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Noninvasive measures of stress response in African buffalo (*Syncerus caffer*) reveal an age-dependent stress response to immobilization

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Fecal glucocorticoid metabolites (FGMs) are commonly used as indicators of an animal's stress response in behavioral and ecophysiological studies. FGM assays provide a noninvasive and efficient means of assessing adrenocortical activity. We used 12 African buffalo (*Syncerus caffer*) temporarily maintained in an enclosure to evaluate 2 commercially available FGM assays as tools for assessing stress levels in buffalo in field studies. We also used the experiment to assess potential adverse effects of immobilizations on the study animals. Buffalo responded rapidly to stimulation with adrenocorticotropic hormone (ACTH), which stimulates the adrenal cortex to secrete glucocorticoids. ACTH-stimulated buffalo had higher plasma cortisol concentrations than saline-injected controls between 5 min and 1 h after injection. The ACTH-induced plasma cortisol peak was detectable in FGMs at 10–20 h post-injection. Both of the commercial test kits we evaluated were capable of detecting the ACTH-induced peak in FGM. However, the radioimmunoassay delivered more consistent detection across weeks than the enzyme immunoassay. We tested whether immobilization and handling elicited a stress response detectable by FGM, by comparing immobilized, saline-injected buffalo with controls that were not immobilized or handled. Adult buffalo mounted a stress response to immobilization and handling, whereas subadults did not, suggesting an age-related difference in response to chemical immobilization. Our study validates use of commercially available kits for quantifying FGMs under field conditions.

Key words: adrenocorticotropic hormone, capture stress, fecal glucocorticoid metabolite, immunoassays, stress physiology

Free-living animals regularly face both acute stressors, such as predator attacks, and chronic stressors, such as food deprivation or climate change (Morton and Sherman 1978; Kotrschal et al. 1998; Lima 1998; Cavigelli 1999; Creel 2001; Goymann et al. 2001; Abbott et al. 2003; Sands and Creel 2004). Chronic stressors can have detrimental impacts on the animals' health, reproductive performance, and survival (Mateo and Cavigelli 2005). Quantifying the stress that an animal is experiencing thus represents a measure of the physiological response to endogenous factors and changes in its environment that can complement behavioral and ecological approaches in assessments of animal population health.

The most direct measure of the physiological stress response is to quantify glucocorticoids in blood (Harlow et al. 1990; Widmaier et al. 1994; Wingfield et al. 1994; Hood et al. 1998; Gregory and Schmidt 2001). Using blood is not always practical in wildlife, because capture and collection of a blood sample may itself be stressful to the animal. The acute stress response to capture can thus hide the signal related to environmental stressors of interest. Capture and immobilization also disrupt the animal's normal behaviors, are expensive, and often may be hazardous for the observer (Hopster et al. 1999; Moberg and Mench 2000). Alternatively, physiological stress can be assessed noninvasively by measuring fecal glucocorticoid metabolites (FGMs)—a method often used in field-based

wildlife studies (Touma and Palme 2005; Keay et al. 2006). FGMs have been assessed in many species including various ungulate species such as our target species, African buffalo (*Syncerus caffer*—Ganswindt et al. 2012), cattle (*Bos taurus*—Palme et al. 2000), North American and European populations of red deer (*Cervus elaphus*—Wasser et al. 2000; Huber et al. 2003), and Chinnadurai et al. (2009) included African ungulate species such as giraffes (*Giraffa camelopardalis*), impala (*Aepyceros melampus*), nyala (*Tragelaphus buxtoni*), kudu (*Tragelaphus strepsiceros*), and blue wildebeest (*Connochaetes taurinus*).

FGMs are mainly determined by competitive immunoassays (Möstl et al. 2005), such as radioimmunoassays (RIAs) and enzyme immunoassays (EIAs), but more recently also by liquid chromatography–mass spectrometry. RIAs have high precision, robustness, and good performance, but because cortisol and corticosterone are absent or rare in the feces of mammals and birds (Palme et al. 2005), these assays rely on the cross-reactivity and recognition by the antibody of the group of metabolites excreted (Möstl et al. 2005). Safety regulations associated with the use of radioactive isotopes, especially the disposal of radioactive material (Sheriff et al. 2011), make RIAs less accessible than EIAs. The group-specific antibodies that have been specifically designed to measure metabolite groups in EIAs allow for use in a broad range of species (Möstl et al. 2005; Touma and Palme 2005; Palme 2012).

Despite the widespread use of FGM assays, there are species-specific differences in hormone metabolite excretion, in terms of the amount and route of FGMs excreted as well as the types of metabolites formed (Palme 2005; Palme et al. 2005; Touma and Palme 2005; Hodges et al. 2010). In ungulates, including African buffalo, the physiological stress response is mediated by the commonly measured glucocorticoid hormone, cortisol (Touma and Palme 2005), and as seen in other ruminants, also includes corticosterone (Möstl and Palme 2002). Validation experiments demonstrating that variation in FGMs reflect changes in cortisol concentration in the blood, and the time scale at which this occurs, are therefore necessary for each new target species. For African buffalo, a validation experiment was performed by Ganswindt et al. (2012), who used an in-house EIA protocol to demonstrate that FGMs correlate to glucocorticoid levels in plasma. However, validation of commercially available kits is useful, because they are accessible to researchers conducting ecological and behavioral field studies (who are not typically in a position to design their own endocrinological assays). Moreover, while the Ganswindt et al.'s (2012) study served as proof of concept for the use of FGMs in African buffalo, it was based on a single adult female and her calf. Typical ecological field studies include animals of both sexes, and across the full age spectrum for the species; as such, a validation study allowing broader inference to natural buffalo populations is desirable. Finally, an estimation of the timing of FGM excretion following a cortisol–corticosterone peak in the blood, and expected variability in this timing, is essential to designing sampling schedules in behavioral and ecological studies, and interpreting FGM data from field-collected samples.

Most often, validation experiments are performed in the target species by administering synthetic adrenocorticotropic hormone (ACTH) and monitoring cortisol production and subsequent peaks in FGM (e.g., see reviews by Touma and Palme 2005; Keay et al. 2006). Experimentally administered ACTH causes a rapid peak in blood cortisol levels followed by return to baseline after a few hours (Norris 2006). The same pattern also occurs in feces, with the onset of the peak excretion delayed by the species-specific excretion lag time (Palme et al. 1999, 2005; Palme 2005; Touma and Palme 2005; Hodges et al. 2010). Here, we report on an ACTH challenge experiment on temporarily confined wild buffalo, aimed at evaluating 2 commercially available kits (RIA and EIA) for FGM quantitation in buffalo.

Buffalo are ecologically important, nonselective bulk grazers, that influence the function of savanna ecosystems by shaping plant communities and consequently affecting other herbivores (Mcnaughton 1978). Buffalo increase plant diversity (Huntley 1991) and have been used in the management and restoration of savanna biodiversity (Olff and Ritchie 1998). They are also an important prey species for lions (*Panthera leo*) and have economic value as a key species in the hunting and tourism industries. Buffalo are highly gregarious and carry many pathogens and parasites, some of which can cause important diseases in livestock (Michel and Bengis 2012). As a result, ecology and behavior of African buffalo are studied in many contexts and a FGM assay will provide a valuable and potentially widely used tool for scientists and wildlife managers interested in this iconic species. Because of their size and fierce nature, any handling of African buffalo for management or research purposes typically involves chemical immobilization. As such, assessing whether immobilizations elicit a stress response is of immediate relevance to the husbandry of African buffalo populations.

Our study objectives were: 1) to evaluate 2 commercially available FGM assays (RIA and EIA) to confirm that FGMs reflect blood cortisol changes expected within buffalo of both sexes and across different age groups, that are experiencing stress; 2) to determine the lag time between the increase of circulating cortisol and the appearance of its metabolites in the feces of buffalo; and 3) to investigate whether immobilization and associated sampling stimulate a cortisol stress response in buffalo.

Materials and Methods

Experimental design and sample collection.—An ACTH challenge was conducted using a holding facility operated by South African National Parks' Veterinary Wildlife Services (VWS) located in Skukuza, Kruger National Park (KNP), South Africa (Fig. 1). The buffalo (n = 12) originated from a population (n = 34) previously part of a Bovine Tuberculosis-free breeding project in an 800-ha enclosure in KNP and were housed in the holding facility for 2 weeks prior to induction of experiment to allow them to become habituated to their new environment. The buffalo were then subdivided into 3 groups of 4 animals 3 days prior to the start of the experiment. Ideally,

experimental groups should match in age–sex composition of subjects; however, we were constrained in our allocation of buffalo to experimental groups by: 1) limited access to matching individuals (similar age and sex groups); 2) practical difficulty of partitioning buffalo into groups without additional immobilizations; and 3) concern about potential aggressive behavior of the older males towards the rest of the animals during confinement. As a result, our study groups are not matched for age and sex (Table 1), with the older males concentrated in a single group—which we designated as our control group (no immobilization). The lack of females in the control group is a limitation of our study design; however, we mitigated the imperfect matching in the other 2 groups by choosing a randomized crossover experimental design, in which each animal

Fig. 1.—Layout of the holding facility during the observation and sample collection of the adrenocorticotropic hormone validation challenge.

Table 1.—Summary of the African buffalo (*Syncerus caffer*) group composition (group, sex, age, treatment trial, body weight, treatment dose, and number of fecal samples collected). ACTH = adrenocorticotropic hormone; NA = not available.

Animal ID	Sex	Age (years)[a]	Treatment trial 1 (week 1)	Treatment trial 2 (week 2)	Body weight (kg)	Treatment dose (ml)	No. of fecal samples collected
Group 1							
B266	Female	9	Saline	ACTH	486	4.86	48
B252	Female	8	ACTH	Saline	490	4.90	33
G46	Female	2.5	ACTH	Saline	328	3.28	49
G53	Male	2	Saline	ACTH	262	2.62	36
Group 2							
G30	Female	2.5	Saline	ACTH	306	3.06	45
G44	Female	2	ACTH	Saline	278	2.78	45
G28	Male	3	ACTH	Saline	378	3.78	34
G29	Male	2	Saline	ACTH	300	3.00	41
Group 3							
B163	Male	9	Control	Control	NA	NA	32
B203	Male	8.5	Control	Control	NA	NA	29
G35	Male	3.5	Control	Control	NA	NA	36
G36	Male	3.5	Control	Control	NA	NA	29

[a] For analyses, all buffalo were categorized into subadults (≤ 3.5 years) or adults (> 5 years).

in turn acts as an experimental individual and as control. The buffalo were fed *Eragrostis tef* and provided with an ad libitum water supply.

A randomized crossover experimental design with an 8-day washout period between the 2 interventions (week 1 and week 2) was used. Groups 1 and 2 were treatment groups and the third group served as a control. During each trial, buffalo in groups 1 and 2 were immobilized and injected intramuscularly with either a saline solution or ACTH. Buffalo that received the saline solution during the first trial were switched to ACTH during the second trial, and vice versa (see Table 1). In both trials, the control buffalo (group 3) were not immobilized and did not receive any treatment. The experiment was designed to: 1) measure the increase in plasma cortisol concentration in response to ACTH stimulation, by comparing plasma cortisol concentration when stimulated with ACTH versus saline treatment; 2) detect the ACTH-induced plasma cortisol spike in FGMs and quantify the characteristic lag time between ACTH stimulation and elevated FGMs using 2 commercially available detection assays (RIA and EIA); and 3) investigate the possible effect of handling stress on FGM excretion, by comparing immobilized saline-injected buffalo against unhandled control animals (group 3).

Immobilization of the buffalo was conducted by VWS wildlife veterinarian, Dr. Peter Buss, according to the South African National Parks Standard Operating Procedures for the Capture, Transportation, and Maintenance in Holding Facilities of Wildlife. Each buffalo received a combination of etorphine hydrochloride (Novartis, Kempton Park, South Africa), azaperone (Janssen Pharmaceutical Ltd., Halfway House, South Africa), and hyaluronidase (Kyron Laboratories, Benrose, South Africa). This drug combination was delivered into the muscle of the rump remotely using a dart propelled by a compressed air rifle (DAN-INJECT, International S.A., Skukuza, South Africa). The doses varied between 2 and 4 mg etorphine, 50 and 80 mg azaperone, and 5,000 i.u. hyaluronidase depending on the size of the animal.

At the first immobilization, the buffalo were weighed. A synthetic ACTH analogue (Norvartis, Synacthen Depot Ampoule, 1 mg/ml) was administered intramuscularly at 0.01 ml/kg (Alam et al. 1986) and the animals treated with saline received an equivalent volume of saline. Blood was collected from the jugular vein into heparinized blood tubes prior to and at 5-min intervals for 1-h post-ACTH or saline administration. Blood samples were stored on ice and processed in the laboratory within 4–8 h of collection. Samples were centrifuged for 10 min at 3,000 rpm and plasma harvested into 3.5-ml cryotubes. Plasma samples were then stored at −20°C until the cortisol analysis was performed.

Individual buffalo were identified with colored tape placed around the horns or in some cases by using distinguishing facial markings and horn shapes. All animals also had a numbered ear tag.

Animals between 2- and 5-years-old were aged according to incisor emergence patterns (Grimsdell 1973). In older buffalo, the tooth wear of the first incisor was evaluated for age determination (Jolles 2007). The control animals that were not immobilized were aged according to Grimsdell (1973) based on body size and horn development. For statistical analysis, all buffalo were categorized into subadults (≤ 3.5 years) or adults (> 5 years) based on their reproductive status for separating the effect of reproductive hormones on glucocorticoids. Females can reach sexual maturity at 3.5 years (Pienaar 1969; Jolles 2007), whereas males can reach maturity as early as 2.5–3 years, but rarely breed until an age of 7–8 years (Pienaar 1969).

At the end of the procedure, naltrexone (the antidote to etorphine hydrochloride; 40 mg/ml, Kyron) was administered intravenously and the animal was kept under observation until fully recovered.

Fecal samples were collected at 3 time periods: prior to a trial, during immobilization, and after each trial for 6 consecutive days (Table 1 shows the number of defecations per buffalo). During immobilization, fecal samples were collected directly from the rectum, while the remaining samples were collected from the ground in the holding facility, using a scooping ladle mounted on a long pole (to avoid the observer having to enter the buffalos' enclosure; Fig. 1). The buffalo were observed continuously for the first 48 h after each trial and thereafter only during daytime. Fecal samples were only collected if: 1) the identity of the buffalo was known and 2) the location of the fecal sample was accessible (Fig. 1 shows enclosure layout and fecal collection procedure). All fecal samples (± 30 g) were taken from the center of the dung pile within 30 min post-defecation, immediately placed in a 50-ml conical tube on ice, and stored at −20°C within 1 h until analysis.

All procedures conducted during this research followed the ASM guidelines (Sikes et al. 2016) and were approved by the South African National Parks Board (Reference No. CALJM728) and Oregon State University's Animal Care and Use Committee (OSU ACUP No. 3822).

Plasma cortisol RIA.—A coated tube cortisol RIA was used to determine plasma cortisol concentrations according to manufacturer's instructions (Siemens, PITKCO-8, 2009-07-16).

Fecal sample extraction for RIA.—To prepare fecal samples for the FGM extraction, each fecal sample was thawed at room temperature and thoroughly mixed. Then a sample of 1 g (± 0.0001 g) was placed in a tube and 3 ml of 80 % methanol (Palme and Mostl 1997) was added. This sample was rotated in a vertical plane for 8 h at room temperature followed by 10-min centrifugation at 1,200 × *g*. The resulting supernatant was withdrawn and stored in cryotubes at −20°C until analysis. To determine the moisture content of each fecal sample, a portion was weighed before and after being placed in a drying oven for 48 h at 60°C.

Fecal glucocorticoid metabolite RIA.—The concentration of steroid metabolites in the extracted fecal samples was measured with a double antibody RIA (^{125}I Corticosterone RIA kit; MP Biomedicals LLC, Santa Ana, California) previously validated in several species, including cattle (*B. taurus*—Morrow et al. 2002), African elephants (*Loxodonta africana*), Roosevelt elk (*Cervus canadensis*—Wasser et al. 2000), giraffes

(*G. camelopardalis*), impala (*A. melampus*), and kudu (*T. strepsiceros*—Wasser et al. 2000; Morrow et al. 2002; Chinnadurai et al. 2009). Sample dilutions were optimized for buffalo feces (the methanolic extracts were diluted 10 times with the manufacturer's steroid diluent, Cat. No. 07-166196). Manufacturer's instructions were followed except for the following step. To account for the 1:200 dilution prescribed for the rat plasma corticosterone, the 6 corticosterone calibrators included in the RIA kit (0–1,000 ng/ml) were pre-diluted to extend the calibration range from 0 to 5 ng/ml, therefore lowering the actual concentration of each calibrator by 200 times. FGM concentrations were expressed as nanograms glucocorticoid metabolites per gram of dry feces (ng/g), by correcting for the moisture content of the feces and dilution ratios. Upon analysis, slopes ($r > 0.98$) for serial dilutions of samples remained parallel to the standard curve and within-assay variation was less than 7%.

Fecal sample extraction for EIA.—To prepare fecal samples for the metabolite extraction, each fecal sample was thawed and > 2 g was dried at 25°C under forced air until dry (approximately 12 h—Mateo and Cavigelli 2005). Once dry, 0.2 g was placed in a 15-ml screw cap tube and 1.5 ml of 99% ethanol was added. The sample was vortexed for 3 s and centrifuged for 20 min at 1,200 × *g* (Palme et al. 2000; Mateo and Cavigelli 2005). The resulting supernatant was withdrawn and stored in 1.5-ml cryotubes at −20°C until final processing within 2 weeks. Due to logistic constraints, this extraction technique differs from the one used for the RIA and follows Mateo and Cavigelli's (2005) field-friendly extraction technique. Because the extraction technique and the detection method differ, we do not directly compare the EIA and RIA but are instead asking if both are capable of detecting FGMs in African buffalo.

Fecal glucocorticoid metabolite EIA.—During final processing, the frozen supernatant was removed and the ethanol evaporated under forced air for 4–6 h. Once all ethanol was evaporated, 1,000 µl of assay buffer was added to dilute the samples to achieve dilutions likely to fall within the range of the standard curve provided in the kit. The assay was performed using the EIA kit per manufacturer instructions (Cortisol ELISA kit; Catalog #: ADI-900-071; Enzo Life Sciences Inc., Farmingdale, New York).

Effect of ACTH treatment on plasma cortisol concentrations.—To test whether buffalo responded to ACTH stimulation by increasing plasma cortisol concentration, we compared ACTH-treated buffalo with placebo buffalo that received saline. We ran the experiment twice, with treatment buffalo in week 1 ($n = 4$) used as placebo animals in week 2, and placebo buffalo in week 1 ($n = 4$) treated with ACTH in week 2. To account for the fact that each buffalo was used once as a placebo animal and once as a treatment animal, we used linear mixed-effect models (LMEM), with individual ID included as a random effect (Bates et al. 2015). We included the following independent variables as possible predictors of plasma cortisol: treatment, time, week, age ($n = 2$ adults and $n = 6$ subadults), and sex ($n = 3$ males and $n = 5$ females). All 2-way interactions containing treatment were included in the model. Visual inspection of residual plots did not reveal any obvious deviations from normality or homoscedasticity. *P*-values were obtained using the lmerTest package (Kuznetsova et al. 2016) and considered statistically significant if $P \leq 0.05$. Plasma cortisol results were expressed as nanomoles per liter (nmol/l).

Effect of ACTH treatment on FGMs.—To test whether the ACTH-induced spike in plasma cortisol was detectable in feces and to determine its time lag, FGMs (for both RIA and EIA results) in ACTH-treated and saline-injected (placebo) buffalo were compared for each 10-h time interval from ACTH or saline injection to 140 h later. LMEM was used, including individual ID as a random effect, and treatment, time, week, age ($n = 2$ adults and $n = 6$ subadults), and sex ($n = 3$ males and $n = 5$ females) as explanatory variables. As above, all 2-way interactions containing treatment were included. Visual inspection of residual plots for normality and homoscedasticity revealed the necessity to log transform the response variable (FGM). FGM results were expressed as nanograms glucocorticoid metabolites per gram of dry feces (ng/g).

We used the raw data (i.e., data not lumped into 10-h time intervals) to calculate the time to peak (in hours) and the mean peak FGM concentration (ng/g) for ACTH-treated buffalo from both RIA and EIA results. A Wilcoxon rank sum test was used to test for weekly differences in the mean peak time and peak FGM concentrations for both RIA and EIA results, as well as whether there was a significant difference in the peak time between assays.

Effect of immobilization on stress response in buffalo.—To test whether buffalo showed elevated FGM due to immobilization and handling stress, immobilized saline-injected (placebo) buffalo were compared against unhandled (group 3) controls. As above, LMEM was used to compare FGMs (for both RIA and EIA results) in saline-injected and control buffalo for each 10-h time interval. Treatment, time, week, age ($n = 4$ adults and $n = 8$ subadults), and sex ($n = 7$ males and $n = 5$ females) were all considered as possible predictors of FGMs. All 2-way interactions containing treatment were included, except for treatment × sex (due to no control females). The response variable (FGM) was log transformed to ensure no violation of the model assumptions and *P*-values were obtained as explained above.

Initial levels.—Initial plasma cortisol level was calculated as the mean plasma cortisol measured at time point 0, which was the blood sample collected before ACTH or saline injection for each immobilized buffalo during week 1 ($n = 8$) and week 2 ($n = 8$). We acknowledge that this initial level may be affected by handling or immobilization of the buffalo, but this is unavoidable for the time point in which a blood sample was obtained.

For the initial FGM level, time point "initial" was calculated as the mean of each individual from the following time points where applicable: 1) fecal samples collected prior to treatment application and 2) rectal fecal sample during immobilization.

Initial levels are shown as $\overline{X} \pm SD$. To test for differences in the mean initial levels between week 1 and week 2, a Wilcoxon Signed Rank test for paired data was used.

The statistical program R was used for statistical analysis (R Core Team 2016), including packages: lme4 (Bates

et al. 2015), lmerTest (Kuznetsova et al. 2016), and ggplot2 (Wickham 2009).

Results

ACTH treatment resulted in a rapid and marked cortisol release, measureable in plasma within 10 min post-injection during both runs of the experiment (Figs. 2a and 2b; Supplementary Data SD1). The plasma cortisol concentrations in experimental animals differed between weeks 1 and 2.

A total of 457 fecal samples were analyzed to determine whether, and when, the ACTH-induced peak in plasma cortisol was detectable in feces. Because fecal samples were collected at variable time intervals (when defecation occurred), we binned samples into 10-h time intervals to assess during which periods post-ACTH injection FGM departed from initial levels. The increase in plasma cortisol in ACTH-treated buffalo was detectable in feces between 10 and 20 h during both weeks for both RIA (Figs. 3a and 3b) and EIA. However, the detectability was not as strong during week 1 for the EIA (Figs. 3c and 3d). After accounting for week, age, sex, and buffalo ID (random effect), the average FGM concentrations (for both weeks) at time point 10.1–20 h ($\overline{X}$ = 258.75 ng/g, SD = 119.03 ng/g, LMEM, $t_{125.17}$ = 4.85, $P < 0.0001$) was 5 times higher than initial levels ($\overline{X}$ = 54.94 ng/g, SD = 21.76 ng/g, LMEM, $t_{125.17}$ = 4.85, $P < 0.0001$) for the RIA, and 14 times higher for the EIA (initial: $\overline{X}$ = 6.71 ng/g, SD = 6.88 ng/g; 10.1–20 h: $\overline{X}$ = 90.40 ng/g, SD = 82.39, LMEM, $t_{117.01}$ = 5.29, $P < 0.0001$) (Supplementary Data SD2). The average FGM concentrations at time point 20.1–30 h ($\overline{X}$ = 119.88 ng/g, SD = 61.65 ng/g, LMEM, $t_{125.13}$ = 2.35, P = 0.02) and 30.1–40 h ($\overline{X}$ = 123.91 ng/g, SD = 115.19 ng/g, LMEM, $t_{125.18}$ = 2.55, P = 0.01) was 2.25 and 2 times higher than initial levels, respectively, for the RIA and 3.67 and 3.75 times higher for the EIA (20.1–30 h: $\overline{X}$ = 19.25 ng/g, SD = 13.11 ng/g, LMEM, $t_{116.98}$ = 3.09, P = 0.002; 30.1–40 h: $\overline{X}$ = 20.23 ng/g, SD = 15.97 ng/g, LMEM, $t_{117.32}$ = 2.79, P = 0.006) (Supplementary Data SD2). Using the raw data (not the 10-h

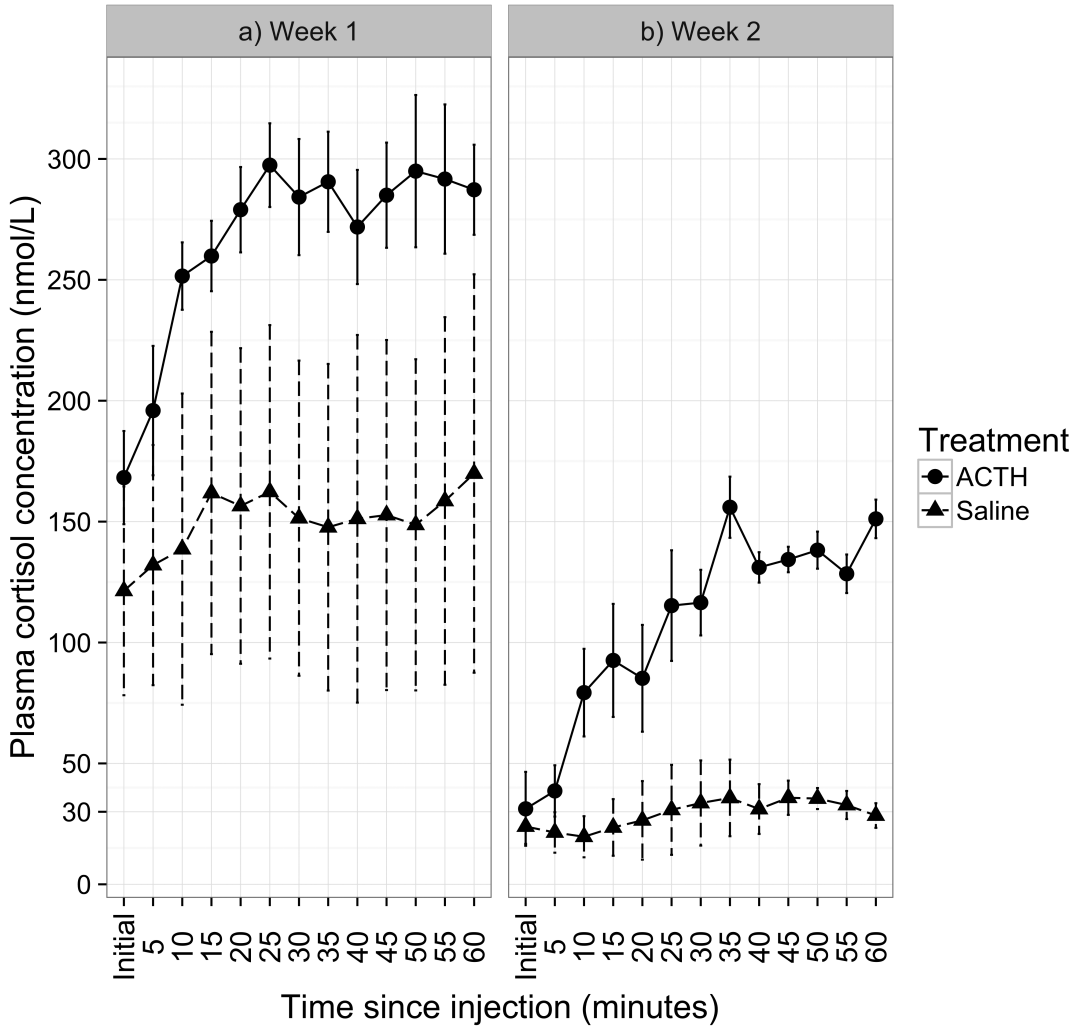

Fig. 2.—a) The mean plasma cortisol concentration as a function of time post-adrenocorticotropic hormone (ACTH) injection during week 1 and b) week 2 for ACTH (circles, solid line) and saline (triangles, dashed line) treatment groups (n = 4 per group). Time point "0" indicates initial level. ACTH-stimulated African buffalo (*Syncerus caffer*) had significantly higher plasma cortisol concentrations than saline-injected buffalo at all time points between 5 min and 1 h since application, after accounting for age, sex, week, and the random effects of individual buffalo (Supplementary Data SD1). Error bars represent *SE*.

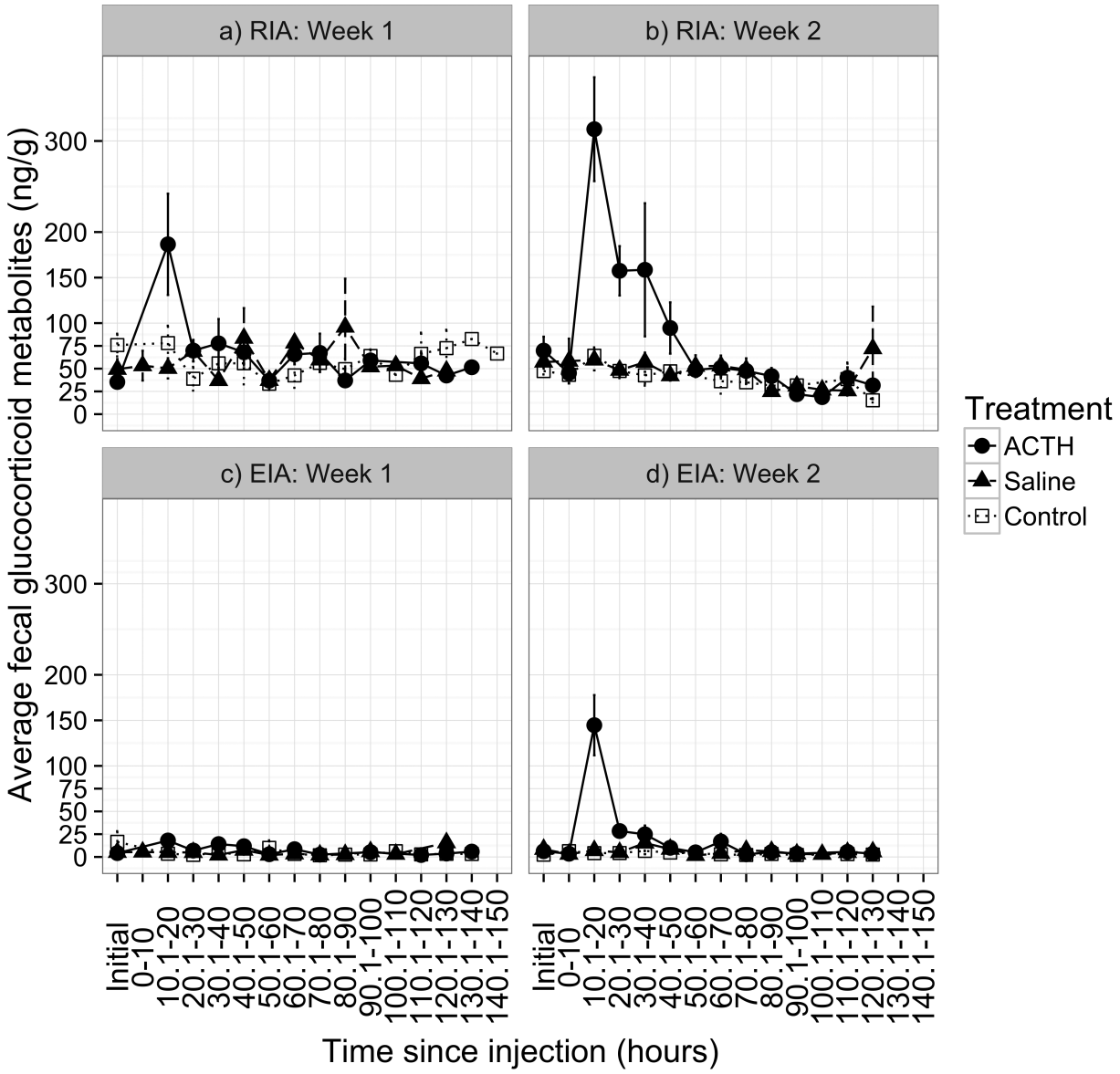

Fig. 3.—a) The mean fecal glucocorticoid metabolite (FGM, ng/g) as a function of time since treatment injection for the adrenocorticotropic hormone (ACTH; circles, solid line) and saline (triangles, dashed line) treatment groups of African buffalo (*Syncerus caffer*), as well as for the control (open squares, dotted line) group ($n = 4$ per group) during week 1, and b) week 2 from the radioimmunoassay (RIA) results. c) The mean FGM (ng/g) as a function of time since treatment injection for the ACTH (circles, solid line) and saline (triangles, dashed line) treatment groups, as well as for the control (open squares, dotted line) group ($n = 4$ per group) during week 1, and d) week 2 from the enzyme immunoassay (EIA) results. Error bars represent *SE*. The RIA detected the ACTH-induced peak in FGM consistently in both weeks. Overall, the EIA recovered less FGM than the RIA. The EIA did detect the ACTH-induced peak in FGM in both weeks, but in week 1, detection levels in all groups were comparatively low (Supplementary Data SD3).

time intervals) to obtain a finer-scale estimate of the lag time between ACTH injection and peak FGM excretion, there was no difference between weeks for either the RIA (week 1: $\bar{X} = 23$ h, $SD = 15.21$ h; week 2: $\bar{X} = 16.75$ h, $SD = 1.5$ h; Wilcoxon rank sum test, $W = 5$, $P = 0.37$; Fig. 4a) or the EIA (week 1: $\bar{X} = 28.5$ h, $SD = 13.92$ h; week 2: $\bar{X} = 16.5$ h, $SD = 1.29$ h; Wilcoxon rank sum test, $W = 3$, $P = 0.14$; Fig. 4b). There was no significant difference in peak time between the RIA and EIA (Wilcoxon rank sum test, $W = 33$, $P = 0.91$). The average time to peak (in hours) of 19.88 (95% confidence interval [*CI*] between 12.57 and 27.18) for the RIA and 22.5 (95% *CI* between 14.76 and 30.24) for the EIA coincides with the lag time found by the LMEM results of 10–20 h for both assays. However, using the raw data to obtain a finer-scale estimate of the peak FGM concentration, week 2 ($\bar{X} = 402.28$ ng/g, $SD = 100.25$ ng/g) was 2 times higher than week 1 ($\bar{X} = 181.4$ ng/g, $SD = 77.3$ ng/g; Wilcoxon rank sum test, $W = 15$, $P = 0.06$ close to significance) for the RIA (Fig. 4c), and 7 times higher for the EIA (week 1: $\bar{X} = 39.06$ ng/g, $SD = 23.28$ ng/g; week 2: $\bar{X} = 285.44$ ng/g, $SD = 162.89$ ng/g; Wilcoxon rank sum test, $W = 16$, $P = 0.03$; Fig. 4d).

The initial level of plasma cortisol was much higher in week 1 ($\bar{X} = 144.8$ nmol/l, $SD = 66.77$ nmol/l; Fig. 2a) than in week

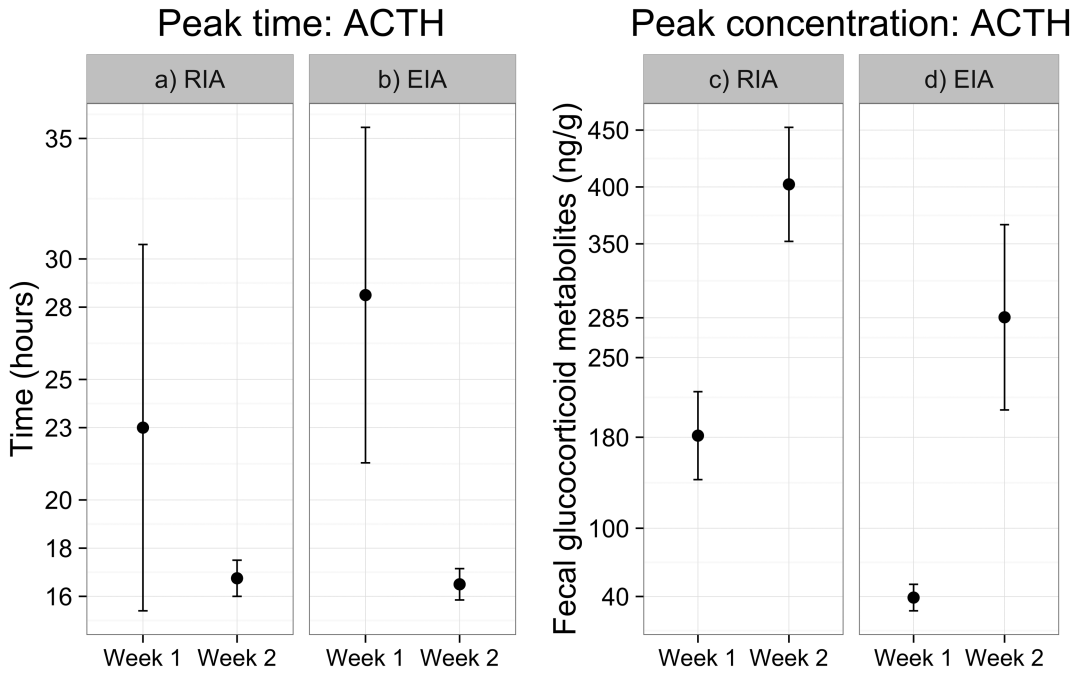

Fig. 4.—a) The average time to peak for adrenocorticotropic hormone (ACTH)-stimulated African buffalo (*Syncerus caffer*) by week for the radioimmunoassay (RIA; $n = 8$) and b) enzyme immunoassay (EIA; $n = 8$) results. Time to peak is not statistically different between weeks for both the RIA and EIA, as well as between assays. c) The average peak fecal glucocorticoid metabolite (FGM) concentration for ACTH-stimulated buffalo by week for the RIA ($n = 8$) and d) EIA ($n = 8$). Error bars represent *SE*. The RIA detected a higher peak concentration of FGMs than the EIA in both weeks, and peak FGM was lower in week 1 than week 2.

2 ($\overline{X}$ = 27.6 nmol/l, *SD* = 22.45 nmol/l; Fig. 2b), (Wilcoxon Signed Rank test, $V = 36$, $P = 0.008$, $n = 8$ pairs), and after accounting for treatment, time, age, sex, and the random effect of each animal, the plasma cortisol response was also significantly higher during week 1 than week 2 (Fig. 2; β = 142.06, $SE = 44.11$, $t_{4.12} = 3.22$, $P = 0.03$; Supplementary Data SD1). This weekly difference in the average plasma cortisol levels was not reflected in FGM, as assessed via RIA (Table 2: log β = 0.18, $SE = 0.14$, $t_{6.29} = 1.25$, $P = 0.26$; Supplementary Data SD2: log β = 0.17, $SE = 0.15$, $t_{6.37} = 1.14$, $P = 0.30$) and EIA (Table 2: log β = −0.04, $SE = 0.24$, $t_{7.14} = -0.16$, $P = 0.88$; Supplementary Data SD2: log β = −0.03, $SE = 0.25$, $t_{6.88} = \beta 0.10$, $P = 0.92$). The initial FGM levels between weeks 1 and 2 also failed to detect the drop in the initial plasma cortisol levels in both RIA (week 1: $\overline{X}$ = 51.48 ng/g, *SD* = 23.83 ng/g; week 2: $\overline{X}$ = 58.4 ng/g, *SD* = 20 ng/g; Wilcoxon Signed Rank test, $V = 39$, $P = 0.64$) and EIA (week 1: $\overline{X}$ = 7.03 ng/g, *SD* = 8.17 ng/g; week 2: $\overline{X}$ = 6.38 ng/g, *SD* = 5.73 ng/g; Wilcoxon Signed Rank test, $V = 36$, $P = 0.43$). However, the magnitude by which the average plasma cortisol levels increased 10–60 min post-ACTH injection from initial levels was lower during week 1 (week 1: $\overline{X}$ = 281.21 nmol/l, *SD* = 14.62 nmol/l, week 2: $\overline{X}$ = 117.68 nmol/l, *SD* = 25.77 nmol/l) than week 2 (2- and 4-fold, respectively; Figs. 2a and 2b): The peak FGM concentration from initial levels was 3.5-fold higher during week 1 (peak: $\overline{X}$ = 181.4 ng/g, *SD* = 77.3 ng/g; initial: $\overline{X}$ = 51.48 ng/g, *SD* = 23.83 ng/g) and 7-fold higher during week 2 (peak: $\overline{X}$ = 402.28 ng/g, *SD* = 100.25 ng/g; initial: $\overline{X}$ = 58.4 ng/g, *SD* = 20 ng/g) for the RIA. The EIA showed a 5.5- and 44-fold increase during week 1 (peak: $\overline{X}$ = 39 ng/g, *SD* = 23.28 ng/g; initial: $\overline{X}$ = 7.03 ng/g, *SD* = 8.17 ng/g) and week 2 (peak: $\overline{X}$ = 285.64 ng/g, *SD* = 162.89 ng/g; initial: $\overline{X}$ = 6.38 ng/g, *SD* = 5.73 ng/g), respectively. We found that higher initial plasma cortisol concentrations (week 1) were associated with a reduced response to ACTH stimulation, compared to week 2. This suggests an upper boundary to plasma cortisol concentrations.

Overall, immobilized saline-injected buffalo showed no significant difference in FGM concentrations compared to unhandled controls for both RIA (log β = −0.31, $SE = 0.33$, $t_{13.13} = -0.93$, $P = 0.37$) and EIA (log β = −0.98, $SE = 0.57$, $t_{14.85} = -1.71$, $P = 0.11$; Table 2). Interestingly, both datasets reveal an interaction effect of age × treatment, with adult animals responding to immobilization with elevated FGM, whereas subadults did not (RIA: log β = 0.55, $SE = 0.25$, $t_{5.25} = 2.18$, $P = 0.08$; EIA: log β = 1.25, $SE = 0.43$, $t_{6.19} = 2.87$, $P = 0.03$; Fig. 5).

Discussion

In this study, we measured FGMs as a noninvasive alternative to measuring plasma cortisol in male and female wild African buffalo aged between 2 and 9 years. The setting and logistics of our study resembled a typical ecophysiological field study, including sample handling, and delays between sample collection, FGM extraction, and detection.

Stimulation of buffalo with ACTH resulted in elevated plasma cortisol concentrations in comparison to saline-injected controls. This plasma cortisol peak was detectable in FGMs 10–20 h later, which is concordant with previous findings in

Table 2.—Summary of linear mixed-effects model for the FGMs (for both assays) comparing control and saline-treated African buffalo (*Syncerus caffer*), accounting for time, age, sex, and random effects of individual buffalo (n = 12) during both trials (weeks) of the validation experiment. Bold font indicates statistical significance. Reference levels of explanatory variables are as follow: treatment = saline; week = week 2; age = adult; sex = female; time = initial FGM level. Sample size for age category = 4 adults and 8 subadults. Sample size for sex category = 7 males and 5 females. FGM = fecal glucocorticoid metabolite; EIA = enzyme immunoassay; RIA = radioimmunoassay.

Predictor	Effect[a]	RIA (n = 162)			EIA (n = 144)		
		Log FGM (β)	t-value	P-value	Log FGM (β)	t-value	P-value
		Estimate ± SE (ng/g)			Estimate ± SE (ng/g)		
Fixed effects							
(Intercept)		**3.93 ± 0.20**	**19.33**	**< 0.0001**	**2.48 ± 0.33**	**7.46**	**<0.0001**
Treatment: control		–0.31 ± 0.33	–0.93	0.3699	–0.98 ± 0.57	–1.71	0.1078
Time: 0–10 h		0.01 ± 0.24	0.06	0.9537	–0.04 ± 0.38	–0.10	0.9221
Time: 10.1–20 h		0.10 ± 0.19	0.53	0.5948	0.05 ± 0.30	0.16	0.8710
Time: 20.1–30 h		0.07 ± 0.20	0.37	0.7089	–0.23 ± 0.31	–0.72	0.4711
Time: 30.1–40 h		–0.07 ± 0.20	–0.36	0.7178	–0.13 ± 0.33	–0.39	0.7007
Time: 40.1–50 h		0.04 ± 0.19	0.19	0.8468	0.22 ± 0.30	0.73	0.4663
Time: 50.1–60 h		–0.21 ± 0.22	–0.95	0.3455	–0.66 ± 0.38	–1.77	0.0805
Time: 60.1–70 h		0.20 ± 0.20	1.02	0.3084	–0.26 ± 0.33	–0.80	0.4258
Time: 70.1–80 h		0.02 ± 0.19	0.12	0.9026	0.08 ± 0.33	0.25	0.8006
Time: 80.1–90 h		–0.12 ± 0.24	–0.46	0.6473	0.11 ± 0.38	0.30	0.7620
Time: 90.1–100 h		–0.25 ± 0.20	–1.23	0.2199	–0.29 ± 0.31	–0.91	0.3641
Time: 100.1–110 h		–0.14 ± 0.24	–0.59	0.5596	–0.58 ± 0.38	–1.54	0.1272
Time: 110.1–120 h	↓	**–0.56 ± 0.22**	**–2.54**	**0.0125**	–0.04 ± 0.38	–0.10	0.9202
Time: 120.1–130 h		0.05 ± 0.27	0.19	0.8533	–0.21 ± 0.42	–0.50	0.6165
Time: 130.1–140 h		–0.15 ± 0.42	–0.36	0.7189	–0.60 ± 0.68	–0.89	0.3750
Time: 140.1–150 h		–0.37 ± 0.42	–0.87	0.3865			
Week: 1		0.18 ± 0.14	1.25	0.2573	–0.04 ± 0.24	–0.16	0.8772
Age: subadult	↓	–0.14 ± 0.18	–0.77	0.4673	**–1.15 ± 0.31**	**–3.73**	**0.0068**
Sex: male		0.07 ± 0.17	0.40	0.6999	–0.05 ± 0.28	–0.19	0.8546
Interaction terms							
Treatment (control):Time (0–10 h)		–0.19 ± 0.34	–0.56	0.5760	–0.30 ± 0.56	–0.54	0.5910
Treatment (control):Time (10.1–20 h)		0.14 ± 0.29	0.49	0.6251	–0.28 ± 0.49	–0.57	0.5683
Treatment (control):Time (20.1–30 h)		–0.34 ± 0.29	–1.15	0.2531	0.01 ± 0.50	0.02	0.9842
Treatment (control):Time (30.1–40 h)		–0.31 ± 0.30	–1.02	0.3113	0.15 ± 0.53	0.28	0.7784
Treatment (control):Time (40.1–50 h)		–0.32 ± 0.28	–1.15	0.2525	–0.42 ± 0.47	–0.90	0.3721
Treatment (control):Time (50.1–60 h)	↓	**–0.80 ± 0.39**	**–2.06**	**0.0411**	0.78 ± 0.64	1.22	0.2270
Treatment (control):Time (60.1–70 h)	↓	**–0.72 ± 0.32**	**–2.28**	**0.0243**	–0.20 ± 0.52	–0.39	0.6979
Treatment (control):Time (70.1–80 h)		–0.29 ± 0.28	–1.03	0.3049	–0.89 ± 0.51	–1.76	0.0815
Treatment (control):Time (80.1–90 h)		–0.34 ± 0.32	–1.06	0.2909	–0.70 ± 0.53	–1.31	0.1915
Treatment (control):Time (90.1–100 h)		–0.17 ± 0.29	–0.59	0.5571	–0.35 ± 0.50	–0.71	0.4774
Treatment (control):Time (100.1–110 h)		–0.66 ± 0.48	–1.36	0.1772	0.63 ± 0.77	0.81	0.4183
Treatment (control):Time (110.1–120 h)		0.15 ± 0.30	0.49	0.6222	–0.44 ± 0.53	–0.82	0.4121
Treatment (control):Time (120.1–130 h)	↓	**–0.81 ± 0.35**	**–2.32**	**0.0221**	–0.26 ± 0.57	–0.46	0.6489
Treatment (control):Week (1)		0.23 ± 0.17	1.31	0.2136	0.08 ± 0.30	0.26	0.7966
Treatment (control):Age (subadult)	↑	0.55 ± 0.25	2.18	0.0781	**1.25 ± 0.43**	**2.87**	**0.0273**
Random effects							
		Variance	*SD*		Variance	*SD*	
Animal ID (intercept)		0.023	0.151		0.068	0.260	
Residuals		0.149	0.385		0.365	0.604	

[a] Effect column indicates the direction of the slope predicted by the model.

an adult female of this species and her calf (Ganswindt et al. 2012). We focused on methodological approaches that could be replicated in ecological and behavioral field studies, utilizing 2 commercially available kits for FGM detection, including an RIA and an EIA kit. Both approaches yielded qualitatively similar results in terms of timing and detectability of the experimentally induced FGM peak, but the RIA delivered more consistent results across our 2 experimental periods.

Overall, 3 minor differences were observed between the 2 approaches. Firstly, the magnitude in change from initial levels to FGM peak was higher in the EIA than the RIA. Secondly, the EIA detected age-related effects more strongly than the RIA. Thirdly, the absolute levels of FGMs detected were higher for the RIA than the EIA, most likely due to the different antibody sets used in the 2 immunoassays and variation in cross-reactivity with excreted FGMs from buffalo. We used different metabolite extraction methods with the 2 kits (80% methanol for RIA, 99% ethanol for EIA) due to logistical constraints, which may also have contributed to quantitative differences in detected FGMs (Palme et al. 2013). However, studies comparing extraction methods for FGMs suggest that variation due to different extraction protocols

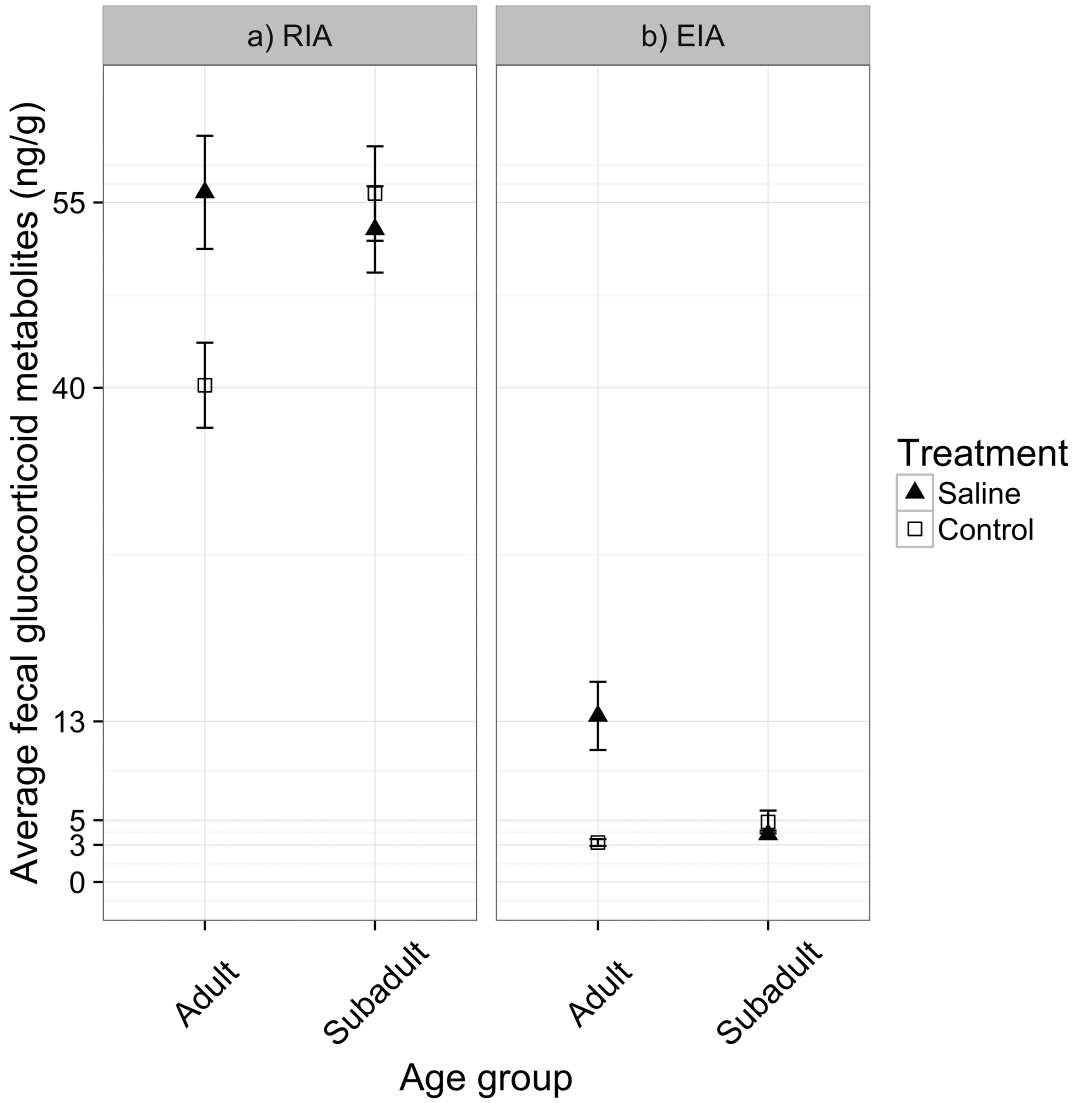

Fig. 5.—a) Summary of the average fecal glucocorticoid metabolite concentrations of immobilized, saline-treated African buffalo (*Syncerus caffer*) (triangles) and unhandled controls (open squares) across different age groups from the radioimmunoassay (RIA) results and b) the enzyme immunoassay (EIA) results shows the significant interaction term between age and treatment (Table 2). Adults, but not subadults, mounted a stress response to immobilization. Error bars represent *SE*.

are probably minor (Mateo and Cavigelli 2005). We conclude that variation in plasma cortisol can be detected, with a delay of 10–20 h, in FGM of male and female African buffalo of various ages, and that both kits we evaluated are suitable for assessing adrenocortical activity in this species under field conditions. Re-emphasizing that we do not directly compare the RIA and EIA due to different extraction techniques and detection methods, the EIA might be more sensitive in that it revealed a higher magnitude of ACTH-induced change in FGM from initial levels, and distinguished responses by different age groups of buffalo more clearly than the RIA. This sensitivity could result in positive outcomes (e.g., detect biologically important differences at a finer scale), but might also introduce more noise in variable datasets, potentially obscuring real differences among groups or time periods. As such, the RIA tended to return more consistent findings across weeks, which may help compensate for the logistical challenges associated with the use of radioactive materials. Moreover, the RIA detected higher FGM concentrations overall, which could be an advantage in situations where FGM levels approach the EIA's lower detection limit.

The passage rate of digesta plays an important role in determining the time course of the excretion of steroids (Palme et al. 1996; Wasser et al. 2000). Seasonal changes in feed intake and pasture digestibility can affect the transit time of digesta passing between the bile duct and the rectum (Möstl et al. 1999; Palme et al. 2000). For example, in an ACTH stimulation experiment in cattle, the median gut passage time was 16.6 h during autumn and 9.8 h during spring, reflected in an ACTH peak in FGMs at 14–18 h in autumn and 8–9 h in spring (Morrow et al. 2002). The faster rate of passage in spring (wet season) was associated with higher consumption of feed, increased pasture digestibility, increased fecal output, and decreased dry matter content (Morrow et al. 2002). In free-ranging buffalo in the dry season, one might thus expect the time delay from plasma cortisol to FGMs to be longer than observed in our study, because

moisture content of forage and water intake in the dry season are lower than those experienced by our experimental animals. In the wet season, free-ranging buffalo may have a quicker gut passage time, and shorter lag between cortisol release and detectable FGM peak than observed here.

Initial and stimulated plasma cortisol concentrations were much higher in week 1 of this study than in week 2. Even though we allowed a 2-week period for the animals to adjust to their confinement prior to initiating the experiment, it is possible that the process of habituation to their situation was ongoing during the experiment, resulting in lower plasma cortisol concentrations in week 2. In addition, they may have been responding to increased human presence during the experiment in week 1 but may have felt less perturbed by our observer by week 2. Alternatively, the observed higher stress levels in week 1 may have been associated with high burdens of coccidian parasites detected in the buffalo at the time. We treated this infection by adding coccidiostatic drugs to the buffalos' drinking water, and by week 2 of the experiment, parasite burdens were greatly reduced.

Animals with elevated baseline glucocorticoid concentrations often show a weaker acute glucocorticoid response (Creel 2001; Romero et al. 2009). We observed a similar pattern, where the higher initial plasma cortisol concentrations during week 1 were associated with a lower change in magnitude post-ACTH injection compared to week 2. Our results are thus consistent with the idea that animals experiencing constitutively less stressful conditions are more able to respond to acute stressors.

The differences in overall plasma cortisol concentrations between week 1 and week 2 were not detected in the FGMs, because the magnitude of differences was small or the variability among animals was large. In this study, the experimental manipulation was detectable, because changes were consistent across animals (e.g., experimental: treatment response across similar conditions), and they were large (change in magnitude from initial levels to peak concentrations), whereas the initial differences between week 1 and 2 were not detectable with FGMs, because they were variable across animals, but relatively small.

Capture, confinement and handling typically increase endogenous glucocorticoid levels in wild animals (Hamilton and Weeks 1985; Cook et al. 1996; Hopster et al. 1999; Harper and Austad 2000; Millspaugh et al. 2001). Indeed, avoiding the immediate stress response due to capture and handling is one of the reasons for measuring stress responses noninvasively in fecal material rather than blood. In this study, we showed that responses to immobilization and handling are age-dependent in African buffalo: immobilized, saline-injected adults showed elevated FGMs compared to control animals that were not immobilized, whereas subadults mounted no detectable stress response to immobilization and handling. The adult buffalo used in this experiment had been captured using chemical immobilization on 8–10 previous occasions (P. Buss, SANPARKS, Veterinary Wildlife Services, Kruger National Park, South Africa, pers. comm.), whereas the subadults were naive to the experience. It is therefore possible that prior capture experience may moderate how stressful a capture event is perceived by buffalo. Similar results were found by Moore et al. (2000), where mature male red-sided garter snakes (*Thamnophis sirtalis parietalis*) showed greater elevation of FGMs following capture stress than younger individuals. The relationship between age and FGM concentrations also varies considerably across studies, from no effect in Alaskan brown bears (*Ursus arctos horribilis*—von der Ohe et al. 2004), African elephants (*L. africana*—Viljoen et al. 2008), Egyptian spiny mice (*Acomys cahirinus*—Nováková et al. 2008), and meerkats (*Suricata suricatta*—Braga Goncalves et al. 2016) to a positive correlation in elk (*C. elaphus*—Creel et al. 2002) and a negative correlation in male Alpine chamois (*Rupicapra* spp.—Corlatti et al. 2014). Therefore, the observed differences in response to capture may simply reflect age-related differences in behavior and stress physiology that are unrelated to the animals' experience of capture and immobilization.

This study confirms that stress responses can be assessed noninvasively via analysis of glucocorticoid metabolites in feces in wild African buffalo of both sexes and across a broad spectrum of ages. Both of the commercially available FGM assay kits we evaluated were suitable for FGM detection in a typical ecological field study context. Our work also points to age- or experience-related variation among buffalo in stress response to chemical immobilization. We believe that our findings will provide a practical contribution facilitating ecophysiological research and conservation management of this key grazer species in sub-Saharan African savanna biomes.

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Supplementary Data

Supplementary data are available at *Journal of Mammalogy* online.

Supplementary Data SD1.—Model output for plasma cortisol concentration results during both trials (weeks) of the experiment.

Supplementary Data SD2.—Model output for the FGMs (for both assays) comparing ACTH- and saline-treated buffalo, during both trials (weeks) of the experiment.

Supplementary Data SD3.—a) The mean FGM (ng/g) as a function of time since treatment injection for the ACTH (circles, solid line) and saline (triangles, dashed line) treatment groups, as well as for the control (open squares, dotted line) group ($n = 4$ per group) during week 1 and b) week 2 from the RIA results. c) The mean FGM (ng/g) as a function of time since treatment injection for the ACTH (circles, solid line) and saline (triangles, dashed line) treatment groups, as well as for the control (open squares, dotted line) group ($n = 4$ per group) during week 1 and d) week 2 from the EIA results. Error bars represent *SE*. Note variable scales on the y-axes.

Literature Cited

Abbott, D. H., et al. 2003. Are subordinates always stressed? A comparative analysis of rank differences in cortisol levels among primates. Hormones and Behavior 43:67–82.

Alam, M. G., H. Dobson, and R. J. Fitzpatrick. 1986. Endocrine response to different doses of ACTH in cows. The British Veterinary Journal 142:239–245.

Bates, D., M. Mächler, B. Bolker, and S. Walker. 2015. Fitting linear mixed-effects models using lme4. Journal of Statistical Software 67:1–48.

Braga Goncalves, I., et al. 2016. Validation of a fecal glucocorticoid assay to assess adrenocortical activity in meerkats using physiological and biological stimuli. PLoS ONE 11:e0153161.

Cavigelli, S. A. 1999. Behavioural patterns associated with faecal cortisol levels in free-ranging female ring-tailed lemurs, *Lemur catta*. Animal Behaviour 57:935–944.

Chinnadurai, S. K., et al. 2009. Validation of fecal glucocorticoid metabolite assays for South African herbivores. Journal of Wildlife Management 73:1014–1020.

Cook, N. J., A. L. Schaefer, P. Lepage, and S. M. Jones. 1996. Salivary vs. serum cortisol for the assessment of adrenal activity in swine. Canadian Journal of Animal Science 76:329–335.

Corlatti, L., R. Palme, and S. Lovari. 2014. Physiological response to etho-ecological stressors in male Alpine chamois: timescale matters! Die Naturwissenschaften 101:577–586.

Creel, S. 2001. Social dominance and stress hormones. Trends in Ecology & Evolution 16:491–497.

Creel, S., J. E. Fox, A. Hardy, J. Sands, B. Garrott, and R. O. Peterson. 2002. Snowmobile activity and glucocorticoid stress responses in wolves and elk. Conservation Biology 16:809–814.

Ganswindt, A., A. S. W. Tordiffe, E. Stam, M. J. Howitt, and F. Jori. 2012. Determining adrenocortical activity as a measure of stress in African buffalo (*Syncerus caffer*) based on faecal analysis. African Zoology 47:261–269.

Goymann, W., et al. 2001. Social, state-dependent and environmental modulation of faecal corticosteroid levels in free-ranging female spotted hyenas. Proceedings of the Royal Society of London, Series B: Biological Sciences 268:2453–2459.

Gregory, L. F., and J. R. Schmid. 2001. Stress responses and sexing of wild Kemp's ridley sea turtles (*Lepidochelys kempii*) in the northeastern Gulf of Mexico. General and Comparative Endocrinology 124:66–74.

Grimsdell, J. J. R. 1973. Age determination of the African buffalo, *Syncerus caffer* Sparrman. African Journal of Ecology 11:31–53.

Hamilton, G. D., and H. P. Weeks, Jr. 1985. Cortisol and aldosterone comparisons of cottontail rabbits collected by shooting, trapping, and falconry. Journal of Wildlife Diseases 21:40–42.

Harlow, H. J., T. D. I. Beck, L. M. Walters, and S. S. Greenhouse. 1990. Seasonal serum glucose, progesterone, and cortisol levels of black bears (*Ursus americanus*). Canadian Journal of Zoology 68:183–187.

Harper, J. M., and S. N. Austad. 2000. Fecal glucocorticoids: a noninvasive method of measuring adrenal activity in wild and captive rodents. Physiological and Biochemical Zoology 73:12–22.

Hodges, K., J. Brown, and M. Heistermann. 2010. Endocrine monitoring of reproduction and stress. The University of Chicago Press, Chicago, Illinois.

Hood, L. C., P. D. Boersma, and J. C. Wingfield. 1998. The adrenocortical response to stress in incubating Magellanic penguins (*Spheniscus magellanicus*). The Auk 115:76–84.

Hopster, H., J. T. Van Der Werf, J. H. Erkens, and H. J. Blokhuis. 1999. Effects of repeated jugular puncture on plasma cortisol concentrations in loose-housed dairy cows. Journal of Animal Science 77:708–714.

Huber, S., R. Palme, W. Zenker, and E. Möstl. 2003. Non-invasive monitoring of the adrenocortical response in red deer. The Journal of Wildlife Management 67:258–266.

Huntley, B. 1991. How plants respond to climate change: migration rates, individualism and the consequences for plant communities. Annals of Botany 67:15–22.

Jolles, A. E. 2007. Population biology of African buffalo (*Syncerus caffer*) at Hluhluwe-iMfolozi Park, South Africa. African Journal of Ecology 45:398–406.

Keay, J. M., J. Singh, M. C. Gaunt, and T. Kaur. 2006. Fecal glucocorticoids and their metabolites as indicators of stress in various mammalian species: a literature review. Journal of Zoo and Wildlife Medicine 37:234–244.

Kotrschal, K., K. Hirschenhauser, and E. Möstl. 1998. The relationship between social stress and dominance is seasonal in greylag geese. Animal Behaviour 55:171–176.

Kuznetsova, A., P. B. Brockhoff, and R. H. B. Christensen. 2016. lmerTest: tests in linear mixed effects models. R package version 2.0-32. https://CRAN.R-project.org/package=lmerTest.

Lima, S. L. 1998. Nonlethal effects in the ecology of predator-prey interactions. BioScience 48:25–34.

Mateo, J. M., and S. A. Cavigelli. 2005. a validation of extraction methods for noninvasive sampling of glucocorticoids in free-living ground squirrels. Physiological and Biochemical Zoology 78:1069–1084.

Mcnaughton, S. 1978. Serengeti ungulates—feeding selectivity influences effectiveness of plant defense guilds. Science 199:806–807.

Michel, A. L., and R. G. Bengis. 2012. The African buffalo: a villain for inter-species spread of infectious diseases in southern Africa. The Onderstepoort Journal of Veterinary Research 79:453.

Millspaugh, J. J., et al. 2001. Fecal glucocorticoid assays and the physiological stress response in elk. Wildlife Society Bulletin 29:899–907.

Moberg, G. P., and J. A. Mench. 2000. The biology of animal stress: basic principles and implications for animal welfare. CABI Publishing, New York.

Moore, I. T., M. P. Lemaster, and R. T. Mason. 2000. Behavioural and hormonal responses to capture stress in the male red-sided

garter snake, *Thamnophis sirtalis parietalis*. Animal Behaviour 59:529–534.

Morrow, C. J., E. S. Kolver, G. A. Verkerk, and L. R. Matthews. 2002. Fecal glucocorticoid metabolites as a measure of adrenal activity in dairy cattle. General and Comparative Endocrinology 126:229–241.

Morton, M. L., and P. W. Sherman. 1978. Effects of a spring snowstorm on behavior, reproduction, and survival of Belding's ground squirrels. Canadian Journal of Zoology 56:2578–2590.

Möstl, E., S. Messmann, E. Bagu, C. Robia, and R. Palme. 1999. Measurement of glucocorticoid metabolite concentrations in faeces of domestic livestock. Journal of Veterinary Medicine Series A 46:621–631.

Möstl, E., and R. Palme. 2002. Hormones as indicators of stress. Domestic Animal Endocrinology 23:67–74.

Möstl, E., S. Rettenbacher, and R. Palme. 2005. Measurement of corticosterone metabolites in birds' droppings: an analytical approach. Annals of the New York Academy of Sciences 1046:17–34.

Norris, D. O. 2006. Vertebrate endocrinology. Elsevier Academic Press, Burlington, Massachusetts.

Nováková, M., R. Palme, H. Kutalová, L. Janský, and D. Frynta. 2008. The effects of sex, age and commensal way of life on levels of fecal glucocorticoid metabolites in spiny mice (*Acomys cahirinus*). Physiology & Behavior 95:187–193.

Olff, H., and M. E. Ritchie. 1998. Effects of herbivores on grassland plant diversity. Trends in Ecology & Evolution 13:261–265.

Palme, R. 2005. Measuring fecal steroids: guidelines for practical application. Annals of the New York Academy of Sciences 1046:75–80.

Palme, R. 2012. Monitoring stress hormone metabolites as a useful, non-invasive tool for welfare assessment in farm animals. Animal Welfare 21:331–337.

Palme, R., P. Fischer, H. Schildorfer, and M. N. Ismail. 1996. Excretion of infused ^{14}C-steroid hormones via faeces and urine in domestic livestock. Animal Reproduction Science 43:43–63.

Palme, R., and E. Mostl. 1997. Measurement of cortisol metabolites in faeces of sheep as a parameter of cortisol concentration in blood. Zeitschrift Fur Saugetierkunde-International Journal of Mammalian Biology 62:192–197.

Palme, R., S. Rettenbacher, C. Touma, S. M. El-Bahr, and E. Möstl. 2005. Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion, and non-invasive measurement in fecal samples. Annals of the New York Academy of Sciences 1040:162– 171.

Palme, R., C. Robia, W. Baumgartner, and E. Möstl. 2000. Transport stress in cattle as reflected by an increase in faecal cortisol metabolite concentrations. The Veterinary Record 146:108–109.

Palme, R., C. Robia, S. Messmann, J. Hofer, and E. Mostl. 1999. Measurement of faecal cortisol metabolites in ruminants: a non-invasive parameter of adrenocortical function. Wiener Tierarztliche Monatsschrift 86:237–241.

Palme, R., C. Touma, N. Arias, M. Dominchin, and M. Lepschy. 2013. Steroid extraction: get the best out of faecal samples. Wiener Tierärztliche Monatsschrift 100:238–246.

Pienaar, U. D. V. 1969. Observation on developmental biology, growth and some aspects of the population ecology of the African buffalo (*Syncerus caffer* Sparrman) in the Kruger National Park. Koedoe 12:29–52.

R Core Team. 2016. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Romero, L. M., M. J. Dickens, and N. E. Cyr. 2009. The reactive scope model—a new model integrating homeostasis, allostasis, and stress. Hormones and Behavior 55:375–389.

Sands, J., and S. Creel. 2004. Social dominance, aggression and faecal glucocorticoid levels in a wild population of wolves, *Canis lupus*. Animal Behaviour 67:387–396.

Sheriff, M. J., B. Dantzer, B. Delehanty, R. Palme, and R. Boonstra. 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia 166:869–887.

Sikes, R. S., and the Animal Care and Use Committee of the American Society of Mammalogists. 2016. 2016 Guidelines of the American Society of Mammalogists for the use of wild mammals in research and education. Journal of Mammalogy 97:663–688.

Touma, C., and R. Palme. 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation. Annals of the New York Academy of Sciences 1046:54–74.

Viljoen, J. J., A. Ganswindt, R. Palme, H. C. Reynecke, J. T. Du Toit, and W. R. Langbauer, Jr. 2008. Measurement of concentrations of faecal glucocorticoid metabolites in free-ranging African elephants within the Kruger National Park. Koedoe 50:18–21.

Von Der Ohe, C. G., S. K. Wasser, K. E. Hunt, and C. Servheen. 2004. Factors associated with fecal glucocorticoids in Alaskan brown bears (*Ursus arctos horribilis*). Physiological and Biochemical Zoology 77:313–320.

Wasser, S. K., et al. 2000. a generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. General and Comparative Endocrinology 120:260–275.

Wickham, H. 2009. ggplot2: elegant graphics for data analysis. Springer, New York.

Widmaier, E. P., T. L. Harmer, A. M. Sulak, and T. H. Kunz. 1994. Further characterization of the pituitary-adrenocortical responses to stress in Chiroptera. The Journal of Experimental Zoology 269:442–449.

Wingfield, J. C., R. Suydam, and K. Hunt. 1994. The adrenocortical responses to stress in snow buntings (*Plectrophenax nivalis*) and Lapland longspurs (*Calcarius lapponicus*) at Barrow, Alaska. Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology 108:299–306.

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