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ORIGINAL PAPER

Comparative patterns of adrenal activity in captive and wild Canada lynx (*Lynx canadensis*)

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Abstract Stress and animal well-being are often assessed using concentrations of glucocorticoids (GCs), a product of the hypothalamic–pituitary–adrenal axis. However, GC concentrations can also be modulated by predictable events, such as changes in season or life history stage. Understanding normative patterns of adrenal activity is critical for making valid conclusions about changes in GC concentrations. In this study, we validated an assay for monitoring fecal glucocorticoid metabolites (FGM) in Canada lynx. We then used this technique to assess patterns of adrenal activity in Canada lynx across several contexts. Our results show that captive lynx have higher FGM concentrations than wild lynx, which may be related to differences in stress levels, metabolic rate, diet, or body condition. We also found that FGM concentrations are correlated with reproductive status in females, but not in males. For males, seasonal increases in FGM expression coincide with the onset of the breeding season, whereas in females, FGM increase toward the end of the breeding season. This information provides a valuable foundation for making inferences about normative versus stress-induced changes in adrenal activity in Canada lynx.

Keywords Fecal metabolites · Glucocorticoids · Non-invasive · Seasonality

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Introduction

Stress physiology is gaining an increasing amount of attention in the literature. The development of fecal glucocorticoid metabolite (FGM) analysis, a method that facilitates non-invasive study design, has helped to fuel the growth of this topic. Glucocorticoids (GCs) are primarily secreted by the adrenal glands and are critical mediators of homeostasis (Sapolsky 2002). Because unexpected threats to homeostasis (stressors) stimulate the hypothalamic–pituitary–adrenal (HPA) axis (Palme 2005; Sapolsky 2002), GC concentrations are often equated with stress. However, GC expression can also be modulated in response to events that are generally not considered stressful, such as predictable changes in homeostatic demand (e.g., circadian rhythm and life history stage; Boonstra 2004; Romero et al. 2009). GCs play a critical role in many physiological processes, such as energy regulation, immune function, and development. Furthermore, although the term "stress" typically has a negative connotation, occasional acute stress responses are adaptive (Boonstra 2004; Möstl and Palme 2002; Romero 2004). The distinction between beneficial and maladaptive stress responses is unclear, in part, because individual patterns

and consequences of GC expression are highly variable (Möstl and Palme 2002; Wielebnowski 2003).

Because so many factors can impact adrenal activity, it is important to establish normative patterns of GC expression before making inferences about the "stress" associated with changes in GC expression. Although the dynamics of stress physiology have been well studied in some species, we only have a rudimentary understanding of the variability in adrenal activity across species, environmental contexts, life-history stages, or among individuals (Boonstra 2004; Palme et al. 2005; Romero 2004; Wielebnowski and Watters 2007). Circulating GC concentrations have been shown to vary with sex, age class, time of day, season, and reproductive status (Romero 2002). Furthermore, several of these factors may also affect metabolism and/or excretion of GCs (Millspaugh and Washburn 2004; Sheriff et al. 2011; Touma and Palme 2005; Wielebnowski and Watters 2007). Therefore, extra care must be taken when interpreting results from studies using FGM analysis and making inferences about "stress" levels. As much as possible, studies should control for normative changes in GC expression or metabolism.

The aim of this study was to establish a basic understanding of adrenal activity in Canada lynx (*Lynx canadensis*) using the non-invasive technique of FGM analysis. We incorporated data from both captive and wild Canada lynx to develop a more comprehensive understanding of normative patterns of GC expression for this species in a variety of contexts. Specifically, our goals were to (1) validate a GC assay for monitoring adrenal activity in Canada lynx via feces, (2) examine the effect of sex, age, and reproductive status (intact, neutered, pregnant) on FGM values, (3) monitor seasonal changes in FGM concentrations, and (4) compare FGM concentrations and patterns of expression between captive and wild Canada lynx populations. Establishing basic information about adrenal activity in Canada lynx provides a foundation for future studies on stress physiology in this species, and may help enhance *ex situ* and in situ management plans.

Methods

Study animals and fecal sample collection

Captive population

This study included 39 captive adult Canada lynx (21 males and 18 females) from 20 institutions. The mean age for the animals sampled was 8.6 years (range 3–18 years). Four males were castrated and seven females were spayed. In addition, five females became pregnant/pseudo-pregnant during the study (see Fanson et al. 2010b for details). All lynx were housed outdoors more than 50% of the time, and thus were exposed to natural photoperiod rhythms. Animal care staff collected fecal samples 2–4 times per week during routine cage cleanings. Duration of sample collection for individual lynx ranged from 2–12 months.

Wild population

Fecal samples were also collected from wild Canada lynx in Colorado (43 males, 56 females), Maine (8 males, 8 females), and Montana (10 males, 10 females). The populations in Maine and Montana are naturally occurring, while the population in Colorado was reintroduced. The mean age was 5.1 years (range 1–12 years). Since all samples came from radio-collared lynx, age was estimated when the radio-collar was put on the lynx. In some cases, the lynx was trapped as a kitten so the year of birth was known, and in other cases, a more precise age estimate was obtained from tooth enamel (collected post-mortem).

All samples were collected by snow-tracking radio-collared lynx between December and April, a time period which includes their breeding season. Samples were collected from 1999 to 2008. Samples were generally collected within 24 h after defecation, and evidence from field experiments suggests that FGM remain stable in winter field conditions for at least 3 days (Fanson 2009). Therefore, we are confident that we were able to obtain meaningful results from samples collected in the field. To ensure that FGM expression in reintroduced lynx was not affected by the translocation, we only included samples that had been collected more than 6 months after a lynx was released.

ACTH challenge

To physiologically validate an enzyme-immunoassay (EIA) and ensure that it detected biologically relevant changes in immunoreactive FGM, an adrenocorticotropic hormone (ACTH) challenge was conducted on five captive lynx (2 males and 3 females). ACTH challenges are a common method of assessing adrenal responsiveness, thereby allowing for validation of GC assays (Palme 2005; Sheriff et al. 2011). They can also be used to determine hormone excretion lag time.

ACTH gel (Corticotropin, Wedgewood Pharmacy, Swedesboro, NJ) was administered as a single intra-muscular injection (20 IU/kg). The two males received the injection while restrained in a squeeze cage, and the three females received the injection while anaesthetized for routine physical examinations. Daily fecal samples were collected for 6 days prior to and 12 days after the injection. For two females, we also collected serial blood samples every 10 min for 2 h following the injection. This allowed

us to ensure that the ACTH injection did indeed elicit an adrenal response. Protocols were approved by the Institutional Animal Care and Use Committee of Purdue University (PACUC: 04-025).

Steroid extraction

To extract steroid metabolites, 5 ml of 80% ethanol was added to 0.5 g of well mixed, wet fecal material in polypropylene tubes. Capped tubes were placed on a rotator overnight and then centrifuged for 15 min at 1,500 rpm. One ml of supernatant was transferred to a new polypropylene tube and diluted with 1 ml assay buffer. Extracts were stored at −20°C.

Assay selection

Circulating GCs are heavily metabolized prior to excretion (Palme et al. 2005; Sheriff et al. 2011). Therefore, the quantification of FGM via EIA relies on the ability of the antibody to cross-react with at least some metabolites. Depending on the ligand and binding site against which the antibody was raised, different antibodies may cross-react with different FGM.

To assess the ability of different EIAs to detect changes in adrenal activity in Canada lynx, we analyzed the samples from the ACTH challenges using five different antibodies. Each specific EIA is identified using the antibody ligand and the initials of the manufacturer [e.g., corticosterone (CJM)]. Two antibodies were raised against corticosterone. One of these was a commercially available kit produced by Assay Designs (AD; Ann Arbor, MI; see below for more details), and the other antibody (Cs6) was provided by CJ Munro (CJM; University of California, Davis, CA; previously described by Narayan et al. 2010). One antibody was raised against cortisol (R4866) and was also provided by CJM (previously described by Young et al. 2004). The remaining two antibodies were developed for specific FGM, and were provided by E Möstl (EM; University of Veterinary Medicine, Vienna, Austria). The two FGM were 5β-androstane-3α, 11β-diol-17-one (hereafter androstanedione; previously described by Frigerio et al. 2004) and 11-oxoetiocholanolone (previously described by Möstl et al. 2002).

All five antibodies were able to detect quantifiable amounts of fecal steroid metabolites, although there was quite a bit of variation in FGM concentrations among assays (Table 1; Fig. 1). For males, all of the assays except the corticosterone (CJM) assay detected a clear response to the ACTH-induced increase in adrenal activity. The androstanedione (EM) and oxoetiocholanolone (EM) assays detected the highest response. Conversely, in females, very few of the assays reliably detected post-ACTH peaks. The corticosterone (AD) assay was the only assay that consistently identified peaks for all three females, but the cortisol (CJM) assay detected a higher response for the two females in which it detected peaks. However, with the cortisol (CJM) assay, baseline values were near or below the detection limit, thereby precluding the reliable quantification of FGM concentrations at baseline levels. The corticosterone (CJM) assay showed the weakest response for both sexes, and was the least reliable for detecting peaks.

Both the corticosterone (AD) and cortisol (CJM) assays seem potentially promising for monitoring adrenal activity in both males and female Canada lynx. However, we chose

Table 1 Comparison of five GC assays for monitoring ACTH-induced increases in adrenal activity

Antibody ligand/manufacturer	Female		Male	
	N	Increase ± SEM	*N*	Increase ± SEM
Corticosterone				
Assay Designs	**3**	3.1 ± 0.6	**2**	7.0 ± 3.3
Corticosterone				
CJ Munro	1	2.5	1	2.5
Cortisol				
CJ Munro	2	7.5 ± 3.5	**2**	5.3 ± 0.2
5β-androstane-3α, 11β-diol-17-one				
E Möstl	1	*	**2**	11.8 ± 0.9
11-oxo-etiocholanolone				
E Möstl	1	*	**2**	10.7 ± 5.2

"*N*" represents the number of lynx in which peaks were detected (out of 3 females and 2 males). Bold type indicates assays for which peaks were detected in all lynx. "Increase" is the fold-increase between an individual's baseline and maximum peak occurring within 4 days of the ACTH injection. Asterisk indicates unreliable baseline estimates due to samples falling below the detection limit. In these cases, peaks were assessed visually

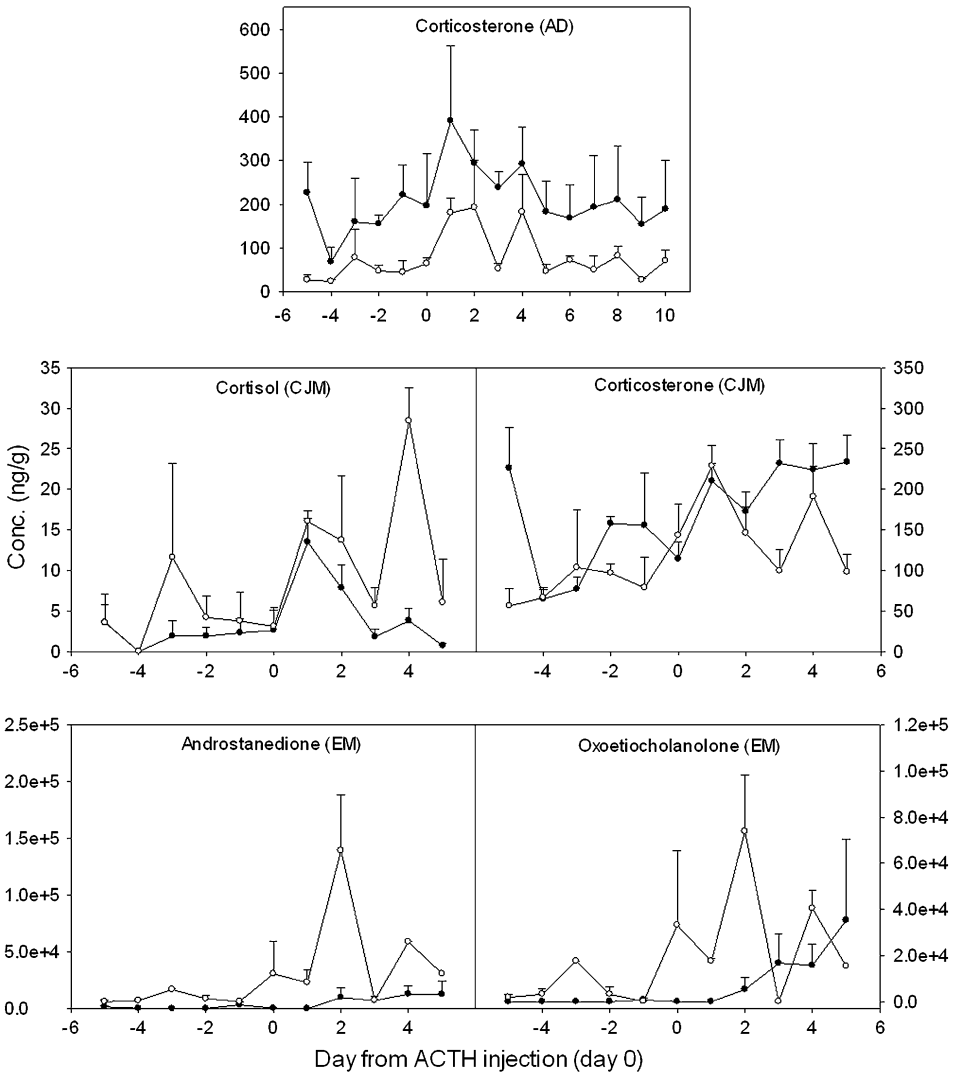

Fig. 1 Comparison of five GC assays for monitoring ACTH-induced increases in adrenal activity (mean + SE). Note that because profiles are averaged, some of the distinct individual peaks become muted

the corticosterone (AD) assay over the cortisol (CJM) assay for the following reasons: (1) it most consistently identified post-ACTH peaks, and (2) it detected quantifiable amounts of FGM, even at baseline levels. Therefore, the corticosterone (AD) assay was used for all subsequent assays and results presented in this paper.

Enzyme-immunoassay

Based on the above results, all samples for this study were analyzed using the Assay Designs Corticosterone EIA (Ann Arbor, MI). Briefly, 100 µl of standard, control, or diluted fecal extract were added to each well, immediately followed by 50 µl of conjugate and antibody. Plates were incubated for 2 h while shaking, then washed three times to remove unbound steroids. Two-hundred microliters of substrate solution were added to each well, and the reaction was stopped after 1 h. Plates were read with a single filter at 405 nm using an optical density plate reader (Dynex MRX Revelation, Dynex Technologies, Chantilly, VA). The antibody cross-reacts with corticosterone (100%), deoxycorticosterone (28.6%), and progesterone (1.7%). All other cross-reactivities were less than 1% (see manufacturer's specifications). All samples were assayed in duplicate. Assay sensitivity was 27 pg/well.

To ensure that the extract medium did not interfere with functioning of the assay, we conducted two biochemical validations: (1) parallelism between serially diluted extracts and the standard curve, and (2) significant (>80%) recovery of exogenous corticosterone added to fecal extracts. To monitor precision and reproducibility, low ($\sim$70% binding) and high ($\sim$30% binding) quality control samples were run on each plate. Intra-assay coefficients of variation (CVs) were 15.5% and 11.4% ($n = 19$) for low and high controls, respectively. The inter-assay CVs were <15%. Data are expressed as ng/g wet fecal weight.

Statistical analysis

All data were analyzed using SAS 9.1 (Cary, NC). Data were log-transformed to meet assumptions of normality and homoscedasticity.

ACTH challenge

To determine whether the assays detected biologically relevant changes in adrenal activity, we tested for the presence of FGM peaks following the ACTH challenge. For each individual, we calculated baseline FGM values through an iterative process excluding all points greater than the mean + 2 SD (Wielebnowski et al. 2002). Peaks were defined as points exceeding the baseline + 2 SD. The magnitude of the post-ACTH increase was also determined by calculating the fold-increase between an individual's baseline and maximum peak occurring within 4 days of the ACTH injection.

For the Assay Designs results, we also ran a repeated-measures ANOVA to test whether FGM concentrations changed significantly following the ACTH injection. A Dunnett's multiple comparison test was used for pairwise comparisons between the "control" (in this case, the average FGM concentration for 7 days preceding the ACTH challenge) and each subsequent day following the ACTH challenge. The model was controlled for individual and sex.

Biological effects and population comparison

An ANCOVA was used to determine the effect of sex, age, and reproductive status (intact, neutered, pregnant, or pseudo-pregnant) on mean individual FGM concentration. The model was also controlled for the effect of population. The four populations (captive, CO, ME, and MT) were considered separately in the model, but pairwise comparisons indicated that the three wild populations were not significantly different from each other. A post hoc linear contrast was used to explicitly test for differences between captive and wild populations. Two-way interactions were excluded if they were not significant. A Tukey–Kramer adjustment was used to correct for multiple pairwise comparisons.

Seasonal effects

A repeated-measures ANOVA was used to test the effect of month on FGM concentration. Only intact, non-pregnant lynx were included in the model, and captive and wild populations were analyzed separately because sampling occurred across different time intervals. The model was also controlled for the effect of sex. Two-way interactions were excluded if they were not significant. Peak FGM values (defined for this part of the study as greater than baseline + 3 SD) were excluded from individual monthly mean calculations. The reason we used different criteria for defining baseline and peak values is because the number of samples and variance in FGM differed between the ACTH challenge and the circ-annual sampling. By nature, the ACTH challenge is associated with large variance in adrenal activity over a short period of time. Consequently, standard deviations are larger and peaks are less likely to be identified, which is why we used a lower criteria for the ACTH challenge.

Results

ACTH challenge

The Assay Designs Corticosterone EIA successfully detected an increase in FGM concentration for both males and females following the ACTH challenge. The ACTH injection elicited a three to tenfold increase in FGM concentrations ($n = 5$) and a four to sevenfold increase in serum GC concentrations ($n = 2$; Fig. 2). FGM concentrations were significantly higher on the first 2 days following the ACTH injection (day 1: $t_{97.1} = 3.65$, $P = 0.004$; day 2: $t_{97.3} = 4.21$, $P < 0.001$), but not on any subsequent days. This indicates an excretion lag time of 24–48 h. Interestingly, for four of the five lynx, there appeared to be a second peak in FGM concentrations ~4 days after the ACTH injection (Figs. 1, 2).

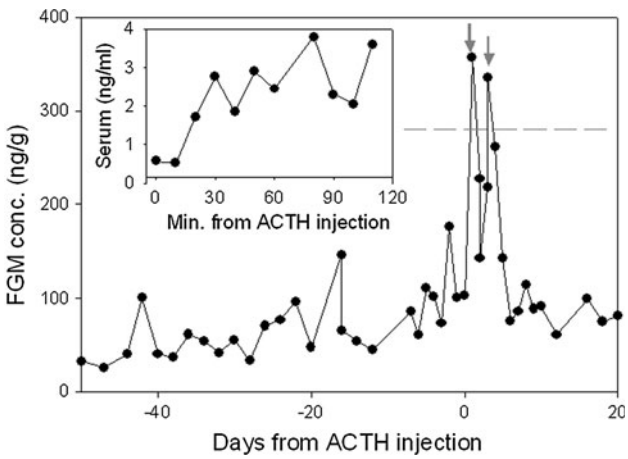

Fig. 2 Serum corticosterone and FGM profiles in response to an ACTH challenge for one representative lynx. Reference line indicates baseline + 2 SD. *Arrows* indicate the two separate post-ACTH peaks detected in FGM

Table 2 Effect of biological factors (age, sex, reproductive status) and population (captive, CO, ME, MT) on mean FGM concentrations

Effect	*df*	*F*	*P*
Population	3,163	7.12	<0.001
Age	1,163	2.13	0.15
Sex	1,163	4.75	0.03
Status	3,163	14.88	<0.001
Status by sex	1,163	4.40	0.04

Biological effects and population comparison

Population had a significant effect on FGM concentrations (Table 2). None of the wild populations were significantly different from each other (all $P > 0.5$). However, captive lynx showed significantly higher FGM concentrations than wild lynx ($t_{167} = 3.42$, $P < 0.001$).

For both captive and wild populations, biological factors influenced FGM concentrations (Table 2; Fig. 3). The effect of reproductive status on FGM concentrations depended on sex. For males, FGM concentrations did not differ significantly between neutered and intact individuals (all $P > 0.5$). Conversely, for females, FGM concentrations varied significantly with reproductive status. Among captive lynx, pairwise comparisons indicated that intact females had significantly higher FGM concentrations than males and spayed females (all $P < 0.05$). Pregnant and pseudo-pregnant females had significantly higher FGM concentrations than all other groups (all $P < 0.05$), but were not significantly different from each other ($t_{166} = -0.52$, $P = 0.99$).

Although there was a trend toward increasing mean FGM concentration with age [$\beta = 0.03 \pm 0.02$ ln (ng/g)/year] the result was not significant (Table 2). There was not a significant interaction between age and sex or age and population.

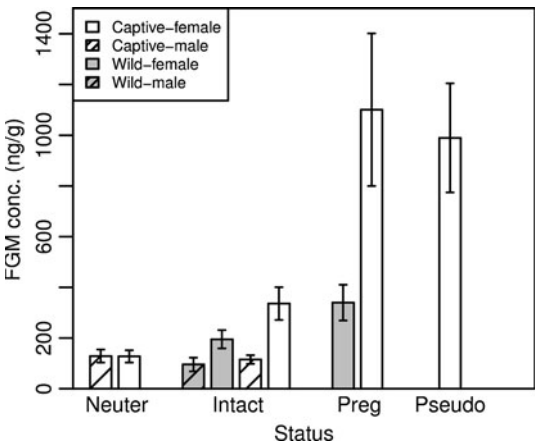

Fig. 3 Effect of sex and reproductive status on FGM concentrations in captive lynx (mean ± SE)

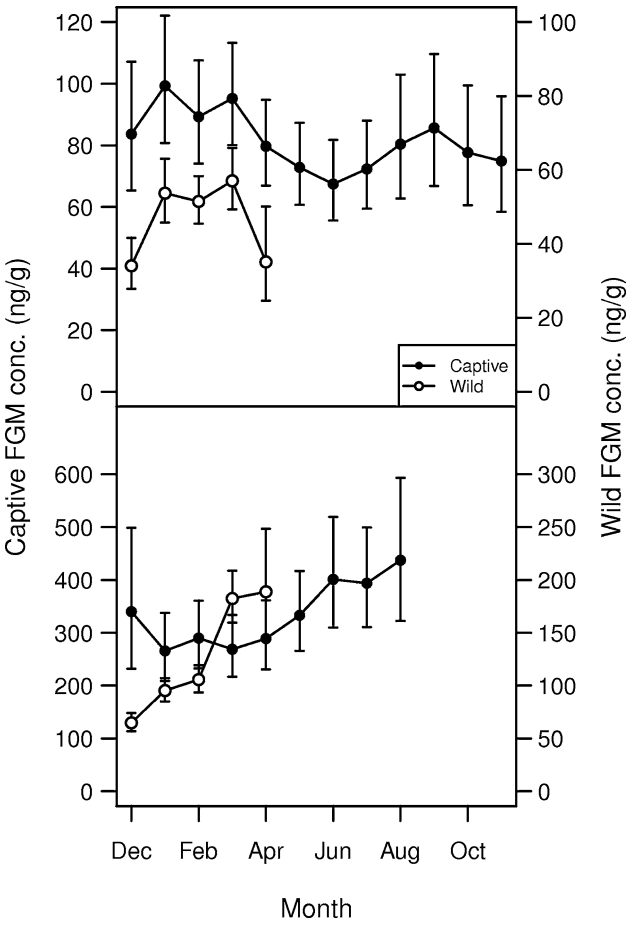

Fig. 4 Seasonal patterns of FGM concentrations for males (**a**) and females (**b**). Data represent back-transformed LS mean ± SE. Data are only plotted for months in which we have data from more than one individual. Note the different scales for captive and wild lynx

Seasonal effects

For both captive and wild populations, patterns of monthly FGM expression differed between the sexes (captive: $F_{11,101} = 2.11$, $P = 0.03$; wild: $F_{4,327} = 2.69$, $P = 0.03$; Fig. 4). For males, captive and wild populations were remarkably similar, both showing an increase in FGM concentrations during the breeding season. For females, the seasonal changes differed between captive and wild populations. Wild females exhibited a pronounced increase in FGM concentrations in early spring (between February and March). In captive females, the increase in FGM was more gradual and occurred later in the year (between April and June).

Discussion

The HPA axis plays a critical role in many physiological processes, including the stress response. Because of this

central role, understanding the normative patterns of adrenal activity can provide valuable insights into basic and comparative physiology. In this study, we describe patterns of adrenal activity for Canada lynx across a variety of contexts.

Circulating GCs can be modified by the liver and by gut bacteria prior to excretion (Palme et al. 2005; Sheriff et al. 2011). Steroid metabolism can vary significantly between species, between individuals, and there is emerging evidence that it can vary within an individual over time (Dehnhard et al. 2010; Palme et al. 2005; Wielebnowski and Watters 2007). Therefore, it is exceedingly important to properly validate an assay and demonstrate that the antibody reacts with FGM that reflect biologically relevant changes in adrenal activity. Following the injection of ACTH to stimulate adrenal activity, we observed detectable peaks (three to tenfold increase) in all five lynx tested. This indicates that the immunoreactive FGM reflect biologically relevant changes in adrenal activity. The ACTH challenge also revealed that FGM excretion lag-time is 1–2 days for Canada lynx. In addition, data collected from lynx that were transferred between locations, moved to new exhibits, or trapped from the wild further indicate that FGM reflect biologically relevant changes in adrenal activity (Fanson 2009).

A unique aspect of this study is our ability to compare FGM concentrations between wild and captive Canada lynx populations. Captive lynx had significantly higher (~2-fold) FGM concentrations than wild lynx. We observed the same trend with fecal reproductive hormone metabolites—captive lynx had higher concentrations than wild lynx (Fanson et al. 2010a, b). However, the magnitude of the population difference was much smaller for FGM than for reproductive steroid metabolites.

Few studies have examined the differences in FGM concentrations between captive and wild populations. Several studies report similar findings, with captive populations having higher FGM levels than wild populations [cheetahs (Terio et al. 2004); starlings (Cyr and Romero 2008); spider monkeys (Rangel-Negrin et al. 2009); but see Stead-Richardson et al. 2010 and Woodruff et al. 2010]. This result is generally attributed to higher levels of stress in captivity. Stress-related pathologies are not widely reported in lynx. However, it is possible that subtle increases in the activity of the HPA axis may cause subpathological changes in physiology. Moderately elevated levels of adrenal activity can impact the hypothalamic–pituitary–gonadal (HPG) axis in many ways and may contribute to reproductive dysfunction (Chrousos et al. 1998; Yildiz and Azziz 2007). Reproductive success in captive Canada lynx is currently quite low (Fanson et al. 2010b), which might suggest that even though clinical indicators of stress are not widely recognized in Canada lynx, they may experience increased levels of stress in captivity.

An alternative, but not mutually exclusive explanation, is that there is an underlying difference in metabolism, diet, or energy regulation between captive and wild lynx that affects steroid production and/or metabolism. These factors are known to impact steroid production and excretion in other species (Goymann 2005; Hajamor et al. 2003; von der Ohe and Servheen 2002). Lynx in captivity generally have more regular access to food, lower energy expenditure, and greater mass than wild lynx (11–20 kg for captive lynx vs. 7–11 kg for wild lynx). GCs are closely linked to energy regulation (Romero et al. 2009), so that major energetic differences between captive and wild lynx could account for differences in FGM concentrations. Indeed, humans diagnosed with the metabolic syndrome (which includes abdominal obesity) exhibit elevated HPA activity (Anagnostis et al. 2009). Essentially, there is a complex network of interactions between GCs, sex steroids, and metabolic hormones (e.g., leptin and insulin; Lordelo et al. 2007). Perturbations to this network may have cascading impacts on other physiological systems. In the face of a growing number of studies that make FGM comparisons between populations, sometimes resulting in specific management actions, it is critical that we develop a better understanding of the variety of factors contributing to observed population differences and underlying causes.

There was a significant interaction between sex and reproductive status on FGM concentration in Canada lynx. In females, FGM concentrations were lowest in spayed females and highest in pregnant or pseudo-pregnant females. In contrast, there was no effect of reproductive status on FGM concentrations in males. Interestingly, FGM concentrations in males were similar to spayed females. This suggests that the measured variation in FGM may to some degree be correlated with ovarian/luteal activity.

Adrenal activity has been shown to vary with female reproductive status in a variety of species. In many species, pregnant females have significantly higher levels of GCs than non-pregnant females [e.g., baboons (Weingrill et al. 2004); cotton-top tamarins (Ziegler et al. 1995); ring-tailed lemurs (Cavigelli 1999); red squirrels (Dantzer et al. 2010)]. Some studies have also reported more subtle changes in GC titers throughout the estrus cycle (Atkinson and Waddell 1997; Cavigelli et al. 2005). Furthermore, anovulatory (Ziegler et al. 1995) and ovariectomized (Seale et al. 2004) females have lower levels of adrenal activity (as measured via circulating GCs) than normally cycling and intact females, respectively. There is a complex interaction between the HPA and HPG axes, and GCs play a critical role in proper functioning of the female reproductive system (Brann and Mahesh 1991; Chrousos

et al. 1998). Given the involvement of GCs in energy regulation (Romero et al. 2009), the link between ovarian and adrenal activity is not surprising. Energetic demands vary across reproductive states, and modulation of the HPA axis may help the body prepare for and cope with those changing demands.

Males and females showed different patterns of seasonality in FGM concentrations. In males, FGM values peaked during the breeding season, then decreased to a nadir during summer. Captive and wild males show remarkably similar patterns of FGM expression. Conversely, FGM concentrations in females were lowest in winter/early spring, and increased toward the end of the breeding season. In wild females, this increase is quite pronounced, whereas in captive females it occurs later and is more gradual.

Seasonal changes in patterns of GC expression have been reported for a number of other species, and are often correlated with changes in food availability/energy regulation or reproduction (Boonstra 2004; Romero 2002; St. Aubin et al. 1996; Touma and Palme 2005). Many species exhibit an increase in GC concentrations during the breeding season, which would coincide with the pattern we see in male lynx. Females show a different trend, with the increase in FGM occurring just after the breeding season. Interestingly, female lynx show similar patterns of expression in both FGM and fecal progestagen metabolites (Fanson et al. 2010b), further indicating a possible link between adrenal and ovarian activity in Canada lynx.

To summarize, we demonstrated that FGM provide a valid measure of adrenal activity in Canada lynx. We then used this technique to describe normative patterns of adrenal activity across a variety of contexts. Captive lynx had significantly higher FGM concentrations than wild lynx, which may be related to differences in adrenal activity, metabolic rate, diet, and/or body condition. For both captive and wild lynx, FGM concentrations were affected by sex and reproductive status. This emphasizes the importance of controlling for such biological factors in studies claiming to investigate "stress" using FGM analysis. Both sexes exhibited seasonal changes in FGM concentration, although the patterns were different for males and females. The non-invasive nature of fecal hormone metabolite analysis makes this a very useful tool for understanding the dynamics of adrenal activity. However, the possible factors contributing to observed FGM variation have to be analyzed and considered carefully. Establishing normative patterns of adrenal activity for a species is important not only for improving management strategies for that species, but also for developing a more comprehensive understanding of basic stress physiology and factors influencing FGM variability.

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