J. KEITH HODGES AND MICHAEL HEISTERMANN

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Field endocrinology: monitoring hormonal changes in free-ranging primates

INTRODUCTION

Field endocrinology can be considered as the application of non-invasive methodologies to examine behavioural–endocrine interactions in primates living in natural conditions and social settings. In bringing together laboratory and field-based research methods, the discipline provides new and exciting opportunities for developing a more integrated approach to studies of primate behavioural ecology.

Traditionally, field studies have relied mainly on visual measures, such as behaviour and/or morphology. Although this has generated a great deal of essential information, its limitation is that interpretation of the observations is often based on assumptions concerning the physiological context in which they were made. The availability of non-invasive methodologies based on measurement of hormones in either urine or faeces now provides us with quantitative measures of physiological status by which the significance of observational data can be gauged. This greatly facilitates the testing of hypotheses concerning the adaptive significance of behavioural and morphological traits and mating systems and is helping to provide new insights into reproductive processes in an evolutionary context. Field endocrinology also facilitates a better understanding of the impact of anthropogenic activities on primate physiology. In combination with studies of the health status of wild primates (Chapter 8), this can help to elucidate the link between environmental stress, health and reproductive parameters and thus to estimate the viability of threatened populations (Pride, 2005; Chapman *et al.*, 2007).

In this chapter, we review the most important aspects fieldworkers need to be aware of when planning and carrying out

Field and Laboratory Methods in Primatology: A Practical Guide, ed. Joanna M. Setchell and Deborah J. Curtis. Published by Cambridge University Press. © Cambridge University Press 2011.

endocrinological studies on free-ranging primates. You should consult Whitten *et al.* (1998a) for a comprehensive overview of the topic of field endocrinology and its application to studies in primatology.

SAMPLE COLLECTION

Urine

Options for collecting urine samples under field conditions include (i) using sheeting, a tray or bowl to collect urine mid-stream (arboreal or semi-arboreal species); (ii) aspiration (using a syringe or pipette) or absorption onto filter paper from foliage; or (iii) recovery (usually using centrifugal force) from samples of earth. Volumes as small as 0.1 ml may be sufficient (provided further reduction during transport and storage does not occur), and more than 1 ml is generally not necessary.

Faeces

Faecal samples are usually easier to identify and locate than urine and you can collect them directly from the ground. A thumb-nail sized amount (roughly 0.5–2 g) provides more than enough material for analysis. In species where the bolus is larger, homogenize the sample on a leaf or other flat surface using your gloved hand or an improvized spatula, and transfer the required amount to the storage container (not more than 50% of total volume). If you store samples and extract them directly in alcohol, don't vary the amount of faeces collected too much, since hormone concentrations related to the mass of faeces may be influenced by the amount of the sample collected (i.e. very small samples lead to proportionately higher hormone concentrations per gram faeces compared to large samples). Avoid the inclusion of large amounts of non-faecal material such as seeds and non-digested foliage.

General points

It is essential that you only collect samples of known origin, in other words only from individuals actually seen to be urinating or defecating. This is particularly important for collection of samples from sleeping sites. It is also essential to avoid sample contamination. Cross-contamination (principally of faeces with urine) is the most likely and can be reduced partially in big samples by collecting an aliquot

from within the faecal bolus. Other sources of contamination are dilution of urine with water and sample-to-sample contamination through use of the same syringe, spatula for homogenization, glove, etc. Where you cannot use disposable materials, rinse the implement with water and wipe it dry, and avoid the use of detergents.

You need to control for time of collection. Diurnal patterns of secretion are particularly pronounced for some hormones (e.g. testosterone, cortisol). Although these patterns are likely to be more evident in urine than in faeces, they may still be seen in the faeces of certain, particularly small-bodied, species in which faecal passage rate is high (e.g. callitrichids, *Callithrix*: Sousa & Ziegler, 1998). Therefore, whenever possible, restrict the period of collection to roughly the same time of each day. If you can only collect samples opportunistically, test the potential effect of time of day on hormone concentration (e.g. by comparing hormone levels in morning and afternoon samples from the same individuals). Remember that urine and faeces, like all other body fluids and tissues from primates, are a potential source of pathogens and thus infection. Handle them with care and take appropriate precautions to reduce the risk of infection at all stages of collection, storage, transport and subsequent analysis.

SAMPLE STORAGE

Whilst most steroids (progesterone is one exception) are relatively robust and stable at ambient temperature for several days, conjugated forms (the predominant form in urine; present in variable amounts in faeces) and creatinine (the protein breakdown marker most often used to index urine concentration) are less so. Steroid breakdown due to bacterial metabolism can be a potential problem in faeces, where gastro-intestinal bacteria are abundant. Thus, in general, keep the interval from sample collection to preservation as short as possible. Immediate preservation at the site of collection is preferable, but otherwise keep the sample cool (in an insulated cool-box) until you preserve it properly after return to camp.

Urine

The principal, most commonly applied, and preferable method of long-term storage of urine is freezing (– 10 °C or below). This may limit long-term sample storage at field sites and, since repeated freezing and thawing should be avoided, also requires the use of dry ice (or ice

packs) to maintain samples in a frozen condition throughout all stages of transport (this can be expensive and certain airlines impose restrictions on transportation of dry ice). Alternative methods are (i) storage as an ethanolic solution, (ii) use of sodium azide (0.1%) as a preservative or (iii) absorption onto filter paper and dry storage. Ethanol is preferable and enables you to store samples at ambient temperatures without deterioration for several weeks. The final solution should be within the range of 30–50% ethanol, and since this must be kept constant for all samples you need accurately measured volumes of urine. One major disadvantage with the use of sodium azide is that it might interfere with the hormone assay step, if you use enzyme immunoassays. Storage of aqueous or ethanolic solutions on filter paper has been shown to be useful, but can introduce a number of potential analytical errors and needs to be very carefully controlled (cf. Shideler *et al.*, 1995).

Faeces

The most effective way of preserving faeces for prolonged periods of time is undoubtedly freezing at −20 °C. In the past, this has generally not been possible for remote field sites, but solar-powered freezers are now available. The most widely used approach for faecal sample storage under field conditions has been to use alcohol (>80% ethanol in a minimum volume to mass ratio of 2.5 : 1), one of the main advantages being that it can be applied at ambient temperature. Although ethanol has the additional advantage that you can take vials or tubes containing a known volume of ethanol into the field, enabling you to transfer samples to the preservation medium immediately after collection, several studies in primate and non-primate species have now shown clearly that this method can markedly alter faecal hormone levels during both short- and long-term storage, even when samples are frozen (see, for example, Khan *et al.*, 2002). This can potentially affect data interpretation, particularly when comparing absolute hormone levels in samples that have been stored for long and/or variable periods before analysis. This storage effect, however, appears to be both species- and hormone-specific and use of ethanol as a preservation medium may thus be less of a problem in some species and/or for the measurement of certain hormones in the species of interest (Fichtel *et al.*, 2007; Daspre *et al.*, 2009). Furthermore, when you are only interested in relative hormone concentrations within individuals, as for example in the case of day-to-day changes in progestogen levels used for timing of ovulation, a potential storage effect is unlikely to lead to

errors in data interpretation since each animal serves as its own control. For some questions, you can also control for the effect of storage on faecal hormone levels during data analysis, for example by calculating average concentrations for certain time periods (e.g. months) or from a cross-section of the study population, thereby generating residual values that can be compared (Pride, 2005). Nevertheless, whenever you plan to use alcohol as the preservative for faeces, you should take into consideration the potential effect of storage on hormone levels.

Additional potential problems with the use of ethanol include evaporation (acceptable in limited amounts) and spillage or leakage. Pay particular attention to the type of storage container used. Many are not as leak-proof as they are claimed to be (particularly when using alcohol) and sample spillage during transport will seriously affect the reliability of your results. Where possible, use screw-cap vials (10–20 ml range) and test them rigorously beforehand with ethanol. As an extra precaution, ensure that sample vials are kept upright at all times. Glass vials are more inert, but are fragile and need careful packing before transport; plastic tubes are safer to transport, although there is a small risk that steroids might stick to the wall during long storage periods.

Alternative methods to freezing and storing faecal samples in alcohol are reviewed by Ziegler and Wittwer (2005). Among these are (i) drying the samples (e.g. using a solar oven) and storing them in moisture-proof packages with desiccant until shipment to the laboratory, (ii) extracting and purifying the sample in the field using alcoholic solutions and filtration and (iii) preserving the extract on octadecylsilane (C-18) cartridges. Although these methods have been shown to stabilize steroids for prolonged times and result in 'samples' that are lighter in weight and meet the requirements for international transport of potentially infectious material better, they require more skill and equipment in the field as well as regular access to organic chemicals. Moreover, as these techniques involve several processing steps, they may result in steroid loss and higher methodological variability. Whatever method of faecal sample storage you use, you should validate it for the species in question and samples should generally be transported to a freezer or the laboratory as soon as possible.

Label sample containers properly, giving animal name/ID number and date (and time of collection, if useful). Special sticky paper labels that resist solvents and freeze–thawing processes are preferable and you can apply clear waterproof tape over the labels

as an additional precaution. Additionally, write relevant information directly on the tube or cap (preferably both), using an indelible, black, waterproof marker. Keep a separate list of samples for each animal for crosschecking purposes. Inappropriate labelling can result in loss of information on sample identity (usually during transport), rendering analysis meaningless. This continues to be one of the major causes of loss of data in this type of work, and of course you can be sure that the unidentifiable samples will always be the ones of particular importance.

LABORATORY SAMPLE PREPARATION

Urine

Steroids in urine are present predominantly in the conjugated form, either as mono- or multi-conjugated sulphate or glucuronide residues. You can measure these either directly in appropriately diluted urine or by first cleaving the conjugate by hydrolysis, followed by an assay designed specifically for the parent compound (e.g. measurement can be either as oestrone-3-glucuronide or after hydrolysis as oestrone). You can perform urine hydrolysis by incubation with an enzyme preparation (e.g. from *Helix pomatia* or *Escherichia coli*) or via a non-enzymatic procedure using organic solvents (solvolysis, see Ziegler *et al.*, 1996 for details). *Helix pomatia* (*HP*) preparations are most commonly used, although since *HP* juice contains enzymes other than glucuronidase/arylsulphatase, you should view its application with caution, particularly for urinary testosterone measurements where hydrolysis using *HP* preparations can result in steroid transformation (e.g. androst-5-ene-3β,17β-diol into testosterone; Hauser *et al.*, 2008). If you use hydrolysis, it is usual to carry out an extraction step, whereby steroids are removed into an organic phase (e.g. diethyl ether), which you reconstitute in aqueous buffer for assay after evaporation. Steroid conjugates are best extracted with aqueous alcohol or (more usually) assayed directly without an extraction step. You need a separate aliquot (usually 0.02–0.05 ml) of each sample to determine creatinine content, either using a creatinine analyser or a micro-titre plate method (see Bahr *et al.*, 2000). More recently, specific gravity measurement has been used as an alternative to creatinine for estimating urine concentration (Anestis *et al.*, 2008). Apart from avoiding potential problems with creatinine degradation, you can also easily measure specific gravity in the field by using a small battery-powered, handheld refractometer (Anestis *et al.*, 2008).

Faeces

An extraction step is always necessary to measure steroids in faeces. Various methods exist for extracting steroids from frozen–thawed faeces, the more commonly used involving agitating (shaking/vortexing) a known weight of sample with an aqueous solution (40–80%) of methanol or ethanol. Allow the suspension to settle (or centrifuge it) and take a portion of the supernatant either for assay or for further purification steps (such as re-extraction or use of Sep-Pak C-18 mini-columns). The final hormone content is expressed per unit mass (g) of wet or dry faeces after determination of the dry mass of the faecal pellet. Use similar procedures with samples stored in ethanol. In our experience, the most efficient way of dealing with these is to homogenize the sample in the original solvent (e.g. by using a metal spatula), shaking it on a vortexer for 15 min (when the amount of faeces collected is relatively small) or on a shaker overnight (when the amount of faeces is large) and, if necessary (depending on the efficiency of the procedure), re-extracting it with additional (e.g. 10 ml) 80% methanol.

Alternatively (our own preferred method), you can freeze-dry frozen faecal samples directly and then pulverize them (usually using a pestle and mortar) and sieve them through a fine wire mesh before methanol extraction (see Heistermann *et al.*, 1995a). Although the combined process is relatively time consuming, the advantages are that: (1) freeze-drying compensates for differences in faecal water content and allows you to remove non-faecal material (e.g. seeds, stones, undigested fibre) easily; (2) pulverization produces a homogeneous powder which you can pre-weigh, aliquot and store further at room temperature and (3) the extraction step is easier and generally does not require further purification steps before assay. With an efficient freeze-drier, you can process up to 150 samples simultaneously, requiring a total of about 72h.

In certain circumstances, you may need chromatographic procedures (thin layer chromatography, TLC; high performance liquid chromatography, HPLC) to detect and/or aid identification of individual steroid components of sample extracts, usually for assay validation purposes (see Bahr *et al.*, 2000).

ASSAY METHODOLOGY

Measurements of hormones and their metabolites are usually carried out by immunological procedures using hormone- or hormone-group-specific antibodies. Two main types of immunoassays are available:

radioimmunoassays (RIA), which use radioactively labelled hormone as the competitive tracer in the quantification process, and enzyme-immunoassays (EIA) in which either enzyme or biotin labelled preparations are employed. Being non-isotopic, EIAs avoid the problems associated with use and disposal of radioactivity and are also cheaper.

Since all immunoassays are highly sensitive, you need to assess assay performance carefully both during the initial set-up phase and during routine use. There are four main criteria of laboratory validation: **sensitivity** (minimum amount of hormone that can be detected); **precision** (within- and between-assay repeatability), **accuracy** (ability to detect the correct amount of hormone in the sample) and **specificity**. The latter has two components: the degree of specificity of the antibody itself and the possible influence of interfering substances excreted with urine and faeces (matrix effects), which you need to control for and remove if present by incorporating additional sample purification steps. Check carefully for the presence of such matrix effects before any routine use of an assay.

Concerning antibody specificity, specific assays may be useful when the identity of the major metabolite is known (or the hormone of interest is not heavily metabolized, e.g. oestrogens) and when you are interested in species comparisons. Since, however, excreta (especially faeces) usually contain numerous metabolites of the parent hormone, a specific measurement is often difficult and might be less useful in cases where the antibody detects only metabolites of low abundance. Group-specific assays use antibodies that cross-react with several metabolites of related structure. Since knowledge of the relative abundance of individual metabolites is not necessary, these assays have advantages in that they can usually be applied to a wider range of species (Heistermann *et al.*, 1995b, Schwarzenberger *et al.*, 1997), thus helping to overcome the problems of species specificity in hormone metabolism. When measuring faecal androgens to assess testicular function, however, group-specific antibodies may lead to problems due to the co-measurement of androgens of adrenal origin (see below).

Available data show that, in the majority of primates, the direct measurement of oestrone conjugates (E1C) and pregnanediol-3-glucuronide (PdG) is most useful for monitoring ovarian function and pregnancy by using urine analysis (see Heistermann *et al.*, 1995b for review). In macaques, you can also use the non-specific measurement of C19/C21-progesterone metabolites, such as androsterone (structurally an androgen) or 20α-hydroxyprogesterone. Urine also provides

a matrix for the measurement of polypeptide and proteohormones (e.g. FSH, LH, oxytocin, prolactin), which you cannot measure in faeces.

Information on the measurement of androgens in primate urine is limited. To date, most studies have measured immunoreactive testosterone, although this is a relatively minor component (Möhle *et al.*, 2002; Hagey & Czekala, 2003). There is some evidence that measurement of 5-reduced androstanes might reflect testicular endocrine activity better (Möhle *et al.*, 2002; Hagey & Czekala, 2003). The measurement of urinary cortisol provides a reliable method for monitoring glucocorticoid output in a variety of primate species (Whitten *et al.*, 1998b; Robbins & Czekala, 1997), but the more abundant 5β-reduced cortisol metabolites with a 3α-hydroxy,11-oxo and 3α,11β-dihydroxy structure might be more suitable, at least in some species (see Bahr *et al.*, 2000).

The most appropriate assays for the measurement of oestrogen and progesterone metabolites in faeces are shown in Table 20.1. Assays for oestradiol-17β or oestrone (or a collective measurement of both) are generally useful for monitoring ovarian function and pregnancy, but oestrogen measurements in faeces appear to be unreliable in some species. The majority of progestogen measurements have been based on the use of non-specific progesterone and pregnanediol assays cross-reacting with a broad range of pregnanediones and hydroxylated pregnanes, which are known to represent abundant progesterone metabolites in the faeces of most mammals, including primates (Schwarzenberger *et al.*, 1997). Which assay reflects female reproductive status best depends on the species of interest (Table 20.1), although faecal progestogen profiles are generally easier to interpret than oestrogen profiles, particularly with respect to monitoring of ovarian cycles and timing of ovulation.

Measurement of adrenal steroids (glucocorticoids) in primate faeces has increased substantially over the past couple of years. In some species (e.g. ring-tailed lemur, *Lemur catta*: Cavigelli, 1999; chimpanzee, *Pan troglodytes*: Whitten *et al.*, 1998b), cortisol assays appear to yield useful information, although cortisol itself is often either barely detectable or not present (Bahr *et al.*, 2000; Heistermann *et al.*, 2006). Since it is impossible to predict whether cortisol is present in substantial amounts in the faeces of any given species, group-specific assays, capable of measuring a range of faecal cortisol metabolites, might be generally more suitable, in that they are more likely to detect at least some of the more abundant metabolites present and also have greater

Table 20.1 *Selected studies in which faecal oestrogen and progestogen assays have been used to assess female reproductive status in primates*

Abbreviations: E_t = total oestrogen; E_2 = oestradiol-17β; E_1C = oestrone conjugates; PdG = pregnanediol(glucuronide); 20α-OHP = 20α-hydroxyprogesterone; P_4 = progesterone; 5-P-3OH = 5α-pregnane-3α-ol-20-one.

Taxa	Species	Oestrogen	Progestin	Reference
Lemuridae	*Eulemur mongoz*	E_t	20α-OHP	Curtis *et al.*, 2000
	Eulemur fulvus rufus		5-P-3OH	Ostner & Heistermann, 2003
	Hapalemur griseus	E_t	5-P-3OH	P. Gerber *et al.*, unpubl.
Indriidae	*Propithecus verreauxi*	E_2	P_4	Brockman & Whitten, 1996
Lorisidae	*Nycticebus coucang*	E_1C	—[a]	Jurke *et al.*, 1997
Cebidae	*Callithrix jacchus*	E_2	Pd, P_4	Heistermann *et al.*, 1993
	Saguinus oedipus	E_2	P_4, Pd	Ziegler *et al.*, 1996
	Saguinus fuscicollis	?[b]	Pd	Heistermann *et al.*, 1993
	Saguinus mystax	E_t	PdG	Löttker *et al.*, 2004
	Leontopithecus rosalia	E_1C	PdG	French *et al.*, 2003
	Callimico goeldii	E_t	?[b]	Pryce *et al.*, 1994
	Cebus apella	—[a]	P_4, Pd	Carosi *et al.*, 1999
	Saimiri sciureus			Moorman *et al.*, 2002
Pitheciidae	*Pithecia pithecia*	E_1C	PdG	Shideler *et al.*, 1994
Atelidae	*Brachyteles arachnoides*	E_2	P_4	Strier & Ziegler, 1997
	Ateles geoffroyi	E_1C	PdG	Campbell *et al.*, 2001
	Alouatta pigra	E_2	P_4	Van Belle *et al.*, 2008
Cercopithecinae	*Macaca fascicularis*	E_1C	PdG	Shideler *et al.*, 1993
	Macaca silenus	E_2	5-P-3OH	Heistermann *et al.*, 2001a
	Macaca fuscata	E_1C	PdG	Fujita *et al.*, 2001

Table 20.1 (*cont.*)

Taxa	Species	Oestrogen	Progestin	Reference
	Macaca sylvanus	E_t	5-P-3OH	Möhle *et al.* 2005
	Cercocebus torquatus	E_2	P_4	Whitten & Russell, 1996
	Papio cynocephalus	E_2	P_4	Wasser *et al.*, 1991
	Papio hamadryas anubis	E_t	PdG	Higham *et al.*, 2008
Colobinae	*Semnopithecus entellus*	E_2	20α-OHP PdG	Heistermann *et al.*, 1995a
	Pygathrix nemaeus	E_t	5-P-3OH	Heistermann *et al.*, 2004
Hylobatidae	*Hylobates lar*	—[b]	5-P-3OH	Barelli *et al.*, 2007
Hominidae	*Pan paniscus*	—[a]	P_4, Pd	Heistermann *et al.*, 1996
	Pan troglodytes	E_2	P_4	Emery & Whitten, 2003
	Gorilla gorilla	E_2	P_4	Miyamoto *et al.*, 2001

[a] Measurement not successful.
[b] No information available.

potential for cross-species application (Heistermann *et al.*, 2006; Fichtel *et al.*, 2007). However, such group-specific assays have the potential to cross-react with structurally related testosterone metabolites (chimpanzee: Heistermann *et al.*, 2006), which can confound the actual glucocorticoid measurement, although the degree to which this occurs may still be acceptable (Heistermann *et al.*, 2006; Fichtel *et al.*, 2007). Nevertheless, take potential co-measurement of metabolites that do not originate from cortisol into account when using faecal glucocorticoid assays.

Information on the metabolism of testosterone and the nature of the metabolites excreted into primate faeces remains surprisingly limited (but see Möhle *et al.*, 2002). As with cortisol, testosterone metabolism is complex and often species-specific, resulting in excretion of a number of metabolites, with native testosterone usually being quantitatively of minor importance or virtually absent in faeces (Möhle

et al., 2002). Nevertheless, testosterone assays have been widely used and in the majority of cases this has yielded informative results (Brockman *et al.*, 1998; Lynch *et al.*, 2002; Setchell *et al.*, 2008). The measurement of 5-reduced androstanes has also been successfully applied to monitor androgen status in male primates (Girard-Buttoz *et al.*, 2009). However, irrespective of the type of assay used, reliable assessment of male testicular androgen secretion in Old World monkey species and great apes might generally be difficult owing to the potential co-measurement of metabolites derived from androgens of extratesticular (e.g. adrenal) origin. For example, dehydroepiandrosterone (DHEA), a weak androgen from the andrenal gland, may potentially confound assessment of male testosterone secretion since it is metabolized to products very similar (if not virtually identical) to those of testosterone itself (Möhle *et al.*, 2002). Specific measurement of testicular endocrine activity (rather than a measurement of overall androgen status) from faecal androgen measurements is therefore likely to remain difficult in these primate taxa.

For a comprehensive overview of references concerning the application of urinary and faecal androgen and glucocorticoid measurements in primates and other vertebrate taxa see Hodges *et al.* (2010).

GENERAL CONSIDERATIONS

Species variation in metabolism and excretion

Steroids circulating in the bloodstream undergo a series of metabolic changes before finally being eliminated from the body. The nature of these changes can vary considerably between species (even closely related ones), resulting in differences not only in the nature and identity of the metabolites themselves, but also in their preferred route of excretion. This can have important consequences for the selection of an appropriate measurement system and correct interpretation of results obtained (Heistermann *et al.*, 2006).

Time lag

Whereas circulating hormones more or less reflect real-time changes in endocrine activity, hormones in excreta reflect events that have occurred in the past. In urine the time lag is usually only about 4–8 h (see Bahr *et al.*, 2000), but even so, with once-daily sampling, this can be enough to delay detection of an event (e.g. peak, defined rise) by

one day. Time lags associated with faecal measurements are longer and more variable, both between and within species, and gut passage times can be affected by a variety of factors including diet, health status and stress level. In most large-bodied species for which data are available, steroids are excreted in faeces 36–48 h after appearance in circulation (see Shideler *et al.*, 1993), although there are exceptions (e.g. 22 h for testosterone and cortisol in the chimpanzee; Möhle *et al.*, 2002; Bahr *et al.*, 2000). In contrast, passage time is quicker in smaller animals (e.g. 4–8 h for testosterone and cortisol in the common marmoset, Möhle *et al.*, 2002; Bahr *et al.*, 2000). The two main consequences of these time lags (especially for faeces) are (i) a delay between the occurrence of a specific event and its detection, which you need to account for when using faecal measurements to determine the timing of events such as ovulation or implantation and (ii) a dampening effect, reducing the amplitude of hormonal changes, and making it more difficult to detect short-lived endocrine responses to acute situations (e.g. stress).

Sampling frequency

Sampling frequency is largely determined by the type of information required, although how often samples can actually be collected is influenced by numerous practical considerations. Information on overall physiological condition/status requires less frequent sampling than information on dynamics or timing of events. As a general rule, to compensate for intra-individual (sample-to-sample) variation (and also for statistical considerations), we recommend that you collect no fewer than 6 and preferably 10 samples per condition (i.e. before vs. after birth; breeding vs. non-breeding season; before and after change in rank; animal 1 vs. animal 2).

Weekly samples should be sufficient to follow the course of pregnancy, although increased sampling (2 per week) may be useful during the period leading up to parturition. To detect the presence of ovarian cycles, twice weekly samples are the absolute minimum, whereas you need more regular samples collected at a higher frequency to define the duration or interval between two events, dependent on the margin of error that is compatible with the objectives of your study. Generally, this will be 1 day at 3–4 samples per week and 2 days at 2 samples per week. To time ovulation, collect daily samples. It is useful to aim for a slightly higher frequency than is necessary, since it is unlikely that you will collect all samples as planned.

In addition to sampling frequency, a variety of other factors can also affect the reliability of the information obtained from hormone assays of field samples. Important among these are assay precision and criteria used to define the events or parameters under investigation. The first refers to the amount of inherent variability in the measurement system and the second is basically a question of how you interpret the results. As no two samples will have identical hormone content, it is essential to differentiate between random variation and patterns of potential significance in relation to the parameters under investigation. One elevated progesterone value doesn't indicate an ovulatory cycle, but how many do, at what intervals and what frequency? What is elevated? Ovulation is best timed retrospectively according to the rise in progesterone that occurs at the onset of the luteal phase. Here, the question is, what is a rise? There are no hard and fixed rules, but in our experience, the increase (and maintenance) above a threshold value determined as the mean plus two standard deviations of preceding (3–5) baseline values provides a statistically significant, useful and informative method for defining a rise in hormone levels (see Heistermann *et al.*, 2001b). Appropriate definitions (providing objective criteria for assessment) are essential to meaningful interpretation of data.

Influence of diet on hormone levels

Many plant species contain phytosteroids, which may potentially interact with an animal's intrinsic physiology and influence reproductive processes. In primates, it has recently been shown that the consumption of certain plants in the genus *Vitex* can confound urinary and faecal measurements of oestrogen and, in particular, progesterone metabolites in baboons and chimpanzees (Higham *et al.*, 2007; Emery Thompson *et al.*, 2008). Effects include increased fluctuations and sustained elevations in hormone levels, making interpretation of results (and assessment of reproductive status) difficult. Since *Vitex* species feature prominently in the diet of many African primates, their effect on female hormone levels may represent a more general phenomenon. To what extent other steroids (glucocorticoids and androgens) may also be affected by consumption of these plants is not really known, but at least for chimpanzees it has been shown that the consumption of *Vitex* does not alter urinary testosterone levels in males (Emery Thompson *et al.*, 2008).

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