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Relationship between androgens, environmental factors and reproductive behavior in male white rhinoceros (*Ceratotherium simum simum*)

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Abstract

We conducted a longitudinal study of the endocrine activity of free-range male white rhinos. An enzyme immunoassay to measure androgens in the feces was developed and validated to show that it can be used to study testicular activity. We identified two fecal metabolites similar to testosterone and dihydrotestosterone. Several lines of evidence suggest that these metabolites clearly reflect testicular activity. Firstly, the stimulation of testicular activity with synthetic GnRH caused a 156% increase in androgen metabolite concentrations in the feces 1 day after treatment. Secondly, androgen metabolite concentrations increased with sexual maturity in rhinos, and finally there was a correlation between testosterone concentrations in plasma and androgen metabolite concentrations in feces. Using the method that we developed, it was possible to establish whether a relationship exists between androgen metabolite concentrations, the behavior and environmental factors. Adult territorial males ($n = 5$) had elevated androgen metabolite concentrations during months of high rainfall (September–February) compared to months of little or no rainfall (March–August). The increase in concentrations coincided with the beginning of the rainy season, suggesting a seasonal trend in reproduction. This trend was confirmed by behavior observations showing both a higher frequency of conceptions within the first 4 months of increased androgen metabolite concentrations, and a higher number of inter-sexual conflicts, reflecting the initial aggression between the sexes during the consort period. It was also evident that males accompanying a receptive female had higher fecal androgen metabolite concentrations compared to being alone. The elevated levels were likely induced by female presence.

Keywords: White rhinoceros; Reproduction; Androgen; Fecal steroids; Seasonal; Social behavior; Enzyme immunoassay; Free-ranging; Mating

Introduction

The breeding success of the white rhinoceros in captivity is very poor (Meister, 1998; Rieches, 1998), and the reasons for this are little understood. Research on breeding of rhinos has mostly been done on females and shows either that they have irregular estrous cycle lengths ranging from 25 to 90 days or that they have a complete lack of cyclic ovarian activity (Brown et al., 2001; Hindle et al., 1992; Patton et al., 1999; Roth et al., 1998; Schwarzenberger et al., 1998), although the reasons for this have yet to be explained. However, very little research has been done on the reproductive activity of male white rhinos, despite the fact that studies of a variety of captive species have shown that male infertility contributes significantly to reproductive failure (Wildt, 1996).

One possible reason for the low reproductive rates in captivity is the absence of certain environmental or social factors which may influence sex steroid concentrations. It is a well-established fact that these factors influence, for example, the androgen status of an individual (Brockman et al., 2001; Bronson, 1989). For example, day length, food availability, rainfall or temperature are known to influence the onset and cessation of sex steroid secretion in a variety of mammals (e.g., day length in fallow deer: Asher et al., 1989; food availability in impala: Brown et al., 1991b; rainfall in African buffalo: Brown et al., 1991a). It is also known that social stimuli such as the sight, sound and smell of a female in estrous can both promote endocrine changes in males, for example, in rhesus monkeys (Bernstein et al., 1977), domestic sheep (Gonzales et al., 1988) and grey lag goose (Kotrschal et al., 2000); and reactivate the sexual activity of a satiated male ("Coolidge effect", Lott, 1991).

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With regard to rhinos, there is also some evidence that environmental factors play an important role in reproductive behavior. The fluctuations in rainfall and food availability in the region south of the Zambesi where white rhinos live (Penny, 1987) suggests that a seasonal pattern of reproduction may occur. Similarly, social factors would seem to be important given the specific characteristics of rhino mating behavior. Studies of free-range rhinos have indicated that only territorial males have the chance to reproduce and that males without a territory adopt a subdominant status and hardly reproduce at all. It has also been shown that male and female rhinos generally live apart and only come together for about a month for mating purposes (Owen-Smith, 1973, 1975). This raises the question whether the absence of seasonal variations in environmental factors or the lack of social stimuli by other males or females can explain the low reproductive rate of rhinos in captivity. To establish this, we conducted a longitudinal study of the endocrine activity of free-range males and investigated firstly, whether the presence of receptive females leads to any changes in the androgen metabolite concentrations of adult territorial white rhinos and secondly whether the testicular activity depends on seasonal rainfall and/or on rainfall-related factors such as fluctuations in the supply and quality of food.

To this end, it was necessary to find a method of investigation which would permit the frequent collection of samples without subjecting the animals to stress, which is known to interfere with hormone concentrations (Brown et al., 1991a; Place and Kenagy, 2000; von Holst, 1998). Previously, non-invasive methods using fecal or urinary steroid metabolites, which are ideal for monitoring the reproductive activity of free-range animals, had only been developed for different species of female rhinoceros (black rhinoceros: Schwarzenberger et al., 1993, 1996; white rhinoceros: Hindle and Hodges, 1990; Meister, 1998; Indian rhinoceros: Schwarzenberger et al., 2000; and Sumatra rhinoceros: Roth et al., 2001). For males, however, the methods previously used had not been fully developed. One study measured testosterone-like immunoreactivity, but without any description of the metabolites (Rachlow et al., 1998). A more recent study reports on fecal androgen measurements in males (Brown et al., 2001), but without establishing the biological relevance of these measurements. For these reasons, to carry out our investigation, we developed a reliable non-invasive method for the assessment of the reproductive status of male rhinoceros and established its biological relevance.

Materials and methods

Study site

Data were collected between March 1997 and April 1999 from a population of individually distinguishable white rhinoceros living on a game farm in the Limpopo Province, South Africa. Name and location of the farm as well as the size of the population are not mentioned to avoid risk of poaching. The area covers 300 km^2 with several dozen rhinos living under natural conditions. The climate of the study area is characterized by wet summer (October–March) and dry winter. The driest months are May and July (EEC, 1998). The average rainfall measured during the study period on the site was 464 mm. The average day temperature ranged from 31.7 °C during summer to 25.9 °C during winter.

Study population

During the first year of the study, animals were identified by individual morphological features such as variations in horn size and shape, body size, hairiness of ears and tails, and folds along the body. In July 1998, all animals of the population were anaesthesized by a veterinarian and individually marked with a number code in the ear allowing to identify sexes and individual animals even from large distances. Rhinos were immobilized with etorphine hydrochloride and azaperone and revived with diprenorphine using the recommended dosage given by the South African Veterinary Foundation (Du Toit, 1998). The rhinos were kept tranquilized for shortest periods possible, maximum 15–30 min, to avoid unnecessary stress.

The age of the rhinos was assessed both by comparing their body and horn size with the size of an adult female using the method of Hillman-Smith (1997) and by comparing these features with pictures of animals of known age. Young males of up to 2 years of age were classified as calves. They stayed together with their mother until her next birth, which usually took place approximately 23.5 months after the previous birth (Kretzschmar, 2003). All males living independently from the mother, which were between 2 and 6 years old, were classified as subadults. The age of adult males is difficult to assess. However, since males usually become territorial with the age of 10 years (Owen-Smith, 1973), and all monitored adult males defended a territory, it is assumed that all of them were 10 years of age or older.

Sample collection and storage of samples

Fecal samples were collected in three different ways. (1) Five adult territorial males were monitored between January 1998 and April 1999 by tracking the animals approximately once a week and a total of 210 fecal samples (41 ± 5 samples per male) were collected. The footprints of individual males were followed with the help of a game tracker and fecal samples were collected along the track, but only when the origin of the sample was certain. The origin of the dung could be determined with certainty by its freshness and by the footprints at the dung. The footprints of individual males were distinguishable by the pattern of lines under-

neath the feet, as well as the sizes of the feet. Tracks dating from the previous night or early morning were followed until the animal was sighted around midday. The time of defecation could be estimated by its freshness and only samples less than 6 h old were collected. This method allowed to monitor the behavior of the males without disturbing them and increased the chance of finding a sample in the field. (2) Fecal samples were also collected by chance when sighting an excreting animal during daily routine patrols and (3) blood and fecal samples were collected from anaesthesized animals (see "Study population"). The fecal samples were obtained out of the rectum of the rhinoceros and the blood samples were collected from the ear vein.

The blood samples were kept in a cooler box until centrifugation at night. The feces were collected from different parts of the dung and mixed in a plastic bag. From this 0.5 g was weighed into a plastic tube and mixed with 5 ml 90% methanol. The tube was stored at −12 °C until processing. The storage time was 7–22 months depending on the time of sample collection. Androgen metabolite concentrations of fecal samples from each individual collected during the same period (January–March) did not differ significantly in concentrations between 1998 and 1999 (Wilcoxon paired sample test $T_{-} = 46.5$, $P = 0.16$, $n = 11$). We therefore assume no effect of storage times on steroid concentrations. This is supported by a recent study of Khan et al. (2002) showing near to initial concentrations at the end of a 6-month storage period.

Determination of androgen concentrations in feces and plasma

Fecal samples were centrifuged (15 min at 1200 × *g*) and the supernatant was transferred into a new tube, diluted 1:1 with water, and aliquot portions of 20 µl were measured for testosterone immunoreactivity by enzyme immunoassays (EIA, see below). For re-extraction, to insure a high recovery of fecal metabolites, the fecal sediment was suspended again in 90% methanol (10 ml) vortexed for 30 min and prepared for EIA as described above. With the second extraction, 30–50% of the metabolites were still recovered. Both extracts were individually analyzed in duplicate and the final androgen metabolite concentration of each fecal sample was calculated by summing up the concentrations of both extracts.

The antibody used (provided by Prof. Meyer, Weihenstephan, Germany) was raised in rabbits immunized against 17α-OH-testosterone-HS-BSA. Testosterone-3-CMO-peroxidase was used as enzyme conjugate. The cross-reactivity of the antibody with other steroids was as follows: 4-androsten-17β-ol-3-one (testosterone: 100%), 5α-androstan-17β-ol-3-one [dihydrotestosterone (DHT): 13.6%], 5α-androst-2-en-17β-ol (1.5%), and <0.1% for 5α-androstan-3β-ol-17-one (epi-androsterone), 5α-androstan-3α-ol-17-one (androsterone), 5α-androst-2-en-17-one.

For EIA, 20 µl sample extract was combined with 100 µl of enzyme label (1:50 000) and 100 µl antibody (1:500 000). After incubation for 24 h at 6–8 °C, the microtitre plates were washed four times before 150 µl of substrate buffer (1.2 mM H_2O_2, 0.4 mM 3,3′,5,5′-tetramethylbenzidine in 10 mM sodium acetate, pH 5.5) was added to each well and incubated for 40 min at 25 °C. The enzymatic reaction was stopped with 50 µl 4 M sulphuric acid to each well and the absorbance was measured at 450 nm.

Fecal sediments were lyophilized and fecal hormone concentrations were expressed as mass/gram dry weight, which rules out the dietary effects on steroid excretion (Wasser et al., 1993).

To determine testosterone in the blood, 0.1 ml of plasma was extracted with 2 ml of solvent consisting of 30% butylmethylether and 70% petroleum ether. After freezing at −80 °C, the organic phase was decanted, dried at 50 °C, dissolved in 1 ml 40% methanol and analyzed with the same EIA technique as described for the fecal samples. The testosterone calibration standards were prepared by dilution with 40% methanol and ranged from 0.2 to 100 pg/well. The sensitivity of the assay was defined as two standard deviations from the signal given by the zero blank. Precision and reproducibility were calculated from multiple measurements of pooled samples. The detection limit of the assay was 0.4 pg/well. Serial dilutions of fecal extracts yielded displacement curves parallel to those of the testosterone standard (Fig. 1). The intra-assay coefficients of variation were 9% ($n = 8$) and 8.9% ($n = 8$) and the inter-assay coefficients were 20.8% ($n = 32$) and 21.3% ($n = 32$) for two pooled samples containing low and high concentrations, respectively. The high and low concentrated quality control was located at the edge of the linear range of the standard curve (80% binding) when the curve already flattened out, which may have caused the high inter-assay coefficients of variation. Since all samples were diluted to fall well within the linear range of the

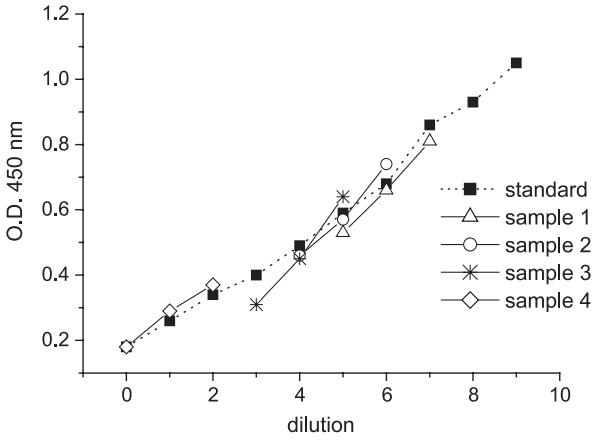

Fig. 1. Parallelism between a dilution of standard testosterone (closed squares) and serial dilutions of four different fecal extracts (open symbols).

standard curve, it can be expected that this amount of variation did not apply to the results. Nevertheless, to reduce methodological variation between individual samples of individual males, if possible, all samples were measured within one plate.

Biological validation of measurements of androgens in rhino feces

Identification of the androgen metabolites measured in the feces

For characterization of fecal metabolites, 500 μl of fecal extracts from different animals was loaded onto an Ultrasep ES100/RP—18/6 μm (Sepserv, Berlin) HPLC column (4 × 150 mm). Steroids were separated by reverse-phase chromatography at a flow rate of 1 ml/min using an isocratic mixture of methanol/water (75:25 v/v). Fractions of 0.3 ml were collected at 20-s intervals over a period of 11 min and diluted with 1 volume of water before 20 μl of the fractions was assayed for testosterone immunoreactivity using the EIA described above. The elution positions of authentic testosterone and DHT on this column had been previously determined in separate HPLC runs. For a further characterization of androgen metabolites, a second separation was carried out with acetonitrile/water (60:40 v/v) on the same column. Androgen standards and extracts were analyzed as described above.

Stimulation of gonadal secretion with synthetic GnRH

To stimulate the gonadal secretion of testosterone, 20 ml Receptal containing 84 μg Buserelin (Hoechst, Midrand, South Africa) was administered to one anaesthesized adult male. All fecal samples were collected by tracking this animal right after injection continuously for 3 days and additionally on the fifth day. In addition, one sample was collected on the tracks 14 days after treatment. Four fecal samples obtained before treatment served as control. Those were taken out of the rectum of the anaesthesized male (n = 1) as well as from sample collections on the tracks 7 days (n = 2) and 8 days (n = 1) before the treatment. Due to the intensive monitoring effort needed to obtain consecutive samples, only one male was chosen for this part of the study. A significant increase in concentrations was defined if the concentrations after administration were higher than the baseline value + 3 interquartile ranges (IQR).

Comparison of testosterone concentrations in plasma and concentrations of fecal androgen metabolites

A total of 12 matched blood and fecal samples was collected from anaesthesized male rhinos (see above) of different ages (adults n = 5, subadults n = 6, calves n = 1). The blood samples were collected immediately after sedation to minimize the influence of stress on testosterone concentrations. The testosterone concentrations in the blood samples of individual males were compared with concentrations of androgen metabolites in feces of the same animal using the Spearman Rank correlation.

Androgen metabolite concentrations in fecal samples of different age classes

Fecal samples of 11 different subadult males and eight male calves were obtained either by chance when sighting an excreting animal or from anaesthesized animals. The age of the animals was assessed as described above. Fecal samples of five adult males (41 ± 5 samples per male) were collected by tracking. For each adult male, the median androgen metabolite concentration was established and compared with concentrations of samples from subadult males and male calves using the Mann–Whitney test. As data of adult males were used for multiple comparisons, a standard Bonferroni correction was applied to adjust P-values.

Presence of receptive females and androgen metabolite concentrations

This study was conducted when tracking five adult territorial males (see "Sample collection and storage of samples"). Whenever tracks of a male and a female were found together for a period of approximately half a day and the same animals were found together again during the next tracking session, it was assumed that the male was courting this female. This definition is based on observations that adult males usually walk on their own, except when courting a female. During that time, he stays for up to a month close together with the female (Owen-Smith, 1973). To be on the safe side, it was established whether the female was receptive during that time. This was managed with the interval between consecutive birth calculated from a pool of females. Females fostering a male calf conceived approximately 7.5 months and those fostering a female calf conceived approximately 18.5 months postpartum (Kretzschmar, 2002). On the basis of these findings, all females with a male calf of 7 months or older and a female calf of 18 months or older were classified as receptive. However, we cannot rule out that some females were receptive beforehand. Courting took place throughout the year and was not restricted to a particular time of year. The median androgen metabolite concentration of fecal samples collected during tracking when a male was walking alone over a period of approximately half a day were compared with the median of fecal samples of the same male accompanied by a receptive female over a similar time period. As consorting and courtship behavior usually takes up to several days or weeks (Owen-Smith, 1973), it is expected that the fecal samples reflect a continued situation rather than a single event. For comparison, a Wilcoxon paired sample test was applied. For the male who was never seen together with a female, the missing value was replaced by randomly choosing three values of the other males and calculating the median out of these values.

Influence of seasonal rainfall on hormones and behavior

Rainfall

Rainfall was measured daily at 21 different weather stations distributed evenly over the study site. All measurements of each month and station were summed and the average monthly rainfall at the study site was calculated.

Androgen metabolite concentrations

Five adult territorial males were monitored between January 1998 and April 1999 by tracking the animals approximately once a week and a total of 210 fecal samples (41 ± 5 samples per male) was collected. Seasonal effects were evaluated by comparing the median fecal androgen metabolite concentration of each individual using samples collected during two 6-month periods. The first period began with the onset of the rainy season in September, and ended in February, at which time the rainfall had almost ceased. The second period (March–August) was one of little or no rainfall. This periodization was chosen to ensure two sets of equally balanced data. For purposes of comparison, monthly medians of the metabolite concentrations in the feces of each individual were calculated, and these medians were in turn used to calculate the medians for each individual over each of the entire period. The median concentrations of each male for each time period (dependent group of data) were then compared using the Wilcoxon paired sample test.

To describe the seasonal variation in androgen metabolite concentrations, monthly medians and IQR (giving 25% and 75% of the data range) of the metabolite concentrations in the feces of each individual were calculated, and these medians were in turn used to calculate the monthly medians of all males. A significant increase in concentrations was defined if a monthly value was higher than the baseline value + 3 IQR. The baseline was defined as the average of lowest values that were not different according to the sensitivity of the assay (8–21%).

Fighting activity

The number of fighting between adult territorial males and between territorial males and adult females was monitored by tracking five territorial males as described before. Signs such as broken trees and long scratch marks on the ground gave clear indications of fights. The size and the pattern of lines underneath the feet gave clues to distinguish the individuals and/or the number of participants. The number of fighting occurring between all five territorial males and between the territorial males and females in the 4 months before the beginning of the rainy season (May–August) and the 4 months thereafter (September–December) were summed and compared. Given the low number of samples due to the known limitations in field studies, no statistical analysis was applied for the number of conflicts and the number of conceptions (see below).

Number of conceptions

The number of conceptions was calculated by subtracting 16 months, the length of pregnancy in white rhinoceros (Owen-Smith, 1973), from the time of birth. The time of birth of a calf was established by direct observations, either of the female with her newborn or of the previous calf having left the mother. A total of 31 births occurred between January 1997 and December 1999; personal data were supplemented by observations of game wardens. The conceptions of subsequent years were summed up for each month and number of conceptions within the 4 months before the beginning of the rainy season (May–August) and the 4 months thereafter were summed and compared.

Data analysis

The data did not fit assumptions of distribution and homogeneity of variance for parametric analysis (Sokal and Rohlf, 1995) and we therefore used nonparametric statistics in accordance with Siegel and Castellan (1988). The statistics were based on two-tailed tests and a probability of ≤ 0.05 was considered significant. The calculation of the statistical tests was carried out using the statistical programs SPSS 8.0 for windows (© SPSS) and SSS 1.0 (Rubisoft Software GmbH). For descriptive statistics, the median and IQR were calculated, giving 25% and 75% of the data range.

Results

Biological validation of the assay

Measurement of androgens in the HPLC fractions of a fecal sample revealed two major metabolites eluting at positions authentic to testosterone and DHT (5.6 and 7.7 ml, respectively, Fig. 2A). In addition, two small peaks of unknown metabolites occurred at 3.5 and 4.7 ml. As shown in Fig. 2B, the presence of testosterone and DHT was confirmed with the second HPLC system. The two minor metabolites (Fig. 2A), however, were not detectable with the second HPLC system.

After administration of Buserelin to one adult male, the androgen metabolite concentrations increased significantly 1 day after treatment (by 156%), from a baseline of 64.8 ng/g feces (IQR = 19.6, $n = 4$) to 165.7 ng/g feces ($n = 1$) (Fig. 3). Within 5 days posttreatment, the values decreased slowly. The concentration on day 14 after administration was comparable to basal concentrations.

A positive correlation was found between plasma hormone and fecal androgen metabolite concentrations both collected at the same time from each of the 12 anaesthesized male rhinos (5 adult, 6 subadult, 1 calf) (Spearman rank correlation: $r_s = 0.594$, $P = 0.042$, $n = 12$, Table 1).

The median androgen metabolite concentration of five adult territorial males was 57.5 ng/g feces (IQR = 8.6, $n =$

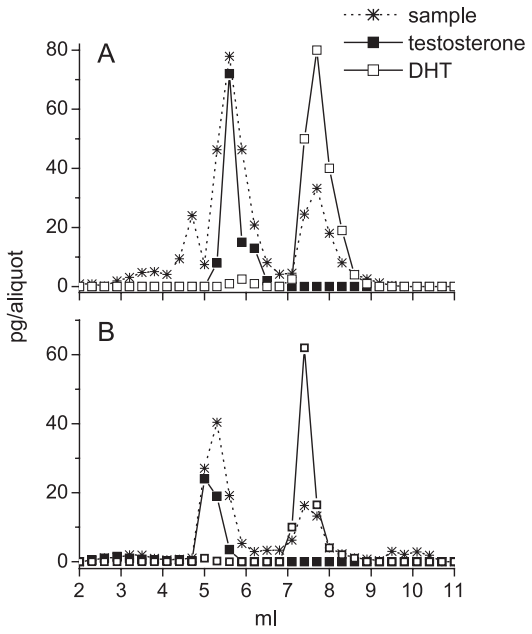

Fig. 2. Characterization of androgen metabolites in a fecal extract from a male white rhinoceros (*Ceratotherium simum simum*) after HPLC separation with a methanol- (A) and an acetonitrile-containing solvent (B). The HPLC fractions were tested in an EIA with an antibody that recognizes testosterone and DHT. The elution profile of the sample (asterisks) was compared with those of testosterone (solid squares) and DHT (open squares) which had been determined previously after injection of both steroids and analysis of the HPLC fractions.

5). This concentration was significantly higher compared to the median androgen metabolite concentrations of male calves (28.5 ng/g feces, IQR = 8.2, n = 8; Mann–Whitney test with a posteriori standard Bonferroni correction: Z = −2.9, P = 0.007, n_a = 5, n_c = 8) and subadult males (28.5 ng/g feces, IQR = 30.5, n = 11; Z = −2.0, P = 0.042, n_a = 5, n_s = 11).

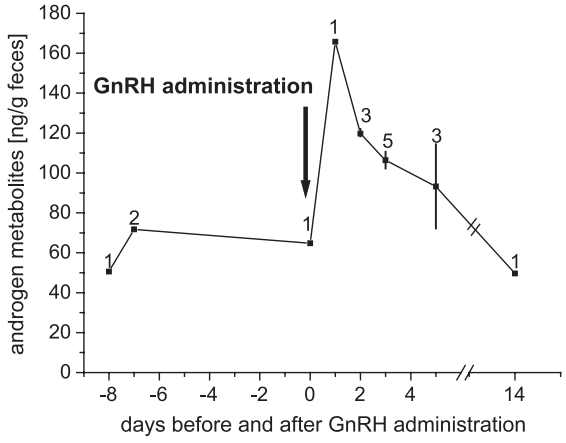

Fig. 3. Daily testosterone metabolite concentrations (median and interquartile ranges) before and after administration of the GnRH analog Buserelin to an adult territorial male. The number of fecal samples per day is indicated on top of each square.

Table 1
Plasma testosterone and fecal androgen metabolite concentrations in samples collected simultaneously from anaesthesized male rhinos of different age

	n	Plasma [ng/ml]		Feces [ng/ml]	
		Range	Median	Range	Median
Adult	5	1.14–2.79	2.3	42.5–100.9	45.9
Subadult	6	0.14–0.9	0.35	20–38.5	22.7
Calf	1	0.17	/	29.3	/

Definition of ages: adults: ≥10 years old, subadults: 2–6 years old, calves: up to 2 years of age.

Presence of receptive females and androgen metabolite concentrations

Median androgen metabolite concentrations of samples collected on the day of sighting territorial males accompanying a receptive female were significantly higher compared to concentrations of individual males being alone (Fig. 4, Wilcoxon paired sample test T_- = 0, P = 0.043, n = 5).

Influence of seasonal rainfall on hormones and behavior

The rainy season began in September and ceased in March. Maximum precipitation was reached in December (Fig. 5B). Individual adult males had significantly higher median androgen concentrations during the period of high rainfall (from September to February on average 70% higher, n = 5) compared to the period of little or no rainfall (from March to August, Wilcoxon paired sample test T_- = 0, P = 0.043, n = 5). Monthly concentrations of the males fluctuated between 30 and 60 ng/g between

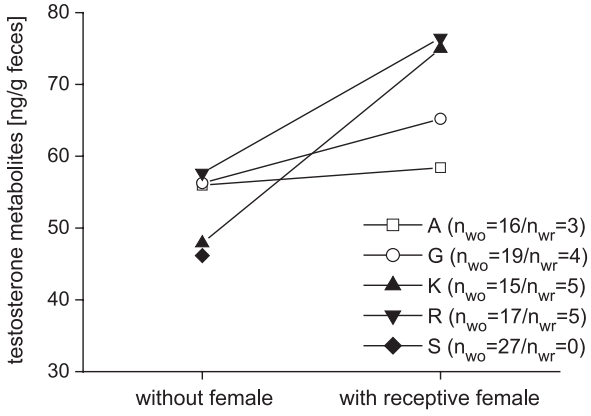

Fig. 4. Median testosterone metabolite concentration of fecal samples collected during tracking when a male was walking alone over a period of approximately half a day compared to median concentrations of the same male accompanied by a receptive female over a similar time period. The receptive phase was defined as the interval between the birth of the previous calf and conception of the following calculated from a pool of females. Capital letters given in the graph indicate the name of each male, the numbers in the parenthesis give the sample size for each male (n_{wo} = without female, n_{wr} = with receptive female).

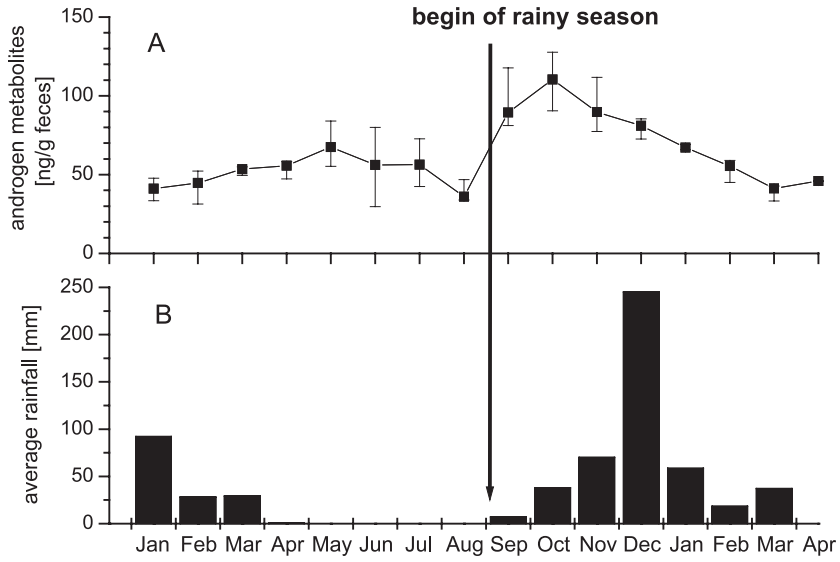

Fig. 5. Androgen metabolite profile (median and interquartile ranges) of five adult males (A) for the time period: January 1998 to April 1999 and monthly rainfall during the same time period (B). The line indicates the beginning of the rainy season.

January and August (Fig. 5A). At the beginning of the rainy season in September, fecal metabolite concentrations increased significantly from 36.0 to 89.5 ng/g feces, reaching a peak in October. The concentrations decreased slowly thereafter, reaching basal concentrations in January. The number of conceptions within the first 4 months of the rainy season was twice as large (September–December: n = 14) as the number of conceptions during the same time span before the beginning of the rainy season (May–August: n = 7). Fighting also occurred (n_{total} = 21) more than twice as often within the first 4 months of the rainy season and after the increase in androgen concentrations (September–December, n = 11) as during the same time span before it (May–August, n = 5). The number of inter-sexual conflicts tripled during the time span of increased androgen metabolite concentrations ($n_{Sep.-Dec.}$ = 6, $n_{May-Aug.}$ = 2) while the number of intra-specific conflicts was only slightly higher ($n_{Sep.-Dec.}$ = 5, $n_{May-Aug.}$ = 3).

Discussion

The overall aim of the study was to explore the interaction between environment, behavior and the androgen metabolite concentrations of free-ranging white rhinoceros, and by this means to contribute to an understanding of why rhinos do not breed well in captivity. Since we used an antibody which was specified to analyze testosterone in blood plasma, and since feces typically contain multiple steroid metabolites with low amounts of native hormones (Palme et al., 1996), it was necessary to establish whether this antibody measured biologically relevant information regarding the androgen status of males. Our results clearly show that the antibody used in the study reflects testicular activity. This conclusion is based on the following observations: firstly, testosterone and DHT were tentatively identified as fecal metabolites when two HPLC systems with different selectivity were used, indicating that our method was capable of measuring androgen metabolite concentrations. Secondly, using this antibody, it was possible to measure the increase in androgen metabolite concentrations following the stimulation of the pituitary with synthetic GnRH. Thirdly, our method verified the previous finding that androgen secretion increases with sexual maturity in mammals (Asa, 1996). Finally, there was a significant correlation between testosterone concentrations in plasma and the androgen metabolite concentrations of fecal metabolites taken at the same time from the same animals. Interestingly, the delay of 1 day in excretion did not influence the correlation, suggesting that there is only low variation between the daily androgen concentrations of individual animals.

Having established the reliability of this method, it was possible to explore two questions. The first was whether the presence of receptive females leads to any changes in the androgen metabolite concentrations of adult territorial white rhinos. The results revealed that androgen metabolite concentrations were generally higher when individual males accompanied a receptive female for half a day than when they were alone. The period during which males accompanied their female partners did not necessarily coincide with the mating season. It is therefore likely that the higher concentrations of androgen metabolites in individual males were induced by female presence rather than by seasonal hormonal fluctuations. Such a relationship is also known for other species, such as rabbits (Agmo, 1976), domestic sheep (Sanford et al., 1974) and grey lag geese (Kotrschal et al., 2000).

This finding is of obvious relevance to the problem of low reproductive rates among rhinos in captivity. In captiv-

ity, males and females seldom have the chance to spend days and nights together. In view of the evidence that the stress resulting from abnormal social relations is likely to influence the hormonal balance and cause infertility in male and female rhinos (Gunn et al., 1998), it would be worth considering whether giving males and females the chance to remain together day and night, as they would do in a natural environment, might not help to stimulate reproductive activity. To achieve greater clarity in this area, one task for future research would be to expose males of known androgen status to females and to establish if the androgen concentration increases.

The second question which we explored was whether white rhinos show a seasonal pattern of reproduction influenced by rain. The longitudinal hormone profiles of individual adult territorial males suggest that this is the case, as they showed significantly higher androgen metabolite concentrations during months of high rainfall (September–February) as compared to months with little or no rainfall (March–August). The increase in androgen metabolite concentrations coincided with the beginning of the rainy season and decreased in the presence of abundant rain. It is likely that testicular activity was induced by the first rain and/or the flush of green grass following the rain, but not directly correlated with it.

This assumption is supported by the evaluation of behavior patterns. During the first 4 months of the rainy season, which coincide with the higher androgen metabolite concentrations, there was an increase both in the number of conceptions and in fighting activity, particularly between males and females. Such inter-sexual conflicts may occur when the male tries to prevent the cow from leaving his territory during the consort period (Owen-Smith, 1973). However, a few matings occurred at other times, suggesting that there may be occasional deviations from the pattern of strict reproductive seasonality in white rhinos.

The evidence that the testicular and mating activity of white rhinos is stimulated by rainfall or rainfall-related factors is in line with observations made in another study of free-living white rhinos in South Africa, which shows an increase in mating and courtship activity in November (Owen-Smith, 1973), the month with the highest rainfall in this area (Downing, 1972cited in Owen-Smith, 1973). Owen-Smith (1973) also considered that there might be a correlation between variations in rainfall patterns and birth patterns, a correlation which has already been shown for other mammals (Blache et al., 2000; Bronson, 1989; Wildt, 1996).

Captive white rhinos, by contrast, do not show any seasonal trend in breeding (Brown et al., 2001; Frädrich, 1997). It would be worth considering whether the simulation of natural conditions might not help to stimulate reproductive activity. Although it would be difficult to simulate rainfall patterns, simulation of the changing food supply and food quality would be an option. To achieve greater clarity in this area, one task for future research would be to put males in a controlled environment which would then be manipulated to see if changes had an effect on testicular steroidogenesis.

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References

Agmo, A., 1976. Serum luteinizing hormone and testosterone after sexual stimulation in male rats. Acta Physiol. Scand. 96, 140–142.

Asa, C.S., 1996. Reproductive physiology. In: Kleiman, D.G., Allen, M.E., Thompson, K.V., Lumpkin, S. (Eds.), Wild Mammals in Captivity. Principles and Techniques. The University of Chicago Press, Chicago, pp. 390–417.

Asher, G.W., Peterson, A.J., Bass, J.J., 1989. Seasonal pattern of LH and testosterone secretion in adult male fallow deer, *Dama dama*. J. Reprod. Fertil. 85, 657–665.

Bernstein, I.S., Rose, R.M., Gordon, T.P., 1977. Behavioral and hormonal responses of male rhesus monkeys introduced to females in the breeding and non-breeding seasons. Anim. Behav. 25, 609–614.

Blache, D., Chagas, L.M., Blackberry, M.A., Vercoe, P.E., Martin, G.B., 2000. Review metabolic factors affecting the reproductive axis in male sheep. J. Reprod. Fertil. 120, 1–11.

Brockman, D.K., Whitten, P.L., Richard, A.F., Benander, B., 2001. Birth season testosterone levels in male Verreaux's sifaka, *Propithecus verreauxi*: insights into sociodemographic factors mediating seasonal testicular function. Behav. Ecol. Sociobiol. 49, 117–127.

Bronson, F.H., 1989. Mammalian Reproductive Biology. The University of Chicago Press, Chicago.

Brown, J.L., Wildt, D.E., Raath, J.R., de Vos, V., Howard, J.G., Janssen, D.L., Citino, S.B., Bush, M., 1991a. Impact of season on seminal characteristics and endocrine status of adult free-ranging African buffalo (*Syncerus caffer*). J. Reprod. Fertil. 92, 47–57.

Brown, J.L., Wildt, D.E., Raath, C.R., de Vos, V., Janssen, D.L., Citino, S.B., Howard, J.G., Bush, M., 1991b. Seasonal variation in pituitary–gonadal function in free-ranging impala (*Aepyceros melampus*). J. Reprod. Fertil. 93, 497–505.

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 noninvasive endocrine monitoring. Zoo Biol. 20, 463–486.

Downing, B.H., 1972. A plant ecological survey of the Umfolozi Game Reserve Zululand. PhD, University of Natal.

Du Toit, J.G., 1998. Rhino Ranching. A Manual for Owners of White Rhinos. Africa Publishers, Pretoria.

EEC, 1998. Vegetation Description and Veld Condition Assessment of the Study Site. Euclea Ecological Consultants CL, Pretoria.

Frädrich, H., 1997. International Studbook for African Rhinoceroses (*Diiceros bicornis*/*Ceratotherium simum*). Zoologischer Garten Berlin AG, Berlin.

Gonzales, R., Poindron, P., Signoret, J.P., 1988. Temporal variation in LH

and testosterone responses of rams after the introduction of oestrus females during the breeding season. J. Reprod. Fertil. 83, 201–208.

Gunn, I.M., Thorne, A., Trounson, A., 1998. Infertility/subfertility in the southern white rhinoceros (*Ceratotherium simum simum*) and the evaluation of their reproductive parameters as to other related species. In: Patton, L., Czekala, N., Lance, V. (Eds.), Workshop on Problems Associated with the Low Rate of Reproduction among Captive-Born Female Southern White Rhinoceros (*Ceratotherium simum simum*). Center for Reproduction of Endangered Species, San Diego, pp. 37–45.

Hillman-Smith, K., 1997. Das Nördliche Breitmaul-Nashorn. In: Nashörner, D. (Ed.), Begegnungen mit urzeitlichen Kolossen. Filander, Fürth, pp. 173–183.

Hindle, J.E., Hodges, J.K., 1990. Metabolism of oestradiol-17β and progesterone in the white rhinoceros (*Ceratotherium simum simum*). J. Reprod. Fertil. 90, 571–580.

Hindle, J.E., Möstl, E., Hodges, J.K., 1992. Measurement of urinary oestrogens and 20α-dihydroprogesterone during ovarian cycles of black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceroses. J. Reprod. Fertil. 94, 237–249.

Khan, M.Z., Altmann, J., Isani, S.S., Yu, J., 2002. A matter of time: evaluating the storage of fecal samples for steroid analysis. Gen. Comp. Endocrinol. 128, 57–64.

Kotrschal, K., Dittami, J., Hirschenhauser, K., Möstl, E., Peczely, P., 2000. Effects of physiological and social challenges in different season on fecal testosterone and corticosterone in male domestic geese (*Anser domesticus*). Acta Ethol. 2, 115–122.

Kretzschmar, P., 2002. Population growth, sex ratio and reproduction of white rhinos in South Africa. In: Schwammer, H.M., Foose, T.J., Fouraker, M., Olson, D. (Eds.), A Research Update on Elephants and Rhinos. Schüling Verlag, Münster, pp. 196–201.

Kretzschmar, P. 2003. Endological, endocrinological and ethological investigations of female mate choice in free-ranging white rhinoceros (*Ceeratotherium simum simum*). PhD, Ernst-Moritz-Arndt-Universität Greifswald.

Lott, D.F., 1991. Intraspecific Variation in the Social Systems of Wild Vertebrates. Cambridge Univ. Press, Cambridge.

Meister, J., 1998. Research on social and reproductive behaviour in captive female white rhinos (*Ceratotherium simum*). PhD, Friedrich Alexander University Erlangen Nürnberg.

Owen-Smith, R.N., 1973. The behavioural ecology of the white rhinoceros. PhD, University of Wisconsin.

Owen-Smith, R.N., 1975. The social ethology of the white rhinoceros *Ceratotherium simum* (Burchell 1817). Z. Tierpsychol. 38, 337–384.

Palme, R., Fischer, P., Schildorfer, H., Ismail, M.N., 1996. Excretion of infused 14C steroid hormones via feces and urine in domestic livestock. Anim. Reprod. Sci. 43, 43–63.

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 fecal pregnane analysis and observations of mating behavior. Zoo Biol. 18, 111–127.

Penny, M., 1987. Rhinos: An Endangered Species. Helm, Bromley, UK.

Place, N.J., Kenagy, G.J., 2000. Seasonal changes in plasma testosterone and glucocorticoids in free-living male yellow-pine chipmunks and the response to capture and handling. J. Comp. Physiol., B 170, 245–251.

Rachlow, J.L., Berkeley, E.V., Berger, J., 1998. Correlates of male mating strategies in white rhinos (*Ceratotherium simum*). J. Mammal. 79, 1317–1324.

Rieches, R., 1998. Concerns with breeding F1 white rhinoceros in zoological institutions. In: Patton, L., Czekala, N., Lance, V. (Eds.), Workshop on Problems Associated with the Low Rate of Reproduction among Captive-Born Female Southern White Rhinoceros (*Ceratotherium simum simum*). Center for Reproduction of Endangered Species, San Diego, pp. 4–5.

Roth, T., Patton, L., Brown, J., Czekala, N., Swaisgood, R., Lance, V., 1998. The North American experience: female hormonal cycles in Southern white rhinoceros. In: Patton, L., Czekala, N., Lance, V. (Eds.), Workshop on Problems Associated with the Low Rate of Reproduction among Captive-Born Female Southern White Rhinoceros (*Ceratotherium simum simum*). Center for Reproduction of Endangered Species, San Diego, pp. 6–8.

Roth, T.L., O'Brien, J.K., McRae, M.A., Bellem, A.C., Romo, S.J., Kroll, J.L., Brown, J.L., 2001. Ultrasound and endocrine evaluation of ovarian cycle and early pregnancy in the Sumatran rhinoceros, *Dicerorhinus sumatrensis*. Reproduction 121, 139–149.

Sanford, L.M., Palmer, W.M., Howland, B.E., 1974. Influence of sexual activity on serum levels of LH and testosterone in the ram. Can. J. Anim. Sci. 54, 579–585.

Schwarzenberger, F., Francke, R., Göltenboth, R., 1993. Concentrations of fecal immunoreactive progestagen metabolites during oestrus cycle and pregnancy in the black rhinoceros. J. Reprod. Fertil. 98, 285–291.

Schwarzenberger, F., Tomásová, K., Holečková, D., 1996. Measurement of fecal steroids in the black rhinoceros (*Diceros bicornis*) using group-specific enzyme immunoassays for 20-oxo-pregnanes. Zoo Biol. 15, 159–171.

Schwarzenberger, F., Waiter, C., Tomásová, K., Vahala, J., Meister, J., Goodrowe, K.L., Zima, J., Straun, G., Lynch, M., 1998. Fecal progesterone metabolite analysis for non-invasive monitoring of reproductive function in the white rhinoceros (*Ceratotherium simum*). In: Patton, L., Czekala, N., Lance, V. (Eds.), Workshop on Problems Associated with the Low Rate of Reproduction among Captive-Born Female Southern White Rhinoceros (*Ceratotherium simum simum*). Center for Reproduction of Endangered Species, San Diego, pp. 10–15.

Schwarzenberger, F., Rietschel, W., Vahala, J., Holečková, D., Thomas, P., Maltzan, J., Baumgartner, K., Schaftenaar, W., 2000. Fecal progesterone, estrogen, and androgen metabolites for noninvasive monitoring of reproductive function in the female Indian rhinoceros. Gen. Comp. Endocrinol. 119, 300–307.

Siegel, S., Castellan, N.J., 1988. Nonparametric Statistics for the Behavioural Sciences, Second ed. McGraw-Hill, New York.

Sokal, R.R., Rohlf, F.J., 1995. Biometry. The principles and Practice of Statistics in Biological Research, Third ed. W.H. Freeman, New York.

von Holst, D., 1998. The concept of stress and its relevance for animal behavior. In: Möller, A.P., Milinski, M., Slater, P.J.B. (Eds.), Stress and Behavior. Academic Press, San Diego, pp. 1–131.

Wasser, S.K., Thomas, R., Nair, P.P., Guidry, C., Southers, J., Lucas, J., Wildt, D.E., Monfort, S.L., 1993. Effects of dietary fibre on fecal steroid measurements in baboons (*Papio cynocephalus cynocephalus*). J. Reprod. Fertil. 97, 569–574.

Wildt, D.E., 1996. Male reproduction: assessment, management, and control of fertility. In: Kleiman, D.G., Allen, M.E., Thompson, K.V., Lumpkin, S. (Eds.), Wild Mammals in Captivity. Principles and Techniques. The University of Chicago Press, Chicago, pp. 429–450.