Short title: UPC^2-MS/MS analysis of Steroids in Mammalian Whiskers

What's in a whisker? High-throughput analysis of twenty-eight C_{19} and C_{21} steroids in mammalian whiskers by ultra-performance convergence chromatography-tandem mass spectrometry

Nico Lübcker[1,*],

Liezl M. Bloem[2],

Therina du Toit[2],

Pieter Swart[2],

P.J. Nico de Bruyn[1],

Amanda C. Swart[2],

Robert P. Millar[3]

[1]Mammal Research Institute, Department of Zoology and Entomology, University of Pretoria, Private Bag X20, Hatfield 0028, South Africa.

[2]Department of Biochemistry, Stellenbosch University, Stellenbosch 7600, South Africa.

[3]Centre for Neuroendocrinology, Department of Immunology, Faculty of Health Sciences, University of Pretoria, Pretoria 0001, South Africa.

*corresponding author: nlubcker@zoology.up.ac.za, +27720370130.

Authors Contributions: NL, RM, NdB, AS, TdT, LB, and PS conceived the ideas and designed methodology, RM, NdB, PS, and AS enabled sample analyses. NL, LB, TdT analyzed the samples and processed the data. NdB maintained the fieldwork programme, allowing access to sampling. All authors assisted with the writing of the manuscript.

Highlights

- We developed a high-throughput assay to quantify 28 steroids from single whiskers.
- This UPC^2-MS/MS method enabled the first quantification of a suite of steroids detectable in mammalian whiskers.
- Sensitive and accurate quantification method at reduced cost and time.
- Whiskers provides biomatrix for longitudinal steroid hormones monitoring.
- Applicable to free-ranging mammals with cryptic life cycles

Abstract

Obtaining longitudinal endocrinological data from free-ranging animals remains challenging. Steroid hormones can be extracted sequentially from non-invasively sampled biologically inert keratinous tissues, such as feathers, nails, hair and whiskers. However, uncertainty regarding the type and levels of steroids incorporated into such tissues complicates their utility in wildlife studies. Here, we developed a novel, comprehensive method to analyze fourteen C_{19} and fourteen C_{21} steroids deposited chronologically along the length of seal whiskers in a single, 6-minute chromatographic step, using ultra-performance convergence chromatography-tandem mass spectrometry. The limits of detection and quantification ranged from 0.01 to 2 ng/mL and from 0.1 to 10 ng/mL, respectively. The accuracy and precision were within acceptable limits for steroids at concentrations ≥2 ng/mL. The recovery (mean = 107.5% at 200 ng/mL), matrix effect and process efficiency of steroids evaluated, using blanked whisker matrix samples, were acceptable. The method was applied to the analysis of steroid hormone levels in adult female whisker segments obtained from southern elephant seals (*Mirounga leonina*), $n = 10$, and two fur seal species, Antarctic fur seals (*Arctocephalus gazella*; $n = 5$) and subantarctic fur seals (*Arctocephalus tropicalis*; $n = 5$), sampled between 2012–2017. In the whisker subsamples

analyzed (n = 71), the median concentration of steroid hormones detected above the LOQ ranged from 2.0–273.7 pg/mg. This is the first extraction of multiple C_{19} and C_{21} steroids, including their C11-oxy metabolites, from the whiskers of mammals. Measuring hormones sequentially along the whisker lengths can contribute to our understanding of the impact of stress associated with environmental/climate changes that affect the health, survival of organisms, as well as to delineate the reproductive cycles of free-living mammals with cryptic life stages.

Keywords

Androgens; progestogens; glucocorticoids; metabolites; keratin and feathers; marine mammal endocrinology; southern elephant seal; vibrissae; whiskers; UPC^2-MS/MS.

1. Introduction

Steroid hormones serve as biomarkers that enable the assessment of organisms' response to intrinsic or extrinsic stressors that modulate trade-offs between reproduction and survival [1,2]. Glucocorticoid steroids produced by the chronic activation of the hypothalamic-pituitary-adrenocortical axis (HPA) have been associated with reproductive impairment [3]. Longitudinal monitoring of an individuals' circulating steroid hormone levels is often associated with ethical and/or practical constraints. Non-invasive steroid monitoring approaches such as repeated saliva or faecal glucocorticoid analyses provide a temporal record of the individuals' circulating steroid levels [4]. However, the requirement for repeated sampling of species with cryptic habits is often unattainable. Steroid levels in biologically inert keratinous tissues (e.g., feathers, nails, hair, whale baleen and whiskers), reflect the "free" (unbound)-steroids and thus the physiologically active steroids in the circulation, which is incorporated into these tissues [5,6,7]. Steroids

contained in biologically inert tissue are insensitive to short term stressors, circadian rhythms, and sample degradation [8,9].

Steroids have previously been extracted from hair [10,11,12,13]. Yet, most of the method development lacks a temporal component and are limited to a few steroids for which enzyme immunoassay kits (EIAs) have been verified [4,14]. Intra-individual variation in the baseline steroid concentrations and complex metabolic pathways suggest that steroid levels should also be assessed in a more comprehensive panel (i.e. more than 2–3 steroids at a time) [15,16].

Southern elephant seals (SES, *Mirounga leonina*) fast on land during the annual breeding season and again during the annual pelage molt [17]. Their new hair growth, which will completely replace the old pelage, is limited to ca. 8–12-weeks of the year before the annual pelage molt [18] and does not span the gestation period. An appropriate, specific tissue matrix is required to investigate whether the intermittent breeding pattern observed in SES [19] and other phocid species [20], is associated with ecophysiological responses to extreme climatic events or with reductions in prey abundances [21,2,22,23]. Consequently, the measurement of stable isotopes, hormones and metabolic indicators from mammalian whiskers for ecological and physiological purposes has increased rapidly (e.g., [24,25]). Yet, only one study has extracted cortisol from mammalian whiskers and quantified the concentration using an EIA kit [24]. Uncertainty regarding steroids incorporated into whiskers, together with their concentrations, limit the potential of this valuable biomatrix in the assessment of ecological and physiological factors impacting reproduction and survival.

Advances in liquid chromatography-tandem mass spectrometry (LC-MS) has facilitated the analyses of comprehensive steroid profiles in various matrices. Ultra-performance convergence chromatography™ (UPC^2) coupled tandem mass spectrometry, is capable of quantifying > 30 different steroid levels in a single chromatographic separation [26]. The advance of supercritical-fluid chromatography (SFC; supercritical CO_2 as mobile phase) combines the benefits of gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-tandem mass spectrometry (LC-MS/MS). This combination enhances chromatographic efficiency while reducing the run times (high throughput) and sample preparation requirements [27].

A novel high-throughput method was developed to analyze multiple C_{19} and C_{21} steroids and their C11-hydroxy and C11-keto metabolites along the length of whiskers sampled from various age-class SES using UPC^2-MS/MS. Whiskers sampled from free-ranging adult female SES, and adult females of two sympatrically occurring fur seal species, Antarctic fur seal (*Arctocephalus gazella*; $n = 5$) and subantarctic fur seal (*Arctocephalus tropicalis*), were used to demonstrate the utility of the method. The validated method provides an excellent analytical tool to ascertain and quantify steroids in seal whiskers. This advance using biologically inert whiskers will facilitate physiological monitoring of factors that affect the reproduction of free-ranging mammals.

2. Methods

2.1. Reagents and Steroid Standards

Steroids were purchased (listed in Table 2) from Steraloids Inc. (Newport, RI, USA) and Merck (Darmstad, Germany), as detailed in Du Toit et al., [26]. Isotopically labelled deuterated steroids,

used as internal standards, were purchased from Cambridge isotopes (Andover, MA, USA), except for testosterone 1,2-D2 (D2T) which was purchased from CDN Isotopes. The internal standards included D2T; 4-androsten-11β-ol-3,17-dione 2,2,4,6,6,16,16-D7 (D7A4); 4-androstene- 3,17-dione 2,2,4,6,6,16,16-D7 (D711OHA4) and progesterone 2,2,4,6,6,17A,21,21,21-D9 (D9PROG). The molecular weights of steroids are provided (Table 2). Chromatographic separation of the steroids was achieved using a fitted ACQUITY UPC^2® ethylene-bridged hybrid (BEH) column (3.0×100 mm, 1.7 µm particle size) with a VanGuard™ pre-column (2.1×5mm, 3.5 µM), purchased from Waters (Waters Corporation, Milford, USA). Details of the other chemicals used, such as FOODFRESH CO_2 (Afrox), formic acid (Sigma-Aldrich; St. Louis, MO, USA), isopropanol (Sigma-Aldrich; St. Louis, MO, USA), and high pressure liquid chromatography (HPLC)-grade methanol (MeOH) 215 SpS (from ROMIL Ltd.; Cambridge, England) were identical to Du Toit et al., [26].

2.2. Sample Collection for Method Validation

Sample collection occurred at Marion Island (46.88° S, 37.87° E) between 2012–2017. A single whisker, representative of all age-class SES, was sampled by cutting as close to the skin as possible. Approximately 3 g of whiskers, sampled from ca. 60 individuals, were pooled for the method development. This method was subsequently tested using chronologically subsampled whiskers (n = 71) obtained from adult females of three seal species, which included SES, n = 10, Antarctic fur seals (n = 5) and subantarctic fur seals (n = 5). Samples were stored in sealed plastic bags and kept at room temperature before analyses.

2.3. Removal of Surface Contaminants from Whiskers

Before steroid extraction, the surface contaminants were removed by washing each whisker once with 10 mL distilled water for 3 minutes at room temperature while shaking gently at 1250 rpm (IKA Vibrax® VXR Basic Orbital Shaker), then washed twice with 10 mL isopropanol. The samples were left to dry for a minimum of 5 hours at room temperature before processing. Different decontamination protocols were considered, and our final sample pre-treatment protocol followed a method previously validated for hair (e.g., [5,15,22]). The washing procedure was sufficient to remove all contaminants and surface steroids without extracting steroids (quantifiable amounts) from the whisker shaft (intrinsic steroids).

2.4. Steroid Extraction from Whiskers

The 78.8 ± 26.3 mg (mean ± SD), 118.8 ± 25.3 mm long cleaned whiskers sampled from the three species were sectioned into 2–7 chronological subsamples, with each segment being 20.0 ± 21.7 mm long. The number of suitable subsamples was determined by the whisker diameter and mass (mean = 3.0 ± 2.5 segments per whisker). Liquid nitrogen was used to freeze the sample prior to the use of a mortar and pestle to grind subsamples to a fine powder. Samples were weighed into glass scintillation vials to allow for analyses per dried mass (mg) and ranged from 8–27.0 mg per whisker subsample. The sample mass depended on the diameter of the whisker segment analyzed and the amount of whisker powder recovered. The dried subsamples were then spiked with four internal standards at a concentration of 0.1 ng/µL (D711OHA4, D9PROG, D7A4) and 0.01 ng/µL (D2T) before extraction. Steroids were extracted using 10 mL 100% MeOH with sample incubation at 37 °C and shaking for 24-hours at 1250 rpm. Thereafter, the suspension was centrifuged at 4500 rpm on a bench centrifuge and the supernatant aspirated. The pellet was washed with 1 mL 100% MeOH for 15 minutes by shaking at 1500 rpm, centrifuged

at 4500 rpm, and the supernatant added to the initial supernatant. Extracted steroids were dried under a gentle stream of nitrogen at 40 °C (Techne® Dri-Block® heater Model DB-3) until completely dry. The sides of the test tubes were rinsed with 1 mL MeOH and vortexed gently to ensure that all the steroids concentrated at the bottom of the test tubes, before being transferred to 2 mL LC-MS/MS vials and dried at room temperature. The dried steroid residue was resuspended in 100 µL 50% MeOH/Water, which was gently vortexed (ca. 40 seconds) before being transferred to LC-MS/MS vial inserts and stored at -20 °C until analyses.

The extraction efficiency of dichloromethane (DCM), the use of which was previously reported by Vanaelst et al., [13], methyl tert-butyl ether (MTBE), and MeOH were compared on both ground and unground whisker segments. Other extraction protocols, such as the extraction of steroids from the whiskers using a Thermo Scientific ™ Dionex™ ASE™ 200 Accelerated Solvent Extractor (ASE; heat 5 min, 5 min static phase, temperature 75°C, pressure 15 MPa, flushed in three cycles to make-up a total of 40% of cell volume) were also considered. However, our exploratory protocols (data not shown) confirmed that the MeOH extraction protocol described above was adequate and reproducible.

2.5. Preparation of Standards for Method Validation and Steroid Quantification

Standards curves were generated from a stock solution of steroids (2 mg/mL), as described in Du Toit et al., [26]. Briefly, standard master mixes of 20, 200, and 1000 ng/mL were prepared in 50% MeOH/Water and used to spike either 50% MeOH/Water (no matrix) or whisker (matrix) to generate two standard curves ranging from 0.01, 0.02, 0.1, 0.2, 1, 2, 10, 20, 100, 200 to 1000 ng/mL. To prepare a standard curve representative of the chemical composition of the whisker sample matrix (matrix-matched calibration; [28]), a ca. 3 g pool of ground whiskers were

‘blanked’ of steroids after extracting the steroids using three repeated 24-hour MeOH extractions, as described above. The resulting matrix of the second and third extraction did not contain detectable steroid concentrations (similarly observed in Macbeth et al., [29] for hair) and was pooled to represent the ‘whisker matrix’ used to prepare the standard curve. The pooled extractions were aliquoted and dried down to attain the concentration resembling the upper-end of the whisker matrix concentrations of the extracted whisker samples (ca. 0.2 mg/μL^{-1}) and subsequently resuspended using the standard master mixes. In addition, blanked whisker powder was required for method validation (see “2.7. Validation Protocol” below). The residual whisker powder remaining after the third extraction was used to represent ‘blanked whisker powder’, similarly described for hair in Gao et al., [15] and Vanaelst et al., [13]. Samples for the method validation were prepared by spiking either the ‘blanked whisker powder’ or 50% MeOH/Water with standards at different steroid concentrations as well as the four internal standards.

2.6. Chromatographic Conditions for Quantification of Steroid Metabolites

Twenty-eight extracted steroids were analyzed using UPC^2-MS/MS, as described in Du Toit et al., [26] and adapted to quantify the whisker steroids and steroid metabolites. Steroids were separated using a UPC^2 ethylene-bridged hybrid (BEH) (3 mm x 100 mm, 1.7 µm particle size) column, the injection volume set to 2 µL with gradient specifications and MS parameters summarized in Tables 1 & 2. Briefly, a Xevo TQ-S triple quadrupole mass spectrometer (Waters, Milford, USA) was used for quantitative mass spectrometric detection. All steroids were analyzed in multiple reaction monitoring (MRM) mode using electrospray ionization in the positive mode (ESI+). The capillary voltage was set to 3.7 kV, using an ion-source temperature of 150 °C, desolvation temperature of 350 °C, desolvation gas flow of 900 $L.h^{-1}$ and cone gas

flow of 150 $L.h^{-1}$ for the analyses. Data were collected with the MassLynx 4.0 software program (Waters Corporation).

Table 1: Gradient specifications of solvent A (CO_2) and solvent B (MeOH) for chromatographic steroid separation and elution.

Step	Time (min)	Solvent A (%)	Solvent B (%)	Curve
1	0	98	2	Initial
2	0.5	98	2	6
3	2.7	90	10	7
4	4.9	75	25	5
5	5.5	75	25	5
6	5.6	98	2	1
7	6	98	2	1

2.7. Validation Protocol

The lowest limit of detection (LOD) and lowest limit of quantification (LOQ) were based on the signal-to-noise ratio (S/N) of the quantifier ion of > 3:1 and > 10:1, respectively [30]. The LODs and LOQs were obtained from replicates of whisker matrix spiked with a range of steroid concentrations, for which the percentage relative standard deviation (%RSD) of the accuracy (%RSD < 20%) and precision (%RSD < 15%) were acceptable. The linearity of the standard curves was expressed using a linear, weighted least trimmed robust squares regression, and was not forced through zero to account for potential high 'blank' values [31].

The accuracy, precision, recovery, matrix effect and process efficiency were tested at two low (0.02 ng/mL and 0.2 ng/mL), a mid (2.0 ng/mL), and a high (200 ng/mL) steroid concentration. Method validation was performed at the lower end of the steroid concentration range since steroids are not generally present at high concentrations in a non-lipid (keratin) matrix. The accuracy %RSDs was based on the variation in the average response of $n = 8$ repeated injections of a single sample comprising steroid spiked whisker matrix (analytical

Table 2: UPC^2-MS/MS parameters for steroid detection and quantification. Settings for steroids and internal deuterated reference standards: cone voltage (CV), collision energy (CE), molecular ion species, multiple reaction monitoring (MRM) mass transitions, and retention time (RT).

Abbreviation	Name		Mass transitions		CV(V)		CE (eV)		RT (min)
		Molecular weight	Quantifier	Qualifier					
11αOHPROG	11α-Hydroxyprogesterone	331.2	331.2 > 295.2	331.2 > 121.0	30	30*	30	15	3.47
11βOHPROG	11β-Hydroxyprogesterone	331.2	331.2 > 121.0	331.2 > 295.0	30	30	20	20	3.26
11KA4	11-Ketoandrostenedione	300.4	301.2 > 257.0	301.2 > 265.2	35	35	25	25	2.56
11KAST	11-Ketoandrosterone	304.4	305.0 > 147.2	305.0 > 173.1	30	30	30	30	3.06
11KDHPROG	5α-Pregnane-3,11,20-Trione	331.2	331.2 > 105.0	331.2 > 147.0	25	25	30	30	1.96
11KDHT	5α-Androstan-17β-Ol-3,11-Dione	304.4	305.2 > 269.0	305.2 > 269.0	30	30	20	20	3.25
11KPROG	11-Ketoprogesterone	329.4	329.2 > 121.0	329.2 > 84.8	15	15*	20	20	2.58
11KT	11-Ketotestosterone	302.4	303.2 > 121.0	303.2 > 267.0	30	30	20	20	3.50
11OHA4	11β-Hydroxyandrostenedione	302.4	303.2 > 267.2	303.2 > 121.0	30	30	30	15	3.13
11OHAST	11β-Hydroxyandrosterone	306.2	289.0 > 271.0	289.0 > 213.0	15	15	15	15	3.42
11OHT	11β-Hydroxytestosterone	304.4	305.3 > 269.0	305.3 > 269.0	35	35	20	15	3.81
16OHPROG	16α-Hydroxyprogesterone	331.2	331.2 > 97.0	331.2 > 108.9	30	30	15	15	3.40
17OHPROG	17α-Hydroxyprogesterone	331.1	331.1 > 97.0	331.1 > 109.0	26	26	22	28	2.82
21-dF	21-Desoxycortisol	347.1	347.1 > 121.0	347.1 > 269.2	20	20	25	15	3.63
3α-diol	3α-Androstanediol	292.4	275.2 > 257.0	275.2 > 175.0	15	15	15	15	3.29
5α-dione	5α-Androstanedione	288.4	289.2 > 253.1	289.2 > 97.2	22	30	16	22	1.04
A4	Androstenedione	286.4	287.2 > 96.9	287.2 > 108.8	30	30	15	15	1.85
AST	Androsterone	290.4	273.2 > 105.3	291.3 > 273.3	30	18	30	8	2.37
Cortisol	Cortisol	363	363.0 > 121.0	363.3 > 97.1	30	26	20	30	3.85
Cortisone	Cortisone	361.2	361.2 > 163.0	361.2 > 163.0	34	30	30	30	3.59
DHEA	5-Androsten-3β-Ol-17-One	271.2	271.2 > 253.2	271.2 > 253.2	30	30	15	15	2.42
DHPROG	5α-Dihydroprogesterone	317	317.0 > 105.2	317.0 > 95.0*	30	30*	30	30	0.92
DHT	5α-Dihydrotestosterone	290.4	291.2 > 255.0	291.2 > 273.0	25	25	15	20	2.32
Pdiol	5α-pregnan-3α,17α-diol-20-one	317.4	317.4 > 299.0	317.4 > 111.0	20	20	25	15	3.33
Pdione	5α-pregnan-17α-ol-3,20-dione	333.4	333.4 > 159.0	333.4 > 137.0	20	20	25	25	2.15
Pregnanetriol	Pregnanetriol	301.2	301.2 > 135.0	301.2 > 81.0	25	25	25	15	3.78
PROG	Progesterone	315.2	315.2 > 97.0	315.2 > 109.0	28	28	20	26	1.74
T	Testosterone	288.4	289.2 > 97.2	289.2 > 109.0	30	30	22	22	2.99

D7A4	4-Androsten-11β-Ol-3,17-Dione 2,2,4,6,6,16,16-D7	293.45	294.3 > 100.0	294.3 > 113.0	25	25	25	25	1.85
D2T	Testosterone 1, 2-D2	290.44	291.0 > 99.1	291.0 > 111.2	30	30	20	20	2.99
D711OHA4	4-Androstene-3,17-Dione 2,2,4,6,6,16,16-D7	309.46	310.2 > 99.8	310.2 > 147.2	25	25	30	25	3.12
D9PROG	Progesterone 2,2,4,6,6,17A,21,21,21-D9	323.52	324.2 > 100.0	324.2 > 113.0	30	30	20	25	1.74

*Secondary Qualifier: DHPROG: 317.0 > 175.3, Cone Voltage 30V, Collision energy 20eV; 11KPROG: 329.2 > 285.0, Cone Voltage 15V, Collision energy 20eV; 11αOHPROG: 331.2 > 295.2, Cone Voltage 30V, Collision energy 15eV.

repeats), for each of the steroid concentrations. Intra- and interday precision (%RSD) was determined by analysing three replicates of independent samples comprising steroid spiked whisker matrix (biological repeats) at the different steroid concentrations, measured on two separate days.

The recovery (%) was determined by comparing the response of the whisker powder, that was blanked of all steroids (stripped of all endogenous steroids) before being spiked (pre-extraction steroid addition) and extracted according to the described extraction protocol, to samples comprising steroid spiked whisker matrix (post-extraction steroid addition). The matrix effect (%) described the impact that interfering analytes, present in the extracted whisker matrix, have on the steroid analyses; values > 100% indicate ion enhancement by the sample matrix and values < 100% suggests ion suppression [28]. The matrix effect was expressed as the difference between the response of the samples prepared by spiking of whisker matrix with relevant steroids (post-extraction steroid addition) and the response of samples prepared by the addition of steroids at the same concentration to a pure solution of 50% MeOH/Water. The difference in response was then divided by the response of the steroids in the pure solution [28]. Lastly, the difference in the response of samples comprising steroid spiked whisker matrix (pre-extraction additional) and the response of steroids prepared in pure solution, provided a measure of the overall process efficiency (%). Three replicates were analyzed for each steroid concentration.

In addition, the influence of ion suppression or enhancement by the whisker matrix on the internal standards was evaluated by extracting four repeats of 20 mg whisker matrix and resuspending the matrix in 50 (final whisker matrix concentration of 0.4 mg/µL), 100 (0.2 mg/µL), 150 (0.13 mg/µL), and 200 µL (0.1 mg/µL) of 50% MeOH/Water. Each extract was

spiked with equal amounts of the four internal standards, representing the concentration of the internal standards in the analyzed whisker samples (0.1 ng/µL and 0.01 ng/µL for D2T).

2.8. Statistical Analyses

Mathematical and statistical computations were performed using R ([32], v. 3.4.4) coupled with the RStudio interface (v. 1.0.153). The *robustbase* package (v. 0.93) in R was used to fit the least trimmed robust squares regression to the standard curves to test linearity. A non-parametric Kruskal-Wallis test was used to compare the median of all the steroid concentrations (pooled) between the three species and between the tip, middle and base of the whisker segments analyzed. A principal component analysis (PCA) was used to visualize the multivariate relationships between the hormone concentrations, using the *FactoMineR* package (v. 1.34) in R [33]. The data were centered and scaled [34], and steroid concentrations (ng/mL) > LOD, but < LOQ, were replaced with the LOD of the steroid to perform the PCA analyses. Steroids detected above the LOQ in ≥30% of our samples were included in the PCA analyses. The median ± standard error (SE) of the measured steroid concentrations are reported.

3. Results

3.1. Separation of Steroid Standards

Separation of 14 C_{19} and 14 C_{21} steroids and metabolites was achieved in a single 6-minute chromatography step, which included C11-oxy C_{19} and C11-oxy C_{21} derivatives together with four deuterated steroid standards. Stereoisomers with similar retention times were successfully differentiated based on different quantifier and qualifiers ion masses used (Table 2). Whisker matrix samples (*n* = 4 repeats) were analyzed to confirm that the residual steroid concentrations

of all steroids were below the LOQ (mean blank steroid concentration = 0.009 ng/mL) and did not interfere with the method development.

3.2. Performance and Validation of the Method

3.2.1. Calibration Range

The LOD and LOQ of each steroid were based on the S/N ratio and the concentration at which the accuracy (%RSD < 20%) and precision (%RSD < 15%) were within acceptable limits. The LOQs ranged from 0.1 to 10 ng/mL (Table 3a). The R^2 of the linear calibration regressions were all > 0.9930, except for 11KAST and Pdione that were 0.9793 and 0.9866, respectively, however acceptable and exhibiting good linearity (Table 3a). Validations based on the two lower steroid concentrations (0.02 ng/mL and 0.2 ng/mL) were either below the LOQs, or not within the acceptable accuracy or precision %RSD ranges and were excluded. Peak saturation was observed for T and PROG at a concentration of 1000 ng/mL.

3.2.2. Accuracy and Precision

The %RSD of the accuracy of steroids measured at mid (2 ng/mL) and high concentrations (200 ng/mL) were acceptable (accuracy %RSDs < 20%; Table 3b). The mean accuracy %RSDs were 6.6% and 5.8% for steroids > LOQ at 2 ng/mL and 200 ng/mL, respectively. The precision of all steroids measured at mid and high concentrations was acceptable on at least one of the two days (precision %RSDs < 15%; Table 3b). The intra-day and interday precision, quantified over two days, had a mean precision %RSDs of 12.3% and 4.2% for steroids at the mid and high concentrations on Day 1, and 9.0% and 4.3% on Day 2. The precision of AST and DHPROG at 2 ng/mL during Day 2 was higher than expected, similarly observed for 11KA4, 11KDHT, 11KT, and 21-dF during Day 1, as well as pregnanetriol during Day 2 at a concentration of 200 ng/mL.

Table 3a: Comprehensive method validation data as determined from least trimmed robust squares regressions: Limit of detection (LOD) and limit of quantification (LOQ) of steroids, calibration range (ng/mL) and linearity (R^2).

Steroid metabolite	LOD (ng/mL)	LOQ (ng/mL)	Calibration range	R^2
11αOHPROG	0.2	1	0.2–1000	0.9998
11βOHPROG	0.01	1	0.1–1000	0.9998
11KA4	0.01	0.2	0.02–1000	0.9996
11KAST	1	10	1–1000	0.9793
11KDHPROG	0.1	10	0.1–1000	0.9997
11KDHT	0.2	1	0.2–1000	0.9994
11KPROG	0.2	1	0.2–1000	0.9998
11KT	0.01	1	0.01–1000	0.9999
11OHA4	0.2	0.2	0.2–1000	0.9999
11OHAST	0.1	10	0.1–1000	0.9998
11OHT	0.1	1	0.1–1000	0.9998
16OHPROG	0.02	0.1	0.02–1000	0.9976
17OHPROG	0.02	0.1	0.02–1000	0.9999
21-dF	0.1	2	0.1–1000	0.9956
3α-diol	0.2	10	0.2–1000	0.9958
5α-dione	0.1	1	0.1–1000	0.9994
A4	0.02	0.1	0.02–1000	0.9998
AST	1	2	2–1000	0.9993
Cortisol	0.1	1	0.1–1000	0.9996
Cortisone	0.1	2	0.1–1000	0.9989
DHEA	0.2	10	0.2–1000	0.9930
DHPROG	1	2	1–1000	0.9997
DHT	0.2	1	0.2–1000	0.9997
Pdiol	1	10	1–1000	0.9997
Pdione	2	10	2–1000	0.9866
Pregnanetriol	1	10	1–1000	0.9987
PROG	< 0.01	0.2	0.01–200	0.9956
T	< 0.01	0.2	0.01–200	0.9999

Table 3b: Comprehensive method validation data continued: Percent relative standard deviation (%RSD) of method accuracy (n = 8 repeats at 2, and 200 ng/mL) and precision (n = 3).

Steroid metabolite	Internal standard	Accuracy (%RSD)		Precision (%RSD)			
				Day 1		Day 2	
		2 ng/mL	200 ng/mL	2 ng/mL	200 ng/mL	2 ng/mL	200 ng/mL
11αOHPROG	D2T	4.6	4.0	4.6	3.1	7.3	3.6
11βOHPROG	D2T	6.5	3.3	10.2	2.0	3.6	2.8
11KA4	D7A4	6.2	4.8	21.9	5.8	13.0	2.7
11KAST	D2T	< LOQ	6.8	< LOQ	0.9	< LOQ	6.1
11KDHPROG	D7A4	< LOQ	3.1	< LOQ	9.0	< LOQ	2.2
11KDHT	D2T	11.8	4.0	43.3	1.7	8.6	1.8
11KPROG	D7A4	4.4	7.0	1.6	9.3	5.3	2.2
11KT	D2T	5.6	5.6	17.4	4.0	12.5	5.2
11OHA4	D711OHA4	5.6	1.5	5.4	1.7	3.8	0.7
11OHAST	D2T	< LOQ	5.2	< LOQ	4.6	< LOQ	3.5
11OHT	D2T	8.5	11.4	6.4	1.0	4.2	9.7
16OHPROG	D2T	3.3	5.3	9.9	2.8	5.0	2.8
17OHPROG	D2T	5.9	2.8	10.4	1.8	6.6	3.7
21-dF	D2T	6.6	17.0	20.7	3.2	8.5	10.0
3α-diol	D2T	< LOQ	4.9	< LOQ	2.7	< LOQ	3.4
5α-dione	D9PROG	8.0	3.4	18	6.0	6.4	1.8
A4	D7A4	2.1	1.0	9.0	5.4	3.2	2.3
AST	D2T	10.6	2.9	8.8	2.4	19.3	2.2
Cortisol	D2T	11.5	11.3	6.2	5.3	9.1	9.9
Cortisone	D2T	8.0	18.0	12.9	3.0	8.5	8.8
DHEA	D9PROG	< LOQ	5.8	< LOQ	4.2	< LOQ	2.2
DHPROG	D9PROG	13.6	2.9	9.7	4.8	41.2	1.3
DHT	D2T	4.3	3.2	8.7	5.0	8.5	2.5
Pdiol	D2T	< LOQ	5.2	< LOQ	2.6	< LOQ	3.4
Pdione	D7A4	< LOQ	6.4	< LOQ	7.3	< LOQ	0.7
Pregnanetriol	D2T	< LOQ	12.7	< LOQ	9.1	< LOQ	22.0
PROG	D9PROG	1.6	1.5	7.5	5.6	2.7	1.7
T	D2T	3.0	1.5	14.2	2.2	1.7	2.2

Table 3c: Comprehensive method validation data continued: Recovery (%, n = 3), matrix effect (%, n = 3), and process efficiency (%, n = 3).

Steroid metabolite	Recovery (%)		Matrix effect (%)		Process efficiency (%)	
	2 ng/mL	200 ng/mL	2 ng/mL	200 ng/mL	2 ng/mL	200 ng/mL
11αOHPROG	78.1	100.6	59.2	-0.2	123.6	100.3
11βOHPROG	91.1	114.0	28.7	5.3	117.3	120.1
11KA4	113.7	127.7	11.5	-19.2	124.9	103.2
11KAST	< LOQ	98.8	< LOQ	-3.1	< LOQ	94.7
11KDHPROG	< LOQ	100.7	< LOQ	-14.7	< LOQ	85.9
11KDHT	32.62	86.8	64.4	4.9	53.7	91.0
11KPROG	84.5	130.5	31.9	-18.5	111.7	106.3
11KT	83.5	100.8	41.4	-6.3	117.2	94.0
11OHA4	114.3	113.6	0.1	-19.2	114.5	91.8
11OHAST	< LOQ	120.3	< LOQ	-27.2	< LOQ	88.6
11OHT	70.4	102.5	61.6	6.1	113.8	107.8
16OHPROG	76.3	105.2	46.8	2.4	111.4	107.5
17OHPROG	88.1	113.4	59.2	4.5	139.5	118.4
21-dF*	105.2	77.3	38.6	36.5	146.0	110.6
3α-diol	< LOQ	127.1	< LOQ	4.1	< LOQ	132.5
5α-dione	65.0	95.7	36.1	-3.8	89.4	92.3
A4	115.7	120.3	13.5	-18.8	131.2	97.7
AST	67.3	118.8	79.9	24.6	112.4	148.4
Cortisol	62.5	77.4	46.9	16.3	92.1	88.0
Cortisone	62.4	90.3	55.9	-8.3	97.6	82.4
DHEA	< LOQ	123.5	< LOQ	-16.3	< LOQ	103.0
DHPROG	56.6	99.1	72.3	-6.9	111.0	92.5
DHT	61.9	90.8	43.9	1.8	89.1	91.8
Pdiol	< LOQ	132.2	< LOQ	-6.5	< LOQ	123.6
Pdione†	< LOQ	105.9	< LOQ	-11.2	< LOQ	94.1
Pregnanetriol	< LOQ	78.9	< LOQ	-11.8	< LOQ	66.3
PROG	131.1	118.1	21.7	-11.8	159.5	104.2
T	78.9	108.7	37.9	3.5	108.8	112.2

*Validation based on n = 2 repeats only at 200 ng/mL. †Validation based on n = 2 repeats only at 2 ng/mL.

Steroids with a precision %RSD > 15% are best interpreted semi-quantitively when detected in samples at a concentration of 2 ng/mL.

3.2.3. Recovery, Matrix Effect and Process efficiency

Recovery at 2 ng/mL ranged from 32.6% for 11KDHT to 131.1% for PROG (Mean = 83.0%), and from 77.3% for 21-dF to 132.2% for Pdiol at 200 ng/mL (Mean = 107.5%; Table 3c). The recovery at the low concentration was, on average, 17.0% lower than expected from 100% steroid recovery (Table 3c). Matrix ion enhancement occurred in the majority of steroids measured at the mid concentration and ranged from 0.1% for 11OHA4 to 79.9% for AST (mean = 42.3%). The matrix suppression and enhancement at the highest steroid concentration ranged from -27.2% for 11OHAST to 36.5% for 21-dF but were less prominent (mean -4.1%) compared to the lower concentration tested. The process efficiency was 114.3% on average for steroids at 2 ng/mL and ranged from 53.7% for 11KDHT to 159.5% for PROG. The process efficiency at the highest steroid concentration ranged from 66.3% for pregnanetriol to 148.4% for AST (mean = 102. 3%; Table 3c).

Different concentrations of whisker matrix did not result in linear increases or decreases in the peak area of the internal standards. Most steroids were calibrated to D2T (Table 3b), which were unaffected by the whisker matrix concentration ($y = 52.8x + 151333$, Fig. 1S). The whisker sample resuspension volume of 100 µL for ca. 20 mg whisker samples are, therefore, not sensitive to variations in whisker sample mass (whisker matrix concentration) over the range of whisker sample masses analysed.

3.3. Steroids Detected in Whisker Samples

Herein, 14 C_{19} and 14 C_{21} steroids were detected >LOD in the whiskers of the three seals species (Table 4). However, 13 of the 28 steroids were detected in less than 15% of the n = 71 whisker subsamples at concentrations above the LOQ (Table 4). Ten steroids were consistently measured above the LOQ in ≥30% of the whisker segments analyzed; these included 3α-diol, 5α-dione, cortisol, AST, and 17OHPROG, with PROG, 11KA4, 11OHA4, T, and A4, above the LOQ in >70% of the whisker segments analyzed (Fig. 1 & 2). The median steroid concentration ranged from 2.0 pg/mg for T measured in subantarctic fur seals to 273.7 pg/mg measured for 3α-diol in the whiskers of SES (Table 5). The steroid profile and pooled median concentration of the steroids detected above the LOQ in ≥30% whisker segments of each species, differed significantly between species (Kruskal-Wallis $\chi^2 = 59.24$, $df = 2$, $P < 0.001$; Fig. 2 & 2S). The SES whisker steroid concentration (41.7 ± 11.6 pg/mg; Median ± SE) was significantly higher than Antarctic fur seals (14.3 ± 10.5 pg/mg; $P < 0.001$) and ($n = 5$) subantarctic fur seals (10.2 ± 13.3 pg/mg; $P < 0.001$), but did not differ between the two fur seal species ($P = 0.06$).

The median steroid concentration measured at the base (26.7 ± 12.7 pg/mg) and tip (27.2 ± 18.7 pg/mg) of the whisker did not differ significantly for all species ($P = 0.504$). The PCA scoring obtained from steroids found in ≥30% of the whisker samples further indicated that the variation in the PCA dimensions was influenced more by the species than the position of the whisker segment (tip, middle, base) analyzed (Fig. 2Sb), but no species or sample position differentiation occurred at the 95% probability intervals plotted (Fig. 2S). The range, and variation in the concentrations of the steroids measured in the tip, middle and base of the whiskers segments of each species varied and is best assessed on a steroid-to-steroid basis. The

Table 4: Frequency (percentage) of steroids detected in n = 71 sequentially subsampled whiskers obtained from adult female seals. SES (*M. leonina*) whiskers, n = 36, sampled from 10 individuals; *A. gazella* whiskers, n = 15, sampled from 5 individuals, and *A. tropicalis whiskers,* n = 20 whisker sampled from 5 individuals. The percentage of samples in which the steroid concentrations were below the LOD are indicated in brackets.

Steroid metabolite	SES (n = 36 segments)		*A. gazella* (n = 15 segments)		*A. tropicalis* (n = 20 segments)		n = 71 segments
	% <LOQ (<LOD)	% >LOQ	% <LOQ (<LOD)	% >LOQ	% <LOQ (<LOD)	% >LOQ	% >LOQ
11αOHPROG	88.9 (72.2)	11.1	80 (60)	20	90 (75)	10	12.7
11βOHPROG	100 (88.9)	0	100 (80)	0	100 (90)	0	0
11KA4	8.3 (8.3)	91.7	60 (60)	40	40 (40)	60	71.8
11KAST	91.7 (88.9)	8.3	100 (93.3)	0	95 (80)	5	5.6
11KDHPROG	97.3 (80.6)	2.8	100 (86.7)	0	100 (85)	0	1.4
11KDHT	97.2 (94.4)	2.8	100 (93.3)	0	100 (90)	0	1.4
11KPROG	80.6 (80.6)	19.4	86.6 (73.3)	13.3	90 (85)	10	15.5
11KT	80.6 (25)	19.4	80 (40)	20	85 (55)	15	18.3
11OHA4	13.9 (13.9)	86.1	40 (40)	60	50 (50)	50	70.4
11OHAST	100 (88.9)	0	93.3 (60)	6.7	95 (35)	5	2.8
11OHT	100 (69.4)	0	100 (40)	0	100 (50)	0	0
16OHPROG	94.5 (55.6)	5.6	93.3 (13.3)	6.7	90 (30)	10	7
17OHPROG	58.4 (55.6)	41.7	26.7 (6.7)	73.3	40 (40)	60	53.5
21-dF	77.8 (72.2)	22.2	80 (66.7)	20	75 (65)	25	22.5
3α-diol	52.8 (50)	47.2	93.4 (86.7)	6.7	85 (65)	15	29.6
5α-dione	58.3 (58.3)	41.7	0 (0)	100	20 (10)	90	67.6
A4	0 (0)	100	0 (0)	100	10 (20)	80	94.4
AST	41.7 (41.7)	58.3	66.7 (66.7)	33.3	50 (50)	50	50.7
Cortisol	58.3 (11.1)	41.7	86.7 (66.7)	13.3	85 (60)	15	28.2
Cortisone	83.3 (11.1)	16.7	93.3 (20)	6.7	100 (10)	0	9.9
DHEA	88.9 (66.7)	11.1	80 (73.3)	20	90 (75)	10	12.7
DHPROG	83.3 (83.3)	16.7	93.3 (93.3)	6.7	60 (60)	40	21.1
DHT	97.2 (83.3)	2.8	93.3 (60)	6.7	95 (50)	5	4.2
Pdiol	97.2 (97.2)	2.8	93.3 (93.3)	6.7	85 (80)	15	7
Pdione	77.8 (77.8)	22.2	93.3 (93.3)	6.7	80 (70)	20	18.3
Pregnanetriol	94.5 (66.7)	5.6	86.7 (60)	13.3	85 (75)	15	9.9
PROG	0 (0)	100	0 (0)	100	5 (0)	95	98.6
T	25 (0)	75	40 (0)	60	25 (0)	75	71.8

Table 5: Steroid analysis of adult female seal whiskers. Steroid levels detected above the limit of quantification in *M. leonina* whiskers, *n* = 36, sampled from 10 individuals, *A. gazella* whiskers, *n* = 15 sampled from 5 individuals, and *A. tropicalis* whiskers, *n* = 20, sampled from 5 individuals) are shown in pg/mg, median ± SE. The number of samples in which each steroid was detected above the LOQ for each species is indicated in brackets. LOD = Limit of detection.

Steroid metabolite	Analyte steroid concentration (pg/mg)		
	SES	*A. gazella*	*A. tropicalis*
11αOHPROG	35.6 ± 6.4 (*n* = 4)	19.4 ± 3.2 (*n* = 3)	54.4 ± 35.4 (*n* = 2)
11βOHPROG	<LOD	<LOD	<LOD
11KA4	33.3 ± 17.1 (*n* = 33)	8.9 ± 1.5 (*n* = 6)	5.6 ± 0.9 (*n* = 12)
11KAST	304.7 ± 27.9 (*n* = 3)	<LOD	322.9 (*n* = 1)
11KDHPROG	281.3 (*n* = 1)	<LOD	<LOD
11KDHT	132.9 (*n* = 1)	<LOD/LOQ	<LOD
11KPROG	46.9 ± 18.3 (*n* = 7)	15.7 ± 7.1 (*n* = 2)	9.7 ± 2.3 (*n* = 2)
11KT	19.8 ± 8.1 (*n* = 7)	18.3 ± 4.4 (*n* = 3)	22.5 ± 8.5 (*n* = 3)
11OHA4	126.4 ± 519.7 (*n* = 31)	3.8 ± 3.0 (*n* = 9)	5.2 ± 1.9 (*n* = 10)
11OHAST	<LOD	81.5 (*n* = 1)	215.0 (*n* = 1)
11OHT	<LOD	<LOD	<LOD
16OHPROG	1.3 ± 0.1 (*n* = 2)	1.7 (*n* = 1)	3.4 ± 2.0 (*n* = 2)
17OHPROG	7.2 ± 1.2 (*n* = 15)	6.7 ± 4.1 (*n* = 11)	3.8 ± 7.9 (*n* = 12)
21-dF	75.5 ± 41.3 (*n* = 8)	49.8 ± 5.7 (*n* = 3)	20.3 ± 29.4 (*n* = 5)
3α-diol	273.7 ± 319.8 (*n* = 17)	128.6 (*n* = 1)	200.1 ± 603.8 (*n* = 3)
5α-dione	47.4 ± 53.4 (*n* = 15)	42.4 ± 168.3 (*n* = 15)	39.7 ± 341.3 (*n* = 18)
A4	63.6 ± 531.5 (*n* = 36)	11.2 ± 3.6 (*n* = 15)	4.9 ± 10.8 (*n* = 16)
AST	65.3 ± 86.7 (*n* = 21)	29.0 ± 10.6 (*n* = 5)	23.9 ± 2.2 (*n* = 10)
Cortisol	14.2 ± 8.2 (*n* = 15)	68.0 ± 57.4 (*n* = 2)	18.5 ± 4.7 (*n* = 3)
Cortisone	24.5 ± 7.7 (*n* = 6)	18.6 (*n* = 1)	<LOD
DHEA	107.4 ± 393.7 (*n* = 4)	99.3 ± 21.5 (*n* = 3)	125.8 ± 81.1 (*n* = 2)
DHPROG	29.1 ± 4.9 (*n* = 6)	193.6 (*n* = 1)	72.1 ± 19.3 (*n* = 8)
DHT	10.9 (*n* = 1)	44.9 (*n* = 1)	34.1 (*n* = 1)
Pdiol	347.8 (*n* = 1)	145.5 (*n* = 1)	103.9 ± 33.0 (*n* = 3)
Pdione	200.4 ± 86.4 (*n* = 8)	138.7 (*n* = 1)	138.7 ± 15.8 (*n* = 4)
Pregnanetriol	302.3 ± 238.4 (*n* = 2)	161.1 (*n* = 2)	216.9 ± 88.8 (*n* = 3)
PROG	55.6 ± 333.4 (*n* = 36)	23.7 (*n* = 15)	12.7 ± 59.0 (*n* = 19)
T	3.3 ± 0.9 (*n* = 27)	4.0 ± 3.0 (*n* = 9)	2.0 ± 9.2 (*n* = 15)

Figure 1: Androgenic steroid pathway of mammals illustrating the interrelation between the fourteen C_{21} (left) and fourteen C_{19} steroids (right) measured sequentially along the length of whiskers sampled from adult female SES (*M. leonina*) and two fur seal species (*A. tropicalis* and *A. gazella*). Steroids detected at concentrations above the LOQ in < 15% of samples, or steroids not included in the method, are presented in grey text. Steroids in black text were detected above the LOQ in >15% of the samples. White boxes indicate steroids detected in ≥30% of the samples above the LOQ, while underscored values indicate steroids prevalent in >70% of the samples.

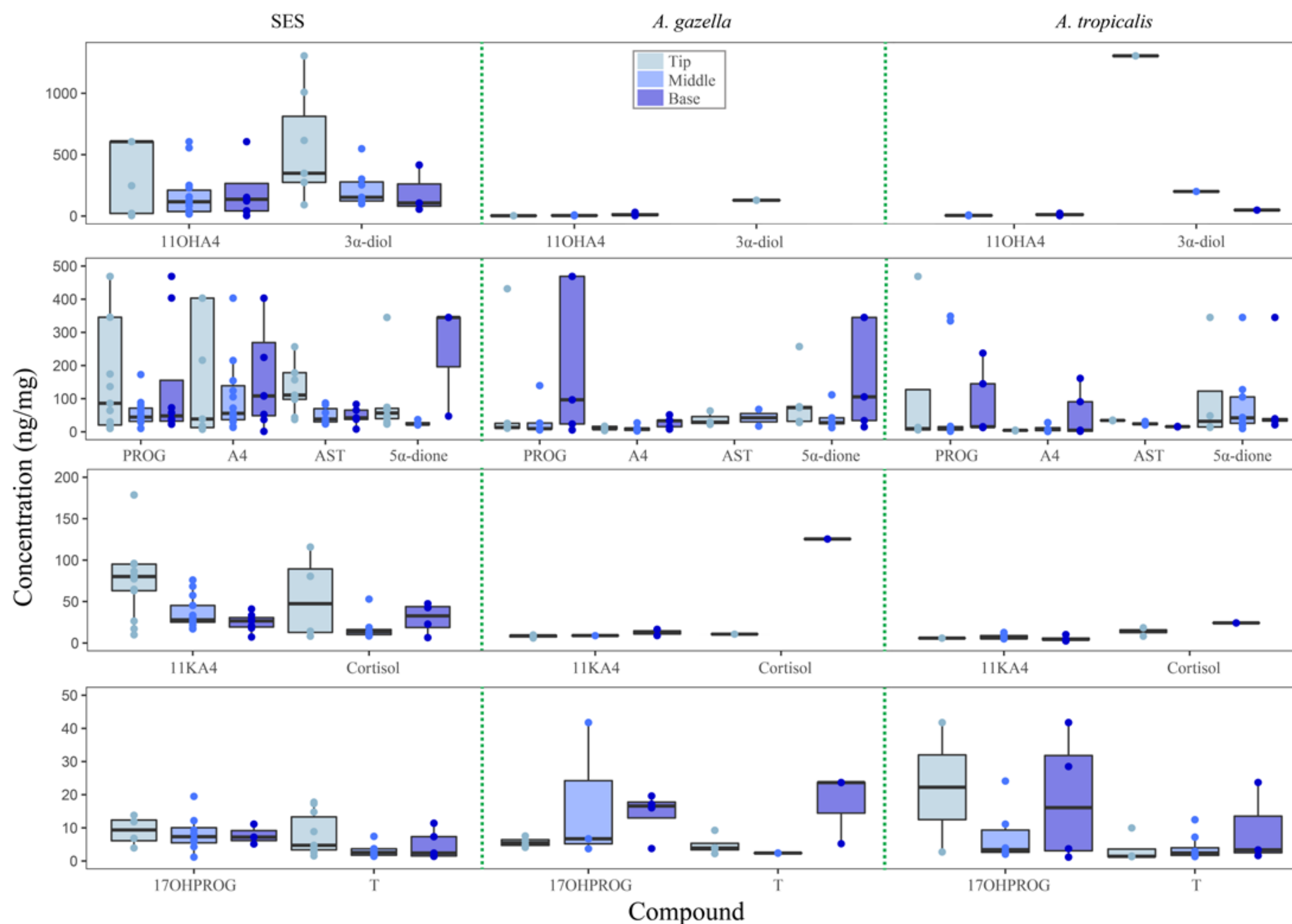

Figure 2: Median (25th and 75th percentiles) of steroids measured above the LOQ in ≥30% of the whisker segments analyzed that were sampled from southern elephant seals (SES, *Mirounga leonina,* left column) and the two fur seals species, *Arctocephalus gazella* (middle column) and *A. tropicalis* (right column). Whisker subsample segments represent the distal (tip, light blue), middle, and base (dark blue) of the sampled whiskers sampled. The boxplot whisker segments extend to include samples with 1.5 times the inter-quartile range (IQR). Steroid levels 3 x IQR were considered outliers for the purpose of the plotting and were replaced with the calculated 3 x IQR level of each steroid. Steroids with levels in the same range are shown together to facilitate visual comparison of concentration differences in the whiskers of the three species.

variation also likely reflects the different physiological states (e.g., fasting vs foraging) spanned by the different whisker segments (Fig. 2).

4. Discussion

We report the first chronological extraction and quantification of 28 steroids from single mammalian whiskers, sampled from SES, and two fur seal species, using UPC^2-MS/MS. Aside from cortisol that was previously extracted from whiskers and quantified using an EIA kit [24], this is the first high-throughput method (6-minutes) developed to sequentially extract cortisol as well as 27 additional C_{19} and C_{21} steroids (including their C11-hydroxy and C11-keto metabolites) along the length of a whisker. Our accuracy and precision are within the acceptable limits at the mid (2 ng/mL) and high (200 ng/mL) concentrations tested (Table 3b), and comparable to the results of two previous reports using a serum matrix [27,26]. The recovery, matrix effects and process efficiency were also comparable, confirming the reliability of our method. Compared to other methods such as EIAs, this analytical approach provides a cost-effective, and sensitive method. Moreover, when compared with EIA it is highly specific and is capable of quantifying multiple steroids simultaneously. Furthermore, sample preparation and steroid extraction are inexpensive, require no sample derivatization and can be performed in most analytical laboratories.

Twenty-eight steroids extracted from whiskers of three seal species were separated in a single chromatographic step. The steroid profiles and concentrations varied and not all steroids were detected in all of the samples. The inclusion of only adult seal females possibly explains the lack of detecting testosterone metabolites at concentrations >LOQ. Nevertheless, 10 steroids were detected in ≥30% of the samples at concentrations above the LOQ; heralding a significant

advance on the number of steroids measured in any keratinous tissue sampled from free-ranging mammals. Our median measured steroid concentrations (2.0–273.7 pg/mg, Table 5) correspond to previous measurements of glucocorticoids in hair, based on EIAs and mass spectrometry, ranging from 5–91 pg/mg [35,5,36]. However, these earlier studies were generally restricted to cortisol and cortisone. Our measured SES cortisol concentration (14.2 ± 8.2 pg/mg) was comparable to the only whisker cortisol measurements available (range 0.3–28.4 pg/mg), which were determined for three phocid species, namely ringed (*Pusa hispida*), spotted (*Phoca largha*) and harbor (*Phoca vitulina*) seals using an EIA kit [24]. Cross-reactivity of structurally similar glucocorticoids and metabolites using EIA kits [16] could influence measured steroid levels and should be considered when quantifying steroid levels from a complex matrix such as whiskers.

The whisker steroid profiles of the three species were comparable (Fig. 2S), although the concentrations of steroids measured in the two fur seal species were significantly lower compared to SES. The median steroid concentration measured in the tip and base of the whiskers did not differ significantly, suggesting the steroids are not leached from the tip of the finer whiskers and appeared to not degrade over the length of the matrix as previously described [24]. This is an important finding and when considered in terms of growth, our data suggest that even exposure to seawater for long periods (ca. 1 year; [37]) does not cause leaching of steroid metabolites from the whisker thus allowing for data collection spanning longer physiological periods than compared to sampling blood or faeces samples, is thus possible. Similarly, cortisol measured in distal and proximate ends of hair sampled from primates did not differ [5,14], yet, steroid leaching remains a possibility [13,24]. In the case of fur seals, it is possible that lower steroid concentrations measured in their whiskers could relate to the species retaining their whiskers longer than SES. The whiskers of the SES are shed during the pelage molt [37], while

the whiskers of otariids (fur seals) are retained for multiple years [38]. Nonetheless, the variation in steroid concentration and profile likely relates to the physiological state of the individual during the time of deposition in tissues which will be the subject of future analysis of the data. Steroids quantified in the two fur seal species are very low and differences in metabolite levels in tip, mid and base segments are not detectable. In SES, steroids can be analyzed over the length of the whisker. In all of the steroids detected above the LOQ, 5α-dione was the only metabolite that was present in higher levels at the base of the whisker.

There are some limitations of the simultaneous detection and quantification of multiple steroids using this method. Firstly, the concentration of individual steroids deposited in the whiskers may vary. Defining a calibration range to accurately quantify all 28 steroids in a single run is challenging. In some cases, the samples could be concentrated and reanalyzed to quantify steroids with poor ionization efficiencies. In addition, the whisker matrix is arguably more complex than other biomatrices such as serum and culture media routinely used in *in vitro* and *in vivo* studies. While matrix effects are often confounding in LC-MS analysis, we ensured that the matrix was included at equal levels in the standard curve and samples analyzed. In addition, equal amounts of internal deuterated standards were added to the samples and the standards curve to compensate for potential loss of steroids during extraction. The matrix effect did not affect the ionization of the internal standards over the reported quantification range (Fig. 1S). However, matrix enhancement was still observed in the lower steroid concentrations, which prevented method validation at the low steroid concentrations (≤ 0.2 ng/mL). When they become commercially available, deuterated standards can be included for each steroid analyzed. Additional sample “clean-up” can be considered prior to the chromatographic step, although

additional pre-columns and steps may alter steroid concentrations unpredictably and were, therefore, not attempted.

5. Conclusion

Whiskers are increasingly recognized as a sought-after matrix for the chronological extraction of biochemical data for ecological and physiological research (e.g., [24]). In the current study we successfully applied a selective and specific, rapid and high-throughput UPC^2-MS/MS method to simultaneously quantify 28 steroids in a single chromatographic step from sequentially analyzed single mammalian whiskers. The study provides the first quantitative data on a suite of steroids that investigators can expect to detect in mammalian whiskers. Our method is capable of extracting more steroids per gram single sample at a higher sensitivity than other techniques reported to date, reducing the time and costs of sample preparation, as well as differentiating between metabolites that have similar immunoreactivity and would have cross-reacted if quantified using EIA kits [16, NL personal observation]. The utility of whiskers as a biomatrix to obtain retrospective longitudinal records of the endocrine profile of free-ranging mammals can contribute to bridging the endocrinological knowledge gap that currently exists for mammals with cryptic life cycles, such as pinnipeds. Our findings have highlighted that ecological and physiological insights may be derived from comprehensive steroid hormone analysis of currently-collected and historic whisker samples [39,24,25] to investigate physiological and pathophysiological endocrine changes related to life history and environmental impacts.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethical approval

The Animal Ethics Committee of the University of Pretoria provided ethical clearance for the research, under AUCC 040827-022, AUCC 040827- 023, and EC030602-016.

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