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RESEARCH ARTICLE

Previous assessments of faecal glucocorticoid metabolites in Cape mountain zebra (*Equus zebra zebra*) were flawed

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Abstract

1. Steroid hormones, especially glucocorticoids (GCs), are widely used to assess physiological responses to stressors. As steroid hormones are heavily metabolised prior to excretion, it is essential to validate enzyme immunoassays (EIAs) for measuring faecal glucocorticoid metabolites (fGCMs). Although problems with unvalidated assays have been raised repeatedly, their use persists widely.
2. Lea et al. (2017) used an unvalidated corticosterone assay (CJM006) to relate fGCM concentrations to habitat quality, demography and population performance in the Cape mountain zebra (*Equus zebra zebra*). Here, we revisit their findings and evaluate the validity of their conclusions using a validated EIA. First, we evaluate the biological sensitivity of six EIAs (three group-specific metabolite assays and three corticosterone assays, including CJM006) through a biological validation experiment (translocation) for two sub-species of mountain zebra, Cape mountain and Hartmann's mountain zebra (*E. z. hartmannae*). Second, we reanalyse the faecal extracts from Lea et al. (2017) using a validated EIA.
3. fGCM concentrations consistently increased following translocation, when using two 11-oxoaetiocholanolone (lab codes: 72T and 72a) and an 11ß-hydroxyaetiocholanolone (69a) EIA, but did not with three different corticosterone EIAs. All corticosterone EIAs (including CJM006) failed to detect an increase in fGCMs within the critical 48–72-h period post translocation. Therefore, the CJM006 EIA utilised in Lea et al. (2017) does not sensitively measure hypothalamic–pituitary–adrenal (HPA) axis activity in CMZ faeces.
4. Using a validated assay (72T), fGCM concentrations were no longer associated with adult sex ratio or habitat quality (measured by grassiness) and these variables were dropped from predictive models. fGCM concentrations now varied between seasons and were negatively associated with female fecundity (foal:mare ratio). Consequently, we can conclude that the results of the previous study are unreliable.
5. We introduce the terms "insensitive" and "sub-optimal" to categorise assays that are tested but fail validation, and assays that are comparatively poor at detecting

relevant hormone changes, respectively. We discuss how both "insensitive" and "sub-optimal" assays could lead to incorrect inferences about population stressors and counterproductive conservation recommendations.

KEYWORDS
11-oxoaetiocholanolone (72T), assay validation, conservation management, corticosterone, enzyme immunoassay, insensitive assay, sub-optimal assay

1 | INTRODUCTION

Physiological measures and biomarkers can provide mechanistic insights into how organisms respond to a dynamic landscape of stressors (Chown & Gaston, 2008). The "functional marginality" (Shultz et al., 2021) and "timeline to collapse" (Cerini et al., 2023) frameworks predict that populations in poor quality conditions should display increased biomarkers or signals associated with negative physiological status and poor fitness respectively.

The hypothalamic–pituitary–adrenal (HPA) axis has received much interest as a key pathway for stress responses (McEwen & Wingfield, 2003; Sapolsky et al., 2000; reviewed in Palme, 2019). Following exposure to a stressor, the adrenal glands release glucocorticoids (GCs): cortisol or corticosterone, depending upon the species. A short-term glucocorticoid response is adaptive as it mobilises energy and alters behaviour (Sapolsky et al., 2000). However, persistent GC elevation or depression may have deleterious effects on individual fitness (Schoenle et al., 2021). While GC levels are not a simple reflection of an organism's "stress" levels (MacDougall-Shackleton et al., 2019), they consistently indicate physiological responses to acute stressors (Shultz et al., 2021).

Enzyme immunoassays (EIAs) can measure glucocorticoids (or their metabolites) in various biological materials, such as blood, feathers, hair, tissues and faeces (Sheriff et al., 2011). Faeces are commonly used to assess HPA axis activity in mammals (Palme, 2019) as they can be collected non-invasively and provide an integrated estimate of HPA activity across the gut retention time of the species (Palme, 2019). Many EIA kits target native, that is unmetabolised steroid hormones. These native EIAs can measure GCs accurately when: (1) the native hormone is present in the biological material, or (2) the antibody has sufficient cross-reactivity with metabolites distinct to the parent hormone (Montiglio et al., 2012). Commercially available EIAs can, therefore, be a highly accessible and useful tool to measure GC metabolites when these two assumptions are valid. However, faeces can pose a problem as very little, or no, unmetabolised hormone molecules remain in excreted faeces of many vertebrates (Palme & Möstl, 1997; Palme et al., 2005) due to steroid metabolism in the liver and gut (Palme, 2019). When native EIAs are used in inappropriate contexts, they can fail to bind to relevant metabolites and do not detect relevant responses. Group-specific metabolite assays were developed to target specific metabolite groups known to occur in the faeces following steroid metabolism (Frigerio et al., 2004; Möstl et al., 1999; Möstl & Palme, 2002). Group-specific metabolite EIAs can, therefore, give a more robust, biologically meaningful approximation of fGCMs (and therefore HPA activity) in faeces following appropriate validation.

Lea et al. (2017) used a corticosterone EIA (CJM006; Watson et al., 2013) to relate glucocorticoid concentrations to habitat quality, demography and population performance in the Cape mountain zebra (*Equus zebra zebra*, CMZ). At the time of publication, several studies had used corticosterone EIAs to assess HPA activity in equid faeces (e.g. Merkies et al., 2016; Yarnell et al., 2016; Yarnell & Walker, 2017; York & Schulte, 2014). However, the validity of these studies was questioned by Palme (2019) as they lacked appropriate biological validation. There have been repeated calls outlining the importance of physiological and biological assay validation (Goymann, 2005, 2012; Palme, 2019; Touma & Palme, 2005) but validation is often not performed appropriately (Palme, 2019). Importantly, inappropriate validation is widespread (Palme, 2019) and is not unique to any specific group, taxonomic or otherwise. Liquid chromatography has shown that negligible traces of unmetabolised corticosterone remain in the faeces of ruminants or equids (Möstl et al., 1999, 2002; Möstl & Palme, 2002). Hinchcliffe et al. (2021) demonstrated that the CJM006 corticosterone EIA did not show the predicted fGCM increase following an acute stress event in horses (*Equus caballus ferus*). It is highly unlikely that corticosterone is present at appreciable levels in CMZ faeces and therefore the corticosterone results from Lea et al. (2017) may be inaccurate.

Here we re-evaluate the apparent ecological correlates of fGCMs in Lea et al. (2017) with different EIAs. We test three group-specific (72T, 72a, 69a) fGCM EIAs, which have been analytically and biologically validated for many equid species (*E. caballus*, Möstl et al., 1999; Merl et al., 2000; *E. hemionus onager*, Vick et al., 2012; *E. quagga*; Seeber et al., 2018) and three corticosterone EIAs (Arbor DetectX®, CJM006 and an in-house assay developed by Palme & Möstl, 1997, Table 1). We first compare the biological sensitivity of these six EIAs in a biological validation experiment for each sub-species, following two separate acute stress events: a translocation of five free-ranging CMZ and another of four captive Hartmann's mountain zebra (HMZ; *E. z. hartmannae*). We then use a validated EIA to re-analyse the samples used in Lea et al. (2017) and attempt to replicate the impacts of ecological and demographic factors on fGCM concentrations. We do not discuss the androgen results in Lea et al. (2017), however, this EIA is also unvalidated for testing androgen levels in CMZ faeces.

TABLE 1 Details of the enzyme immunoassays (EIAs) used. Comparison of group-specific EIAs is available in Ganswindt et al. (2003).

Enzyme immunoassay	"Native" versus group-specific EIAs	Metabolites assessed	Reference
Arbor DetectX® Corticosterone	Native	Unknown	https://www.arborassays.com/documentation/inserts/K014-H.pdf
Corticosterone (Palme & Möstl, 1997)	Native	fGCMs with an 11β,21-diol-20-one structure	Palme and Möstl (1997)
Corticosterone (Munro, CJM006)	Native	fGCMs with an 11β,21-diol-20-one structure	Watson et al. (2013)
11-oxoaetiocholanolone (lab code 72a)	Group-specific	11,17-dioxoandrostanes (11,17-DOAs)	Palme and Möstl (1997)
11-oxoaetiocholanolone (lab code 72T)	Group-specific	fGCMs with a 5β-3α-ol-11-one structure (3α,11-oxo-CMs)	Möstl et al. (2002)
11β-hydroxyaetiocholanolone (lab code 69a)	Group-specific	fGCMs with a 5β-3 α,11β-diol structure (3α,11β-diol-CMs)	Frigerio et al. (2004)

Note: "Native" EIAs identify parent hormones whereas group-specific EIAs identify a specific group of metabolites originated from parent hormone.

2 | MATERIALS AND METHODS

2.1 | Biological validation experiments with *Equus zebra*

Human handling and transportation are well-established methods for biologically validating GC assays in animals, as they dramatically increase HPA axis activity across species (Dickens et al., 2010; Palme, 2019). We predicted that if an assay is biologically reliable, fGCM concentrations should increase between 24 and 72 h post-translocation reflecting the approximate gut retention time (24–36 h) of *Equus zebra* (Steuer et al., 2011).

We used two separate management interventions to assess the relationship between translocation and measured fGCM concentrations. First, we assessed three HMZ females that were translocated to a new enclosure within a zoo. Samples were collected daily 7 days prior to the move until 7 days after the move. We also assessed fGCM concentration in a single male HMZ that was transported between zoos and introduced to the enclosure with the three females. Samples were collected for the male on the day of transportation, prior to any disturbance and every day for 7 days post-introduction to the new enclosure.

Second, five CMZ (1 male and 4 females) were translocated between two neighbouring properties (9 September 2018). Sanbona Wildlife Reserve (SWR) (33.87° S, 20.53° E) and Koktyls Private Reserve (KOK) (33.83° S, 20.67° E) are privately owned reserves within the Western Cape of South Africa, with vegetation dominated by the Fynbos and Succulent Karoo biomes. KOK and SWR are adjacent, separated by game fencing and thus are ecologically similar in terms of climate, vegetation communities, elevation and absence of predator communities. The five CMZ individuals were moved from KOK to the predator-free southern section of SWR. Individuals were darted from a helicopter, loaded onto vehicles for transportation, driven to the adjacent property and released upon waking. Following translocation, all individuals behaved similarly, quickly running to the fence line between the two reserves, staying close to the fence boundary, avoiding roads and fleeing at the sight of vehicles for the subsequent 8 days. Ethical approval for this work was granted by the University of Manchester CAT D non-licenced procedure ethics panel (ref 0031).

Faecal samples were collected non-invasively by watching the animal defecate and collecting the samples from the ground within ~30 min of deposition. Defecating animals were photographed for identification and the stripe/pattern recognition software 'hotspotter' was used to confirm individual identifications (Crall et al., 2013). Faecal samples were collected approximately 12 days prior to translocation to provide baseline GC levels of undisturbed CMZ. Faecal samples from the translocated individuals were collected 24–72 h post translocation. Each sample was thoroughly homogenised in a plastic bag and stored in a cooler at ~4°C in the field. Steroid extraction and storage (drying) were performed within 8 h of collection.

Methods of faecal collection and experimental design for the Lea et al. (2017) study can be found in the original publication. Demographic data from Lea et al. (2017) are provided in Table S1 in the Supporting Information.

2.2 | Extraction and assay selection for fGCM analysis

For the biological validation, GC metabolites were extracted from faecal samples by adding 5 mL of 80% methanol to 0.5 g of faeces (Palme et al., 2013), after which the mixture was shaken by hand for 5 min, left to settle for 30 min (following Shutt et al., 2012) and the supernatant decanted into Eppendorf tubes. All extracts were completely evaporated at 50°C using a waterbath and were stored frozen at −20°C in the field and −80°C in the laboratory.

Lea et al. (2017) extracted steroids in the field using a modified extraction technique (Edwards et al., 2014) with HyperSep™ octyl bonded silica (C8) cartridges (Thermo Fisher Scientific, UK). After initial extraction, samples were exported to the UK on cartridges, extracted and then stored as liquid extracts at −20°C until EIA analysis. We assessed the effect of extraction method and storage (HyperSep™ octyl bonded silica (C8) cartridges vs methanol extraction and drying) in our translocation samples to ensure the cartridge extraction method did not influence the trends observed in Lea et al. (2017). We describe this experiment in more detail in Text S1 in the Supporting Information. In summary, we show a strong positive correlation in concentrations measured using the different extraction and storage methods (r=0.85–0.94, Figure S1 in the Supporting Information) and so the modified extraction method did not influence the trends observed in fGCM concentration. However, storing on HyperSep™ octyl bonded silica (C8) cartridges resulted in significantly lower fGCM concentrations than drying alone although the trend following translocation did not change (Figure S2 in the Supporting Information).

Sample extracts from Lea et al. (2017) were stored for an additional ~2.5–3 years at −20°C before reanalysis. Storing liquid extracts in frozen, dark conditions is well established for long-term storage, although changes can occur after 50 weeks of storage due to evaporation (Kalbitzer & Heistermann, 2013). Authors acknowledge that storage may have influenced sample concentration. However, evaporation is unlikely to impact our findings as samples were dried and resuspended in the same volume of assay buffer as the original liquid extract before analysis. Moreover, we stored all samples in identical conditions and hence assume any changes in concentration are equivalent across samples, maintaining overall trends. Validation samples were stored dry for ~6 months at −80°C.

Immunoreactive fGCM concentrations were measured using several EIAs (Table 1). All samples were dried and redissolved in EIA buffer and stored at −20°C until analysis. EIA buffer was consistent for all metabolite specific assays, CJM006 and the in-house corticosterone EIA. The EIA buffer for Arbor DetectX® Corticosterone EIA was included in the commercial kit. Analyses were performed within 2 days of resuspension. We confirmed antibody binding of Arbor DetectX® corticosterone, CJM006, 72T and 72a EIAs via parallelism using pooled faecal extracts, which is presented in Figure S3 of the Supporting Information.

For fGCM assays, we also compared results from non-concentrated versus concentrated samples using diethyl ether and 5% sodium bicarbonate (Merl et al., 2000). We included this step because metabolite traces may be under the detection limit of the EIA. We provide the results for concentrated versus unconcentrated EIAs in Figures S4 and S5 in the Supporting Information. In short, we can conclude the diethyl ether concentration step was not necessary for CMZ faecal extracts. Details on each EIA protocol, buffer composition, labels, reagents, intra and inter assay variation is provided in the Text S2 in the Supporting Information. We do not discuss the results of the corticosterone EIA measuring fGCMs with a 11β,21-diol-20-one structure (Palme & Möstl, 1997) in the main text as several samples had concentrations below the detection limit.

2.3 | Data analysis

We tested for increases in fGCM concentrations (pre- vs. post-translocation) using one-tailed paired t-tests. For both sample sets, we calculated the percentage increase from pre-translocation baseline levels (mean of all pre-translocation samples across all individuals in free-ranging CMZ and mean of all pre-translocation samples per individual in HMZ). A summary of the descriptive statistics is available in Table S2 in the Supporting Information. We also calculated the time required for fGCM levels to return to baseline. For our sample set of translocated free ranging CMZ, we also calculated z scores (increase measured by the average number of standard deviations away from the pre-translocation mean across all individuals, Bashaw et al., 2016).

We ran mixed effect models using the *lme4* package (Bates et al., 2015) in R version 4.3.1 (R Core Team, 2023). We replicated the analysis from Lea et al. (2017) using concentrations from 11-oxoaetiocholanolone (72T) EIA and compared the results to those of the original study. In short, we assessed whether fGCM concentrations from the 72T EIA varied between populations, using reserve as a fixed effect and zebra ID and sampling trips as random effects. We then built a global linear mixed model for the 72T EIA to investigate the relative impacts of demographic and ecological factors. This linear mixed model included grass abundance, season (spring/summer/autumn), rainfall type (seasonal/non-seasonal), log transformed group size (number of individuals) and population sex ratio. We evaluated the relative predictive performance of each variable by calculating the Akaike information criterion (AIC) (Akaike, 1973) and the change in AIC after dropping each variable sequentially (ΔAIC).

We also analysed the impacts of ecological and demographic factors on fGCM concentration in males and females separately. Males and females can metabolise glucocorticoids at different rates and EIAs may cross-react with metabolites of different reproductive hormones (Goymann, 2012). We used linear mixed effect models to assess the impact of male social position (herd stallion vs. bachelor) and female's maternal state (foal at foot vs. no foal) using both 72T and CJM006 EIAs. We included individual ID included as a random effect.

Finally, we assessed the relationship between average fGCM levels (averaged across both visits) and both foal:mare ratio and population growth rate using one-tailed Spearman's rank correlation coefficient. We extended Lea et al.'s (2017) analysis with linear regression models and compared the goodness-of-fit. Foal:mare ratio and population growth rate were log-transformed for the linear regression analysis.

3 | RESULTS

3.1 | Biological validation

The three group-specific fGCMs EIAs displayed large percentage increases following the acute stressor, in contrast to three corticosterone EIAs, which did not increase consistently during (i) an introduction to a new enclosure for captive HMZ (Figure 1) or (ii) a translocation of free-ranging CMZ (Figure 2). In the zoo translocation of HMZ, faecal GCMs measured by the 69a EIA displayed the greatest average % increase and the greatest overall % (Table 2). However, 72a and 72T also displayed a strong increase in response to transportation with a peak between 1 and 4 days post translocation across all individuals (Table 2 and Figure 1). In the translocation of

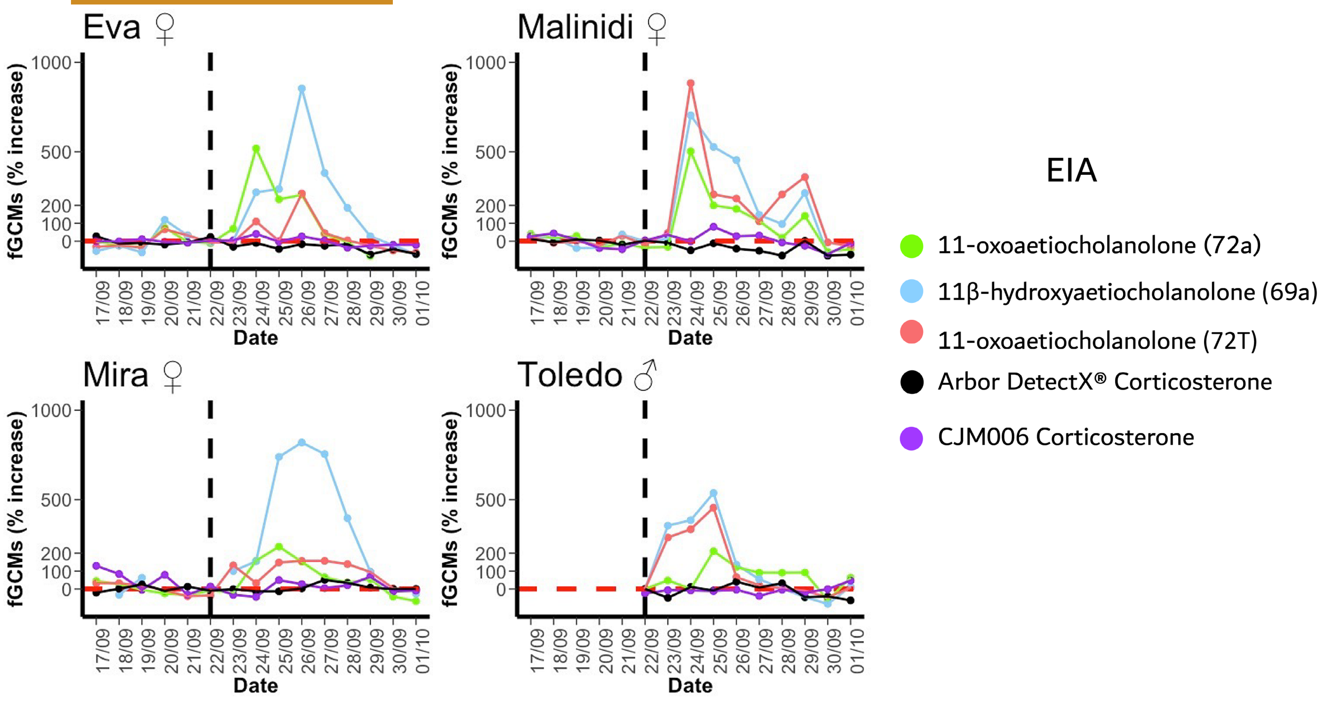

FIGURE 1 Biological validation of fGCM assays in four captive Hartmann's mountain zebra. Comparison of fGCM concentrations in four mountain zebra with their % response to an acute stressor (translocation) serving as biological validation. Name and sex of each individual is presented in top left corner of each graph. The red dashed line denotes the baseline set as the mean of all pre samples per individual. Black dashed line denotes the date of the translocation event. Baseline is estimated as the mean of the pre samples per individual. Results of the corticosterone EIA measuring fGCMs with a 11β,21-diol-20-one structure are not included in the figure as several samples had values below the detection limit and therefore were not accurately measurable.

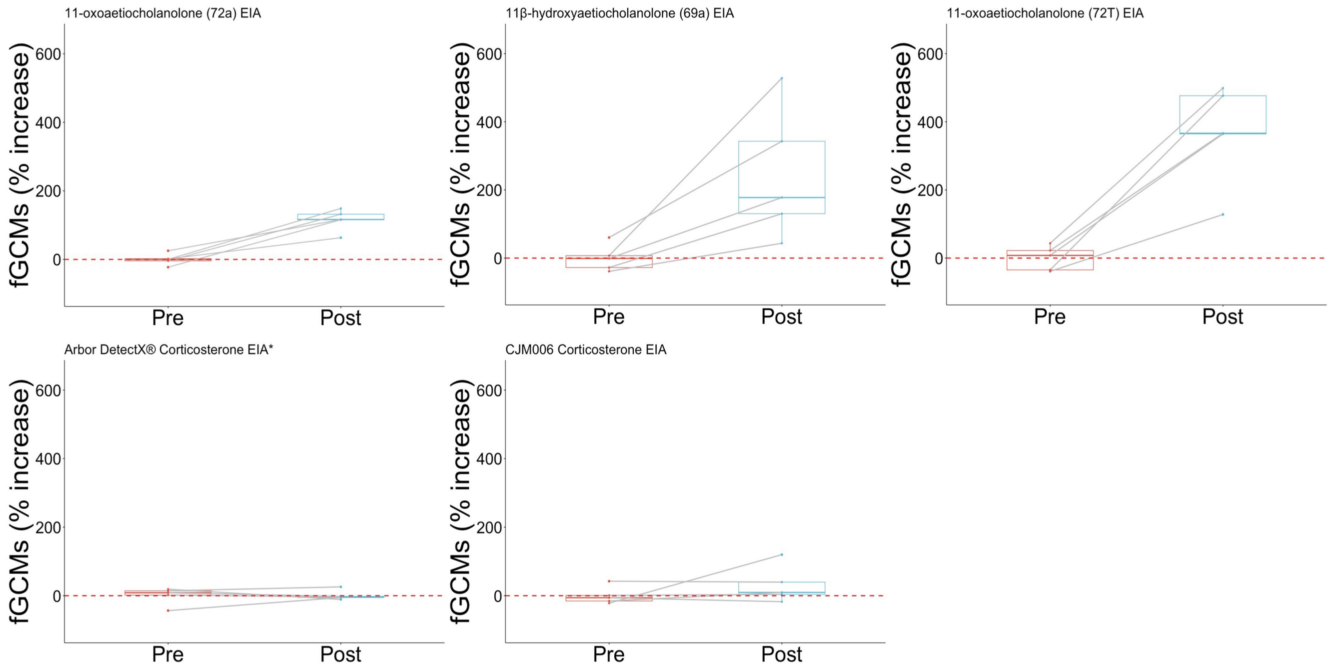

FIGURE 2 Comparison of fGCM concentrations in Cape mountain zebra per assay in response to an acute stressor (translocation) used for the purpose of biological validation. 'Pre' represents fGCM levels approximately twelve days before translocation. All 'post' samples were collected 24–72 h post-translocation. Red line denotes baseline as the mean of all pre samples (all individuals) per assay. Results of the corticosterone EIA measuring fGCMs with a 11β,21-diol-20-one structure are not included in the figure as several samples had values below the detection limit and therefore were not accurately measurable.

TABLE 2 Comparison of individual Hartmann's mountain zebra responses to transport measured by native and three metabolite-specific EIAs with methanol extraction.

EIA	Measure	Mountain zebra 1 (Eva ♀)	Mountain zebra 2 (Malindi ♀)	Mountain zebra 3 (Mira ♀)	Mountain zebra 4 (Toledo ♂)
Arbor DetectX Corticosterone, targets unknown but designed for unmetabolised corticosterone	% increase of peak in critical period	No increase	No increase	No increase	No increase
	Return to baseline from peak	No increase	No increase	No increase	No increase
CJM006 corticosterone	% increase of peak in critical period	40% (48 h post)	80% (48 h post)	No increase	No increase
	Return to baseline from peak	1 day	3 days	No increase	No increase
11-oxoaetiocholanolone, targets 11,17-DOAs (72a)	% increase of peak in critical period	519% (48 h post translocation)	503% (48 h post translocation)	237% (72 h post translocation)	212% (72 h post translocation)
	Return to baseline from peak	7 days	8 days	8 days	8 days
11-oxoaetiocholanolone, targets 3α,11-oxo-CMs (72T)	% increase of peak in critical period	109% (24 h post translocation)	884% (48 h post translocation)	148% (72 h post translocation)	455%(72 h post translocation)
	Return to baseline from critical period	7 days	7 days	9 days	4 days
11ß-hydroxy-aetiocholanolone, targets 3α,11β-diol-CMs (lab code 69a)	% increase of peak in critical period	291% (48 h post translocation)	704% (24 h post translocation)	738% (72 h post translocation)	537% (72 h post translocation)
	Return to baseline from peak	7 days	8 days	8 days	5 days

Note: Critical period denotes 24–72 h following translocation and is the species-specific range which where we would predict a peak in fGCM concentration. We calculated %increase in critical period using ($X_{\text{highestincriticalperiod}}/M_{\text{pre}}X_{\text{starting sample}}$). *X* denotes concentration and *M* denotes Mean.

free-ranging CMZ, fGCM concentrations measured by the 72T EIA showed the greatest average increase and highest *Z* score between pre- and post-translocation values ($t = 6.2$, 95% CI = 37.6 to inf, average increase = 397%, z score = 11.2, $p = 0.002$), followed by 69a ($t = 3.2$, 95% CI = 13.8 to inf, average increase = 245%, z score = 5.0, $p = 0.02$) and 72a ($t = 6.9$, 95% CI = 4.5 to inf, average increase = 115%, z score = 4.4, $p = 0.001$, Figure 3).

The fGCM levels from the Arbor DetectX and CJM006 corticosterone EIAs did not significantly increase post-translocation, after either an enclosure change or translocation (Figures 1 and 2). The in-house corticosterone EIA measuring fGCMs with a 11β,21-diol-20-one structure did not detect fGCM concentrations above the EIA detection limit and so metabolite levels were too low to be measured accurately. The fGCM concentrations assessed with three group-specific EIAs (72T, 72a, 69a) were highly correlated ($r = 0.5$–0.83), whereas none were significantly correlated with the DetectX corticosterone or CJM006 EIAs ($r = -0.11$–0.28; Figure S6 in the Supporting Information).

As none of the corticosterone EIAs demonstrated a consistent increase to either acute stress event, we conclude that they do not measure HPA axis activity in CMZ faeces. As we do not know which steroid hormones or metabolites are binding to the corticosterone EIAs, we refer to concentrations from these EIAs as unspecified "faecal steroid" concentrations from this point onwards. Concentrations from validated assays are referred to as fGCMs as they are sensitive to HPA axis activity.

3.2 | Ecological and demographic predictors change using a validated EIA on Lea et al.'s samples

We reanalysed samples from Lea et al. (2017) with the 72T EIA, because it showed the greatest average increase and highest Z score in the CMZ translocation. The ecological and demographic predictors of elevated fGCMs changed substantially between the assays. When controlling for individual and sampling season, we found fGCMs measured by the 72T EIA varied between reserves ($F_{6,159} = 13.96$, $p < 0.001$), as did unspecified faecal steroid hormone concentrations assessed using the CJM006 corticosterone EIA ($F_{6,159} = 10.57$, $p < 0.001$). However, each reserve changed relative position when ranked by concentration between CJM006 corticosterone and 72T EIAs (Figure S7 in the Supporting Information).

Habitat grassiness was a strong predictor for unspecified faecal steroid concentrations (CJM006 EIA) across all individuals (Table 3),

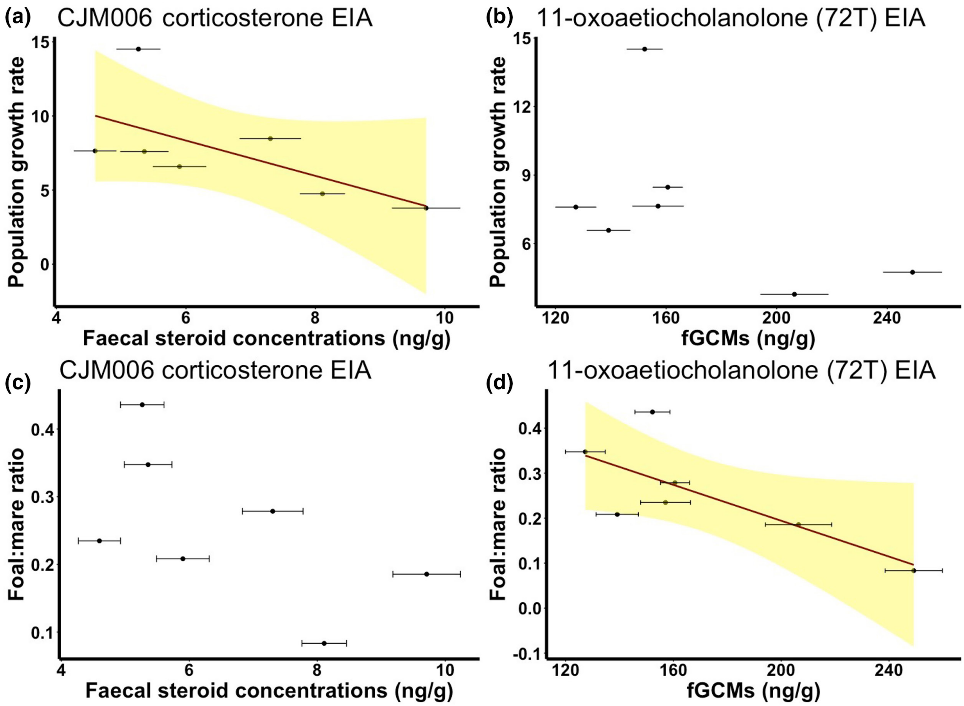

FIGURE 3 Unspecified steroid concentrations from the insensitive CJM006 corticosterone EIA and fGCMs assessed with the 72T EIA are not consistently associated with Cape mountain zebra population growth rate (a, b) and foal:mare ratios (c, d). Error bars represent ± SE of mean. Trend lines and error are displayed for significant linear regression.

but was not a strong predictor (ΔAIC < 2, p > 0.05) for fGCMs (72T EIA) across all samples or when assessing females and males separately (Table 3). Sex ratio was an important predictor for unspecified faecal steroid concentrations (CJM006 EIA), but was a poor predictor (ΔAIC < 2, p > 0.05) of fGCM concentrations (72T EIA) in all models (Table 3).

3.2.1 | Males

Ecological and demographic drivers of unspecified steroid concentrations (CJM006 corticosterone EIA) and fGCMs (72T EIA) were not the same in males (Table 3). In males, unspecified faecal steroid concentrations (CJM006 EIA) were high in low grassiness habitats (Lea et al., 2017). This effect was not found in fGCMs (72T EIA; Table 2). Although including group size improved model fit (ΔAICc >2), group sizes did not significantly impact fGCMs (72T EIA) in males (Table 3). Group size did not improve model fit and was non-significant for unspecified steroid concentrations (CJM006 EIA).

Male fGCMs (72T EIA) were lower in sites with summer rainfall (Table 3, Figure S8 in the Supporting Information), and varied with male social position (bachelor vs herd stallion) ($F_{1,90}$ = 10.3, p = 0.002). On average, herd stallion males had higher levels of fGCMs than bachelors when using the 72T EIA (Figure S9 in the Supporting Information). Unspecified faecal steroid concentrations (CJM006 EIA) did not vary between male social position ($F_{1,90}$ = 0.006, p = 0.94).

3.2.2 | Females

Ecological and demographic drivers of unspecified steroid concentrations (CJM006 EIA) and fGCMs (72T EIA) were also not the same in females (Table 3). In females, unspecified steroid concentrations (CJM006 EIA) were high in low-grassiness habitats. This effect was not found for fGCMs (72T EIA), although grassiness improved model fit (ΔAICc >2; Table 3). In females, unspecified steroid concentrations were higher in high male sex-biased populations, again this effect was not found for fGCMs (72T EIA; Table 3).

Females had elevated fGCM (72T EIA) concentrations compared to males, while sex had no effect on unspecified steroid concentrations (CJM006 EIA; Table 3). Female fGCM (72T EIA) concentrations were lowest in summer compared to spring and autumn (Table 3).

3.3 | fGCM concentrations (72T EIA) decline with foal:mare ratio, but not population growth rate

Using the 72T EIA, fGCMs were negatively correlated with female fecundity (foal:mare ratio; ρ: −0.68, S = 94, p = 0.055), but not with long-term population growth rate (ρ: −0.39, S = 78, p = 0.20, n = 7; Figure 3).

Using linear regression, average unspecified faecal steroid concentrations (CJM006 EIA) were lower in populations with high growth rate but were unrelated to foal:mare ratio (β = −0.18 ± 0.069, t = −2.5, adjusted R^2 = 0.48, p = 0.05, and

TABLE 3 Linear mixed effect model coefficients AIC change with sequentially dropping terms and *p* values for the 72T and CJM006 corticosterone assays.

	Factor	Categories	72T	CJM006	ΔAIC 72T	ΔAIC CC	*p* value 72T	*p* value CC	Model changes
All individuals	Season	Spring-Autumn	−0.27 ± 0.37	0.33 ± 0.09	21.50	41.17	0.001	<0.001	Sign Change
		Summer-Autumn	−2.94 ± 0.62	−0.411 ± 0.14					
	Grassiness		−1.28 ± 2.12	−1.93 ± 0.42	1.70	18.58	0.55	<0.001	Factor dropped—AIC and *p* value
	Rainfall seasonality	Aseasonal-Summer	0.15 ± 1.07	0.69 ± 0.22	6.24	7.21	0.03	<0.001	No change
		Aseasonal-Winter	−1.91 ± 0.72	−0.43 ± 0.14					
	Sex ratio		−1.19 ± 10.01	−0.20 ± 0.10	0.78	−1.01	0.137	0.05	Factor dropped *p* value
	Group size		−1.19 ± 10.01	0.001 ± 0.20	1.24	−3.36	0.25	0.99	N.S.
	Sex		−0.99 ± 0.36	−0.04 ± 0.07	5.48	−5.11	0.01	0.52	Factor added—AIC and *p* value
Males	Androgens		3.16 ± 0.92	0.66 ± 0.22	45.29	10.06	0.001	0.004	AIC change
	Season	Spring-Autumn	0.66 ± 0.52	0.31 ± 0.10	6.43	6.61	0.06	0.013	Significance lost
		Summer-Autumn	−1.34 ± 0.89	−0.15 ± 0.17					
	Grassiness		−0.66 ± 2.73	−1.35 ± 0.55	1.90	4.46	0.8	0.017	Factor dropped—AIC and *p* value
	Rainfall seasonality	Aseasonal-Summer	−1.20 ± 1.36	0.29 ± 0.27	13.77	−4.63	0.001	0.059	Factor added—AIC and *p* value
		Aseasonal-Winter	−3.16 ± 0.92	−0.22 ± 0.18					
	Sex ratio		0.54 ± 0.66	−0.24 ± 0.13	−0.34	−0.89	0.42	0.22	N.S.
	Group size		−1.81 ± 1.13	0.29 ± 0.24	2.53	−1.53	0.11	0.38	Sign change, Factor added AIC
Females	Season	Spring-Autumn	−0.75 ± 0.52	0.42 ± 0.13	19.52	33.97	0.001	<0.001	Sign change. AIC change
		Summer-Autumn	−3.93 ± 0.81	−0.57 ± 0.20					
	Grassiness		−0.09 ± 3.10	−2.14 ± 0.60	2.09	10.69	0.98	0.001	Significance lost. AIC change
	Rainfall seasonality	Aseasonal-Summer	0.59 ± 1.56	0.97 ± 0.32	0.77	3.64	0.93	0.011	Factor dropped—AIC and *p* value
		Aseasonal-Winter	−0.23 ± 1.10	−0.33 ± 0.21					
	Sex ratio		0.66 ± 0.79	−0.34 ± 0.15	0.05	1.04	0.41	0.028	Significance lost
	Group size		0.46 ± 1.95	−0.37 ± 0.38	1.22	−1.14	0.82	0.355	N.S.

Note: Yellow shading indicates substantial changes to model using validated assay (relative coefficients have changed direction (Sign change), factors have been added or dropped or the AICc has changed by more than two). Grey shading highlights little change between assays. No shading is where factors were neither significant nor impacted on AIC.

$\beta = -0.17 \pm 0.11$, $t = -1.6$, adjusted $R^2 = 0.20$, $p = 0.17$, respectively, $n = 7$). Average fGCM concentrations (72T EIA) were lower in populations with high foal:mare ratio but were not related to population growth rate ($\beta = -0.01 \pm 0.0029$, $t = -3.6$, $R^2 = 0.67$, $p = 0.015$ and $\beta = -0.006 \pm 0.003$, $t = -1.86$, $R^2 = 0.29$, $p = 0.12$, respectively, $n = 7$).

4 | DISCUSSION

We took advantage of two translocation events and the wide variety of available EIAs to test whether Lea et al. (2017) had used a valid EIA and if not, whether their results could be replicated with a valid EIA. Of the six assays tested here, the three group-specific assays (72T, 72a, 69a) demonstrated an acute stress response to translocation. In contrast, none of the corticosterone assays (including CJM006) detected an increase in fGCMs within the predicted timescale, despite animals being tranquilised, moved and handled upon release. Corticosterone EIAs are likely insensitive because there is very little or no unmetabolised corticosterone in equid faeces following steroid metabolism (Möstl et al., 1999). We demonstrate that the CJM006 EIA does not measure biologically meaningful changes in fGCMs in Mountain zebra faeces and thus the results of Lea et al. (2017) are unreliable. The authors wish to note that any EIA whether commercial or in-house, group-specific or native can be a useful scientific tool when used in the correct context. For example, native corticosterone assays can out-perform group-specific assays in some contexts and in some species (see Benhaiem et al., 2012; Goymann et al., 1999). If an EIA is not valid in one species, it does not mean that it is inappropriate for another. Thus, it is critical to ensure that there is sufficient validation of an assay on the focal species or at the very minimum from closely related species.

4.1 | Ecological and demographic predictors change with a validated assay

We could not replicate two major findings from Lea et al. (2017): fGCM concentrations from the 72T assay did not vary with either habitat quality (grassiness) nor demography (adult sex ratio). Seasonal effects remained significant but changed direction of effect. We cannot be sure what the CJM006 corticosterone EIA is binding to so we can only speculate what drives the few similar patterns between assays. Given that native steroid hormones and their metabolites have closely related molecular structure, cross-reactivity between native assays and steroid metabolites may occur across biologically distinct pathways. Hinchcliffe et al. (2021) demonstrated insensitive 'native' corticosterone EIAs may cross-react with faecal metabolites from reproductive physiology pathways. Hence, associations between unspecified faecal steroid hormones from CJM006 EIA may be due to cross-reactivity with metabolites from completely different physiological pathways.

The elevated fGCM levels (72T) found in herd stallions may be due to the increased social stress of maintaining, or being part of, a breeding group. Our results suggest larger group size may decrease HPA activity in males, as group size improved model fit when using the 72T EIA.

4.2 | Glucocorticoids as a biomarker of population health and resilience

Lea et al. (2017) found negative relationships between unspecified steroid hormone concentrations (CJM006 EIA), foal:mare ratio and population growth rate. We also found that fGCM concentrations (72T EIA) were negatively correlated with foal:mare ratio, but not with population growth rate. However, a similar relationship in both EIAs does not demonstrate that they measure the same physiological activities. If the CJM006 corticosterone EIA is binding non-specifically, the measured concentrations may be a 'composite' of a range of physiological functions that *could* inadvertently measure something equivalent to allostatic load. However, until we determine which metabolites the EIA is binding to and which physiological processes it can predictably measure, the EIA cannot be used to make meaningful inferences about the physiological state of CMZ. We can also conclude that the associations between population performance and unspecified steroid hormone concentrations found in Lea et al. (2017) were not due to differences in HPA axis activity as initially suggested.

Moreover, the mechanistic relationship between fGCMs, ecological challenges and population performance are also difficult to disentangle. GCs have complex effects on processes such as metabolism (Sapolsky et al., 2000), immune function (Cain & Cidlowski, 2017), reproduction (Dulude-de Broin et al., 2020), cognition, behaviour (Packard et al., 2016; Raulo & Dantzer, 2018) and development (MacLeod et al., 2021). GC concentrations have been associated with long-term population dynamics and fitness (Bonier et al., 2009), but the mechanism which drives this may be complex and multifaceted. An increase in circulating GCs is not necessarily an indication of a compromised physiological state (Romero & Ursula, 2022) and chronic GC responses may only evolve in species when they increase fitness (Boonstra, 2013). However, these complex effects also mean that GCs can potentially provide broad-brush insights into physiology, life history and behavioural responses to environmental perturbations (Boonstra, 2005; Crespi et al., 2013). We recommend that future studies use multiple validated biomarkers to identify mechanistic associations between population performance and environmental challenges (Shultz et al., 2021).

4.3 | Insensitive and sub-optimal assays

Our results demonstrate the importance of assay validation. Assay validation is essential and should be a prerequisite for all non-invasive studies of HPA axis responses (Palme, 2005). ACTH and dexamethasone challenges, which directly stimulate or suppress the HPA axis activity, are the gold standard physiological validations (Touma & Palme, 2005). If an EIA does not detect an increase or decrease in measured metabolites following these interventions, it is clearly not appropriate. However, these physiological challenges require invasive procedures and invoke an intense response. It is also vital to ensure that the assay is biologically sensitive enough to measure a relevant level of response (Palme, 2019) as an EIA that shows a mild to moderate increase to an ACTH challenge may not be sensitive enough to detect ecological stressors. Biological validation, where animals experience a known, acute stress event (such as capture, translocation and handling), can provide direct evidence that an assay is sensitive enough to pick up 'real world' stressors (Touma & Palme, 2005). Importantly, analytical validation demonstrating repeatability, precision and appropriate dilution curves does not indicate that the assay is measuring compounds or physiological processes of interest (Palme, 2019). For example, a common misconception is that a parallelism is a sufficient validation, but a parallelism only demonstrates a dose–response relationship that is, that when more of a metabolite is present (a higher dose) the assay detects a higher level (a higher response; Möstl et al., 2005). We demonstrated that an assay could show a "successful" parallelism but fail biological validation (as seen in the CJM006 and DetectX corticosterone EIA; Figure S3, see Fanson et al., 2017 for similar findings). Assay validation can be time consuming and logistically challenging, however it is essential to prevent non-interpretable results or false conclusions.

The previous study on CMZ (Lea et al., 2017) is far from the only study to use an unvalidated assay. Palme (2019) collated a comprehensive list of 1329 studies evaluating GC concentrations and found that ~37% of the studies lacked adequate physiological or biological validation. Five years following its publication, despite numerous citations and an additional ~750 papers collated (now 2105 papers), the proportion of studies using unvalidated methods has not improved (38%; Palme, 2023). Poor assay validation is not unique to GC EIAs (see Pribbenow et al., 2016 for the importance of validation for assays assessing male reproductive hormones in faeces). Hence, despite numerous publications on the importance of validation (e.g. Goymann, 2005, 2012; Palme, 2019; Touma & Palme, 2005), there remains a consistent validation problem in the field of wildlife endocrinology.

Currently, the term 'unvalidated' denotes both untested assays and assays which have been (later) tested but fail biological validation (such as the CJM006, Arbor DetectX® and in-house corticosterone EIAs used in this study). Currently, there is also no term that distinguishes between the biological sensitivity of validated assays. We believe this could confuse the literature and does not convey the importance of validation and optimal assay choice. We suggest using 'insensitive' to refer to an assay that has been tested but failed validation and 'suboptimal' to refer to an assay which is valid but has comparatively poor biological sensitivity. *Unvalidated* would then only be used for untested assays.

4.4 | Impacts of insensitive assays: A cautionary tale

From a conservation perspective, using insensitive assays may result in inappropriate recommendations. Glucocorticoids have been linked to major areas of conservation interest such as physiological effects of invasive species (Santicchia et al., 2018), human impacts on animal populations (Rehnus et al., 2014) and the impacts and effectiveness of conservation interventions (Shultz et al., 2021). Lea et al. (2017) recommended reducing apparent social stress by removing males and balancing adult sex ratios. Our validated and sensitive 72T assay, however, did not find evidence that sex-biased populations had higher fGCM concentrations, so this recommendation is no longer justified. Some recommendations made by Lea and colleagues, such as reserve expansion and translocation of individuals into better quality habitat, are supported by other empirical data such as demographic (Kerley et al., 2020; Lea et al., 2016), niche and diet analyses (Britnell et al., 2024). However, elevated HPA axis activity cannot be used as evidence for these recommendations and cannot provide a physiological explanation for the mechanism. More broadly in a conservation context, if habitat suitability is estimated based on insensitive assays, conservation mistakes can be made, particularly given the widespread incorrect perceptions of species ecologies (Britnell et al., 2021).

Lea et al. (2017) conducted the experiments and assay selection in good faith, based on available recommendations. Their findings and interpretations were also published in good faith. Although, the importance of assay validation was highlighted in the literature, it only became clear after publication that an insensitive assay had been used and previous results were potentially unreliable. Going forward, all users and developers of assays for biomarkers must ensure adequate biological validation to prevent similar occurrences in the future. As conservation science has both biological and social aspects, scientists should adapt to emerging best practice as it is developed. This could include publishing or disclosing all validation results, comparing multiple assays to identify the most appropriate and revisiting findings if more sensitive assays are identified, as done here.

5 | CONCLUSIONS

Our findings show that the CJM006 EIA, and corticosterone EIAs in general, do not assess short-term HPA axis activity from faecal material in mountain zebra. Hence, the results of Lea et al. (2017) are inaccurate. We, like many before us, highlight how critical assay validation is and discuss potential impacts of using insensitive assays. We hope this contribution acts as a case-study and cautionary example for others in wildlife endocrinology and urges conservation scientists to apply only validated assays when developing evidence-based conservation management or policy.

AUTHOR CONTRIBUTIONS

J.A.B, and S.S. analysed the data for the manuscript. R.P. provided substantial expertise on the assay selection. J.A.B. performed hormone extractions and sample preparation for reanalysed samples under the supervision of R.P. Metabolite assays were performed in laboratory of R.P. J.A.B. collected samples for biological validation. R.P. performed assays for biological validation experiment except for the CJM006 and Arbor DetectX corticosterone EIA which were performed by J.A.B. J.A.B. and S.S. wrote the initial draft. R.P., G.I.H.K., S.S. provided substantial input on draft structure and writing. All authors contributed critically to the revisions of the manuscript. All authors agreed to submission.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data and code to regenerate results are available on Dryad at doi: https://doi.org/10.5061/dryad.vq83bk42d and on Zenodo at: https://doi.org/10.5281/zenodo.12582244.

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DATA SOURCES

Britnell, J. A., Palme, R., Kerley, G. I. H., Jackson, J., & Shultz, S. (2024). Data and code for: Previous assessments of faecal glucocorticoid metabolites in Cape Mountain zebra (*Equus zebra zebra*) were flawed. *Dryad*. (Forthcoming). https://doi.org/10.5061/dryad.vq83bk42d

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1. Descriptive statistics of sex ratio, foal:mare ratio and population growth rate of Cape mountain zebra reserve. Statistics for habitat grassiness is available in Lea et al., 2016.

Table S2. Descriptive statistics of fGCM response in free-ranging Cape mountain zebra following translocation for each enzyme immunoassay and diethylether concentration step.

Text S1. Impacts on concentration and correlations of storage.

Text S2. Further Information on the enzyme immunoassays.

Figure S1. Correlation between three metabolite enzyme immunoassays across both sample sets using dried and cartridge storage techniques.

Figure S2. Faecal glucocorticoid metabolite (fGCMs) responses of two mountain zebra to transport as measured by the three different group-specific enzyme immunoassays (EIAs) with different storage protocols.

Figure S3. Parallelism for two 11-oxoaetiocholanolone (72a and 72T), CJM006 Corticosterone and Arbor DetectX® Corticosterone EIAs.

Figure S4. Faecal glucocorticoid metabolite (fGCM) percentage increases of four mountain zebra to transport as measured by 5 different enzyme immunoassays (EIA including concentration after diethylether extraction marked with *).

Figure S5. Biological validation of fGCM assays in free-living mountain zebra following translocation.

Figure S6. Correlation matrix of metabolites across both sample sets.

Figure S7. Boxplot graphs of concentrations of unspecified faecal steroid metabolites from the CJM006 corticosterone EIA and fGCMs assessed by the 11-oxoaetiocholanolone (72T) EIA across reserves.

Figure S8. Cape mountain zebra unspecified steroid hormone concentrations from CJM006 corticosterone EIA (left) and fGCM concentrations (right) from 11-oxoaetiocholanolone (72T) EIA showed seasonal variation and differed depending on the timing of peak rainfall.

Figure S9. Cape mountain zebra bachelor and stallion faecal glucocorticoid metabolite concentrations from validated 11-oxoaetiocholanolone (72T) EIA.

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