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Male reproductive success is correlated with testosterone in the eastern black rhinoceros (*Diceros bicornis michaeli*)

Katie L. Edwards [a,b,*], Susanne Shultz [c], Mark Pilgrim [b], Susan L. Walker [b]

[a] Institute of Integrative Biology, University of Liverpool, Liverpool L69 7ZB, UK
[b] North of England Zoological Society, Chester Zoo, Chester CH2 1LH, UK
[c] Faculty of Life Sciences, University of Manchester, Manchester M13 9PT, UK

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ABSTRACT

Among natural populations of polygynous species, males often vary in their lifetime reproductive success. However, in managed populations of endangered species, either *in situ* or as part of captive breeding programmes, it is important to understand why differences in reproductive success occur. The European captive population of the critically endangered eastern black rhinoceros is currently under-performing relative to their wild counterparts, with low reproductive output and high reproductive skew limiting growth and genetic diversity. To investigate why over 40% of captive males fail to breed, faecal samples were collected weekly from 23 males at 12 institutions across Europe for 4–32 months. Testosterone metabolite concentration was compared between proven and non-proven males and a number of intrinsic and extrinsic factors that could influence reproductive success were also investigated. Males that sired within the last 3½ years had significantly higher androgen concentrations than non-proven males, and average testosterone was positively correlated with the number of offspring sired per year spent in the reproductive age class. Proven and non-proven males did not differ in their body condition, or in average faecal glucocorticoid concentration. Differences in individual temperament were associated with adrenal activity, but did not correlate with reproductive category. Highest testosterone concentrations were observed in proven males that were housed with females during oestrus, and lowest concentrations in non-proven females not housed with females at all during the study period. Further work is necessary to determine whether proven males had higher testosterone due to underlying differences associated with quality, or whether external stimuli such as access to females could influence testosterone concentration and increase a male's chances of becoming a successful breeder.

1. Introduction

Loss and degradation of habitat, unsustainable hunting and the introduction of invasive species are among the threats that endangered species must contend with, often driving species towards extinction without a combination of *in situ* and *ex situ* conservation efforts. Although captive breeding programmes have the potential to contribute to conservation, problems with sustainability are common (Leus et al., 2011; Long et al., 2011). One species in need of such insurance populations is the black rhinoceros (*Diceros bicornis*), with only around 5000 individuals remaining across continental Africa (Emslie, 2013), and poaching is once again threatening the fragile conservation successes of recent years. However, recent analyses have revealed that the long-term viability of captive black rhinoceros populations is limited by low rates of reproduction and high reproductive skew. In Europe at the end of 2010, 42% of males and 49% of females of reproductive-age were yet to reproduce, and growth rates remained below that of wild populations (Edwards, 2013; Edwards et al., unpublished results). In captive black rhinoceros, we have recently demonstrated that intrinsic physiological and behavioural differences are apparent between parous and nulliparous females, potentially underlying differences in reproductive success (Edwards et al., 2014b). However, as yet, differences between breeding and non-breeding male black rhinoceros have not been investigated.

In wild populations of polygynous species, reproductive skew among males is common, with a small number of males monopolising a high proportion of matings (Clutton-Brock, 1989). Across species, such variation in reproductive success may reflect differences in fitness, with better quality males more able to compete

* Corresponding author at: Center for Species Survival, Smithsonian Conservation Biology Institute, Front Royal, VA 22630, USA
E-mail addresses: katieedwards787@gmail.com, edwardskl@si.edu (K.L. Edwards).

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against rivals (Alonso-Alvarez and Velando, 2001), defend territories or leks (Alatalo et al., 1996; Bro-Jorgensen and Durant, 2003) or afford the costs of elaborate ornamentation (Canal et al., 2011) or other sexually selected signals (Wyman et al., 2008). Under natural conditions variation in reproductive success may result in lower quality males not contributing their genetics to future generations. However, different selection pressures in captivity could result in inadvertent selection and the loss of key phenotypes that may be better suited to wild conditions, reducing the potential for future adaptation. Furthermore, with finite population sizes and individuals distributed across multiple locations, non-reproductive individuals pose problems for small population management, automatically limiting the breeding opportunities of conspecifics with which they are housed. Indeed, observed differences in reproductive success could merely reflect differences in opportunity, as some males may be provided with fewer or less suitable opportunities to breed. If reproductive skew is to be minimised, it is first important to understand why differences in reproductive success may exist, whether they are merely due to differences in opportunity, or whether they could reflect underlying physiological differences.

In wild black rhinoceros, reproductive skew among males has been reported in a number of populations. In the Save Valley Conservancy, Zimbabwe, dominant males achieved higher reproductive success and a single male sired over half of all offspring during a 10-year period, whereas 64% of adult males (7/11 males) failed to sire at all (Garnier et al., 2001). Similarly, paternity analyses of three Kenyan black rhinoceros populations revealed high variance in reproductive success among males (Cain et al., 2014), with more heterozygous males holding larger territories and siring more offspring. These findings suggest that some males are able to out-compete their rivals in achieving successful matings, perhaps due to underlying differences between males. However, male black rhinoceros in captivity do not have to compete for access to females as they would under natural conditions, meaning that variation in reproductive success observed in captivity may not reflect differences in competitive ability or dominance status *per se*. The observed reproductive skew in captivity could be due to the constraints of small population management, or intrinsic differences between males may exist that could otherwise have resulted in certain males acquiring both dominance and reproductive success.

Endocrinology can be an important tool in understanding differences in reproductive success, as androgens play an important role in both physiological and behavioural components of male reproduction. In a variety of species, testosterone concentration is positively correlated with reproductive success (Alatalo et al., 1996; Peters et al., 2008; Swanson et al., 2003). Similar to wild black rhinoceros, dominant white rhinoceros males also achieve more matings than subordinate males (Owen Smith, 1977), and territorial males also exhibit higher testosterone concentrations than non-territorial males (Rachlow et al., 1998). This increased testosterone concentration could confer a breeding advantage to males in a number of ways. For example, spermatogenesis is highly dependent on testosterone concentration, and testes size is often correlated with both testosterone concentration and reproductive success (Preston et al., 2012). Additionally, a number of sexually-selected traits such as sexual ornamentation (Zuk et al., 1995), weaponry such as horns and antlers (Malo et al., 2009), and both intra- and inter-sexual behaviour (Holmes and Wade, 2005; Muller and Wrangham, 2004) are all associated with testosterone concentration. Increased testosterone concentration could therefore confer an advantage over rivals.

Variation in testosterone concentration between males may be due to intrinsic differences between males, or could result from differences in external stimuli (Kempenaers et al., 2008). Across species, the presence of females has been demonstrated to impact aspects of male reproduction including testosterone concentration (Rosa et al., 2000), testicular growth (Jean Faucher et al., 1978) and copulatory behaviour (Orgeur et al., 1984). Indeed, differences in testosterone concentration between captive male black rhinoceros in North America have previously been attributed to the sociosexual environment (Christensen et al., 2009); as higher testosterone concentrations were observed in males housed with a greater number of conspecifics, compared to those housed singly. Similarly, in the white rhinoceros, the presence of receptive females is associated with higher androgen concentration, both in the wild (Kretzschmar et al., 2004), and in captivity (Christensen et al., 2009). The social environment in captivity is often different to that which black rhinoceros would encounter under natural conditions. If testosterone concentration is correlated with reproductive success and increased by specific social stimuli, this could have important implications for how best to manage this species in captivity to enhance reproductive success.

In addition to variation in testosterone concentration, there may be other intrinsic factors that could influence reproduction. A similar degree of reproductive skew exists among captive black rhinoceros females (Edwards, 2013; Edwards et al., unpublished results), where a number of intrinsic differences have been identified between parous and nulliparous females (Edwards et al., 2014b). For example, nulliparous females have higher body condition scores than parous females, and a similar finding has also been reported in captive elephants (Freeman et al., 2009). So far, much of the focus on obesity and reproductive dysfunction in captive wildlife has been on females, but obesity can also affect male fertility. Excess adipose tissue increases the conversion of testosterone to oestradiol, resulting in reproductive axis suppression and reduced testosterone concentration (Michalakis et al., 2013). Furthermore, oxidative stress resulting from fat accumulation has also been linked to decreased spermatogenesis (Michalakis et al., 2013).

Reproduction can also be disrupted due to hypothalamic–pituitary–adrenal (HPA) axis activation in response to potential challenges. HPA activity can inhibit gonadotropin release; affecting gonadotropin-release hormone (GnRH) and luteinising hormone (LH) pulsatility at the level of the hypothalamus and pituitary (Kalantaridou et al., 2010). Increases in glucocorticoid secretion from the adrenal gland also inhibit testosterone-biosynthetic enzyme activity (Orr et al., 1994), leading to a reduction in testosterone secretion (Hardy et al., 2005). There is also evidence that stress may have a direct impact on fertility through reduction in the number and function of Leydig cells (Hardy et al., 2005), and reduced sperm count due to direct effects upon the seminiferous epithelium (Fenster et al., 1997). Cumulatively, these effects can lead to diminished libido and fertility (Phillips et al., 1989). Changes in adrenal activity have previously been highlighted as a potential issue affecting the health and mortality of captive black rhinoceros (Carlstead and Brown, 2005; Dorsey et al., 2010; Munson et al., 1998). Certain aspects of the captive environment are considered to be potential stressors in this species (Carlstead and Brown, 2005), which could additionally impact reproduction. When considering the impact of potential stressors upon reproductive function or captive welfare, it is important to also consider the behavioural adaptations that act as the primary mechanism for coping with potential challenges (Wielebnowski, 2003). Individual black rhinoceros are reported to vary in their temperament (Edwards, 2013), which may mediate their behavioural and physiological responses to potential challenges. Indeed, temperament differences in female black rhinoceros in this population have been demonstrated to correlate with both adrenal activity and prior reproductive success (Edwards et al., 2014b).

The aim of this research was therefore to investigate whether differences in reproductive success between males may be correlated with faecal testosterone metabolite concentration, as

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opposed to merely differences in opportunity. In addition, a number of intrinsic and extrinsic differences were investigated, using a similar approach to that used to investigate differences between parous and nulliparous females (Edwards et al., 2014b). Faecal glucocorticoids and ratings of individual temperament were used to assess how individuals respond to potential challenges in their environment, and differences in body condition were assessed. Finally, extrinsic factors, namely the social environment, were investigated to determine whether testosterone differences may be apparent according to social stimuli, and whether differences between males could help explain the observed variation in reproductive success.

2. Methods

2.1. Study population

This study included 23 male eastern black rhinos situated at 12 zoological institutions across Europe (Table 1), between the ages of 2 years 10 months and 32 years 6 months. This represents around 92% of males in the European Endangered Species Breeding Programme (EEP) population that had been at or approaching reproductive age during the study period.

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, males were categorised by their age, with those between the ages of 7 and 32 considered to be of breeding age ($N = 17$), and those under seven classed as immature ($N = 6$). Males in the reproductive age class were then further categorised as proven breeders if they had sired a calf by the end of the sample collection period ($N = 11$; age range 12–32 years), whereas those that had never sired a calf were considered non-proven ($N = 6$; age range 8–19 years). Although non-proven males were younger on average than their proven counterparts during the study period (mean age 11.7 years compared to 21.0 years; $t = -2.739$, df = 15, $P = 0.015$), there was no difference between the current age of non-proven males and the age of proven males when they first sired offspring (mean age 11.7 years compared to 10.0 years; $t = 0.858$, df = 15, $P = 0.404$).

All but one of the males included in the study were housed at the same institution as at least one female, with composition ranging from one adult male and one adult female to four adult males and eight adult females (Biddle and Pilgrim, 2011; Pilgrim, 2009). This solitary male had been housed with a female for over 11 years, but a lack of reproductive behaviour and failed introduction attempts led to the transfer of the female to another institution. Breeding management in this population 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). However, a common problem in this population is the lack of overt oestrus behaviour from certain females (Edwards et al., 2014b), which together with the often aggressive nature of black rhinoceros introductions (Fouraker and Wagener, 1996; Hutchins and Kreger, 2006) means that despite an intention to breed from all individuals (Biddle and Pilgrim, 2013), breeding opportunities are not always strictly equal.

2.2. Faecal sample collection and preparation

A total of 1455 faecal samples were collected over a sample collection period that ranged between 4 and 32 months. Faecal samples were collected at least weekly across the monitoring period. 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.

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

Table 1
Summary of males from which faecal samples were collected as part of the study, including their age and reproductive category during the period of sample collection, and faecal testosterone metabolite concentration (mean and standard deviation).

SB #	Name	Location[a]	Age[b]	Breeding status[c]	Faecal testosterone metabolite concentration (ng/g)		
					Mean	Standard deviation	*N*
955	Asani	Chester	2.8	*i*	27.96	8.42	84
714	Magadi	Chester	12.8	*P*	40.62	9.45	134
750	Sammy	Chester	12.0	*P*	53.58	15.82	140
483	Baringo II	Dvur Kralove	17.5	*P*	76.75	13.09	11
877	Davu	Dvur Kralove	4.2	*i*	48.05	4.56	12
926	Dzanti	Dvur Kralove	2.5	*i*	39.74	7.44	11
268	Isis	Dvur Kralove	32.5	*P*	55.77	9.51	13
283	Jimm	Dvur Kralove	31.2	*P*	72.92	7.48	11
659	Mweru	Dvur Kralove	13.7	*P*	56.21	5.47	14
928	Kito	Ebeltoft	4.4	*i*	28.65	4.49	80
927	Thabo	Ebeltoft	4.3	*i*	31.33	5.26	80
349	Kifaru II	Hannover	27.9	*P* (>10)	66.28	18.54	95
890	Vungu	Howletts	8.4	NP	45.44	9.21	44
533	Taco	Koln	15.5	NP	34.28	17.21	27
528	Usoni	Krefeld	15.8	*P*	81.61	21.56	23
653	Madiba	Magdeburg	20.1	*P*	73.59	16.79	50
438	Jakob	Pont Scorff	19.5	NP	55.13	7.98	56
341	Kingo	Port Lympne	27.5	*P*	48.80	9.65	68
892	Manyara	Port Lympne	8.6	NP	52.36	6.95	22
951	Monduli	Port Lympne	5.3	*i*	38.29	6.33	64
430	Quinto	Port Lympne	20.4	*P* (>10)	50.24	15.36	144
903	Zambezi II	Port Lympne	8.3	NP	43.45	9.96	65
857	Jeremy	Zurich	9.8	NP	45.96	15.94	207

[a] Current location when samples were collected for study.
[b] Age at the end of the sample collection period.
[c] *P* = proven – has sired at least one calf (>10 = has sired a calf, but not for over a decade), NP = non-proven – has never sired a calf, *i* = immature.

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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 598*g*. 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 androgen (Edwards et al., 2014a) and glucocorticoid (Watson et al., 2013) metabolites. Each EIA utilised an antiserum (polyclonal testosterone R156/7 or corticosterone CJM006; C.J. Munro, University of California, Davis); 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 (1:20 for testosterone and corticosterone EIAs), and run in duplicate (50 µl) on the respective EIA's.

The cross reactivities for testosterone and corticosterone antisera have been reported elsewhere (de Catanzaro et al., 2003; Watson et al., 2013). EIAs were biochemically validated for measuring testosterone-reactive and corticosterone-reactive metabolites in male black rhino faecal extracts through parallelism ($R^2 = 0.997$, $F_{1,7} = 2563.486$. $P < 0.001$ and $R^2 = 0.987$, $F_{1,7} = 537.761$, $P < 0.001$, respectively) and matrix interference assessment ($R_2 = 0.996$, $F_{1,7} = 1668.608$, $P < 0.001$ and $R^2 = 0.995$ $F_{1,7} = 1471.256$, $P < 0.001$, respectively). The testosterone EIA was biologically validated prior to this study by showing clear increases in faecal androgen metabolite concentration following a gonadotropin release hormone (GnRH) challenge (Edwards, 2013). The corticosterone EIA has previously been biologically validated for assessing adrenal status via faecal glucocorticoid metabolites in male black rhinoceros following an adrenocorticotrophic hormone (ACTH) challenge (Santymire et al., 2012). 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, as previously described in Edwards et al. (2014b). This scoring system involves the assessment of seven key regions of the body: neck, shoulder, ribs, spine, rump, abdomen and tail base. The index uses 0.5 increments between 1 (poor/emaciated) to 5 (excellent/heavy). Concurrent with faecal sample collection, 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 ($N = 9$), or from photographs alone ($N = 14$).

2.5. Questionnaire

Similar to the methodology used by Edwards et al. (2014b), keepers that spent the most time working directly with each rhinoceros (minimum of two years' experience) were asked to provide information on the behaviour and social environment of study subjects. Firstly, keepers were asked to score each male on the frequency with which they would express certain behaviours, and how they would typically respond to certain events or situations (Edwards, 2013). They were then asked to rate the temperament of each individual, based on how consistent these behavioural responses were from day to day, selecting from 'almost always behaves the same', 'sometimes can be unpredictable', or 'very unpredictable'. Secondly, information regarding the social environment for each male was collected, specifically whether the male was housed in the same enclosure as a female for any part of the study period (all the time; during oestrus only; some of the time, but not limited to oestrus; or not at all), and the number of conspecifics (males and/or females) in close proximity to the subject. The total number of males and females at each institution during the study period was also determined from the studbook (Biddle and Pilgrim, 2011; Pilgrim, 2009).

2.5.1. Data analysis

To investigate differences in testosterone metabolite concentration between males, faecal samples collected at least weekly were analysed for hormone metabolite concentration, and compared using generalised linear mixed models (GLMM's) in MLwiN version 2.02 (Rasbash et al., 2005). GLMMs allow nested random effects to be incorporated into the model (Bolker et al., 2009) to control for non-independence of data, such as repeated faecal samples per subject. Hormone data were transformed where necessary, using $\log_{10}$ transformations to improve the distribution of data. Seasonal differences in androgen concentration were investigated in a subset of males ($N = 7$) where faecal samples were collected at least weekly across all months of the year. An average concentration was calculated for each month, for each male and then compared using a GLMM including individual ID as a random effect.

To investigate differences in $\log_{10}$ faecal testosterone metabolite concentration ($\log_{10}$Tt) between individuals, random (date of sample collection and subject ID) and fixed effects were incorporated into the GLMM. Firstly, age was fitted as a continuous fixed effect to determine the relationship between age and $\log_{10}$Tt. Secondly, among reproductive-age individuals (7–32), $\log_{10}$Tt was compared between males according to their prior reproductive history, categorised as follows. Of the 11 males that had previously sired at least one calf, nine had sired within the last 3½ years; whereas two males had not sired for over a decade (11.3 and 12.5 years, respectively). Therefore to allow for potential differences associated with the length of time since the last reproductive event, males were divided into three categories: proven males that had bred within the last 3½ years ($P < 3½$; $N = 9$), proven males that had not bred for over 10 years ($P > 10$; $N = 2$), and non-proven males (NP; $N = 6$).

In addition, a multivariate approach was used to investigate how the social environment may contribute to the observed variation in faecal testosterone metabolite concentration in adult males. In this case, all fixed effects (Table 2) were entered into the GLMM together before non-significant terms were dropped sequentially until only those that explained significant variation in $\log_{10}$Tt concentration remained. All statistics reported are taken from this minimal model. Finally, each dropped term was re-entered to the minimal model individually to obtain their level of non-significance. The interaction of reproductive category and access to females during the study period was also investigated in the GLMM. A normal error structure was used for all models of $\log_{10}$Tt 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.

The relationship between testosterone metabolite concentration and reproductive success was investigated using a bivariate correlation. For each male aged 7–32 ($N = 17$), average testosterone metabolite concentration was calculated from all samples collected during the study period. As age is likely to have a strong influence on the number of calves sired due to differences in opportunity, average testosterone metabolite concentration was correlated with a measure of reproductive success, calculated as

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Table 2
Generalised linear mixed model (GLMM) of $\log_{10}$ faecal testosterone metabolite concentration in reproductive-age male black rhinoceros. A multivariate approach was used to investigate how the social environment may contribute to the observed variation in faecal testosterone metabolite concentration in breeding and non-breeding males. For categorical variables, a reference category was assigned (†), to which all other categories were compared. Negative effect size means $\log_{10}$Tt was lower than the reference category, positive effect size means $\log_{10}$Tt was higher than the reference category. Interaction terms were tested individually, and are included where significance was observed; alpha was set to $P < 0.05$ in all cases.

Fixed term	Categories	df	Effect	SE	Wald	*P*
Significant terms within the minimal model		5			13.272	**0.021**
Reproductive category[a]	†Proven breeder, sired calf within 3½ last years ($P < 3½$)					
	Proven breeder, has not sired for over 10 years ($P > 10$)	1	−0.011	0.070	0.027	0.869
	Non-proven breeder	1	−0.100	0.043	5.256	**0.022**
Housed with female(s)[a]	†During oestrus only					
	Some of the time, not limited to oestrus	1	−0.085	0.063	1.846	0.174
	All the time	1	−0.091	0.093	0.954	0.329
	Not at all	1	−0.122	0.054	5.058	**0.025**
Non-significant terms						
Number of other males at institution[b]		1	0.007	0.011	0.434	0.510
Number of females at institution[b]		1	0.001	0.004	0.031	0.860
Number of males nearby[b]		1	0.035	0.026	1.754	0.185
Number of females nearby[b]		1	−0.006	0.031	0.038	0.845
Interaction						
Reproductive category * housed with female[c]	†$P < 3½$ * during oestrus only	7			23.913	**0.001**
	$P < 3½$ * some of the time, not limited to oestrus	1	−0.181	0.067	7.325	**0.007**
	$P < 3½$ * all the time	1	−0.141	0.084	2.822	0.093
	$P < 3½$ * not at all	1	−0.177	0.061	8.489	**0.004**
	Non-proven * during oestrus only	1	−0.241	0.080	9.131	**0.003**
	Non-proven * some of the time, not limited to oestrus	1	−0.152	0.079	3.679	0.055
	Non-proven * not at all	1	−0.270	0.058	21.847	**<0.001**
	$P > 10$ * some of the time, not limited to oestrus	1	−0.147	0.065	5.129	**0.024**

[a] Significance of fixed term within minimal model.
[b] Fixed terms non-significant in minimal model, but re-entered individually to obtain their level of non-significance.
[c] Interaction terms were tested individually, only significant interactions are shown.

the total number of offspring sired divided by the number of years that particular male had spent within the reproductive age class.

Adrenal activity was compared between males using the mean, standard deviation (SD), and coefficient of variation (CV) calculated from faecal glucocorticoid metabolite (fGCM) concentrations for each individual. These three measures of adrenal activity were compared among the three categories $P < 3½$, $P > 10$ and NP using a one-way ANOVA. In addition, fGCM measured from longitudinal faecal samples collected from each individual were also compared using a GLMM, with date of sample collection and subject ID fitted as random effects. Reproductive category and individual temperament were then fitted individually as categorical fixed effects, with proven males that sired within the last 3½ years, 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, the distribution of temperament and body condition scores across reproductive categories were investigated using non-parametric Kruskal Wallis *H* tests to compare the three groups $P < 3½$, $P > 10$ and NP).

3. Results

3.1. Testosterone and reproductive success

Mean and standard deviation in faecal testosterone metabolite concentrations for the 23 males included in this study are presented in Table 1. Testosterone concentration was highly variable within individual males, but in seven males where faecal samples were collected for at least one year, no differences in $\log_{10}$Tt were observed according to month (GLMM $\chi^2 = 11.446$, df = 11, $P = 0.41$). Age was a significant predictor of faecal testosterone metabolite concentration, with $\log_{10}$Tt increasing with age (GLMM coefficient = 0.009, SE = 0.002, $\chi^2 = 16.213$, df = 1, $P < 0.001$). However, among males of reproductive age (7–32 years) and incorporating reproductive category into the GLMM, age was no longer a significant predictor of faecal testosterone metabolite concentration ($P = 0.48$). Males that had produced a calf in the last 3½ years had significantly higher $\log_{10}$Tt than non-proven males (GLMM coefficient = −0.128, SE = 0.046, $\chi^2 = 7.609$, df = 1, $P = 0.006$; Fig. 1). The two previously proven males that had not sired offspring for over a decade exhibited intermediate $\log_{10}$Tt concentration relative to those that have never sired a calf, and those that had bred more recently ($P = 0.16$ and $P = 0.66$ respectively).

Furthermore, among males of reproductive age (7–32 years), faecal testosterone was positively correlated with the number of calves sired per year that the male was represented in the reproductive age class ($r = 0.667$, $N = 17$, $P = 0.003$; Fig. 2).

3.2. Adrenal activity, temperament and body condition

There was no difference in $\log_{10}$ fGCM concentration among proven males that had successfully sired in the last 3½ years, those that had not sired for over a decade or non-proven males (GLMM $\chi^2 = 0.492$, df = 2, $P = 0.78$). Similarly, there were no differences in mean glucocorticoid concentration ($F = 0.065$, df = 2, $P = 0.938$), or either measure of variation, SD ($F = 3.382$, df = 2, $P = 0.0.063$) or CV ($F = 2.551$, df = 2, $P = 0.114$) among males categorised as whether or not they had sired a calf during the last ten years.

Although there were no overall differences in fGCM between males according to their prior reproductive history, adrenal activity did vary according to keeper's ratings of individual temperament. Among males of all ages, those rated as 'almost always behaves the same' ($N = 16$) had significantly lower $\log_{10}$ fGCM concentration across the study period (GLMM $\chi^2 = 8.815$, df = 2, $P = 0.012$) than males that were rated as 'sometimes can be unpredictable' ($N = 3$; GLMM coefficient = 0.086, SE = 0.041, $\chi^2 = 5.504$, df = 1, $P = 0.035$) or 'very unpredictable' ($N = 2$; GLMM coefficient = 0.0.109, SE = 0.046, $\chi^2 = 5.504$, df = 1, $P = 0.019$). There

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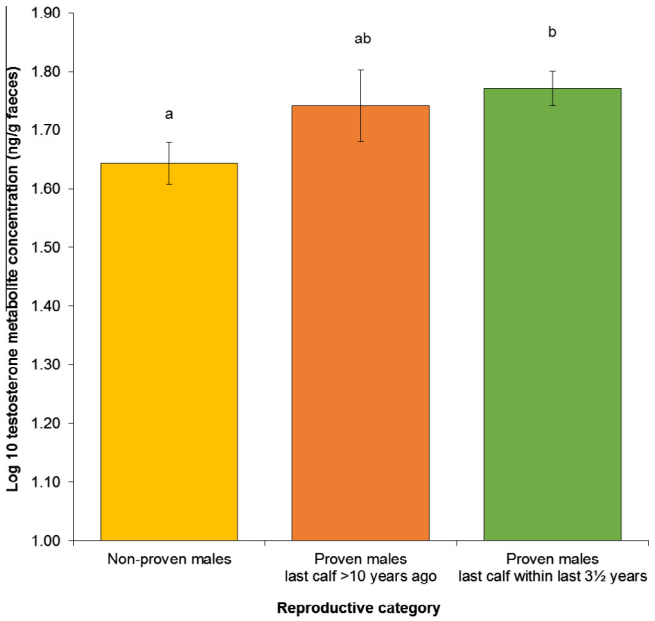

Fig. 1. Log_{10} transformed faecal testosterone metabolite concentration (±s.e.m) in males that have never bred (non-proven), compared to those that have bred but not in the last 10 years, and those that have bred within the last 3½ years. Data are predicted from a GLMM controlling for repeated faecal samples within individuals. Letters represent significant differences ($P < 0.05$).

were no consistent differences in temperament between breeding and non-breeding males ($P = 0.604$).

BCS was highly consistent between reproductive-age males, with all but one individual scored as 4.0. There was no relationship between BCS and either reproductive success, testosterone concentration or adrenal activity ($P > 0.05$).

3.3. Social environment

After accounting for the differences in faecal testosterone metabolite concentration between males that had sired a calf within the last 3½ years and those that had not, males that were housed with females during oestrus had significantly higher log_{10}-Tt than males that were not housed with females at all during the study period (Table 2). The interaction of reproductive category and whether a male had been housed with a female revealed that this effect was primarily driven by proven males that had sired within the last 3½ years and were housed with a female during oestrus only (Fig. 3). These males had significantly higher log_{10}Tt than non-proven males housed with females during oestrus only, males not housed with females at all, and both groups of proven males ($P < 3½$ and $P > 10$) housed with females not limited to oestrus periods. There was no effect either of the total number of conspecifics at the same institution, or the number of males or females housed nearby with visual, auditory and olfactory contact.

4. Discussion

As expected, male testosterone concentration increased with age (Christensen et al., 2009; Kretzschmar et al., 2004), with immature males exhibiting significantly lower faecal testosterone metabolite concentrations than mature males. Interestingly, within reproductive-age males there were clear differences with reproductive history, with males that had sired offspring within the last 3½ years exhibiting significantly higher faecal testosterone metabolite concentrations than non-proven males. Males that had not sired for at least 10 years tended to have testosterone concentrations intermediate to those that had sired more recently and those that had never sired, although the small sample size in this category perhaps precluded a more robust test. Furthermore, males that had sired more calves per year spent in the reproductive age-class had higher average faecal testosterone metabolite concentration across the study period. This indicates that differences in androgen concentration could be one possible explanation for the reproductive skew observed within this population. However, we cannot yet determine whether higher testosterone in proven males is related to their prior breeding experience, influenced by external stimuli, or whether underlying differences between males may result in both higher testosterone and greater reproductive success.

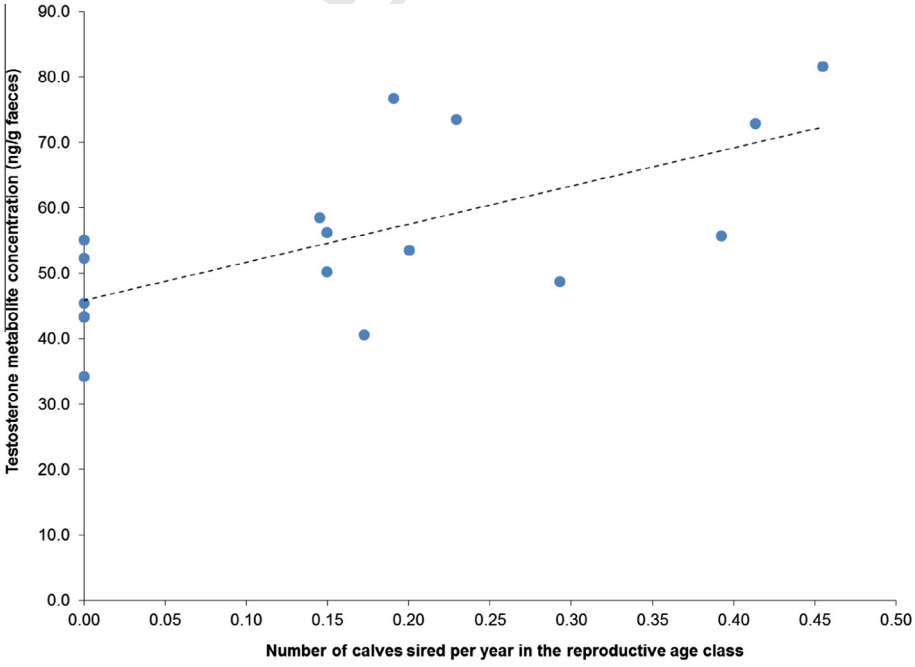

Fig. 2. Correlation between average faecal testosterone metabolite concentration and reproductive success, measured as the number of calves sired per year in the reproductive age class (7–32 years).

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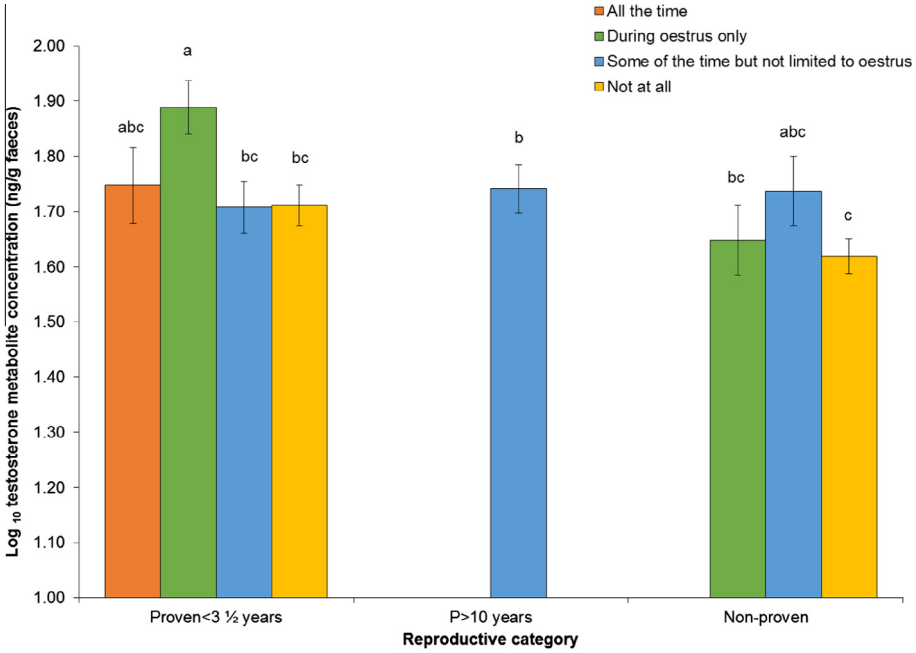

Fig. 3. Log_{10} transformed faecal testosterone metabolite concentration (±s.e.m) in male back rhinoceros, categorised according to their prior reproductive history and access to females during the study period. Data are predicted from a GLMM controlling for repeated faecal samples within individuals. Letters represent significant differences ($P < 0.05$).

In polygynous species such as the black rhinoceros (Garnier et al., 2001; Hutchins and Kreger, 2006), males compete for access to receptive females, with dominant males maintaining larger home ranges (Cain et al., 2014) and monopolising more matings (Garnier et al., 2001). Increased testosterone concentration plays an important role in male:male competition (Gleason et al., 2009) and is associated with dominance in a range of species, including chacma baboons, Père David's deer, bison, fur seals and white rhinoceros (Beehner et al., 2006; Li et al., 2004; Mooring et al., 2004; Negro et al., 2010; Rachlow et al., 1998). Although the relationship between dominance, testosterone and reproductive success has yet to be determined in wild black rhinoceros, increased testosterone could confer a similar advantage in maintaining dominance and monopolising matings, ultimately leading to higher reproductive success. However, in captive populations, mature male black rhinoceros are generally kept apart, suggesting that intra-sexual competition is unlikely to explain the differences in testosterone observed here, or the differences in reproductive success between males.

If testosterone concentration is a marker of male quality, then mate choice could play a role in the sub-optimal reproduction occurring in captivity. In wild black rhinoceros, females are almost exclusively dominant in breeding encounters (Berger and Cunningham, 2008), which could allow active mate choice by females. As black rhinos have relatively poor eyesight, and scent marking is common in both males and females (Estes, 1991), presumably signalling about quality, and possibly testosterone, would be through olfaction. This has recently been demonstrated in goats, where oestrus females consistently selected males with higher testosterone concentration in choice tests over those with lower concentration (Longpre and Katz, 2011). However, if this were the case in the black rhinoceros, there would presumably need to be a threshold concentration that signalled acceptable quality, as females rarely have the opportunity for direct comparison, especially in the captive setting. Further investigation could elucidate whether testosterone concentration is variable over the lifetime of an individual or is correlated with a fitness measures such as heterozygosity.

However, differences in testosterone may not necessarily represent differences in male quality, but could also be stimulated by external stimuli (Kempenaers et al., 2008). Sociosexual signals have been demonstrated to stimulate the neuroendocrine control of reproduction in both males and females (Delgadillo et al., 2009; Martin et al., 2004; Ungerfeld and Silva, 2004). In pampas deer, males maintained in groups with females had significantly higher faecal testosterone concentration and higher sperm quality and motility than males held in isolation (Villagran and Ungerfeld, 2013), indicating a positive relationship between social stimulation and measures of reproductive function. Similarly, serum testosterone concentration in captive black and white rhinoceros in North America was correlated with the presence of increasing numbers of female conspecifics. Although differences in reproductive success were not investigated by Christensen et al. (2009), males with increased testosterone concentration due to more social stimulation may also have a reproductive advantage. In the current study, we found differences in testosterone according to the social environment, with males that had sired within the last 3½ years and were housed with females during oestrus exhibiting higher testosterone concentrations than those that were not housed with females at all during the study period. However, non-proven males housed with females during oestrus did not exhibit higher testosterone concentrations, indicating that proximity to females alone does not explain the variation in testosterone concentrations observed between proven and non-proven males in this population.

Unlike the previous study by Christensen et al. (2009), we found no relationship between the total number of male or female conspecifics housed at the same institution and testosterone concentration. One possible explanation for this difference may be related to the social groupings under investigation between the two studies. In the current study, only one male was housed without a female at the same institution, compared to 15 males housed in isolation in the study by Christensen et al. (2009). This lone non-breeding male exhibited the lowest average testosterone concentration observed in the current study, and if the isolated males in the North American population were also non-breeding, findings

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from the two studies could be consistent. Interestingly, however, the two males with the highest testosterone concentrations in the current study were housed in very different social environments; as a single male with a single female (1 male:1 female), and with three additional males and four females (4 males:4 females). The relatively low sample size for these social groupings may impact our ability to investigate differences in testosterone between isolated males and those housed with multiple conspecifics, especially when the differences in concentration between breeding and non-breeding males are also considered.

Higher testosterone concentration and greater reproductive success could also be due to prior experience. Serum testosterone increased as a result of experience with reproductive females in bulls (Lunstra et al., 1989), rams (Borg et al., 1992) and mice (Kamel et al., 1977). This relationship between mating and testosterone concentration may be the result of copulation itself (Sanford et al., 1974), or associated with courtship behaviours such as nasogenital investigation (Borg et al., 1992; Lunstra et al., 1989). An increase in testosterone has also been observed in anticipation of mating in sexually experienced mice (Kamel et al., 1975). Therefore, males that have previously been introduced to females and previously mated and sired offspring could exhibit increased testosterone concentration because of their previous reproductive success.

It is also important to note that the categorisation of proven and non-proven breeders used in this study could not take into account whether all individuals had equal opportunities to reproduce. If a non-proven male and non-proven female were housed at the same institution, the two individuals' failure to reproduce may be related. For example, if a female was not cycling regularly, and not exhibiting overt signs of oestrus, as is common for non-proven females (Edwards et al., 2014b), the male may not have had suitable opportunities to reproduce. Similarly, differences in breeding opportunities may exist depending on the number, or the identity of conspecifics with which a male is housed during any given time-period. For example, a male housed with the same female for five years will have the opportunity to sire only one or at most two calves, whereas a male that has been housed with two females over the same period will have twice the opportunity to sire offspring. Alternatively, if this male were transferred between institutions and had access to a different female each year, his opportunity to breed would be much higher. However, making successful introductions is a significant management concern in this population, as introducing new pairs for breeding purposes can be difficult to achieve, perhaps taking several attempts before a mating occurs. Therefore, a previously successful breeding pair might in fact have better opportunities to produce offspring by staying together at the same institution than a male that is introduced to several different females but without a successful mating. This variety in true opportunities for breeding is very difficult to quantify, but could have a profound effect on an individual's reproductive success.

This variation in access to females could potentially influence androgen concentration if certain males are not receiving the stimulation needed to increase testicular activity. In wild giant pandas, testicular activity is quiescent until a male is stimulated by interaction with females or potentially male competitors (Nie et al., 2012), and in wild white rhinoceros, testosterone concentration is elevated during the period of most copulations, and when accompanying a receptive female (Kretzschmar et al., 2004). If males require some form of stimulation to increase their testicular activity, this is an important factor to consider when investigating the underlying differences in reproductive success. Testosterone mediates breeding behaviour and libido in inter-sexual encounters (Deen, 2008; Gleason et al., 2009; Roser, 2008), and as such, low concentration in non-breeding males may result in reduced motivation or expression of breeding behaviour needed for successful introductions and mating success to then occur. If male reproductive function could benefit from social stimulation, then ensuring non-proven males are provided with a suitable social environment may be one approach to minimising the reproductive skew in captivity. Alternatively, simulating a more complex social environment with olfactory cues within faeces or urine may be an alternative strategy to encourage reproductive behaviours, as previously explored in both white (Kretzschmar et al., 2002) and Indian rhinoceros (Stoops et al., 2014).

In addition to any variation in testosterone between males, there may be other intrinsic factors that affect normal reproductive function, and individual differences could result in some males being more susceptible to disruption than others. Both male (this study) and female black rhinos (Edwards et al., 2014b) that were judged to be more unpredictable and more reactive in their behaviour had significantly higher faecal glucocorticoid concentration, suggesting that individuals may respond differently to challenges in their environment. However, unlike females, there were no consistent differences in temperament between breeding and non-breeding males. There were also no differences in average or variability in faecal glucocorticoids between proven and non-proven males, indicating that chronic adrenal activity does not appear to be compromising reproduction. Additionally, unlike females in this population (Edwards et al., 2014b), there were no differences in body condition between proven and non-proven males, indicating that differences in body weight do not appear to be impacting reproductive success in males. However, body condition scores between males were less variable than we have previously seen between females in the same population, and seemed to show little variation according to season in a subset of males where BCS was assessed repeatedly across the year (Edwards et al., unpublished results).

5. Conclusion

With the growth and long-term viability of captive breeding programmes often limited by low rates of reproduction and high reproductive skew, it can be beneficial to investigate the underlying causes of variation in reproductive success. In the black rhinoceros we have previously demonstrated intrinsic differences between breeding and non-breeding females (Edwards et al., 2014b), and we now add to this differences between breeding and non-breeding males. Males that had sired offspring within the last 3½ years had significantly higher faecal testosterone metabolite concentration than those that had never sired offspring, and average testosterone positively correlated with the number of calves sired per year that the male was of reproductive age. These results are the first to indicate that androgen concentration is correlated with reproductive success in this species. However, we cannot yet determine whether these proven males have higher androgen concentration because of their previous mating success, due to more suitable social stimulation, or whether underlying differences in quality may drive both testosterone concentration and greater reproductive success. Our data support the findings of Christensen et al. (2009) that the sociosexual environment may play a role in stimulating testosterone production in male black rhinoceros, but our data also revealed that this effect was most prominent in males that had sired offspring within the last 3½ years. To reduce the reproductive skew among males, it would be beneficial to utilise potential fitness measures to determine whether the observed variation in testosterone could reflect differences in quality, or whether male reproductive success could be improved through altering the social environment. However, longitudinal analyses are required to investigate the variability in individual androgen concentration, both over time and in response to social stimulation and mating opportunities.

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Uncited references

Brown (1997), Folstad and Karter (1992) and Kempenaers (2007).

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