The successful non-invasive endocrine monitoring of oestrous cycles in Banteng (Bos javanicus)

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The use of endocrinology to monitor reproduction and fertility is an important tool in our efforts to conserve and support sustainable populations of endangered species. In this research we describe the use of an enzyme linked immunosorbent assay (EIA) to assess the faecal progesterone metabolite concentrations in female banteng (*Bos Javanicus*) at Chester Zoo, UK. An oestrous cycle length of 21 days (n=5 cycles) with reproductive synchronicity was observed. This work outlines a non-invasive method for collecting female banteng reproductive physiology data, which can be applied on an individual or herd level. This data could be utilised to support future Global Species Management Plan (GSMP) *ex situ* breeding efforts by confirming reproductive status of female banteng and feeding this information into husbandry and breeding management.

Introduction

The reproduction and fertility of a species is fundamental for survival, for conservation efforts to be successful it is vital that there is a solid understanding of reproductive processes (Sontakke, 2018). When breeding is being managed though timed introductions or techniques such as artificial insemination, an accurate assessment of reproductive parameters such as species oestrous cycle length and estimated ovulation can be key to a successful captive breeding program (Herrick, 2019).

Circulating blood hormone concentrations are the most accurate indicators of reproductive state. However, most wildlife species are intractable or require extensive training which makes repeated blood collection very difficult (Kersey and Dehnhard, 2014). A less invasive alternative to monitoring hormone concentrations in the blood is measuring concentrations in faeces (Lasley and Kirkpatrick, 1991). The non-invasive monitoring of reproductive hormones has the added benefits of negating short-term fluctuations through pooled samples, permitting routine sampling over long periods of time and having little or no contact with the animal (Kersey and Dehnhard, 2014). The analysis of mainly unconjugated faecal progesterone metabolites is a well-known approach for monitoring mammal reproductive function in farm, wild and zoo animals (Schwarzenberger et al., 1996).

Reproductive Endocrinology of Cattle

The reproductive physiology of cattle is of major economic importance in both the dairy and beef industry and therefore the reproductive physiology and subsequently endocrinology of female cattle has been explored extensively (Garverick and Smith, 1993). The mean duration of the oestrous cycle in the domestic cow is 21 days (range 18-24 days) and consists of four phases, pro-oestrous, oestrous, metoestrus and dioestrus (Noakes, 1997). Hormone monitoring is not routinely used to manage breeding in domestic cattle; rather visual observation is the most traditional and commonly used form of oestrus detection (and presumed ovulation) but has labour costs and human error. Hormone monitoring of wild cattle species for conservation purposes is a little explored area of research and has the potential to be an important tool for captive breeding programmes.

Banteng (*Bos javanicus*) are an endangered species of wild bovid from South-east Asia. The species is considered to have a decreasing population trend which is estimated to range

between 4,000 and 8,000 individuals (IUCN, 2014). The major cause of decline is due to hunting, habitat destruction and fragmentation, as well as being subject to hybridization and disease from domestic livestock (Sansinena et al., 2005). Bali cattle are a domesticated descendent of the wild banteng and represent 27% of the total cattle population in Indonesia, where artificial insemination is practiced in limited locations (Purwantara et al., 2012). In banteng, serum estradiol and progesterone profiles have previously shown a mean cycle length of 20 days ± 0.68 (Asa et al., 1993). Urinary non-invasive endocrine monitoring has also previously been used successfully to assess fertility control techniques (Kirkpatrick et al., 1995). The aim of the current study is to add to the body of knowledge on female banteng reproductive physiology and develop a non-invasive method to measure oestrous cycles and estimate periods of oestrus through faecal progesterone metabolite concentrations.

Materials and Methods

Faecal Sample Collection

The banteng herd used for this research comprised 14 individuals housed separately in two distinct groups at Chester Zoo, UK. The first group consisted of 1 mature male, 4 adult females and 3 calves; the second group is consisted of 4 adult females and 2 juvenile females.

Faecal samples (n=44) were collected 2-3 times per week from two sexually mature parous females (age 3 and 4 years) over a 162 day period. The two individuals were housed in the second group in a small, off-show herd away from the mature male. In order to ensure the samples were collected from the specified individual, the herd were observed for two hours on any given day and samples were collected if either of the individuals defecated and stored in individually marked bags. Individuals were identified by unique physical features and ear tags. Samples were stored at -20°C immediately following collection.

Faecal Extraction and EIA

Faecal samples were extracted using a wet-weight extraction technique adapted from Walker et al. (2002) and described elsewhere (Watson et al., 2013; Edwards et al., 2014), whereby 0.5 g of faecal matter was extracted with 5 ml of 90% methanol, shaken overnight, dried and reconstituted in 1ml of 100% methanol, and stored at−20°C until being analysed with a progesterone enzyme immunoassay (CL425; supplied by Coralie Munro, University of California Davis, CA, USA). The progesterone CL425 cross-reactivities are published elsewhere (Watson et al., 2013).

The progesterone antibody was diluted (1:10,000) in coating buffer (0.05 M $NaHCO_3$, pH 9.6), loaded 50 µl/well on a 96-well Nunc-Immuno MaxiSorp microtitre plate (Thermo-Fisher Scientific, UK), covered with a microplate sealer and incubated overnight at 4°C. Plates were washed five times (0.15 M NaCl, 0.05% Tween 20), and the entire plate loaded with 50 µl/well of progesterone standard (P0130 Sigma–Aldrich, UK) in EIA buffer (0.1 M $NaPO_4$, 0.149 M NaCl, 0.1% bovine serum albumin, pH 7.0), or Banteng faecal extract (diluted 1:100 in EIA buffer) immediately followed by 50 µl/well of horseradish peroxidase conjugate (diluted at 1:35,000 in EIA buffer). Following incubation for 2h at room temperature (RT), plates were washed 5 times and incubated with 100 µl/well substrate [0.4 mM 2,20-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt (ABTS), 1.6 mM H_2O_2, 0.05 M citrate, pH 4.0), until average optical density (OD) reached 0.8 to 1.0. The resulting OD of all individual wells was then measured at 405 nm. Intra- and inter-assay coefficients of variation (CVs) were <15% for high- and low-binding synthetic controls.

Biochemical Validation

A parallelism was used to confirm progesterone metabolites present in the faecal extracts behave in a similar way to the synthetic progesterone to which the antibody was raised. A serial dilution of faecal extract was run in duplicate on the EIA, alongside a serial dilution of the synthetic standard. The parallelism was also used to determine the correct dilution to run biological samples. To obtain accurate results, ideally

faecal samples will be run on the EIA at a dilution to give approximately 50% binding, as this is the most sensitive and accurate portion of the calibration curve as it is steep and linear.

A matrix interference assessment was used to determine whether the sample matrix causes any interference to sample measurement. A serial dilution of synthetic standard was spiked with an equal volume of diluted faecal extract. Once the background has been accounted for, the observed concentration was compared to the expected concentration.

Both parallelism and matrix interference data were subject to regression analysis.

Results

The progesterone EIA was biochemically validated for measuring progesterone metabolites in female banteng faecal extracts through parallelism with the standard curve (R2=0.99, F1,7=923.966, P <0.00) and no matrix interference (R2=0.99, F1,7=460.85. P <0.00; Figure 1). An oestrous cycle length (based on the number of days between observed baseline faecal progesterone metabolite concentrations) was determined to be of 21 days for both individuals (Figure 2).

Faecal progesterone metabolite concentrations of the two females also follow a similar profile over the collection period, indicating reproductive synchronicity (Figure 2).

Discussion

This study has both chemically and biologically validated a non-invasive technique to monitor ovarian activity in female banteng. The data presented here indicate a cycle duration of 21 days which is in agreement with other cattle species (Noakes, 1997). One challenge during the current study was the ability to routinely collect known faecal samples from individuals. To improve the frequency of collection the use of indigestible markers (for example dye or cracked corn) for identifying individual faeces could be utilised in future attempts (Fuller et al., 2010). Additionally, monitoring could be updated to include behavioural observations to confirm oestrus and ensure sexual behaviours are aligning with the rise in progesterone metabolite concentrations from baseline.

The current study also demonstrated that faecal progesterone metabolite concentrations of the two females were synchronized. In a commercial setting, reproductive synchronicity is

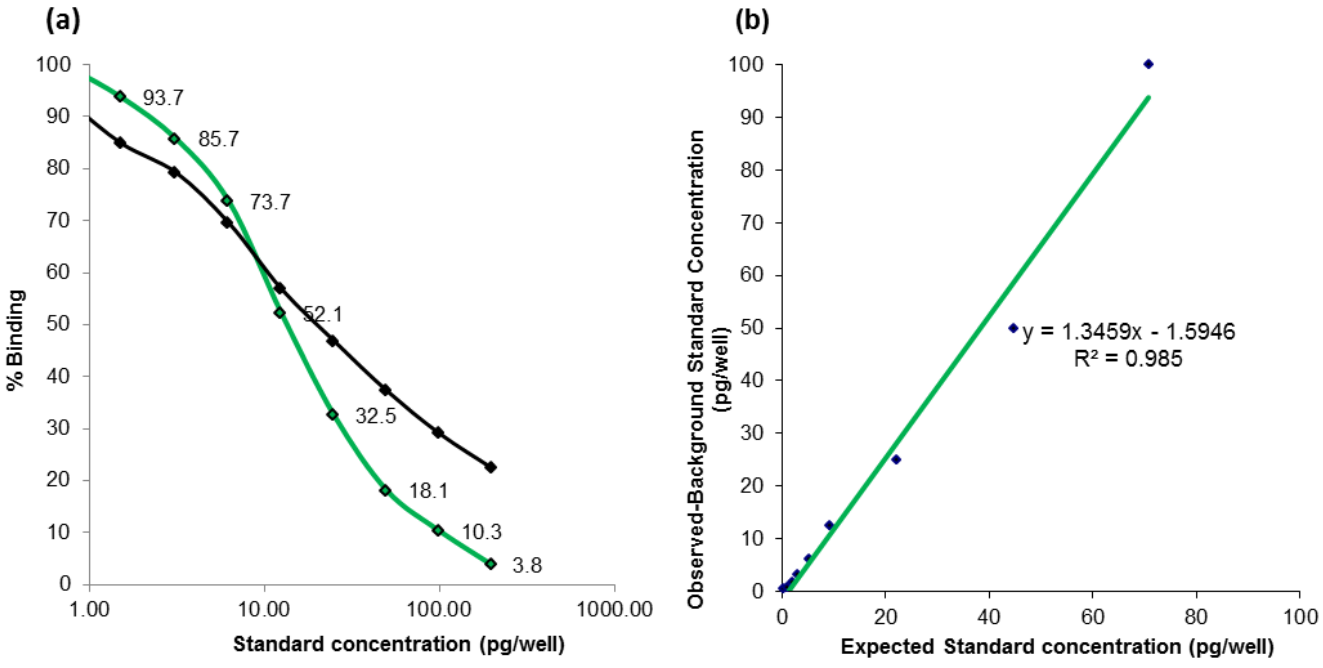

Figure 1. (A) Female banteng faecal pooled extract (green) parallelism with progesterone synthetic standard curve (black) on the progesterone EIA (CL425 antibody) and (B) Assessment of matrix interference of female banteng extract when spiked with progesterone synthetic standards on the progesterone EIA (CL425 antibody).

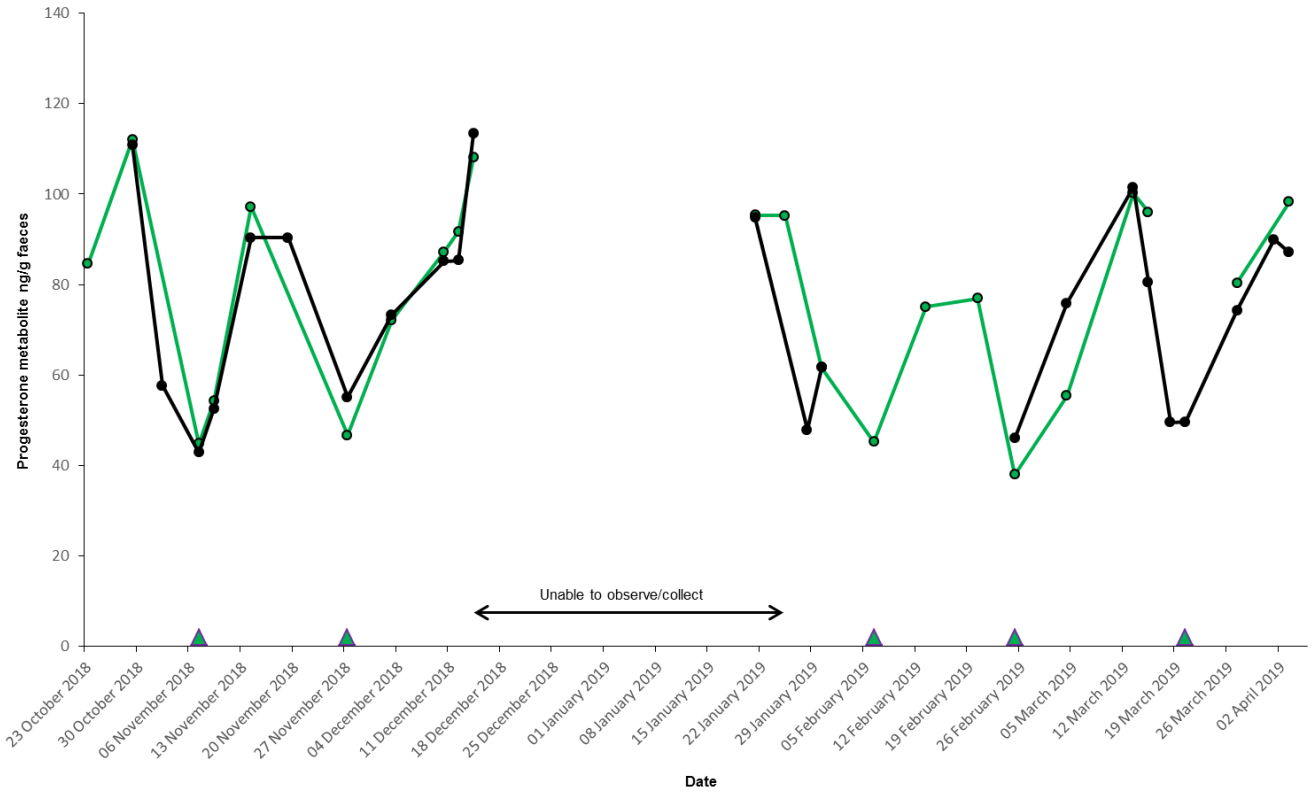

Figure 2. Faecal progesterone metabolite concentrations (ng/g) from two female banteng (individual 1 (○), individual 2 (●)) (n = 44 samples) on the progesterone EIA (CL425 antibody) over 162 days and estimated periods of oestrus (△).

advantageous for increasing produce yield and such synchrony is usually artificially maintained. Cattle have been shown to exhibit highly synchronised behaviour in such activities as feeding and resting (Bouissou et al., 2001). It is well known that reproductive synchronicity exists in certain mammals and has been demonstrated in other mammals such as the captive African elephant (*Loxodonta africana*) (Weissenböck et al., 2009; Edwards et al., 2016). However, there has been limited exploration into this potentially adaptive advantage in wild or captive bovid herds. Given the small sample size of the current study, further sample analysis is required to confirm if reproductive synchrony is commonly observed in this and other banteng herds.

In conclusion, this work outlines a non-invasive method for collecting female banteng reproductive physiology data, which can be applied on an individual or herd level. This methodology could be utilised to realise future banteng GSMP objectives (Metzler et al., 2016) by providing further data to support natural breeding attempts, timed introductions or artificial insemination within the ex situ population.

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