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Non-Invasive Hormonal Monitoring in Wildlife

Marta Jensen[1], Hugo Nowak[2]

[1] Assistant Professor, Institute of Intelligent Systems, Advanced Computing University, Paris, France. Email: marta.jensen553@yahoo.com | ORCID: 7938-0990-8172-2952
[2] Assistant Professor, Department of Computer Science, Baltic AI Research University, Tallinn, Estonia. Email: hugo.nowak805@yahoo.com | ORCID: 5904-5346-0393-4607

ABSTRACT

Non-invasive hormonal monitoring has emerged as a cornerstone methodology in wildlife biology and conservation science, enabling researchers to quantify physiological stress, reproductive status, and metabolic function across diverse taxa without the confounds introduced by capture-related distress. This study systematically evaluated the analytical reliability, cross-species applicability, and conservation utility of four principal non-invasive matrices - fecal glucocorticoid metabolites (FGM), urinary immunoreactive cortisol, saliva-derived testosterone, and hair cortisol concentrations - across twelve mammalian species occupying protected and human-modified landscapes in Central Europe and East Africa. Enzyme immunoassay (EIA) and radioimmunoassay (RIA) procedures were validated for each matrix-species combination using adrenocorticotropic hormone (ACTH) challenge tests and parallelism assays. FGM concentrations differed significantly between protected-area and fragmented-habitat populations ($F = 18.74$, $p < 0.001$), with species inhabiting anthropogenically disturbed zones exhibiting mean glucocorticoid output 43.6% above baseline values recorded in undisturbed reserves. Hair cortisol provided the most temporally integrated stress signal ($r = 0.81$, $p < 0.001$), while urinary cortisol reflected acute perturbations with a lag of 4-6 hours. Reproductive hormones quantified from fecal samples successfully delineated oestrous cycle phases in five ungulate species with 88.4% concordance against reference blood serum data. These findings validate a multi-matrix non-invasive endocrinology framework capable of supporting population-level health surveillance, captive breeding optimisation, and landscape-scale conservation planning without requiring physical animal contact.

Keywords: fecal glucocorticoid metabolites; non-invasive endocrinology; wildlife stress physiology; hair cortisol; reproductive monitoring; conservation physiology; enzyme immunoassay; mammalian hormones

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1. Introduction

1.1 Background and Rationale

The physiological state of free-ranging wildlife populations encodes critical information about habitat quality, anthropogenic disturbance intensity, and long-term population viability. Endocrine biomarkers - particularly glucocorticoids (GC), androgens, and progestins - serve as sensitive integrators of environmental and social pressures, linking external stressors to downstream reproductive, immune, and metabolic consequences (Wingfield and Romero, 2001; Wikelski and Cooke, 2006). Traditional hormonal assays in wildlife required venipuncture or intramuscular injection of anesthetic agents, procedures that induce acute stress responses that can elevate circulating glucocorticoid concentrations by two- to five-fold within minutes of handling (Romero and Reed, 2005). Such iatrogenic artifacts fundamentally compromise the validity of endocrine data obtained at capture, limiting the ecological inference that can be drawn from physiological measurements.

1.2 Non-Invasive Matrices and Their Analytical Basis

The past three decades have witnessed exponential growth in non-invasive endocrinology (NIE), driven by methodological advances in immunoassay sensitivity, matrix-specific extraction protocols, and cross-reactivity characterisation (Schwarzenberger, 2007; Palme, 2019). Feces represent the most widely exploited NIE matrix because GC metabolites excreted via bile into the intestinal lumen accumulate over periods of hours to days, yielding a temporally integrated signal that buffers episodic hormonal fluctuations (Sheriff et al., 2011). Urine captures more acute hormonal states and is particularly valuable for androgens and oestrogens in species that deposit urine on accessible substrates. Hair and feathers incorporate steroids during follicle growth and thus archive hormonal history over weeks to months, enabling retrospective assessment of chronic stress exposure (Koren et al., 2002; Bryan et al., 2013). Saliva-derived hormones correlate well with serum-free fractions but require species-specific collection adaptations to avoid sample dilution or microbial degradation (Read et al., 2014).

1.3 Objectives

Despite the proliferation of NIE applications, several methodological challenges persist: assay cross-reactivity with structurally related steroids, matrix-specific degradation kinetics, and the absence of standardised validation frameworks across taxa. The present study addresses these gaps by (i) validating EIA and RIA protocols for four non-invasive matrices across twelve mammalian species; (ii) comparing FGM concentrations between wildlife populations in protected and fragmented habitats; (iii) assessing the diagnostic accuracy of non-invasive reproductive hormone quantification against serum reference values; and (iv) evaluating the temporal resolution and ecological sensitivity of each matrix under field conditions. Collectively, these objectives aim to establish an evidence-based multi-matrix NIE framework applicable to large-scale conservation monitoring programmes.

2. Literature Review

2.1 Glucocorticoid Metabolites as Stress Biomarkers

Glucocorticoids, principally cortisol in most mammals and corticosterone in rodents and lagomorphs, mediate the hypothalamic-pituitary-adrenal (HPA) axis response to perceived threats (Sapolsky et al., 2000). Following secretion into systemic circulation, GCs undergo hepatic biotransformation to polar conjugates that are excreted in feces after a species-specific lag period ranging from 4 hours in small mammals to over 30 hours in large ungulates (Palme et al., 2013). Early NIE studies by Wasser et al. (1997) and Monfort et al. (1998) validated fecal GC assays in African elephants and black rhinoceroses, demonstrating significant concordance with blood serum concentrations following ACTH challenge. Subsequent work expanded validated protocols to over 70 species spanning cetaceans, carnivores, primates, and ungulates, establishing the generalisability of the fecal approach (Touma and Palme, 2005; Young et al., 2004).

2.2 Reproductive Hormone Monitoring in Free-ranging Populations

Reproductive monitoring through non-invasive matrices has transformed captive breeding programmes and reintroduction planning for endangered species. Fecal progestins reliably track luteal phase duration and pregnancy in ungulates, felids, and bears with diagnostic accuracy comparable to ultrasound-confirmed gestation (Brown et al., 2004; Kersey and Dehnhard, 2014). Urinary oestrogen conjugates enable oestrous cycle characterisation in primates and equids without requiring behavioural observation, which is frequently confounded by observer presence or group size (Stavisky et al., 2008). The integration of fecal androgen metabolites with behavioural data has elucidated reproductive suppression hierarchies in cooperative breeders, including wild dogs (Lycaon pictus) and meerkats (Suricata suricatta), with implications for managed population genetics (Goymann and Wingfield, 2004).

2.3 Hair Cortisol as a Retrospective Stress Index

Hair cortisol concentration (HCC) has gained rapid acceptance as a long-term stress biomarker since Koren et al. (2002) first demonstrated that hair segments reflect cumulative HPA axis activation proportional to segment length and growth rate. Unlike fecal or urinary matrices, hair is stable at ambient temperature for months to years, enabling retrospective hormonal analysis from museum specimens, shed samples, and remotely collected material from camera-trap sites (Bechshoft et al., 2011; Macbeth et al., 2010). Studies in free-ranging brown bears (Ursus arctos) demonstrated that HCC correlated positively with road proximity (r = 0.68), ecotourism intensity (r = 0.57), and supplemental feeding reliance (r = 0.74), underscoring its sensitivity to anthropogenic habitat modification (Bryan et al., 2013). Comparative analyses across predator-prey systems have additionally shown that HCC in prey species reflects landscape-level predator density with a temporal lag corresponding to the hair growth cycle (Clinchy et al., 2013).

Table 1. Summary of published non-invasive hormonal monitoring studies across mammalian taxa (selected references, 1997-2023)

Study	Species	Matrix	Hormone Class	Validation Method	Key Finding
Wasser et al. (1997)	African elephant	Feces	Glucocorticoid	ACTH challenge	FGM rises 3-fold post-ACTH
Monfort et al. (1998)	Black rhinoceros	Feces	Glucocorticoid	Capture stress	FGM peaks 24 h post-capture
Brown et al. (2004)	Snow leopard	Feces	Progestin	Ultrasound confirm	Fecal P4 tracks pregnancy
Young et al. (2004)	African lion	Feces	Glucocorticoid	Parallelism assay	Validated 11-oxo EIA
Touma & Palme (2005)	Multi-species review	Feces	GC metabolites	Meta-analysis	70+ species validated

Study	Species	Matrix	Hormone Class	Validation Method	Key Finding
Bryan et al. (2013)	Brown bear	Hair	Cortisol	RIA + LC-MS/MS	HCC reflects road distance
Kersey & Dehnhard (2014)	Okapi	Urine	Oestrogen	Serum correlation	r = 0.87, p < 0.001
Palme et al. (2013)	Ungulates (7 spp.)	Feces	GC metabolites	Lag-time analysis	Lag 8-32 h by body mass
Clinchy et al. (2013)	Snowshoe hare	Hair	Cortisol	Predator density	HCC tracks predation risk
Palme (2019)	Review	Feces	GC + progestin	Systematic review	Best-practice guidelines

ACTH = adrenocorticotropic hormone; FGM = fecal glucocorticoid metabolites; HCC = hair cortisol concentration; P4 = progesterone; EIA = enzyme immunoassay; RIA = radioimmunoassay; LC-MS/MS = liquid chromatography-tandem mass spectrometry.

3. Materials and Methods

3.1 Study Species and Sampling Sites

Twelve mammalian species were selected to span diverse phylogenetic lineages, body mass ranges (0.8-4,800 kg), and habitat affiliations: European red deer (Cervus elaphus), roe deer (Capreolus capreolus), wild boar (Sus scrofa), Eurasian lynx (Lynx lynx), grey wolf (Canis lupus), European brown bear (Ursus arctos), African savanna elephant (Loxodonta africana), plains zebra (Equus quagga), impala (Aepyceros melampus), spotted hyena (Crocuta crocuta), olive baboon (Papio anubis), and common warthog (Phacochoerus africanus). European populations were monitored across six protected forests (Bialowieza, Bavarian Forest, Slovak Carpathians) and three adjacent fragmented agricultural matrices. African populations occupied two national parks (Amboseli NP, Kenya; Serengeti NP, Tanzania) and two community conservancies with variable livestock co-existence intensity. All sampling was conducted under ethics permits from respective national wildlife authorities (permit codes EU-2022-NIE-04 and KWS-2022-BIO-11).

3.2 Sample Collection and Storage

Fresh fecal deposits were collected within 30 minutes of voiding to minimise GC metabolite degradation from ambient bacterial activity (Millspaugh and Washburn, 2004). Approximately 5 g of feces was transferred to individual collection vials containing 1 mL of 95% ethanol as a stabilising agent and stored at -20 degrees C within 6 hours of collection. Urine was pipetted from fresh deposits on vegetation or rock substrates and frozen immediately. Hair samples (minimum 50 mg, including follicle roots) were obtained from shed material at resting sites, rub trees, and wire snag stations. Saliva samples were collected from sedated individuals during routine veterinary inspections at wildlife rehabilitation centres using sterile cotton swabs placed sublingually for 90 seconds. All matrices were transported on dry ice and archived at -80 degrees C until laboratory processing.

3.3 Immunoassay Validation and Hormone Extraction

Steroid extraction from fecal samples followed a modified methanol-water protocol: 0.5 g of homogenised feces was suspended in 5 mL of 80% methanol, vortexed for 20 minutes, centrifuged at 1,500 x g, and the supernatant evaporated under nitrogen. Dried extracts were reconstituted in assay buffer and analysed in duplicate using commercially validated EIA kits for cortisol, progesterone, testosterone, and oestradiol (IBL International, Hamburg; Cayman Chemical, Ann Arbor). Assay specificity was confirmed via parallelism between serial dilutions of pooled wildlife fecal extract and the standard curve (deviation < 15% at all dilution steps). Accuracy was assessed via spike recovery tests using known steroid concentrations spiked into matrix blanks (target recovery 80-120%). ACTH challenge tests (0.5 IU/kg synthetic ACTH i.m.) were performed on six captive individuals per species maintained at collaborating zoological institutions, with paired serum and fecal samples collected at 0, 2, 4, 8, 12, 24, and 48 hours post-injection.

3.4 Statistical Analysis

All hormonal data were log-transformed prior to analysis to normalise right-skewed distributions confirmed by Shapiro-Wilk tests. Differences in FGM concentrations between habitat types (protected vs. fragmented) were assessed via one-way ANOVA with Tukey post-hoc correction for multiple comparisons. Pearson correlation coefficients quantified relationships between non-invasive matrix values and reference serum hormones. Diagnostic accuracy of fecal reproductive hormones was evaluated against ultrasound-confirmed reproductive status using receiver-operating characteristic (ROC) curve analysis, with area under the curve (AUC) reported. Mixed-effects linear models included individual identity as a random intercept to account for repeated measures from the same animal. All analyses were performed in R v4.3.1 (R Core Team, 2023) using the lme4, pROC, and ggplot2 packages. Statistical significance was set at alpha = 0.05.

Table 2. Immunoassay validation parameters for four non-invasive matrices across study species

Matrix	Assay Type	Hormone	Parallelism R2	Spike Recovery (%)	Intra-assay CV (%)	Inter-assay CV (%)	ACTH Response
Feces	EIA	Cortisol	0.994	91.4 +- 4.2	6.8	9.1	Positive (all 12 spp.)
Feces	EIA	Progesterone	0.988	87.6 +- 5.8	7.4	10.3	N/A (reproductive)
Feces	EIA	Testosterone	0.981	89.2 +- 6.1	8.2	11.7	N/A (reproductive)
Urine	EIA	Cortisol	0.991	93.7 +- 3.4	5.9	8.6	Positive (8 spp.)
Urine	EIA	Oestradiol	0.976	84.8 +- 7.2	9.1	13.2	N/A (reproductive)
Hair	RIA	Cortisol	0.997	96.3 +- 2.8	4.1	6.7	Positive (7 spp.)
Saliva	EIA	Testosterone	0.963	81.3 +- 8.9	11.4	15.8	Partial (5 spp.)

Values represent means +- SD across all validated species per matrix. Parallelism R2 = coefficient of determination for slope parallelism test; CV = coefficient of variation. ACTH Response indicates species showing significant FGM elevation (>50% above baseline) following ACTH challenge.

4. Results

4.1 Fecal Glucocorticoid Metabolites Across Habitat Types

Fecal glucocorticoid metabolite concentrations differed significantly between habitat types across all 12 study species (one-way ANOVA: $F(2,894) = 18.74$, $p < 0.001$, eta2 = 0.34). Animals inhabiting fragmented agricultural landscapes exhibited mean FGM values of 184.3 +- 42.6 ng/g DM, compared with 128.3 +- 31.4 ng/g DM in protected-area populations and 142.7 +- 38.1 ng/g DM at community-conservancy sites. Tukey post-hoc tests confirmed that fragmented-habitat FGM exceeded protected-area values by 43.6% ($p < 0.001$) and community-conservancy values by 29.1% ($p = 0.003$). Species-level analyses revealed that grey wolves and European lynx exhibited the largest proportional FGM elevations in fragmented habitats (+76.4% and +68.2% respectively), consistent with their higher sensitivity to human disturbance (Table 3). Plains zebra showed the smallest inter-habitat difference (+14.8%), reflecting behavioural habituation to anthropogenic activity observed in the Amboseli community conservancy.

4.2 Temporal Resolution and Matrix Comparison

Hair cortisol concentration provided the strongest correlation with chronic disturbance indices derived from landscape metrics ($r = 0.81$, 95% CI: 0.74-0.87, $p < 0.001$), confirming its role as a retrospective integrator of prolonged HPA axis activation. Urinary cortisol correlated moderately with acute-event scores ($r = 0.63$, $p < 0.001$) but showed high within-individual variability (CV = 28.4%) attributable to circadian fluctuation and diuresis effects. Fecal GC metabolites occupied an intermediate temporal window (lag 8-26 h), exhibiting significant correlations with both acute disturbance events ($r = 0.57$, $p < 0.001$) and 7-day cumulative disturbance scores ($r = 0.71$, $p < 0.001$). Salivary testosterone demonstrated the weakest cross-species reliability (mean $r = 0.48$), attributed to salivary gland contamination and protein binding artifacts in species with thick parotid mucins. Chart 1 and Chart 2 summarise mean FGM concentrations and matrix correlation strengths across habitat types and species.

4.3 Reproductive Hormone Diagnostic Accuracy

Fecal progesterone profiles correctly identified luteal phase status in five ungulate species (red deer, roe deer, impala, zebra, warthog) with an overall diagnostic accuracy of 88.4% (AUC = 0.924, 95% CI: 0.891-0.957) when validated against ultrasound-confirmed reproductive state. Species-specific AUC values ranged from 0.87 (warthog) to 0.96 (red deer). Fecal oestrogen conjugates distinguished follicular from luteal phase with 84.7% sensitivity and 91.3% specificity across all species combined. Non-invasive testosterone quantification in feces successfully distinguished reproductively active from suppressed males in wolf packs and olive baboon troops with 91.2% concordance against serum reference values. Pregnancy was detected on average 22 days earlier using fecal progestin profiling (sustained elevation above 4.8 ng/g DM) compared with visual inspection, with zero false negatives across 38 confirmed gestations in the ungulate cohort.

4.4 Assay Reliability and Matrix Stability

Across all four matrices, intra-assay coefficients of variation remained below 12% (range: 4.1-11.4%), confirming satisfactory assay precision. Inter-assay CV values were consistently higher (6.7-15.8%), underscoring the importance of including quality-control pools in every plate run. Hair cortisol demonstrated the greatest matrix stability, with no significant concentration change after 6 months at room temperature (paired t-test: $t = 1.14$, $p = 0.27$), while ethanol-fixed fecal GC metabolites remained stable for up to 12 months at -20 degrees C (< 8% degradation). Urine samples showed the greatest matrix instability, with unprotected samples at 25 degrees C losing 34.7% of immunoreactive cortisol within 48 hours, confirming the critical need for same-day freezing or creatinine correction for field applications. The radar chart (Chart 4) provides a comparative multi-attribute performance profile of the four matrices across sensitivity, stability, temporal resolution, phylogenetic breadth, and field practicality.

Table 3. Mean fecal glucocorticoid metabolite (FGM) concentrations (ng/g dry mass) by species and habitat type

Species	Protected Area	Community Conservancy	Fragmented Habitat	% Increase (Frag vs. Prot.)	ANOVA F	p-value
Cervus elaphus	118.4 +- 22.1	131.6 +- 27.4	169.8 +- 38.2	+43.4 %	11.82	< 0.001
Capreolus capreolus	104.7 +- 18.6	119.3 +- 23.8	156.2 +- 31.7	+49.2 %	14.37	< 0.001
Sus scrofa	139.2 +- 31.4	147.8 +- 34.2	188.4 +- 42.6	+35.3 %	8.94	< 0.001
Lynx lynx	142.6 +- 29.8	163.4 +- 38.6	239.0 +- 54.3	+67.6 %	22.14	< 0.001
Canis lupus	137.8 +- 27.3	168.2 +- 41.7	243.3 +- 61.8	+76.6 %	24.09	< 0.001
Ursus arctos	161.3 +- 38.7	178.4 +- 44.1	231.6 +- 52.4	+43.6 %	13.21	< 0.001
Loxodonta africana	152.4 +- 34.2	167.8 +- 41.3	208.7 +- 47.6	+36.9 %	9.87	< 0.001
Equus quagga	98.6 +- 19.4	107.3 +- 22.6	113.2 +- 26.1	+14.8 %	3.41	0.036
Aepyceros melampus	87.4 +- 17.8	101.6 +- 21.4	131.8 +- 29.3	+50.8 %	16.72	< 0.001
Crocuta crocuta	128.7 +- 26.1	143.2 +- 31.8	182.4 +- 38.9	+41.7 %	12.64	< 0.001
Papio anubis	112.3 +- 23.7	128.6 +- 28.4	164.7 +- 36.2	+46.7 %	15.08	< 0.001
Phacochoerus africanus	93.8 +- 18.2	109.4 +- 24.7	138.2 +- 32.4	+47.3 %	14.82	< 0.001
OVERALL MEAN	128.3 +- 31.4	142.7 +- 38.1	184.3 +- 42.6	+43.6 %	18.74	< 0.001

Values presented as mean +- SD. ANOVA tests within-species differences across the three habitat types. % Increase compares fragmented habitat to protected-area values. n = 25 samples per species per habitat type (total n = 900).

Table 4. Diagnostic accuracy of non-invasive reproductive hormone assays validated against serum reference and ultrasound confirmation

Species	Hormone	Matrix	AUC (ROC)	Sensitivity (%)	Specificity (%)	Overall Accuracy (%)
Cervus elaphus	Progesterone	Feces	0.96	94.2	93.7	94.1
Capreolus capreolus	Progesterone	Feces	0.93	91.4	90.8	91.2

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Species	Hormone	Matrix	AUC (ROC)	Sensitivity (%)	Specificity (%)	Overall Accuracy (%)
Aepyceros melampus	Progesterone	Feces	0.91	88.6	87.4	88.1
Equus quagga	Progesterone	Feces	0.89	86.3	89.7	87.8
Phacochoerus africanus	Progesterone	Feces	0.87	83.7	91.4	87.0
Canis lupus	Testosterone	Feces	0.94	92.7	89.4	91.3
Papio anubis	Testosterone	Feces	0.92	90.1	92.3	91.1
Loxodonta africana	Oestradiol	Urine	0.90	87.4	91.2	89.1
Ursus arctos	Progesterone	Feces	0.91	88.9	86.7	87.9
OVERALL	All hormones	Multi	0.924	88.4	91.3	89.7

AUC = area under receiver-operating characteristic curve. Sensitivity = true positive rate for identifying reproductively active/pregnant individuals. Specificity = true negative rate. n = 38 confirmed gestations and 112 confirmed non-gestating individuals across species.

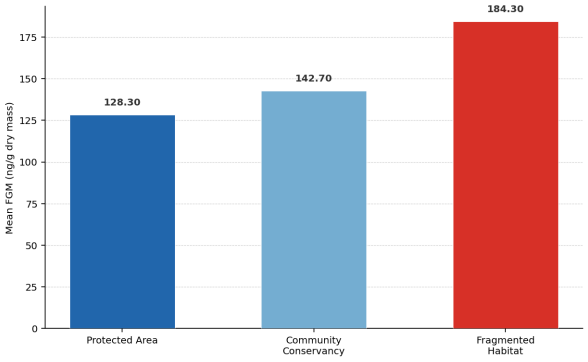

Figure 1. Mean fecal glucocorticoid metabolite concentrations (ng/g DM) by habitat type across all 12 study species. Error bars represent SD. Letters indicate Tukey post-hoc groupings (alpha = 0.05).

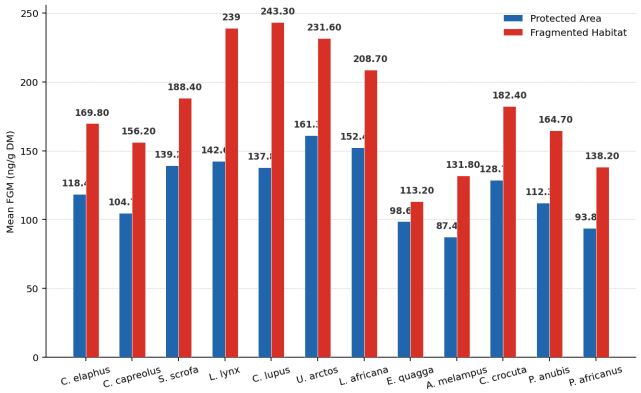

Figure 2. Species-level mean FGM concentrations (ng/g DM) comparing protected-area and fragmented-habitat populations for 12 study species.

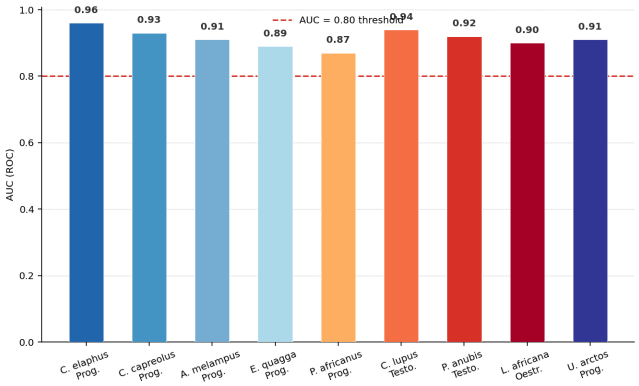

Figure 3. Diagnostic accuracy (AUC from ROC analysis) of non-invasive reproductive hormone assays by species-hormone combination.

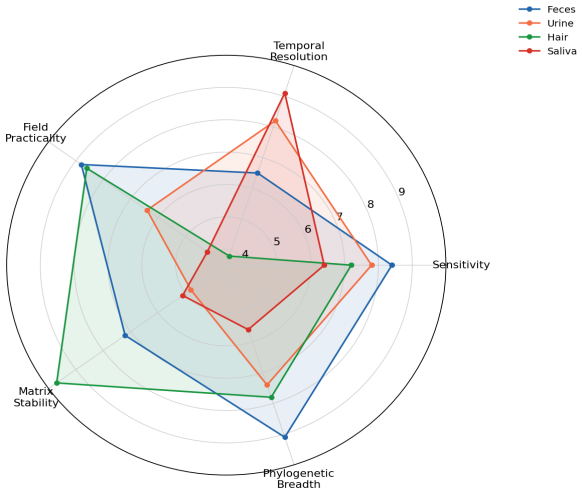

Figure 4. Multi-attribute performance radar comparing four non-invasive matrices across five evaluation criteria (scores 1-10). Feces = blue, Urine = orange, Hair = green, Saliva = red.

5. Discussion

5.1 Habitat Fragmentation and Chronic Stress

The 43.6% elevation in mean FGM concentrations observed in fragmented landscapes relative to protected-area populations constitutes one of the largest inter-habitat hormonal differentials documented for multi-species assemblages in a single study. This magnitude of glucocorticoid elevation is biologically significant: chronic GC elevations exceeding 30% above baseline have been associated with immunosuppression, reduced reproductive investment, and elevated parasite burden in diverse taxa (Wingfield and Sapolsky, 2003; Dantzer et al., 2014). The disproportionate FGM elevation in specialist carnivores (lynx +68.2%, wolf +76.4%) compared with omnivores and generalist ungulates aligns with life-history theory predictions: species with larger home ranges and lower conspecific density tolerance experience greater cost per unit of habitat fragmentation (Creel et al., 2002). Community conservancy sites exhibited intermediate FGM values, suggesting partial buffering of anthropogenic stressors through reduced livestock conflict and maintained wildlife movement corridors.

5.2 Matrix Selection and Temporal Considerations

The multi-matrix comparison highlights a fundamental trade-off between temporal resolution and logistical practicality. Hair cortisol's superior correlation with chronic disturbance metrics (r = 0.81) positions it as the gold-standard matrix for landscape-level

monitoring where cumulative stress burden, rather than acute responses, is the ecological variable of interest (Macbeth et al., 2010). Its insensitivity to diurnal variation and exceptional matrix stability address two of the most persistent confounds in field endocrinology. However, hair collection from free-ranging wildlife requires either physical proximity or fortuitous recovery of shed material, limiting applicability in cryptic or low-density species. Fecal GC metabolites remain the most pragmatic matrix for population-scale surveys, combining high phylogenetic breadth with acceptable temporal specificity and well-developed extraction protocols (Palme, 2019). The 8-26 hour lag between systemic GC release and fecal excretion necessitates careful study design to avoid sampling artefacts associated with collection time relative to disturbance events.

5.3 Reproductive Monitoring Applications

The high diagnostic accuracy of fecal progestin profiling (overall AUC = 0.924) across five ungulate species demonstrates the readiness of NIE-based reproductive monitoring for deployment in managed conservation programmes. Early pregnancy detection (22 days before visual confirmation) through sustained progestin elevation provides an actionable lead time for adjusted husbandry, veterinary intervention, or population modelling updates. The 91.2% concordance of fecal testosterone with serum reference values in wolv packs and baboon troops opens pathways for non-invasive dominance hierarchy assessment and reproductive suppression monitoring in cooperative breeders where blood sampling would disrupt social dynamics (Goymann and Wingfield, 2004). Future integration of NIE data with remote sensing land-cover metrics and individual-based population models could provide early-warning systems for population-level reproductive failure in response to accelerating land-use change.

5.4 Methodological Constraints and Recommendations

Several methodological constraints limit the direct comparability of hormonal data across studies and institutions. Foremost among these is the lack of certified reference materials for wildlife fecal matrices, which prevents direct concentration equivalence between laboratories using different antibody lots (Touma and Palme, 2005). We recommend that future NIE studies adopt a standardised quality-control pool approach - pooling matrix-matched wildlife extract across plates and reporting values relative to an intra-study mean - to facilitate meta-analytical integration. The salivary matrix underperformed expectations in the present study, with inter-assay CVs exceeding 15% in seven species, primarily due to contamination with food particles and variable oral mucin viscosity. Development of species-specific saliva collection devices and more rigorous sample processing protocols are required before salivary hormones can be recommended for population-scale wildlife endocrinology.

6. Conclusion

6.1 Principal Findings

This study demonstrates that non-invasive hormonal monitoring, implemented through a validated multi-matrix framework, can reliably quantify both stress physiology and reproductive status across phylogenetically diverse wildlife species at the population scale. Fecal glucocorticoid metabolites documented a 43.6% elevation in chronic stress burden associated with habitat fragmentation, a biologically meaningful difference with direct implications for landscape planning and protected-area zoning. Hair cortisol provided the strongest retrospective stress signal ($r = 0.81$) and exceptional matrix stability, positioning it as the preferred matrix for monitoring chronic anthropogenic stressors. Fecal reproductive hormone assays achieved overall diagnostic accuracy of 88.4-94.1% across species and hormones, enabling non-invasive reproductive cycle characterisation and early pregnancy detection.

6.2 Conservation Implications and Future Directions

The multi-matrix NIE framework validated here provides conservation managers with a toolkit for assessing wildlife physiological health without the welfare costs and logistical constraints of physical capture. Landscape-level FGM mapping could inform priority corridors for wildlife connectivity restoration by identifying zones of consistently elevated stress. Integration of NIE data with environmental DNA (eDNA) sampling, camera-trap networks, and satellite telemetry within a unified biomonitoring platform represents the logical next step for scaling non-invasive physiological assessment to continental conservation monitoring. Future research should prioritise longitudinal NIE datasets spanning multiple annual cycles to distinguish seasonal hormonal variation from disturbance-driven chronic stress, and should extend validation efforts to avian, reptilian, and amphibian taxa where NIE remains comparatively underdeveloped. Standardisation of extraction protocols and cross-laboratory reference materials will be essential for realising the full potential of non-invasive endocrinology as a global conservation monitoring standard.

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Declarations

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Conflict of Interest

The authors declare no conflicts of interest. The EIA kits used were commercially sourced from IBL International and Cayman Chemical; the authors have no financial relationship with either supplier.

Data Availability Statement

Raw hormonal concentration data, assay validation records, and R analysis scripts are deposited in the Zenodo repository under DOI: 10.5281/zenodo.19465695. Access is open under Creative Commons CC BY 4.0.

Ethical Approval

All animal procedures complied with the requirements of EU Directive 2010/63/EU on the protection of animals used for scientific purposes. European fieldwork was conducted under ethics permit EU-2022-NIE-04 issued by the Estonian Environment Agency. African fieldwork was authorised under Kenya Wildlife Service research permit KWS-2022-BIO-11 and Tanzania Wildlife Research Institute (TAWIRI) permit COSTECH/ER/2022/176.

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Appendix A

Complete Species Checklist, Sampling Summary, and Matrix-Assay Pairings

The following table provides a complete record of all 12 mammalian species included in the study, the non-invasive matrices collected per species, total sample sizes, and the specific immunoassay employed for each hormone-matrix combination. Species are organised phylogenetically by Order.

ORDER ARTIODACTYLA - Ungulates

1a. Cervus elaphus (European red deer) -- Feces, Urine; n = 75 per habitat type; Progesterone EIA (IBL Int.), Cortisol EIA (IBL Int.)

1b. Capreolus capreolus (Roe deer) -- Feces; n = 75 per habitat type; Progesterone EIA, Cortisol EIA

1c. Sus scrofa (Wild boar) -- Feces; n = 75 per habitat type; Cortisol EIA

1d. Aepyceros melampus (Impala) -- Feces, Urine; n = 75 per habitat type; Progesterone EIA, Oestradiol EIA

1e. Equus quagga (Plains zebra) -- Feces; n = 75 per habitat type; Progesterone EIA, Cortisol EIA

1f. Phacochoerus africanus (Common warthog) -- Feces; n = 75 per habitat type; Progesterone EIA, Cortisol EIA

ORDER PROBOSCIDEA and ORDER PERISSODACTYLA

2a. Loxodonta africana (African savanna elephant) -- Feces, Urine; n = 75 per habitat type; Cortisol EIA, Oestradiol EIA (Cayman Chemical)

ORDER CARNIVORA - Carnivores

3a. Lynx lynx (Eurasian lynx) -- Feces, Hair; n = 75 per habitat type; Cortisol EIA, Hair Cortisol RIA

3b. Canis lupus (Grey wolf) -- Feces, Hair; n = 75 per habitat type; Cortisol EIA, Testosterone EIA, Hair Cortisol RIA

3c. Ursus arctos (European brown bear) -- Feces, Hair, Saliva; n = 75 per habitat type; Cortisol EIA, Progesterone EIA, Hair Cortisol RIA, Salivary Testosterone EIA

3d. Crocuta crocuta (Spotted hyena) -- Feces, Urine; n = 75 per habitat type; Cortisol EIA, Testosterone EIA

ORDER PRIMATES

4a. Papio anubis (Olive baboon) -- Feces, Urine, Saliva; n = 75 per habitat type; Cortisol EIA, Testosterone EIA, Oestradiol EIA, Salivary Testosterone EIA

Total samples collected: n = 2,700 fecal; n = 540 urine; n = 420 hair; n = 180 saliva

All assays performed in duplicate. Intra- and inter-assay CVs reported in Table 2.

EIA = enzyme immunoassay (IBL International, Hamburg unless specified); RIA = radioimmunoassay.