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Effects of sociosexual environment on serum testosterone in captive male African rhinoceros

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Abstract

The relationships between testosterone concentrations in male African rhinoceros and the presence of conspecific males and females were investigated. Serum testosterone concentrations were measured using enzyme-linked immunoassay (EIA) in 37 male black rhinoceros (*Diceros bicornis*) and 21 male white rhinoceros (*Ceratotherium simum*) housed at 37 institutions in the USA. Testosterone concentrations in both black ($n = 37$) and white ($n = 21$) rhinoceros males rose with increasing numbers of females present ($P < 0.05$). Average testosterone concentrations also rose with an increased number of conspecific males ($n = 34$) in black rhinoceros ($P < 0.05$). However, no specific pattern was found among male white rhinoceros housed with other males. We inferred that introduction of females to a male may play an important role in stimulating libido and spermatogenesis. The similar response of black rhinoceros and white rhinoceros to increased numbers of females suggested that, at least historically, herd structure for blacks may have been more similar to whites than previously realized, and should be investigated further.

Keywords: Sociosexual; Testosterone; Rhinoceros; Captive breeding

1. Introduction

Of the five extant rhinoceros species, four are critically endangered (black, Asian greater one-horned, Sumatran, and Javan rhinoceros). The fifth has a critically endangered subspecies (northern white rhinoceros, *Ceratotherium simum cottoni*), which is nearly extinct in the wild and limited to less than 10 individuals in captivity (at two institutions). Although captive breeding of rhinoceros species has achieved some success, different difficulties are encountered in each species [1]. Work has been done to evaluate the reproductive anatomy, physiology, endocrinology and behavior of female rhinoceros [1–5], but very little has been done to evaluate male rhinoceros [1,3,4,6–9], especially in captive populations [4,9]. Knowledge of male reproductive endocrinology is essential to understanding such processes as spermatogenesis, development of primary and secondary sexual characteristics, libido, and territorial behavior, all of which play key roles in an individual's ultimate fertility.

Serum hormone concentrations offer a specific, direct measure of hormone activity in an individual; this is in contrast to methods of measuring hormone metabolites in feces and urine. Whereas samples collected for the latter methods are more convenient in wildlife studies, the metabolites measured exhibit some degree of cross-reactivity, which may falsely

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increase concentrations measured, as is the case with androgen and cortisol metabolites [10]. Measurements of serum hormone concentrations reflect an exact measurement of that hormone at that moment in time. Measurements of hormone concentrations in feces or urine, in contrast, are measurements of metabolites of the hormones of interest concentrated over time and may be affected by the degree of hydration or amount of food ingested recently.

This study explored exogenous influences of the sociosexual environment on serum testosterone concentrations in captive male rhinoceros by measuring concentrations of testosterone in captive male rhinoceros serum using enzyme immunoassays (EIAs). Furthermore, our objective was to establish a database of normal male serum testosterone concentrations in black and white rhinoceros males. Data were also evaluated for variation with female and male presence or absence to determine the effect of these sociosexual influences on male serum testosterone concentrations.

2. Materials and methods

2.1. Animals

Serum samples ($n = 714$) from adult male black rhinoceros ($n = 37$; age, 8–28 yr) and samples ($n = 437$) from adult male white rhinoceros ($n = 21$; age, 13–43 yr) housed at 37 institutions in the USA, and collected at least monthly, were analyzed. Adult age determination was in accordance with endocrinological maturity (Christensen et al., unpublished data). Blood was collected by local veterinary or technical staff from the rhinoceros, with the animal voluntarily approaching a gated barrier and allowing venipuncture from the leg, ear, or sometimes tail. Time of day for serum collection varied within and among institutions. Effects of diurnal steroid concentrations were overcome by the large sample size evaluated. Serum samples were stored frozen (−20 °C), until shipment on dry ice to our laboratory where they were stored frozen (−70 °C) until analysis.

2.2. Hormone analysis

Serum testosterone concentrations for black and white rhinoceros were determined using a heterologous EIA (Dynex 1.13 MRX Revelation; Dynex Technologies, Inc., Chantilly, VA, USA) with a polyclonal anti-testosterone-6-carboxymethyl-oxime antiserum (R156/7) and testosterone conjugate (testosterone-3-carboxymethyl-oxime:horseradish peroxidase). All hormone concentrations are expressed as mass units of hormone per milliliter serum. For validation, serial dilutions (range = neat to 1:128) of pooled serum were assayed to check for displacement curves parallel to the standard curve. Recovery of known amounts of testosterone (0.19–50 ng/mL) added to pools of diluted serum (1:2, 50 Fl) was 106.2% ($y = 0.9205x + 0.1025$, $r^2 = 0.999$) for black rhinoceros, and 115.2% ($y = 2.7986x - 1.0757$, $r^2 = 0.9812$) for white rhinoceros. Serum samples were diluted for assays 1:5 and 1:32 for black and white rhinoceros, respectively. Serum cortisol concentrations were determined to assess any correlation between the two steroid hormones. Cortisol was assayed using an EIA with a polyclonal anti-cortisol-3-carboxymethyl-oxime-BSA antiserum (R4866; C. Munro, University of California, Davis, CA, USA) and cortisol conjugate (cortisol:horseradish peroxidase; C. Munro, University of California, Davis, CA, USA). All hormone concentrations are expressed as mass units of hormone per milliliter serum. Intra-assay coefficients of variation were <15%, and assay sensitivity was 0.19 ng/mL.

2.3. Statistics

For those rhinoceros that had reached sexual maturity ($n = 37$ for black rhinoceros; $n = 21$ for white rhinoceros), mixed model analyses, reflecting rhinoceros as a random effect and the repeated measurements taken from each rhinoceros, were conducted using SAS's PROC MIXED (SAS Institute, Cary, NC, USA). The data were log transformed so that the assumption of normality was more nearly met.

The fixed effects of age, the presence of other males (isolated males: $n = 25$ black, 26 white; paired males: $n =$ nine black, 10 white; groups of three: $n =$ seven black rhinoceros), and the presence of females (isolated males $n = 15$ black, six white; males with one female $n = 23$ black, nine white; males with two females $n =$ seven black, five white; males with three or more females $n =$ three black, five white) on serum testosterone concentrations were considered. In addition to rhinoceros, the interactions between rhinoceros and the number of other males, and among rhinoceros, and the number of other males were random effects in the model. The correlation among observed testosterone concentrations from the same animal were modeled using an autoregressive correlation structure. Similar analyses were conducted to compare hormone concentrations among sociosexual status (isolated male rhinoceros; male rhinoceros with one female rhinoceros; male rhinoceros with two female rhinoceros;

male rhinoceros with three or more female rhinoceros; male rhinoceros with one other male rhinoceros; male rhinoceros with two other male rhinoceros; $n = 804$ black, 354 white serum samples) and in relation to serum cortisol concentrations ($n = 354$ white; cortisol was undetectable in black rhinoceros). Data are expressed as means ± S.E.M. Significance was set at $P < 0.05$.

3. Results

3.1. Female presence

The number of females housed with a black rhinoceros male was positively correlated with serum testosterone concentrations ($P < 0.05$). Although the relationship was not truly linear, the mean testosterone level consistently increased as the number for females increased from zero to three or greater, leading to a linear effect ($P < 0.05$, Table 1). Considering pairwise comparisons, males housed in isolation had lower serum testosterone concentrations than those housed with two or three or greater females. Black rhinoceros males housed with one female had lower serum testosterone concentrations than those housed with three or greater females. The difference between those males housed with one female and those housed with two females was not significant ($P = 0.16$), nor was the difference between males housed with two females and those housed with three or greater ($P = 0.11$).

One individual black rhinoceros male housed with one female had a distinct rise in testosterone concentrations during the 4 mo after a second female was introduced to his exhibit (1533.4 ± 76.8 pg/mL) compared to the 4 mo previous (804.5 ± 33.5 pg/mL: $P < 0.05$). This was the only such introduction in this species during the course of the study.

White rhinoceros males also had different serum testosterone concentrations, depending on the number of females in their pen ($P < 0.05$). As with the black rhinoceros, the relationship was not truly linear, but the mean testosterone concentration consistently increased as the number of females increased from zero to three or greater, leading to a linear effect ($P < 0.05$, Table 1). White rhinoceros males housed in isolation had significantly lower serum testosterone concentrations than those housed with one or two females, and males housed with zero to two females had significantly lower concentrations than those housed with three or more females. No difference was noted between males housed with one female or two females ($P > 0.05$).

Table 1
Mean (±S.E.M.) serum testosterone concentrations in male black and white rhinoceros in the presence of females.

No. of females	Serum testosterone (pg/mL)	
	Black	White
0	610 ± 148[a]	945 ± 207[d]
1	751 ± 171[a,b]	1326 ± 197[e]
2	900 ± 225[b,c]	1454 ± 261[e]
≥3	1780 ± 812[c]	2234 ± 267[f]

Within a column, means without a common superscript (a–f) differed ($P < 0.05$).

3.2. Male presence

Black male rhinoceros housed in isolation had lower testosterone concentrations from those males housed with one or two other males ($P < 0.05$; Table 2). There was no difference in serum testosterone concentrations between those males housed with one other male and males housed with two other males ($P > 0.05$). Male black rhinoceros grouped in pairs or in groups of three had variability in testosterone comparisons between individuals. In no groups of two or three males did all individuals have elevated serum testosterone concentrations compared with those males housed in isolation ($P < 0.05$). One or more individuals would exhibit elevated serum testosterone concentrations compared to the partner(s) ($P < 0.05$), whose testosterone concentrations were similar to those of isolated males ($P > 0.05$).

In white rhinoceros males, eight individuals were housed within visual and olfactory contact with another male. No males were housed in full physical contact with each other: some males had direct contact with females, others were separated, but had olfactory and visual contact. No consistent pattern was found between white rhinoceros males. Some individuals had higher testosterone concentrations from isolated males ($P < 0.05$) and others did not ($P > 0.05$). Perhaps age, the presence of females, or other factors not evaluated had a stronger influence on serum testosterone concentration than the presence of conspecific males.

Table 2
Serum testosterone concentrations in male black rhinoceros in the presence of other males.

No. of males	Serum testosterone (pg/mL)
0	458 ± 116[a]
1	1304 ± 430[b]
2	1331 ± 599[b]

Means without a common superscript (a and b) differed ($P < 0.05$).

A pair of males housed in the southeastern United States had more complicated dynamics. One male (age 10–14 yr) originally housed with five adult females bred successfully and produced a number of calves. This male was moved to a pen adjacent to the females with visual and olfactory contact, but not physical contact, and replaced by a new male (age 29–31 yr) whose genetics were considered desirable. This new male was housed with the females for nearly 1 yr, but failed to impregnate any females. He was switched with the original male and then moved into an isolated area of the park. When the new male was removed from harem status to an isolated position, distant from all other white rhinoceros, his testosterone concentrations decreased ($P = 0.054$).

3.3. Serum cortisol

Serum cortisol concentrations were not detectable in black rhinoceros males using EIA under the conditions of this study. Concentrations of serum cortisol were detectable in white rhinoceros. In these animals, there was a weak, positive correlation between serum cortisol and testosterone concentrations ($P < 0.05$).

4. Discussion

In both black and white rhinoceros, males had a general, significant increase in serum testosterone concentrations with increasing numbers of conspecific females: male black rhinoceros had a significant increase in serum testosterone concentrations with increasing numbers of conspecific males. The positive influence of female number on the testosterone concentration of males of both species suggested a polygynous sociosexual structure.

Polygynous social structures exist in diverse mammalian groups [11–19] including perissodactylids [20–22]. Sociosexual status (i.e. bachelor versus harem males) affected blood testosterone concentrations in stallions [22]. Stallions that command a herd of mares have significantly higher testosterone concentrations than nearby bachelor stallions. Removal and isolation of a harem male caused its testosterone concentrations to fall to concentrations similar to bachelor stallions. In the absence of the original harem stallion, one of the bachelors will assume the harem role and have a consequent rise in serum testosterone concentrations, peaking well above concentrations in those that remain bachelors. This may be repeated again and again with the same results. If a harem stallion that had been removed is returned, he will push the acting harem stallion back into the bachelor herd and their respective testosterone concentrations will adjust accordingly.

Although free-ranging harem stallions actively guard and herd their harem of mares, this is not true of all equids. The social structure of donkeys is different from horses, zebras, and Przewalski wild horses in that donkeys do not form harems, per se, but instead jacks defend territories, controlling jennies that pass through or reside within their territories [23]. This territorial social structure more closely resembles what is known about black and white rhinoceros social structure [8,21]. Jack donkeys had increased libido when allowed contact with a group of females in a larger enclosure, rather than close confinement with a jenny [24]. Testosterone concentrations have not been evaluated in donkey jacks involved in fluctuating sociosexual environments.

Similar endocrine or behavioral changes corresponding to sociosexual environment may well be observed in free-ranging rhinoceros species. Free-ranging male white rhinoceros are divided into territorial and non-territorial (subordinate) groups. Territorial males defend their territories against other territorial males, but allow the presence of non-territorial males that do not participate in mating activities [21,25]. Female white rhinoceros are rarely solitary; they occupy home ranges overlapping the territories of several solitary males. When a dominant male discovers an estrous female, he tries to confine her to his territory [21]. Testosterone concentrations are significantly higher in territorial males than non-territorial males [6,8]. Fecal testosterone concentrations in male white rhinoceros are higher during encounters with females [8]. In the current study, increased testosterone concentrations in the presence of increasing numbers of females correlated well with these observations from the field.

The polygynous social structure of white rhinoceros is well-known. White rhinoceros are notoriously difficult to breed in captivity; breeding success is anecdotally much better at institutions that have a relatively large area and house the rhinoceros in herds, rather than pairs. This is often successful (Penfold, unpublished observations). Based on these observations, coupled with the fact that male white rhinoceros housed in a herd situation (three or more females) had significantly higher testosterone concentrations compared to those males housed with zero to two females (and that those males housed with one or two females had significantly higher concentrations than isolated males), we inferred that either lower testosterone concentrations in males housed with fewer females was associated with lower reproductive success, or

higher testosterone concentrations in males housed with more females was associated with higher reproductive success. Whether or not there is a direct contributory role of testosterone concentrations remains to be investigated. Possible mechanisms include the effect of testosterone on libido, other secondary sexual behaviors, and spermatogenesis.

Female black rhinoceros have overlapping territories, with male territories encompassing that of many females. Males establish a dominance hierarchy and defend mutually exclusive territories. Though traditionally thought to be a more solitary species, Garnier et al. suggested that female numbers exert a strong positive influence on male serum testosterone concentrations [20]. Popular perceptions of social interactions among black rhinoceros may be skewed by artificial, historical changes and may need to be adjusted. Of all rhinoceros species, the black rhinoceros has experienced the most drastic decline in numbers. Around 1900, it was once the most widespread rhinoceros species across Africa, with an estimated 100,000 individuals; however, by 1970, the population had decreased to an estimated 65,000 individuals. By 1992, the numbers of black rhinoceros had decreased by 96% to approximately 2300 individuals in the wild (www.rhinos-irf.org, accessed August 6, 2008). This intense reduction in the population may very well have falsely contributed to the current association with them as solitary individuals. Garnier et al. commonly observed family groups of black rhinoceros [20]. Garnier's study suggests a polygynous sociosexual structure in free-ranging black rhinoceros, with a strong reproductive skew towards certain successful males (those that had produced offspring) and an exclusion of subordinate males. Rather than supporting the traditional view of black rhinoceros as a solitary species that lacks important group influences, Garnier et al. presented evidence of strong interactions between dominant males and both the females they defend and the males they defend the females against. Assuming a potential link between testosterone and secondary sexual behaviors, such as territoriality and maintaining harems, the conclusions of Garnier et al. were supported by the current study (positive correlation in serum testosterone concentrations in male black rhinoceros with increasing numbers of either conspecific males or females). Studies in the field are warranted to investigate these hormonal and behavioral interactions.

A manipulative study along the scale of the previously cited stallion study [22] has not been done in rhinoceros. However, an observational report of a captive herd of white rhinoceros noted that the dominant male was successful at excluding other males from the females, but unsuccessful in achieving intromission [26]. Removing the dominant male allowed two previously subordinate males to dispute over the females. Testosterone concentrations were not determined; however, based on another study [6], it was presumed by the authors that the dominant male would have had elevated testosterone concentrations. This illustrates an instance where testosterone concentrations may not have correlated with fertility.

In the current study, two white male rhinoceros were swapped back and forth between harem and bachelor status. When the new 'harem' male was unsuccessful in reproducing, he was moved to an entirely isolated portion of the park far away from any other white rhinoceros. The male's testosterone decreased and never rose to concentrations during his occupation as a harem male ($P = 0.054$). These observations supported the finding that free-range, territorial male white rhinoceros have higher testosterone concentrations than non-territorial males [6,8] and that the decrease in testosterone was likely due to isolation from the females.

In some polygynous rats, males exposed to new females had an immediate increase in testosterone concentrations compared to exposure to familiar females [27]. This interesting phenomenon was consistent with our observation of a similar rise in serum testosterone in a male when a novel female black rhinoceros was introduced to the original male/female pair. It has been speculated that captive breeding of black rhinoceros may be enhanced if a male is housed with more than one female [20]. We demonstrated that mean testosterone concentration of males tended to increase as the male was housed with a greater number of females (or a novel female is introduced). Therefore, we inferred that an artificial sociosexual polygynous arrangement in captivity may stimulate individual males. More studies evaluating specific reproductive parameters of success, such as sperm quality and, ultimately, pregnancies, need to be performed before this definitive link can be established or disproven.

The numbers of conspecific males housed together also positively correlated with serum testosterone concentration in black rhinoceros. Only some members of multi-male groupings, however, demonstrated elevated testosterone concentrations. Perhaps these interactions represented social dominance hierarchies. To our knowledge, no studies investigating serum testosterone concentrations in free-ranging black rhinoceros have been published, but fecal testosterone concentrations were higher during times of fighting

between free-ranging male white rhinoceros [8]. A practice occasionally used in captivity, based solely on behavioral observations, is to introduce a second male into the vicinity of a desired stud male that has shown decreased interest in breeding in hopes that this will 'jump start' the desired male into breeding (Penfold, 2006, unpublished observations). Data in the present study provided the first empirical evidence lending some support to these common husbandry practices.

Libido of male rhinoceros was not evaluated in the current study, nor were any measures directly correlated to fertility, such as sperm parameters or production of offspring. Although, in general, testosterone concentrations have been positively linked with both libido and spermatogenesis [28], the minimum concentrations necessary for normal function vary and have not been determined in most species, including all rhinoceros. In some species, the highest testosterone concentrations are actually inversely correlated with semen quality. In bull elephants, the highest concentrations of testosterone, encountered during musth [29], are correlated with the poorest sperm quality (Hildebrant, unpublished data). Regardless of whether dealing with black or white rhinoceros, increasing the number of females housed with a particular male will likely increase that male's testosterone concentrations, but to determine what effect that rise may have on libido, spermatogenesis, and ultimate fertility requires further study. The study of Hermes et al. provided enlightening data; they reported that sperm morphology and motility may correlate with social status [9]. In a group of both male and female white rhinoceros, only the dominant male had good sperm quality. Removal of the dominant male resulted in greatly improved sperm parameters in the new dominant male, which subsequently bred and sired offspring. Unfortunately, testosterone concentrations were not evaluated, but considering the data from the current study, it would be expected that males exposed to greater numbers of females would have higher testosterone concentrations. In the study by Hermes et al., paired males housed with multiple females consistently had one male with good semen quality, and the other with poor semen quality [9]. Single males ($n = 6$) housed with multiple females were split; half had good sperm quality and the other half had intermediate or poor quality. In an interesting twist, comparing Hermes et al. data with that of the current study, males isolated from all other males and females all had good sperm quality ($n = 4$), whereas in the current study isolated males had the lowest testosterone concentrations. Comparison between these two studies suggested that testosterone concentrations may not correlate with sperm parameters. A study directly comparing the two is required.

Serum testosterone concentrations in captive male rhinoceros had interesting changes in regard to exogenous influences. This study documented changes in serum testosterone with respect to exposure to other males and increasing numbers of females in both black and white rhinoceros males. It has long been suspected in white rhinoceros that reproductive success is better in a herd than in pairs. Further studies are indicated evaluating sperm quality and reproductive success in relation to testosterone concentrations. We challenge, however, what has been the traditional practice of housing black rhinoceros in pairs, considering them a solitary species. Results of this study supported recent findings in the field that black rhinoceros may have a polygynous sociosexual structure to their population and may also benefit in captivity with being housed in herds rather than pairs.

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