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RESEARCH ARTICLE

Effects of Management Strategies on Glucocorticoids and Behavior in Indian Rhinoceros (*Rhinoceros unicornis*): Translocation and Operant Conditioning

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The ex situ Indian rhino population experienced a decrease in genetic diversity indicating that the breeding program could possibly benefit from novel reproductive management strategies to ensure population sustainability. We sought to determine how management tools used for reproductive management, specifically translocation and operant conditioning, impact physiological and behavioral measures of welfare in Indian rhinos. First, an adrenocorticotropic hormone challenge performed in an adult male resulted in a 38-fold increase in urinary and a 3.5-fold increase in fecal glucocorticoid metabolites (FGM). Mean and peak FGM differed among three females, but all demonstrated elevated ($P < 0.0001$) concentrations for variable durations after translocation that lasted up to 9 weeks. Lastly, behavioral and adrenal responses of two females to operant conditioning to stand during transrectal ultrasound exams were monitored and rhinos differed in their mean and peak FGM concentrations. However, FGM were not different before versus during training or on pasture versus in the barn. One female exhibited more stereotypic behavior during training in the barn than on pasture ($P < 0.05$); although, stereotypies (1.73% of time) were relatively uncommon overall. In summary, individual variation exists in FGM both at baseline levels and in response to a stressor. In addition, while a transient rise in glucocorticoid activity post-translocation indicated that Indian rhinos have a physiological response to changes in their environment, minor alterations in daily routines using operant conditioning only resulted in minimal changes in behaviors and FGM. Zoo Biol. 33:131–143, 2014.

Keywords: behavior; glucocorticoids; Indian rhinoceros; operant conditioning; stress; transport

INTRODUCTION

The Indian rhino (*Rhinoceros unicornis*), considered vulnerable to extinction by the IUCN [Talukdar et al., 2008], has ex situ breeding limitations due to the decreasing number of genetic founders in the North American population [Foose and Wiese, 2006], variable and occasional anovulatory cycles [Stoops and Roth, 2003; Stoops, Pairan, and Roth, 2004], and a history of aggressive breeding interactions [Lindburg and Fitch-Snyder, 1994; Stoops and Roth, 2003]. This decrease in genetic diversity indicates a need for recommended translocations among facilities and alternative breeding strategies to ensure population sustainability [Foose and Wiese, 2006]. Intensive reproductive monitoring has contributed to available reproductive knowledge [Radcliffe, Bommarito, and Osofsky, 1996; Stoops and Roth, 2003; Stoops, Pairan, and Roth, 2004], and recently aided in achieving successful artificial insemination in the Indian rhino [Stoops et al., 2007]. These technological developments significantly benefit zoo populations because managers can use hormone

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and ultrasound results to identify candidates for breeding [Hildebrandt, Goritz, and Hermes, 2005] and artificial insemination, and to increase the efficacy of timed breeding to maintain genetic variation. However, the potential impacts of ex situ management strategies, including translocation and intensive reproductive monitoring for enhanced population management, on behavior and stress physiology in this species have not been examined.

Although difficult to define, a stressor can be described as any stimulus that challenges homeostasis [Morgan and Tromborg, 2007]. As a primary reaction to a stressor, a stress response is initiated by stimulating the hypothalamic pituitary–adrenal axis [Möstl and Palme, 2002]. The stressor is first perceived in the brain and triggers a response by the hypothalamus, stimulating secretion of corticotrophin releasing hormone, and ultimately, adrenocorticotropic hormone (ACTH) from the anterior pituitary. In vertebrates, ACTH acts on the adrenal gland to initiate an increase in glucocorticoids (GC), followed by a return to baseline [Norris, 2006; Sapolsky, Romero, and Munck, 2000; Wasser et al., 2000; Wielebnowski et al., 2002]. The biological response to GC is the redirection of energy from functions that can be continued later such as reproduction, growth, or metabolism, to one of an immediate coping mechanism [Moberg, 2000]. In addition, GCs are released in positive situations, or eustress, such as reproduction, courtship, and hunting, which affect homeostasis by initiating energy mobilization and altering behavior [Möstl and Palme, 2002].

Animals that encounter stimuli that challenge their behavior and physiology can acclimate to the challenge, or become susceptible to longer-term effects, such as biological exhaustion [Moberg, 2000]. While short-term increases in GC are normal, prolonged increases can lead to deleterious effects, including compromised metabolism, immune responses, growth, reproduction, and behavior [Carlstead, 1996; Carlstead and Brown, 2005; Eberhardt, Keverne, and Meller, 1980; Moberg, 1985; Morgan and Tromborg, 2007; Rideout et al., 1985]. Ex situ population management includes a variety of positive external stimuli, such as environmental enrichment [Morgan and Tromborg, 2007; Shepherdson, Mellen, and Hutchins, 1998] that challenge physiology and behavior, resulting in normal fluctuations in GC metabolite activity. Animals can also encounter aversive stimuli such as perceived lack of control, lack of natural spatial or social configurations, confinement, or lack of opportunity to develop natural behaviors [Carlstead, 1996; Morgan and Tromborg, 2007]. Management decisions, such as translocation among facilities and intensive reproductive management, are necessary tools to optimize genetic diversity of ex situ populations by varying social groups, and breeding pairs. However, it is not yet known whether these practices may stimulate significant or prolonged increases in GC activity in Indian rhinos.

Translocation is a common and necessary practice in both in situ [Dinerstein and Price, 1991; Sale and Singh, 1987] and ex situ populations. There was a precipitous decline of the in situ Indian rhino population between 1,600 and 1,900 due to a reduction of its original distribution range [Talukdar et al., 2008]. Despite recent population increases in some areas, numbers continue to decrease in areas such as Nepal and there is a need to maintain a healthy and genetically diverse population in zoological facilities [Emslie, Amin, and Kock, 2009]. Thus, research on ex situ translocations is necessary to optimize rhino management and husbandry and contribute to the available knowledge of post-transport effects on zoo animal welfare [Hill and Broom, 2009]. Knowledge gained through ex situ research regarding the influence of transport on physiology may also help to create informed management decisions and more detailed transport plans for both free ranging and zoo populations [Singh, Sharma, and Talukdar, 2012]. In addition, it may equip managers to prepare animals in a proactive, rather than a reactive approach to a change in their environment [Hill and Broom, 2009]. Previous studies have focused on the impact of translocation on GC activity in black and white rhinos [Linklater et al., 2010; Turner, Tolson, and Hamad, 2002]; however, similar studies have not yet been conducted on the Indian rhino.

Operant conditioning is another common ex situ wildlife management tool that facilitates advanced husbandry procedures, such as blood collection and ultrasonography, presumably without adversely affecting animal well-being and causing undue stress. Because operant conditioning can help an animal acclimate to novel situations through desensitization [Laule and Whittaker, 1999], it enables animals to voluntarily participate in husbandry and medical procedures without the use of anesthesia. Operant conditioning, in conjunction with restraint chutes, have been successfully used to condition rhinos to stand for ultrasounds [Radcliffe, Bommarito, and Osofsky, 1996; Roth, 2001; Stoops, Pairan, and Roth, 2004] and perform successful artificial insemination procedures without anesthetics [Shaffstall, 2007; Stoops et al., 2007]. While it is assumed that using positive reinforcement operant conditioning to prepare individuals for reproductive monitoring without anesthesia enhances well-being [Stoops, Pairan, and Roth, 2004; Stoops et al., 2007], the behavioral and physiological effects of conditioning have not been monitored.

Welfare can be difficult to define and to measure. In general, welfare can be described as an animal's state while attempting to cope with its environment [Broom, 1986; Hill and Broom, 2009]. A multidisciplinary approach using multiple measures to monitor welfare in zoo-managed animals can provide valuable data to create enhanced management plans. Collecting urine and fecal samples is a reliable, non-invasive method for assessing fluctuations in GC activity, one measure of welfare, in a variety or mammalian and avian species [Wasser et al., 2000] and are an advantageous alternative to blood sampling, which may result in data confounded by human influence. Urine and fecal sampling also provides an aggregate measure of GC activity over several hours, rather than representing an instantaneous snapshot of the stress response when measured

in blood. Although salivary cortisol has been validated for the Indian rhino [Menargues, Urios, and Mauri, 2008], assays to detect GC in urine and feces through non-invasive monitoring have not been validated for this species. Another approach is to use behavioral observations gathered in a standardized manner using an ethogram to gain insight to subtle changes in animal physical, social, or physiological states [Watters, Margulis, and Atsalis, 2009]. Objectively determining an animal's behavioral repertoire over time provides information about potential individual management challenges [Watters, Margulis, and Atsalis, 2009] such as, responses to changes in exhibits, social dynamics, or breeding partners. The effects of operant conditioning on behavioral repertoires have not yet been studied in any rhino species.

The relationship between breeding management practices, glucocorticoids, and behavior have not been examined in Indian rhinos. Thus, the specific goals of this study were (1) to validate methods for non-invasive monitoring of GC metabolites using an adrenocorticotropic (ACTH) challenge; (2) to examine the effects of translocation on fecal glucocorticoid metabolites (FGM); and (3) to examine the behavioral and GC response to introducing a positive reinforcement operant conditioning program and regular reproductive monitoring via transrectal ultrasound. We predicted that translocation among zoological facilities would affect adrenal physiology as demonstrated by a transient rise in FGM in female Indian rhinos. We also expected that operant conditioning would result in consistent, voluntary participation of the rhinos in the collection of reproductive data (e.g., rectal ultrasounds) without an associated increase in GC activity or change in overall behavioral repertoire.

MATERIALS AND METHODS

Subjects and Facilities

For both experiments, all procedures were reviewed and approved by Animal Care and Use Committees at the Wilds and the Cincinnati Zoo and Botanical Gardens (CZBG) and by management staff at White Oak Conservation Center and Mesker Park Zoo where only non-invasive sample collection occurred.

Experiment 1: ACTH challenge and translocation

An ACTH challenge was performed in a single, adult male Indian rhino (Rhino 1, age 32 years), who resided at his current facility since 1996. Transport subjects were three adult female Indian rhinos housed at separate zoological facilities (Table 1). Female rhinos were transported among facilities to fulfill management recommendations in November and December 2009. Anecdotal keeper notes were collected from the sending and receiving institutions to track only significant events (crate training, day of arrival, medical exams, introduction to conspecifics, estrus behaviors) occurring during the study period. The subjects had a range of previous experience with transport and varying amounts of preparation for the loading and transport process (Table 1). Prior to this study, Rhino 2 experienced two previous transports, had access to a crate and was conditioned to enter it before the shipment. Rhino 3 was wild born and had experienced four previous translocations. She had access to the shipping crate before her translocation and azaperone (200 mg, IM; ZooPharm, Wildlife Pharmaceuticals, Inc., Windsor, CO) was administered to facilitate transport. Rhino 4 had not yet experienced transport or separation from her dam. In preparation for transport, she was housed in a stall with visual access to a transport crate, but did not have physical access to enter the crate until the day of the shipment. Azaperone (100 mg, IM; Janssen Pharmaceutica, Beerse, Belgium) and butorphanol (20 mg, IM; Fort Dodge, IA) were administered to facilitate transport. Any pharmaceutical agents administered to facilitate transport were given at the discretion of the attending veterinary staff at each facility and were not given for the purpose of this study.

Experiment 2: operant conditioning

Experiment 2 subjects were two female Indian rhinos that were proven breeders. Rhino 5 was 10 years of age, and Rhino 6 was 13 years of age at the time of the study. Both rhinos were captive-born and resided at their current facility since 2004 and 2001, respectively. From April to November 2009, prior to the start of the project, Rhinos 5 and 6 were together with their 2.5-year-old calves, another 3-year-old female, and a 32-year-old male in a 101-acre mixed species pasture (subsequently referred to as "pasture") with 24 hr access to one another, and had limited interactions with keepers. During the winter months, the rhinos were moved off pasture to an indoor stall (subsequently referred to as "barn") with an adjacent outdoor holding yard. For the entire winter, all rhinos were housed in separate stalls, except Rhinos 5 and 6 that each shared a stall with their calves. The daily winter routine included feeding, moving among stalls for cleaning, and more frequent interactions with keepers. The indoor stalls

TABLE 1. Indian rhino subjects and details about translocations among North American zoological facilities

Subject	Age	Crate-conditioned?	Pharmacological agent administered?	Distance (miles)	Approximate travel time (hr)	No. of previous transports
Rhino 2	13	Yes	None	780	12	2
Rhino 3	23	Yes	Azaperone	750	12	4
Rhino 4	4	No	Azaperone, butorphanol	200	3.5	0

were adjacent to one another allowing head to head interactions between animals. If the temperature or wind-chill factor fell below −9.0°C, the rhinos were not given access to the outdoor yard.

The rhinos were accessible from indoor holding areas that were equipped with a chute specifically designed for temporary restraint to perform advanced husbandry, medical, and research-related procedures. The restraint device had dual manual entry doors with hydraulic moveable sides to squeeze the chute and reduce the amount of space on either side of the rhinoceros. The subjects had only limited exposure to the chute prior to this study. The goal of the training was to condition the rhinos to enter the chute and to stand stationary for 15 min during transrectal ultrasound examinations. The conditioning progressed gradually over a 5-month period (December 2009 to April 2010) from a simple target command to position the rhino to voluntarily participate in frequent reproductive examinations. The training protocol was developed for each rhino to determine daily goals and expected outcomes. Sessions occurred 6–7 times weekly, lasting approximately 15–30 min, until the desired behaviors were consistently achieved. The primary reinforcement was small quantities of favorite food items, such as peanut butter, apples, sweet potatoes, and alfalfa cubes. Initially, the rhinos were separated from their calves during training to allow them to focus on the target behavior. The calves were occupied in adjacent stalls with a portion of their diet.

After each rhino acclimated to eating a grain ration in the chute, the back chute door was closed. This stage indicated a significant distinction between rhinos, which resulted in the need for divergent methods for the remainder of the training program. Rhino 5 demonstrated nervous behaviors and refused to re-enter the chute after being temporarily closed in, so the front chute door was left open while the back door was closed behind her. This change allowed Rhino 5 to stay calm and remain in the chute. Rhino 6 demonstrated reluctance to leave her calf, so her calf was allowed access to the head end of the chute when temperatures permitted outside access. This flexibility allowed trainers to proceed with the rest of the conditioning stages on schedule. As the desired behavior of standing calmly in a closed chute became daily practice, the program progressed to include the touch command, gradually touching on the side and moving caudally to eventually perform rectal palpations. Once they remained calm for rectal palpations, ultrasound equipment was introduced by first allowing the rhinos visual access to the machine. Finally, reproductive examinations were conducted once weekly throughout the luteal phase and then daily during the follicular phase for a total of 12 weeks.

Behavior Data Collection

For Experiment 2 only, behavior data were collected using an ethogram (Table 2) created from previous studies [Fouraker and Wagener, 1996; Laurie, 1982; Mueller, 2008].

TABLE 2. Indian rhinoceros ethogram

Event behaviors	
Affiliative	
Chin rest	Rests chin on object or conspecific (not in breeding position)
Climb	Puts front feet on stall bars, water bowl, or conspecific
Lie together	Lying on ground, touching another rhino
Moo-grunt	Mouth open or closed, short grunt from the throat, sometimes a contact call from calves
Nuzzling	Nose to nose contact
Rub/lick	Rubs or licks conspecific
Spar	Horn-to-horn or head contact with conspecific, offensively or defensively
Estrus	
Urine-squirt	Projects urine in distinct squirts
Whistling	Half aspiratory, half vocal shrill squeak followed by a sharp exhale
Investigate	
A/G investigation	Sniffs anogenital region of another
Flehmen	Raises head and curls upper lip back
Object investigation	Manipulating or moving an object with head or horn
Rub/lick object	Rubs or licks inanimate object
Urine/feces investigation	Sniffs urine or feces
Stereotypies	
Chain	Moves chain on stall bars up and down with mouth/face in a repetitive sustained motion
Horn rub	Rubs horn against an object
Pacing	Back and forth locomotion in a repetitive, sustained pattern, repeating path at least three times
Sway	Rubs horn against stall bars in a repeated sustained motion, while swaying body side to side
State behaviors	
Alert	Standing stationary with head at or above shoulder level
Drink	Ingestion of water
Eat	Ingestion of food
Elimination	Urination or defecation
Locomote	Movement in a forward or backward direction
Rest	Stationary, standing with head down or lying with eyes open or closed; not demonstrating any other simultaneous behaviors

To simplify analyses, event behaviors were grouped into related categories (affiliative, estrus, investigate, and stereotypies); state behaviors including drink, eat, eliminate, locomote, rest, and alert, were analyzed individually. Behavioral data collection began while rhinos were on pasture (November 2009) and continued through the 5-month (December 2009 to April 2010) operant conditioning program. Observations were conducted between 0700 and 1500 hr, 5–7 times/week, alternating between morning and afternoon sessions. Data were scored on a check sheet using interval time sampling (interval = 1 min) for 30-min per observation session [Altmann, 1974]. The project ended in April, coinciding with the seasonal release from the holding barns to their semi-free ranging summer pasture. Immediately following the completion of the 5-month conditioning program, the females moved into a breeding situation on pasture, thus preventing collection of comparable post-training baseline data.

Pharmacological Validation

The ability of a cortisol assay to measure adrenal activity in Indian rhinos was validated using an ACTH challenge of an adult male. Urine and fecal samples were collected daily for 2 weeks prior to the exogenous hormone injection. A single IM injection (3,000 IU) of a slow-release ACTH gel (Premier Pharmacy Labs, Inc., Weeki Watchee, FL) was administered at 1,500 hr. Every urine and fecal void was collected immediately post-voiding for 72 hr post-injection. Following the 72 hr continuous sample collection, daily urine and fecal samples were collected for an additional week.

Sample Collection and Processing

Urine samples were aspirated from a clean floor immediately after voiding, and then stored in sealed tubes at −20°C. To correct for water content in the samples, urinary glucocorticoid metabolites (UGM) were indexed by creatinine (Cr) concentration and expressed as ng/mg Cr [Gronwall and Price, 1985]. Fresh fecal samples (<12 hr old) were collected from pasture or stall floors, and placed in sealed plastic bags. Both types of samples were stored at −20°C until analysis (<3 months), and thawed immediately prior to extraction. Both types of samples were collected for the ACTH challenge, but only fecal samples were collected for Experiments 1 and 2 due to ease of collection. For Experiment 1, fecal samples were collected a minimum of every 48 hr from the three adult females for at least 6 weeks prior to transport (range 40–50 days) and approximately 11 weeks after transport (range 76–85 days). For Rhino 3, post-transport sample collection did not begin until 14 days after her arrival to the new facility. For Experiment 2, fresh fecal samples (<12 hr old) were collected 5–7 times/week from stall floors at 0700 hr, placed in plastic bags, and sealed. All samples were immediately stored at −20°C, and only thawed prior to extraction.

Fecal hormones were extracted using a procedure described by Merl et al. [2000] for measuring GC in equine feces. Briefly, 0.5 g of wet feces were weighed and placed in a 15-ml conical polypropylene tube (Cat# 352096, Becton Dickenson, Franklin Lakes, NJ) and adding 5 ml 80% methanol. The samples were homogenized on a rotator-shaker overnight, centrifuged at 1,500*g* for 15 min, and 1 ml supernatant transferred to a new 15 ml tube. Next, 5 ml of diethyl ether (Cat# 60-29-7 Sigma Chemical Corporation, St. Louis, MO) and 250 μl of 5 M sodium bicarbonate (Cat # S8875, Sigma Corp.) were added to each tube and vortexed vigorously for 60 sec. The samples were placed at −80°C for 30 min to freeze the aqueous layer, and then the supernatant was poured off into a new, labeled tube. The supernatant was dried overnight under a stream of air. The residues were reconstituted by adding 500 μl of assay buffer (0.1 M phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA)) and sonicating the samples for 15 min. The resulting extract was transferred to a new polypropylene tube and stored at −20°C until analyzed (within 2 months of extraction). Final hormone concentrations are expressed as ng/g feces.

Enzyme Immunoassay

Concentrations of GC metabolites in urine and feces were quantified by enzyme immunoassay (EIA) using an anti-cortisol antiserum (R4866) and cortisol-horseradish peroxidase (HRP) ligand obtained from Coralie Munro (University of California, Davis, CA). The polyclonal antiserum was raised in rabbits against cortisol-3-carboxymethyloxime (CMO), linked to bovine serum albumin that cross-reacted with cortisol (100%), prednisolone (9.9%), prednisone (6.3%), cortisone (5%) and <1% with androstenedione, androsterone, corticosterone, desoxycorticosterone, 11-desoxycortisol, 21-desoxycortisone and testosterone [Munro and Lasley, 1988]. The EIA procedure was completed according to methods established by Munro and Lasley [1988]. Briefly, 96-well microtitre plates (Nunc-Immuno Maxisorp, Fisher Scientific, Pittsburgh, PA) were coated with cortisol antiserum (50 μl/well, diluted 1:20,000 in bicarbonate coating buffer, pH 9.6), sealed, and incubated overnight at 4°C. The next day, plates were washed (0.9% saline + 0.05% Tween 20) three times and then duplicate standards (250 to 3.9 pg/well), samples, and three internal controls (representing 70%, 50%, and 20% of the optical density of the assay buffer or 5, 20, and 120 pg/well, respectively) as well as enzyme conjugate (cortisol-3-CMO:HRP; diluted 1:20,000 in assay buffer: 0.1 M PBS containing 1% BSA, pH 7.0) were added to the plate. After incubation at room temperature (2 hr), plates were washed three times.

Freshly prepared substrate solution (100 μl; 0.05 M citrate, 1.6 mM hydrogen peroxide, 0.4 mM 2,2-azino-di-3-ethylbenzithiazoline sulfonic acid diammonium salt, pH 4.0) was added to all wells and color was allowed to develop to an optical density of 1.0. Absorbance was measured at 405 nm.

Serially diluted urine and feces from Indian rhino displayed parallelism with (and were not significantly different from) the standard curve (urine $T\text{-stat}_{(12)} = 0.16$, $P = 0.34$; feces $T\text{-stat}_{(12)} = 0.42$, $P = 0.34$). Urine was diluted 1:40 and fecal extracts were diluted between 1:20 and 1:5 in assay buffer. Percent recoveries averaged 93% in urine samples and 84% in fecal extracts spiked with high and low controls and intra- and inter-assay coefficients of variation were 4.9 and 10.9, respectively.

Data Analysis

Experiment 1

Baseline concentrations of FGM were calculated for each female using an iterative process [Brown et al., 1999]. Briefly, data points exceeding two standard deviations (SDs) from the mean concentration of FGM, respectively, were removed. Averages were then recalculated, and the elimination process repeated until no values exceeded the mean $\pm$ 2 SD. To compare overall differences before and after translocation, FGM raw values were log transformed and analyzed using a general linear-mixed model (PROC MIXED, SAS, version 9.2, SAS Institute, Cary, NC) with individual animal as a random effect and time (before or after transport) included in the model as a fixed effect. For comparisons between rhinos before and after treatment, a repeated measures analysis of variance was used (RM ANOVA; PROC GLM) with individual and time as a fixed effect. The sources of between-individual heterogeneity were controlled by a mixed-effects model to provide a more accurate measurement of within-individual patterns in longitudinally measured data [Nussey et al., 2008]. Because model data included repeated measures from individual rhinos, animal identity was incorporated as a random effect and data were grouped by subject [Wittemyer, Ganswindt, and Hodges, 2007]. Post hoc comparisons between individuals either before or after transport were analyzed using least-squared means and Tukey's HSD test for multiple comparisons.

Experiment 2

To analyze differences in FGM and proportion of behaviors over time with respect to the operant conditioning, the study was divided into five stages: (1) while the rhinos were out on pasture (20 days), (2) a period of acclimation to the barn, prior to operant conditioning (16 days), (3) introduction to operant conditioning, including teaching a target behavior and preparation for entering the chute on cue (31 days), (4) daily conditioning sessions that include standing in the restraint chute and introduction to hands-on preparation, but not yet rectal palpations (26 days), and (5) gathering reproductive data via transrectal palpations and ultrasound on a regular basis (90 days). Additionally, FGM values were delayed by 24-hr to correspond with behavioral observations to adjust for excretion delay.

Concentrations of FGM were log transformed and analyzed for changes over time using a general linear regression within individual. Differences in FGM concentrations between the two Indian rhinos were analyzed using an RM ANOVA (PROC GLM). Differences in FGM concentrations with respect to stage of conditioning (1–5) were evaluated with a general linear-mixed model (PROC MIXED). Because, these stages encompassed many factors (e.g., differences in location and training goals) that could impact FGM concentrations and the behavioral repertoire of the rhinos, comparisons between pasture (Stage 1) and barn (Stages 2–4), and before (Stages 1–2) and during (Stages 3–5) operant conditioning were also analyzed with PROC MIXED. When significant differences were present, a post hoc Tukey's pairwise comparison of the least-squared means was performed to compare differences between stages, location, or training. Because, there were differences in FGM output between the two rhinos, post hoc tests comparing FGM between stages of training were also performed within-subject.

All behaviors within a category were summed and the number of times the behavioral category or state behavior was displayed per 30-min observation session was calculated; results were presented as the proportion of observations each behavior was performed. To investigate differences between rhinos and among the behaviors, mean proportions of time were analyzed in a two-way ANOVA to avoid pseudoreplication. Because, behavioral data were not normally distributed, a generalized linear-mixed model (PROC GLIMMIX) was used to analyze the effects of stage of training, pasture versus barn, and before versus during training. All behaviors were analyzed using a gamma distribution and log link function, with the exception of "eat," which was analyzed using a log normal distribution and an identity link function in SAS. All behavioral analyses were performed within rhino.

For all analyses, behavioral and FGM, $P < 0.05$ was considered significant. Mixed model data were presented as the model estimate $\pm$ SE in the tables and all other data were presented as mean $\pm$ SEM in the text and figures. All analyses other than mixed models were conducted using SigmaPlot (v. 11.0; Systat, Inc., 2008). A Shapiro–Wilk test was used for normality assumption testing and the Levene median test for equal variance assumption testing.

RESULTS

Experiment 1

ACTH challenge

A 38-fold increase in UGM concentrations (baseline: 56.73 ± 4.12 vs. peak: 2,179.60 ng/mg Cr) was observed approximately 16 hr after the ACTH injection in Rhino 1 (Fig. Fig. 1). A 3.5-fold increase in FGM concentrations (baseline: 9.66 ± 0.59 vs. peak: 35.28 ng/g feces) was observed approximately 22 hr after the ACTH injection

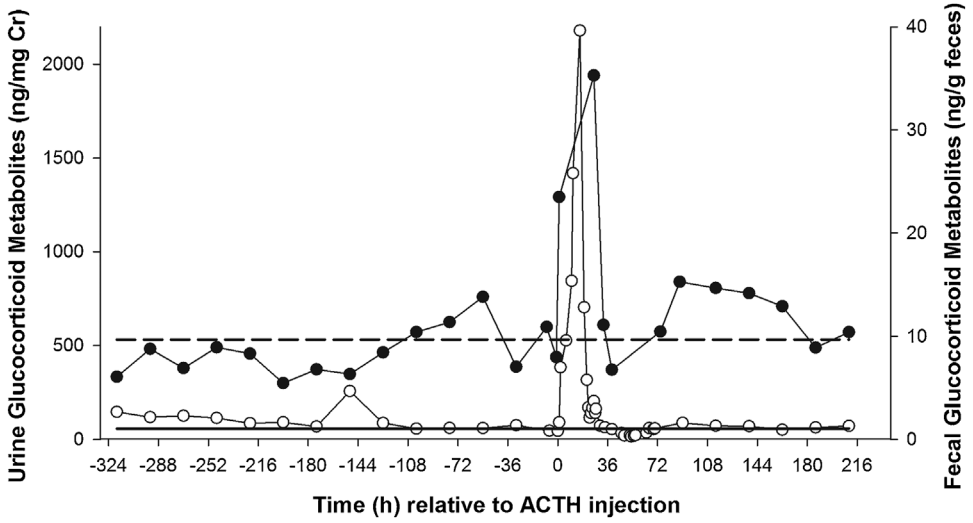

Fig. 1. Open circles represent changes in urinary glucocorticoid metabolites (UGM) and closed circles represent changes in fecal glucocorticoid metabolites (FGM) relative to the hour of injection of a slow-release ACTH gel (3,000 IU, i.m.) in an adult, male Indian rhinoceros. The solid and dashed lines represent baseline concentrations of UGM and FGM, respectively.

(Fig. Fig. 1). Concentrations of GC metabolites returned to and remained below 2 SD of baseline after 30 hr in urine and 88 hr in feces.

Translocation

There was a significant effect of transport (Table 3) on FGM in female Indian rhinos. Mean concentrations differed among the rhinos prior to transport ($F_{2,94} = 28.49$, $P < 0.0001$) and post-transport ($F_{2,136} = 4.05$, $P < 0.05$), with the lowest concentrations observed in the youngest, Rhino 4 (Table 4). Peak concentrations after translocation also differed, with an approximate fourfold increase above baseline for the two older females, Rhinos 2 and 3, and an eightfold increase in the younger Rhino 4 (Fig. Fig. 2).

Individually, the rhinos demonstrated responses to transport that varied in both mean concentration ($P < 0.05$; Table 4) and duration (Fig. Fig. 2). Prior to transport, Rhinos 2 and 4 did not exhibit variations in FGM more than mean baseline + 2 SD. Post-translocation, Rhino 2 demonstrated variable increases in FGM that lasted approximately 6 weeks (Fig. Fig. 2A). Rhino 3 had a variable FGM profile with multiple peaks above baseline + 2 SD both before and after transport. However, elevated FGM (lasting several days in a row) were detected for 9 weeks post-translocation (Fig. Fig. 2B), before returning to baseline. Despite having the lowest baseline concentrations of FGM (Rhino 2: 13.11 ± 0.54 ng/g feces; Rhino 3: 12.83 ± 0.42 ng/g feces; Rhino 4: 8.49 ± 0.35 ng/g feces), Rhino 4 exhibited the most pronounced response to translocation, with significant (twofold to eightfold) elevations in GC for up to 8 weeks post-translocation (Fig. Fig. 2C).

TABLE 3. Linear-mixed model depicting the effects of transport on FGM for three female Indian rhinos transported among North American zoological facilities

	Estimate ± SEM	*t*-Value	*P*-value	AIC and BIC
Intercept	1.01 ± 0.06	16.00	0.0039	−39.6
Transport	0.2291 ± 0.03	7.94	<0.001	−42.3

Experiment 2

Fecal glucocorticoid metabolites

Overall concentrations of FGM did not change over time for Rhino 5 ($F_{1,142} = 0.36$, $P = 0.551$; $r^2 = 0.0025$; Fig. Fig. 3A) or Rhino 6 ($F_{1,141} = 0.0001$, $P = 0.95$; $r^2 < 0.001$; Fig. Fig. 3B). Individually, Rhino 5 (10.7 ± 0.27 ng/g feces) excreted lower ($F_{143} = 323.85$, $P < 0.001$) overall mean FGM concentrations than Rhino 6 (21.64 ± 0.68 ng/g feces). In contrast to overall changes in FGM within a rhino, concentrations of FGM varied with respect to conditioning stage ($F_{4,\ 280} = 2.82$, $P = 0.03$). Within-subject analyses revealed that concentrations of FGM were lower (Tukey's $P < 0.05$) during Stage 2 (acclimation to the barn) compared to all other stages for Rhino 6 only (Table 5); no other differences in FGM between stages were observed. In spite of the effects among the stages, we found no difference in FGM concentrations between the period Indian rhinos spent on pasture (13.66 ± 1.45 ng/g feces) versus in the barn (15.18 ± 1.46 ng/g feces; $F_{1,283} = 3.16$, $P = 0.07$), or before (13.89 ± 1.45 ng/g feces) and during (13.83 ± 1.46 ng/g feces) operant conditioning ($F_{1,283} = 0.01$, $P = 0.93$).

Behavior

There were differences among the proportion of time the rhinos spent performing the various behaviors ($F_{1,9} = 20.50$, $P < 0.001$). Both rhinos spent a greater

TABLE 4. Mean ± SEM fecal glucocorticoid metabolite concentrations (ng/g feces) in three Indian rhinos before and after transport among North American zoological facilities

Subject	Before transport	After transport	DF	*t*-Value	*P*-value (within row)
Rhino 2	11.32 ± 0.90^{a}	19.08 ± 0.97^{a}	76	6.82	<0.0001
Rhino 3	15.65 ± 1.15^{b}	21.12 ± 2.10^{a}	63	2.00	<0.0500
Rhino 4	6.75 ± 0.51^{c}	17.32 ± 1.50^{b}	89	5.97	<0.0001

Superscript letters indicate significant differences within a column ($P < 0.05$).

($F_{1,9} < 0.001$, $P = 0.99$) proportion of time eating than engaging in all other behaviors except for affiliative events (Tukey's $P > 0.05$; Fig. Fig. 4). Affiliative events occurred more often than all other behaviors except locomote and alert (Tukey's $P < 0.05$). There were no differences among the proportions of time rhinos spent performing the other behaviors (Tukey's $P > 0.05$).

Among the five stages of training, the only behavior that differed was alert. Rhino 6 spent a smaller proportion of time exhibiting an alert behavior during Stage 3 (0.095 ± 0.027) compared with Stage 5 (0.236 ± 0.034; Table 6); no further differences were found among stages of training for either rhino. However, Rhino 5 exhibited more stereotypic behaviors in the barn (0.031 ± 0.000) compared to on pasture (0 ± 0) and during training (0.034 ± 0.000) compared to before training (0 ± 0; Table 7). No other differences in behaviors were observed between pasture and barn, or before versus during operant conditioning for either rhino.

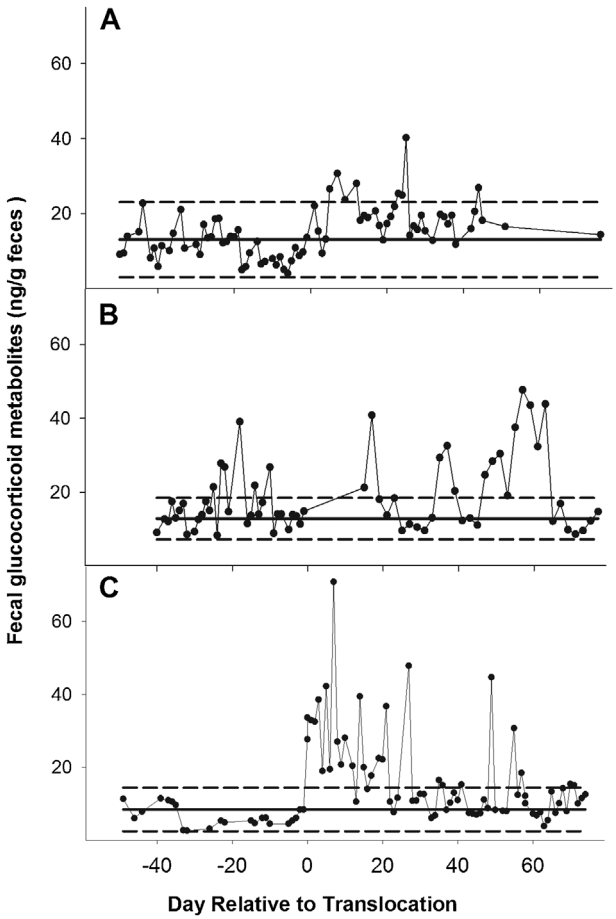

Fig. 2. Changes in FGM before and after translocation of three Indian rhinos: (**A**) Rhino 2, (**B**) Rhino 3, and (**C**) Rhino 4 among North American zoological facilities. Day "0" indicates day of transport. The solid and dashed black lines indicate baseline and ±2SD of the baseline concentrations of FGM for each individual, respectively.

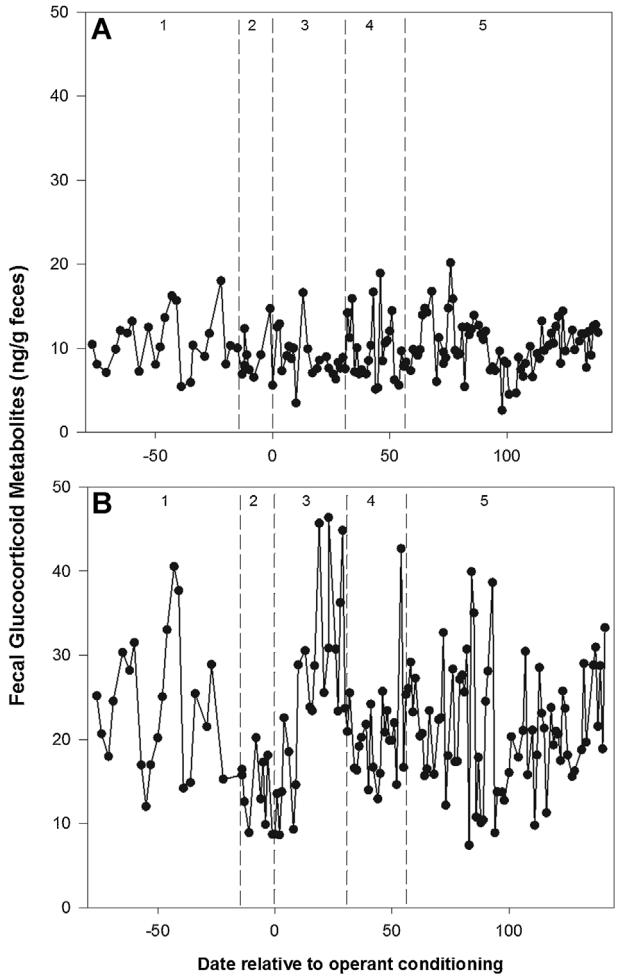

Fig. 3. Changes in FGM concentrations in two female Indian rhinoceros (**A**) Rhino 5, and (**B**) Rhino 6 at a North American zoological facility over the course of an operant conditioning study. The five stages of operant conditioning training are depicted with dashed lines and numbers at the top of each panel.

TABLE 5. Linear-mixed model of differences in FGM concentrations between five stages of an operant conditioning program in Rhino 6

	Estimate ± SE	*t*-Value	*P*-value	AIC and BIC
Intercept	3.00 ± 0.05	66.79	<0.001	
Stage				
1[a]	0.10 ± 0.09	1.12	0.264	134.3
2[b]	−0.41 ± 0.13	−3.15	0.002	137.2
3[a]	0.11 ± 0.09	1.20	0.230	
4[a]	−0.02 ± 0.09	−0.19	0.850	
5[a]	0			

Different superscripts denote significant differences among least-squared means between stages (Tukey's, $P < 0.05$). There were no differences in FGM between stages for Rhino 5 (not shown).

DISCUSSION

Knowledge gained about the adrenal and behavioral responses to management tools (e.g., translocation and operant conditioning) can provide insight into the best practices for Indian rhinos. This study was the first time an ACTH challenge was conducted to test the validity of urine samples as an acceptable matrix for measuring glucocorticoids in any rhino species, in addition to the validation of UGM and FGM in the Indian rhino. Transport of female Indian rhinos to a new facility resulted in a significant but transient rise in FGM that lasted for up to 9 weeks. The operant conditioning experiment was the first study to longitudinally and non-invasively examine its effects on the daily behavioral repertoire and glucocorticoid output in any ungulate. Positive reinforcement operant conditioning was a successful method for training two female Indian rhinos to stand for regular ultrasounds, confirming previous studies [Stoops, Pairan, and Roth, 2004; Stoops et al., 2007]. Although some differences in FGM activity were noted for Rhino 6, the results suggested that the training methods did not elicit an elevated or prolonged GC response in these Indian rhinos. The proportion of time that Rhino 5 spent exhibiting stereotypic behavior and Rhino 6 spent alert varied over the course of the study and may have been influenced by the operant conditioning program or environmental variables. The differences in behavioral repertoire as well as mean and peak concentration of FGM among the Indian rhinos in these studies highlighted a distinct individual variability in relation to management practices.

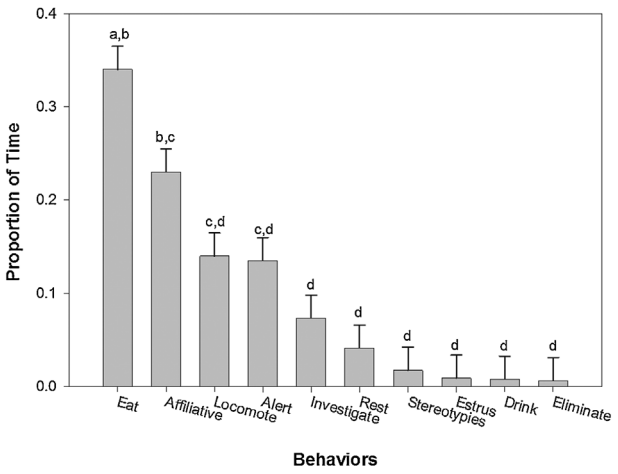

Fig. 4. Mean ± SE proportions of time behaviors were performed by two Indian rhinoceros at a North American zoological facility. Significant differences between behaviors are designated with superscripts (Tukey's, $P < 0.05$).

ACTH Challenge

Despite the opportunity to conduct an ACTH challenge being limited to only a single Indian rhino, results were similar to studies in other rhino species [Brown et al., 2001; Turner, Tolson, and Hamad, 2002]. Comparable to the results of the current study where FGM peaked at 22 hr post-ACTH administration, Brown et al. [2001] conducted an ACTH challenge for black rhinos, and concluded that peak FGM were excreted about 24 hr post-injection. The authors also reported no gender differences with respect to GC concentrations in either black or white rhinos [Brown et al., 2001]. In addition, Turner, Tolson, and Hamad [2002] reported 6.9-fold increases in FGM in white rhinos after transport that are comparable to that observed for the Indian rhinos in this study.

A greater increase in UGM was observed as a result of the ACTH challenge compared to FGM, even at a lower dilution factor than the fecal extracts. While this suggests that urine may be a more sensitive matrix for measuring glucocorticoid activity with the cortisol EIA, urine was not an optimal choice for our experiments, which included collecting samples from both barn housing and large pasture habitats, because it was more difficult to acquire. Although urine requires less sample processing compared to feces, immediate access to the void is required in order to prevent contamination or loss of the sample. In situ research could benefit from the ease of remotely collecting fecal samples versus urine.

Experiment 1

Translocation has been reported as a stressor in many mammals, such as domestic dairy cattle [Morrow et al., 2002;

TABLE 6. Generalized linear-mixed model of differences in frequency of alert behavior of between five stages of an operant conditioning program in Rhino 6

	Estimate ± SE	*t*-Value	*P*-value	AIC and BIC
Intercept	0.21 ± 0.02	9.74	<0.001	
Stage				
1[a,b]	−0.16 ± 0.07	−2.23	0.028	
2[a,b]	−0.09 ± 0.05	−2.67	0.139	−53.22
3[a]	−0.12 ± 0.04	−2.98	0.004	−37.47
4[a,b]	−0.11 ± 0.04	−2.88	0.010	
5[b]	0			

Different superscripts denote significant difference among least-squared means of stages (Tukey's, $P < 0.05$). There were no differences in alert behavior between stages for Rhino 5 (not shown).

TABLE 7. Generalized linear-mixed model of changes in stereotypic behavior in Rhino 5 on pasture versus in the barn and before versus during and operant conditioning program

	Estimate ± SE	*t*-Value	*P*-value	AIC and BIC
Stereotypies				
Intercept (pasture)	$1.48 \times 10^{-12} \pm 0.02$	0.00	1.000	290.89
Barn	0.0304 ± 0.03	2.01	0.045	283.02
Stereotypies				
Intercept (before)	$1.94 \times 10^{-12} \pm 0.02$	0.00	1.000	−287.94
During	0.033 ± 0.02	2.11	0.037	−280.07

There were no differences in stereotypic behavior throughout the study in Rhino 6 (not shown).

Palme et al., 2000], Grevy's zebra (*Equus grevyi*) [Franceschini et al., 2008] as well as black and white rhino [Linklater et al., 2010; Turner, Tolson, and Hamad, 2002] based on FGM monitoring. Comparable to the current study, wild white and black rhinos displayed increased concentrations of FGM for 6 weeks after arrival and acclimation to a free ranging wildlife preserve [Turner, Tolson, and Hamad, 2002]. Even after the 6-week adjustment period, black rhinos excreted higher FGM concentrations than rhinos that had been in their new surroundings for more than 1 year, suggesting that more than 6 weeks are required for a complete acclimation. Indian rhinos in this study exhibited similarly elevated FGM concentrations that lasted up to 9 weeks.

Individuals respond differently to transport according to prior experience [Grandin, 1997]. In the present study, difference in previous experience with transport could have contributed to variations in FGM responses to translocation. Rhinos 2 and 3 had transport experience and crate conditioning prior to translocation; however, FGM remained elevated for 6 and 9 weeks, respectively. Rhino 4 was the only female without prior transport experience or crate conditioning, and had the highest peak FGM response of the three rhinos. The observed difference could be attributed to lack of previous experience, separation from her dam, or her younger age compared to the other females. Age plays a role in adrenal responsiveness in mammals [Reeder and Kramer, 2005]; however, it typically increases with age [Born et al., 1995; Creel et al., 2002; Reeder and Kramer, 2005] contrary to what was observed in the current study. Therefore, age alone likely did not contribute to the FGM response in the youngest female Indian rhino.

The administration of therapeutic agents to Rhinos 3 and 4, but not Rhino 2 may have contributed to variability in individual FGM responses to translocation. Despite administration of azaperone and/or butorphanol, the FGM response to translocation was not diminished in magnitude (Rhino 4) or duration (Rhinos 3 and 4), compared to Rhino 2. However, the lack of data for the first 14 days after transport from Rhino 3 prevents forming solid conclusions relative to the use of therapeutic agents to mitigate the stress response and aid in transport in this species. Further studies with additional animals are needed.

While translocation resulted in a transient rise in GC activity in three female rhinos, keeper observation notes indicated that several GC peaks more than 2 SD above baseline coincided with introductions to conspecifics. Fecal glucocorticoid responses to the introduction of a new sable antelope (*Hippotragus niger*) varied between the resident animal and the individual that was new to the zoo [Loeding et al., 2011]. The resident sable antelope had consistently lower FGM than the sable that was being introduced [Loeding et al., 2011]. Although introduction to conspecifics was not the focus of this project, it may warrant future investigation, and animal managers should consider the possible impact of introductions within the context of acclimation to a new environment.

Experiment 2

Prior to the present study, it has only been suggested that operant conditioning is a non-stressful means of achieving animal participation in basic husbandry, such as blood collection [Grandin, 2000; Philips et al., 1998], and advanced medical procedures like transrectal ultrasound [Stoops, Pairan, and Roth, 2004] and artificial insemination [Stoops et al., 2007]. In the current study, there was evidence that FGM levels were no different during training than on large open pastures with minimal human interaction for Rhino 6 and did not vary at all for Rhino 5, suggesting that operant conditioning had no effect on FGM levels. It is worth noting that Rhino 6 was reluctant to leave her calf during early stages of training and would periodically leave the chute to check on her. Additionally, Rhino 6 progressed more slowly through the operant conditioning program and the added variability of FGM during Stages 3 through 5 could reflect a difference in acclimation to reproductive monitoring compared to Rhino 5. These results may provide evidence for variability in the coping response to novel stimuli.

The few other studies that investigated an adrenal response to operant conditioning programs did not compare individual variability, but did support the notion that trained animals cope better with management practices. Nyala (*Trageaphus angasii*) and bison (*Bison bison*) conditioned for restraint had lower serum cortisol values [Grandin, 2000] than previously published data on unconditioned, restrained domestic cattle [Grandin, 1997] and unsedated, restrained wild antelope [Grandin, 2000]. Plasma cortisol in conditioned, unsedated bongo (*Tragelaphus euryceros*) was lower

than previously reported values from restrained, immobilized antelope species [Philips et al., 1998]. In contrast to the present study, Philips et al. [1998] and Grandin [2000] made comparisons of current plasma GC values in conditioned animals to previously published GC values in unconditioned animals as evidence of a lower stress response from conditioned individuals. However, comparison of current and previously published plasma GC data may not be directly comparable due to differences in laboratory methods. Our study on Indian rhinos made direct comparisons in FGM among the same individuals before and after conditioning. Additionally, Philips et al. [1998] monitored serum cortisol, which only measures the immediate, acute stress response, whereas our longitudinal non-invasive assessment in female Indian rhinos compared pooled values of FGM.

Systematic evaluations of operant conditioning programs on welfare have not yet been investigated in ungulate species. This study is the first to provide coordinated physiological and behavioral evidence that positive reinforcement operant conditioning is not stressful and sets the stage for research into positive welfare benefits of operant conditioning in ungulates. Considering the Indian rhinos moved from pasture to barn in the operant conditioning study and were introduced to transrectal ultrasounds, the behavioral repertoire remained fairly consistent throughout the program. Both rhinos spent the majority of the total time observed eating, performing affiliative behaviors, locomoting, and being alert. Stereotypies, estrus, drinking, and eliminating all occurred infrequently, less than 2% of the time each. The frequency of these results was similar to Indian rhinos observed in an activity budget study in Orang National Park, (India) which also demonstrated high frequencies of feeding, vigilance, locomotion, and wallowing that also vary seasonally [Hazarika and Saikia, 2011]. Each rhino exhibited only one behavioral change throughout the course of the study. Rhino 5 demonstrated increased stereotypy; primarily horn rubbing, from 0% of observations on pasture and before training to approximately 3% of observations in the barn and during training. Horn rubbing is often observed in rhinos [Hutchins and Kreger, 2006]. Though the effect of operant conditioning on horn rubbing cannot be ruled out, this finding was most likely due to the change in location and surroundings during the study. Rhino 5's horn was already significantly worn down prior to the observations, indicating that the behavior was not a novel response to operant conditioning. The stereotypic behavior may also have been related to anticipation of receiving the majority of grain diet during the conditioning session. For example, increasing meal ration frequency throughout the day reduced oral stereotypies in horses, but increased locomotive and head movement stereotypies in anticipation to feeding time [Cooper et al., 2005]. Overall, stereotypic behaviors were observed infrequently throughout the study.

Rhino 6 exhibited an "alert" behavior twice as often during the transrectal ultrasound stage of training than when being introduced to the chute. This result was most likely influenced by Rhino 6 exhibiting a high proportion of alert (50–90%) for 3 days following an intramuscular injection by veterinary staff during Stage 5. If these three data points were removed from the analysis, alert behaviors no longer differed among the stages of training.

Overall, the differences in behavior observed in the current study were justified by factors other than the operant conditioning program. These findings in conjunction with the FGM data suggest that operant conditioning provided a non-stressful means of achieving advanced husbandry behaviors. It is worth considering whether participation in a daily program that challenges individuals to meet new goals every day and widens the repertoire of regular activities can be an example of positive welfare. Cognitive research activities and training offered a substitution for negative self- and keeper-directed behaviors in chimpanzees (*Pan troglodytes*), thus having a positive impact on welfare [Herrelko, Vick, and Buchanan-Smith, 2012]. The present study also demonstrated that the reproductive assessments conducted using operant conditioning [Stoops, Pairan, and Roth, 2004; Stoops et al., 2007] were a non-stressful means to enhance captive breeding efforts, although additional research is needed to elucidate the nature of observed behavioral and adrenal changes.

Management Implications

There was considerable intra-species variability in GC with respect to changes in management, specifically translocation and operant conditioning. Individual variability in FGM responses to management have been observed in other mammalian species, including perissodactyla. White rhinos (*Ceratotherium simum*) displayed inter-individual variation in basal FGM concentrations [Metrione and Harder, 2011]. Persian onagers (*Equus hemionus onager*) also varied their GC activity after moving from large pastures to smaller yards with increased exposure to humans [Vick et al., 2012], similar to the transition from a large open enclosure, to a smaller barn that the Indian rhinos experienced. Indian rhinos and Asian elephants displayed increased but variable salivary GC activity in response to a zoo opening to the public [Menargues, Urios, and Mauri, 2008]. The variable FGM response among rhinos reported in the current study and literature indicates that management decisions based on an individual animal's observed response to novel situations, or perhaps even FGM profiles, could be preferable to making generic, species-wide assumptions.

Considering the challenges faced by the current ex situ Indian rhino population with respect to maintaining reproduction and genetic diversity, effective management strategies such as the ones described in this study can help enhance sustainability. Knowledge gained from ex situ population management practices and enhanced breeding success can augment in situ conservation initiatives by providing a more genetically stable population. Thus, this study enhanced our knowledge about the effects that common

reproductive management practices have on Indian rhino behavior and physiology.

CONCLUSION

1. An ACTH challenge was used to validate an EIA assay for UGM and FGM in the Indian rhino.
2. Female Indian rhinos demonstrated a transient increase in FGM concentrations that continued up to 9 weeks after transport to a new facility, suggesting that translocation is a significant, yet transient stressor and acclimation time should be included in management plans before introducing new factors such as conspecifics.
3. Positive reinforcement operant conditioning can obtain voluntary participation in advanced reproductive examinations via transrectal ultrasound within a 5-month time period with female Indian rhinos.
4. Although some differences in FGM were found among the stages of training, the overall findings suggest that positive reinforcement operant conditioning did not negatively impact female Indian rhinos.
5. There was considerable variability in individual hormone responses to the management strategies of translocation and operant conditioning.

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