A Practical Field Extraction Method for Non-invasive Monitoring of Hormone Activity in Animals

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Non-invasive hormone analysis of captive and wild animals can be beneficial in understanding an animal's adrenal and reproductive status, which can be beneficial to in situ and ex situ conservation, and to understanding individuals' responses to their environment. However, direct sampling from the animals can be difficult and thus the analysis of faecal samples is common place away from controlled laboratory conditions. However, the use of faecal samples presents many challenges in terms of sample stability, health and safety issues, and transportation issues in particular when electricity is not readily available for in situ analysis to occur. This article will look at the various modes by which a sample can be stored and subsequently analysed and how the use of solid phase extraction has radically altered the approach to sample handling in the conservation of animals.

Researchers are gaining an insight into the reproductive and adrenal activity of a wide variety of species [1,2] by monitoring specific hormone levels. Investigating the relationship between hormone levels and animal behaviour [3-5] gives an insight into reproductive processes [1,6], indices of stress [7,8], life-history traits [9], as well as understanding the evolution of physical and behavioural traits [10], and how individuals respond to both natural and anthropomorphic changes in their environment [11-13]. For endangered species both in the wild and captivity, these approaches can be hugely beneficial to gain a better understanding of species biology, optimise welfare and aid conservation [14,15].

Faecal measures of hormones are becoming widely used as they are relatively easy to obtain and can be collected non-invasively, minimising the impact of sampling on the animal [16]. However faecal material has a greater level of metabolites which are vulnerable to bacterial and environmental degradation, which can further alter the metabolites present [17,18]; but as long as faecal samples are collected relatively soon after defecation, the hormone metabolites present can be a reliable indicator of the individuals' physiological state [17,18].

The best option for sample preservation is to freeze samples immediately following excretion. However, this is not a practical option for most field researchers. Several other approaches have been utilised including storing faecal material in alcohol [19,21,22] drying either faecal material [4,22,23] or faecal extract in the field [24], and storing faecal extract on filter paper [25]. Each method though has potential constraints, either due to health and safety issues or due to transportation or to the reliability of the data produced.

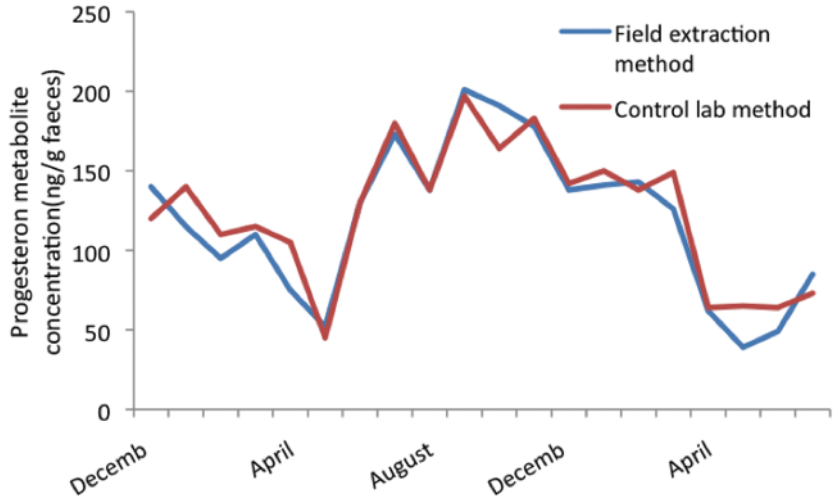

Figure 1: Female black rhino faecal progesterone metabolite concentration (ng/g) over one oestrus cycle; samples extracted according to either the field extraction method or control lab method.

The solution employed successfully by Chester Zoo is to use solid phase extraction (SPE) cartridges as a temporary storage and transportation vessel. In the current study, there was a desire to develop a technique for researchers who may have to travel long distances over long periods of time, to locations where electricity supplies may be unreliable or non-existent to track individual animals. Additionally, whereas existing methodologies are often validated techniques for a single hormone, there was a desire to look at multiple hormones in animals; to understand the reproductive state of individuals in situ, and to investigate adrenal activity between individuals and populations.

Further development was therefore

warranted, so accordingly the aim of this study was to develop a field method that required minimal equipment, and could be used where no electricity was available. The technique was required to:

- provide reasonable recovery of all synthetic and faecal hormone metabolites of interest
- provide accurate and repeatable quantitative results for faecal reproductive and adrenal hormone metabolites
- be stored at ambient temperatures in the field, without the risk of faecal hormone metabolite degradation before further processing in the laboratory
- deliver qualitatively comparable results to controlled laboratory protocols.

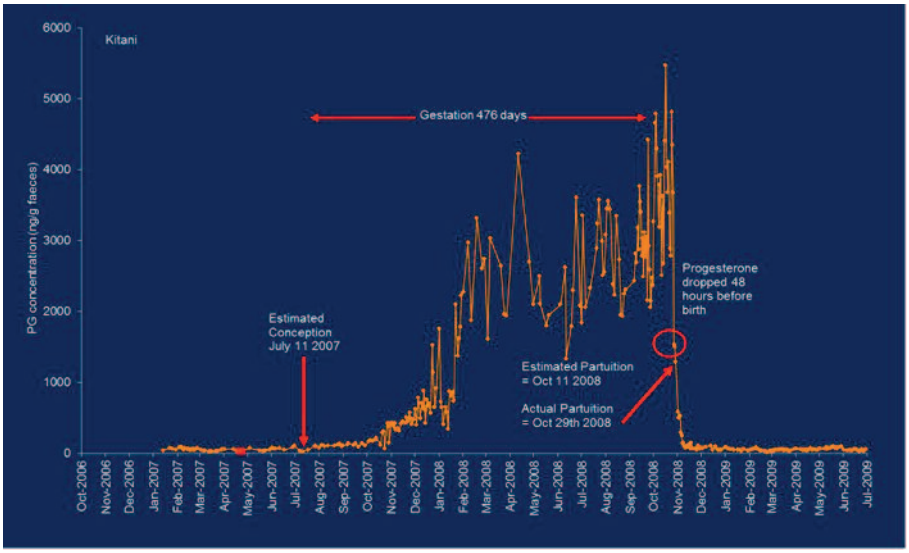

Figure 2: Black rhino faeces extracted and analysed using enzyme immunoassays (EIA) looking at the progesterone concentration of a female black rhino during conception, gestation and partuition.

Methods

An SPE method for the analysis of a range of hormones, corticosterone, progesterone and testosterone was developed on a HyperSep™ octyl bonded silica (C8) 500 mg/3ml (Thermo Fisher Scientific, UK).

Each sample was thoroughly mixed and 0.5g (±0.05g) weighed using portable battery operated scales (3000g x 0.05g; Salter Brecknell, UK), suspended in 4ml of 90% methanol / 10% water, and individually hand-shaken for five minutes. To separate the extract from the faecal material, samples were filtered using moistened filter paper (Grade 4, Whatman, UK). The resultant faecal extract was then ready to load onto SPE cartridges, according to the conditions outlined below.

SPE Cartridges and Optimal Loading Solvent

The cartridges were primed according to the manufacturer's instructions with 4ml methanol followed by 4ml distilled water, with a flow rate of 1 ml/min. Once primed, the cartridges were loaded with the filtered faecal extract at a flow rate of 0.5 ml/min (the optimal loading solvent is determined below). Cartridges were washed with 2ml distilled water (1 ml/min) and sealed with parafilm to prevent the solid-phase from drying out during storage. Once ready for elution off the cartridge, 5ml 100% methanol was pushed through the column (0.5 ml/min), collected, dried under air, re-suspended in 1 ml 100% methanol and stored at -20°C until analysis.

The optimum solvent concentration for loading the extract onto the cartridge was determined by loading (0.5 ml/min) synthetic hormones progesterone (P0130, Sigma Aldrich, UK; 200ng), testosterone (T1500, Sigma Aldrich, UK; 300ng) and corticosterone (C2505, Sigma Aldrich, UK; 500ng) in 4ml H_2O onto a primed C8 cartridge. Hormones were then eluted using a 10% stepwise increase in methanol concentration (5ml; 10 - 100%). Each resulting 5ml fraction was collected separately, dried under air, re-suspended in 1ml 100% methanol and quantified on the respective enzyme immunoassay (EIA). The percentage recovery of each synthetic hormone was calculated from the total concentration observed across all fractions eluted, as a percentage of the total mass of hormone expected. It was observed that corticosterone eluted at between 50-60% methanol with water, followed by testosterone at a methanol water ratio of 70/30 and progesterone at a ratio of 80/20 from the HyperSep C8 SPE cartridge. Therefore, to maximise the binding of hormone metabolites to the solid phase, and minimise the risk of metabolites being simultaneously eluted in the loading step, the optimal loading solvent concentration for all subsequent experiments was set to 40% methanol.

Faecal Samples and Extraction

The method was originally tested on faecal samples were collected from two male (age 11 and 12 years) and three female (age 8, 12 and 22 years) captive black rhinoceros D. b. michaeli, housed at Chester Zoo, UK. Samples collected and analysed as previously stated. The data collected from a single female faecal samples over a month period is shown in Figure 1. Two approaches were utilised based around the method described previously. Using this information it is possible to determine the optimum breeding time for a female, which was then used to aid the breeding programme at Chester Zoo

The levels of progesterone measured using the EIA approach for a female from conception to partuition are shown in Figure 2. It can be seen that after conception the concentration of hormone increases dramatically, and at birth it drops to pre-conception levels. Finally the results of the studies performed at Chester Zoo are shown in Figure 3.

Using this approach the zoo has been incredibly successful in improving its breeding programme for black rhinos. From 1987 – 1997 there were 0 births, which was prior to hormone monitoring being established, after which the zoo has seen 5 new arrivals;

- Asani (rebelious)
- Chauna (Blossom)
- Dakima (the joy of living)
- Bashira (Joyful)
- Enbu

Discussion

The ability to monitor non-invasively hormone levels in animals can be a useful tool for researchers, conservationists and animal managers alike to help understand reproduction and adrenal activity, and investigate a wide range of hormone-behaviour interactions [3,7,1]. This approach has been applied to several different species and the pioneering work performed at Chester Zoo is being carefully monitored by many other conservationist groups located around to world. The outcome of this study

Figure 3: One of the products of the testing of the female black rhinos at Chester Zoo

was a practical method by which faecal samples can be extracted under field conditions, without the need for extensive equipment or electricity, and loaded onto C8 HyperSep™ SPE cartridges. These cartridges can then be stored at ambient temperatures for up to 6 months prior to re-extraction and enzyme immunoassay, without degradation of hormone metabolites. SPE cartridges are compact and lightweight and can be easily transported back to the laboratory following faecal extraction eliminating the need for approaches that require electricity or have safety issues.

Acknowledgements

The authors would like to thank collaborators at Zoological Society of London; Hannah Andrews, Raj Amin and Benson Okita for testing earlier versions of this method; Thermo Scientific for supplying cartridges and advice on how to improve recovery; and the rhino team at Chester Zoo for collecting faecal samples.

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