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A simplified method for monitoring progestagens in African elephants under field conditions

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Summary

1. Hormone analyses are frequently used to support management of wildlife; however, current techniques are not very field-friendly. *In situ* hormone monitoring is often expensive, time consuming and logistically difficult. Thus, a new method for assessing ovarian cycle activity non-invasively in free-ranging African elephants was developed.

2. The technique involves handshaking faecal samples in common organic solvents, use of environmentally stable antibody-coated microtitre plates and assessment of progestagen concentrations based on a visual colour change.

3. Studies using *ex situ* African elephants determined that handshaking faeces in a solution of isopropyl alcohol was effective for extracting the faecal progestagens (efficiency $> 90\%$).

4. Antibody-coated plates were stable for up to 3 months under a range of temperatures (4 to > 38 °C) and the resulting faecal oestrous cycle progestagen profiles corresponded significantly to those of serum ($r = 0{\cdot}89$, $P < 0{\cdot}01$).

5. This field-friendly technique provided qualitative hormone data without the need for expensive equipment. Although developed for progestagen analyses in elephants, this approach should be adaptable to other steroids in a myriad of species. As such, it could facilitate how hormones are measured in species under field conditions and provide new tools for making sensible conservation management decisions.

Key-words: enzyme immunoassay, faeces, hormone extraction, *Loxodonta africana*, non-invasive, progestagens

Introduction

Hormone analyses are frequently used to support *in situ* and *ex situ* management of wildlife. Although measuring circulating, free steroid concentrations provides a clear snapshot of endocrine function, longitudinal collection of blood can be difficult to obtain if animals are not properly trained (Graham *et al.* 2001; Monfort 2003). Blood collection is particularly problematic from free-ranging wildlife as it requires anaesthesia or physical restraint, which is impractical for longitudinal analyses (Kirkpatrick *et al.* 1993; Brown & Wildt 1997; Whitten, Brockman, & Stavisky 1998). Additionally, the stress of physical manipulation could alter circulating hormone concentrations and create a false picture of the underlying physiological state of the animal (Lasley & Kirkpatrick 1991; Monfort 2003). As an alternative, steroid metabolites excreted in urine or faeces can provide a non-invasive measure of hormone activity without having to disturb the animal (Lasley & Kirkpatrick 1991; Monfort 2003). Moreover, urine and faeces provide a 'pooled' measure of the gonadal and/or adrenal status of the animal, dampening the pulsatile and diurnal fluctuations of many circulating hormones (Monfort 2003). Lastly, faecal and urinary hormone metabolites are frequently higher than circulating concentrations, which facilitates the development of non-instrumented (or 'field') kits (Lasley & Kirkpatrick 1991). In field settings, faeces are typically easier and less risky to collect than urine (Wasser *et al.* 1996; Graham *et al.* 2001; Monfort 2003). The ease of faecal collection facilitates longitudinal (even daily) analyses, which enhance statistical power when monitoring hormone activity such as oestrous cycles and pregnancies (Monfort 2003).

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While hormone monitoring is a useful conservation tool, sample processing and assay techniques often utilize specialized and expensive equipment (Kirkpatrick *et al.* 1993). These factors limit the facilities where hormone analyses can take place, especially in range countries (MacDonald *et al.* 2008; Santymire & Armstrong 2009), and make it nearly impossible to do on-site endocrine assessments in the field. Thus, field researchers must send samples to off-site laboratories and wait weeks or months for results. In addition, for many species there are restrictions and extensive protocols that must be followed before urine, faecal and blood samples can be exported from overseas (e.g. to control for diseases like foot and mouth). If assay techniques were simplified and made more 'field-friendly', real-time qualitative results could be generated without the need to ship samples elsewhere for analysis. This would greatly expand the types and scope of studies conducted *in situ*, and facilitate management of species in range countries without endocrine laboratories.

Endocrine techniques are frequently used as a tool to monitor the physiological state of *in situ* (e.g. Poole *et al.* 1984; Hodges *et al.* 1997; Ganswindt *et al.* 2005; Wittemyer, Ganswindt, & Hodges 2007; Rasmussen *et al.* 2008) and *ex situ* (see review: Brown 2000) elephants. As early as 1984, Poole *et al.* (1984) investigated musth in male African elephants by measuring testosterone in urine, and so was the first to apply non-invasive endocrine techniques to free-ranging wildlife (Lasley & Kirkpatrick 1991; Monfort 2003). Endocrinology continues to play a role in conservation-related issues as they apply to elephants, including, but not limited to, the needs for population control in some areas (Fayrer-Hosken *et al.* 2000; Delsink *et al.* 2006; Kerley & Shrader 2007; Kirkpatrick 2007). Conservation managers could benefit from a quick endocrine test that provides information on the pregnancy status of elephants and/or the effectiveness of contraceptive techniques (Lasley & Kirkpatrick 1991). Although field methods have been developed for extracting hormones from faeces of wildlife (MacDonald *et al.* 2008; Santymire & Armstrong 2009), they have not been tested on elephants and do not address the stability of enzyme immunoassays (EIAs) in the field.

The oestrous cycle of the elephant is 14–16 weeks in duration, with an 8- to 12-week luteal phase and a 4- to 6-week follicular phase (Plotka *et al.* 1988; Hodges 1998; Brown 2000). The cycle is characterized by assessing luteal activity; however, the elephant is unique among mammals in that the major circulating luteal steroid is not progesterone as in other mammals, but reduced pregnanes like 5α-pregnane-3,20-dione and 5α-pregnane-3-ol-20 one (Hodges *et al.* 1997; Brown 2000). Fifty-five percent of metabolized progestagens in elephants are excreted in faeces (Wasser *et al.* 1996) and faecal progestagen concentrations closely correspond to serum analyses with a 2-day lag time (Wasser *et al.* 1996; Graham *et al.* 2001).

This study describes the development and validation of a field progestagen EIA using matched serum and faecal samples collected from *ex situ* African elephant (*Loxodonta africana*) females. The goals were to create field-friendly methods that (i) used simple faecal extraction methods and locally available solvents and (ii) produced real-time progestagen results derived from qualitative determination of progestagen concentrations based on visual assessment of colour change alone. Achieving these goals would eliminate the need for sample exportation, when qualitative results will suffice, and the use of expensive instrumentation to obtain valuable hormone data. When used in conjunction with behavioural and demographic observations, these simplified, field-friendly techniques provide a powerful conservation tool for monitoring free-ranging populations of African elephants, and could therefore help to improve strategies for elephant population management and control.

Materials and methods

STUDY ANIMALS AND SAMPLE COLLECTION

For development of the field assay, matched serum and faecal (lagged by 48 h) samples were collected weekly for 6 months (i.e. two ovarian cycles) from African elephants (n = 8, 22–37 years of age) at three North American institutions. Samples also were collected from a 25-year-old elephant during the last 2 months of gestation. Once the techniques were validated in the laboratory, a blind comparison between quantitative (using a portable spectrophotometer) and qualitative hormone assessments based solely on colour change in a non-laboratory setting was conducted on seven females housed at two additional facilities. Faecal and serum samples were stored frozen until the time of analysis.

FAECAL EXTRACTION AND EIA VALIDATION

An extraction method developed for rhinoceros (MacDonald *et al.* 2008) was used as a model to create a field faecal extraction technique for elephants. The extraction efficiencies of alcohols that should be relatively easy to purchase in any country, 70% propanol (isopropyl alcohol or rubbing alcohol) and 50% ethanol (100-proof vodka), were compared to the gold standard 80% methanol (MacDonald *et al.* 2008). Faecal samples were thawed and 0·5 g of wet faeces were weighed on a Mettler-Toledo (Columbus, OH, USA) battery-powered balance (#PL202-S/00, accurate to 0·01 g) and placed into an 8 mL o-ring, plastic tube (#60·542·077; Sarstedt Inc., Nümbrecht, Germany). A total of 5 mL of alcohol solution, either 80% methanol, 50% ethanol or 70% propanol was added; tubes were capped and then shaken vigorously by hand for 30 s. The tubes were allowed to sit overnight at room temperature (23 °C). The next day, samples were shaken by hand again and allowed to settle for 5 min. A 1:100 dilution was made by taking faecal extract taken from the top of the vial and adding it to distilled water (dH_2O).

A progestagen EIA, previously validated for elephants (Graham *et al.* 2001), was modified to withstand field conditions such as heat, humidity and long-term storage. For the assay, microtitre plates (Nunc MaxiSorp, flat wells, #439454; Thermo Scientific, Waltham, MA, USA) were coated with a monoclonal progestagen antibody (1:10 000 CL425; C. Munro, University of California-Davis, CA, USA), stored at 4 °C overnight and then washed once. To enhance the storage life of the assay, a commercially available buffer (Coating Stabilizer and Blocking Buffer, #J16430D; Biodesign International, Saco, ME, USA) was added (200 μL) to each well and the plate incubated at room temperature for 30 min. Excess buffer was shaken from the plates without washing and they were placed upside down on top of a layer of drierite (#23001; W.A. Hammond Drierite Company, Xenia, OH, USA) in a sealed tub placed under vacuum for 2 h. The dried, buffered microtitre plates bound with progestagen antibody

were sealed in 3″ × 5″ Ziploc bags with two desiccant packets until used. For the assay, progesterone standards (200–0.78 pg/mL, #P0130; Sigma Aldrich, Inc., St. Louis, MO, USA) or sample (50 μL) were added to each well, followed immediately by addition of horseradish peroxidase-conjugated label (1:40 000; C. Munro). After an hour of incubation at room temperature, the plates were washed using a 0·02% solution of Tween 20 (#P2287; Sigma Aldrich, Inc., St. Louis, MO, USA) and substrated with a phosphate-citrate (#P4560; Sigma Aldrich, Inc.) and 3,3′,5,5′-Tetramethylbenzidine dihydrochloride (TMP) (#T3405; Sigma Aldrich, Inc.). Assay sensitivity was 0·78 pg/well and intra- and inter-assay coefficients of variation (CV) were < 10%.

To determine the stability of the plates under the various simulated, field conditions, the buffered/antibody-bound microtitre plates were stored at four temperatures: (i) in a refrigerator (4 °C), (ii) at room temperature (23 °C), (iii) in an incubator (38 °C) and (iv) in an automobile from June through September to simulate extreme fluctuating temperatures (15–50 + °C) that could be found in some field settings. These plates were evaluated at five time intervals: 1 day, 2 weeks, 1 month, 2 months and 3 months by comparing the shape of the standard curves between the different time and temperature treatments.

The faecal extraction and EIA methods were validated for African elephants by demonstrating: (i) parallelism between faecal progestagen profiles using the field method and serum progestagen profiles generated by a solid-phase ^{125}I-radioimmunoassay (RIA) (Coat-A-Count; Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA – Brown & Lehnhardt 1995) and (ii) significant recovery of exogenous progesterone added to faecal extracts.

QUALITATIVE HORMONE ANALYSIS BASED ON COLOUR CHANGE

The progestagen EIA was modified to provide qualitative hormone determinations based on colour change. Microtitre plates were coated and buffered as described above. The quantitative, 10-point standard curve (200–0 pg/well) was reduced to six points (100–0 pg/well). Each of the six standards has a distinct colour (ranging from nearly clear to dark blue) that corresponds to a hormonal concentration. These 'field' standards were designed to provide qualitative hormone levels (high, medium or low) without using a spectrophotometer (Fig. 1).

Accuracy of the qualitative, 'field' hormone assessment was tested by conducting blind trials for 1 week at two North American facilities. These two facilities were chosen due to the large number of females they housed (n = 7) and the possibility of capturing several different cycles, including pregnancies. Matched serum and faecal samples were collected daily. Faeces were extracted on site using 5 mL of 70% propanol (bought at a local drug store) and the hand-shaking method. Ten minutes after the phosphate citrate/TMB substrate was added to each well, a visual comparison of the colour concentration of the unknown samples was made to the standards. Unknown samples were ranked as having a high (50–100 pg/well), medium (25 pg/well) or low (0–12·5 pg/well) hormone concentration (Fig. 1). Results of the qualitative, 'field' analyses were compared to quantitative hormone values obtained using the standard laboratory assay method with the 10-point curve and a portable spectrophotometer (Graham *et al.* 2001).

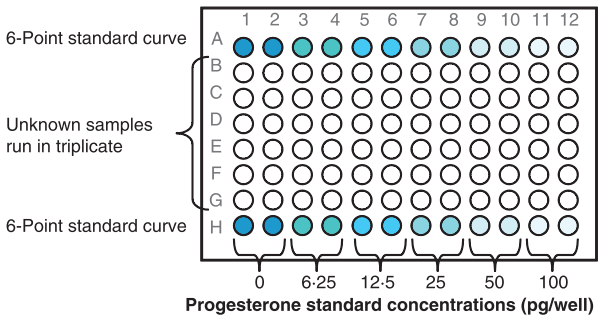

Fig. 1. Template for the qualitative, 'field' assay depicting the layout for the six standards and unknown samples run in triplicate.

EFFECT OF STORAGE ON FAECAL EXTRACT STEROID CONCENTRATION

Although the purpose of developing these methods was to produce real-time results, it may not always be feasible or even cost-effective to analyse the faecal extracts within a day or two of collection. Thus, the long-term stability of the faecal extracts was tested. Faecal propanol extracts from the week-long visits to North American facilities were stored at room temperature for 1 year. At 1, 3, 6, 9 and 12 months, the faecal samples that were stored in 70% propanol were vortexed for 30 s and a 1:100 dilution in dH_2O was made after the sample settled for 5 min. Hormone concentrations were quantified spectrophotometrically using buffered/antibody-bound progestagen EIA microtitre plates and a 10-point standard curve.

STATISTICAL ANALYSES

Coefficient of variation was used to compare standard curves measured on the buffered/bound progestagen microtitre plates that were stored under various temperatures (4 °C, 23 °C, 38 °C and fluctuating 15–50 + °C) for different lengths of time, and for extracts stored for up to a year before analysis. Pearson's product moment correlations (Sokal & Rohlf 1981) were used to compare the progestagen concentrations between the matched serum and faecal samples, while Spearman rank order correlation was used to compare the quantitative and qualitative, 'field' progestagen concentrations in the blind test. The effect of long-term storage of the faeces in 70% alcohol was determined using a two-way repeated measures (RM) ANOVA and Tukey's *post hoc* analysis comparing the progestagen concentration with respect to sample collection date and length of storage. Mean quantitative progestagen concentrations were compared to the high, medium and low qualitative designations using an ANOVA on ranks and Dunn's Method for *post hoc* analyses. The CVs were calculated using Excel 2007 (Microsoft Corp., Redmond, WA, USA), while all correlations were conducted using SIGMAPLOT (2008, v 11·0; Systat Software, Inc., San Jose, CA, USA).

Results

Faecal extractions using three different alcohols, 50% ethanol (100-proof vodka), 70% propanol (isopropyl alcohol) and 80% methanol resulted in progestagen profiles that were highly correlated: ethanol and propanol (r = 0·94, P < 0·01), ethanol and methanol (r = 0·90, P < 0·01) and propanol and methanol (r = 0·96, P < 0·01; Fig. 2). The extraction efficiencies for the methanol and propanol extracted samples were > 90%, while the efficiency for ethanol extraction ranged from 40% to 50%. Because 70% propanol is readily available in most countries and has a similar extraction efficiency to methanol, it was used in all subsequent analyses.

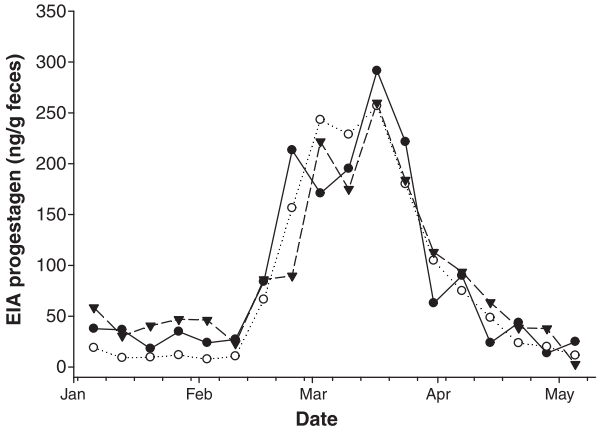

Fig. 2. Progestagen profile for a female African elephant using faecal samples extracted with 50% ethanol (●), 70% propanol (○) and 80% methanol (▼).

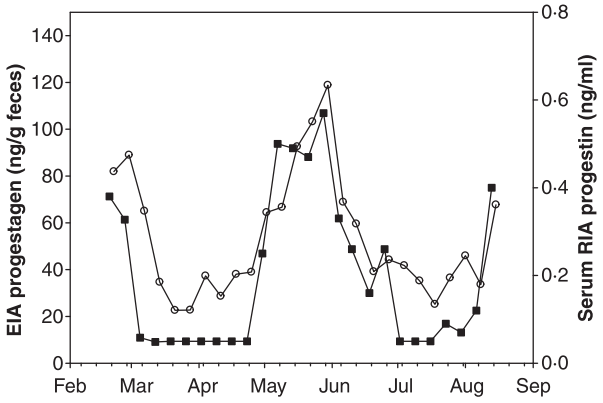

Fig. 4. Comparison of hormone profiles from an adult female African elephants. Profiles were generated from faeces (○) using 70% propanol and a precoated EIA microtitre plates stored for 1 month at room temperature, and from serum (■) using a solid-phase ^{125}I RIA.

Progestagen concentrations were measured from faecal extracts stored at room temperature and analysed after 1 day, 1 month, 3 months, 6 months, 9 months and 12 months. Hormone values did not differ with respect to the day of the faecal sample collection ($F_{5,10}$ = 0·73, P = 0·62), most likely because the daily samples were all collected within the same week. However, mean progestagen concentrations declined significantly with length of storage time ($F_{5,10}$ = 15·59, P < 0·01). The mean progestagen values did not vary between 1 day, 1 month and 3 months; however, they were significantly higher than the hormone concentrations measured after 6, 9 and 12 months of sample storage (Tukey's; Fig. 3). Additionally, there was a significant interaction between date of sample collection and length of storage ($F_{25,50}$ = 1·75, P < 0·05).

Storage conditions of the precoated EIA microtitre plates did not have a significant effect on the progesterone standard curve. Characteristics of standard curve binding remained consistent for microtitre plates stored in 3 × 5 sealed plastic bags with desiccant packs for up to 3 months (CV <15%) and under four different temperatures (CV <10%).

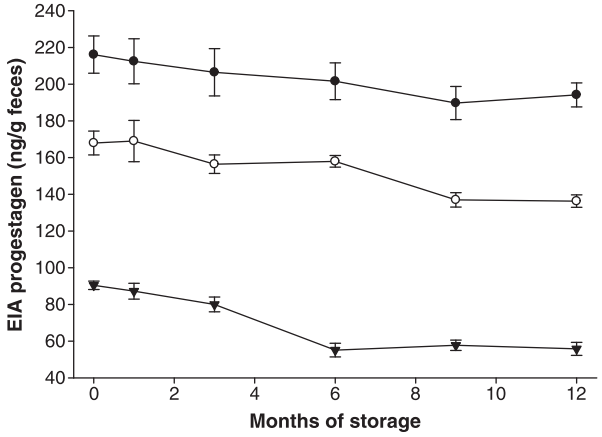

Fig. 3. Mean ± SE of progestagen concentration for a week of faecal samples (n = 7) collected from three adult female African elephants. The faeces were extracted with 70% propanol and analysed periodically for up to 12 months. Hormone concentrations measured after 1 day, 1 month and 3 months of storage were significantly higher than measurements taken after 6, 9 and 12 months of storage (two-way repeated measures ANOVA and Tukey's *post hoc* analysis).

Progestagen profiles of faecal samples extracted with 70% propanol and analysed using precoated EIA microtitre plates stored at room temperature for 1 month were positively correlated with serum progestagen profiles (r = 0.89, P < 0·01; Fig. 4).

Results from the qualitative, 'field' analysis of high, medium and low progestagen concentrations correlated with the quantitative values obtained using a spectrophotometer (r = 0·86, P < 0·01). The actual mean progestagen concentrations calculated for each sample (ng/g faeces) varied significantly (H_2 = 80·12, P < 0·01) between those identified via colour change as having high, medium and low hormone concentrations (Table 1). The dark wells had significantly lower progestagen concentrations than the medium and light collared wells (Table 1; Dunn's P < 0·05). Similarly, the medium collared wells had significantly lower progestagen concentrations than the light collared wells (Dunn's P < 0·05). In a blind, visual assessment, it was possible to successfully distinguish among

Table 1. Mean ± SE progestagen concentrations of African elephant faecal samples evaluated both quantitatively and qualitatively in a blind test using the buffered/bound progestagen EIA microtitre plates

Qualitative hormone assessment	Colour intensity	Corresponding EIA progestagen concentrations: Mean ± SE (pg/well)	Corresponding EIA progestagen concentrations: Mean ± SE (ng/g faeces)
Low	Dark	11·4 ± 0·6^a	56·5 ± 3·1^a
Medium	Medium	23·9 ± 1·4^b	116·8 ± 6·9^b
High	Light	48·7 ± 2·0^c	240·3 ± 9·4^c

Letters denote significant differences between mean values (Dunn's P < 0·05).

faecal samples collected from acyclic, cycling and pregnant African elephants.

Discussion

Using a commercial blocking buffer, we were able to develop an EIA method that does not require refrigeration of antibody-bound plates and is stable under simulated field conditions, for at least 3 months. We further report a simplified extraction technique for elephant faeces using locally purchased alcohol (70% isopropyl) and handshaking. These faecal extracts provided consistent quantitative hormone values when stored without refrigeration for up to 3 months. After that time, the quantitative values decline significantly but the qualitative results (high, medium or low) persist for at least a year. Last, a qualitative method of estimating high, medium and low hormone concentrations based on visual assessment of colour changes was developed. This rapid faecal extraction and EIA technique now offers the ability to produce real-time results in the field without the need for expensive instrumentation or sample exportation. As such, it can provide data about the reproductive state of individuals in a short enough time frame for managers to make informed decisions based on sound scientific data.

In a laboratory setting, the EIA progestagen method used previously (Graham *et al.* 2001) involves coating microtitre plates with antibodies and storing them wet in the refrigerator until they are used. Microtitre plates in this condition are stable for a short time and must be used within a week, in comparison to our method, which is stable for several months. Using these microtitre plates, visual assessment of colour changes corresponding to high, medium and low concentrations of hormone successfully distinguished between cycling, noncycling and pregnant African elephant females. Additionally, hormones are typically extracted from faecal samples that are dried to account for differences in water content and the quantity of undigested material (Wasser *et al.* 1996; Monfort 2003). The field extraction method presented here used wet faecal samples from zoo animals, which likely have a more stable diet than free-ranging elephants that face seasonal variation in food quality. However, the wet faecal hormone profiles from this study closely matched those produced by the more intensive procedures (Wasser *et al.* 1996; Graham *et al.* 2001; Ganswindt *et al.* 2002, 2003).

While techniques such as these facilitate monitoring elephant progestagen activity in the field, care needs to be taken in collecting samples that can provide relevant biological data. One shortcoming for monitoring elephants is that oestrous cycle activity cannot be readily distinguished from a pregnancy using a single faecal sample. Longitudinal, at least monthly, sample collection is necessary to distinguish between a nonpregnant luteal phase and pregnancy. While pregnancy could be detected with longitudinal sampling, field applications such as these are not appropriate for predicting parturition or rapidly determining loss of the foetus (MacDonald *et al.* 2008). The 2-day lag time for circulating progestagen concentrations to be represented in the faeces (Wasser *et al.* 1996) combined with the extraction and assay incubation time (MacDonald *et al.* 2008) limit the utility of these techniques when hormone assessments are required within less than 36 h of an event. Thus, field techniques such as these are not appropriate for all applications.

Although the techniques presented here were developed for elephants, they should be adaptable to studying a variety of other species in the field. The hormone extraction method presented in this study was adapted from one used for monitoring pregnancy in wild rhinoceros (MacDonald *et al.* 2008). This rhinoceros technique relies on using 80% methanol for extracting hormones from the faeces and qualitatively measuring hormone concentrations using microtitre plates coated with antibody in the field. We have demonstrated that faeces can be extracted with methanol as well as other solvents that may be more readily available in field settings. Additionally, our buffered microtitre plates are stable under a variety of field conditions. Field extraction methods also have been developed recently for measuring hormones in the faeces of African wild dogs (*Lycaon pictus*) using a battery operated sample homogenizer (Santymire & Armstrong 2009). The simpler, handshaking method requires less equipment to obtain similar results.

The methods presented in this paper should be adaptable to measuring faecal steroidal concentrations in a variety of species, but only if metabolites actually are excreted in faeces. For instance, these techniques would not be useful for assessing follicular activity in elephants because 95% of oestrogen metabolites are excreted in urine (Wasser *et al.* 1996). Quantitative measures of adrenal activity also may be more useful because variations in glucocorticoid concentrations often are more subtle than differences in gonadal hormone levels during oestrus and pregnancy. Under the right circumstances, field faecal extraction and EIA techniques are useful for evaluating endocrine levels in free-ranging wildlife, but only after they have been properly validated for each species (Lasley & Kirkpatrick 1991; MacDonald *et al.* 2008; Santymire & Armstrong 2009).

The relative ease and non-invasive nature of these techniques will enable wildlife managers to monitor individual reproductive status or population level reproductive rates in free-ranging populations. The interpretation of the data generated can help target conservation initiatives to optimize the use of limited resources towards the greatest gains (MacDonald *et al.* 2008). Integrating non-invasive endocrine monitoring with behavioural and ecological research enhances our understanding of the reproductive strategies (Lasley & Kirkpatrick 1991; Ganswindt *et al.* 2002), evolutionary biology (Santymire & Armstrong 2009) and life-history requirements of endangered species (Monfort 2003). Additionally, field endocrine techniques could be used in a more applied nature to determine the impact of poaching, culling and contraceptives (Wasser *et al.* 1996), human–elephant conflict and translocations (Monfort 2003) on oestrous cycle activity of free-ranging elephants. Field endocrine techniques could transform endangered species research under field conditions and provide new tools for making sensible conservation management decisions based on real-time hormone results.

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