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Factors influencing degradation of DNA and hormones in maned wolf scat

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Abstract

The ability to noninvasively detect the presence of species and assess physiological health by DNA and hormone analysis makes scat a valuable tool for ecology and conservation. We assessed factors associated with DNA and hormone degradation in a four-season study that employed detection dogs to collect scats from maned wolf (*Chrysocyon brachyurus*) in the Brazilian Cerrado, a tropical savanna landscape mosaic. Fecal DNA sample viability was assessed by attempting polymerase chain reaction amplification of a mitochondrial DNA (mtDNA) locus (~ 246 bp) and a nuclear DNA zinc finger protein gene (~ 195 bp). We assessed how extraction method, environmental exposure, and amount of odor, moisture and diet items in the sample influenced DNA amplification and allelic dropout rates. Samples that amplified mtDNA were assayed for glucocorticoids and thyroid hormone. Amount of odor and moisture (indicating freshness) predicted mtDNA amplification success, as well as mean hormone levels. While factors related to sample condition were negatively correlated with lower mean hormone levels, samples comprised mainly of fruit had higher levels of glucocorticoids and lower levels of thyroid hormone, and we thus interpret this result as biologically meaningful. In summary, DNA and hormone degradation are predicted by measures of sample freshness, making the assessment of sample quality an important criterion for sample collection as well to manage measurement error in analyses of hormone concentration associated with environmental disturbance.

Introduction

The advent of improved efficiency in molecular methods makes the use of noninvasively collected samples increasingly feasible for presence–absence, demographic, hybridization and physiological studies (Foran, Crooks & Minta, 1997; Wasser *et al*., 2004; Adams, Lucash & Waits, 2007; Ball *et al*., 2007). Noninvasive genetic sampling has been extremely useful for identifying species and individuals in an area, evaluating distribution, determining sex ratio and estimating population size (Kohn *et al*., 1999; Bellemain *et al*., 2005; Waits & Paetkau, 2005; Solberg *et al*., 2006), and fecal hormone metabolites may be used to assess physiological health and the disturbance response of populations (Creel *et al*., 2002; Rolland *et al*., 2006; Gobush, Mutayoba & Wasser, 2008). Scat is particularly enticing for studies of species of conservation concern because it may be collected noninvasively, without having to capture, handle or observe animals. When scats are subject to DNA and/or hormone extraction and analysis, however, poor sample quality can increase measurement error, wasting laboratory costs on extraction and amplification of degraded DNA or hormones. Degradation of hormones in samples is particularly problematic since these are quantitative measures and thus necessitate the removal of variation in hormone levels due to degradation from that resulting from disturbance variables of interest (e.g. noise, habitat degradation). Factors that influence sample quality can be considered to minimize effort collecting and analyzing samples unlikely to yield high-quality DNA, as well as to account for variation due to sample condition when analyzing sample hormone levels.

Variables influencing condition of samples in the field have been shown to influence DNA amplification success and include age of sample (Lucchini *et al*., 2002; Piggott, 2004; Santini *et al*., 2007), weather conditions (Farrell, Roman & Sunquist, 2000; Lucchini *et al*., 2002; Piggott, 2004), diet (Murphy, Waits & Kendall, 2003; Maudet *et al*., 2004) and intestinal slough rate, which may vary among species and within species as diets vary by individual or season (Farrell *et al*., 2000; Maudet *et al*., 2004). These studies have suggested that success rates will be highest when samples are as fresh as possible and climatic condi-

tions are either dry (Farrell *et al.*, 2000; Piggott, 2004) or very cold (Lucchini *et al.*, 2002). The need to address questions of conservation concern, however, often necessitates sampling across large, heterogeneous regions where sample age at time of collection is unknown and conditions are neither dry nor cold (i.e. in the tropics). Operational assessments of sample condition from field-collected samples that could be used to improve genotyping success are therefore needed in order to make noninvasive genetic sampling more accurate and affordable.

Old or poor-quality samples may also influence the amount of measurable hormone from a sample (Millspaugh & Washburn, 2004). While previous work has shown that sample preservation methods may influence hormone levels (Hunt & Wasser, 2003; Galama, Graham & Savage, 2004), studies examining the relationship of sample quality in field-collected samples and hormone levels are lacking (Washburn & Millspaugh, 2002). Also, dietary intake may influence fecal excretion of hormone metabolites independent of stress or nutritional status (Goldin *et al.*, 1981; Lewis & Heaton, 1997; von der Ohe *et al.*, 2004). While diet is typically controlled by removing water from the sample (Wasser *et al.*, 1993), von der Ohe & Servheen (2002) postulated that large dietary differences will not be completely adjusted for during lyophilization since dietary intake may impact the degree of reabsorption of metabolites, time of pooling and exogenous augmentation of glucocorticoid levels.

We present results from a 4-year study that employed detection dogs to survey scats of wild maned wolves (*Chrysocyon brachyurus*) in Brazil (Vynne *et al.*, 2011). Scat detection dogs enable efficient sampling of wildlife populations (Long *et al.*, 2007; Vynne *et al.*, 2011), yet the effectiveness of this sampling method means that a large number of old and degraded samples are encountered. Because factors influencing ability to extract DNA from maned wolf scat had not been studied, and because samples collected in the tropics may be particularly susceptible to degradation of DNA and hormones, we sought to quantify factors affecting sample quality of field-collected scat. We examined the influence of extraction method, habitat and season in which the sample was collected, sample condition (moisture level, strength of odor, presence of mold or invertebrates on the sample) and food items (amount of fruit) on the ability to amplify DNA. To determine whether sample quality influences hormone levels, we measured fecal glucocorticoid metabolites and thyroid hormone levels in samples and analyzed these mean values with respect to whether or not the sample amplified nuclear DNA (nDNA), and the amount of odor, moisture and fruit in the sample.

Methods

Sample collection

We employed specially trained detection dogs to locate scat from maned wolves (*C. brachyurus*) in a protected area and on private lands in the Cerrado of Brazil between August of 2004 and April of 2008 (Vynne *et al.*, 2011). Average annual precipitation was approximately 1500 mm during the wet season (September–May), which had an average of 14 days per month with rain, and < 1 cm of rain fell in the dry season (June–August). The dry season average temperatures were 23° during the dry season (average high was 29° and average low was 16°) and 25° during the wet season (average high was 29° and average low was 20°). Our search area spanned 700–900 m elevation.

When we located a sample, we recorded the habitat and sample condition, including moisture level, odor strength, presence of mold and presence of invertebrates. We did not collect samples that were both odorless (to human observer) and formless (e.g. consisted only of a scattering of undigested animal material or seeds). Otherwise, we preserved a portion of the sample for fecal DNA extraction in a 40-mL vial with 25 mL of 20% dimethyl sulfoxide buffer (DMSO; Frantzen *et al.*, 1998). When the scat was intact, we collected from the outside of the sample (Stenglein *et al.*, 2010). We then collected the remainder of the sample with a gloved hand and retained this portion for subsequent diet, hormone and DNA analysis. We kept samples on ice packs and froze them upon returning from the field until shipment (on dry ice) to the US. Samples from 2006 experienced a 3-week delay in shipment and were subjected to a complete thaw.

DNA extraction, purification and amplification

We conducted DNA extractions in a laboratory dedicated to noninvasive DNA samples and spatially separated from polymerase chain reaction (PCR) products. 1536 putative maned wolf samples were extracted in duplicate or triplicate to control for uneven distribution of DNA in scat (Wasser *et al.*, 1997). Fecal DNA preserved with DMSO (approximately 0.5 g) was extracted and processed using the Qiagen QiaAmp® stool mini kit & Dneasy® 96 blood and tissue kit (Qiagen, Inc., Valencia, CA, USA) and DNA extracts were then purified using the Geneclean® III kit (Q-BIOgene Inc., Carlsbad, CA, USA), both with modified protocols (Vynne, 2010). To test the efficacy of extracting DNA from the mucosal layer of the feces, we swabbed a subset of 143 maned wolf samples preserved by freezing (not DMSO) from the 2007 and 2008 field seasons to collect the epithelial and immune cells. Swabbing was done with a cotton-tipped applicator dipped in 10x phosphate-buffered saline (PBS) buffer applied with light pressure to the outside of the sample in an effort to remove the mucosal cells and avoid fecal material (Ball *et al.*, 2007; Rutledge *et al.*, 2008; Wasser *et al.*, 2011). The applicator tip was then placed in a 2-mL tube containing 300 mL Qiagen ATL standard lysis buffer and 33 mL Proteinase K, and incubated at 21° for 1 h. Another 33 mL Proteinase K was then added and samples were incubated at 21° overnight before being extracted using the Qiagen DNAeasy® 96 blood and tissue kit (Qiagen, Inc.) following the recommended protocol for DNA extraction of tissue with the following modifications:

(1) 366 mL AL was added and incubation of 21° was for 1 h.
(2) 366 mL ethanol was added.
We included one negative control (no scat material added to the extraction) for every 11 extraction tubes.

The species test consisted of fragment analysis from PCR amplification of the mitochondrial control region (D-loop) using unlabeled HSF21 (GTACATGCTTATATGCAT GGG) and 5′6-FAM-labeled LTPROB13 (CCACTAT TAACACCCAAAGC) primers (Wasser *et al.*, 1997) with methods detailed in Vynne (2010). To ascertain the taxonomic identity of the fecal samples, we compared band sizes with known control samples from maned wolf, which amplified at a fragment length of 151 bp, and other sympatric carnivores (Supporting Information).

We subjected all confirmed maned wolf samples ($n = 936$) to a nDNA test to determine sex. For this, we used a primer set designed for kit foxes (*Vulpes macrotis mutica*, Ortega *et al.*, 2004). This primer amplifies a short (195 bp) fragment of the zinc finger (*Zfx* and *Zfy*) protein genes and contains a *Taq*I digestion site unique to the *Zfy* gene (Ortega *et al.*, 2004). We PCR amplified all extracts twice; those loci that were heterozygous (indicating male) in both replicates were scored as reliable and genotypes were recorded. All homozygous and uncertain genotypes (due to amplification failure or to allelic dropout) were PCR amplified an additional four times. We discarded samples that could be not reliably typed after six amplifications. To ensure accurate calling given the potential for allelic dropout of the Y band, we only called a sample as female once we had seen three female (and no male) bands. Known female, male and negative controls were run in all PCR-amplified assays and band sizes were as in Ortega *et al.* (2004).

Hormone extraction and assays

We prepared samples for hormone assays by freeze drying for 48 h or until all moisture was removed from the sample and then sifting the sample through a steel-mesh colander to remove nonfecal material. We recorded pre- and postfreeze dry weights to determine the per cent moisture in a sample and we expressed hormone concentrations per gram dry mass to control for dietary effects on hormone excretion rates (Wasser *et al.*, 1993). For extraction of glucocorticoids, we added 2.0 mL of 90% ethanol to 0.2 g freeze-dried and thoroughly homogenized fecal powder. For thyroid extractions, 15 mL of 70% ethanol was added to 0.1 g powder (Wasser *et al.*, 2010). We then vortexed, centrifuged and removed the supernatant from the tube containing the fecal pellet for storage at –20°C (Wasser *et al.*, 2010). Before assaying, fecal extracts were diluted in PBS (1:120 for glucocorticoids, 1:20 for thyroid).

Radiolabeled infusion studies have shown that maned wolves are predominantly fecal excretors of steroid hormones (Velloso, 1996; Velloso *et al.*, 1998) and adrenocorticotropic hormone challenge studies of fecal glucocorticoids produced biologically meaningful results on related taxa (gray wolves, Sands & Creel, 2004; spotted hyena, Goymann *et al.*, 1999). Fecal hormones were assayed with commercial [^{125}I] assay kits (MP Biomedicals 200-tube Corticosterone Double Antibody RIA kit: 07-120103 and MP Biomedicals 200-tube coated antibody Total T3 RIA kit: 06-B256447, MP biomedicals, Solon, OH, USA). The fecal glucocorticoid assay was previously used by Cummings *et al.* (2007) to study the relationship between environmental enrichment and fecal glucocorticoids in captive maned wolves and the thyroid hormone assay was validated for recovery by our laboratory (Wasser *et al.*, 2010).

Assays showed excellent parallelism and accuracy for maned wolf feces. All samples were assayed in duplicate, with nonspecific binding and blank controls in quadruplicate, a full standard curve and both manufacturer and study low and high controls in duplicate. Any samples falling outside the range of 15–85% bound or > 10% coefficient of variation between duplicates were re-assayed. Intra- and inter-assay variation was < 12%.

We measured glucocorticoids in 519 samples, which is less than the total number of maned wolf samples confirmed because some hormone samples had to be discarded when unforeseen circumstances (car breakdowns, power failures) prevented proper treatment and storage of samples in the field. Also, some samples did not have adequate fecal material for both DNA and hormone analysis. A reduced number ($n = 384$) of samples were analyzed for thyroid hormone since this assay was developed and validated later in our study and thus it did not include samples from 2004.

Predictors of DNA amplification and mean hormone levels and statistical analyses

To understand the relative influence and contribution of suspected important covariates related to sample condition and environment on DNA amplification success, we used classification trees, computationally intensive methods that facilitate data inspection and selection of explanatory variables (Breiman *et al.*, 1984; De'ath & Fabricius, 2000; Crawley, 2002). Classification tree partitioning allowed us to distinguish break points for scalar variables as well as to distinguish and group habitats for more parsimonious analysis without running each habitat type as a separate variable. From all possible splits of explanatory variables, we selected the one that maximized the homogeneity of the two resulting groups (mostly amplified, mostly failed to amplify; for further explanation of classification tree analysis and results, please see Vynne, 2010 or Supporting Information). We used these results to inform a generalized linear model (GLM) that predicted amplification success based on the suite of categorical variables assessed via classification tree analysis.

Exposure to humidity and ultraviolet light were expected to adversely affect sample quality (Murphy *et al.*, 2007). We therefore looked at the influence of season and habitat in which the sample was found on DNA amplification success. Based on the classification tree output, we considered four broad habitat classifications: closed-canopy woodlands and forests, open-canopy grasslands and shrublands, marshlands and converted (pasture and croplands).

Table 1 Description of categorical variables used to understand factors influencing quality of maned wolf (*Chrysocyon brachyurus*) scat samples collected in the Cerrado of Brazil

Variable	Character	Values
Odor	C	1 (none), 2 (some), 3 (strong)
Moisture	C	1 (completely dry), 2 (some moisture), 3 (moist/fresh)
Mold	C	1 (throughout), 2 (some), 3 (none)
Invertebrates	C	1 (throughout, > 20), 2 (some/none)
Habitat	L	closed canopy, open canopy, marshland, converted
Season	S	wet, dry
Fruit diet	F	1 (all fruit), 2 (some), 3 (none)

The character of variables is denoted by C, condition; L, location, S, season; F, food items.

We used a qualitative classification to define sample condition at time of collection by assessing odor strength, moisture level, presence of mold and amount of invertebrates on the sample prior to collection. These categories had previously been demonstrated to influence genotyping rates and are more accurate than age estimation (Piggott, 2004; Wasser *et al*., 2004; Gebhardt *et al*., 2009). We recorded the amount of odor in 1 of 6 categories: very strong, strong, moderate, weak, none or earthy. We assigned level of moisture to 1 of 4 categories ranging from fresh/moist to dry throughout unless the sample was wet due to rain, in which case this was noted. The project leader assured interobserver reliability by periodically cross-checking all teams for scoring consistency. For data analysis purposes and to help minimize potential scoring differences between observers, however, we collapsed categories into three levels each for odor and moisture (Table 1).

We also expected DNA amplification to be affected by the presence of fruit in the samples, since fiber decreases gut passage time and bacterial degradation and increases sloughing of cells in a sample (Burrows *et al*., 1982; Wasser *et al*., 1993; Murphy *et al*., 2003) and may also contain DNA inhibitors. For data analysis purposes, therefore, we grouped samples according to amount of fruit in the sample (Table 1).

We analyzed amplification success for mitochondrial DNA (mtDNA) and nDNA (separately) through a GLM using binary logistic regression (Crawley, 2002). We predicted amplification success as a function of the categorical variables (Table 1) to determine the variance explained and the relative importance of each factor. Model fit was determined through maximum likelihood estimation (Burnham & Anderson, 1998) after transforming the dependent into a logit variable, such that the GLM estimated the probability of amplification given the independent variables related to sample condition and environment (Table 1). The response variables for candidate models were, for mtDNA, successful amplification from at least one of duplicate extracts (binary response of yes or no), and for sex identification, successful determination based on our conservative calling protocol. We then used a step function to sequentially remove variables and analyze the resultant change in model performance, evaluated on the basis of the Akaike Information Criterion.

To test for influence of sample quality on mean hormone levels, we used linear regression with amount of hormone measured as $\log_{10}$ ng/g as the dependent variable and suspected influential covariates (amount of odor and moisture in sample at time of collection, presence of fruit in the sample, sex identification success or failure and % water weight lost in drying) as independent variables. We used Student's *t*-tests to compare means of all levels within a variable, and for all statistical tests, significance was measured at the $P = 0.05$ level (Zar, 1999).

Results

Over the four seasons of study, an average of 84% of samples amplified mtDNA (Supporting Information). Sixty-one per cent of the samples analyzed for sex yielded positive results and the sex ratio was not different from 50:50 ($X_1^2 = 0.1650$, $P = 0.6846$, 213 males, 192 females), as expected (Jácomo *et al*., 2009).

Influence of extraction method on amplification success

For 143 samples extracted from DMSO solution as well as by swabbing, Fisher's exact test showed a significant difference in mtDNA amplification between the two extraction methods; mucosal swabbing of frozen samples yielded better amplification (105 samples amplified) than samples extracted from DMSO solution (76 samples amplified; $P = 0.001$). Only 3% (4 of 118) of samples extracted a third time resulted in a positive species identification not previously confirmed by the duplicate pair extracts. Even in samples that passed screening for mtDNA, the mucosal swab method of extraction improved nDNA amplification success ($P = 0.023$; 83% of swabbed samples amplified for gender vs. 70% of DMSO-preserved samples).

Allelic dropout in field-collected samples, which was calculated by dividing the number of errors detected by the total number of cases in which an error could have been detected (Creel *et al*., 2003; Broquet & Petit, 2004; Vynne, 2010), occurred in 32% of samples (68 male band dropouts in 213 confirmed male samples). Of 44 confirmed male samples extracted with both swabbing and DMSO methods, allelic dropout occurred in seven of the DMSO-extracted and three of the swabbing extracted samples.

Influence of sample condition, exposure and contents on amplification success

Significant contributing factors to the GLM model fit for amplification of mtDNA from putative maned wolf scat were amount of odor and moisture in the sample, as well as habitat where the sample was found (Table 2). The most parsimonious GLM for predicting amplification success for

Table 2 Parameter estimates from statistical model showing factors contributing to probability of mtDNA and nDNA amplification of maned wolf (*Chrysocyon brachyurus*) scats collected in the Cerrado of Brazil between August 2004 and April 2008

	mtDNA				nDNA			
Term	Parameter estimate	SE	*z*-value	*P*-value	Parameter estimate	SE	*z*-value	*P*-value
(Intercept)	1.135	0.260	4.356	< 0.001	1.103	0.266	4.140	< 0.001
Odor	0.326	0.154	2.118	0.034	0.106	0.153	0.691	0.489
Moisture	0.539	0.170	3.178	0.001	0.125	0.127	0.984	0.325
Mold	–0.501	0.310	–1.615	0.106	–0.677	0.302	–2.242	0.025
Invertebrates	–0.391	0.372	–1.053	0.292	–0.597	0.371	–1.612	0.107
Habitat	–0.699	0.312	–2.243	0.025	–0.480	0.239	–2.007	0.045
Season	0.738	0.544	1.357	0.174	–0.160	0.346	–0.461	0.645
Fruit diet	–0.089	0.152	–0.588	0.556	–0.747	0.154	–4.864	< 0.001

mtDNA included habitat, odor, moisture, mold and season only (i.e. exclude diet and invertebrates). Amplification success was positively correlated with the amount of odor and moisture (Fig. 1), and was negatively correlated with closed-canopy habitats (Table 2; Supporting Information).

Presence of fruit, mold and habitat type drive variance in the GLM for sex determination in samples that previously amplified mtDNA (Table 2). Presence of fruit negatively predicted amplification success as did occurrence of mold throughout a sample and samples found in closed-canopy and marsh habitats were collectively less likely to amplify nDNA than samples found in other vegetation types (Supporting Information).

Factors influencing mean hormone levels

The per cent moisture lost during in the drying process was not correlated with mean hormone levels ($R^2 < 0.002$, $P = 0.384$ for glucocorticoids; $R^2 < 0.006$, $P = 0.149$ for thyroid) and samples that thawed in shipment did not have different mean hormone levels than samples that arrived frozen ($F_1 = 0.940$, $P = 0.334$ for glucocorticoids and $F_1 = 1.335$, $P = 0.249$ for thyroid). Samples that successfully amplified for sex had higher mean levels of glucocorticoids ($F_1 = 5.321$, $P = 0.022$) and thyroid hormone ($F_1 = 19.229$, $P = 0.001$) than samples that failed to amplify nDNA. Amount of odor in the sample was also positively correlated with mean glucocorticoids ($F_2 = 22.284$, $P = 0.001$) and thyroid hormone concentrations ($F_2 = 3.630$, $P = 0.028$), as was the amount of moisture in a sample ($F_2 = 3.037$, $P = 0.049$ glucocorticoids; $F_2 = 5.882$, $P = 0.003$ thyroid). Samples that were fresh or retained moisture had higher mean hormone levels than samples that that were entirely dry or wet due to rain (Fig. 2). The influence of odor remained when the sample set was constrained to samples that had positively amplified nDNA ($F_2 = 13.780$, $P < 0.001$ glucocorticoids; $F_2 = 4.600$, $P = 0.011$ thyroid). Samples that contained fruit material had higher mean glucocorticoid levels than samples without fruit ($F_1 = 4.272$, $P = 0.039$). For thyroid, samples comprised entirely of fruit had lower mean hormone levels than samples with some or no fruit ($F_2 = 7.699$, $P = 0.001$).

Discussion

Mucosal swabbing of samples for DNA extraction yielded improved amplification success over buffer-preserved samples and reduced allelic dropout. This is likely due to a greater number of epithelial cells being present on the outside of the scat as well as because this method reduces the amount of inhibitors in the PCR (Wasser *et al.*, 2011). Since our samples were mixed and experienced some freeze thawing during storage and transport prior to implementing our swabbing protocol, we expect the genotyping success could be further improved by swabbing the outside of fresh samples in the field (Rutledge *et al.*, 2008; Stenglein *et al.*, 2010). The mucosal swabbing method is also significantly less costly, since it does not require the use of the Qiagen® Inhibitex tablets or the Geneclean® purification process, and the higher DNA quality will reduce the number of required repeat-amplifications for confirming alleles.

Our amplification results were similar to those of Miotto *et al.* (2007) for puma in another region of Brazilian Cerrado, to Michalski *et al.* (2011) for Amazonian carnivores and to those of to Farrell *et al.* (2000) for puma and jaguar in Venezuela. A decrease of at least 20% from mtDNA success rates to sex or individual identifications with nDNA is typical for scat (Frantzen *et al.*, 1998; Kohn *et al.*, 1999; Ernest *et al.*, 2000) and consistent with our findings. Our relatively poor amplification and high allelic dropout rates of nDNA in samples that were more degraded at time of collection supports use of condition as a proxy for sample age, which controlled studies have shown influences error and amplification at rates comparable to ours (Dallas *et al.*, 2000; Piggott, 2004; Murphy *et al.*, 2007). The poor amplification and high error rates of DNA-poor samples in our study made our attempts at amplification of microsatellite loci for genotyping individuals of all samples cost-prohibitive, which is a limitation to this study. Prioritization of high-quality samples, however, would improve feasibility since initial testing of a few samples selected based on our condition analyses amplified consistently across loci and with fewer errors.

Other studies have hypothesized that diet may influence ability to extract DNA from scats (Reed *et al.*, 1997; Farrell

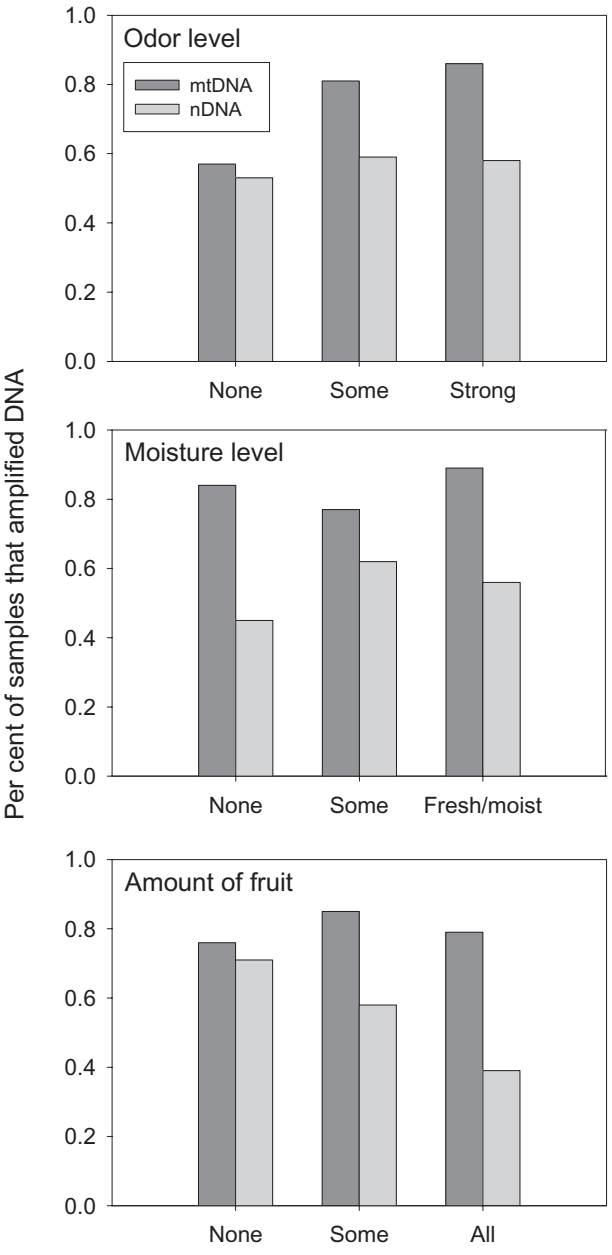

Figure 1 Proportion of putative maned wolf (*Chrysocyon brachyurus*) scat samples that amplified for mtDNA and nDNA based on amount of odor, moisture and fruit in the sample.

et al., 2000). While potentially complicated by the fact that we did not analyze for nDNA samples that failed to yield mtDNA, our results showed that that nDNA, but not mtDNA, was sensitive to diet type. This is consistent with the controlled study of Murphy *et al.* (2003) and should be considered in both study design and data analysis for studies of species with highly varied diets such as maned wolves. Although their sample size was small, Panasci *et al.* (2011) found that preservation method influenced DNA amplification depending on the food item in the scat. This warrants further exploration, and while we caution researchers to consider the influence of individual dietary preferences and seasonal abundances of certain food items in study design and analysis, mucosal swabbing of scats is likely to eliminate problems due to fecal inhibitors that vary by diet type (Wasser *et al.*, 2011).

The influence of sample quality at the time of collection on hormone levels is of considerable importance and will be increasingly important to address as interest in the collection of noninvasive measures increases. Measurement error in the quantifiable amount of hormone will affect ability to interpret main effects if not accounted for in statistical analyses (Vynne, 2010). That our results demonstrated a significant influence in presumably older samples (samples that were dried or had little odor) suggests the importance of collecting samples that are as fresh as possible. Ensuring consistent measurement of sample quality at time of collection will be essential for accounting for this error in analysis.

Correlations showing differences in food items in a scat and hormone levels could be either due to fiber affecting excretion and the amount of hormone in the sample or due to a physiological difference the animal is experiencing that is affecting food item choice by the animal. We believe that the results of this study are biologically meaningful because prior work has been shown to control for the effects of fiber (Wasser *et al.*, 1993); fiber-rich scats were associated with positive amounts of one hormone and negative amounts of another hormone, and the results are as would be expected from related work that correlates landscape use, diet and physiology in maned wolves (Vynne, 2010). More specifically, while maned wolves regularly consume large amounts of fruit (Jácomo *et al.*, 2004) and a fruit-rich diet does not necessarily indicate an animal is in poor health, diet analysis from our study showed that the most commonly consumed fruit was *Solanum lycocarpum,* which has been shown to have antihelmintic properties (Cruz *et al.*, 2008). This fruit was more commonly consumed in disturbed habitats and when other food resources were less available (Vynne, 2010). Our results thus match expectations that individuals experiencing high parasite loads and/or nutritional stress would consume more fruit (Vynne, 2010) and have lower levels of thyroid hormone (van der Heyden *et al.*, 1986; Eales, 1988; Wasser *et al.*, 2010) and higher levels of glucocorticoids (Wasser *et al.*, 2004; Pereira, Duarte & Negrão, 2006).

Similar effects of diet on glucocorticoids were also shown for grizzly bear by von der Ohe *et al.* (2004), although they were unable to explain these trends at the time. Glucocorticoids were lowest in grizzly bears eating berries, second lowest in bears eating flesh (salmon) and highest in bears eating grass. These dietary differences are precisely what one would predict in a nutrition model of glucocorticoid secretion (Romero, Dickens & Cyr, 2009), given carbohydrate-rich berries and the relatively low nutritional value of grass. By contrast, fiber impacts on steroid excretion could not explain these trends since berries and grass are both much higher in fiber than in flesh and samples were freeze dried and expressed per gram dry weight to control for those

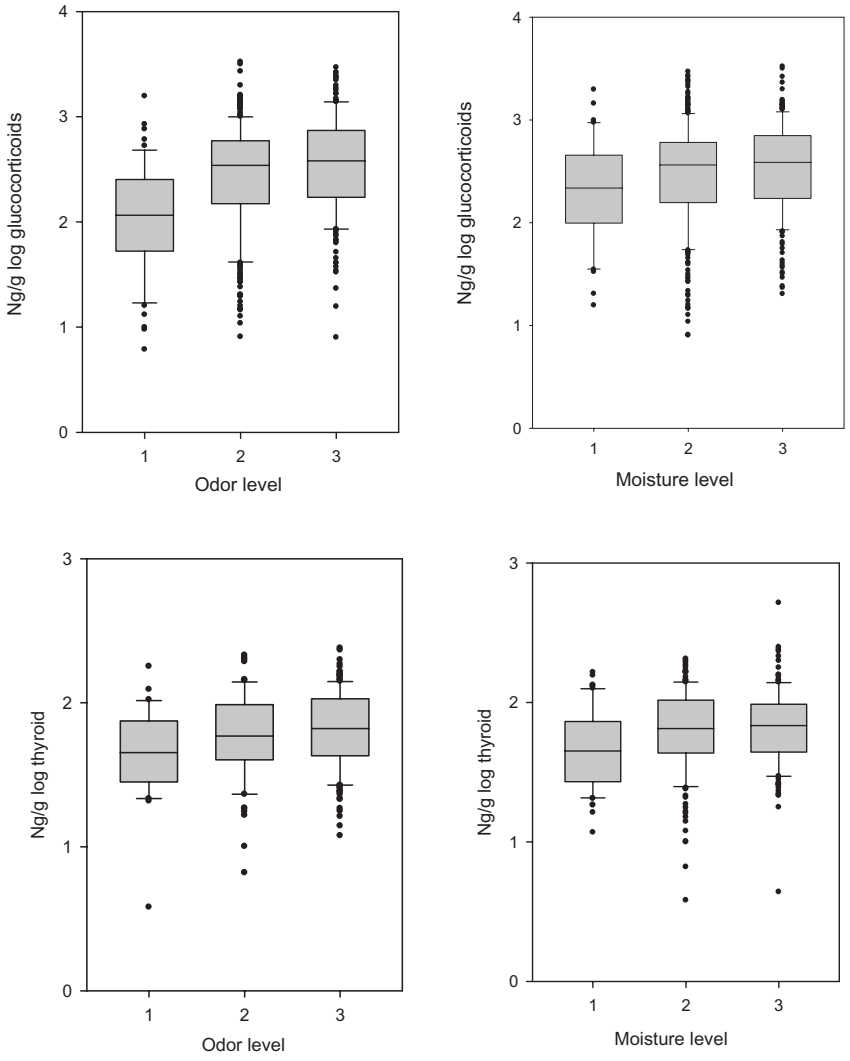

Figure 2 Median hormone levels of maned wolf (*Chrysocyon brachyurus*) scats with respect to varying levels of odor (1 = none, 2 = some, 3 = strong) and moisture (1 = none, 2 = some, 3 = moist/fresh) at time of collection.

effects (Wasser *et al*., 1993). A fourth diet group in the bear study, termed mixed, varied in composition from grass and other vegetation early in the season to a combination of berries and flesh late in the season. Glucocorticoids sharply declined across the season in those mixed samples, which is in concordance with nutritional expectations. Both the von der Ohe *et al*. (2004) and our study were correlational, and the possibility that fiber is influencing hormone amounts thus cannot be ruled out. To our knowledge, however, there is no reason to expect that excretion rate would influence one hormone in a positive manner and another in a negative manner, as occurred with our study. Nevertheless, it would be wise to conduct a dietary trial and/or correlate fecal hormones with independent measures of stress (blood, behavior, visual assessment of body condition) to rule out the possibility that fiber is not directly influencing hormone level measurements in maned wolf scats.

The results of this study highlight the importance of using consistent, quantifiable and repeatable measures of sample quality when collecting samples of unknown age in the field. While samples may be collected and stored relatively inexpensively, laboratory processing of samples requires large amounts of time and often comprises a large proportion of a field project's budget. Being able to prioritize samples for laboratory analyses that are most likely to yield positive DNA results for all but the rarest of species is thus likely to benefit most investigators. Sample quality categories may be compared with available budgets and then used to prioritize samples for extraction for DNA analysis. While other studies have suggested using mtDNA as a screening for further nDNA analysis, our results show that further prioritization and associated cost savings of avoiding repeat amplification and/or error due to allelic dropout may be done by selecting only the highest quality samples even of those that positively yielded mtDNA.

This study supports a small but growing body of evidence that demonstrates the feasibility of extracting DNA from samples of unknown age that are collected in challenging environmental conditions, including the tropics (Michalski *et al*., 2011). It also emphasizes the importance of using

consistent indices of sample condition at time of collection. While comparable studies for hormone analyses do not to our knowledge exist, it is reasonable to expect that the species of origin and environment in which the sample is deposited will influence the degree to which sample condition affects measurable hormone levels. We thus recommend that researchers measure and account for both environmental and sample quality factors that may contribute to measurement error when testing for main effects. Increasing understanding for how sample quality influences hormone levels, as well as if and how this varies by species, in particular, will enable researchers seeking to employ noninvasive measures of animal physiology to better measure and understand main effects and crucial questions for species of interest. We strongly encourage other researchers to measure and report results in future studies.

In summary, the ability to monitor wide-ranging species over large landscapes is increasingly important for conservation planning (Boyd *et al.*, 2008). Scat provides a survey and monitoring tool that is attractive since it may be collected without having to disturb study animals. Samples that are collected without directly observing the target species, however, may be days or weeks old (Smith *et al.*, 2003), and environmental conditions may degrade DNA and influence hormone levels. To effectively and efficiently apply noninvasive scat sampling methods in large-scale studies, therefore, it is important to identify the variables that impact PCR amplification success. To control for hormone variation due to sample quality and allow for accurate testing of biological effects, we recommend that sample quality covariates be included in analyses to exclude variation due to sample quality. Our results show that operational assessments of sample condition in the field can be reliably made by multiple observers and provide more refined predictors of DNA and hormone degradation to reduce genotyping costs and errors, as well as remove nonbiological variance in endocrine assessments of biological impacts.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Online S1. Species controls of some sympatric carnivores in the region of Emas National Park, Brazil and their band lengths as analyzed using HSF21 and LTPROB13 primers and fragment length polymorphisms.
Online S2. Mitochondrial DNA amplification success and species assignments from scat samples collected from putative maned wolf.
Online S3. Classification tree outputs showing all variables that split (i.e. there was a significant break between the factors of the variable) for mtDNA in maned wolf scats. Any variables not represented in the figures were uniform.
Online S4. Classification tree outputs showing all variables that split for nDNA in maned wolf scats. Any variables not represented in the figures were uniform.
Online S5. Classification trees of the most parsimonious models for mtDNA (a) and nDNA (b) amplification from maned wolf scats. Significant break points for mtDNA are evident in odor (break between samples having none and any (some or strong), and moisture (samples that were entirely dry and samples that had some moisture or were fresh), whereas diet alone dominated the model for nDNA amplification.

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