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
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 buf-
falo 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 immo-
bilizations; 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 randomised 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.](https://academic.oup.com/jmammal/article-abstract/98/5/1288/4096804/Noninvasive-measures-of-stress-response-in-African)

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<th>Treatment trial 2 (week 2)</th>
<th>Body weight (kg)</th>
<th>Treatment dose (ml)</th>
<th>No. of fecal samples collected</th>
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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 (Kyro 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 (Novartis, Synachten 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, PTKCO-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 ≤ 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 X ± 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, measurable 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 ($\bar{X} = 258.75$ ng/g, $SD = 119.03$ ng/g, LMEM, $t_{125.17} = 4.85, P < 0.0001$) was 5 times higher than initial levels ($\bar{X} = 54.94$ ng/g, $SD = 21.76$ ng/g; 10.1–20 h: $\bar{X} = 90.40$ ng/g, $SD = 82.39$, LMEM, $t_{17.01} = 5.29, P < 0.0001$) (Supplementary Data SD2). The average FGM concentrations at time point 20.1–30 h ($\bar{X} = 119.88$ ng/g, $SD = 61.65$ ng/g, LMEM, $t_{25.14} = 2.35, P = 0.02$) and 30.1–40 h ($\bar{X} = 123.91$ ng/g, $SD = 115.19$ ng/g, LMEM, $t_{25.18} = 2.55, P = 0.01$) 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: $\bar{X} = 19.25$ ng/g, $SD = 13.11$ ng/g, LMEM, $t_{16.98} = 3.09, P = 0.002$; 30.1–40 h: $\bar{X} = 20.23$ ng/g, $SD = 15.97$ ng/g, LMEM, $t_{17.32} = 2.79, P = 0.006$) (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.](#)
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 (\(\bar{X} = 27.6\) 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; \(t = 0.14, SE = 0.24, t_{6.14} = 3.22, P = 0.03;\) Supplementary Data SD1). This weekly difference in the average plasma cortisol levels was not reflected in FGM, as assessed via RIA (Table 2: log \(\beta = 0.18, SE = 0.14, t_{6.26} = 1.25, P = 0.26;\) Supplementary Data SD2: log \(\beta = 0.17, SE = 0.15, t_{6.37} = 1.14, P = 0.30) and EIA (Table 2: log \(\beta = −0.04, SE = 0.24, t_{6.12} = −0.16, P = 0.88;\) Supplementary Data SD2: log \(\beta = −0.03, SE = 0.25, t_{6.98} = 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: \(\bar{X} = 51.48\) ng/g, \(SD = 23.83\) ng/g; week 2: \(\bar{X} = 58.4\) ng/g, \(SD = 20\) ng/g; Wilcoxon Signed Rank test, \(V = 39, P = 0.64\) and EIA (week 1: \(\bar{X} = 7.03\) ng/g, \(SD = 8.17\) ng/g; week 2: \(\bar{X} = 6.38\) 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: \(\bar{X} = 281.21\) nmol/l, \(SD = 14.62\) nmol/l; week 2: \(\bar{X} = 117.68\) 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: \(\bar{X} = 181.4\) ng/g, \(SD = 77.3\) ng/g; initial: \(\bar{X} = 51.48\) ng/g, \(SD = 23.83\) ng/g) and 7-fold higher during week 2 (peak: \(\bar{X} = 402.28\) ng/g, \(SD = 100.25\) ng/g; initial: \(\bar{X} = 58.4\) ng/g, \(SD = 20\) ng/g) for the RIA. The EIA showed a 5.5- and 44-fold increase during week 1 (peak: \(\bar{X} = 39\) ng/g, \(SD = 23.28\) ng/g; initial: \(\bar{X} = 7.03\) ng/g, \(SD = 8.17\) ng/g) and week 2 (peak: \(\bar{X} = 285.64\) ng/g, \(SD = 162.89\) ng/g; initial: \(\bar{X} = 6.38\) 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 \(\beta = −0.31, SE = 0.33, t_{6.13} = −0.93, P = 0.37\) and EIA (log \(\beta = −0.98, SE = 0.57, t_{6.45} = 1.71, P = 0.11;\) Table 2). Interestingly, both datasets reveal an interaction effect of age \(\times\) treatment, with adult animals responding to immobilization with elevated FGM, whereas subadults did not (RIA: log \(\beta = 0.55, SE = 0.25, t_{6.25} = 2.18, P = 0.08\); EIA: log \(\beta = 1.25, SE = 0.43, t_{6.19} = 2.87, P = 0.03;\) 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

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**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.
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.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Effect</th>
<th>RIA (<em>n = 162</em>)</th>
<th>EIA (<em>n = 144</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Log FGM (β)</td>
<td>t-value</td>
<td>P-value</td>
</tr>
<tr>
<td>(Intercept)</td>
<td>Estimate ± SE (ng/g)</td>
<td>&lt; 0.0001</td>
<td>-0.98 ± 0.57</td>
</tr>
<tr>
<td>Treatment: control</td>
<td>-0.31 ± 0.33</td>
<td>-0.93</td>
<td>0.3699</td>
</tr>
<tr>
<td>Time: 0–10 h</td>
<td>0.01 ± 0.24</td>
<td>0.06</td>
<td>0.9537</td>
</tr>
<tr>
<td>Time: 10.1–20 h</td>
<td>0.10 ± 0.19</td>
<td>0.53</td>
<td>0.5948</td>
</tr>
<tr>
<td>Time: 20.1–30 h</td>
<td>0.07 ± 0.20</td>
<td>0.37</td>
<td>0.7089</td>
</tr>
<tr>
<td>Time: 30.1–40 h</td>
<td>-0.07 ± 0.20</td>
<td>-0.36</td>
<td>0.7178</td>
</tr>
<tr>
<td>Time: 40.1–50 h</td>
<td>0.04 ± 0.19</td>
<td>0.19</td>
<td>0.8468</td>
</tr>
<tr>
<td>Time: 50.1–60 h</td>
<td>-0.21 ± 0.22</td>
<td>-0.95</td>
<td>0.3455</td>
</tr>
<tr>
<td>Time: 60.1–70 h</td>
<td>0.20 ± 0.20</td>
<td>1.02</td>
<td>0.3084</td>
</tr>
<tr>
<td>Time: 70.1–80 h</td>
<td>0.02 ± 0.19</td>
<td>0.12</td>
<td>0.9026</td>
</tr>
<tr>
<td>Time: 80.1–90 h</td>
<td>-0.12 ± 0.24</td>
<td>-0.46</td>
<td>0.6473</td>
</tr>
<tr>
<td>Time: 90.1–100 h</td>
<td>-0.25 ± 0.20</td>
<td>-1.23</td>
<td>0.2199</td>
</tr>
<tr>
<td>Time: 100.1–110 h</td>
<td>-0.14 ± 0.24</td>
<td>-0.59</td>
<td>0.5596</td>
</tr>
<tr>
<td>Time: 110.1–120 h</td>
<td>↓ -0.56 ± 0.22</td>
<td>-2.54</td>
<td>0.0125</td>
</tr>
<tr>
<td>Time: 120.1–130 h</td>
<td>0.05 ± 0.27</td>
<td>0.19</td>
<td>0.8533</td>
</tr>
<tr>
<td>Time: 130.1–140 h</td>
<td>-0.15 ± 0.42</td>
<td>-0.36</td>
<td>0.7189</td>
</tr>
<tr>
<td>Time: 140.1–150 h</td>
<td>-0.37 ± 0.42</td>
<td>-0.87</td>
<td>0.3865</td>
</tr>
<tr>
<td>Week: 1</td>
<td>0.18 ± 0.14</td>
<td>1.25</td>
<td>0.2573</td>
</tr>
<tr>
<td>Age: subadult</td>
<td>↓ -0.14 ± 0.18</td>
<td>-0.77</td>
<td>0.4673</td>
</tr>
<tr>
<td>Sex: male</td>
<td>0.07 ± 0.17</td>
<td>0.40</td>
<td>0.6999</td>
</tr>
<tr>
<td>Interaction terms</td>
<td>Treatment (control): Time (0–10 h)</td>
<td>-0.19 ± 0.34</td>
<td>-0.56</td>
</tr>
<tr>
<td>Treatment (control): Time (10.1–20 h)</td>
<td>0.14 ± 0.29</td>
<td>0.49</td>
<td>0.6251</td>
</tr>
<tr>
<td>Treatment (control): Time (20.1–30 h)</td>
<td>-0.34 ± 0.29</td>
<td>-1.15</td>
<td>0.2531</td>
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<tr>
<td>Treatment (control): Time (30.1–40 h)</td>
<td>-0.31 ± 0.30</td>
<td>-1.02</td>
<td>0.3113</td>
</tr>
<tr>
<td>Treatment (control): Time (40.1–50 h)</td>
<td>-0.32 ± 0.28</td>
<td>-1.15</td>
<td>0.2525</td>
</tr>
<tr>
<td>Treatment (control): Time (50.1–60 h)</td>
<td>↓ -0.80 ± 0.39</td>
<td>-2.06</td>
<td>0.0411</td>
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<td>Treatment (control): Time (60.1–70 h)</td>
<td>↓ -0.72 ± 0.32</td>
<td>-2.28</td>
<td>0.0243</td>
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<tr>
<td>Treatment (control): Time (70.1–80 h)</td>
<td>-0.29 ± 0.28</td>
<td>-1.03</td>
<td>0.3049</td>
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<tr>
<td>Treatment (control): Time (80.1–90 h)</td>
<td>-0.34 ± 0.32</td>
<td>-1.06</td>
<td>0.2909</td>
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<tr>
<td>Treatment (control): Time (90.1–100 h)</td>
<td>-0.17 ± 0.29</td>
<td>-0.59</td>
<td>0.5571</td>
</tr>
<tr>
<td>Treatment (control): Time (100.1–110 h)</td>
<td>-0.66 ± 0.48</td>
<td>-1.36</td>
<td>0.1772</td>
</tr>
<tr>
<td>Treatment (control): Time (110.1–120 h)</td>
<td>0.15 ± 0.30</td>
<td>0.49</td>
<td>0.6222</td>
</tr>
<tr>
<td>Treatment (control): Time (120.1–130 h)</td>
<td>↓ -0.81 ± 0.35</td>
<td>-2.32</td>
<td>0.0221</td>
</tr>
<tr>
<td>Treatment (control): Week (1)</td>
<td>0.23 ± 0.17</td>
<td>1.31</td>
<td>0.2136</td>
</tr>
<tr>
<td>Treatment (control): Age (subadult)</td>
<td>↑ 0.55 ± 0.25</td>
<td>2.18</td>
<td>0.0781</td>
</tr>
<tr>
<td>Random effects</td>
<td>Variance</td>
<td>SD</td>
<td>Variance</td>
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<tr>
<td>Animal ID (intercept)</td>
<td>0.023</td>
<td>0.151</td>
<td>0.068</td>
</tr>
<tr>
<td>Residuals</td>
<td>0.149</td>
<td>0.385</td>
<td>0.365</td>
</tr>
</tbody>
</table>

* Effect column indicates the direction of the slope predicted by the model.

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...
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

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.
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; Millsapgh 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 naïve 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—von der Ohe et al. 2004), African elephants (Loxodonta africana—Viljoen et al. 2008), Egyptian spiny mice (Acomys cahirinus—Novákova 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.

ACKNOWLEDGMENTS
Thanks to the assistance from South African National Parks (SANPARKS) for allowing us to conduct this project in Kruger National Park (KNP). We are grateful to all assistance from the Game Capture Team (KNP) as well as Veterinary Wildlife Services and Dr. K. Thompson. Thanks to E. Caldo for her assistance and support during the observation trials and to R. Spaan for his invaluable support and assistance. Thanks to S. Carrera for her assistance in running the cortisol extractions for the EIA. The EIA portion of this research was funded by the United States Department of Agriculture-National Institute of Food Agriculture Agriculture and Food Research Institute Grant (2013-67015-21291). This research was supported in part by a National Science Foundation Ecology of Infectious Diseases Grant awarded to VOE and AEJ (DEB-1102493/ EF-0723928 and EF-0723918). JMS was supported through an International Fellowship for Women in Science from UNESCO-L’Oreal.

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.

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MOORE, I. T., M. P. LEMASTER, AND R. T. MASON. 2000. Behavioral and hormonal responses to capture stress in the male red-sided...


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