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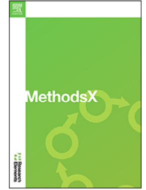

Method development: Assigning sex to African clawless otter spraints and assessing stability of faecal androgen and progestagen metabolites post-defaecation ☆

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A R T I C L E   I N F O

Method name:
Using African clawless otter spraints to quantify reproductive hormones

Keywords:
Non-invasive
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A B S T R A C T

Monitoring reproductive physiology in wildlife can be a useful tool for assessing population dynamics for conservation and management purposes. Utilizing non-invasive approaches for this, such as quantifying reproductive hormone metabolites from faeces, can be challenging when defaecation events are not observed, or when cryptic species like African clawless otters (*Aonyx capensis*) are involved. Additionally, test systems for quantifying hormone metabolites in a species for the first time must first be reliably validated prior to use. Our results indicate that Epiandrosterone and Progesterone EIAs are most suitable for determining fAM and fPM concentrations in African clawless otter spraints. The fAM:fPM ratio and respective thresholds are more reliable in sex identification compared to the separate use of individual hormone classes. Sex-related hormone metabolite concentrations remained comparable for up to 12hrs post-defaecation in both sexes.

- We screened two androgen and two progestagen enzyme-immunoassays (EIAs) for suitability and reliable quantification of faecal androgen metabolites (fAM) and faecal progestagen metabolites (fPM) in African clawless otters.
- We assessed whether the ratio of fAM:fPM concentrations can be used to assign sex to faecal samples from unknown individuals.
- We tested the stability of fAM and fPM concentrations post-defaecation to determine the effects of environmental exposure and bacterial metabolism.

Specifications table

Subject area:	Agricultural and Biological Sciences
More specific subject area:	Zoology; Endocrinology
Name of your method:	Using African clawless otter spraints to quantify reproductive hormones
Name and reference of original method:	Original research
Resource availability:	Endocrine Research laboratory University of Pretoria, South Africa EIAs evaluated: Progesterone, 5α-Progesterone, Testosterone, Epiandrosterone (see table 1).

☆ **Related research article:** New method

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Background

Studying reproductive physiology requires an integrative approach to investigate the relationships between sex, age, season, reproductive status, social structure, and behaviour [1,2]. Respective findings can inform species population size and dynamics, and assess reproductive success [1]. Reproductive function can be monitored by quantifying reproductive hormones [3-5]. When longitudinal fPM measurements can be taken from the same individual over several reproductive cycles to account for seasonal differences in fPM concentrations, reproductive hormone patterns in individuals can be used to establish sex [3]. However, these types of studies are rare, as they require access to the same individuals over multiple seasons and years. Additionally, almost daily sampling, often achieved through invasive techniques including chemical/physical restraint are required to obtain blood samples which can be costly and logistically challenging to perform [3]. Repeated chemical immobilisation and handling pose a risk to general health of the individual under investigation and may in fact influence hormone levels being examined [6].

Although male and female reproductive hormones are produced in both sexes, the site of production, concentrations, and interactions with target tissues and organs are sex-specific [7]. Consequently, reproductive hormones or their metabolite concentrations should facilitate the allocation of sex to a utilized hormone matrix (e.g. faecal samples). Reproductive steroid secretion, metabolism, and excretion are species- and sex-specific [8-12]. Environmental conditions, gut bacterial enzymes, and age of faecal samples can affect immunoreactive faecal hormone metabolite concentrations post-defaecation [10,13]. As a prerequisite for non-invasive investigations of reproductive physiology, test systems quantifying reproductive hormone metabolites must first be validated for reliability in any species investigated for the first time [8,14,15]. An appropriate assay should be established through analytical, physiological, and biological validation [15]. A biological validation involves monitoring and comparing reproductive hormones in both sexes to assess the reliability of established EIAs for quantifying reproductive hormone metabolites.

If hormone metabolite concentrations can be determined in faecal samples from known individuals, a threshold, based on the ratio of faecal androgen:faecal progestagen metabolites (fAM:fPM), could allocate the correct sex to analysed samples. This approach can then be applied to samples from unknown individuals that were collected without observation of the defecation event to successfully assign sex [1,16]. Faecal samples from known-sex African clawless otters can be used to test available EIAs specific to different hormone classes for suitability and detection of respective hormone metabolites. By calculating and comparing the ratio of fAM:fPM concentrations from known sex individuals, distinct thresholds can therefore be used to allocate sex to faecal samples from unknown individuals. Potential alterations in immunoreactive hormone metabolite concentrations post-defecation for each sex can be determined by exposing various sub-sets of a faecal samples to different environmental conditions and subsequently testing samples at defined time intervals [16-19]. Once validated, this method allows sex determination of free-ranging individuals from faecal samples without observation or the need to capture, restrain, or handle individuals, an approach not yet established for African clawless otters.

African clawless otters are widespread in Africa [20]. They are predominantly found in freshwater habitats, but also inhabit certain coastal intertidal zones if access to fresh water sources is available [20]. African clawless otters utilize latrines throughout their range [21,22]. This serves the purpose of scent marking and facilitates olfactory communication between conspecifics [23]. African clawless otters are predominantly active during dawn and dusk, but also hunt during the day or night [22]. African clawless otters are carnivorous, generalist feeders, incorporating a variety of food items in their diet [24]. The reproductive biology of African clawless otters is largely unknown. In this regard, there is no clear consensus on mating season, whether ovulation is induced or spontaneous, the length of oestrus cycle or how frequently they reproduce [20].

This study aimed to determine suitable enzyme immunoassays for reliable quantification of faecal androgen and progestagen metabolites in African clawless otters. Additionally, it aimed to use the respective hormone metabolite concentrations to determine sex-specific fAM:fPM thresholds, which could be used to allocate sex to free-ranging samples. Further, the study aimed to establish appropriate timeframes for faecal steroid hormone metabolite stability post-defecation. Validation of this approach allows for reliable collection of faecal samples to facilitate the monitoring of reproductive physiology, providing researchers with the means to non-invasively investigate population dynamics. Information from such research will provide much needed conservation guidance for the management of elusive and vulnerable wildlife species such as African clawless otters.

Method details

Sample collection

A total of 73 spraint samples were collected from captive individuals to evaluate whether fAMs and fPMs can be reliably quantified in African clawless otters and assess the feasibility of determining sex from otter spraints (faecal samples) via sex-specific hormone ratios and respective thresholds. From 17 September – 18 November 2021, spraint samples were collected from an adult male (n=38) housed at Johannesburg City Parks and Zoo, South Africa (JCPZ) and one captive adult female (n=35) housed at the Hartbeespoort Dam Snake and Animal Park, South Africa (HSAP) from 30 September – 4 November 2021.

To assess the viability of determining sex from free-ranging otter spraints using sex-specific hormone ratios and respective thresholds, a total of 202 African clawless otter faecal samples were collected from free-ranging individuals between July 2021 – January 2022. All faecal material was collected from three study sites across South Africa, namely Kalkfontein Dam Nature Reserve in the Free State province, Millstream Farm in Mpumalanga province, and Rietvlei Nature reserve in Gauteng province. Sprainst were identified based on physical characteristics including size (diameter=~25mm), shape, characteristic smell and tracks surrounding latrines [25,26]. As the species is crepuscular [21], all latrines were inspected daily between 06h00 and 09h00 to ensure the collection of fresh faeces (<3 hours) and minimise faecal exposure to environmental conditions (e.g., sunlight or wind). If any uncertainty ex-

Table 1
Specificities of the four enzyme immunoassays (EIAs) screened for quantification of respective hormone metabolites in faeces, including labels, antibodies, standards, sensitivities, inter- and intra-assay variances, and first descriptions in the literature.

EIA Name	Progesterone	5α-Progesterone	Testosterone (T-3CMO)	Epiandrosterone
Biotin-Label	5α-pregnane-3β-ol-20-one-3HS[1]: DADOO-biotin[2]	5α-pregnan-3β-ol-20-one-3-HS-HRP[3]	5α-androstane-3β,17β-diol-3-HS:DADOO-biotin	5α-androstane-3,17-dionethioether: DADOO-biotin
Antibodies raised against	5β-pregnane-3αol-20-one-3HS:BSA[4]	5α-pregnan-3β-ol-20-one-3-HS-BSA	Testosterone-3-CMO[5]:BSA	5α-androstane-3αol-17-one-HS:BSA
Standard	Progesterone (5α-pregnan-3βol-20-one)	5α-pregnan-3β-ol20-one	Testosterone (17β-Hydroxy-3-oxo-4-androstene)	Epiandrosterone (5α-androstan-3βol-17-one)
Sensitivity DW (ng/g)	9.6	6	1.2	7.2
Intra-assay CV (%)	5.55	4.36	5.57	4.96
	5.73	6.67	6.87	5.09
Inter-assay CV (%)	7.14	4.77	4.61	8.17
	8.52	6.48	8.38	12.83
First described in	(Schwarzenberger et al., 1996)	(Dehnhard et al., 2010)	(Palme and Möstl, 1993)	(Palme and Möstl, 1993)

[1] HS: hemisuccinate.
[2] DADOO-biotin: N-biotinyl-1,8-diamino-3,6-dioxaoctane.
[3] HRP: horseradish peroxidase.
[4] BSA: bovine serum albumi.
[5] CMO: carboxymethyloxime.

isted related to species or sample age, samples were not collected. Given the potential dilution effects of rain on hormone stability post-defaecation, spraint deposits at known latrines were flattened to prevent the collection of old samples. The majority of spraint samples from free-ranging individuals were collected from previously identified latrine sites, and on rare occasions from observed defaecation events, using anal jelly texture, moisture content, temperature [27], and the above criteria to assign freshness to samples. All samples were collected from the centre of the faecal pile to avoid contamination by substrate. Faecal deposits were homogenised *in situ* using latex gloves and samples were placed in individually labelled plastic screw-top containers and kept cool in a portable cooler box with ice bricks. All samples were frozen within three hours of collection and remained frozen during transport by road to the University of Pretoria's Endocrine Research Laboratory, where they were stored at -20°C until further processing [28].

Steroid extraction and quantification

Frozen faecal samples were lyophilized, pulverised, and sieved to remove debris and undigested particulate matter. As outlined by [28], after lyophilisation and pulverisation, 0.10-0.11g of faecal powder was extracted by vortexing each sample for 15 minutes with 3ml of 80% ethanol in water to ensure proper mixing. Each suspension was then centrifuged at 1500g for 10 minutes and the supernatant decanted into microcentrifuge Eppendorf tubes and stored at -20°C until analysis.

To identify the most suitable EIAs for fAM and fPM quantification respectively, a total of 15 samples from the captive male and 16 samples from the captive female were analysed using four available enzyme immunoassays. The Testosterone [29] and Epiandrosterone [29] EIAs were used to quantify fAM concentrations, while the Progesterone [30] and 5α-Progesterone [31] EIAs were used to determine fPM concentrations. Respective assay characteristics, including sensitivity, intra- and inter-assay coefficients of variation (CV) together with first description in the literature for each EIA are provided in Table 1. The Epiandrosterone (for fAMs) and Progesterone (for fPMs) EIA combination yielded more distinct and homogenous ranges belonging exclusively to male or female individuals and were therefore deemed most suitable for further fAM and fPM quantification. The serial dilutions of the faecal extracts showed a displacement curve that was parallel to the standard curve with a relative variation of the slope of the trend line being <5% for both the Epiandrosterone and the Progesterone EIAs (See Appendix; Fig. 3 & 4). Steroid concentrations are expressed per mass (μg/g) of dry faecal matter. All faecal steroid extractions and analyses were conducted at the University of Pretoria's Endocrine Research Laboratory, South Africa.

Calculating and comparing fAM:fPM ratios

Based on results from the biological validation of the EIAs, Epiandrosterone:Progesterone and Epiandrosterone:5α-Progesterone EIA ratio combinations were tested.

Stability of reproductive faecal hormone metabolite concentrations post-defaecation

To determine the extent of faecal hormone metabolite degradation post-defaecation, pooled fresh faecal samples obtained from observed defaecation events by the captive male (n=38) and female (n=35), were separately homogenised. Sex-specific subsets were subsequently divided into 54 subsamples, 27 subsamples were placed outside under full shade conditions, while the other 27

subsamples were exposed to full sunlight. Sex-specific triplicate subsets from respective treatments were then frozen at 0h as a control and subsequently at 1h, 3h, 6h, 9h, 12h, 24h, 48h, and 72h. Subsamples were kept frozen at -20°C until further processing, extraction, and analyses.

Data analysis

To examine the suitability of using EIAs for quantifying fAM and fPM concentrations, the difference between the fAM and fPM concentrations between the two sexes were tested for all four EIAs. The fAM and fPM concentrations and residuals followed a positively skewed distribution for all four EIAs tested, necessitating $\log_{10}$ transformation prior to statistical analysis. Sex-specific differences between the fAM and fPM concentrations were tested for each EIA using a student's t-test. From these results (see method validation below), linear models that included sex and an Epiandrosterone:Progesterone or Epiandrosterone:5α-Progesterone ratio combination were respectively assessed. The difference between the respective hormone ratio combinations between sexes was tested using repeated measures one-way analysis of variance (ANOVA). Thresholds obtained from the Epiandrosteron:Progesterone EIA ratios were used to determine the sex of the free-ranging samples.

Time-dependent hormone metabolite (fAM and fPM) concentrations for each subsample were expressed as a percentage using the triplicate mean value of subsamples at t=0 as baseline concentrations (representing 0% increase). The fAM, fPM concentrations and residuals followed a positively skewed distribution, necessitating $\log_{10}$ transformation prior to statistical analysis. Linear models that included time and the interaction between time and the condition (full sun/full shade) were analysed separately for each sex and for each hormone metabolite class (fAM and fPM) respectively. The difference between the respective hormone metabolite concentrations at each time point in relation to t=0 was tested using repeated measures one-way analysis of variance (ANOVA). This was followed by a post-hoc Pairwise t-test with a Bonferroni correction to determine which time point(s) were significantly different from t=0.

All statistical analysis were conducted using R (R Core Team 2022). For all models, normality was assessed by testing the model residuals using the Levene's test and visually using quantile plots. Statistical significance was determined at $p<0.05$. All concentrations are presented as mean ± standard deviation (SD) μg/g DW.

Results and discussion

Method validation

EIA reliability of quantifying fAMs and fPMs

The fAM concentrations were not significantly different between the male and female when the Testosterone EIA was used ($F_{(1,29)}$= 0.0849, n=31, p=0.7728). However, with the Epiandrosterone EIA, fAM concentrations were significantly higher ($F_{(1,29)}$= 6.8728, n=31, p=0.0138) in the male compared to the female, with an overall median difference of 43%. Consequently, the Epiandrosterone EIA was selected for further determination of fAM concentrations in remaining samples. The fPM concentrations did not differ statistically between the sexes when using the Progesterone ($F_{(1,29)}$=2.1548, n=31, p=0.1529) or 5α-Progesterone ($F_{(1,29)}$= 2.8553, n=31, p=0.1018) EIA. The overall median fPM concentration for the female was 7% higher compared to the male for the Progesterone EIA, and 14% higher for the 5α-Progesterone EIA. Both Progesterone and 5α-Progesterone data sets for the females show distinctively greater variance (296% and 495% respectively) compared to those for the males, which is interpreted as a reflection of cyclicity in non-pregnant females [32]. Considering this, both assays are reliable for measuring fPM concentrations to determine fAM:fPM ratios. Both fPM EIAs were then assessed in combination with Epiandrosterone to evaluate the feasibility of determining sex from otter spraints using the thresholds obtained from sex-specific hormone ratios.

Sample sex determination using thresholds obtained from fAM:fPM ratios

For evaluating fAM:fPM ratios, Epiandrosterone:Progesterone ($F_{(1,29)}$= 11.296, n=31, p= 0.002) and Epiandrosterone:5α-Progesterone ($F_{(1,29)}$= 7.5251, n=31, p=0.010) combinations were assessed. As the Epiandrosterone:Progesterone EIA combination performed best overall, and yielded more distinct and homogenous ranges belonging exclusively to male or female, this approach was used to determine the sex of samples from unknown free-ranging animals. Of the various different hormones and ratios tested by [33], the fAM:fPM ratio combination was the most successful in sex identification across different reproductive classes. Previous studies conducted on carnivores also utilised fAM:fPM ratios to distinguish between sexes and also found fAM:fPM ratios of males to be significantly higher than their female [33,34].

Using the results from the fAM:fPM ratios determined from captive (n=32) known sex African clawless otters, two approaches were assessed for accuracy. In the first approach, a distinct threshold of <3.63 exclusively indicated female (n=5), while a threshold of >9.85 exclusively indicated male (n=7) (Fig. 1). Samples that fall between a threshold of 3.63-9.85 could indicate either male or female as a result of individual variability (age, sex, reproductive status) and were therefore labelled as unknown (n=19). In the second approach that used a higher threshold (<5.29) for females, more samples (ten of 12 assessed) could be assigned to females, but with lower confidence.

When the method was applied to unknown samples from free-ranging individuals and the strict approach was used in which female threshold was <3.63; and male was >9.85, 92 of the 202 free-ranging faecal samples collected, could be characterised as female, and 54 samples could be characterised as male, resulting in a 72% successful allocation rate (Fig. 2). When the second approach was used (fAM:fPM threshold of <5.29 for females), an additional 24 samples could be characterised as female, with then only 32 samples being categorised as unknown, improving the allocation rate (84%), but lowering confidence in allocation rate. For more

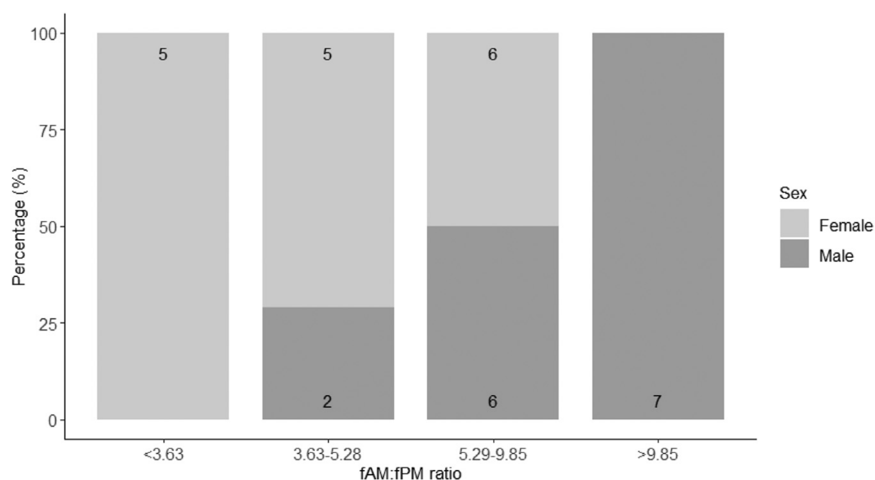

Figure 1. Vertical bar plot depicting the number of samples (seen on each bar) and the percentage (on the y-axis) of each allocated sex with a specific threshold obtained from the Epiandrosterone:Progestagen (fAM:fPM) ratios (on the x-axis). Samples (n=31) were all obtained from one captive male (n=15) and one female (n=16) African clawless otter housed at the Johannesburg City Parks and Zoo and Hartbeespoort Snake and Animal Park respectively.

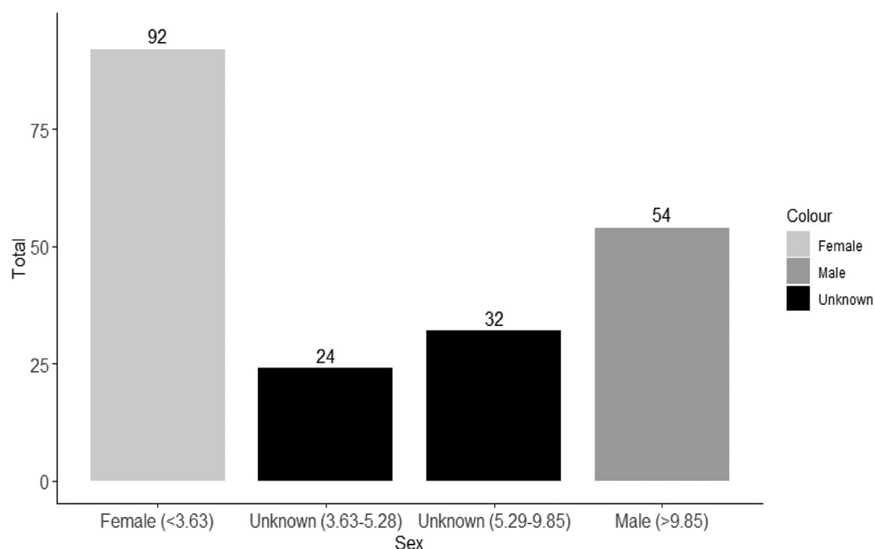

Figure 2. Validated enzyme immunoassays for fAM:fPM quantification applied to faecal samples collected from free-ranging African clawless otters with 72% certainty. There was a total of 92 females (<3.63) and 54 males (>9.85).

conclusive results, future studies should include molecular genetic techniques in combination with the abovementioned approach to determine the sex of African clawless otters [34]. In addition, the intra-specific comparison of different reproductive classes (e.g., proestrus, follicular phase, the luteal phase, pregnancy, lactation, etc.) should also be considered [35]. It is challenging to determine sex-specific variation among the different reproductive classes when only one captive female is available for investigation, as was the case for this study, and is further challenged by the lack of information related to general reproductive biology of the species [20,36]. Further investigation that includes a larger sample size and one that investigates seasonal hormone profiles in breeding and non-breeding individuals could be helpful to further explore the use of reproductive hormones to distinguish between sexes.

Stability of hormone metabolite concentrations post-defaecation

Alterations in fAM and fPM concentrations post-defaecation in the male and female can be seen in Fig. 3A-D. In subsamples from the male exposed to full sun conditions, faecal androgen metabolite concentrations differed significantly ($F_{(8,18)}$=29.082; n=27; p<0.001) over time post-defaecation (Fig. 3A). Respective fAM increased significantly after 24 hours (147.91 ± 56.96μg/g DW, p<0.001) compared to t=0. Under full shade conditions, fAM concentrations in male subsamples differed significantly ($F_{(8,18)}$=36.844; n=27; p<0.001) over time post-defaecation (Fig. 3A). Respective fAM concentrations increased significantly after 24 hours (118.71 ± 26.32μg/g DW, p<0.001) compared to t=0.

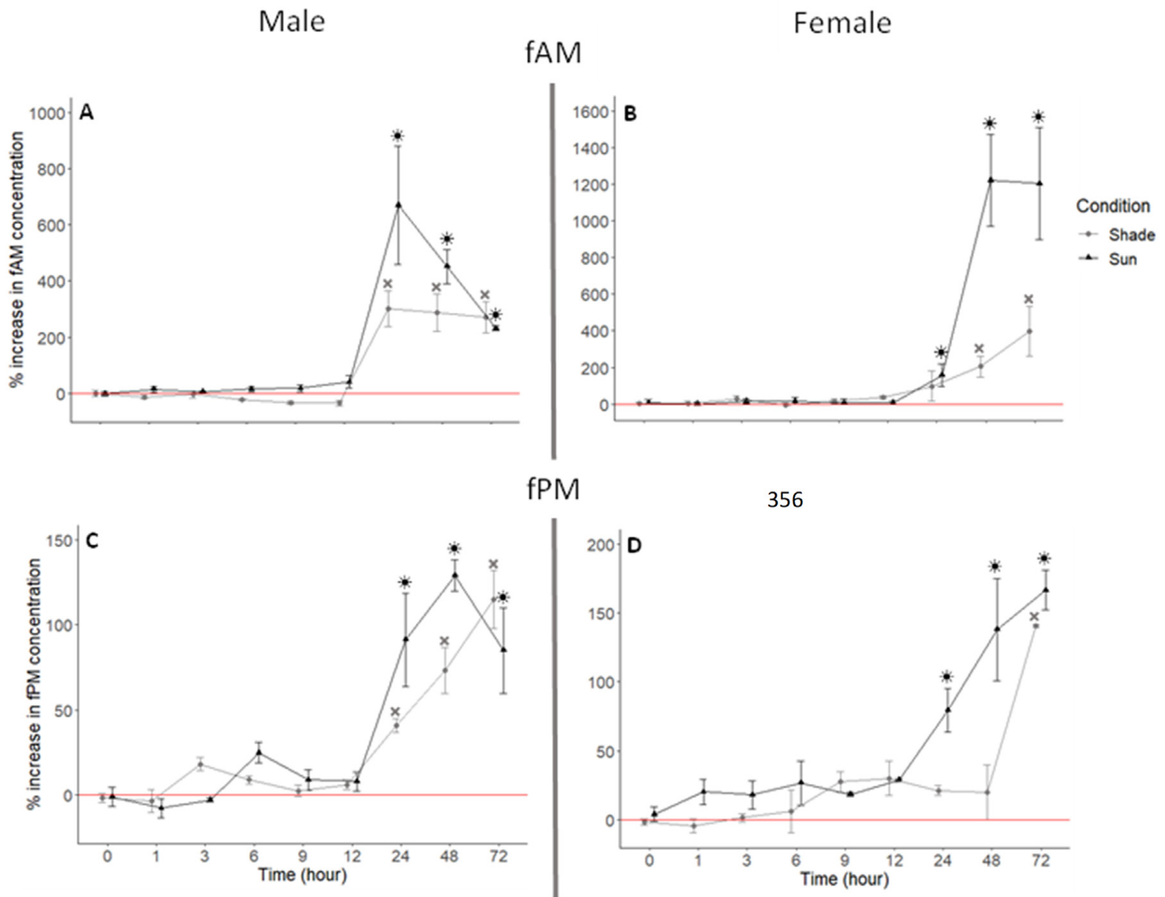

Figure 3. A-D: Changes in hormone concentrations (fAM: top; fPM: bottom) post-defaecation in the male (left) and female (right) African clawless otter. Percentage increase in hormone concentrations is shown on the y-axis. Respective time intervals (0 – 72 hours) are shown on the x-axis and full sun (solid black line) and full shade (solid grey line) conditions are depicted in all graphs. The red line indicates 0% increase using t=0 as a baseline. Statistically significant difference ($p<0.05$), compared to t=0, is indicated with respective symbols for full sun (☀) and full shade (x). Respective points indicate mean hormone percentage increase, while lines above and below the point indicate ± SE.

In the female subsamples exposed to full sun conditions, fAM concentrations also differed significantly ($F_{(8,18)}=33.896$; n=27; $p<0.001$) over time post-defaecation (Fig. 3B). Respective fAM concentrations increased significantly after 24 hours (32.60 ± 11.36µg/g DW, p=0.036) compared to t=0. In the female, fAM subsamples placed in full shade differed significantly ($F_{(8,18)}=6.0883$; n=27; $p<0.001$) over time post-defaecation (Fig. 3B). Although not significant, there was an 89% increase in fAM concentration after 24 hours. The fAM concentrations significantly increased to 191% after 48 hours (34.79 ± 9.51µg/g DW, p=0.0288) compared to t=0.

The fPM concentrations differed significantly ($F_{(8,18)}=17.17$; n=27; $p<0.001$) over time post-defaecation in subsamples from the male exposed to full sun conditions (Fig. 3C). Respective fPM significantly increased after 24 hours (3.00 ± 0.61µg/g DW, $p<0.001$) compared to t=0. Similarly, in subsamples from the male exposed to full shade conditions, fPM concentrations differed significantly ($F_{(8,18)}=30.065$; n=27; $p<0.001$) over time post-defaecation (Fig. 3C). Respective fPM concentrations significantly increased after 24 hours (2.36 ± 0.10µg/g DW, $p<0.001$) compared to t=0.

In subsamples from the female exposed to full sun conditions, fPM concentrations differed significantly ($F_{(8,18)}=15.854$; n=27; $p<0.001$) over time post-defaecation (Fig. 3D). Respective fPM concentrations significantly increased after 24 hours (2.86 ± 0.35µg/g DW, p=0.0021) compared to t=0. In subsamples from the female exposed to full shade condition, fPM concentrations differed significantly ($F_{(8,18)}=9.8936$; n=27; $p<0.001$) over time post-defaecation (Fig. 3D). Respective fPM concentrations significantly increased after 72 hours (3.85 ± 0.02µg/g DW, $p<0.001$) compared to t=0.

Inter-sample variability of fAM and fPM concentrations between triplicates, increased considerably over time post-defaecation for both sexes (Fig. 3A-D). An increase in variation of faecal steroid hormone concentrations over time is commonly seen in faecal hormone degradation studies [13,27,37]. Bacterial enzymes within the faeces, are most likely responsible for continuous hormone

metabolism post-defaecation [13,17,38]. Increased exposure to environmental conditions over time allows the bacteria present in the sample to continuously alter the composition of faecal steroid hormones, resulting in higher variability [38,39].

These findings demonstrate that fAM and fPM concentrations determined in both sexes remained stable and comparable for at least 12 hours post-defaecation regardless of the conditions (full sun/full shade) to which the samples were exposed. Taking this into account, future studies focused solely on reproductive hormones (progestagens or androgens) should collect African clawless otter faecal material from latrines within 12 hours of dawn and dusk, regardless of sex or environmental conditions.

This was the first study to use fAM and fPM concentrations to assign sex to faecal material of African clawless otters with a success rate of >70%. This study also provides a more comprehensive overview of how environmental exposure of faecal material affects the rate of reproductive hormone metabolite (fAM and fPM) alteration of both male and female African clawless otters over time, providing a more accurate collection window for future studies. We therefore recommend that samples for fPM and fAM analysis be collected within 12 hours post-defaecation regardless of sex or environmental condition.

Limitations

We do note that for more conclusive results, future studies should include molecular genetic techniques in combination with the abovementioned approach to determine the sex of African clawless otters. In addition, the intra-specific comparison of different reproductive classes (e.g., proestrus, follicular phase, the luteal phase, pregnancy, lactation, etc.) should also be considered [35].

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.

CRediT authorship contribution statement

Marli Burger: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization, Project administration. **Andrea B. Webster:** Conceptualization, Methodology, Writing – review & editing, Visualization, Supervision. **Tshepiso L. Majelantle:** Conceptualization, Methodology, Formal analysis, Writing – review & editing, Supervision. **Juan Scheun:** Conceptualization, Writing – review & editing, Visualization, Supervision. **Andre Ganswindt:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Visualization, Supervision, Funding acquisition.

Data availability

Data will be made available on request.

Ethics statements

This study was conducted with the approval of the University of Pretoria's Research and Animals Ethics and Care Committees (NAS087/2020) in compliance with animal diseases from the Department of Agriculture, Land Reform and Rural Development (DALRRD; section 20; SDAH-Epi-21110813130) and the Johannesburg City Parks and Zoo (JCPZ) Animal Ethics Committee (JHBZOOESC-21/015).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2024.102883.

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