General and Comparative Endocrinology 289 (2020) 113376

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Irregular ovarian cyclicity is associated with adrenal activity in female eastern black rhinoceros (*Diceros bicornis michaeli*)

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ARTICLE INFO

Keywords:
Acyclicity
Faecal glucocorticoids
Luteal-phase
Nulliparous
Oestrous cycle
Seasonal

ABSTRACT

To achieve self-sustaining and genetically diverse populations *ex situ*, captive breeding programmes must ensure good overall rates of reproduction, and equal contribution across individuals. Previous research in the critically endangered eastern black rhinoceros (*Diceros bicornis michaeli*) revealed a high incidence of irregular oestrous cyclicity; in particular extended cycle duration among nulliparous females and acyclic periods in parous females that have not bred for several years. Irregular ovarian activity could play a role in reduced reproductive output; however, the mechanisms underlying these anomalies are poorly understood. The aim of this study was to measure faecal glucocorticoid metabolite (fGCM) concentrations and variability prior to and during periods of regular and irregular ovarian activity, and determine if adrenal activity influences the occurrence of different cycle types in this species. Faecal samples were collected every other day from parous (N = 6) and nulliparous (N = 12) females at eight European institutions for periods of 9–15 months. Concentration and variability in fGCM were compared between periods of regular and irregular cyclicity and between different cycle types (< 20 days, 20–40 days, > 40 days, acyclic) using generalized linear mixed models. Concentrations of fGCM were influenced by season and higher during the luteal than the follicular phase of the oestrous cycle. Taking this into account, fGCMs were increased during periods of irregular cyclicity (all types combined and during cycles > 40 days in length) compared to 20–40 day cycles. This was predominantly driven by nulliparous females. The variation in fGCM concentration also differed between periods of regular and irregular cyclicity; higher standard deviation in fGCM preceded irregular cycles and > 40 day cycles compared to 20–40 day cycles. These results suggest that although fGCM concentrations fluctuate across the oestrous cycle in this species, changes in adrenal activity at specific times could be one factor associated with irregular ovarian activity in the black rhinoceros.

1. Introduction

Among female black rhinoceros, both *in situ* (Garnier et al., 2002) and *ex situ* (Brown et al., 2001; Edwards, 2013; Edwards et al., 2015a), irregular oestrous cycles can negatively impact population sustainability. Four cycle types have been identified, including those that are both shorter and longer than the average of 26–27 days, as well as extended periods of acyclicity where progestagen concentrations remain at baseline. Indeed, previous results have indicated that irregular cyclicity may be correlated with reproductive success (Edwards, 2013; Edwards et al., 2015a); specifically, longer cycles are more common in nulliparous females, and acyclic periods are more common in parous females that have not bred for at least 7 years. However, the hormonal basis of these irregular cycles is poorly understood, and a better understanding of the physiology underlying periods of regular and irregular cyclicity in the black rhinoceros is required.

Irregular ovarian cyclicity and acyclicity have been reported in captive populations of a number of endangered species, including the white rhinoceros (*Ceratotherium simum*) (Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001), Indian rhinoceros (*Rhinoceros unicornis*) (Gomez et al., 2004; Stoops et al., 2004), Asian (*Elephas maximus*) and African (*Loxodonta africana*) elephants (Brown et al., 2004; Proctor et al., 2010a; Dow et al., 2011), and a number of felid species including the cheetah (*Acinonyx jubatus*) (Brown et al., 1996; Schwarzenberger and Brown, 2013). Results of previous studies suggest that abnormal adrenal activity may play a role in some of these fertility issues. For example in the white rhinoceros, non-cycling females had more variable faecal glucocorticoid metabolite (fGCM) patterns than

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https://doi.org/10.1016/j.ygcen.2019.113376
Received 28 April 2019; Received in revised form 30 October 2019; Accepted 22 December 2019
Available online 24 December 2019
0016-6480/

cycling females (Carlstead and Brown, 2005), non-cycling female cheetahs exhibited higher fGCM concentrations overall (Jurke et al., 1997), and periods of acyclicity in female douc langurs (*Pygathrix nemaeus*) were associated with increased fGCM concentrations (Heistermann et al., 2004). In captive elephants, although no differences in serum cortisol concentrations have been observed between cycling females and those that exhibit irregular or complete acyclicity (Proctor et al., 2010b), abnormal adrenal activity is associated with hyperprolactinemia (Rivier, 1995; Sobrinho, 2003); a condition observed in acyclic African elephants (Dow and Brown, 2012).

When an individual perceives a potential threat to homeostasis, activation of the hypothalamic–pituitaryadrenal (HPA) axis results in the secretion of glucocorticoids from the adrenal gland (Matteri et al., 2000). This neuroendocrine response to stress is an adaptive mechanism, facilitating the mobilisation of energy stores to allow the body to respond accordingly and maintain homeostasis (Moberg and Mench, 2000). However, prolonged or repeated activation, or at certain key times, can lead to detrimental consequences including the disruption of reproductive cyclicity and reduced fertility (Dobson and Smith, 2000; Tilbrook et al., 2000). We have previously demonstrated that reproduction in the European captive population of eastern black rhinoceros (*Diceros bicornis michaeli*) does not appear to be disrupted by chronic stress, as no differences in average or variation in fGCMs were apparent between parous and nulliparous females (Edwards et al., 2015a). However, this does not mean that adrenal activity has no impact on reproductive function in this species; rather a different approach may be needed to investigate changes in fGCM concentration within individuals over time (Fanson et al., 2014) and in relation to more short-term changes in adrenal activity.

The physiological basis of irregular oestrous cycles in captive black rhinoceros is not yet understood, but disruption of regular ovarian activity may contribute to subfertility. Reduced reproductive output has previously been suggested as a contributing factor to the limited growth and viability of *ex situ* black rhinoceros populations (Smith and Read, 1992; Carlstead et al., 1999a; Carlstead et al., 1999b; Edwards, 2013; Edwards et al., 2015b). Furthermore, with a proportion of females failing to produce offspring (Edwards, 2013; Edwards et al., 2015b), reproductive skew could have consequences for maintenance of genetic diversity and long-term population viability. The aim of this study was to retrospectively assess adrenal activity prior to and during periods of regular and irregular ovarian activity to determine how it is related to different cycle types in this species. Specifically, fGCM concentrations and their variability were compared between different cycle types (20–40 days (considered normal in this species), > 40 days, < 20 days, and periods of acyclicity), and between regular and irregular periods of cyclicity combined, to investigate whether differences in adrenal activity are associated with abnormal ovarian function.

2. Methods

2.1. Study population

We examined a sub-set of females from a larger study of the reproductive status of the European captive population of eastern black rhinoceros (Edwards, 2013; Edwards et al., 2015a) that included 18 female black rhinoceros housed at eight zoological institutions across Europe. This research was approved by the European Endangered Species Breeding Programme (EEP), The North of England Zoological Society (NEZS), management at each participating institution, and where applicable, was reviewed and approved by zoo research committees. The full reproductive history of each individual was determined from the European Association of Zoos and Aquaria (EAZA) studbook (Pilgrim, 2009; Biddle and Pilgrim, 2011), and of these females, six were categorized as parous (age range 7y 8mo – 27y 10mo) and previously produced at least one live calf, and 12 were categorized as nulliparous (age range 4y 6mo – 21y 0mo), having never produced a live calf.

2.2. Faecal sample collection and preparation

Faecal samples were collected from each female every other day for between 9 and 15 months. Samples were collected by keepers as soon as possible after defecation, frozen at −20 °C, and stored before shipment to Chester Zoo, UK for analysis. Steroid metabolites were extracted from faecal samples according to an established wet-weight shaking extraction method (Walker et al., 2002; Edwards et al., 2013). In brief, each sample was thawed, thoroughly mixed, and weighed (0.5 g ± 0.003 g) before adding 5 ml 90% methanol, vortexing and shaking overnight on an orbital shaker. Each sample was then vortexed and centrifuged for 20 min at 598g. The supernatant was decanted, dried under air, re-suspended in 1 ml 100% methanol and the resulting faecal extract stored at −20 °C until analysis.

2.3. Enzyme immunoassay

Previously described enzyme immunoassays adapted from Munro and Stabenfeldt (1984), were used with some modifications to measure faecal progestagen metabolite (fPGM) (Edwards et al., 2014) and fGCM (Watson et al., 2013) metabolites. Each EIA utilized an antiserum (monoclonal anti-progesterone CL425 or polyclonal anti-corticosterone CJM006, respectively; C.J. Munro, University of California, Davis), and corresponding horseradish peroxidase (HRP) conjugated label (C.J. Munro, University of California, Davis) and standards (Sigma-Aldrich, UK) on a Nunc-Immuno Maxisorp (Thermo-Fisher Scientific, UK) microtitre plate. Black rhino faecal extracts were diluted as necessary in EIA buffer (1:70 for progesterone or 1:20 for corticosterone), and 50 µl run in duplicate on the respective EIA's. Samples collected at least every other day were analysed for fPGM concentration (N = 2684), and samples collected at least weekly over the sample collection period were analysed for fGCM concentration, with samples collected at least every other day analysed for fGCM around periods of regular and irregular cyclicity (N = 2090).

The cross reactivities for progesterone and corticosterone antisera have been reported elsewhere (Walker et al., 2008; Watson et al., 2013). EIAs were biochemically validated for measuring progestagen and glucocorticoid metabolites in female black rhino faecal extract through parallelism (R^2 = 0.969, y = 0.851x + 2.014, $F_{1,7}$ = 222.140, P < 0.001; and R^2 = 0.982, y = 0.971x – 0.873, $F_{1,7}$ = 377.007, P < 0.001, respectively) and matrix interference assessment (R^2 = 0.998, y = 0.775x + 0.807, $F_{1,7}$ = 4338.484, P < 0.001; and R^2 = 0.999, y = 1.082x + 2.267, $F_{1,7}$ = 7133.701, P < 0.001, respectively). The progesterone EIA was shown to be biologically valid for black rhinoceros faeces (Edwards et al., 2014), and was validated prior to this study by showing clear increases in faecal progestagens during pregnancy and oestrous cycles (Edwards, 2013). The corticosterone EIA was biologically validated for assessing adrenal status via fGCM changes in female black rhinoceros following translocation (Edwards, 2013; Watson et al., 2013). Intra- and inter-assay coefficients of variation (CVs) were < 10% and < 15%, respectively, for high- and low-binding synthetic and biological controls for both assays.

2.4. Data analysis

Oestrous cycles were determined from measures of fPGM and characterized according to a previously established method (Brown et al., 1994; Brown et al., 2001; Edwards et al., 2015a). All non-pregnant samples were used to calculate the mean and standard deviation (SD) for each individual female. An iterative process was then used to remove all samples > 1.5 SD above the mean, before the mean was recalculated and the process repeated until no values exceeded 1.5 SD from the mean. These values were considered baseline and represented the follicular phase of the cycle. The onset of the luteal phase was

considered to be the first sample where fPGM concentration exceeded 1.5 SD above the mean, and the end of the luteal phase was when at least two consecutive samples were below the threshold of 1.5 SD of the mean. As we were interested in any potential disruption that may occur during the follicular phase (period of follicle development prior to ovulation) and/or the subsequent luteal phase, a complete cycle was characterized as the first follicular phase sample to the last luteal phase sample. Oestrous cycles were defined according to Edwards et al. (2013, 2015a), with cycles < 20 days considered short, 20–40 days as normal, > 40 days as long, and sustained periods (> 10 days; Brown et al. (2001)) where fPGM concentrations remained at baseline without any increase above 1.5 SD above the mean categorized as acyclic. Any cycles with insufficient data points for categorization, i.e. missing data points, or cycles at the start or end of the study period where only follicular or luteal phases were represented, were removed from subsequent analyses.

Generalized linear mixed models (GLMMs) were used to investigate differences in fGCM between cycle types using MLwiN version 2.02 (Rasbash et al., 2005). Normality tests were first conducted, and hormone data were $\log_{10}$ transformed to improve distribution ($\log_{10}$ fGCM). To account for repeated sampling within and between females, and the inclusion of multiple females from the same facilities, random effects (date of sample collection, subject ID and institution) were incorporated into the GLMM. Differences in fGCM were observed across seasons (χ^2 = 138.856, df = 3, P < 0.001), being higher in winter (mean ± SE: 22.7 ± 0.6 ng/g faeces) and lower in summer (18.6 ± 0.5 ng/g faeces), compared to spring (20.5 ± 0.5 ng/g faeces) and autumn (20.8 ± 0.5 ng/g faeces); therefore season was included as a covariate in all subsequent models. Fixed effects of oestrous cycle phase (follicular or luteal), season (spring, summer, autumn, and winter), and either cycle category (regular [20–40 day cycles] or irregular [< 20 day, > 40-day cycles and acyclic periods combined]), or cycle type (20–40 days, < 20 days, > 40 days or acyclic) were added individually. As these were categorical GLMM, a reference category was assigned to which all other categories were compared, using follicular phase, spring, regular periods of cyclicity, or 20–40-day cycles as the reference category, respectively. fGCM concentration was significantly higher during the luteal phase (21.7 ± 0.5 ng/g faeces) compared to the follicular phase (19.5 ± 0.5 ng/g faeces) of the cycle (χ^2 = 75.417, df = 1, P < 0.001); therefore cycle phase was included as a covariate in subsequent models investigating $\log_{10}$ fGCM according to cycle category and cycle type. Comparisons were first made using samples from all females (N = 18); the GLMM was then repeated separating parous females (N = 6) and nulliparous females (N = 12) to investigate whether differences existed according to prior reproductive success. Differences between the two parity groups are reported only where significant. The variability in fGCM, calculated as the standard deviation (SD) in $\log_{10}$ fGCM, was compared between cycle types and cycle phases, with institution, subject ID and cycle number (1–17) as random effects and season as a covariate.

Finally, both the mean and SD of $\log_{10}$ fGCM were compared during the luteal phase preceding each cycle type. Institution, subject ID and cycle number were incorporated into the model as random effects, and season as a covariate. Again, fixed effects of either cycle category (regular or irregular) or cycle type (20–40 days, < 20 days, > 40 days or acyclic) were added individually, and reference categories assigned as previously described. All GLMMs utilized a normal distribution, and the significance of each fixed effect was determined using the Wald statistic and chi-squared (χ^2) distribution, with alpha set to 0.05.

3. Results

3.1. Oestrous cycles

A total of 172 oestrous cycles were observed across the study period; 121 cycles in nulliparous females and 51 cycles in parous females (Table 1). Of these, 51 were < 20 days, 88 were 20–40 days, and 33 were > 40 days. Additionally, 24 acyclic periods were observed, occurring in 12 (5 parous and 7 nulliparous) out of 18 females, with fPGM concentrations remaining at baseline for between 14 and 133 days. All 18 females exhibited periods of both regular (20–40 days) and irregular cyclicity (< 20 days, > 40 days or acyclic) during the sampling period. Representative profiles of regular and irregular cyclicity patterns, and corresponding fGCM concentrations are presented in Fig. 1, and individual profiles are provided in Supplementary data. For all further analyses, samples representing these 196 periods of potential oestrous cyclicity were used (N = 2053).

3.2. Glucocorticoids and irregular cyclicity

After controlling for differences according to season and cycle phase, fGCMs were significantly higher during periods of irregular compared to regular cyclicity (χ^2 = 5.861, df = 1, P = 0.015). Furthermore, fGCMs were significantly higher during cycles > 40 days (χ^2 = 5.767, df = 1, P = 0.016), and tended to be higher during cycles < 20 days (χ^2 = 3.325, df = 1, P = 0.068) compared to 20–40 day cycles. However, there were no differences between 20-40 day cycles and acyclic periods (χ^2 = 0.435, df = 1, P = 0.510), or between any of the irregular cycle types (Fig. 2a).

Data were then separated by reproductive category, and patterns of fGCMs differed according to parity. In nulliparous females, irregular periods of cyclicity were associated with significantly higher fGCM (χ^2 = 7.203, df = 1, P = 0.007). Furthermore, fGCMs were significantly higher during long cycles (> 40 days: χ^2 = 8.876, df = 1, P = 0.003) and tended to be higher during short cycles (< 20 days: χ^2 = 3.665, df = 1, P = 0.056) compared to 20–40 day cycles, but not during acyclic periods (χ^2 = 0.103, df = 1, P = 0.748). In contrast, there were no differences in fGCMs in parous females between periods of regular and irregular cyclicity (P = 0.765), or between the four cycle types (P > 0.570).

The variation in fGCM between cycle types, represented by the standard deviation calculated for each cycle, did not differ between regular and irregular periods of ovarian activity (χ^2 = 1.405, df = 1, P = 0.236). However, < 20 day cycles had significantly lower fGCM SD than both 20–40 day (χ^2 = 4.427, df = 1, P = 0.035) and > 40 day (χ^2 = 5.888, df = 1, P = 0.015) cycles. Finally, there were no differences between regular and irregular periods (χ^2 = 0.113, df = 1, P = 0.737), or between cycle types (P > 0.688) in mean fGCM during the preceding luteal phase. However, SD in fGCM during the luteal phase preceding the current cycle was higher prior to irregular cycle types (χ^2 = 3.965, df = 1, P = 0.046) when compared to 20–40 day cycles. Specifically, fGCM SD was higher during luteal phases prior to > 40 day cycles compared to 20–40 day cycles (χ^2 = 6.199, df = 1, P = 0.013) and acyclic periods (χ^2 = 4.949, df = 1, P = 0.026) (Fig. 2b), and there was a tendency for higher fGCM SD in < 20 day cycles compared to both 20–40 day cycles (χ^2 = 3.351, df = 1, P = 0.067) and acyclic periods (χ^2 = 3.026, df = 1, P = 0.082).

4. Discussion

Although there are likely to be multiple factors underlying irregular ovarian activity within and between species, we provide the first clear evidence in female black rhinoceros that adrenal activity could be involved. All of the females included in this study exhibited both regular (cycles 20–40 days in length), and irregular periods of ovarian cyclicity (cycles < 20 or > 40 days in length, or acyclicity) over the course of a year. These data support previous findings (Brown et al., 2001; Edwards, 2013, Edwards et al., 2015a) that irregular ovarian activity is a relatively common observation in captive females of this species. We have previously demonstrated that the longer cycle type is more common among nulliparous females, and acyclic periods are more common among females that have not bred for at least 7 years

Table 1
Number and type of oestrous cycles characterized in each female black rhinoceros.

				Number of Cycles				
ID	Institution	Age	Parity	Total	20–40 days	< 20 days	> 40 days	Acyclic
1	A	6y 8mo	Nulliparous	13	10	2	1	
2	B	7y 8mo	Parous	13	7	3	2	1
3	B	13y 1mo	Parous	12	4	5	2	1
4	C	13y 7mo	Nulliparous	16	6	3	1	6
5	B	5y 6mo	Nulliparous	15	6	7	1	1
6	C	27y 10mo	Parous	8	3	2	2	1
7	B	11y 11mo	Nulliparous	10	3	4	3	
8	B	20y 7mo	Nulliparous	10	5	2	2	1
9	D	21y 0mo	Nulliparous	9	5		1	3
10	E	18y 9mo	Parous	9	3	4		2
11	D	9y 10mo	Nulliparous	11	9	2		
12	F	9y 0mo	Nulliparous	13	5	4	2	2
13	E	20y 1mo	Parous	9	5	2	1	1
14	G	20y 8mo	Parous	9	1	3	2	3
15	H	18y 2mo	Nulliparous	9	4	1	3	1
16	A	15y 6mo	Nulliparous	12	6	3	3	
17	F	13y 7mo	Nulliparous	10	3	3	3	1
18	B	4y 6mo	Nulliparous	8	3	1	4	

(Edwards, 2013; Edwards et al., 2015a). This study adds to these findings that although overall fGCM concentrations may not differ between parous and nulliparous females (Edwards et al., 2015a), the occurrence of irregular oestrous cycles was accompanied by increased fGCM compared to 20–40 day cycles. This was particularly evident in nulliparous females. In addition, increased variability in fGCM during the luteal phase of the preceding cycle was also associated with irregular cyclicity. Together these results suggest that increased adrenal activity may indeed be related to the occurrence of irregular ovarian activity in this species.

In the white rhinoceros, acyclicity is deemed to be an abnormal, albeit relatively common phenomenon (Roth et al., 2018), and non-cycling females have previously been demonstrated to exhibit more variable fGCM concentration than cycling females (Carlstead and Brown, 2005). However, the debate regarding the presence of two distinct cycle types has continued for some time, with arguments in favour of both the shorter 30–35 day and longer 65–70 day cycles being physiologically normal (Radcliffe et al., 1997; Schwarzenberger et al., 1998; Patton et al., 1999; Brown et al., 2001). Although the longer cycle type appears to be fairly widespread amongst captive white rhinoceros, successful pregnancies have only been reported following breeding associated with the shorter cycle type, leading to the current consensus that perhaps only the 30–35 day cycle is fertile in this species. Until now, there has been little attention on the consistent occurrence of different cycle types in the black rhinoceros, despite their being reported in both captive (Brown et al., 2001; Edwards et al., 2015a) and wild (Garnier et al., 2002) populations. We provide the first evidence to suggest that in the black rhinoceros, both the shorter (< 20 days in length) and the longer oestrous cycle type of > 40 days in length, as well as acyclic periods, may not be physiologically normal, as indicated by elevated fGCM concentrations compared to 20–40 day cycles. However, the potential cause of increased adrenal activity associated with these different cycle types, and the mechanisms by which higher adrenal activity could be disrupting normal ovarian activity, remain unclear.

It is important to note that although elevated glucocorticoids have the potential to have a suppressive effect on reproduction (Dobson and Smith, 2000; Tilbrook et al., 2000), they also play an important role in normal reproductive function, including follicle maturation, ovulation, and luteinisation (Brann and Mahesh, 1991; Tetsuka, 2007). Indeed, glucocorticoid concentrations vary predictably across the oestrous cycle in many species, such as ewes (Sosa et al., 2013), giant pandas (Kersey et al., 2011) and elephants (Fanson et al., 2014). Cortisol concentrations also change across the menstrual cycle in women, being lower during the follicular phase when oestrogens are high, compared to the luteal phase (Kirschbaum et al., 1999). Indeed, disruption of the cyclic release of glucocorticoids can be associated with ovarian dysfunction (Brann and Mahesh, 1991; Tébar et al., 1995; Mahesh and Brann, 1998). Similar to the pattern in humans, this study showed a consistent difference across the cycle, with fGCM concentrations significantly higher during the luteal than the follicular phase. This was true for the 20–40 day cycles and both shorter and longer cycle types, and so should be taken into consideration when assessing the impact of adrenal activity on ovarian function. However, the increased concentrations observed during irregular cycles presumably is not solely related to normal reproductive function because fGCMs were significantly higher during irregular cycles compared to normal 20–40 day cycles, and more specifically in cycles of abnormal duration.

Abnormal adrenal activity has the potential to negatively impact reproduction in a number of ways, including follicle development and ovulation via the disruption of gonadotrophins (Moberg, 1985, Dobson and Smith, 2000). If follicles do not develop appropriately, ovulation could fail completely resulting in prolonged baseline concentrations of progestagens (Dobson and Smith, 2000). Alternatively ovulation may occur but result in short cycles due to sub-optimal quality of oocytes (Badinga et al., 1993), perhaps related to altered LH pulsatility (Jones, 1991). A third scenario is the luteinisation of a non-ovulatory follicle, resulting in erratic or prolonged elevations in progestagen concentrations, as previously reported in the black rhinoceros (Radcliffe et al., 2001). Elevated fGCM concentration during cycles > 40 days in length could also indicate disruption to the process of luteolysis and the normal regression of the corpus luteum (Liptrap, 1993). Experimental evidence from other species, including the cow, sheep and rat (Cooke and Benhaj, 1989; Wang et al., 1993; Lee et al., 2007) suggests that abnormally high fGCMs can block luteolysis through the disruption of prostaglandins. Although we cannot yet determine if this is the case for black rhinoceros, prolonged fPGM concentrations that often exceeded those during the normal 20–40 day cycle (Edwards et al., 2015a) in conjunction with increased fGCM concentrations point towards retention of the corpus luteum as a potential reproductive anomaly in this species, and should be investigated further with the addition of ultrasonography in tandem with endocrine analyses.

In addition to differences in fGCM during ovarian cycles of different length, these data also suggest that variability in fGCM may influence the subsequent ovarian cycle. Research from other species has indicated that correct priming during the previous cycle is important for follicular

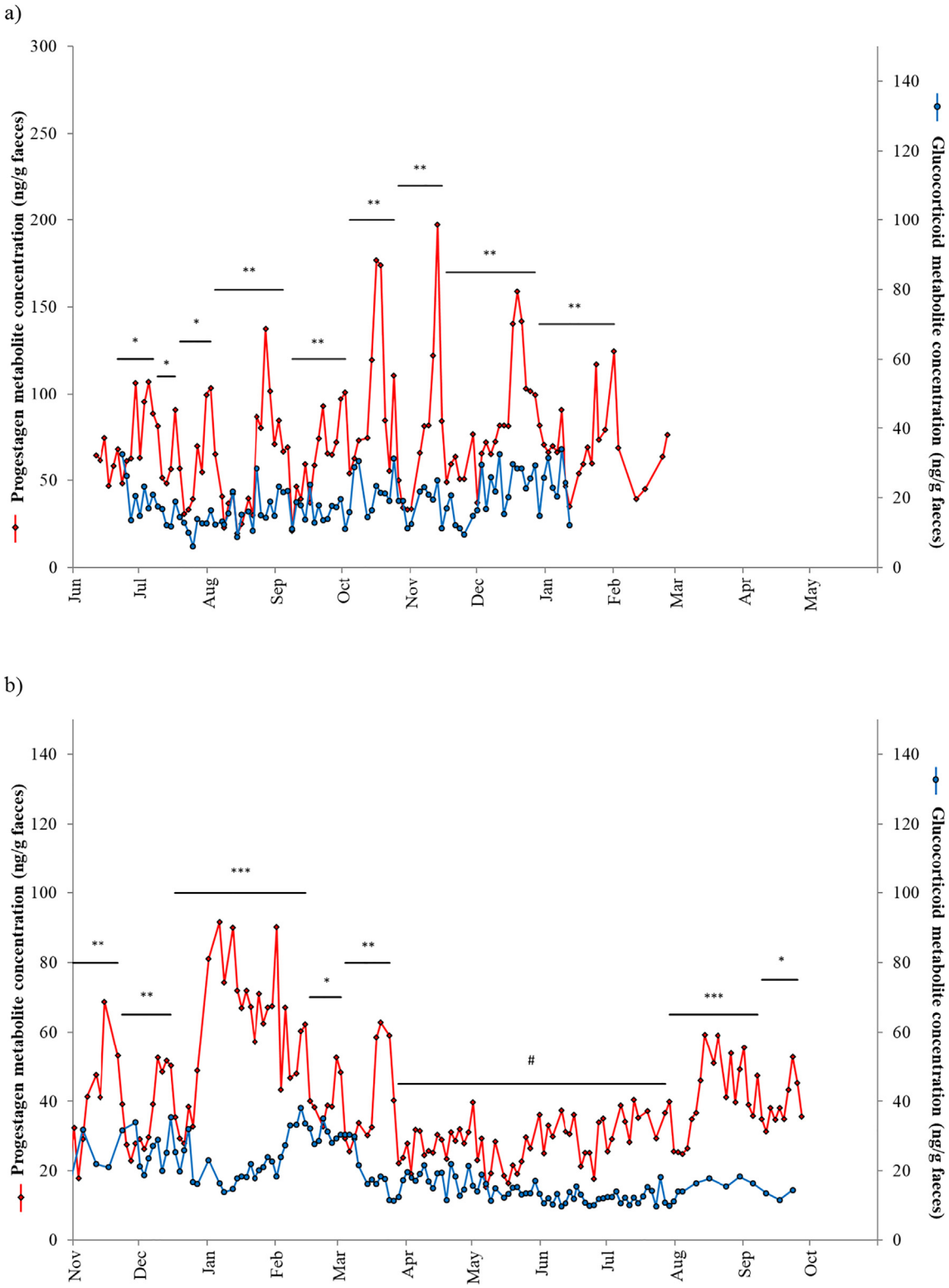

Fig. 1. Example profiles showing faecal progestagen (red) and glucocorticoid (blue) metabolite concentration in a) female 13, a multiparous female black rhinoceros age 20 years, and b) female 17, a nulliparous female age 14 years. Cycle types include those < 20 days (*), 20–40 days (**) and > 40 days (***) in length, and acyclic periods (#).

development (Strott et al., 1970; Sherman and Korenman, 1974; Smith et al., 1985), oestrus behaviour (Carrick and Shelton, 1969; Stevenson et al., 1989; Blache et al., 1994; Véliz et al., 2009), conception rates (Cuervo-Arango and Clark, 2010) and embryo survival (Cuervo-Arango et al., 2018). Studies in post-partum dairy cows have also demonstrated that the resumption of normal ovarian activity is faster, and subsequent fertility is better, in females that exhibited a normal luteal phase following post-partum ovulation (Shrestha et al., 2004; Kawashima et al., 2006) compared to those that exhibited anovulatory cycles or prolonged luteal phases. Although none of these previous studies investigated the role of adrenal activity during the preceding cycle, it is clear that appropriate priming may be important for normal

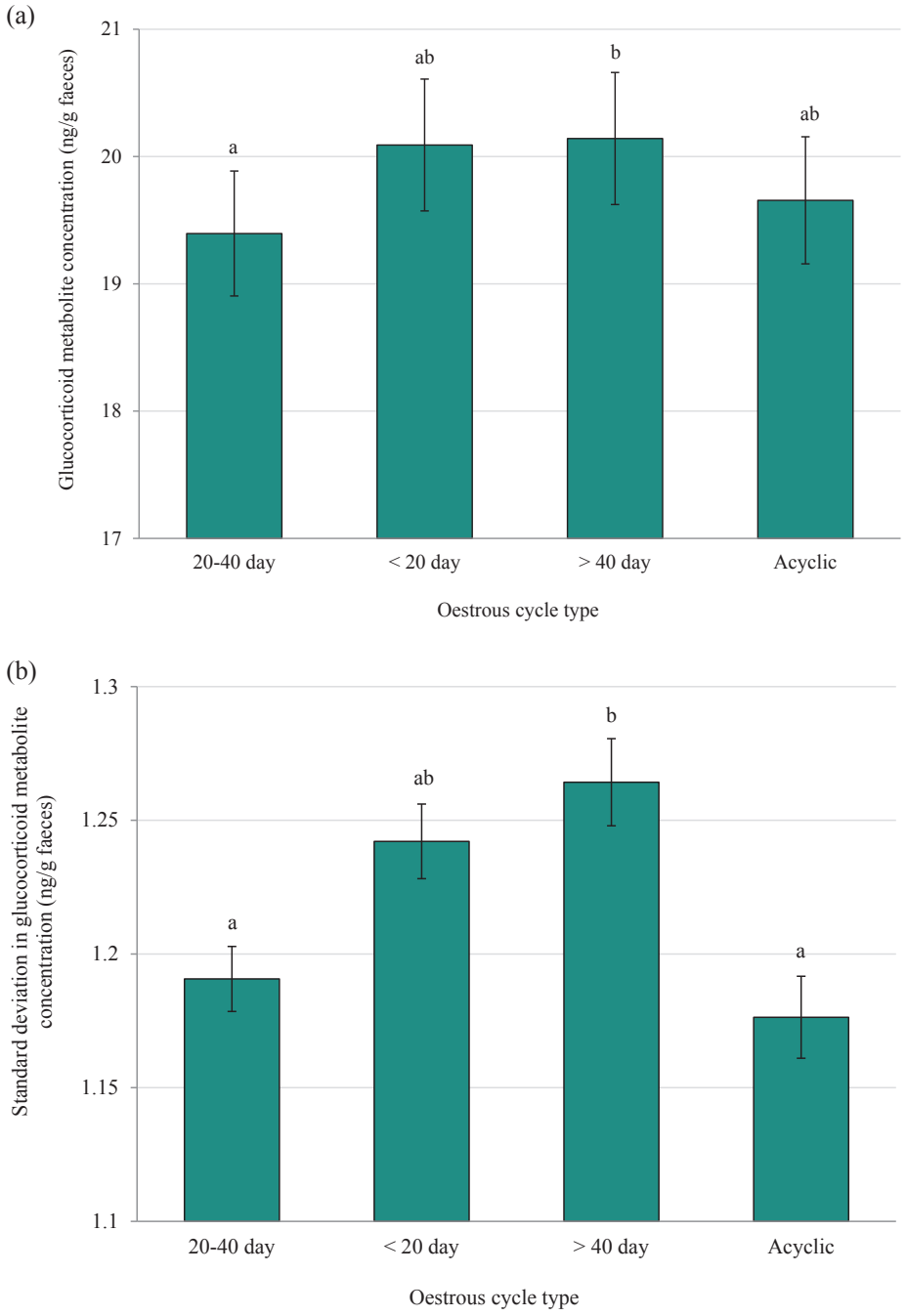

Fig. 2. a) Faecal glucocorticoid metabolite concentration (fGCM) during, and b) standard deviation in fGCM during the luteal phase preceding 20–40 day, < 20 day and > 40 day cycles, and acyclic periods. Bars represent the back-transformed mean prediction from the GLMM +/−1SE; letters reflect significant differences.

reproductive function, and our data suggest that increased fGCM variability could result in sup-optimal conditions that lead to the development of abnormal cycles.

A further possibility for extended luteal phase concentrations of progestagens, together with an increase in fGCM concentration, is that of conception and early pregnancy loss. This issue has been reported in all of the four rhinoceros species currently maintained in captivity (Berkeley et al., 1997; Radcliffe et al., 1997; Patton et al., 1999; Roth et al., 2001; Stoops et al., 2014). In the white rhinoceros, a single female exhibited two cycles of 73 and 78 days in length, both of which were confirmed by ultrasound to be the result of embryonic loss during the first month of gestation (Radcliffe et al., 1997). Although the role of glucocorticoids in pregnancy loss has yet to be fully investigated in any rhinoceros species, elevated concentrations can result in both implantation failure and early embryonic loss in other species (Nepomnaschy et al., 2006; Thorpe et al., 2013). However, in most of the females included in this study, long oestrous cycles were observed without prior mating (Edwards et al., 2015a). For example, the female represented in Fig. 1b exhibited a cycle lasting 60 days (mid-December to mid-February), despite not being successfully introduced to a male for the duration of the study period due to behavioural incompatibility and a lack of overt oestrus behaviour. At least in unmated females, the increase in fGCMs during long cycles cannot be associated with pregnancy loss, and the extended luteal concentration of fPGM must have some other physiological basis.

In both the white rhinoceros (Patton et al., 1999) and horse (Hughes et al., 1979; Daels and Hughes, 1993), variable oestrous cycle lengths have been attributed to the existence of reproductive pathologies. Such

problems in the rhinoceros include pyometra, leiomyoma, endometritis and hyperplasia (Roth, 2006). Although the prevalence of reproductive pathologies has not been determined in this population, the well-characterized role of glucocorticoids in inflammatory and immune responses (Sapolsky et al., 2000) means it is another feasible explanation for elevated fGCM concentrations observed during irregular cycles. In nulliparous female rhinoceros, long periods without reproducing are believed to contribute to the development of pathologic lesions, which begin to develop around the age of 15 years (Hermes et al., 2006). However, irregular oestrous cycles have so far been observed in females between the ages of 5 and 30 years (this study and Edwards et al. (2015a)), making it unlikely to be the sole explanation for increased glucocorticoids and irregular ovarian cyclicity.

Whatever the mechanism by which glucocorticoids could be impacting ovarian activity in captive rhinoceros, the cause of increased fGCM concentration needs to be addressed, as increased adrenal activity has been considered to be an underlying cause of poor reproductive performance in this species for some time (Brown et al., 2001). Previous research suggests social and environmental factors can constitute potential stressors in captive black rhinoceros (Carlstead and Brown, 2005), including aggression between breeding pairs and exposure to visitors. Furthermore, individual differences in temperament may moderate the adrenal response (Edwards, 2013; Edwards et al., 2015a); if some females are more reactive to potential stressors, this may be associated with increased variability in fGCM as demonstrated here, and could explain why some females are more prone to exhibiting irregular cycles. Indeed, females rated as more behaviourally unpredictable exhibited higher fGCM concentrations (Edwards et al., 2015a), nulliparous females tend to be rated as more unpredictable in their temperament than parous females, and are more likely to exhibit abnormal oestrous cycles. This highlights the need to investigate changes in fGCM concentrations over time and in reference to particular events. Although no differences in average fGCM concentration were apparent between parous and nulliparous females in this population (Edwards et al., 2015a), differences between cycle types suggest that the timing of changes in fGCMs may be important, and could contribute to the reproductive skew previously observed in this population.

The European Endangered Species Breeding Programme (EEP) for the black rhinoceros is currently experiencing good growth rates of 4.7% per annum (Biddle and Pilgrim, 2019), more than triple that observed between 2001 and 2010 (Edwards et al., 2015b). Nonetheless, for genetically healthy, self-sustaining captive populations to be achieved, a better understanding of why these irregular cycles occur, particularly in nulliparous females and those that have not bred for > 7 years, is required. Higher fGCM concentration and variation have been observed during irregular cycles and the luteal phase prior to irregular cycles, respectively; however, the significance of this finding is unknown. Further investigation is warranted to determine whether external factors, perhaps relating to the social or physical environment, an individual's own susceptibility to potential challenges, or whether other internal factors such as reproductive pathology could be involved in increased adrenal activity. As longer cycle types are characterized by a prolonged luteal phase, we suggest that one possible explanation is the disruption of normal luteolysis, and retention of the progestagen-secreting corpus luteum. However, further research is required to investigate other possibilities for extended cycle duration and for the occurrence of shorter than normal cycles and periods of acyclicity. Of high priority is to investigate the physiology and/or pathology involved with different cycle types, and factors within the captive environment that could lead to increased adrenal activity.

Acknowledgements

The authors would like to thank the animal keepers, curators, veterinarians and assistants at the following institutions for collection of faecal samples: Chester Zoo, UK; Bioparc Zoo de Doué la Fontaine, France; Zoo Hannover, Germany; Paignton Zoo, UK; Howletts Wild Animal Park, UK; Zoo Zürich, Switzerland; Zoologischer Garten Magdeburg, Germany; and Zoo de Pont-Scorff, France. We would also like to thank Helen Massey, Jenny Hardy, Vicki Norton, Hannah McArthur, Leanne Harrington, Thijs van den Houten, Rebecca Watson, Rebecca Purcell, Taylor Harrison, Sarah Crosby, Katherine Cho, and Aaron Marshall for assistance preparing and extracting faecal samples. This work was funded by a NERC CASE studentship, and the North of England Zoological Society, Chester Zoo, with contribution from the Thriplow Charitable Trust and the Association of British and Irish Wild Animal Keepers (ABWAK). Funding organizations had no input into the study design, data analyses or manuscript preparation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2019.113376.

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