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Research paper

Analysis of hair steroid hormones in polar bears (*Ursus maritimus*) via liquid chromatography–tandem mass spectrometry: comparison with two immunoassays and application for longitudinal monitoring in zoos

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ABSTRACT

Analysis of hair cortisol concentrations (HCCs) is a promising method for monitoring long-term stress in mammals. However, previous measurements of HCCs in polar bears (*Ursus maritimus*) have yielded highly variable results, which are likely due to different methodological approaches. In this study, hair samples of zoo-housed polar bears were analyzed for cortisol with two independent immunoassays [an enzyme-linked immunoassay (EIA) and a chemiluminescence assay (CLIA)] and liquid chromatography–tandem mass spectrometry (LC–MS/MS). HCC measurements depended significantly on assay type applied, sample processing (cutting vs. powdering hair) and their interaction. Best agreement was observed between LC–MS/MS and CLIA ($R^2 = 0.81$ for powdered hair) and sample processing had a minor, albeit significant, effect on obtained HCC measurements in these assays ($R^2 > 0.9$). EIA measurements were consistently higher than with the other assays. HCC measurement was validated biologically for CLIA and LC–MS/MS in one male polar bear that experienced considerable stress for a prolonged period of time (> 18 weeks). Subsequently, by using the validated LC–MS/MS the measurement of cortisol could be complemented by the analysis of other steroids including cortisone, testosterone and progesterone levels from hair samples collected over a 9-month period (5–13 months) from six zoo-housed polar bears (five males, one female). No seasonal steroid variation was observed except in male progesterone levels. For all steroids except cortisone, a strong body region effect (neck or paw) was observed. Cortisol and cortisone, as well as progesterone and testosterone, concentrations were positively correlated. We show that hair steroid concentrations can be used to longitudinally measure stress and reproductive hormone axes in polar bears. The data established herein provide important basic information regarding methodology and study design for assessing hair steroid hormones.

1. Introduction

Understanding species-specific endocrine function and interactions within a greater ecological context are key prerequisites for targeted conservation measures (see reviews by Koren et al., 2019; Kumar and Umapathy, 2019). Steroid hormones, primarily those linked to reproductive and adrenal function, have been measured in a variety of different sample matrixes (see reviews by Dantzer et al., 2014; Koren et al., 2019; Madliger and Love, 2014; Romano et al., 2010; Sheriff et al., 2011). Characterization of hypothalamic–pituitary–gonadal (HPG) axis activity, routinely done by monitoring sexual steroid hormones such as progesterone and testosterone, can provide valuable information on the

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social and reproductive status of an animal. This knowledge can contribute to successful breeding programs, which often require effective control of gonadal function either to enhance or prevent reproductive outcomes. Additionally, hypothalamic–pituitary–adrenocortical (HPA) axis activity can be assessed by measuring adrenal glucocorticoid (GC) hormone production: cortisol in most mammals (and fish) and corticosterone in rodents, reptiles, amphibians and birds. Under the influence of a stressor, GCs are rapidly released via the HPA pathway into the blood to initiate a stress response. While crucial for maintaining homeostasis and survival, chronically elevated GC levels can have detrimental effects upon the health, reproduction and stability of a population (Boonstra et al., 1998; Moberg, 2000; Sapolsky et al., 2000).

Recently, hair has become a favored substrate for endogenous steroid hormone analyses, having been used in more than 40 species (see review by Koren et al., 2019), including polar bears (*Ursus maritimus*). Since polar bears are exposed to several anthropogenic and environmental stressors (Patyk et al., 2015; Vongraven et al., 2012), studies have focused primarily on the measurement of HPA axis activity. However, cortisol levels reported in previous polar bear hair studies differ substantially. Bechshoft et al. detected hair cortisol concentrations (HCCs) in East Greenland polar bears (2012; 2011) that were 30-fold higher than levels measured in polar bears from Southern Hudson Bay and James Bay (Macbeth et al., 2012) or those from Western Hudson Bay (Mislan et al., 2016). Similar low levels were observed by Weisser et al. (2016) in a pooled hair sample from four East Greenland polar bears.

Varying cortisol levels may derive from biological variation among individuals and subpopulations that may experience different levels of stress or have evolved different coping mechanisms. However, even within the same ecoregion and therefore the same subpopulation (East Greenland), HCCs were variable, suggesting methodological differences to be more likely the cause of HCC variation (Kroshko et al., 2017). In these previous polar bear hair studies, different sample preparation (e.g., washing procedure, cutting or powdering of hair) and assay types, including various immunoassays (IAs) and liquid chromatography–tandem mass spectrometry (LC–MS/MS; only in Weisser et al., 2016) were used to determine HCCs. While IAs are most commonly used for analyzing hormones in mammals because of their sensitivity and rapid and cost-effective application, LC–MS/MS techniques have greater specificity and sensitivity and can measure multiple steroids simultaneously but require expensive equipment and technical expertise (see reviews by Gao et al., 2016; Murtagh et al., 2013).

A standardized methodology of measuring steroids in hair that allows cross-study comparisons is highly desirable, especially in species such as polar bears where extensive sampling is cost-intensive. Apart from methodological issues, the hair matrix offers several advantages that are particularly valuable during field work in remote habitats such as the Arctic: in contrast to more invasively sampled matrixes such as blood or saliva, which provide point estimates of hormone levels, hair enables a retrospective assessment of long-term integrated hormone concentrations over weeks and months by means of only a few samples. As lipophilic substances, hormones are thought to be incorporated continuously into the growing hair shaft, proportionally to their unbound (biologically active) fraction in the blood (see reviews by Russell et al., 2012; Stalder and Kirschbaum, 2012). The main route of hormone incorporation is likely to be by passive diffusion from blood capillaries around the hair root, although the exact mechanisms of incorporation are not fully known. However, incorporation from surrounding tissues or external sources and local hormone production in the hair follicle are possible (reviewed by Koren et al., 2019; Meyer and Novak, 2012; Russell et al., 2012; Stalder and Kirschbaum, 2012). The speed and duration of active hair growth define the amount of systemic steroid that can be deposited into the hair within a certain time. While in humans and other primates hair growth rates average 1 cm/month (Carlitz et al., 2014; Stalder and Kirschbaum, 2012; Wennig, 2000) growth rates and cycles are not well described for polar bears. Although there are multiple sources of anecdotal evidence, no exact data exist on the timing of hair growth in polar bears. However, most sources agree that molting and regrowth likely happens between early summer and late autumn (Amstrup and DeMaster, 2003; Derocher, 2012; Kolenosky, 1987; Macbeth et al., 2012; Pedersen, 1945; and personal observations, see 4.3 Longitudinal measurement of steroid hormones in hair and assessment of hair growth rates). Once incorporated, steroids remain stable within the intact hair shaft (Koren et al., 2019; Meyer and Novak, 2012; Russell et al., 2012). However, decreases in hormone concentrations in the distal hair segments compared to the proximal segments have been detected in some cases (Carlitz et al., 2015; Dettenborn et al., 2010; Kirschbaum et al., 2009; but see Bennett and Hayssen, 2010; Carlitz et al., 2014; Davenport et al., 2006; Heimbürge et al., 2020; Malcolm et al., 2013; Manenschijn et al., 2011). Nonetheless, hair steroid levels could be measured in 1500-year-old Peruvian mummies (Webb et al., 2010, 2015), in Siberian woolly mammoths (*Mammuthus primigenius*; Koren et al., 2018), and in museum hides of polar bears (Bechshoft et al., 2012) at similar levels to modern samples (humans, polar bears), indicating that steroids remain stable in the hair over time. In addition, the simple storage conditions for hair offer another advantage, particularly for field-based work: aside from keeping samples dry and dark, no cooling or special preservation techniques are necessary.

Overall, hair steroid analysis seems a promising tool to better understand the activities and relations of the HPA and HPG axes and can help obtain a broader picture of polar bear endocrinology. This is the basis for many health and welfare assessments, breeding programs or conservation measures—in managed as well as in free-ranging polar bear populations (Koren et al., 2019; Kumar and Umapathy, 2019; Patyk et al., 2015). The aim of the present study was to investigate the effect of different types of sample processing (cutting vs. powdering) and assays (IA vs. LC–MS/MS) on HCC measurement in polar bears. Cut and powdered hair samples from zoo-housed polar bears were analyzed for cortisol with two different IAs [one enzyme-linked immunosorbent assay (EIA) and one chemiluminescence immunoassay (CLIA)] and LC–MS/MS. Furthermore, a biological validation of HCC measurement was performed in one bear using CLIA and LC–MS/MS, and hair growth rates were accessed in one polar bear. Finally, the previously validated LC–MS/MS method was applied for longitudinal measurements of cortisol as well as cortisone, testosterone and progesterone, in hair of six zoo-housed polar bears. The main objectives were to identify possible effects of seasonal variation and sampled body region on steroid hormone levels. To our knowledge, this is the first longitudinal study concurrently investigating HPA and HPG axis activity in polar bear hair.

2. Materials and methods

2.1. Subjects and sample collection

Including all parts of the study, hair samples were collected from a total of 13 polar bears (nine males and four females) from 12 European zoos. The participating zoos were all members of the European Association of Zoos and Aquaria (EAZA) and housing conditions were comparable for all bears (e.g., similar diet, predominant use of outdoor enclosures). Each zoo received a starter kit containing the necessary material for sample collection: a polar bear training manual, detailed hair sampling instructions and a simple protocol for recording sampling conditions (see Supplementary Material I, II; III Table 1).

All samples consisted of guard hair and undercoat in varying proportions and were not further separated, since no effect of hair type on HCCs could be detected in polar bear hair (Macbeth et al., 2012; and results of a pre-test, see Supplementary Material III Table 2).

For the assay comparison, hair samples sufficient for repeated measurements were collected opportunistically (e.g., during anesthesia for medical procedures, routine checks of cubs, necropsies or from enclosures; $\geq$ 1.5 g per sample, see Fig. 1). Pooled samples containing hair from different individuals (e.g., from shared enclosures) were included in this part of the study in order to get higher sample weights.

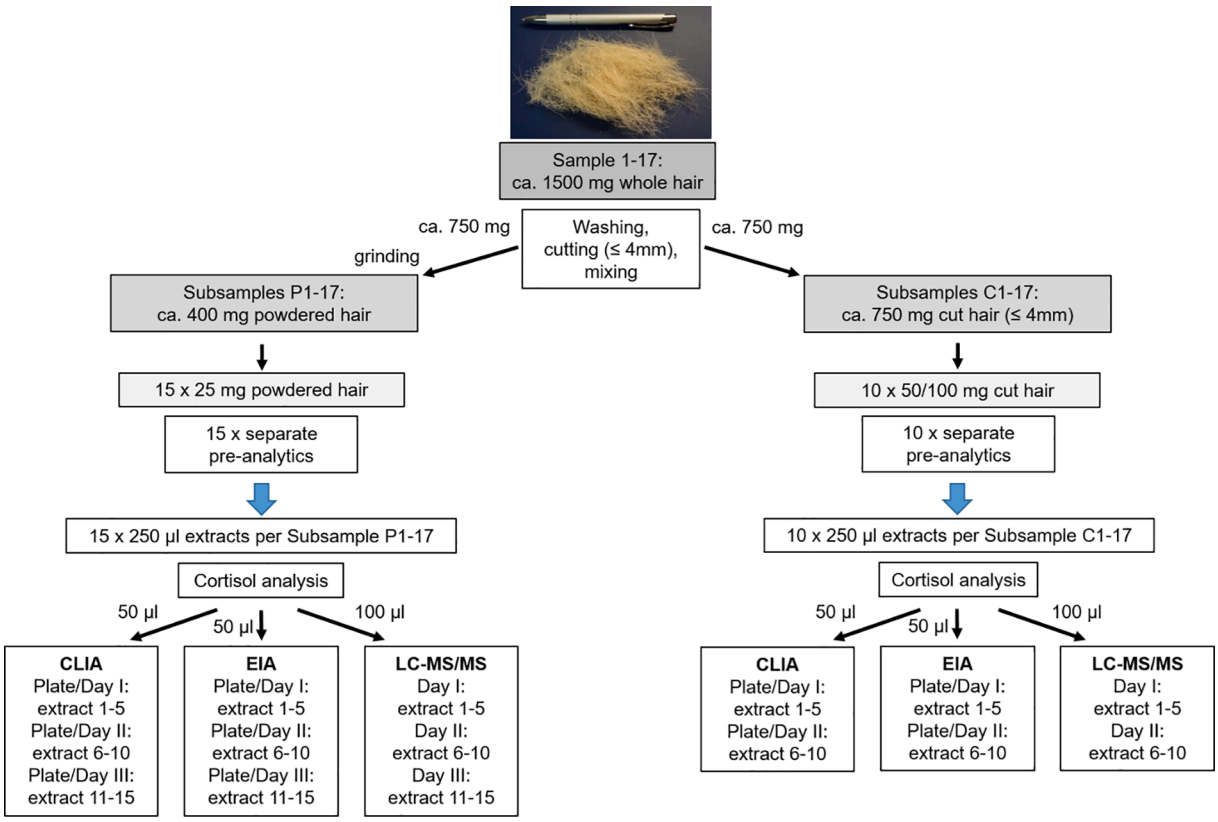

Fig. 1. Overview of the method comparison: measurement of cortisol concentrations in 17 polar bear hair samples by CLIA, EIA and LC–MS/MS using powdered or cut hair.

For longitudinal measurement of HCCs, trained bears were shaved repeatedly at the same spot at regular intervals (just before the shaved area could no longer be distinguished from the adjacent unshaved area, approximately every 3–8 weeks) to observe the effects of sampling time.

Hair was shaved and not plucked to avoid the addition of follicles to the sample and to prevent possible blood contamination and skin irritation (potentially leading to local hormone content or production, see Bechshoft et al., 2011; Cattet et al., 2017; Salaberger et al., 2016; reviewed by Sergiel et al., 2020). Samples were taken as close to the skin as possible from a 6 × 7-cm area, using disposable shavers. To minimize contamination of the sample with "non-regrown hair" from the peripheral area, the inner 4 × 5 cm was always shaved first and only hair from this area used for analysis. Afterwards, the spot was enlarged to the initial 6 × 7 cm (see Supplementary Material II).

Hair samples were air-dried if necessary and stored dry and dark in paper envelopes at room temperature until analysis. All experiments were performed in accordance with the German Animal Welfare law, following paragraph 7(2); no special permits were necessary.

2.2. Study design

2.2.1. Comparison of HCC measurement using different types of assays and sample processing

For the method comparison, a total of 17 polar bear hair samples were analyzed (from six males and four females; some bears were sampled repeatedly). Approximately 1.5 g of hair was washed (as described in 2.3 Hair sample preparation and steroid extraction), cut into pieces of ≤ 4 mm and divided into two 750-mg subsamples (see Fig. 1). One subsample was left as it was (Subsample C) while the second was ground to a fine powder in a Retsch Mixer Mill MM 400 for 5 min (one ball of 1 cm, 30 Hz; resulting in approximately 380 mg hair powder, Subsample P). Each Subsample P was then further divided into 15 aliquots of 25 mg (±0.5 mg) hair powder, using a Sartorius AZ124 precision balance (Sartorius AG, Göttingen, Germany). For each aliquot, steroid extraction, methanol evaporation and resuspension steps were run separately (see 2.3 Hair sample preparation and steroid extraction). After reconstituting with 250 µl Aqua bidest (Rotisolv®, LC-MS grade, Carl Roth GmbH & Co. KG, Karlsruhe, Germany; to have a sufficient volume for all three assay types), the resulting 15 extracts were analyzed in parallel by CLIA, EIA and LC–MS/MS (for assay details see 2.4 Hair steroid hormone analysis). Five extracts each were run on three different IA plates on three different days. LC–MS/MS of the five extracts each were also run on three different days. Each Subsample C was divided into 10 aliquots of 50 or 100 mg (depending on availability). Again, all pre-analytical steps were run separately for each aliquot. Differing from the final protocol (2.3 Hair sample preparation and steroid extraction), 3.6 ml methanol was used for the extraction of 100 mg samples and 2 ml methanol evaporated after 24 h. All resulting extracts were analyzed in parallel by CLIA, EIA and LC–MS/MS, on two different IA plates (days) with five extracts per plate (day).

2.2.2. Biological validation

To biologically validate the measurement of HCCs in polar bears, hair samples were collected from one adult male polar bear that was transported from one zoo to another and repeatedly received dental treatments in the new zoo (see Fig. 2). Hair was collected from the side of the neck four weeks before transport and on the day of transport during anesthesia (Samples 0 + 1). Approximately seven weeks later, hair was sampled from the same spot and additionally from the medial thigh during anesthesia for dental treatment (Samples 2 + 3). Almost 11 weeks later, another dental treatment was necessary, and hair was collected from the same spot at the medial thigh (Sample 4). Transport itself and activities related to it (e.g., crating, unloading) have been shown to be stressful for polar bears (Hein et al., 2020). Furthermore, the new environment and socialization (Hein et al., 2020) with a female and chronic pain (two broken canines; see Malcolm et al., 2013) were

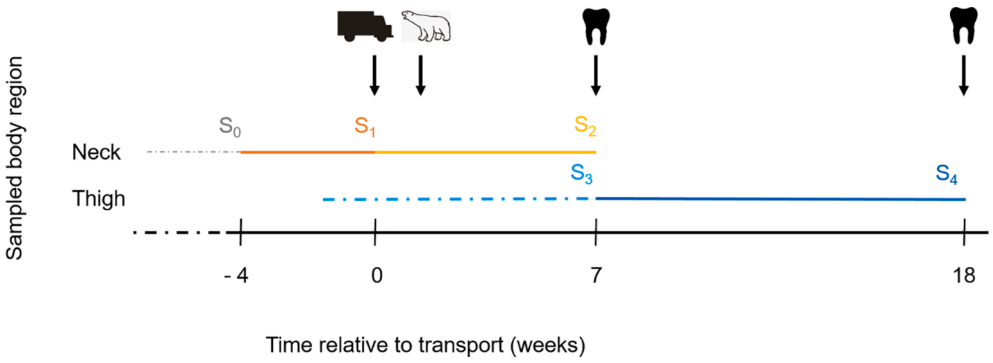

Fig. 2. Overview of hair samples collected from an adult male polar bear at the day of transport (0) and the following 18 weeks. S_0–S_4: Samples 0–4, dotted lines indicate unknown periods of hair growth and cortisol incorporation, solid lines indicate known periods of hair growth and cortisol incorporation. Symbols: truck = transport, polar bear = socialization, tooth = dental treatment. (Symbols taken from the Wikimedia Commons: 'SHSM_truck.svg', 'Tooth_-_The_-Noun_Project.svg', ' Ice-bear-161992.svg').

assumed to be stressful. We therefore expected an increase in HCCs in regrown hair of the samples collected after the transportation event (Samples 2 ± 4) compared to the sample collected on the day of transport (Sample 1; Sample 0 was not available for analysis). All samples were analyzed for cortisol via CLIA and LC–MS/MS, (see 2.3 Hair sample preparation and steroid extraction) from 25 mg hair powder (samples were already prepared until this processing step).

2.2.3. Longitudinal measurement of hair steroid hormones and assessment of hair growth rates

To assess individual hair steroid profiles, hair was collected repeatedly from the same animals. Six polar bears (five males and one female) were trained to be shaved at regular intervals (approximately every 3–8 weeks) from the same body region: either the neck, about a hand's width below the ear (right or left; see Supplementary Material III Fig. 1 + 2), or the dorsal side of the paw (right or left front leg, preferably through a paw cage; see Supplementary Material III Fig. 3 + 4). Both body regions have proved to be best accessible during training and were chosen by the keepers working with the bears. Two male bears were sampled from the neck, two other males from the paw, and one male and one female from neck and paw, according to individual accessibility (see Table 1). Hair was collected for 5–13 consecutive months (mean = 9 consecutive months; longest total period = 23 months) and a basic sampling protocol was filled out by the keepers (see Supplementary Material III, Table 1). Samples were examined for cortisol, cortisone, testosterone and progesterone using the validated LC–MS/MS.

To estimate individual polar bear hair growth rates, the length of regrown hair from samples obtained by shave and reshave was measured. Three samples of one male adult polar bear were available for this part of the study, collected from a previously shaved area at the side of the neck as close to the skin as possible. Hair from each sample was collected opportunistically and represented growth over a 27- or 21-day period: Sample 1 roughly corresponded to the last three weeks of June and the first week of July, Sample 2 to the last three weeks of July and Sample 3 to the subsequent three weeks in August. Hair was sampled just before it reached the length of the surrounding unshaved hair. Average hair growth rates were determined based on length measurements of 20 single hairs per sample.

2.3. Hair sample preparation and steroid extraction

Sample size varied between 50 and 2000 mg hair, with smaller samples from the timed shavings and larger samples from opportunistic hair sampling. After cleaning from external material such as dried mud and plant matter, samples were thoroughly mixed and transferred into glass jars with isopropanol (3–6 ml for samples weighing 50 mg to 1 g), shaken for 3 min at room temperature and the isopropanol subsequently discarded (Bechshoft et al., 2011). This washing procedure was performed twice to further remove possible contamination by external hormone sources or blood and fat. However, most hair samples were free of obvious external contaminants and generally less stained than hair samples from free-ranging polar bears (Bechshoft et al., 2011). Washed samples were dried overnight under an exhaust hood and prepared as in Kirschbaum et al. (2009) or Bechshoft et al. (2011) with minor changes: hair of each sample was cut with a pair of scissors into pieces of ≤ 4 mm and 50 ± 0.5 mg weighed into 20-ml glass jars. Then 1.8 ml methanol (LC–MS-grade; Carl Roth GmbH & Co. KG) was added for incubation at room temperature. After 24 h, 1.6 ml methanol was transferred into a 3-ml plastic tube and the methanol evaporated at 50 °C and 0.1 bar under nitrogen for at least 30 min or until dry.

After complete evaporation, 120 µl Aqua bidest was added for reconstitution, resulting in a 13.3-fold concentration of steroids. Each tube was then vortexed for at least 30 s and the extract analyzed by LC–MS/MS and CLIA. Each sample was processed in triplicates (separate pre-analytics from weighing until extracting), resulting in three extracts per sample.

Table 1
Overview of polar bears, samples and body regions during longitudinal measurement of steroid hormones in hair.

PB	Sex	Reproductive status	Housing conditions	Age[a] [years]	Shaved body region (s)	Number of hair samples	Sampling period [consecutive months]
A	M	Fertility proven	Temporarily housed with female	11	Neck	19	13 (23)[b]
B	M	Fertility proven	Temporarily housed with males	16	Neck	11	12
C	M	Fertility unknown	Housed with D	6	Paw	4	6
D	M	Fertility unknown	Housed with C	6	Paw	4	6
E	M	Fertility proven	Housed with F	5	Neck + paw	3 + 6	11
F	F	Fertility proven[c]	Housed with E	5	Neck + paw	3 + 3	5

Abbreviations: F, female; M, male; PB, polar bear.
[a] at start of sampling phase.
[b] in total three sampling periods of 13, 5 and 5 consecutive months.
[c] stillbirth of an immature fetus during monitored period.

2.4. Hair steroid hormone analysis

2.4.1. Immunoassays

HCCs were determined using two commercially available immunoassays. A salivary cortisol EIA (Salimetrics, State Collage, PA, USA) that was previously applied to HCC measurement in polar bears (Bechshoft et al., 2012, 2011) was used for method comparison only. A salivary cortisol CLIA (IBL, Hamburg, Germany) was used for method comparison and biological validation. In both assays, 50 µl of the extracts were pipetted into the wells of a microtiter plate and further processed according to manufacturer protocols (for cross-reactivities of the IAs see Supplementary Material III, Table 3). Intra- and interassay coefficients of variation (CVs) of the assays were below 10% (based on quality control samples provided with the kit). Immuno-specificity for cortisol measured in polar bear hair could be observed in both assays by parallelism between serially diluted hair extracts and cortisol standards provided by the manufacturer. Limits of detection (LODs) were 3 nmol/l (CLIA) and 0.2 nmol/l (EIA). Results below these thresholds were assigned a value half way between 0 and the detection limit (Macbeth et al., 2012, 2010).

2.4.2. LC–MS/MS

The applied LC–MS/MS system consisted of a Shimadzu LC-20AD high-performance liquid chromatography (HPLC) unit, a Shimadzu SIL-20AC autosampler and a Shimadzu CTO-20AC column temperature oven (Shimadzu, Canby, OR, USA). For method comparison these devices were connected to an AB Sciex API 5000 triple quadrupole tandem mass spectrometer equipped with an ion source (Turbo V™) for atmospheric pressure chemical ionization (APCI; AB Sciex, Foster City, CA, USA). For biological validation and longitudinal steroid analysis, a QTRAP® 6500 + with electrospray ionization (ESI; AB Sciex) was used, having the advantage of higher sensitivity, faster processing and a lower injection volume needed (only 50 µl compared to 100 µl in the API 5000). Online solid-phase extraction (SPE) was performed for sample purification, as reported by Gao et al. (2013). Further operation steps, details of chemicals and hormones and the chromatographic and mass spectrometric settings are provided in Gao et al. (2013). The LODs for both systems were 0.13 pg/mg for cortisol, 0.05 pg/mg for cortisone, 0.11 pg/mg for testosterone and 0.10 pg/mg for progesterone. Results below this threshold were assigned a value half way between 0 and the LOD. Intra- and interassay CVs were below 12% (based on quality control samples included in each LC–MS/MS run).

2.5. Data analysis

2.5.1. Comparison of HCC measurement using different types of assays and sample processing

In ten of the 34 subsamples (17 independent cut and 17 independent powdered subsamples) the weight was below 1.5 g, so that material was insufficient to prepare the full number of 15 (powdered hair) or ten (cut hair) aliquots for these subsamples. Nonetheless, each subsample was measured 8–15 times by each assay type. A total of 1203 measurements were conducted and the effect of processing (cutting vs. powdering) and assay type (EIA, CLIA or LC–MS/MS) on HCC measurements was evaluated using linear mixed-effect models. Sample (i.e., hair sample ID) was included as a random effect to account for repeated measurements. The fixed effect predictors in the full model were assay type, processing and their interaction. HCC measurements were log10-transformed, which we found appropriate through examination of residual plots. Model selection was based on an exhaustive screening of all candidate models nested within the full model (see Supplementary Material III, Table 4). The model with the lowest Akaike Information Criterion (AIC) value was selected. Reported p-values are based on likelihood ratio tests. A post hoc pairwise comparison with Bonferroni–Holm correction was carried out to test between which assays HCC measurements differed significantly. In addition, the three assay types and the two types of sample processing were compared using pairwise correlations (Pearson correlation coefficients) and linear regressions of the log10-transformed HCC measurements.

2.5.2. Longitudinal measurement of hair steroid hormones

We assessed how hair steroid levels depended on seasons and body region using linear mixed-effect models. Season was defined as a factor variable with four levels: pre-breeding (December to February), breeding (March to May), post-breeding (June/July) and non-breeding (August to November; see Hein et al., 2020). This analysis was restricted to samples from male polar bears, since longitudinal samples could be collected from all seasons from five males, whereas for females only data from one bear and two seasons (non- and post-breeding) was available. For each of the four hormones analyzed (cortisol, cortisone, testosterone and progesterone) separate models were calculated. Full models contained the log10-transformed hormone concentration as response variable and season and body region as explanatory variables. For the assignment of measured steroid levels to the appropriate seasons, hair growth periods rather than sampling dates were used. Growth periods were defined as the times between two shavings. Since the total regrown hair was analyzed, steroid concentrations measured in the shaved hair presented a mean value for the corresponding growth period. Thus, hair steroid concentrations were referred to the midpoint of the corresponding growth period, which was at the same time the criterion for the assignment to a season. Body region was either paw or neck. To account for grouping in the data, sampled individual and sampling date were included as random effects. Model selection was based on an exhaustive screening of all candidate models nested within the full model. The model with the lowest AIC value was selected. Reported p-values were based on likelihood ratio tests. Additionally, we evaluated Pearson correlations between log10-transformed hormone concentrations.

All statistical analyses were conducted in R version 3.5.0 (R Development Core Team, 2018) using packages “nlme” and “multcomp”.

3. Results

3.1. Comparison of HCC measurement using different types of assays and sample processing

Table 2 provides a summary of the 1203 HCC measurements using the different methods. One of the hair samples consistently produced the highest HCC values (above 20 pg/mg) of all samples (cut and powdered) in the immunoassays, but resulted in HCC levels below the detection

Table 2
Overview of hair cortisol concentrations (pg/mg hair) of captive polar bears determined using different analytical techniques (CLIA, EIA, LC–MS/MS) and different sample processing (cutting vs. powdering hair). Number of samples = 16 (17)[a]; number of measurements = 1128 (1203)[a]; number of bears = 10.

Method	HCCs of cut hair [pg/mg]			HCCs of powdered hair [pg/mg]		
	CLIA	EIA	LC–MS/MS	CLIA	EIA	LC–MS/MS
Min[b]	0.50	2.31	0.19 (0.07)[a]	0.84	3.46	0.56 (0.07)[a]
Max[b]	17.46 (21.35)[a]	14.20 (21.54)[a]	14.77	26.12	20.03 (24.00)[a]	17.09
Mean[b]	2.67 (3.81)[a]	5.06 (6.07)[a]	1.71 (1.61)[a]	3.75 (5.10)[a]	7.24 (8.34)[a]	2.38 (2.24)[a]
Median[b]	1.40 (1.48)[a]	4.31 (4.50)[a]	0.83 (0.82)[a]	1.46 (1.54)[a]	6.13 (6.53)[a]	1.23 (1.17)[a]

Abbreviations: CLIA, chemiluminescence immunoassay; EIA, enzyme-linked immunosorbent assay; HCCs, hair cortisol concentrations; LC–MS/MS, liquid chromatography tandem mass spectrometry.

[a] including outlier, indicated only if different.

[b] calculated by the mean HCC of each sample.

limit (0.013 pg/mg) in LC–MS/MS (outlier sample marked as a red dot in Figs. 3 and 4). Cross-reactivity measured with the IAs is the most likely reason for the differing values in this sample (see 4.1 Comparison of HCC measurement using different types of assays and sample processing). It was excluded from all statistical analyses. A linear mixed model analysis of the remaining 1128 measurements of 16 independent samples revealed that HCC analyses were influenced by sample processing, assay type and their interaction (Table 3; Supplementary Material III Table 4). Perfect agreement among methods requires that there is no random error and no constant or proportional bias. To assess agreement between the three assays as well as between the two types of sample processing we performed pairwise comparisons. Perfect agreement between log10-transformed measurements corresponds to a coefficient of determination (R^2) of 1 (no random error), a linear regression intercept of 0 (no constant bias on the log10-scale) and a linear regression slope of 1 (no proportional bias on the log10-scale). The greatest concordance was observed between CLIA and LC–MS/MS (with an $R^2 = 0.81$, an intercept of 0.16 and a slope of 0.97 for powdered hair samples; Fig. 3). Agreement of the EIA with the other assays was poor (Fig. 3). EIA measurements were systematically higher than the measurements obtained with the other assays (intercept of 0.73 for the comparison with LC–MS/MS and of 0.68 for the comparison with the CLIA; Fig. 3). Thus, both IAs resulted in statistically significantly elevated measurements as compared to LC–MS/MS (intercept of 0.16 and standard error of 0.04 for CLIA vs. LC–MS/MS; intercept of 0.73 and standard error of 0.03 for EIA vs. LC–MS/MS), but this difference was much more pronounced for the EIA than for the CLIA.

Powdering as compared to cutting hair resulted in elevated HCCs for LC–MS/MS, EIA and CLIA by factors of 1.51, 1.26 and 1.23, respectively (see Table 3). These differences are statistically significant (Table 3), but are smaller than the differences between assay types (Table 3). The pairwise comparisons confirm that there is substantial agreement between cut and powdered samples for the CLIA and LC-MS/MS (with an $R^2 = 0.91$, an intercept of 0.11 and a slope of 0.86 for CLIA and an $R^2 = 0.90$, an intercept of 0.16 and a slope of 0.72 for LC–MS/MS; Fig. 4).

For polar bear hair samples, intra- and interassay CVs were higher (CVs of 15–20%) than those based on the quality control samples provided by the manufacturers.

3.2. Biological validation

Cortisol levels in regrown hair samples from a polar bear collected after a transport event for dental treatments (Sample 2: 6.23 pg/mg (CLIA) and 5.52 pg/mg (LC–MS/MS) and Sample 4: 5.64 pg/mg (CLIA) and 4.36 pg/mg (LC-MS/MS); see Table 4) were higher than those before translocation (Sample 1: 0.89 pg/mg (CLIA) and 0.61 pg/mg (LC–MS/MS)). At the time of the first dental treatment cortisol levels were approximately 8-fold higher than on the transport day (Sample 2, CLIA and LC–MS/MS, respectively; Table 4). At the time of the second dental treatment (Sample 4) cortisol levels were approximately 6.7-fold higher (CLIA and LC–MS/MS) compared to those on the transport day—however measured from a different body region (medial thigh vs. neck). Due to the low sample size further analysis was not performed, and the statistical significance of the results could not be determined.

3.3. Longitudinal measurement of hair steroid hormones and assessment of hair growth rates

Longitudinal measurements of hormone concentrations in hair samples obtained from either the paw or neck of males did not yield evidence for seasonal variation, but strong evidence for an effect of body region sampled. Body region was retained as a predictor variable in the best-fitting models for cortisol, testosterone and progesterone (Supplementary Material III, Table 5). Cortisol measurements were 0.26-fold lower in paw than in neck samples ($p < 0.0001$, Table 5). Testosterone and progesterone measurements in paw samples exceeded those of neck samples by factors of 2 ($p < 0.0001$) and 2.4 ($p < 0.0001$), respectively (Table 5). A significant effect of season on hormone concentrations was found only for progesterone (Table 5; Supplementary Material III, Table 5). Progesterone concentrations were lowest in the breeding period and highest in the post-breeding period, when they exceeded concentrations during the breeding period by a factor of 3.1 ($p = 0.02$; Table 5).

Significant positive correlations between log10-transformed hormone concentrations for cortisol and cortisone ($R = 0.52$, $p < 0.001$; Fig. 5), as well as for progesterone and testosterone ($R = 0.84$, $p < 0.001$; Fig. 5), were observed.

Measured hair progesterone levels for the female were 6.3 pg/mg (hair grown in July), 6.2 pg/mg (hair grown in August) and 2.7 pg/mg (hair grown from September to November). For detailed hair steroid concentrations of the longitudinally monitored male polar bears see Table 6 in Supplementary Material III.

The measurement of regrown hair resulted in growth rates of 1.6, 1.5 and 0.9 cm (Samples 1 to 3) referring to a 21-day period for this polar bear (see Supplementary Material III Table 7).

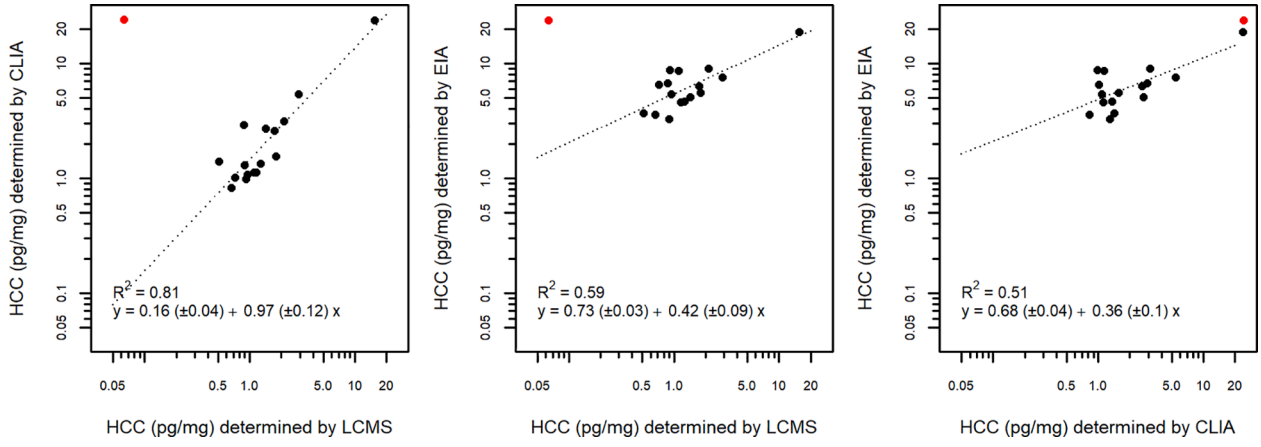

Fig. 3. Correlations between hair cortisol concentrations (HCCs) determined by three analytical techniques for powdered hair samples. Plotted data include an outlier (marked by a red dot) with high HCC as determined by CLIA and EIA but low HCC as determined by LC–MS/MS. The coefficient of determination R^2 and the equations of the linear regression analysis of the log10-transformed HCC measurements are given after removal of this outlier (numbers in brackets are standard errors). CLIA, chemiluminescence immunoassay; EIA, enzyme-linked immunosorbent assay; LC–MS/MS, liquid chromatography tandem mass spectrometry.

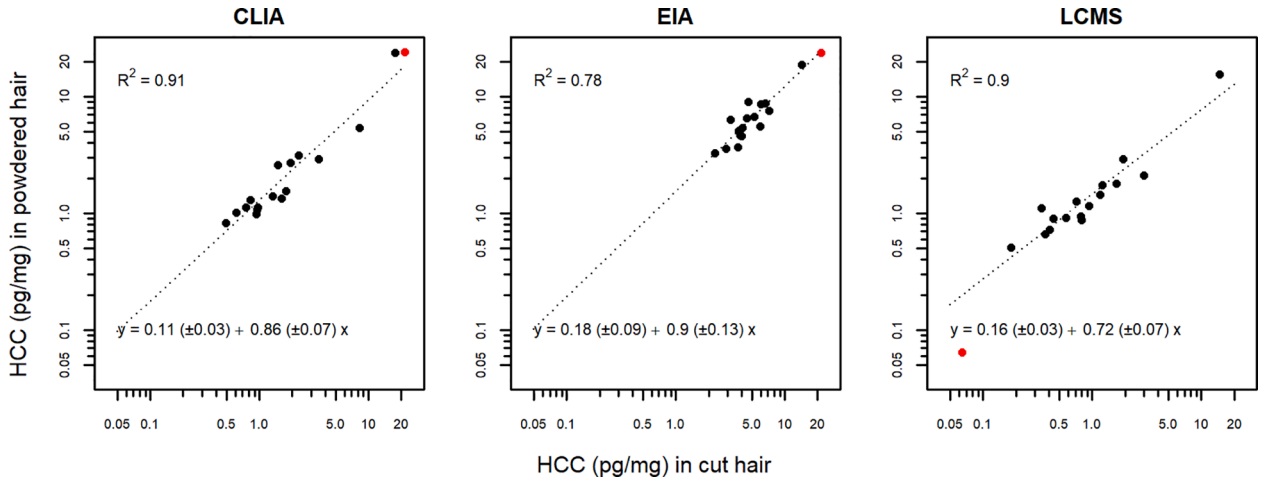

Fig. 4. Effect of sample processing on hair cortisol concentration (HCC) measurements in each of the three analytical technique. Plotted data include an outlier (marked by a red dot) with high HCC as determined by CLIA and EIA but low HCC as determined by LC–MS/MS. The coefficient of determination R^2 and the equations of the linear regression analysis of the log10-transformed HCC measurements are given after removal of this outlier (numbers in brackets are standard errors). CLIA, chemiluminescence immunoassay; EIA, enzyme-linked immunosorbent assay; LC–MS/MS, liquid chromatography tandem mass spectrometry.

Table 3
Post hoc comparisons for the linear mixed model explaining log10-transformed HCC measurements by analytical technique, sample processing and their interaction. Reported p-values are adjusted using the Bonferroni–Holm procedure.

Comparison		Effect on log10-scale	10^Effect size	SD	Z	Adjusted p
EIA vs. LC–MS/MS	cut hair	0.73	5.37	0.022	33.15	<0.001
	powdered hair	0.65	4.47	0.018	35.81	<0.001
CLIA vs. LC–MS/MS	cut hair	0.26	1.82	0.022	11.7	<0.001
	powdered hair	0.17	1.48	0.018	9.17	<0.001
EIA vs. CLIA	cut hair	0.47	2.95	0.022	21.45	<0.001
	powdered hair	0.48	3.02	0.019	25.92	<0.001
powdered vs. cut hair	LC–MS/MS	0.18	1.51	0.02	9.06	<0.001
	EIA	0.1	1.26	0.02	5.04	<0.001
	CLIA	0.09	1.23	0.02	4.4	<0.001

Abbreviations: CLIA, chemiluminescence immunoassay; EIA, enzyme-linked immunosorbent assay; HCCs, hair cortisol concentrations; LC–MS/MS, liquid chromatography tandem mass spectrometry.

Table 4
Overview of hair samples collected from an adult male polar bear at the day of transport and the following 18 weeks. Resulting cortisol concentrations (in pg/mg hair) were measured by CLIA and LC–MS/MS and show clear increases in samples collected after transport compared to samples collected before.

Sample no.	Sampling time	Body region	HCCs [pg/mg]		x-fold increase of HCCs compared to levels at DT	
			CLIA	LC–MS/MS	CLIA	LC–MS/MS
0	DT – 4 weeks	Neck	NA	NA	–	
1	DT	Neck	0.89	0.61	–	
2	DT + 7 weeks (day 49)	Neck	6.23	5.52	7.0	9.0
3	DT + 7 weeks (day 49)	Medial thigh	7.02[a]	5.17[a]	–	
4	DT + 18 weeks (day 126)	Medial thigh	5.64	4.36	6.3[b]	7.1[b]

Abbreviations: CLIA, chemiluminescence immunoassay; DT, day of transport; HCCs, hair cortisol concentrations; LC–MS/MS, liquid chromatography tandem mass spectrometry; NA, not available.
[a] Sample 3: first shave at this body region, thus no exact temporal correlation of measured HCCs.
[b] note: different body region than at DT.

4. Discussion

4.1. Comparison of HCC measurement using different types of assays and sample processing

Measurements of split samples processed by either cutting or powdering hair indicate that sample processing had a minor effect on HCC measurements. While all assays yielded significantly higher HCCs from powdered hair than from cut hair, these differences were relatively small as compared to differences arising between assay types (Table 3). Importantly, we observed substantial correlation between powdered and cut hair measurements, particularly for the CLIA and LC–MS/MS ($R^2 \geq 0.9$; see Fig. 4). This indicates that both processing techniques produce consistent datasets, but that care must be taken when comparing HCC measurements obtained from differently processed samples. Similar correlations between HCCs and other steroids measured in pulverized and non-pulverized human hair were described by Gao et al. (2013) and Stalder et al. (2012) for both LC–MS/MS and CLIA. The higher values resulting from powdered hair are probably due to a slightly more efficient steroid extraction from powdered hair where the hair matrix is readily accessible for the extraction solution (Gao et al., 2016). Considering that powdering requires an extra working step and entails the risk of material loss due to static (up to 50%, authors personal experience; and Gao et al., 2016), the advantage of a higher extraction efficiency is negligible. This demonstrates that steroid levels are reliably detectable even if only relatively small amounts of hair, insufficient for powdering, are available—which is often the case when samples are collected via shave and reshave or, e.g., via barbed wire snags from free-ranging bears (Cattet et al., 2017).

Higher HCCs measured by IA as compared to LC–MS/MS demonstrated a method-dependent influence on polar bear hair cortisol quantification. One sample that showed high HCCs as determined by both IAs but very low HCCs as determined by LC–MS/MS (mean values

Table 5
Effect of body region and season on log10-transformed concentrations of cortisol, cortisone, testosterone and progesterone in five male polar bears. For predictor variables that remained in the best-performing models the likelihood ratio test statistic and resulting p-value is given. For post hoc comparisons the test statistic is the z score, which is compared to a normal distribution to derive the p-value (p-values of post hoc comparisons were Holm–Bonferroni adjusted). Significant effects and post hoc comparisons (adjusted p-value < 0.05) are highlighted in bold print.

Response variable (log10-transformed)	Predictor or post hoc comparison	Effect on log10-scale	10^Effect size	SD	Test Statistic	P-value
Cortisol	**body region**				17.39	<0.0001
	paw vs. neck	–0.59	0.26	0.13		
Cortisone	intercept					
Testosterone	**body region**				23.52	<0.0001
	paw vs. neck	0.3	2.0	0.058		
Progesterone	**season**				9.10	0.028
	non-breeding vs. breeding	0.34	2.2	0.17	2.05	0.20
	post-breeding vs. breeding	0.49	3.1	0.16	3.01	0.02
	pre-breeding vs. breeding	0.11	1.3	0.24	0.45	0.99
	post-breeding vs. non-breeding	0.15	1.4	0.15	0.92	0.99
	pre-breeding vs. non-breeding	–0.23	0.58	0.24	–0.98	0.99
	pre-breeding vs. post-breeding	–0.38	0.42	0.24	–1.6	0.43
	body region				32.66	<0.0001
	paw vs. neck	0.38	2.4	0.061		

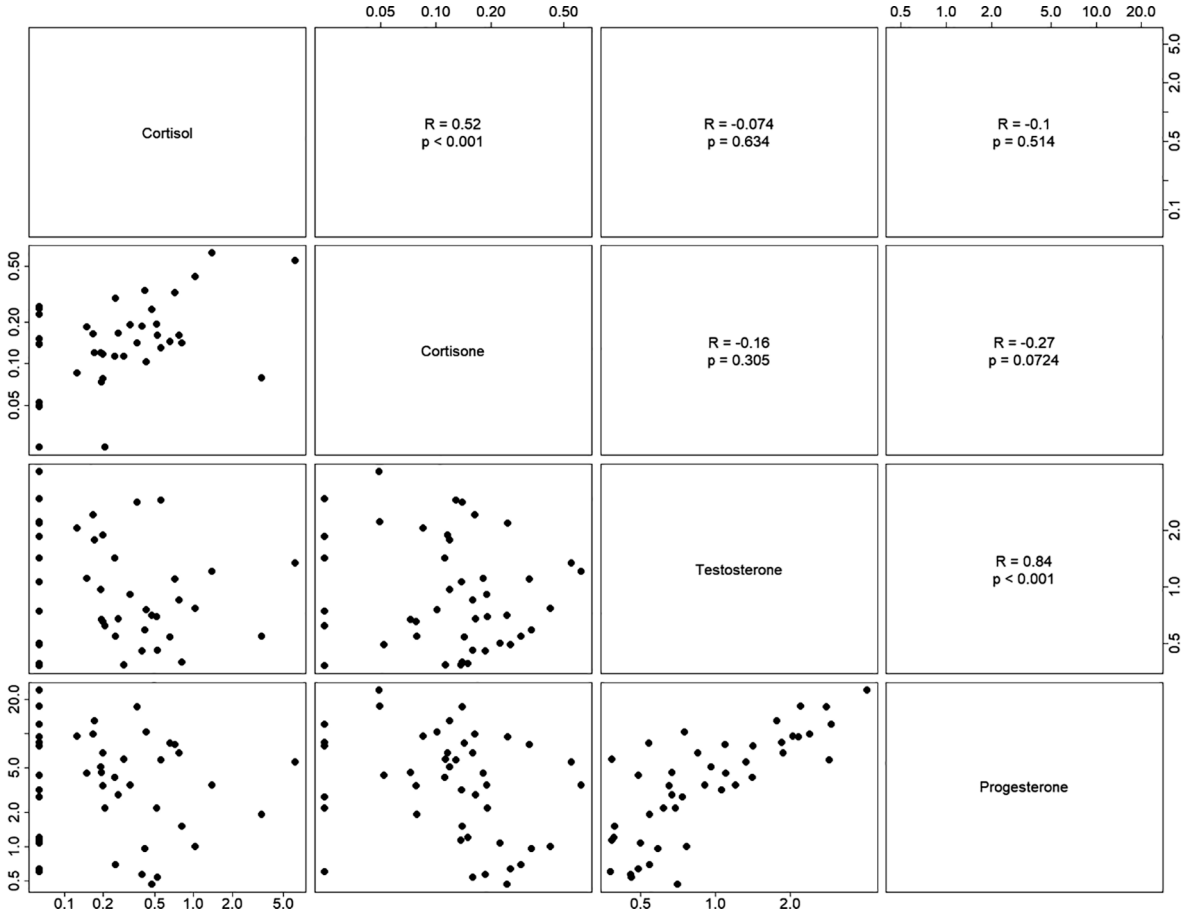

Fig. 5. Correlations between steroid hormone concentrations measured in hair samples using LC–MS/MS. The numbers in the upper right panels are the Pearson product moment correlation coefficients (R) and corresponding p-values. LC–MS/MS, liquid chromatography tandem mass spectrometry.

of 21.4 pg/mg (CLIA) and 21.5 pg/mg (EIA) in cut and 24.3 pg/mg (CLIA) and 24.0 pg/mg (EIA) in powdered hair, respectively, vs. 0.013 pg/mg in cut and powdered hair measured by LC–MS/MS) was removed as an outlier. After removing this outlier, method comparability and correlation were acceptable between CLIA and LC–MS/MS (Fig. 3; Supplementary Material III Fig. 5).

A method-dependent impact on measured steroid levels has been well documented in several studies on (mainly) human endocrinology: IAs often lead to an overestimation of hormones compared to results from LC–MS/MS systems, which are considered the gold standard for quantification of most steroids (Gao et al., 2016; Gust et al., 2010; Keevil, 2013; Kirschbaum et al., 2009; Preis et al., 2011; Russell et al., 2015). Also, in the field of animal endocrinology LC–MS/MS has been recognized as a powerful analytical tool and preferred over IAs for steroid analysis in different matrixes (Di Francesco et al., 2017; Hauser et al., 2011; Murtagh et al., 2013). Nevertheless, comparability and correlation among IAs and LC–MS/MS are often concentration-dependent and partly insufficient (Fanelli et al., 2011; Jewgenow

et al., 2020; Koal et al., 2012; Wang et al., 2004). Commercial cortisol IAs are usually developed to detect one specific analyte in a defined material: most commonly serum or/and saliva. When the assay antibody binds structurally related substances in a sample (cross-reactivity) it can lead to overestimation of the analyte concentration (see review by Gao et al., 2016; Kirschbaum et al., 2009; Miller et al., 2013; Murtagh et al., 2013). Especially at low concentrations of the target analyte, cross-reactive effects result in poor specificity (Moal et al., 2007; Taieb et al., 2002). This problem is exacerbated when IAs are applied to steroid detection in materials other than those for which the assays were originally developed: potentially confounding metabolites might be present in higher concentrations than the target analyte. Furthermore it should be taken into account that substances from the different matrixes might interfere with the kit antibodies (see review by Murtagh et al., 2013). Antibody design and binding characteristics vary across different IAs, which further impedes direct comparison of analyte concentrations even when measured from the same samples. Cross-reactivity of assay antibodies with structurally related substances is most likely the explanation for those samples that produced highly discrepant results in the IAs versus the LC–MS/MS analyses due to significantly greater specificity of the latter analytical method. It should be noted that in case of discordant results, concentrations measured by IAs were always higher than the LC–MS/MS result for the respective sample. Thus, for future hair steroid measurements we suggest LC–MS/MS to avoid potential cross-reactivities and to achieve better comparability between studies. Another advantage of this method is that it offers simulatenous measurement of different hormones (which reduces the 3- to 5-fold higher costs of LC–MS/MS compared to IAs). If IAs are used we strongly recommend selecting an appropriate assay depending on its specificity toward the hormones of interest, e.g., by previous separation of the steroid extracts via HPLC (Jewgenow et al., 2020; Kersey and Dehnhard, 2014; Kumar and Umapathy, 2019) and to include testing for matrix effects.

4.2. Biological validation

The higher HCCs in samples collected during and after longer periods of stress support the biological relevance of hair cortisol measurement in polar bears, although a biological validation could only be performed for one individual and a bigger sample size should be intended in future validation studies. Cortisol levels peaked in hair Sample 2 (see Table 4 and Fig. 2), reflecting the 7-week period after translocation. During that time transport itself, a new environment, socialization to a female and painful dental conditions (two broken canines) occurred, all of which have been identified as stressors (Davenport et al., 2006; Fairbanks et al., 2011; Hein et al., 2020; Joyce-Zuniga et al., 2016; Malcolm et al., 2013; Van Uum et al., 2008). It is also noteworthy that cortisol levels from both body regions sampled 7 weeks after translocation (Sample 2: neck, Sample 3: medial thigh) were similar via both CLIA and LC–MS/MS. The exact growth period of Sample 3 was unknown. However, since the sample was taken at the end of May, a major portion of the hairs in Sample 3 were in anagen phase for several weeks before and at the time of collection. Thus, measured HCCs should reflect integrated HPA axis activity in the weeks just before collection and correspond roughly to the HCCs measured in Sample 2.

There are only a few studies that have biologically validated HCC analysis in different species, however with results comparable to ours (increased HCCs after a stressful event; for an overview see Koren et al., 2019). During the whole monitored period no glucocorticoid-containing medication was administered.

4.3. Longitudinal measurement of hair steroid hormones and assessment of hair growth rates

To our knowledge there are no other studies measuring multiple steroids longitudinally in polar bear hair. In other mammals, only few studies have examined multiple hair steroids (Bryan et al., 2013, 2015; Cattet et al., 2018; Schell et al., 2017; Tennenhouse et al., 2017; Terwissen et al., 2014), whereas longitudinal measurements were conducted by Cattet et al. (2017) only in brown bears (*Ursus arctos;* cortisol, testosterone, progesterone and estradiol).

Whereas progesterone concentrations were lowest in the breeding period and highest in the post-breeding period in the current study, Cattet et al. (2017) found little variation in hair progesterone concentrations throughout the year in male brown bears. However, progesterone levels tended to be lowest during hibernation (October to March). In American black bears (*Ursus americanus*) no seasonal differences in plasma progesterone levels for either sex were observed (Harlow et al., 1990).

Contrary to previous work we did not observe seasonal variation in testosterone levels, which is reported for serum and fecal testosterone in polar bears, with higher levels during spring linked to increasing day length, enhanced spermatogenesis and breeding activity, and lower levels during the non-mating season in the fall (Curry et al., 2012; Howell-Skalla et al., 2002; Palmer et al., 1988). In hair samples of male and female brown bears, testosterone levels were highest at the beginning of the breeding season and subsequently decreased until the post-breeding season and hibernation in fall and winter (Cattet et al., 2017). Due to the different life and breeding cycles of brown or black bears and the small sample size in both hair studies (Cattet et al., 2017 and current study) further interpretation remains difficult. Also, molting patterns and therefore times of active hair growth and hormone incorporation are highly influenced by species, sex, age, reproduction, latitude, and food availability (Macbeth et al., 2010). In the current study, different age classes, the unknown reproductive status in two males (see influence of age/sexual maturity on hair steroids in Cattet et al., 2018) and small sample sizes, combined with uneven distribution across seasons (see Table 1), could be further possible explanations for the lack of clear seasonal trends in hair steroid levels. However, highest testosterone levels were observed in one young male polar bear (bear E, Table 1) that was known to have successfully bred. From two of the other males no data on breeding activity were available; the two remaining males were siblings and were housed together without a female throughout the study.

Contrary to free-ranging polar bears, no significant seasonal differences in hair cortisol (and cortisone) concentrations were observed in the current study, which agrees with longitudinal investigation of fecal cortisol or glucocorticoid metabolites (FGMs), respectively, of polar bears under human care (Bryant and Roth, 2018; Hein et al., 2020). Zoo-housed and free ranging polar bears experience considerably distinct living environments that likely influence hair cortisol production. In free-ranging polar bears higher cortisol levels could be measured in serum and hair samples during the later phases of the onshore fasting period when there is less sea ice coverage and thus poor access to their primary prey, bearded and ringed seals (Erignatus barbatus, Pusa hispida; Hamilton, 2008; Macbeth et al., 2012). Also, Boonstra et al. (2020) and Bechshoft et al. (2013) found links between free serum cortisol levels or HCCs, respectively, and climate change as well as sea ice coverage. Body condition is closely linked to nutritional stress, which was correlated negatively with HCCs in wild polar and brown bears (Cattet et al., 2014; Macbeth et al., 2012; Mislan et al., 2016). Even though in the current study some polar bears tended to eat less during the summer months (authors' personal experience and communication with polar bear keepers of participating zoos: S. Krüger, Nuremberg Zoo; J. Bartunek, Vienna Zoo; D. van Appeldoorn, Ouwehands Zoo)—despite of a constant food availability throughout the year in the participating zoos—body condition of the monitored bears varied only between 3 and 4 (average to fat; see Supplementary Material III Table 1), suggesting that zoo-housed polar bears experience little to no nutritional stress. Moreover, in contrast to free-ranging bears, other stressors such as aggressive encounters due to seasonally high competition for food or mating partners or temporary high energetic demands are practically

non-existent in a zoo environment. Nevertheless, other factors such as social tension, environmental changes (e.g., change of enclosure) and other disturbances such as construction work lead to an increase of FGMs in zoo polar bears (Hein et al., 2020); these were not accounted for in the current study but are probably too short-lasting to be reflected in hair samples.

Although we could obtain only very few hormone data from one female polar bear, it should be mentioned that progesterone levels were more than twice as high during July and August than those from September to November when a dead cub (immature fetus) was found in mid-November. The higher progesterone levels during pregnancy of this female were similar to mean hair progesterone levels reported for pregnant female brown bears, while the lower progesterone value corresponding to abortion is similar to mean levels measured in non-breeding female brown bears (receiving megestrol acetate for contraception; Cattet et al., 2017), thus further supporting the biological relevance and diagnostic use of hair steroid measurement in polar bears.

Body region has been shown to influence hair steroid hormone levels in various mammals, including Canada lynx (*Lynx canadensis*) and brown bears (*(Ursus arctos)*; Ashley et al., 2011; Carlitz et al., 2015; Heimbürge et al., 2020; Macbeth et al., 2010; Terwissen et al., 2013). Whereas variation in HCCs of free-ranging polar bears from Southern Hudson Bay could not be explained by the body region sampled (Macbeth et al., 2012), in the current study we found strong evidence for an effect of body region, not only for hair cortisol but also for testosterone and progesterone levels. However, these findings have to be interpreted with care, since only a limited number of simultaneously taken paw and neck samples were available (see Table 1) and the resulting imbalance in the dataset may have influenced the analysis. Similar to our findings, Macbeth et al. (2010) found the highest HCCs in samples from the neck of brown bears, and variation across body regions was greater in undercoat than in guard hair (which was not tested for in the current study). Interestingly, however, for testosterone and progesterone we observed the opposite pattern, with higher levels in paw compared to neck samples. Apart from one study on coyote pups (*Canis latrans*; Schell et al., 2017) where hair testosterone (and cortisol) levels were not affected by body region, no comparative data are available regarding progesterone and testosterone (or cortisone, respectively). Differences in hair type, hair growth cycles and rates, skin blood flow, and distribution and density of locally steroid producing skin cells could all result in a body region effect (Carlitz et al., 2015; Cattet et al., 2017; Fourie et al., 2016; Macbeth et al., 2010). Also, body-region-dependent exposure to rain, UV radiation, mechanical irritation (Salaberger et al., 2016) or contamination by urine, feces or saliva could possibly lead to diverging hair steroid levels (Acker et al., 2018; Carlitz et al., 2015; and review by Heimbürge et al., 2019).

The hair growth rates observed in one male polar bear were similar to the results of Felicetti et al. (2004), who determined a hair growth rate of about 1.5 cm/month for brown bears. Samples of the monitored polar bear very likely consisted of hair in different growth phases (anagen, catagen or telogen) and of different hair types (underfur and guard hair), explaining the different lengths by asynchronous growth (Fourie et al., 2016; Harkey, 1993). Furthermore, our findings suggest a seasonal growth cycle with presumably higher growth rates toward the beginning of the growth phase in spring/early summer. Though there were no lengths available from hair sampled at the end of the year, we observed considerably slower or no hair growth between around November to March in all monitored bears, which resulted in longer shaving intervals during the winter months, as the hair took longer to reach the minimum required sample length. While the applied method gave a rough estimation of hair growth rates at selected times in this individual bear, its results should be interpreted with caution. Inaccuracy was introduced due to difficulty of shaving the hair as close to the skin as possible and to collect the entire hair shaft in one pass. In addition to seasonality, hair growth rates have been reported to be highly variable depending on other biological factors (e.g., age, sex, latitude, species, body region) and individual differences (Feldhamer et al., 2003; Fourie et al., 2016; Macbeth et al., 2010; Pearson, 1975; Sergiel et al., 2020).

However, as long as more precise methods to investigate growth rates are not available or feasible (e.g. using radionuclide or dye markers; see review by Chamberlain and Dawber, 2003), the shave and reshave procedure is a good approach for collecting recently grown hair in individuals under human care—even if single hairs might not reflect exactly the same growth period.

5. Conclusions

The current study is the first to show a method-dependent impact on HCC measurement in paired samples of polar bears. For the first time a biological validation of HCC analysis via CLIA and LC–MS/MS was performed in a polar bear and the latter used to longitudinally monitor cortisol and other steroids in the hair of zoo-housed polar bears.

Our results suggest that LC–MS/MS is preferable to IAs for measuring hormones in hair in order to obtain a higher degree of accuracy and specificity. LC–MS/MS also allows for the simultaneous analysis of multiple hormones from sample sizes acquirable in the field. No clear benefit of powdering hair prior to LC-MS/MS analysis was observed, suggesting that this step can be omitted from hormone analysis, particularly if samples are small. However, it should be taken into account that analysis of cut hair might deliver significantly lower measurement results (compared to powdered hair and depending on assay type), which could be an issue in individuals whith very low hormone concentrations. Thus, uniform sample processing (cutting or powdering) is necessary.

The applied shave-reshave method and observed hair growth rates can guide future hair sampling of zoo-housed polar bears and help in the interpretation of HCCs from free-ranging bears. In order to control for the body region effect in future zoo or field studies, hair should be sampled preferably via shaving from the same region. If shed hair is used (e.g., when collected from enclosures) we recommend larger hair sample sizes and uniform hair collection procedures (e.g., barbed wire snags at different heights) and periods (e.g., fall), to obtain seasonally harmonized samples from varying body regions. A possible effect of hair type (undercoat vs. guard hair) on steroid levels at different body regions should be investigated in future studies.

While our study highlights important basic aspects of methodology and study design, a larger sample size will be needed in order to fully understand the longitudinally measured hair steroid levels in polar bears of different sex, age and reproductive states in different seasons and depending on body region.

Nonetheless, we demonstrate the feasibility of longitudinal studies of stress and reproductive hormone levels using hair steroid concentrations in polar bears.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2021.113837.

References

Acker, M., Mastromonaco, G., Schulte-Hostedde, A.I., 2018. The effects of body region, season and external arsenic application on hair cortisol concentration. Conserv. Physiol. 6, 1–9.

Amstrup, S.C., DeMaster, D., 2003. Polar bear, *Ursus maritimus*. In: Feldhamer, G.A., Thomson, B.C., Chapman, J.A. (Eds.), Wild mammals of North America: biology, management, and conservation, 2nd ed. Johns Hopkins University Press, Baltimore, pp. 587–610.

Ashley, N.T., Barboza, P.S., Macbeth, B.J., Janz, D.M., Cattet, M.R.L., Booth, R.K., Wasser, S.K., 2011. Glucocorticosteroid concentrations in feces and hair of captive caribou and reindeer following adrenocorticotropic hormone challenge. Gen. Comp. Endocrinol. 172, 382–391.

Bechshoft, T., Riget, F., Sonne, C., Letcher, R., Muir, D., Novak, M., Henchey, E., Meyer, J., Eulaers, I., Jaspers, V., Eens, M., Covaci, A., Dietz, R., 2012. Measuring environmental stress in East Greenland polar bears, 1892–1927 and 1988–2009: what does hair cortisol tell us? Environ. Int. 45, 15–21.

Bechshoft, T., Sonne, C., Dietz, R., Born, E., Novak, M., Henchey, E., Meyer, J., 2011. Cortisol levels in hair of East Greenland polar bears. Sci. Total Environ. 409, 831–834.

Bechshoft, T., Sonne, C., Rigét, F., Letcher, R., Novak, M., Henchey, E., Meyer, J., Eulaers, I., Jaspers, V., Covaci, A., 2013. Polar bear stress hormone cortisol fluctuates with the North Atlantic Oscillation climate index. Polar Biol. 36, 1525–1529.

Bennett, A., Hayssen, V., 2010. Measuring cortisol in hair and saliva from dogs: coat color and pigment differences. Domest. Anim. Endocrinol. 39, 171–180.

Boonstra, R., Bodner, K., Bosson, C., Delehanty, B., Richardson, E.S., Lunn, N.J., Derocher, A.E., Molnár, P.K., 2020. The stress of Arctic warming on polar bears. Global Change Biol. 26, 4197–4214.

Boonstra, R., Hik, D., Singleton, G.R., Tinnikov, A., 1998. The impact of predator-induced stress on the snowshoe hare cycle. Ecol. Monogr. 68, 371–394.

Bryan, H.M., Darimont, C.T., Paquet, P.C., Wynne-Edwards, K.E., Smits, J.E.G., 2013. Stress and reproductive hormones in grizzly bears reflect nutritional benefits and social consequences of a salmon foraging niche. PLoS ONE 8, e80537-e80537.

Bryan, H.M., Smits, J.E., Koren, L., Paquet, P.C., Wynne-Edwards, K.E., Musiani, M., 2015. Heavily hunted wolves have higher stress and reproductive steroids than wolves with lower hunting pressure. Funct. Ecol. 29, 347–356.

Bryant, J., Roth, T., 2018. Annual fecal glucocorticoid metabolite concentrations in pregnant and pseudopregnant polar bears (*Ursus maritimus*) in North American zoos. J Zoo Aqua Res 6, 6–11.

Carlitz, E.H., Kirschbaum, C., Miller, R., Rukundo, J., van Schaik, C.P., 2015. Effects of body region and time on hair cortisol concentrations in chimpanzees *(Pan troglodytes)*. Gen. Comp. Endocrinol. 223, 9–15.

Carlitz, E.H., Kirschbaum, C., Stalder, T., van Schaik, C.P., 2014. Hair as a long-term retrospective cortisol calendar in orang-utans (*Pongo spp.*): new perspectives for stress monitoring in captive management and conservation. Gen. Comp. Endocrinol. 195, 151–156.

Cattet, M.R., Macbeth, B.J., Janz, D.M., Zedrosser, A., Swenson, J.E., Dumond, M., Stenhouse, G.B., 2014. Quantifying long-term stress in brown bears with the hair cortisol concentration: a biomarker that may be confounded by rapid changes in response to capture and handling. Conserv. Physiol. 2 https://doi.org/10.1093/conphys/cou1026.

Cattet, M.R., Stenhouse, G.B., Boulanger, J., Janz, D.M., Kapronczai, L., Swenson, J.E., Zedrosser, A., 2018. Can concentrations of steroid hormones in brown bear hair reveal age class? Conserv. Physiol. 6, (1): coy001; 010.1093/conphys/coy1001.

Cattet, M.R., Stenhouse, G.B., Janz, D.M., Kapronczai, L., Anne Erlenbach, J., Jansen, H. T., Nelson, O.L., Robbins, C.T., Boulanger, J., 2017. The quantification of reproductive hormones in the hair of captive adult brown bears and their application as indicators of sex and reproductive state. Conserv. Physiol. 5 (cox032) https://doi.org/10.1093/conphys/cox1032.

Chamberlain, A.J., Dawber, R.P., 2003. Methods of evaluating hair growth. Australas. J. Dermatol. 44, 10–18.

Curry, E., Roth, T.L., MacKinnon, K.M., Stoops, M.A., 2012. Factors influencing annual fecal testosterone metabolite profiles in captive male polar bears (*Ursus maritimus*). Reprod. Domest. Anim. 47, 222–225.

Dantzer, B., Fletcher, Q.E., Boonstra, R., Sheriff, M.J., 2014. Measures of physiological stress: a transparent or opaque window into the status, management and conservation of species? Conserv Physiol 2, cou023.

Davenport, M.D., Tiefenbacher, S., Lutz, C.K., Novak, M.A., Meyer, J.S., 2006. Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. Gen. Comp. Endocrinol. 147, 255–261.

Derocher, A.E., 2012. Polar bears: a complete guide to their biology and behavior. JHU Press, Baltimore, Maryland, US.

Dettenborn, L., Tietze, A., Bruckner, F., Kirschbaum, C., 2010. Higher cortisol content in hair among long-term unemployed individuals compared to controls. Psychoneuroendocrinology 35, 1404–1409.

Di Francesco, J., Navarro-Gonzalez, N., Wynne-Edwards, K., Peacock, S., Leclerc, L.-M., Tomaselli, M., Davison, T., Carlsson, A., Kutz, S., 2017. Qiviut cortisol in muskoxen as a potential tool for informing conservation strategies. Conserv. Physiol. 5 (cox052) https://doi.org/10.1093/conphys/cox1052.

Fairbanks, L.A., Jorgensen, M.J., Bailey, J.N., Breidenthal, S.E., Grzywa, R., Laudenslager, M.L., 2011. Heritability and genetic correlation of hair cortisol in vervet monkeys in low and higher stress environments. Psychoneuroendocrinology 36, 1201–1208.

Fanelli, F., Belluomo, I., Di Lallo, V.D., Cuomo, G., De Iasio, R., Baccini, M., Casadio, E., Casetta, B., Vicennati, V., Gambineri, A., 2011. Serum steroid profiling by isotopic dilution-liquid chromatography–mass spectrometry: comparison with current immunoassays and reference intervals in healthy adults. Steroids 76, 244–253.

Feldhamer, G.A., Thompson, B.C., Chapman, J.A., 2003. Wild mammals of North America: biology, management, and conservation. JHU Press.

Felicetti, L.A., Schwartz, C.C., Rye, R.O., Gunther, K.A., Crock, J.G., Haroldson, M.A., Waits, L., Robbins, C.T., 2004. Use of naturally occurring mercury to determine the importance of cutthroat trout to Yellowstone grizzly bears. Can J Zool 82, 493–501.

Fourie, N.H., Brown, J.L., Jolly, C.J., Phillips-Conroy, J.E., Rogers, J., Bernstein, R.M., 2016. Sources of variation in hair cortisol in wild and captive non-human primates. Zoology 119, 119–125.

Gao, W., Kirschbaum, C., Grass, J., Stalder, T., 2016. LC–MS based analysis of endogenous steroid hormones in human hair. J. Steroid Biochem. Mol. Biol. 162, 92–99.

Gao, W., Stalder, T., Foley, P., Rauh, M., Deng, H., Kirschbaum, C., 2013. Quantitative analysis of steroid hormones in human hair using a column-switching LC–APCI–MS/MS assay. J. Chromatogr. B 928, 1–8.

Gust, M., Vulliet, E., Giroud, B., Garnier, F., Couturier, S., Garric, J., Buronfosse, T., 2010. Development, validation and comparison of LC–MS/MS and RIA methods for quantification of vertebrates-like sex-steroids in prosobranch molluscs. J. Chromatogr. B 878, 1487–1492.

Hamilton, J., 2008. Evaluation of indicators of stress in populations of polar bears (*Ursus maritimus*). and grizzly bears (*Ursus arctos*). University of Waterloo.

Harkey, M.R., 1993. Anatomy and physiology of hair. Forensic Sci. Int. 63, 9–18.

Harlow, H.J., Beck, T.D., Walters, L.M., Greenhouse, S.S., 1990. Seasonal serum glucose, progesterone, and cortisol levels of black bears (*Ursus americanus*). Can J Zool 68, 183–187.

Hauser, B., Mugisha, L., Preis, A., Deschner, T., 2011. LC–MS analysis of androgen metabolites in serum and urine from east African chimpanzees (*Pan troglodytes schweinfurthii*). Gen. Comp. Endocrinol. 170, 92–98.

Heimbürge, S., Kanitz, E., Otten, W., 2019. The use of hair cortisol for the assessment of stress in animals. Gen. Comp. Endocrinol. 270, 10–17.

Heimbürge, S., Kanitz, E., Tuchscherer, A., Otten, W., 2020. Within a hair's breadth – Factors influencing hair cortisol levels in pigs and cattle. Gen. Comp. Endocrinol. 288, 113359.

Hein, A., Palme, R., Baumgartner, K., von Fersen, L., Woelfing, B., Greenwood, A.D., Bechshoft, T., Siebert, U., 2020. Faecal glucocorticoid metabolites as a measure of adrenocortical activity in polar bears (*Ursus maritimus*). Conserv. Physiol. 8 (coaa012) https://doi.org/10.1093/conphys/coaa1012.

Howell-Skalla, L., Cattet, M., Ramsay, M., Bahr, J., 2002. Seasonal changes in testicular size and serum LH, prolactin and testosterone concentrations in male polar bears (*Ursus maritimus*). Reproduction 123, 729–733.

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 (coaa009) https://doi.org/10.1093/conphys/coaa1009.

Joyce-Zuniga, N.M., Newberry, R.C., Robbins, C.T., Ware, J.V., Jansen, H.T., Nelson, O. L., 2016. Positive Reinforcement Training for Blood Collection in Grizzly Bears (*Ursus arctos horribilis*) Results in Undetectable Elevations in Serum Cortisol Levels: A Preliminary Investigation. J Appl Anim Welf Sci 19, 210–215.

Keevil, B.G., 2013. Novel liquid chromatography tandem mass spectrometry (LC-MS/MS) methods for measuring steroids. Best Practice & Research Clinical Endocrinology & Metabolism 27, 663–674.

Kersey, D.C., Dehnhard, M., 2014. The use of noninvasive and minimally invasive methods in endocrinology for threatened mammalian species conservation. Gen. Comp. Endocrinol. 203, 296–306.

Kirschbaum, C., Tietze, A., Skoluda, N., Dettenborn, L., 2009. Hair as a retrospective calendar of cortisol production—increased cortisol incorporation into hair in the third trimester of pregnancy. Psychoneuroendocrinology 34, 32–37.

Koal, T., Schmiederer, D., Pham-Tuan, H., Röhring, C., Rauh, M., 2012. Standardized LC–MS/MS based steroid hormone profile-analysis. J. Steroid Biochem. Mol. Biol. 129, 129–138.

Kolenosky, G.B., 1987. Polar bear. In: Novak, M., Baker, J.A., Obbard, M., Malloch, B. (Eds.), Wild furbearer conservation and management in North America. Ontario Fur Trappers Association, North Bay, Ontario, Canada, pp. 475–485.

Koren, L., Bryan, H., Matas, D., Tinman, S., Fahlman, Å., Whiteside, D., Smits, J., Wynne-Edwards, K., 2019. Towards the validation of endogenous steroid testing in wildlife hair. J. Appl. Ecol. 56, 547–561.
Koren, L., Matas, D., Pečnerová, P., Dalén, L., Tikhonov, A., Gilbert, M.T.P., Wynne-Edwards, K.E., Geffen, E., 2018. Testosterone in ancient hair from an extinct species. Palaeontology 61, 797–802.
Kroshko, T., Kapronczai, L., Cattet, M.R.L., Macbeth, B.J., Stenhouse, G.B., Obbard, M.E., Janz, D.M., 2017. Comparison of methanol and isopropanol as wash solvents for determination of hair cortisol concentration in grizzly bears and polar bears. MethodsX 4, 68–75.
Kumar, V., Umapathy, G., 2019. Non-invasive monitoring of steroid hormones in wildlife for conservation and management of endangered species—A review. Indian J. Exp. Biol. 57, 307–314.
Macbeth, B.J., Cattet, M.R.L., Obbard, M.E., Middel, K., Janz, D.M., 2012. Evaluation of hair cortisol concentration as a biomarker of long-term stress in free-ranging polar bears. Wildl. Soc. Bull. 36, 747–758.
Macbeth, B.J., Cattet, M.R.L., Stenhouse, G.B., Gibeau, M.L., Janz, D.M., 2010. Hair cortisol concentration as a noninvasive measure of long-term stress in free-ranging grizzly bears (*Ursus arctos*): considerations with implications for other wildlife. Can J Zool 88, 935–949.
Madliger, C.L., Love, O.P., 2014. The need for a predictive, context-dependent approach to the application of stress hormones in conservation. Conserv. Biol. 28, 283–287.
Malcolm, K.D., McShea, W.J., Van Deelen, T.R., Bacon, H.J., Liu, F., Putman, S., Zhu, X., Brown, J.L., 2013. Analyses of fecal and hair glucocorticoids to evaluate short- and long-term stress and recovery of Asiatic black bears (*Ursus thibetanus*) removed from bile farms in China. Gen. Comp. Endocrinol. 185, 97–106.
Manenschijn, L., Koper, J.W., Lamberts, S.W., van Rossum, E.F., 2011. Evaluation of a method to measure long term cortisol levels. Steroids 76, 1032–1036.
Meyer, J.S., Novak, M.A., 2012. Minireview: Hair cortisol: a novel biomarker of hypothalamic-pituitary-adrenocortical activity. Endocrinology 153, 4120–4127.
Miller, R., Plessow, F., Rauh, M., Groschl, M., Kirschbaum, C., 2013. Comparison of salivary cortisol as measured by different immunoassays and tandem mass spectrometry. Psychoneuroendocrinology 38, 50–57.
Mislan, P., Derocher, A.E., Louis, V.L.S., Richardson, E., Lunn, N.J., Janz, D.M., 2016. Assessing stress in Western Hudson Bay polar bears using hair cortisol concentration as a biomarker. Ecol. Indicators 71, 47–54.
Moal, V., Mathieu, E., Reynier, P., Malthièry, Y., Gallois, Y., 2007. Low serum testosterone assayed by liquid chromatography-tandem mass spectrometry. Comparison with five immunoassay techniques. Clin. Chim. Acta 386, 12–19.
Moberg, G.P., 2000. Biological response to stress: implications for animal welfare. In: Moberg, G.P., Mench, J. (Eds.), The Biology of Animal Stress: Basic Principles and Implications for Animal Welfare. CABI Publishing, London, pp. 1–21.
Murtagh, R., Behringer, V., Deschner, T., 2013. LC-MS as a method for non-invasive measurement of steroid hormones and their metabolites in urine and faeces of animals. Wien Tierärztl Monat-Vet Med Austria 100, 247–254.
Palmer, S., Nelson, R., Ramsay, M., Stirling, I., Bahr, J., 1988. Annual changes in serum sex steroids in male and female black (*Ursus americanus*) and polar (*Ursus maritimus*) bears. Biol. Reprod. 38, 1044–1050.
Patyk, K.A., Duncan, C., Nol, P., Sonne, C., Laidre, K., Obbard, M., Wiig, Ø., Aars, J., Regehr, E., Gustafson, L.L., 2015. Establishing a definition of polar bear (*Ursus maritimus*) health: a guide to research and management activities. Sci. Total Environ. 514, 371–378.
Pearson, A.M., 1975. The northern interior grizzly bear Ursus arctos L. Environment Canada, Wildlife Service.
Pedersen, A., 1945. Der Eisbär (*Thalarctos maritimus phipps*): Verbreitung und Lebensweise. E. Bruun & Co, Copenhagen, Denmark.
Preis, A., Mugisha, L., Hauser, B., Weltring, A., Deschner, T., 2011. Androgen and androgen metabolite levels in serum and urine of East African chimpanzees (*Pan troglodytes schweinfurthii)*: Comparison of EIA and LC–MS analyses. Gen. Comp. Endocrinol. 174, 335–343.
R Development Core Team, 2018. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
Romano, M.C., Rodas, A.Z., Valdez, R.A., Hernandez, S.E., Galindo, F., Canales, D., Brousset, D.M., 2010. Stress in wildlife species: noninvasive monitoring of glucocorticoids. Neuroimmunomodulation 17, 209–212.
Russell, E., Kirschbaum, C., Laudenslager, M.L., Stalder, T., de Rijke, Y., van Rossum, E.F. C., Van Uum, S., Koren, G., 2015. Toward standardization of hair cortisol measurement: results of the first international interlaboratory round robin. Ther. Drug Monit. 37, 71–75.
Russell, E., Koren, G., Rieder, M., Van Uum, S., 2012. Hair cortisol as a biological marker of chronic stress: current status, future directions and unanswered questions. Psychoneuroendocrinology 37, 589–601.
Salaberger, T., Millard, M., El Makarem, S., Möstl, E., Grünberger, V., Krametter-Frötscher, R., Wittek, T., Palme, R., 2016. Influence of external factors on hair cortisol concentrations. Gen. Comp. Endocrinol. 233, 73–78.
Sapolsky, R., Romero, L.M., Munck, A.U., 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocr. Rev. 21, 55–89.
Schell, C.J., Young, J.K., Lonsdorf, E.V., Mateo, J.M., Santymire, R.M., 2017. Investigation of techniques to measure cortisol and testosterone concentrations in coyote hair. Zoo Biol 36, 220–225.
Sergiel, A., Cattet, M., Kapronczai, L., Janz, D.M., Selva, N., Bartoń, K.A., Swenson, J.E., Zedrosser, A., 2020. Do follicles matter? Testing the effect of follicles on hair cortisol levels. Conserv. Physiol. 8 (coaa003) https://doi.org/10.1093/conphys/coaa1003.
Sheriff, M.J., Dantzer, B., Delehanty, B., Palme, R., Boonstra, R., 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia 166, 869–887.
Stalder, T., Kirschbaum, C., 2012. Analysis of cortisol in hair–state of the art and future directions. Brain. Behav. Immun. 26, 1019–1029.
Stalder, T., Steudte, S., Miller, R., Skoluda, N., Dettenborn, L., Kirschbaum, C., 2012. Intraindividual stability of hair cortisol concentrations. Psychoneuroendocrinology 37, 602–610.
Taieb, J.L., Benattar, C., Birr, A.S., Lindenbaum, A., 2002. Limitations of steroid determination by direct immunoassay. Clin. Chem. 48, 583–585.
Tennenhouse, E.M., Putman, S., Boisseau, N.P., Brown, J.L., 2017. Relationships between steroid hormones in hair and social behaviour in ring-tailed lemurs (*Lemur catta*). Primates 58, 199–209.
Terwissen, C., Mastromonaco, G., Murray, D., 2014. Enzyme immunoassays as a method for quantifying hair reproductive hormones in two felid species. Conserv. Physiol. 2 https://doi.org/10.1093/conphys/cou1044.
Terwissen, C.V., Mastromonaco, G.F., Murray, D.L., 2013. Influence of adrenocorticotrophin hormone challenge and external factors (age, sex, and body region) on hair cortisol concentration in Canada lynx (*Lynx canadensis)*. Gen. Comp. Endocrinol. 194, 162–167.
Van Uum, S.H.M., Sauvé, B., Fraser, L.A., Morley-Forster, P., Paul, T.L., Koren, G., 2008. Elevated content of cortisol in hair of patients with severe chronic pain: A novel biomarker for stress. Stress 11, 483–488.
Vongraven, D., Aars, J., Amstrup, S., Atkinson, S.N., Belikov, S., Born, E.W., DeBruyn, T. D., Derocher, A.E., Durner, G., Gill, M., Lunn, N., Obbard, M.E., Omelak, J., Ovsyanikov, N., Peacock, E., Richardson, E., Sahanatien, V., Stirling, I., Wiig, Ø., 2012. A circumpolar monitoring framework for polar bears. Ursus 23 (1–66), 66.
Wang, C., Catlin, D.H., Demers, L.M., Starcevic, B., Swerdloff, R.S., 2004. Measurement of total serum testosterone in adult men: comparison of current laboratory methods versus liquid chromatography-tandem mass spectrometry. J. Clin. Endocrinol. Metab. 89, 534–543.
Webb, E., Thomson, S., Nelson, A., White, C., Koren, G., Rieder, M., Van Uum, S., 2010. Assessing individual systemic stress through cortisol analysis of archaeological hair. J Archaeol Sci 37, 807–812.
Webb, E.C., White, C.D., Van Uum, S., Longstaffe, F.J., 2015. Integrating cortisol and isotopic analyses of archeological hair: reconstructing individual experiences of health and stress. Am. J. Phys. Anthropol. 156, 577–594.
Weisser, J.J., Hansen, M., Bjorklund, E., Sonne, C., Dietz, R., Styrishave, B., 2016. A novel method for analysing key corticosteroids in polar bear (*Ursus maritimus*) hair using liquid chromatography tandem mass spectrometry. J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 1017–1018, 45–51.
Wennig, R., 2000. Potential problems with the interpretation of hair analysis results. Forensic Sci. Int. 107, 5–12.