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Short Communication

Stress steroid levels and the short-term impact of routine dehorning in female southern white rhinoceroses (*Ceratotherium simum simum*)

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Rhinoceros populations in Africa are under severe threat as a result of surging poaching rates and risk-mitigation strategies are continuously adapted in an attempt to ensure the survival of the species. This study compared faecal glucocorticoid metabolite (fGCM) levels of two age classes of limited free-ranging female white rhinos with fGCM levels of adult free-ranging female white rhinos. Subsequently, fGCM alterations in the limited free-ranging animals were monitored following routine dehorning as a measure of the animals' short-term physiological stress response. Baseline fGCM levels differed significantly between tested groups, with both free-ranging and limited free-ranging adult animals showing significantly higher fGCM levels compared with limited free-ranging juvenile females. In contrast, baseline fGCM levels did not differ significantly between limited free-ranging and free-ranging adult individuals. Routine dehorning procedures resulted in a short-term stress response expressed by a significant increase in fGCM levels 48 h post-dehorning, with stress steroid levels returning to pre-dehorning concentrations 72 h after the procedure.

Keywords: faecal glucocorticoid metabolites, non-invasive hormone monitoring, physiological stress, South Africa

White (*Ceratotherium simum*) and black (*Diceros bicornis*) rhinoceros populations in South Africa are under severe threat as a result of surging poaching rates (Thomas 2010). Official statistics released by the Department of Environmental Affairs, South Africa, reveal that the number of rhinos killed in South Africa increased annually between 2007 and 2014 from 13 to 1 215. In 2015 a slight decrease was seen, with 1 175 rhinos reportedly killed in the country (Save the Rhino International 2016).

Risk-mitigation strategies are continuously adapted by rhinoceros owners in an attempt to ensure the survival of these animals. As a result of the imminent nature of the threat, the short- and long-term physiological effects of such interventions on rhinos are often largely unknown at the time of implementation. The controversial practice of horn infusion with indelible dye and ectoparasiticides has been criticised for lack of evidence-based research on animal health, welfare and legal aspects, as well as overall effectiveness (Ferreira et al. 2014). Keeping groups of rhinos under limited free-ranging conditions allows access control and security monitoring, but may have social, welfare and ecological implications (Linsey et al. 2012). A strategy that recently regained popularity is the trimming of horns of live, immobilised rhinos as a poaching deterrent. Pioneered in 1989 in vulnerable black rhino populations in Namibia, several variations of the original method have been applied over the years (Pinchin 1993). The aim is to remove the bulk of the horn in a humane fashion without damaging the growth plate, allowing regrowth. Although poaching of dehorned rhinos has been reported, the rationale exists that the risk for poachers to be caught may outweigh the benefit of attempting removal of remaining horn bases from such animals (Pinchin 1993). However, the perception of the dehorning procedure as a stressor for rhinos has not been examined so far.

Stress can be defined as a generic term for any stimulus that threatens or appears to threaten homeostasis of an individual (Selye 1936; Wielebnowski 2003). A stress response is a series of adaptive mechanisms aimed at protecting an individual and restoring homeostasis (Wielebnowski 2003). The primary hormones involved in a stress response are glucocorticoids and catecholamines, and the levels of these hormones can be determined as a parameter of adrenal activity and thus as a measure of stress (Möstl and Palme 2002). Measurement of faecal glucocorticoid metabolite (fGCM) concentrations to monitor adrenocortical function provides a practical, non-invasive and feedback-free alternative to glucocorticoid determination in blood, saliva or milk (Möstl and Palme 2002; Touma and Palme 2005). FGCM concentrations, unlike rapidly-fluctuating blood cortisol/corticosterone levels, reflect cumulative secretion and elimination of hormones over an extended

time period. Such a delayed, time-averaged response to a stressor is dependent on species-specific gut-passage times (Touma and Palme 2005). For both black and white rhinos, remotely collected faeces has been shown to be a reliable resource for monitoring stress-related alterations in GCM concentrations, with respective immunoreactivity measurable in faeces within 2 d after perceiving a stressor (Brown et al. 2001; Turner et al. 2002).

This study aimed to determine fGCM concentrations in adult and juvenile female white rhinos living under limited free-ranging conditions and to compare these values to fGCM concentrations of adult white rhino females roaming freely. A second aim was to examine fGCM alterations following routine dehorning of limited free-ranging female white rhinos, as a measure of the animals' short-term physiological stress response.

Data collection was performed at a privately owned breeding facility for southern white rhinos in the North West province of South Africa from October to November 2014. Rhinos at the facility were kept under limited free-ranging conditions in camps that ranged between 400 and 500 ha of natural *Cymbogon/Themeda*-type vegetation. Individual breeding camps were populated with predominantly adult females, their offspring and two dominant breeding bulls, at an average stocking density of 9 ha per rhino.

From an approximate age of 24 months, depending on horn length, rhinos at the facility were routinely dehorned with an average interval of 20 months between consecutive dehornings. Rhinos were chemically immobilised in camps with a combination of etorphine hydrochloride and azaperone (doses based on estimated body weight), administered intramuscularly by a dart and remote delivery system. Horn trimming was done with an electrical saw at 100 mm and 25 mm above the skin-horn interface of the rostral and caudal horns, respectively, to prevent damage to growth plates and sinuses (Figure 1). Intravenous naltrexone (at 10 times the etorphine dose) was administered as reversal immediately after horn trimming. All applicable international, national and/or institutional guidelines for the care and use of animals were followed.

A total of 138 faecal samples for steroid analysis were collected from 22 female rhinos, including 15 adults (>72 months) and seven juveniles (<72 months). All 15 adult animals and two juvenile animals had been dehorned on previous occasions. Individual sample collection periods included the two days preceding dehorning (D−2 and D−1), per rectum collection from the immobilised individual on the day of dehorning (D), and the four days following dehorning (D+1 to D+4). Sample collection pre- and post-dehorning took place opportunistically in the respective camps following spontaneous defecation. Depending on terrain, rhinos were followed either on foot or in a vehicle at an appropriate distance during daylight hours. Faecal material was immediately placed on ice following collection, frozen at −20 °C within 3 h, and kept frozen until further processing at the Endocrine Research Laboratory, University of Pretoria, South Africa. Frozen faeces was then lyophilised, pulverised and sifted using a metal mesh strainer to remove fibrous material (Ganswindt et al. 2010). Between 0.10 and 0.11 g faecal powder was extracted by vortexing for 15 min with 80% ethanol in water (3 mL). Following centrifugation for 10 min at 1 500 g, supernatants were transferred into microcentrifuge tubes and stored at −20 °C until further analysis.

For comparison, steroid extracts of 45 faecal samples from nine adult free-ranging female white rhinos (average five samples per individual; range: 3–7), collected between 2008 and 2012 in Lapalala Wilderness, a 36 000 ha privately owned nature reserve in Limpopo, South Africa, were reanalysed for fGCM content (van der Goot et al. 2015).

Steroid extracts were measured for immunoreactive fGCM concentrations using a 5α-pregnane-3β,11β,21-triol-20-one enzyme immunoassay detecting 3β,11β-diol-CM.

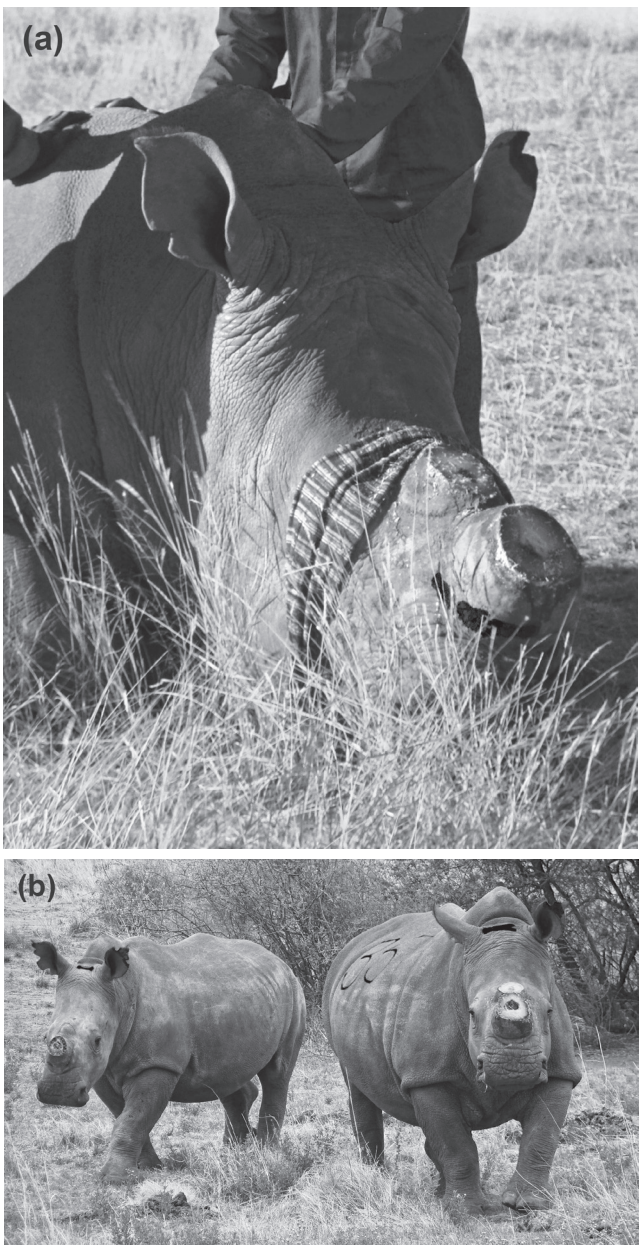

Figure 1: (a) Chemically immobilised adult white rhino after trimming of both horns. (b) Juvenile and adult white rhinos after horn-trimming. Note that the caudal horn of the juvenile animal was not long enough to safely trim

Detailed assay characteristics including cross-reactivities are given by Touma et al. (2003). The assay was biologically validated for white rhinoceroses by comparing transport-related fGCM values of two animals (first day post-transport: 1.27 and 0.70 μg g^{-1} dry weight (DW); 19 days post-transport: 0.46 and 0.31 μg g^{-1} DW; a decrease of 64% and 56%, respectively) as well as parturition-related fGCM values of two free-ranging females (non-pregnant: 0.56 and 0.49 μg g^{-1} dry DW; around parturition: 0.93 and 0.89 μg g^{-1} DW, an increase of 66% and 82%, respectively). Serial dilutions of steroid extracts gave displacement curves parallel to the standard curve of the assay. Sensitivity of the assay at 90% binding was 2.4 ng g^{-1} faeces. Intra- and inter-assay coefficients of variation, determined by repeated measurement of high- and low-value quality controls, ranged between 6.7% and 13.0%. The enzyme immunoassay was performed on microtiter plates as described by Ganswindt et al. (2002).

For the limited free-ranging animals, individual median fGCM concentrations were calculated for the monitoring period of D−2, D−1 and D, representing the pre-dehorning (baseline) fGCM concentration of each individual. Likewise, individual median fGCM concentrations were calculated for each of the four days post-dehorning (D+1 to D+4), if more than one sample was collected. For the free-ranging animals the median fGCM concentration was calculated for each individual's sample set.

For the limited free-ranging animals, differences in individual pre-dehorning/baseline fGCM levels between age groups were determined using the Mann–Whitney rank sum test. Subsequently, individual median fGCM concentrations of free-ranging animals were compared with respective individual fGCM concentrations of each age class separately, using the Mann–Whitney rank sum test. Individual dehorning-related changes in fGCM concentrations were calculated by setting the respective individual pre-dehorning value 100%. Differences in fGCM concentrations between pre-dehorning and the four days post-dehorning were tested using one-way repeated measures ANOVA with a *post-hoc* Bonferroni test. Data were log transformed to ensure normal distribution. Analytical statistics were performed using SigmaPlot 12.5 (Systat Software, San Jose, CA, USA).

For the limited free-ranging animals, baseline fGCM concentrations were significantly different ($T_{15,7}$ = 40, p = 0.005) between the two age groups tested, with limited free-ranging adults showing higher fGCM baseline concentrations compared to the juvenile individuals (Figure 2). FGCM concentrations of each age group were therefore separately compared with fGCM concentrations of the free-ranging animals. No differences in fGCM concentrations were observed between free-ranging and limited free-ranging adults ($T_{9,15}$ = 112, p = 1.0), but fGCM concentrations of juveniles ($T_{9,7}$ = 35, p = 0.011) were again significantly lower (Figure 2).

When comparing fGCM concentrations of limited free-ranging adult females (n = 15), which had all been dehorned on previous occasions, overall median fGCM concentrations increased by 17.1% (range: −7.1 to 81.0%) on D+1, as well as by 31.8% (range: −7.0 to 293%) on D+2, 3.2% (range: −38.5 to 125%) on D+3, and 16.0% (range: −24.3 to 41.8%) on D+4. The difference was significant when comparing individual pre-dehorning fGCM concentrations with respective steroid hormone concentrations on D+2 (F_4 = 4.8, p = 0.003; *post-hoc*: p = 0.007, Figure 3).

When comparing fGCM concentrations of limited free-ranging juvenile females (n = 7), of which only two animals had been dehorned on previous occasions, overall median fGCM concentrations increased by 14.7% (range: −8.9 to 26.6%) on D+1, as well as by 32.9% (range: 13.5 to 174%) on D+2, 5.5% (range: −5.5 to 29.2%) on D+3, and −10.5% (range: −34.1 to 24.6%) on D+4. Again, the difference was significant when comparing individual pre-dehorning fGCM concentrations with respective steroid hormone concentrations on D+2 (F_4 = 3.3, p = 0.035; *post-hoc*: p = 0.014).

Variation in function of the hypothalamic-pituitary-adrenal axis with age is well documented and higher basal glucocorticoid levels in older animals have been reported for several mammalian species (Reeder and Kramer 2005). Furthermore, deleterious effects of chronic stress on reproductive performance have been documented for animals in captivity, including rhinos (Carlstead and Brown 2005; van der Goot et al. 2015). In this study, baseline fGCM levels of adult limited free-ranging female rhinos did not differ significantly from those of adult free-ranging female individuals, potentially contributing to the frequent reproductive success noted for this particular limited free-ranging population (RH Emsley and K Adcock, IUCN SSC African Rhino Specialist Group RMG SAVC Rhino Management Group, pers. comm., 2015).

Significant short-term increases in fGCM levels following capture and radio-collar fitting have been described for free-ranging deer (Munerato et al. 2015), and routine claw trimming of physically restrained domestic cows has resulted in significant increases in fGCM levels

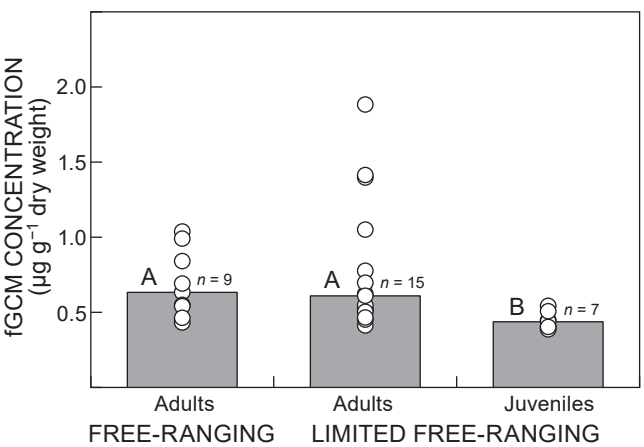

Figure 2: Individual (symbols) and overall median (bars) faecal glucocorticoid metabolite (fGCM) concentrations of limited free-ranging (n = 22 individuals, 15 adult and seven juvenile animals, average 2.1 samples per animal, range 1–3 samples) and free-ranging (n = 9 individuals, average 5.0 samples per animal, range 3–7 samples) white rhinos. Samples from the free-ranging population were collected across seasons and years. Different upper-case letters indicate statistically significant differences between groups (p < 0.05)

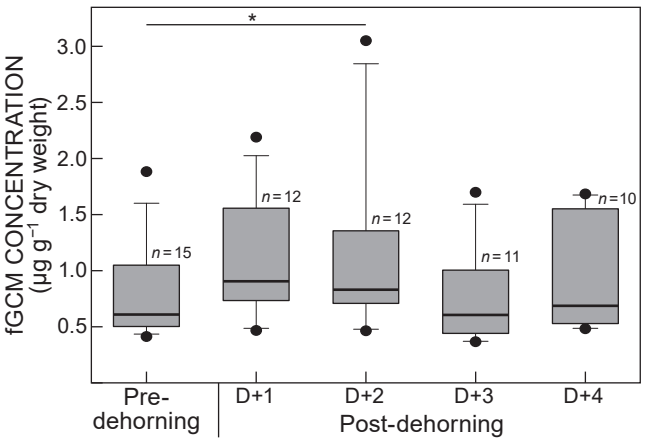

Figure 3: Boxplots of overall faecal glucocorticoid metabolite (fGCM) concentrations of 15 adult female white rhinos monitored prior to (D−2, D−1 and D) and after (D+1 to D+4) dehorning. Individual pre-dehorning fGCM values were median steroid concentrations from samples collected on D-2, D-1 and D (average 2.1 samples per individual, range: 1–3 samples). Individual fGCM concentrations post-dehorning were median values per day (0.98 samples per individual per day, range: 0–3 samples). The boxes show the median value and the upper and lower quartile values; the whiskers show the 10th and 90th percentiles of the values and the dots are outliers. The asterisk indicates a statistically significant difference between groups ($P < 0.05$)

(Pesenhofer et al. 2006). Chemical immobilisation and trimming of the horn are two inseparable components inherent to the dehorning process of rhinos. The observed increase in fGCM levels 48 h post-dehorning may be attributed to combined effects of these processes on stress steroid secretion by dehorned rhinos, and the lag time in appearance of the respective signal in faeces corresponds with previously reported patterns for rhinos (Brown et al. 2001; Clauss et al. 2005). Pinchin (1993) suggested that the stress related to dehorning of rhinos was 'very minimal' and that rhinos suffered no long-term adverse effects as a result of being immobilised for dehorning. Our results show that rhinos do indeed have a significant stress response following dehorning, regardless of previous dehorning experience. This response, however, appears relatively short-lived, with fGCM levels starting to decrease 48 h after the procedure. In contrast, increased fGCM levels have been reported for longer than 75 days in female white rhinos following immobilisation and relocation (Linklater et al. 2010). As data collection of the current study did not continue beyond the fourth day post-dehorning, conclusions regarding the long-term physiological effects of neither immobilisation nor horn removal can be made. Furthermore, it has been suggested that social behaviour and survivability may be affected in dehorned individuals (Berger and Cunningham 1996). These subjects warrant further investigation in future studies in order to gain an overall understanding of the effects of dehorning on rhinos.

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