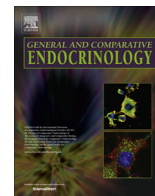




Contents lists available at ScienceDirect

General and Comparative Endocrinology

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

Irregular ovarian activity, body condition and behavioural differences are associated with reproductive success in female eastern black rhinoceros (*Diceros bicornis michaeli*)

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ARTICLE INFO

Article history:

Received 26 May 2014

Revised 26 July 2014

Accepted 28 July 2014

Available online xxx

Keywords:

Adrenal activity

Cyclicity

Estrus

Ex situ

Parity

Temperament

ABSTRACT

Ex situ populations of endangered species such as the black rhinoceros play an important role in global conservation strategies. However, the European captive population of eastern black rhinoceros is performing sub-optimally, with growth rates and genetic viability limited by low birth rates and high reproductive skew. We investigated several intrinsic differences between parous and nulliparous females that may underlie differences in reproductive success, including ovarian cyclicity, adrenal activity, behaviour and body condition. Faecal samples were collected from 39 females (17 parous, 15 nulliparous and 7 pre-reproductive) at 11 zoological institutions, every other day for between 4 months and 6 years. Progestagen metabolite concentration indicated that although all non-pregnant females exhibited ovarian activity, irregular cyclicity was common. Longer cycles (>40 days) were more common in nulliparous females and periods of acyclicity observed more often in females that had not bred for at least 7 years. Even when endocrine data indicated clear ovarian activity, overt behavioural signs of oestrus were not always apparent, particularly among nulliparous females. Faecal glucocorticoids did not differ between parous and nulliparous females, although did differ according to individual temperament. More unpredictable temperaments were associated with higher glucocorticoids, and nulliparous females tended to be rated as more unpredictable. Finally, nulliparous females had higher body condition scores than parous females. This is the first comprehensive survey of the reproductive physiology of this European captive population, and highlights a number of intrinsic differences related to parity, which may underlie differences in reproductive success among captive female black rhinoceros.

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1. Introduction

The European captive population of eastern black rhinoceros (*Diceros bicornis michaeli*) currently consists of around 10% of the global population of this critically endangered subspecies. However this population is only marginally self-sustaining and 49% of reproductive-age females are yet to reproduce (Biddle and Pilgrim, 2011, 2012, 2013). Recent analyses illustrate that low birth rates and high reproductive skew not only limit growth, but also the long-term genetic viability of this population (Edwards, 2013). Similarly, inconsistent rates of reproduction are amongst the factors that limit the sustainability of captive populations of

black rhinoceros in North America (Carlstead et al., 1999; Foose and Wiese, 2006). To create genetically healthy, self-sustaining *ex situ* populations of this species, it is essential to investigate why differences in reproductive success occur, so that reproductive skew can be minimised and overall birth rates improved.

In situ populations of eastern black rhinoceros have a much lower level of female reproductive skew than in captive populations (Edwards, 2013), suggesting that some aspect of captivity could be involved in differential reproductive success. However, nulliparous females are distributed across multiple institutions, often alongside parous females (Edwards, 2013), which suggests that differences in husbandry or physical conditions between institutions may not fully explain reproductive skew. Although variation in reproductive success could merely reflect differences in mating opportunities, there may be underlying physiological differences that could lead to variance in reproductive rates.

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A number of studies have been conducted both *in situ* (Brett et al., 1989; Garnier et al., 2002; MacDonald et al., 2008) and *ex situ* (Berkeley et al., 1997; Czekala and Callison, 1996; Graham et al., 2001; Hindle et al., 1992; Radcliffe et al., 2001; Ramsay et al., 1987; Schwarzenberger et al., 1993, 1996) to establish basic reproductive parameters in female black rhinoceros, such as characterising reproductive cyclicity and pregnancy determination. Brown et al. (2001) revealed that cyclicity was quite erratic in the North American captive population, with longer and shorter cycles than normal, as well as periods of acyclicity observed. This suggests that population growth rates may be limited by underlying issues with reproductive function. However, the potential causes of irregular cyclicity and their effects on fecundity in black rhinoceros have not yet been fully determined, nor has the prevalence of these different cycle types been investigated in the European captive population.

Ovarian activity can be disrupted by increased adrenal activity as a result of perceived stressors experienced in captivity (Hodges et al., 2010). In captive white rhinoceros, non-cycling females were found to have higher variability in faecal glucocorticoid concentration than cycling females (Carlstead and Brown, 2005), and in captive black rhinoceros, differences in adrenal activity have been associated with mortality (Carlstead and Brown, 2005). However, to investigate the role of potential stressors in differential reproductive success, we also need to take into account the behavioural adaptations involved in coping with stress (Wielebnowski, 2003), as these may moderate how an individual may perceive and respond to such challenges (Sapolsky, 1994).

Natural diets are often difficult to replicate in captivity, with alternatives often over nutrient-rich (Berkeley et al., 2011; Dierenfeld, 1997). In a range of wildlife species, unsuitable diets in captivity lead to higher than ideal body mass (Clauss et al., 2009; Goodchild and Schwitzer, 2008; Mustonen et al., 2009; Vester et al., 2008), and in captive female elephants body mass is related to a higher prevalence of acyclicity (Freeman et al., 2009). Furthermore, in the horse, obesity and related metabolic conditions are associated with altered oestrous cycle duration (Fitzgerald et al., 2003; Sessions et al., 2004), the presence of anovulatory follicles (Vick et al., 2006) and altered follicle development (Sessions-Bresnahan and Carnevale, 2014). Over-condition has also been suggested as a possible contributory factor in reproductive issues in the white rhinoceros (Berkeley et al., 2011). The black rhinoceros is predominantly a browsing species, and over-feeding of unsuitable energy-rich, roughage-low food types in captivity can lead to obesity (Clauss and Hatt, 2006), which may in turn impact reproductive function.

To facilitate captive breeding programmes, it also is important to understand behavioural cues associated with receptivity (Lindburg and Fitch-Snyder, 1994), as they can play a key role in successful mating. Captive black rhinoceros breeding pairs are often housed separately, and only introduced during oestrus. Although several behaviours associated with oestrus have been described, behaviours in the absence of a male are often difficult to distinguish and can be highly variable (Fouraker and Wagener, 1996; Radcliffe et al., 2001). Even when a female is receptive, courtship can be very aggressive, and full oestrus may be preceded by mock charges and defensive displays (Hutchins and Kreger, 2006). Introductions can therefore be difficult to manage, and reduced fecundity could be the result of insufficient opportunities to mate.

This study used a multi-institutional approach to investigate different factors that could influence ovarian activity and reproductive success in captive eastern black rhinoceros. Firstly, the overall prevalence of irregular cyclicity in this population was determined, before comparing different cycle types between parous and nulliparous females. Secondly, a number of intrinsic factors were investigated and compared between parous and

nulliparous females, namely adrenal activity, temperament, body condition and the behavioural expression of oestrus.

2. Methods

2.1. Study population

This study included 39 female eastern black rhinoceros at 11 zoological institutions across Europe, between the ages of 1 year 3 months and 40 years 9 months. This represents 89% of females in the European Endangered Species Breeding Programme (EEP) that had been at or approaching reproductive-age during the study period. All females were housed at the same institution as at least one male (Biddle and Pilgrim, 2011; Pilgrim, 2009). However, breeding management varies according to both individual behaviour and the facilities available at each institution. Compatible pairs or groups may be housed together continuously until conception occurs, or alternatively, pairs may only be introduced during oestrus (Pilgrim and Biddle, 2014).

The full reproductive history of each individual between their birth (or capture in the case of wild-caught founders) and the end of 2010 was determined from the European Association of Zoos and Aquaria (EAZA) studbook (Biddle and Pilgrim, 2011; Pilgrim, 2009). Individuals were categorised as follows. Firstly, females were categorised by their age, with those between the ages of 5–32 considered to be of breeding age ($N = 31$); females aged 5 years and under classed as immature ($N = 7$), and individuals aged 33 years and over classed as post-reproductive ($N = 1$). Females in the reproductive age class ($N = 31$) were then further categorised as parous ($N = 17$, age range 8–28 years) if they had ever produced a live calf, whereas those that had never produced a live calf were considered nulliparous ($N = 15$, age range 5–21 years). However, to distinguish between females that were currently breeding and those that may have bred previously but not reproduced for some time, parous females were further categorised according to whether they had produced a calf within the last 7 years. This timescale represents double the period during which a female would ideally have produced a subsequent calf based on the average inter-calving interval of this population of 3½ years (Biddle and Pilgrim, 2011; Pilgrim, 2009). Therefore breeding age individuals were also categorised as either (1) parous females that had produced a calf within the last 7 years ($P < 7$; $N = 11$; age range 8–28), (2) parous females that had not produced a calf within the last 7 years ($P > 7$; $N = 6$; age-range 18–27 years), and (3) nulliparous (NP).

2.2. Faecal sample collection and preparation

A total of 9743 faecal samples were collected over a sample collection period that ranged between 4 months and 6 years. Faecal samples were collected at least every other day from females across the monitoring period. In 18 non-pregnant females (5 parous and 13 nulliparous), samples were collected over at least 12 consecutive months, to investigate any seasonal differences in cyclicity patterns or glucocorticoid metabolite concentrations. Samples were collected by keepers as soon as possible after defecation, immediately frozen at -20°C and stored before shipment to Chester Zoo, UK for analysis.

Hormone metabolites were extracted from faecal samples according to an established wet-weight shaking extraction method (Edwards et al., 2013). In brief, each sample was thawed, thoroughly mixed and weighed ($0.5\text{ g} \pm 0.003\text{ g}$), 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 immunoassays

Previously described enzyme immunoassays (EIAs) adapted from Munro and Stabenfeldt (1984), were used with some modifications to measure faecal progesterone (Edwards et al., 2014) and glucocorticoid (Watson et al., 2013) metabolites. Each EIA utilised an antiserum (monoclonal progesterone CL425 or polyclonal corticosterone CJM006; C.J. Munro, University of California, Davis); corresponding horseradish peroxidase (HRP) conjugated label (C.J. Munro, University of California, Davis); and standards (0.78–200 pg/well and 3.9–1000 pg/well, respectively, 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 1:1050 during pregnancy) and 1:20 for corticosterone), and run in duplicate (50 μl) on the respective EIA's.

The cross reactivities for progesterone and corticosterone antisera have been reported elsewhere (Walker et al., 2008 and Watson et al., 2013, respectively). EIAs were biochemically validated for measuring progesterone and glucocorticoid metabolites in female black rhinoceros 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 has previously been 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 progesterone concentration during pregnancy (Edwards, 2013). The corticosterone EIA was biologically validated for assessing adrenal status via faecal glucocorticoid metabolites in female black rhinoceros in a previous study (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. Body condition scoring

A 5-point body condition scoring index previously developed for black rhinoceros (Reuter and Adcock, 1998) was used, which incorporates assessments of seven key regions of the body: neck, shoulder, ribs, spine, rump, abdomen and tail base. The index was modified slightly to allow for 0.5 increments between 1 (poor/emaciated) and 5 (excellent/heavy). Each participating institution supplied a set of three standardised photographs taken from the front, side and rear. A single investigator then scored each rhinoceros using a combination of direct observation and photographs ($N = 24$), or from photographs alone ($N = 15$).

2.5. Keeper assessments of behaviour

Concurrent with faecal sample collection, keepers that spent the most time working directly with the rhinoceros (minimum of two years' experience) were asked to complete a number of questions regarding the behaviour of study females. Keepers were asked to select from a list of potential behaviours (Table 1) that may be expressed by the female during oestrus and the regularity (monthly, irregular, not observed) with which that female was considered to be in oestrus during the study period. Additionally, to obtain a measure of each individual's temperament, keepers were asked to score each female on the frequency with which they would express certain behaviours, and how they would typically

Table 1

Potential behaviours exhibited by the female black rhinoceros when in or approaching oestrus.

Behaviour:
+/- Restlessness/pacing ^d
+/- Vocalisations ^{e,f}
+/- Increased urine spraying ^{a,b,c,d}
+/- Tail up ^e
+/- Swelling of vulva ^{b,c}
+/- Vulva winking ^d
+/- Discharge from vulva ^{b,f}
+/- Less cooperative with keepers (or) more cooperative with keepers
+ Interest in male
+ Female presenting hind-quarters to male ^b
+ Female standing for male ^{b,d}

+/- may be observed with or without male present; + only observed when male present.

^a Hutchins and Kreger (2006).

^b Goeltenboth et al. (1995).

^c Fouraker and Wagener (1996).

^d Berkeley et al. (1997).

^e Hitchins and Anderson (1983).

^f Pilgrim and Biddle (2014)

respond to certain events or situations (for details on all behaviours assessed, see Edwards (2013)). Keepers were then asked to rate how consistent these behavioural responses were from day to day, selecting from 'almost always behaves the same', 'sometimes can be unpredictable', or 'very unpredictable'.

2.6. Data analyses

Faecal progesterone metabolite concentration (fPGM) was used to characterise oestrous cycles according to a previously established method (Brown et al., 2001, 1994). Samples with baseline hormone concentrations were distinguished from those with elevated hormone concentrations using an iterative process. To calculate a baseline fPGM concentration for each female, sample concentrations that exceeded the mean plus 1.5 standard deviations (SD) were removed and the process repeated until no samples exceeding 1.5 SD from the mean remained. These baseline samples represented the follicular phase of the cycle. The onset of the luteal phase was considered to be the first of at least two consecutive samples where fPGM concentration exceeded 1.5 SD above the mean, and the end of the luteal phase was considered to be when at least two consecutive samples were below the threshold of 1.5 SD of the mean. This cyclic pattern of fPGM represents one oestrous cycle. Cycle length was calculated from the last sample of the follicular phase prior to the increase in fPGM concentration, to the same point on the following cycle. As per previously established criteria (Brown et al., 2001), sustained periods (>10 days) where fPGM concentration remained at baseline without any increase above this threshold of 1.5 SD above the mean were categorised as acyclic periods.

Differences in the relative proportions of different cycle types observed between parous and nulliparous females, or among $P < 7$, $P > 7$, and NP females were then investigated using cross-tabulation with Pearson's chi-square test. The percentage of each cycle type across reproductive categories was compared using z-tests, with Bonferroni corrected P -values.

Adrenal activity was compared between parous and nulliparous females (non-pregnant samples only) using the mean, standard deviation (SD), and coefficient of variation (CV) calculated from faecal glucocorticoid metabolite (fGCM) concentrations for each individual using independent samples t -tests, and among the three categories $P < 7$, $P > 7$ and NP using a one-way ANOVA. In addition, fGCM measured from longitudinal faecal samples collected from each individual were also compared using generalised linear mixed

models (GLMM's) in MLwiN version 2.02 (Rasbash et al., 2005), where date of sample collection and subject ID were fitted as nested random effects to control for non-independence of data. Reproductive category and individual temperament were then fitted individually as categorical fixed effects, with parous females or $P < 7$, and 'almost always behaves the same' as the reference categories, respectively. A normal error structure was used for all models of \log_{10} fGCM concentration, 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.

Finally, to investigate any potential differences in temperament, body condition scores, or regularity of oestrus across reproductive

categories, comparisons were made using either Mann Whitney U tests (parous vs. nulliparous) or Kruskal Wallis ($P < 7$ vs. $P > 7$ vs. NP).

3. Results

3.1. Reproductive cyclicity

Based on fPGM analyses, all reproductive-age females exhibited oestrous cyclicity (e.g. Fig. 1a), with the exception of one female that was pregnant and another that exhibited postpartum anoes-

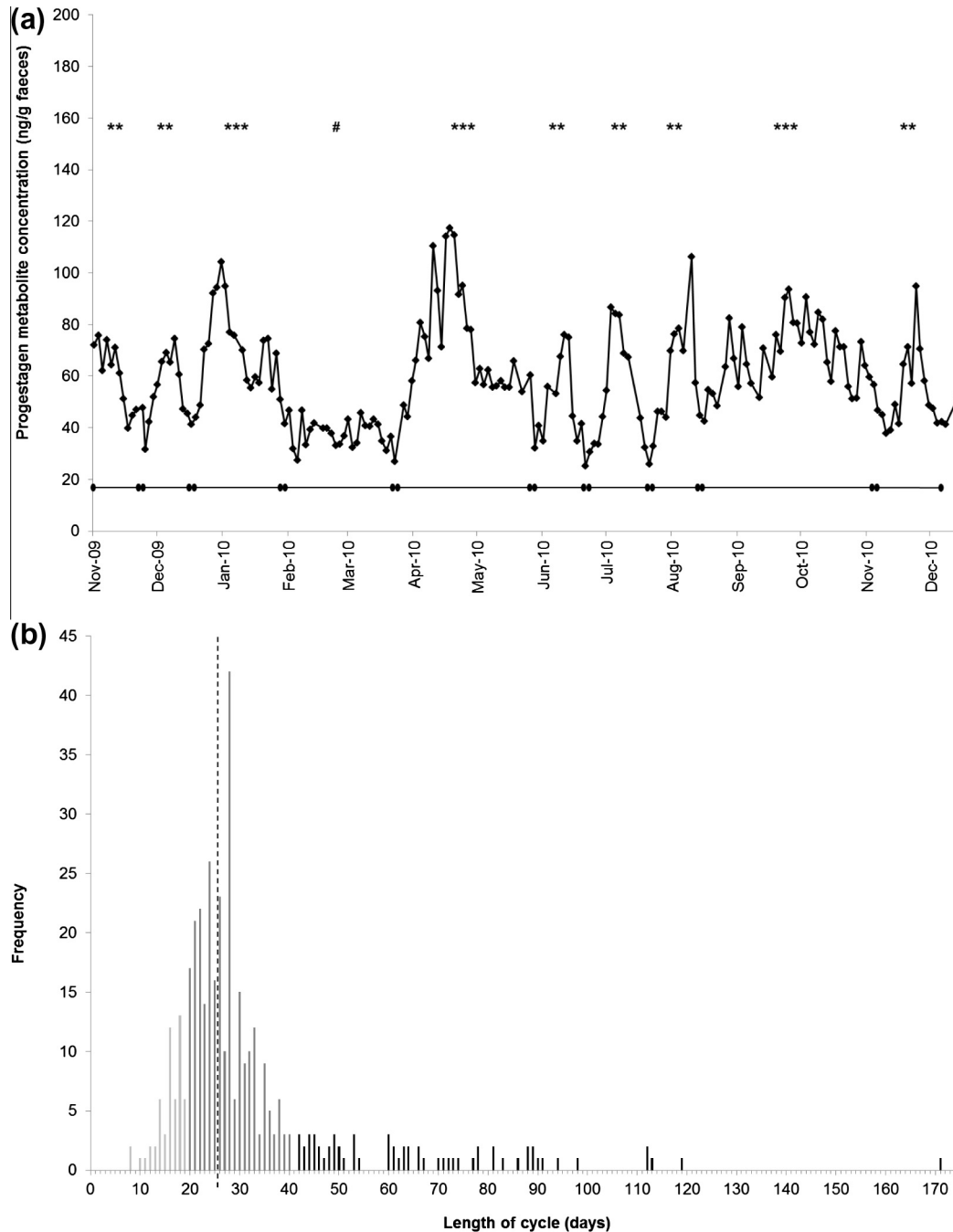


Fig. 1. Oestrous cycles in the black rhinoceros as determined from faecal progesterone metabolite concentration: (a) representative profile from a single nulliparous female, illustrating cycles 20–40 days in length (**), and periods of irregular cyclicity, including long cycles >40 days in length (***) and an acyclic period (#); (b) distribution of oestrous cycle lengths observed from all females during the study period, categorised as <20 days (pale grey), 20–40 days (mid-grey) or >40 days (black) in length. Mean (\pm SD) cycle length of 27.1 (\pm 5) days is represented by a dashed line.

trus. In the immature age class, four females aged between 3 years 8 months and 4 years 11 months were already showing clear signs of cyclicity and the single post-reproductive female still showed signs of cyclicity at age 40.

A total of 436 oestrous cycles were characterised, with cycles observed during all months of the year, indicating no seasonal differences in cycle occurrence. However, there was substantial variation in cycle lengths (Fig. 1b). The majority of cycles ranged from 20 to 40 days (63.3%), with average length 27.1 days (standard deviation 5.0 days). However, irregular cyclicity was also observed, including short (<20 days; 12.4%) and long (>40 days; 14.7%) cycles, and prolonged periods where faecal progesterone metabolite concentration remained at baseline (hereafter termed acyclic periods), ranging from 12 to 127 days (9.6%).

Within females of reproductive age, 411 oestrous cycles were characterised during the study period; 263 were categorised as regular cycles of 20–40 days in length, whereas 148 were categorised as irregular cycles (<20 days, >40 days and acyclic periods combined) (Table 2). The overall occurrence of these regular and irregular cycles were compared according to reproductive history, but there was no difference between parous and nulliparous females ($\chi^2 = 0.548$, $df = 1$, $P = 0.459$).

However, when comparing the occurrence of each of the four different cycle types across reproductive categories, the relative proportion did vary between parous and nulliparous females ($\chi^2 = 9.116$, $df = 3$, $P = 0.028$). There was no difference in the proportion of <20d cycles, 20–40 days cycles or acyclic periods according to reproductive category ($P > 0.05$), but a higher percentage of longer cycles (>40 days in length) were observed in nulliparous females ($P < 0.05$; Table 2). Furthermore, when comparing these four cycle types between parous females that had produced a calf during the last 7 years, and parous females that had not produced a calf during the last 7 years, again there was a difference in the proportion of different cycle types observed ($N = 140$ cycles) ($\chi^2 = 15.282$, $df = 3$, $P = 0.002$), with acyclic periods more common in females that had not reproduced during the last 7 years ($P < 0.05$; Table 2), but no difference in the proportion of other cycles types ($P > 0.05$).

Oestrous cycle characteristics across the parity groups were further evaluated by totalling the number of days that a particular female was exhibiting cycles <20, 20–40 and >40 days in length, and calculating the proportion of total study days during which type of cycle was observed. Overall, nulliparous females exhibited normal cycles only 49% of the time compared to 63% for parous females.

3.2. Adrenal activity

There were no differences in \log_{10} fGCM concentration between parous and nulliparous females (GLMM $\chi^2 = 2.869$, $df = 1$, $P = 0.09$),

or between nulliparous females and parous females regardless of whether they had bred during the last 7 years (GLMM $\chi^2 = 3.346$, $df = 2$, $P = 0.188$). Similarly, there were no differences in mean glucocorticoid concentration ($t = -1.256$, $df = 29$, $P = 0.219$; $F = 0.763$, $df = 2$, $P = 0.476$), or either measure of variation, SD ($t = -0.723$, $df = 29$, $P = 0.476$; $F = 0.315$, $df = 2$, $P = 0.733$) or CV ($t = 0.629$, $df = 29$, $P = 0.534$; $F = 0.386$, $df = 2$, $P = 0.683$) between parous and nulliparous females, or between $P < 7$, $P > 7$ or NP females, respectively.

Adrenal activity varied according to keeper's ratings of individual temperament. Females rated as 'almost always behaves the same' had significantly lower \log_{10} fGCM concentration across the study period ($\chi^2 = 26.499$, $df = 2$, $P < 0.001$) than females rated as being 'sometimes can be unpredictable' ($P = 0.007$) or 'very unpredictable' ($P < 0.001$). Although not quite reaching significance, nulliparous females tended to be rated as more unpredictable in their behaviour than parous females ($P = 0.09$).

3.3. Body condition

Body condition scores (BCS) ranged from 3.0 to 4.5, and there were no differences in BCS according to age (Mann Whitney $U = 92.500$, $P = 0.157$). However, among reproductive-age females, nulliparous females had higher BCS than parous females (Mann Whitney $U = 52.500$, $P = 0.004$), with nine out of 15 nulliparous females scored as 4.5, compared to only one out of 17 parous females. When categorised according to whether they had bred during the last 7 years, again NP females scored higher than $P < 7$ females (Kruskal Wallis = 9.000, $P = 0.009$), but there was no difference between $P > 7$ females to either $P < 7$ (Kruskal Wallis = -0.900, $P = 1.000$) or NP females (Kruskal Wallis = 8.100, $P = 0.117$).

3.4. Expression of oestrus behaviours

The regularity of behavioural expression of oestrus among reproductive-age females ($N = 31$) varied according to reproductive category. Oestrus was observed monthly more often in parous females than nulliparous females (Mann Whitney $U = 56.000$, $P = 0.006$). There was also a difference in regularity of observed oestrus across the reproductive categories of $P < 7$, $P > 7$, and NP, (Kruskal Wallis = 10.299, $df = 2$, $P = 0.006$; Fig. 2), as females that had bred during the last 7 years exhibited oestrus more regularly than nulliparous females (Mann Whitney $U = 9.812$, $P = 0.007$). However, there was no difference between parous females whether they had reproduced during the last 7 years or not (Mann Whitney $U = -1.845$, $P = 1.000$), or between females that had not bred during the last 7 years and those that had never bred (Mann Whitney $U = 7.967$, $P = 0.168$).

Table 2

Number of cycles of each type, and total number of days these cycle types were exhibited by females in each reproductive category (parous vs. nulliparous; and parous females that had produced a calf during the last 7 years ($P < 7$), parous females that had not produced a calf during the last 7 years ($P > 7$)) during the study period.

	Parous		Nulliparous (NP)		Parous: had produced a calf during the last 7 years ($P < 7$)		Parous: had not produced a calf during the last 7 years ($P > 7$)	
	Number of cycles (%)	Number of days (%)	Number of cycles (%)	Number of days (%)	Number of cycles (%)	Number of days (%)	Number of cycles (%)	Number of days (%)
<20	21 (15%)	333 (9%)	28 (10%)	449 (5%)	15 (14%)	241 (8%)	6 (19%)	92 (12%)
20–40	92 (67%)	2449 (63%)	170 (63%)	4638 (49%)	75 (71%)	2031 (65%)	17 (53%)	418 (54%)
>40	12 (9%)	737 (19%)	52 (19%)	3583 (37%)	11 (10%)	695 (22%)	1 (3%)	42 (5%)
Acyclic	13 (9%)	358 (9%)	21 (8%)	892 (9%)	5 (5%)	143 (5%)	8 (25%)*	215 (28%)
Total cycles/days	138	3877	271	9562	106	3110	32	767
Number females	14	14	15	15	9	9	5	5

* Denotes differences between parous and nulliparous females, significant at $P < 0.05$ level.

** Denotes differences between $P < 7$, $P > 7$ and NP, significant at $P < 0.05$ level.

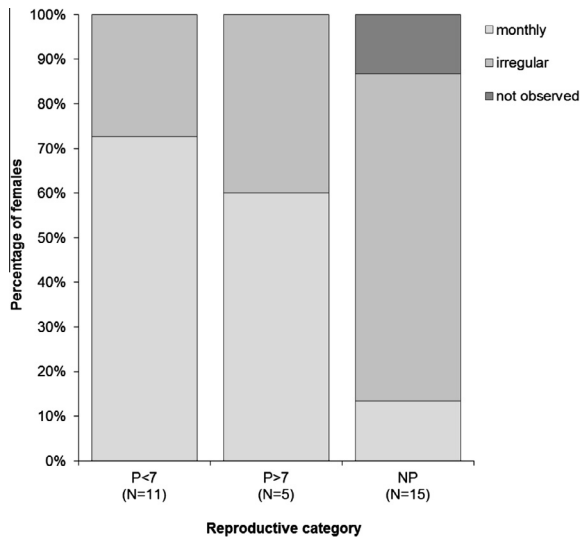


Fig. 2. Percentage of females that keepers reported as being in oestrus monthly, irregularly or not observed in oestrus, amongst parous females that had produced a calf during the last 7 years ($P < 7$), parous females that had not produced a calf during the last 7 years ($P > 7$), and nulliparous (NP) females.

Oestrus behaviours were reported as being observed monthly in 73% of females that had reproduced within the last 7 years, and 60% of females that have bred previously but not produced a calf in the last 7 years, compared to only 13% of nulliparous females. Among reproductive-age nulliparous females, a further 73% only exhibited oestrus behaviours irregularly, and 13% of nulliparous females were recorded as never exhibiting clear behavioural signs of oestrus (Fig. 2).

Although the regularity with which oestrus behaviours were typically observed tended to be related to the proportion of the study period characterised as normal 20–40 days cycles (Mann Whitney $U = 138.0$, $P = 0.053$), this relationship was not consistently observed. All of the females that exhibited only 20–40 days cycles during the study period were reported as exhibiting oestrus behaviour regularly on a monthly basis. In contrast, when behavioural oestrus was only observed irregularly or not at all, normal oestrous cycles were exhibited for between 22% and 97% of the study period; indicating that some females may have been exhibiting clear ovarian cycles based on endocrine data, but behavioural oestrus was not always apparent.

4. Discussion

The average cycle length of female eastern black rhinoceros in the European captive population was comparable to that previously published, both *in situ* (Garnier et al., 2002) and *ex situ* (Berkeley et al., 1997; Brown et al., 2001; Hindle et al., 1992; Radcliffe et al., 2001; Schwarzenberger et al., 1993). Similar to the survey of captive black rhinoceros in North America by Brown et al. (2001), cyclicity in the European captive population was quite erratic, with cycles characterised as both longer and shorter than average, as well as periods of acyclicity. Periods of regular and irregular cyclicity were observed in both parous and nulliparous females, but cycles longer than 40 days in length were exhibited more often in nulliparous females, and females that had not reproduced during the last 7 years exhibited more periods of acyclicity than those that had bred more recently. This is the first comprehensive survey of reproductive physiology in this population, and the higher prevalence of irregular cyclicity patterns in non-breeding females suggests that abnormal ovarian activity could be related to sub-optimal reproductive performance. Fur-

thermore, we highlight a number of intrinsic factors that may be correlated with reproductive success, providing a number of areas that warrant further investigation in this species.

In captive white rhinoceros females, two cycle types have previously been identified (Brown et al., 2001; Patton et al., 1999; Schwarzenberger et al., 1998). Generally the shorter cycle length (30–35 days) is considered to be fertile (Patton et al., 1999; Radcliffe et al., 1997; Schwarzenberger et al., 1998), and the longer cycle (65–70 days) to be abnormal since no conceptions have been known to result from these cycles (Brown et al., 2001; Roth, 2006; Schwarzenberger et al., 1998). In the Indian rhinoceros (*Rhinoceros unicornis*), cycle lengths have also been reported to be highly variable ranging from 37 to 86 days in length (Gomez et al., 2004), but are not considered to reflect aberrant ovarian function (Roth, 2006). Although we cannot yet state that longer cycles observed in the black rhinoceros are abnormal, no conceptions have been recorded during these periods of irregular cyclicity (K.L. Edwards, unpublished results). At the very least, longer oestrous cycles, which often last 2–3 times the length of an average cycle reduce opportunities for mating and conception. This has important consequences for breeding management, as nulliparous females exhibited the normal 20–40 days cycle type for just under half of the study period. However, the etiology of these different cycle types is still not clearly understood, and the question remains as to what other factors may be involved in their occurrence.

In wild black rhinoceros, births are often seasonal according to rainfall (Garnier et al., 2002), and seasonality has previously been proposed as an explanation for the presence of anovulatory follicles and erratic progesterone profiles in captive black rhinoceros (Radcliffe et al., 2001). Anovulatory follicles and reduced progesterone concentration have also been observed in captive Indian rhinoceros, with reduced light exposure considered to be an associated factor in their occurrence (Stoops et al., 2004). In the horse, luteolytic failure and extended luteal-phase progesterone concentration have also been reported to occur during the transition between breeding and non-breeding seasons (King et al., 2010). This phenomenon resembles more closely the progesterone profiles observed in the black rhinoceros, where the luteal phase is extended during longer cycle types. However, both longer cycles of >40 days and the more typical 20–40 days cycles were observed across all months of the year, similar to that reported in the North American population of black rhinoceros (Brown et al., 2001), and for white rhinoceros (Brown et al., 2001; Patton et al., 1999; Schwarzenberger et al., 1998), suggesting that the irregular cyclicity patterns observed are not likely to be a normal seasonal occurrence in captive rhinoceros.

Another possibility for the occurrence of these different cycle types is that they may be related to age, or more specifically to pathologies related to asymmetric reproductive aging (Hermes et al., 2004). This phenomenon has been reported in captive black (Godfrey et al., 1991), white (Hermes et al., 2006) and Indian (Hermes et al., 2014) rhinoceros, where long periods without reproduction can have detrimental effects on the reproductive system, leading to the development of pathologies and reduced fertility. In the white rhinoceros, there appears to be a period between the ages of 15–19 years, during which the ovarian activity of nulliparous females begins to decrease (Hermes et al., 2006). Although we cannot rule out the possible contribution of asymmetric reproductive ageing in older nulliparous black rhinoceros females, irregular cycles were observed in females between the ages of 5 and 30 years, indicating that age alone cannot explain the reproductive issues apparent in this population.

Early pregnancy loss is a further reproductive issue described in all four rhinoceros species kept in captivity, which has been reported within the first 4–14 weeks of gestation (Berkeley et al., 1997; Patton et al., 1999; Radcliffe et al., 1997; Roth et al., 2001;

Stoops et al., 2014). In the black rhinoceros, the initial 3–4 months of gestation is characterised by progestagen concentrations comparable to the luteal-phase, after which progestagen concentration increases markedly (Brown et al., 2001). It is therefore conceivable that the extended cycles observed in the black rhinoceros, where luteal concentrations are maintained for between 15 and 125 days could in fact indicate early pregnancy loss. In the wild black rhinoceros, cycles of >40 days with sustained high progestagen concentrations were attributed to early pregnancy loss after approximately 3–3.5 months of gestation in two females (Garnier et al., 2002). However, during this study, this was only suspected in one case: a primiparous female that typically cycled at intervals of around 26 days exhibited elevated fPGM concentration for 51 days following mating, before returning to baseline. However, in the majority of other cases females exhibited long cycles with prolonged luteal phase progestagen concentrations without first being mated.

Unlike previous research (Carlstead and Brown, 2005), we found no evidence for chronic stressors having a detrimental effect on captive black rhinoceros in this population. Nulliparous females did not exhibit consistently higher fGCM concentration or greater variability across the study period, indicating they may not be chronically stressed compared to parous females. However, individual temperament traits that underlie and modulate the expression of behaviour (McDougall et al., 2006) can be linked to differences in the physiological response to potential challenges (Koolhaas et al., 1999). Differences in temperament between female black rhinoceros were indeed correlated with adrenal activity, as females scored as being more reactive and unpredictable had significantly higher fGCM concentration than those rated as calmer and more predictable. Nulliparous females also tended to be rated as more unpredictable in their behaviour than parous females, and although this did not quite reach significance, may indicate differences in how these individuals respond to potential challenges in their environment. If certain behavioural phenotypes are better adapted to captivity, they may be less susceptible to disruption and therefore have higher reproductive success (Carlstead, 1996; Frankham et al., 1986). This possibility requires further investigation, but raises an important question as to how we cater for a range of behavioural phenotypes within captive breeding programmes, perhaps highlighting the need to investigate alternative exhibit conditions, under which all individuals can thrive and reproduce successfully.

This study has also indicated that body condition could play a role in differential reproductive success, as nulliparous females were scored as having higher body condition than parous females. Research from other species has demonstrated that excess body weight can lead to reduced reproductive success due to a number of factors ranging from reduced oocyte quality to the increased risk of failed implantation and pregnancy loss (Brewer and Balen, 2010; Ferguson, 2005). There is also evidence that body weight can be associated with changes in oestrous cycle length, with excess body weight associated with longer oestrous cycles in both horses (Vick et al., 2006), and in humans (Harlow and Matanoski, 1991). As longer oestrous cycles were more common in nulliparous black rhinoceros females, who also scored higher body condition scores, this is one potential factor that could be involved in reduced reproductive success among females. Alternatively, these females may carry more weight because they have not paid the nutritional demands associated with successful pregnancy and lactation (Emery Thompson et al., 2012; Sand, 1998). Nonetheless, since body condition has previously been linked to health problems in black rhinoceros (Clauss and Hatt, 2006) it would be beneficial to maintain females in ideal condition. In wild black rhinoceros, breeding females mostly range between BCS 3–4.5, with scores of 3.5–4 being more common (K. Adcock, personal communication).

Therefore, aiming to maintain females in captivity at 3.5–4.0 may better reflect natural body condition.

Many captive breeding programmes rely on the accurate detection of oestrus to time breeding introductions. However, in the black rhinoceros, nulliparous females displayed overt behavioural signs of oestrus less often than parous females. Furthermore, some females failed to demonstrate clear behavioural signs of receptivity despite faecal progestagen metabolite profiles indicating that they were in oestrus. The absence of overt oestrus behaviour has been reported in other rhinoceros species (Patton et al., 1999; Roth, 2006; Roth et al., 2001), and is a commonly reported issue in this population (M. Pilgrim, personal communication). This could have potential management implications if females that are not expressing suitable behaviours are not introduced to potential mates. Alternatively, if introductions do occur but the female is not receptive to the male, aggression can ensue. This could potentially result in fewer, less successful, introductions, leading to reduced breeding opportunities and a lower chance of conception. The inconsistency in expression of oestrus behaviour has so far received little attention in the black rhinoceros, but constitutes a significant management concern. One avenue that should be explored is whether captive breeding programmes could benefit from housing greater numbers of rhinoceros to offer a choice of mating opportunities (Asa et al., 2011), or using male faecal samples in the days preceding oestrus to stimulate the expression of oestrus behaviours (Stoops et al., 2014). Additionally, using endocrine analysis of ovarian activity concurrent with behavioural observations can be beneficial to indicate when females are likely to be receptive even when behaviours are not a reliable indicator of oestrus.

5. Conclusion

This is the first comprehensive survey of reproductive physiology in the European captive population of eastern black rhinoceros, encompassing 89% of reproductive-age females, and has revealed a high prevalence of irregular cyclicity among this population, similar to that previously reported in North America. However, this study is the first to identify a correlation between irregular oestrous cyclicity and reproductive success in this species, suggesting that differences in ovarian activity may underlie some of the issues with reproduction. Of particular concern is the occurrence of long cycles and extended luteal phases in nulliparous females, and periods of acyclicity observed in females that have not bred for some time.

In addition, this study has taken an important step in identifying other intrinsic factors that could contribute to irregular cyclicity and reproductive skew in captive female black rhinoceros. Not only do nulliparous females exhibit more extended oestrous cycles, potentially coming into oestrus less often, but are also less likely to exhibit overt behavioural signs of oestrus. Nulliparous females do not exhibit either higher or more variable faecal glucocorticoid concentrations than parous females, suggesting that chronic stressors do not appear to play a role in differential reproductive success in captive black rhinoceros. However, nulliparous females do tend to be more unpredictable in their temperament, and this behavioural trait is associated with increased adrenal activity.

These results are an important step in understanding reproductive problems in this species, and highlight a number of measures that could be taken by animal managers to minimise reproductive skew. In particular, behavioural differences should be taken into account, both in terms of minimising undue disturbances for females that may be considered more reactive, and using alternative measures of oestrus detection in females where behavioural signs are less reliable. Finally, maintaining female black rhinoceros at lower body condition scores may be advantageous, not only due

to any potential relationship with reproductive success, but to maximise the overall health and welfare of this species in captivity.

Acknowledgments

The authors would like to thank the animal keepers, curators, veterinarians and assistants at the following institutions, for collection of faecal samples and completion of questionnaires: Chester Zoo, UK; Bioparc Zoo de Doué la Fontaine, France; Zoo Dvůr Králové, Czech Republic; Ree Park Ebeltoft Safari, Denmark; Zoo Hannover, Germany; Zoo Krefeld, Germany; Zoologischer Garten Köln, Germany; Paignton Zoo, UK; Port Lympne Wild Animal Park, 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, with contribution from The Thriplow Charitable trust and the Association of British and Irish Wild Animal Keepers (ABWAK). S. Shultz is supported by a Royal Society University Research Fellowship.

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