

Declining and low fecal corticoids are associated with distress, not acclimation to stress, during the translocation of African rhinoceros

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Abstract

Concentrations of adrenal steroid metabolites in feces are routinely used to assess the welfare of animals that are the subject of conservation efforts. The assumption that low and declining corticoid concentrations indicate the absence of stress and acclimation, respectively, is often made without experimental support or wild-animal comparisons, although intrinsic control of adrenal steroids might occur even under ongoing stress and distress. We adopted the capture and 11-week captivity of 18 black (*Diceros bicornis*: 11 males, seven females) and 52 white (*Ceratotherium simum*: 22 males, 30 females) rhinoceros as an experimental test of the relationship between corticoid concentrations and stress (translocation) and measured for suppressed gonad function as an indicator of distress – the biological cost of cumulative stressors. Fecal samples collected from the rectum at capture and during captivity were stored frozen and their corticoid, and androgen (in males) or progestin (in females), concentrations determined by radioimmunoassay. Corticoid profiles followed the expected pattern of being two to five times pre-capture levels (ng g^{-1} : black rhino: female 24.5 ± 3.7 , male 23.9 ± 2.2 ; white rhino: female 16.3 ± 1.6 , male 12.3 ± 2.4) for up to 17 days after capture and declined with time in captivity. Black rhinoceros and male white rhinoceros corticoids declined below pre-capture values and were associated with suppressed levels of androgens and progestins with increased time in captivity. Declining corticoids could not be interpreted as acclimation or the absence of stressors, without also measuring for distress in African rhinoceros. White rhinoceros female corticoid values remained elevated, although their gonad steroid levels were also suppressed. We discuss our findings for the management of rhinoceros in the wild and captivity.

Introduction

Distinguishing empirically between stress and distress (Moberg, 2000) is key to assessing animal welfare (Wielebnowski, 2003). Stress, the biological response to a homeostatic threat, might be indicated by elevated concentrations of circulating adrenal steroids like glucocorticoids or their excreted metabolites, called corticoids (Sapolsky, Romero & Munck, 2000). Distress is a chronic condition from repeated or cumulative stress. It is distinguished from stress by its biological cost because resources are diverted from core functions (e.g. reproduction, Moberg, 2000).

The stress–distress dichotomy has important implications for species management by translocation (Teixeira *et al.*, 2007). Translocations are often unsuccessful (e.g. 74% unsuccessful, Fischer & Lindenmayer, 2000) but the role of

stress and distress in post-release survival and fecundity is rarely considered (Letty, Marchandea & Aubineau, 2007). Stressors might impair cognitive processes, including learning and memory, and combine during translocation to cause distress that impairs immune and reproductive function (Teixeira *et al.*, 2007).

Translocations are fortuitous experiments for validating biological indicators of stress and distress. In particular, the hypothesis that a decline in adrenal steroids after stress is evidence of acclimation to stressors, or their absence, needs to be tested. Declines in adrenal steroids might also occur through intrinsic controls which prevent them from being elevated for prolonged periods (i.e. negative feedback mechanism, Smith & Dobson, 2001) even under ongoing stress and distress; however, authors continue to interpret declines

in adrenal steroid levels as acclimation (e.g. Turner, Tolson & Hamad, 2002; Millspaugh *et al.*, 2007; Franceschini *et al.*, 2008).

Although important to species recovery, the success of rhinoceros translocations also vary (e.g. Linklater & Swaisgood, 2008), probably because translocations involve several life-threatening stressors (Letty *et al.*, 2007; Teixeira *et al.*, 2007). Capture, transportation, captivity and release have immediate physiological (Kock *et al.*, 1990, 1999) and behavioral (Linklater *et al.*, 2006) effects but the role of stress and distress in translocation success is unknown. In captivity poor survival and reproduction are associated with higher, more variable corticoid levels (Brown *et al.*, 2001; Carlstead & Brown, 2005) but they may be unrelated. Moreover, the classic adrenal steroid spike and decline after arrival in captivity (Turner *et al.*, 2002), currently interpreted as evidence of acclimation, could actually be intrinsic control under ongoing distress. *Ex situ* conservation efforts currently lack physiological parameters from wild-animal counterparts for comparison and, therefore, would benefit from *in situ* experiments.

We took advantage of routine translocations of black (*Diceros bicornis*) and white (*Ceratotherium simum*) rhinoceros as fortuitous science-by-management experiments in stress and distress. We describe the physiological response of African rhinoceros to capture and captivity, and address two questions of general interest: (1) do elevated corticoid concentrations indicate stress and distress? and (2) are elevated corticoid concentrations associated with a measurable biological cost, in this case suppressed gonad activity?

Materials and methods

During 2004 18 black (11 males and seven females) and 52 white (22 males and 30 females) rhinoceros were captured from Ezemvelo KwaZulu-Natal Wildlife reserves, South Africa, by remote chemical immobilization from a helicopter (drugs and protocol used described in Linklater *et al.*, 2006). All white, and 11 black, rhinoceros were captured from Hluhluwe-iMfolozi Park (HIP: 28°16'54.60"S, 31°59'13.42"E), and four black rhinoceros from Ithala (27°31'46.19"S, 31°19'11.68"E), two from Eastern Shores (28°5'49.63"S, 32°32'1.31"E), and one from Tembe (26°57'0.43"S, 32°27'35.33"E) reserves. White rhinoceros were captured between 19 March and 3 June, and black rhinoceros from 14 August to 14 October, and accommodated in enclosures (boma) in HiP until transport for release into other reserves (e.g. Linklater *et al.*, 2006). Black rhino were fed freshly cut browse and lucerne hay and white rhino lucerne and meadow hay twice a day, and both diets supplemented with pelleted domestic ungulate food. All rhino adjusted to this diet within the first few days after arrival.

In rhinoceros, fecal corticoid concentrations reflect adrenal activity (Brown *et al.*, 2001; Carlstead & Brown, 2005) and metabolites of androgen and progesterone in feces are indicators of reproductive function in males (Rachlow, Berkeley & Berger, 1998; Kretzschmar, Ganslosser & Dehn-

hard, 2004) and females (Kock *et al.*, 1991; Patton *et al.*, 1996; MacDonald *et al.*, 2008). Five ounce samples of feces were taken from each rhino's rectum at capture and from fresh dung piles attributed to individuals while in boma. Samples were stored in a freezer within 3 h of collection, and transported on ice to San Diego for radioimmunoassay. Metabolites of adrenal and gonad steroids in feces are robust and decay only slowly after deposition in feces (Linklater *et al.*, 2000; Turner *et al.*, 2002). The clearance of corticoid metabolites into feces peaks at 1.5–2 days and is elevated for up to the third day after ACTH challenge (Brown *et al.*, 2001), and at 1 day but up to at least 5 days for androgen metabolites following GnRH stimulation (Kretzschmar *et al.*, 2004) in black rhino. Thus, rectal fecal samples measured steroid levels during the previous 1–3 days before capture.

Radioimmunoassay

Fecal samples were lyophilized, sifted, weighed, extracted with ether, dried, resolubilized in 1 mL ethanol, capped and refrigerated (as detailed in Patton *et al.*, 1999). We used the ¹²⁵I corticosterone RIA kit (ICN Biomedical, Costa Mesa, CA, USA), similarly to others (Brown *et al.*, 2001). Solubilized extracts were brought to room temperature, duplicate 50 µL samples pipetted into 12 × 75 mm test tubes, and allowed to evaporate in a water bath (37 °C) for 15 min. Five hundred microliters of steroid diluent was added to each sample and vortexed for 1 min. Duplicate 100 µL of six corticosterone calibrators (25–1000 ng mL⁻¹) and 100 µL high and low controls were also pipetted into 12 × 75 mm test tubes and 400 µL of steroid diluent added to each tube. Two hundred microliters of corticosterone ¹²⁵I was added to all tubes and 200 µL of anti-corticosterone was added to all tubes except the total counts and non-specific binding tubes. The assay was vortexed and incubated at room temperature (22–25 °C) for 2 h. After incubation, 500 µL of precipitant solution was added to all tubes, vortexed and centrifuged at 1000 × g for 15 min. Supernatant was decanted and test tubes blotted before placing on absorbent paper. Precipitate was counted in a gamma counter (Wallac 1470 Automatic, Wallac, Waltham, MA, USA) and results are presented as nanograms per gram feces.

Validation of the assay was tested by comparing parallelism in a serial dilution of fecal extract with the corticosterone standard curve ($r_{\text{white}} = 0.980$ and $r_{\text{black}} = 0.989$). Extraction efficiency of added ¹²⁵I corticosterone was > 55% for both species. Assay sensitivity was 3.09 pg tube⁻¹ (calculated as mean pg tube⁻¹ at 90% B/BO, $n = 10$). Buffer blanks were below sensitivity. Accuracy was determined for white rhinoceros as $Y = 22.527 + 0.3(X)$, $R^2 = 0.956$ and for black rhinoceros as $Y = 17.431 + 0.459(X)$, $R^2 = 0.979$ by recovery of five known quantities of standard equivalent to the quantities used in the standard curve and 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%. The protocols and performance of

radioimmunoassays to measure progestin and androgen metabolite concentrations in the same samples of feces are described in Patton *et al.* (1999, 2001), respectively.

Data analysis

Sampling constraints lead to unequal and uncoordinated sampling of individuals. We used, therefore, trends in maximum observed values over three time periods (1–15, 16–35 and 36–60 days) to test for temporal changes in adrenal and gonad activity after capture, and tested interspecific and sex differences in those trends using a multivariate repeated measures ANOVA (type IV sums of squares and Greenhouse–Geisser tests for non-sphericity in univariate tests). Pregnant females (progestin levels consistently $> 250 \text{ ng g}^{-1}$, MacDonald *et al.*, 2008) were treated in separate analyses. Differences in baseline corticoid levels between pregnant and non-pregnant females were tested using Student's *t*-test. MANOVAs and Student's *t*-test were conducted using general linear models' procedure of SPSS (SPSS Inc., 2005). A *P*-value of 0.05 was the threshold of statistical confidence and 0.1 as indications of uncertain biological trend.

Results

Temporal patterns in fecal corticoid concentrations were different between the species and sexes. Two general patterns emerged: a species difference and a different corticoid response in female white rhinoceros. Fecal corticoid levels in some samples were two to three times pre-capture levels in both male and female black rhinoceros up to 17 days after capture but declined thereafter. Levels gradually declined lower than pre-capture baseline in both sexes (Fig. 1a and b). Male white rhinoceros also demonstrated elevated corticoid levels after capture three to five times pre-capture levels

but, unlike black rhino, levels remained elevated up to 75 days. Female white rhinoceros had high corticoid levels during captivity such that a spike immediately after capture could not be discerned (Fig. 1c and d). Conjugate concentrations of progestin, in non-pregnant females, and androgen, in males, after capture were similar. Levels in both species and sexes underwent a spike immediately after capture for 4–16 days and declined thereafter (Fig. 2). Significant changes in maximum values of corticoids and sex hormones with increasing time in captivity and relative to pre-capture baseline were observed (ANOVA, multivariate within-subjects Wilks' λ , *d.f.* = 4, 74, time: $F = 4.922$, $P = 0.001$). Those changes were different between the species and sexes (ANOVA, multivariate within-subjects Wilks' λ , *d.f.* = 4, 74, time \times species: $F = 2.953$, $P = 0.025$, time \times sex: $F = 5.366$, $P = 0.001$) but no significant interaction was found between the three variables. Univariate tests revealed changing patterns in corticoid and sex hormones through time after capture. Increasing time in captivity suppressed corticoid levels (ANOVA, time: $F_{2,38} = 6.050$, $P = 0.014$) in black rhinoceros and white rhinoceros males and levels in white rhino females actually increased with time in captivity such that there was a significant time \times species interaction ($F_{2,38} = 6.311$, $P = 0.012$). The interaction between time \times sex and three-way interaction were not significant (Fig. 3a). After capture, sex hormone levels in males and females of both species declined below pre-capture values (time: $F_{2,38} = 9.168$, $P = 0.004$). Gonad hormone suppression in white rhino was greater and immediate whereas black rhino sex hormones were initially elevated, particularly in females (time \times sex: $F_{2,38} = 5.938$, $P = 0.017$; Fig. 3b). The three-way interaction was not significant.

One black rhino and four white rhino were pregnant. Pregnant black and white rhino did not differ from non-pregnant females in their pre-capture corticoid levels (non-pregnant, $n = 30$, mean \pm 1 SE: 18.2 ± 2.6 ; pregnant, $n = 8$,

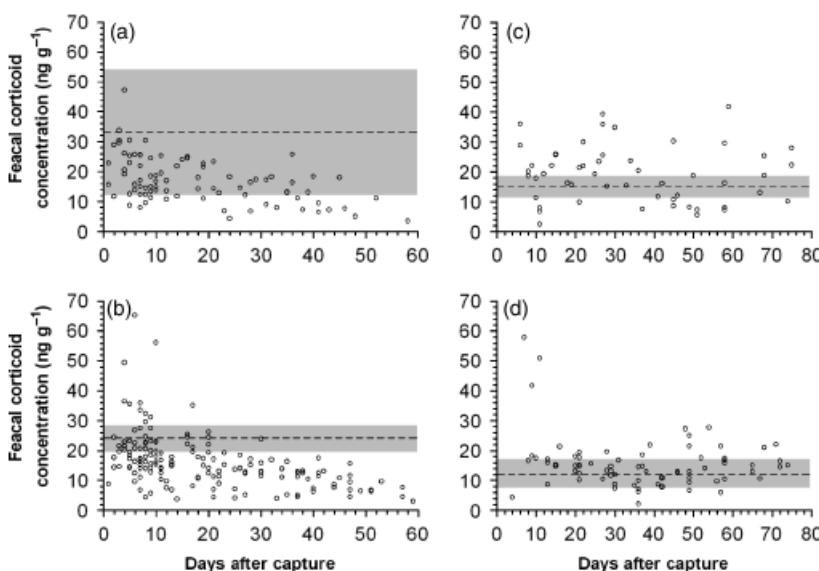


Figure 1 Fecal corticoid concentrations measured among five (not pregnant) female (a) and 10 male (b) black rhinoceros and 23 female (c) and 19 male (d) white rhinoceros during captivity. Average pre-capture levels and their 95% confidence intervals are indicated by the dashed line and gray-shaded box, respectively.

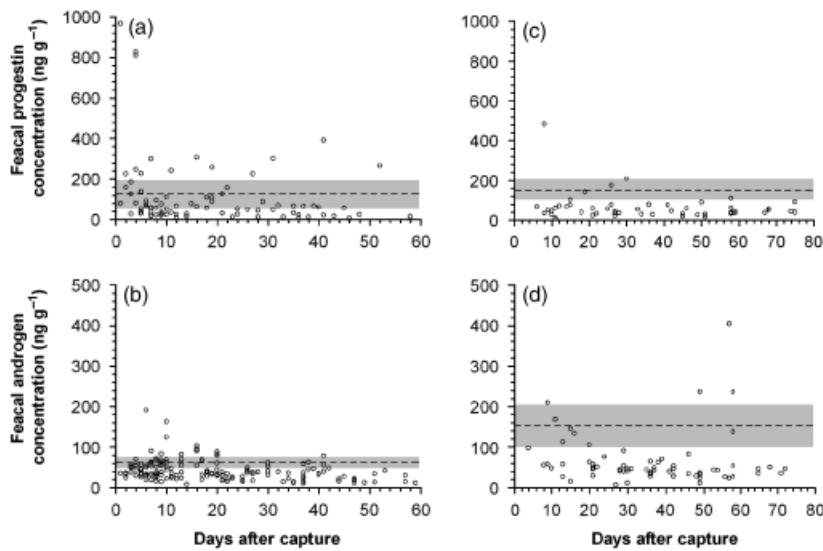


Figure 2 Fecal progestin concentration among five (not pregnant) female black (a) and 23 white (c) rhinoceros, and fecal androgens among 10 male black (b) and 19 male white (d) rhinoceros during temporary captivity. Average pre-capture levels and their 95% confidence interval are indicated by the dashed line and gray-shaded box, respectively.

22.1 ± 3.1 ; Student's *t*-test: $t_{36} = 0.511$, $P = 0.612$). Progestin levels in at least two white rhino declined below the non-pregnant threshold ($< 250 \text{ ng g}^{-1}$), perhaps indicating pregnancy loss. Those declines coincided with the highest corticoids levels observed in any of the females (Fig. 4). Overall, pregnant females also showed elevated corticoid levels after capture and decline in progestin levels.

Discussion

Profiles of adrenal and gonad hormone conjugates in feces illustrated stress and distress in rhinoceros after capture and during captivity for translocation. Corticoids were temporarily elevated after capture (as observed previously in rhinoceros, Kock *et al.*, 1990, and other species, Morton *et al.*, 1995), although the response was weaker in females of both species. Corticoid levels gradually declined with time in captivity, but instead of returning to pre-capture levels became suppressed below pre-captive levels. Corticoid suppression was strongest in black rhinoceros and not evident in female white rhinoceros.

Corticoid suppression could be interpreted in two ways: as evidence for (1) acclimation or (2) intrinsic control. The former assumes stressors subside while the latter could occur even if stressors continue and lead to distress (Smith & Dobson, 2001). Some choose to consider only the first explanation (e.g. Millspaugh *et al.*, 2007; Franceschini *et al.*, 2008). In our case, the first scenario is unlikely because daily boma cleaning and twice-daily feeding by teams of staff using heavy vehicles, among other disturbances, continued to create aggressive and flight responses among rhino throughout their time in captivity. More significantly, however, corticoid suppression was matched by suppressed gonad hormone levels in the sexes of both species. Translocation stressors, therefore, had a biological cost. Wild African rhinoceros, therefore, undergo a classic stress–distress response with implications for their welfare and under-

standing reproductive performance and survival in captivity and after release.

Acclimation to captivity?

While we show elevated corticoid levels with stress, low levels were associated with distress; a worse management problem. We question, therefore, the use of corticoid levels to conclude that there is acclimation to captivity. For example, Turner *et al.* (2002) used declining corticoid levels as evidence for acclimation to captivity by black rhinoceros but without a comparative pre-capture corticoid value, and did not measure distress. Wild black rhinoceros in our study, however, had relatively high corticoid levels before capture, capture caused values to increase but then become suppressed, and their decline was also associated with suppressed gonad function. Our findings, therefore, indicate an entirely different interpretation of Turner *et al.*'s (2002) data is possible. Declines in corticoid levels could have measured the intrinsic control of adrenal function during ongoing distress, not acclimation. We caution that corticoid declines and comparatively low corticoid levels cannot be confidently used as evidence for acclimation without other measures of biological function to exclude the possibility of distress.

Rhinoceros survival and fecundity in captivity is poor, primarily as a consequence of disease (Dennis *et al.*, 2007), low progesterone levels (Brown *et al.*, 2001; Carlstead & Brown, 2005) and irregular reproductive cycling (Patton *et al.*, 1999; Brown *et al.*, 2001; Roth, 2006), respectively. The role of stress–distress in poor immune and reproductive function has been considered, but without a wild baseline for comparison (Roth & Vance, 2007). Our results support the case for stress temporarily elevating, and distress suppressing, gonad function to explain highly variable and poor reproductive function among captive rhino (Brown *et al.*, 2001; Carlstead & Brown, 2005).

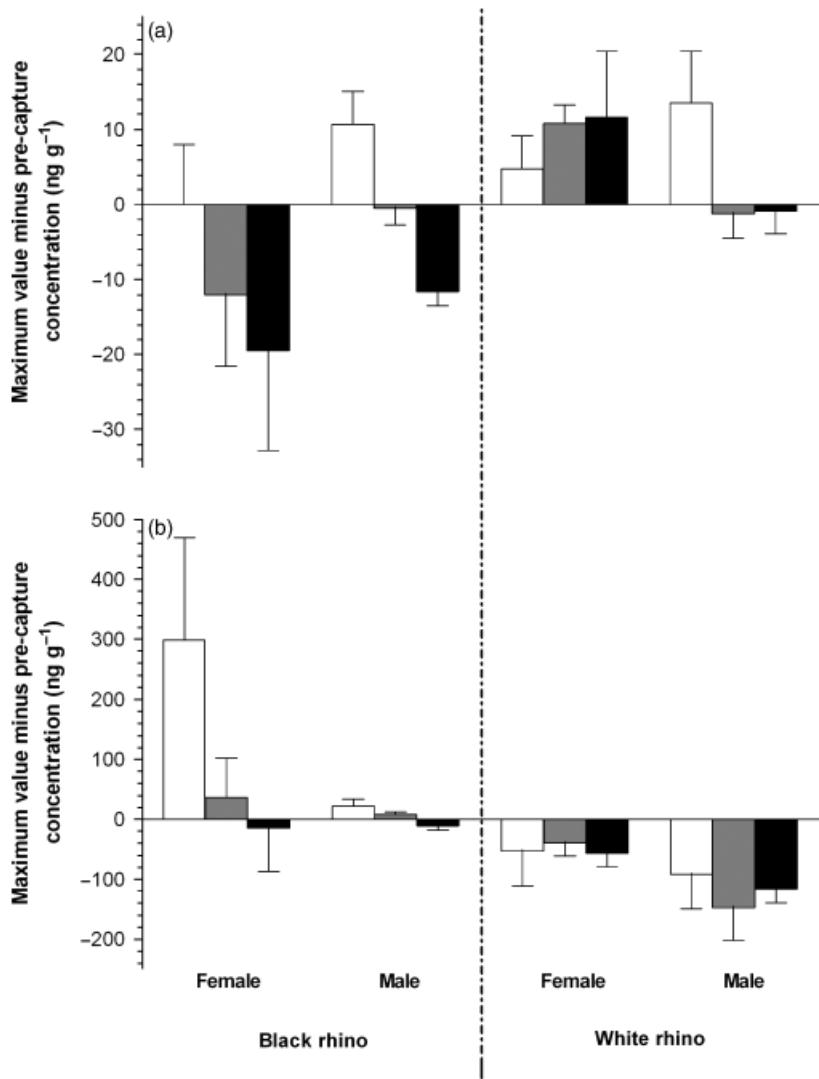


Figure 3 The average (± 1 se) difference between the maximum value and pre-capture concentration of fecal (a) corticoid and (b) sex hormone conjugates (progesterins in females and androgen in males) in individual rhino during three time periods after capture: 1–15 (clear bar), 16–35 (gray bar) and 36–60 (black bar) days. A negative value indicates that maximum values recorded during that time period were lower than pre-capture values. ses indicate variation among individuals.

Captivity, distress and post-release breeding delay

Translocation protocols usually include temporary captivity in purpose-built enclosures (a.k.a. boma) near the site of capture or release, and sometimes both. The time that animals spend in the boma may be unavoidable during long-distance translocation, but may also allow animals to recover from capture, acclimate to the new environment, especially its foods, or socialize with release mates (Rushen, 2000). Some refer to a 'boma effect,' that is, the time taken for a rhinoceros to 'calm down' before release. Differences in opinion about the time required in the boma mean that various agencies use from 1 to 8 weeks or more in South Africa and Namibia (pers. obs.). Temporary captivity and its associated benefits are thought to result in a 'soft release.' Hard releases, where captured animals are transported for immediate release, may be associated with a greater flight response (Raath & Hall-Martin, 1989; Geldenhuys, 1991,

see also Thompson *et al.*, 2001), increased encounters with hazards like fences and other rhino, or heightened propensity to fight, and so have historically not been favored.

Our data reveal the usefulness of the stress–distress dichotomy for considering the value of temporary captivity during translocation. Captivity should be just long enough to allow animals to recover from capture, adapt or socialize, but not so long that stressors accumulate to cause distress that might impact on post-release survival and fecundity. Translocation stressors, like restraint and transportation, disrupt ovulation (Dobson & Smith, 2000; Sapolsky *et al.*, 2000) and might delay the return to normal reproductive function after release. We demonstrate evidence for suppressed gonad function occurring before 15 days in captivity and further deterioration up to at least 60 days. Prolonged captivity during translocation, therefore, may not improve animal welfare in the way expected and the 'boma effect' may not be due to calmer, acclimated rhino but distressed rhino whose behavior is abnormally suppressed.

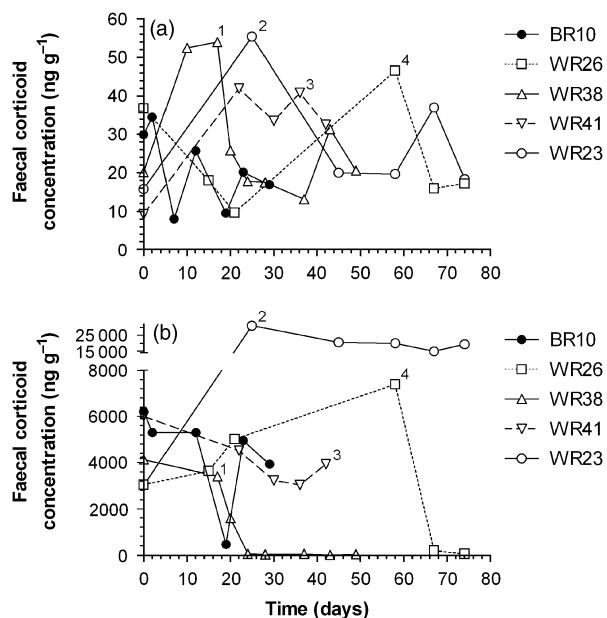


Figure 4 Fecal corticoid (a) and progestin (b) profiles for one black (BR10) and four white (WR23, 26, 38 and 41) rhinoceros pregnant at capture. Progestin levels indicate that WR38 and 26 lost pregnancies after elevated corticoid levels indicated by numbers 1 and 4 on the graphs. WR23 did not lose its pregnancy despite the corticoid peak indicated at 2. WR41's record is not long enough to determine whether the elevated corticoid levels indicated resulted in pregnancy loss. The corticoid levels for the single pregnant black rhinoceros were not elevated relative to pre-capture baseline.

Post-release breeding by the females described in this study provides tentative evidence for a translocation-induced reproductive delay. Of the seven females included in our dataset and released into Mun-ya-wana Reserve (Linklater *et al.*, 2006), four have given birth. One birth was a pregnancy carried through translocation (i.e. BR10, Fig. 4). The remaining three births were conceived 18, 28 and 31 months after release (estimated by back-dating 460 days gestation from birth), indicating a substantial reproductive delay for a spontaneous, and year round ovulator (Roth, 2006). Similarly, after translocations in East Africa a quarter of potential mothers had not calved and those that did took 4.6 years to calve (i.e. 3.3 years to conceive, Brett, 1998). Translocation appears, therefore, to delay breeding, although to what degree distress from prolonged captivity, and the other characteristics of translocation contribute, remains unknown. Our results support the case for shortening the period from capture to release beyond the first or second week to reduce the period of distress and its biological costs during translocation.

Conclusions toward future progress

The difficulties and complexities of assessing wild animal welfare during conservation programs means workers quickly adopted fecal indices of adrenal function (e.g.

corticoids, Turner *et al.*, 2002; Millspaugh *et al.*, 2007; Franceschini *et al.*, 2008). Our finding that distress was associated with post-stressor decline and lower than usual corticoid levels contributes a substantial challenge to their interpretation and application. Low corticoid concentrations might not be evidence of low stress, but distress. Declines in corticoid levels after a stressor-induced peak have been used to indicate recovery or acclimation (e.g. Turner *et al.*, 2002; Franceschini *et al.*, 2008), although corticoid levels might decline through intrinsic control even when the animal is under ongoing stress and distress (Moberg, 1987; Smith & Dobson, 2001). We demonstrate both of these problems in rhinoceros and, therefore, add empirical evidence to the cautions of others about an over-reliance on measures of adrenal steroids to quantify an animal's welfare, and the importance of distinguishing between stress and distress. We concur with Brown *et al.* (2001) that it is important to pair measures of corticoids with a measure of biological cost; that is, distress. Brown *et al.* (2001) recommends gonad function, and we used sex hormone concentrations in this paper, but it could be any validated measure of biological cost including, but not restricted to, measures of appetite, body condition or immune function. We urge that authors consider applying the stress-distress paradigm and move beyond studies that measure only adrenal steroids as indicators of animal well-being.

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