

Pattern of faecal 20-oxopregnane and oestrogen concentrations during pregnancy in free-ranging plains zebra mares

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ABSTRACT

Regulative endocrine mechanisms influence the reproductive behaviour and success of mammals, but they have been studied predominantly in domestic and captive animals. The study aims at describing the pattern of faecal 20-oxopregnane and oestrogen concentrations during pregnancy in wild plains zebra *Equus quagga chapmani*. Data were collected during wet and dry seasons 2007-2009. Enzyme Immunoassays were used to determine 20-oxopregnane and oestrogen concentrations in faecal samples (n=74) collected from individual mares (n=32) whose dates of foaling were known through long-term monitoring. Hormonal profiles were described with a General Additive Model (GAM: Hormone ~ Days to Foaling). Faecal 20-oxopregnanes have a complex cycle during pregnancy (GAM, n=70, $R^2=0.616$, $p<0.001$). From -250 days to foaling, faecal 20-oxopregnane concentrations were above the baseline levels found in non-pregnant mares, peaking in the last 50 days. Faecal oestrogen levels showed a clear peak in mid-pregnancy (GAM, n= 62, $R^2=0.539$, $p<0.001$). The sex of the foetus and season had no detectable effect on hormone concentrations during pregnancy. High levels (>200ng/g DW) of faecal 20-oxopregnanes associated with high (>160ng/g DW) faecal oestrogen levels indicate mid-pregnancy in c.90% of cases (16/17). High faecal 20-oxopregnanes (>200ng/g DW) and low faecal oestrogen levels (<160ng/g DW) indicate late pregnancy, again in c.90% of cases. Two faecal samples would allow the stage of pregnancy to be determined with confidence.

Keywords: Oestrogen; 20-oxopregnane; reproduction; non-invasive method; pregnancy diagnosis; *Equus quagga*.

1 INTRODUCTION

Endocrine mechanisms regulate the reproductive physiology of mammals, which in combination with genetic, social and ecological factors influence individual fitness and reproductive behaviour. As a consequence, variation in reproduction can influence population survival such that an understanding of reproduction is essential for conserving species, populations and, indirectly, ecosystem functioning. Although reproductive physiology has been extensively studied in domestic and captive mammals [33], less is known about reproduction in wild populations, largely due to sampling difficulties. Non-invasive hormone monitoring methods using faecal samples have been used increasingly over the last 20-25 years both because of logistic feasibility and for welfare considerations since the welfare implications of collecting blood samples from wild animals are increasingly recognized, e.g. [15,35]. Faecal sample analysis is now considered as a reliable method after being tested on several mammal species in captivity [4,7,14], and has been used for pregnancy diagnosis in the field [22].

Among non-human mammals, most work on the physiology of reproduction has been done on Ruminantia (including bovids) and Perissodactyla (including equids); two major groups of ungulates which have evolved in parallel [36]. The Perissodactyla, including seven extant species of equids, are much less studied than the Ruminantia, with hormonal cycles only described in detail for the horse [1]. Among wild species, the basic patterns are known in the endangered Grevy's zebra *Equus grevyi*, where oestrogen levels have been observed to be 10 times higher in pregnant mares than in non-pregnant females and in male conspecifics, similar to the horse [3,31]. These patterns in faecal oestrogens are similar to plasma measurements, with a marked increase in levels of oestrogen after the first trimester [24]. Consequently, a study of four captive *E. quagga antiquorum* mares showed that total unconjugated oestrogen concentrations measured by RIA could be used to diagnose pregnancy during the second and third trimester [37].

Progesterone levels increase above baseline levels during mid-pregnancy [16,26], but can show a biphasic pattern with an initial rise followed by a gradual decline, with a later second peak in both horses and zebras, e.g. [16,17]. Such a pattern may be associated with the formation of a secondary corpora lutea [17].

The plains zebra is an important component of large herbivore savannah communities, and is widely distributed throughout East and Southern Africa [11]. However, during the last decades, plains zebra populations have been extirpated from several parts of their range [12]. So far, studies on plains zebra have focused on social behaviour and reproduction [19,20,21,40], and little is known of their population dynamics [10,23], and, in particular, survival and reproductive rates [12]. Moreover, the patterns of reproductive hormone concentrations have not been described in wild plains zebra. Shot specimens have been used in some studies to determine the reproductive status of animals and to estimate fecundity rates [10,40], but non-invasive methods are clearly preferable. Enzyme Immunoassays (EIAs) for faecal progestagens and oestrogens have been shown to be a reliable method to determine the reproductive status of other equids, including Grevy's zebra [3,6], wild and feral horses [4,22], and domestic horses [14,32], producing patterns that mirror plasma measurements, e.g. [16,24]. The overall aim of the present study was to examine the endocrine function of wild plains zebra mares, by describing the patterns of 20-oxopregnane and oestrogen concentration during pregnancy. Using measurements of faecal oestrogens and 5β -pregnane- 3α -ol-20-one concentrations, the study further aims to determine whether these techniques can be used to diagnose pregnancy in wild plains zebra, information essential to determine reproductive rates in wild populations.

2 MATERIALS AND METHODS

2.1 Study area

The study was conducted in the Hwange National Park (14 651 km²), centred at 18⁰ 45'S and 26⁰ 45'E in south-western Zimbabwe. The plains zebra population investigated ranged within 40 km of Main Camp, about a fifth of the reserve. Altitude ranges from 900m to 1100m. Rainfall averages 640mm annually and is distributed within a wet summer season from November to April, with a precipitation peak reached in February, and a dry winter season [5]. The study area is generally flat and characterised by Kalahari sands, with occasional sand dune relicts, and includes vleis and patches of open grasslands. The vegetation consists mainly of woodlands and bush-lands dominated by *Baikiaea plurijuga*, *Combretum* spp., *Acacia* spp., and *Colophospermum mopane* [41].

2.2 Field observations and sample collection

The study employed an individual-based technique to investigate the endocrine status of mares (n=32) from a sample population (N=208) of free ranging plains zebra with known life histories. The individuals were identified by their unique stripe patterns that serve as a reliable key [28]. The field study began in 2004 and spans both wet and dry seasons. Faecal samples for endocrine analysis were collected from January 2007 till February 2009. The study animals were habituated to the presence of vehicles, facilitating behavioural observations and sample collection. As re-sightings of individuals were opportunistic, the foaling date was taken as the mid-point between the earliest and latest possible dates of birth estimated from the size of the foal when it was first seen, using the criteria of Smuts [39] and Penzhorn [27]. The precision was less than ± 30 days

for almost all samples except two.

In heavily pregnant mares, the swelling of udders was used as an indication of closeness to foaling and thereafter re-sighting efforts were increased to try and estimate the date of foaling accurately. Suckling and maternal behaviour were used to determine maternity [18]. Since conception dates were not known, the number of days to foaling was estimated by backdating by the mean gestation period of the plains zebra (375 days [21]) from the estimated date of foaling.

In total, 74 samples were collected between 2007 and 2009 from 32 mares (mean \pm SE = 2.31 \pm 1.26 samples per mare; range = 1 to 6 samples per mare). In each case, mares were observed defecating, and no stallion had urinated on their faeces. A representative sub-sample of the faecal bolus was collected in the field, amounting to 100g each and dried within 48 hours, either by air or in a low heat (40⁰C) field oven. The samples were packed in plastic bags and stored dry at room temperature until assayed (as in [14]).

2.3 Faecal extraction and hormone assays

Dried faecal samples were pulverized and sifted using a nylon mesh strainer to remove fibrous material as described by Fieß et al. [9]. Approximately 0.1 g of the faecal powder was extracted by vortexing for 15 minutes with 80% ethanol in water (3 ml). Following centrifugation for 10 minutes at 1500 g_n, supernatants were transferred to glass tubes, ready for hormone analysis. The resulting extracts were measured for unconjugated oestrogens and 20-oxopregnanes using enzyme immunoassays for oestrogens (E_t, [25]) and 5 β -pregnane-3 α -ol-20-one (5 β -20-one, [34]), which have been previously used to reliably diagnose pregnancy in mares of domestic equids [26]. Assay procedures followed standard protocols (e.g. described by Schwarzenberger

[34]). Sensitivities (90% binding) of the assays were 1.0 pg/well for E_t and 0.3 pg/well for 5 β -20-one, respectively. Intra- and interassay coefficients of variation ranged between 6.4% and 9.3% for E_t , and 6.4% and 16.7% for the 5 β -20-one measurements.

2.4 Statistical Analysis

A Generalised Additive Model (GAM) [Hormone (Y) ~ Days to Foaling (X)] was used to describe the profiles of 20-oxopregnane and oestrogen concentrations during gestation. The GAM is described as $E(Y) = s_0 + s_i(X_i) + \dots + s_j(X_j)$, where $s_i(X)$, $i = 1, \dots, j$ are smooth functions [13]. The model computes a general non-parametric function that relates the predicted Y values to the predictor values [38]. The GAM was necessary in order to deal with pseudoreplication and to separate any effects on hormone levels of the various independent variables.

The day of foaling was designated Day 0. Samples were described with reference to the day of foaling. Four data points with abnormally low 20-oxopregnane values in late pregnancy were excluded as repeat samples of the same animals showed high levels, with three of the repeats within 2 weeks in the same pregnancy.

Since the hormonal data were not normally distributed, they were log transformed by $Y' = \text{Log}_{10}(Y)$. As a result of the complex nature of the 20-oxopregnanes distribution curve, a local General Linear Mixed-effect model (GLMM) was used to test the effects of fixed factors on levels of 20-oxopregnanes, and take into account the variability caused by repeated measures on individual mares. The pregnancy period was analysed in two phases, during early to mid-pregnancy (-375 to -100 days to foaling) and late pregnancy (-100 to 0 days to foaling). A GLMM was first used to test the effect of season and sex of the foetus as fixed factors and mare

identity as a random variable on concentrations of 20-oxopregnanones. In addition, another GLMM with a 2nd order term was used to test for the fixed effects of sex of the foetus and season on oestrogen during pregnancy; and mare identity was included as a random effect. Statistical significance of all tests was set at $\alpha = 0.05$ and computed using R-version 2.9.0 software [30].

3 RESULTS

3.1 20-oxopregnane pattern for pregnant and non-pregnant mares

The relationship between the stage of pregnancy and concentration of faecal 20-oxopregnanones was non-linear (estimated degrees of freedom, >1.0). The GAM showed a significant effect of days to foaling (DTF) ($p < 0.001$) and explained 65.9% of the deviance (Table 1). It allowed us to describe a complicated polynomial relationship between faecal 20-oxopregnane concentration and DTF, throughout the pregnancy and post-partum (Figure 1). The levels of faecal 20-oxopregnanones increased from -250 days to foaling, with a peak in the last 50 days. Immediately after foaling, levels of faecal 20-oxopregnanones declined sharply, with levels reaching baseline values almost immediately.

3.2 Effects of season and sex of foetus on levels of 20-oxopregnanones

According to the pattern of faecal 20-oxopregnane levels described in the previous analysis, the effects of season and sex of the foetus were tested on the two main stages of pregnancy (early-mid and late) with contrasting patterns of this hormone, using Generalised Linear Mixed Models (GLMM) to take into account individual variability. In these GLMM, the effects of DTF, sex of

the foetus and season (dry/wet) were tested. There was a significant effect of DTF in early-mid ($p=0.001$) and late ($p=0.030$) pregnancy stages. Sex of the foetus ($p=0.654$) and season ($p=0.151$) had no significant effect on the levels of faecal 20-oxopregnanes in early-mid pregnancy or in late pregnancy (sex ($p=0.382$) and season ($p=0.470$)).

3.3 Oestrogen pattern for pregnant and non-pregnant mares

The GAM for oestrogen also showed a significant effect of DTF ($p<0.001$), and was efficient in describing the levels of faecal oestrogen in mares, explaining 56.8% of the deviance (Table 2). The relationship was polynomial (order 2), with a clear peak in faecal oestrogen levels in mid-pregnancy (Figure 2).

3.4 Effects of season and sex of the foetus on oestrogen levels

According to the pattern of faecal oestrogen concentrations described in the previous analysis, the next analysis was done during pregnancy only ($n=45$) using GLMM with a 2nd order term, in order to take into account individual variability. We tested for the effects of the sex of the foetus and season. The GLMM indicated a significant effect of DTF ($p=0.001$) and squared DTF ($p<0.001$). The sex of the foetus ($p=0.671$) and season ($p=0.170$) had no significant effect.

4 DISCUSSION

We describe for the first time the patterns of faecal 20-oxopregnanes and oestrogen concentrations throughout pregnancy in wild plains zebra. The gradual increase of faecal 20-

oxopregnane concentrations to levels above baseline values (<200ng/g DW for non-pregnant mares) during mid-pregnancy (-250 to -100 days to foaling), resembles the pattern of plasma progesterone levels observed in domestic horses [26,32]. In addition, the high 20-oxopregnane (>200ng/g DW) levels were maintained throughout mid-pregnancy, reaching a peak in late pregnancy (-100 to 0 days to foaling), as observed in horses [32], where they maintain pregnancy, suppressing foetal tissue rejection. The fall in the level of faecal 20-oxopregnanes immediately after foaling can be attributed to the loss of the foetal-maternal interface [1,8], since a greater proportion of progesterone during gestation is released by the allantochorion lining of the myometrium [43]. Furthermore, the biphasic faecal progestagen pattern we recorded has previously been reported in zebras [17] and may be associated with the formation of a secondary corpora lutea.

In contrast to 20-oxopregnane, faecal oestrogen levels during pregnancy rise above baseline levels of non-pregnant mares (<160ng/g DW) after about 250 DTF, reaching a peak in mid-gestation, followed by a gradual decline until parturition; a trend consistent with the pattern observed in horses [4,32]. The rise and fall of faecal oestrogen levels in mid-pregnancy (Figure 2) coincides with the enlargement and subsequent regression of foetal gonads in equids [26]. A similar pattern in oestrogen concentrations during pregnancy has been observed in Grevy's zebra [3,6,31], where foetal gonads synthesize large amounts of dehydroepiandrosterone, which initiates the production of oestrogen by the placenta [32]. The pattern of faecal oestrogen levels found in these wild zebras is similar to that described for the captive ones [37], although we found a gradual decline in levels from mid-pregnancy to foaling. Faecal extracts therefore appear to be an adequate source of steroid hormones for describing the gestational patterns of 20-oxopregnanes and oestrogen in zebras, as in other mammals.

Few studies have investigated the effects of seasonality on steroid hormone concentrations. In this study of wild zebras, in spite of the strongly seasonal environment, no seasonal effects were found in faecal 20-oxopregnane or oestrogen levels. Contrasting results have been found in other large herbivores: in the savannah elephant *Loxodonta africana*, faecal 5 α -pregnane-3-ol-20-one levels increase in the wet season coinciding with an increase in food availability [42]. In Holstein–Friesian cows in Europe, different amounts of grass intake did not influence faecal progesterone metabolite concentrations significantly [29]. Another study showed that rats on a high plane of feeding had excreted more oestrogen in the faeces [2], and Schwarzenberger [32] postulates that factors such as compartmentalization, turnover rates, permeability and nutritional stress influence levels of faecal steroid metabolites in horses. Therefore, the potential seasonal effect of faecal progestagen and oestrogen metabolite excretion in wild zebra needs more attention, because out of the 52 samples from pregnant females, only 16 were from the dry season.

The GAM described the pattern of each hormone well, accounting for over 60% of the variance in the data. The models describing patterns of both hormones enable the detection of the reproductive status of plains zebra mares. High levels of 20-oxopregnanes (>200ng/g DW) associated with high oestrogen levels (>160 ng/g DW) indicated mid-pregnancy in 90% of cases (16/17, from data in from Figures 1 & 2) and high 20-oxopregnane and low oestrogen levels (<160 ng/g DW) indicate late pregnancy, again in 90% of cases. Two faecal samples collected three months apart should therefore allow the pregnancy status of mares to be ascertained with confidence.

ACKNOWLEDGEMENTS

This work is part of the CNRS Hwange Environmental Research for Development programme directed by Dr. Hervé Fritz and administered by the CNRS UMR 5558: we are most grateful for valuable scientific advice and effective administration. We are very grateful to the CNRS Institute of Ecology and Environment for long-term support. The work was conducted within the framework of the Research Platform – Production and Conservation in Partnership (RP-PCP). We thank the *Ministère Français des Affaires Etrangères* for supporting Hlengisizwe Ncube through the French Embassy in Zimbabwe (RP-PCP grant ECOL#2). We are most grateful to the Zimbabwe Parks and Wildlife Management Authority permission to conduct research in Hwange National Park, and in particular to Mr A. Musakwa for support and encouragement. We thank Prof. Peter Mundy for helping to supervise this MPhil. study and commenting on the drafts of this paper. We also thank Alison Duncan and Stéphanie Periquet for their help in collecting faecal samples, and Stefanie Muenscher for excellent expert help in laboratory techniques.

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FIGURE CAPTIONS

Figure 1: Log transformed concentrations of faecal 20-oxopregnanes (ng/g DW) in plains zebra mares before and after foaling.

Figure 2: Log transformed concentrations of faecal oestrogens (ng/g DW) in plains zebra mares before and after foaling.

Table 1: Summary table for the Generalised Additive Model of the faecal 20-oxopregnane levels in plains zebra mares

Formula	20-Oxopregnanes ~ s(Days to foaling)				
Parametric coefficients: (Intercept)	Estimate	Std. Error	t value	Pr(> t)	
	2.41655	0.04899	49.32	<2e-16 ***	
Significance of smooth terms- s(Days to foaling)	Edf		F	p-value	
	7.738		13.24	3.11e-11 ***	
	R-sq.(adj.)	Deviance explained	Generalised cross validation score	Scale est.	N
Model	0.616	65.9%	0.19199	0.16802	70

Table 2: Summary table for the Generalised Additive Model of faecal oestrogen levels in plains zebra mares

Formula	Oestrogen ~ s(Days to foaling)			
Parametric coefficients: (Intercept)	Estimate	Std. Error	t value	Pr(> t)
	1.9766	0.0444	44.52	$<2e-16$ ***
Significance of smooth terms: s(Days to foaling)	Edf		F	p-value
	4.830		14.55	$4.89e-09$ ***
Model	R ² -(adj.)	Deviance explained	Generalised Cross Validation score	N
	0.539	56.8%;	0.12221	62

Figure 1

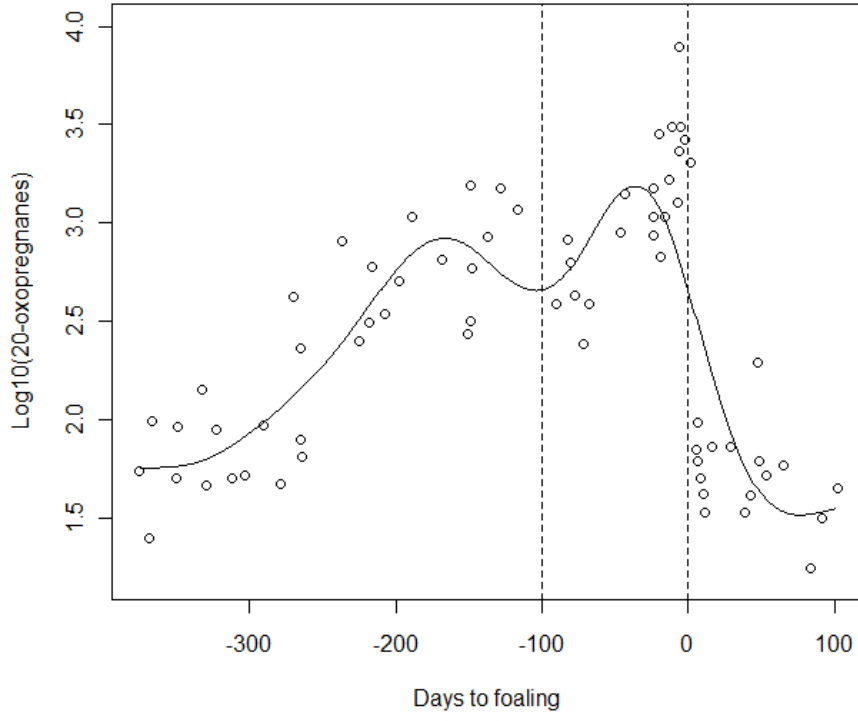


Figure 2

