

Research article

## Male behaviours signal the female's reproductive state in a pair of black rhinoceros housed at Lincoln Park Zoo

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**Abstract**

The black rhinoceros (rhino; *Diceros bicornis*) is a Critically Endangered species due to habitat loss and poaching. Zoological institutions' breeding programs have been minimally successful over the years. Our goal was to introduce and breed an eastern black rhino (*Diceros bicornis michaeli*) pair at Lincoln Park Zoo (LPZ; Chicago, IL USA). The specific objectives were to: 1) use faecal hormone metabolite and serum analyses to monitor the female's reproductive state (via progesterone) and male's testosterone production; 2) determine the behaviours that signalled the female was in oestrus; and 3) use these data to determine the optimal time to introduce the pair. Faecal samples and behavioural observations were collected daily. Sera were collected without restraint during routine veterinary procedures. Results demonstrated that the male's faecal and serum testosterone concentrations changed with the female's reproductive state. Specifically, testosterone increased when the female became sexually mature but there were no physical introductions and during her pregnancy. The behaviours most indicative of the female's oestrous state were the male investigation of female faeces and urine and erect penis. Using these behavioural signals, the staff introduced the rhinos 77% of the time during her follicular phase, when oestrus would occur, versus luteal phase. After 52 days of introduction spanning 1 year and 16 oestrous cycles, the pair successfully bred and a male calf was born approximately 15 months later. This study is an example of how zoo science and management collaborations can improve the breeding success of zoo-housed endangered species.

**Introduction**

The black rhinoceros (rhino; *Diceros bicornis*) is one of the rarest of the large, terrestrial mammals. For almost 20 years, it has been listed as Critically Endangered with a population decline of 96% between 1970 and 1992. Currently there are only 5,050 individuals in the wild (Emslie 2013). Habitat loss and increased poaching to meet the high demands for rhino horn from Middle East and China are the main threats to the species (Emslie 2013). Efforts to ensure the survival of the black rhino have focused on reducing poaching, restoring habitat and breeding rhinos in captive facilities either *in situ* or in zoos. However, managing black rhinos in zoos can be challenging. Because the black rhino is a browser, nutritional problems due to limited natural dietary options can lead to health issues such as iron overload disease (Schook et al. 2015) and obesity due to a high carbohydrate/low roughage diet, which may lead to poor reproduction (Claus and Hatt 2006).

In addition to nutritional issues, environmental and social stressors also can impede reproduction. Because black rhinos are semi-social and territorial (Hutchins and Kreger 2006), it

can be difficult to house them in zoos. For example, stress may be induced by facility design due to limited space because black rhinos have to be housed separately (rather than as a herd); this limits the number of rhinos a zoo can house, thus reducing options for breeding (Carlstead et al. 1999a). Reproductive failure in zoos has also been linked to certain male black rhino behaviours including 'olfactory' and 'dominance' and certain female behaviour including 'chasing/stereotypy/mouthing' (Carlstead et al. 1999b).

To maintain a healthy and successful zoo population, furthering the knowledge of important behavioural cues that signal an individual's reproductive state is imperative (Lindburg and Fitch-Snyder 1994). The use of non-invasive physiological monitoring techniques, such as faecal hormone metabolite analysis, can facilitate reproduction in zoo-housed individuals (Monfort 2013) and can provide information about the onset of sexual maturity, ovulation, pregnancy, parturition and foetal/neonatal loss (Berkeley et al. 1997; Garnier et al. 2002). Several studies have examined the reproductive physiology of both wild and zoo-housed black rhinos using faecal hormone metabolite analysis. Reproductive issues including variability

in length (ranging from 14 to 60 days) and consistency of oestrous cyclicity in zoo-housed black rhinos (Brown et al. Roth 2001) have been observed. This can make predictability of pairing individuals difficult. Variation in oestrous cycle length also has been observed in wild black rhinos (*Diceros bicornis minor*). Oestrous cycles were classified as Type I if they were less than 40 days in length with an average of about 27 days, Type IIa if cycles were longer than 40 days with longer luteal phases, or Type IIb if cycles were longer than 40 days with longer follicular phases (Garnier et al. 2002). Whether this variability is a result of environmental issues, such as nutritional stress, or a characteristic of the species is unknown.

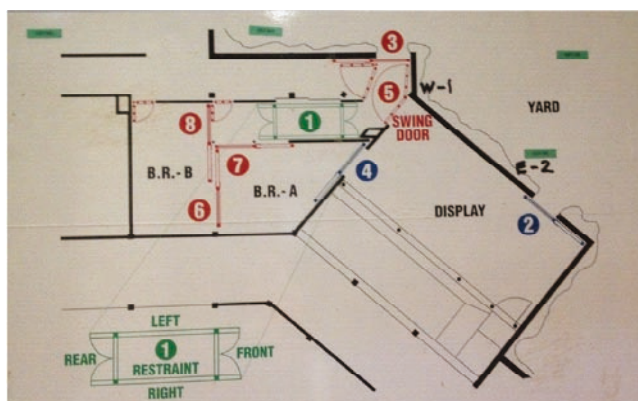
In addition to female reproduction, faecal androgen metabolite monitoring has been used to elucidate characteristics of male black rhino reproductive behaviour and physiology both *in situ* and *ex situ* (Brown et al. 2001; Freeman et al. 2014a, b). For zoo-housed black rhinos, androgen analysis has been used to determine sexual maturity and the influence of social structure on physiology and behaviour (Garnier et al. 2001; Christensen et al. 2009). Seasonal effects on faecal androgen metabolites have been observed in wild males (Freeman et al. 2014b).

Because black rhinos are semi-social, individuals are often housed separately and males are introduced to females only during oestrus. Although several oestrus-associated behaviours have been described, such as mounting behaviour (Berkeley et al. 1997) and mating behaviour (Patton et al. 1999), behaviours in the absence of direct contact with a male are often difficult to identify (Fouraker and Wagener 1996; Edwards et al. 2015). Therefore, the objectives of this study were to: 1) use faecal hormone metabolite and serum analyses to determine and monitor the female's reproductive state (via faecal progesterone metabolites; FPM) and male's testosterone production; 2) determine the behaviours that signalled the female was in oestrus; and 3) use these data to determine the optimal time to introduce the pair, with the goal of successfully introducing and breeding an eastern black rhino (*Diceros bicornis michaeli*) pair at the Lincoln Park Zoo (LPZ; Chicago, IL USA).

## Methods

### Animals and housing conditions

At the beginning of this study, the male and female were 23 and three years of age, respectively. In September 2008, the female was transferred to LPZ and housed in the south barn with



**Figure 1.** Floor plan of the black rhinoceros barn at Lincoln Park Zoo (Chicago, IL USA). Numbers 2 through 8 indicate hydraulic doors that open and close for shifting purposes. 'B.R.-A' indicates the holding area known as Bedroom A. 'B.R.-B' indicates the holding area known as Bedroom B. 'Display' indicates a third holding area. Number 1 is a restraint within a hallway that has hydraulic doors, 'front' and 'rear'; and hydraulic walls, 'left' and 'right'.

**Table 1.** Ethogram categories used to develop an Animal Keeper Questionnaire for eastern black rhinoceros during a breeding introduction at Lincoln Park Zoo (Chicago, IL USA)

Flehmen	Lip up and extended, mouth open in response to urine or faeces
Investigation	Sniff, lick, horn smash, shuffle feet through faeces and/or urine
Erection	Unsheathed penis with an erection
Presenting genitals	Female presents rear end towards male
Feet shuffle	Dragging feet across floor rather than picking them up
Defaecation	Defaecating
Resting	Eyes closed, lying down
Excited/Nervous	Pacing, wide eyes, startles easily
Avoidance	Actively stays away from other individual
No interest	Demonstrates no interest in conspecific
Chin rest (bars)	Resting chin/head on horizontal bars
Horn wrestle	Touching, rubbing horns
Aggressive	Mock charging/charging, snorting. Behaviour geared toward other rhino or keeper staff
Climbing bars	Attempting to step up onto horizontal separation bars
Urine spraying	Spurts of urine used to mark objects and noting frequency
Urine dribbling	Small amounts of urine trickling out of urethra through the penis and vulva.
Vulva changes	Swelling and reddening of the vulva with or without mucus secretion.

the male, separated by one stall which allowed only visual and olfactory contact. By September 2009, the animals were moved to the north barn, which consists of two bedrooms, a chute, and a larger dayroom separated by hydraulic doors for full introductions (Fig. 1). Once transferred to the north barn, the rhinos had visual access but minimal physical contact through the separating doors; however, they were allowed to enter each other's holding stalls in the morning before cleaning began and alternated stalls overnight. This allowed the individuals to investigate each other's scent. During this time, the female (now four years of age) was not cycling (based on FPM data). Additionally, another adult male (12 years old) was housed in an exhibit located in the south barn, which is not connected to the north barn. He arrived in October 2009, but was not involved in the study.

### Behavioural observations

Before formal introductions and after we observed fluctuations in FPMs indicative of cyclicity, staff used an ethogram (Table 1) that was modified from Carlstead et al. (1999b) and Carlstead and Brown (2005) to monitor reproductive behaviours that were indicative of female cycling. Staff began documenting behaviours in both individuals. Then, the most common behaviours were used for a simplified behaviour questionnaire that could be completed by animal care staff daily (Table 2). For the female, the questionnaire asked about vulvar changes such as swelling, winking and/or mucus discharge. Her behaviour was also closely monitored for interest in the male's urine or faeces, as well as if

**Table 2.** Black rhinoceros reproductive behaviour questionnaire for the animal care staff. Staff monitored behaviour throughout the day and then recorded a 'yes' or 'no' if the black rhino had been observed demonstrating the behaviour.

Week of: Date	Day of Week						
	Mon.	Tues.	Wed.	Thurs.	Fri.	Sat.	Sun.
<i>Female</i>							
Vulvar swelling, reddening with or without mucus secretions?							
Increased dribbling urine or urine-spraying along with signs of agitation?							
Investigating male urine/faeces, showing flehmen?							
<i>Male</i>							
Showing interest in female?							
Penis unsheathed (partial erection, facing anterior, not associated with urination) or erect?							
Investigating female's urine/faeces or showing flehmen?							
Aggressive towards female and/or staff?							

she stayed in the vicinity of the male when he was around. The male was monitored for interest in the female's urine or faeces, flehmen response, penile unsheathing or erection, and his behaviour toward staff. Specifically, aggression toward the female and staff members were noted (Table 2). Animal care staff filled out the behaviour questionnaire daily from 13 September 2010 to 24 June 2012.

In April 2011 formal introductions began with a separation barrier, which was a secure door with horizontal bars that allowed for both olfactory and visual contact in a controlled setting. These introductions began and lasted anywhere from 15 minutes to 1 hour and started no later than 0900. In the interest of safety (i.e. to reduce the likelihood of a rhino slipping and falling), staff picked up all but a small amount of faeces, leave urine, and then swap the rhinos into the stalls the other had occupied overnight. This gave the staff an opportunity to monitor rhino behaviour in the presence of urine and faeces. Staff collected and recorded their observations on rhinos before and during these introductions. Initially, the same five staff members were present for all introductions to facilitate consistency. No food was given before the introductions so that the rhinos would concentrate on each other. Physical introductions occurred based on the observations of staff during the introductions behind the barrier and the timing in relation to the female's reproductive cycle was confirmed by hormonal data at a later time. The pair was never left unobserved and introductions only occurred during the day. The occurrence of introductions was based on the behaviours that the staff observed using the introductions with the barrier.

#### **Faecal sample collection, processing and analysis**

To determine the female's reproductive state and monitor the male's testosterone production, faecal samples were collected three to seven times per week. In the morning, the freshest sample was collected in a sealed, labelled plastic bag with date and time collected and then kept frozen at -20° C until analysis at the LPZ endocrinology laboratory. Banked serum samples were used to quantify the male's testosterone across his tenure at LPZ.

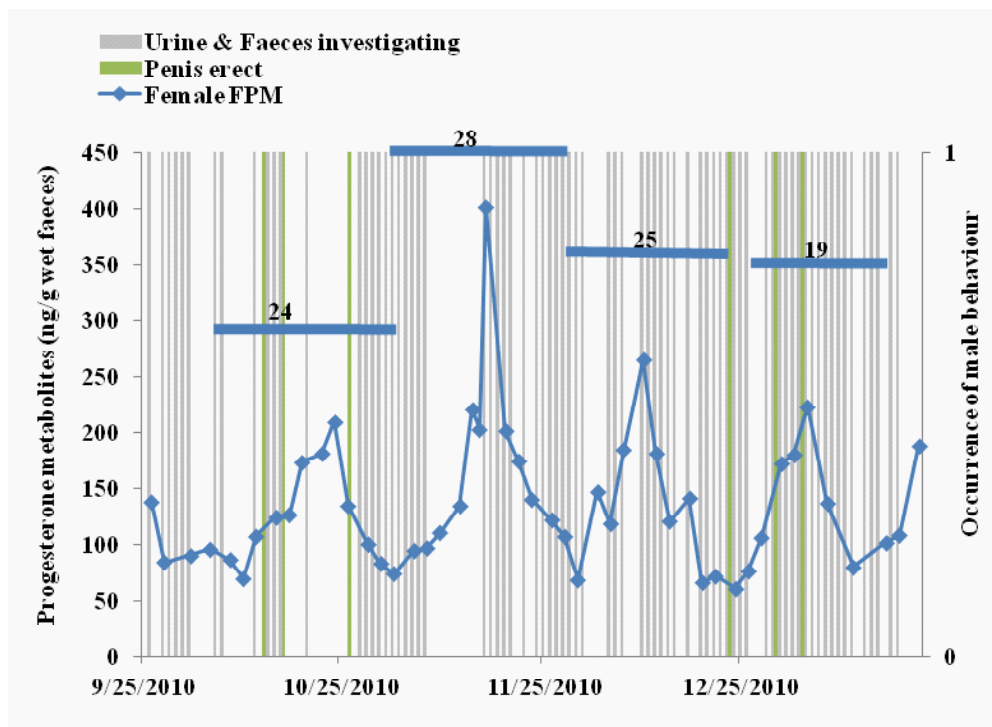
Hormones were extracted using methods from a previous publication (Loeding et al. 2011). Extracted samples were diluted using phosphate-buffered saline (PBS; 0.01 M PO<sub>4</sub>, 0.14 M NaCl, 0.05% BSA, 0.01% NaN<sub>3</sub>, pH 7) for hormonal analysis using enzyme immunoassays (EIA). Serum and faecal androgen metabolites (FAM) were analysed by a testosterone EIA (Santymire and Armstrong 2010). The testosterone horseradish peroxidase (HRP) ligands and polyclonal antiserum (R156/7) were provided by C.

Munro (Davis, California USA). The testosterone EIA was validated by demonstrating: 1) parallelism between binding inhibition curves of faecal extract dilutions (1:20-1:5120;  $y = 0.90x + 13.38$   $R^2 = 0.976$ ;  $P < 0.001$ ) and 2) significant recovery (> 90%) of exogenous testosterone added to faecal extracts (1: 5,000;  $\hat{y} = 1.23x - 0.50$ ,  $R^2 = 0.999$ ;  $P < 0.001$ ). Assay sensitivity was 2.3 pg/well, and intra- and inter-assay coefficients of variation were <10%.

Faecal progestagen metabolites (FPM) were analysed using EIA (Loeding et al. 2011). Progestagen polyclonal antiserum (CL425) and HRP were provided by C. Munro (Davis, California USA). The progestagen EIA was previously validated biochemically (1:1000;  $y = 1.04x - 2.10$ ;  $R^2 = 0.999$ ;  $P < 0.001$ ) and physiologically (via pregnancy; Freeman et al. 2014a). Assay sensitivity was 0.78 pg/well and intra- and inter-assay coefficients of variation were <10%.

#### **Data analysis**

All statistical analyses were performed using Microsoft Excel (MS Office XP) and Sigma Stat Version 3.0 (SPSS Inc., Chicago, IL, USA) using a threshold level of significance of  $P < 0.05$ . A Kolmogorov-Smirnov test was used for normality assumption testing and the Levene median test for equal variance assumption testing. Values were reported as the mean  $\pm$  standard error (SEM). For the female hormonal analysis, the FPM baseline value was calculated using an iterative process (Moriera et al. 2001). Elevated FPM was defined as any sample with a value greater than two standard deviations above the mean baseline value. A complete oestrous cycle length was determined by taking the date of the first elevated FPM value and counting the number of days until the first elevated value of the next cycle. To be considered the end of an oestrous cycle phase, hormone values must have returned to baseline values for at least 1 day. Single-day drops were excluded if the subsequent sample returned to elevated hormone concentrations (Loeding et al. 2011). The luteal phase was calculated by counting the number of days that FPM was continuously elevated within an oestrous cycle. The follicular phase was calculated by counting the number of days FPM was not elevated (Loeding et al. 2011). When overlaying the behaviour onto the hormonal data, FPM values were adjusted by setting the dates back three days from the date of sample collection. This adjustment was based on the results of an adrenocorticotrophic challenge (Santymire et al. 2012), when it was determined that peak faecal glucocorticoid metabolites (FGMs) occurred at approximately 72 hours. Others (Wasser et al. 2000; Brown et al. 2001) found FGMs peaked at 24 and 48 hours later; however, we choose to use 72 hours because



**Figure 2.** A subset of the female black rhinoceros faecal progesterone metabolites during the study depicting oestrous cyclicity. Hormonal data was backed up by 3 days and then male behaviours that significantly indicated the female was becoming receptive were overlaid. If the behaviour was observed on a certain day, then it was scored as '1'. The horizontal bars indicate length of each documented oestrous cycle in days.

it was the result from a study conducted at LPZ and with the same male. Because diet and husbandry practices, such as how often individuals are fed, can play a role in when and how faecal hormones are excreted, we believe it was best to use the results from the same facility. For male hormonal analysis, a one-way repeated measure ANOVA with a Student-Newman-Keuls post-hoc test was used to determine the effects of female reproductive state (sexually immature, regular cyclicity and pregnancy) on testosterone production (both faecal and serum concentrations).

For the behavioural data analysis, the number of days when each behaviour occurred was totalled and divided by the total number of observation days to determine the percentage of time that each behaviour was observed across time. Any days that were missing a response on the behaviour questionnaire were excluded from the analysis. To determine which behaviours were indicative of the female in oestrus, we used chi-square analysis to compare the proportion of both male and female behaviours observed during the follicular and luteal phases of the female's oestrous cycle.

## Results

### Female reproductive hormones

The female black rhino was not reproductively active when she first arrived at LPZ in September 2008. Her FPM values were monitored from September 2009 through January 2010, during which her FPM values were at baseline ( $87.88 \pm 3.78$  ng/g faeces). Samples were not collected again until July 2010, when she had begun to cycle. So, between January 2010 and July 2010 she became sexually mature at the age of 4.5–5 years old. Samples were collected from July 2010 during the introduction, through pregnancy and concluded with parturition on 26 August 2013. Mean FPM during her oestrous cycles was  $152.36 \pm 3.28$  ng/g faeces (Fig. 2). During the follicular and luteal phases, mean FPM was  $88.54 \pm 2.50$  ng/g faeces and  $170.70 \pm 3.60$  ng/g faeces,

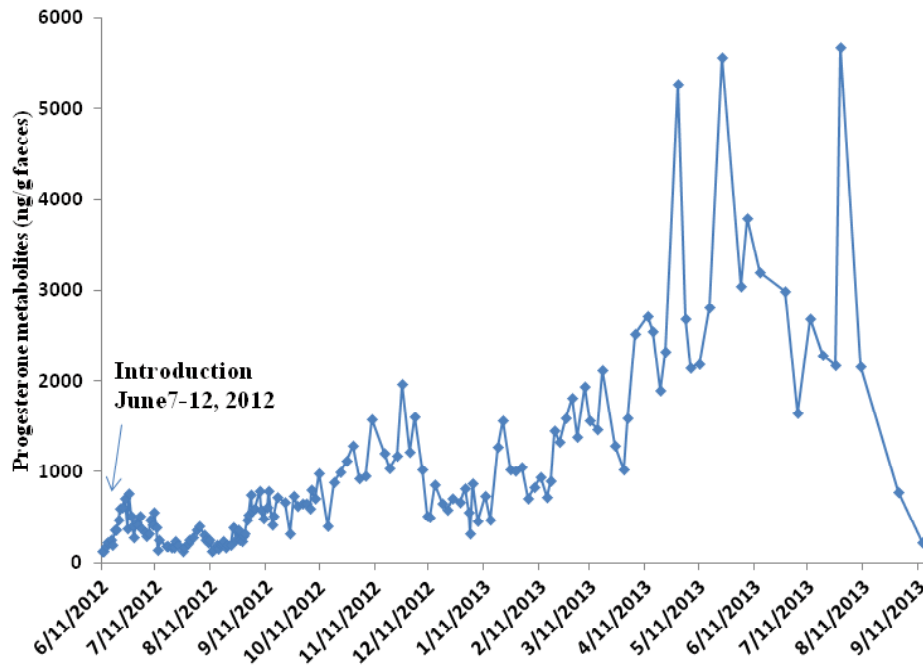
respectively. Mean oestrous cycle length was  $22.6 \pm 0.97$  days (range 11–32 days;  $n=28$  cycles) with a follicular phase length of  $5.59 \pm 0.44$  days (range 2–12 days) and luteal phase length of  $17.11 \pm 0.93$  (range 5–26 days). The female mated with the male on 11 June 2012 which resulted in a 441-day gestation period (Fig. 3). Mean FPM during the pregnancy was  $942.29 \pm 77.02$  ng/g faeces (range 122.0–5678.5 ng/g faeces).

### Male reproductive hormones

Overall mean serum testosterone was  $1.00 \pm 0.04$  ng/ml for the male. When the new female arrived but still was not sexually mature, the male's mean serum testosterone was  $0.96 \pm 0.08$  ng/ml (Fig. 4). When she started cycling, his testosterone concentrations ( $1.35 \pm 0.16$  ng/ml) were higher ( $P=0.031$ ) than prior to the female reaching sexual maturity and the initiation of her oestrous cyclicity. For faecal hormonal analysis, the male's overall mean FAM was  $355.57 \pm 15.58$  ng/g faeces. The male's FAM values ( $290.73 \pm 15.68$  ng/g faeces) recorded before the female was sexually mature were lower ( $P<0.001$ ) than his FAM values when the female was cycling but there were no introductions ( $540.24 \pm 47.11$  ng/g faeces) and when she was pregnant ( $403.58 \pm 26.01$  ng/g faeces;  $P=0.002$ ), but similar ( $P>0.05$ ) to when she was sexually mature and the pair was being physically introduced (Fig. 5). The male's FAMs were highest ( $P<0.001$ ) when the female was cycling but no physical introductions were occurring (Fig. 5)

### Behaviour

Overall, the female rarely showed definitive behavioural signs of oestrus, although periodically she did stay in close proximity to the male, horn wrestled with the male and showed some interest in him and/or presented to him. There was only one observation of the female spraying or dribbling urine throughout the entire study. This type of urination was different from simply elimination of waste. It is more frequent, every 5–10 minutes,

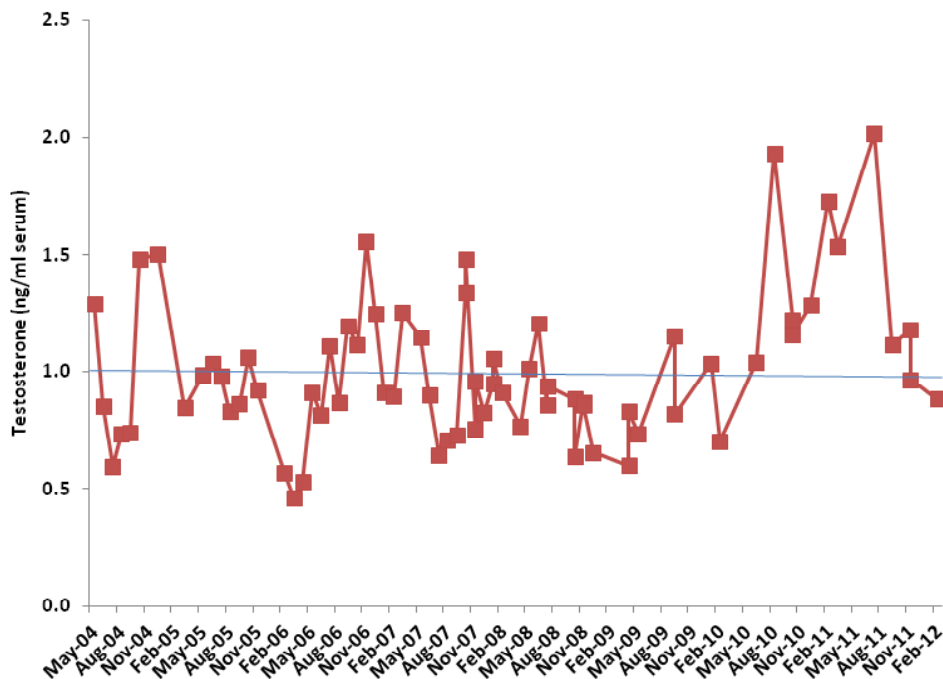


**Figure 3.** Female black rhinoceros faecal progesterone metabolite values from the time that she bred (June 11, 2012) with the male until she gave birth on August 26, 2013.

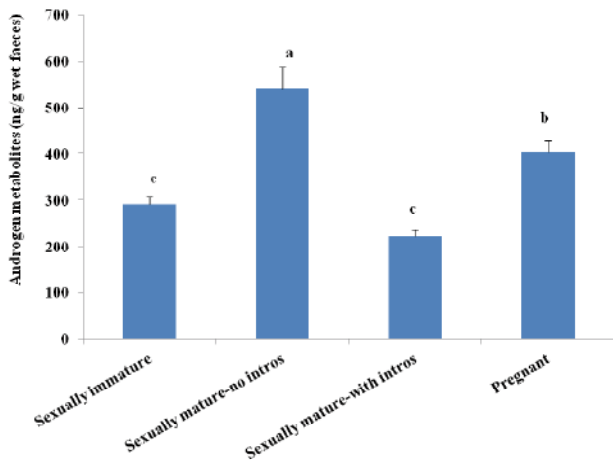
and associated with agitation and pacing. Additionally, there was a very low occurrence of vulvar changes. Specifically, during the female's follicular phase, similar changes in the vulva were observed ( $\chi^2_1=0.279$ ;  $P=0.597$ ) across her oestrous cycle (8.5%, or 13 of 153 days, in the follicular phase; 10.4%, or 42 of 402 days, in the luteal phase; Fig. 5). Similarly, there was no difference ( $\chi^2_1=0.0123$ ;  $P=0.912$ ) in the percentage of days that the female

investigated urine or faeces between the follicular phase (11.7% or 18 of 154 days) and the luteal phase (11.6% or 47 of 406 days) of her oestrous cycle.

In general, most of the behaviours on the keeper questionnaire were observed in the male. For example, the male showed interest in the female on 306 days out of the 22 months of this study. Specifically, the male had a tendency ( $\chi^2_1=2.966$ ;  $P=0.085$ )



**Figure 4.** Male black rhino serum testosterone values over his tenure at Lincoln Park Zoo. The horizontal line is the mean testosterone over the years in month per year. The female black rhino became reproductively mature between January and July 2010. Formal introductions between the pair began in April 2011.



**Figure 5.** Mean ( $\pm$  SEM) faecal androgen metabolite values (FAM; ng/g wet faeces) from the male black rhino during different phases of the female's reproductive state. The female black rhino became reproductive mature between January and July 2010 and formal introductions between the pair began April of 2011. The pair mated on 11 June 2012, which resulted in a pregnancy with a male calf born on 26 August 2013. Superscripts indicate a difference ( $P < 0.05$ ) in the male's FAM among the female's reproductive states.

to show interest in the female more often during her follicular phase (58.4% of the time; 97 of 166 days) than her luteal phase (50.2%; 209 of 417 days) of her oestrous cycle (Fig. 5). Behaviours indicative of his interest in the female included resting his chin on the introduction bars and attempting to climb the bars. The male exhibited an erect penis more often ( $\chi^2_1=4.339$ ;  $P=0.037$ ) during the female's follicular phase (21.6%; 33 of 153 days) than during her luteal phase (12.7%; 53 of 417 days). Another frequently observed behaviour was investigation by the male of the female's urine and/or faeces, which occurred on 380 days out of the 22 months of this study. Specifically, he investigated her faeces more often ( $\chi^2_1=215.989$ ;  $P < 0.001$ ) during the female's follicular phase (73.9%; 122 of 165 days) compared to her luteal phase (61.7%; 258 of 418 days). During these investigations the male exhibited a flehmen response to the female's urine and investigated her faeces by smashing his horn or face into her faecal pile, shuffling his feet through her faeces, or defaecating himself. Finally, the male displayed aggressive behaviour towards the female and the animal care staff lasting  $5.5 \pm 1.0$  days (range 1–11 days). The aggressive behaviour was displayed similarly ( $\chi^2_1=1.063$ ;  $P=0.303$ ) across the female's oestrous cycle (follicular phase, 20.9%, or 32 of 153 days; luteal phase, 26.3%, or 103 of 392 days; Fig. 5). Using the male's behaviours, over the course of one year (June 2011–June 2012) the animal care staff physically introduced the male to the female on 52 days and 16 oestrous cycles; 77% ( $n=40$ ) of the introductions occurred during her follicular phase and 23% ( $n=12$ ) occurred during her luteal phase.

## Discussion

The combination of faecal hormone metabolite analysis and behavioural observations allowed us to determine that the male's erect penis and his investigation of the female's faeces and urine indicated that the female was in the follicular phase of her oestrous cycle. By identifying these key male behaviours the animal care staff were able to confidently introduce the male and female for breeding, which resulted in a successful pregnancy. Using faecal hormone metabolite analysis is an effective tool to determine

reproductive state in zoo-housed and free-ranging black rhinos (Brown et al. 2001; Garner et al. 2002; Edwards et al. 2015). It allows for monitoring the variability and consistency of cyclicity, which is particularly important for a species like the black rhino where oestrous cycles have varied among individuals (Brown et al. 2001; Edwards et al. 2015). Here, the female had regular oestrous cycles that were approximately every 23 days, which is similar to previous reports (approximately 27 days, ranging from 14 to 60 days; Brown et al. 2001); and 24 and 26.5 days; Schwarzenberger et al. 1993).

Previous characterisation of the black rhino oestrous cycle has shown that FPMs are 1.3 to 2 times higher in the luteal phase compared to the follicular phase (Schwarzenberger et al. 1993; MacDonald et al. 2008). Here, the female's FPMs were twice as high post-ovulation. Pregnancy detection is feasible in this species as reports have indicated that FPMs rise to four to 10 times higher (up to six times here) after the first three months of pregnancy and remain elevated throughout the rest of the gestational period (Schwarzenberger et al. 1993, 1996; Berkeley et al. 1997; Garnier et al. 1998; MacDonald et al. 2008). During pregnancy, this female's FPM values were similar to previous reports (Berkeley et al. 1997; Garnier et al. 2002) but lower than others (10–20  $\mu\text{g/g}$  faeces; Brown et al. 2001).

For the male, FAMs appear to be influenced by the female's reproductive state. This male's FAMs (approximately 300–360 ng/g faeces) were higher than what has previously been reported for zoo-housed black rhinos (approximately 30 ng/g faeces, Brown et al. 2001; and 30–80 ng/g faeces, Edwards et al. 2015) and wild adult males (*Diceros bicornis bicornis*; approximately 140 ng/g faeces) from South Africa (Freeman et al. 2014a). The wild males had elevated FAMs in the season prior to peak conceptions (Freeman et al. 2014b). In zoo-housed black rhinos, a positive relationship between age and serum testosterone was found with testosterone peaking at eight years of age and then stabilising through advanced age (Christensen et al. 2009). Interestingly, zoo-housed males can sire offspring as young as 4.5 years of age, most likely due to nutrition and social factors, since males in the wild do not usually sire before seven years of age (Garnier et al. 2001). Recently, proven males (sired within the last 3.5 years) have been shown to have higher FAMs than non-proven male black rhinos (Edwards et al. 2015). Serum testosterone levels were also found to increase when females or other males were present (Christensen et al. 2009). Here, we observed increases in serum testosterone when the female began cycling and in FAMs when the female was sexually mature but no physical introductions had occurred, and also when the female was pregnant. A similar relationship has been observed in white rhinos (Kretzschmar et al. 2004) and black rhinos (Edwards et al. 2015), with the presence of a receptive female corresponding with elevated FAMs in the male. Interestingly, the male's FAM values were highest when the female had started cycling but he had no physical access to her. When the male was being physically introduced to the female approximately every month when she was cycling, his FAMs dropped back down to values during the time period when the female was sexually immature. It seems that the male's testosterone increases during the time period when he would want to establish a territory around the female to prevent other males from breeding or interacting with her. These findings suggest that female presence might be important for reproductive activity and spermatogenesis in males. Results also suggest that black rhino breeding success in zoos may be improved by housing multiple individuals at a facility to allow for olfactory communication; however, further investigation is needed to confirm this notion.

One limitation of using faecal hormone metabolite analysis for determining oestrus is the time it takes to process and analyse samples. Additionally, the hormone metabolites in the faeces are a

reflection of what has already occurred, more than 24 hours earlier. In the black rhino, the lag time for the production, metabolism and excretion of glucocorticoids ranges from 24 to 75 hours (Brown et al. 2001; Wasser et al. 2000; Santymire et al. 2012). So, even if the sample was processed and analysed the same day as defaecation, the results reflect a physiological state that occurred 1–3 days ago. This method is not effective for oestrous monitoring since the oestrus period in black rhinos is about five days. Therefore, either analysing serum hormone concentrations (if blood can be collected with minimal stress) or determining behavioural cues that signal oestrus may be a more effective management tool. Here, we began with daily faecal sample collection and a keeper behavioural questionnaire. The faecal hormonal results were set back by three days (based on Santymire et al. 2012) and behaviours were overlaid onto the hormonal profile. This process allowed us to determine the behaviour that was the oestrus signaller.

Because of their territoriality, it is difficult to introduce black rhinos to each other for breeding purposes in zoos. This incentivised our determination of the behaviours that signalled when the female was entering into her receptive phase. Edwards et al. (2015) determined that nulliparous females rarely demonstrate the oestrus-associated behaviours that have been described. Similarly, in this study, the female rarely exhibited behavioural signs of oestrus, making it difficult to introduce the pair based on her behaviour alone. However, the male demonstrated behaviours that were indicative of the female's reproductive state. When the female was in her follicular phase, the male had an erect penis and demonstrated interest in the female's excrement significantly more often than when she had ovulated and entered into the luteal phase of her oestrous cycle. The staff also used the male's aggressive behaviour as the indicator that the female was entering oestrus. The male was usually aggressive towards the female and animal care staff for 5–6 days, after which the staff would introduce him to the female. Using these behaviours, the staff introduced the rhinos to each other during the female's follicular phase 77% of the time over a period of one year. Other research has determined that male black rhinos perform more olfactory behaviours including urine spraying and scent investigation and may rely on these signals more than females (Carlstead et al. 1999). Therefore, it is cautioned that zoo staff be careful about using disinfectants as they may interfere with this necessary social communication (Carlstead et al. 1999b). On the contrary, it has been determined that males exhibiting more olfactory behaviours and dominance-related behaviours are less successful breeders than other males (Carlstead et al. 1999b).

To mark their territory, black rhinos will defaecate near other rhino dung in middens. Rhinos will also scrape their back feet through dung piles to spread odours that advertise their presence (Hutchins and Kreger 2006). In the wild this scraping behaviour is observed in both males and females (Freeman et al. 2014a). Interestingly, this behaviour shows an inverse relationship with FPM concentrations (Freeman et al. 2014a) and appears to advertise a female's reproductive state. Males scraped longer than females, and this behaviour increased with age, but it was not related to FAMS. Although scraping was not included in the present study, the effectiveness of this behaviour in identifying zoo-housed female black rhinos' reproductive state should be investigated further.

## Conclusion

The North American zoo-housed black rhino population has had challenges with both health (Schook et al. 2015) and reproductive success (Carlstead et al. 1999a; Fosse and Wiese 2006). Several factors may be limiting their success including behavioural, social and environmental causes (Carlstead et al. 1999a). By combining

non-invasive hormone monitoring and the collection of behavioural data, we were able to determine the specific male behaviours that were signalling when the female was becoming receptive to the male. Interestingly, the male's testosterone production also fluctuated with the female's reproductive state. Therefore, how the individuals are housed may play an important role in reproductive success. In conclusion, this study is an example of how zoo science and management can work in tandem to improve the breeding success of zoo-housed endangered species.

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## References

- Berkeley E.V., Kirkpatrick J.F., Schaffer N.E., Bryant W.M., Threlfall W.R. (1997) Serum and faecal steroid analysis of ovulation, pregnancy and parturition in the black rhinoceros (*Diceros bicornis*). *Zoo Biology* 16: 121–132.
- Brown J.L., Bellem A.C., Fouraker M., Wildt D.E., Roth T.L. (2001) Comparative analysis of gonadal and adrenal activity in the black and white rhinoceros in North America by non-invasive endocrine monitoring. *Zoo Biology* 20: 463–486.
- Carlstead K., Brown J.L. (2005) Relationships between patterns of fecal corticoid excretion and behavior, reproduction, and environmental factors in captive black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros. *Zoo Biology* 24: 215–232.
- Carlstead K., Fraser J., Bennett C., Kleiman D.G. (1999a) Black rhinoceros (*Diceros bicornis*) in U.S. zoos: II. Behavior, breeding success, and mortality in relation to housing facilities. *Zoo Biology* 18: 35–52.
- Carlstead K., Mellen J., Kleiman D.G. (1999b) Black rhinoceros (*Diceros bicornis*) in U.S. zoos: I. Individual behaviour profiles and their relationship to breeding success. *Zoo Biology* 18: 17–34.
- Christensen B.W., Troedsson M.H.T., Young L.J., Oliva M., Penfold L.M. (2009) Effects of sociosexual environment on serum testosterone in captive male African rhinoceros. *Theriogenology* 71: 1105–1111.
- Edwards K.L., Shultz S., Pilgrim M., Walker S.L. (2015) Irregular ovarian activity, body condition and behavioural differences are associated with reproductive success in female eastern black rhinoceros (*Diceros bicornis michaeli*). *General and Comparative Endocrinology* 214: 186–194.
- Emslie R. (2013) *Diceros bicornis*. In: *IUCN 2013. IUCN Red List of Threatened Species. Version 2013.2*. [www.iucnredlist.org](http://www.iucnredlist.org) (downloaded on 4 June 2014).
- Foose T.J., Wiese R.J. (2006) Population management of rhinoceros in captivity. *International Zoo Yearbook* 40: 174–196.
- Fouraker M., Wagener T. (1996) *AZA Rhinoceros Husbandry Resource Manual*. Fort Worth: Fort Worth Zoological Park.
- Freeman E.W., Meyer J.M., Adendorf J., Schulte B.A., Santymire R.M. (2014a) Age and faecal gonadal metabolite concentrations affect scraping behaviour of black rhinoceros (*Diceros bicornis bicornis*) in Addo Elephant National Park, South Africa. *Journal of Mammalogy* 95: 340–348.
- Freeman E.W., Meyer J.M., Adendorf J., Schulte B.A., Santymire R.M. (2014b) Impacts of environmental pressures on the reproductive health of subpopulations of black rhinoceros (*Diceros bicornis bicornis*) in Addo Elephant National Park, South Africa. *Conservation Physiology* 2(1): DOI:10.1093/conphys/cot034.
- Garnier J.N., Bruford M.W., Goossens B. (2001) Mating system and reproductive skew in the black rhinoceros. *Molecular Ecology* 10: 2031–2041.
- Garnier J.N., Green D.I., Pickard A.R., Shaw H.J., Holt W.V. (1998) Non-invasive diagnosis of pregnancy in wild black rhinoceros (*Diceros bicornis minor*) by faecal steroid analysis. *Reproduction, Fertility and Development* 10: 451–458.
- Garnier J.N., Holt W.V., Watson P.F. (2002) Non-invasive assessment of oestrous cycles and evaluation of reproductive seasonality in the female wild black rhinoceros (*Diceros bicornis minor*). *Reproduction* 123: 877–889.
- Hutchins M., Kreger M.D. (2006) Rhinoceros behaviour: Implications for captive management and conservation. *International Zoo Yearbook*

40: 150–173.

- Kretzschmar P., Gansloßer U., Dehnhard M. (2004) Relationship between androgens, environmental factors and reproductive behaviour in male white rhinoceros (*Ceratotherium simum simum*). *Hormones and Behaviour* 45: 1–9.
- Lindburg D.G., Fitch-Snyder H. (1994) Use of behaviour to evaluate reproductive problems in captive mammals. *Zoo Biology* 13: 433–445.
- Loeding E., Bernier D., Thomas J., Santymire R.M. (2011) Investigating the impacts of a female to female introduction on hormone levels in the sable antelope (*Hippotragus niger*). *Journal of Applied Animal Welfare Science* 14: 220–246.
- MacDonald E.A., Linklater W.L., Steinman K.J., Czekala N.M. (2008) Rapid colour-change pregnancy test for rhinoceros using faeces. *Endangered Species Research* 4: 277–281.
- Monfort S.L. (2003) Non-invasive endocrine measures of reproduction and stress in wild populations. In: Holt W.V., Pickard A.R., Rodger J.C., Wildt D.E. (eds). *Conservation Biology: Reproductive Science and Integrated Conservation*. Cambridge, UK: Cambridge University Press, 147–165.
- Moriera N.E., Montiero-Filho L.A., Moraes W., Swanson W.F., Graham L.H., Pasquali O.L., Gomes M.L.F., Morias R.N., Wildt D.E., Brown J.L. (2001) Reproductive steroid hormones and ovarian activity in felids of the *Leopardus* genus. *Zoo Biology* 20: 103–116.
- Patton M.L., Swaisgood R.R., Czekala N.M., White A.M., Fetter G.A., Montagne J.P., Rieches R.G., Lance V.A. (1999) Reproductive cycle length and pregnancy in the southern white rhinoceros (*Ceratotherium simum simum*) as determined by faecal pregnane analysis and observations of mating behaviour. *Zoo Biology* 18: 111–127.
- Santymire R., Freeman E.W., Lonsdorf E., Heintz M., Armstrong D. (2012) Assessment of adrenocortical activity after ACTH challenge in diverse African wildlife species. *International Journal of Animal and Veterinary Advances* 4: 99–108.
- Schook M.W., Wildt D.E., Raghanti M.A., Wolfe B.A., Dennis P.M. (2015) Increased inflammation and decreased insulin sensitivity indicate metabolic disturbances in zoo-managed compared to free-ranging black rhinoceros (*Diceros bicornis*). *General and Comparative Endocrinology* 217–218: 10–19.
- Schwarzenberger F., Francke R., Goltenboth R. (1993) Concentrations of faecal immunoreactive progesterone metabolites during the oestrous cycle and pregnancy in the black rhinoceros (*Diceros bicornis michaeli*). *Journal of Reproduction and Fertility* 98: 285–291.
- Schwarzenberger F., Tomášová K., Holecková D., Matern B., Möstl E. (1996) Measurement of faecal steroids in the black rhinoceros (*Diceros bicornis*) using group-specific enzyme immunoassays for 20-oxo-pregnanes. *Zoo Biology* 15: 159–171.
- Wasser S.K., Hunt K.E., Brown J.L., Cooper K., Crockett C.M., Bechert U., Millsaugh J.J., Larson S., Monfort S.L. (2000) A generalized faecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. *General and Comparative Endocrinology* 120: 260–275.