Hair cortisol analyses in different mammal species: choosing the wrong assay may lead to erroneous results

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Wild animals are faced with a broad range of environmental stressors and research is needed to better understand their effect on populations. Hormone analysis based on enzyme immunoassays (EIAs) can provide valuable information on adrenocortical activity (stress), and assessment of cortisol in hair may allow the quantification of cortisol production. To validate hair hormone analysis, we compared two EIAs based on antibodies against cortisol-3-CMO-BSA and cortisol-21-HS-BSA for hair glucocorticoid (hGC) measurements in Egyptian mongoose, Iberian lynx, Alpine marmot, Asiatic black bear, spotted hyena and cheetah, with results obtained by liquid chromatograph coupled with tandem mass spectrometry (LC-MS/MS) measurements. Both EIAs were also characterized by HPLC immunograms. Our results revealed that the cortisol-21-HS EIA measured 2.3- to 12-fold higher hGC concentrations than the cortisol-3-CMO assay. In dependence of the species, high-performance liquid chromatography (HPLC) immunograms showed that up to 70% of immunoreactivities determined by the cortisol-21-HS constituted of unknown unpolar compounds leading to an overestimation of hGC. The cortisol-3-CMO EIA expressed a better specificity, with 32.1–67.4% of immunoreactivity represented by cortisol and cortisone. The LC-MS/MS analyses (gold standard) revealed that the cortisol-3-CMO EIA also resulted in an (up to 3-fold) overestimation of hGC, but EIA results were correlated with LC-MS/MS in the mongoose, the lynx, the spotted hyena and the marmot. No correlation was obtained for Asiatic black bears. As a result of our study, we strongly recommend to test any cortisol EIA for its specificity towards extracted hair components. In all analyzed species, except the Asiatic black bear, cortisone and cortisol were simultaneously present in hair extracts; consequently, an appropriate EIA should cross-react to these two glucocorticoid hormones and express negligible affinity towards substances with less polarity than corticosterone. Choosing the wrong EIA for hGC analyses may lead to overestimations of hGC or—in the worst case—to results that do not mirror real adrenocortical activity.

Key words: glucocorticoids, hair, HPLC, immunogram, LC-MS/MS

Editor: Ben Dantzer

Received 22 August 2019; Revised 5 November 2019; Editorial Decision 22 January 2020; Accepted 22 January 2020

Cite as: Jewgenow K, Azevedo A, Albrecht M, Kirschbaum C, Dehnhard M (2020) Hair cortisol analyses in different mammal species: choosing the wrong assay may lead to erroneous results. Conserv Physiol 8(1): coaa009; doi:10.1093/conphys/coaa009.
Introduction

Wild animals are faced with a broad range of environmental stressors, and one physiological process helping vertebrates to cope with any challenge is the stimulation of the hypothalamic pituitary axis (HPA) leading to the release of glucocorticoid (GC; corticosterone/cortisol) hormones. These metabolic hormones not only are involved in the maintenance of energetic balance but also act as key components of the acute stress response. Baseline levels of GC in free-living animals may vary during development and throughout the year. Thus, annually varying GC levels reflect the physiological response of animals to meet seasonal needs, which are thought to be different between sexes (Romero, 2002; Bauer et al., 2014).

In case of acute stress events, however, increased levels of GC are essential to shift energy towards the functions demanded to rapidly cope with the stressor (amongst others dominance behaviour, intra- and inter-species competition, escape from predators and human–wildlife conflicts) (McEwen et al., 2003). Usually, these changes are of limited duration, and thereafter GC levels return to baseline, but if adverse environmental conditions act permanently, continuously elevated GC secretion may cause deleterious consequences by altering individual fitness, immune function and reproductive success (Terio et al., 1999; Tilbrook et al., 1999; Ludwig et al., 2019) and eventually reduce survival (Wingfield et al., 2003). Thus, the possible impact of an environmental stressor, either of anthropogenic or of natural origin on an animal’s fitness, can only be detected if physiological baselines are known and reliable measurements of GC are performed on a regular and large-scale basis.

Determination of cortisol as an indicator of the hypothalamic–pituitary–adrenal (adrenocortical) activity is often used to measure stress. Since serum GC concentrations are characterized by diurnal pulsatile fluctuations and capture/restraint for blood collection induce increases of GC concentration within a few minutes (Carnes et al., 1988; Sheriff et al., 2011), alternative approaches that use matrices like urine, faeces or hairs have been developed and validated for wild animals.

Urine and faeces can be collected non-invasively from terrestrial mammals without causing stress from capture, restraint and venipuncture procedures, mirroring adrenocortical (stress) status of the preceding hours or days. Urine, however, requires special sampling procedures in captive animals. Faecal samples are easy to obtain from wild animals and DNA-based technologies can be applied (Sheriff et al., 2011) to assign them to a species or even to individuals (Mohd Salleh et al., 2017; Mengulluoglu et al., 2019). In particular, faecal glucocorticoid metabolites (fGMs) are described as reliable indicators of GC excreted by the adrenal glands during the previous 12–24 h (depending on defecation rates). For some species, the delay may be even longer (e.g. reptiles). They are less affected by episodic fluctuations or the pulsatility of hormone secretion and are therefore useful to evaluate adrenal activity in an integrated manner (Sheriff et al., 2011; Palme, 2019). However, successful measurement of fGM relies on the development of immunoassays that detect the species-specific hormone metabolites of cortisol or corticosterone. The native hormones are, if at all, represented in minor quantities in faecal samples, as has been shown, e.g. for carnivores (Young et al., 2004). In most cases, fGM still cross-react with antibodies primarily produced to measure the respective unmetabolized GC in the plasma. However, experiments dealing with the physiological and biological validity of fGM analyses are essential to prove whether an immunoassay is able to reflect an animal’s endocrine status. The most widely used approach is to stimulate adrenocortical activity with an adrenocorticotropic hormone (ACTH) challenge test or to measure an increase of fGM in response to a known stressor experienced by the animal (Touma et al., 2004; Young et al., 2004).

In contrast to fGM, assessment of cortisol in hair may reflect circulating cortisol levels over extended time periods, although the mechanism of incorporation is still a matter of debate. It is suggested that cortisol is incorporated into hairs by passive diffusion from the bloodstream and therefore may serve as a unique biomarker of HPA activity providing an integrated value of circulating hormones covering several weeks or even months (Davenport et al., 2006; Accorsi et al., 2008; Macbeth et al., 2010; Ashley et al., 2011; Gonzalez-de-la-Vara et al., 2011; Terwissen et al., 2013; Mastromonaco et al., 2014). Consequently, hair cortisol is thought to be insensitive to the impact of sampling procedures, ultradian rhythms and the effect of any acute stressor, which certainly offers great potential as an indicator of chronic stress. However, a recent radio-metabolism study using labelled cortisol demands a cautious interpretation of the retrospective time frame to record stress in relation to the hair growth rate (Kapoor et al., 2018). The authors also showed that radiolabeled cortisol was incorporated into hair of rhesus monkey as cortisol and cortisone and as other unknown metabolites, whereas in the guinea pig radiolabeled cortisol was incorporated mainly as cortisone (Keckeis et al., 2012). Raul et al. (2004) suggested for human that the enzyme 11β-hydroxysteroid-dehydrogenase (HSD) (in)activates GCs converting cortisol to cortisone, and a conversion of cortisol to cortisone before its incorporation in hair (Raul et al., 2004). Thus, in addition to cortisol, cortisone may also be regarded as a useful biomarker for stress research.

The authentic hormones should be detectable when applying an immunoassay. However, the application of antibody-based methods requires a thoughtful validation before they can provide useful information on the effect of stressors on animals. For example, the body regions from which samples are collected may influence the concentration of cortisol present in hair (Terwissen et al., 2013; Acker et al., 2018). Mechanical irritation by scratching might enhance local hair cortisol production as well (Salaberger et al., 2016). Moreover, hair growth pattern might be highly
variable between individuals, which may affect between subject comparisons (Kapoor et al., 2018). In addition, the precise mechanisms by which lipophilic steroid hormones are incorporated into hair are still not fully understood. In vitro, an equivalent of the central HPA in the hair follicle of humans has been demonstrated (Ito et al., 2005). Data from a radiometabolism study performed in guinea pigs (Keckeis et al., 2012) demonstrated only small amounts of injected 3H-cortisol in the hair matrix, but surprisingly high amounts of unlabelled cortisol, assuming a local synthesis and quite likely an autonomous production of cortisol (Cirillo et al., 2011). In addition, reversed-phase high-performance liquid chromatography (HPLC) of hair samples from guinea pig and wool samples from sheep demonstrated depending on the applied GC enzyme immunoassay (EIA)-the presence of cortisol, cortisone and corticosterone as well as of several unknown cortisol immunoreactivities (Keckeis et al., 2012, Salaberger et al., 2016).

These unknown steroid hormones may hamper the determination of GC in hair samples (hGC) leading to an overestimation of GC content in hair samples due to unforeseeable cross-reactivities with the antibody. The application of high-performance liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) for exact quantification of hGC is therefore preferable to any immune assay. It combines the physical separation of liquid chromatography (LC) paired with the specificity of mass analysis capabilities of tandem mass spectrometry (MS/MS) allowing the sensitive detection and quantification of multiple compounds. Until now, only a few LC–MS/MS methods for the simultaneous identification and quantitation of steroids in hair have been described (Stalder et al., 2013). In contrast to an EIA, LC-MS/MS equipment is quite expensive and demands high analytical skills. Therefore, it is often unavailable to smaller laboratories and, in particular, to researchers working on wildlife. For them, immunoassays are still the method of choice.

We recently validated an EIA to determine hair cortisol concentrations in wild caught Egyptian mongoose (Azevedo et al., 2019). In the present follow-up study, we applied two different cortisol EIA, and obtained different cortisol concentrations in identical hair extracts in the mongoose and five other wildlife species. By conducting HPLC immunograms, we determined the immunoreactive components in hairs and related them to known steroids, in particular to GC. This allowed us to identify the predominant hGC component in the six species. Finally, we assessed the steroid composition by LC-MS/MS analyses (gold standard) and analyzed the EIA quantification in light of the LC-MS/MS results.

Materials and methods

All chemical reagents were purchased from Sigma-Aldrich (Taufkirchen, Germany) unless stated otherwise and were of the highest purity available.

Sample collection

Hair samples were obtained from six mammalian species in the framework of different research programs: Egyptian mongoose (Herpestes ichneumon), Iberian lynx (Lynx pardinus), cheetah (Acinonyx jubatus), Alpine marmot (Marmota marmota), Asiatic black bear (Ursus thibetanus) and the spotted hyena (Crocuta crocuta).

Hair samples from the individual Egyptian mongoose (Herpestes ichneumon; \( n = 294 \); 140 males and 154 females; 147 adults and 97 juveniles and sub-adults) were collected from hunting activities (Bandeira et al., 2016) and were previously used to determine baseline variation in hair cortisol levels (Azevedo et al., 2019). Following death, specimens were frozen at \(-20^\circ C\) until the date of sample collection. After thawing, hair was clipped with scissors from a standard area between the shoulders and stored in paper envelopes until further processing (Azevedo et al., 2019). Samples from individual Iberian lynx (Lynx pardinus; 93 adult females, 6 adult males) were obtained during routine health checks within the Iberian lynx conservation breeding program in Spain and Portugal. The hair was clipped from the inner surface of hind legs.

All other hair samples were obtained from the IZW hair collection compiled over several years. Hair samples from 20 alpine marmot (M. marmota) were clipped from an area between shoulders (5 adult males, 4 adult female, 11 juveniles) housed at the Feldstation Niederfinow 70 km north of Berlin. The samples from adult spotted hyenas (C. crocuta; \( n = 7 \); 2 males, 5 females) and from the one adult male cheetah were collected from the tail region, whereas those from adult Asiatic black bears (U. thibetanus; \( n = 14 \); 9 males, 5 females) were obtained from the inner surface of hind legs. All hair samples were stored in envelopes at room temperature.

From all Iberian lynx and Egyptian mongoose hair samples, an aliquot of 20 mg was separated and washed twice with 2 ml of 90% methanol by vortexing for 5–10 s to remove surface contaminations. Thereafter, the samples were dried for 1 h at \( 70^\circ C \) and aliquots (~10 mg) from each Egyptian mongoose (\( n = 294 \)) and female Iberian lynx (\( n = 93 \)) were taken and milled to a fine power with ceramic beads in a tissue homogenizer as described before (Azevedo et al., 2019). Then, 400 μl of 90% methanol was added to the powder and shaken at room temperature for 30 min. Following centrifugation (3 min, \( 1000 \times g \)), the supernatant was collected and transferred into a new tube, diluted 1:2 with water and kept frozen until EIA analysis and the preparation of HPLC immunograms. From hair samples of 14 mongoose (7 males, 7 females) and 12 lynxes (6 males, 6 females), additional extracts were produced for LC-MS/MS analyses (see below) following the same protocol.

For hair samples from the Asiatic black bear, the alpine marmot, the cheetah and the spotted hyena, the washing procedure was modified utilizing aliquots of 50–60 mg that...
were washed twice with 4.5 ml of 90% methanol. Thereafter, the samples were extracted as described by Carlitz et al. 2014. In brief, before washing and drying, hairs were cut into 10-mm pieces and afterwards 50 mg of the dried hairs were incubated with 1.8 ml of methanol for 18 h at 45°C. Subsequently, the extract was aspirated, dried for 20 min at 60°C under a constant stream of nitrogen and resolved in 1 ml 90% methanol. An aliquot of 100 μl was diluted 1:2 with water for EIA and kept frozen. The remaining 900 μl was subjected to LC-MS/MS analyses or were pooled for HPLC-immunograms (see below).

**GC analyses**

We used two in-house immunoassays based on polyclonal antibodies (rabbit) directed against (i) cortisol-3-CMO-BSA and (ii) cortisol-21-hemisuccinate (HS)-BSA. The corresponding label of cortisol was cortisol-3-CMO-peroxidase and cortisol-21-HS-peroxidase as previously described in (Ludwig et al., 2013) and in (Kelm et al., 2016), respectively. The antibody cross-reactivities of the cortisol-3-CMO assay were as follows: cortisol, 100%; cortisone, 19.5%; corticosterone, 6.3%; desoxycorticosterone, 0.1%; progesterone, 0.1%; estradiol, 0.1%; testosterone, 0.1%. Those of the cortisol-21-HS EIA were cortisol 100%; cortisone, 17.5%; progesterone, 13.2%; corticosterone, 10.4%; desoxycorticosterone, 7.9%; estradiol, 0.1%. The principle of our in-house EIA procedure has been described in detail in Finkenwirth et al. (2010). All EIA measurements were conducted in 20 μl duplicates and results expressed as pg/mg hair weight.

For both EIA applied, serial dilutions of hair extracts showed parallelism to the cortisol standard with no significant difference in slopes (P > 0.05). The sensitivity of the assays was 0.40 pg/well. The inter-assay and intra-assay coefficients of variation were determined on two pooled extracts. For cortisol-3-CMO EIA, the pools contained 0.25 and 0.6 ng/ml cortisol. Inter-assay and intra-assay coefficients of variation were 11.3% (n = 7) and 9.5% (n = 7), and 6.7% (n = 16) and 5.4% (n = 16), respectively. For the cortisol-21-HS assay, the inter-assay and intra-assay coefficients of variation were determined on two different extraction pools, which were composed of 0.25 ng/ml and 0.5 ng/ml cortisol. The corresponding inter- and intra-assay variations were 14.6% (n = 7) and 9.8% (n = 8), and 12.8% (n = 16) and 5.9% (n = 16), respectively.

**High-performance liquid chromatography**

To characterize the compounds detected by the respective EIA, we ran HPLC immunograms from pooled hair extracts that were obtained using two different extraction procedures (see above). From the hair extracts of 294 mongooses and 93 female lynxes, aliquots of 150 and 460 μl of each extract were pooled corresponding to ~550 and ~350 mg of hairs, respectively. For the marmots (n = 20) and black bears (n = 14), 50 mg hair of each individual was extracted as described and 900-μl aliquots of each extract were combined, resulting in a pool of ~900 and 630 mg of hairs, respectively. From each individual hyena samples (n = 7), two different hair aliquots of 50 mg were extracted, whereas from the one cheetah 20 hair aliquots of 50 mg were taken. From each extraction, 900 μl was pooled, so the pools for hyena and cheetah represented ~630 and 700 mg of hairs, respectively. All pools were purified on Octadecyl C18 columns (0.5 ml, J.T. Baker, BAKERBOND SPE™ 7020-01) as described in Dehnhard et al. (2010) and Azevedo et al. (2019). Eluates were evaporated in a heater at 55°C under nitrogen and reconstituted in 200 μl of 40% methanol. One hundred to 150 μl portions of purified hair extract were used for HPLC analysis on a reverse-phase Ultrasep ES100/18-μm HPLC column (4 × 250 mm, Sepserv, Berlin). Compounds were separated using a methanol + water mixture with the following gradient: 60% methanol over 5 min, 60–90% methanol over 10 min and 90–100% methanol over another 10 min. The flow rate was 1 ml/min. Fractions of 0.33 ml were collected at 20-s intervals over a period of 25 min. All fractions were lyophilized and re-suspended in 200 μl 40% methanol before 20-μl aliquots were determined by both cortisol-EIA. The elution positions of native cortisone, cortisol, corticosterone, 11-hydroxyetiocholanolone, testosterone (T), epi-androsterone, dihydrotestosterone (DHT) and progesterone (P4) on this column had been previously determined in separate HPLC runs (Ludwig et al., 2013; Pribbenow et al., 2015).

**Liquid chromatography coupled with tandem mass spectrometry**

Hair extracts from individual mongooses (n = 14), Iberian lynxes (n = 12), marmots (n = 20), Asiatic black bears (n = 14) and spotted hyenas (n = 7) were adjusted to an absolute content about 150 pg cortisol. To achieve this, cortisol concentrations of extracts were determined by 3-CMO EIA, and the required volume of each species was determined according to the mean cortisol concentration (Table 2). Thus, for mongoose 300 μl (~7.5 mg hair), for Iberian lynx 200 μl (~5 mg hair), for marmots 600 μl (~30 mg hair) and for black bears 800 μl (~40 mg hair) of each extract were transferred to 1.5-ml reaction tubes, dried down for 90 min at 60°C under a constant stream of nitrogen and dissolved in 225 μl 50% methanol (LC-MS/MS grade). The samples were sent to the Department of Biopsychology, Technical University of Dresden, Germany, for LC-MS/MS analyses. Details on liquid chromatography methodology and mass spectrometric conditions are described in Carlitz et al. (2016) and Gao et al. (2013).

**Statistical methods**

Determination of mean values ± SD, comparison of cortisol amounts between both EIA as well regression analysis were
Table 1: hGC concentrations in five different mammalian species determined by two different cortisol EIA, a cortisol-3-CMO and a cortisol-21-HS. Represented are means ± SD. For correlation analysis, Pearson’s correlation coefficients (r) were determined

| Species (Number)        | Cortisol-21-HS in pg/mg Mean ± SD (Range) | Cortisol-3-CMO in pg/mg Mean ± SD (Range) | Relation | correlation coefficient P value |
|-------------------------|----------------------------------------|----------------------------------------|----------|---------------------------------|
| Egyptian mongoose (294) | 159.7 ± 46.4 (61.9–394.6)              | 20.0 ± 8.5 (0.0–114.2)                 | 8:1      | r = 0.324, P = 0.000            |
| Iberian lynx (12)       | 87.7 ± 46.9 (32.2–174.3)               | 37.3 ± 13.2 (21.1–60.5)                | 2.3:1    | r = 0.863, P = 0.000            |
| Alpine marmot (20)      | 60.6 ± 18.6 (36.3–111.5)               | 6.0 ± 1.9 (4.1–11.5)                  | 10:1     | r = 0.190, P = 0.423           |
| Asiatic black bear (14) | 44.6 ± 20.2 (15.5–76.2)                | 3.7 ± 2.3 (0.8–10.6)                  | 12:1     | r = 0.619, P = 0.009           |
| Spotted hyena (7)       | 28.9 ± 16.8 (18.0–60.7)                | 12.1 ± 2.7 (6.6–17.5)                 | 2.3:1    | r = 0.507, P = 0.246           |

Differences in mean for cortisol levels determined by cortisol-21-HS and cortisol-3-CMO EIA were highly significant (P < 0.001) for the Egyptian mongoose, the Iberian lynx, the marmot and the Asiatic black bear, whereas in case of the spotted hyena the difference was significant (P = 0.036). The respective t values (degree of freedom) in paired T test were 56.039 (293), −4.833 (11), −13.400 (17), 7.830 (13) and −2.684 (6) for the mongoose, lynx, marmot, black bear and hyena, respectively.

Results

Determination of cortisol content in hair samples using EIA

Within a framework of other projects, 294 hair samples from Egyptian mongoose were analyzed with both cortisol EIA revealing significant differences between both EIA (Table 1, Supplement Table 1, paired t test: t = 56.039, df = 293, P < 0.0001). The mean cortisol content determined with cortisol-21-HS EIA was about eight times higher compared to the results obtained with the cortisol-3-CMO assay; both data sets were correlated with each other (Table 1, Fig. 1, r = 0.324, P < 0.0001). By applying linear mixed fixed models for data obtained with cortisol-3-CMO EIA it was shown that age, sex and storage time had an effect on hair cortisol, but season did not. The full data analysis was reported in Azevedo et al. (2019). In contrast, the same analysis performed on hGC concentrations determined with cortisol-21-HS EIA revealed that only age had an effect (Table 2, Supplement Table 3, Supplement Fig. 1).

In the Iberian lynx and the spotted hyena (Table 1, Supplement Table 2), the difference between the two cortisol EIA was less pronounced with less than half cortisol determined when using the cortisol-3-CMO assay (lynx: t = −4.833, df = 11, P < 0.0001; hyena: t = −2.684, df = 6, P = 0.036). From the samples from marmots and Asiatic black bears (Table 1, Supplement Table 2), we also obtained significantly different hGC measurements between the two EIA. Analyses with the cortisol-21-HS EIA revealed 10- and 12-fold higher hGC concentrations in the marmot (t = −13.400, df = 19, P < 0.0001) and the black bear (t = 7.830, df = 13 P < 0.0001), respectively. Although being significantly

Discussion

Parents and offspring of the Egyptian mongoose were analyzed with cortisol-21-HS EIA revealing significantly different hGC concentrations between both EIA (Table 1, Supplement Table 1, paired t test: t = 56.039, df = 293, P < 0.0001). The mean cortisol content determined with cortisol-21-HS EIA was about eight times higher compared to the results obtained with the cortisol-3-CMO assay; both data sets were correlated with each other (Table 1, Fig. 1, r = 0.324, P < 0.0001). By applying linear mixed fixed models for data obtained with cortisol-3-CMO EIA it was shown that age, sex and storage time had an effect on hair cortisol, but season did not. The full data analysis was reported in Azevedo et al. (2019). In contrast, the same analysis performed on hGC concentrations determined with cortisol-21-HS EIA revealed that only age had an effect (Table 2, Supplement Table 3, Supplement Fig. 1).
Figure 1: X-Y-diagram of cortisol data determined with 3-CMO EIA versus 11-HS EIA for all samples from mongoose, combined with box-plots of both EIA.

Table 2: Significance of model terms age, season, sex and storage time, calculated according to Azevedo et al. (2019) using parametric bootstrapped likelihood ratio tests, with results of cortisol-21-HS and cortisol-3-CMO EIAs, respectively

| Variable     | Cortisol-21-HS |       | Cortisol-3-CMO |       |
|--------------|----------------|-------|----------------|-------|
|              | Likelihood ratio test value | P value | Likelihood ratio test value | P value |
| Age          | **3.052**      | **0.029** | 19.38          | **0.001** |
| Season       | 2.039          | 0.109 | 5.71           | 0.187 |
| Sex          | 0.086          | 0.769 | **5.05**       | **0.028** |
| Storage time | 0.180          | 0.671 | **9.51**       | **0.020** |

Likelihood ratio test value is the value of the likelihood ratio test value generated from the true data, which was then compared to likelihood ratio test values simulated with parametric bootstrapping. Significant terms ($\alpha = 0.05$) are in bold.

different between the EIAs, results of both EIA were still correlated in the Egyptian mongoose, the Iberian lynx and the Asiatic black bear, whereas no relation between both EIA was found in alpine marmots and spotted hyenas (Table 1).

**LC-MS/MS analyses**

LC-MS/MS allows the selective detection of different GCs in hairs combining the physical separation of liquid chromatography (HPLC) paired with the specificity of tandem mass spectrometry (MS/MS) and was therefore used to assess the results obtained by the two different EIA. In our study, quantitative LC-MS/MS determinations of cortisone, cortisol and corticosterone were carried out in a total of 68 hair samples from the above mentioned species (limit of detection [LOD] = 0.32 pg/mg, limit of quantification [LOQ] = 1.05 pg/mg). In all samples, the cortisol content was determined by both EIA as well (Table 3, Supplement Table 2) making species differences apparent.

Highest cortisone and cortisol concentrations were found in the lynx followed by lower concentrations in the mongoose and the hyena. Mean cortisol concentrations below 1.0 pg/mg were obtained in the marmot and the black bear. Moreover, cortisol levels in 9, 3 and 10 samples were below detection limit in the mongoose, the marmot and the black bear, respectively. Except from the black bear, low corticosterone concentrations were obtained in all species and were completely undetectable in the lynx and the marmot. Although almost close to detection level, corticosterone was measurable in all black bear samples. Whether corticosterone represents the main hGC in the black bear needs to be confirmed.
Comparison between cortisol EIA and LC-MS/MS analyses

Altogether, LC-MS/MS analyses revealed similar species differences compared to the analyses with both EIA (Table 3, Fig. 2). In the lynx, the highest amounts of hGC were detected, and the range of LC-MS/MS concentrations expressed as the sum of cortisone + cortisol (38.16 ± 12.27 pg/mg) matched with the mean values obtained by the 3-CMO EIA (37.3 ± 13.2 pg/mg, Table 3).

In hair samples of mongoose (Table 3), the LC-MS/MS cortisol + cortisone content was determined to be 11.43 ± 12.63 pg/mg. In comparison, the 3-CMO EIA overestimated hGC twice (20.6 ± 15.0 pg/mg), whereas the cortisol-21-HS EIA revealed an about 10-fold higher overestimation (193.15 ± 68.65 pg/mg) of the same sample set. Similar results were obtained for the marmots, where 21-HS EIA concentrations (60.6 ± 18.6 pg/mg, Table 3) did not reflect the hGC determined by LC-MS/MS at all, whereas the use of the 3-CMO EIA revealed three times higher values (2.03 ± 2.1 pg/mg LC-MS/MS vs 6.04 ± 1.92 pg/mg 3-CMO, Table 3). In the hyena, the mean 3-CMO EIA amount (13.58 ± 4.12 pg/mg) was just double, whereas the 21-HS EIA amount was 4-fold higher than the amount determined by LC-MS/MS (7.44 ± 2.24 pg/mg). In the black bear samples, almost no cortisone and cortisol were detectable by LC-MS/MS. This coincides with values obtained by the 3-CMO EIA that were the lowest for all examined species (Table 3), whereas the results obtained by 21-HS EIA were more than 10 times higher (50.8 ± 22.6 pg/mg) and thus were in the same range as those determined for lynx, hyenas and marmots with this assay (Fig. 2, Table 3).

With the limited samples available, we also performed a regression analysis between results obtained by 3-CMO EIA, 21-HS EIA and the LC-MS/MS concentration of the sum of cortisone + cortisol (Table 3). The results indicated for a significant relationship between LC-MS/MS and 3-CMO analysis in case of the mongoose, the lynx, the hyena and the marmot (Table 3), but only for the mongoose and the lynx a significant relationship between LC-MS/MS and 3-CMO was found between results obtained by 3-CMO EIA, respectively.

HPLC analyses of immunoreactive GC in hair samples

To further elucidate the differences between both cortisol-EIA, we performed HPLC immunograms to characterize the immunoreactive compounds in hair extracts of six mammalian species (Fig. 3). For the six species analyzed, very different results were obtained, reflecting the specificity of both cortisol antibodies towards different compounds extracted from mammalian hairs. In all samples, the 3-CMO EIA...
detects immunoreactive substances in Fraction 11 and, more pronounced, in Fraction 13–14, which coincide with elution peaks of cortisone and cortisol, respectively. The cortisol-21-HS antibody, however, has been found to be less specific, since it additionally cross-reacted with unknown compounds in Fractions 3–7, and depending on species, with substantial amounts of unpolar substances in fractions above 33.

In detail, in mongoose hair extracts (Fig. 3A) the proportions of cortisone and cortisol together represent only 10.5% of the total immunoreactivity when using the cortisol-21-HS EIA. Despite a major peak between Fractions 3–7, most immunoreactivity was found contributing to an unclear profile with peaks in Fractions 45, 50 and 54 not corresponding to any of our steroid standards. Moreover, some of them were less polar than our most unpolar standard in Fraction 45 (progesterone). Clearly different results were achieved with the cortisol-3-CMO assay, identifying cortisol (Fraction 13/14; 34.6%) and cortisone (Fraction 11; 10.9%) as the most prominent peaks. In addition, a polar peak in Fractions 3–6 has been found (11.8%), probably representing conjugated GCs. Following two minor peaks in Fractions 20 and 24, the profile nearly runs on baseline without the presence of questionable unpolar compounds. No significant peaks were detected on the position of corticosterone (Fraction 23). HPLC-immunograms from the lynx (Fig. 3B) clearly revealed two major peaks in Fractions 11 and 13 representing the elution positions of cortisone and cortisol, respectively, when using the cortisol-3-CMO assay, escorted by marginal amounts of additional compounds. For comparison, the cortisol-21-HS assay detects similar quantities of cortisone and cortisol; however, substantial amounts of additional less polar immunoreactivities were recognized in Fractions 18–22, 31, 33–35 and between 43–50. None of these immunoreactivities fits with the elution position of any of our standards. Altogether, the cortisol-3-CMO EIA specifically identifies cortisone and cortisol, both representing 67.4% of total immunoreactivity.

Similar deviating immunograms were found when checking both EIA in samples from the cheetah (Fig. 3C). As in the lynx the cortisol-21-HS EIA depicts considerable amounts of unknown compounds, whereas the use of the corresponding 3-CMO EIA increases the specificity towards cortisone + cortisol up to 65.4%.

A similar difference between both cortisol-EIA was found for hair samples from the spotted hyena (Fig. 3D). Again,
Figure 3: High-performance liquid chromatography (HPLC; reversed phase) separations of immunoreactive cortisol metabolites in pooled hair samples from six different mammalian species. The obtained fractions were analyzed with a cortisol-3-CMO EIA (blue lines) and a cortisol-21-HS EIA (green lines). The elution positions of reference standards are indicated by vertical lines; 11: C (cortisone); 13/14: HC (cortisol); 23: CC (corticosterone); 26: 11-OH (11-hydroxyetiocholanolone); 36/37: T (testosterone); 41: epi-A (epi-androsterone); 42: DHT (dihydrotestosterone); 45: P4 (progesterone). Pooled samples of A: Egyptian mongoose (Herpestes ichneumon, n = 294, 550 mg hair), B: Iberian lynx (Lynx pardinus, n = 93, 530 mg hair), C: cheetah (Acinonyx jubatus, n = 1, 900 mg hairs), D: spotted hyena (Crocuta crocuta, n = 7, 630 mg hair), E: marmot (Marmota marmota, n = 20; 900 mg hair) and F: Asiatic black bear (Ursus thibetanus, n = 14, 630 mg hair) were used.
Figure 4: Comparison of HPLC immunograms from six mammal species showing the percentage of compounds contributing to the HPLC-immunograms. Fractions 3–7: polar, probably conjugated GCs; Fractions 11–14 cortisol and cortisone; Fractions 34–54: unpolar compounds.}

the cortisol-21-HS assay cross-reacted with unpolar compounds eluting between Fractions 33 and 49 and peaking in Fraction 44 close to the elution position of progesterone. The cortisol-3-CMO assay only depicts cortisol and cortisone which together make up 60.6% of the total immunoreactivity.

In the marmot, the differences between both EIA were even more profound (Fig. 2E). From the total amount of immunoreactivity detected by the 21-HS assay, only a very small proportion (3.5%) was comprised of cortisol (Fraction 13) and probably cortisol conjugates (Fraction 4–6). Moreover, the 21-HS assay detected unpolar immunoreactivities with a peak in Fraction 44. Again, the 3-CMO assay was found to be more specific detecting cortisol and cortisone, which together represent 32.1% of the total immunoreactivity.

In the black bear (Fig. 3F), however, no distinct peak of immunoreactivity could be assigned to cortisone and cortisol, respectively, neither when testing the 21-HS assay nor the 3-CMO assay. This might be due to very low cortisol concentrations in hairs of black bears, as determined by LC-MS/MS (Table 3). The 21-HS assay cross-reacts with unknown compounds that would falsify the results. By contrast, it appeared that the 3-CMO assay detected small amounts of cortisol (the lowest of all species studied so far) which can be suggestively seen at the position of the cortisol standard (Fraction 13) apparently unaffected by other compounds.

Summary of all HPLC-immunograms: a species comparison

Based on the HPLC immunograms of the investigated species (Fig. 3A–F), the cortisol-3-CMO EIA was determined to be the assay of choice when compared with the corresponding 21-HS assay. An exception might be the black bear where the results were less clear. All results are summarized in Fig. 4, whereby Fractions 11–14 correspond to the sum of cortisone and cortisol, Fractions 3–7 to presumed polar conjugates of GC and Fractions 34–54 to compounds less polar than corticosterone found to be elevated in all HPLC immunograms causing falsification of measurements.

Except in the lynx, both assays detected the proportion of the most polar immunoreactivities in Fractions 3–7 in a...
similar order. Huge differences existed regarding the unpolar compounds between Fractions 34 and 54, with a distinctly smaller or absent affinity of the 3-CMO assay towards these compounds in all species, simultaneously improving the specificity of the 3-CMO assay towards cortisone and cortisol above 60% in the lynx, the cheetah and the hyena.

Discussion

Our comparative hGC analysis in six mammalian species demonstrate that results of cortisol EIA may lead to an over-estimation of hGCs and that in dependence of chosen immune assay erroneous results might be obtained. The method of choice to exactly quantify hGC is LC-MS/MS (Russell et al., 2015; Carlitz et al., 2016). The LC-MS/MS technique recognizes the analyte by the combined actions of liquid chromatography separation and the specific composition of mass/charge ratio of fragments generated during mass spectrometry allowing the simultaneous quantification of cortisol, cortisone and corticosterone and their differentiation from putative precursors or metabolites and from other steroids, such as testosterone and progesterone. Unlike when using EIA, this high specificity prevents an impact of similar steroids or non-steroidal compounds that may bias the quantification of cortisol, cortisone and corticosterone. In addition, LC-MS/MS methods have shown good reproducibility and high specificity and can be used to analyze multiple steroids simultaneously (Parikh et al., 2018).

Hair has been recognized as a biomaterial that accumulates GC hormones (Davenport et al., 2006; Kirschbaum et al., 2009; Macbeth et al., 2010) and is supposed to reflect average blood serum level. In contrast to faeces, authentic hormones are present within the inert hair matrix as shown for human and many other species, although the precise mechanisms by which steroid hormones are incorporated into hair are still not fully understood (Ito et al., 2005). In addition to systemic origin, a local synthesis of GC by the hair follicle is suggested (Cirillo and Prime, 2011) which may impact the steroid pattern in hairs. Our LC-MS/MS quantification of hGC in different mammalian species revealed different relations between cortisol, cortisone and corticosterone. For the hair of mongoose, hyena and marmots, cortisol is present in identical amounts compared to cortisone, whereas in the Iberian lynx and the cheetah cortisone is almost twice as high as cortisol. Corticosterone was found at very low levels. In the hair of Asiatic black bear, however, corticosterone was identified being the only GC measurable in this species (Table 3). Different serum levels as well as cortisol/corticosterone ratios were also described in different wildlife species (Koren et al., 2012a), which were also unstable across individuals. In addition, diverging hair matrices may also contribute to species specific incorporation and storage patterns (e.g. impact of structural differences, different depositions of oils or pheromones and climatic conditions).

The relation between the different GC in blood serum might be an indicator for endocrine misbalances (Parikh et al., 2018) or physiological stages like hibernation, and the cortisol/cortisone ratio in serum is not necessarily reflected in hGC. In human serum, the cortisol/cortisone ratio is about 5 : 1 (250 ng/ml cortisol vs. 50 ng/ml cortisone) (Eisenhofer et al., 2017), whereas in hairs the relationship was found to be opposite with 7 pg cortisol vs. 25 pg cortisone per mg hair (1 : 3.5) (Stalder et al., 2013). It is known that the cortisol/cortisone ratio is regulated by the enzymes 11β-HSD1 and 11β-HSD2. While 11β-HSD2 is responsible for producing cortisone from cortisol, 11β-HSD1 works predominantly in the opposite direction, reducing cortisone to cortisol in the liver, adipose tissue and other tissues (Liu et al., 2019). Thus, the cortisol/cortisone ratio is not determined by GC production from the adrenal, but rather by the metabolic activity of target cells, including hair follicles.

Both EIA presented in this study were in-house made based on a polyclonal antiserum against cortisol, and both assays were also validated for non-invasive monitoring of fGMs. Those against cortisol-3-CMO have been previously utilized to non-invasively monitor changes in adrenal activity in faecal samples from hyenas (Benhaiem et al., 2012); those against cortisol-21-HS were previously used for cortisol analyses in blood (Voigt et al., 2004) and to non-invasively monitor adrenal activity in faeces from bats (Kelm et al., 2016). Antibodies against cortisol-3-CMO are used quite commonly to monitor adrenal activity in several primate species such as Barbary macaques (Macaca sylvanus), lowland gorillas (Gorilla gorilla) and common marmosets (Callithrix jacchus) (Heistermann et al., 2006), and in domestic dogs (Canis lupus familiaris) (Schatz et al., 2001). Remarkably, both EIA were found unsuitable to monitor adrenal activity in the Iberian lynx based on fGM (Pribbenow et al., 2014).

Faecal samples from carnivores usually contain only minor amounts of the original hormone (Young et al., 2004); thus, it is not surprising that EIA directed towards the original steroids are unsuitable in some species. In hair extracts, however, we expect to find the non-metabolized hormones, thus cortisol assays should be able to detect cortisol accordingly. Our present data, however, demonstrate that cortisol-EIA based on different antibodies may lead to substantially deviating data, not only in respect of the hormone quantities determined but also providing misleading results. We analyzed the baseline variation in hair cortisol of Egyptian mongooses and highlight the importance of accounting for influences of age, sex and storage time by applying the 3-CMO EIA (Azevedo et al., 2019). Determination of hGC with the 21-HS EIA was performed on the same sample set. When comparing both EIA (Table 1), higher putative amounts of GC were detected when using the cortisol-21-HS test. These results might lead to the assumption that cortisol-21-HS was superior compared to cortisol-3-CMO, but our HPLC immunograms revealed exactly the opposite. A pronounced cross-reactivity towards unknown substances extracted from hair was found to be very obvious, instead
of a higher specificity toward hGC (Fig. 3). The results of both assays were significantly correlated (Fig. 1), but when we replicated the models used to the assess the effect of age, sex, season and sample storage time (Azevedo et al., 2019), the model using the 21-HS EIA data set did not show an effect of sex and storage time (Table 2, Supplement Table 3).

In the Iberian lynx, we achieved the best concordance between both EIA, with mean levels that are only 2.3 times higher when using the cortisol-21-HS assay (Table 1). Despite this apparently acceptable difference, the HPLC-immunogram showed very similar amounts of cortisol and cortisone (Fig. 3B) but also increasing amounts of unknown compounds between Fractions 33 and 55 with an additional peak in Fraction 34 in case of the cortisol-21-HS assay. Nevertheless, concentrations measured with both EIA do significantly correlate with each other. When applying the cortisol-3-CMO assay, however, the hGC concentrations almost exactly matched with the cortisol + cortisone amount determined by LC-MS/MS, verifying this EIA as more appropriate for hGC analyses in the lynx. In contrast, hair samples collected from Egyptian mongooses and marmots revealed 8–10-fold higher hGC concentrations when using the cortisol-21-HS EIA. In addition, comparative HPLC analysis (Fig. 3) confirmed that in these species cortisol-3-CMO was the more appropriate assay, even if hGC concentrations were still double to 3-fold compared to LC-MS/MS cortisol + cortisone amounts (Table 3, Fig. 2), still indicating an overestimation of hGC by this EIA. This agrees with data from Russell et al. (2015). They determined hGC in human hairs with four immunoassay methods, which were highly and positively correlated with two LC-MS/MS methods. However, LC-MS/MS hGC concentrations were between 2.5 and 50 times lower in comparison to the EIA results. The authors suggested employing a correction factor for each lab to achieve similar ranges of normal levels.

Our data on hair analysis in wildlife species contribute to the growing data base of studies that aim to use hGC (Koren et al., 2002; Davenport et al., 2006; Accorsi et al., 2008; Comin et al., 2011) to evaluate chronic stress, in most of which assays based on antibodies directed against cortisol have been applied (Koren et al., 2008; Ashley et al., 2011; Carlitz et al., 2014; Peric et al., 2018). According to our results, different antibodies generated different immunograms indicating that besides cortisol and cortisone, unknown immunoreactive compounds can be extracted from hair in different quantities. Additionally, external factors on regions of growing hair may influence the composition of immunoreactive substances (Salabarger et al., 2016). The comparison of HPLC immunograms established from sheep hairs exposed to dexamethasone fluid revealed significantly different compositions, when applying a cortisol-3-CMO and a cortisol-21-HS antibody, respectively.

It might be suggested that the method of steroid extraction influences the outcome of HPLC immunograms. Although we did not compare the two extraction methods for hair cortisol directly, our results indicate that irrespectively of the extraction method, high amounts of interfering unpolar components were determined with the cortisol-21-HS EIA. This applies to hairs from the mongoose and the lynx where hairs were milled and extracted for 30 min as well as from the other four species, where overnight extractions at 45°C were carried out. Independently of the extraction method, all HPLC immunograms were characterized by distinct amounts of interfering unpolar components between Fractions 34–54 (Fig. 3) when using the cortisol-HS-21 EIA, but obviously better results were achieved with the cortisol-3-CMO assay. Best results with more than 60% of immune reactivity contributing to cortisol + cortisone were obtained for hyena and the cheetah (no milling) and for the pooled milled Iberian lynx samples (Fig. 3).

The large amounts of unknown immunoreactivities between Fractions 34 and 54 might be caused by pooling and concentrating the extracts prior to HPLC-analyses, which uses extracts arisen from 500–1000 mg of hairs. Pooling is suggested to enhance inclusion of potential interfering compounds from the matrix (Koren et al., 2012b). Against this suggestion argues the parallelism of hair extracts in case of both EIA (data not shown), and the observation that all HPLC-immunograms showed comparable amounts of cortisone and cortisol at their corresponding elution positions (Fraction 11 and Fraction 13/14, respectively) when applying both cortisol EIA (Fig. 3). The unknown unpolar compounds, however, were exclusively detected by the cortisol-21-HS EIA. These unknown immunoreactivities lead to a substantial overestimation of hGC concentration when using the cortisol-21-HS EIA, which was up to 12-fold for African black bear (Table 1). Similar results were obtained by Kroshko et al. (2017) when quantifying hGC in grizzly bears applying different commercial ELISA kits commonly used for hGC determinations. Mean concentrations between 0.70 and 9.35 pg/mg of hairs were measured, resulting in a difference up to 13-fold between kits. Because nearly all commercial ELISA kits are originally designed for diagnostic use in plasma or serum, adapting them for quantification of hGC introduces potential for cross-reactions of unknown substances extracted from hair as shown in our comparison between the HPLC immunograms. Thus, absolute hGC values amongst studies of the same species that use different ELISAs should be viewed with caution.

As a result of our study, we decided to use the cortisol-3-CMO EIA for hGC analysis of mammalian hair extracts. In four of five tested species (mongoose, Iberian lynx, hyena, marmot), the determined amount of hGC nicely correlated with the LC-MS/MS determination of cortisol + cortisone, despite the overestimations in the mongoose, the hyena and the marmot (Table 3). This was not the case in the black bear where both EIA failed to produce reasonable results, possibly due to a very low content of hGC. The suitability of the 3-CMO EIA was demonstrated based on its strong specificity towards cortisone and cortisol, and low cross-reaction with other (unknown) mainly unpolar compounds.
extracted from hair. Distinct species differences not only in mean hGC concentration but also in the cortisol/cortisone ratio were demonstrated by both LC-MS/MS and HPLC immunograms.

Although we just used two different cortisol EIA, overall recommendation towards EIA selection for hGC analysis in wildlife can be deducted. In all investigated species, except the Asiatic black bear, we show that cortisone and cortisol were simultaneously present in hair extracts. Consequently, an appropriate EIA should cross-react to at least these two GC to account for any change of the cortisol/cortisone ratio in respect to physiological changes (Koren et al., 2012a, Parikh et al., 2018) or species-specific mechanisms of GC incorporation and storage within hair matrices. A selected antibody (EIA) should also express negligible affinity towards unknown substances that are less polar than corticosterone probably generating overestimations that could be misinterpreted as increased adrenocortical activity. Exemplarily for the mongoose we demonstrated that choosing the wrong EIA for hGC analyses lead to results that confound interpretation of adrenocortical activity. This validation can only performed by applying HPLC immunograms. Finally and most importantly, for any non-invasive stress related analysis, a proper pharmacological or biological validation is inevitable to demonstrate that data obtained are in relation to the adrenocortical activity (stress). Irrespective the source of hair GC, local or systemic, experiments in dairy cattle (Gonzalez-de-la-Vara et al., 2013), goats (Endo et al., 2018), Canada lynx (Terwissen et al., 2013) and chipmunks (Mastromonaco et al., 2014) showed that repeated ACTH treatments from 2 weeks to 2 months were sufficient to affect hGC. However, HPA activation by a single ACTH doses has been shown to be insufficient (Ashley et al., 2011). This clearly supports hGC as a biomarker of repeated or even long-term stress events. The use of validated EIA or even the gold standard LC-MS/MS will generate more studies on wild animals and will also elucidate further species specific differences for this attractive non-invasive approach.

Supplementary material

Supplementary material is available at Conservation Physiology online.

Acknowledgements

Thanks are due to Carlos Fonseca and Victor Bandeira of the Department of Biology & CESAM, University of Aveiro, Portugal, for hair samples of mongoose from the project ‘Genetic assessment of a successful invasion: Population genetics of the Egyptian mongoose (Herpestes ichneumon) in Portugal’ (PTDC/BIA-BEC/104401/2008), Iberian lynx samples were provided by the Environmental Council of the Government of Andalusia (201599900206971-03/06/2015) and the Iberian lynx conservation breeding program in Donana (Spain) and Silves (Portugal). In particular we are very thankful to Antonio Rivas and Francisco Villaespesa, as well as Rodrigo Serra, who supported hair sample collection during annual health checks. Our thanks go also to the IZW staff, and in particular to our colleagues Dr Marion East, Dr Sylvia Ortmann and Dr Bettina Wachter for contributing to our hair collection from several mammalian species; and to Katrin Paschmionka and Michal Ciok for their excellent technical assistance. We also acknowledge Liam Bailey for the statistical analysis of mongoose data. The publication of this article was funded by the Open Access Fund of the Leibniz Association.

Funding

The mongoose project was supported by Centre for Environmental and Marine Studies (CESAM): UID/AMR/50017/2019; by national funds of Fundação para a Ciência e a Tecnologia; and cofunded by FEDER, within the PT2020 Partnership Agreement and Compete 2020.

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