The effects of short term confinement on glucocorticoids and sex steroids in black and white rhinoceros

Edith A.MacDonald[1] and Wayne L. Linklater[2,3]

[1]Wellington Zoo, edy.macdonadl@wellingtonzoo.com
[2]Centre for Biodiversity and Restoration Ecology, Victoria University of Wellington, P.O. Box 600, Wellington 6005, New Zealand. wayne.linklater@vuw.ac.nz
[3]Centre for African Conservation Ecology, Department of Zoology, Nelson Mandela Metropolitan University, Port Elizabeth, South Africa.

***Abstrac*t**

The captive rhinoceros population is effected by several reproductive problems: low birth rate by the F1 generation, male skewed birth sex ratio and reproductive pathologies. It has been suggested that stress may play a role in these problems. We monitored faecal hormone metabolites (glucocorticoid, progestogen, and androgen) in black and white free-ranging rhinos experiencing an extreme stress event: i.e. capture and confinement in a boma for 10 weeks. Hormone patterns differed between the species and did not display the expected stress response. The findings may have implications for captive management and breeding of rhinoceros.

1. Introduction

Over the last decade, rhinoceros conservation has had marked achievements but some species continue to decline (Amin *et al.* 2006). Viable captive rhino populations are an essential 'insurance' against extinction. However, the captive population is not self sustaining and there are reproductive challenges for the species (Roth 2006). Several possible mechanisms have been suggested for poor fertility in captive rhinos including reproductive pathology (Hermes *et al.* 2006), social stressors (Carlstead and Brown 2005), and behaviour (Swaisgood *et al*. 2006). Understanding and managing the effects of captivity on rhinoceros reproductive health is essential not only for the captive population but also because short-term captivity is a common occurrence in the management of fragmented wild populations during reintroduction and re-stocking events (i.e., translocation, (IUCN 1987)).

Stress is the physiological response to external stimuli that are harmful or potentially harmful, e.g., a predator (Norris, 1997). Physiological systems, i.e. increased heart rate, respiration, and blood sugar, play an immediate role in removing or avoiding the stressor (e.g., running away from the predator) i.e. heart rate, respiration, and blood sugar. The physiological systems that do not play a direct role in removing the stressor are suppressed, e.g., immune function and reproduction. Once the stressor is removed the body returns to homeostasis. The classic stress response involves an increase in stress-related hormones and a decrease in reproductive hormones as shown in Figure 1. However if the stressor is not removed and stress continues for longer, i.e. is chronic, the continual increased heart rate, respiration and blood sugar and long term suppression of digestion, immunity and reproduction can manifest into distress (Breazile 1987) and eventually illness. For example, chronic stress in humans contributes to heart disease skin disorders, diabetes, and gastrointestinal disorders (Bennett, 1995) and viral infection (Cohen et al., 1991).

A common method to measure the magnitude and duration of the stress response is to measure glucocorticoids, a steroid hormone that increases in concentration in the blood during a stress response (cortisol is the primary human glucocorticoid). High concentrations of glucocorticoids in the blood are often associated with decreasing sex hormones (i.e., progesterone in females and androgens such as testosterone in males). Thus, when there is chronic stress, continual elevated levels of glucocorticoids may suppress reproductive hormones resulting in poor fertility (Sapolsky 1987)

The objective of this study was to measure what happens to the sex hormones of wild rhinos during the extreme stress response of capture and short-term confinement during their translocation to other game reserves. This process has already shown to have dramatic consequences for reproduction (Linklater in press). One of the first steps towards understanding the factors that may be contributing to poor fertility in captive populations is to establish baseline conditions and the stress responses for wild rhinos.

2. Methods

Study animals (48 white rhinoceros, *Ceratotherium simum simum,* and 18 black rhinoceros, *Diceros bicornis minor)* were free ranging rhinoceros in KwaZulu-Natal province, South Africa. Approximately 5 ozs samples of feces were gathered either directly from the rectum while rhino were chemically immobilized at capture or from fresh dung piles while they were temporarily housed in boma (reinforced enclosures) after capture but prior to transportation for release at another reserve during the period August-October, 2004 (See also Linklater et al. (2006) for more detailed descriptions of capture, translocation and sample collection). Fecal samples taken from the rectums of rhino at capture provide an estimate of pre-capture levels of hormones because that fecal material has already completed it passage through the gut and formed during the day prior to capture.

While in the bomas samples were collected from fresh piles of dung weekly up to the 7th week after capture. Dung could be correctly attributed to each black rhinoceros because they were housed individually but white rhino were housed together in larger boma. Thus, only the dung piles that were observed being deposited were sampled for white rhino to ensure samples were attributed to the correct donor. Samples were placed in plastic specimen cups and frozen immediately prior to assaying. Fecal samples were transported on ice to San Diego for analysis via radioimmunoassay (RIA).

2.1 Extraction

Fecal samples were extracted and processed according to Patton et al (1999). Briefly, samples were lyophilized, sifted, weighed, and flash frozen. The extract was resolublized in 1 mL ethanol, capped and refrigerated until further use. The extract was used for all subsequent assays.

2.2 Glucocorticoid assay

To analyze fecal extracts, we used the ^{125}I Corticosterone RIA kit (ICN Biomedical, Costa Mesa, CA), similar to previous studies of glucocorticoids in rhinoceros (Brown *et al.* 2001; Carlstead and Brown 2005). In brief, the reconstituted extracts were brought to room temperature for approximately 30 minutes. Fifty microliters of the sample in duplicate was

pipetted into 12x75 test tube and allowed to evaporate in a water bath (20° C) for approximately 15 minutes. Five hundred microliters of steroid dilutent was added to each sample and vortexed for 1 minute. Standards, controls, corticosterone ^{125}I , and antibody were added according to manufacture directions and incubated for two hours at room temperature. After incubation, 500 µl of precipitant solution was added to all tubes and vortexed. Samples were centrifuged at 2300-25000 rpm (1000g) for 15 minutes at room temperature. Supernatant was decanted and test tubes blotted before placing on absorbent paper. Precipitate was counted in a gamma counter and results are presented as nanograms per gram.

The assay was validated by comparing parallelism in a serial dilution of fecal extract with the corticosterone standard curve (r_{white} = 0.98 and r_{black} = 0.989). Extraction efficiency of added ^{125}Icortiocosterone was >55% for both species. Assay sensitivity was 3.09 pg/tube (calculated as mean pg/tube at 90% B/BO, n = 10). Buffer blanks were below sensitivity. Accuracy was determined for white rhinoceros as Y=22.527+.3(X), R^2=0.956 and for black rhinoceros as Y=17.431+.459(X), R^2=0.979 by recovery of five known quantities of standard that were equivalent to the quantities used in the standard curve added to a pool of feces extract. Intra-assay variation was 6.5% for immunoreactive high control and 10.07% for the immunoreactive low control. Inter assay coefficient of variation was < 6%.

2.3 Progestogen assay

The progestogen RIA assay has been previously used successfully on rhinos (Patton *et al.* 1999) and due to limited space will not be summarized here.

2.4 Androgen assay

The androgen radioimmunoassay (RIA) was a modification of MacDonald et al. (2006) with 10µl of fecal extract assayed.

The assay was validated by comparing parallelism in a serial dilution of fecal extract with the androgen standard curve (r_{white} = 0.975 and r_{black} = 0.985). Extraction efficiency of added ^{3H}Testosterone was >70% for both species. Assay sensitivity was 7.56 pg/tube (calculated as mean pg/tube at 90% B/BO, n = 10). Buffer blanks were below sensitivity. Accuracy was determined for white rhinoceros as Y = 2.549+1.056(X) R^2=0.996 and for black rhinoceros as Y=-11.94+1.562(X) R^2=0.961 by recovery of five known quantities of standard that were equivalent to the quantities used in the standard curve added to a pool of feces extract. Intra-assay variation was 3% for immunoreactive high control and 6% for the immunoreactive low control. Inter assay coefficient of variation was < 5%.

3. Results

3.1 Stress hormones

Both male and female white rhinos had comparable concentrations of fecal glucocorticoids at capture and exhibited the classic stress response (Figure 2a), initially elevated levels of glucocorticoids that eventually returned to pre-capture levels. Interestingly females showed a second peak in glucocorticoid concentrations during week four returning to pre-capture levels during the fifth week.

The male and female black rhino glucocorticoid response was different to that observed of the white rhino (Figure 2b). While both males and females had capture samples that were similar

in glucocorticoid concentration, black rhino concentrations at capture were twice as high as white rhinos. The black rhinos did not show the classic response to stress. Instead glucocorticoids continued to decrease over the seven weeks, reducing by nearly 2.5 times their initial concentration.

3.2 Reproductive hormones

Fecal progestogen concentration in both white and black rhino females showed significant decreases by the second week of confinement. Black rhino fecal concentration decreased to approximately 66% and white rhino by 50% of pre-capture levels. The progestogen concentration in black rhino females continued to remain low and by week 7 was less than half the concentration of samples taken at capture. In white rhino females progestogen concentrations remained at week 2 levels until week 7 when a marked increase was observed, almost returning to capture levels. It is not possible to establish if the rhinos became acyclic during confinement as only weekly samples were collected. To definitely identify the follicular and luteal phase samples must be collected at least every four days over the course of 30 days or longer.

Similar to the females, the sex hormones in male black and white rhinos also decreased during the seven weeks of confinement. However, fecal androgen concentrations in black males decreased to 66% of their pre-capture concentration by week 5 but fluctuated at week 2 to near capture levels. In contrast, white males decreased to 80% of their pre-capture levels by week two and remained at this concentration through out the remaining study period.

4. Discussion

Although black and white rhinos are in the same taxonomic family, they exhibit different physiological responses to the stress of capture and short-term confinement. These findings reinforce the need to study each species independently and not extrapolate physiological processes between similar species. White rhinos demonstrated the classic stress response, albeit females took an additional two weeks for glucocorticoids concentrations to return to pre-capture levels. In contrast, black rhinos have a continual glucocorticoid decline that does not follow the standard stress response.

Interestingly, black rhino pre-capture glucocorticoids were twice those of white rhino, perhaps indicating a fundamental difference between the species in their physiological base-line and functions of glucocorticoids. Black and white rhino have very different social systems. White rhino are comparatively social, whereas black rhino are regarded as solitary with a reputation for aggression. Moreover, black rhino also have a reputation for being more playful and responsive to training and human contact in boma than white rhino that would appear to contradict people's experience of them in the wild. However, it is possible that higher base-line glucocorticoid levels in black rhino are related to their solitary and spacing behaviour (expressed as conspecific and anti-predator aggression). Therein may also lay the reason for their different response to stress and different behavioural outcomes. The playful and inquisitive black rhino's high stress hormone levels plummet in captivity whereas those for white rhino are elevated. The effect is a '*Jekyll-and-Hyde*'-like response by the two species but in the opposite direction that might be explained by the difference and changes in their stress hormone levels between the wild and captivity.

5. Conclusion

This study has established baseline levels of glucocorticoids and monitored the stress response. This is the first step in unravelling the role stress may have in the poor fertility of captive rhinos. Knowing the response to stress of wild rhino allows researchers to evaluate the captive population, both at a baseline level and in response to stressors (i.e. movement between zoos, public observation). A future study that compares glucocorticoids and sex steroids in wild and captive rhinos through out the year and during stressors may help elucidate the issues of sub-fertility in the captive population.

The rhinos involved in this study are part of two larger studies looking at translocation in black rhinos and reproductive physiology in white rhinos. The black rhinos in this study were released into a new reserve in October 2004 and since then have been seen mating, however we have not had the opportunity to quantify post-release reproductive behaviour yet. The results shall be forth coming and contribute to increasing our understanding of the role stress plays in rhinos and possible implications for the captive environment.

6. Acknowledgements

Our thanks to WWF for funding the Black Rhino Range Expansion Project, under whose auspices these black rhino were monitored in boma and Ezemvelo KZN Wildlife for permitting the research to take place (Reserve permits ZC/101/01 and ZC/097/05). The research was made possible through the financial support of the Ammerman Foundation, a Millennium Post-doctoral Fellowship to WLL at the Conservation & Research for Endangered Species (CRES) of the Zoological Society of San Diego (Institutional Animal Care and Use Committee approval number 169), and granted funds from the U.S. Fish & Wildlife Service administered Rhinoceros and Tiger Conservation Act of 1994 (grant agreement numbers 98210-2-G363 and 98210-4-G920). We would like to thank the following people for their assistance: Dale Airton, Kirsten Bond, Jacques Flamand, Chris Kelly, Craig Reid, Quinton Rochet, Ilse Schmit, Angela White, Ron Swaisgood, and Sue van Rensberg. A special thank you to Nancy Czekala who supported EAM through out this study.

7. References

Amin, R., Thomas, K., Emslie, R., Foose, T. & Van Strien, N. (2006) An overview of the conservation status of and threats to rhinoceros species in the wild. *International Zoo Yearbook*, **46**, 96-117.

Bennett, P. (1995) Disorders of the gut. in *Psychology: Process and Applications (2nd edn).* A. Broome and S. Llewelyn (eds). London: Chapman and Hall.

Breazile J (1987) Physiologic basis and consequences of distress in animals. *Journal of the American Veterinary Medical Association* **191**, 1212-1215.

Brown, J.L., Bellem, A., Fouraker, M., Wildt, D. & Roth, T. (2001) Comparative analysis of gonadal adrenal activity in the black and white rhinoceros in North American by noninvasive endocrine monitoring. *Zoo Biology*, **20**, 463-86.

Carlstead, K. & Brown, J.L. (2005) Relationship between patterns of fecal corticoid excretion and behavior, reproductive, and environmental factors in captive black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros. *Zoo Biology*, **24**, 215-32.

Cohen, S., Tyrell, D.A., & Smith, A.P. (1991) Psychological stress in humans and susceptibility to the common cold. *New England Journal of Medicine*, **325,** 606-12.

Hermes, R., Hildebrandt, T., Walzer, C., Goritz, F., Patton, M.L., Silinski, S., Anderson, M., Reid, C., Wibbelt, G., Tomasova, K. & Schwarzenberger, F. (2006) The effect of long non-reproductive periods on the genital health in captive female white rhinoceroses (*Ceratotherium simum simum, C.s. cottoni*). *Theriogenology*, **65**, 1492-515.

IUCN (1987) 'Position statement on the translocation of living organisms: Introductions, re-introductions, and re-stocking.' IUCN, The World Conservation Union, Gland, Switzerland and Cambridge, UK.

Linklater W (in press) Translocation reverses birth sex ratio bias depending on its timing during gestation: evidence for the action of two sex allocation mechanisms. *Reproduction, Fertility and Development*.

Linklater W, Flammand J, Rochet, Q, Zekela, N, MacDonald, EA, Swaisgood, RR, Airton, DF, Kelly, CP, Bond, K, Schmidt, I, Morgan, S. (2006) Preliminary analyses of the free-release and scent-broadcasting strategies for black rhinoceros re-introduction. *Ecological Journal* **7**, 26-34.

MacDonald, E.A., Czekala, N., Pengyan, W., Gual-Sill, F. & Nakao, T. (2006) Urinary testosterone and cortisol metabolites in male giant pandas (*Ailuropoda melanoleuca*) in relation to breeding, housing, and season. *Acta Zoologica Sinica,* **52***,* 242-49

Norris, D.O. (1997) *Vertebrate Endocrinology (3rd edn)*. San Diego, CA: Academic Press Inc.

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 Biology*, **18**, 111-27.

Sapolsky, R.M. (1987) Stress, social status, and reproductive physiology in free-living baboons. in *Psych biology of Reproductive Behavior: an Evolutionary Perspective.* D. Crews (ed). Englewood, NJ: Prentice Hall

Swaisgood, R.R., Dickman, D.M., & White, A.M. (2006). A captive population in crisis: Testing hypotheses for reproductive failure in captive-born southern white rhinoceros females. *Biological Conservation*. **129**, 468-476

Roth, T.L. (2006) A review of the reproductive physiology of rhinoceros species in captivity. *International Zoo Yearbook*, **40**, 130-43.

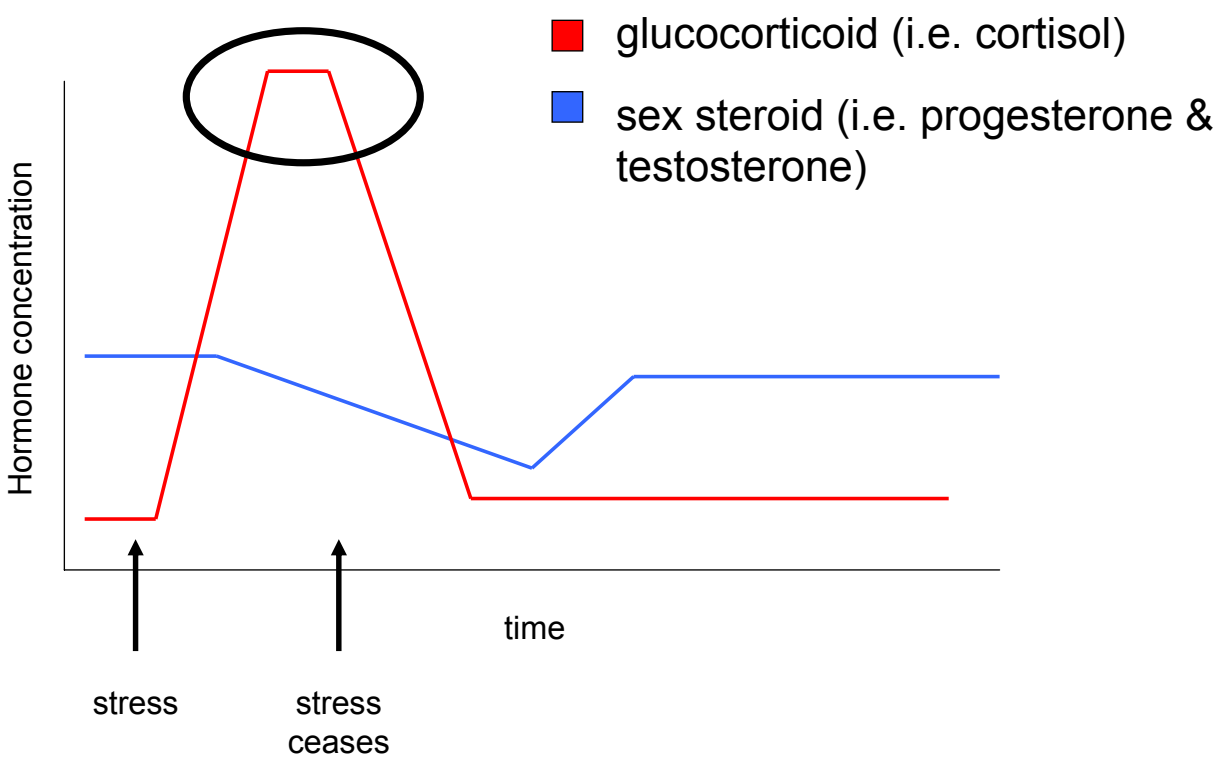

Figure 1. At the onset of a stressor (i.e. being chased by a predator), glucocorticoids increase in concentration and sex hormones are suppressed. Upon removal of the stressor, both glucocorticoids and sex hormones return to pre-stressor concentration. This is physiological process is known as the classic stress response. However, during chronic stress the stressor is not removed (the circle) and glucocorticoid concentrations remain elevated and the sex hormones remain suppressed. This can result in health problems.

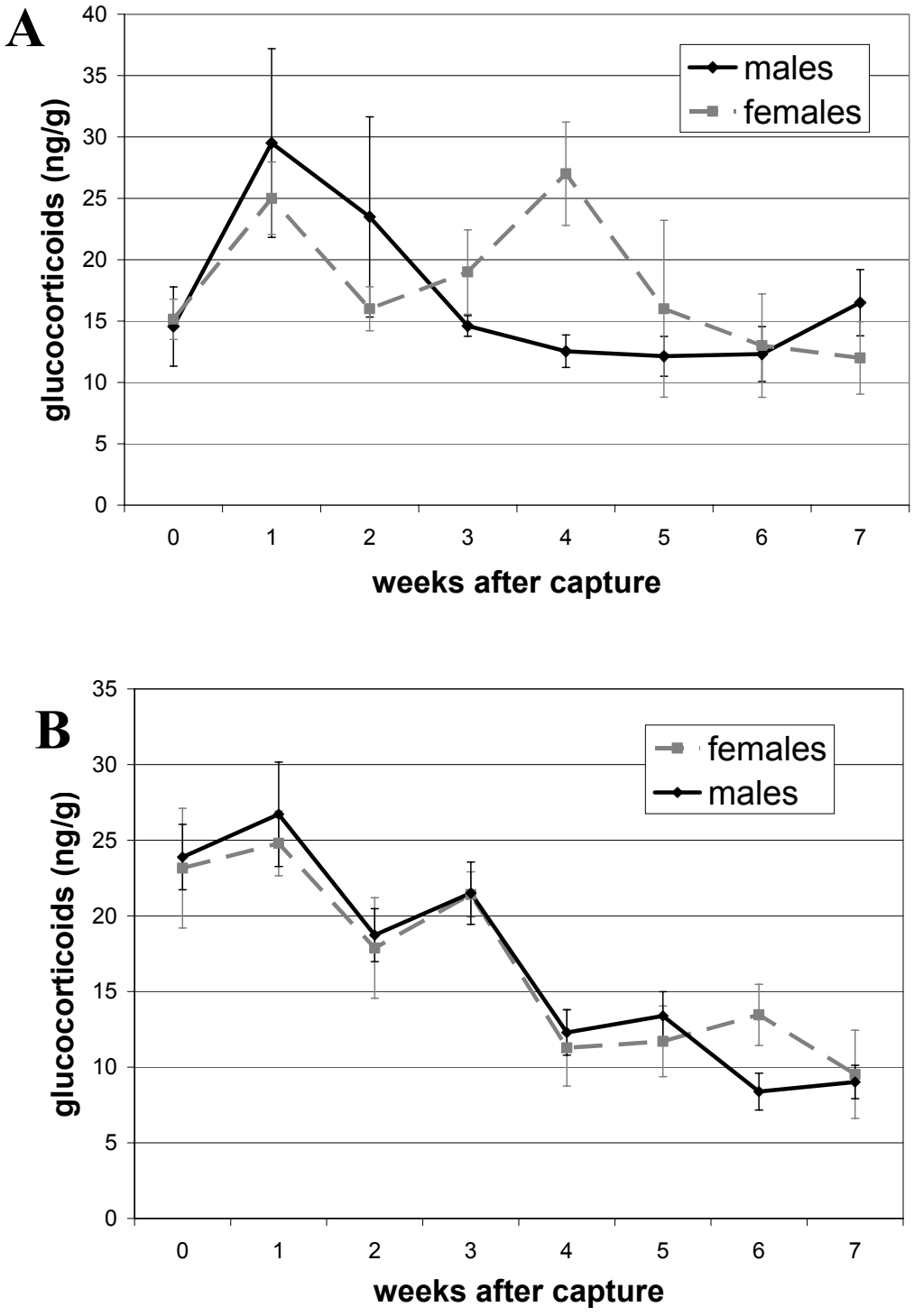

Figure 2. Average fecal stress hormone (glucocorticoid) concentration in male and female (a) white rhinos and (b) black rhinos. Bar represents the standard error of the mean. Week 0 is date of capture.

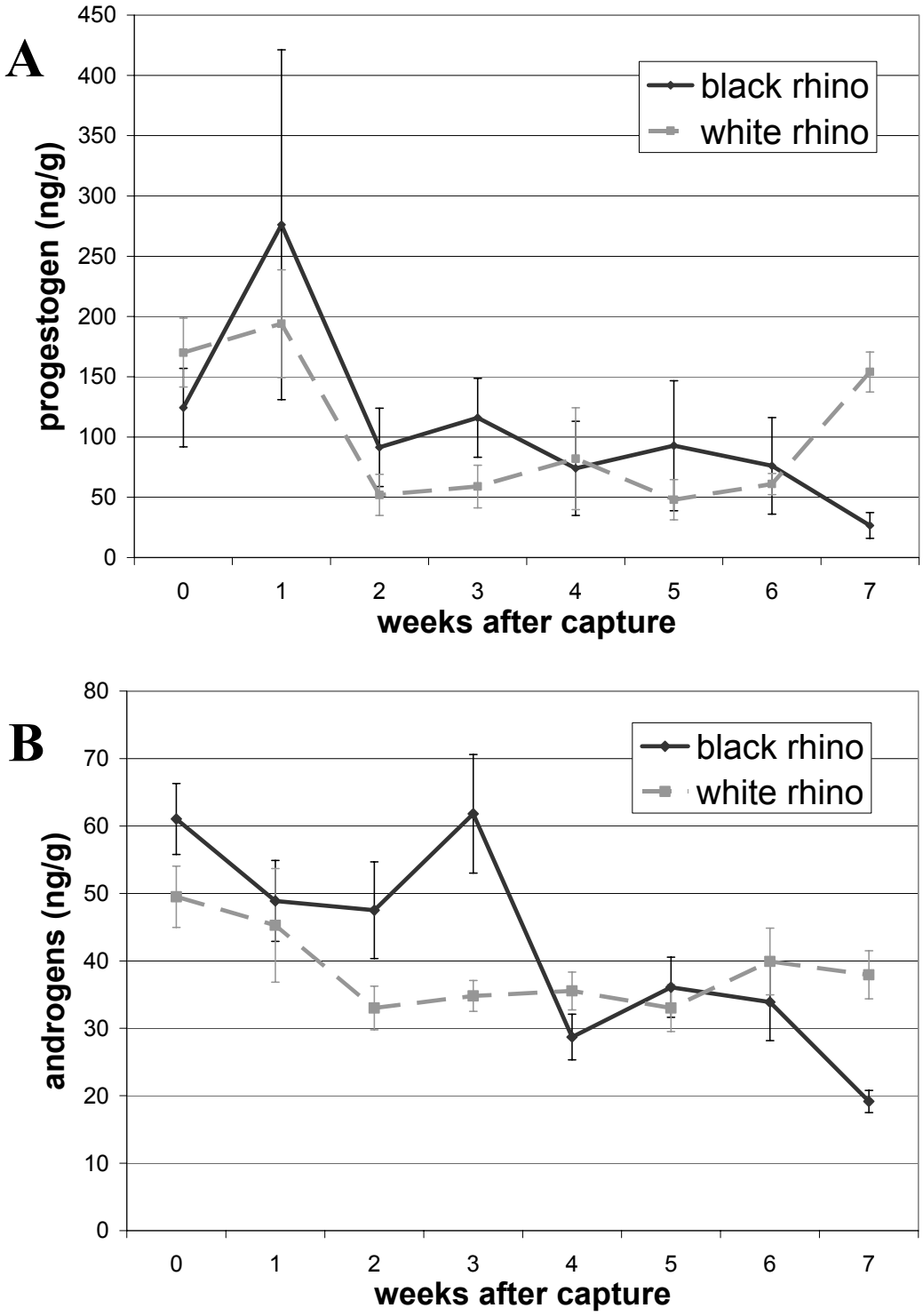

Figure 3. Average fecal sex hormone concentration in black and white (a) female and (b) male rhinos. Bar represents the standard error of the mean. Week 0 is date of capture.