Non-invasive Assessment of Stress Hormones, Parasites, and Diet, Using Scat of Five Felid Species in Belize, Central America

Jose Bernardo Mesa Cruz

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Marcella J. Kelly, Chair
Janine L. Brown
William A. Hopkins

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Abstract

Many Neotropical felid species, such as jaguars, are threatened with extinction due to habitat fragmentation and/or human persecution. Human activities around protected areas in Belize, Central America, are increasing and so are levels of human-felid conflict. Potential consequences of this conflict are an increase in stress impacting health, diet shifts, or heightening of animal aggression. The goal of this work was to assess the effects of human-modified habitats on native felids by comparing fecal glucocorticoid metabolite (FGM) concentrations, endoparasite species richness (ESR), and diet using non-invasive scat sampling in a protected forest vs. surrounding non-protected areas in Belize. Field studies relying on non-invasive fecal hormone monitoring are subject to potential hormone degradation in samples exposed to the environment. Therefore I conducted immunoassay and environmental validations for measuring FGM in jaguars (*Panthera onca*).

In the field, I collected scat using a detector dog, identified samples using DNA, retrieved parasite propagules with a flotation technique, and identified prey remains by morphology. I detected five felids: jaguar, puma, ocelot, jaguarundi and domestic cat. FGM concentrations were higher in pumas and jaguarundis than in the other felids. I found no livestock remains in felid scats. ESR was similar across felid species. Domestic cats were found only in human-modified areas. This results provide a baseline on adrenal activity, prey consumption, and endoparasites in felids of Belize. These findings could be used for comparisons to populations thought to be affected by human activities across Belize and in neighboring countries.

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Attributions

Dr. Janine L. Brown is a research scientist and director of the Endocrine Laboratory of the Center for Species Survival at the Smithsonian Conservation Biology Institute. Dr. Brown has conducted endocrine research to better understand reproductive and stress biology of endangered wildlife, including several felid species, for more than 15 years. Dr. Brown contributed supplies and facilities for hormonal analysis in this work as well as provided significant input in study design and analysis approaches.

Dr. Marcella J. Kelly is an associate professor in the Department of Fish and Wildlife Conservation at Virginia Tech. Dr. Kelly's research program uses population dynamics framed in an ecological context and cutting edge technologies to promote wildlife conservation. Dr. Kelly has studied felids in Belize for over 12 years. Dr. Kelly contributed financial support and provided significant input in study design and analysis approaches in this work.

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Introduction

Humans threaten several carnivore species with extinction across the globe and Neotropical felids are no exception. Currently, habitat destruction and killing in retaliation for livestock losses are the most important factors contributing to population declines. Although Belize has relatively healthy felid populations, very little is known about the effects of human disturbances on these populations. Human activities near and inside forests, along with human-felid conflict, are occurring more and more frequently in Belize. Therefore it is a high priority to determine whether anthropogenic activities are affecting the well-being of Neotropical Belizean felids. The overall goal of this work is to evaluate four different biological aspects of Neotropical felids that might be altered by human disturbances: species distribution, stress hormones, parasitic diseases, and prey consumption. Such disturbances can decrease the capacity of individuals, and thereby populations, to survive and persist in highly impacted environments. Nevertheless, obtaining biological information directly from wild Neotropical felids is very challenging. Recent advances in non-invasive carnivore monitoring using scat has made it possible to evaluate the potential effects of human disturbances on felids at the population level. Despite such advances, there are several limitations in data acquisition and interpretation when information is collected by non-invasive means. In the following review I address and discuss applications, advantages, and limitations of three different non-invasive methodologies that use scat to measure degrees of disturbance in free-ranging carnivores: endocrine assessments to measure stress hormones, disease assessments, and diet assessments.

Endocrine assessment

Description

Hormones play a crucial role in a myriad of physiological processes that allow an animal to maintain homeostasis and cope with its environment. Hormones influence growth, immune

function, behavior, response to environmental cues or stressors, and regulate reproduction (Boonstra, 2005; Moberg, 1985). Today, the emerging discipline of conservation physiology seeks to understand the physiological responses of animals to environments altered by human disturbance and endocrinology is a crucial component of this field (Wikelski and Cooke, 2006).

Traditionally, studies of endocrine function in wildlife relied on collecting blood to measure circulating hormones. There are evident disadvantages in using this method in the field to study carnivores. Besides the inherent difficulties in capture and handling of carnivores, trapping and handling elicit a stress response in the animal that is measurable in blood within minutes after capture in many species - masking the hormonal concentrations that are often of interest (Creel, 2001; Place and Kenagy, 2000; Romero et al., 2008). In addition, concentrations of hormones like cortisol in blood vary naturally according circadian patterns, sometimes as much as sevenfold in a single day (Hess et al., 1981; Thun et al., 1981).

Currently, non-invasive hormone monitoring (NHM) tools are being increasingly employed to monitor wildlife populations and individuals; especially in the fields of conservation endocrinology and behavioral ecology (Berger et al., 1999; Cockrem, 2005; Foley et al., 2001; Garnier et al., 2002; Sands and Creel, 2004). Immunoassays for biological samples collected non-invasively, such as scat (Barja et al., 2008; Creel et al., 1997; Wasser et al., 1988), urine (Braun et al., 2009; Thompson and Wrangham, 2008), saliva (Queyras and Carosi, 2004), feathers (Bortolotti et al., 2008), and hair (Bechshøft et al., 2012; Koren et al., 2002) have been used to measure the concentration of select hormones and their metabolites.

Two types of steroid hormones are commonly assessed in non-invasive studies of wildlife endocrinology: adrenal and gonadal. The measurement of adrenal hormones including glucocorticoids (GCs) (*e.g.,* cortisol and corticosterone), also referred as stress hormones, is commonly used as an indicator of overall physiological condition of the individual (Sheriff et al., 2011). One timely application of this measurement is to quantify the effect of specific anthropogenic influences on wildlife, such as habitat modification (Spercoski et al., 2012;

Wasser et al., 1997), motorized winter recreation (Creel et al., 2002), and human presence (Barja et al., 2007; Creel et al., 2013; von der Ohe et al., 2004). Gonadal hormones, such as progestagens, estrogens, and androgens, have been used to non-invasively determine puberty, estrous, ovulation, pregnancy, abortion, and sex (Brown and Wildt, 1997; Dehnhard et al., 2008; Graham et al., 2006; Herrick et al., 2010; Morato et al., 2004; Sanson et al., 2005). Methods to determine these reproductive stages have been well established in captive populations, *ex-situ* conservation programs, and domestic livestock, and have successfully been applied to investigations on free-ranging wildlife (Barja et al., 2007; Dloniak et al., 2004; Wasser et al., 1997).

The general physiological mechanism by which hormones are excreted and then detected in feces is described here. Some noxious stimulus, like a winter storm, first causes the activation of the hypothalamic-pituitary-adrenal (HPA) axis and subsequent release of GCs and other synergistic hormones (*e.g.,* catecholamines) into the bloodstream. GCs are metabolized in both liver and kidneys and excreted in the bile and urine, respectively. Hormone metabolites secreted in the bile are further metabolized in the gut by microflora, and finally excreted in feces (Möstl and Palme, 2002). The concentration of steroid hormones, have a much longer lag time of appearance in feces than in blood. This time is determined by metabolic clearance rates and gut transit time. In medium and large sized carnivores, that lag can take anywhere from 12 hours to 2 days after the endocrine event (Schwarzenberger et al., 1996). Fecal hormone monitoring has several advantages, including: 1) no induction of stress through animal capture for sample collection, and 2) hormonal concentrations in scat represent a "pool" that dampens the effects of normal circadian and pulsatile secretory fluctuations (Brown and Wildt, 1997), circumventing the caveat of fluctuating dynamics of GCs in blood that can complicate interpretation of results (Creel, 2001; Palme, 2005).

In biological samples, hormones can be assessed through both quantitative and qualitative techniques. The first (*e.g.,* immunoassays and spectrometric), are capable of detecting small concentrations. The second (*e.g.,* commercial bench-top assay kits), identify presence or absence

of the hormone. Nonetheless, quantitative techniques such as immunoassays are the most widely used method in wildlife physiology. The 1960s saw the development of both radioimmunoassays (RIA) by Yalow and Solomon (1960), and enzyme-linked immunosorbent assays (ELISA), also known as enzyme immunoassays (EIA), developed by Perlmann, *et al.* (1971) (Lequin, 2005). Since then, the reliability of these techniques has improved tremendously for the study of wildlife physiology. Today EIA/ELISAs and RIAs are capable of recognizing a suite of steroid hormones and their metabolites; an ideal scenario for NHM because results can reflect physiological conditions in a given species with greater specificity. In contrast, spectrometric techniques like mass spectrometry are not routinely used in this field, despite the great applications in metabolite identification and utility in hormone concentration measurement for applied endocrinology. Economic costs of equipment and intensive sample processing have delayed the application of this technique in non-invasive endocrinology. Yet these spectrometric techniques hold great promise for future applications in wildlife endocrinology.

Data collection and storage

Techniques that measure steroid metabolites excreted in urine or feces provide an avenue for NHM research on the physiology of free-ranging carnivore populations. Unfortunately, urine is often impractical to procure from wild, free-ranging carnivores except in some cases taking advantage of behavioral particularities of a species. For instance, dwarf mongooses (*Helogale parvula*) tend to urinate on rubber pads for scent marking (Creel et al., 1993) and urine soaked sand collected from human-habituated meerkats (*Suricata suricatta*) was used to measure reproductive steroids (Moss et al., 1991). Due to its limited use, non-invasive urine collection will not be further discussed here. In fact, fecal hormone monitoring is often preferred for non-invasive endocrine assessment in free-ranging populations for several reasons. Metabolic studies in captive populations show that adrenal and gonadal steroid metabolites are predominantly excreted at measureable concentrations in feces of several carnivore species (Brown and Wildt, 1997; Brown, 2006; Young et al., 2004). Additionally, scat is relatively abundant in the environment and improvements in scat detection techniques have made sample collection more

feasible (Kerley, 2010). This method allows long-term longitudinal study designs, especially in instances where paired studies or impact assessments are necessary.

There are important aspects of scat collection and processing that need to be considered to ensure sample quality and obtain biologically relevant data about the individual or population. Scats should be mixed, or homogenized, prior to lab analysis to ensure representative hormonal concentrations in the sample. This is important because hormones and metabolites may be unevenly distributed in the scat (Millspaugh and Washburn, 2003). Moreover, after defecation, microflora present in the scat can still produce enzymes that further metabolize steroids altering concentrations (up or down) of target steroids (Macdonald et al., 1983; Shackleton et al., 2008). Therefore, several field methods have been proposed to arrest microbial and enzymatic activity in the short (hours to days) and long (weeks to months) term such as drying (using oven or desiccants) and freezing, respectively (Lynch et al., 2003; Millspaugh and Washburn, 2004; Möstl and Palme, 2002). Freezing is recommended even if the samples have been dried or hormones extracted (methodology discussed below), unless metabolite stability over time is tested beforehand for the species of interest.

When conducting field scat collection for NHM studies, it is extremely important to know the age of the sample and hormonal degradation rates, as they are generally hormone- and species-specific. A number of environmental factors can affect the concentration of hormones/ metabolites in a scat sample. Steroid hormones in feces can be degraded by ultraviolet light, humidity, and temperature. Additionally, degradation rates are influenced by species-specific intrinsic intestinal flora and diet (Schwartz and Monfort, 2008; Touma and Palme, 2005).

Validation

An important aspect in performing NHM is to validate the technique in order to ensure biologically meaningful results. Laboratory procedures should be developed and validated for the species of interest prior to undertaking the study in the field. This is due to the fact that metabolism of steroid hormones is different for each species. In addition to different degradation

rates (noted above), both type and concentration of metabolites are unique for each species and for each biological sample type (*e.g.,* urine or feces).

Traditionally, validation involves three components: evaluation of the species endocrine physiology, hormonal extraction procedures, and immunoassay selection. First, evaluation of endocrine physiology can be done by either stimulating endocrine organs with exogenous hormones (*e.g.,* adenocorticotropic hormone (ACTH) challenge) or by monitoring physiological events (*e.g.,* acclimatization, recovering from surgical procedures, pregnancy, ovarian cycles). Subsequently, a metabolite analysis using high-performance liquid chromatography (HPLC) or mass spectrometry, on samples (feces) collected from the stimulation or physiological event, will allow the selection of candidate immunoassays for validation, as well as provide information about metabolism and gut transit time. For this component, the use of captive individuals under controlled settings is strongly recommended. Second, hormonal extraction procedures are required to solubilize steroid hormones present in feces. There are two main extraction methods that are commonly used in carnivore research, agitation and heating, to dissolve the hormones in an alcohol based media (*e.g.,* ethanol or methanol). Agitation is a practical method for field conditions and ranges from simple hand agitation to more powerful industrial homogenizers (Santymire and Armstrong, 2010). Heating extraction, also known as “boiling”, is considered the best method of extraction, and is often used to corroborate results of agitation methods. Third, immunoassay selection is made based on both affinity of the antibody to the desirable metabolite (obtained from HPLC analysis) and cross-reactivity of the antibody with other hormonal metabolites and substances present in the extract (Keay et al., 2006; Millspaugh and Washburn, 2004; Palme, 2005; Touma and Palme, 2005; Young et al., 2004).

A fourth critical, but sometimes not considered, source of variance in NHM that is important to validate is the impact of environmental conditions on hormone profiles. Most of the research on NHM in wild populations of elusive species has occurred in temperate environments, yet the effects of the environment on hormonal metabolite concentrations have not been directly assessed in the majority of these studies. For instance, a survey of fecal glucocorticoid

metabolites (FGM) of Alaskan brown bears (*Ursus arctos horribilis*) conducted during the summers months in Katmai National Park, Alaska, adopted a 2 day interval as prudential time for hormonal concentration stability (von der Ohe et al. 2004). Other studies have been performed strictly during the snow-filled winter, thus avoiding any type of change in hormone metabolite concentrations, which are stable when frozen (e.g. elk, *Cervus elaphus* and wolves, *Canis lupus* in Yellowstone National Park- Creel et al. 2002; capercaillie, *Tetrao urogallus* - Thiel et al. 2008).

While there have been some attempts to evaluate the effects of environment on hormone concentration for NHM (Table 1.1), in general the studies have been fairly limited. Additionally there are multiple methodologies as researchers have assigned different names to procedures for assessing environmental effects on steroid metabolites concentrations, despite the shared objective of establishing a period of time over which such concentrations remain constant, or stable, in samples found in a certain habitat type. Some current techniques have been described as: washing-out experiments (Rehnus et al., 2009), effects of fecal age and seasonality (Abáigar et al., 2010), field stability experiments (Fanson, 2009), and the rate of FGM degradation (Hulsman et al., 2011). Therefore, I propose the term *environmental validation* to collectively refer to systematic experimental methods that aim to assess the effects of natural environmental conditions on fecal steroids present in the excreta of animals that have been exposed to such conditions over a known amount of time. Environmental validation fits within the already established framework that ensures appropriate validation for NHM techniques (discussed above). For further details on validation see also (Touma and Palme 2005).

To achieve biologically meaningful results, implementation of environmental validation should include:

a. Assessment of steroid hormone concentrations of a control sample, with limited exposure to the environment, and subsequent subsamples with known time of exposure to known environmental conditions.

b. Assessment of environmental effects on samples in areas of the species' range. Currently, validations are often established in geographic regions where the species of interest is not native. Yet, environmental features and patterns differ drastically across the globe, potentially having different effects on steroid metabolite concentrations.

c. Monitoring of relevant environmental factors and characteristics. This is particularly important for determining which environmental conditions are most favorable to steroid metabolites, especially if the geographic area does not have marked seasonality. Measures of environmental variables should include: temperature, relative humidity and rainfall, exposure to the sun and ultraviolet ray intensity, soil substrate, and any other habitat feature relevant to the species of interest.

d. Conducting field tests to determine the range of natural climatic conditions that are disadvantageous to stability of hormonal concentrations (*e.g.,* rain/moisture, UV light, high heat). Age assignment of samples using morphological characteristics (and photographs) over time could be attempted to assess reliability of using such characters in scat age determination.

e. In field tests with known age scats, exposure of excreta to the environment for 3 times as long as the gastrointestinal passage of the species of interest. For example, gut passage for a jaguar is about 36 hours, therefore field trials should be a minimum of 4.5 days. The longer the excreta are exposed to the environment in field trials, the longer the time horizon for establishing a period of hormonal concentration stability.

f. Evaluating at least two immunoassays for biological relevance. Uncertainty in biotransformation processes of steroid hormone metabolites and the interaction of byproducts with the immunoassay antibody could be decreased by testing more than one immunoassay. This practice will increase the chance of capturing or confirming relevant changes in the concentrations of steroid metabolites.

g. The use of dried feces for efficient analysis. Excreta can gain or lose water content depending on the relative humidity of the environment. Relative humidity in the environment can

change drastically on ultradian or circadian bases, and this increases variability in the final steroid metabolite concentration, potentially masking the initial concentration.

Stress assessment

Stress is considered as the physiological and/or behavioral response that occurs when an individual perceives a threat to completion of its normal life cycle (McEwen and Wingfield, 2003; Romero, 2004). This response could be classified as either acute or chronic depending on the length of exposure, intensity of the stressor (*i.e.,* threat), and the ability of the individual to find a physiological balance (*i.e.,* acclimatization/acclimation). The acute stress response is highly conserved phylogenetically across vertebrate taxa (Romero, 2004) thus the mechanism is considered adaptive (Boonstra, 2005; Nelson, 2005). In fact, acute stress allows an organism to modulate its metabolism, redirecting resources from innate processes like digestion, growth, immune function, and reproduction, to counter an immediate threat (Nelson, 2005). When a stress response is sustained over extended periods of time, and the individual is not capable of finding , the organism is said to be experiencing chronic stress, also known as distress. However, high complexity of interactions between physiological and pathological processes acting during stress has led to controversies among researchers on how stress is defined and studied. Nevertheless, stress could be framed in a general form under three approaches. First, stress could be framed based on an earlier definition under the homeostatic model, which implies that physiological stages remain fairly constant over time (Cannon, 1932). Therefore the animal recognizes a stressor as a threat against homeostasis, then a cascade of physiological events occurs to neutralize the stressor and reestablish homeostasis (Nelson, 2005). When the stress response has adverse effects in the animal's well-being, then the animal experiences "distress" (Moberg, 2000).

Second, stress can be framed based on another definition under the allostatic model, which implies that physiological stages are maintained through change (*i.e.,* life stage and environment). Therefore there is a "normal" fluctuation in concentration and bioactivity of hormones mediating stress called allostatic load. Once those normal fluctuations reach a

threshold producing distress or allostatic overload, detrimental effects become evident in the animal (McEwen and Wingfield, 2003). More recently, Romero *et al* (2009) proposed a third stress model that merges both former models, the Reactive Scope Model. The later overcomes the limitations of both homeostasis and allostatic models, by expanding the physiological mediators to more than energetic constrains and accounting for circadian and circannual variations in physiological responses to stress.

It is clear from studies in other species, however, that detrimental effects on behavioral, reproductive, metabolic, immune, and neurological function are associated with chronic exposure to high concentrations of stress hormones, and that this results in a decreased capacity of individuals to survive and persist in highly impacted environments (*Mammals*- Maccari and Morley-Fletcher, 2007; Pride, 2005; Sapolsky et al., 2000; Ward et al., 2008; Zhao et al., 2007; *Birds*- Ellenberg et al., 2007; Kitaysky et al., 2006; *Reptiles*- French et al., 2008; Romero and Wikelski, 2001). GCs are controlled and produced by the HPA axis in response to physiological and pathological demands, and environmental disturbances. In general, the HPA axis is self-regulated by a series of cascade effects and feedback controls. The Hypothalamus produces corticotropin-releasing hormone (CRH), which stimulates the anterior pituitary to produce adenocorticotropic hormone (ACTH). Then, ACTH stimulates the adrenal cortex to produce and secrete of GCs. Cortisol and corticosterone are the main GCs produced in the adrenal cortex (Martin and Crump, 2003). These steroid hormones circulate in the blood stream looking for target organs to perform their physiological function, such as control of circadian rhythms, metabolism of carbohydrates, proteins and lipids, enhancing cardiocyte activity, and immune-modulation, among others (Martin and Crump, 2003). After the effect of GCs is performed, GCs can be metabolized either by the liver, where they are conjugated with glucoronic acid (glucoronidation), or by the kidneys, where a sulfate radical is added (sulfation) (Möstl and Palme, 2002).

Moberg (1985) noted that the neuroendocrine response to stress has the greatest potential to indicate the impact of stress on an animal's overall well-being. Wildlife endocrinology has,

therefore, focused on correlating the physiological responses of individuals and populations to human caused disturbances in ecosystems. For example, Wasser *et al.* (1997) showed the use of such a proxy in their study on the effects of logging disturbance on the northern spotted owl (*Strix occidentalis caurina*). Male owls in logged areas had higher concentration of fecal glucocorticoid metabolites (FGM) than those in undisturbed areas. Creel *et al.* (2002) observed higher FGM concentrations in elk and wolves in relation to the intensity of winter snowmobile activity in Yellowstone National Park. Barja *et al.* 2007 showed a direct correlation between unregulated tourism and the level of FGM in the European pine marten (*Martes martes*). These studies demonstrated that FGM can be measured as an indicator of the physiological response of wild animals to anthropogenic activities. As such, endocrine sampling provides an effective, low cost tool to conservation practitioners and land managers, facilitating the identification of potential threats to the well-being of carnivore populations.

Despite the advances obtained in this field using correlative inference, to date, these studies do not generally address cause and effect or ultimate consequence. The direct link between elevated GCs and FGM and individual fitness continues to be difficult to ascertain. However, potential improvement could be attained by designing paired and/or manipulative studies, increasing sample size (*i.e.,* study sites), and using the support from other disciplines such as behavioral ecology, reproductive biology, and disease ecology.

Behavioral ecology

Another common application for non-invasive FGM sampling is for investigating the role of stress hormones in social structure and behavior of wild carnivores. Interesting studies in wolves (*Canis lupus*), African wild dogs (*Lycaon pictus*), dwarf mongoose (*Helogale parvula*), meerkat (*Suricata suricata*), and otters (*Lutra lutra*) have contributed to the understanding of social dynamics through behavior and NHM (Barrette et al., 2012; Creel, 2001; Kalz et al., 2006; Sands and Creel, 2004).

Primatologists have contributed considerably in expanding our collective understanding of behavioral endocrinology in free-living wildlife populations. Their focus lies in understanding the interaction between social structure, food availability, and gonadal steroids (Chapman et al., 2006; Thompson and Wrangham, 2008). In these species it is relatively easy to collect feces at the time of defecation while watching animals and their social interactions. For free-ranging carnivores, longitudinal sampling of known individuals combined with behavioral observations are rare, but it has been employed in spotted hyenas (*Crocuta crocuta*) (Van Meter et al., 2009). This approach could be used on carnivores that occur in open habitat types such as African savannahs or for more visible species such as gray wolves in North America.

Challenges and limitations of non-invasive hormone monitoring

Great strides have been made in the past three decades toward the development and application of NHM to better understand the physiology of wild carnivore species. Field endocrinology holds enormous potential for conservation. The field however has a few logistical drawbacks and interpretation of results is not always straightforward. Here I outline some of the challenges that confront practitioners of these methods:

- Hormone concentrations change in the environment and can decrease or increase over time. Therefore, an environmental validation should be conducted from known age scats that are sub-sampled for several days under field conditions.
- In general, specialized equipment is not required for extraction, and in fact it is possible to perform hormone extractions in the field. For instance, baboon hormone fecal extracts can be stored at room temperature in Ambasely, Kenya for up to 40 days before assay, circumventing the need for refrigeration in the field (Lynch et al. 2003). If, however, RIAs are employed rather than EIAs, hormone measurement could involve some logistical complications. For instance, laboratory facilities need adequate infrastructure to handle and dispose of radioactive materials.
- Considerable caution must be taken when interpreting results of the measured hormone concentrations in the target population. While gonadal steroid hormones are relatively

straightforward and generally uncomplicated by extrinsic factors or individual heterogeneity, FGM, on the other hand, can be influenced by a plethora of intrinsic and extrinsic factors. These factors include: individual, age, sex, reproductive status, torpor, diet, social status, previous exposure to stressors (*i.e.*, conditioning), personality, exposure to multiple stressors simultaneously, context in which the stressor occurs, and neonatal exposure to stress (Cockrem, 2007; Goymann, 2012; Romero, 2004; von der Ohe and Servheen, 2002). For example, it is common to observe natural diurnal and seasonal variability in baseline FGM (von der Ohe and Servheen, 2002). Bonier et al. (2004) showed a significant difference between captive individuals, such that some individuals had a behavioral response to a stressor which was coupled with an adrenal response. Whereas, individuals that did not show any change in behavior to the same stressor did not exhibit an adrenal response either. This example highlights the potential heterogeneity among individuals (or populations) to an identical stressor.

- Because FGM are influenced so many factors (see above), determining baseline FGM is also complicated. It would require repeated sampling of the same (free-living) individual accounting for ecological and physiological contexts to determine baselines as well as detecting acute versus chronic stress levels (Bonier et al., 2009).
- Finally, while we know animals experience stress that can be measured as FGM concentrations, we do not fully understand the threshold levels for elevated FGM that impact fitness through immune-reproductive suppression. This is an important avenue of discovery especially as more researchers link human-caused stressors to elevated FGM in carnivore populations.

Parasite assessment

Description

Despite the inherent global need to understand wildlife disease and processes driving transmission, data on parasite, and host ecology are scant. Important aspects of parasite diagnostics such as difficulties obtaining samples *antemortem*, and limitations in interpretation of

serologic surveys, have delayed the understanding of wildlife disease dynamics. For example, serologic surveys (*i.e.,* detection of antibodies in blood) in wildlife are not necessarily indicative of ongoing infection. Instead, they indicate only that the individual has, or has not, been previously exposed to a pathogen, but disease development cannot be confirmed until the pathogen is either detected directly or isolated from the animal and/or pathological (macro and microscopically) evidence is demonstrated.

The expansion of both agricultural frontiers in developing countries and suburban areas in developed countries has led to increased contact between wild carnivores and domesticated animals like dogs, cats, and livestock (Bradley and Altizer, 2007; Daszak et al., 2000). In fact, several carnivore populations have contracted diseases transmitted from domestic animals causing devastating impacts. For instance, populations of African wild dogs (*Lycaon pictus)* and Ethiopian wolves (*Canis simensis*) were drastically reduced in numbers and in some areas even faced local extinction as a result of rabies outbreaks originating from domestic dogs (Woodroffe, 1999). Similarly, African lions (*Panthera leo*) in the Serengeti were reduced two fold after contracting a canine distemper virus originated in domestic dogs (Cleaveland et al., 2000; Roelke-Parker et al., 1996).

Zoonotic diseases are those that can be transmitted either from wildlife to humans or from humans to wildlife species. Globally, 60,000 people die a year due to rabies transmitted from domestic and free-living animals (WHO, 2013). Recently concern regarding zoonotic diseases stems from epidemiologic evidence demonstrating the important role of wildlife-human interactions in emerging and re-emerging diseases in humans (*e.g.,* Nipah virus encephalitis, Ebola hemorrhagic fever, and influenza). For example, *Baylisascaris procyonis*, a nematode that naturally affects the small intestine of racoons (*Procyon lotor*), can cause fatal, or devastating cases of larval invasion to the central nervous system in humans, who are an accidental host (Gavin et al., 2005). Direct contact with raccoon feces is the most important risk factor, and proximity of humans to raccoons has dramatically increased the numbers of cases in the United States and Europe (Gavin et al., 2005; Okulewicz and Buńkowska, 2009). Moreover, urban

bobcats (*Lynx rufus*) might be involved in indirect transmission of *Giardia* to humans in California (Carver et al., 2012).

While human health is impacted by zoonotic diseases, wildlife species also face health threats from infectious diseases caused by anthropogenic activities (see de Castro and Bolker, 2005). Examples include accelerated global climate change leading to range shifts of hosts and parasites (Polley and Thompson, 2009; Raffel et al., 2012; Slenning, 2010), habitat modification such as loss and subsequent fragmentation (Cottontail et al., 2009; Estrada-Peña et al., 2010; Goldberg et al., 2008), production of pollutants (Blitzer et al., 2012; Noyes et al., 2009), and illegal traffic of fauna (Gómez and Aguirre, 2008; Karesh et al., 2005).

Wildlife diseases in carnivores are not only a concern for human health or endangered species conservation, but many are also of economic interest. For example, badgers (*Meles meles*) and opossums have been linked to epizootics of tuberculosis in cattle (Courtenay et al., 2006). Coyotes, raccoons and many other carnivores could potentially play an important role in neosporosis, a widespread disease produced by a protozoan (*Neospora caninum*) that causes abortion and is transmitted by either becoming infected from livestock-sourced *Neospora* or by transmitting neosporosis to cattle (Dubey et al., 2007).

Many infectious diseases can be detected through non-invasive approaches in free-ranging carnivores. Macroparasites (*e.g.,* intestinal worms) such as nematodes, cestodes, and trematodes have been studied in several wild carnivore species (Table 1.2). Some studies have examined parasite richness in relation to spatial or population dynamics. For instance, urban and rural coyotes (*Canis latrans*) have different intestinal parasite species richness and diversity in Alberta. Rural coyotes exhibited higher parasite richness. Some parasites such as *Toxascaris leonina* and *Cystoisospra* spp. were most prevalent in urban sites while Taenids and *Trichuris* spp. were more common in rural areas. Factors such as diet, behavior, and environmental factors could be affecting this disparity (Watts and Alexander, 2012). Recently, other studies have identified species of macroparasites in carnivore scat samples. *Echinococcus*, a cestode that

affects the small intestine of many mammals, and uses herbivores and humans as intermediate hosts, has been found in scats of African lions (*P. leo*). Interestingly, infections in this felid are not caused by the same species that affects other mammals (*e.g., E. granulosus* in cattle, camels, sheep, goats, pigs and humans; *E. equinus* in zebras; *E. canadensis* in goat, cattle, camel, sheep, pig, and dogs) (Hüttner and Romig, 2009). Instead infections are cause by *E. felidis*, which seems to be very specific to lions (Hüttner et al., 2008).

Protozoa and fungi (microparasites) have not being widely studied in wild carnivores. However, some diseases caused by protozoa (*e.g.,* neosporosis and thrichomoniasis) have important economic implications for farmed animals and could be of interest for endangered species. For instance, *Tritrichomonas foetus* has been characterized in feces of domestic cats by PCR, simultaneously this protozoan is of relevance for cattle and other livestock abortion episodes (Stauffer et al., 2008).

Use of scat for parasite assessment

Virtually all macro and micro parasites affecting the gastrointestinal tract are subject to analysis in scats. Scats should be collected as fresh as possible to avoid degradation of the macro and micro parasites present in the sample.

There are several techniques to assess parasites non-invasively, but special attention should be given to specific characteristics of the methodologies such as sensitivity (ability to detect a positive when positive), and specificity (ability to detect a negative when negatives) while selecting the best option to accomplish the goal of the study. For instance, using a double centrifugation sugar fecal flotation, considered to be a highly sensitive technique, to detect gastrointestinal parasites in coyotes had different performance according to the parasite species examined (Liccioli et al., 2012). Therefore, a diagnostic technique should be tested on the parasite species of interest.

Diseases and their drivers are intricate and complexity increases when using non-invasive means to study disease. Effects of environment on biological samples and parasite shedding dynamics must be taken into account. The rate that parasites and propagules are shed in host feces can be influenced by disease state, immunocompetence, diet, time of day, and seasonality. Additionally, scats that are collected opportunistically are subject to environmental contamination and pathogen degradation. The lack of basic information on these topics somewhat limits the use of scat samples to make inferences about the impacts of disease on wildlife populations, but it also opens the door for much basic research on the topic.

Several methods to investigate macroparasite (*e.g.,* intestinal worms) infection in wildlife have been in use for decades and involve isolation and counting of parasite propagules found in animal feces. Detection of gastrointestinal parasites has been done using classical techniques of flotation or sedimentation, which are widely applied to scats (Gompper et al., 2003; Lesmeister et al., 2008; Patton and Rabinowitz, 1994), and in some special cases in hair (Roddie et al., 2008) (e.g. *Toxocara spp*). Molecular techniques are rising in popularity to identify eggs that have indistinguishable morphology under optical microscopes or to determine specific transmission pathways among potential hosts (Archie et al., 2009; Khademvatan et al., 2013; Trachsel et al., 2007). Disease ecologists have employed non-invasive monitoring to understand evolutionary life strategies of pathogens as well as elucidate dynamics of infectious diseases through molecular markers that allow specific identification of viral and bacterial strains. These strains can be traced to establish both the species in which the virus cycles naturally and how it is transmitted to other hosts (Archie et al., 2009). In addition, ecological analyses related to species richness and community diversity of parasites provide further insights in parasite-host relationships. Those species of carnivores that prey on a great variety of species in different ecoregions (*e.g.,* gray wolf) are expected to have a wide diversity of parasites with indirect life cycles (*i.e.,* that require more than one host to reach adulthood) such as segmented worms (*e.g., Taenia spp*), flat worms (e.g. *Alaria spp*), and round worms (e.g. *Trichinella spp*). In fact, a meta-analysis performed by Craig and Craig (2005) studied intestinal worms in different wolf populations and found that wolves in temperate climates have higher helminthic diversity than

those ranging the tundra. In addition, tape worms (*Taenia hydatigena*), parasites with indirect life cycles, were the most prevalent helminth, achieving up to 79% of the total intestinal parasite composition. Another interesting aspect of ecology of directly transmitted diseases (*i.e.,* transmission from individual to individual) is the study of individual heterogeneities in study subjects that predict parasite load and/or individuals that are disproportionately responsible for the majority of the disease transmission in a population ("super spreaders"). Even though, the pathogen super spreading theory has not been demonstrated in free-ranging wildlife, there is important evidence in the epidemiology of Severe Acute Respiratory Syndrome (SARS) supporting this theory, where a few people highly connected to many individuals infected a great number of individuals in the Hong Kong outbreak (Small et al., 2006).

Disease surveillance

Disease surveillance is important in conservation medicine. This is especially true at the human-wildlife interface, where domestic species can introduce novel pathogens to native fauna. The World Organization for Animal Health (OIE) defines surveillance as "the systematic ongoing collection, collation, and analysis of information related to animal health and the timely dissemination of information to those who need to know so that action can be taken". Non-invasive monitoring is important for collecting data relevant to disease surveillance in carnivores. Particular efforts have been directed to study multihost pathogens because most of the infectious diseases (*e.g.,* viral and bacterial) affecting carnivore species are usually shared not only with domestic animals but also with other wild carnivores. For instance, infection of domestic dog parvovirus in large felids has been demonstrated (Steinel et al., 2000). To date, most surveillance efforts are directed towards zoonotic diseases such as rabies and influenza in developed countries, whereas surveillance for human and wildlife diseases in developing countries is virtually nonexistent. Disease surveillance programs could be highly beneficial providing baseline information about prevalence of native macro and micro parasites in a particular ecoregion. Once this information is acquired, monitoring "spillovers" from livestock to

wildlife and "spillbacks" from wildlife to livestock and other epidemiologic dynamics can certainly improve efficiency in strategies to control and/or eradicate pathogens.

Lastly, developing studies focused on epidemiology such as herd immunity, threshold population size for pathogen invasion, control and prevention of outbreaks, spillovers and spillbacks, are often desirable to establish management actions. I recommend consulting and engaging epidemiologists (*e.g.,* veterinarians, biologists, ecologists, human doctors) and authorities involved in wildlife disease issues such as the Natural Resources Commissions at the local level, the Center for Disease Control and Prevention (CDC), USDA's Animal and Plant Health Inspection Service, and the World Organization for Animal Health (OIE).

Challenges and limitations of non-invasive parasite assessments

- Interpretation of results from non-invasive parasite detection techniques should be approached with caution because different factors affect the probability of detecting parasites when they are present (*i.e.,* sensitivity). Parasites are detectable only during the shedding phase; hence, stages of early infection, latency, and post-infection will usually be negative. Additionally, the environment can degrade parasite material in feces, providing false negatives in old samples. Therefore, controlled degradation studies to assess the impact of the environment on the parasite material over time are imperative for free-ranging carnivore parasite monitoring.
- Parasite loads and disease outbreaks are dynamic, changing over time. Seasonal changes in weather, reproduction, and social status can impact carnivore parasite loads. For instance, rabies outbreaks in skunks have seasonal peaks during the fall in Eastern U.S. and during the spring in central U.S. These patterns could be attributed to an increase in contact rates with raccoons (a known reservoir of rabies) during the dispersal of juvenile skunks in the fall in eastern US and during breeding activity in early spring in the central U.S. (Altizer et al., 2006).

- PCR methodologies are characterized by their great specificity and sensitivity, however there are some inhibitors in fecal samples that could block the detection of viral nucleic acids obtaining a false negative result. This limitation could be surmounted by employing co-agglutination tests to improve DNA replication in the biological sample (Mendes Ribeiro and Pessoa Araújo, 2009).
- Lessons from domestic animal research demonstrated that assumptions about the direct relationship between macroparasite load (*e.g.,* intestinal worms) and the number of eggs, embryos, or immature forms estimated in feces are often inaccurate. Therefore, there could be some cases where parasite load is high (*i.e.,* high number of adults) but the number of eggs shed is low due host immunity, or conversely, parasite load is low but many eggs could be shed due to density dependent factors and environmental cues (Coadwell and Ward, 1982; Stear et al., 1995). However, egg counts can be used to evaluate qualitatively degrees of infections (*e.g.,* low, moderate, or high) if the relationship of parasite numbers and number of eggs counted in feces is validated for the specific parasite.

The future for non-invasive wildlife disease research is wide open. By nature, the field is interdisciplinary utilizing expertise in numerous subjects including parasitology, virology, bacteriology, human and veterinary epidemiology, molecular ecology and wildlife physiology and ecology (Rohr et al., 2011). The use of captive wildlife is suggested to develop, validate, and apply non-invasive parasite monitoring techniques for free-ranging populations. Long-term multidisciplinary approaches integrating specialists from various fields will be necessary to achieve effective, practical, and holistic solutions for issues concerning diseases and carnivore populations.

Diet assessment

Description

Diet studies in carnivores generally employ scats, stomach contents, or kill sites to determine ingested species. Prey species are mostly identified, under dissecting and regular microscopes,

using prey remains such as hairs, bones, teeth, claws, skins, and feathers. However, this method is difficult to implement due to the need for keys and reference collections to perform adequate prey species identification. More recently, molecular techniques such as *mt*DNA, barcoding, and stable isotope analysis have helped overcome those limitations of identifying prey remains using microscopy.

Diet assessments are extensively performed in carnivores to elucidate their relationships with potential prey species and how they relate to their environment. Therefore, for this review I will focus mostly on studies applied to felids, which can also be applied to other carnivores.

The most elementary information that could be inferred is determination of prey species in an area, including their size and relative consumption. For instance, Emmons (1987), used scat to establish a baseline of jaguar, puma, and ocelot prey items in a protected area in Peru. Consumed prey richness was high in this area, reflecting opportunistic hunting strategies of these felids.

Prey selection assessments are possible through a combination of techniques including identification of consumed items in scat and population prey estimations such as density and availability. Prey selection is actually a measure of deviance between the proportion of a prey item in the diet and the availability of that prey item relative to all possible prey items in the area (Nilsen et al., 2012). The definition of prey availability is a common limitation in these type of studies (Nilsen et al., 2012). Contrary to the general misconception, availability is not always directly associated to prey abundance (Molinari-Jobin et al., 2004). Prey availability is, in fact, influenced by multiple factors such as prey antipredator behavior, life stage of prey, life stage of predator, seasonality, habitat types and possibly many others (Nilsen et al., 2012). Therefore, prey availability is extremely complicated to account for in non-invasive studies. Despite these limitations, prey availability for jaguars and pumas has been estimated using prey densities obtained from line-transect sampling in the Maya-Biosphere Reserve in Guatemala (Novack et al., 2005). Additionally, Weckel et al. (2006) used camera trapping arrays to assess prey selection and general feeding ecology of jaguars in Belize. In the latter study, camera trap surveys

provided relevant information on prey activity while accounting for predator-prey temporal overlap, habitat overlap, and spatial distribution of both predators and prey.

Quantification of niche breath and overlap among top and mesocarnivores ranging in pristine and modified habitats also have been obtained from diet analysis in scat (Guerrero et al., 2002; Moreno et al., 2006; Novack et al., 2005; Polisar et al., 2003; Silva-Pereira et al., 2011). There are different indices and estimators of these ecological interactions. Possibly the most widely used to estimate niche breath are the Shannon-Wiener (*H'*) and Levins' (*B*) indices. Niche overlap has been traditionally assessed with Pianka's index. Alternatively, there are other methods of analysis that allow a graphical visualization of resource partitioning at the community level such as correspondence analysis (Nilsen et al., 2012).

Most recently, diet analyses have provided insight into conservation issues such as shifts in diet after a sympatric predator is extirpated from an area or when non-native introduced species become prey of native predators. For instance, ocelots prey on larger mammal species in regions of Panama where jaguars have been extirpated than in those areas where they still remain sympatric (Moreno et al., 2006). Furthermore, introduced species can become part of the diet of predators in the absence of their native prey, such as the case of introduced red deer (*Cervus elaphus*), wild boar (*Sus scrofa*), and native predator, pumas (*Puma concolor*), in some regions of Patagonia, Argentina (Novaro et al., 2000). However, when native prey is still present in the area, pumas prefer to prey on their native species such as guanaco (*Lama guanicoe*) and the Patagonian mara (*Dolichotis patagonum*) (Zanón Martínez et al., 2012). These predator-prey interactions are of particular interest in conservation programs to evaluate the ecological role of decimated native populations and the growing populations of introduced species.

Data collection, storage and analysis

Scat surveys can be designed in several ways including random transects in predetermined plots, opportunistic finds, radio-tracking animals to follow their movements, and with scat detector dogs (Cascelli de Azevedo, 2008; Long et al., 2007; Rabinowitz, 1986).

Different storage and treatment procedures can be adopted once scats are located (Table 1.3). However, scat drying is probably the most common and easiest field method to preserve prey remains in the scat. Scat drying could be achieved by exposing the sample to either atmospheric air, silica gel, or laboratory ovens. These methods are widely used for traditional and more modern methods of prey item identification (see below) (Codron et al., 2007; Foster et al., 2010).

Prey species or carnivore diet composition can be identified from scat using three different techniques: microscopy, DNA, and stable isotopes. Microscopy, the most widely used, focuses on analyzing hair (medulla and cuticle) and feather (barbs, barbules, and villi) patterns under the microscope, and bigger structures such as teeth, bones, nails, claws, invertebrate exoskeletons and seeds under a dissecting scope. Prey items can be identified using classification keys, reference materials from museums, and samples collected from the study site (Nilsen et al., 2012). Diet research in Neotropical felids is predominantly performed using microscopy (Table 1.3).

Molecular techniques are being used with more frequency in vertebrate diet studies. Mitochondrial DNA (*mt*DNA) has been used to identify prey species from the scat of carnivores. Farrell et al. (2000) compiled sequences of *mt*DNA from potential prey species as well as sympatric predators to analyze the diet of felids. This technique has been used in the field to identify predator species such as jaguars (Weckel et al., 2006). While *mt*DNA provides information for identifying predator species at the population level, very little analysis had been done at the individual level. Nuclear DNA (*n*DNA) such as microsatellites can help elucidate the effects of individual preference for certain prey species and also would aid with pseudoreplication issues related to survey designs. For example, scats of individual coyotes were identified with *n*DNA markers in the Olympic National Park. Some individual coyotes preyed with higher pressure on Olympic marmots than others (*Marmota olympus*). Furthermore, a single individual coyote represented about half of the total coyote samples analyzed in that study (Witczuk et al., 2013).

Stable isotopes, particularly carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$), have been readily used to elucidate trophic interactions (Kelly, 2000). Most of the samples for stable isotope analyses are acquired from hair (Darimont and Reimchen, 2002; Roth and Hobson, 2000), bone, muscle and other tissues (Polischuk et al., 2001; Roth and Hobson, 2000). However, a few studies have measured stable isotopes in hair remains found in carnivore scats. For instance, main prey groups were identified in the scat of lions and hyenas in Kruger National Park. Lions and hyenas preyed more on impala and large grazers of the African savanna (Codron et al., 2007). Scat drying is also an effective method for stable isotope examination. Once the scat is homogenized it is combusted in mass spectrometers, where solid organic compounds are converted into gaseous inorganic compounds which can be separated and identified (Ben-David and Flaherty, 2012) (Ben-David and Flaherty, 2012). Ben-David and Flaherty (2012) present a detailed introduction of isotope estimation and the applications to ecological studies.

Challenges and limitations of non-invasive diet assessments

- Assigning species of origin to the scat is difficult and there is considerable human bias when using morphology because decisions often are made based on scat diameter and "circumstantial evidence" (Garla et al., 2001). This becomes very limiting when analyzing scat of sympatric carnivores that overlap in body size and prey consumption. This challenge could be overcome by analyzing bile acids, as reported for jaguars and pumas (Taber et al., 1997) or by performing DNA analysis to determine species (Farrell et al., 2000).
- Microscopic techniques to identify prey species demand an extensive reference collection of all possible prey items, which often times is not readily available. Laboratory personnel need to be highly trained to identify hair and feathers. Lastly some of prey species that leave trace remains in the scat can be not detected or are underestimated (Nielsen et al., 2012).
- DNA techniques are extremely powerful for prey item identification. However, they are expensive and, in areas with high prey diversity such as the tropics, it is time consuming to

acquire DNA sequences of all prey items. Moreover, DNA tends to degrade rather quickly in habitat types with high humidity and high UV-ray exposure.

- In general, inherent outcomes of stable isotope analysis to identify diet are of broad spectrum and might have very little application when one or a small group of prey species are of concern. Extensive calibration trials and spatio-temporal variation in isotopes need to be accounted for in this type of analysis. Boecklen et al. (2011) present a detailed review of potential applications, limitations, and sources of variation in isotope analysis.

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Figures and tables

Table 1.1. Summary of study designs assessing the impact of steroid metabolite concentrations in fecal samples exposed to the environment. FEM: fecal estrogen metabolites; FPM: fecal progestagen metabolites; FAM: fecal androgen metabolites; FGM: fecal glucocorticoid metabolites. Temp: temperature; RH: relative humidity; d: day; h: hour.

Species	Testing Setting	Target Steroid Metabolites	Weather Variables	Environmental Conditions	Time of Exposure	Concentration Stability	Reference
Canada lynx (*Lynx canadensis*)	Field - Natural Range	FEM, FPM, FAM, FGM	none reported	Sunny and shade	8 d	3 d	(Fanson, 2009)
White-tailed deer (*Odocoileus virginianus*)	Laboratory	FGM	temp. and rainfall	Wet and dry seasons	7 d	7 d (Dry) unsuitable (Wet)	(Washburn and Millspaugh, 2002)
Mountain hare (*Lepus timidus*)	Laboratory	FGM	temp. and rainfall	Wet and dry seasons	3 d	3 d (Dry) unsuitable (Wet)	(Rehnus et al., 2009)
Brown hyena (*Hyaena brunnea*)	Field - Natural Range	FGM	temp. and rainfall	Outside on grass	72 h	72 h	(Hulsman et al., 2011)
Iberian lynx (*Lynx pardinus*)	Field - Natural Range	FEM, FPM, FAM	temp., RH, and rainfall	Spring, Summer, Winter, and Fall	7 d	PEM, FPM & FAM: 7 d	(Abaigar et al., 2010)
Mhorr gazelle (*Gazella dama mhorr*)	Field – Outside of Range	FEM, FPM, FAM	temp., RH, and rainfall	Spring, Summer, Winter, and Fall	7 d	FPM & FAM: 2 d FEM: 6 h	(Abaigar et al., 2010)
Saharan Barbary sheep (*Ammotragus lervia sahariensis*)	Field – Outside of Range	FEM, FPM, FAM	temp., RH, and rainfall	Spring, Summer, Winter, and Fall	7 d	FPM & FAM: 7 d FEM: 6 h	(Abaigar et al., 2010)

Table 1.2. Brief compilation of macro and microparasite assessment in free-ranging carnivores using non-invasive means.

Parasite Type	Biological Sample	Carnivore Species	Diagnostic Technique	Reference
Macroparasites				
Baylisascaris procyonis	Feces	Raccoon *(Procyon lotor)*	Flotation	(Sexsmith et al., 2009)
Echinicoccus spp., Taenia spp.	Feces	Red fox (*Vulpes vulpes*)	PCR	(Trachsel et al., 2007)
Echinicoccus spp., Taenia spp.	Feces	Badger (*Meles meles*)	PCR	(Trachsel et al., 2007)
Echinicoccus felidis	Feces	Lion (*Panthera leo*)	PCR	(Hüttner et al., 2009)
Toxocara canis	Hair	Red fox (*Vulpes vulpes*)	Sedimentation	(Roddie et al., 2008)
Toxocara spp	Feces	Pallas' Cats (*Otocolobus manul*)	Flotation	(Brown et al., 2005)
Nematodes	Feces	Spotted skunk (*Spilogale putorius*)	Flotation	(Lesmeister et al., 2008)
Intestinal worms	Feces	Coyote (*Canis latrans*)	Flotation	(Gompper et al., 2003)
Intestinal worms	Feces	Gray wolf (*Canis lupus*)	ELISA/PCR	(Craig and Craig, 2005)
Intestinal worms	Feces	Gray wolf (*Canis lupus*)	Flotation/ PCR	(Bryan et al., 2012)
Intestinal worms	Feces	Spotted Hyenas (*Crocuta crocuta*)	Flotation	(Engh et al., 2003)
Intestinal worms	Feces	Jaguar (*Panthera onca*)	Flotation	(Patton and Rabinowitz, 1986)
Intestinal worms	Feces	Jaguarundi (*Puma yagoaroundi*)	Flotation	(Patton and Rabinowitz, 1986)
Intestinal worms	Feces	Ocelot (*Leopardus pardalis*)	Flotation	(Patton and Rabinowitz, 1986)
Intestinal worms	Feces	Puma (*Puma concolor*)	Flotation	(Patton and Rabinowitz, 1986)
Intestinal worms	Feces	Tiger (*Panthera tigris*)	Flotation	(Marathe et al., 2002)
Intestinal worms	Feces	Bobcat (*Lynx rufus*)	Flotation/ PCR	(Carver et al., 2012)
Microparasites				
Isospora	Feces	Coyote (*Canis latrans*)	Flotation	(Miller et al., 2009)
Isospora	Feces	Pallas' Cats (*Otocolobus manul*)	Flotation	(Brown et al., 2005)
Criptosporidium sp.	Feces	Gray wolf (*Canis lupus*)	Immunofluorescence	(Bryan et al., 2012)
Criptosporidium sp. and	Feces	Bobcat (*Lynx rufus*)	Immunofluorescence/ PRC	(Carver et al., 2012)
Giardia sp.	Feces	Coyote (*Canis latrans*)	ELISA	(Gompper et al., 2003)
Giardia sp.	Feces	Bobcat (*Lynx rufus*)	Immunofluorescence/ PRC	(Carver et al., 2012)

Table 1.3. Sample storage, predator, and prey identification techniques used in scat diet analyzes of free-ranging Neotropical felids.

Neotropical felid	Sample storage	Predator identification	Prey identification	Reference
Jaguar (*Panthera onca*)	Formalin 10% - Ethanol 70%	Size, jaguar hair in scat, tracks	Microscopy	(Emmons, 1987)
	Air dried	Chromatography bile acids	Microscopy	(Taber et al., 1997)
	Air dried in paper envelopes	Size, jaguar hair in scat, tracks, circumstantial evidence	Microscopy	(Garla et al., 2001)
	Air dried on sterile paper	*mt*DNA	*mt*DNA	(Farrell et al., 2000)
	Not reported	*mt*DNA, tracks	Microscopy	(Polisar et al., 2003)
	Air dried in paper envelopes	*mt*DNA	Microscopy	(Novack et al., 2005)
	Not reported	Jaguar hair in scat, local expert opinion, *mt*DNA	Microscopy	(Weckel et al., 2006)
	Dried in silica gel	*mt*DNA	Microscopy	(Foster et al., 2010)
Puma (*Puma concolor*)	Formalin 10% - Ethanol 70%	Size, puma hair in scat, tracks	Microscopy	(Emmons, 1987)
	Air dried	Chronatography bile acids	Microscopy	(Taber et al., 1997)
	Air dried on sterile paper	*mt*DNA	*mt*DNA	(Farrell et al., 2000)
	Not reported	*mt*DNA, tracks	Microscopy	(Polisar et al., 2003)
	Air dried in paper envelopes	*mt*DNA	Microscopy	(Novack et al., 2005)
	Not reported	Size, odor, tracks	Microscopy	(Moreno et al., 2006)
	Dried in silica gel	*mt*DNA	Microscopy	(Foster et al., 2010)
	Air dried in paper envelopes	Size, color, shape	Microscopy	(Zanón et al., 2012)
Ocelot (*Leopardus pardalis*)	Formalin 10% - Ethanol 70%	Size, tracks	Microscopy	(Emmons, 1987)
	Air dried on sterile paper	*mt*DNA	*mt*DNA	(Farrell et al., 2000)
	Not reported	Size, odor, tracks	Microscopy	(Moreno et al., 2006)
	Not reported	Ocelot hair in scat	Microscopy	(Silva-Pereira et al., 2011)
Jaguarundi (*Puma yagouaroundi*)	Air dried	Tracks, size, shape, odor	Microscopy	(Guerrero et al., 2002)
	Dried	Jaguarundi hair in scat	Microscopy	(Tofoli et al., 2009)
	Oven dried	Jaguarundi hair in scat	Microscopy	(Bianchi et al., 2011)
	Not reported	Jaguarundi hair in scat	Microscopy	(Silva-Pereira et al., 2011)
Oncilla (*Leopardus trigrinus*)	Not reported	Oncilla hair in scat	Microscopy	(Silva-Pereira et al., 2011)
Margay (*Leopardus wiedii*)	Oven dried	Margay hair in scat	Microscopy	(Bianchi et al., 2011)

Chapter 2: Effects of Natural Environmental Conditions on Fecal Glucocorticoid Metabolite Concentrations in Jaguars (*Panthera onca*) in Belize

J. Bernardo Mesa Cruz [a, b], Janine L. Brown [a], Marcella J. Kelly [b]

[a] Center for Species Survival, Smithsonian Conservation Biology Institute 1500 Remount Rd, Front Royal, VA, 22630 USA

[b] Department of Fish and Wildlife Conservation, Virginia Polytechnic and State University 100 Cheatham Hall, Blacksburg, VA, 24061 USA

Abstract

In situ studies relying on non-invasive fecal hormone monitoring are subject to problems due to potential hormone degradation in samples exposed to field conditions. In this study, we conducted an environmental validation for measuring fecal glucocorticoid metabolites (FGM) in jaguars (*Panthera onca*). We collected fresh feces from jaguars (6 males and 4 females), housed at the Belize Zoo, and exposed them randomly to two environmental conditions: shade and sun. A control (first subsample) was immediately frozen, after which subsamples were frozen daily over a 5-day period in both the dry and wet seasons. We quantified FGM using a cortisol EIA and a corticosterone RIA, both capable of identifying relevant metabolites. Results indicated that FGM assessed with the cortisol EIA were stable for 5 days during the dry season, but less than 1 day during the wet season, while FGM assessed with the corticosterone RIA were stable for 5 and 4 days during the dry and wet seasons, respectively. Exposure of jaguar feces to sun or shade had no effect on FGM degradation, despite significant differences in weather parameters. Fecal morphology analysis proved unreliable in identifying fecal age. We conclude that the corticosterone RIA is suitable for assessing FGM in free-ranging jaguars by surveying the same transects every 3-4 days in both seasons. The cortisol EIA can be used during the dry season, but there are possible shifts in metabolite immunoactivity under wet conditions. Assessing adrenal activity in jaguars ranging areas of varying human disturbance is a timely application of this methodology in Belize.

Keywords

validation; hormone degradation; fecal glucocorticoid metabolites; non-invasive monitoring; jaguar.

Highlights

- We validated two immunoassays to assess jaguar fecal glucocorticoid metabolites
- We assessed glucocorticoid stability in jaguar scat under natural field conditions
- We determined hormone metabolite stability to be about 4 days, with the corticosterone RIA
- Aging scat by morphology is not accurate for jaguar scat
- Environmental validation is needed to determine field sample collection protocols

Introduction

Non-invasive hormone monitoring (NHM) is widely used in a variety of wildlife species, but requires rigorous testing to ensure validity of techniques employed to assess steroid metabolites in excreta such as feces (Palme, 2005; Touma and Palme, 2005; Möstl et al., 2005). The applicability of this powerful tool has been demonstrated in several areas of animal research, such as ethology, reproductive physiology, and animal health (Goymann et al., 2001; Franceschini et al., 2008; Wielebnowski et al., 2002; Brown, 2006; Dorsey et al., 2010). Most of the studies employing NHM, however, are performed in *ex situ* settings such as zoos or research centers, where samples can be collected fresh and exposure to environmental conditions is limited. Whereas a substantial amount of research using NHM has been conducted in free-ranging populations of primates, very little has been done in other species of mammals, reptiles, and amphibians (Schwarzenberger, 2007). Reasons for this disparity are attributed to limitations in observing fresh defecations without introducing "human induced noise" in results, difficulties in assessing sample age and/or assigning collected samples to an individual, and instability of steroid hormone concentrations in feces after defecation (Sheriff et al., 2011; Möstl and Palme, 2002). However, recent advances in the use of remote tracking have proven valuable for following individuals to collect scat without disturbance (Ganswindt et al., 2010). Molecular techniques, such as DNA detection in animal excreta, are now employed to monitor populations non-invasively and assign individual identity or gender to animals from scat samples (Barja et al., 2008; Piggott et al., 2006). The remaining challenge is ensuring that fecal metabolite concentrations are stable, within the timeframe of collection, after deposited scat is exposed to natural environmental conditions.

The increased interest in employing NHM to aid wildlife conservation efforts has provided incentive to develop sampling methods that are robust under field conditions. For instance, primatologists have tested various sample storage conditions to avoid changes in hormonal concentrations and facilitate transport of excreta and hormone extracts from the field to the laboratory (Khan et al., 2002; Lynch et al., 2003; Pappano et al., 2010; Shutt et al., 2012). Recent

efforts in other mammals have targeted the design of "field-friendly" techniques to increase the applicability of NHM under field settings. Some of these methods include hormone extraction, sample storage, and sample immunoanalyses in the field (Freeman et al., 2010; Santymire and Armstrong, 2010). It is widely accepted that hormonal metabolites in feces are affected by factors such as temperature, humidity and ultraviolet rays, which can influence the presence of flora that alter hormonal concentrations directly and indirectly through biotransformation processes (Macdonald et al., 1983; Schwartz and Montfort, 2008; Touma and Palme, 2005) . There is evidence that steroid hormones can increase or decrease in concentration after exposure to the environment (Abáigar et al., 2010; Washburn and Millspaugh, 2002), although it is not always clear if steroid hormone metabolites actually are changing in concentration or if there is a conversion of metabolites to forms that have higher or lower affinity to the antibody. The lack of this information for most species hampers our ability to use NHM in *in situ* wildlife studies (Kelly et al., 2012). Another caveat arises in accurately aging fecal samples exposed to environmental conditions. Most field-based studies determine fecal age through subjective judgment based on scat morphology, such as categorizing samples based on condition, color, moisture level, odor strength, presence of mold, and presence of invertebrates (e.g., Vynne et al., 2012). However, these sample characteristics can vary dramatically based on environmental conditions other than simply scat age.

Most of the research utilizing NHM in wild populations of elusive species has occurred in temperate environments, yet the effects of the environment on hormonal metabolite concentrations have not been directly assessed in the majority of these studies. For instance, a survey of fecal glucocorticoid metabolites (FGM) of Alaskan brown bears (*Ursus arctos horribilis*) conducted during the summer months in Katmai National Park, Alaska, considered 2 days as prudential time for hormonal concentration stability (von der Ohe et al., 2004). Other studies have been performed strictly during the snow-filled winter, thus avoiding any type of change in hormone metabolite concentrations, which are stable when frozen (e.g. elk, *Cervus elaphus* and wolves, *Canis lupus* in Yellowstone National Park- Creel et al., 2002; capercaillie, *Tetrao urogallus* - Thiel et al., 2008). While there have been some attempts to evaluate the effects

of environment on hormone concentration for NHM, in general the studies have been fairly limited (Abáigar et al., 2010; Fanson, 2009; Hulsman et al., 2011; Rehnus et al., 2009).

The objectives of this study were two-fold: to validate two immunoassays for measuring FGM in jaguars (*Panthera onca*); and to conduct an environmental validation as a means of assessing the effects of a natural environment, with its climate variations (dry and wet seasons), on the degradation of FGM present in jaguar scat.

Material and methods

Animals and sample collection

Jaguars (*Panthera onca*) included in this study were kept at the Belize Zoo, Belize, Central America. All individuals were wild born adults (5 males and 4 females, approximately between 6 and 10 years of age), with the exception of one captive-born male (2 years of age). Jaguars were housed individually; some enclosures had common yards where individuals are rotated through, one at the time. Most jaguars were not exposed to the public, except for 2 wild born adults and the captive born male. The diet consisted mainly of chicken carcasses and other animal products when available. Water was provided *ad libitum*.

Study site and environment

The natural vegetation in Belize, a Neotropical country, is predominantly moist and wet broadleaf forest. In some areas there are pine forests and more open pine savannahs. The climate follows bimodal tropical conditions separated by a cool transitional period. The dry season, from February to May, is characterized by higher temperatures ranging from 24-33 °C and scarce rainfall (≤100 mm per month). The wet season, from June to November, has slightly lower temperatures (18-28 °C) and predominant rains, ~60% of the annual precipitation, which on average fluctuates between 1,500-2,000 mm. The cool transition period occurs between November and January. Relative humidity oscillates around 80% year-round, with some fluctuations between the dry and wet seasons (Fuller and Wilson, 2002).

Environmental validation

Study design

We collected fresh jaguar fecal samples (e.g., no older than 8 hours) from each individual at the Belize Zoo in both the wet and dry seasons over a 3-day period in each season. We placed samples in plastic bags and moved to the field site at the Tropical Education Center (TEC) within 30 min, where they were exposed to two environmental conditions: shade (N 17.35707 W 088.54159; 41 m elevation); and sun (N 17.35735 W 088.54121; 39 m elevation). Fecal samples assigned to the shade treatment were placed under broadleaved trees that provided shade during daylight periods, whereas samples assigned to the sun treatment were placed under direct exposure to the sun. We thoroughly mixed collected feces before subsampling over time. A control (first subsample) was immediately frozen at -20 °C to arrest degradation. Thereafter, we collected daily subsamples from each scat over a 5-day period in both seasons. We assigned scats of individual jaguars at random to seasons and treatments. Subsamples were immediately frozen for later analysis. We took photographs of scats daily and assessed for morphological aging using the following parameters: color (dark brown, light brown, white) and moisture (scale: 1=dry through 5=very moist).

Weather variables

We measured temperature (°C), relative humidity (%), and dew point (°C) every 5 minutes with one data logger (Lascar Electronics, EL-USB-2) placed at ground level under each environmental condition. We used a handheld unit (Omega, HHUVA1) to measure UV-rays ($W \cdot m^{-2}$) every 3 hours at ground level during collection periods.

Extraction of steroids from feces

At the Smithsonian Conservation Biology Institute (SCBI), we freeze-dried, homogenized, and pulverized feces. We extracted dry feces (0.19 ± 0.01 g) in 5 mL of ethanol 90% (v/v) by boiling in a water bath (90-100 °C) for 20 min, and centrifuging at 500 *g* for 15 min. We recovered supernatants and pellets resuspended in 5 mL of ethanol 90% (v/v), vortexed for 30 s, and centrifuged again. We combined and air-dried supernatants overnight. We resuspended

extracts in 1 mL of methanol and placed in an ultrasonic cleaner for 10 min. We diluted extracts (1:1 v/v) with steroid buffer (0.1 M NaPO4, 0.149 M NaCl, pH 7.0) and stored at -20 °C until analysis.

Glucocorticoid metabolite immunoassays

We tested two immunoassays validated for other carnivore species by Young et al. (2004) to identify the best method for measuring FGM in jaguar feces. The cortisol enzyme immunoassay (EIA) employed a cortisol-horseradish peroxidase ligand and antiserum (No. R4866; C.J. Munro, University of California, Davis, CA) and cortisol standards (hydrocortisone; Sigma-Aldrich Inc., St. Louis, MO). The polyclonal antiserum was raised in rabbits against cortisol-3-carboxymethyloxime linked to bovine serum albumin. We performed the EIA in 96-well microtiter plates (Nunc-Immuno™, Maxisorp™ Surface; Fisher Scientific, Pittsburgh, PA), coated 14-18 h previously with cortisol antiserum (50 µl per well; diluted 1:20,000 in coating buffer; 0.05 M $NaHCO_3$, pH 9.6). We analized fecal extracts, diluted 1:8 in steroid buffer, in duplicate. We combined cortisol standards (50 µl, range 3.9-1000 pg/well, diluted in assay buffer, 0.1 M $NaPO_4$, 0.149 M NaCl, 0.1% BSA, pH 7.0) and samples (50 µl) with cortisol-horseradish peroxidase (50 µl, 1:8,500 dilution in assay buffer). Following incubation at room temperature for 1 h, we washed plates five times before adding 100 µl substrate buffer (0.4 mM 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) diammonium salt, 1.6 mM H_2O_2, 0.05 M citrate, pH 4.0) to each well. After incubation on a shaker for 10-15 min at room temperature, we measuerd absorbance at 405 nm.

We also analyzed fecal extracts using a double-antibody ^{125}I corticosterone radioimmunoassay (RIA) (MP Biomedicals, Orangeburg, NY) according to the manufacturer's instructions, except all reagent volumes were halved. The polyclonal antiserum was raised in rabbits against corticosterone-3-carboxymethyloxime coupled to bovine serum albumin. We diluted fecal extracts 1:250 in steroid diluent and analyzed in duplicate. Inter-assay coefficient of variation (CV) of quality control samples run in each assay was less than 10% (cortisol EIA and corticosterone RIA: CV dose high control: 6.42% and 4.08%; CV dose low control: 9.65% and

8.04%; n = 14 and 3, respectively). FGM concentrations are expressed as nanograms per gram dry fecal matter ($ng \cdot g^{-1}$).

Immunoassay validation

We used fecal extracts from fresh samples (i.e., controls for environmental validation, see section 2.3.1) to conduct parallelism, exogenous corticosteroid accuracy recovery, high-performance liquid chromatography (HPLC), and a biological validation for each immunoassay.

Parallelism

We performed a test for parallelism between the assay standards and a pool of fecal extracts for both the corticosterone and cortisol immunoassays. We pooled an equal amount of fecal extract from each fresh sample and diluted serially, two-fold, in steroid buffer. We analyzed serial dilutions in duplicates for each assay.

Exogenous corticosteroid accuracy recovery

We tested exogenous corticosteroid accuracy recovery by adding a known amount of either cortisol or corticosterone, according to the immunoassay, to a pool of jaguar fecal steroid extracts. We analyzed serial dilutions of the spiked combinations in duplicate. We calculated percentage steroid recovery based on observed and expected concentrations.

HPLC

We performed a reverse phase HPLC to analyze the immunoreactivity of a pool of fecal steroid extracts with known radioactive glucocorticoid tracers: ^{3}H-cortisol, ^{3}H-corticosterone and ^{3}H-deoxycorticosterone. We filtered and dried a pool of fecal steroid extracts and spiked with radioactive hormone markers. We separated 55 µL of sample extract on a Microsorb column (Reverse Phase Microsorb™ MV 100 C-18, 5 µm diameter particle size; Varian Inc., Woburn, MA) using a gradient of 20-100 % (water/methanol) over 80 min (1.0 mL/min flow rate, 1 mL fractions) as described by Young et al. (2004). We used a 100 µL aliquot of each fraction to analyze radioactivity (cpm). We air-dried overnight the remaining 900 µL per fraction and reconstituted in 150 µL of distilled water for immunoreactivity analysis.

Biological validation of immunoassays

We compared individuals that had not been exposed to a stressor to those that were assumed to undergo periods of high adrenal activity, *i.e.*, a "stressful" situation. The Belize Zoo has a jaguar rehabilitation program, which brings free-ranging, "problem jaguars" into captivity to avoid the killing of jaguars by ranchers or villagers in retaliation for livestock losses. Free-ranging jaguars that are brought to captivity experience adrenal challenges, such as transport, confinement, appetite loss, and potentially self-injury. As jaguars acclimate to the new captive environment, their appetite increases, aggressive behaviors towards keeper-staff decrease, and jaguars spend less time hiding (Sharon Matola, Director of the Belize Zoo, pers. comm.). Jaguars are thought to take less than one year to acclimate, at least behaviorally, to captive conditions (Matola, pers. comm.). Furthermore, jaguars that spend more than 1 year in captivity show little or no aggressive behaviors and even affiliative expression such as, body rubbing and vocalizations (*e.g.*, gurgle and prusten) towards keeper staff (Matola, pers. comm.). Therefore, we grouped fecal samples from each jaguar in the study based on time spent in captivity: less than 1 year (n = 2); between 1 and 4 years (n = 4); more than 5 years (n = 3); and captive-born (n = 1). We averaged four fecal samples per individual for statistical analysis. We expected jaguars recently brought to captivity (e.g., ≤ 1 year) to have higher FGM than those individuals that were captive-born or had been exposed to long-term captivity (e.g., >5 years).

Statistical analysis

We implemented all analyses, except for the environmental validation assessment, in the statistical software JMP Pro 10 (Version 10.0.0; SAS Institute Inc., Cary, NC. 2012). We tested all data for normality before applying a statistical test. We assessed normality with the Shapiro-Wilk goodness of fit test (α=0.05). We log transformed data distributed in a non-normal fashion. We performed multiple comparisons when appropriate using Tukey-Kramer HSD multiple comparisons test (α=0.05). We used a one-way ANOVA to assess biological validation by

grouping FGM concentrations of jaguars by time spent in captivity. We tested for parallelism with a multiple linear regression of log transformed concentration and the binding percentage of both the standard and serially diluted extracts; we contrasted linearly the least squares means of the regression by standard and serially diluted samples, as suggested by Grotjan and Keel (1996). We used a simple linear regression for each immunoassay to test expected and observed concentrations of the exogenous corticoid accuracy recovery analysis. Using the statistical software SAS (Version 9.2, SAS Institute Inc., Cary, NC. 2002-2009), we constructed and ran a generalized linear mixed model to test environmental validation, where FGM concentrations as repeated measures were a function of season, environmental condition, time, and their respective interactions. We condensed weather variables as daily averages. We analyzed condensed data in a nested model, where every weather variable was a function of the environmental condition (sun or shade) factor, which was nested in the season factor (wet and dry).

Results

Immunoassay validation

Parallelism

Both immunoassays showed parallel displacement between dilutions of steroid standard and fecal extracts: cortisol EIA ($t=1.47$, $P=0.16$); and corticosterone RIA ($t=0.54$, $P=0.6$).

Exogenous corticoid accuracy recovery

Results indicate that the cortisol EIA and the corticosterone RIA had an accuracy recovery of 93.97% and 112.13%, respectively; both immunoassays had a strong positive relationship between the expected and observed concentrations (cortisol EIA: $P<0.0001$, $R^2=0.9717$; corticosterone RIA ($P<0.0001$, $R^2=0.9937$).

HPLC

For the cortisol EIA, we observed the majority of immunoactivity in fractions 4 and 7 - 25 indicating the presence of polar glucocorticoid metabolites in jaguar fecal steroid extracts (Figure 2.1). We observed another peak at fraction 35. An immunoreactive peak at fraction 46 co-eluted

with ^{3}H-corticosterone. We observed similar immunoreactivity patterns for the corticosterone RIA, with the exception of an additional peak at fractions 49-50. Again, we observed most of the cross-reactivity in fractions 4 - 25 indicating the presence of more polar glucocorticoid metabolites (Figure 2.1).

Biological validation of immunoassays

FGM concentrations of jaguars were not normally distributed for either the cortisol EIA or the corticosterone RIA, but achieved normality after log transformations (cortisol EIA: $W=0.8855$, $P>0.05$; corticosterone RIA: $W=0.9359$, $P>0.05$). Both immunoassays revealed that "problem jaguars" recently captured from the wild and brought into captivity excreted 5-fold more FGM than long-term captive jaguars (cortisol EIA: $F=12.79$, $P<0.0069$, $R^2=0.81$; corticosterone RIA: $F=19.70$, $P=0.0023$, $R^2=0.868$) or a captive-born individual. Additionally, FGM concentrations declined significantly with increasing time that jaguars spent in captivity, <1 year, between 1 and 4 years, and >5 years (Figure 2.2).

Environmental validation

Weather variables

All weather variables were significantly different between seasons (wet and dry) and environmental conditions (sun or shade) (temperature: $df=3$, $F=28.16$, $P<0.0001$, $R^2=0.725$; relative humidity: $df=3$, $F=32.88$, $P<0.0001$, $R^2=0.755$; dew point: $df=3$, $F=49.10$, $P<0.0001$, $R^2=0.822$; UVA: $df=3$, $F=30.69$, $P<0.0001$, $R^2=0.742$) (Figure 2.3).

FGM measures

FGM concentrations achieved normality after log transformation for both assays (cortisol EIA: $W=0.8869$, $P<0.05$; corticosterone RIA: $W=0.8971$, $P<0.05$). Statistical analysis indicated that FGM, measured with the cortisol EIA, were overall more stable in the dry season than in the wet season ($df=24$, $F=6.93$, $P=0.0146$). By contrast, we did not find a statistical difference between seasons in FGM measured with the corticosterone RIA ($df=24$; $F=2.46$; $P=0.13$).

Furthermore, time after collection was a highly significant factor contributing to stability in FGM concentrations for both assays (cortisol EIA: df=120, F=6.78, P<0.0001; corticosterone RIA: df=120, F=7.48, P<0.0001). FGM concentrations measured with the cortisol EIA during the dry season were stable for 5 days (df=120, t=-0.22, Adjusted P=0.93), while stability of FGM concentrations during the wet season was less than 1 day (df=120, t=-5.46, Adjusted P<0.0001). FGM concentrations measured with the corticosterone RIA during the dry season were also stable for 5 days (df=120, t=3.23, Adjusted P=0.0664), while stability of FGM during the wet season was 4 days (df=120, t=4.22, Adjusted P=0.0027). Lastly, environmental conditions (i.e., samples exposed to sun or shade) did not have significant effects on the stability of FGM concentrations for either assay (cortisol EIA: df=24, F=1.70, P=0.204; corticosterone RIA: df=24, F=0.32, P=0.575) (Figure 2.4).

Scat age assessment

We were unable to accurately determine age of scat exposed to different environmental conditions. Moisture, and color to a lesser degree, changed markedly after a scat was exposed to the environment. Scats gained moisture after rainfall causing the color to turn darker. In general, scat samples became dryer and whiter through time in the dry season, but no such trend was observed in the wet season (Figure 2.5).

Discussion

We validated two immunoassays to measure FGM in jaguar scat: a cortisol EIA (R-4866 antibody) and a corticosterone RIA (MP Biomedicals). Both showed satisfactory parallel displacement, and evidence of multiple glucocorticoid metabolites based on HPLC analysis, including an important amount of immunoactivity associated with more polar metabolites, similar to that observed in other felid species (i.e., cheetah, domestic cat, clouded leopard; Young et al., 2004). Contrary to those species, jaguar extracts also contained immunoactive peaks that co-eluted with ^{3}H-corticosterone, which is feasible for the RIA that uses a corticosterone antibody; however, the cortisol antibody cross-reacts only 0.7% with this glucocorticoid, so it is unlikely to be the native steroid, instead it could be metabolite that cross reacts in that fraction.

We demonstrated biological validity of both assays by showing a significant difference in FGM among individuals in relation to the time spent in captivity. As expected, individuals that had spent less than 1 year in captivity exhibited higher adrenal activity than those with multiple years in captivity or a captive-born individual. These results are also consistent with behavioral differences among jaguars relative to time in captivity noted by the Belize Zoo director and staff (Matola, pers. comm.). The biological validity of the corticosterone RIA is further supported by results of a previous study of captive jaguars that were challenged with exogenous ACTH (Conforti et al., 2012). Although different steroid extraction techniques were used, boiling (present study) vs. shaking (Conforti et al., 2012), FGM concentrations assessed with the corticosterone RIA of the captive-born jaguar and the individuals that spent less than 1 year in captivity at the Belize Zoo were similar to the baseline and the ACTH challenged jaguars respectively in the study by Conforti et al. (2012). Thus, based on laboratory and biological validations, both immunoassays appear to be effective at measuring FGM in fresh jaguar scat samples.

A few investigators have conducted fecal degradation studies related to measurements of steroid metabolites, although the terminology differs among studies: e.g., washing-out experiment (Rehnus et al., 2009), effects of fecal age and seasonality (Abáigar et al., 2010), field stability experiment (Fanson, 2009), and rate of FGM degradation (Hulsman et al., 2011). Regardless of terminology, systematic experimental assessments of the effects of natural environmental conditions on hormone metabolites in excreta of wild animals are crucial to ensure methodological validity. In our environmental validation, results differed somewhat for the two immunoassays. FGM concentrations did not vary between seasons using the corticosterone RIA. By contrast, the cortisol EIA indicated that jaguar FGM immunoreactivity changes, possibly due to steroid degradation, at faster rates and with more variability during the wet season than during the dry season. Overall FGM concentrations also fluctuated more during the wet season for both immunoassays. However, a stronger effect was seen in the cortisol EIA, where FGM in older samples could mistakenly identify an individual as having higher adrenal activity. Both immunoassays showed FGM concentrations changed over time when exposed to

the environment; however, the effects were variable. FGM concentrations with corticosterone RIA were different only at day 5 of exposure during the wet season. In contrast, FGM concentrations measured with the cortisol EIA were stable for less than 1 day of environmental exposure during the wet season. During the dry season, little change in FGM concentrations over time were observed for both the cortisol EIA and corticosterone RIA. Selecting the appropriate immunoassay, in this case, for measuring FGM in Belizean free-ranging jaguars, ultimately depends on the research question and goals. The corticosterone RIA is robust against the effect of climate seasonality while the cortisol EIA should only be used for scat field surveys during the dry season. Contrasting outcomes from these immunoassays suggest the importance of evaluating more than one immunoassay in environmental validations. This practice could increase the chance of capturing or confirming relevant changes in steroid metabolite concentrations.

Warmer and dryer weather appeared to minimize variation in FGM concentrations. Cooler and wetter weather possibly creates a more hospitable environment for microorganisms with steroid biotransformation capacities, as previously suggested (Washburn and Millspaugh, 2002). Within season, vast differences in scat exposure to UV-rays among environmental conditions (*e.g.*, 30 and 10 times higher UV intensity in sun vs. shade treatments in dry and wet seasons, respectively) did not appear to have an important effect on FGM concentrations. These results are perhaps explained by the low capacity of UV-rays to penetrate solid structures such as feces (for example, Abdelrahman et al., 1979). Therefore, the effects of UV-ray exposure on FGM degradation might be limited to the surface of the scat (*i.e.*, depth of micrometers), whereas fecal steroids are widely distributed throughout the entire fecal sample.

Scat aging by assessing morphology in relation to hormone stability was not successful because of high variation in color and moisture changes after rain events. Additionally, scats turn white very quickly in dry conditions, likely leading to overestimates of sample age. Furthermore, we observed that humidity drastically altered the morphology of fecal samples, making aging scats in the field by morphology inaccurate. Instead, we recommend adjusting survey regimens

to incorporate results of environmental validations by resurveying an area at intervals that ensure stability in hormonal concentrations in excreta as described below.

We suggest that researchers interested in NHM sampling for jaguars in a tropical country like Belize should conduct surveys in the dry season by using either the cortisol EIA or the corticosterone RIA; while surveys in the wet season should only use the corticosterone RIA. We found that scats cannot be aged accurately by morphology, but because FGM concentrations are stable up to 5 days, sampling can involve an initial clearing of all scats from the target area and, to be conservative, resurveying every 3-4 days to ensure accuracy in hormone concentrations. A different sampling regime could be adopted during the wet season, if the cortisol EIA is used, resurveying would need to take place daily; however, strict caution in the interpretation of data should be taken due to the rapid change in FGM concentrations. In addition, daily surveys may be impractical under field conditions.

Future directions

Assessing the degree of stress in wild jaguars ranging across areas of varying human disturbance is a timely application of these methodologies. Belize has experienced a progressive increase in human activities such as hunting, housing developments, forest eradication, land conversion for agriculture, and hence increasing levels of human-jaguar conflict. Our methods will ensure physiologically relevant FGM concentrations in feces and could advance conservation physiology for jaguars by exploring linkage of habitat fragmentation and human-wildlife conflict to measures of adrenal activity in free-living jaguars.

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Figures and tables

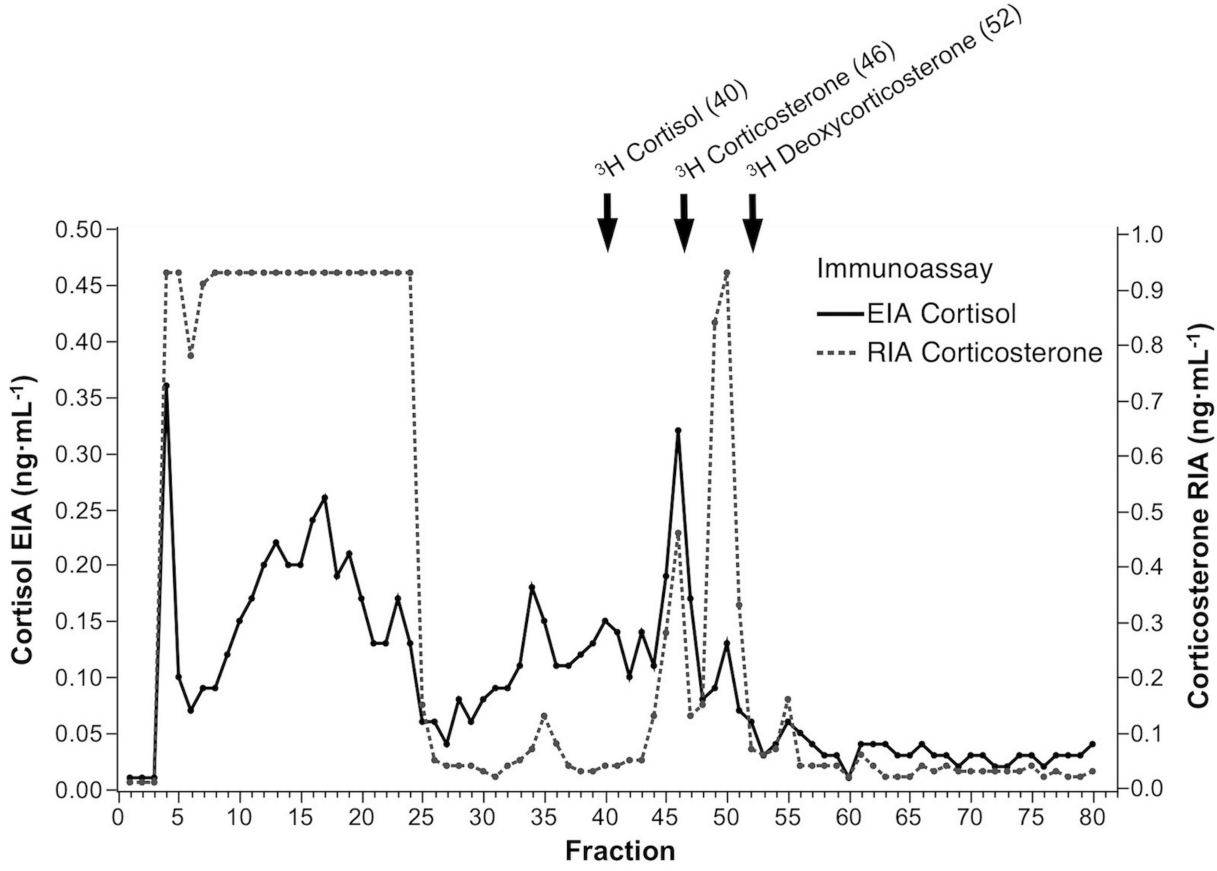

Figure 2.1. HPLC profile of jaguar FGM using two immunoassays, cortisol EIA (solid line) and corticosterone RIA (dotted line). Three radiolabeled hormones were included: ^{3}H cortisol, ^{3}H corticosterone, and ^{3}H deoxycorticosterone (solid arrows). Pooled fecal steroid extract was separated in a reverse phase column (C18 μm diam. particle size) using a gradient of 20-100% methanol over 80 min (1.0 mL/min flow rate, 1 mL/fraction).

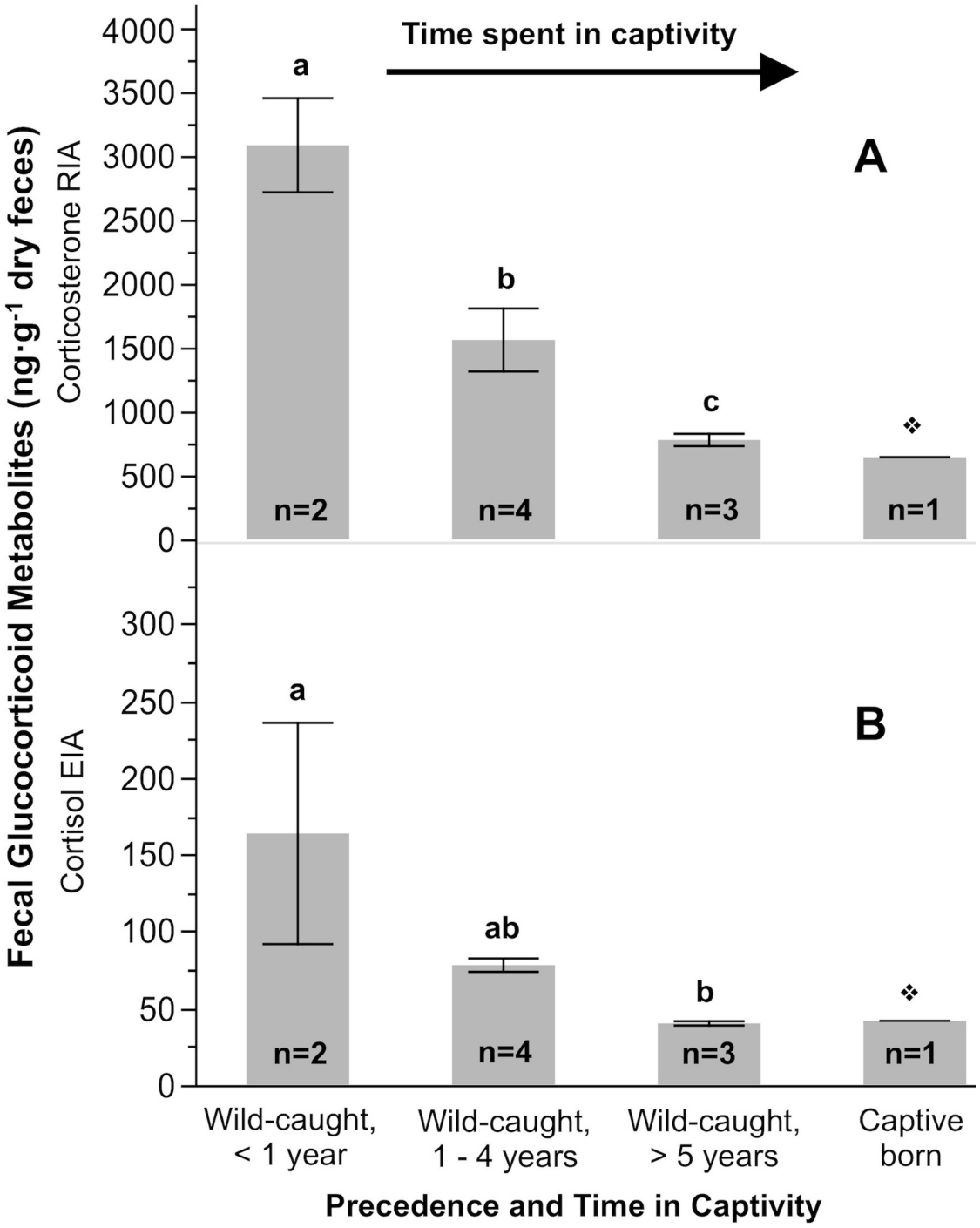

Figure 2.2. Biological validation of jaguar FGM using two immunoassays, corticosterone RIA (A) and cortisol EIA (B). Mean FGM concentrations in jaguars exposed to different time in captivity after capture. Error bars represent 1 SE. Bars with different letters are significantly different from each other; n = number of individuals included in each category; v = captive born male not included in statistical test.

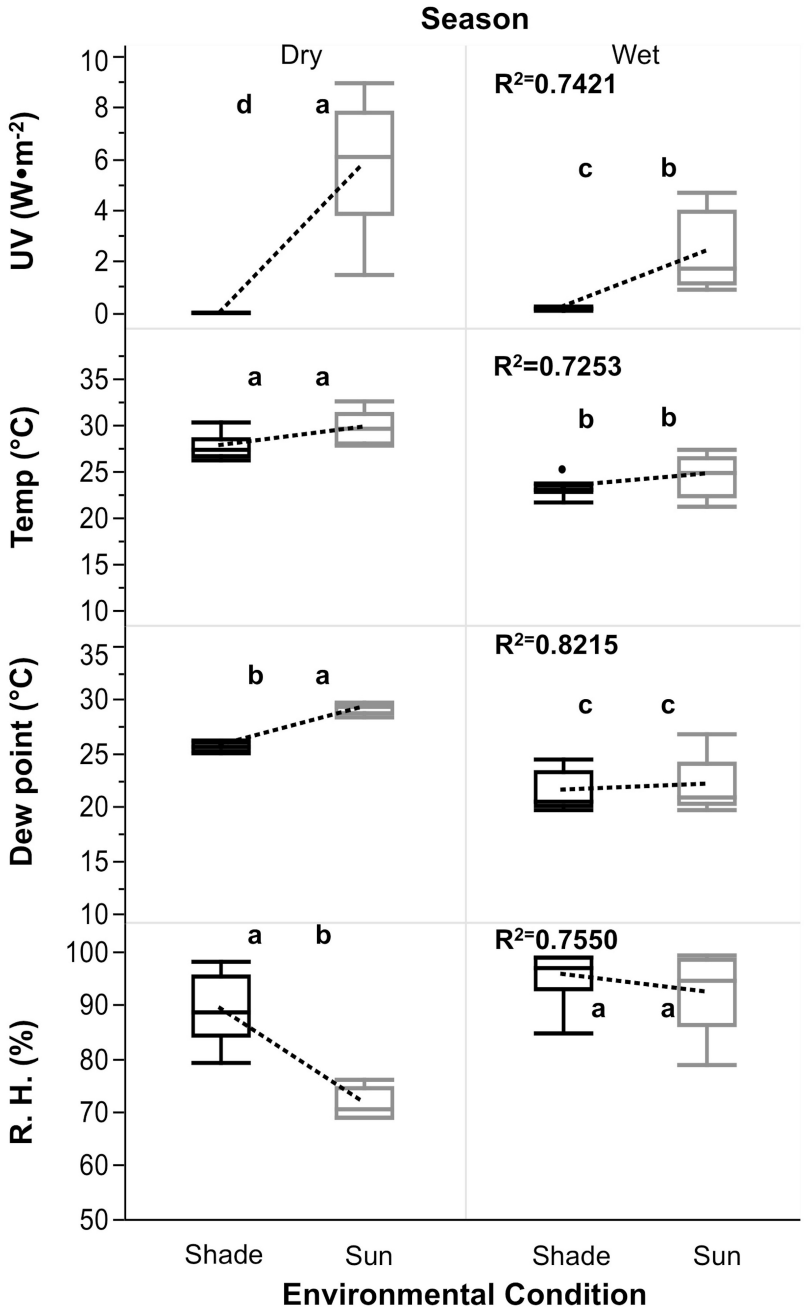

Figure 2.3. Climate variables, summarized in box and whisker plots, associated to environmental validation of jaguar FGM. R^2 is the value of the root square of the significant model: weather variable = environmental condition (sun or shade) nested within a season (wet or dry) + error. Different letters denote significant differences. Dotted lines represent trends in environmental conditions within a season.

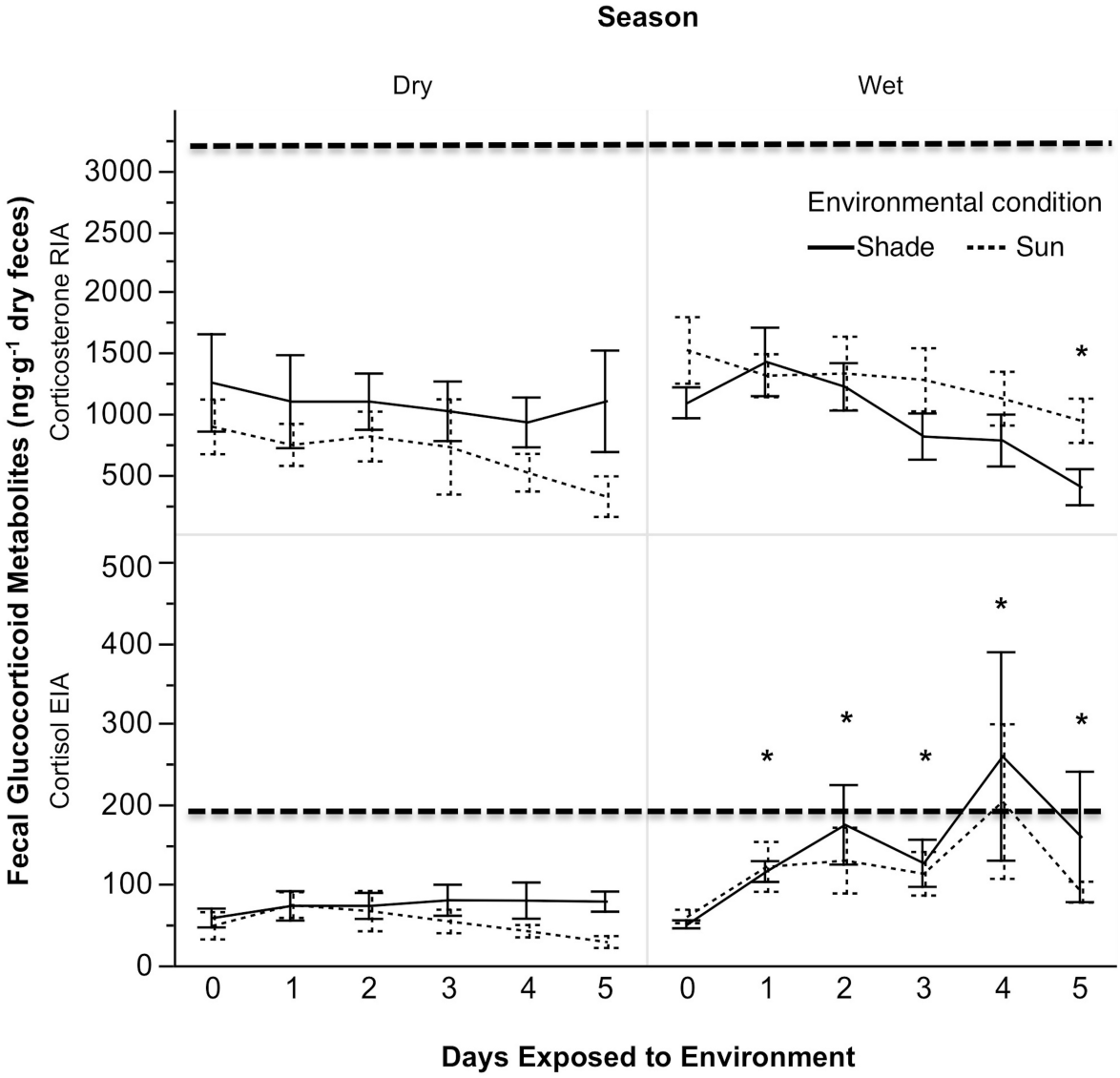

Figure 2.4. FGM concentrations assessed by two immunoassays, corticosterone RIA (top pane) and cortisol EIA (bottom pane) in jaguar fecal samples exposed for 5 days to two environmental conditions: sun (black dashed line) and shade (black solid line), in two seasons: wet and dry. Error bars represent 1 SE. * = significant difference from control sample or day 0 of exposure. Horizontal black dashed lines represents average FGM of individuals with high adrenal activity (i.e., less than 1-yr captivity) from biological validation.

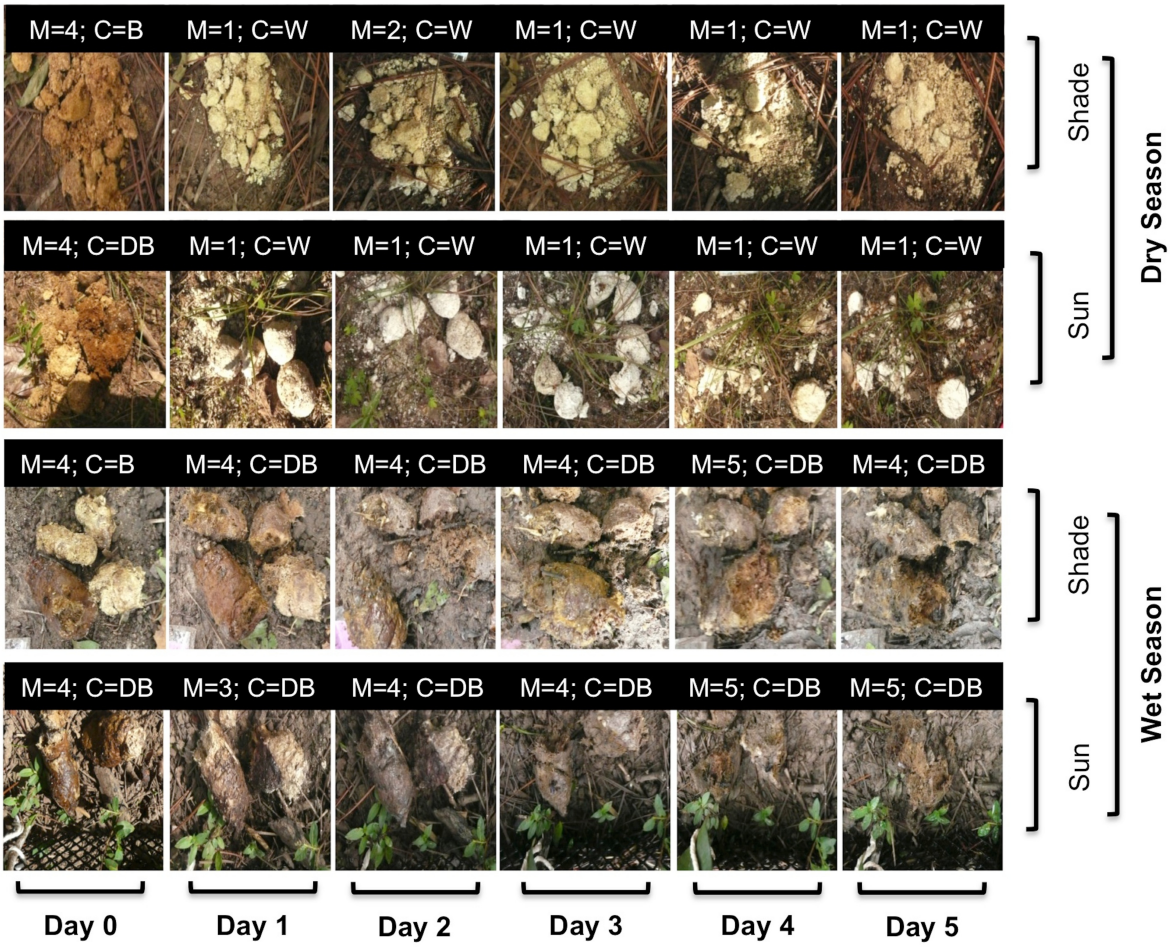

Figure 2.5. Morphology of jaguar scat during 5 days of exposure during the wet and dry seasons under two environmental conditions: sun and shade. An example of four scats photographed over time (row direction) is shown under every environmental combination. Morphological characteristic scores: M: moisture, from driest (1) to moistest (5); C: color (DB: dark brown; B: brown; W: white) are presented in the black boxes.

Chapter 3: Population Assessment of Belizean Felids in a Mosaic Landscape Through Non-invasive Genetic, Parasite, Diet, and Stress-Hormone Analyses.

J. Bernardo Mesa Cruz [a, b], Janine L. Brown [a], Marcella J. Kelly [b]

[a] Center for Species Survival, Smithsonian Conservation Biology Institute 1500 Remount Rd, Front Royal, VA, 22630 USA

[b] Department of Fish and Wildlife Conservation, Virginia Polytechnic and State University 100 Cheatham Hall, Blacksburg, VA, 24061 USA

Abstract

Many Neotropical felid species, such as jaguars, are threatened with extinction due to direct effects of habitat destruction and/or human persecution. However indirect effects that may contribute to these threats remain unknown. Human activities around protected areas in Belize, Central America, are increasing and so are levels of human-felid conflict. Potential consequences of this conflict are an increase in stress that negatively impacts health, diet shifts, or heightening of animal aggression resulting in more conflict. The goal of this study was to assess the potential effects of human-disturbed habitats on the five native Belizean felids by comparing fecal glucocorticoid metabolites (FGM) concentrations, endoparasite species richness (ESR), and diet using non-invasive felid scat sampling in protected forest vs. non-protected areas, including areas highly modified by humans outside the core forest. We collected scat using a detector dog, and analyzed DNA to assign individual identity to the scats. We retrieved parasite propagates with a fecal floatation technique, identified diet items using morphological characteristics at the macro and microscopic level, and we measured FGM with the ^{125}I corticosterone RIA (MP Biomedicals). We collected 336 scat samples. DNA amplification success was low at 30%. We detected five felid species: jaguar, puma, ocelot, jaguarundi and domestic cat. FGM concentrations were higher in pumas and jaguarundis. We found no evidence of livestock in the scats of large felids. ESR did not differ across felid species, but the domestic cat had overall the

highest number of endoparasites. Geographical distribution of samples and physiological measures suggested that the degrees of landscape modification in the study areas did not compromise overall animal well-being. Results of this study provide a baseline on adrenal activity, prey consumption, and endoparasite loads in felids in the Rio Bravo Conservation and Management Area (RBCMA) in Belize Central America. These findings could be used to compare to populations thought to be affected by human activities not only across Belize, but also in neighboring countries.

Introduction

Belize is a Neotropical country in Central America and hosts over 150 species of mammals, including five species of wild felids: jaguar (*Panthera onca*), puma (*Puma concolor*), ocelot (*Leopardus pardalis*), jaguarundi (*Puma yagouaroundi*) and margay (*Leopardus wiedii*) (Meerman, 2005). Within protected areas, Belize has relatively large jaguar, puma, and ocelot populations (Dillon and Kelly, 2007; Kelly et al., 2008; Silver et al., 2004), yet little is known about the ecology or what factors impact the well-being of these secretive species outside of protected areas (Dillon and Kelly, 2007; Foster et al., 2010; Konecny, 1989; Laundré and Hernández, 2010). Additionally, there is scarce biological information about margays and jaguarundis throughout their entire range.

Despite having a large proportion of protected territory, ranging from 26- 36% (Meerman and Wilson, 2005; Young, 2008), in the past two decades, Belize has experienced a progressive increase in human activities, such as hunting, housing developments, forest eradication, and conversion to agriculture (Barbier, 2009; Davis and Holmgren, 2000; Emch et al., 2005; Kay and Avella, 2010; Whitman et al., 1997; Wyman and Stein, 2010). Consequently, the levels of human-felid conflict (H-FC) have been positively associated with the increase of human activities (Inskip and Zimmermann, 2009). The conflict between humans and both jaguars and pumas has been well described. Latin American communities are not generally concerned about the risk of felid attacks on humans, but rather livestock depredation is the strongest factor

provoking the conflict (Michalski et al., 2006; Polisar et al., 2003). Interestingly, livestock depredation has been confirmed in jaguars but not in pumas in Belize (Foster et al., 2010; Rabinowitz, 1986). This has led to jaguar and puma killing in retaliation for livestock loses. Today, human-jaguar and -puma conflict is present throughout the species' geographic range. In contrast, human conflict with ocelots, jaguarundis, and margays is considered low; however the dynamics of this conflict remain poorly studied (Inskip and Zimmerman, 2009).

While habitat loss and poaching are directly related to felid population declines, other contributing factors (*e.g.,* indirect effects) could exacerbate such declines. Potential consequences of this H-FC could be an increase in adrenal activity that negatively impacts reproductive rates and animal health as well as heightening of animal aggression resulting in more H-FC. Additionally, a decrease in native prey availability could result in livestock predation, such as cattle for the larger cats and on poultry for the smaller cats, which could increase retaliatory hunting pressure on these felids. Finally, there may be interactions among these factors. Therefore, to achieve effective conservation, it is important to understand basic felid health status in human modified habitats compared to such status in protected areas.

Levels of stress can be monitored through the evaluation if glucocorticoids (GCs), adrenal hormones produced in response to physiological and environmental disturbances. The detrimental effects of chronic GC exposure are well documented and include suppression of reproductive, immune, and neurological functions (*Mammals*- Maccari and Morley-Fletcher, 2007; Pride, 2005; Sapolsky et al., 2000; Ward et al., 2008; Zhao et al., 2007; *Avian*- Ellenberg et al., 2007; Kitaysky et al., 2006; *Reptiles*- French et al., 2008; Romero and Wikelski, 2001). Therefore, there has been an increasing interest in evaluating physiological responses of individuals and populations to human caused disturbances in ecosystems. GCs have been measured as fecal glucocorticoid metabolites (FGM) in several free-living species (Chapman et al., 2006; Cyr and Romero, 2008; Fanson et al., 2012; Garcia Pereira et al., 2006; Muehlenbein et al., 2012), as well as captive Neotropical felids (Bonier et al., 2004; Morato et al., 2004; Moreira et al., 2007; Romano et al., 2010). For example, Wasser *et al.* (1997) showed the use of

such a proxy in their study on the effects of logging on the northern spotted owl (*Strix occidentalis caurina*). Male owls in logged areas had higher concentrations of FGM than those in undisturbed areas. Creel *et al.* (2002) observed higher FGM concentrations in feces of elk and wolves in relation to the intensity of winter snowmobile activity in Yellowstone National Park. Barja *et al.* (2007) showed a direct correlation between unregulated tourism and the levels of FGM in the European pine marten (*Martes martes*). To our knowledge there are no reports of FGM assessments in any free-living Neotropical felid in their native habitat to address similar concerns.

Anthropogenic disturbances, such as the expansion of agricultural frontiers in developing countries, and of suburban areas in developed countries have led to increased contact between wild carnivores and domestic animals such as dogs, cats, and livestock. In fact, some carnivore populations have contracted diseases transmitted by domestic animals causing devastating impacts. For instance, as result of rabies outbreaks originating from domestic dogs, populations of African wild dogs (*Lycaon pictus)* in some areas faced local extinction, and Ethiopian wolves (*Canis simensis*) were drastically reduced in numbers (Woodroffe, 1999). Moreover, coprological studies present a great opportunity to monitor parasitic infection dynamics at the population level (Lafferty, 1997; Weyher et al., 2006). In this context, Belizean felids have not been the exception. A scat survey in the Cockscomb Basing, Belize, identified 14 different types of endoparasites present in the scat of jaguars, pumas, ocelots, and jaguarundis (Patton and Rabinowitz, 1986). However, periodic monitoring is necessary to develop effective disease surveillance and determine trends in wildlife-parasite dynamics and health (Artois et al., 2012).

The diet of jaguars and pumas in human modified environments has been fairly well studied across their range, but to a lesser extent in Belize. Pumas and jaguars, ranging hunted and non-hunted areas in Guatemala, had low dietary overlap and were not found to predate on livestock (Novack et al., 2005). In Venezuela, it appears that pumas exert greater pressure on livestock than jaguars (Polisar et al., 2003). In contrast, jaguars were found to prey more on livestock than pumas in the Iguaçu National Park area (Cascelli de Azevedo, 2008). In Belize, Rabinowitz

(1986) reported that most jaguars roaming in proximity of human modified landscapes, around Cockscomb Basin, did not prey on livestock. Most recently, however, in the same region, 1 out of 10 jaguar scats contained evidence of cattle as part of their diet, while pumas did not appear to prey on livestock. However, detection of puma scat was very low in human modified areas in that study (Foster *et al.*, 2010). Diet of smaller felids such as ocelots, margays, and jaguarundis has been assessed in protected forests only (*Brazil*- Bianchi et al., 2011; Silva-Pereira et al., 2011; *Panama*- Moreno et al., 2006; *Venezuela*- Farrell et al., 2000). However, limitations in assigning species of origin to scats of smaller felids remain the biggest challenge for estimating the diet of these felines.

Obtaining biological data from wild Neotropical felids is challenging due to their secretive nature, thick habitat, and the perceived or real risks of aggression towards humans. Nevertheless, recent advances in non-invasive hormone and DNA analysis techniques have improved the feasibility of long-term field monitoring (Wultsch et al., In press), Additionally, the use of detector dogs to find scat, has been shown to greatly improve the efficiency of sample collection (Kerley, 2010; Long et al., 2007; Wasser et al., 2004; Wultsch et al., In review).

The goal of this study was to assess the potential indirect effects of human-modified habitats on the five native Belizean felids by comparing FGM concentrations, endoparasite species richness (ESR), and diet using non-invasive felid scat sampling in protected forest vs. non-protected areas, including areas with high human disturbance outside the core forest. We hypothesize that felids ranging in areas highly modified by humans (*e.g.*, habitat fragmentation, agricultural conversion, cattle ranching) will have higher FGM concentrations, indicative of increased adrenal activity and possibly stress. Additionally, we expected that felids would consume livestock and have higher ESR in the non-protected areas.

Methods

Study site

This study took place in northwestern Belize, Central America. Scat surveys were conducted in the Rio Bravo Conservation and Management Area (RBCMA); which is the largest private protected area in Belize and one of the largest protected areas in the country (104,897 hectares), accounting for 4% of the total protected land in Belize. Lowland broad-leaf moist forest is the predominant ecotype at RBCMA. In this area, jaguars are found at densities of ~6 individuals per 100 km^2 (Waight, 2010), while the density of other felids is unknown. RBCMA is adjacent to highly modified land, dominated by cropland (*e.g.*, corn, soy bean, sugar cane, onion, and tropical fruits) and cattle ranching. In addition, there are several settlements with populations of less than 300 people, such as Indian Church, San Carlos and Blue Creek, and bigger settlements, with about 1000 or more inhabitants such as Indian Creek, Shipyard, and San Felipe (Castillo, 2010) (Figure 3.1).

Field survey

From March to July 2011 a scat collection team, with a professionally trained detector dog (PackLeader LLC Dog Training - http://www.packleaderdogtraining.net), trained to detect scat of jaguar, puma, ocelot, jaguarundi, and margay, located felid scat across a mosaic landscape of protected and human modified land in an opportunistic fashion. This survey adopted a systematic approach to ensure the reliability of FGM measures in scat collected under Belizean conditions (see chapter 2). Each transect (5-10 km) was surveyed three times at a 4 day interval. Samples found in the first visit were cleared off the trails areas and were not included in the FGM analysis due to unknown age and hence possible degradation of steroid metabolites. We chose this sample regime due to significant shifts documented in FGM concentrations in jaguar scat exposed to 4 or more days to the environment in Belize (Mesa et al., In Prep.). Scats found in subsequent visits were considered optimal for FGM analysis, unless drastic morphological signs of degradation were evident in a sample. Information at each scat was collected such as GPS location, habitat features (trail width (m), distance of the scat to main trail (m), habitat type,

percent tree canopy cover (%), understory vegetation type, and ground cover type) and reference photographs were taken.

Molecular species identification

We used protocols for DNA preservation, extraction, and genotyping previously developed and validated for Belizean felids (Wultsch *et al.*, In press). We removed a small portion (~0.5 g) of the outer surface of the scat, which contains intestinal epithelial cells, suspended in DET buffer (20% v/v DMSO, 0.25M EDTA, 0.1M tris, pH 7.5, NaCl saturated) and stored at room temperature until analysis. We extracted fecal DNA with the QIAamp® DNA Stool Mini Kit (Qiagen Inc., Valencia, CA), according to manufacturer protocol. PCR, *n*DNA (microsatellites) genotyping, and *mt*DNA sequencing were performed at the University of Idaho Conservation Genetics Laboratory. We used *n*DNA to determine species, individual identity, and sex in all scat samples collected; while *mt*DNA was assessed in those samples with low quality *n*DNA samples and in two random samples in each defined genetic group for species confirmation classification obtained from *n*DNA frequencies.

*n*DNA

A set of 7 microsatellite primers (FCA043NED, FCA090, FCA096, F124NED, FCA126, FCA275VIC, FCA391) and 1 sex marker (Zn-finger) specific to felids were used in this study (Pilgrim et al., 2005; Wultsch et al., In press). We performed PCR reactions (7μL) under the following conditions: 3.5 μL 2X Master mix, 0.7 μL 5X Q solution, 10μM each primer (including sex marker Zn), and 1.8 μL of template. PCR amplifications included one denaturation cycle (15 min 95 °C), 13 cycles (30 s at 94 °C, 90 s at 60 °C, 60 s at 72 °C), 37 cycles (30 s at 94 °C, 90 s at 50 °C, 60 s at 72 °C), and one concluding step, (30 min at 60 °C). We then separated PCR products in an automated genetic analyzer (3130*xl* Applied Biosystems Foster City, CA) and used GeneMapper® software (Applied Biosystems, v3.7 2004) for allele determination.

*mt*DNA

Carnivore specific mitochondrial cytochrome b primers (146 b.p.) were employed in procedures slightly modified from Farrell et al. (2000). We performed PCR reactions (15 µL) under the following conditions: 1.5 µL 10X buffer, 25 mM $MgCl_2$, 10mM dNTPs, 10 µM each primer, 30 $mgmL^{-1}$ BSA, 5 $unitsµL^{-1}$ Amplitaq Gold® DNA polymerase (Applied Biosystems Foster City, CA) and 1 µL of template. PCR amplifications included one denaturation cycle (10 min 95 °C), 55 cycles (30 s at 92 °C, 45 s at 50 °C, 40 s at 72 °C), and two concluding steps, (2 min at 72 °C, and 30 min at 4 °C). After amplification, we run a small fraction of all products on 2% agarose gels. We cleaned PCR products (10 µL) of samples presenting a positive band with SAM™ and XTerminator™ solutions (45 µL and 10 µL, respectively), by agitating for 30 min followed by centrifugation at 1000 *g* for 2 min. We sequenced PCR products in an automated genetic analyzer (3130*xl* Applied Biosystems, Foster City, CA). We used GeneMapper® (Applied Biosystems v3.7 2004) to edit the sequences and aligned those in the NCBI's basic local alignment search tool (BLAST®, http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome, accessed May 2013). Furthermore, we used additional *mt*DNA markers to confirm *n*DNA results for species assignment to the number of groups (*i.e.*, species) detected by program STRUCTURE. Two samples were selected at random from each distinct genetic group and tested for Carniv, 12Sv, 16S, 16Sco, and ATP6 *mt*DNA makers.

Endoparasite analysis

In the field, we preserved a subsample of each scat (1-3 g) in buffered formalin (10%, pH 7) and stored subsamples at room temperature until analysis. We retrieved parasite eggs and larvae with a modified Wisconsin fecal flotation test as described by Zajac and Conboy (2012). In the laboratory, we mixed preserved feces with distilled water (1:5 v/v) and filtered them through gauze into a conical centrifuge tube. This solution was centrifuged for 10 min at ~550 *g*. We removed supernatant and resuspended the pellet in 15 mL of Sheather's sugar solution (1.27 specific gravity, Jorgensen Laboratories Inc., Loveland, CO). Thereafter, we centrifuged samples for 10 min at 550 *g*. We observed and identified endoparasite eggs, oocysts, and larvae present in

the supernatant under the microscope; we recorded genera and number of propagules. We calculated prevalence and endoparasite species richness (ESR) as the total number of species observed in each individual and each felid species.

FGM analysis

Once subsamples for DNA and parasites were collected in the field, we stored remaining fecal material in plastic bags, transported it in a cooler to camp, and froze it at -20 °C until processing at the Smithsonian Conservation Biology Institute (SCBI). At SCBI, we freeze-dried feces in a lyophilizer, and subsequently homogenized and pulverized the dry product. We separated the dry fecal powder (0.19 ± 0.01 g) from prey remains and extracted fecal steroids in 5 mL of ethanol by boiling in a water bath (90-100 °C) for 20 min and centrifuging at 500 *g* for 15 min. We recovered supernatants and resuspended pellets in 5 mL of ethanol, vortexed for 30 s, and centrifuged again. Supernatants were combined and air-dried overnight. We resuspended extracts in 1 mL of methanol and placed in an ultrasonic cleaner for 10 min. We diluted extracts (1:1 v/v) with steroid buffer (0.1 M NaPO4, 0.149 M NaCl, pH 7.0) and stored extracts at -20 °C until analysis. We assessed extraction efficiency recovery by adding radiolabeled cortisol (^{3}H cortisol, 2500 dpm) to all samples prior to boiling extraction. We used the double-antibody ^{125}I corticosterone radioimmunoassay (RIA) (MP Biomedicals, LLC, Orangeburg, NY, USA) to estimate FGM concentrations. This assay has been validated for jaguar, ocelot, margay, and puma (Bonier et al., 2004; Conforti et al., 2012; Dias et al., 2008; Young et al., 2004). We validated the ^{125}I corticosterone RIA for jaguarondi by testing for parallelism between the assay standards of and a pool of jaguarundi fecal extracts.

Diet analysis

We analyzed prey remains such as teeth, hair fibers, bones, claws, feathers, and scales at the macroscopic and microscopic level and identified as previously described in jaguars (Foster *et al.*, 2010). Our reference sample collection consisted of hair, claws, teeth and bone samples from

over 30 potential prey species. We collected these reference samples at the Belize Zoo, farms around the study sites, and through opportunistic sampling from road killed animals observed during the scat survey. We also used other reference materials such as books, scientific papers, and manuals to identify prey remnants in scats not accounted for in our collection (-*Teeth and bone morphology*- Avenue and Kingdom, 2009; Best et al., 1995; Engilis et al., 2012; Frey, 2007; García and Sánchez-González, 2013; Goodwin, 1969; Hunt et al., 2004; Lawlor, 1982; Musser et al., 1998; Schmidt et al., 1989; Tribe, 2005 -*Hair morphology*- Baca Ibarra and Sanchez-Cordero, 2004; Debelica and Thies, 2009; Juarez et al., 2010; Lungu et al., 2007; Pech-Canche et al., 2009). We cleaned fragments of bone, teeth, and claws and observed such remains under a dissecting scope. We submerged hair and feathers in xylene for 2 hours and then mounted hairs on microscope slides to observe medulla and cuticle casts. A similar procedure was performed with feathers, but only barbules and villi were characterized (Dove and Koch, 2010). We performed diet analysis on all samples with felid genetic identity and also on those felid samples with no genetic identity. The latter were classified by both scat detector dog positive identification and morphology as "large felid" (*e.g.*, jaguar or puma).

Statistics

We used the software GeneAlEX 6.5 (Peakall and Smouse, 2012) to find matches in consensus genotypes to identify recaptured individuals. We formed matches with samples having common genotypes at a minimum of 5 loci. We used program *STRUCTURE* 2.3.4 (Falush et al., 2003; Pritchard et al., 2000) to analyze the number of distinct genetic groups, k (*i.e.*, species); one genotype per individual was included in the analysis (100,000 burn-in period and 400,000 MCMC repetitions after burn-in; frequency for metropolis update for Q: 10). We used both, the largest average probability of k given the simulated data (Ln P(D)) and the *ad hoc* statistic Δk (Evanno et al., 2005) to determine k. We used a contingency analysis to compare DNA amplification success in protected and non-protected areas.

We estimated prevalence of endoparasite genera as the relative frequency of identification for each parasite (*i.e.*,). To avoid pseudoreplication, we combined results of parasite analysis and averaged for each individual. ESR was compared across felid species with a one-way ANOVA. Additionally, we grouped parasites in four categories related to target organ or system of infection (*e.g.*, generalized, respiratory, liver, and gastro-intestinal) to evaluate how these parasites are distributed in each felid species through contingency analysis.

We summarized the frequency of occurrence of prey item in scat of each felid species as: We analyzed the diet results by examining the relationship between predator species (*i.e.*, felids) and prey species consumed through correspondence analysis. Prey species were grouped in 6 different categories: reptiles, insects, birds, and mammals - small (<1kg body mass), medium (1-5 kg body mass) and large (>5 kg body mass).

We used a test for parallelism between the RIA and jaguarundi fecal extracts with a multiple linear regression of log transformed concentration and the binding percentage of both the standard and the serially diluted extracts. We applied linear contrasts of the least squares means of the regression to standard and serially diluted samples, as suggested by Grotjan and Keel (1996). We adjusted all FGM concentrations based on the extraction efficiency recovery. We combined and averaged FGM concentrations, if multiple samples from the same individual were suitable for hormonal analysis. We compared FGM across species using a one-way ANOVA and a simple linear regression to determine the relationship between ESR and FGM concentrations for each felid species. We assessed all data for normality using the Shapiro-Wilk goodness of fit test (α=0.05) before applying a statistical test. Data distributed in a non-normal fashion were log transformed. We conducted all statistical analyses, except for the genetic assessments, in the statistical software JMP Pro 10 (Version 10.0.2; SAS Institute Inc., Cary, NC. 2012).

Results

During the entire field season we surveyed approximately 420 km in different habitat types with equivalent effort between protected and non-protected areas. We collected 336 scats samples from wild felids distributed across the study areas (Figure 3.1).

Genetics

Five different genetic groups were identified by (Ln P(D)) and Δk methods and confirmed by *mt*DNA, corresponding to jaguar (*Panthera onca*), puma (*Puma concolor*), ocelot (*Leopardus pardalis*), jaguarundi (*Puma yagouaroundi*) and domestic cat (*Felis s. catus*) (Figure 3.2). Table 3.1 summarizes the number of individuals detected and number of recaptures by species, sex, and technique used to identify these felids. We found most jaguar, puma, and ocelot samples in the protected area (RBCMA), whereas most jaguarundi and all domestic cat samples were found in non-protected areas.

Thirty three percent of the total number of samples was positive to the gene markers (*i.e.*, amplification success), *n*DNA and *mt*DNA. Marked differences in DNA amplification success were observed in scat samples found in RBCMA and in non-protected areas. Scats found in RBCMA were three times more likely to amplify for DNA than samples found in non-protected areas (χ^2=40.690; P<0.0001). *Ad hoc* tests (*i.e.*, multiple linear regressions and contingency analyses) examining the relationship among habitat features and DNA amplification success revealed that tree canopy cover was significantly associated with amplification success. DNA amplification success was highest (65%) in samples found under canopy cover that was greater than 70%, while amplification success was lowest (28%) for those samples with very little cover 0-34% (Figure 3.3A). Scats found in non-protected areas usually were located in areas with very little canopy cover (Figure 3.3B).

Endoparasites

We identified a total of 24 genera of endoparasites and one unknown (*i.e.*, unidentified) species (Table 3.2). Most scat samples were positive for nematodes (60%), trematodes (70%), and protozoans (85%). ESR across felid species was not significantly different (df=40; F=0.43 P=0.782). However, the domestic cat had overall the highest number of parasite species identified (18) while the jaguarundi had the lowest (12) (Table 3.2). Numbers of parasite eggs were highly variable within and among felid species. Two trematode species, *Spirometra sp*. and *Platynosomum sp*, were found at the highest prevalence across the felid species. However, it was not possible to classify to the genus level one parasite species affecting one female ocelot ranging in the RBCMA. This unidentified parasite is most likely from the Troglotrematidae family.

It appears that all felid species were similarly affected by parasites in feces with no significant difference in ESR (χ^2=8.1; P=0.777). As expected, gastro-intestinal parasite eggs were most common as compared to parasites producing respiratory, liver, or generalized infections (Figure 3.4).

Diet

We identified a total of 35 animal prey species in felid scats in this study (Table 3.3). Jaguars preyed with more frequency on peccaries and armadillo, whereas pumas preyed more on cervids (e.g., red brocket and white-tailed deer). While cotton rats were the most frequent prey item for jaguarundis, ocelots, and domestic cats. Correspondence analysis indicated that puma and jaguar were more associated with medium and large prey, ocelots were associated with small mammal prey and marginally with birds, jaguarundis were associated with birds and reptiles, and domestic cats were associated with the consumption of small mammals and insects (Figure 3.5).

We found a total of 20 large cat samples (*i.e.,* those samples where DNA did not amplify but were classified as “large cat” by morphology and scat dog positive identification), 8 in the

RBCMA and 12 in human modified areas. Small-sized prey were more commonly found in human modified areas (small 50%; medium: 40%; large: 10%) of the prey items), while medium prey were more frequent in the protected area (small 25%; medium: 75%).

Livestock or other domestic animals were not found in any scat of these felid species, including those recognized by the scat dog and categorized by morphology as large cats.

FGM

The test for parallelism between the corticosterone RIA and a pool of jaguarundi fecal extracts showed that the curves were not significantly different from each other (t=-0.33, P=0.748). Therefore, jaguarundi FGM can be measured with the corticosterone RIA. Extraction efficiency recoveries were on average above 84% for all species (jaguar: 84.5% ± 2.2; puma: 85.8% ± 1.1; ocelot: 84.4% ± 2.1; jaguarondi: 84.5 ± 3.1; domestic cat: 86.7% ± 0.7). Figure 3.6 summarizes the results of FGM concentrations in all felid species, individuals represented with more than one scat were averaged for the analysis. Interestingly, pumas and jaguarundis exhibited significantly higher concentrations of FGM than other felids (df=40, F=13.07, P<0.0001). Some domestic cat, jaguar, ocelot and puma individuals had multiple samples (*e.g.,* genetic captures) suitable for FGM analysis, hormonal concentration distributions of those individuals are shown in Figure 3.7. Additionally, no linear associations between ESR and FGM concentrations were observed in any of the felid species (Figure 3.8).

Discussion

This study demonstrates the feasibility of surveying endoparasites, adrenal activity, and diet of free-ranging Belizean felids by using non-invasive scat sampling. To our knowledge, this is the first study using non-invasive scat surveys to identify felid *n*DNA in non-protected areas in Belize. Sample numbers were somewhat limited because of sample degradation and environmental effects, especially outside of the protected area. Low DNA amplification success

of scat samples in the human modified areas impeded our ability to compare adrenal activity, ESR, and prey consumption between those felids ranging in the RBCMA versus those ranging in the human-modified surrounding areas. The overall 33% DNA amplification success was low compared to a previous study by Wultsch et al. (In press), which was ~60%. However, that study collected scat only in protected areas. When DNA amplification success was calculated by status of land protection, our results are similar, being 63% for samples collected in the RBCMA compared to only 24% in scat found in human-modified areas. Lack of canopy cover in human-modified areas was closely associated with low DNA amplification success (*i.e.*, highly degraded DNA). Scats found in protected areas were usually sheltered from the sun by trees. Thus, direct exposure to the sun (*e.g.*, UV rays) and possibly higher temperatures appear to affect the viability of DNA in feces, as has been previously reported in brown bears (Murphy et al., 2007). Despite this limitation, some important spatial distribution trends were observed. Jaguars, pumas, and ocelots were present in both the RBCMA and in human-modified habitats surrounding the RBCMA, whereas jaguarundis were detected only in the human-modified areas. This is consistent with previous reports that found jaguarundis ranging in heterogeneous habitats with intermingled closed and open areas (Caso, 2013; Sunquist and Sunquist, 2002).

The absence of margay detections in both the RBCMA and human-modified areas was not surprising. Although margays are known to be present in the RBCMA (Kelly, unpublished data), their secretive nature makes them extremely difficult to study. Margays are mostly arboreal (Sunquist and Sunquist 2002), perhaps depositing their feces in areas out of the detector dog's scent reach. Additionally, margays are thought to be very sensitive to human disturbance (Carvajal-Villarreal et al., 2012), which may reduce the likelihood of them inhabiting the non-protected areas.

Domestic cats were found only in human-modified habitats, despite the close proximity to the RBCMA. This finding is important for local wildlife conservation efforts at RBCMA because it suggests that domestic cats have not colonized this protected forest as they have in other areas

across the world (Kays and DeWan, 2004; Woods et al., 2003). A reason for this finding is that the native carnivore guild might be out competing domestic cats from the protected forest. This is particularly important since feral cats are known to consume important numbers of small mammals and birds in Australia, United States, and many islands worldwide (Loss et al., 2013; Medina et al., 2011; Risbey et al., 2000).

A previous survey of felid endoparasites in Belize found 13 genera in feces of wild Belizean felids (Patton and Rabinowitz 1986), while we found almost twice that number in the same felid species. We speculate this is due to an increased sensitivity the modified (double centrifugation) Wisconsin flotation technique we used compared to the standard Wisconsin method, as previously suggested (Zajak and Conboy, 2012). Egg morphology of the unidentified parasite species resembled those of the trematode *Paragonimus sp.* (Family: Troglotrematidae) displaying a thick and clear operculum at the larger end (ridge). However, the egg dimensions were much smaller than a *Paragonimus sp.*, (90 x 50 μm vs. 45 x 25 μm). We recommend using different diagnostic techniques, other than morphometrics, such as ELISA or molecular genetic determination to identify this parasite species. We suggest that future studies adopt the modified Wisconsin technique to assess gastrointestinal parasites in felid scat to obtain higher sensitivity and comparable results.

Estimating parasite richness (*e.g.,* ESR) can provide a coarse estimation of the ability of the host to control infections (Muehlenbein, 2006). Despite the gradient obtained in ESR values, there was no association with adrenal activity based on FGM. However, it is unclear if the range of ESR for these Neotropical felids is “normal” since there are no previous reports of ESR in Belize. Nevertheless, taking into account the results of ESR, their relationships with FGM and the overall parasite community structure, our data suggest that these endoparasite loads might not be causing important pathogenic effects on these felids. A larger sample size would provide more insight into the relationship between parasites and parasite loads, especially as they relate to richness and freshness of the samples. Future research is needed to assess the effects of

environmental variables such as humidity, temperature, and time on parasite egg degradation and egg hatching rates of the parasite species found in this study. The overall parasite community structure affecting different organs and systems did not differ across of all five felid species.

It appears that the dietary resources are well partitioned across wild felid species. Results of this study are congruent with previous reports of these felids across their range in that pumas and jaguars prey more on deer, and paca (*Cuniculus paca*) and peccary (Tayassuidae) and armadillo (*Dasypus novemcintus*), respectively, where as the smaller cats relied more on smaller mammals, birds and reptiles (Aranda et al., 1996; Cascelli de Azevedo, 2008; Foster et al., 2010; Núñez et al., 2000). Not surprisingly, scat samples from large cats, in human-modified areas, contained predominantly remains of smaller sized mammals followed by medium-sized prey. This finding provides support for the opportunistic nature that jaguars and pumas have in their predatory behavior. This suggests that the degree of habitat modification outside of RBCMA might be influencing a slight change on the diet of the large felids; however, it is not strong enough for these felids to shift their diets to livestock. Furthermore, it appears that native prey still exists in these areas and that large cats prefer them. In contrast, it is possible that domestic cats are competing for smaller prey, such as small mammals, with wild ocelots and jaguarundis present in the human modified areas.

We validated a radioimmunoassay to measure FGM in jaguarundies. We observed differences in FGM across species, with higher concentrations in pumas and jaguarundis compared to the other felids in this study. Romano et al (2010) and Bonier et al (2004) also observed high FGM concentrations in captive jaguarundis and pumas, respectively. Even though, Romano et al (2010) used a different immunoassay from our corticosterone RIA, jaguarundis exhibited almost twice the FGM concentrations than ocelots and margays. Therefore, higher FGM in these two species does not appear to be related to high adrenal activity. Instead, there could be some glucocorticoid metabolic particularity, either in the steroid conjugation step for excretion or in the gut breakdown, that is shared by these two felids; especially since they are clustered in the

same phylogenetic lineage (Pecon Slattery and O'Brien, 1998). Multiple samples from a single individual displayed narrower FGM concentration ranges compared to all individuals of the same species. However, one male puma presented great variability across two samples. Future studies with larger sample sizes could evaluate the precision and accuracy of FGM measures in free-ranging carnivores to provide more insight on the applicability of this technique for elusive species.

In summary, this study provides a baseline for FGM, diet, and endoparasites in Belizean wild felids. However, poor amplification success hampered the ability to compare adrenal activity, endoparasite richness, and prey consumption of felids ranging in the RBCMA to those in human modified habitats. We demonstrated that non-invasive surveys are feasible to address these aspects of Neotropical felids, however, experimental designs must account for low amplification success outside protected areas.

Future directions

Results from this study can be used as a baseline to compare felid populations thought to be affected by human activities, not only across Belize, but also in neighboring countries, thereby providing a template for expanding this approach to future studies across the entire Mesoamerican Biological Corridor, also known as the Path of the Jaguar (Paseo Panthera) (Weber and Rabinowitz, 1996).

Future field surveys could increase sampling effort in human-modified landscapes to overcome sample degradation issues. This increase in sampling effort could be addressed in three ways: 1. by adding one or two dog detection teams; 2. revisiting transects at two-day intervals instead of four; 3. by extending the duration of field surveys.

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Figures and tables

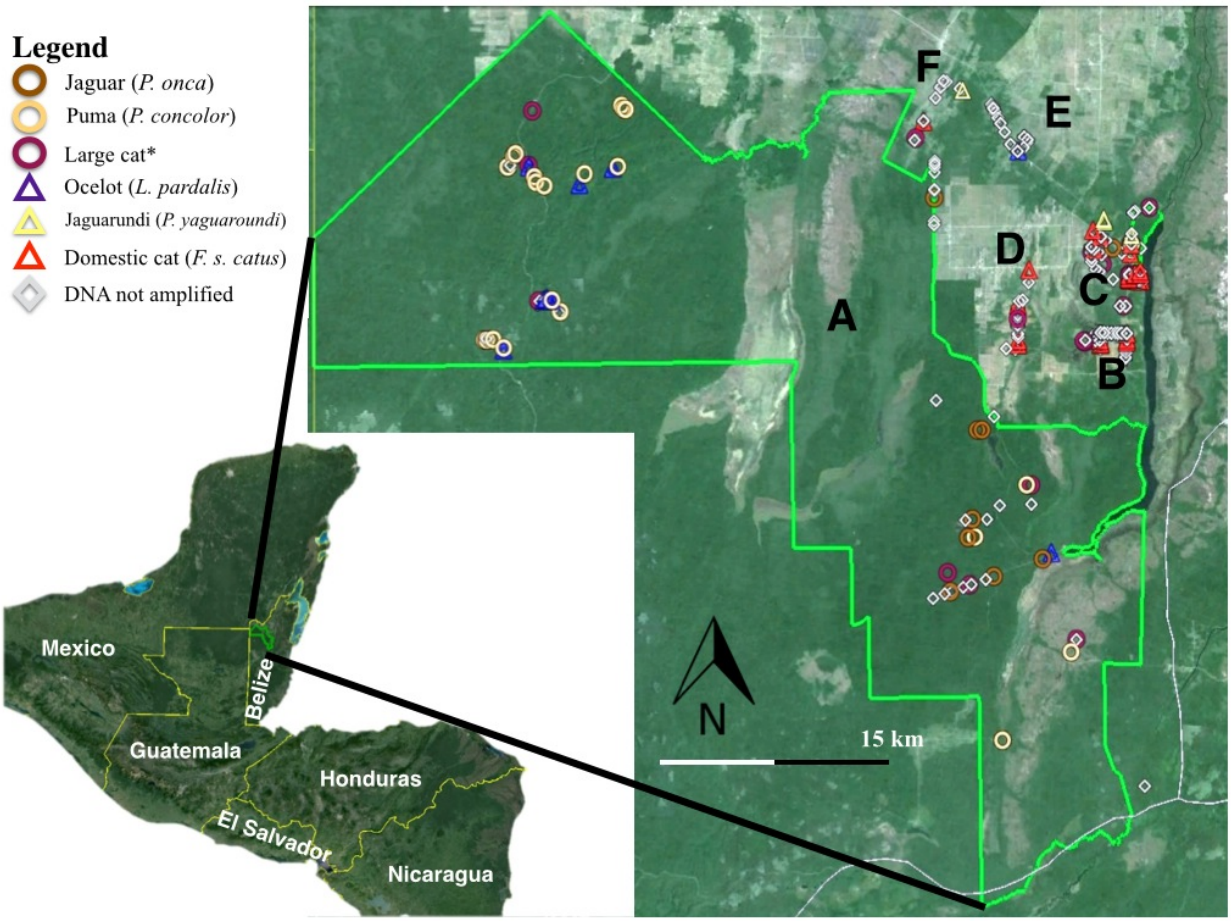

Figure 3.1. Map of study area in Belize, Central America with location of collected scats classified as felid species using molecular techniques. * Samples classified as "Large cat" were identified by morphology due to failure to amplify DNA via molecular techniques. Study sites included a protected area, A. Rio Bravo Conservation and Management Area – RBCMA (outlined in light green), and human-modified non-protected areas, B. San Carlos Village; C. Indian Church Village; D. Indian Creek Village; E. Shipyard Village; F. San Felipe Village. Map images modified from Google Earth 2014.

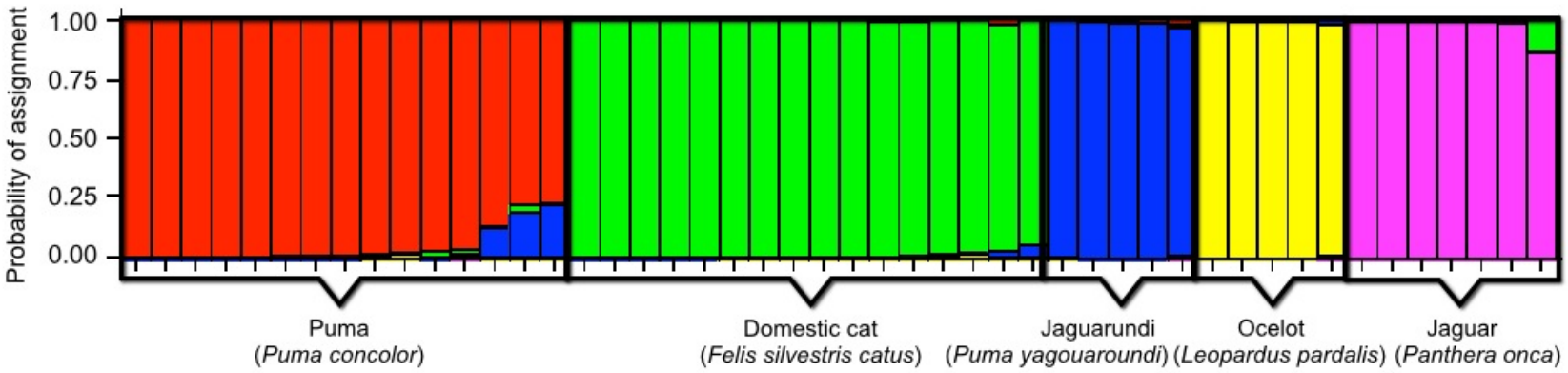

Figure 3.2. Estimated distinct felid genetic groups, based on *n*DNA frequency, obtained from program *structure* k=5. Each individual is represented by a vertical line, partitioned into five color segments that represent the probability of belonging one of the five felid species. Species assignments were confirmed with five *mt*DNA markers in two random samples from every color segment.

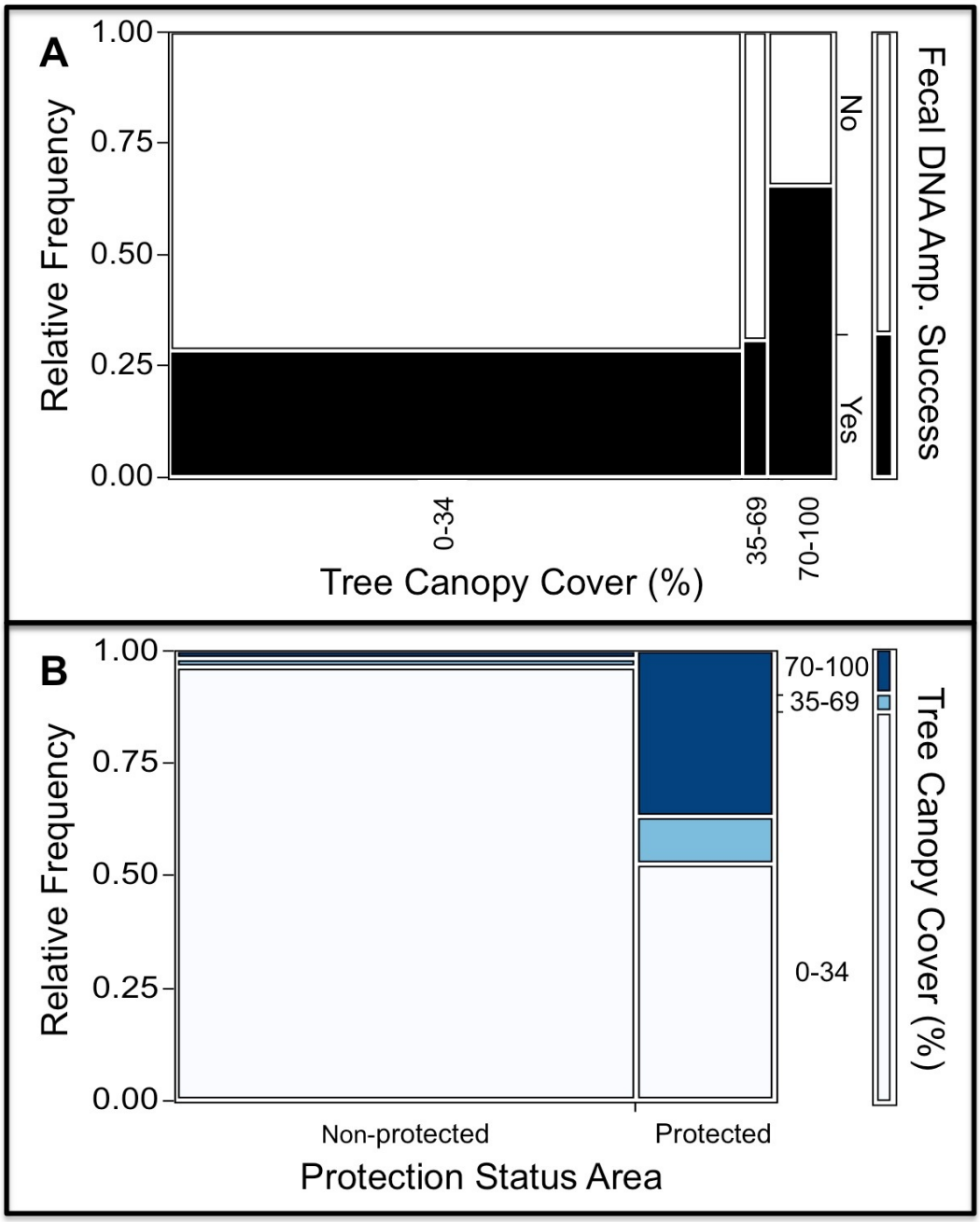

Figure 3.3. Mosaic plots representing relationships between fecal DNA amplification success, tree canopy cover (TCC), and protection status of surveyed areas. A. Relative frequency of felid scats with successful DNA amplification (Yes) or did not amplify (No), found under different TCC (0-34, 35-69, and 70-100%); fecal DNA amplification success increased significantly as TCC of scat location increased (χ^2=18.275; P<0.0001). B. TCC structure in protected and non-protected surveyed areas; non-protected areas, which were modified by humans, had significantly lower TCC than the protected area, RBCMA (χ^2=98.915; P<0.0001).

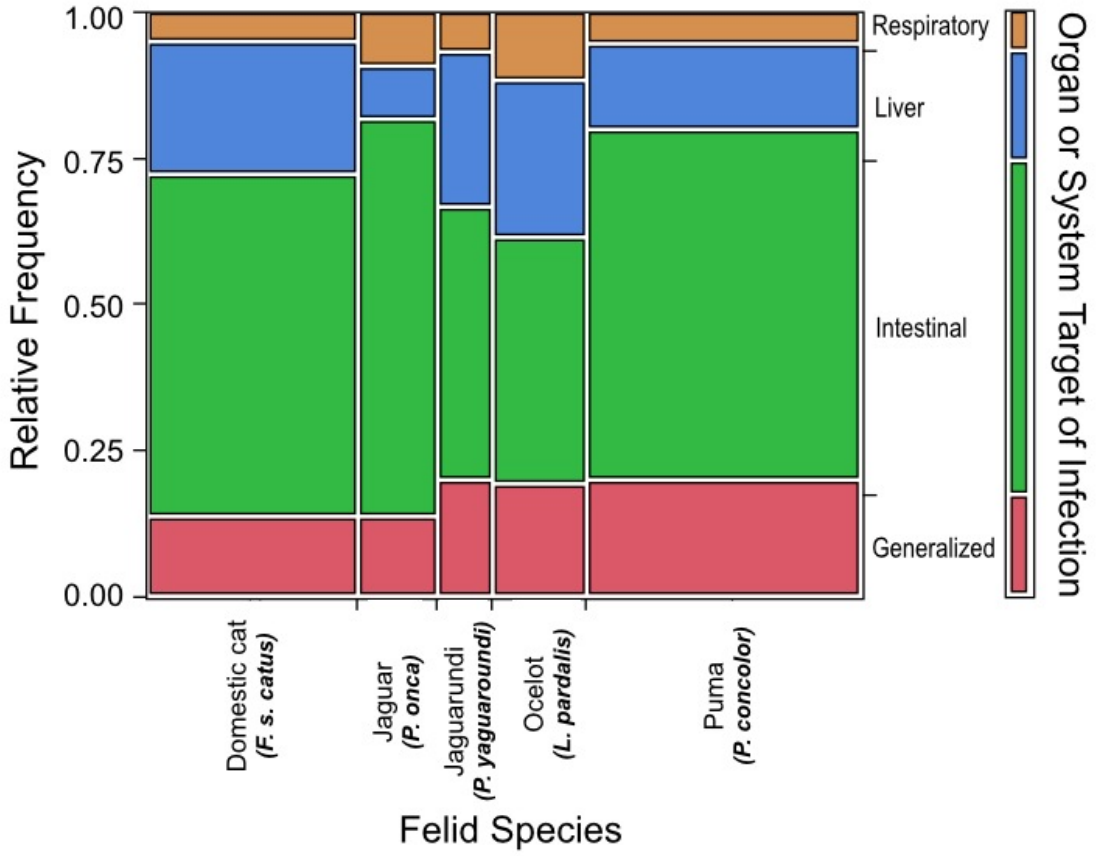

Figure 3.4. Distribution of organs and systems affected by endoparasites identified in five sympatric Neotropical felid species. All felids shared similar proportions of parasites affecting their different organs and systems.

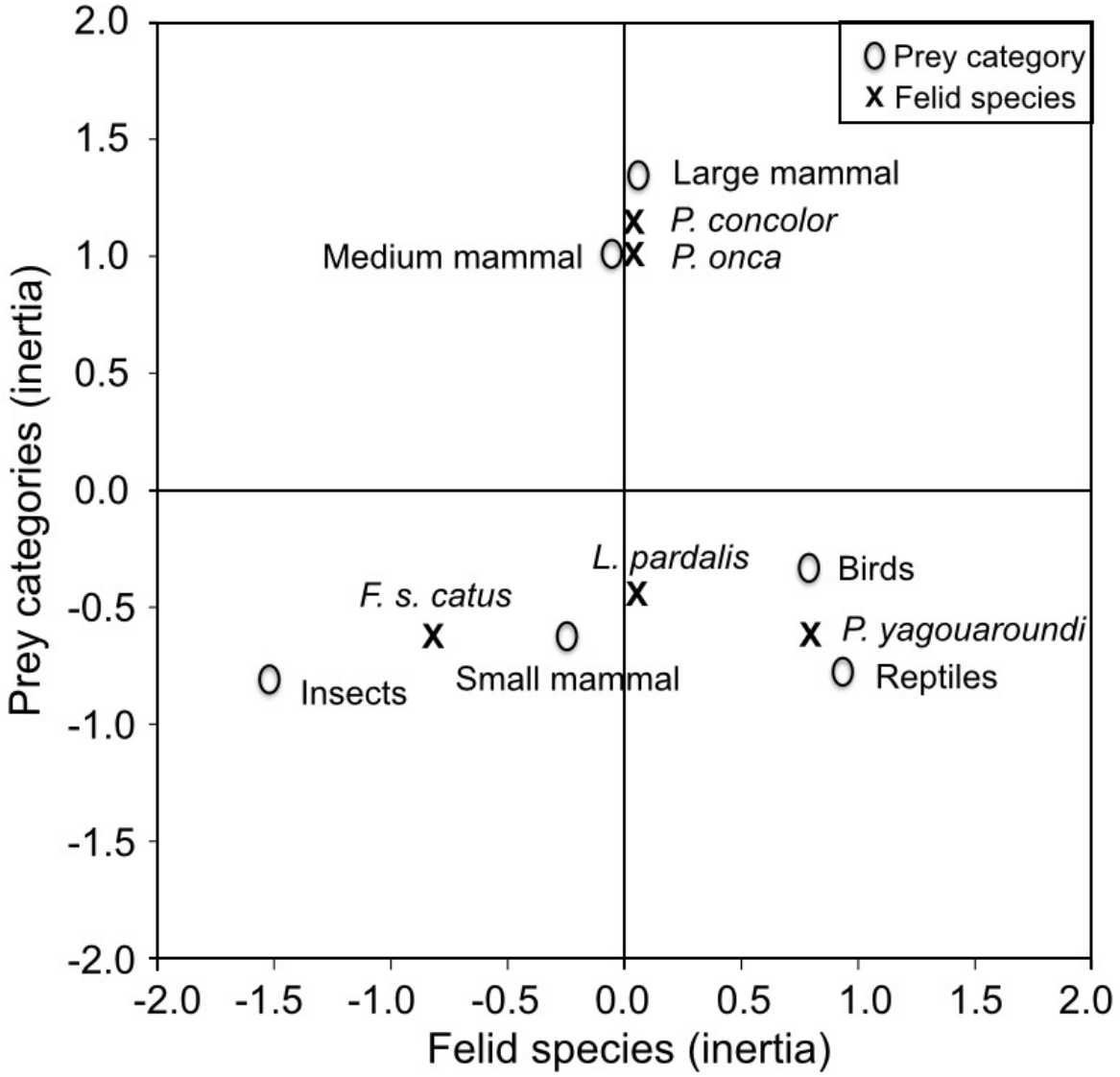

Figure 3.5. Correspondence analysis among five sympatric Neotropical felid species and their diets. There was a significant relationship between prey species (mammals -small <1kg body mass, medium 1-5 kg body mass, and large >5 kg body mass-, birds, reptiles, and insects) and felid species (Jaguar -*P. onca*-, puma -*P. concolor*-, ocelot -*L. pardalis*-, jaguarondi -*P. yagouraoundi*-, domestic cat -*F. s. catus*-). Correspondence analysis indicates that the *x* and *y* axes for felid and prey species explain 27 and 67 % of the model variation, respectively.

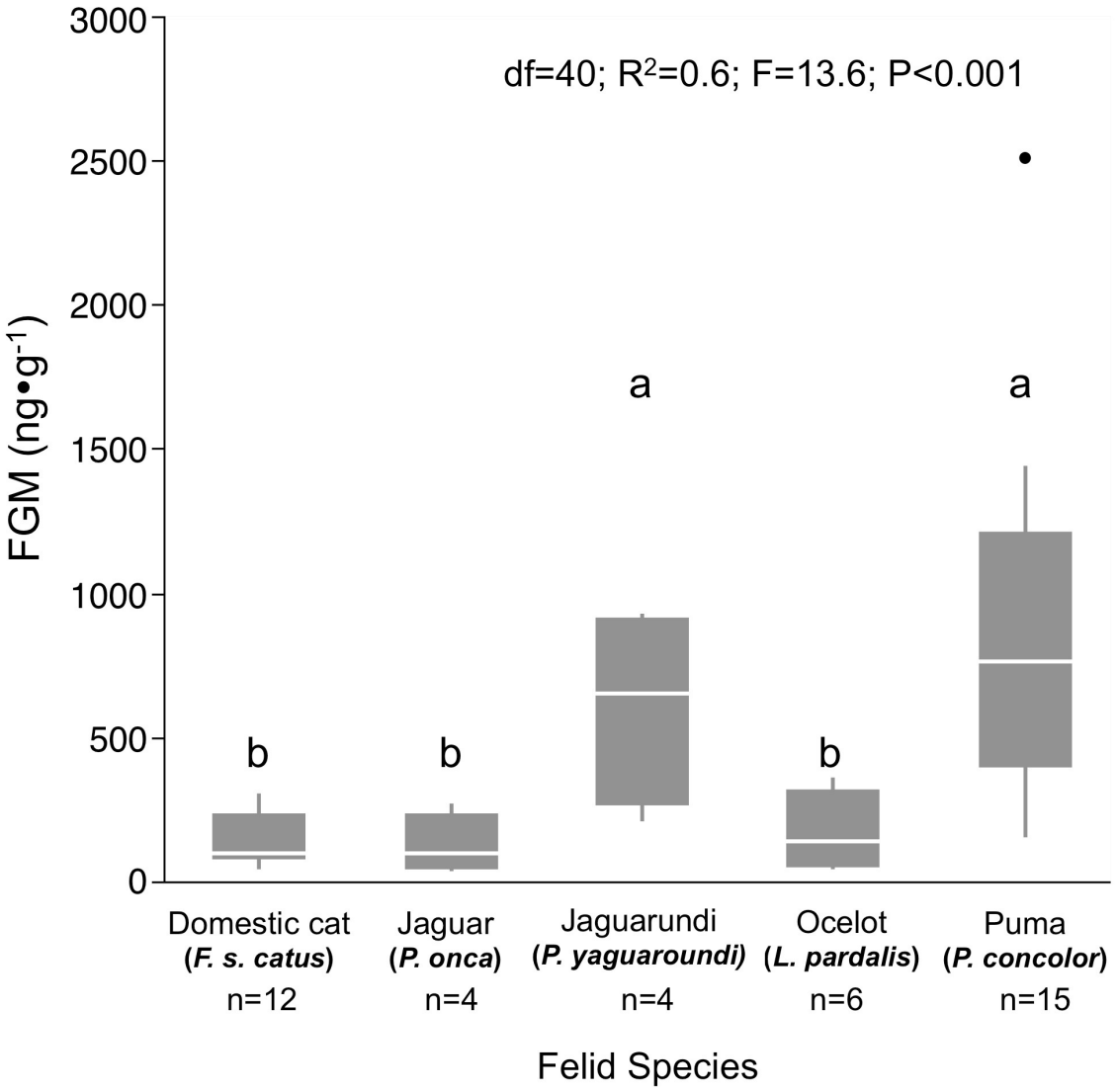

Figure 3.6. Fecal glucocorticoid metabolites (FGM) of five sympatric free-raging felids. Samples from every individual were averaged before statistical analysis; *n* corresponds to genetically unique individuals. Box plots designated with a different letter are significantly different.

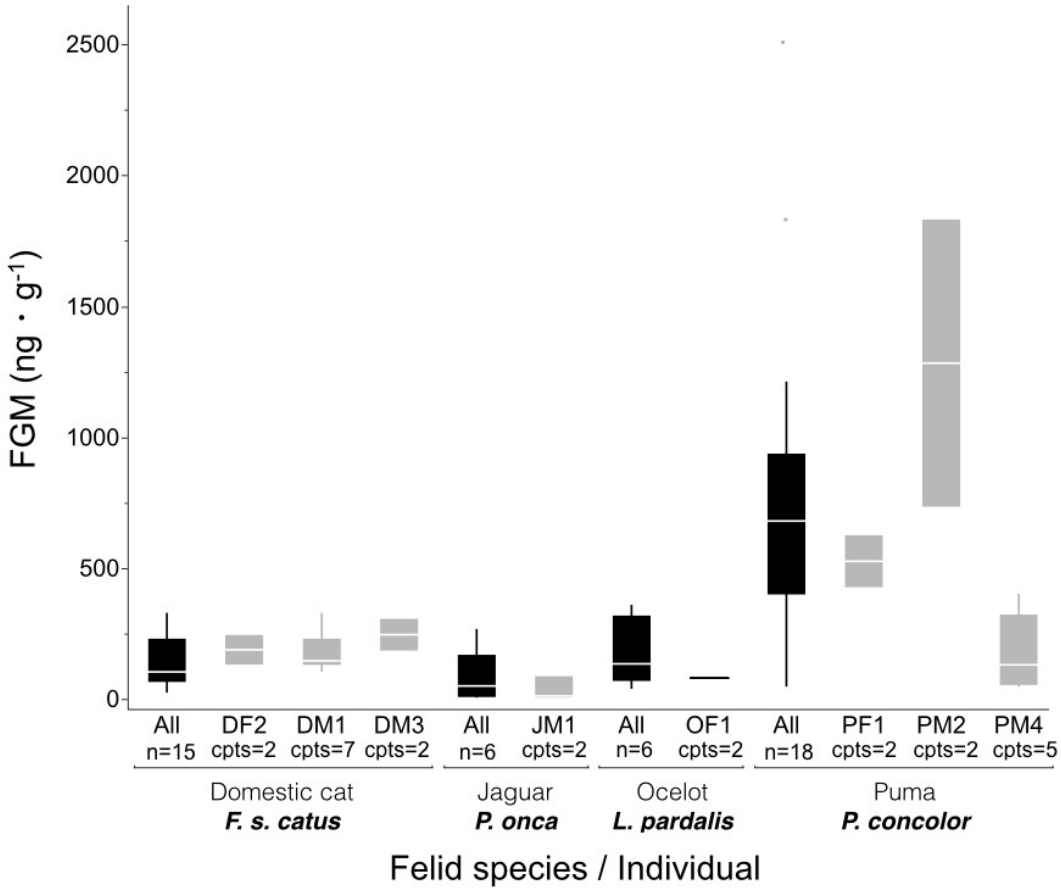

Figure 3.7. Fecal glucocorticoid metabolites (FGM) of individual felids with multiple genetic captures (e.g., multiple scat samples). Black box plots represent all individuals for each species; grey box plots represent values from each individual; n is the number of individuals; cpts is the number of captures, or scat samples from each individual. Individuals are referenced with three characters: species, sex, and unique number.

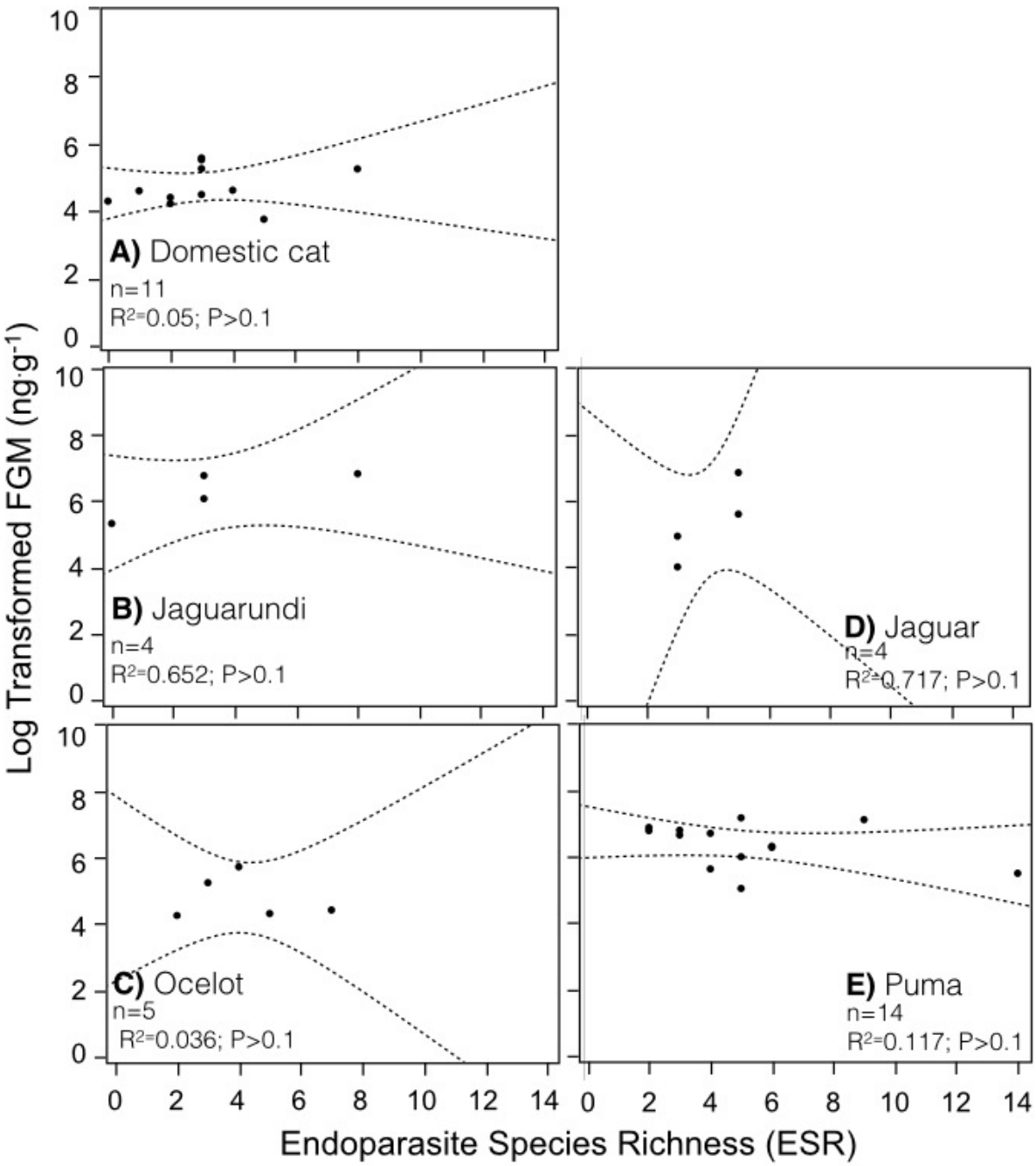

Figure 3.8. Linear regressions between endoparasite species richness (ESR) and FGM in five sympatric felid species. No linear relationship was observed in any felids. Black dots represent each sample; dotted lines represent a 95% confidence interval. FGM values were log transformed due to non-normality.

Table 3.1. Total number of Neotropical felid samples identified with *n*DNA and *mt*DNA and morphology. * Scat samples identified by scat detector dog and were compatible by morphology with a large felid, but had low quality DNA and did not amplify for molecular species assignment.

Species	Number of Identified Samples						
	*n*DNA (Number of individuals)					*mt*DNA	Morphology*
	Sex		Total	Recaptures		*Sex*	*Sex*
	Males	Females		Individual	Times	Unknown	Unknown
Domestic cat (*Felis s. catus*)	9	4	13	2 males 1 female	1 & 6 1	13	
Jaguarundi (*Puma yagouaroundi*)	2	3	5	-	-	0	
Ocelot (*Leopardus pardalis*)	2	3	5	1 female	1	4	
Puma (*Puma concolor*)	10	6	16	2 males 2 females	1 & 5 1 each	1	
Jaguar (*Panthera onca*)	6	1	7	1 male	2	0	
Large cat*							20

Table 3.2. Summary of nematode, trematode, and protozoan eggs, larvae, and oocysts identified in feces of five sympatric Neotropical felid species. Number of eggs was calculated per gram of feces and averaged if more than one sample per individual was analyzed. Prevalence was calculated as . Endoparasite species richness (ESR) is the total number of parasite species affecting a single individual. Dashes indicate that the parasite was not observed for that felid species.

Parasite type	Species	Domestic cat *Felis s. catus* (n=16)		Jaguarundi *Puma yagouaroundi* (n=5)		Ocelot *Leopardus pardalis* (n=5)		Puma *Puma concolor* (n=14)		Jaguar *Panthera onca* (n=5)	
	Parasite	Mean [eggs/g]	Prevalence (%)	Mean [eggs/g]	Prevalence (%)	Mean [eggs/g]	Prevalence (%)	Mean [eggs/g]	Prevalence (%)	Mean [eggs/g]	Prevalence (%)
Nematode											
	Aelurostrongylus sp.	-	-	7	20	-	-	-	-	-	-
	Ancylostoma sp.	-	-	2	20	-	-	4	7.1	1	20
	Aonchotheca sp.	-	-	-	-	-	-	-	-	11	20
	Capillaria sp.	2	18.8	-	-	1	60	1	21.4	1	20
	Physaloptera sp.	8	18.8	-	-	4	20	1	42.9	1	20
	Spirocerca sp.	6	6.3	3	40	-	-	1	14.3	115	80
	Strongylid	1	6.3	2	20	-	-	1	21.4	1	20
	Toxascaris sp.	1	25	2	20	1	20	1	14.3	3	40
	Toxocara sp.	1	6.3	9	20	5	20	16	71.4	-	-
	Trichuris sp.	-	-	-	-	1	20	-	-	-	-
	Uncinaria sp.	19	18.8	-	-	28	60	2	28.6	4	20
Protozoa											
	Eimeria sp.	1	12.5	-	-	-	-	-	-	-	-
	Giardia sp. (trophozoite)	36	6.3	-	-	-	-	-	-	-	-
	Isospora sp.	9	62.5	2	40	4	40	2	53.3	4	40
	Like - *Toxoplasma sp.*	1	12.5	1	20	1	60	1	21.4	-	-
Trematode											
	Alaria sp.	-	-	-	-	-	-	-	-	1	20
	Diphyllobothrium sp.	3	6.3	-	-	-	-	-	-	-	-
	Dipylidium sp.	1	18.8	-	-	-	-	1	6.7	-	-
	Nanophyetus sp.	-	-	1	20	-	-	-	-	-	-
	Opisthorchis sp.	3	25	1	20	1	40	1	21.4	-	-
	Paragonimus sp.	-	-	-	-	-	-	-	-	56	20
	Platynosomum sp.	15	50	86	60	102	80	26	57.1	352	40
	Spirometra sp.	184	62.5	20	20	150	60	287	100	35	40
	Taenidae	1	18.8	9	20	-	-	5	50	1	20
Unknown		-	-	-	-	9	20	-	-	-	-
Mean ESR (95% confidence interval)		4.4 (3.1-5.6)		5 (2-8)		4.3 (2.5-6.2)		5.8 (3.7-7.8)		4.4 (3.2-5.6)	
Total parasite species identified		18		13		12		15		14	

Table 3.3. Summary of prey species consumed by five sympatric Neotropical felid species, organized by kingdom, class, order and family. Bold numbers in brackets are the number of species observed as prey items in each class. Frequency of occurrence prey species (%) = and were calculated for each felid species. + Represents positive identification of remains in scat, but frequency of occurrence was complied in a broader classification group such as genus or family. * Represents scat samples that were compatible by morphology with a large felid and had positive identification by the scat detector dog, but had low quality DNA and did not amplify for molecular species assignment.

Prey Species						Frequency of occurrence prey species (%) by felid					
Kingdom	Class	Order	Suborder	Family	Species	Domestic cat *Felis s. catus* (n=33)	Jaguarundi *Puma yagouaroundi* (n=5)	Ocelot *Leopardus pardalis* (n=10)	Puma *Puma concolor* (n=27)	Jaguar *Panthera onca* (n=9)	*Large cat** (n=20)
Animalia	**Aves**					**[≥2]**	**[≥1]**	**[1]**	**[≥2]**	**[≥1]**	**[1]**
		Galliformes		Cracidae	*Crax rubra*				3.7		
		Galliformes		Unknown	-	3					
		Passeriformes		Unknown	-	3	60	10	3.7		
		Struthioniformes		Tinamidae	Unknown					11.1	5
		Unknown				3				11.1	
	Bivalvia					**[0]**	**[0]**	**[0]**	**[0]**	**[1]**	**[0]**
		Unionoida		Unknown	Freshwater mussel					11.1	
	Mammalia					**[13]**	**[1]**	**[≥7]**	**[≥8]**	**[≥5]**	**[≥11]**
		Artiodactyla		Cervidae	*Mazama americana*				+		
				Cervidae	Unknown				29.6		
		Carnivora		Canidae	*Urocyon cinereoargenteus*			10			
				Mephitidae	*Conepatus sp.*						5
				Procyonidae	*Nasua narica*					22.2	5
		Cingulata		Dasypodidae	*Dasypus novemcintus*				14.8	44.4	
		Didelphimorphia		Didelphidae	*Philander opossum*	3			3.7		5
		Insectivora		Soricidae	*Cryptotis sp.*	3					
		Lagomorpha		Leporidae	*Sylvilagus floridanus*	6.1		10			
		Perissodactyla		Tayassuidae	*Pecary tajacu*					+	5
				Tayassuidae	Unknown					44.4	
		Pilosa		Myrmecophagidae	*Cyclopes didactylus*	3		10	3.7		
					Tamandua mexicana				7.4		20
		Primates		Atelidae	*Ateles geoffroyi*				11.1		
					Unknown				+		5
		Rodentia									
				Cuniculidae	*Cuniculus paca*			10	14.8		10
				Cricetidae	*Nyctomys sumichrasti*	6.1		10		11.1	
					Oligoryzomys fulvencens	3					
					Oryzomys couesi	+					
					Oryzomys sp.	27.3					10
					Peromyscus mexicanus	+					
					Peromyscus sp.	9.1					
					Reithrodontomys sp.	6.1					
					Rhipidomys sp.						5
					Sigmodon hispidus	+	+	+			+
					Sigmodon toltecus	+		+			
					Sigmodon sp.	51.5	60	30			15
					Tyomlys nudicaudus	3		10			
				Dasyproctidae	*Dasyprocta punctata*				14.8	11.1	10
				Heteromyidae	*Heteromys desmarestianus*	3					
				Muridae	*Rattus rattus*	3					
		Unknown				6.1		10			
	Insecta					**[≥1]**	**[0]**	**[0]**	**[0]**	**[0]**	**[0]**
		Orthoptera		Gryllidae	Unknown	27.3					
	Osteichthyes					**[≥1]**	**[0]**	**[0]**	**[0]**	**[≥1]**	**[0]**
		Unknown				12.1				11.1	
	Reptilia					**[0]**	**[≥1]**	**[≥2]**	**[0]**	**[0]**	**[≥1]**
		Squamata	Lacertilia		Unknown		40	10			
		Squamata	Serpentes		Unknown			30			5
Plantae		Poales		Poaceae	Grass	12.1				22.2	5
		Fabales		Fabaceae	Red bean	3					