion (between blood GCs and CM in excreta; there are usually two CM peaks in response to stress due to CM in urine and faeces, see below) and because measurements in blood provide information about GC values at the precise moment of blood sampling, whereas CM levels in excreta always reflect values averaged over a certain time span. These 'mean' values might be an advantage of non-invasive methods, which offer more representative information on GC secretion over time (TOUMA and PALME, 2005).

To date, relatively little is known about CBG during the development of nestlings (WADA et al., 2007) and nothing is known about its effects on patterns and concentrations of CM metabolites. It is probable that a change in excreted CM during development is not limited to birds: steroid secretion (e.g. extremely high cortisol levels after birth, PRYCE et al., 2002), gut flora, nutrition and amounts of CBG also change over development in mammals (SÉRALINI, 1996), which could affect patterns of CM.

Hydrolysis of CM metabolites has the strongest effects in 3–4 days old nestlings, with a notable difference observed between the sexes. This once more underlines the importance of validating the analytical method with samples of both sexes and all age classes of interest. Due to differences in CM patterns, analytical steps may affect metabolites and thus antibody-metabolite interactions unevenly. While hydrolysis of samples of 3–4 days old male nestlings leads to an impressive increase in CM as detected by assay 1, this was not the case for female nestlings. Assay 2 revealed the opposite pattern (Fig. 2). The difference relates to differences in the cross-reactivity to CM of the antibodies used in assays 1 and 2 (Tab. 2). In both assays the effects of hydrolysis were stronger the younger the birds. We suggest including a hydrolysis step for processing nestling droppings to increase the comparability between samples (by reducing uncontrolled changes in metabolite pattern due to bacterial activity, see methods). The hydrolysis steps could be omitted when dealing with samples from adult blue tits. Each step in sample processing increases errors and variation in CM concentrations due to sample manipulation (pipetting, evaporation, changing vials etc.). Whether hydrolysis should be performed thus depends on the species, the age of the individuals and the assay system.

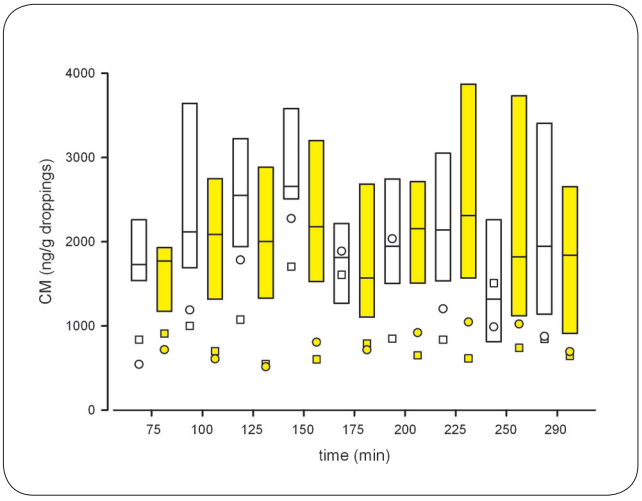

Fig. 3: CM (ng/g dropping) of adult blue tits ($n_{females}$=4, n_{males}=2) in response to a catching/handling challenge. White box plots show amounts of CM determined using assay 1, yellow box plots those measured with assay 2. Box plots show the median and the interquartile range from the 25th to the 75th percentile of the data of the females. The data of the two males are indicated by circles (male 1, white circles: assay 1, yellow circles: assay 2) and squares (male 2, white squares: assay 1, yellow squares: assay 2). Time (min) after challenge is given on the x axis.

The results of comparisons of the absolute amounts of CM measured with assays 1 and 2 after capture and handling stress in adult blue tits reveal that assay 2 is suitable only to a limited extent. In males, assay 2 detected almost no increase in excreted CM after the handling challenge and in some of the females only slight increases were observed (Fig. 3). It seems that the antibody of assay 2 binds preferably to something other than the predominant CM excreted in the droppings of adult blue tits. As a consequence, the assay is not very sensitive at detecting stress loads and measures an increase in CM only if stressors are strong. To increase the robustness of assay 1 it might be possible to use a different chemical bridge for the biotinylated label (MÖSTL et al., 2005). The label of

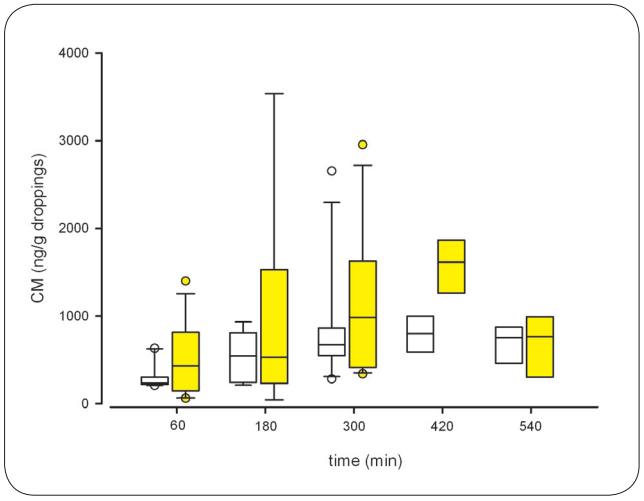

Fig. 4: Amounts of CM (ng/g dropping) excreted after an ACTH challenge in female adult chickens (n=12), determined with two different assays. White box plots represent results with assay 1, yellow box plots those with assay 2. Time (min) after ACTH administration is shown on the x-axis.

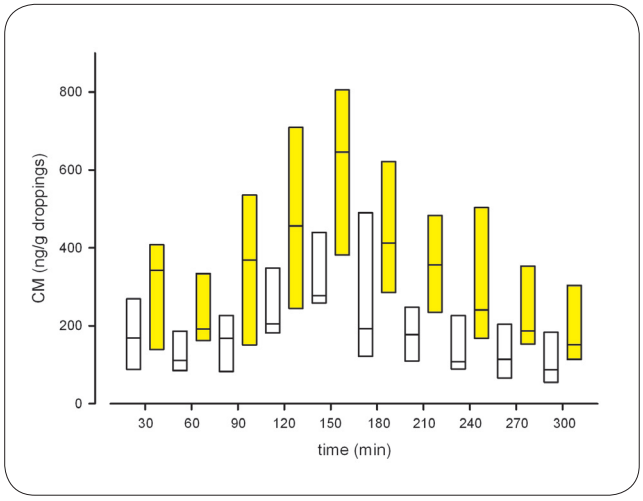

Fig. 5: Amounts of CM (ng/g dropping) excreted by adult male quails (n=8) in response to an ACTH challenge. CM concentrations were determined with two different assays (assay 1: white box plots; assay 2: yellow box plots). Time (min) after ACTH injection is given on the x-axis.

the present assay uses 4-pregnene-17α,21-diol-3,11,20-trione linked at position C-20 to carboxymethyloxime (CMO, RETTENBACHER et al., 2004). Substituting this label with biotin-labelled cortisone-21 glucuronide could improve assay 1 due to bridge heterology instead of site heterology (MÖSTL et al., 2005). We are currently testing this possibility.

Both assays – although assay 2 to a larger extent – measured a second peak in CM that was excreted almost four hours after the handling stress (Fig. 3). In most birds, urine and faeces are excreted together as droppings. After a stressful event, an increase in CM in urine is observed before peak CM values appear in the faeces, so there are two peaks of CM (RETTENBACHER et al., 2004), although whether both peaks are seen naturally depends on the sampling intervals and pooling of the droppings. CM excreted in urine and faeces can differ in metabolite structure and in concentration (PALME et al., 2005). The second peak was higher with assay 2 than with assay 1, again underlining that different metabolites are detected by the different antibodies.

While assay 2 cannot be fully recommended for measuring stress response in adult blue tits, it proved to be a suitable alternative to assay 1 in female chickens and male quails (females should also be tested). This highlights once more that the suitability of an assay is highly species dependent. Especially in chickens there was a large variation between individuals with regard to the time course of CM excretion (Fig. 4). At a given time point, some individuals excreted peak CM levels, while others showed only an increase. When the later birds excreted maximum CM in response to the challenge, the early birds were already on the way back to baseline levels. Factors such as the proximity to blood vessels of the ACTH injection site and the availability of CBG binding sites may contribute to the individual differences in time between ACTH administration and CM peak excretion. Stress response patterns due to the handling during ACTH administration were superimposed with the results of the physiological challenge (ACTH), so it is unlikely that differences in stress responses were due to individual differences in coping with the handling stress during ACTH injection.

CM excretion in response to the ACTH challenge peaked much earlier in quails than in chickens (5–7 hours vs 2–3 hours), which indicates species differences in metabolism and time of gut passage (in both species ACTH was injected in the major pectorialis muscle). HIRSCHENHAUSER et al. (2012) also observed a faster CM excretion in quails than in chickens.

Our results show that changing the antibody charge (a different immunization) may have a remarkable impact on antibody cross-reactivity (see Tab. 2). Different antibody cross-reactions may have a huge impact on the biological sensitivity of an assay and on its suitability for measuring CM. The findings of the present study highlight the importance of using exactly the same method for all samples in a project and the need for a careful re-validation if new antibodies are utilized, even if the same antigen is used for immunization. Besides different antibody cross-reactivities, the concentrations of cross-reacting substances in the samples are of major importance. An illustrative example of how measurements are dramatically affected if cross-reacting substances are present in much higher concentrations than the substance in question is the confounding effect of gestagens on the measurement of corticosterone in egg yolk of chickens and other bird species (RETTENBACHER et al., 2009, 2013). When using

assays produced in-house it is no problem to keep track of whether the antibodies used originate from the same immunization. But such detailed information is usually lacking from commercially available kits. The antigen used for immunization is frequently unknown (although the position where the steroid had been linked crucially affects antibody cross-reactivity, MÖSTL et al., 2005), information on antibody cross-reactivity is limited to the very few steroids tested (in relation to the range of steroid metabolites present in excreta) and potential differences between batches (whether they exist and if so to what extent) are not mentioned. The differences in antibody-metabolite interactions between test kits of different producers explain the huge variation in hormone values measured by diverse labs (ZEUGSWETTER et al., 2013). The dissimilarities could be significantly reduced by the use of liquid chromatography-mass spectrometry, which determines the quantities of each metabolite separately (for details see MURTAGH et al., 2013). The method is very accurate but quite elaborate, which could create problems when sample sizes are large. Assays based on group-specific antibodies work well if the antibodies show a high cross-reactivity with the predominant CM excreted after stressful events. The better the match between CM and antibody cross-reactivity, the more sensitive the assay will be to small changes in amounts of CM excreted, leading in optimal cases to an assay suitable for assessing an animal's hormonal response to minor stressors (i.e. FRIGERIO et al., 2004).

In conclusion, it is necessary to validate the suitability of the assay for all groups of individuals involved (age, sex differences in CM patterns) before the assay is used. The key reagents (labels and antibodies) should be exactly the same throughout the entire project. In the event of even minor changes, the suitability of the assay system should be re-tested. When planning to examine individual differences in patterns of stress response, it is advisable to test whether sex and age could confound the results. It is preferable to compare animals of the same category (age, sex, social rank, social support, season, reproductive status). It is also important to bear the social context in mind (Who was present/absent during the stressful event? Did changes in group composition occur? etc.). In view of the notable effects single individuals may have on the behaviour and the GC secretion of other group members (e.g. SAPOLSKY, 1992; SACHSER et al., 1998; CREEL et al., 2013), particularly in studies on zoo animals (which in contrast to most field situations can neither escape nor choose group members) it is prudent to monitor different groups, to avoid generalizing patterns that might be very specific to the group observed.

The cross-reactions of an antibody used in an immunoassay have a huge impact on the results of a study. It is well worth taking time to test and look for the most suitable assay system.

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***corresponding author's address:**
Mareike Stöwe,
University of Veterinary Medicine Vienna,
Veterinärplatz 1, 1210 Vienna, Austria
e-mail: m_stoewe@hotmail.com,
mareike.stoewe@vetmeduni.ac.at