Journal of Mammalogy, 103(2):394–406, 2022
https://doi.org/10.1093/jmammal/gyab168
Published online January 27, 2022

Validation and effects of drying and prey hair on fecal hormone concentrations in spotted hyenas

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As fecal steroid methods increasingly are used by researchers to monitor the physiology of captive and wild populations, we need to expand our validation protocols to test the effects of procedural variation and to identify contamination by exogenous sources of steroid hormones. Mammalian carnivore feces often contain large amounts of hair from the prey they consume, which itself may contain high concentrations of hormones. In this study, we report first a validation of two steroid hormone antibodies, corticosterone and progesterone, to determine fecal concentrations of these hormones in wild spotted hyenas (*Crocuta crocuta*). Next, we expand on these standard validation protocols to test two additional metrics: (i) whether hair from consumed prey or (ii) the specific drying method (oven incubation vs. lyophilization) affect steroid hormone concentrations in feces. In the first biological validation for the progesterone antibody in this species, progesterone concentrations met our expectations: (i) concentrations of plasma and fecal progesterone were lowest in immature females, higher in lactating females, and highest in pregnant females; (ii) across pregnant females, fecal progesterone concentrations were highest during late pregnancy; and (iii) among lactating females, fecal progesterone concentrations were highest after parturition. Our additional validation experiments indicated that contamination with prey hair and drying method are hormone-specific. Although prey hair did not release hormones into samples during storage or extraction for either hormone, its presence appeared to "dilute" progesterone (but not corticosterone) measures indirectly by increasing the dry weight of samples. In addition, fecal progesterone, but not corticosterone, values were lower for lyophilized than for incubated samples. Therefore, in addition to the standard analytical and biological validation steps, additional methodological variables need to be tested whenever we measure fecal hormone concentrations, particularly from predatory mammals.

Key words: glucocorticoid, hair, progesterone, radioimmunoassay, spotted hyena, validation

During the past three decades, noninvasive monitoring of fecal hormones has become a powerful tool for researchers and managers studying mammalian physiology and behavior in captivity and the wild. Analyses of fecal hormones and their metabolites can help researchers assess the reproductive status and the effects of social, environmental, and anthropogenic stressors on sampled individuals. Fecal hormone extraction and assay methods allow researchers to assess hormone measures without the risks and costs associated with invasive sampling via capture, handling, and chemical immobilization, which themselves can elevate hormone concentrations in blood samples. Researchers can use a variety of methods to extract hormones from fecal material, but common procedures involve adding methanol or ethanol to samples, vortexing and centrifuging, and finally measuring the hormone content in the supernatant via standard assay techniques (Wielebnowski and Watters 2007; Sheriff et al. 2011). In contrast to serum or salivary samples, fecal hormone values provide researchers with an integrated average of hormones that an animal has secreted into the blood over a daylong or multiday interval (Schwarzenberger et al. 1996; Palme et al. 2005; Sheriff et al. 2011). Experiments on storage effects show that degradation of fecal steroid hormones over time often is negligible, allowing this method to be used as a valuable tool in long-term monitoring projects, in

which samples cannot be processed immediately (Millspaugh and Washburn 2004).

As fecal hormone extraction and assay methods have become more popular, researchers recognize that they need to validate and standardize methods used in sample collection, storage, and processing. For example, several reviews highlight the importance of validating species-specific fecal hormone extraction and assay methods and the need to maintain methodological consistency in longitudinal studies (Wielebnowski and Watters 2007; Sheriff et al. 2011). Such validations have two main components. First, analytical validation is necessary to demonstrate that the antibody and assay have the accuracy, precision, and sensitivity to measure concentrations of the hormone of interest within a determined range of values. Second, biological validation is necessary to demonstrate that physiological changes in hormone concentrations are reflected in the biological matrix within which it is being measured (e.g., blood, urine, feces, etc.; Möstl et al. 2005). Because the effects of each methodological step often show species- and hormone-specific differences, researchers must undertake both analytical and biological validations for each study system and hormone of interest. However, researchers often do not test different methods for carrying out these two validations. For example, consider studies that use a "drying" step for the initial extraction. In one study on cheetahs (*Acinonyx jubatus*), researchers found that the effect of different drying methods was hormone-specific with fecal samples dried by lyophilization (i.e., freeze drying) showing significantly *higher* concentrations of glucocorticoid and estrogen metabolites and significantly *lower* concentrations of progesterone metabolites than samples dried using a solar or conventional oven (Terio et al. 2002).

Analyses of hormones in the feces of mammalian carnivores present an additional problem that is not an issue for nonpredator taxa: mainly, the hair of prey species often is present in the feces of their predators. One way that prey hair could influence hormone values measured in predator fecal samples is by adding to the overall weight of samples, effectively "diluting" the concentration of hormone determined to be in the sample. For example, the amount of indigestible material, including hair, in the fecal samples of wild wolves (*Canis lupus*) significantly affected fecal glucocorticoid values (Sands and Creel 2004). However, this material also included shards, which both are heavier than hair and far easier to remove completely from samples. Furthermore, the amount of hair in different samples is unlikely to be equal, due to variation in prey availability and differential access to meat, based on age, sex, or social status. Hair might introduce a significant source of unmeasured variation in fecal hormone concentrations. In one study, the highest coefficients of variation in samples collected from four felid species were those containing large amounts of hair (Brown et al. 1994). Although the presence of hair in carnivore fecal samples is common, researchers rarely report how they address it (for an exception, see Umapathy et al. (2007) who reported that they removed hair in the fecal samples of lions, *Panthera leo persica*, prior to incubation).

An additional concern when fecal samples contain hair is that the hair of prey species may actually "leach" their own steroid hormones into carnivore fecal samples during storage or extraction. In the last decade, we have learned that hair contains hormones in amounts proportional to what was circulating during the hair growth period (Koren et al. 2002; Davenport et al. 2006; Russell et al. 2012). Although this offers a new method to some researchers, it simultaneously presents a confounding variable for others, because we do not know how hormones in prey hair contained in a predator's feces may combine with, and therefore contaminate, the hormonal signature of the predator. To remedy the potential problem, researchers can remove hair from samples, but this can significantly add to sample processing time, and in practice it rarely is possible to completely remove all hair. Moreover, if the release of hormones from prey hair into predator fecal samples occurs during storage (i.e., before extraction), then removing hair from samples prior to extraction will not solve the problem.

In this study, we provide validations of corticosterone and progesterone antibodies for fecal hormone assays in wild spotted hyenas (*Crocuta crocuta*), and then we extend these validations to test whether drying method or the presence of prey hair affect our measurements of these hormone concentrations. Although fecal progesterone assays have been developed for some mammalian carnivores, the majority of studies have focused on captive animals, for example, felids (Brown et al. 1994; Dalerum et al. 2006), or cooperatively breeding carnivores, who often show patterns specific to reproductive suppression of nonbreeding individuals, for example, Iberian wolves (Barja et al. 2008), African wild dogs (Creel et al. 1997), and meerkats (Moss et al. 2001). The ability to use noninvasive fecal sampling to repeatedly measure concentrations of progesterone in spotted hyenas will allow us to address unanswered questions about the reproductive biology of this species. We still lack basic information about the hyena estrous cycle, such as cycle length and whether ovulation is spontaneous or induced, because females show no conspicuous signs of receptivity or reproductive state, such as changes in genital swelling or color. In fact, it is nearly impossible to visually determine if a female hyena is in late pregnancy because female body mass can increase by up to 18 kg after an individual eats a single meal (Henschel and Tilson 1988).

To validate a progesterone antibody for fecal hormone assays in spotted hyenas, we assayed hormones from both fecal and plasma samples of immature, pregnant, and lactating female hyenas, and we expected that variation in fecal progesterone concentrations would mirror those measured in plasma. We predicted that progesterone concentrations (both from feces and plasma) would be lowest in immature females and highest in pregnant females, as is the case in other mammals (Al-Hozab and Basiouni 1999; Rolland et al. 2005; Stephens and Wallen 2013). Within pregnant females, we predicted that progesterone concentrations would be highest late in gestation, based on an earlier study of hormonal changes in pregnant captive spotted hyenas (Licht et al. 1992). Finally, we predicted that progesterone concentrations in lactating females would decrease over the course of lactation, as they do in other species (Maestripieri and Megna 2000; French et al. 2004).

To examine the potential effects of drying method and presence of prey hair on the measured concentrations of corticosterone and progesterone in hyena fecal samples, we carried out a series of additional experiments. First, we investigated whether the method of drying samples, oven incubation or lyophilization, affected hormone concentrations. Second, we examined whether the presence of prey hair affected predator fecal hormone concentrations. We reasoned that if the presence of hair does not affect fecal hormone concentrations, the sample hormone concentrations would not be affected either by adding or removing hair. If hair leaches hormones into fecal samples during extraction, we predicted that hormone concentrations would be lower in samples from which hair had been removed than in matched controls. However, even if we detected no effect of hair removal during extraction, this would not rule out the possibility that the hair had already released hormones in the feces during storage prior to extraction. To investigate this possibility, we added hair to samples that naturally contained very little hair and compared hormone concentrations in these samples to samples with no hair added. If hair secretes hormones into fecal samples during storage, we predicted that hormone concentrations would be higher in samples to which hair had been added than in matched controls. Finally, hair could affect hormone concentrations simply by adding to the sample's dry weight. If this is the case, we predicted that hormone concentrations would be lower than control samples when hair was added and higher than control samples when hair was removed (Table 1).

Materials and Methods

Study site and population.—We collected samples from spotted hyenas inhabiting the Masai Mara National Reserve, Kenya. This is a tropical savanna ecosystem covered primarily by open grassland and grazed year-round by several resident ungulate species and by the annual migration of wildebeest (*Connochaetes taurinus*) and zebra (*Equus quagga*) from Serengeti National Park in Tanzania. Our sampled individuals lived in one social group, referred to as a "clan," that has been continuously monitored since 1988 by personnel from the Mara Hyena Project. We individually recognized all clan members by spot patterns, ear damage, and other distinguishing characteristics, and all samples therefore were from known individuals. Spotted hyena clans are matrilineal, female-dominated societies whose members defend a stable communal territory. Social rank determines priority of access to resources, and resource access affects reproduction in female hyenas (Holekamp et al. 1996). We observed clans daily during two observation periods, a morning period from 0600 to 1000 h, and an evening period from 1600 to 2030 h. We determined sex based on the morphology of the erect phallus (Frank et al. 1990), and estimated birthdates from the size, pelage, and behavior of cubs when they first were seen (Holekamp et al. 1996).

We assigned females to one of three reproductive categories: immature, pregnant, or lactating. We considered females to be immature from birth until their first conception. Although hyenas are reproductively mature at around 24 months, age at first conception varies greatly with social rank and resource availability (Holekamp et al. 1996). In the field, we estimated the date of a female's first parturition based on observations of tearing and scarring of the female's phallus, which occur during the birth process (Frank and Glickman 1994), or observations of a female's first litter of cubs. We then could determine the conception date by counting backwards 110 days, the length of the gestation period of this species (Kruuk 1972). We considered females to be pregnant from the estimated conception date of each litter until parturition. We divided pregnancy into three equal stages to analyze variation in excreted hormone concentrations across gestation. Early pregnancy was from conception to day 37 of gestation, middle pregnancy was from day 38 to day 74 of gestation, and late pregnancy was from day 75 until parturition. We considered females to be lactating from the day after parturition until weaning, disappearance of the litter, or conception of the subsequent litter, whichever came first. Age at weaning is highly variable in this species, ranging from 7 to 24 months, and can be dramatically affected by the mother's social rank, litter composition (singleton vs. twin litters), and prey availability (Holekamp et al. 1996). We calculated weaning dates based on mother–offspring nursing conflicts or as the day halfway between the last time cubs were observed nursing and the first time cubs were seen with their mother but were not nursing (Holekamp et al. 1996, 1999). We defined three stages of lactation for lactating females (early, middle, late) by dividing the total number of days a mother nursed her litter into three time periods of equal length. Thus, lactation stages were one-third of each female's entire lactation period, which varied across females based on the timing of weaning (Holekamp et al. 1996).

Fecal sample collection and storage.—Fecal samples used in this study were collected in 1993–2011. We collected samples during morning or evening observation periods whenever a known hyena was observed defecating. We collected a large portion of the sample into a plastic bag within 30 min of excretion and recorded the hyena's identity and time of defecation. The time of defecation was recorded because fecal steroid hormone concentrations have been found to vary with time of day in this species (Van Meter et al. 2008, 2009). Within 12 h, each sample was brought to camp, mixed, and aliquoted into 2 ml cryovials, which then were frozen and stored in liquid nitrogen until they could be transported on dry ice to Michigan

Table 1.—Predictions for hormone concentrations relative to a matched control of each of two test conditions.

Test condition	Hair does not affect sample	Hair adds hormone into sample during extraction	Hair adds hormone into sample during storage	Hair adds to dry weight but not hormone of sample
Hair removed	Same	Lower	Same	Higher
Hair added	Same	Higher	Same or higher	Lower

State University for storage at −20°C or colder. Samples then were transported to the Beehner Endocrinology Laboratory at the University of Michigan where all hormone assays were run. The time between collection and freezing and between collection and assay has not been shown to significantly affect concentrations of other fecal steroid hormones in this species (Dloniak 2004; Van Meter et al. 2009) and we assume here the same is true for progesterone, because others have argued that this finding should generalize to similar fecal steroid hormones (Van Meter et al. 2008).

Fecal hormone extraction and analysis.—We used the same extraction procedure described in previously published research (Dloniak 2004; Dloniak et al. 2006; Van Meter et al. 2008, 2009). Briefly, samples were dried, either by oven incubation or lyophilization (see procedure below), ground using a mortar and pestle, and shaken overnight for 14 h in 5 ml of 100% ethanol. Samples then were boiled and centrifuged, resulting in an ethanol supernatant (which was poured off into a new tube) and a pellet (which was discarded). The ethanol supernatant was evaporated under compressed air in a warm water bath using an Evaporac. Dried samples were reconstituted with 1.0 ml phosphate-buffered saline (PBS; pH 5.0), transferred to microcentrifuge tubes, and frozen at −20°C until assay.

For fecal corticosterone analysis, we assayed samples in duplicate using an I^{125} radioimmunoassay kit (ImmuChem Double Antibody Corticosterone ^{125}I RIA kit; MP Biomedicals [formerly ICN], Solon, Ohio). We diluted extracts in a 1:20 ratio with the steroid diluent provided in the kit. The primary antibody in this kit cross-reacts 100% with corticosterone, 0.34% with desoxycorticosterone, 0.1% with testosterone, 0.05% with cortisol, 0.03% with aldosterone, and 0.02% with progesterone. The minimum detection limit of the assay was 10 ng g^{-1}. We excluded from further analysis any sample with a coefficient of variation (CV) between duplicates higher than 15%.

For fecal progesterone analysis, we assayed samples in duplicate using the CL425 first antibody (C. Munroe, University of California Davis, diluted 1:12,000 in PBS), a Goat Anti-Mouse IgG as the second antibody (Equitech-Bio GAMG-0100, diluted 1:100 in PEG; Equitech-Bio Inc., Kerrville, Texas), and ^{125}I labeled tracer and standards (MP Biomedicals). The CL425 antibody is monoclonal, raised against 4-Pregnen-3α-ol-20-dione (P4) hemisuccinate:bovine serum albumin (Grieger et al. 1990) with known cross-reactivity with several other progesterone metabolites (e.g., 4-Pregnen-3a-ol-20-one: 188%; 5a-Pregnan-3b-ol-20-one: 94%; pregnanediol: 0.1%). These antibodies have been used to assay progesterone in the feces of several other vertebrate species (Graham et al. 2001; North and Harder 2008; Lu et al. 2010). The minimum detection limit of the assay was 0.02 ng g^{-1}. Samples collected from immature female hyenas were assayed at a 1:20 dilution, samples collected from pregnant females were assayed at a 1:2,000 dilution, samples collected from lactating females were assayed at a 1:500 dilution, and pooled samples used in the drying and hair tests were assayed at a 1:160 dilution. We excluded from further analysis any sample with a CV between duplicates higher than 15%.

Blood collection and plasma progesterone assay.—From 1990 to 2005, hyenas in our study population were anesthetized with 6.5 mg kg^{-1} total of tiletamine–zolazepam (Telazol; Zoetis, Parsippany, New Jersey) diluted with 2.5 ml distilled water via a lightweight plastic dart fired from a CO_2 rifle. Immobilizations took place during the morning hours and occurred opportunistically when hyenas were resting, and numerous body and dental measurements were taken while the animal was anesthetized (van Horn et al. 2003). Within 10–17 min of the Telazol injection, we drew a blood sample from the jugular vein into a heparinized vacutainer tube. Hyenas usually recovered within 60 min. At our field camp, blood was centrifuged at 1,000*g* for 10 min. Plasma was drawn off and stored in liquid nitrogen until it could be brought on dry ice to Michigan State University, where plasma was stored at −80°C until assayed in K. E. Holekamp's laboratory at Michigan State University.

For plasma progesterone analysis, we assayed samples using an I^{125} radioimmunoassay kit (Diagnostic Products Corp., Coat-A-Count ^{125}I RIA kit; Diagnostic Products Corporation, Los Angeles, California). The primary antibody in this kit cross-reacts 100% with progesterone, 2% with 20-alpha-dihydroprogesterone, 1.7% with 11-deoxycorticosterone, 2.4% with 11-deoxycortisol, and 1.3% with 5-pregnan-3,20-dione. The minimum detection limit of the assay was 0.02 ng ml^{-1}. We assigned the minimum detection limit of the assay to 30 plasma progesterone samples collected from immature females because the concentration determined by the assay fell below this minimum value. We excluded three samples from our data set because they were collected at the hyenas' necropsies, and we cannot rule out the possibility that the circumstances of collection resulted in abnormal hormone profiles or in elevated secretion of progesterone from the adrenal glands due to the stress of a fatal injury (Hueston and Deak 2014). For plasma progesterone, our intraassay CV was 3.15% and our interassay CV was 12.29% ($N = 11$ assays).

Analytical validation of corticosterone and progesterone assays.—To validate the corticosterone and progesterone antibodies for hyena fecal extracts, we characterized both assays with respect to precision (intraassay CV), accuracy, and parallelism. To calculate precision for the cortisterone antibody, we calculated an intraassay CV from a fecal pool generated by mixing many samples together (1:1 dilution, binding at 36%). We ran this control eight times within the same assay. To calculate precision for the progesterone CL425 antibody, we generated a fecal pool by mixing many samples together and used a low (1:32,000 dilution, binding at ~75%) and a mid (1:800 dilution, binding at ~50%) control to calculate an intraassay CV. We ran the low and the mid controls eight times within the same assay.

We tested accuracy across the range of binding by spiking each of the MP Biomedicals standards with an aliquot of a pooled fecal sample extract. In the corticosterone assay validation, 80 µl of six different standards were spiked with 20 µl of fecal pool with a concentration of 175.94 ng ml^{-1} ($SE = 5.91$). In the progesterone assay validation, 30 µl of five different standards were spiked with 20 µl of fecal pool with a concentration of 4.55 ng ml^{-1} ($SE = 0.57$). Accuracy was calculated as the observed values divided by the expected values.

We tested for parallelism in the progesterone and corticosterone assays by modeling the percent binding as a function of the known concentrations of a serial dilution of a fecal extract pool and of the respective standard curve. For each hormone, we used a linear model to test for a significant interaction between the known concentrations and the type of sample (pool vs. standard). Lack of a significant interaction would indicate parallelism, meaning that the slopes of these lines do not significantly differ.

Biological validation of fecal progesterone assay.—If our assay can be used to measure progesterone in the feces of spotted hyenas, fecal progesterone concentrations should show biologically meaningful variation in a predictable pattern across female reproductive states, and fecal and plasma progesterone values should vary similarly among these reproductive states. Because our fecal and blood sampling was purely opportunistic, we did not have adequate repeated sampling from individual females to track changes in progesterone concentrations across reproductive cycles within females or to directly compare plasma and fecal progesterone concentrations collected from a single female on the same day (see Table 2 for sample sizes). Instead, we examined how the average concentrations of fecal and plasma samples collected from immature, pregnant, and lactating females vary based on these reproductive states. In addition, we compared progesterone concentrations across stages both of pregnancy and lactation (early, middle, or late). For our analysis of lactation stages, we excluded samples collected from females who had died before weaning their cubs or whose cubs died before being weaned because these factors shortened their overall lactation period. To determine whether fecal progesterone concentrations show a circadian pattern, as they do in other steroid hormones in this species (Van Meter et al. 2008, 2009), we also investigated the effect of time of day (morning vs. evening) on fecal progesterone concentrations. This was not possible for plasma progesterone concentrations, because all of our immobilizations and blood draws took place during the morning hours. A biological validation of our corticosterone assay using an adrenocorticotropic hormone (ACTH) challenge was carried out earlier for spotted hyenas (Dloniak 2004), so we did not repeat that here.

Drying method experiment.—We tested whether the method of drying wet samples, namely oven incubation versus lyophilization, affected corticosterone and progesterone concentrations. We aliquoted 1 g each of 11 wet fecal samples, then incubated half of each sample (0.5 g) overnight at 37°C in a drying oven and lyophilized the other half (0.5 g) overnight. After drying, each incubated or lyophilized replicate was ground and weighed, such that each replicate for a sample weighed the same (between 0.202 and 0.237 g). All lyophilized samples then were mixed and divided into 10 identical aliquots weighing 0.2 g each; the same was done for the incubated samples. Extraction and assays for corticosterone and progesterone then were undertaken as described above.

Table 2.—Number of fecal and plasma samples analyzed for females in each reproductive state.

Reproductive state	# Fecal samples	# Plasma samples
Immature females	188	92
Pregnant females (total)	78	27
Early	25	4
Middle	27	11
Late	26	12
Lactating females (total)	336	31
Early	125	11
Middle	95	9
Late	116	11

Hair removal experiment.—We selected 12 samples that contained average to high quantities of prey hair based on visual inspection. We thoroughly mixed 1 g wet weight of each of these samples to create a pool, and incubated these pools overnight at 37°C. After incubation, we ground and mixed the pooled samples, then divided them into two halves. To create our two treatment conditions, we removed as many hairs as possible from one half of the pool using forceps, and did not remove hair for the other half of the pool. Hairs were cleaned with kimwipes and set aside for the *Hair addition* experiment. We then weighed ten 0.2 g aliquots from each half, creating 10 "Hair Removal" replicates and 10 "No Hair Removal" replicates for comparison. Extraction and assays for corticosterone and progesterone then were undertaken as described above.

Hair addition experiment.—We selected 12 samples that contained virtually no hair, based on visual inspection. We thoroughly mixed 1 g wet weight from each of these samples to create a pool. To create our two treatment conditions, we divided the pooled wet samples into two halves then added and thoroughly mixed in the hairs set aside from the *Hair removal* experiment into one half. We incubated both the "Hair Addition" and "No Hair Addition" pools overnight at 37°C. We then weighed ten 0.2 g aliquots from each half, creating 10 "Hair Additions" replicates and 10 "No Hair Addition" replicates for comparison. Extraction and assays for corticosterone and progesterone were then performed as described above.

Data analysis.—The details for the analytical validation of our progesterone antibody are listed above. We carried out all our statistical analyses in R (R Development Core Team 2019). For the biological validation of our progesterone antibody, we built linear mixed models using the "lmer" function (lme4 package; Bates et al. 2015). Progesterone concentration (either in fecal or plasma samples) was the response variable, and values were log-transformed to more closely approximate a normal distribution. Because we had multiple samples from some individuals, individual ID was entered as a random effect in all models. For models of fecal progesterone concentrations, we also entered time of day when each sample was collected (morning or evening) as a fixed factor in all models.

First, we tested for the effects of reproductive state on progesterone concentrations by entering reproductive state (immature, pregnant, or lactating) as a fixed factor. To determine a parameter estimate for reproductive state, we used a likelihood ratio test to compare models with and without the reproductive state variable. Next, we restricted our analysis to pregnant females and tested whether progesterone concentrations increased over the course of gestation by entering stage of pregnancy (early, middle, late) as a fixed factor. To test whether progesterone changed over the course of lactation, we restricted our analysis

to samples from lactating females and entered stage of lactation (early, middle, late) as a fixed factor. We used Tukey's post hoc tests for pairwise comparisons in the R package "multcomp" (Hothorn et al. 2008) to test for differences among reproductive states and among stages of pregnancy or lactation. All response variables were inspected for outliers using Cook's distance (R package "influence.ME"; Nieuwenhuis et al. 2012) and visual inspection. Because our response variable was log-transformed in these models, beta coefficients reported here should be interpreted as the ratio of the change in hormone concentrations as a function of the change in the predictor variable.

To test whether the drying methods and the hair addition/removal experiments altered measurements of progesterone and corticosterone in hyena fecal samples, we built three linear models using the "lm" function (lme4 package; Bates et al. 2015), one for each experiment, and entered condition as a predictor variable. Our data for the progesterone hair removal experiment were not normally distributed according to a Shapiro test. However, a nonparametric Wilcoxon signed-rank test showed similar results, so we report the linear model results here for consistency.

Ethical note.—The study was carried out in compliance with guidelines of the American Society of Mammalogists for the use of wild mammals in research (Sikes et al. 2011) and was approved by the Michigan State University IACUC and the Kenyan National Commission on Science, Technology and Innovation.

Results

Analytical validation.—For our analytical validation of fecal hormone antibodies, the intraassay CV (precision) for our fecal pool was 1.64% for corticosterone and 7.82% for progesterone. Our additional low pool for progesterone yielded an intraassay CV of 13.32%. We ran only one assay for corticosterone, and therefore do not have an interassay CV to report. For progesterone, our interassay CV was 9.11% for the mid pool ($N = 20$ assays) and 17.65% for the low pool ($N = 20$ assays). Mean accuracy for corticosterone was 89.59% ± 5.19 ($N = 6$) and for progesterone was 80.00% ± 16.00 ($N = 4$). Both the corticosterone and progesterone antibodies demonstrated parallelism; that is, there was no significant interaction between hormone concentration and type of sample (pool vs. standard) for corticosterone ($t = 0.102$, $P = 0.208$) or for progesterone ($t = -1.357$, $P = 0.246$).

Biological validation of the progesterone assay.—When we examined whether our progesterone antibody produced the expected progesterone values across hyena reproductive states, we found that, indeed, reproductive state predicted progesterone levels in a meaningful way for fecal values ($\chi^2 = 1{,}091.7$, $P < 0.001$; Fig. 1). Reproductive state also predicted plasma progesterone values ($\chi^2 = 231.72$, $P < 0.001$) and variation in fecal and plasma progesterone concentrations showed the same pattern across reproductive states (Fig. 2). Tukey's post hoc comparisons indicated that pregnant females had significantly higher progesterone concentrations than either lactating females (fecal: β = 1.38, $SE = 0.08$, $P < 0.001$; plasma: β = 4.00, $SE = 0.32$, $P < 0.001$) or immature females (fecal: β = 4.43, $SE = 0.09$, $P < 0.001$; plasma: β = 6.30, $SE = 0.26$, $P < 0.001$). Lactating females had significantly higher progesterone concentrations than immature females (fecal: β = −3.05, $SE = 0.07$, $P < 0.001$; plasma: β = 2.30, $SE = 0.25$, $P < 0.001$). Time of day was a near-significant predictor of fecal progesterone values (β = −0.08, $SE = 0.05$, $P = 0.096$), with samples showing slightly higher progesterone concentrations in the morning than in the evening.

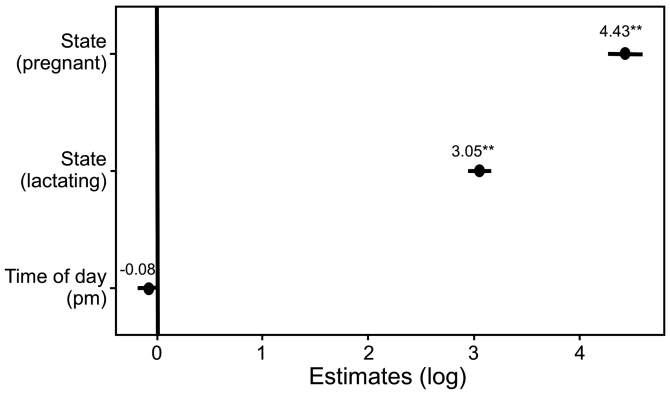

Fig. 1.—A forest plot showing the size of the coefficient for each of the main effects in the model of fecal progesterone concentrations. Coefficients are on the log scale. The reference level for reproductive state is immature and the reference level for time of day is morning (AM). Points falling to the right of the line indicate higher fecal progesterone concentrations and points falling to the left indicate lower fecal progesterone concentrations for that predictor. The 95% confidence interval for each estimate is shown. Samples for this analysis were collected from spotted hyenas (*Crocuta crocuta*) living in the Maasai Mara National Reserve, Kenya in 1993–2011. $^{**}P < 0.001$.

For fecal progesterone results across pregnancy, we found that progesterone concentrations during the early stage of pregnancy did not differ significantly from concentrations during the middle stage of pregnancy (β = 0.25, $SE = 0.17$, $P = 0.274$; Fig. 3A). Fecal progesterone concentrations were significantly lower during the early stage of pregnancy than during the late stage of pregnancy (β = 0.47, $SE = 0.17$, $P = 0.017$). Fecal progesterone concentrations did not differ between females in the middle and late stages of pregnancy (β = 0.21, $SE = 0.16$, $P = 0.382$). Among pregnant females, time of day was not a significant predictor of fecal progesterone concentrations (β = −0.03, $SE = 0.13$, $P = 0.794$).

For plasma progesterone results across pregnancy, we found that progesterone concentrations during the early stage of pregnancy did not significantly differ from concentrations during the middle stage of pregnancy (β = 0.21, $SE = 0.30$, $P = 0.765$; Fig. 3B). Plasma progesterone concentrations were significantly lower during the early stage of pregnancy than during the late stage of pregnancy (β = 0.79, $SE = 0.30$, $P = 0.020$). Plasma progesterone concentrations were significantly lower in the middle stage of pregnancy than during the late stage of pregnancy (β = 0.58, $SE = 0.21$, $P = 0.017$).

For fecal progesterone results across lactation, we found that progesterone concentrations during the middle stage of lactation were significantly lower than during the early stage of lactation (β = −0.41, $SE = 0.09$, $P < 0.001$), and progesterone concentrations during the late stage of lactation were

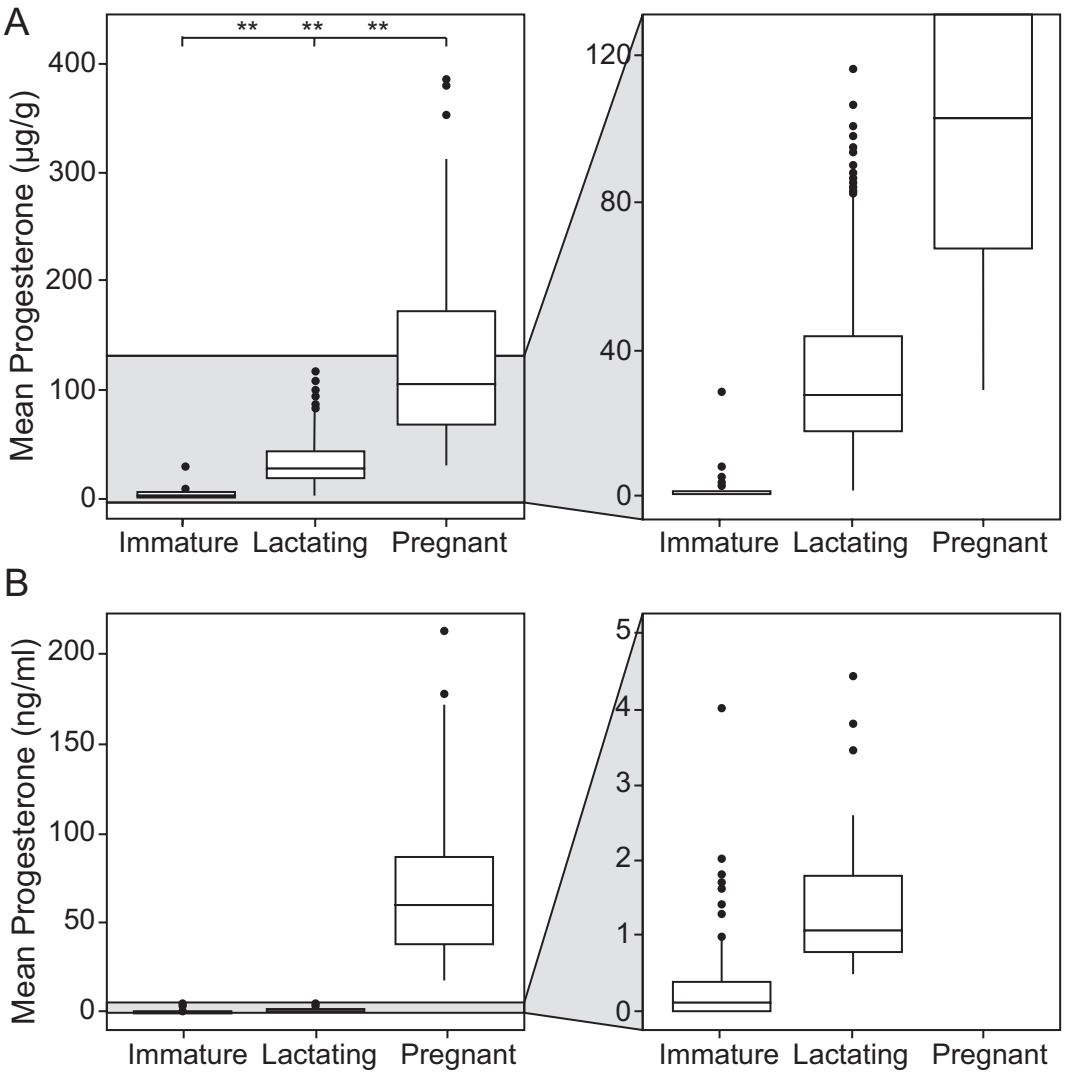

Fig. 2.—Boxplots showing concentrations of fecal progesterone concentrations (A) and plasma progesterone concentrations (B) in immature (fecal: $N = 188$, plasma: $N = 94$), lactating (fecal: $N = 336$, plasma: $N = 31$), and pregnant females (fecal: $N = 78$, plasma: $N = 27$). Samples were collected from spotted hyenas (*Crocuta crocuta*) living in the Maasai Mara National Reserve, Kenya in 1993–2011 (fecal) and 1990–2005 (plasma). $^{**}P < 0.001$.

marginally lower than during the early stage of lactation ($\beta = -0.19$, $SE = 0.09$, $P = 0.063$; Fig. 4A). Fecal progesterone concentrations during the middle stage of lactation were significantly lower than during the late stage of lactation ($\beta = 0.22$, $SE = 0.08$, $P = 0.026$). Among lactating females, time of day was not a significant predictor of fecal progesterone concentrations ($\beta = -0.06$, $SE = 0.07$, $P = 0.379$).

For plasma progesterone results across lactation, we found that there were no significant differences in plasma progesterone concentrations between females in the middle and early stage of lactation ($\beta = -0.22$, $SE = 0.23$, $P = 0.661$), between females in the late and early stage of lactation ($\beta = -0.42$, $SE = 0.22$, $P = 0.187$; Fig. 4B), and between females in the middle and late stage of lactation ($\beta = -0.20$, $SE = 0.23$, $P = 0.652$).

Drying method experiment.—Although fecal corticosterone values tended to be higher in oven-incubated (mean = 54.49 ng g^{-1}, $SE = 3.06$) than lyophilized (mean = 52.42 ng g^{-1}, $SE = 2.68$) samples, this difference was not statistically significant ($\beta = -2.07$, $SE = 1.29$, $P = 0.125$; Fig. 5A). Drying method significantly affected hormone values for progesterone ($\beta = -105.91$, $SE = 17.34$, $P < 0.001$; Fig. 5B). Progesterone concentrations were significantly higher when samples were oven-incubated (mean = 32.20 µg g^{-1}, $SE = 1.50$) than when they were lyophilized (mean = 25.59 µg g^{-1}, $SE = 3.08$).

Hair removal experiment.—Removing hair from the pooled fecal samples did not significantly affect values of either corticosterone ($\beta = -0.07$, $SE = 3.64$, $P = 0.986$) or progesterone ($\beta = 90.07$, $SE = 46.49$, $P = 0.073$; Fig. 6). Corticosterone concentrations were not significantly different when hair was removed (mean = 42.57 ng g^{-1}, $SE = 3.39$) as compared to control samples (mean = 42.63 ng g^{-1}, $SE = 4.27$). Progesterone concentrations were not significantly different when hair was removed (mean = 171.02 µg g^{-1}, $SE = 124.97$) as compared to control samples (mean = 80.95 µg g^{-1}, $SE = 40.92$).

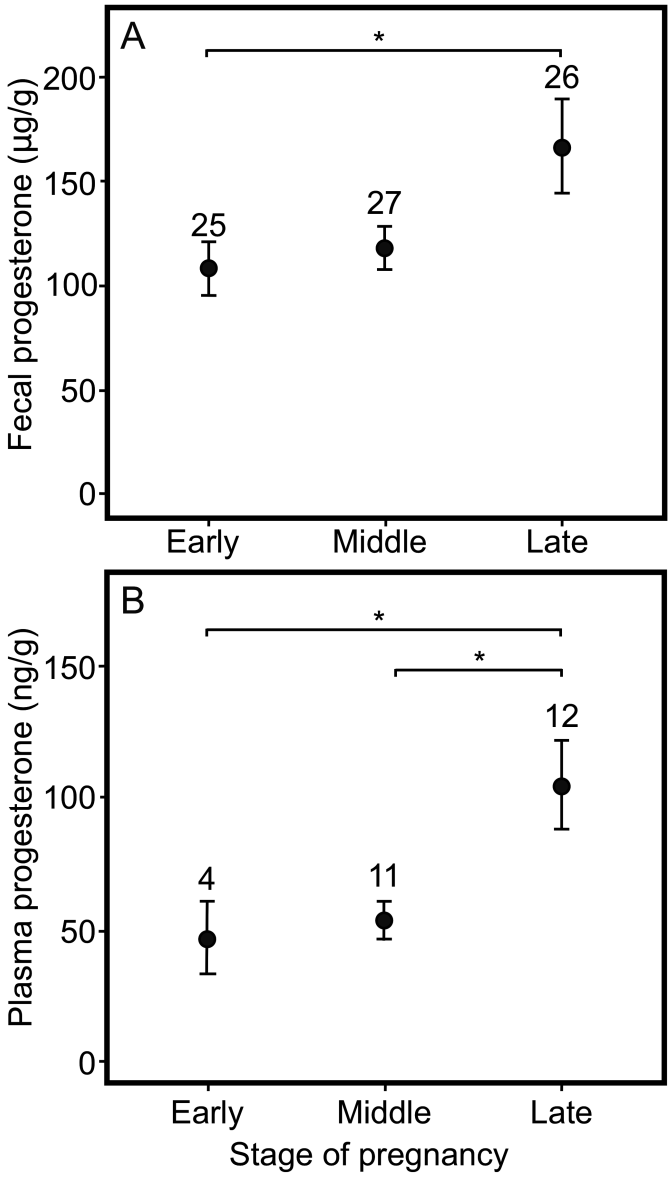

Fig. 3.—Mean fecal progesterone concentrations (A) and plasma progesterone concentrations (B) for pregnant females. Labels indicate the number of samples in each stage of pregnancy. *$P < 0.05$. We defined three stages of pregnancy by dividing the length of spotted hyena gestation (110 days) into three equal periods of 37 days each. Samples were collected from spotted hyenas (*Crocuta crocuta*) living in the Maasai Mara National Reserve, Kenya in 1993–2011 (fecal) and 1990–2005 (plasma).

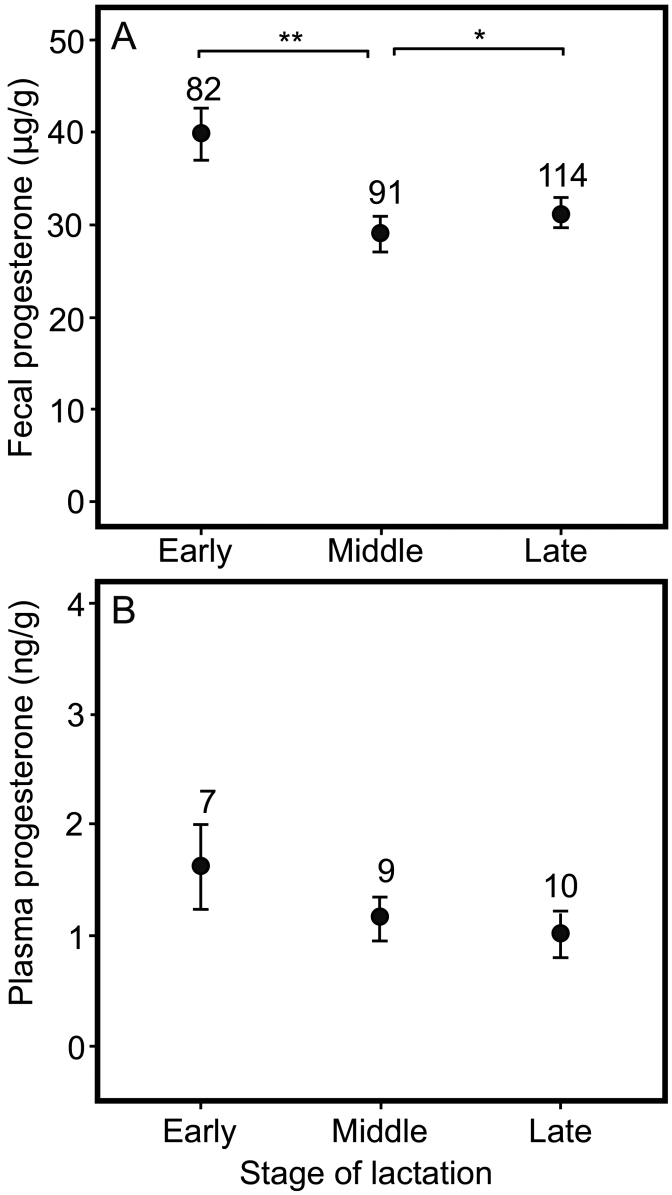

Fig. 4.—Mean fecal progesterone concentrations (A) and plasma progesterone concentrations (B) for lactating females. Labels indicate the number of samples in each stage of lactation. **$P < 0.001$, *$P < 0.05$. To define periods of lactation, we divided the total number of days a mother nursed into three periods of equal length. Samples were collected from spotted hyenas (*Crocuta crocuta*) living in the Maasai Mara National Reserve, Kenya in 1993–2011 (fecal) and 1990–2005 (plasma).

Hair addition experiment.—Adding hair did not affect hormone values for corticosterone ($\beta = -1.22$, $SE = 1.47$, $P = 0.419$; Fig. 7A) but did significantly affect hormone values for progesterone ($\beta = -36.06$, $SE = 11.91$, $P = 0.007$; Fig. 7B). Corticosterone concentrations were not significantly different when hair was added (mean = 22.90 ng g^{-1}, $SE = 1.77$) as compared to control samples (mean = 22.18 ng g^{-1}, $SE = 0.92$). Progesterone concentrations were significantly lower when hair was added (mean = 250.53 µg g^{-1}, $SE = 21.86$) than in control samples (mean = 286.59 µg g^{-1}, $SE = 30.68$).

Discussion

Our results demonstrate that we can measure biologically meaningful variation in fecal progesterone values in spotted hyenas using the CL425 progesterone antibody. Fecal progesterone concentrations showed significant variation with reproductive state, being highest in pregnant females, intermediate in lactating females, and lowest in immature females. This mirrors the pattern we observed in plasma progesterone concentrations and mirrors the expected patterns across many mammalian taxa (Hellgren et al. 1991; Strier and Ziegler 1997; Gudermuth et al.

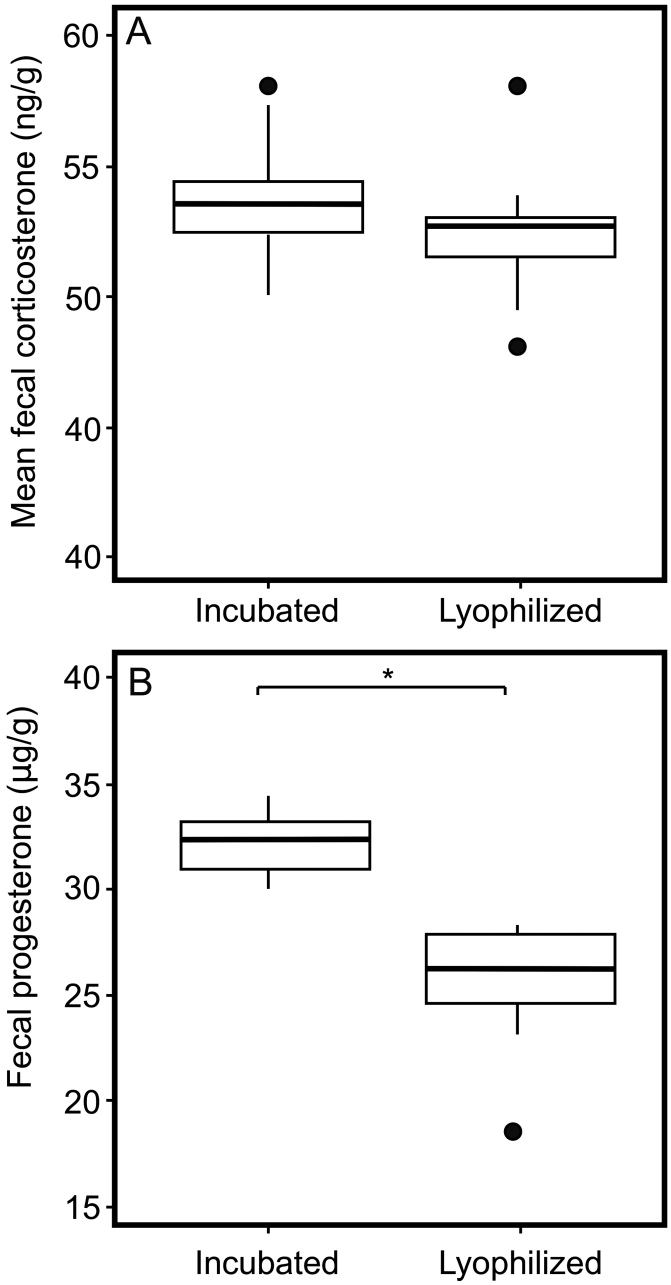

Fig. 5.—Boxplots showing average hormone concentrations in replicate samples dried using incubation and lyophilization for fecal corticosterone (A) and fecal progesterone (B). Samples were replicates of pooled fecal samples collected from spotted hyenas (*Crocuta crocuta*) living in the Maasai Mara National Reserve, Kenya in 1993–2011. *$P < 0.05$.

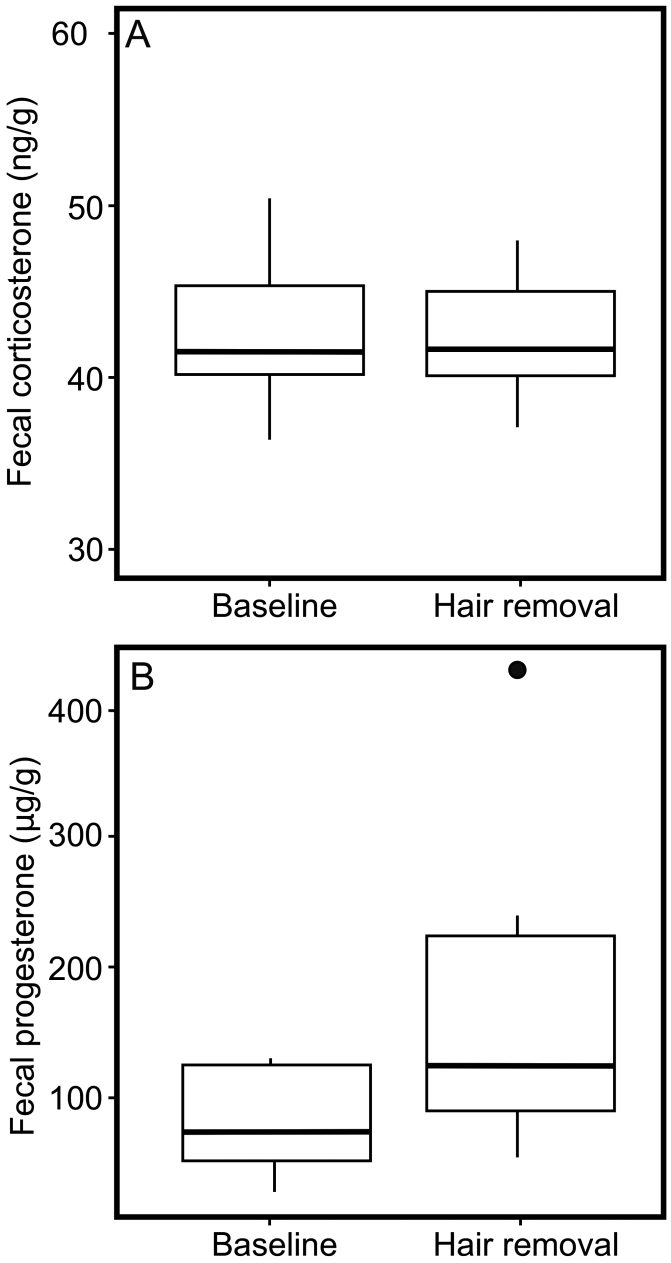

Fig. 6.—Boxplots showing average hormone concentrations in replicate samples when hair was removed from samples versus a baseline condition in which no hair was removed for fecal corticosterone (A) and fecal progesterone (B). Samples were replicates of pooled fecal samples collected from spotted hyenas (*Crocuta crocuta*) living in the Maasai Mara National Reserve, Kenya in 1993–2011.

1998; Raggi et al. 1999; Graham et al. 2001; Borque et al. 2005; Lu et al. 2010; Bergfelt et al. 2011). We found that fecal progesterone concentrations were considerably lower in adult females before than after conception of their first litter, which mirrors the pattern of fecal estrogen concentrations in adult females (Van Meter et al. 2008). In captive spotted hyenas, estrogen concentrations begin to rise at 23 months (around the age of reproductive maturity), as measured in plasma (Glickman et al. 1992).

Among pregnant females, late trimester fecal and plasma progesterone concentrations were significantly higher than first trimester concentrations. This pattern is identical to the pattern of fecal estrogen concentrations across trimesters of pregnancy in spotted hyenas (Van Meter et al. 2008) and is consistent with the increase in plasma progesterone over the course of gestation documented in captive spotted hyenas (Licht et al. 1992). Fecal and plasma progesterone concentrations declined after parturition, with concentrations being higher earlier than later during lactation.

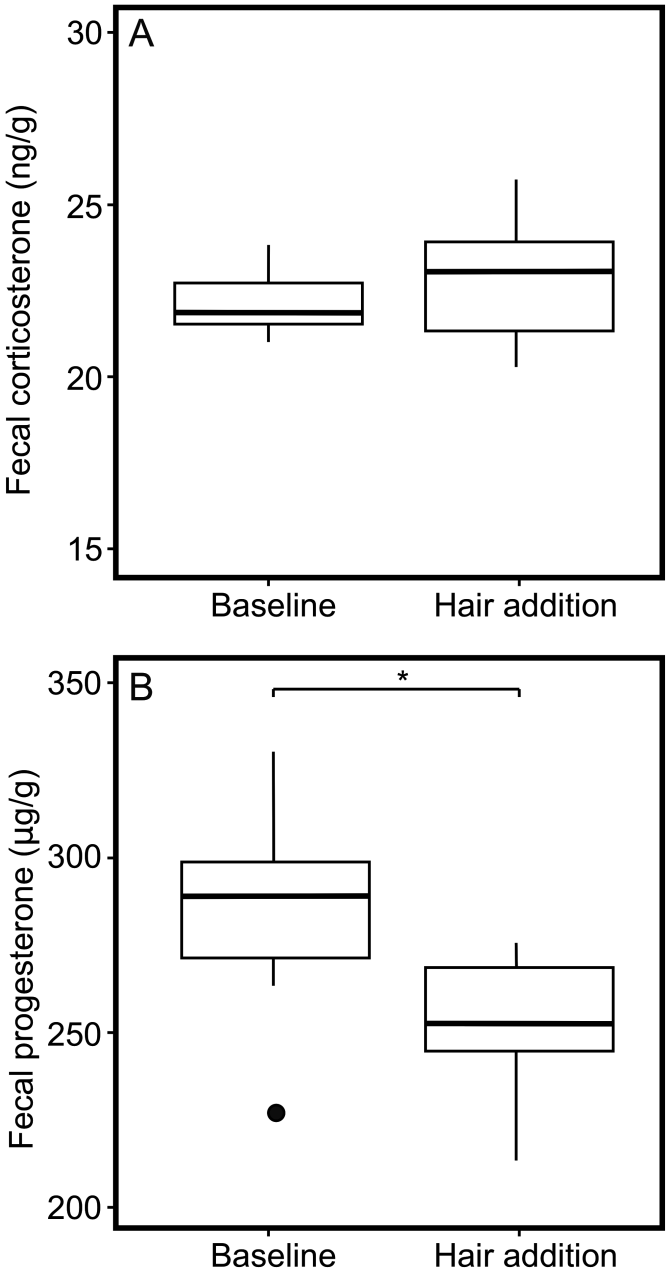

Fig. 7.—Boxplots showing average hormone concentrations in replicate samples when hair was added to samples versus a baseline condition in which no hair was added for fecal corticosterone (A) and fecal progesterone (B). Brackets and asterisks indicate significant differences. Samples were replicates of pooled fecal samples collected from spotted hyenas (*Crocuta crocuta*) living in the Maasai Mara National Reserve, Kenya in 1993–2011. *$P < 0.05$.

Although we saw the same general patterns in fecal and plasma progesterone across the three stages of pregnancy and lactation (i.e., an increase across pregnancy and a decrease across lactation), there were some differences between plasma and fecal values. For example, both fecal and plasma progesterone increased across pregnancy, but only plasma showed a significant increase for all three stages. Among lactating females, although fecal and plasma progesterone concentrations showed the same general pattern of being highest in early lactation, there only were significant differences across stages of lactation in fecal progesterone concentrations. We are not surprised by these differences given that our fecal and plasma samples were not collected from the same individuals on the same day. Had we been able to collect paired fecal and plasma samples, we would expect more consistency in these stage-by-stage comparisons; but given that we could only compare groups and that our plasma progesterone sample size was relatively small, we believe the similarity in patterns between fecal and plasma progesterone concentrations across stages of pregnancy supports the biological validity of our fecal progesterone assay.

Finally, whether it is necessary to control for circadian variation when analyzing fecal progesterone concentrations needs further study. Samples tended to show slightly higher fecal progesterone concentrations when collected during the morning than during the afternoon, although there was no significant difference related to time of day when analyzing pregnant or lactating females separately. In this species, fecal glucocorticoids (Dloniak 2004) and estrogens (Van Meter et al. 2008), but not fecal androgens (Dloniak et al. 2004), show this same circadian pattern of variation, with higher values found in the morning than late afternoon. Because hyenas are crepuscular (Holekamp and Dloniak 2010), we might expect circadian patterns in hormone secretion to differ from those in the diurnal mammals whose patterns have been more thoroughly studied (Jha et al. 2015).

The ability to measure progesterone concentrations noninvasively by repeated sampling of known individuals offers a valuable tool for investigating many aspects of spotted hyena biology in both captive and wild populations, especially when paired with our continuous demographic and behavioral monitoring of wild hyenas. First, by measuring fecal progesterone and estrogen concentrations while monitoring a female's reproductive state and mating activity, we may better understand of the mode of ovulation and estrous cyclicity in this species. This will add to our understanding of comparative reproductive biology across mammals. Second, because there was high variation in fecal progesterone concentrations during late pregnancy, it would be interesting to further examine the social and ecological correlates of late pregnancy progesterone levels within this free-living study population. Late gestation elevations in progesterone can be critical in mediating the onset of maternal behavior postpartum (Bridges et al. 1978), and, in some mammalian species, variation in progesterone concentrations (or the ratio of estrogen to progesterone) during late pregnancy is correlated with variation in maternal care (Fleming et al. 1997; Jarcho et al. 2012; Glynn et al. 2016). Female spotted hyenas demonstrate significant individual variation in maternal care (Greenberg 2017), and such behavioral variation could be mediated by differences in progesterone and other hormones. Third, in addition to its role in reproduction, progesterone has been found to mediate certain prosocial and cooperative behaviors and to help regulate aggression in other species (Huck et al. 1979; Fraile et al. 1987; Wirth and Schultheiss 2006; Brown et al. 2009). Linking our data on fecal progesterone concentrations in known individuals to long-term behavioral records could help us to better understand whether progesterone mediates the significant interindividual variation in cooperative, aggressive, or affiliative behavior in this species.

Our findings regarding the effects of drying method, as well as the addition and removal of hair from samples, suggest that these variables need to be considered when establishing a method of fecal hormone analysis and/or when considering a change in equipment or laboratories. The effects of these procedural variables were hormone-specific. First, drying method significantly affected progesterone (and to some extent, corticosterone) values, with oven-incubated samples showing higher readings than lyophilized samples. Second, hair removal did not affect the values for either hormone, suggesting that prey hair does not release its own hormone content into predator fecal samples during the extraction process. We also can exclude the possibility that hair releases its hormone content into samples during storage, because adding hair also did not result in higher values of either hormone. Indeed, mean fecal progesterone concentrations were *lower* in samples with hair added compared to controls (Fig. 6B). Together, these manipulations demonstrate that, for the extraction procedure used here, we do not need to be concerned about fecal hormone values of the predator being contaminated by hormones in prey hair. On the other hand, if enough hair is present in samples, it may actually "dilute" hormone concentrations by inflating the dry weight (in other words, the prey hair is adding to the dry weight without adding correspondingly to the hormone content). For the typical range of prey hair mass present in our spotted hyena samples, progesterone appears more sensitive than corticosterone to this effect. It is possible that the overall higher concentrations of corticosterone than progesterone in our samples simply make it less likely that hair will have this "diluting" effect when measuring corticosterone than progesterone.

Our routine storage and extraction procedures also likely minimized the possibility of detecting hormones from the hair in our samples. First, although little is known about how long-term storage conditions may affect the hormone content in hair samples, most studies store hair samples at room temperature for less than 1 year before hormone assay (Sheriff et al. 2011). Our fecal samples, on the other hand, are stored in liquid nitrogen or freezers, which could reduce the likelihood of prey hair leaching their hormone content. Second, extracting hormones from hair typically involves pulverizing the hairs with a ball mill to increase the surface area of the hairs and the efficiency of hormone extraction (Sheriff et al. 2011). Although our dried fecal samples were ground with a mortar and pestle, this does not thoroughly cut up the hairs in the same way as when pulverized with a ball mill. Third, most procedures used for extracting hormones from hair samples call for a longer period of agitation in alcohol than what we use in our fecal hormone extraction (16–24 h for most hair extractions vs. 14 h for our procedure; Sheriff et al. 2011). Therefore, it is possible that our shorter agitation time reduced the likelihood of extracting hormones from the hair contained in fecal samples. It may be important for researchers studying hormones of mammalian predators to consider how closely their fecal hormone extraction procedures mimic the extractions necessary for hair samples. Researchers may consider using procedures that include long-term storage of fecal samples in freezers, minimizing any grinding of fecal samples that could pulverize prey hair contained in the sample, and, for alcohol extractions, minimizing the amount of time samples are agitated in alcohol. It would also be useful to experimentally test the effects of manipulating these components of extraction procedures to investigate whether they affect extraction of hormones from hair contained in fecal samples.

Our results regarding the effects of drying method and presence of prey hair may not be generalizable to all mammalian carnivore species, all hormones, or all extraction and assay procedures. We therefore suggest that the potential effects of hair should be routinely tested when validating hormone extraction and assay methods for mammalian predators, and that researchers should explicitly state in their methods how they dealt with hair in their samples. Reporting these methods should increase the likelihood of reproducibility across time and among studies, but such reporting is rarely done. The hormone-specific effect of drying method also implies that researchers should not alter drying procedures without first determining that doing so does not significantly affect hormone concentrations.

Acknowledgments

We thank the Kenyan National Commission for Science, Technology, and Innovation, the Kenyan National Environmental Monitoring Agency, the Narok County Government, the Naboisho Conservancy, the Maasai Mara University, and the Kenya Wildlife Service for permission to carry out this research and to export fecal samples. We also thank all those who assisted the Mara Hyena Project with long-term data collection in the field. We thank Teera Losch for assistance in the Beehner Endocrinology Laboratory at the University of Michigan, Karen Hubbard for laboratory assistance at Michigan State University, and John B. Graham for assistance with figures.

FUNDING

Sample collection was supported by National Science Foundation (NSF) grants BNS9021461, IBN9309805, IBN9906445, IBN0113170, IBN0343381, IOB0618022, IOB0819437, IOS1121474, OISE1853934, and IOS1755089 to KEH and by a NSF Graduate Research Fellowship to JRG. JRG also was supported by fellowships from the College of Natural Sciences and from the program in Ecology, Evolutionary Biology, and Behavior at Michigan State University. Sample extraction and assay were supported by NSF grant DEB1353110 to KEH and JCB.

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Submitted 26 May 2020. Accepted 12 December 2021.

Associate Editor was Jack Hopkins.