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For the degree of Doctor of Philosophy

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For the degree of Doctor of Philosophy

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STRESS AND REPRODUCTIVE PHYSIOLOGY IN CANADA LYNX (*LYNX CANADENSIS*): IMPLICATIONS FOR *IN-SITU* AND *EX-SITU* CONSERVATION

A Dissertation

Submitted to the Faculty

of

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LIST OF ABBREVIATIONS

ACTH	adrenocorticotropic hormone
CL	corpus luteum
EIA	enzyme immunoassay
fA	fecal androgen metabolites
fE	fecal estrogen metabolites
fGC	fecal glucocorticoid metabolites
fP	fecal progestogen metabolites
GC	glucocorticoid
HPA	hypothalamic-pituitary-adrenal axis
HPLC	high performance liquid chromatography

ABSTRACT

Fanson, Kerry V. Petterson. Ph.D., Purdue University, December 2009. Stress and Reproductive Physiology in Canada Lynx (*Lynx canadensis*): Implications for *In-situ* and *Ex-situ* Conservation. Major Professors: Jeffrey R. Lucas and Nadja C. Wielebnowski.

Species reintroductions are a valuable conservation tool, but such efforts often fail, and we have a poor understanding of why. Understanding the role of stress physiology in reintroductions may be critical for enhancing their success rates. The goal of my dissertation was to validate a technique for monitoring stress and reproductive physiology in Canada lynx, and apply this technique to a reintroduced lynx population. The non-invasive technique of fecal hormone analysis was used to accomplish four objectives: 1) investigate the effect of environmental and methodological factors on the quantification of fecal steroid metabolite concentrations, 2) establish normative patterns of male and female reproductive physiology in captive and wild lynx populations, 3) describe patterns of stress physiology in captive and wild lynx populations, and 4) examine changes in stress physiology of lynx throughout the reintroduction process, and investigate patterns of individual variation in stress responses.

Validation experiments indicated that fecal steroid metabolites remain relatively stable in winter field conditions, even when exposed to repeated freezing and thawing. Thus biologically meaningful results can be obtained from fecal samples collected in the field. Canada lynx are highly seasonal breeders, and I found that both males and females exhibit seasonal increases in reproductive hormones (estrogens and androgens, respectively), which coincide with the breeding season. Progestogens, which are typically useful for monitoring pregnancy, were not useful indicators of pregnancy in Canada lynx. Nonetheless, they may accurately reflect luteal activity, as corpora lutea in *Lynx* species may persist for some time, although the dynamics of luteal activity are still poorly understood. Canada lynx are solitary in the wild, and group-housing in captivity appears to alter both reproductive and stress physiology. Population comparisons revealed that captive lynx exhibit higher concentrations of all four steroid hormones than wild populations, which may be linked to differences in energy regulation or metabolism. Finally, in

reintroduced lynx, the magnitude of a lynx' stress response is a significant predictor of post-release survival; lynx with larger stress responses have shorter post-release survival.

Thus, fecal hormone analysis provides a valuable tool for monitoring the reproductive and stress physiology of Canada lynx. Furthermore, information gained using this technique can be used to guide the development of stronger management plans for captive and wild populations, thereby improving animal well-being and conservation.

CHAPTER 1. ENVIRONMENTAL AND METHODOLOGICAL FACTORS AFFECTING MEASURED FECAL HORMONE METABOLITE CONCENTRATIONS FOR CANADA LYNX

Introduction

The technique of fecal hormone analysis has revolutionized the fields of behavioral endocrinology, physiological ecology, animal welfare, and conservation biology because it allows researchers to address questions that could not be studied previously (Wasser et al. 2000, Brown et al. 2001, Schwarzenberger 2007). The non-invasive nature of this technique provides several advantages over traditional methods of studying animal physiology. First, it does not require any handling or manipulation of the subject. Therefore, we can obtain physiological data for animals that are hard to capture, sensitive to stress, or protected by conservation status. This has opened the door for investigations into the reproductive and stress physiology of species we previously knew very little about [e.g., cheetahs (Brown et al. 1996, Wielebnowski and Brown 1998, Terio et al. 2004), right whales (Rolland et al. 2005, Hunt et al. 2006), and spotted owls (Tempel and Gutierrez 2004, Washburn et al. 2004, Wasser and Hunt 2004)]. Furthermore, samples can be collected more frequently without altering hormone expression or negatively impacting the study subject (e.g., handling stress, hypovolemia). This has allowed investigators to monitor longitudinal patterns of hormone expression much more extensively than with traditional techniques. Finally, fecal hormone analysis provides a "pooled" endocrine value rather than a "snapshot" value provided by serum analysis; consequently fecal hormone concentrations can provide a superior measure of long-term physiological condition than serum hormone analyses (Palme et al. 2005, Wielebnowski and Watters 2007).

However, fecal hormone analysis is a rather "noisy" technique. There are numerous factors acting on hormones between the time they are secreted and the time they are analyzed (e.g., hepatic metabolism, bacterial metabolism, gut passage time, route of excretion, sample condition and characteristics, storage conditions, and extraction technique; see Millspaugh and Washburn 2004, Touma and Palme 2005, Wielebnowski and Watters 2007). We know that all of these factors have the potential to influence the measurement of fecal hormone metabolites, but at

this time, we do not fully understand the magnitude or directionality of the effect of each of these factors, nor the implications for obtaining biologically meaningful results. Our lack of understanding is due in part to the fact that this technique is still relatively young, but also because some of the experiments required to understand these factors are expensive, time consuming, and sometimes prohibitive in nature (especially in a species with conservation concerns). Some of these factors can be minimized or controlled for by scientists; others simply need to be taken into consideration when interpreting the results. Fortunately, much research has already been conducted on a variety of these factors in more tractable species, such as farm and domestic animals, and some inferences can cautiously be made across species (Brown et al. 1994, Bahr et al. 2000, Palme et al. 2005). However, there is considerable variation between species, between individuals, and even within an individual with regards to the effect of internal (biological) and external (environmental/ methodological) factors on measured hormone concentration (Millspaugh and Washburn 2004, Touma and Palme 2005, Wielebnowski and Watters 2007, Dehnhard et al. *in review*). Some internal factors, such as metabolic variation, cannot be controlled for at this point and can only be taken into account by using inference, ensuring proper biological hormone assay validation, and practicing careful interpretation of ensuing findings. However, the impact of several external factors, such as sample collection and storage, can be readily assessed prior to the start of a field project.

Canada lynx (*Lynx canadensis*), a North American felid, contribute significantly to ecosystem dynamics in the boreal forest (Ruggiero et al. 2000, Poole 2003). Lynx have recently been listed as a threatened species in the continental US (USFWS 2000). Fecal hormone analysis could be applied to free-ranging lynx to provide valuable physiological insights that may aid conservation efforts. However, it is critical to understand how the field conditions under which samples are collected might affect the quantification of steroid metabolites. The goal of this study was to investigate the effect of several external factors on measured steroid metabolite concentrations in Canada lynx. Specifically, we examined 1) the stability of fecal steroid metabolites in winter field conditions, 2) the effect of repeated freezing and thawing on measured hormone concentration, 3) the effect of several fecal characteristics (total sample weight, water content, and non-fecal material) on measured hormone concentration, and 4) the correlation between wet-weight and dry-weight extraction methods. Since classes of hormone metabolites can be affected differently by external factors, this study examined both reproductive hormones [fecal metabolites of estrogens (fE), progestogens (fP), and androgens (fA)] and "stress" or adrenal hormones (fecal glucocorticoid metabolites, fGC).

Methods

Winter Field Stability Experiment

Trackers can snow-track individual lynx during the winter, but samples are generally at least 12 h old before they are collected. To assess the stability of fecal hormone metabolites in winter field conditions, fecal samples were collected from wild Canada lynx that were temporarily housed in holding pens in southwestern Colorado. One sample per individual was collected from 5 males and 7 females in March 2005. Since the lynx defecate primarily at night and samples were frozen by the time the care-taker arrived in the morning, samples were stored frozen (-20°C) until all samples for the experiment had been collected.

Once sample collection was completed, each sample was divided into six sub-samples. With the exception of a 0 d control which was immediately frozen at -20°C, sub-samples were placed outside on the snow. Each sample had been randomly assigned *a priori* to one of two spatial treatments: 1) sunny location or 2) shady location. All sub-samples from a given sample were placed in the assigned location type. Sub-samples were then collected at the following time intervals: 0.5 d (12 h), 1 d, 2 d, 4 d, and 8 d. After collection, sub-samples were stored in zip-lock plastic bags and frozen at -20°C. The experiment was conducted in early April near Creede, CO (elevation ~2700 m); thus, field conditions are representative of conditions under which fecal samples would be collected. All samples were shipped overnight on ice to Brookfield Zoo for analysis. All sub-samples were extracted and analyzed in duplicate.

A repeated measures ANOVA was used to test for the effects of time in the field, exposure (whether the sample was exposed to sun or shade), and sex of the lynx. Each hormone was analyzed separately, and models included all two-way interactions. The residuals for each model were tested for dependence across time, and when present, the covariance matrix of the residuals was modeled to include this correlation (see Littell et al. 2006 for details). Additionally, non-constant variance required further modeling of the covariance matrix for some models (see Table 1-1 for the data transformation and covariance structure used for each hormone). Fit statistics (AICc and BIC) were used to determine the best models. After acquiring the final model for each hormone, pairwise comparisons between the control sample and each time-point were made using a Dunnett's test.

Freeze-Thaw Experiment

To assess the effect of freeze-thaw cycles on fecal hormone metabolites, fecal samples were collected from captive Canada lynx. Four males and four females were included in the study, and each lynx resided at a different North American institution. One sample was collected from each lynx as soon as possible after defecation. Samples were stored in zip-lock bags and frozen at -20°C. All samples were shipped overnight on ice to Brookfield Zoo for the experiment.

Upon arrival at Brookfield Zoo, samples were well mixed and one 0.5 g aliquot was immediately re-frozen at -20°C to serve as the control. The entire remaining sample was then exposed to a series of four freeze-thaw cycles. Samples were thawed for ~10 h at room temperature (23°C) inside their original collection container (i.e., a zip-lock bag). The samples were then re-frozen at -20°C for at least 15 h before the next thaw. At the end of each "thaw" period, one 0.5 g aliquot was taken and stored in a tightly capped, polypropylene vial at -20°C.

To assess the effect of freeze-thaw cycles, we used a random coefficient model (Littell et al. 2006). For this model, the slope of the change in hormone concentration across freeze-thaw cycles is calculated for each sample. The model then tests whether this population of slopes is significantly different from zero. The model also controlled for sex of the lynx. All hormones were analyzed separately, and no correlation was found among the residuals over time. All hormone concentrations were log transformed to meet assumptions of normality and homoscedasticity.

Effect of Fecal Characteristics

To determine how different sample characteristics affect the measured hormone concentration, the following variables were recorded for each sample: total weight of the fecal sample, hair content, and water content. Each fecal sample was weighed to the nearest 0.01 g prior to extraction. Hair content was ranked on a scale from 1-6 (1=no visible hair, all fecal material; 3=some hair, but still plenty of fecal material; 6=all hair, barely any fecal material). Similarly, water content was ranked on a scale from 1-7 (1=dry, crumbly; 4= well-formed, malleable; 7=not formed, runny). This experiment included a total of 3064 samples collected from 50 lynx, although we did not measure titers of all four hormones for each sample (fE = 1963 samples, fP = 1728 samples, fA = 1015 samples, fGC = 2437 samples).

A mixed linear model was used to test for the effects of total fecal weight, hair content, and water content on fecal metabolite concentrations. All factors were treated as continuous variables, and each hormone was analyzed separately. Because there were multiple samples from the same individual, lynx ID was included as a random factor to account for this correlation. Hormone concentrations were log transformed to meet assumptions of normality and homoscedasticity.

Comparison of Wet and Dry Weight Extraction Methods

To examine the difference between dry-weight and wet-weight extraction methods for Canada lynx, fecal samples (14-30) were collected from 4 female and 1 male lynx. For each sample, one 0.5 g aliquot of wet fecal material was weighed out in a polypropylene vial and extracted for analysis (see below). The remainder of each sample was then dried in a lyophilizer (Labconco, Kansas City, Missouri) for 8 days (-20°C). After drying, samples were crushed and sifted to remove non-fecal debris (e.g., bones, hair, etc.). One 0.1 g aliquot of dried fecal material was weighed out in a polypropylene vial and extracted.

To test whether the results obtained using dry weight extractions differed notably from wet weight extractions, Pearson's correlation coefficients were calculated for each lynx and each hormone. All hormone concentrations were log transformed.

Extraction and analysis of steroid metabolites

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 1500 rpm. One ml of supernatant was transferred to a new polypropylene tube and diluted with 1 ml assay buffer. The extracts were stored at -20°C, and all samples were analyzed within three months of extraction.

Fecal extracts were analyzed using enzyme-immunoassays (EIAs), and all samples were assayed in duplicate. Reproductive steroids were quantified using antibodies and corresponding HRP-conjugates provided by C. Munro (University of California-Davis, Davis, CA; see Appendix 1 for details). The testosterone antibody R156/7 was used to quantify fecal androgen metabolites (fA), the estradiol-17β antibody R4972 was used for fecal estrogen metabolites (fE),

and the progesterone antibody CL425 was used for fecal progestogen metabolites (fP). Fecal glucocorticoid metabolites (fGC) were quantified using a commercially available Corticosterone EIA (Assay Designs, Ann Arbor, MI; see Appendix 2 for details).

Results

Winter Stability

All four types of fecal steroid metabolites remained stable for at least three days in winter field conditions (Fig. 1-1). Results of the ANOVA indicated that "time in the field" did not have a significant effect on measured fP and fGC concentrations (Table 1-1). For fE and fA, "time" did have a significant effect on measured hormone concentration, but the Dunnett's pairwise comparisons revealed that concentrations did not change significantly until day 8 for fE, and day 4 for fA. Note that sample size is smaller on day 8 because two samples did not have sufficient fecal material to collect a final sample.

Fecal progestogens were the most stable; fP concentrations did not change significantly over time in either exposure treatment. Similarly fGC concentrations did not change significantly over time, but did differ between exposure treatments. Concentrations of fGC were significantly lower for the "sun" treatment than for the "shade" treatment. This is probably related to sampling variation and not necessarily an effect of exposure, because the controls were significantly different, too. Although it was not significant, fGC concentration decreased in the first 12 h in both exposure treatments. Both fE and fA exhibited similar patterns of stability in winter field conditions (Table 1-1, Fig. 1-1). Concentrations did not change significantly until day 8 for fE (in the shade, only), and until day 4 for fA (in the sun, only).

Freeze-Thaw

There was no significant effect of number of freeze-thaw cycles (up to four) on hormone metabolite concentration for any of the hormones (β±SE – fE: 0.019±0.013 ln(ng/g)/cycle, t_7=1.55, P=0.17; fP: 0.009±0.020 ln(ng/g)/cycle, t_7=0.46, P=0.66; fA: 0.037±0.021 ln(ng/g)/cycle, t_7=1.76, P=0.12; fGC: 0.026±0.015 ln(ng/g)/cycle, t_7=1.73, P=0.13).

Not surprisingly, sex had a significant effect on concentration for all hormones except fA (fE: $F_{1,24}$=5.97, P=0.02; fP: $F_{1,24}$=28.74, P<0.0001; fA: $F_{1,24}$=0.91, P=0.35; fGC: $F_{1,24}$=10.90, P=0.003). Two of the males randomly selected for this experiment had very low fA levels compared to other males, and one of the females had particularly high fA levels (probably related to the fact that she was pregnant). This could explain the lack of variation in fA between males and females.

Fecal Characteristics

The magnitude of the effects of the three fecal characteristics on measured hormone concentration varied between hormones, although trends were generally similar (Table 1-2). In general, measured hormone concentration decreased with increasing water content, but the effect of water was influenced by hair content (Fig. 1-2). For samples with little hair, the effect of water was the strongest, while samples with lots of hair were only slightly affected by changing water content. On average (across the range of hair content), there was a two- to three-fold difference in measured hormone concentration depending on water content.

The effect of fecal weight on measured hormone concentration was dependent on water content. Drier samples had a slightly positive relationship between fecal mass and hormone concentration. As water content of the sample increased, this relationship became increasingly negative. The average magnitude of the effect of weight was two-fold (range = 1.5 to 3 fold).

Wet vs. Dry Weight Extraction Methods

Measured hormone concentrations were highly correlated between wet weight and dry weight extraction methods (mean r±SD – fE: 0.95±0.02; fP: 0.83±0.07; fA: 0.76±0.16; fGC: 0.90±0.05; Fig. 1-3). Furthermore, all correlations between measured “wet” and “dry” hormone concentrations for each lynx were highly significant (all P<0.01).

Discussion

Winter Stability

Canada lynx populations are declining, and physiological insights provided by fecal hormone analysis may prove useful to conservation efforts. Due to the solitary nature of lynx, it is possible for biologists to track - and collect fecal samples from - known individuals in the winter. Several studies have found that fecal steroid metabolites degrade rapidly in the field, and therefore samples should be collected immediately and stored at -20°C (Touma and Palme 2005, Wielebnowski and Watters 2007). For Iberian lynx, fecal steroid metabolites degraded significantly within 24 h in a Mediterranean climate (T. Ancin, pers. comm.). Unfortunately, immediate collection is not always feasible in the field, especially when it is desirable to have a minimal impact on or avoid detection by the animal. However, most of these studies have been conducted at ambient temperatures between 20-30°C (Masunda et al. 1999, Beehner and Whitten 2004, Galama et al. 2004). Degradation is primarily caused by fecal bacteria (Touma and Palme 2005, Wielebnowski and Watters 2007), so storage of the sample at -20°C can inhibit bacterial activity and preserve steroid metabolites. This suggests that hormone metabolites in samples deposited at sub-zero temperatures may remain stable between defecation and collection (generally less than 3 d). However, degradation may also be caused by UV radiation (Terio et al. 2002), which can alter chemical bonds. Our first goal was to investigate the effects of sun-exposure and time in the field on measured fecal hormone metabolite concentrations.

Our findings indicate that measured steroid metabolite concentrations do not change significantly in winter field conditions for at least 3 days in either sun or shade. While there is variability in mean hormone concentration over time, there is not much systematic change in hormone expression over time. The exception is fGCs, which decrease slightly over time.

In general, trends were quite similar in both sun and shade. The one exception is the "day 8 – shade" treatment, which shows a notable increase, particularly for fE and fA. We found that the "shade" treatment tended to be higher than the "sun" treatment for all four hormones. However, this difference appears to be an artifact of sampling variation, as even the Time 0 controls were different.

It must be pointed out that within-sample variability may have contributed to differences in hormone concentration between time points, because the fecal samples could not be mixed prior to sub-sampling. While it would have been preferable to homogenize the samples before

starting the experiment, the hair content of the samples prevented any mixing. Hormones may not be distributed homogeneously within a sample (Brown et al. 1994, Millspaugh and Washburn 2003, Wielebnowski and Watters 2007). Indeed, when aliquots were taken from different parts of a Canada lynx fecal sample, variability was up to 50-70%CV (K. Fanson, unpubl. data). Therefore, quite a bit of the variation in concentration over time may be related to intra-sample variability, and not necessarily to environmental exposure.

Even though concentrations varied over time and across sub-samples, we were still able to detect known biological trends. For example, females have higher fGC concentrations than males, and this difference remained distinct across all treatments. Because there are so many sources of noise associated with fecal hormone analysis, this technique is only appropriate for monitoring coarse differences in patterns of hormone expression (Wielebnowski and Watters 2007). Consequently, it is not particularly sensitive to much of the variance caused by methodological factors, unless the factor has a particularly strong effect. Although it is often possible to find a statistically significant effect of a methodological or environmental factor, qualitative conclusions are fairly robust to these sources of variation (Lynch et al. 2003).

Freeze-Thaw

Samples may experience some thawing in the field or during transport between institutions. Fecal bacteria may become dormant at sub-zero temperature, thereby halting enzymatic activity (Palme et al. 2005, Wielebnowski and Watters 2007). However, sub-zero temperatures are not sufficient to destroy enzymes, and thus bacterial metabolization may be re-initiated upon thawing. Therefore, it is important to understand the effect of freeze-thaw cycles on hormone metabolite concentrations. Millspaugh et al. (2003) found that the effect of thawing varies between species. After one long thaw cycle, fGC concentrations in deer samples did not change significantly, but they decreased significantly in elk samples.

We found no evidence that repeated freeze-thaw cycles have a significant effect on measured hormone concentration. In fact, there was remarkably little variation across freeze-thaw cycles. Although freezing at -20 may not kill fecal bacteria or destroy bacterial enzymes, it does inactivate them. Samples were thawed for 10 h, which was ample time for them to thaw. However, we did not monitor temperature, and it is possible that the temperature either did not get warm enough or was not warm for a sufficient period of time to allow bacteria to become

active. In any case, the degree of thawing that would occur during a typical day in the field does not appear to pose a problem for quantifying fecal hormone metabolites in Canada lynx feces.

Fecal Characteristics

In general, hormone concentration decreased with increasing water content. As water content (and thus weight) increases, the 0.5 g aliquot for a sample contains less actual fecal material. However, the effect of water is muted in samples with higher hair content. We suspect that this is because hair is so light and does not absorb much water, so even with wet samples, a lot of material is required to reach the target weight. It has been suggested that samples with lots of hair or bone may underestimate hormone concentration (Wielebnowski and Watters 2007), but we found no evidence that hairy samples had lower hormone concentrations.

We also found that there was a subtle negative relationship between total weight of the fecal sample and measured hormone concentration. However, this relationship was influenced by water content of the sample. For drier samples, this relationship became slightly positive, and for wetter samples the relationship became more negative. It has been documented in animals with small fecal samples that steroid metabolite concentrations may be related to the total size of the feces, in part because fecal size may reflect variations in gut passage time or defecation frequency. For example, in birds and mice, very small fecal samples have proportionately higher hormone concentrations (Millspaugh and Washburn 2004, Tempel and Gutierrez 2004). It is generally assumed that fecal size does not matter for animals with large fecal samples, although this idea has not been well tested in mammals. Our results indicate that even with large scat, size may have some effect on hormone concentration. As mentioned above, this may reflect gut passage time, or it may also reflect dietary changes, which are known to affect fecal metabolite concentrations (Touma and Palme 2005). The effect of size was fairly minimal (approximately two-fold), and although it was significant for fA and fGC, it does not seem to be particularly meaningful.

While each individual exhibited a range of fecal characteristics, the individual means fell within a narrow range (e.g., water content was scored on a scale from 1 to 7, but mean water content only ranged from 2.4 to 4.6). Therefore, as long as multiple samples are collected for an individual, fecal characteristics are unlikely to influence biological results. Indeed, we did not observe any change in qualitative results when water content was included in the statistical models (see Chapters 2, 3, and 4).

Wet vs. Dry Weight Extraction Methods

Traditional extraction methods called for fecal samples to be lyophilized in order to control for variable water content between fecal samples (Wasser et al. 2000, Terio et al. 2002). However, subsequent tests have shown that the amount of noise introduced by variable water content is minimal, and thus does not significantly impact the results (Moreira et al. 2001, Palme et al. 2005, Pettitt et al. 2007). As a result, many people are now employing the simpler, cheaper, and thus more efficient method of wet-weight extractions.

Indeed, we found that longitudinal profiles yielded very similar patterns, and wet-weight and dry-weight concentrations were highly correlated. Our results indicate that both water and hair content contribute to sample variability. Thus, it might be concluded that samples should be dried and sifted in order to minimize variation. However, while wet-weight samples tended to have lower concentrations than dry-weight samples (see Fig. 1-3), the qualitative patterns and ensuing conclusions are the same for both methodologies. Furthermore, the process of drying samples may also affect steroid metabolites and thereby contribute to sample variability.

We emphasize that fecal hormone analysis is a coarse measure of endocrine activity, and as such, is fairly robust to small sources of variation. It is known that numerous factors contribute to variation in measured fecal hormone metabolite concentration (Millspaugh and Washburn 2004, Touma and Palme 2005, Wielebnowski and Watters 2007). Studies on the effect of a specific factor will often yield statistically significant results, but "statistically significant" is not the same as "meaningful." By no means do we wish to discourage or undermine the importance of validation studies. However, we encourage scientists to consider not only the statistical significance of the treatment effect, but also the implications for qualitative conclusions drawn from the results.

Concluding Remarks

We investigated the effect of several environmental and methodological factors on the quantification of fecal hormone metabolites. The key findings of our study include:

1) Fecal hormone metabolites appear to remain stable in winter field conditions for at least three days.
2) Repeated freeze-thaw cycles do not have a significant effect on fecal metabolite concentrations.

3) Although water content is correlated with hormone concentration, it does not have a dramatic effect and wet-weight extraction is appropriate for monitoring fecal steroid metabolites in Canada lynx.

Thus, fecal hormone analysis appears to be a valid tool for application to Canada lynx in the field. While we did find that some external factors had a significant effect on measured fecal metabolite concentrations, it is important to remember that there are several sources of "noise" inherent in this technique, and that fecal hormone metabolite analysis is only appropriate for measuring gross patterns of hormone expression.

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Literature Cited

Atsalis, S., S. W. Margulis, A. Bellem, and N. Wielebnowski. 2004. Sexual behavior and hormonal estrus cycles in captive aged lowland gorillas (*Gorilla gorilla*). American Journal of Primatology 62:123-132.

Bahr, N. I., R. Palme, U. Mohle, J. K. Hodges, and M. Heistermann. 2000. Comparative aspects of the metabolism and excretion of cortisol in three individual nonhuman primates. General and Comparative Endocrinology 117:427-438.

Beehner, J. C. and P. L. Whitten. 2004. Modifications of a field method for fecal steroid analysis in baboons. Physiology & Behavior 82:269-277.

Brown, J. L., L. H. Graham, N. Wielebnowski, W. F. Swanson, D. E. Wildt, and J. G. Howard. 2001. Understanding the basic reproductive biology of wild felids by monitoring of faecal steroids. Advances in Reproduction in Dogs, Cats and Exotic Carnivores:71-82.

Brown, J. L., S. K. Wasser, D. E. Wildt, and L. H. Graham. 1994. Comparative aspects of steroid-hormone metabolism and ovarian activity in felids, measured noninvasively in feces. Biology of Reproduction 51:776-786.

Brown, J. L., D. E. Wildt, N. Wielebnowski, K. L. Goodrowe, L. H. Graham, S. Wells, and J. G. Howard. 1996. Reproductive activity in captive female cheetahs (*Acinoyx jubatus*) assessed by faecal steroids. Journal of Reproduction and Fertility 106:337-346.

Dehnhard, M., K. Fanson, F. Göritz, S. Naidenko, A. Vargas, and K. Jewgenow. *In review.* Comparative metabolism of gestagens and estrogens in the four lynx species, the Eurasian (*Lynx lynx*), the Iberian (*Lynx pardinus*), the Canada lynx (*Lynx canadensis*) and the bobcat (*Lynx rufus*). General and Comparative Endocrinology.

Galama, W. T., L. H. Graham, and A. Savage. 2004. Comparison of fecal storage methods for steroid analysis in black rhinoceroses (*Diceros bicornis*). Zoo Biology 23:291-300.

Hunt, K. E., R. A. Rolland, S. D. Kraus, and S. K. Wasser. 2006. Analysis of fecal glucocorticoids in the North Atlantic right whale (*Eubalaena glacialis*). General and Comparative Endocrinology 148:260-272.

Littell, R. C., G. A. Milliken, W. W. Stroup, R. D. Wolfinger, and O. Schabenberger. 2006. SAS for Mixed Models, 2nd ed. SAS Institute Inc., Cary, North Carolina.

Lynch, J. W., M. Z. Khan, J. Altmann, M. N. Njahira, and N. Rubenstein. 2003. Concentrations of four fecal steroids in wild baboons: short-term storage conditions and consequences for data interpretation. General and Comparative Endocrinology 132:264-271.

Masunda, B., C. Mutisi, H. Hamudikuwanda, and J. G. O. Agumbah. 1999. The concentration of faecal progestins during the oestrous cycle in Nkone cows and the effect of duration of storage of faecal samples at room temperature on faecal progestin levels. Tropical Animal Health and Production 31:373-381.

Millspaugh, J. J. and B. E. Washburn. 2003. Within-sample variation of fecal glucocorticoid measurements. General and Comparative Endocrinology 132:21-26.

Millspaugh, J. J. and B. E. Washburn. 2004. Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. General and Comparative Endocrinology 138:189-199.

Millspaugh, J. J., B. E. Washburn, M. A. Milanick, R. Slotow, and G. van Dyk. 2003. Effects of heat and chemical treatments on fecal glucocorticoid measurements: implications for sample transport. Wildlife Society Bulletin 31:399-406.

Moreira, N., E. L. A. Monteiro-Filho, W. Moraes, W. F. Swanson, L. H. Graham, O. L. Pasquali, M. L. F. Gomes, R. N. Morais, D. E. Wildt, and J. L. Brown. 2001. Reproductive steroid hormones and ovarian activity in felids of the *Leopardus* genus. Zoo Biology 20:103-116.

Palme, R., S. Rettenbacher, C. Touma, S. M. El-Bahr, and E. Möstl. 2005. Stress hormones in mammals and birds - Comparative aspects regarding metabolism, excretion, and noninvasive measurement in fecal samples. Annals of the New York Academy of Science 1040:162-171.

Pettitt, B. A., C. J. Wheaton, and J. M. Waterman. 2007. Effects of storage treatment on fecal steroid hormone concentrations of a rodent, the Cape ground squirrel (*Xerus inauris*). General and Comparative Endocrinology 150:1-11.

Poole, K. G. 2003. A review of the Canada lynx, *Lynx canadensis*, in Canada. Canadian Field-Naturalist 117:360-376.

Rolland, R. M., K. E. Hunt, S. D. Kraus, and S. K. Wasser. 2005. Assessing reproductive status of right whales (*Eubalaena glacialis*) using fecal hormone metabolites. General and Comparative Endocrinology 142:308-317.

Ruggiero, L. F., K. B. Aubry, S. W. Buskirk, G. M. Koehler, C. T. Krebs, K. S. McKelvey, and J. R. Squires. 2000. Ecology and conservation of lynx in the United States. University Press of Colorado, Boulder, Colorado.

Schwarzenberger, F. 2007. The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species. International Zoo Yearbook 41:52-74.

Tempel, D. J. and R. J. Gutierrez. 2004. Factors related to fecal corticosterone levels in California Spotted Owls: Implications for assessing chronic stress. Conservation Biology 18:538-547.

Terio, K. A., J. L. Brown, R. Moreland, and L. Munson. 2002. Comparison of different drying and storage methods on quantifiable concentrations of fecal steroids in the cheetah. Zoo Biology 21:215-222.

Terio, K. A., L. Marker, and L. Munson. 2004. Evidence for chronic stress in captive but not free-ranging cheetahs (*Acinonyx jubatus*) based on adrenal morphology and function. Journal of Wildlife Diseases 40:259-266.

Touma, C. and R. Palme. 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: The importance of validation. Annals of the New York Academy of Science 1040:54-74.

USFWS. 2000. Endangered and threatened wildlife and plants: final rule to list the contiguous United States distinct population segment of the Canada lynx as a threatened species. Federal Register 63 Number 58.

Washburn, B. E., D. J. Tempel, J. J. Millspaugh, R. J. Gutierrez, and M. E. Seamans. 2004. Factors related to fecal estrogens and fecal testosterone in California Spotted Owls. Condor 106:567-579.

Wasser, S. K. and K. E. Hunt. 2004. Noninvasive measures of reproductive function and disturbance in the barred owl, great horned owl, and northern spotted owl. Pages 109-137 *in* Workshop on Analysis of Hormones in Dropping of Birds and Optimality in Bird Migration, Seewiesen, Germany.

Wasser, S. K., K. E. Hunt, J. L. Brown, K. Cooper, C. M. Crockett, U. Bechert, J. J. Millspaugh, S. Larson, and S. L. Monfort. 2000. A generalized fecal glucocorticoid assay for use in a diverse array of nondomestic mammalian and avian species. General and Comparative Endocrinology 120:260-275.

Wielebnowski, N. and J. L. Brown. 1998. Behavioral correlates of physiological estrus in cheetahs. Zoo Biology 17:193-209.

Wielebnowski, N. and J. Watters. 2007. Applying fecal endocrine monitoring to conservation and behavior studies of wild mammals: Important considerations and preliminary tests. Israel Journal of Ecology & Evolution 53:439-460.

Table 1- 1. Stability of fecal hormone metabolites in winter field conditions. Data were transformed as indicated to meet model assumptions. "Structure" refers to the covariance structure of the model. Sample collection factors that had a significant effect are in bold. "Variance" shows the amount of residual variance in the model, and is listed separately for each group when there was non-constant variance among treatments.

	fE			**fP**			**fA**			**fGC**		
Transformation:	log			square-root			log			log		
Structure:	compound symmetry			spherical			compound symmetry			power		
Effect	DF	F	P	DF	F	P	DF	F	P	DF	F	P
Sex	1, 7.9	0.40	0.54	1, 5.06	7.36	0.04	1, 8	1.43	0.27	1, 9.3	17.10	0.002
Exposure	1, 7.9	2.87	0.13	1, 4.87	0.35	0.58	1, 8	0.66	0.44	**1, 8.7**	**6.90**	**0.03**
Time in field	5, 43	2.05	0.09	5, 31.8	0.85	0.53	5, 43	2.22	0.07	5, 14.2	2.12	0.12
Sex by Exposure	1, 7.9	3.07	0.12	1, 4.87	0.50	0.51	1, 8	0.01	0.93	1, 9.7	0.08	0.78
Sex by Time	5, 43	0.25	0.94	5, 32.1	0.85	0.53	5, 43	0.64	0.67	5, 14.6	0.35	0.87
Exposure by Time	**5, 33.6**	**7.05**	**<0.001**	5, 13.2	1.80	0.18	**5, 43**	**8.67**	**<0.001**	5, 26.8	1.26	0.31
Variance			Estimate			Estimate			Estimate			Estimate
	Female		0.12	Female		2137.56	Residual		0.15	Female		0.48
	Male		0.05	Male		4.14				Male		0.57

Table 1- 1. Effect of fecal characteristics on measured fecal steroid concentrations. Slope and standard error obtained from ANOVA models (run separately for each hormone). "Weight" is the total weight of the fecal sample. Single asterisk indicates $P<0.05$, double asterisk indicates $P<0.001$.

Effect	**fE**	**fP**	**fA**	**fGC**
Hair	0.24±0.11*	-0.30±0.19	-0.29±0.11*	0.08±0.07
Water	-0.09±0.05	-0.28±0.08**	-0.52±0.08**	-0.26±0.04**
Weight (g)	-0.0006±0.005	-0.007±0.01	-0.01±0.007	-0.01±0.004*
Hair by Water	0.04±0.02	0.10±0.04*	0.15±0.04**	0.06±0.02*
Hair by Weight	-0.004±0.002	0.003±0.004	-0.001±0.003	-0.001±0.002
Water by Weight	0.0001±0.001	-0.0003±0.002	0.004±0.002*	0.002±0.001*

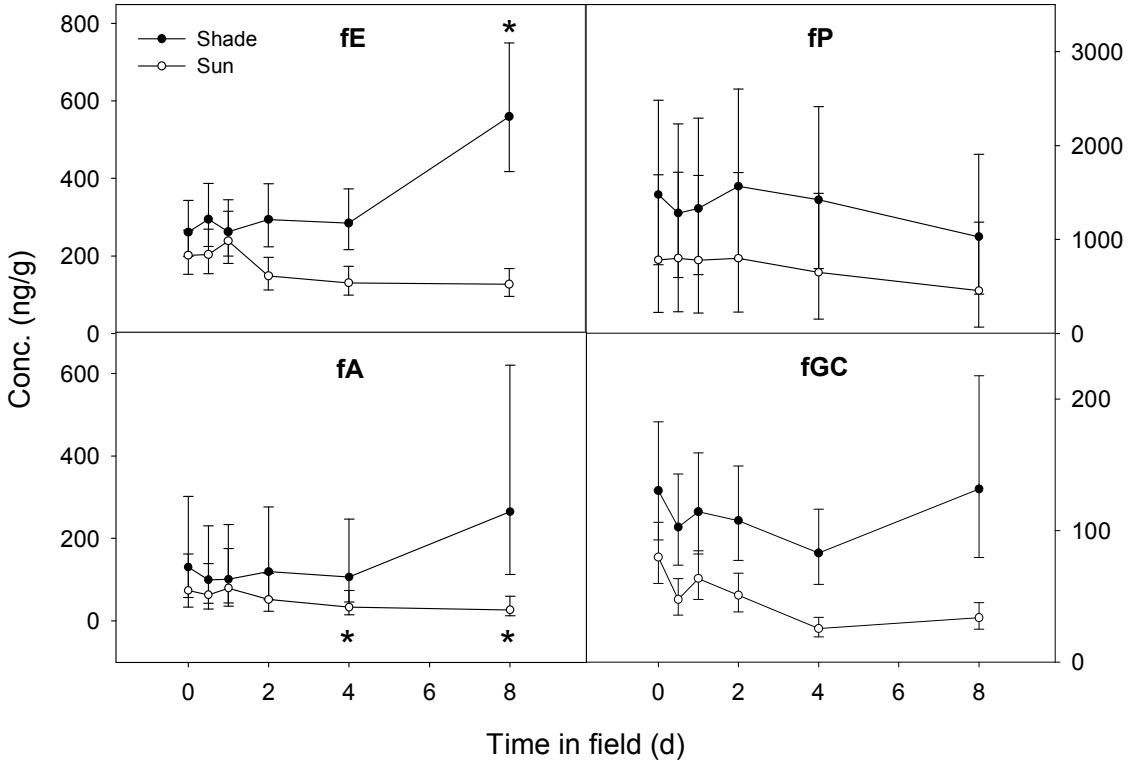

Fig. 1- 1. Stability of fecal hormone metabolites in winter field conditions (back-transformed LSM±SE). Asterisk indicates points that are significantly different from Time 0 (control). The "day8-shade" point for fA does not significantly differ from the control because this treatment had higher standard error due to missing values. All four panels represent the same dataset assayed for different hormones.

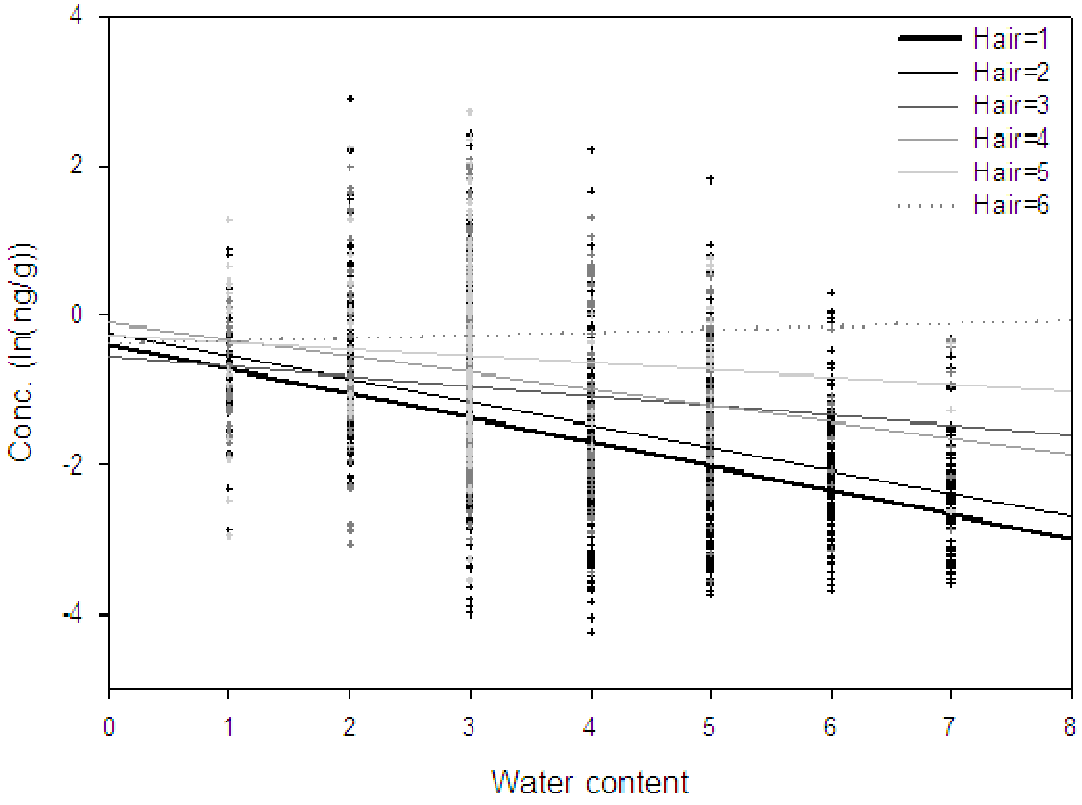

Fig. 1- 2. Representative figure illustrating the effect of water and hair content on measured hormone concentration. For both variables, increasing scores represent increasing content of either hair or water. Figure depicts fE, but trends were the same for all four hormones.

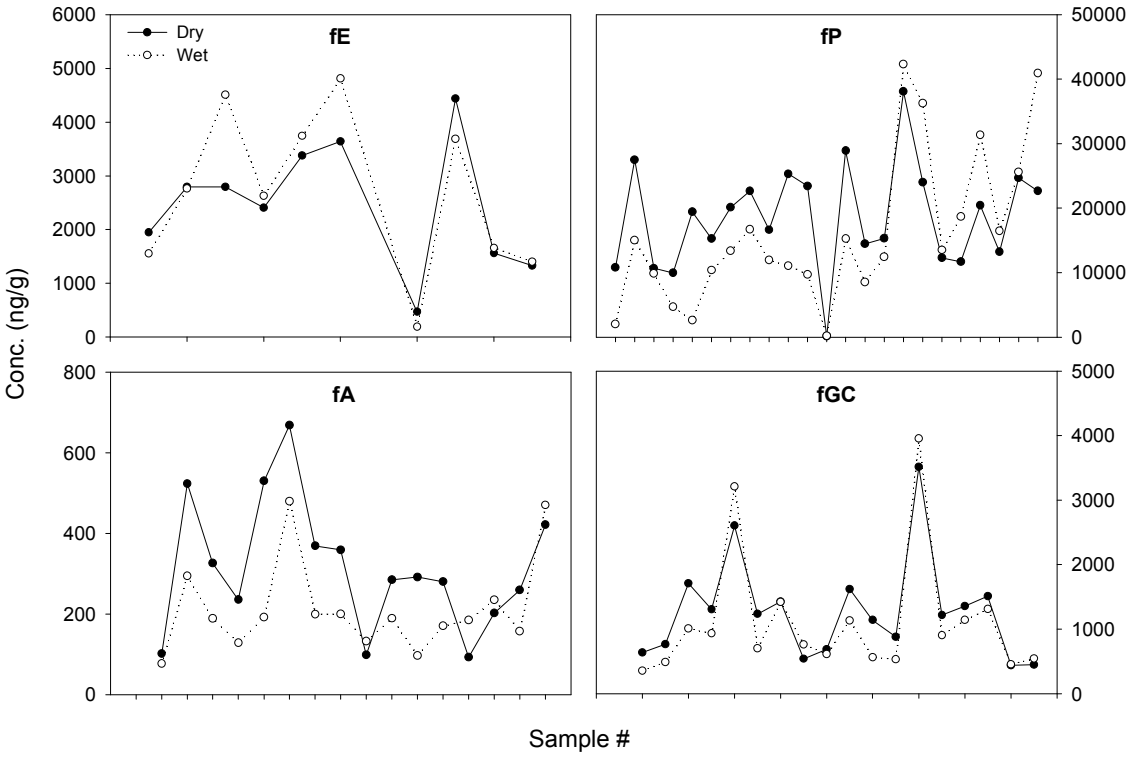

Fig. 1- 3. Comparison of longitudinal hormone profiles between wet-weight and dry-weight extraction methods. Each panel depicts a different individual.

CHAPTER 2. MONITORING PATTERNS OF TESTICULAR ACTIVITY IN CAPTIVE AND WILD CANADA LYNX

Introduction

Canada lynx (*Lynx canadensis*) are still the most abundant felid species inhabiting North America's boreal forest. However, southern populations of lynx have declined dramatically in the last century, and in 2000, they were listed as a threatened species by the US Fish and Wildlife Service (USFWS 2000). While lynx historically extended well into the northern continental US, anthropogenic activities (e.g. trapping, habitat destruction) and climate change have dramatically reduced most US populations (Ruggiero et al. 2000, Poole 2003). Recent conservation efforts have aimed to reverse this trend, but our lack of knowledge about this species hinders the development of effective management strategies (Murray et al. 2008).

One very poorly understood aspect of lynx biology is their reproductive physiology. In order to develop effective conservation strategies and management plans for Canada lynx, it is critical that we develop a stronger understanding of this species' reproductive biology and physiology. Compared to other felids, Canada lynx experience unique constraints on reproduction imposed by pronounced seasonal climate changes and extreme fluctuations in prey populations. Reproductive success and population recruitment fluctuates dramatically both throughout the year, and throughout their 10-year population cycle in the north (Poole 2003).

Although it is well documented that Canada lynx experience both annual and decadal restrictions to breeding, we have a very poor understanding of what cues mediate reproductive behavior or breeding success (e.g. photoperiod, body condition/prey availability, social interactions with conspecifics/population density, etc.). Furthermore, it is unclear whether these breeding restrictions are mediated solely by changes in female physiology, or whether males also exhibit physiological changes that may impact reproduction. Consequently, it is unclear how climate change, anthropogenic activities, or captive housing situations may impact lynx reproduction. In order to develop effective conservation strategies and management plans for lynx, it is critical that we develop a stronger understanding of their reproductive physiology. Furthermore, Canada lynx are closely related to the other three *Lynx* species: the bobcat (*Lynx*

rufus), the Eurasian lynx (*Lynx lynx*), and the Iberian lynx (*Lynx pardinus*), which is the most critically endangered felid species. These four species share several similarities in their reproductive physiology, and thus information about Canada lynx reproduction may help advance research and knowledge for the other three species.

This study integrates information from both captive and wild lynx populations to establish basic knowledge about the reproductive physiology of male Canada lynx. Since hormones are critical mediators of reproductive physiology and behavior, we used the non-invasive technique of fecal hormone analysis to establish normative patterns of hormone expression in male lynx. Specifically, our objectives were to 1) validate an assay for monitoring testicular activity non-invasively, 2) characterize normative patterns of testicular activity, particularly around the breeding season, 3) compare patterns of hormone expression between captive and wild lynx, and 4) examine the effect of breeding status and housing situation on levels of fecal androgen metabolites (fA) in captive animals. This study provides the first longitudinal data on androgen levels in wild and captive Canada lynx. As such, it serves as an important foundation for future studies and the development of stronger management plans.

Methods

Animals and Fecal Sample Collection

Captive – This study included a total of 28 captive Canada lynx males from 19 institutions (3 juveniles, 7 castrated males, and 18 intact, sexually-mature males; Appendix 3). All lynx were housed outdoors at least 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. Most samples were collected during the breeding season (February – April), but sampling extended into other parts of the year for several individuals. When multiple lynx were housed together, icing dye (Wilton Industries, Wilton, IL) was delivered to each individual in a food treat, and then color was used to distinguish between feces. All samples were stored in zip-lock bags at -20°C and shipped on ice to Brookfield Zoo for processing and hormone metabolite analysis.

Wild – Fecal samples were also collected from wild adult males in Colorado (44 lynx, 339 samples), Maine (7 lynx, 51 samples), and Montana (9 lynx, 39 samples). The populations in

Maine and Montana are naturally-occurring, while the population in Colorado has been reintroduced. 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. The age of the fecal sample (and thus the potential for metabolite degradation) may impact measured fecal hormone concentrations. Since samples could not be collected immediately after defecation in the field, this is an important consideration. However, evidence from field experiments suggests that fA metabolites remain stable in winter field conditions for at least four days (see Chapter 1). Furthermore, for logistical reasons, trackers often followed fresh tracks (<24 h old), ensuring that fecal metabolites had probably not degraded much. Based on the rate at which tracks melted-out (and thus could not be confidently distinguished from other species' tracks) or were covered by fresh snowfall, it is unlikely that any samples were older than four days old. Therefore, we are confident that we were able to obtain meaningful results from samples collected in the field. To ensure that fA expression in reintroduced lynx was not affected by the translocation, we only included samples that had been collected more than six months after a lynx was released.

Steroid Extraction and Analysis

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 1500 rpm. One ml of supernatant was transferred to a new polypropylene tube and diluted with 1 ml assay buffer. Prior to extraction, the water content of each sample was ranked on a scale from 1-7 (1=dry, crumbly; 4= well-formed, malleable; 7=not formed, runny). Extracts were stored at -20°C.

Fecal androgen metabolites (fA) were quantified using a single-antibody enzyme-immunoassay (see Appendix 1 for assay procedures; also see deCatanzaro et al. 2003). Testosterone polyclonal antibody R156/7 and the corresponding horseradish peroxidase (HRP) conjugate were obtained from C. Munro (University of California, Davis, CA). The testosterone antibody had the following cross-reactivities: 100% testosterone, 57% 5α-DHT, 0.3% androstenedione, and <0.1% androsterone, DHEA, cholesterol, 17β-estradiol, progesterone and pregnenolone (deCatanzaro et al. 2003, Dloniak et al. 2004). Assay sensitivity was 0.039 ng/well. All samples were assayed in duplicate, and data are expressed as ng/g wet fecal weight.

The assay was biochemically validated for Canada lynx by demonstrating 1) parallelism between serially diluted extracts and the standard curve, and 2) significant (>80%) recovery of exogenous testosterone added to fecal extracts. To monitor precision and reproducibility, low (65% binding) and high (30% binding) quality control samples were run on each plate. Intra-assay coefficients of variation were 9.5% and 7.9% (n=14) for low and high controls, respectively. The inter-assay coefficients of variation were 20.2% and 22.4% (n=97), respectively. Although this degree of inter-assay variation is relatively high due to the extended duration of the study, the amount of inter-assay variation for a given individual was minimized by analyzing all of their samples at the same time, whenever feasible.

Statistical Analysis

All data were analyzed using SAS 9.1 (Cary, NC) and hormone data were log transformed to meet assumptions of normality and homoscedasticity. Means provided in text and figures are least-squares means that have been back-transformed. One captive male (M13) showed unusually low fA values (similar to values observed in juveniles), even though he was an intact adult housed with an intact female. Therefore, he was excluded from all analyses except seasonality, in which he was included because his general pattern of fA expression was similar to other intact males.

Previous experiments revealed that both wet and dried (lyophilized) fecal samples yielded very similar longitudinal profiles (see Chapter 1). Consequently, we adopted a wet-weight extraction protocol. However, further analysis indicated that estimated water content of the sample *did* have a systematic effect on measured concentration; concentration decreased with increasing water content (see Chapter 1). To account for the effect of water, mean water content was included in all analyses. For the samples that were missing an estimate of water content (captive: n=1; wild: n=41), we interpolated values using median water content.

Biological validation

To assess the biological relevance of the assay, overall mean fA values were compared between juvenile (n=3), castrated (n=7), and intact, sexually mature (n=17) males using a one-way ANOVA. Only captive males were included, since nearly all wild males were adults.

Because two of the juveniles were housed together and their samples were indistinguishable, they were combined for statistical analysis (M26). A Tukey-Kramer adjustment was used to correct for multiple pairwise comparisons.

Population and age

We tested the effect of population and age using an ANCOVA. Mean age was calculated for each individual, and only intact, adult (≥2 years old) males were included in this analysis. Captive lynx were treated as a single population, and the three wild populations (Colorado, Maine, and Montana) were considered separate populations. Captive males ranged in age from 3-18 years old, and wild males ranged from 2-12 years old. Two-way interactions were excluded if they were not significant. A Tukey-Kramer adjustment was used to correct for multiple pairwise comparisons between populations.

Unlike captive lynx, we had data spanning multiple years for several wild individuals (n=15, difference in age=2-5 years). To see if there was any effect of age on fA *within* an individual, we ran a one-sample t-test using the difference in fA concentrations between the youngest and oldest age points for an individual as the response variable.

Seasonality

We conducted a repeated measures ANOVA to test the effect of month on fA values for both captive and wild populations. The model also controlled for the effect of year (sample collection from wild lynx spanned 10 years; captive collection only spanned 4 years). Only intact, sexually mature males were included.

To test for differences between breeding and non-breeding seasons in captive males, we performed a post hoc linear contrast of monthly means. (This comparison was not possible for wild lynx due to the limited window of sample collection.) For this analysis, we compared the average fA concentrations during the breeding season (February and March) to the average of four non-breeding months for which we had the most data (May, June, July, and August).

For the captive males, we also ran a simple regression to test the effect of latitude on the timing of peak fA values. Since reproductive seasonality in many temperate felids is influenced by photoperiod (Brown et al. 2002), we were interested if patterns of fA expression varied by

latitude. This test included 13 males with the most complete fA profiles. Each individual's mean weekly fA values were used to determine when their maximum fA occurred (weeks were numbered 1-52, with week 1 starting January 1 of the year sampling occurred).

Past breeding success and housing situation

Keepers at each captive institution were asked to provide information about each male's past breeding success and housing situation. Past breeding success was scored as either proven breeder (has sired at least one litter), non-breeder (has been housed with a suitable mate for at least two years, but has not sired a litter), or unsure (not housed with suitable mate for at least two years). Information about housing included number, sex, and status (intact or neutered) of cage-mates.

Using mean fA levels as the response variable, we initially ran an ANOVA model with intact males housed *with* a cage-mate. Because of the limited number of housing situations, it was not possible to include interactions in the model. Neither the number of cage-mates, nor the sex of the cage-mate(s) had a significant effect on baseline fA levels ($P>0.4$), so these variables were removed from the model. For the final model, we created a variable called 'housing situation' with three levels (housed alone, with an intact cage-mate, or with a spayed/castrated cage-mate). The final ANOVA included breeding success and housing situation. A Tukey-Kramer adjustment was used to correct for multiple comparisons between housing situations.

Results

Biological validation

Male status had a significant effect on fA expression ($F_{2,22}=18.15$, $P<0.001$; Fig. 2-1). Both juveniles and castrated males had significantly lower mean fA values than intact adult males ($t_{22}=5.39$, $P<0.001$ and $t_{22}=2.52$, $P=0.04$, respectively). Interestingly, juveniles also had significantly lower fA concentrations than castrated males ($t_{22}=2.91$, $P=0.02$).

Population

Fecal androgen levels differed significantly between populations ($F_{3,64}$=23.78, P<0.001; Fig. 2-1). The captive population had significantly higher fA values than all three wild populations (CO: t_{64}=8.41, P<0.001; ME: t_{64}=4.75, P<0.001; MT: t_{64}=4.97, P<0.001), but the wild populations were not significantly different from each other (CO vs. ME: t_{64}=0.07, P=0.99; CO vs. MT: t_{64}=0.99, P=0.76; ME vs. MT: t_{64}=0.74, P=0.88).

Age

Age did not have a significant effect on fA concentrations (β=0.01±0.03 ln(ng/g)/year of age, $F_{1,64}$=0.11, P=0.74; Fig. 2-2). Although not statistically significant, the two populations exhibited opposite trends for the effect of age: in captive males, there was a positive relationship between age and fA, while in wild males, it was negative. The one-sample t-test, which examined individual-level changes in fA with age, also indicated there was not a significant relationship between fA concentration and age (t_{14}=0.12, P=0.91, n=15).

Seasonality

For both populations, month strongly affected fA expression in intact male lynx (captive: $F_{11,68.1}$=3.87, P<0.001; wild: $F_{4,127}$=3.54, P=0.009; Fig. 2-3). There were also significant differences in fA concentrations among years for wild lynx ($F_{9,120}$=3.06, P=0.003), but not for captive lynx ($F_{3,48.3}$=0.07, P=0.97). There was no obvious trend to the changes in fA between years.

In the captive population, fA concentrations were significantly higher during the breeding season than the non-breeding season (breeding – February/March: mean=773 ng/g, 95%CI=400 – 1493 ng/g; non-breeding – May to August: mean=399 ng/g, 95%CI=206 – 789 ng/g; t_{77}=4.77, P<0.001). While this comparison was not possible for wild lynx, the monthly trends are similar, with the highest fA concentrations in February and March. Latitude did not have a significant effect on the timing of an individual's maximum weekly fA in captive lynx ($F_{1,11}$<0.001, P=0.99).

Past breeding success and housing situation

Past breeding success did not have a significant effect on mean fA levels in intact males ($F_{2, 11}$=0.76, P=0.49). However, housing condition did significantly affect mean fA levels ($F_{2, 11}$=13.02, P=0.001). Males housed with intact cage-mates, either male or female, had higher fA levels than males housed alone (t_{11}=3.89, P=0.006) or with neutered cage-mates (t_{11}=3.81, P=0.008). Mean fA levels were not significantly different between intact males housed alone or with a neutered cage-mate (t_{11}=0.27, P=0.96). Since the initial model revealed that sex of the cage-mate did not have a significant effect on fA levels, it was not included in the final model.

Discussion

Biological validation

The fact that intact, reproductively mature males had significantly higher fA levels than castrated or juvenile males indicates that our assay was successful at detecting biologically relevant differences in testicular activity. However, there were still detectable levels of fA in juvenile and castrated males. This suggests that some of the androgen metabolites detected by the assay may have been of adrenal origin. While androgens are primarily secreted by the testes, they are also secreted by other glands, such as the adrenals (Meikle et al. 1991). Prior to excretion, steroid hormones undergo extensive metabolism by the liver and intestinal bacteria (Palme 2005). Therefore, it is the steroid hormone *metabolites* rather than parent hormone molecules that are being measured in the feces. In fact, studies on other felids have revealed that very little testosterone is excreted in its native form (Brown et al. 1996, Jewgenow et al. 2006b). It was beyond the scope of this study to determine whether some immunoreactive androgen metabolites may indeed have been of adrenal origin. Nonetheless, we are confident, based on the overall results, that our assay primarily measured testicular activity.

Population

While there was no difference in fA levels among the three wild populations, captive males had dramatically higher fA levels (~7-fold) than wild males. It is unlikely that this

difference is related to differences in gonadal activity, but rather other biological factors affecting fecal hormone metabolites, such as diet, metabolic rate, and/or body condition (von der Ohe and Servheen 2002, Wielebnowski and Watters 2007). Individuals in captivity have more regular access to greater quantities of food, and their energy expenditure is much lower – a difference which is reinforced by the fact that captive lynx are generally heavier (11-20 kg; institutions provided individual lynx weights) than wild lynx (7-11 kg; T. Shenk, unpubl. data). Interestingly, female Canada lynx showed a similar trend: both fecal estrogens and progestogens were found to be significantly lower in wild lynx (see Chapter 3).

A unique aspect of this study is our ability to compare fecal hormone concentrations between wild and captive populations. Very few studies have considered this comparison. For the few studies that do exist, results are quite variable depending on the species *and* the hormone (Ziegler et al. 1997, Shiho Fujita 2001, Terio et al. 2004). In contrast to our results, Terio et al. (2004) found that fA concentrations were lower in captive than wild cheetahs. However, they also found that fecal corticoids were higher in captive cheetahs, and this, in conjunction with other physiological data obtained during medical exams, suggested that captive cheetahs experienced chronic stress that resulted in suppression of testicular activity. In a study of spotted hyenas, there was no notable difference in fA concentrations between captive and wild populations (S. Dloniak, pers. comm.). Given our lack of understanding about the impact of different biological factors on fecal steroids and the multitude of variables involved, results must be interpreted very carefully when comparing across populations. Further experimental studies are needed to examine the biological significance of these interesting population differences.

As mentioned previously, one of the intact adult males (M13) was excluded from most analyses because his fA levels were very low compared to the rest of the captive males. Even compared to castrated males, he would have been an outlier. Interestingly, his fA values fell within the range of wild male fA values, and he exhibited normative seasonal changes in fA expression. It is not entirely clear why his fA values were so low: he was born in captivity, his feeding schedule and quantity of food were similar to other males, his weight is on the lower side but he was not the lightest male, he has bred successfully in the past (although he did not successfully sire any litters the year we collected samples).

One possible explanation is that his low fA values were indicative of testicular dysfunction. In both domestic cats and lions, lower circulating testosterone is associated with teratospermia (Wildt et al. 1987, Howard et al. 1990). However, in these species, testosterone levels in teratospermic males are only 25-60% lower than in normospermic males, not ~95%

lower as is the case for M13. Furthermore, in clouded leopards, severe testicular dysfunction is associated with unusually high serum testosterone values, rather than low values (Wildt et al. 1986). We feel that this explanation is unlikely, especially since he exhibited normal seasonal fA fluctuations.

We suspect a more likely explanation is that his metabolism and/or body condition were more similar to wild lynx. M13 was located in Newfoundland, Canada and had the largest enclosure (4600 m^2) of all males (the second largest enclosure was only 500 m^2, and the average enclosure size for males, excluding M13, was 300 m^2). Given the climatic conditions and enclosure size, his activity levels/energetic demands may more closely approximate those of wild lynx. In addition, his diet contained more fish than diets of other captive males. His female cage-mate also had low fecal steroid concentrations compared to other captive females, but similar to wild females (see Chapter 3).

Both the case of M13 and the difference between captive and wild males indicate that there may be a link between fecal hormone levels and energy regulation. This may be an artifact of the methodology, or may reflect differences in circulating steroid concentrations. In humans, obesity, or the metabolic syndrome, is associated with changes in steroid production and binding globulin concentrations (Hajamor et al. 2003). This includes not only glucocorticoids, which have obvious connections to energy regulation, but also sex hormones, which can impact metabolism, adipose tissue, and energy regulation, as well (Tchernof et al. 1996). Essentially, there is a complex network of interactions between glucocorticoids, sex steroids, and metabolic hormones (e.g., leptin and insulin), and perturbations to this network may have cascading impacts on other physiological systems. Because steroid over-production is associated with metabolic dysfunction and other pathologies, it is important to develop a better understanding of the significance and potential implications of elevated steroid hormone levels in captive lynx.

Age

Age did not have a significant effect on fA levels for either population. Even at the individual level, there was no evidence for an effect of age on androgen expression. Male lynx reach sexual maturity at two years of age (Poole 2003), so all males in this study should have been reproductively mature. Although the effect of puberty on androgen expression has been

extensively documented in many species, there is very little literature that describes the trajectory of androgen expression beyond the point of sexual maturity in non-human species. In sun bears, older males have higher fA than younger (but still reproductively mature) males (Hesterman et al. 2005). Younger leopards (3-7 years old) exhibit higher sperm quantity and quality compared to older males, but hormone expression was not monitored (van Dorsser and Strick 2005).

Seasonality

Male lynx show strong seasonal changes in androgen expression. There was no obvious trend to the changes in fA between years, and we suspect that these differences primarily reflect sampling variation. Fecal androgen increased dramatically in February and remained high in March. The biological significance of this pattern of androgen expression is unclear, because androgen expression does not always correlate with other reproductive parameters (Brown et al. 1996). In some felids, testosterone expression varies by season, but sperm production and quality remain constant (Wildt et al. 1986, Byers et al. 1990). However, in other species, increases in androgen expression are associated with increases in testicular volume and increases in seminal quality 1-2 months later [Pallas' cats (Brown et al. 1996, Newell-Fugate et al. 2007); Eurasian lynx (Göritz et al. 2006, Jewgenow et al. 2006a); snow leopards (Johnston et al. 1994); ocelots, tigrinas, margays (Morais et al. 2002)]. Based on North American Canada lynx studbook records (Goff 2008), females conceive around mid-March. If fA expression *is* related to other reproductive parameters in lynx, then the increase in fA concentrations in February may result in higher sperm quality by the time the female becomes receptive.

Many felid species exhibit some degree of reproductive seasonality (Brown 2006). Male felids often exhibit some variation in at least one reproductive parameter (testosterone expression, testicular volume, sperm quantity or quality) throughout the year [domestic cats (Blottner and Jewgenow 2007); jaguars (Morato et al. 2004); ocelots, tigrinas, margays (Morais et al. 2002); leopards (van Dorsser and Strick 2005); clouded leopards (Wildt et al. 1986)]. However, in many felid species, these fluctuations do not preclude successful breeding throughout the year, and are much less tightly regulated than changes in females (Spindler and Wildt 1999). From an evolutionary perspective, it may be advantageous for males to maintain reproductive functionality throughout the year in case a breeding opportunity arises, given the presumed low cost of gametogenesis for males. However, in species inhabiting more northerly regions with harsh winters, breeding opportunities may be so tightly restricted that even the minimal cost of

spermatogenesis still outweighs the potential benefits of maintaining reproductive function outside the breeding season. This is supported by the fact that seasonal changes in male reproductive physiology are much more pronounced in more temperate species [Pallas' cats (Brown et al. 1996, Newell-Fugate et al. 2007); Eurasian lynx (Göritz et al. 2006, Jewgenow et al. 2006a, Jewgenow et al. 2006b); snow leopards (Johnston et al. 1994)].

Lynx species have the shortest breeding seasons among felids (Tumlison 1987, Jewgenow et al. 2006b). Pronounced male seasonality has been documented in all four lynx species [bobcats, *L. rufus* (Crowe 1975); Eurasian lynx, *L. lynx* (Göritz et al. 2006, Jewgenow et al. 2006a, Jewgenow et al. 2006b); Iberian lynx, *L. pardinus* (Jewgenow et al. 2006b)]. Harsh winter conditions and other environmental constraints may prevent females from becoming receptive outside of the breeding season, and contribute to the seasonality in males. However, there is one interesting caveat. In Eurasian lynx, if a female looses a litter, she can begin cycling again in June or July (Göritz et al. 2006, Jewgenow et al. 2006a). Correspondingly, male Eurasian lynx have a second increase in testosterone expression and testicular size in May and June (Göritz et al. 2006, Jewgenow et al. 2006a). There is some anecdotal evidence that female Canada lynx can also give birth in August, which means there would be a second period of estrous in May or June (J. Vashon and J. Tremblay, pers. comm.). However, we did not see a second increase in fecal androgens in May or June for any of the males in the study. The frequency of this "second estrous" in females is not known for either species. However, given the differences in androgen expression between male Eurasian lynx and male Canada lynx, we can speculate that a second estrous is more common in Eurasian lynx. It is interesting that Iberian lynx and bobcats also exhibit seasonal breeding patterns, despite the fact that they inhabit milder climates.

Peak fA levels did not vary significantly with latitude in captive lynx. The range of latitudes represented a change in photoperiod from ~4.5h for the southernmost male, to ~8h for the northernmost male. While this is still much less than the 16+h change in photoperiod experienced by lynx in the northern part of their range, it nonetheless represents a fairly wide range. If peak fA was regulated by photoperiod in lynx, we would have expected to see some effect of latitude. There are two, non-mutually exclusive explanations for why we did not observe an effect of latitude. First, it is possible that photoperiod may affect a component of androgen expression that we were not able to characterize for enough males (e.g. onset or duration of elevated androgen expression). Alternatively, androgen levels may peak in response to different cues, such as female receptivity. Other mammalian males are known to exhibit peak

androgen levels when they are exposed to a female or when they copulate (Kretzschmar et al. 2004, Hesterman et al. 2005, Dloniak et al. 2006). In such cases, changes in androgen expression are a response to (rather than preparation for) a breeding opportunity/situation. Dloniak (2006) speculated that when breeding opportunities are unpredictable, this endocrine response may help synchronize male and female behavior/physiology. Given that lynx are solitary and can be widely dispersed, this could be a possible mechanism for ensuring successful breeding encounters. Males in this study were permanently housed with their cage-mates, so the peak in fA would not have been triggered by the presence of the female alone. We only received notes about breeding activity for three males. Peak fA values for one of the males did coincide with mating activity. However, for the other two males, peak fA levels occurred 1-2 weeks after mating was recorded.

While there was no correlation between latitude and peak fA timing, there was much more variation in the timing of peak fA values in the south than in the north (low latitudes 34-36°: n=6, range=11 weeks; middle latitudes 39-42°: n=5, range=5 weeks; high latitudes 47-50°: n=2, range=2 weeks). Due to small sample sizes at northern latitudes, we were not able to analyze this statistically. However, the observed trend may suggest that the breeding season does not shift across latitudes; rather, it may be more constricted at northern latitudes.

Past breeding success and housing situation

Past breeding success was not significantly related to fA concentrations. Even if we sub-divided proven breeders into 'historic breeders' (had successfully sired litters in the past, but not during the study) and 'current breeders' (sired a litter during the study period), there was still no relationship between fA and breeding status. Similar to our findings, neither serum testosterone levels nor semen quality were correlated with breeding status in cheetahs (Wildt et al. 1993). Conversely, in a survey of eight different felid species, Swanson et al. (2003) found that reproductive status was significantly correlated with serum testosterone; successful breeders had higher testosterone levels. Note that when large and small cats (such as lynx) were analyzed separately, this trend became only marginally significant for large cats, but remained significant for small cats.

Housing situation did have a significant effect on baseline fA levels. Males housed with intact cage-mates had higher fA levels than males housed alone or with castrated cage-mates. Interestingly, it did not appear to matter whether the cage-mate was male or female. Androgens,

which mediate both reproductive and aggressive behaviors, may be notably modulated by social environment (Hirschenhauser and Oliveira 2006). Although lynx are solitary in the wild (Ruggiero et al. 2000, Poole 2003), they are not reported to be very aggressive towards conspecifics, and seem to be more tolerant of group-housing situations than other solitary mammals. This may explain why we did not observe testicular suppression in males housed together.

Black rhinoceros males exhibit similar patterns of androgen expression in captivity: serum testosterone is higher in males housed with females *or* males than in those housed alone (Christensen et al. 2009). Conversely, in greylag geese, singleton males had higher fA levels than paired males, but these individuals were free-ranging (Hirschenhauser et al. 1999). In a survey of six small felid species, Swanson (Swanson et al. 2003) found that housing situation did not affect serum testosterone levels, but did affect other reproductive parameters. Males housed alone or with a single female had increased testis size, semen quality, and sperm count compared to males housed with another male or in larger groups. Since we did not measure other reproductive parameters, we do not know if sex of the cage-mate may have affected other traits in lynx.

It should be noted that fA concentrations were significantly lower in males housed with neutered or spayed cage-mates of either sex. This suggests that lynx use more than just the physical presence or absence of conspecifics to assess their socio-sexual situation. Potential cues for such an assessment could be behavioral (e.g., aggressive behaviors from males or receptive behaviors from females) or chemical (e.g., pheromones) in nature. Future experimental studies in captive populations could help shed more light on this interesting topic and further aid in proper management of this species.

Concluding Remarks

We were able to validate a fecal androgen EIA for non-invasive monitoring of testicular activity in Canada lynx. Using this technique, we provide the first description of testicular activity for this species, and also provide a unique comparison between captive and wild males. Our key findings include:

1) Male Canada lynx exhibit seasonal increases in fA during the breeding season, but patterns of seasonality are not strongly affected by latitude.
2) Captive males have much higher fA concentrations than wild males, which may be related to differences in metabolic rate, diet, or body condition.

3) A male's housing situation can influence fA expression; specifically, males housed with an intact cage-mate exhibit higher fA concentrations.

Understanding not only the basic reproductive physiology of males, but also the degree of plasticity in these reproductive traits, is critical for developing successful captive breeding programs and designing sound conservation strategies. Furthermore, this information can be used to understand the reproductive limitations of this genus in the face of environmental and climatic changes.

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Literature Cited

Blottner, S. and K. Jewgenow. 2007. Moderate seasonality in testis function of domestic cat. Reproduction in Domestic Animals 42:536-540.

Brown, J. L. 2006. Comparative endocrinology of domestic and nondomestic felids. Theriogenology 66:25-36.

Brown, J. L., L. H. Graham, J. M. Wu, D. Collins, and W. F. Swanson. 2002. Reproductive endocrine responses to photoperiod and exogenous gonadotropins in the Pallas' cat (*Otocolobus manul*). Zoo Biology 21:347-364.

Brown, J. L., K. A. Terio, and L. H. Graham. 1996. Fecal androgen metabolite analysis for non invasive monitoring of testicular steroidogenic activity in felids. Zoo Biology 15:425-434.

Byers, A. P., A. G. Hunter, U. S. Seal, E. F. Graham, and R. L. Tilson. 1990. Effect of season on seminal traits and serum hormone concentrations in captive male Siberian tigers (*Panthera tigris*). Journal of Reproduction and Fertility 90:119-125.

Christensen, B. W., M. H. T. Troedsson, L. J. Young, M. Oliva, and L. M. Penfold. 2009. Effects of sociosexual environment on serum testosterone in captive male African rhinoceros. Theriogenology 71:1105-1111.

Crowe, D. M. 1975. Aspects of aging, growth, and reproduction of bobcats from Wyoming. Journal of Mammalogy 56:177-198.

deCatanzaro, D., C. Muir, E. Beaton, M. Jetha, and K. Nadella. 2003. Enzyme immunoassay of oestradiol, testosterone and progesterone in urine samples from female mice before and after insemination. Reproduction 126:407-414.

Dloniak, S. M., J. A. French, and K. E. Holekamp. 2006. Faecal androgen concentrations in adult male spotted hyaenas, *Crocuta crocuta*, reflect interactions with socially dominant females. Animal Behaviour 71:27-37.

Dloniak, S. M., J. A. French, N. J. Place, M. L. Weldele, S. E. Glickman, and K. E. Holekamp. 2004. Non-invasive monitoring of fecal androgens in spotted hyenas (*Crocuta crocuta*). General and Comparative Endocrinology 135:51-61.

Goff, D. 2008. North American Regional Canadian Lynx Studbook (*Lynx canadensis*), Bridgeport, CT: Connecticut's Beardsley Zoo.

Göritz, F., K. Neubauer, S. V. Naidenko, J. Fickel, and K. Jewgenow. 2006. Investigations on reproductive physiology in the male Eurasian lynx (*Lynx lynx*). Theriogenology 66:1751-1754.

Hajamor, S., J. P. Despres, C. Couillard, S. Lemieux, A. Tremblay, D. Prud'homme, and A. Tchernof. 2003. Relationship between sex hormone-binding globulin levels and features of the metabolic syndrome. Metabolism-Clinical and Experimental 52:724-730.

Hesterman, H., S. K. Wasser, and J. F. Cockrem. 2005. Longitudinal monitoring of fecal testosterone in male Malayan Sun bears (*U. malayanus*). Zoo Biology 24:403-417.

Hirschenhauser, K., E. Möstl, and K. Kotrschal. 1999. Seasonal patterns of sex steroids determined from feces in different social categories of greylag geese (*Anser anser*). General and Comparative Endocrinology 114:67-79.

Hirschenhauser, K. and R. F. Oliveira. 2006. Social modulation of androgens in male vertebrates: meta-analyses of the challenge hypothesis. Animal Behaviour 71:265-277.

Howard, J. G., J. L. Brown, M. Bush, and D. E. Wildt. 1990. Teratospermic and normospermic domestic cats - Ejaculate traits, pituitary gonadal-hormones, and improvement of spermatozoal motility and morphology after swim-up processing. Journal of Andrology 11:204-215.

Jewgenow, K., F. Göritz, K. Neubauer, J. Fickel, and S. V. Naidenko. 2006a. Characterization of reproductive activity in captive male Eurasian lynx (*Lynx lynx*). European Journal of Wildlife Research 52:34-38.

Jewgenow, K., S. V. Naidenko, F. Göritz, A. Vargas, and A. Dehnhard. 2006b. Monitoring testicular activity of male Eurasian (*Lynx lynx*) and Iberian (*Lynx pardinus*) lynx by fecal testosterone metabolite measurement. General and Comparative Endocrinology 149:151-158.

Johnston, L. A., D. L. Armstrong, and J. L. Brown. 1994. Seasonal effects on seminal and endocrine traits in the captive snow leopard (*Panthera uncia*). Journal of Reproduction and Fertility 102:229-236.

Kretzschmar, P., U. Ganslosser, and M. Dehnhard. 2004. Relationship between androgens, environmental factors and reproductive behavior in male white rhinoceros (*Ceratotherium simum simum*). Hormones and Behavior 45:1-9.

Meikle, A. W., R. A. Daynes, and B. A. Araneo. 1991. Adrenal Androgen Secretion and Biologic Effects. Endocrinology and Metabolism Clinics of North America 20:381-400.

Morais, R. N., R. G. Mucciolo, M. L. F. Gomes, O. Lacerda, W. Moraes, N. Moreira, L. H. Graham, W. F. Swanson, and J. L. Brown. 2002. Seasonal analysis of semen characteristics, serum testosterone and fecal androgens in the ocelot (*Leopardus pardalis*), margay (*L. wiedii*) and tigrina (*L. tigrinus*). Theriogenology 57:2027-2041.

Morato, R. G., I. T. N. Verreschi, M. Guimaraes, K. Cassaro, C. Pessuti, and R. C. Barnabe. 2004. Seasonal variation in the endocrine-testicular function of captive jaguars (*Panthera onca*). Theriogenology 61:1273-1281.

Murray, D. L., T. D. Steury, and J. D. Roth. 2008. Assessment of Canada lynx research and conservation needs in the southern range: Another kick at the cat. Journal of Wildlife Management 72:1463-1472.

Newell-Fugate, A., S. Kennedy-Stoskopf, J. L. Brown, J. F. Levine, and W. F. Swanson. 2007. Seminal and endocrine characteristics of male Pallas' cats (*Otocolobus manul*) maintained under artificial lighting with simulated natural photoperiods. Zoo Biology 26:187-199.

Palme, R. 2005. Measuring fecal steroids - Guidelines for practical application. Annals of the New York Academy of Science 1040:162–171.

Poole, K. G. 2003. A review of the Canada lynx, *Lynx canadensis*, in Canada. Canadian Field-Naturalist 117:360-376.

Ruggiero, L. F., K. B. Aubry, S. W. Buskirk, G. M. Koehler, C. T. Krebs, K. S. McKelvey, and J. R. Squires. 2000. Ecology and conservation of lynx in the United States. University Press of Colorado, Boulder, Colorado.

Shiho Fujita, F. M., Hideki Sugiura, Keiko Shimizu,. 2001. Measurement of urinary and fecal steroid metabolites during the ovarian cycle in captive and wild Japanese macaques, *Macaca fuscata*. American Journal of Primatology 53:167-176.

Spindler, R. E. and D. E. Wildt. 1999. Circannual variations in intraovarian oocyte but not epididymal sperm quality in the domestic cat. Biology of Reproduction 61:188-194.

Swanson, W. F., W. E. Johnson, R. C. Cambre, S. B. Citino, K. B. Ouigley, D. M. Brousset, R. N. Morals, N. Moreira, S. J. O'Brien, and D. E. Wildt. 2003. Reproductive status of endemic felid species in Latin American zoos and implications for ex situ conservation. Zoo Biology 22:421-441.

Tchernof, A., F. Labrie, A. Belanger, and J. P. Despres. 1996. Obesity and metabolic complications: Contribution of dehydroepiandrosterone and other steroid hormones. Pages S155-S164 *in* International Symposium on DHEA Transformation into Androgens and Estrogens in Target Tissues - Intracrinology, Quebec, Canada.

Terio, K. A., L. Marker, and L. Munson. 2004. Evidence for chronic stress in captive but not free-ranging cheetahs (*Acinonyx jubatus*) based on adrenal morphology and function. Journal of Wildlife Diseases 40:259-266.

Tumlison, R. 1987. *Felis lynx*. Mammalian Species:1-8.

USFWS. 2000. Endangered and threatened wildlife and plants: final rule to list the contiguous United States distinct population segment of the Canada lynx as a threatened species. Federal Register 63 Number 58.

van Dorsser, F. J. D. and J. A. Strick. 2005. Semen characteristics and sperm morphology in the Arabian leopard (*Panthera pardus nimr*) and how these vary with age and season. Reproduction Fertility and Development 17:675-682.

von der Ohe, C. G. and C. Servheen. 2002. Measuring stress in mammals using fecal glucocorticoids: Opportunities and challenges. Wildlife Society Bulletin 30:1215-1225.

Wielebnowski, N. and J. Watters. 2007. Applying fecal endocrine monitoring to conservation and behavior studies of wild mammals: Important considerations and preliminary tests. Israel Journal of Ecology & Evolution 53:439-460.

Wildt, D. E., J. L. Brown, M. Bush, M. A. Barone, K. A. Cooper, J. Grisham, and J. G. Howard. 1993. Reproductive status of cheetahs (*Acinonyx jubatus*) in North-American zoos - the benefits of physiological surveys for strategic-planning. Zoo Biology 12:45-80.

Wildt, D. E., M. Bush, K. L. Goodrowe, C. Packer, A. E. Pusey, J. L. Brown, P. Joslin, and S. J. Obrien. 1987. Reproductive and genetic consequences of founding isolated lion populations. Nature 329:328-331.

Wildt, D. E., J. G. Howard, P. K. Chakraborty, and M. Bush. 1986. Reproductive physiology of the clouded leopard 2. A circannual analysis of adrenal-pituitary-testicular relationships during electroejaculation or after an adrenocorticotropin hormone challenge. Biology of Reproduction 34:949-959.

Ziegler, T. E., C. V. Santos, A. Pissinatti, and K. B. Strier. 1997. Steroid excretion during the ovarian cycle in captive and wild muriquis, *Brachyteles arachnoides*. American Journal of Primatology 42:311-321.

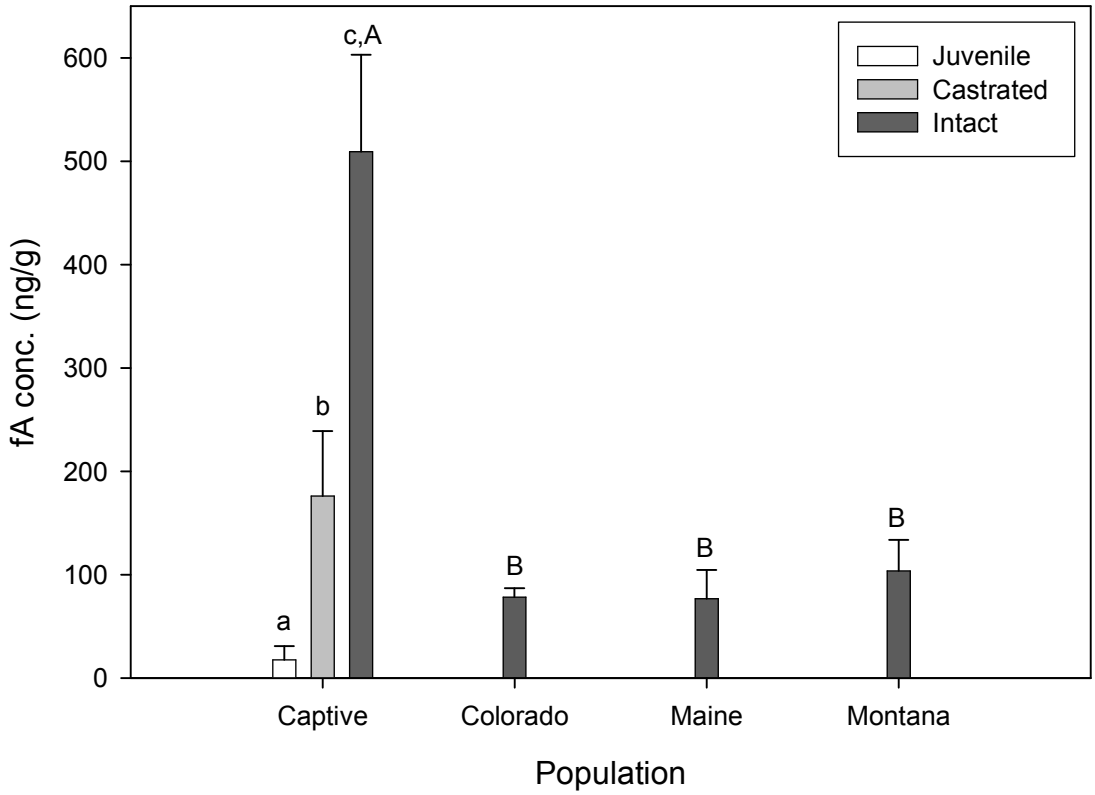

Fig. 2- 1. Population and status differences in fA expression (back-transformed LS mean ± SE; concentrations were adjusted for water content). Letters indicate statistically significant differences ($P<0.05$): lower-case letters pertain to status comparisons within the captive population, and upper-case letters apply to population comparisons.

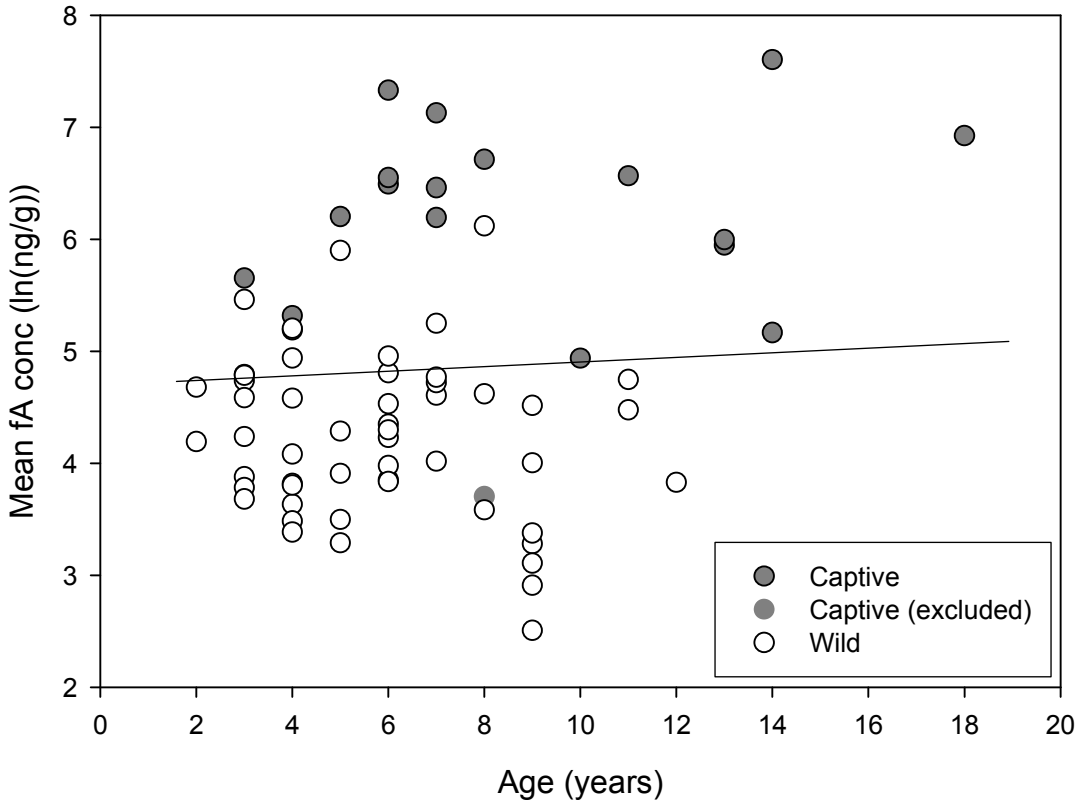

Fig. 2- 2. Effect of age on fA concentration. See text for discussion of excluded male M13. Note that the y-axis is in log-scale.

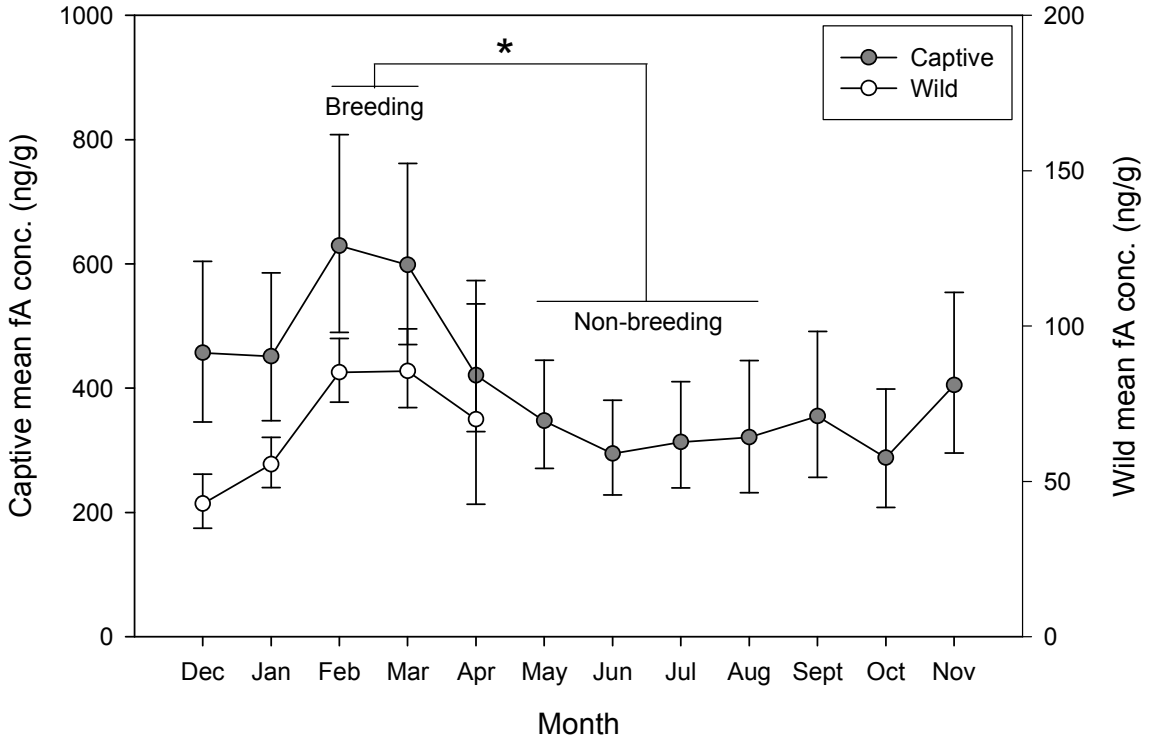

Fig. 2- 3. Seasonal patterns of androgen expression (back-transformed LS mean ± SE). Asterisk indicates a statistically significant difference between breeding and non-breeding seasons for captive males.

CHAPTER 3. MONITORING PATTERNS OF OVARIAN AND LUTEAL ACTIVITY IN CAPTIVE AND WILD CANADA LYNX

Introduction

Canada lynx (*Lynx canadensis*) populations have suffered notable declines in the past 50 years. There is relatively little information about basic lynx physiology, particularly reproductive physiology, and such knowledge may aid the development of conservation strategies. Specifically, there are two characteristics of lynx reproduction that must be considered in relation to population dynamics, captive breeding efforts, and conservation programs. First, Canada lynx are highly seasonal breeders (Poole 2003). Their breeding season lasts from late February to early April and they give birth primarily in May, with a gestation period of 60-70 days (Nowak 1999, Ruggiero et al. 2000). Even in the southern part of their range and in captivity, females only breed once per year and most kittens are born in May. Canada lynx can therefore be regarded as a truly seasonal felid species. Although the species' breeding season lasts about two months, individual females may have a much more restricted window of mating opportunity. Captive females exhibit signs of estrus or receptive behavior for one week or less. Little is known about estrus in wild lynx, but behavioral indicators suggest that females are only receptive for a short period. Mating pairs only remain together for several days and females presumably mate with only one male (Ruggiero et al. 2000). There is no published information about the duration or the length of estrus in captive or wild Canada lynx (Nowak 1999).

The second unique characteristic of lynx reproduction is that recruitment fluctuates dramatically with snowshoe hare abundance, especially in the northern part of their range (Ruggiero et al. 2000). Hare abundance correlates positively with pregnancy/conception rates, birth rates, average litter size, and kitten survival, and negatively with female age at sexual maturity (Ruggiero et al. 2000, Poole 2003). Biologists have also speculated that females ovulate spontaneously when prey densities are high, but become induced ovulators when prey densities are low (Ruggiero et al. 2000). Thus, lynx reproduction is closely linked to snowshoe hare population dynamics.

Canada lynx face a unique combination of breeding restrictions compared to other felids. Physiological adaptations to these annual and decadal breeding restrictions may impact captive breeding and conservation programs. Historically, it has been widely assumed that Canada lynx breed well in captivity, in part because of their prominent place in the fur industry. However, recent data do not support these claims. Between 2000 and 2008, there has only been one successful lynx birth (defined as survival of kittens beyond two weeks) at an institution accredited by American Association of Zoos and Aquariums (Goff 2008). Additionally, the number of private lynx breeders has been declining recently due to erratic breeding results (L. Culver, pers. comm.). Mounting evidence suggests we must re-evaluate the notion that Canada lynx "typically do reproduce prolifically in captivity" (Mellen 1991); we cannot take successful breeding for granted.

The goal of this study is to establish basic knowledge about the reproductive physiology of captive and wild female Canada lynx. Using the non-invasive technique of fecal hormone metabolite analysis, we monitored concentrations of fecal estrogen (fE) and progestogen (fP) metabolites. Specifically, our objectives were to 1) validate an assay for monitoring ovarian/luteal activity non-invasively, 2) characterize normative patterns of estrogen and progestogen expression, 3) compare patterns of hormone expression between captive and wild lynx, and 4) examine the effect of breeding status and housing situation on fE and fP in captive animals. As the first longitudinal study of estrogen and progestogen expression in wild and captive Canada lynx, our results provide a foundation for future studies of this kind and may also inform the development of stronger management plans for this species that can now include such basic physiological information.

Methods

Animals and Fecal Sample Collection

Captive – This study included a total of 26 captive Canada lynx females from 17 institutions (9 spayed females and 19 intact females; Appendix 1). [Note: two females were spayed during the study, so they are counted in both groups.] Three of the females became pregnant during the study and each gave birth to a single kitten. We also identified three possible pseudo-pregnancies, defined as confirmed mating associated with an increase in fP values, but no

subsequent parturition. 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. Sample collection was primarily centered around the breeding season (January – May), but we also obtained circ-annual data for several individuals. When multiple lynx were housed together, a marker (e.g., millet, icing dye, seed beads) was delivered to each individual in a food treat and used to distinguish between feces. All samples were stored in zip-lock bags at -20°C, and were shipped on ice to Brookfield Zoo for analysis.

Wild – Fecal samples were also collected from wild females in Colorado (56 lynx, 601 samples), Maine (8 lynx, 45 samples), and Montana (10 lynx, 52 samples). This dataset includes samples collected during 18 pregnancies (16 females), as confirmed by the observation of kittens. The populations in Maine and Montana are naturally-occurring, while the population in Colorado is reintroduced. All samples were collected by snow-tracking radio-collared lynx between December and April. Samples were collected from 1999 to 2008. Age of the sample is an important consideration with fecal hormone analysis, and samples could not be collected immediately after defecation in the field. However, evidence suggests that in winter field conditions, fE and fP remain stable for at least 4 days (see Chapter 1). Furthermore, for logistical reasons, trackers often followed fresh tracks (<24 h old), ensuring that fecal metabolites had probably not degraded and we were able to obtain meaningful results from these samples.

For most analyses involving lynx in Colorado, we only included samples that had been collected more than six months post-release in order to minimize any effect of translocation on hormone expression. However, for the pregnancy analysis, we included a subset of data collected while the lynx were housed in temporary holding pens (6 non-pregnant and 6 pregnant females, all in holding pens concurrently). All lynx reintroduced to Colorado were trapped in Canada or Alaska, transported to Colorado, and held in holding pens for 1-4 months prior to their release. In the first year of the reintroduction, Colorado received six pregnant lynx, which was confirmed by radiography (Shenk 2001). As the females were released prior to giving birth (early-mid May), the outcome of the pregnancies is unknown. However, based on radio- and snow-tracking data, there was no indication that any of the females established dens or were traveling with kittens; these six pregnancies were most likely unsuccessful.

Steroid Extraction and Analysis

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 1500 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.

Single-antibody enzyme-immunoassays (EIAs) were used to quantify fecal estrogen (fE) and progesterone (fP) metabolites (see Appendix 1 for assay procedures; also see Graham et al. 2001, deCatanzaro et al. 2003, Atsalis et al. 2004). Both antibodies and corresponding horseradish peroxidase (HRP) conjugates were obtained from C. Munro (University of California, Davis, CA). The assays were biochemically validated for Canada lynx by demonstrating 1) parallelism between serially diluted extracts and the standard curve, and 2) significant (>80%) recovery of exogenous estradiol/progesterone added to fecal extracts. To monitor precision and reproducibility, low (~70% binding) and high (~30% binding) quality control samples were run on each plate.

The estradiol-17β antibody R4972 was used to quantify fE. This antibody had the following cross-reactivities: 100% estradiol-17β, 3.3% estrone, 1% testosterone, 1% androstenedione, and 0.8% progesterone (deCatanzaro et al. 2003). Assay sensitivity was 0.39 ng/well. Intra-assay coefficients of variation were 13.9% and 11.5% (n=14) for low and high controls, respectively. The inter-assay coefficients of variation were 14% and 13% (n=144), respectively.

The progesterone monoclonal antibody CL425 was used to quantify fP. The progesterone antibody had the following cross-reactivities: 100% progesterone, 55% 5α-pregnen-3,20-dione and <0.1% pregnanediol, androstenedione, corticosterone (see Graham et al. 2001 for a more comprehensive list). Assay sensitivity was 0.05 ng/well. Intra-assay coefficients of variation were 8.5% and 6.4% (n=14) for low and high controls, respectively. The inter-assay coefficients of variation were 22% and 14% (n=143), respectively. Inter-assay variation for a given individual was minimized by analyzing all of their samples at the same time, when feasible.

[Note: Because the biological validation was not unequivocal (see below), we tried using several different assays with different antibody cross-reactivities which might react with other fecal hormone metabolites. To monitor fecal estrogens, we also tried an estrone conjugates antibody (E1C, C. Munro, University of California, Davis, CA) and an estrogen antibody (E. Möstl, University of Veterinary Medicine, Vienna, Austria). Both assays yielded similar results as the estradiol R4972 assay. For fecal progestogens, we also tried a polyclonal progesterone

antibody (R5864, C. Munro, University of California, Davis, CA), a pregnanediol glucuronide antibody (PdG, C. Munro, University of California, Davis, CA), and two commercial progesterone assays (Assay Designs, Ann Arbor, MI and Cayman Chemicals, Ann Arbor, MI). While longitudinal profiles varied slightly between assays, none of the other progesterone assays could be more clearly validated than the progesterone CL425 antibody (particularly in regards to pregnancy), so we did not include any of those results here.]

Statistical Analysis

All data were analyzed using SAS 9.1 (Cary, NC). We were not able to manipulate conditions for the lynx, which resulted in a somewhat unbalanced dataset and dictated the statistical analyses we could run. For all analyses, we modeled fE and fP separately. Hormone data were log transformed, and residuals for each model were analyzed to confirm that assumptions of normality and homoscedasticity were met. Means provided in text and figures are least-squares means that have been back-transformed. One captive female (F15) had unusually low fE and fP values (see Discussion for additional information). Therefore, she was excluded from all analyses except seasonality, in which she was included because her general pattern of hormone expression was similar to other females.

Previous experiments revealed that both wet and dried (lyophilized) fecal samples yielded very similar longitudinal profiles (see Chapter 1). Consequently, we adopted a wet-weight extraction protocol. However, further analysis indicated that estimated water content of the sample *did* have a systematic effect on measured concentration; concentration decreased with increasing water content (see Chapter 1). To account for the effect of water, mean water content was included in all analyses. For the samples that were missing an estimate of water content (~1/4 of the wild samples), we interpolated values using median water content.

Population

One-way ANOVAs were used to determine whether hormone concentrations varied between the four populations (one captive population and three wild populations: CO, ME, and MT). Only adult, intact, non-pregnant individuals were included in the model. A Tukey-Kramer adjustment was used to correct for multiple pairwise comparisons between populations.

Status and age

To examine the effect of reproductive status on fecal metabolite values, we compared fE and fP values between spayed, intact/non-pregnant (hereafter referred to as "intact"), pregnant, and pseudo-pregnant females using an ANCOVA. Mean hormone concentrations were calculated for each individual by status, as a few individuals were sampled in multiple reproductive states. For this analysis we treated them as independent observations. To control for potential effects of age, we also included each individual's mean age in the models. Captive females ranged in age from 3-18 years old, holding pen females ranged from 1-11 years old, and wild females ranged from 1-12 years old. For captive lynx, we found that 2-year old females had significantly lower concentrations of both fE and fP, so they were excluded from this analysis (see Discussion for further treatment of this topic). Because the wild populations did not include any spayed individuals and we could not identify pseudo-pregnancies, separate models were run for captive and wild populations. The "wild" model also included data from 12 females (6 pregnant and 6 non-pregnant) in holding pens, so we controlled for location (wild or holding pen) in this model. Two-way interactions were excluded if they were not significant. A Tukey-Kramer adjustment was used to correct for multiple comparisons within the captive population.

Although sample sizes were small, we ran a paired t-test for the two lynx that were spayed during the course of the study. Since they were both 2 years old, they were excluded from the ANCOVA described above, which is why we ran a separate analysis to examine the effect of spaying within an individual.

For several wild females, we had data spanning multiple years (n=22, difference in age=2-7 years). To determine if there was any effect of age on fE or fP *within* an individual, we ran a random coefficients model, which estimates a slope and intercept for each lynx and tests if the population of slopes and intercepts differs from zero (Littell et al. 2006).

Seasonality

We conducted a repeated measures ANOVA to test the effect of month on fE and fP values in captive and wild lynx. The model also controlled for the effect of year (sample collection from wild lynx spanned 10 years; captive collection only spanned 3 years). Only intact, non-pregnant females were included, and captive and wild populations were run in

separate models because sampling in the wild occurred during a more restricted window of time (December – April).

To specifically test for differences between breeding and non-breeding seasons in captive lynx, we performed a post hoc linear contrast of monthly means. For this analysis, we compared fE and fP concentrations in March and April (breeding season) to concentrations in June, July, August, and September (non-breeding months for which we had the most data).

Since reproductive seasonality in many temperate felids is influenced by photoperiod (Brown et al. 2002), we were interested in whether patterns of fE expression varied by latitude. We ran a simple regression to test the effect of latitude on the timing of peak fE values in captive females. (This model was not run for fP because increases in fP did not coincide with the breeding season.) This test included seven intact females with the most complete fE profiles during the breeding season. We excluded females who were pregnant, pseudo-pregnant, or exhibited ovarian suppression (see "Results – Housing Situation"). Each individual's mean weekly fE values were used to determine when their maximum fE occurred (weeks were numbered 1-52, with week 1 starting January 1 of the year sampling occurred).

Housing situation

Keepers at each captive institution were asked to provide information about each female's past breeding success and housing situation. Past breeding success was scored as either proven breeder (has given birth to at least one litter), non-breeder (has been housed with a suitable mate for at least two years, but has not successfully given birth), or unsure (not housed with suitable mate for at least two years). Information about housing included number, sex, and status (intact or neutered) of cage-mates.

Because past breeding success was too closely correlated with housing situation, we could not include both factors in this model. However, when past breeding success was analyzed separately, there was no significant difference in fE or fP between breeders and non-breeders. Additionally, since all intact females were housed with intact cage-mates, we excluded cage-mate status from the model. The final ANOVA included cage-mate sex and number, and only included intact females. As with the age/status model, the 2-year old females were excluded since their fE and fP levels were lower and they were all housed with females, which might influence the

results of cage-mate sex. A Tukey-Kramer adjustment was used to correct for multiple comparisons between housing situations.

Results

Longitudinal profiles

Looking at longitudinal profiles, we were unable to associate individual fE peaks (defined as points that fell more than 2.5 SD above baseline) with reported estrus behaviors or known ovulations (defined as specific mating events that resulted in pregnancy). However, the majority of fE peaks occurred during March or April, which coincides with the breeding season and with the general timeframe of any observed estrus behaviors and matings. Some females exhibited a few discrete fE peaks during the breeding season, while other females showed a more sustained elevation of fE values throughout the breeding season. Although most peaks occurred during the breeding season, we also observed some peaks later in the year.

Another unexpected finding was that fE and fP show correlated patterns of expression. This correlation is particularly prominent during the breeding season; trajectories of the two hormones tend to diverge later in the year (Fig. 3-1).

It was difficult to identify pregnancies based on longitudinal fP profiles (Fig. 3-2). Although there was a trend towards higher fP values in pregnant females, this difference was rather subtle. One female (F1) had higher fP concentrations towards the end of her pregnancy, while another female (F17) exhibited more of an increase in the middle of her pregnancy. Both of these females were experienced breeders, but each only gave birth to one kitten which was pulled quickly to be hand-reared. The youngest female (F4), who was also an inexperienced breeder, showed the most obvious increase in fP throughout her pregnancy, although it was still not nearly as pronounced as pregnancy profiles published for other felid species (Graham et al. 1995, Brown 2006). This female also gave birth to just one kitten, and it died shortly after birth. Also, fE remained elevated or even increased during pregnancy, in a parallel manner to fP. For pregnant females in holding pens, fP profiles were quite variable, but patterns were generally similar to those of pregnant females in captivity (Fig. 3-2).

We identified three pseudo-pregnancies among captive females, defined as mating followed by an increase in fP, but no parturition. Hormone profiles were quite similar for pseudo-

pregnancies and pregnancies. Both fE and fP values tended to be higher and were sustained for the duration of a typical pregnancy. The clearest difference between non-pregnant and pregnant/pseudo-pregnant hormone profiles appeared to be the duration of increase in fE (Fig. 3-3). In non-pregnant females (particularly when housed with males), fE levels often increased early in the breeding season and decreased by late March. In contrast, pregnant/pseudo-pregnant females maintained elevated fE levels for the duration of a typical pregnancy. While it is difficult to distinguish between pregnant and non-pregnant profiles, it is even more difficult to distinguish between pregnant and pseudo-pregnant profiles.

In several females, including females housed alone or with a female cage-mate, we observed dramatic increases in fP concentrations (Fig. 3-1). This suggests that female lynx are capable of ovulating spontaneously. Furthermore, the prolonged duration of elevated fP levels following such ovulations highlights the persistent nature of the corpora lutea (CLs). Following this elevation, fP levels occasionally return to baseline, but we are unable to identify the underlying cause of these drops. Similar decreases in fP are observed in other species during pregnancy, and do not seem to indicate a problem with luteal or placental functioning.

Population

Population had a significant effect on both fE and fP levels (fE: $F_{3,87}$=26.61, P<0.001; fP: $F_{3,87}$=7.96, P<0.001; Fig. 3-4). Pairwise comparisons revealed that captive lynx had higher fE and fP concentrations than all three wild populations (fE: vs. CO, t_{87}=8.11, P<0.001; vs. ME, t_{87}=3.25, P=0.009; vs. MT, t_{87}=7.23, P<0.001; fP: vs. CO, t_{87}=3.86, P=0.001; vs. ME, t_{87}=3.90, P=0.001; vs. MT, t_{87}=3.48, P=0.004). However, none of the wild populations were significantly different from each other (fE: CO vs. ME, t_{87}=1.43, P=0.48; CO vs. MT, t_{87}=1.99, P=0.20; ME vs. MT, t_{87}=2.49, P=0.07; fP: CO vs. ME, t_{87}=1.90, P=0.23; CO vs. MT, t_{87}=0.99, P=0.75; ME vs. MT, t_{87}=0.89, P=0.81).

Status

In captive females, status had a significant effect on fE ($F_{3,20}$=22.38, P<0.001; Fig. 3-5a) and fP concentrations ($F_{3,18}$=90.60, P<0.001; Fig. 3-5b). For both fE and fP, spayed females had

the lowest concentrations, and pregnant and pseudo-pregnant females had the highest concentrations.

Among wild and penned lynx, status had a significant effect on fE ($F_{1,83}$=17.07, P<0.001) and fP concentrations ($F_{1,83}$=9.21, P=0.003; Fig. 3-4 and 3-5). In holding pens as well as in the wild, pregnant females exhibited higher fE and fP levels than non-pregnant females. Females in holding pens had significantly higher fE and fP concentrations than wild females (fE: $F_{1,83}$=107.70, P<0.001; fP: $F_{1,83}$=15.71, P<0.001).

For the two lynx that were spayed during the study, both fE and fP concentrations decreased after they were spayed. This difference was not significant for fE ($F_{1,1}$=20.36, P=0.14), but was significant for fP ($F_{1,1}$=1809.88, P=0.02). Again, the sample size is quite small, but this result is a testament to how strong the difference in fP is between spayed and intact individuals.

Age

Age did not have a significant effect on fE or fP levels for captive females >2 years old (fE: β=0.05±0.03 ln(ng/g)/year, t_{20}=1.55, P=0.14; fP: β=0.04±0.03 ln(ng/g)/year, t_{18}=1.08, P=0.29), nor did it have a significant effect for wild females of all ages (fE: β= -0.03±0.02 ln(ng/g)/year, t_{83}=1.27, P=0.21; fP: β=0.07±0.06 ln(ng/g)/year, t_{83}=1.08, P=0.28; Fig. 3-6). At the individual level for the wild females, the random coefficients model indicated that neither fE ($F_{1,70}$=0.06, P=0.81) nor fP ($F_{1,70}$=0.14, P=0.71) changed significantly with age (n=22 lynx).

Seasonality

Month had a significant effect on fE and fP in both populations (fE: captive, $F_{11,73.8}$=3.00, P=0.002, wild, $F_{4,184}$=4.17, P=0.003; fP: captive, $F_{11,76}$=2.24, P=0.02, wild, $F_{4,191}$=8.85, P<0.001; Fig. 3-7). Year also had a significant effect on fE and fP in captive females (fE: $F_{3, 77.2}$=5.08, P=0.003; fP: $F_{3,82.8}$=4.07, P=0.009), but not in wild females (fE: $F_{9.170}$=1.62, P=0.11; fP: $F_{9,172}$=1.19, P=0.31).

Captive females had significantly higher fE values during breeding months than non-breeding months ($t_{76.5}$=4.66, P<0.001). Interestingly, fP concentrations were significantly lower during breeding months than later in the year ($t_{78.9}$=2.61, P=0.01).

For the seven females that we included in the analysis, latitude had a significant effect on timing of peak fE values (β=0.21±0.08 weeks/degree, $F_{1,5}$=7.67, P=0.04). An individual's highest weekly mean fE occurred about 5 weeks earlier at lower latitudes (early March) than at higher latitudes (mid-April; also see Fig. 3-8).

Housing situation

The sex of a female's cage-mate had a significant effect on fE concentrations ($F_{2,10}$=6.22, P=0.02) but not on fP concentrations ($F_{2,10}$=1.33, P=0.31). Lynx housed with another female had significantly lower fE concentrations than lynx housed with a male (t_{10}=3.46, P=0.02). The number of cage-mates a female was housed with did not have a significant effect on fE ($F_{1,10}$=0.01, P=0.99) or fP concentrations ($F_{1,10}$=0.57; P=0.47).

Furthermore, when we overlay longitudinal fE profiles for females housed together (including the 2 year old females that were excluded from the above statistical model), there is evidence of ovarian suppression. In two of the three "female only" groups (Alaska Zoo and Wildlife Science Center), one of the females had dramatically lower fE values during the breeding season, and exhibited little to no seasonal increase (Fig. 3-8).

Discussion

Longitudinal profiles

Although fE surges can be used to identify estrus in some other felid species (see Brown 2006), we failed to detect fE peaks associated with documented estrous behavior or known ovulations. We did observe some distinct peaks in fE during the breeding season, but cannot confirm the significance of these peaks because we were unable to correlate them with behavioral notes, known ovulations (pregnancy), or subsequent increases in fP, which would have confirmed ovulation and formation of a corpus luteum (CL). Other researchers have documented similar fE results in other *Lynx* species [Eurasian lynx, *L. lynx* (Jewgenow et al. 2006a, Dehnhard et al. 2008); Iberian lynx, *L. pardinus* (Pelican et al. 2006, Braun et al. 2009); bobcat, *L. rufus* (Shille 1991)]. Fecal hormone metabolite analysis does not lend itself readily for identifying clear

associations between behaviors and individual hormone peaks due to variability in hormone metabolism and metabolite excretion time within and across individuals (Palme et al. 2005). Analyses of such specifics can be challenging, and has posed difficulties in species that do not show regular estrus cycles, such as felids.

We also observed that fE and fP show correlated patterns of expression, particularly during the breeding season. This is contrary to what we would expect to see, because luteal production of progesterone typically suppresses estrogen secretion, so these hormones should be inversely related. However, this trend has also been observed in other *Lynx* species (Jewgenow et al. 2006b, Pelican et al. 2006, Dehnhard et al. 2008). Additionally, covariance between fE and fP has also been reported during estrus in some non-*Lynx* species [e.g., ocelot and jaguar (Brown 2006)], and it has been speculated that some fP may be follicular in origin (Brown 2006).

The use of fecal hormone metabolite analysis to monitor pregnancy has become somewhat routine in several exotic felid species (e.g., Graham et al. 2006, Putranto et al. 2007, van Dorsser et al. 2007). In those species, pregnancy is characterized by a significant increase in fP a few days after mating, which is sustained until parturition. Although we observed a subtle increase in fP during pregnancy, the overall signal was quite noisy and not particularly useful as a diagnostic tool.

One possible explanation for the unusual fP profiles during pregnancy (i.e., the lack of increase in fP concentrations) is that the pregnancies we sampled were not "typical" pregnancies. All three captive females only gave birth to one kitten, as opposed to the normal litter size of 2-5 kittens (Poole 2003), and one of the females contracted an undiagnosed illness in the first trimester. Furthermore, one of the kittens died shortly after birth. The other two survived, but were pulled within a few days and hand-reared, so we do not know what their fate would have been had they remained with the mother. For the six lynx that were pregnant in the holding pens, the process of being captured and translocated during the early stages of pregnancy could certainly have impacted hormone expression. Since all the lynx in holding pens were released prior to parturition, the outcome of those pregnancies is largely unknown. However, movement patterns and tracking data indicate that even if these females gave birth, they were not able to raise the kittens for any length of time because there was no evidence that the females were accompanied by cubs. Thus, the nine pregnancies for which we were able to obtain longitudinal hormone data were arguably not "normal." However, several wild females did appear to have "normal" pregnancies, as defined by multiple kittens/litter and kitten survival. Although wild females had drastically lower fP concentrations overall compared to "captive" and "holding pen"

lynx, the relative fP increase in pregnant females is similar for all three populations. This suggests that the lack of increase in fP concentrations during pregnancy is not necessarily associated with the outcome of the pregnancy.

Interestingly, fP are not useful for identifying pregnancy in any of the other three *Lynx* species, either. In the Iberian lynx, neither fecal nor urinary progestogens increase during pregnancy (Pelican et al. 2006, Braun et al. 2009). While Eurasian lynx exhibit a slight increase in fP during pregnancy, the difference was not particularly clear, and fP concentrations did not decrease at parturition (Jewgenow et al. 2006a, Dehnhard et al. 2008). In bobcats, peaks in fP occur just prior to mid-gestation, but concentrations also return to baseline during pregnancy, so again, it is difficult to confidently identify pregnancy (Miller 1995).

Pseudo-pregnancies are quite common in felid species, but it is unclear whether pseudo-pregnancies are strictly a hormonal signal, or whether they are associated with abortion or reabsorption of a fetus. In several felid species, pseudo-pregnancies can be distinguished from pregnancies based on the length of time that fPs are elevated: pseudo-pregnancies are usually about half as long as regular pregnancies (Brown 2006). However, in Canada lynx, we were unable to distinguish between pregnancies and pseudo-pregnancies. Once again, this coincides with findings in other *Lynx* species [Eurasian lynx (Jewgenow et al. 2006a, Dehnhard et al. 2008); Iberian lynx (Pelican et al. 2006, Braun et al. 2009)].

Thus, there seem to be two unusual characteristics of fE and fP in *Lynx* species compared to other felids: 1) fE and fP can follow parallel trajectories, and 2) although fP (and fE) concentrations increase during pregnancy, the increase is not substantial enough to provide a useful indicator of pregnancy. In light of these findings, an obvious question is whether or not fecal hormone metabolites accurately reflect biological signals in *Lynx* species. There are several factors that may affect quantification of fecal hormone metabolites: sources of circulating steroids, metabolism and excretion of steroids, and the ability of an assay to recognize excreted hormone metabolites.

A radiometabolism study with Eurasian lynx revealed that steroid metabolites are primarily excreted in the feces (K. Jewgenow, unpubl. data), as is the case for most felids (Palme et al. 2005). Estradiol was excreted as ~50% authentic estradiol/estrone and ~50% polar conjugates. Progesterone was fairly heavily metabolized, and excreted almost entirely as polar conjugates; there was very little authentic progesterone in the feces (Dehnhard et al. 2008, Dehnhard et al. *in review*). This matches findings from other studies that very little parent hormone is excreted (Brown et al. 1994, Palme et al. 2005). Subsequent immunoassays revealed

that the estradiol and progesterone antibodies used still reacted with several of the metabolites derived from their respective hormones (Dehnhard et al. 2008, Dehnhard et al. *in review*), and thus were appropriate for assessing patterns of circulating estradiol and progesterone expression.

For any given hormone, antibodies can have notable differences in their ability to bind with metabolites of that hormone (Möstl and Brunner 1997). To determine whether improper antibody binding was interfering with our ability to detect a pregnancy signal, we tested five different progesterone antibodies (see Methods). While some antibodies indicated a slight increase in fP towards the end of the pregnancy, none showed the characteristic elevation seen in other felid species (Graham et al. 1995, Brown 2006). Furthermore, different progesterone assays have been used for all four *Lynx* species, yet all studies obtained similar results (Dehnhard et al. 2008, Braun et al. 2009). Finally, a comparative HPLC study revealed that metabolism of estrogens and progestogens was conserved among *Lynx* based on elution profiles, and that estrogen and progesterone assays exhibited a high degree of immunoreactivity with several of their respective metabolites (Dehnhard et al. *in review*).

Since steroid metabolism, excretion, and immunoreactivity do not appear to be inhibiting quantification of fE and fP, we must consider the source of circulating estrogens and progestogens. There is some evidence in Canada lynx that fE may be of adrenal origin. We observed fE spikes that coincided with physical exams and particularly aggressive fights between cage-mates. We also observed fE spikes in spayed females. Furthermore, the fact that spayed females still have quantifiable levels of fE suggests that there is a non-ovarian source of estrogens. In humans, the adrenal cortex is known to produce androstenedione which is then converted to estrone by the liver (Lievertz 1987).

Another possible source of estrogens and progestogens is the corpus luteum (CL). The primary source of circulating progesterone in most female mammals is the CL. In felids, CLs persist for the duration of the pregnancy, or for one-third to one-half the length of gestation during non-pregnant luteal phases/pseudo-pregnancies (Brown 2006). The placenta is not a major source of progestogens in most felids, and thus pregnancy is maintained through progesterone production by CLs (Verstegen et al. 1992).

There is growing evidence that CLs persist for extensive periods of time in all four *Lynx* species [Canada lynx (Nellis et al. 1972); Eurasian lynx (Kvam 1990, Dehnhard et al. 2008); bobcats (Duke 1949, Pollack 1950, Johnson and Holloran 1985, Woshner et al. 2001)]. Given this, it seems that fPs do accurately reflect luteal activity in *Lynx*. Of the four *Lynx* species, CL life history and function has perhaps best been described for bobcats (Duke 1949, Woshner 1988,

Miller 1995). The bobcat retains luteal bodies between cycles, perhaps for life, and these CLs are capable of producing progesterone (Woshner 1988). These CLs might also contribute to circulating estrogens. Duke (1949) proclaims "[t]he life history of the corpus luteum of the bobcat is an intriguing puzzle." Indeed, this puzzle seems to be relevant to the entire *Lynx* genus.

The function of these persistent CLs is not clear, nor is it known how females might accommodate this prolonged elevation of circulating progesterone while maintaining normal patterns of reproduction. However, it has been speculated that the existence of luteal bodies from previous cycles actually facilitates successful reproduction in bobcats (Miller 1995). Persistent CLs might also help regulate seasonal changes in reproductive physiology and maintain the strong seasonality observed in lynx. Clearly, the basic reproductive endocrinology of *Lynx* and the role/contribution of persistent CLs merit much more attention.

Population

Captive females have dramatically higher fE and fP levels than wild populations. This same trend is also seen in fecal androgen metabolites in male Canada lynx (see Chapter 2). There are very few studies that compare fecal hormone concentrations between wild and captive populations, and results are variable between studies (Ziegler et al. 1997, Shiho Fujita 2001, Terio et al. 2004). Similar to our findings, Shiho Fujita et al. (2001) found that fecal progestogens and estrogens were both lower in wild Japanese macaque females than in captive ones. There was no clear methodological reason for this trend, and the authors attributed it to unknown dietary, ecological, or social factors. Terio et al. (2004) also found that fecal estrogens were lower in wild female cheetahs, but they attributed this to sampling error (specifically, some of the wild samples might have been from males).

We suspect that the population differences observed in Canada lynx may reflect differences in metabolic rate or body condition. Fecal hormone metabolites can be affected by diet, metabolic rate, or body condition (von der Ohe and Servheen 2002, Wielebnowski and Watters 2007). Given their regular access to food and often limited capacity for movement/exercise, it is quite probable that captive lynx have a different metabolic rate and/or body condition than wild animals. It is worth noting that wild lynx in holding pens for 21 – 128 days have fE and fP concentrations similar to captive lynx (Fig. 3-4 and 5). In the holding pens, lynx almost certainly have lower activity levels than in the field and they also have more regular access to food, similar to captive lynx.

In addition, one captive female (F15) was excluded from most analyses due to low steroid metabolite concentrations. Interestingly, her fE and fP values were similar to wild females. Her male cage-mate also had unusually low fecal androgen metabolites compared to other captive males (see Chapter 2). Based on our findings with other captive lynx, the fact that F15 was housed with a male predicts that she should have higher fE values. However, this pair was housed in Newfoundland in one of the largest enclosures (4600 m^2; the second largest enclosure was only 500 m^2, and the average enclosure size for females, excluding F15, was 300 m^2). Also, their diet contained more fish than other captive lynx, which may affect metabolism. Furthermore, F15 had a low body weight compared to other captive females (although we did not find a significant relationship between body weight and fE or fP; K. Fanson, unpubl. data). Given the climatic conditions and enclosure size, her activity levels/energetic demands may more closely approximate those of a wild lynx. This may suggest that the observed population difference may be linked to differences in energy regulation.

The implications of this difference are unclear. It is well known that diet, metabolism, and body condition can affect dynamics of steroid endocrinology (Tchernof et al. 1996, Hajamor et al. 2003), and that steroid dysfunction may result in different pathologies. In light of the recent poor breeding success in captivity, the elevated steroid levels in captive lynx and their connection to energy regulation merit closer examination. Two private lynx breeders have independently informed us that diet is critical during the breeding season, although the specifics of that requirement are unclear (L. Culver and R. Wood-Samman, pers. comm.). One breeder adds any red meat to the female's diet during pregnancy, and another specifically prefers beaver. Successful fur farms have developed a special diet for lynx (L. Culver, pers. comm.). Interestingly, the one captive pair with hormone concentrations similar to wild lynx have not successfully bred, and they have been paired for seven years. The female conceived one year, but gave birth to one still-born kitten. However, this pair also has more fish (and less red meat) in their diet. Thus, dietary and metabolic differences between captive and wild lynx may affect a variety of other factors, such as reproduction and overall health of captive animals. The sensitivity of the hypothalamic-pituitary-gonadal (HPG) axis to changes in prey type or availability may be an evolutionary response to the 10-year snowshoe hare cycle, and may help regulate energy allocation. However, further research is required to understand the mechanisms underlying this potential link between reproduction and diet, the evolutionary significance, and implications for captive management.

Status

Spayed vs. Intact

Spayed females had significantly lower fE concentrations than intact females, suggesting that the assay does detect biologically relevant changes in ovarian activity. However, spayed females still had a considerable amount of immunoreactive estrogen metabolites. This suggests that some of the estrogen metabolites detected by the assay may have been produced by another source, such as the adrenal glands. While non-ovarian sources of estrogen must be considered when interpreting results, the observed intact-spay results confirm that ovarian activity is reflected in fE.

Levels of fP were also significantly lower in spayed females. In fact, for the 2 females that were spayed during the study, fP concentrations decreased more dramatically than fE concentrations following the ovariectomy. Corpora lutea (CLs) are a primary source of circulating progestogens in many mammals. Based on our knowledge of luteal activity in other felids, fP concentrations should be quite low except during pregnancies or pseudopregnancies / non-pregnant luteal phases (Graham et al. 1995, Brown 2006). Therefore, we would not expect such a dramatic difference in baseline fP between intact and spayed females. Other felid species also exhibit lower fP values in ovariectomized and pre-pubertal females as compared to intact females (~1/6 to 1/3 lower; Graham et al. 1995). However, the difference is not as dramatic as we observed in Canada lynx. The fact that baseline fP levels are so high in intact/non-pregnant females is further evidence of the existence of persistent CLs, which contribute to circulating progesterone levels.

One critical note is that many of the intact females had never been mated, or even housed with a male, including the two females that were spayed during the study. Therefore, if fP are indeed an index of luteal activity as our results suggest, Canada lynx must ovulate spontaneously, at least on occasion. Felids exhibit an incredible diversity in ovulatory mechanisms (Brown 2006). Although they have historically been thought of as induced/reflex ovulators, recent studies have revealed not only that some species can ovulate spontaneously, but that some individuals may alternate between the two strategies. Indeed, it has been speculated that Canada lynx ovulate spontaneously at high population densities and reflexively at low population densities (Ruggiero et al. 2000). We were not able to identify the frequency at which induced and spontaneous ovulations occurred. However, the existence of spontaneous ovulation in Canada

lynx is supported by the fact that 1) virgin females exhibited a dramatic decline in fP when their ovaries (and consequently CLs) were removed, and 2) fP concentrations increased in unmated females following the breeding season.

Intact vs. Pregnant

It is surprising that fP concentrations were not higher in pregnant females. Although the difference between intact and pregnant females was significant for wild and holding-pen lynx, we expected the magnitude of this difference to be greater. In other felids, fP values increase 5 to 30-fold during pregnancy (Graham et al. 1995), whereas in all three Canada lynx populations, fP values only doubled (Fig. 3-5). Given the subtlety of the increase in fP concentrations, this does not appear to be a useful diagnostic method for identifying lynx pregnancies.

Pregnant females also had higher fE concentrations, although this difference was not significant for captive females. Typically, fE concentrations return to baseline during pregnancy. As mentioned above, the covariance between fE and fP has also been documented in other *Lynx* species (Pelican et al. 2006, Dehnhard et al. 2008). This may also indicate that CLs are a source of fE in lynx (Brown 2006).

Age

With the exception of captive 2-year old females, age did not have a significant effect on fE or fP levels. This suggests that although CLs may persist for some time, there must either be some turn-over in luteal bodies between breeding seasons, or that progesterone production by CLs changes over time. Indeed, in bobcats, Woshner et al. (2001) found that older CLs did produce lower levels of progesterone than fresh CLs. Again, we emphasize that further studies are needed to better understand the "life history" of lynx CLs and the role of chronically elevated progesterone in lynx reproduction.

We also found it interesting that captive 2-year old females had lower fE and fP concentrations, because we did not observe this trend in wild lynx. Canada lynx are generally reproductively mature by 9 months of age (first breeding season), although when prey density is low, they may not become reproductively mature until their second winter (Ruggiero et al. 2000).

Therefore, all females in this study should have been reproductively mature. There are a few possible explanations for why young captive females had lower fE and fP levels.

First, it may be a characteristic part of the maturation process. Pelican et al. (2006) also found that 2-year old Iberian lynx had significantly lower fE and fP values than older females. However, we did not observe a similar result in wild females, and there were even younger females included in that population. One possibility is that reproductive maturity is delayed in captive females.

Alternatively it could be a consequence of social situation. All of the 2-year old females were housed in "female only" groups. We found that lynx housed with a female cage-mate had lower fE concentrations, and although it was not significant, there was a trend toward lower fP concentrations as well. The effect of housing a solitary species in same-sex situations may lead to ovarian suppression or delayed onset of reproductive maturity (see below). Ovarian suppression has been documented in other felid species when females are housed together (Wielebnowski et al. 2002).

Seasonality

Fecal estrogen metabolites exhibited a clear increase associated with the breeding season. Although this pattern is somewhat muted in the wild population, it is still present. However, in the wild populations, the highest fE values occur in Feb/Mar (coinciding with peak levels of fecal androgens in wild males), while in captive females, the highest fE values occur in Mar/Apr (one month later than peak androgens in captive males). In looking at longitudinal fE profiles in captive females, we see quite a bit of variability in timing and duration of this seasonal increase in ovarian activity – particularly between institutions. At least some of this variability appears to be due to latitudinal variation. Pelican et al. (2006) found less variation in timing of ovarian activity in Iberian lynx, but all study animals were housed at the same institution.

Several other felid species exhibit some degree of reproductive seasonality (Brown 2006). Photoperiod appears to be the primary mediator of seasonality in these species, and reproductive cycles can be manipulated to some degree through changes in lighting regime (Brown et al. 2002, Brown 2006).

Interestingly, despite the fact that we see fE and fP track each other in some situations (specifically pregnancy), we see opposite patterns of expression circ-annually. Mean fP concentrations are lowest just prior to the breeding season, increase in March and April, and

remain elevated through December. Even if we exclude the 2-year old females, this pattern still remains. This again is consistent with the persistence of CLs and the occurrence of spontaneous ovulation. Five of the seven females housed in a "female only" group exhibited an increase in fP after the breeding season (e.g., see Fig. 3-1). The wild females also exhibit a pronounced increase in fP concentrations in March/April, but because we were unable to obtain any samples after April, we do not know the trajectory of fP concentrations for the remainder of the year.

Iberian lynx also exhibit a prolonged elevation of fP following the breeding season (Pelican et al. 2006). However, in Iberian lynx, fP values decreased around October and remained low until the following breeding season. We did not observe a decrease in fP concentrations for Canada lynx until January. Eurasian lynx exhibit a post-partum increase in not only fP, but also fE (Dehnhard et al. 2008). However, different antibodies were used for the fecal hormone analyses in the three species, which may account for some of the observed differences.

We also saw that "year" had a significant effect on fE and fP concentrations in captive females. This result is probably driven by sampling differences and changes in housing situation between years. Year was not a significant factor for the wild population.

Housing situation

Although the number of cage-mates did not affect hormone expression, cage-mate sex did significantly affect fE expression. Specifically, lynx housed with another female had significantly lower fE concentrations. When we examined female pairs, we often found that one of the females had lower fE concentrations and also failed to exhibit a seasonal increase in fE. Interestingly, the female that exhibits this ovarian suppression is the subordinate female, as ranked by keepers (K. Fanson, unpubl. data). In one of the female pairs, fE levels were generally similar between both females, and they both exhibited a seasonal increase around the breeding season. However, one female had much higher peak fE values, and she was also reported to exhibit more pronounced signs of estrus than her cage-mate.

For the fecal hormone studies on Eurasian and Iberian lynx, females were housed separately and only paired with a male for mating (Pelican et al. 2006, Dehnhard et al. 2008). Therefore, a cross-species comparison with respect to housing situation is not possible. However, ovarian suppression in female cats housed together has been documented in other studies (Wielebnowski et al. 2002).

It is well-known that social structure can have a tremendous impact on reproductive function, and social suppression has been documented in a wide variety of species (Wasser and Barash 1983, Mellen 1991). While social suppression of reproduction may help maintain social dynamics in wild populations, this phenomenon can become problematic in captive breeding populations. In a cross-institutional study including several small felid species, Mellen et al. (1991) found that females maintained in groups larger than a single male-female pair were less likely to reproduce and rarely succeeded in rearing any offspring produced. In light of these findings, we reiterate Mellen's suggestion that Canada lynx be housed in male-female pairs, and possibly separated outside of the breeding season.

Concluding Remarks

This study provides the first longitudinal investigation of female reproductive hormones in captive and wild Canada lynx. We were able to validate a fecal estrogen EIA for monitoring gross patterns of ovarian activity, although it does not appear appropriate for monitoring distinct ovarian events. Similarly, the fecal progestogen EIA we used may not be particularly useful for monitoring pregnancy, but appears to accurately reflect luteal activity. Our key findings are:

1) Females exhibit seasonal increases in fE during the breeding season, but this increase is shifted later in captive females.
2) Canada lynx appear to be capable of spontaneous ovulation, although the frequency of this is unknown.
3) Corpora lutea (CLs) are retained for an unusually long duration.
4) Captive females show much higher fE and fP levels than wild females, which may be associated with differences in energy regulation.
5) Female group housing may contribute to ovarian suppression and/or delayed reproductive maturity.

The unusual CL dynamics in Canada lynx and other *Lynx* species raise several interesting questions relevant to both basic and applied reproductive biology. The first and most apparent question is regarding the function and evolutionary significance of persistent CLs. At this point, it is unclear what role they play in lynx physiology. Second, it is unclear if there are implications of chronic elevation or progesterone (and possibly other steroids) for other aspects of lynx physiology. Finally, it would be interesting to examine how nutrition/body condition affects the

life history of the CL. If at least part of the function of persistent CLs is to restrict breeding and enforce strict seasonality, it would be interesting to know how they are affected by the 10-year lynx-hare cycle. Understanding the dynamics and function of CL activity in lynx is critical for the development of assisted reproductive technologies in this species.

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Literature Cited

Atsalis, S., S. W. Margulis, A. Bellem, and N. Wielebnowski. 2004. Sexual behavior and hormonal estrus cycles in captive aged lowland gorillas (*Gorilla gorilla*). American Journal of Primatology 62:123-132.

Braun, B. C., A. Frank, M. Dehnhard, C. C. Voigt, A. Vargas, F. Göritz, and K. Jewgenow. 2009. Pregnancy diagnosis in urine of Iberian lynx (*Lynx pardinus*). Theriogenology 71:754-761.

Brown, J. L. 2006. Comparative endocrinology of domestic and nondomestic felids. Theriogenology 66:25-36.

Brown, J. L., L. H. Graham, J. M. Wu, D. Collins, and W. F. Swanson. 2002. Reproductive endocrine responses to photoperiod and exogenous gonadotropins in the Pallas' cat (*Otocolobus manul*). Zoo Biology 21:347-364.

Brown, J. L., S. K. Wasser, D. E. Wildt, and L. H. Graham. 1994. Comparative aspects of steroid-hormone metabolism and ovarian activity in felids, measured noninvasively in feces. Biology of Reproduction 51:776-786.

deCatanzaro, D., C. Muir, E. Beaton, M. Jetha, and K. Nadella. 2003. Enzyme immunoassay of oestradiol, testosterone and progesterone in urine samples from female mice before and after insemination. Reproduction 126:407-414.

Dehnhard, M., S. Naidenko, A. Frank, B. Braun, F. Göritz, and K. Jewgenow. 2008. Non-invasive monitoring of hormones: A tool to improve reproduction in captive breeding of the Eurasian lynx. Reproduction in Domestic Animals 43:74-82.

Dehnhard, M., K. Fanson, F. Göritz, S. Naidenko, A. Vargas, and K. Jewgenow. *in review.* Comparative metabolism of gestagens and estrogens in the four lynx species, the Eurasian (*Lynx lynx*), the Iberian (*Lynx pardinus*), the Canada lynx (*Lynx canadensis*) and the bobcat (*Lynx rufus*). General and Comparative Endocrinology.

Duke, K. L. 1949. Some notes on the histology of the ovary of the bobcat (*Lynx*) with special reference to the corpora lutea. Anatomical Record 103:111-131.

Goff, D. 2008. North American Regional Canadian Lynx Studbook (*Lynx canadensis*), Bridgeport, CT: Connecticut's Beardsley Zoo.

Graham, L., F. Schwarzenberger, E. Möstl, W. Galama, and A. Savage. 2001. A versatile enzyme immunoassay for the determination of progestogens in feces and serum. Zoo Biology 20:227-236.

Graham, L. H., A. P. Byers, D. L. Armstrong, N. M. Loskutoff, W. F. Swanson, D. E. Wildt, and J. L. Brown. 2006. Natural and gonadotropin-induced ovarian activity in tigers (*Panthera tigris*) assessed by fecal steroid analyses. General and Comparative Endocrinology 147:362-370.

Graham, L. H., K. L. Goodrowe, J. I. Raeside, and R. M. Liptrap. 1995. Non-invasive monitoring of ovarian-function in several felid species by measurement of fecal estradiol-17-beta and progestins. Zoo Biology 14:223-237.

Hajamor, S., J. P. Despres, C. Couillard, S. Lemieux, A. Tremblay, D. Prud'homme, and A. Tchernof. 2003. Relationship between sex hormone-binding globulin levels and features of the metabolic syndrome. Metabolism-Clinical and Experimental 52:724-730.

Jewgenow, K., A. Frank, F. Göritz, A. Vargas, S. Naidenko, and M. Dehnhard. 2006a. A comparative analysis of the endocrine patterns of the Eurasian and the Iberian lynx in captivity. Pages 116-117 *in* Iberian lynx ex-situ conservation seminar series, Doñana.

Jewgenow, K., S. V. Naidenko, F. Goeritz, A. Vargas, and A. Dehnhard. 2006b. Monitoring testicular activity of male Eurasian (*Lynx lynx*) and Iberian (*Lynx pardinus*) lynx by fecal testosterone metabolite measurement. General and Comparative Endocrinology 149:151-158.

Johnson, N. F. and D. J. Holloran. 1985. Reproductive activity of Kansas bobcats. Journal of Wildlife Management 49:42-46.

Kvam, T. 1990. Ovulation Rates in European Lynx, *Lynx lynx*, from Norway. Zeitschrift Fur Saugetierkunde-International Journal of Mammalian Biology 55:315-320.

Lievertz, R. W. 1987. Pharmacology and pharmacokinetics of estrogens. American Journal of Obstetrics and Gynecology 156:1289-1293.

Littell, R. C., G. A. Milliken, W. W. Stroup, R. D. Wolfinger, and O. Schabenberger. 2006. SAS for Mixed Models, 2nd ed. SAS Institute Inc., Cary, North Carolina.

Mellen, J. D. 1991. Factors influencing reproductive success in small captive exotic felids (*Felis* spp.): A multiple regression analysis. Zoo Biology 10:95-110.

Miller, D. L. 1995. Feasibility of embryo transfer using the bobcat (*Felis rufus*). PhD. Mississippi State University.

Möstl, E. and I. Brunner. 1997. Comparison of different progestagen assays for measuring progesterone metabolites in faeces of the bitch. Journal of Veterinary Medicine Series a-Physiology Pathology Clinical Medicine 44:573-578.

Nellis, C. H., S. P. Wetmore, and L. B. Keith. 1972. Lynx-prey interactions in central Alberta. The Journal of Wildlife Management 36:320-329.

Nowak, R. 1999. Walker's Mammals of the World. Johns Hopkins University Press, Baltimore, Maryland.

Palme, R., S. Rettenbacher, C. Touma, S. M. El-Bahr, and E. Möstl. 2005. Stress hormones in mammals and birds - Comparative aspects regarding metabolism, excretion, and noninvasive measurement in fecal samples. Annals of the New York Academy of Science 1040:162–171.

Pelican, K., T. Abaigar, A. Vargas, J. M. Rodriguez, J. Bergara, A. Rivas, F. Martinez, D. Rodriguez, J. L. Brown, and D. E. Wildt. 2006. Hormone profiles in Iberian lynx: Application to in situ and ex situ conservation. Pages 113-115 *in* Iberian lynx ex-situ conservation seminar series, Doñana.

Pollack, E. M. 1950. Breeding habits of the bobcat in northeastern United States. Journal of Mammalogy 31:327-330.

Poole, K. G. 2003. A review of the Canada lynx, *Lynx canadensis*, in Canada. Canadian Field-Naturalist 117:360-376.

Putranto, H. D., S. Kusuda, K. Inagaki, G. Kumagai, R. Ishii-Tamura, Y. Uziie, and O. Doi. 2007. Ovarian activity and pregnancy in the Siberian tiger, *Panthera tigris altaica*, assessed by fecal gonadal steroid hormones analyses. Journal of Veterinary Medical Science 69:569-571.

Ruggiero, L. F., K. B. Aubry, S. W. Buskirk, G. M. Koehler, C. T. Krebs, K. S. McKelvey, and J. R. Squires. 2000. Ecology and conservation of lynx in the United States. University Press of Colorado, Boulder, Colorado.

Shenk, T. M. 2001. Post-release monitoring of lynx reintroduced to Colorado. USFWS.

Shiho Fujita, F. M., Hideki Sugiura, Keiko Shimizu,. 2001. Measurement of urinary and fecal steroid metabolites during the ovarian cycle in captive and wild Japanese macaques, *Macaca fuscata*. American Journal of Primatology 53:167-176.

Shille, V. M., G. V. Kollias, M. J. Thatcher, S. Waterman. 1991. Determination of reproductive status in the serval and bobcat using a validated, direct radioimmunoassay of fecal estradiol. Biology of Reproduction 44(suppl):121.

Tchernof, A., F. Labrie, A. Belanger, and J. P. Despres. 1996. Obesity and metabolic complications: Contribution of dehydroepiandrosterone and other steroid hormones. Pages S155-S164 *in* International Symposium on DHEA Transformation into Androgens and Estrogens in Target Tissues - Intracrinology, Quebec, Canada.

Terio, K. A., L. Marker, and L. Munson. 2004. Evidence for chronic stress in captive but not free-ranging cheetahs (*Acinonyx jubatus*) based on adrenal morphology and function. Journal of Wildlife Diseases 40:259-266.

van Dorsser, F. J. D., D. I. Green, W. V. Holt, and A. R. Pickard. 2007. Ovarian activity in Arabian leopards (*Panthera pardus nimr*): sexual behaviour and faecal steroid monitoring during the follicular cycle, mating and pregnancy. Reproduction Fertility and Development 19:822-830.

Verstegen, J. P., K. Onclin, L. D. M. Silva, P. Woutersballman, P. Delahaut, and F. Ectors. 1992. Regulation of progesterone during pregnancy in the cat - studies on the roles of corpora lutea, placenta, and prolactin secretion. Pages 165-173 *in* 2nd International Symposium on Canine and Feline Reproduction, Liege, Belgium.

von der Ohe, C. G. and C. Servheen. 2002. Measuring stress in mammals using fecal glucocorticoids: Opportunities and challenges. Wildlife Society Bulletin 30:1215-1225.

Wasser, S. K. and D. P. Barash. 1983. Reproductive suppression among female mammals - Implications for biomedicine and sexual selection theory. Quarterly Review of Biology 58:513-538.

Wielebnowski, N. and J. Watters. 2007. Applying fecal endocrine monitoring to conservation and behavior studies of wild mammals: Important considerations and preliminary tests. Israel Journal of Ecology and Evolution 53:439-460.

Wielebnowski, N. C., K. Ziegler, D. E. Wildt, J. Lukas, and J. L. Brown. 2002. Impact of social management on reproductive, adrenal and behavioural activity in the cheetah (*Acinonyx jubatus*). Animal Conservation 5:291-301.

Woshner, V. M. 1988. Aspects of reproductive physiology and luteal function in the bobcat (*Felis rufus*). M.S. Mississippi State University.

Woshner, V. M., D. L. Miller, S. J. Waldhalm, N. M. Cox, H. A. Jacobson, and B. D. Leopold. 2001. Progesterone in luteal bodies of bobcats. Pages 427-435 *in* 55th Annual Conference of the Southeastern-Association-of-Fish-and-Wildlife-Agencies, Louisville, Ky.

Ziegler, T. E., C. V. Santos, A. Pissinatti, and K. B. Strier. 1997. Steroid excretion during the ovarian cycle in captive and wild muriquis, *Brachyteles arachnoides*. American Journal of Primatology 42:311-321.

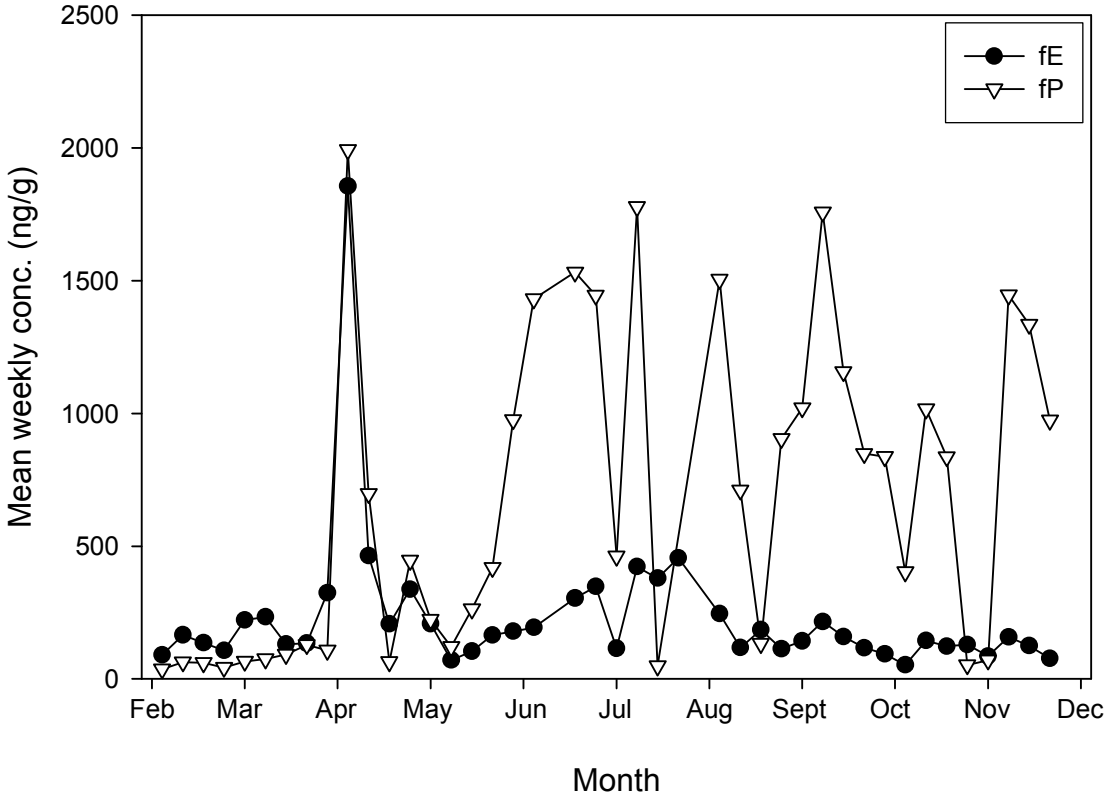


Fig. 3- 1. Longitudinal patterns of fE and fP expression in a 2-year old captive female Canada lynx. During the breeding season, the two hormones are strongly correlated, and then diverge later in the year. She was housed with another female, so the magnitude and the duration of the fP increase suggest that she ovulated spontaneously and that the corpus luteum persisted for the remainder of the year.

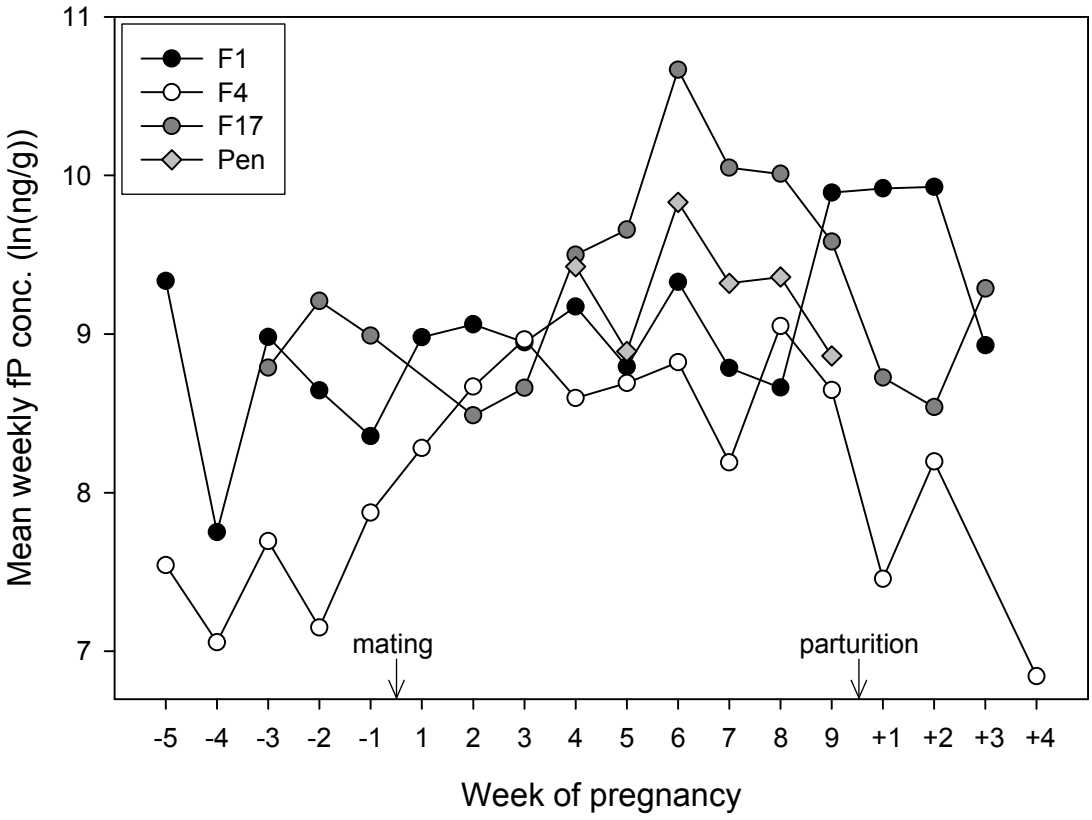


Fig. 3- 2. Longitudinal fP profiles during pregnancy for the three captive females and one reintroduced female in a holding pen. The female in the holding pen conceived in the wild, so date of conception is estimated based on radiograph data. Note that the y-axis is in log-scale.

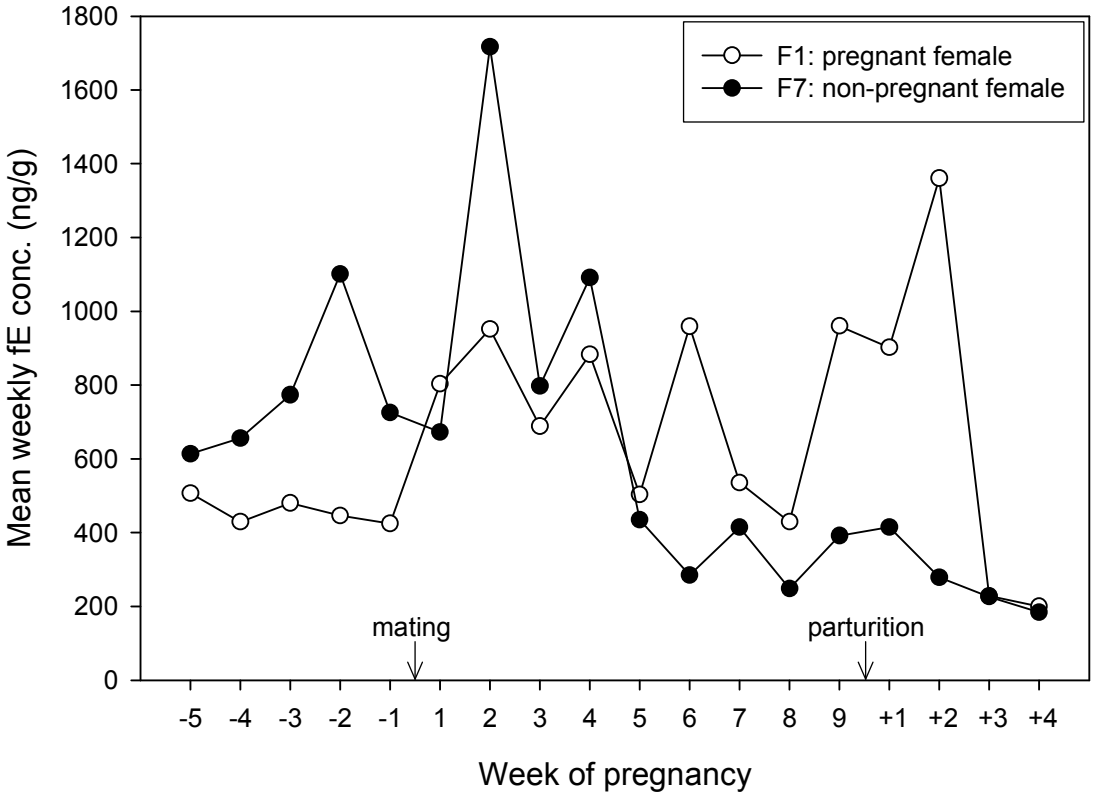


Fig. 3- 3. Longitudinal fE expression in a non-pregnant and a pregnant female. Profiles are aligned by date; "mating" and "parturition" only applies to pregnant female. Both females showed an increase in fE during the breeding season, but fE concentrations declined sooner in the female that had not mated.

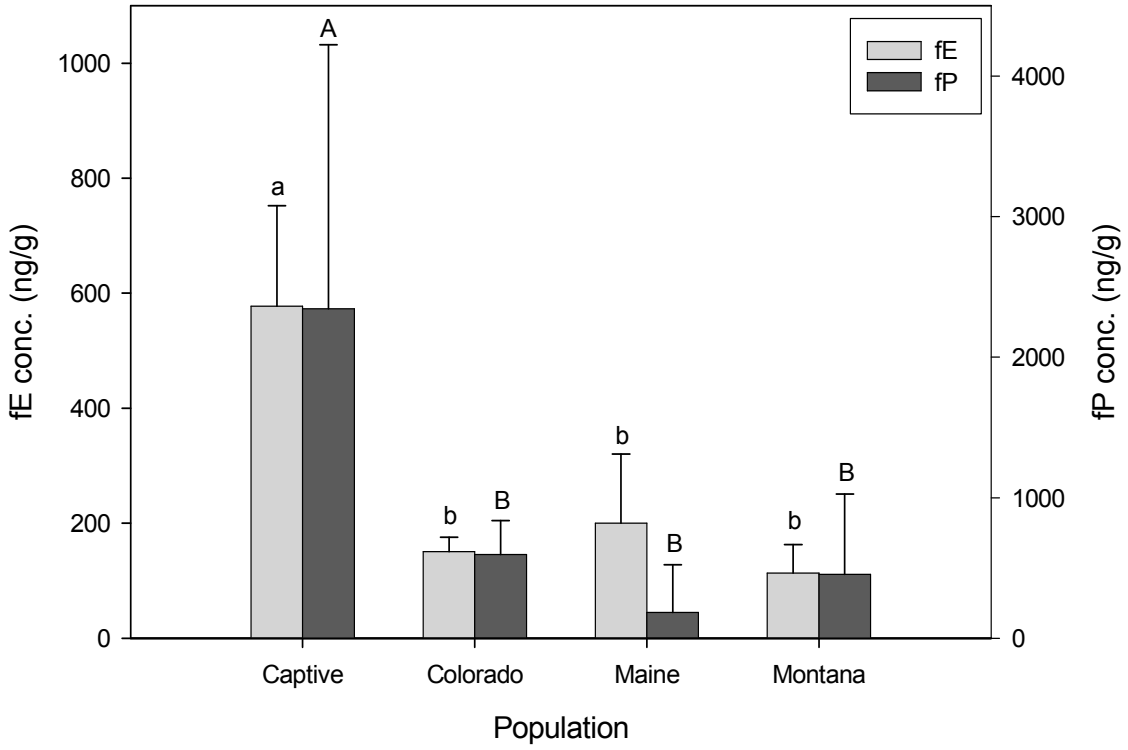
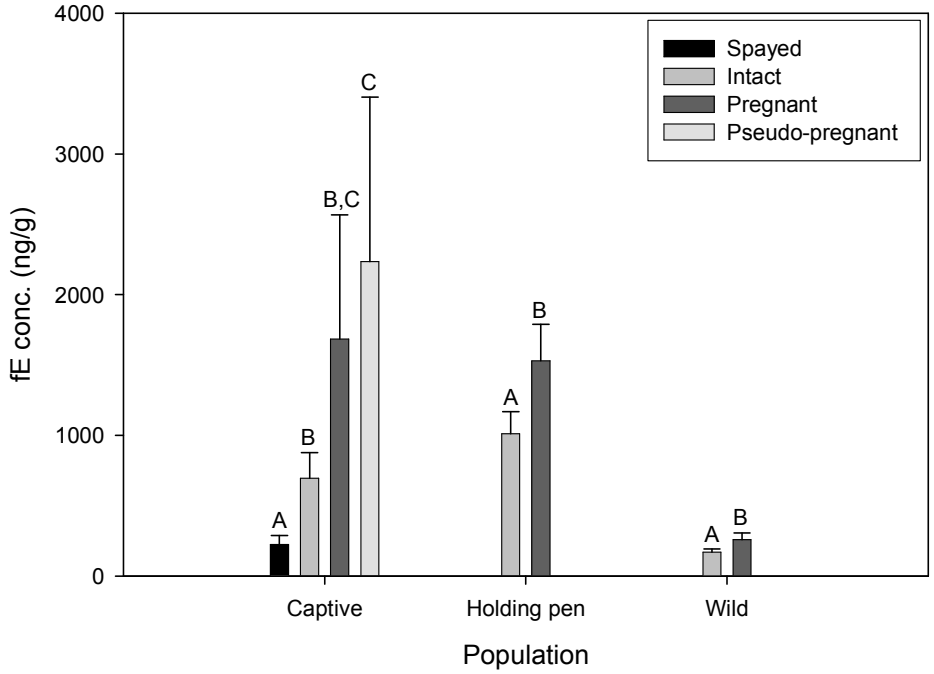


Fig. 3- 4. Population differences in fE and fP expression (back-transformed LS mean + SE). Only data from non-pregnant, intact females were included. Lower-case letters indicate statistically significant differences in fE; upper-case letters indicate statistically significant differences in fP ($P<0.05$).

a)



b)

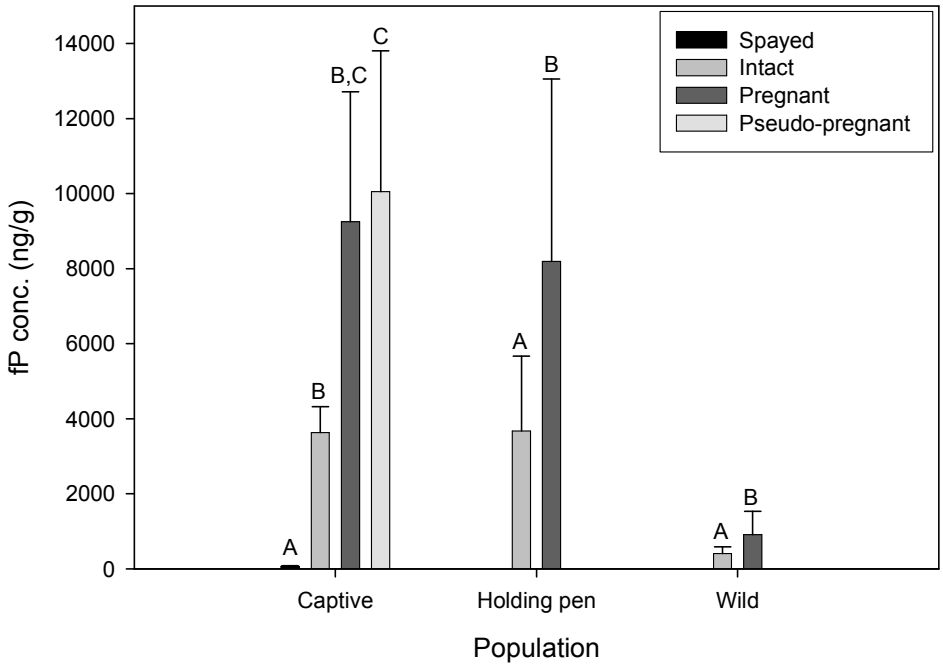
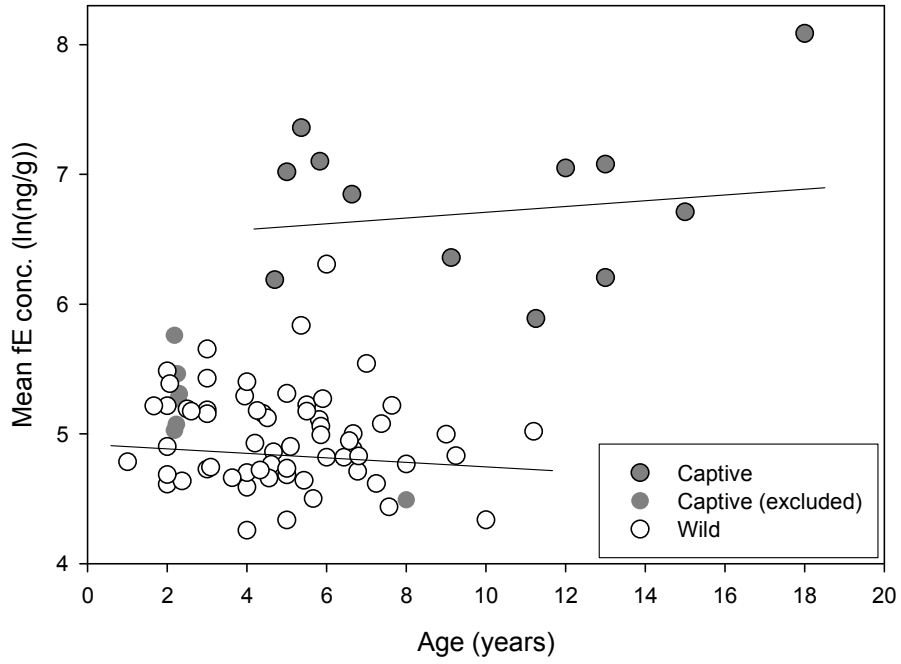


Fig. 3- 5. Effect of female status on fE (a) and fP (b) concentrations (back-transformed LS mean + SE). Letters indicate statistically significant differences within a population ($P<0.05$).

a)



b)

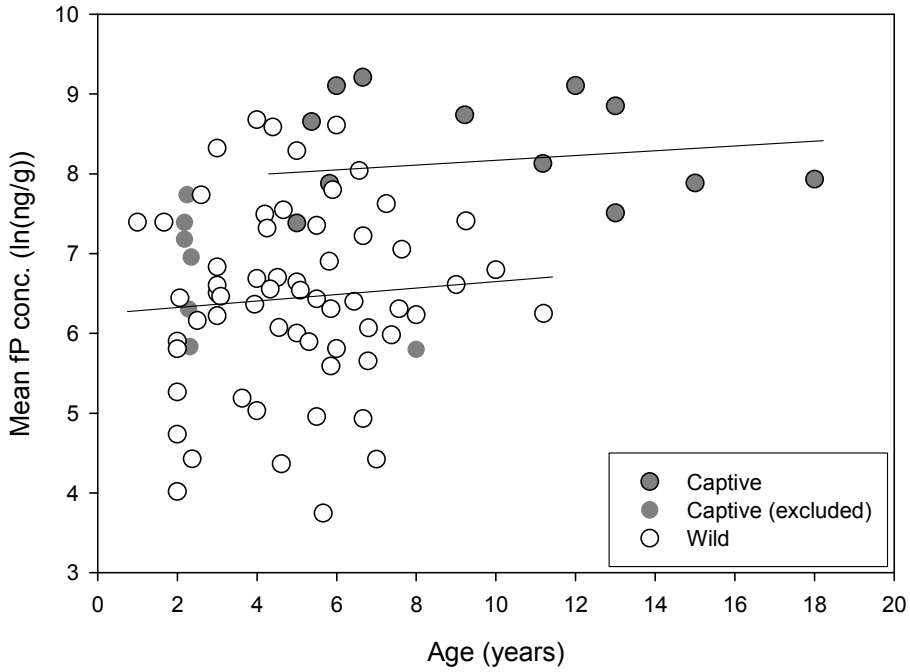
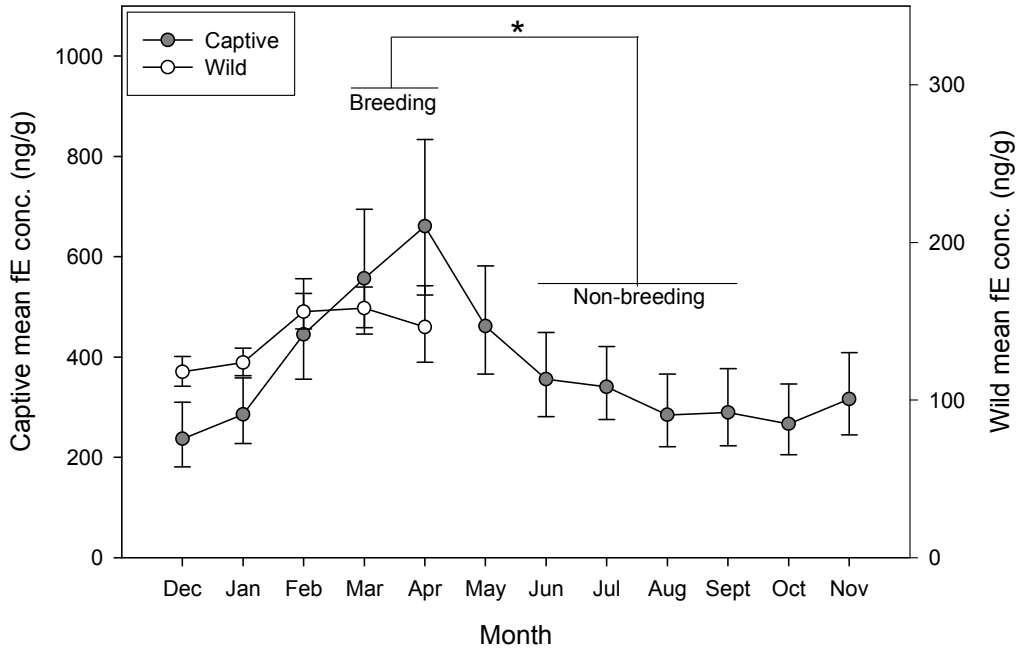


Fig. 3- 6. Effect of age on fE (a) and fP (b) concentrations. See text for discussion of points that were excluded from analysis. Note that the y-axis is in log-scale.

a)



b)

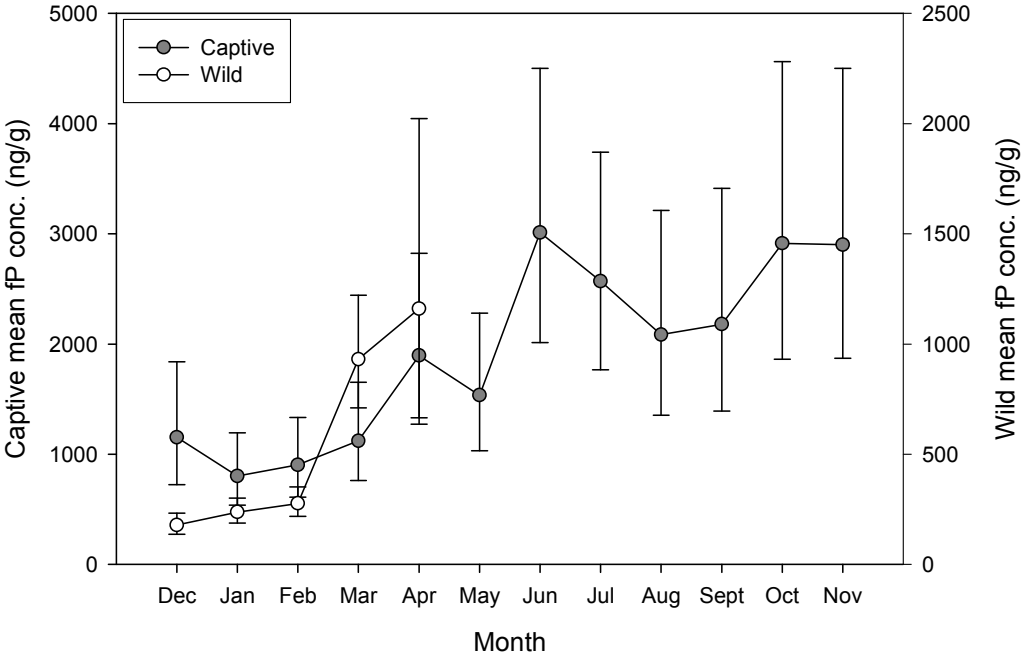
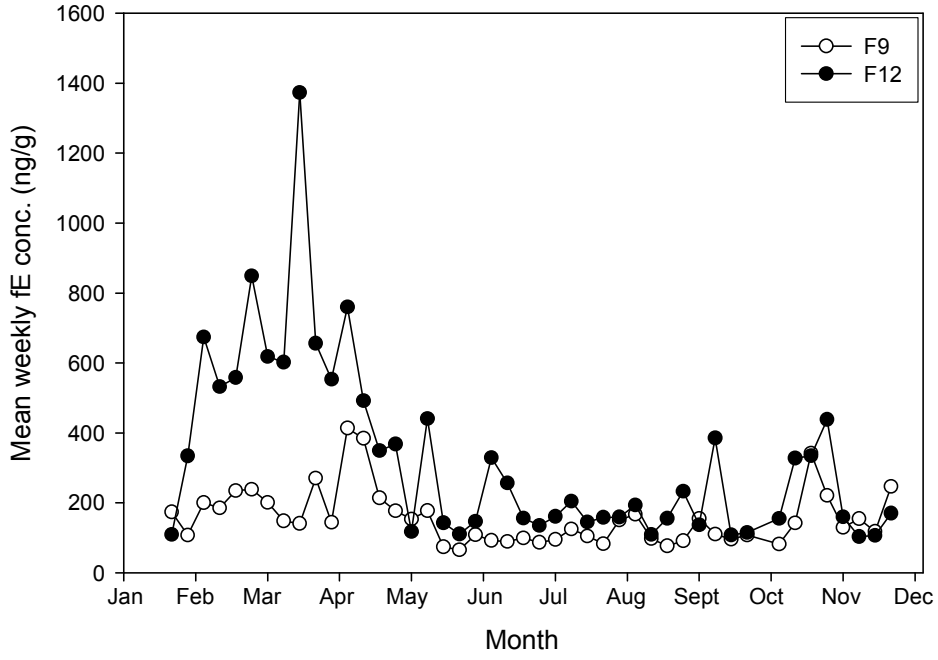


Fig. 3- 7. Seasonal patterns of fE (a) and fP (b) expression (back-transformed LS mean ± SE). Asterisk indicates a statistically significant difference between the breeding and non-breeding seasons for captive females.

a)



b)

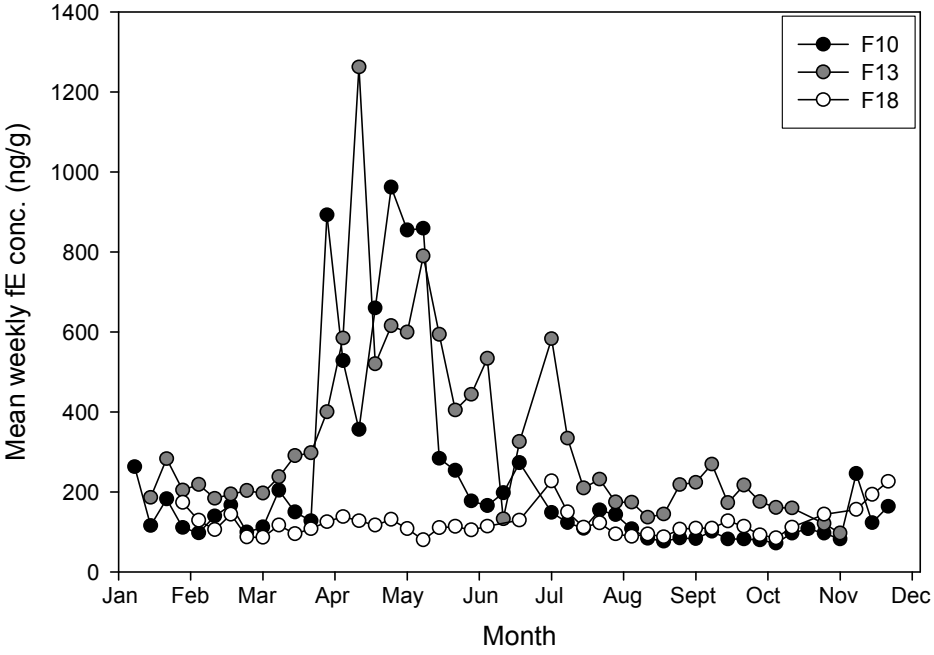


Fig. 3- 8. Longitudinal fE profiles for females housed together. a) Two-female group housed at 45° latitude. b) Three-female group housed at 61° latitude.

CHAPTER 4. MONITORING PATTERNS OF ADRENAL ACTIVITY IN CANADA LYNX IN RELATION TO BIOLOGICAL FACTORS, POPULATION TYPE, AND HUSBANDRY PRACTICES

Introduction

Fecal glucocorticoids provide a valuable window into the condition of an animal, and are becoming a common technique for assessing an individual's physiological state. Glucocorticoids (GCs) are a product of the hypothalamic-pituitary-adrenal (HPA) axis, which is one of the primary components of the vertebrate stress response and is activated by adverse or potentially threatening stimuli (Sapolsky 2002, Palme et al. 2005). Although the term "stress" typically has a negative connotation, occasional acute stress responses are adaptive (Möstl and Palme 2002, Romero 2004). Thus 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).

Understanding the stress physiology of a species and identifying normative patterns of adrenal activity is useful for management of captive and wild populations, as well as answering basic biological questions (Wielebnowski 2003, Schwarzenberger 2007). Although the dynamics of stress physiology have been well studied in some species, we only have a shallow understanding of the variability in stress physiology between species and individuals, as well as across contexts and life-history stages (Romero 2004, Palme et al. 2005, Wielebnowski and Watters 2007). Some species and individuals seem particularly prone to stress-induced pathologies in captivity (Clubb and Mason 2007). However, even among species that do not exhibit pathological manifestations of stress, there may be sub-pathological variations in adrenal activity that contribute to the health, reproductive success, and overall well-being of the animal.

Canada lynx have a long history in captivity, both in public (e.g., zoos) and in private (e.g., fur-industry, pets) sectors. They are common as pets and ambassador/educational animals because of their 'easy-going' temperament. Because they are relatively easy to maintain *ex-situ*, little attention has been devoted to understanding factors that contribute to the well-being of lynx in captivity. However, with a recent trend toward lower reproductive success in the captive North

American population (Goff 2008), and an increasing interest in animal welfare, it is important to consider factors that contribute to lynx well-being. Canada lynx in the North American population are maintained in a wide range of housing situations, from small artificial enclosures with multiple individuals, to large naturalistic enclosures with a single pair. In the wild, lynx are solitary (Ruggiero et al. 2000), but it is unknown how factors such as group-housing may affect them.

The aim of this study was to establish a basic understanding of stress physiology in Canada lynx using the non-invasive technique of fecal glucocorticoid metabolite (fGC) analysis. By incorporating data collected from both captive and wild lynx, we are able to develop a more comprehensive understanding of stress physiology in Canada lynx. Specifically, our goals were to 1) validate an fecal glucocorticoid assay for monitoring adrenal activity in Canada lynx, 2) examine the effects of sex, status (intact, neutered, pregnant), and age on fGC values in both captive and wild populations, 3) monitor seasonal changes in fGC expression in the wild and in captivity, and 4) investigate the relationship between fGC levels and housing conditions / husbandry practices. Establishing basic information about adrenal activity in Canada lynx may help enhance *ex situ* and *in situ* management plans, and provide a foundation for future studies on stress physiology in lynx.

Methods

Animals and Fecal Sample Collection

Captive – This study included a total of 45 captive Canada lynx (21 males and 24 females) from 22 institutions. The mean age was 7.6 years (range: 2-18 years). Four males were castrated, and seven females were spayed. In addition, six females became pregnant/pseudo-pregnant during the study (see Chapter 3 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, and duration of sample collection for individual lynx ranged from 2-12 months.

Keepers were asked to record potentially stressful events or stimuli throughout the duration of the study. These events were then matched with longitudinal hormone profiles to help assess the biological relevance of the assay. Physiological stress responses were defined by the

presence of fGC peaks that exceeded the baseline plus three standard deviations. There were six lynx that were either moved to a new enclosure during the study or experienced a notable change in their current enclosure (specifically loss of a cage-mate). We were also able to obtain data from one male that was transferred between two institutions for breeding purposes in 2006. Fecal samples were collected from him for three months prior to and 25 days after the transfer.

Wild – Fecal samples were also collected from wild adult males 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 has been reintroduced (see Chapters 2 and 3 for collection details). The mean age was 5.1 years old (range: 1-12 years). To ensure that fGC expression in reintroduced lynx was not affected by the translocation, we only included samples that had been collected more than six months after a lynx was released.

ACTH Challenge

To ensure that the corticosterone assay detected biologically relevant changes in adrenal activity, an ACTH challenge was conducted on five lynx (2 males and 3 females). Adrenocorticotropic hormone (ACTH) is produced by the anterior pituitary and stimulates the adrenal cortex to release glucocorticoids (Sapolsky 2002, Palme et al. 2005). ACTH challenges are a common method of assessing adrenal responsiveness, and thereby allowing for validation of corticoid assays (Palme 2005). They can also be used to determine the lag time between changes in circulating hormone levels and changes in fecal hormone metabolite levels.

ACTH gel (Corticotrophin, 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 six days prior to the challenge and 10 days following the challenge. Following the injection, every fecal sample was collected for 48 hours. Furthermore, for two females, we also collected serial blood samples every 10 minutes for 2 hours following the injection. This allowed us to ensure that the ACTH injection elicited an adrenal response and also allowed us to compare circulating and excreted glucocorticoid levels.

To assess the ability of different assays to detect changes in adrenal activity, we ran the samples from the ACTH challenge on five assays. One was a commercial EIA kit produced by

Assay Designs (referred to as "AD Cort"; see Steroid Extraction and Analysis for details). Two assays were provided by CJ Munro (University of California, Davis, CA): cortisol R4866 (referred to as "CM Cortisol"; Munro and Lasley, 1988, Young et al 2004) and corticosterone Cs6 ("CM Cortn"). The other two assays were developed specifically for glucocorticoid metabolites, and were provided by E. Möstl (University of Veterinary Medicine, Vienna, Austria): 5β-androstane-3α, 11β-diol-17-one (hereafter "EM Andro"; Frigiero et al. 2004) and 11-oxo-etiocholanolone ("EM Oxo"; Möstl et al. 2002).

Keeper Surveys

Questionnaires were distributed to each institution to obtain information about the individual history, housing situation, and husbandry routines for each lynx. When possible, the North American Canada Lynx Studbook and personal visits to the institutions were used to corroborate information.

There were seven variables included in final analyses: number of cage-mates, sex of cage-mates, number of dens/nest boxes, number of keepers, visitor proximity, frequency of enrichment, and "density" (Table 4-2; see Appendix 2 for a complete list of husbandry variables). Note that density was not calculated in the traditional way (number of animals per unit space), but rather was calculated as the size of the enclosure (ft^2) divided by the number of lynx. Visitor proximity was defined as the closest possible distance between the lynx and visitors. Frequency of enrichment was determined as the number of days per week that the lynx received an enrichment item/activity (as defined by the keeper).

Steroid Extraction and Analysis

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 1500 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.

Fecal glucocorticoid metabolites (fGC) were quantified using a commercially available Corticosterone EIA (Assay Designs, Ann Arbor, MI; see Appendix 2 for assay procedure). 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 (~70% binding) and high (~30% binding) quality control samples were run on each plate. Intra-assay coefficients of variation were 15.5% and 11.4% (n=19) for low and high controls, respectively. The inter-assay coefficients of variation were 27.5% and 27.1% (n=120), respectively. Data are expressed as ng/g wet fecal weight.

Statistical Analysis

All data were analyzed using SAS 9.1 (Cary, NC). For each individual, we calculated five fGC summary statistics: mean, baseline, peak, %CV, and response magnitude. Baseline was calculated through an iterative process excluding all points greater than the mean+3 SD (Wielebnowski et al. 2002). Mean peak was the average of all points excluded from the baseline calculation. Response magnitude (hereafter referred to as "magnitude") was calculated as mean peak divided by baseline. Because there was considerable variation in overall fGC values between individuals, this ratio provides a standardized measurement of an individual's adrenal reactivity when exposed to a stressor. Data were log-transformed as necessary to meet assumptions of normality and homoscedasticity.

Validation – ACTH Challenge

The ability of assays to reflect adrenal responsiveness was assessed by the detection of fGC peaks (i.e. points excluded from the baseline calculation) following an ACTH injection. For the ACTH challenge data, the baseline was calculated using 2 SD, in contrast to the 3 SD mentioned above for calculating an individual's overall baseline. Because we only analyzed a subset of each individual's samples on all five assays, there were fewer points and thus higher SD values, which meant that peaks were less likely to be identified. Therefore, we lowered the criteria to see if there were any detectable peaks post-ACTH injection.

Biological Effects – Sex, Status, Age and Population

An ANCOVA was used to determine how fGC values vary with sex, age, and status (intact, neutered, pregnant, or pseudo-pregnant). We also controlled for population, as this model included samples from captive lynx, as well as lynx in Colorado, Montana and Maine. The four populations were considered separately in the model, but pairwise comparisons indicated that the wild populations were not significantly different from each other, so a post-hoc linear contrast was used to test for differences between captive and wild populations. In this model, fGC mean was the only response variable examined, because there was not sufficient data for many of the wild lynx to calculate the other four fGC statistics. We found that captive females <3 years old had notably lower fGC levels than older intact females, which is similar to patterns seen in other hormones (Chapter 3). Therefore, these "2-yr old" females were excluded from these analyses. To control for variable water content in the samples (see Chapter 1), each individual's mean water content was included in all analyses. For the samples that were missing an estimate of water content, we interpolated values using median water content. Two-way interactions were excluded if they were not significant. 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 fGC values. 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. Captive 2-year old females were excluded because in addition to having lower fGC concentrations, their patterns of seasonality were also slightly different. The model also controlled for sex and each individual's mean monthly water content. Two-way interactions were excluded if they were not significant. Peak fGC values (>baseline + 3SD) were excluded from monthly mean calculations.

Housing and Husbandry Effects

The final objective of this study was to perform an exploratory analysis of factors that may affect fGC levels, and thereby highlight avenues of research for future studies. It was

beyond the scope of this project to move or manipulate lynx in order to experimentally examine housing/husbandry effects. As a consequence, several variables were correlated. Therefore, we first controlled for the effect of biological factors that can have a strong affect on adrenal activity (Palme 2005, Wielebnowski and Watters 2007), and then examined the effect of housing factors. This was accomplished by using the residuals of the biological model as the response variables in the husbandry model. The qualitative results obtained using the residuals were similar to those obtained using the raw data.

Among the response variables, there was a strong positive correlation among mean, baseline, and mean peak, and they yielded similar results, so only the baseline results are presented. Similarly, magnitude and %CV, which both reflect dynamics of an individual's stress response, were positively correlated, so only results for magnitude are presented. The residuals of the baseline model are referred to as 'Rbase' and residuals of the magnitude residuals are referred to as 'Rmag.' Because the same individuals are included in the calculation of baseline and magnitude, it would be ideal to analyze both response variables in a global model to account for the covariance between them. However, there were several individuals that did not have detectable fGC peaks, and consequently we only have response magnitude data for about 60% of the lynx. Since sample sizes were quite different and this is an exploratory analysis, the two response variables were considered separately. Samples collected during a pregnancy or pseudo-pregnancy were excluded from this study. We also excluded two lynx (M13 and F15) that had low fGC and reproductive hormone concentrations, as well as some housing and dietary differences (see Chapters 2 and 3 for further discussion).

To identify potentially influential husbandry factors, we screened the 26 husbandry variables by running all possible one- and two-factor ANOVAs. These results, in conjunction with graphical analyses, were used to identify 12 variables that had a statistically significant effect on Rbase and Rmag. Correlation coefficients were then calculated for these 12 variables to examine issues of multicollinearity and identify clusters of variables. Two husbandry variables (number of annual visitors and public/private institution) were eliminated because they were correlated with nearly all of the other variables. From the ten remaining variables, a final model was constructed using a backward step-wise procedure with a criteria of $P>0.1$. Tukey-Kramer adjustment was used to correct for multiple pairwise comparisons.

Housing Changes

There were seven lynx for which we had hormone data before and after a major housing change. For each case, we compared pre- and post-change fGC concentrations using a t-test assuming unequal variance. Since the effect of the housing change was assessed for a given individual, the inferential space for these t-tests is limited to the particular lynx.

Results

Validation – ACTH Challenge

All five of the corticoid assays tested for the ACTH challenge were able to detect quantifiable amounts of fecal steroid metabolites, although there was quite a bit of variation in the number of metabolites each assay detected (Table 4-1, Fig. 4-1; see Methods for assay abbreviations). For males, all of the assays except CM Cortn detected a clear response to the ACTH-induced increase in adrenal activity. The EM Andro and EM Oxo assays detected the highest response. Conversely, in females, very few of the assays reliably detected post-ACTH peaks. The AD Cort assay was the only assay that consistently identified peaks for all three females, but the CM Cortisol assay detected a higher response for the two females in which it detected peaks. However, with the CM Cortisol assay, baseline values were near or below the detection limit, thereby precluding the reliable quantification of fGC concentrations at baseline levels. The CM Cortn assay showed the weakest response for both sexes, and was the least reliable for detecting peaks.

Both the AD Cort and CM Cortisol assays seem potentially promising for monitoring adrenal activity in both males and females. However, the AD Cort assay appears to be the most promising because 1) it most consistently identified post-ACTH peaks, and 2) it detected quantifiable amounts of fGC, even at baseline values. Therefore, the AD Cort assay was used for all subsequent assays and results presented in this paper.

The ACTH injection elicited a 3- to 10-fold increase in fGC levels and a 4- to 7-fold increase in serum corticoid levels (Fig. 4-2). The peak in fGC levels occurred 1 to 2 days after the injection, indicating an excretion lag time of 24-48 hours. There was some individual variation in both the timing and the magnitude of the post-ACTH peak. Interestingly, the post-

ACTH increase in fGC levels was biphasic in four of the five lynx, and this pattern was present in the other corticoid assays, as well. The second peak occurred 3-4 days following the ACTH injection.

Biological Effects

Population had a significant effect on fGC levels ($F_{3,163}$=3.52, P=0.02; Fig. 4-3). None of the wild populations were significantly different from each other (CO vs. ME: t_{163}=0.43, P=0.67; CO vs. MT: t_{163}=0.69, P=0.49; ME vs. MT: t_{163}=0.79, P=0.43). Therefore, we used a contrast statement to compare the captive population to all wild populations. Captive lynx had significantly higher fGC concentrations than wild lynx (t_{163}=2.87, P=0.005).

All biological factors had a significant effect on fGC concentrations. The effect of sex on baseline fGC depended on the reproductive status of the lynx ($F_{1,163}$=3.76, P=0.05). There were no significant differences between intact males, castrated males, and spayed females (P=1.0; Fig. 4-4a). However, intact females had significantly higher fGC values than intact males (t_{165}=8.33, P<0.001) and spayed females (t_{165}=3.19, P=0.02), and they had marginally significantly higher fGC values than castrated males (t_{165}=2.77, P=0.07; Fig. 4-4a). Pregnant/pseudo-pregnant females had significantly higher fGC values than all other groups (P<0.001; see Fig. 4-4b for comparison with intact females). Pregnant and pseudo-pregnant females were not significantly different from each other (t_{165}=0.46, P=0.99). Mean fGC concentration increased with age (β=0.047±0.02 ln(ng/g)/year, $F_{1,163}$=5.57, P=0.02; Fig. 4-5).

Seasonal Effects

For both captive and wild populations, patterns of monthly fGC expression differed between the sexes (Captive: $F_{11,99.9}$=2.05, P=0.03; Wild: $F_{4,327}$=3.41, P=0.01; Fig. 4-6). For males, captive and wild populations were remarkably similar, both showing an increase in fGC values during the breeding season. Wild females exhibited a pronounced increase in fGC concentrations in early spring (between February and March). In captive females, the increase in fGCs was more gradual and occurred later in the year (between April and June).

Housing and Husbandry Effects

For the baseline residuals (Rbase) model, there were five factors included in the final model, four of which were significant (Table 4-2). Baseline fGC values were negatively correlated with number of cage-mates, density, and number of keepers. They were positively correlated with visitor proximity; lynx had lower fGC values when visitors were allowed to get closer to the enclosure. There was also a significant effect of cage-mate sex on Rbase. Lynx housed with both a male and a female had higher baseline fGC values than lynx housed with either a male or a female (Fig. 4-7). Lynx housed alone had the lowest fGC values.

For the magnitude residuals (Rmag) model, seven factors were retained in the final model, although only five were significant at the $P<0.05$ level (Table 4-2). Similar to Rbase results, there was a negative relationship between an individual's fGC response magnitude and the number of cage-mates and keepers. However, the effects of density and visitor proximity were opposite to those obtained for Rbase. The magnitude of fGC peaks increased with space per lynx, and decreased with visitor proximity. Cage-mate sex also had a different effect on magnitude than it did on baseline fGC values (Fig. 4-7). In both lynx housed alone (the group with the lowest fGC levels) and lynx housed with both sexes (which had the highest fGC levels), fGC magnitude was the lowest. Additionally, the frequency of enrichment had a marginally significant effect on fGC magnitude. For both Rbase and Rmag, the effect of density was driven by large enclosures (>1000 ft^2/lynx).

Housing Changes

We were able to match several enclosure changes with physiological stress responses (as defined by peaks greater than baseline+3SD). Movement between institutions elicited a strong adrenal response (Fig. 4-8), and movement to a new enclosure was also associated with fGC peaks.

For the seven lynx that experienced a change in housing during the study, support for the housing model was equivocal (Table 4-3). In general, the results for males supported the findings of the model, while results for females were in the opposite direction than the model predicted. For two of the females, samples were collected from the original housing situation when they were young (<3 years) and from the new housing situation when they were adults. Since young females have lower fGC concentrations than adults, the predictions from the biological model

conflict with predictions from the housing model, and biological factors may have a stronger influence on fGCs than environmental factors. With the exception of lynx M17, sample collection was much shorter in the second housing situation, so it could be argued that post-housing change fGC values are inflated by the stress associated with a novel environment, and are not reflective of true baseline.

Discussion

Validation – ACTH Challenge

Circulating glucocorticoids are often modified by the liver and gut bacteria prior to excretion (Palme et al. 2005). Therefore, a "parent" hormone is often excreted as numerous different metabolites, and antibodies against circulating corticoids may not react with fecal corticoid metabolites. Steroid metabolism can vary significantly between species, between individuals, and there is emerging evidence that it can even vary within an individual over time (Palme et al. 2005, Wielebnowski and Watters 2007, Dehnhard et al. *in review*). Therefore, it is exceedingly important to validate corticoid assays used to quantify fecal hormone metabolites and ensure that they actually reflect adrenal activity. The injection of ACTH to stimulate adrenal activity is a common method of validating fGC assays.

We conducted ACTH challenges on five lynx and tested five different corticoid assays to quantify fGCs. There was quite a bit of variability in the performance of the five assays, particularly between sexes. Even within a given assay, there was quite a bit of individual variation, which is common in other species as well (Palme 2005). However, most assays detected some degree of adrenal activity following the ACTH injection. The Assay Designs Corticosterone assay was the most consistent at detecting post-challenge adrenal activity. The ACTH challenge revealed that the lag-time in corticoid excretion is 1-2 days.

Population

Captive lynx had significantly higher fGC concentrations than wild lynx. This is the same trend that we see in reproductive hormones (see Chapters 2 and 3), although the magnitude of the difference is much smaller for fGC values. One possible explanation for this trend is that captive lynx are chronically stressed, which results in dysfunction of the hypothalamic-pituitary-gonadal axis and causes overproduction of sex steroids. Indeed, in a study of captive and wild cheetahs, Terio et al. (2004) determined that high fGC concentrations in captive cheetahs, in conjunction with other physiological data, were indicative of chronic stress. However, they found different trends in reproductive hormone values (Terio et al. 2004). In contrast to our findings, they found that fecal androgens were significantly lower in captive male cheetahs, suggesting stress-induced reproductive suppression. Similar to our findings, they found that fecal estrogens were higher in captive female cheetahs.

Another possible explanation is that there is an underlying difference in metabolism, diet, or energy regulation between the two populations that causes a complete shift in steroid hormone production and/or metabolism. These factors are known to impact steroid production and excretion in other species (Hajamor et al. 2003, Palme 2005, Wielebnowski and Watters 2007). Based on lynx weights reported by each institution, captive lynx on average weigh twice as much as wild lynx.

Very few studies have examined the differences in fGC concentrations between populations. In addition to Terio et al. (2004), the only other study comparing fGC values between captive and wild populations that the authors are aware of at this time is with spotted hyenas, in which the researchers found no evidence of a difference in fGCs between captive and wild hyenas (S. Dloniak, pers. comm.). In the face of a growing number of studies that make fGC comparisons between populations, sometimes resulting in management actions, it is critical that we develop a better understanding of what factors contribute to these population differences.

Sex and reproductive status

We found that intact females had significantly higher fGC concentrations than males and spayed females. Sex differences in fGC concentrations have been reported for several species (Palme et al. 2005). Sex differences in measured fGCs may reflect differences in production of glucocorticoids, binding capacity of binding globulins in the blood stream, and or metabolism and

excretion of steroids (Touma and Palme 2005, Wielebnowski and Watters 2007). However, it is interesting that neutered females had lower fGC values than intact females; they were similar to males. Furthermore, we observed instances in intact females where fGCs track fecal progesterone metabolites (K. Fanson, unpubl. data). This suggests that fGCs reflect ovarian/luteal activity to some extent.

It is possible that this result may be an artifact of the methodology. Since circulating steroids are metabolized prior to excretion (Palme et al. 2005), it is possible that metabolites of ovarian steroids (specifically progestogens) are recognized by the antibody we used. Although this antibody's reported cross-reactivity with progesterone is low (see Methods), manufacturers do not test the cross-reactivity of an antibody against all steroid metabolites/derivatives. Such a task is not only financially and logistically impractical, but is also hindered by a dwindling steroid library against which to test an antibody (R. Palme, pers. comm.).

Alternatively, this result may reflect a real biological signal. Ovaries may contribute to circulating glucocorticoid levels either directly (they are a source of glucocorticoids), or indirectly (they stimulate the adrenal glands or alter the binding capacity of binding globulins). Indeed, sex steroids are known to modify activity of the HPA axis (von der Ohe and Servheen 2002). It was not possible for us to conduct either a radiolabel metabolism study or tissue hormone analysis, which could help us understand the biological significance of this result. It has been reported for other female mammals that fGC concentrations vary with reproductive status (Touma and Palme 2005). The primary explanation for this currently is that changes in circulating estrogen or progesterone can alter the production or function of binding globulins, which in turn alters the amount of "free" corticoids that are excreted (Touma and Palme 2005).

Nonetheless, we feel that measured fGC levels accurately reflected adrenal activity in intact females. Following known stressors (e.g., ACTH challenge or housing transfer), there is an appropriate increase in fGC concentrations, while there is no concurrent increase in fecal progestogens. While fGCs and fPs may track each other sometimes, fGCs behave independently in response to stressors. Additionally, when intact females are analyzed separately from the other groups in the husbandry model, the qualitative results of both models are the same.

Age

In regards to age, there was generally a significant increase in fGC values with age. The slope of this relationship was fairly similar for both captive and wild females, as well as for

captive males. However, wild males exhibited very little change in fGC concentration with age. In wandering albatrosses, baseline corticosterone increases to some extent with age/breeding success, and then decreases when the female becomes reproductively senescent (Angelier et al. 2007). In clouded leopards, there does not appear to be a significant effect of age on fGC concentration (Wielebnowski et al. 2002).

Although they were excluded from analysis, we observed a notable increase in fGCs in captive females between 2 and 3 years of age. This increase was not observed in the wild population, which included even younger females, nor was it observed in either male population. The fGC increase in females coincides with a similar increase in reproductive hormones, which may be associated with reproductive maturity (gonadarche) and/or the maturation of the adrenal cortex (adrenarche). Adrenarche has been documented in other species (Cutler et al. 1978), however, there is still little information on this phenomenon in non-human species.

Seasonal Effects

Males and females showed differing seasonal changes in fGC concentrations. In males, fGC values peak during the breeding season, then decrease to a nadir during summer. Captive and wild males show remarkably similar patterns of fGC expression. Conversely, females had the lowest fGC levels in winter/early spring, and levels then increased towards the end of the breeding season. In wild females, this increase is quite pronounced, whereas in captive females it is much more gradual and also occurs later. However, when the captive 2-year old females were included, the seasonal changes in captive females more closely resembled the pattern seen in wild females.

Seasonal changes in patterns of corticoid expression have been reported for a number of other species (Romero 2002, Touma and Palme 2005). In many species, corticoid concentrations increase during the breeding season, which would coincide with the pattern we see in male lynx. Females show a different trend, with the increase in fGCs occurring just after the breeding season. However, there is quite a bit of variation in the seasonal dynamics of corticoid expression among species, and neither the mechanisms nor the function are well understood (Touma and Palme 2005). Interestingly, female lynx show similar patterns of expression in both fGCs and fecal progestogens. This may further indicate that fGCs reflect some ovarian activity in Canada lynx.

Housing and Husbandry Effects

We found five variables that had a significant effect on fGC baseline and/or response magnitude: "density" (enclosure size/lynx), cage-mate sex, number of cage-mates, number of keepers, and visitor proximity. Due to limited housing arrangements, it was not possible to tease apart all the interactions and confounding relationships between husbandry variables. For example, we excluded two factors (annual number of visitors to the institution, and whether or not the institution was open to the public) because they were highly correlated with many other factors. These two factors, as well as their association with other factors (e.g., budget or staff load), may be important contributors to an individual's fGC levels. However, we provide a preliminary examination of husbandry factors that influence adrenal activity in Canada lynx, and merit further attention in regards to the well-being of captive lynx.

Group size has been widely documented as an important contributor to the well-being of captive animals (Price and Stoinski 2007). Species-inappropriate group sizes, either too small or too large, can have severe repercussions for the health and reproductive success of the animals. Our findings reveal that for Canada lynx, adrenal activity is not simply affected by group size, but more specifically by the sex-composition of the group and the size of the group relative to the size of the enclosure.

"Density" (size/lynx) had a significant effect on fGC expression in Canada lynx. Baseline fGCs decreased with density, but response magnitude increased, particularly for large enclosures. Animals with more than 1000 ft^2/lynx had notably lower fGC levels. However, among lynx that had <1000 ft^2/lynx, the effect of density was negligible. It has been documented in felids that larger group sizes are associated with poor reproductive success (Mellen 1991). Increased density is also associated with increased fGC in captive Pere David's deer, as well as changes in behavior (Li et al. 2007).

Cage-mate sex was related to baseline fGC and response magnitude in different ways. Individuals housed alone had the lowest fGC levels, and also exhibited smaller fGC increases. Conversely, individuals housed with both a male and a female had the highest fGC levels, but the magnitude of their stress response was still small. The latter may possibly indicate signs of chronic stress which could result in a muted acute stress response if the adrenal glands have been negatively affected by chronic overproduction of glucocorticoids. Alternatively, lynx housed with both males and females may be more habituated to stressors and are consequently less responsive to their environment. Lynx housed with either male or female cage-mates had intermediate baseline fGC values. These two groups also had the greatest response magnitude.

Based on these findings, it appears that solitary housing is associated with the lowest level of adrenal activity, both baseline and response, and thus may be the optimal housing arrangement for lynx. This agrees with our knowledge of the natural history of this species (Ruggiero et al. 2000), and with findings for other felids (Mellen 1991).

Interestingly, the closest possible distance of visitors was negatively correlated with fGC magnitude; as visitors were kept further away, the magnitude of the lynx's stress response decreased. The effect of visitors on zoo animals has been somewhat equivocal (Davey 2007). However, there is certainly evidence that visitors can impact the stress physiology of some species. Clouded leopards on public display had higher fGC concentrations than those off display (Wielebnowski et al. 2002). In koalas, fGC expression is not related to visitor numbers, but is associated with the pattern of visitation (K. Webster, pers. comm.). Even though they were exposed to higher numbers of visitors overall, koalas exposed to a constant stream of visitors had lower fGC levels than koalas exposed to periodic pulses of visitor groups. While visitor proximity was the only visitor variable that was significant in our final model, future studies should further examine how visitor dynamics affect fGC concentrations (and the underlying stress physiology) in lynx. This is particularly pertinent because lynx are often used as ambassador/educational animals. However, lynx are sometimes "retired" from this position at a young age due to behavioral changes (J. Maynard and B. Roe, pers. comm.). It is possible that the stress of intense visitor interactions, especially at young ages, may affect the temperament and behavior of some individuals.

Both fGC baseline levels and magnitude were negatively associated with the number of keepers. This is in contrast to findings in other species about the effect of keeper number on fGCs (Wielebnowski et al. 2002). One thing to note is that the quantification of keepers may have varied among institutions; some institutions may have included only primary keepers, whereas others might also have included relief keepers. Determining the number of keepers, especially over periods extending up to a year, is not always as straightforward as it might seem. Furthermore, the number of keepers may be confounded with other factors. For example, the number of keepers may be inversely related to keeper workload, and thus the amount of time keepers spend with the lynx. It may also be related to institutional budget. Before we can conclusively determine the effect of keeper number on lynx well-being, further investigation is required to understand the nature and quality of the lynx-keeper interaction.

Effect of Events and Housing Changes

In recent years, there has been an increasing focus on animal welfare and well-being in zoos. However, it is often difficult to quantify an animal's well-being. Fecal corticoids have been one tool used to increase our understanding of how environmental factors impact an individual's physiology. The challenge with the post-hoc nature of this method of assessing stress physiology is identifying stressors that are relevant to the animal. A keeper's perception of the environment is likely to be much different than an animal's, and it can be difficult to determine what led to a stress response retrospectively. We found that several of the potentially stressful events that keepers recorded were not associated with fGC peaks, and vice-versa. In general, we found that visitor activity and special zoo events were not associated with fGC peaks. However, particularly in males, nearby construction was associated with increased adrenal activity.

The process of being transferred between enclosures or institutions was associated with an increase in adrenal activity. For the male that was moved between institutions, fGC values increased dramatically following the transfer, reaching a peak 5 days post-transport. His fGC levels returned to baseline fairly quickly (~2 weeks) after returning to his home institution. It has been documented in other felids that movement to a novel enclosure results in a significant, though relatively short-lived (~1-2 weeks), increase in glucocorticoid expression (Carlstead et al. 1992, Carlstead et al. 1993, Dembiec et al. 2004). Furthermore, this GC increase is associated with changes in behavior, generally reduced activity and increased hiding in felids (Carlstead et al. 1993, Dembiec et al. 2004).

Additionally, a change in cage-mates, even without being moved to a new enclosure, was often associated with changes in adrenal activity. However, in cases where biological and environmental factors were predicted to have opposite effects of fGC expression, the biological factor appeared to outweigh the environmental factor. This finding further highlights the importance of group size, and reinforces our suggestion that lynx be housed in small group sizes.

Concluding Remarks

In this study, we biochemically and biologically validated a fecal glucocorticoid EIA for non-invasive monitoring of adrenal activity in Canada lynx. We then used this technique to

monitor patterns of adrenal activity in lynx in relation to biological factors, population type, and husbandry practices. Our key findings include:

1) Although fGCs provide a valid measure of adrenal activity, they may also reflect some degree of ovarian/luteal activity in females.
2) For males, seasonal increases in fGC expression coincide with the onset of the breeding season, whereas in females, fGCs increase toward the end of the breeding season
3) Captive lynx have higher fGC concentrations than wild lynx, which may be related to differences in metabolic rate, diet, or body condition.
4) Husbandry factors, particularly the size of the enclosure relative to the number of lynx and the sex of the cage-mates, are associated with different patterns of fGC expression.

The non-invasive tool of fecal hormone analysis provides a useful tool for understanding the dynamics of stress physiology, as well as fitness consequences of differential patterns of adrenal activity. Establishing normative patterns of adrenal activity for a species is useful not only for improving management plans for that species, but also for developing a more comprehensive understanding of basic stress physiology.

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Literature Cited

Angelier, F., H. Weimerskirch, S. Dano, and O. Chastel. 2007. Age, experience and reproductive performance in a long-lived bird: a hormonal perspective. Behavioral Ecology and Sociobiology 61:611-621.

Carlstead, K., J. L. Brown, S. L. Monfort, R. Killens, and D. E. Wildt. 1992. Urinary monitoring of adrenal responses to psychological stressors in domestic and nondomestic felids. Zoo Biology 11:165-176.

Carlstead, K., J. L. Brown, and J. Seidensticker. 1993. Behavioral and adrenocortical responses to environmental-changes in leopard cats (*Felis bengalensis*). Zoo Biology 12:321-331.

Clubb, R. and G. J. Mason. 2007. Natural behavioural biology as a risk factor in carnivore welfare: How analysing species differences could help zoos improve enclosures. Applied Animal Behaviour Science 102:303-328.

Cutler, G. B., M. Glenn, M. Bush, G. D. Hodgen, C. E. Graham, and D. L. Loriaux. 1978. Adrenarche – Survey of rodents, domestic animals, and primates. Endocrinology 103:2112-2118.

Davey, G. 2007. Visitors' effects on the welfare of animals in the zoo: A review. Journal of Applied Animal Welfare Science 10:169-183.

Dehnhard, M., K. Fanson, F. Göritz, S. Naidenko, A. Vargas, and K. Jewgenow. *In review.* Comparative metabolism of gestagens and estrogens in the four lynx species, the Eurasian (*Lynx lynx*), the Iberian (*Lynx pardinus*), the Canada lynx (*Lynx canadensis*) and the bobcat (*Lynx rufus*).

Dembiec, D. P., R. J. Snider, and A. J. Zanella. 2004. The effects of transport stress on tiger physiology and behavior. Zoo Biology 23:335-346.

Frigerio, D., J. Dittami, E. Möstl, and K. Kotrschal. 2004. Excreted corticosterone metabolites co-vary with ambient temperature and air pressure in male Greylag geese (*Anser anser*). General and Comparative Endocrinology 137:29-36.

Goff, D. 2008. North American Regional Canadian Lynx Studbook (*Lynx canadensis*), Bridgeport, CT: Connecticut's Beardsley Zoo.

Hajamor, S., J. P. Despres, C. Couillard, S. Lemieux, A. Tremblay, D. Prud'homme, and A. Tchernof. 2003. Relationship between sex hormone-binding globulin levels and features of the metabolic syndrome. Metabolism-Clinical and Experimental 52:724-730.

Li, C., Z. Jiang, S. Tang, and Y. Zeng. 2007. Influence of enclosure size and animal density on fecal cortisol concentration and aggression in Père David's deer stags. General and Comparative Endocrinology 151:202-209.

Mellen, J. D. 1991. Factors influencing reproductive success in small captive exotic felids (*Felis* spp.): A multiple regression analysis. Zoo Biology 10:95-110.

Möstl, E. and R. Palme. 2002. Hormones as indicators of stress. Domestic Animal Endocrinology 23:67-74.

Palme, R. 2005. Measuring fecal steroids - Guidelines for practical application. Annals of the New York Academy of Science 1040:75-80.

Palme, R., S. Rettenbacher, C. Touma, S. M. El-Bahr, and E. Möstl. 2005. Stress hormones in mammals and birds - Comparative aspects regarding metabolism, excretion, and noninvasive measurement in fecal samples. Annals of the New York Academy of Science 1040:162-171.

Price, E. E. and T. S. Stoinski. 2007. Group size: Determinants in the wild and implications for the captive housing of wild mammals in zoos. Applied Animal Behaviour Science 103:255-264.

Romero, L. M. 2002. Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates. General and Comparative Endocrinology 128:1-24.

Romero, L. M. 2004. Physiological stress in ecology: lessons from biomedical research. Trends in Ecology & Evolution 19:249-255.

Ruggiero, L. F., K. B. Aubry, S. W. Buskirk, G. M. Koehler, C. T. Krebs, K. S. McKelvey, and J. R. Squires. 2000. Ecology and conservation of lynx in the United States. University Press of Colorado, Boulder, Colorado.

Sapolsky, R. M. 2002. Endocrinology of the stress-response. Pages 409-450 *in* J. B. Becker and S. M. Breedlove, editors. Behavioral Endocrinology. MIT Press, Cambridge, Massachusetts.

Schwarzenberger, F. 2007. The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species. International Zoo Yearbook 41:52-74.

Terio, K. A., L. Marker, and L. Munson. 2004. Evidence for chronic stress in captive but not free-ranging cheetahs (*Acinonyx jubatus*) based on adrenal morphology and function. Journal of Wildlife Diseases 40:259-266.

Touma, C. and R. Palme. 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: The importance of validation. Annals of the New York Academy of Science 1040:54-74.

von der Ohe, C. G. and C. Servheen. 2002. Measuring stress in mammals using fecal glucocorticoids: opportunities and challenges. Wildlife Society Bulletin 30:1215-1225.

Wielebnowski, N. 2003. Stress and distress: Evaluating their impact for the well-being of zoo animals. Journal of the American Veterinary Medical Association 223:973-977.

Wielebnowski, N. and J. Watters. 2007. Applying fecal endocrine monitoring to conservation and behavior studies of wild mammals: Important considerations and preliminary tests. Israel Journal of Ecology & Evolution 53:439-460.

Wielebnowski, N. C., N. Fletchall, K. Carlstead, J. M. Busso, and J. L. Brown. 2002. Noninvasive assessment of adrenal activity associated with husbandry and behavioral factors in the North American clouded leopard population. Zoo Biology 21:77-98.

Table 4- 1. Comparison of five corticoid assays for monitoring ACTH-induced increases in adrenal activity (see Methods for assay abbreviations). "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 injection. Asterisk indicates unreliable baseline estimates due to samples falling below the detection limit. In these cases, peaks were assessed visually.

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

Table 4- 2. Parameter estimates for the effect of housing and husbandry on fGC expression in captive lynx.

		Rbase: fGC Baseline residuals (ln(ng/g))				Rmag: fGC Magnitude residuals (fold-increase)			
Effect	**Mean ± STD**	**Slope**	**DF**	**F**	**P**	**Slope**	**DF**	**F**	**P**
Cage-mate sex	N.A.	N.A.	3, 35	8.71	<0.001	N.A.	3, 14	8.52	0.002
# Cage-mates	1.25± 0.87	-0.39± 0.14	1, 35	7.42	0.010	-1.96± 0.85	1, 14	5.34	0.037
Density (ft^2) (*size / # lynx*)	1095± 1824	-0.0004± 0.0001	1, 35	14.18	<0.001	0.003± 0.001	1, 14	12.53	0.003
# Keepers	3.19± 1.33	-0.13± 0.04	1, 35	10.21	0.003	-0.71± 0.22	1, 14	10.10	0.007
Visitor proximity (ft)	4.76± 3.73	0.04± 0.03	1, 35	3.08	0.088	-0.58± 0.15	1, 14	14.28	0.002
Enrichment frequency(d/wk)	3.29± 2.62	----				-0.21± 0.09	1, 14	4.56	0.051
# Dens	1.98± 0.99	----				1.08± 0.59	1, 14	3.31	0.090

Table 4- 3. Effect of housing change on an individual's fGC levels. First letter of lynx ID indicates sex (F=female, M=male).

Lynx	Housing Change	Predicted Δ in fGC	Actual Δ in fGC	Δ in fGC (ng/g)	Test statistics
M3	Reduction in cage-mates ***Size/lynx*** - increase ***Cage-mate sex*** - 'both' to 'male'	Decrease Decrease	Decrease	-84.99	t_{50} =4.73 P <0.001
F25	Moved to new enclosure ***Size/lynx*** - increase ***Cage-mate sex*** - 'male' to 'alone'	Decrease No change	Increase	18.52	t_{20} =1.01 P =0.324
F10	Reduction in cage-mates ***Size/lynx*** - increase	Decrease	Increase	253.11	t_{101}=10.30 P <0.001
F18	Reduction in cage-mates ***Size/lynx*** - increase	Decrease	Increase	269.62	t_{93} =6.73 P <0.001
F13	Moved to new enclosure ***Size/lynx*** - increase ***Cage-mate sex*** - 'female' to 'alone'	Decrease Decrease	Increase	114.22	t_{94} =2.06 P =0.042
M1	Moved between institutions *not sufficient info for post-transfer enclosure		Increase	102.06	t_{61} =1.10 P =0.275
M17	Moved to larger enclosure ***Size/lynx*** - increase	Decrease	Decrease	-41.30	t_{146}=4.96 P <0.001

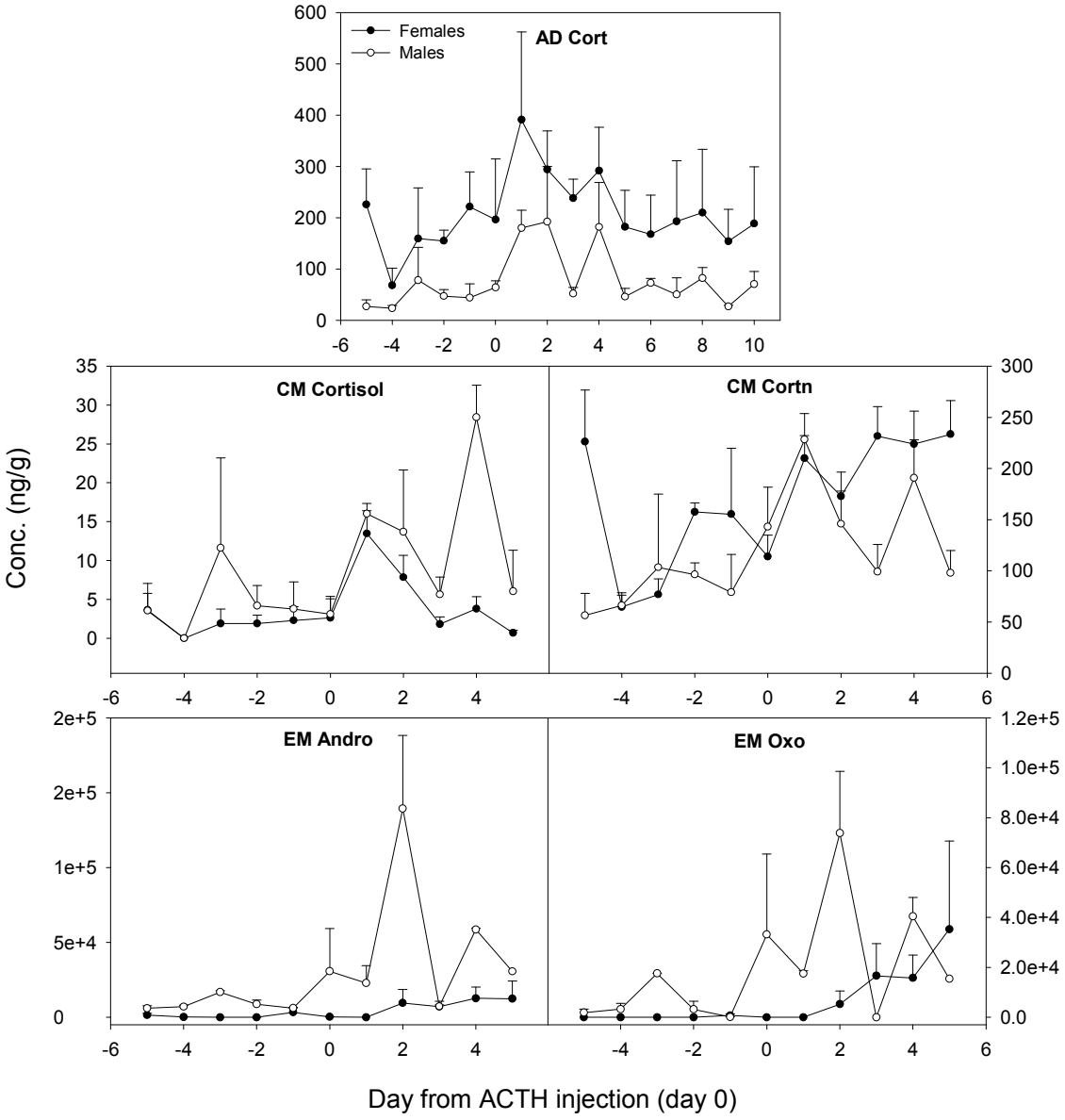


Fig. 4- 1. Comparison of five corticoid assays for monitoring ACTH-induced increases in adrenal activity (see Methods for assay abbreviations). Error bars represent 1 SEM. Note that because profiles are averaged, some of the distinct individual peaks become muted (see Fig. 4-2 for an example of clearer individual peaks).

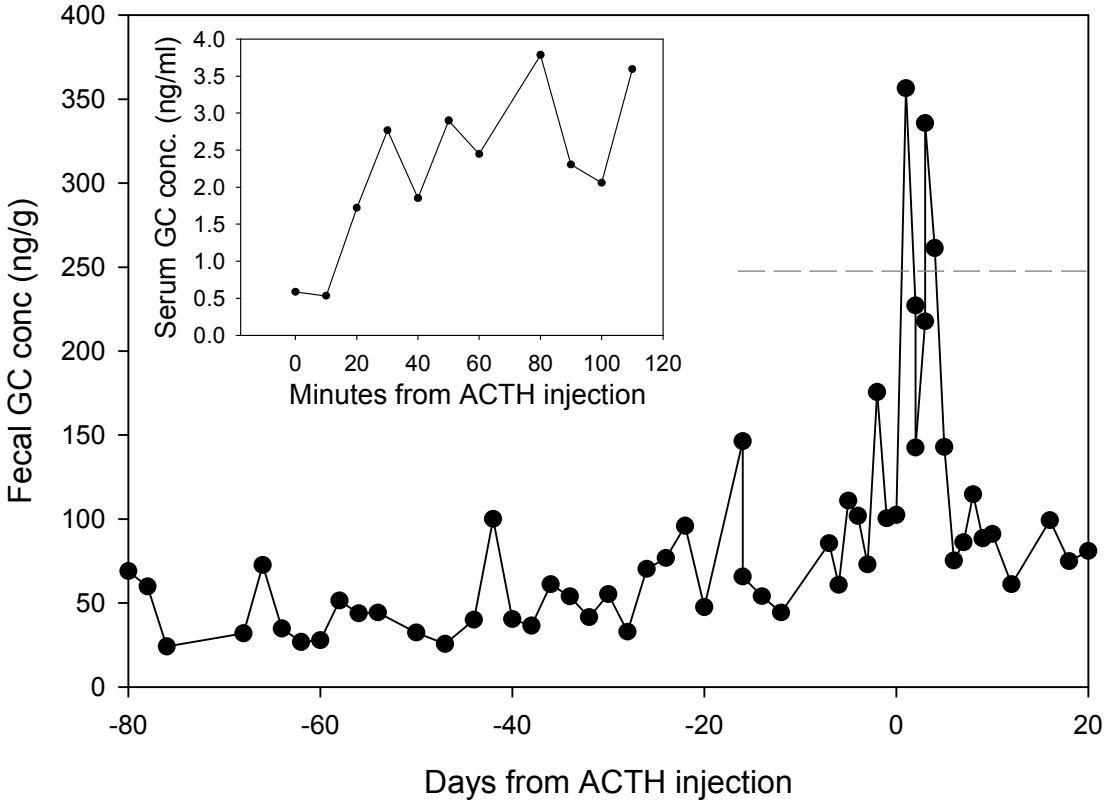


Fig. 4- 2. Representative serum and fecal corticoid profiles for one individual. Reference line indicates baseline+2SD.

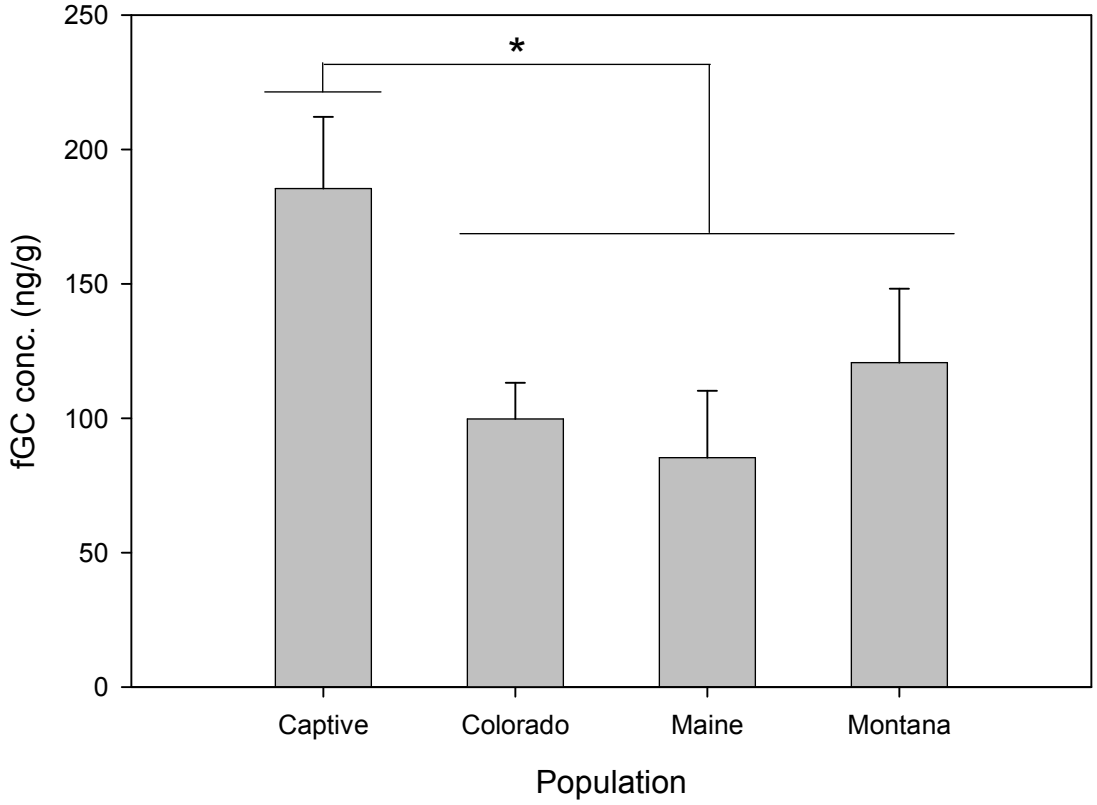
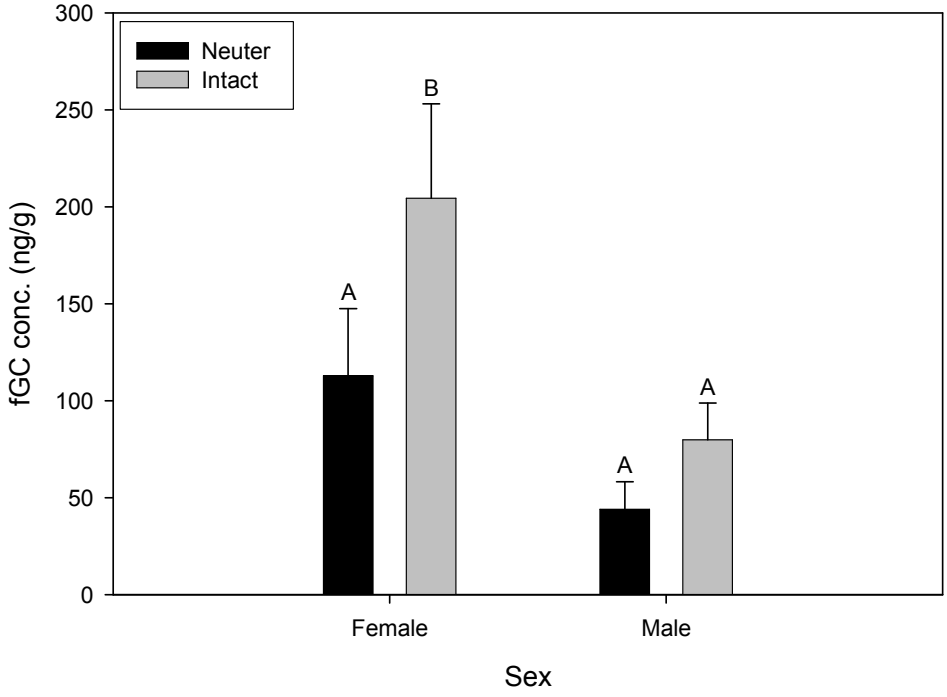


Fig. 4- 3. Differences if fGC concentrations between populations (back-transformed LS mean ± SE). Asterisk indicates a significant difference between groups.

a)



b)

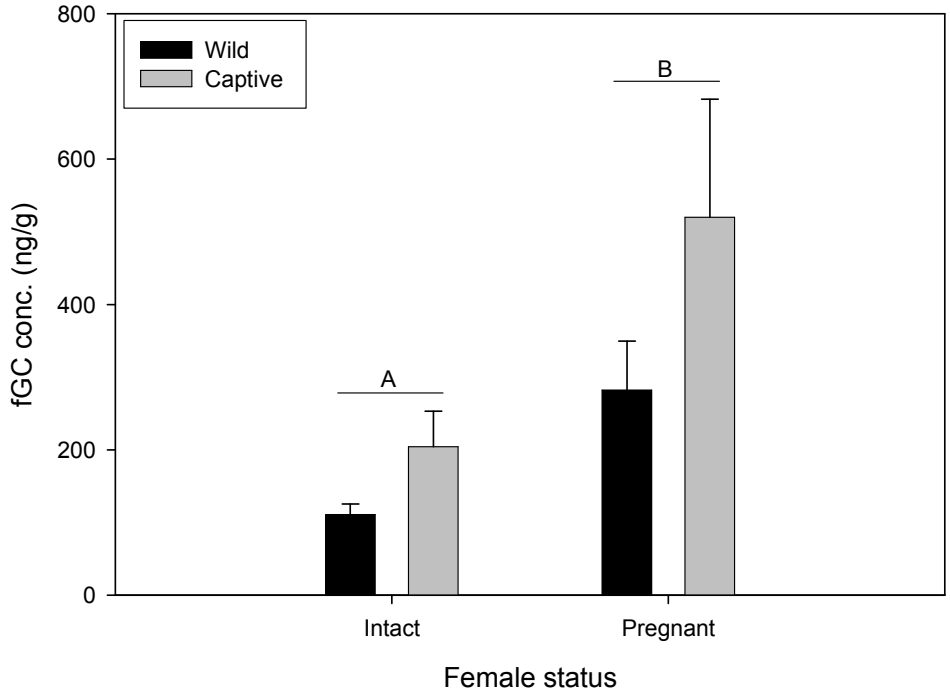


Fig. 4- 4. Effect of sex and status on fGC concentration (back-transformed LS mean ± SE). a) Comparison of captive females and males that are intact vs. castrated/spayed. b) Comparison of non-pregnant and pregnant females in captivity and the wild. Letters indicate statistically significant differences.

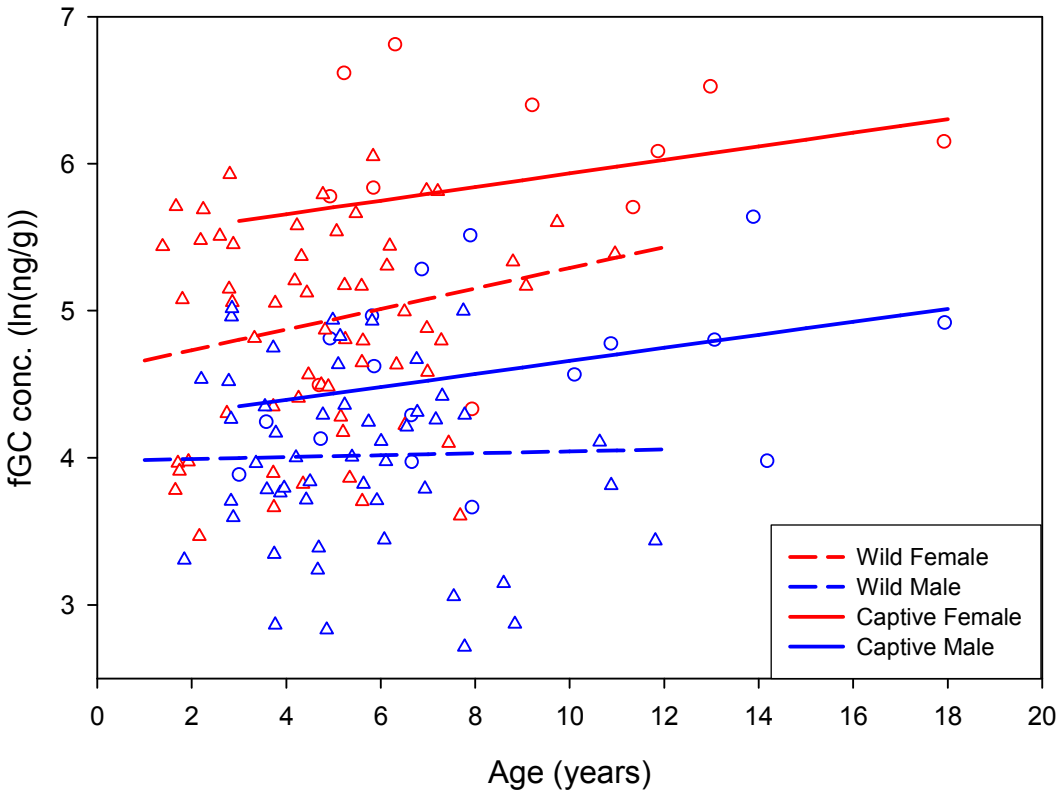
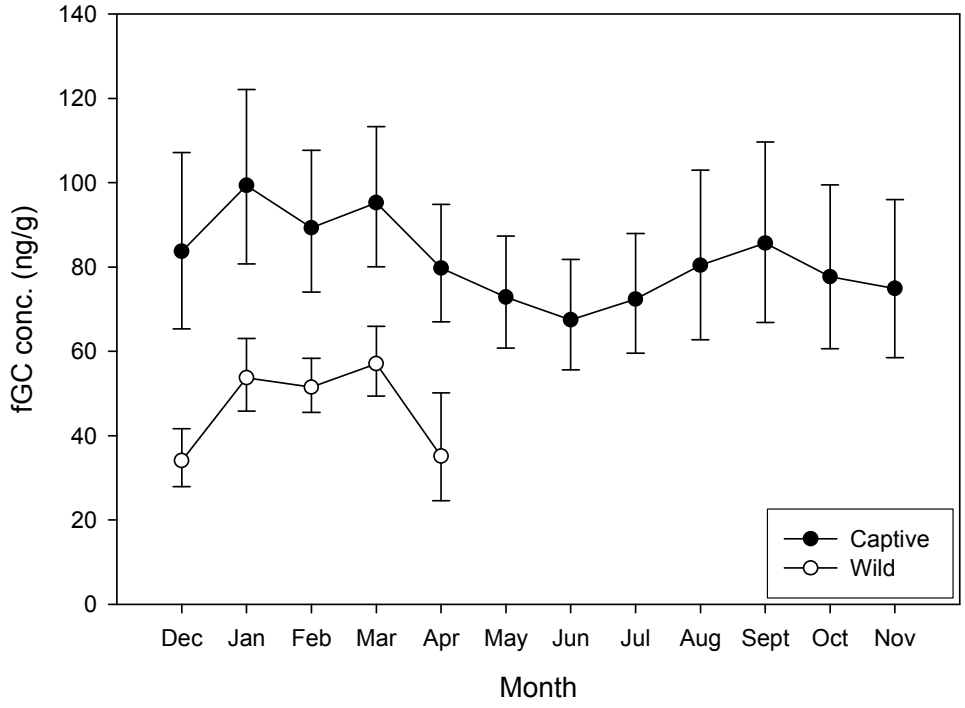


Fig. 4- 5. Effect of age on fGC concentration. Slope and intercept estimates obtained from biological-effects ANOVA. Note that the y-axis is in log-scale. Triangles and dashed lines represent wild lynx, circles and solid lines represent captive lynx, blue represents males, and red represents females.

a)



b)

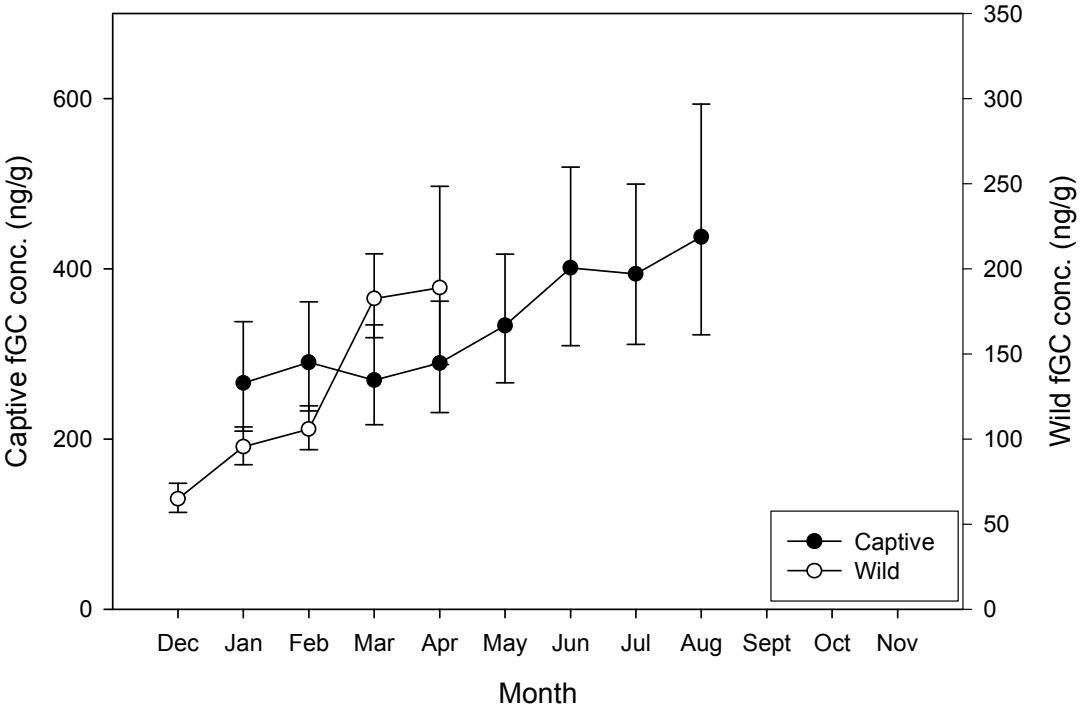


Fig. 4- 6. Seasonal patterns of fGC concentrations in males (a) and females (b). Data represent back-transformed LS mean ± SE. Data are only plotted for months where we have data from more than 1 individual. Note the different scales for captive and wild females (b).

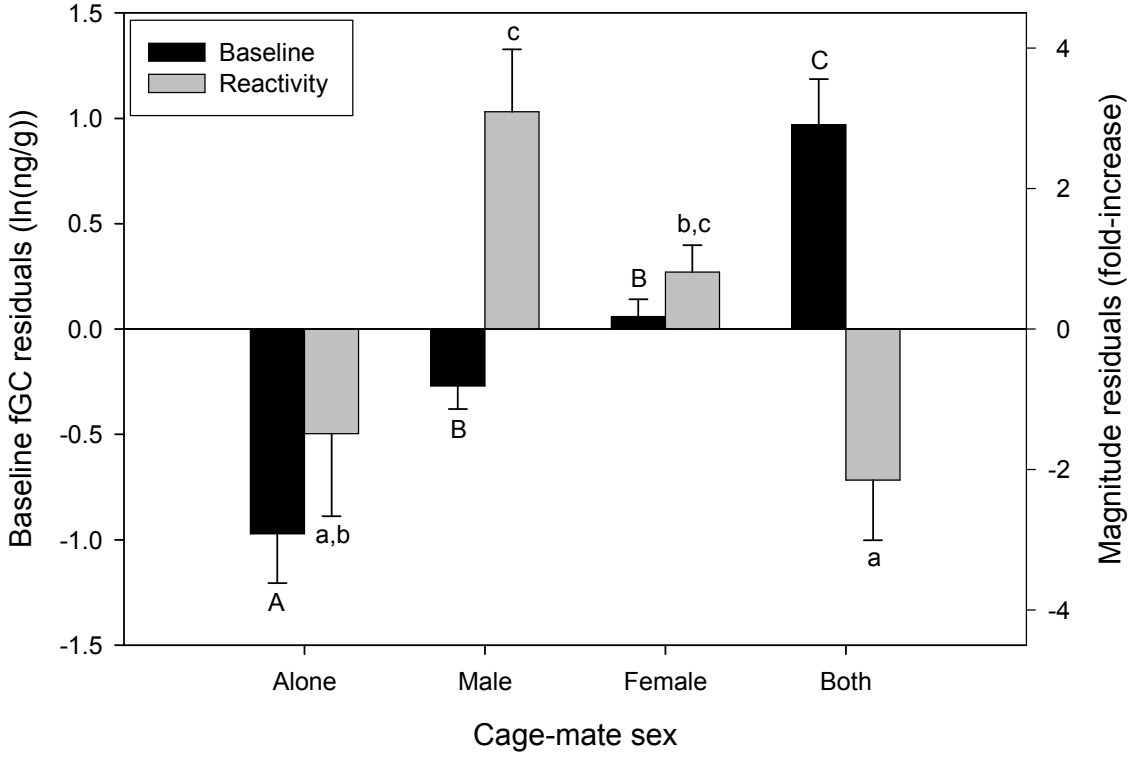


Fig. 4- 7. Effect of cage-mate sex on baseline fGC (Rbase) and response magnitude (Rmag) values (LS mean ± SE). The y-axis is expressed as residuals from the biological model. Letters indicate significant differences: upper-case letters used for Rbase and lower-case letters used for Rmag.

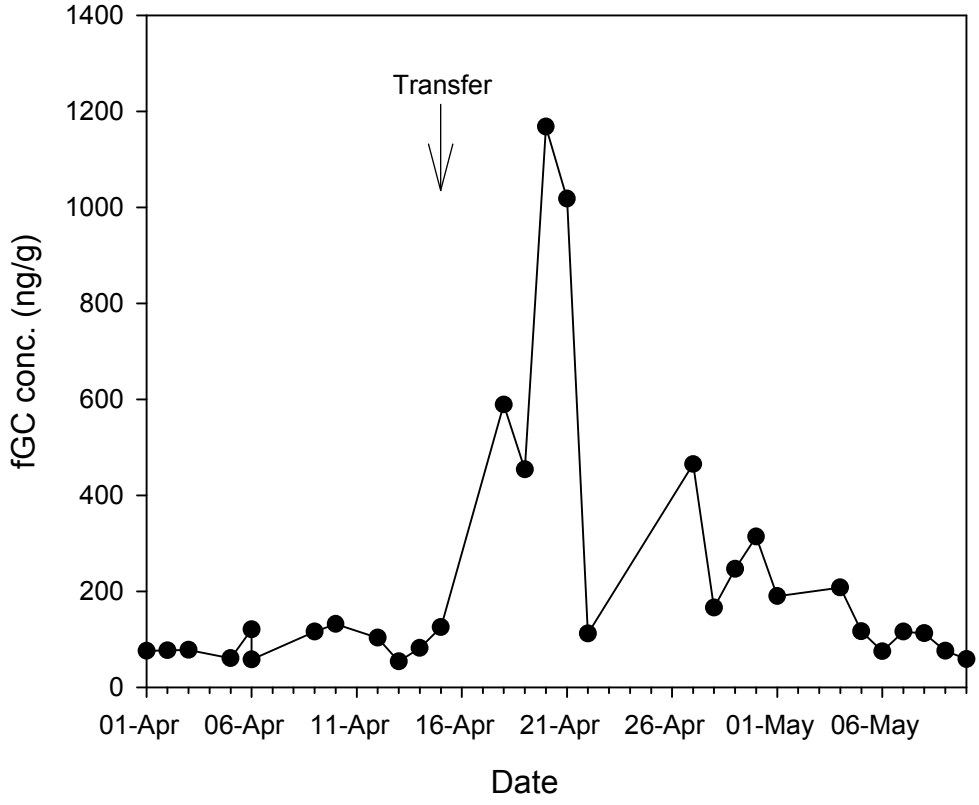


Fig. 4- 8. Longitudinal fGC profile for a male that was transferred between two institutions that were about 450 miles apart.

CHAPTER 5. PATTERNS OF STRESS PHYSIOLOGY IN REINTRODUCED CANADA LYNX AND IMPLICATIONS FOR REINTRODUCTION SUCCESS

Introduction

Reintroductions are critical for preserving endangered populations, as well as the integrity of ecosystems. As the number of threatened or endangered species continues to increase, so does the number of reintroduction efforts initiated world-wide (IUCN 1996, Fischer and Lindenmayer 2000). Unfortunately, the success of reintroductions is highly variable, and our ability to predict whether or not any given effort will succeed is very low (Saltz and Rubenstein 1995, Fischer and Lindenmayer 2000, Bar-David et al. 2005). Given the ecological value of reintroductions and the economic costs associated with such endeavors, we must gain a clear understanding of what contributes to the success or failure of a reintroduction. Recent advocacy for intensified post-release monitoring of reintroductions (IUCN 1996) has led to a number of behavioral and demographic studies (e.g., Meriggi and della Stella 2004, Bar-David et al. 2005; also see Fischer and Lindenmayer 2000). However, there is very little information about how the reintroduction process affects an animal's physiology; it is important to understand this because physiology can ultimately determine reintroduction success by influencing both demography and behavior.

Reintroductions are inherently associated with an array of potentially stressful stimuli (i.e. capture, handling, restraint, transport, novel environment). In response to stressful stimuli - both physical and psychological - vertebrates initiate a cascade of physiological changes that help them cope with the stressor (Sapolsky 2002). A critical component of this physiological stress response is activation of the hypothalamic-pituitary-adrenal (HPA) axis, which causes an elevation in circulating levels of glucocorticoids (GCs). While occasional, acute increases in GC levels are essential for survival, chronic increases and frequent acute increases in GC titers can have detrimental consequences for an individual, including suppressed reproduction, decreased immune function, altered metabolism, and compromised growth (Sapolsky 2002, Palme et al. 2005). The array of stressful stimuli associated with reintroductions may elicit a chronic stress response that impacts individual survival and reproduction, and consequently affects the

demographic parameters of a reintroduced population (Morton et al. 1995, Hartup et al. 2005, Teixeira et al. 2007, Zidon et al. 2009).

Furthermore, it has been widely documented among vertebrates that two distinct stress coping styles exist among individuals (Koolhaas et al. 1999, Sih et al. 2004, Overli et al. 2007). When confronted with the same stressor, some individuals exhibit high HPA axis reactivity / responsiveness, while others exhibit weak HPA reactivity. These differences in the magnitude of the stress response have been associated with distinct behavioral patterns, disease susceptibility, and differences in survival (Blas et al. 2007, Overli et al. 2007). However, there are very few empirical studies that examine how individual coping styles contribute to translocation outcomes. The little research that has been conducted suggests that individual variation in coping styles may be a critical determinant of the success of such endeavors. For example, Cabezas et al. (2007) found that rabbits that exhibited higher GC levels in response to a stressor also had a higher probability of survival post-release.

The effect of reintroduction events on stress physiology, as well as individual differences in response to these events, may be important contributors to reintroduction success. Indeed, a number of studies have documented a "reintroduction effect," in which individual survival and/or reproductive success is considerably lower during the early years of a reintroduction effort (e.g., Saltz and Rubenstein 1995, Meriggi and della Stella 2004, Bar-David et al. 2005). Unfortunately, the mechanisms causing this "reintroduction effect" are unknown. It is plausible that a non-adaptive physiological stress response may underlie the "reintroduction effect" (Saltz and Rubenstein 1995, Bar-David et al. 2005). If we can identify individual differences in stress coping styles that are related to post-release survival, then we may be able to "screen" individuals prior to release and thereby facilitate the successful establishment of translocated populations. Thus, understanding the role of stress physiology in reintroductions may be critical for enhancing their success rates.

The recent reintroduction of Canada lynx to southern Colorado provides a valuable opportunity to study stress physiology throughout the reintroduction process. The Colorado reintroduction is unique in that biologists have adopted an intensive post-release monitoring program, providing extensive information about both population demographics and individual behavior (see Shenk 2001, 2006, 2008). Furthermore, field biologists are able to collect fecal samples from known lynx, allowing us to monitor patterns of stress physiology using the non-invasive technique of fecal hormone analysis. Rarely is it possible, physically or financially, to collect as much post-release information as has been acquired through this project. With this

extensive database, we can synthesize physiological, behavioral, and life-history data for individual lynx to obtain a more holistic understanding of how individuals respond to the reintroduction process. By integrating the existing post-release data with fecal GC analysis, we are able to adopt a multi-disciplinary approach to understanding how certain stimuli affect an individual's stress physiology, and how that may in turn affect behavior and population demographics. We are in a unique position to study this process because behavioral data, ecological information and fecal samples have been collected from known individuals during the entire reintroduction effort.

This study had three objectives: 1) to monitor patterns of hormone expression while lynx are temporarily held in holding pens, and use this information to develop indices of stress reactivity for individual lynx, 2) to examine how individual variation in stress physiology is linked to post-release fitness, and 3) to describe how adrenal activity changes post-release and in relation to ecological correlates. To monitor stress physiology in reintroduced lynx, we employed the non-invasive technique of fecal hormone analysis. The ultimate goal of this project is to improve reintroduction success rates by developing a better understanding of how individuals respond physiologically to the reintroduction process.

Methods

Animals and Reintroduction Details

Canada lynx (*Lynx canadensis*) are elusive, small cats (9–11 kg) that inhabit North America's boreal forests. They are primarily found in coniferous forests with a mixture of forest age classes (Poole 2003). Lynx once roamed throughout much of the northern United States, but trapping and habitat destruction have dramatically reduced most US populations (Ruggiero et al. 2000). They feed primarily on snowshoe hares and squirrels (Nowak 1999). In the northern part of their range, their dependence on snowshoe hares is so strong that their population size fluctuates with snowshoe hare density, leading to the classic 10-year snowshoe hare/lynx cycle (Ruggiero et al. 2000, Poole 2003). Lynx are solitary, but there is some overlap of home ranges between males and females (Ruggiero et al. 2000).

Canada lynx breed from late February to early April and give birth primarily in May (Nowak 1999, Ruggiero et al. 2000). Mating pairs remain together for several days and females

presumably mate with only one male. Gestation is 70 days (Nowak 1999). Lynx kittens are altricial and remain with their mother until the next breeding season, but siblings may maintain contact and hunt together through the following summer. It is possible that related females maintain social contact throughout adulthood, but this is unclear (Ruggiero et al. 2000).

In 1999, the state of Colorado initiated a substantial Canada lynx reintroduction program. The native lynx population in Colorado is believed to have been extirpated; the last sighting of a non-reintroduced lynx was in 1974 (Ruggiero et al. 2000). Therefore, the entire lynx population in Colorado is most likely comprised of reintroduced individuals. All reintroduced lynx were caught from healthy wild populations (Alaska, British Columbia, Manitoba, Quebec, and Yukon). Lynx were trapped between November and March and transported to Colorado after a brief holding period and initial exam. Lynx were reintroduced in a series of releases that occurred between 1999 and 2006. Post-release data were obtained via satellite-, aerial-, and snow-tracking. (See Shenk 2001, 2006, 2008 for further details about the reintroduction.)

Upon arrival in Colorado, lynx were kept in holding pens (15 m^2) for 1-4 months. This holding period served to monitor the health of the lynx and improve body condition prior to release. Lynx were primarily housed individually, although some lynx were housed in same-sex pairs. They were fed daily, and the majority of their diet consisted of domestic rabbits. Human presence was minimized as much as possible. All lynx were fitted with a radio- or satellite-collar and released in the spring.

After they are released, all lynx are closely monitored using radio- or satellite-collars equipped with a mortality signal. When a mortality signal is detected, the carcass is retrieved as quickly as possible and a thorough necropsy is performed at Colorado State University. In most cases, there is specific information about length of survival and cause of death for each individual. There was considerable variation in survival post-release, with the shortest survival being ~20 days and the longest being at least seven years post-release.

Fecal samples have been collected from known lynx throughout the reintroduction process. Therefore, we were able to monitor stress physiology during two distinct phases of the reintroduction process: temporary holding prior to release ("holding") and in the field post-release ("field"). In this paper, we consider four variables related to aspects of the reintroduction, hereafter called "reintroduction factors": source population, duration of time in holding pens, release year/cohort, and date of release (see Tables 5-1 and 5-2). We also include the biological factors sex and age. Age was estimated for all lynx during initial exams. Additionally, when

lynx died, a lower canine was sent to Matson Labs (Missoula, Montana) to obtain a more accurate age estimate based on tooth enamel.

Fecal Sample Collection

Pen collection

Lynx are temporarily housed in holding pens in Colorado prior to release. During this time, lynx are exposed to several known stressors (specifically transport, physical exams, and periods of intense human activity). Thus we can assess an individual's physiological response to these events and develop an index of stress reactivity for each lynx by analyzing fecal samples collected while in holding pens. Care-takers collected fecal samples as soon as possible after defecation and immediately froze them (total = 1886 samples; Table 5-2).

Field collection

Fecal samples were collected from free-ranging lynx during snow-tracking sessions. During the winter months (December to April), biologists used radio-telemetry to estimate the lynx' location, and then circled the signal until they found a track. The track was followed away from the lynx as far as possible, which allowed biologists to collect fecal samples and ecological data for each individual. Although most samples were collected within 24 hours, previous experiments indicate that hormone metabolites remain stable for up to 4 days in winter conditions (see Chapter 1). A total of 1085 samples were collected in the field from 102 lynx over the course of 10 years. All samples were stored in zip-lock bags at -20°C, and were shipped on ice to Brookfield Zoo for analysis.

Ecological data

Radio- and snow-tracking have generated valuable information about the behavior and ecology of each individual. This database includes information about habitat use, movement

patterns, diet, mating encounters, and reproductive success. In this paper, we include three categories of ecological factors: habitat, human, and trophic (Table 5-1).

To obtain habitat data, biologists conducted vegetation analysis at numerous intervals during a tracking session, including when a fecal sample was located. Vegetation plots were 144 m^2, and included quantification of understory, overstory, and snow depth (see Shenk 2006 for further details on vegetation analysis). In addition, the slope of the terrain, the aspect, and the elevation (m^2) were recorded at each site. Here, we only consider the following vegetation variables: dominant tree species in the plot, and total number of trees per plot (reflection of tree density).

Throughout a tracking session, biologists also recorded information about overall activity of humans ("human" factors) and other species ("trophic" factors). Human activity included road density, snowmobiling, snowshoeing, and skiing, and activity level was ranked on a scale from 1 (low) to 3 (high). If evidence of a given activity was not observed by the field biologists, it was assigned a score of 0 (none). Presence of other species was primarily estimated through tracks, although visual observations were also obtained occasionally. Scoring levels were either 1 (single track/observation) or 2 (multiple tracks/observations). Similar to human activity, if a species was not detected, it was assigned a score of 0 (none). For this analysis, species were either scored as present or absent, and the number of each species within a trophic group (carnivore, prey, ungulate) was the response variable.

Steroid Extraction and Analysis

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 1500 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.

Fecal glucocorticoid metabolites (fGC) were quantified using a commercially available Corticosterone EIA (Assay Designs, Ann Arbor, MI), which has been previously validated for Canada lynx (see Chapter 4; see Appendix 2 for assay procedures). To monitor precision and reproducibility, low (~70% binding) and high (~30% binding) quality control samples were run on each plate. Intra-assay coefficients of variation were 15.5% and 11.4% (n=19) for low and high controls, respectively. The inter-assay coefficients of variation were 23.9% and 21.8%

(n=83), respectively. All samples were assayed in duplicate. Data are expressed as ng/g wet fecal weight.

Statistical Analysis

All analyses were conducted using SAS 9.1 (Cary, NC). See Fig. 5-1 for a schematic diagram of the analyses considered in this chapter.

fGC Variables and Characterization of Hormonal Profiles

A key component of this study was summarizing the dynamic glucocorticoid response. As can be seen in Figs. 5-2 and 5-4, patterns of fGC expression are quite variable among lynx while in holding pens. Therefore, we took a multivariate approach to distill the key characteristics of each lynx' fGC profile. We summarized each individual's profile in the pen using the following seven statistics: mean, median, baseline, mean peak, coefficient of variation (%CV), reactivity, and slope (also see Table 5-3). Baseline was calculated through an iterative process in which points >3 SD of the mean were removed until no points exceeded this threshold, and the remaining data were averaged. Mean peak was calculated as the average of all points excluded from the baseline. If a lynx did not have any detectable peaks, their mean peak was set as their mean fGC. Reactivity was calculated as the mean peak divided by baseline, and reflected the average magnitude of an individual's stress response. Slope reflected change in fGC expression over time in the holding pen, and was determined by fitting a regression line to an individual's baseline fGC profile.

To reduce the number of fGC variables, we conducted a principal component analysis (PCA) on the correlation matrix of the seven variables. After performing a varimax rotation, we identified three interpretable principal components (PCs) of corticoid expression in the pens (Table 5-3): PC1 ('Mean') represents mean level of fGC and provides an index of overall HPA axis activity; PC2 ('Reactivity') reflects the magnitude of increase in fGC concentration in response to a stressor, and thus provides an index of HPA axis reactivity; and PC3 ('ΔfGC') is the slope of the change in baseline fGC values while in the pens. Interestingly, we found that the standard deviation was split between PC1:Mean and PC2:Reactivity. This result is due to the fact that as mean fGC increases, variation generally increases as well. Additionally, if a lynx reacts

strongly to a stressor, standard deviation increases drastically due to the square penalty function of variance estimates. Therefore, the PCA splits this contribution between PC1:Mean and PC2:Reactivity. Hereafter, we refer to these three pen corticoid components as "PenCCs."

Pen fGC Profiles

The first goal of the study was to describe the effect of biological factors (age and sex) and time in holding pen on fGC expression in lynx temporarily held in holding pens. (For a description of fGC expression in wild/free-ranging lynx, see Chapter 4). For this analysis, we examined how two different aspects of HPA activity changed over time: baseline fGC concentration and variation in fGC expression.

To determine if baseline fGC concentrations changed over time in the holding pens, we constructed a random coefficient model, which estimates individual slopes for lynx and then estimates the overall slope for the population from these individual slopes (see Littell et al. 2006). In addition to the length of time in the holding pens and the biological factors of age and sex, the model also controlled for the effects of source population and release year. To estimate the effect of time in pen on baseline hormone level, we initially included a linear and quadratic term in the model, but the quadratic term was removed because it was not significant. We assumed an unstructured covariance matrix for the intercept and slopes. Denominator degrees of freedom were adjusted using a Satterthwaite approximation. Two-way interactions were excluded if they were not significant. We log-transformed fGC concentration to satisfy model assumptions.

In a separate analysis, we explored the pattern of variance in fGCs throughout the time in the pen. High variability in fGCs may reflect repeated activation of the adrenal glands, possibly due to stress, and the purpose of this analysis was to determine if the dynamics of adrenal activity changed depending on the length of time in captivity. To obtain variance estimates over time, an individual's time in the holding pen was divided into 10-day intervals, and the standard deviation of fGC concentration was calculated for each interval. Individuals with fewer than 3 samples/interval and fewer than 5 intervals were excluded. (Note: this fGC variable was not included in other analyses because there were too many missing points.) We then constructed a random coefficient model as above, except using the log of standard deviation as the response variable. For all models, normality and homoscedasticity assumptions were satisfied.

Pen fGC Profiles and Post-Release Survival

Our second objective was to examine how dynamics of an individual's adrenal activity in the pens correlated with post-release survival. To test if pen hormone profiles predict survival success, we performed a parametric survival analysis assuming a Weibull distribution. This distribution was chosen based on an approximately straight log-log plot of the survival function distribution (Cantor 2003). In the model, we included source population, cohort, lynx age at release, length of time in pen, sex, and the three PenCCs. We censored lynx still alive at the time of last database update (April 2008).

Post-Release Patterns of fGC Expression

An individual's pattern of fGC expression in the field may be influenced by several factors, including an individual's known pattern of adrenal activity (determined while in the holding pens), factors associated with the reintroduction, and ecological conditions (see Table 5-1).

First, to understand how patterns of fGC expression in the pen related to post-release patterns of fGC expression, we tested for the effects of PenCCs on the difference in mean fGC concentrations between pen and post-release periods. We conducted a general linear model with the difference between an individual's pen and field mean fGC as the response variable. The model also controlled for sex, age, and duration of time in holding pens. We had a reduced sample size (N=32) for this analysis, for various reasons: lynx died before fecal samples could be collected, trackers were unable to locate lynx post-release, fecal samples were not collected in the pen.

Next we explored how fGC concentration changed with time from release. First, we averaged all of an individual's samples for each year since release. We then conducted a repeated measures model, in which we treated year since release as a categorical variable. Since samples from the same lynx are correlated and samples from adjoining years may be more correlated, we tested three covariance structures: 1) compound symmetry, 2) first order autoregressive, and 3) first order autoregressive with lynx id as a random effect (Littell et al. 2006). Using BIC and AICC values, the assumptions of compound symmetry fit the best. In addition to time since release, we also included sex, age, duration of time in holding pens, and the year and month of sample collection. Unfortunately, due to sampling limitations, there were very few lynx for

which we had data from the holding pens and across multiple years post-release; therefore, we could not include the PenCCs.

Finally, we explored the relationship between environmental correlates and fGC levels through an exploratory analysis. We divided the environmental correlates into three categories: habitat, human and trophic (Table 5-1). Dividing the correlates accomplished two objectives: 1) we could do a more thorough analysis within each category and 2) we retained a larger dataset for each category due to missing data across categories. As the correlations among the variables within each category were $r<0.5$, we did not implement any variable reduction techniques. We tested all possible variable combinations and two-way interactions within each category. Response variable was log-transformed fGC concentration. Each model treated sex, as well as the month and year the sample was collected, as fixed, categorical effects. Additionally, we treated lynx id as a random factor. Each linear mixed model was fitted using maximum likelihood estimation and denominator degrees of freedom were adjusted using a Satterthwaite approximation (Littell et al. 2006). We then used an information-theoretic approach for model comparison, based on Akaike weights (Burnham and Anderson 2002). We ranked all ordinal variables (e.g. carnivore density, skiing, roads) using a scale from 0 to 4, and treated them as numeric variables. For the final models, we confirmed that normality and homoscedasticity assumptions were valid. To visualize the main results, we ran a linear mixed model with sex, tracking year, month collected, and lynx id, and then used the residuals from this model for all figures.

Results

Pen fGC Profiles

We first analyzed how biological factors affected fGC profiles while in the pen (Table 5-4). Females had higher baseline fGC concentrations (5.7±0.12 v. 3.6±0.17 ln(ng/g)) and overall fGC variance (5.6±0.13 v. 3.4±0.11 ln(ng/g); Fig. 5-4) than males. Baseline fGC values increased with age, but the effect of age on fGC variation differed between the sexes. In females, fGC variation increased with age (0.56±0.11 ln(ng/g)/year, $t_{20.8}=5.37$, $P<0.001$; Fig. 5-5), but in males, there was little or no effect of age (-0.02±0.05 ln(ng/g)/year, $t_{19.1}=-0.46$, $P=0.65$; Fig. 5-5).

We also considered how fGC expression changed over time in the holding pens (Table 5-4). Baseline fGC concentration increased for both sexes in a similar manner (0.01±0.002 ln(ng/g)/day; Fig. 5-4). However, fGC variance increased more steeply for females (0.21±0.05 ln(ng/g)/day, $t_{33.6}$=4.2, P<0.001; Fig. 5-4) than males (0.05±0.03 ln(ng/g)/day, t_{37}=1.52, P=0.14; Fig. 5-4). Neither source population nor cohort had a significant effect on fGC baseline or variance (Table 5-4).

Pen fGC Profiles and Post-Release Survival

We found that both the lynx's age at release and PC2:Reactivity predicted survival post-release (Table 5-5, Fig. 5-6). At least above a threshold, lynx with high reactivity to a stressor had lower expected lifespan. To test the sensitivity of the model to the highest PC2:Reactivity values, we removed the two highest values and reran the model; the qualitative conclusions remained the same (PC2, P=0.016). We also found that older lynx had lower survival rates than younger lynx.

Post-Release Patterns of fGC Expression

Overall, lynx showed a pronounced drop in mean fGC levels post-release (Fig. 5-7). Lynx with higher PC1:Mean values exhibited a larger decrease in fGC after they were released (Table 5-6). In addition, lynx that were held in the holding pens longer also showed a larger decrease in fGC values. Male lynx had a smaller decrease in fGC concentrations than females, although the difference was not significant. Proportionally, male fGC values decreased by less than half, whereas female fGC values decreased approximately three-fold.

We did not find any evidence that fGC expression changed with time since release (P>0.8; Table 5-7, Fig. 5-7). Although males and females exhibit slightly different trends across years, there was no significant interaction between sex and time since release. Some of the variability between years may be explained by differences in sampling and sample sizes across years.

The habitat analysis revealed a suite of likely models and in general, the best models included slope of the terrain (β=0.04 ± 0.02 ln(ng/g)/degree, P=0.07), slope aspect (β= -0.0008 ± 0.0008 ln(ng/g)/degree, P=0.35), and snow depth (β=0.005 ± 0.006 ln(ng/g)/cm, P=0.46) (Table

5-8). The best model suggested a negative interaction between snow and slope (β= -0.0007 ± 0.0003 ln(ng/g)/degree, P=0.05).

For the human activity results, the best model included skiing and snowmobiling and their interaction (Table 5-8). To understand this interaction, we plotted the mean residuals for all possible combinations of skiing and snowmobiling levels (Fig. 5-9). In general, the lowest fGC concentrations are seen in habitats with no snow-mobiling activity, and as snow-mobiling increases, so do fGC values. However, in habitats with the highest snow-mobiling and highest skiing activity, fGC concentrations are particularly low. To examine the strength/robustness of this trend, we reran the final model, treating skiing as a categorical variable and using a linear contrast. We found statistical support that the snowmobile slope at the highest skiing level (which was negative) differed from the other three slopes (which were positive; difference=1.6 ± 0.61 ln(ng/g)/level, P=0.01).

For the trophic analysis, the best model included a positive relationship between fGC and carnivore activity (β=0.33 ± 0.15 ln(ng/g)/level, P=0.04) and a negative relationship between fGC and prey activity (β= -0.58 ± 0.32 ln(ng/g)/level, P=0.08) (Table 5-8; Fig. 5-10). This model was twice as likely as the next best model.

Discussion

A potential key to reintroduction success is understanding which biological attributes affect post-release survival. This study provides a valuable perspective on stress physiology throughout the reintroduction process, and also examines the importance of individual variation in dynamics of adrenal activity. The intensive monitoring of reintroduced Canada lynx in Colorado has provided a wealth of information about individual behavior and fitness post-release. By integrating fecal hormone analysis with the existing monitoring program, we are able to explore how the dynamics of glucocorticoid (GC) expression are influenced by the reintroduction process.

Pen fGC Profiles

We found that fGC concentrations were higher in females than males, and that fGC values increased with age. This matches patterns of fGC expression in wild (naturally-occurring)

and captive lynx (see Chapter 4 for further discussion). The significance of these differences is unclear, as they may reflect differences in GC production, concentrations of GC binding globulins in circulation, or the metabolism and excretion of GCs.

For both males and females, baseline fGC concentrations increase over time in the holding pens. Note that this is *baseline* fGC (i.e., peaks are excluded). Many lynx exhibited an initial peak in fGC concentrations in response to the transport process, and then fGC values declined within a few days, indicating some degree of acclimation. Thus, lynx exhibit a similar fGC profile in response to capture and transport as other species (Franceschini et al. 2008, Bosson et al. 2009). The increase in baseline fGC concentration is independent of peaks, and this trend appears to be linear; there was not strong support for a quadratic fit to the slope. Furthermore, fGC variance also increased with time, especially for females.

As fGCs are a valid measure of adrenal activity (see Chapter 4), this finding may imply that lynx become increasingly stressed the longer they are in captivity. Indeed, elevated GC values in temporarily confined animals is often attributed to the stress associated with captivity/confinement (Franceschini et al. 2008, Dickens et al. 2009). For Canada lynx, this pattern could either be a response to the process of being confined itself, or could be associated with patterns of activity around the holding pens. In general, human activity is minimized as much as possible around the pens. However, human activity increases toward the end of the holding period in preparation for the releases (physical exams, press coverage, preparing animals for transport).

Alternatively, this increase in adrenal activity could be related to metabolic or dietary factors. Not only are fGC concentrations higher in holding pens compared to wild (naturally-occurring) lynx, but we also found that concentrations of reproductive hormone metabolites were elevated as well (K. Fanson, unpubl. data). Furthermore, the values observed for all steroid metabolites in penned lynx were similar to values observed in permanently captive Canada lynx. We suspect that factors associated with energy regulation underlie these population differences (see Chapters 2 and 3). While in the holding pens, lynx have a more constant food supply, lower activity levels, and body mass generally increases. However, if energy regulation is associated with changes in steroid production/metabolism, this most likely affects baseline fGC values, and not fGC variance. The fact that fGC variance also increases throughout holding suggests that fGCs still reflect changes in an individual's stress-axis state.

Pen fGC Profiles and Post-Release Survival

The dynamic nature of the HPA axis can be difficult to quantify, and consequently researchers often over-simplify patterns of adrenal activity by just measuring the mean/baseline and absolute peak. However, these measures are correlated to some degree: if an individual's baseline fGC values are high, then their peaks, by definition, must also be high. The inverse is not necessarily true – an individual with a low baseline may have either high or low fGC peaks. For Canada lynx, we found a high degree of correlation between baseline and peak fGC values. By using the ratio between baseline and peak fGC values, we are able to isolate the magnitude of the increase and remove any correlation with an individual's overall level of HPA activity. Indeed, we found that this measure of reactivity was not correlated with mean or baseline. Furthermore, this was the most significant fGC parameter for predicting post-release survival.

The relative magnitude of an individual's fGC response, or their HPA axis reactivity, was a good predictor of post-release survival. Individuals that exhibited a strong fGC response to stressors in the holding pen had a higher rate of post-release mortality. Increasing evidence suggests that there are two distinct stress coping styles that are manifested across species, and that these coping styles are associated with a suite of behavioral and physiological characteristics (Koolhaas et al. 1999, Sih et al. 2004). "Proactive" individuals typically respond to stressors by taking action; they fight, flee, build nests, etc. (Koolhaas et al. 1999). Conversely, "reactive" individuals respond by freezing, or decreasing their activity level. These two behavioral responses are associated with distinct patterns of HPA activity, as well. Proactive individuals have lower baseline fGC as well as smaller fGC peaks than reactive individuals (Koolhaas et al. 1999). The fact that Canada lynx with higher HPA reactivity in the pens also had higher post-release mortality may suggest that these individuals had a "reactive" stress coping style and were less inclined to search for new home ranges, try new food sources, or take other actions necessary to survive in a novel environment.

Very few studies have examined the importance of individual coping styles in relation to the success of reintroductions and other conservation efforts. Similar to our study, Cabezas et al. (2007) found that dynamics of GC expression in European wild rabbits held temporarily were associated with short-term post-release survival. However, in contrast to our findings, they found that mean serum and fecal GC concentrations were *positively* correlated with post-release survival. We did not find any relationship between mean fGC values and survival. The trophic differences between the two study species (i.e., lynx are a predator and rabbits are prey) may account for the conflicting results between the two studies. Whereas it may be adaptive for a

predator to be proactive and exploratory in a novel habitat, this behavioral strategy may be detrimental to a prey species. However, in swift foxes, "bold" or proactive behavior styles are associated with high post-release mortality (Bremner-Harrison et al. 2004). Patterns of GC expression were not monitored for the swift foxes, and we were unable to monitor behavior in Canada lynx, so we are unable to make direct comparisons between these studies. Therefore, further research is needed before we can make generalizations across trophic levels about which stress coping style favors the highest post-release survival in translocations and reintroductions.

Although they did not investigate individual differences in HPA activity, Dickens et al. (2009) found that events associated with translocation had additive impacts on stress physiology in chukar partridges. Specifically, translocated birds exhibited lower baseline corticosterone and an impaired ability to mount a stress response. The post-translocation recapture rate in this study is unclear, but it would be interesting to know whether recaptures could have been biased, possibly toward individuals with a particular stress coping style. Regardless, accumulating evidence highlights the fact that stress physiology may contribute to the outcome of conservation efforts such as translocations and reintroductions.

Post-Release Patterns of fGC Expression

We found that fGC concentrations decreased significantly after lynx were released, but did not change over subsequent years post-release. However, we were unable to capture their initial response after being released because the first post-release samples were collected a minimum of six months post-release due to constraints of snow-tracking and also to minimize potential impacts of tracking on the lynx. Similar to our findings, Franceschini et al. (2008) found that compared to pre- and post-translocation values, fGC concentrations were elevated in Grevy's zebras temporarily held during the translocation process. After release, fGC concentrations returned to pre-translocation values within 11-18 weeks. We were unable to obtain pre-reintroduction samples, so it is unclear how post-release fGC values in lynx compare to pre-capture values; however, they are similar to fGC concentrations observed in wild (naturally-occurring) lynx (see Chapter 4). In contrast, Zidon et al. (2009) found that when captive Persian fallow deer were released, fGC concentrations increased and remained elevated for up to 200 days.

Patterns of fGC expression in the field were associated with several environmental factors, particularly patterns of human activity and presence of other species. In general, fGC

concentrations increased with snow-mobile activity, while skiing, snow-shoeing, and road density did not appear to have a consistent effect on fGC values. However, in habitats that had high levels of snow-mobiling and high levels of skiing, fGC concentrations were particularly low. Sample size was small for this group, consisting of only four points, and so we cannot be certain this effect is real. Levels of human activity are scored by trackers based on their perception of human activity throughout a tracking session, and thus do not apply to the specific location the scat was collected. Furthermore, due to excretion lag-time, fecal steroid metabolites reflect physiological events that occur 24-48 h prior to defecation. If samples are collected from lynx when they are within their core-use area, then fGC values likely reflect the lynx' perception of the general habitat. However, if samples are collected when lynx are traveling between core-use areas, as may be the case in high snow-mobile/high skiing habitats, then fGCs are poor indicators of the lynx' perception of that habitat.

A growing number of studies have found that human activities are correlated for GC concentrations in wild animals. Similar to our findings, Creel et al. (2002) found that fGC concentrations in elk and wolves were positively associated with snow-mobile activity. Grouse also exhibit higher fGC values in response to high levels of human disturbance associated with snow-sport (Arlettaz et al. 2007). In a study on hyenas, human population density and pastoralist activities (but not tourism) are associated with increased fGC values (Van Meter et al. 2009). Furthermore, mean adult GC concentrations are not always the best index of the effect of human activity on a population. In Magellanic penguins, adrenal activity in adults exposed to high levels of eco-tourism do not appear to be affected compared to an undisturbed population, but chicks show a stronger adrenocortical response to high levels of eco-tourism (Walker et al. 2005). As of yet, it is unclear how differences in HPA activity associated with human disturbance may affect individual fitness or population viability.

Concluding Remarks

We monitored patterns of adrenal activity in Canada lynx throughout the reintroduction process. The amount of information we were able to obtain for known individuals, as well as the fact that we were able to monitor an actual reintroduction (as opposed to a simulated reintroduction with a model species) make this study unique, and yielded several important findings:

1) Both fGC baseline concentrations and variance increase over time in holding pens, and may reflect changes in stress-axis state.
2) Individual differences in stress physiology in holding pens is related to post-release survival; individuals with greater HPA reactivity (larger fGC peaks) experience higher rates of mortality.
3) In the field, fGC concentration is positively associated with snow-mobile activity and abundance of competitor species; it is negatively related to abundance of prey species.

This study has enhanced our understanding of how reintroduction events impact an individual's stress physiology, and how stress physiology may in turn impact the outcome of the reintroduction. This information can be used to enhance the success of future reintroduction efforts, particularly of closely related species such as the Iberian lynx, which is the most endangered felid species.

Acknowledgements

This project would not have been possible without the on-going support of the Colorado Division of Wildlife and particularly Dr. Tanya Shenk. Numerous people have contributed to every dimension of this project, and undoubtedly we will miss some people, but we cannot emphasize enough how much we appreciate the hard work of every person who made this project possible. We greatly appreciate the hard work of everyone involved in collecting fecal samples (*Frisco Creek Wildlife Rehabilitation Center*: Susan and Herman Dieterich, Kate Goshorn; *Field Crews (1999-2008)*: Steve Abele, Brandon Barr, Bryce Bateman, Todd Bayless, Ryan Besser, Mandi Brandt, Keith Bruno, Brad Buckley, Patrick Burke, Paula Capece, Matthew Chappell, Stacey Ciancone, Doug Clark, John DePue, Shana Dunkley, Tim Hanks, Matt Holmes, Allie Hunter, Andy Jennings, Susan Johnson, Paul Keenlance, Darrin Kite, Tony Lavictoire, Clay Miller, Denny Morris, Kieran O'Donovan, Gene Orth, Chris Parmater, Bob Peterson, Jake Powell, Jeremy Rockweit, Britta Schielke, Josh Smith, Adam Strong, Dave Unger, David Waltz, Andy Wastell, Lyle Willmarth, Leslie Witter, Kei Yasuda, Jennifer Zahratka). Much thanks to Anne Trainor and Laurie Baeten for logistical support. Funding for this project was provided by the Chicago Zoological Society/Chicago Board of Trade, Colorado Division of Wildlife, Purdue University, and PEO International.

Literature Cited

Arlettaz, R., P. Patthey, M. Baltic, T. Leu, M. Schaub, R. Palme, and S. Jenni-Eiermann. 2007. Spreading free-riding snow sports represent a novel serious threat for wildlife. Proceedings of the Royal Society B-Biological Sciences 274:1219-1224.

Bar-David, S., D. Saltz, T. Dayan, A. Perelberg, and A. Dolev. 2005. Demographic models and reality in reintroductions: Persian fallow deer in Israel. Conservation Biology 19:131-138.

Blas, J., G. R. Bortolotti, J. L. Tella, R. Baos, and T. A. Marchant. 2007. Stress response during development predicts fitness in a wild, long lived vertebrate. Proceedings of the National Academy of Sciences of the United States of America 104:8880-8884.

Bosson, C. O., R. Palme, and R. Boonstra. 2009. Assessment of the stress response in Columbian ground squirrels: Laboratory and field validation of an enzyme immunoassay for fecal cortisol metabolites. Physiological and Biochemical Zoology 82:291-301.

Bremner-Harrison, S., P. A. Prodohl, and R. W. Elwood. 2004. Behavioural trait assessment as a release criterion: Boldness predicts early death in a reintroduction programme of captive-bred swift fox (*Vulpes velox*). Animal Conservation 7:313-320.

Burnham, K. P. and D. R. Anderson. 2002. Model selection and inference: A practical information theoretic approach, 2nd edition. Springer-Verlag, New York.

Cabezas, S., J. Blas, T. A. Marchant, and S. Moreno. 2007. Physiological stress levels predict survival probabilities in wild rabbits. Hormones and Behavior 51:313-320.

Cantor, A. 2003. SAS Survival analysis techniques for medical research, 2nd edition. SAS Press, Cary, NC.

Creel, S., J. E. Fox, A. Hardy, J. Sands, B. Garrott, and R. O. Peterson. 2002. Snowmobile activity and glucocorticoid stress responses in wolves and elk. Conservation Biology 16:809-814.

Dickens, M. J., D. J. Delehanty, and L. M. Romero. 2009. Stress and translocation: alterations in the stress physiology of translocated birds. Proceedings of the Royal Society B-Biological Sciences 276:2051-2056.

Fischer, J. and D. B. Lindenmayer. 2000. An assessment of the published results of animal relocations. Biological Conservation 96:1-11.

Franceschini, M. D., D. I. Rubenstein, B. Low, and L. M. Romero. 2008. Fecal glucocorticoid metabolite analysis as an indicator of stress during translocation and acclimation in an endangered large mammal, the Grevy's zebra. Animal Conservation 11:263-269.

Hartup, B. K., G. H. Olsen, and N. M. Czekala. 2005. Fecal corticoid monitoring in whooping cranes (*Grus americana*) undergoing reintroduction. Zoo Biology 24:15-28.

IUCN. 1996. IUCN/SSC guidelines for reintroductions. *in* 41st Meeting of the IUCN Council. IUCN, Gland, Switzerland.

Koolhaas, J. M., S. M. Korte, S. F. De Boer, B. J. Van Der Vegt, C. G. Van Reenen, H. Hopster, I. C. De Jong, M. A. W. Ruis, and H. J. Blokhuis. 1999. Coping styles in animals: current status in behavior and stress-physiology. Neuroscience and Biobehavioral Reviews 23:925-935.

Littell, R. C., G. A. Milliken, W. W. Stroup, R. D. Wolfinger, and O. Schabenberger. 2006. SAS for Mixed Models, 2nd ed. SAS Institute Inc., Cary, North Carolina.

Meriggi, A. and R. M. della Stella. 2004. Dynamics of a reintroduced population of red-legged partridges *Alectoris rufa* in central Italy. Wildlife Biology 10:1-9.

Morton, D. J., E. Anderson, C. M. Foggin, M. D. Kock, and E. P. Tiran. 1995. Plasma-cortisol as an indicator of stress due to capture and translocation in wildlife species. Veterinary Record 136:60-63.

Nowak, R. 1999. Walker's Mammals of the World. Johns Hopkins University Press, Baltimore, Maryland.

Overli, O., C. Sorensen, K. G. T. Pulman, T. G. Pottinger, W. Korzan, C. H. Summers, and G. E. Nilsson. 2007. Evolutionary background for stress-coping styles: Relationships between physiological, behavioral, and cognitive traits in non-mammalian vertebrates. Neuroscience and Biobehavioral Reviews 31:396-412.

Palme, R., S. Rettenbacher, C. Touma, S. M. El-Bahr, and E. Möstl. 2005. Stress hormones in mammals and birds - Comparative aspects regarding metabolism, excretion, and noninvasive measurement in fecal samples. Annals of the New York Academy of Science 1040:162-171.

Poole, K. G. 2003. A review of the Canada lynx, *Lynx canadensis*, in Canada. Canadian Field-Naturalist 117:360-376.

Romero, L. M. 2004. Physiological stress in ecology: lessons from biomedical research. Trends in Ecology & Evolution 19:249-255.

Ruggiero, L. F., K. B. Aubry, S. W. Buskirk, G. M. Koehler, C. T. Krebs, K. S. McKelvey, and J. R. Squires. 2000. Ecology and conservation of lynx in the United States. University Press of Colorado, Boulder, Colorado.

Saltz, D. and D. I. Rubenstein. 1995. Population-dynamics of a reintroduced Asiatic wild ass (*Equus hemionus*) herd. Ecological Applications 5:327-335.

Sapolsky, R. M. 2002. Endocrinology of the stress-response. Pages 409-450 *in* J. B. Becker and S. M. Breedlove, editors. Behavioral Endocrinology. MIT Press, Cambridge, Massachusetts.

Shenk, T. M. 2001. Post-release monitoring of lynx reintroduced to Colorado. Colorado Division of Wildlife, Fort Collins, Colorado.

Shenk, T. M. 2006. Post-release monitoring of lynx reintroduced to Colorado. Colorado Division of Wildlife, Fort Collins, Colorado.

Shenk, T. M. 2008. Post-release monitoring of lynx reintroduced to Colorado. Colorado Division of Wildlife, Fort Collins, Colorado.

Sih, A., A. M. Bell, J. C. Johnson, and R. E. Ziemba. 2004. Behavioral syndromes: An integrative overview. Quarterly Review of Biology 79:241-277.

Teixeira, C. P., C. S. De Azevedo, M. Mendl, C. F. Cipreste, and R. J. Young. 2007. Revisiting translocation and reintroduction programmes: the importance of considering stress. Animal Behaviour 73:1-13.

Van Meter, P. E., J. A. French, S. M. Dloniak, H. E. Watts, J. M. Kolowski, and K. E. Holekamp. 2009. Fecal glucocorticoids reflect socio-ecological and anthropogenic stressors in the lives of wild spotted hyenas. Hormones and Behavior 55:329-337.

Walker, B. G., P. D. Boersma, and J. C. Wingfield. 2005. Field endocrinology and conservation biology. Integrative and Comparative Biology 45:12-18.

Zidon, R., D. Saltz, L. S. Shore, and U. Motro. 2009. Behavioral changes, stress, and survival following reintroduction of Persian fallow deer from two breeding facilities. Conservation Biology 23:1026-1035.

Table 5- 1. Table of predictor variables. All "Human" factors are scored as none, low, medium, high, and "Trophic" factors are quantified as the number of species within a trophic group for which tracks were observed.

Type	Variable	Description
Biological	sex	Male or female
	age	estimated age
Reintroduction	source population	source population for the lynx (Alaska, British Columbia, Manitoba, Quebec, Yukon)
	pen duration	length of time spent in holding pen in CO before being released
	cohort	group of lynx released in a given year; year lynx was released
	release date	date lynx was released into the wild
Ecological		
Habitat	dominant tree species	tree species with the most stems in 144 m^2 plot
	total number of trees	total number of trees in 144 m^2 plot
	elevation	elevation at which sample was collected (m)
	slope	degree of slope of terrain
	snow	Depth of snow pack (cm) at site of fecal sample
Human	road	road density
	skiing	intensity of skiing activity
	snowmobiling	intensity of snowmobile activity
	snowshoeing	intensity of snowshoe activity
Trophic	carnivore	observed carnivore tracks (e.g., coyote, fox, bobcat, mountain lion, bear, pine marten)
	prey	observed tracks of lynx prey (e.g., hares, rabbits, squirrels, grouse, mice, beaver, marmot)
	ungulate	observed ungulate tracks (e.g., deer, moose, bighorn sheep, elk)

Table 5- 2. Descriptive table of predictor variables for lynx while in holding pens. Data only provided for lynx with hormone data in the holding pens. "Source" refers to the US state or Canadian province where lynx were trapped.

	Sex		Age (y)		Pen Duration (d)		Source				
Cohort	**Female**	**Male**	**Mean**	**Range**	**Mean**	**Range**	**AK**	**BC**	**YK**	**QU**	**MB**
1999	18	0	2.5± 2.8	0.9-10.9	35.3± 10.1	21.0-67.0	13	1	4	0	0
2000	4	7	2.8± 1.7	0.7-6.7	79.4± 4.5	78.0-93.0	0	11	0	0	0
2003	3	3	2.7± 0.6	1.7-3.7	92.3± 11.9	70.0-102.0	0	6	0	0	0
2004	6	6	3.4± 1.4	0.6-4.8	93.3± 20.7	65.0-128.0	0	6	0	6	0
2005	11	10	5.1± 2.7	0.9-9.9	78.2± 33.0	35.0-117.0	0	8	6	4	3
2006	6	8	4.9± 2.1	1.8-9.8	80.6± 26.9	46.0-104.0	0	9	5	0	0
Total	48	34	3.8± 2.5	0.6-10.9	72.6± 30.1	21.0-128.0	13	41	15	10	3

Table 5- 3. Identified components of adrenal activity. Results of factor analysis with seven fGC statistics/variables.

fGC Variable	Description	PC1 - Mean (Eigen=3.9)	PC2 - Reactivity (Eigen=2.6)	PC3 - ΔfGC (Eigen=1.0)
Mean	as implied	0.95	0.30	-0.03
Median	as implied	0.99	-0.04	-0.02
Baseline	mean excluding all points >3 SD	0.98	-0.07	-0.00
Mean Peak	average of points > baseline + 3SD	0.78	0.53	-0.03
% CV	standard deviation / mean	0.08	0.94	0.05
Standard Deviation	as implied	0.68	0.69	-0.04
Reactivity	magnitude of increase from baseline to mean peak	0.02	0.92	0.09
Slope	slope of baseline fGC while in holding pen	-0.03	0.08	1.00

Table 5- 4. Trends in fGC baseline and variance in holding pens. Statistical results from random coefficient model testing for trends in baseline and variance while in the pen.

	Baseline			Variance		
Variable	**DF**	**F-value**	**P-value**	**DF**	**F-value**	**P-value**
Sex	1,21.4	134.05	<.001	1,37.4	4.46	0.04
Age	1,40.2	9.58	0.004	1,17.8	20.23	<.001
Age by Sex		.	.	1,20.8	28.87	<.001
Source Population	4,35.1	0.61	0.66	2,29.9	0.59	0.56
Cohort	3,27.7	1.96	0.14	1,14.1	1.23	0.29
Day in Pen	1,50.6	30.65	<.001	1,40.4	23.35	<.001
Day in Pen by Sex		.	.	1,33.6	10.31	0.003

Table 5- 5. Survival Analysis Results. Parameter estimates are provided for the continuous variables.

Variable	Estimate	DF	Wald Chi-Square	P-value
Sex		1	1.65	0.20
Age	-0.19±0.08	1	5.24	0.02
PC1:Mean	-0.53±0.34	1	2.40	0.12
PC2:Reactivity	-0.62±0.16	1	16.03	<.001
PC3:ΔfGC	0.003±0.24	1	0.00	0.99
Cohort		3	4.55	0.21
Source Population		4	3.13	0.54
Pen Duration	-0.01±0.02	1	0.30	0.58

Table 5- 6. Change in mean fGC concentration between holding pen and field. Parameter estimates for the difference in pre-and post-release fGC values are provided for continuous variables.

Variable	Estimate	DF	F-Value	P-value
Sex		1,12	0.77	0.40
Age	-32.0±23.3	1,12	1.88	0.19
PC1:Mean	181.3±33.4	1,12	29.43	<0.001
PC2:Reactivity	-174.7±79.9	1,12	4.78	0.049
PC3:ΔfGC	-27.0±38.2	1,12	0.50	0.49
Pen Duration	3.0±1.2	1,12	6.16	0.03

Table 5- 7. Model results for change in fGC expression with time since release.

Variable	DF	F-Value	P-value
Sex	1,18.74	4.51	0.047
Age	1,24.70	2.31	0.14
Time since release	6,70.49	0.87	0.52
Pen Duration	1,21.57	0.19	0.67
Year	9,70.54	0.90	0.53
Month	5,75.52	0.85	0.52

Table 5- 8. Model results for ecological correlates. Three separate model selection processes were conducted for each ecological category. Each model included lynx sex, and year and month of sample collection. All possible combinations of variables, including one-way and two-way interactions were run. The top five models are listed below for each category.

Category	Model Variables	AIC	ΔAIC	Model Likelihood	Akaike Weight
Habitat	aspect slope|snow	255.9	0.0	1.00	0.16
	aspect elevation|slope snow	255.9	0.0	0.99	0.16
	aspect elevation slope|snow	256.3	0.4	0.82	0.13
	aspect slope	257.3	1.3	0.51	0.08
	aspect slope snow	257.9	2.0	0.37	0.06
Human	skiing|snowmobiling	278.6	0.0	1.00	0.23
	road skiing|snowmobiling	279.3	0.7	0.69	0.16
	skiing|snowmobiling snowshoeing	280.3	1.8	0.41	0.10
	road skiing|snowmobiling snowshoeing	281.2	2.6	0.27	0.06
	road snowmobiling	281.8	3.2	0.20	0.05
Trophic	carnivore prey	278.2	0.0	1.00	0.31
	carnivore prey ungulate	279.7	1.5	0.47	0.15
	carnivore|prey	279.8	1.7	0.44	0.14
	prey ungulate	280.1	1.9	0.39	0.12
	carnivore ungulate	280.9	2.7	0.26	0.08

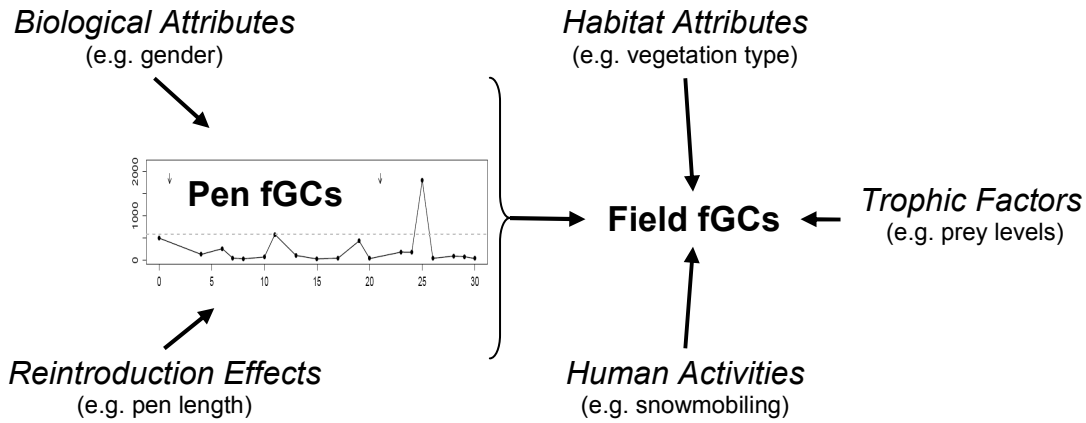


Fig. 5- 1. Schematic diagram of factors explored in this study.

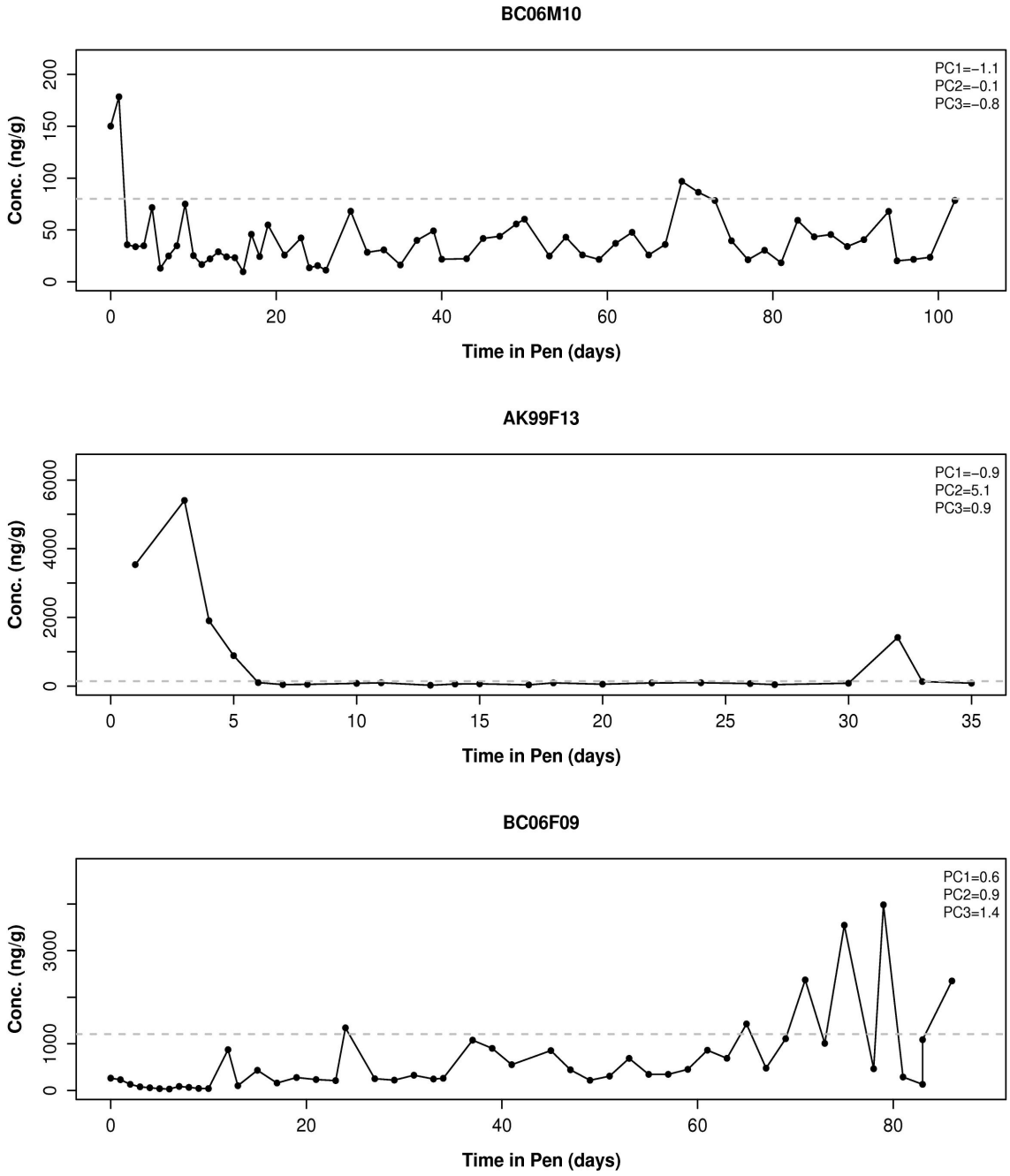


Fig. 5- 2. Representative fGC profiles showing different patterns of adrenal activity in the holding pens. The profiles illustrate differences in the three PenCCs - PC1:Mean, PC2:Reactivity, PC3:ΔfGC (principle component scores for the three factors are provided on each profile). These three lynx are identified in figures depicting PenCCs (Fig. 5-3 & 5-6).

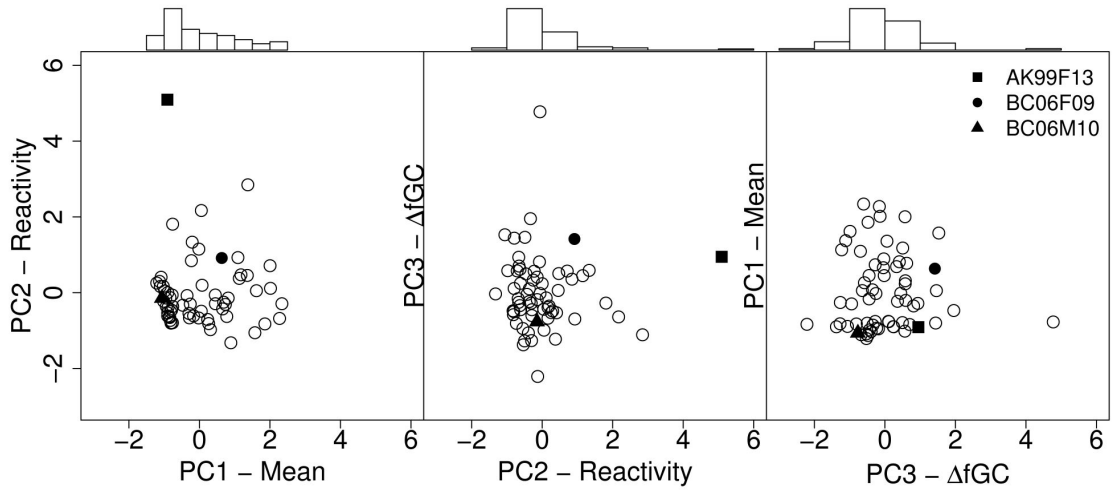


Fig. 5- 3. Principal components describing fGC profiles in holding pens (PenCCs). For each figure, the representative lynx from Fig. 5-2 have different symbols. Histograms illustrate the distribution of lynx across each of the PenCCs.

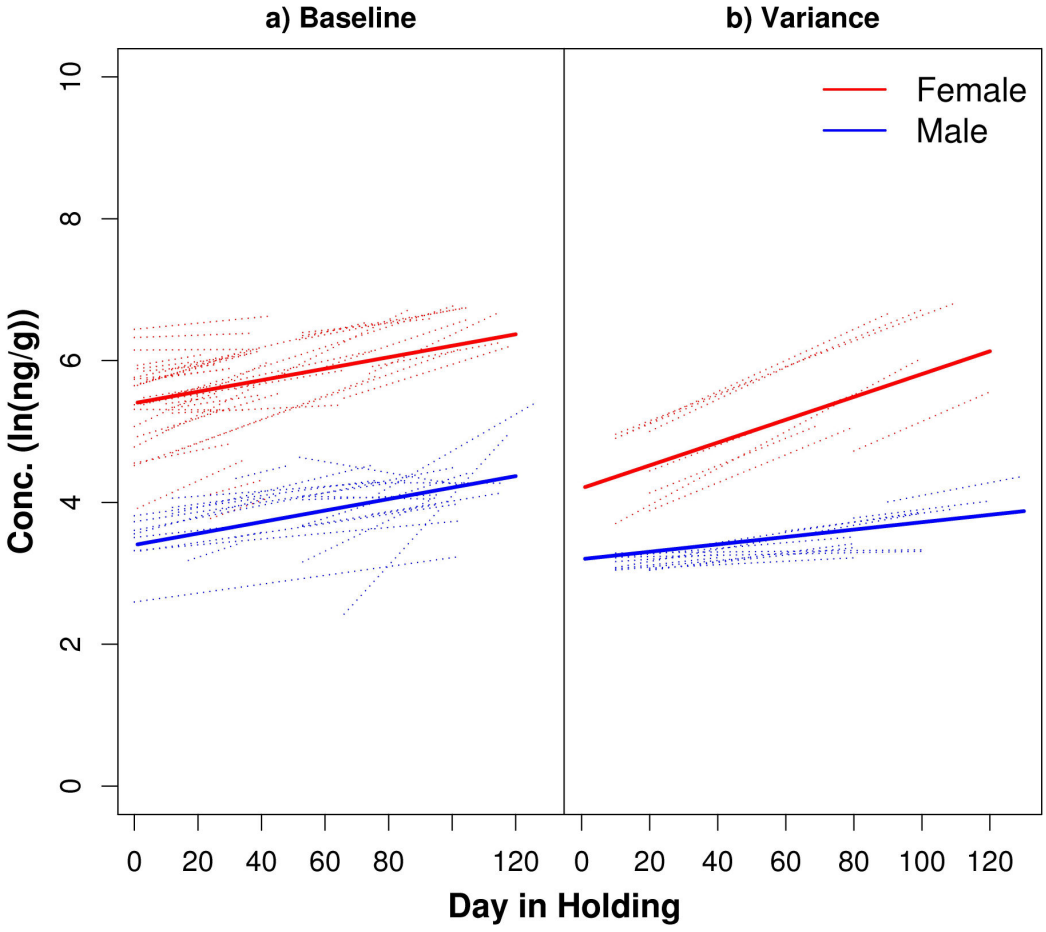


Fig. 5- 4. Trends in fGC baseline concentration (a) and variance (b) while in holding pens. Dotted lines represent slopes for individual lynx, and solid lines show the population trend estimated from the random coefficient model (see Methods for details). For variance, females had a significantly greater increase than males (Table 6-4).

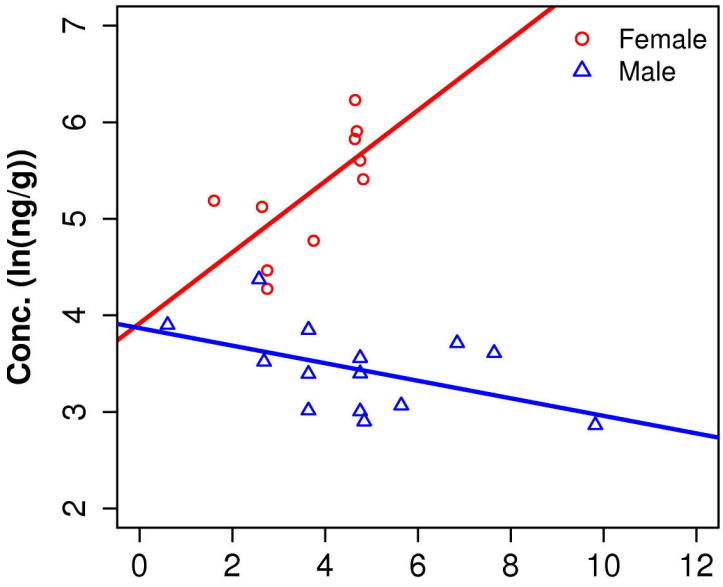


Fig. 5- 5. Effects of sex and age on fGC variance (standard deviation) while in holding pens.

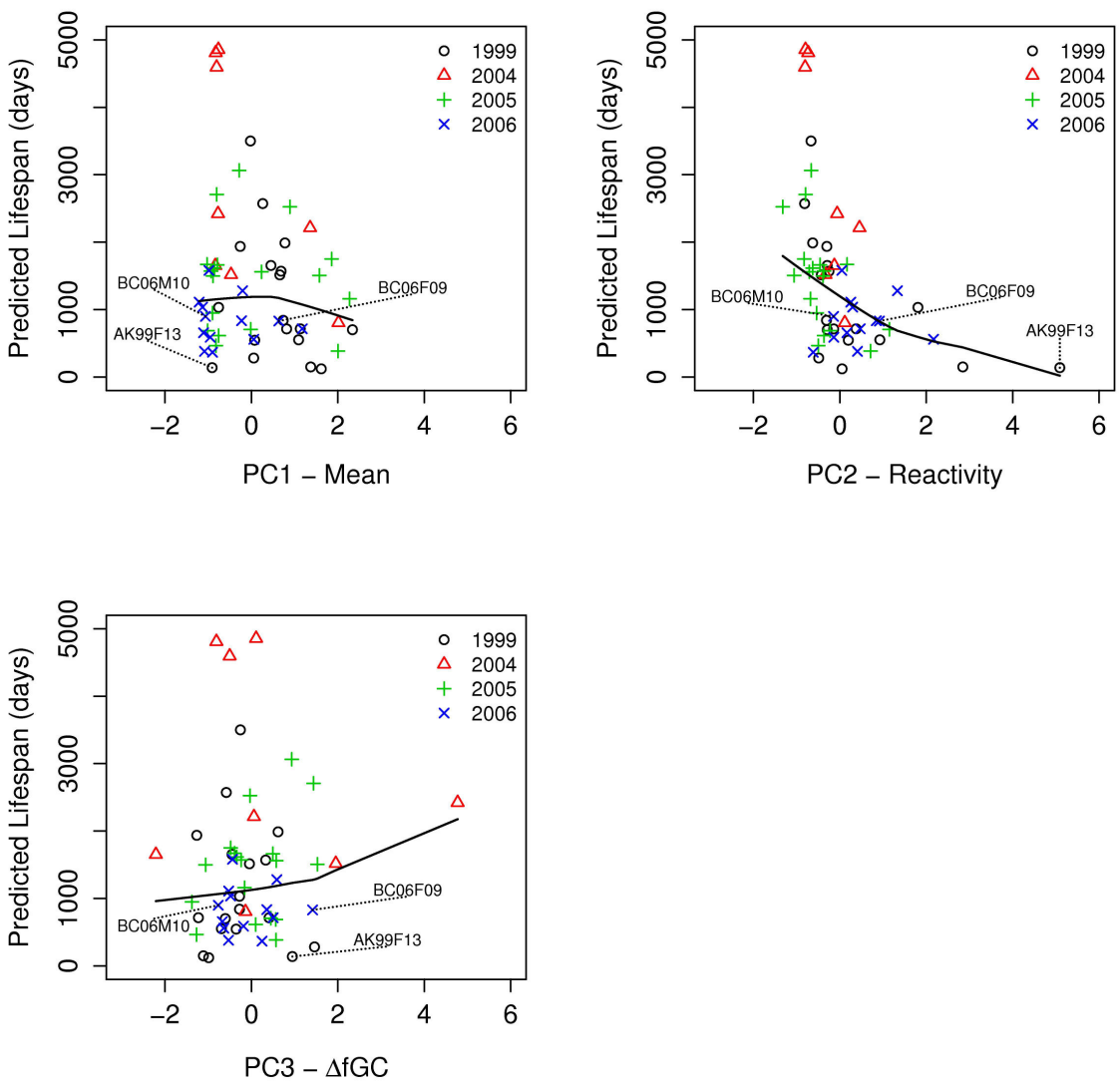


Fig. 5- 6. Survival analysis results for each PenCC. Representative individuals from Fig. 5-2 are shown in each figure. Each figure is plotted with a smoother line to show trends.

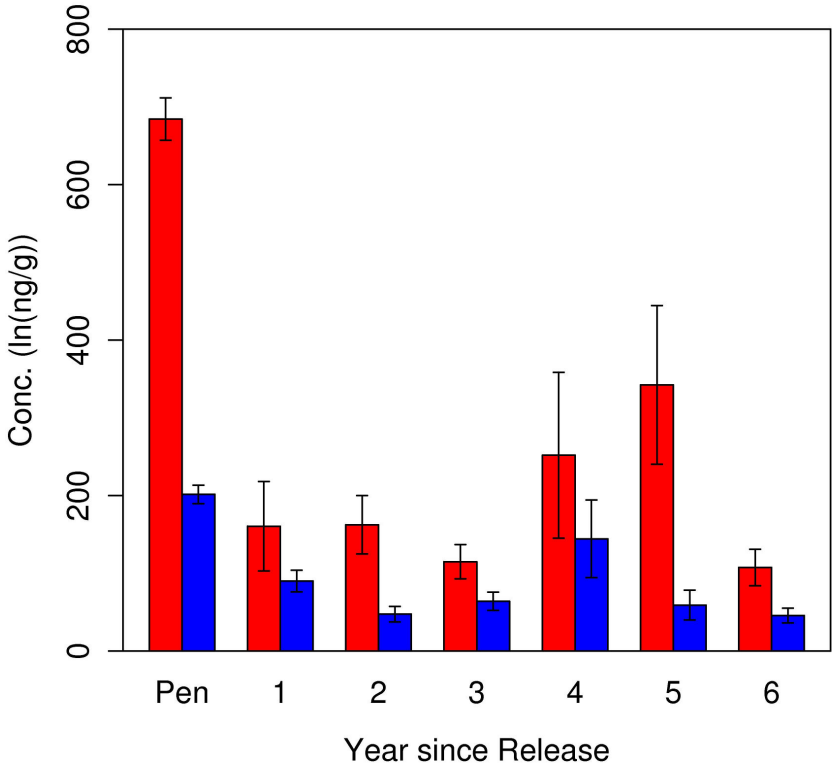
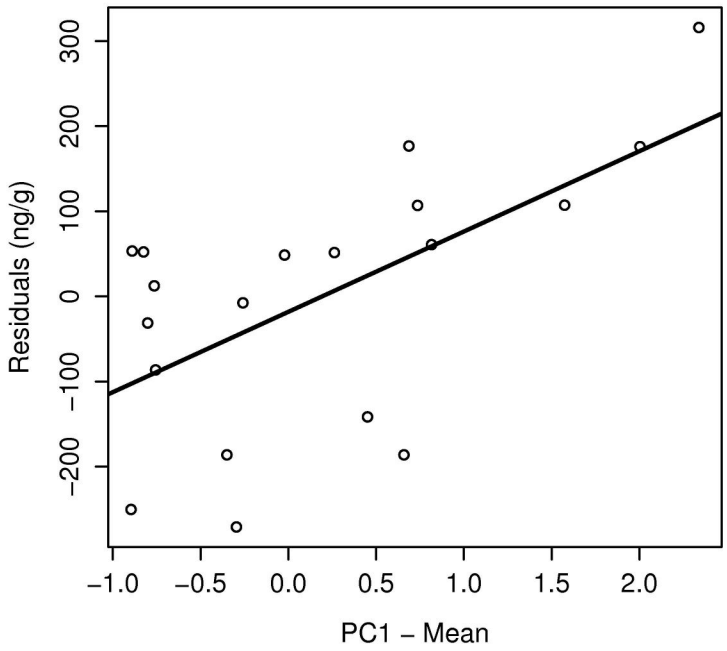


Fig. 5- 7. Mean fGC concentration in holding pens and in subsequent years since release. Red bars represent females and blue bars represent males. Error bars represent 1 SE.

a)



b)

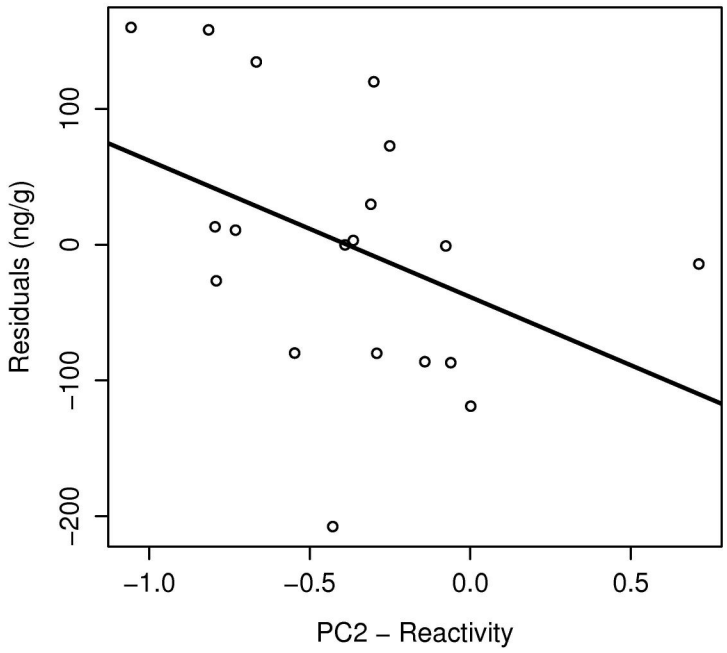


Fig. 5- 8. Effect of PC1:Mean (a) and PC2:Reactivity (b) on an individual's change in mean fGC between holding pen and field. Figures plot the residuals of a mixed linear model including sex, age, and duration of time in holding pen.

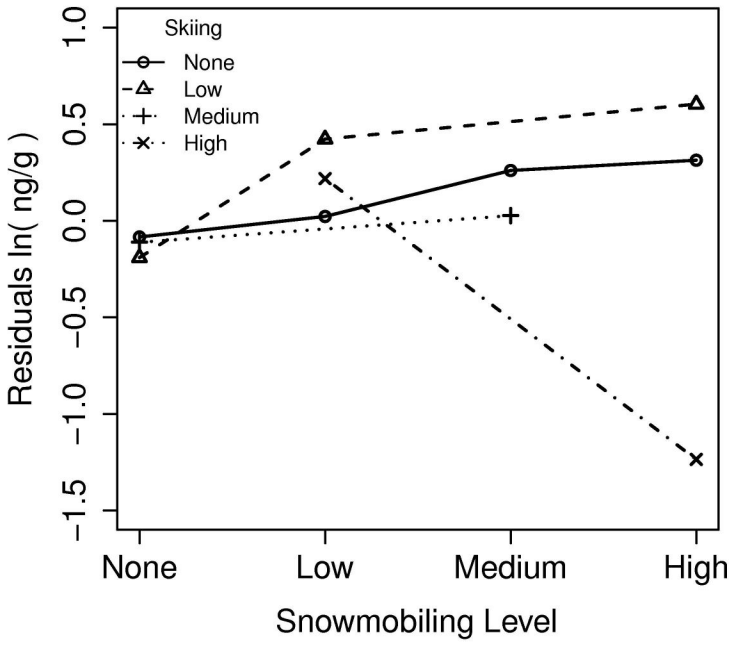
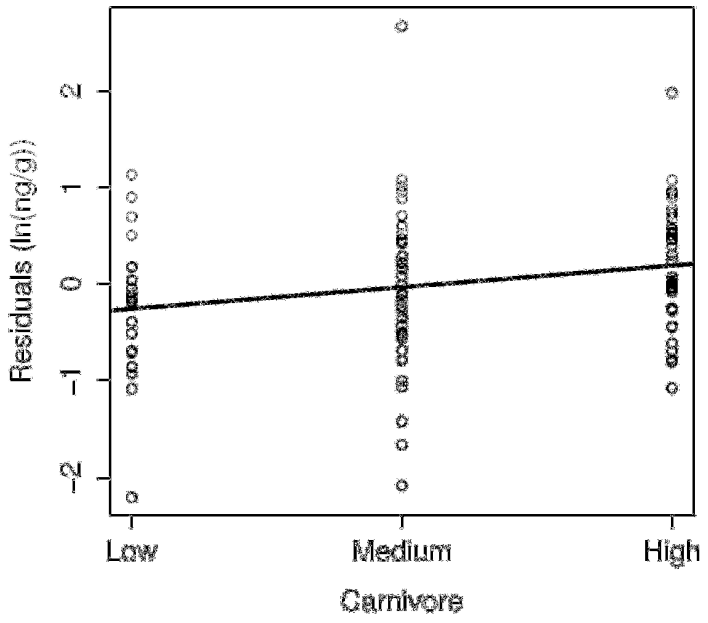


Fig. 5- 9. Mean fGC concentration in relation to skiing and snowmobiling activity. The best AIC model for human activity revealed a snowmobiling by skiing interaction. The figure shows that this interaction is driven by the different pattern seen in high snow-mobiling, high skiing areas.

a)



b)

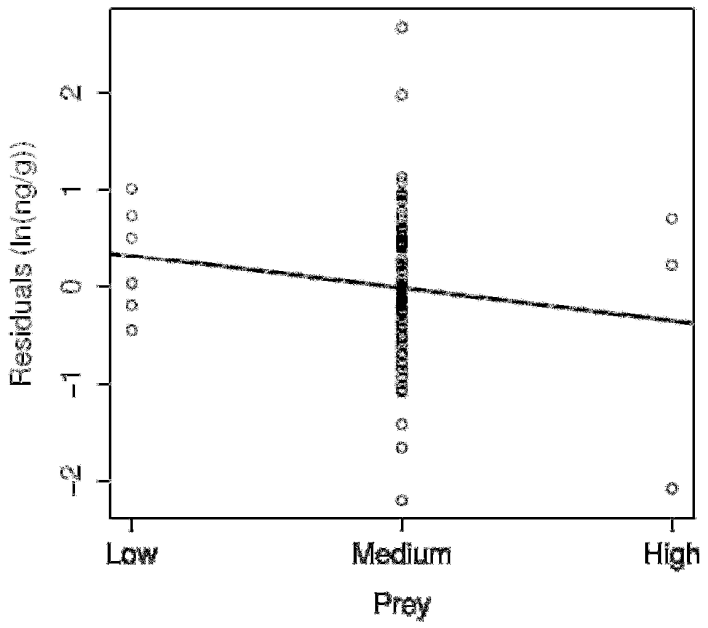


Fig. 5- 10. fGC concentration in relation to carnivore (a) and prey (b) abundance. Both figures plot the residuals from a mixed linear model including lynx id, sex, sample month, and tracking year. The regression line is from a simple regression model.

APPENDICES

Appendix A. Reproductive Steroid Enzyme-Immunoassay Protocol

All antibodies and corresponding conjugates for reproductive steroid EIAs were obtained from Coralie Munro (University of California-Davis, Davis, California, USA). Assay instructions and solution recipes based on protocols established at Brookfield Zoo's Endocrine Lab.

Assay	**Antibody**	**Conjugate**
Estradiol (E2)	Estradiol-17β R4972	estradiol-horseradish peroxidase (HRP)
Progesterone (P4)	Progesterone CL425	progesterone-HRP
Testosterone (T)	Testosterone R156/7	testosterone-HRP

Assay Instructions

1) Plate Coating
- Use flat-bottom 96-well microtiter plates (Nunc Maxisorp; Thermo Fisher Scientific, Rochester, NY).
- Prepare antibody solution.
  E2: working dilution = 1:5,000 (100 µl antibody stock (1:100, -20°C) / 5 ml coating buffer)
  P4: working dilution = 1:6,000 (83.3 µl antibody stock (1:100, -20°C) / 5 ml coating buffer)
  T: working dilution = 1:20,000 (25 µl antibody stock (1:100, -20°C) / 5 ml coating buffer)
- Add 50 µl of antibody solution to each well except column 1.
- Tap plates gently to ensure that coating solution covers well bottom.
- Label and cover tightly with a plate sealer to avoid evaporation.
- Incubate overnight in refrigerator (4°C).

2) Standards
- Prepare standards by using high standard to perform 8 two-fold serial dilutions.
  E2: high standard = 100 ng/ml
  P4: high standard = 12.5 ng/m
  T: high standard = 10 ng/ml
- Use assay buffer as 0 standard.

3) Samples/Controls
- Dilute samples in assay buffer to the appropriate dilution based on parallelism.
- Use high control as is.
- For low control, dilute high control 4-fold in assay buffer.

4) HRP
- Prepare HRP solution immediately before loading plate.
  E2 & P4: working dilution = 1:50,000 (10 µl HRP stock (1:100, 4°C) / 5 ml assay buffer)
  T: working dilution = 1:25,000 (20 µl HRP stock (1:100, 4°C) / 5 ml assay buffer)

- Do not freeze diluted HRP.

5) Plate Washing
- Wash the plate 5 times with wash solution and blot on paper towel to remove excess wash solution.
- Do not allow coated plates to dry!!
  E2 & P4: Add 50 µl of assay buffer per well. Incubate at RT for 2-5 h before loading
  T: Proceed to "Plate Loading" immediately

6) Plate Loading
- Add standard, control, or sample to each well as quickly and accurately as possible, according to plate map.
  E2: Add 20 µl per well
  P4: Add 50 µl per well
  T: Add 100 µl per well
- Immediately add 50 µl of HRP solution to each well.
- Cover plates tightly with plate sealer and incubate at room temperature for 2 h.

7) Plate Washing
- Wash the plate 5 times with wash solution and blot on paper towel.
- Plates are stable at this point and can be left upside down until all plates are washed.

8) Substrate
- Prepare ABTS substrate solution immediately before use.
- Add 40 µl 0.5 M H_2O_2 and 125 µl 40 mM azino-bis-ethylbenzthiazoline sulfonic acid to 12.5 ml substrate buffer and mix well.
- Add 100 µl of substrate solution to each well.
- Cover tightly and incubate at room temperature for 30-60 min, shaking.

9) Plate Reading
- Read at 405 nm using an optical density plate reader (Dynex MRX Revelation, Dynex Technologies, Chantilly, VA).
- Maximum OD should be about 1.0.

Solution Recipes

Wash Solution

87.7 g NaCl (Sigma S-9625)
5.0 ml Tween 20 (Sigma P-1379)
Add to 1 L distilled water. Dilute 10-fold for working wash solution.

Coating Buffer

1.59 g $Na_2CO_3 \cdot H_2O$ (Sigma S-9140)
2.93 g $NaHCO_3$ (Sigma S-6014)
Bring up in 1 L distilled water and pH to 9.6. Store at 4°C.

Assay/Phosphate Buffer

5.42 g $NaH_2PO_4 \cdot H_2O$, monobasic (Sigma S-9638)
8.66 g Na_2HPO_4, dibasic (Sigma S-9763)
8.70 g NaCl (Sigma S-9625)
1.00 g BSA (Sigma A-7906)
Bring up in 1 L distilled water, stir until salts dissolve and pH to 7.0. Store at 4°C.

Substrate/Citrate Buffer

9.61 g citric acid, anhydrous (Sigma C-0759)
Bring up in 1 L distilled water and pH to 4.0. Store at 4°C.

Appendix B. Glucocorticoid Enzyme-Immunoassay Protocol

A commercially available Corticosterone EIA kit was obtained from Assay Designs, Inc. (Ann Arbor, Michigan, USA). The following protocol is based on kit instructions provided by manufacturer.

Assay Instructions

*Bring all reagents to room temperature for at least 30 minutes prior to opening.
*Dilute provided assay buffer concentrate 1:10 and wash solution concentrate 1:20 to prepare proper working concentrations.

1) Standards
- Prepare high standard (20,000 pg/ml) by adding 100 µl of provided standard to 900 µl Assay Designs assay buffer and mix well.
- Prepare remaining 4 standards by using high standard to perform serial five-fold dilutions.
- Use assay buffer as 0 standard.

2) Samples/Controls
- Dilute samples in assay buffer to the appropriate dilution based on parallelism.
- For low control, dilute high control 4-fold in assay buffer.

3) Plate Loading
- Add 150 µl of assay buffer to non-specific binding wells (1E,F).
- Add 100 µl standard, control, or sample to each well, according to plate map.
- Add 50 µl of provided blue conjugate into each well, except wells 1A-D.
- Add 50 µl of provided yellow antibody into each well, except wells 1A-F.
- Cover plates tightly with plate sealer and incubate at room temperature for 2 h, shaking.

4) Plate Washing
- Wash the plate 3 times with 400 µl Assay Designs wash solution and blot on paper towel.
- Plates are stable at this point and can be left upside down until all plates are washed.

5) Substrate
- Prepare ABTS substrate solution immediately before use.
- Add 5 µl of provided blue conjugate to total activity wells (1C,D).
- Add 200 µl of provided substrate solution to each well.
- Cover tightly and incubate at room temperature for 1 h.

6) Stop Reaction
- Add 50 µl of provided stop solution to every well. The plate should be read immediately.

7) Plate Reading
- Read at 405 nm using an optical density plate reader (Dynex MRX Revelation, Dynex Technologies, Chantilly, VA).
- Maximum OD should be about 1.0.

Appendix C. Table of captive lynx included in the study.

Lynx ID	Institution Name	N*	Collection Dates	Age (yrs)	Lat.	Status	Breed. success	Cage-mate(s)		
								#	sex	status
F1	NOAH Feline Refuge	167	Jan-May '05 Jan-May '06	6	34º33	Intact/ Pregnant/ Pseudo	Proven breeder	2	both	intact
F2	Assiniboine Park Zoo	19	Feb-Apr '05	12	49º53	Intact/ Pseudo	Non-breeder	1	male	intact
F3	Wild Trax Feline Refuge	20	Mar-Apr '05	4	35º14	Intact	Non-breeder	1	male	intact
F4	Zoo America	54	Feb-Jun '06	5	40º16	Intact/ Pregnant	Proven breeder	1	male	intact
F5	Wild Trax Feline Refuge	24	Jul '04; Mar-Apr '05	5	35º14	Intact	Non-breeder	1	male	intact
F6	Brec's Baton Rouge Zoo	15	Mar-Apr '06	18	30º35	Intact	Proven breeder	1	male	intact
F7	NOAH Feline Refuge	159	Jan-May '05 Jan-May '06	5	34º33	Intact/ Pseudo	Proven breeder	2	both	intact
F8	Buttonwood Park Zoo	133	Feb '05-Jan '06	2	41º38	Intact	Unsure	1	female	intact
F9	Wildlife Science Center	215	Jan-Dec '05, Jul '07	2	45º16	Intact/ Spayed	Unsure	1	female	intact
F10	Alaska Zoo	149	Jan-Dec '06, Jul '07	2	61º13	Intact	Unsure	2	female	intact
F11	Scovill Zoo	87	Jan-May '06, Jul '07	9	39º49	Intact	Unsure	0	---	---
F12	Wildlife Science Center	223	Jan-Dec '05, Jul '07	2	45º16	Intact/ Spayed	Unsure	1	female	intact
F13	Alaska Zoo	138	Jan-Dec '06, Jul '07	11	61º13	Intact	Unsure	2	female	intact
F14	Buttonwood Park Zoo	115	Feb '05-Jan '06	2	41º38	Intact	Unsure	1	female	intact
F15	Salmonier Nature Park	43	Feb-May '06	8	47º23	Intact	Proven breeder	1	male	intact
F16	Big Cat Rescue	18	Mar-Apr '06	13	27º58	Intact	Non-breeder	0	---	---
F17	Private	26	Feb-Jul '07	13	53º33	Intact/ Pregnant	Proven breeder	1	male	intact
F18	Alaska Zoo	138	Jan-Dec '06, Jul '07	2	61º13	Intact	Unsure	2	female	intact
F19	Big Cat Rescue	20	Mar-Apr '06	15	27º58	Intact	Non-breeder	0	---	---
F20	Zoo Sauvage de St.Félicien	40	Mar-May '06	9	48º38	Spayed	---	2	both	both
F21	The Wildcat Sanctuary	10	Jul '07	6	46º07	Spayed	---	1	male	neuter
F22	Pueblo Zoo	37	Jan-Apr '06	5	38º15	Spayed	---	0	---	---
F23	The Wildcat Sanctuary	8	Jul '07	11	46º07	Spayed	---	3	both	neuter

Lynx ID	Institution Name	N*	Collection Dates	Age (yrs)	Lat.	Status	Breed. success	Cage-mate(s)		
F24	Toronto Zoo	28	Mar-Jul '06	10	43º46	Spayed	---	1	male	intact
F25	Walk on the Wildside	51	Jan-May '06	8	34º33	Spayed	---	2	male	intact
F26	Zoo Sauvage de St.Félicien	42	Mar-May '06	6	48º38	Spayed	---	2	both	both
M1	NOAH Feline Refuge	64	Jan-May '06	8	34º33	Intact	Proven breeder	2	female	intact
M2	Assiniboine Park Zoo	20	Feb-Apr '05	11	49º53	Intact	Non-breeder	1	female	intact
M3	Walk on the Wildside	52	Jan-May '06	7	34º33	Intact	Unsure	2	male	intact
M4	Philadelphia Zoo	104	Jun '06 - Jun '07	6	39º57	Intact	Unsure	1	male	intact
M5	Connecticut's Beardsley Zoo	37	Feb-Apr '05	14	41º11	Intact	Proven breeder	1	female	intact
M6	Dakota Zoo	14	Dec '05 - Jan '06	12	46º48	Intact	Non-breeder	0	---	---
M7	NOAH Feline Refuge	67	Jan-May '05	6	34º33	Intact	Proven breeder	2	female	intact
M8	Zoo Sauvage de St.Félicien	42	Mar-May '06	3	48º38	Intact	Unsure	2	female	neuter
M9	Brec's Baton Rouge Zoo	14	Mar-Apr '06	18	30º35	Intact	Proven breeder	1	female	intact
M10	Wild Trax Feline Refuge	17	Jul '04; Mar-Apr '05	4	35º14	Intact	Non-breeder	1	female	intact
M11	Toronto Zoo	27	Mar-Jul '06	10	43º46	Intact	Proven breeder	1	female	neuter
M12	Philadelphia Zoo	101	Jun '06 - Jun '07	6	39º57	Intact	Unsure	1	male	intact
M13	Salmonier Nature Park	45	Feb-May '06	8	47º23	Intact	Proven breeder	1	female	intact
M14	Zoo America	104	Feb '06 - Mar '07	5	40º16	Intact	Proven breeder	1	female	intact
M15	Minnesota Zool. Gardens	22	Jun-Jul '07	14	44º44	Intact	Non-breeder	0	---	---
M16	Wild Trax Feline Refuge	21	Jul '04; Mar-Apr '05	5	35º14	Intact	Non-breeder	1	female	intact
M17	Feline Conserv. Center	150	Jun '04 - May '05	3	34º51	Intact	Unsure	0	---	---
M18	Dirt Willy Game Bird Farm	11	Mar-Jul '07	13	53º33	Intact	Proven breeder	1	female	intact
M19	Utah's Hogle Zoo	32	May-Jul '06	17	40º46	Castrated	---	0	---	---
M20	Animals for Awareness	6	Aug '07	1	41º39	Castrated	---	2	both	neuter
M21	The Wildcat Sanctuary	12	Jul-Aug '07	11	46º07	Castrated	---	1	female	neuter

Lynx ID	Institution Name	N*	Collection Dates	Age (yrs)	Lat.	Status	Breed. success	Cage-mate(s)		
M22	Animals for Awareness	4	Aug '07	7	41º39	Castrated	---	2	both	neuter
M23	The Wildcat Sanctuary	11	Jul-Aug '07	3	46º07	Castrated	---	3	both	neuter
M24	The Wildcat Sanctuary	12	Jul-Aug '07	1	46º07	Castrated	---	3	both	neuter
M25	Cincinnati Zoo & Botanical Gardens	39	Feb-Apr '05	8	39º06	Castrated	---	0	---	---
M26	Zoo Sauvage de St.Félicien	6	Mar '06	<1	48º38	Juvenile				
M27	Dirt Willy Game Bird Farm	1	Jul '07	<1	53º33	Juvenile				

*N = number of fecal samples collected

VITA

VITA

EDUCATION

Degree Programs

2009 Ph.D. – Purdue University, Department of Biological Sciences (W. Lafayette, IN)
Dissertation: Stress and reproductive physiology in Canada lynx (*Lynx canadensis*): Implications for *in-situ* and *ex-situ* conservation
Advisors: Drs. Jeffrey R. Lucas and Nadja C. Wielebnowski

2000 B.A. – Kalamazoo College, Department of Biology (Kalamazoo, MI)
Senior Thesis: Foraging time and vigilance in rock hyrax (*Procavia capensis*), a social forager
Advisor: Dr. Joel S. Brown

International Experience

2007 Karolinska Instituet (Sweden) – Graduate course entitled "Neuroendocrinology"

2005 Organization for Tropical Studies (Costa Rica) – Graduate course entitled "Tropical Biology: An Ecological Approach"

1998-99 University of Nairobi (Kenya) – Undergraduate Study Abroad

PROFESSIONAL EXPERIENCE

Jan 2009-present Reproductive Biologist – Taronga Western Plains Zoo, Australia

Aug 2008-present Honorary Research Associate – Macquarie University, Australia

OTHER RESEARCH EXPERIENCE

2007 Brookfield Zoo – Research Assistant
- Behavioral and hormonal responses of felids and pachyderms to construction activity

2004 Purdue University, Dr. Peter Waser – Field technician
- Dispersal patterns and population genetics of bannertail kangaroo rats (*Dipodomys spectabilis*) in southeastern Arizona

2004 Purdue University, Dr. Alan Konopka – Intern
- Microbial community dynamics in contaminated soil collected from central Indiana

2003 Geo-Marine, Inc. – Field technician
- Impact of stealth airplanes on the population dynamics and behavior of Mexican spotted owls (*Strix occidentalis*) in western New Mexico

2002 University of Wyoming – Field technician
- Population dynamics and habitat use of snowshoe hares (*Lepus americanus*) in Colorado

2002 Hamer Environmental – Field technician
- Survey of marbled murrelets (*Brachyramphus marmoratus*) on the Olympic Peninsula, Washington

2002 Colorado Division of Wildlife – Field technician
- Causes of juvenile mortality in Rocky Mountain elk (*Cervus elaphus*) in south-western Colorado

2001 National Resource Research Institute – Field technician
- Survey of the Canada lynx (*Lynx canadensis*) population in northern Minnesota

1999 Kalamazoo College – Principal Investigator
- Understanding how various ecological factors affect the vigilance behavior of rock hyrax (*Procavia capensis*) using structural equation modeling

1999 Kalamazoo College – Co-Principal Investigator
- Using patch use theory to study the foraging behaviour of the African unstriped ground squirrel (*Xerus rutilus*) and the potential consequences on community dynamics

1999 Kalamazoo College – Co-Principal Investigator
- Exploring the effects of indirect cues of predation risk on the habitat use of two rodent species inhabiting a rock kopje in Tsavo National Park, Kenya

1998 University of Illinois, Chicago – Field technician
- Foraging behavior of black rhinos (*Biceros dicornous*) in Aberdares National Park, Kenya

1998 Colorado Division of Wildlife – Field technician
- Seasonal habitat use of greater sage grouse (*Centrocercus urophasianus*) in northwestern Colorado

TEACHING EXPERIENCE

Purdue University, West Lafayette, IN

Fall 2007	Biology Resource Seminar – Instructor
Fall 2005	Biology I: Diversity, Ecology and Behavior – Lecture assistant
Summer 2005	Organisms and Populations – Course development
Fall 2004	Biology for Elementary School Teachers – Lab instructor
Spring 2004	Introduction to Ecology Lab – Lab instructor
Fall 2003	Biology I Lab: Practical Skills in Biological Sciences – Lab instructor

Kalamazoo College, Kalamazoo, MI

Fall 1999 Evolution Laboratory – Lab assistant

Other

Summer 2001 Head Naturalist – Kantishna Roadhouse, AK

Summer 2000 Hiking Guide – Kantishna Roadhouse, AK

UNDERGRADUATE MENTORING

2006 - 2008 Lauren Kapernaros – Individual variation in behavior and physiology of Canada lynx
- Howard Hughes Undergraduate Summer Research Fellow
- Sandy and Zippy Ostroy Research Experience for Undergraduates Award
- Senior Honors Thesis

2007 Katy Kindig – Relationship between husbandry styles and personality of Canada lynx

2007 Bret Moore – Behavior types of Canada lynx

2006 Natalie Rappaport – Monitoring fecal hormones in Canada lynx

PUBLICATIONS

Book Chapters

Fanson, K.V., N.C. Wielebnowski, and J.R. Lucas. (*In Press*) Reproductive physiology of Canada lynx (*Lynx canadensis*). In: Iberian Lynx Ex-situ Conservation: An interdisciplinary approach. Eds. A. Vargas, U. Breitenmoser, and C. Breitenmoser.

Peer-reviewed manuscripts

Fanson, K.V., J.R. Lucas, and N.C. Wielebnowski. (*In prep.*) Environmental and methodological factors affecting measured fecal hormone metabolite concentrations for Canada lynx. Journal of Wildlife Management.

Fanson, K.V., N.C. Wielebnowski, T. Shenk, and J.R. Lucas. (*In prep*.) Monitoring patterns of testicular activity in captive and wild Canada lynx (*Lynx canadensis*) using fecal hormone analysis. General and Comparative Endocrinology.

Fanson, K.V., N.C. Wielebnowski, T. Shenk, and J.R. Lucas. (*In prep*.) Monitoring patterns of ovarian and luteal activity in captive and wild Canada lynx (*Lynx canadensis*) using fecal hormone analysis. General and Comparative Endocrinology.

Dehnhard, M., K. Fanson, F. Göritz, S. Naidenko, A. Vargas, and K. Jewgenow. (*In Review*) Comparative metabolism of gestagens and estrogens in the four lynx species, the Eurasian (*Lynx lynx*), the Iberian (*Lynx pardinus*), the Canada lynx (*Lynx canadensis*) and the bobcat (*Lynx rufus*). General and Comparative Endocrinology.

Fanson, B.G., K.V. Fanson, and J.S. Brown. (*In Review*) Foraging ecology of *Xerus rutilus* and its potential consequences for community composition. African Journal of Ecology.

Fanson, B.G., K.V. Fanson, and J.S. Brown. 2008. Foraging behaviour of a community of rodents inhabiting a kopje in Tsavo West National Park, Kenya. African Zoology 43:184-191.

Other Scientific Publications

Che, J. and K. Fanson. 2005. Like a fish out of water: Examining the ability of juvenile cichlids to assess variable costs associated with a unique predator avoidance behavior. Organization for Tropical Studies 2005-01: 103-109.

Fanson, K. and E. Gillam. 2005. Altitudinal variation in natural selection for stomatal and trichome density. Organization for Tropical Studies 2005-01: 111-116.

Cole, R. and K. Fanson. 2005. Saponins as a potential mechanism of herbivore defense in young *Pentaclethra macroloba* leaves. Organization for Tropical Studies 2005-01: 155-159.

Fanson, K., E. Gillam, and G. Pahad. 2005. Does density matter?: A test of the resource concentration hypothesis in *Calathea marantifolia*. Organization for Tropical Studies 2005-01: 235-241.

Petterson, K.V. 2000. Foraging time and vigilance in rock hyrax (*Procavia capensis*), a social forager. Kalamazoo College, Undergraduate thesis.

Popular Press and Outreach

Petterson, K. 2005. Prized poop: Using fecal hormone analysis to monitor reintroduced Canada lynx. Feline Conservation Federation Magazine.

PRESENTATIONS

Research Seminars

2008 University of Chicago, Animal Behavior Research Group – Chicago, Illinois
"Studying stress and reproduction in reintroduced Canada lynx"

2007 Wabash College, Department of Biology – Crawfordsville, Indiana
"The scoop on poop: Using fecal hormone analysis to study Canada lynx physiology"

2006 Purdue University, Department of Biological Sciences – West Lafayette, Indiana
"All that glitters is not gold: Using fecal hormone analysis to study stress and reproduction in Canada lynx"

2006 Leibniz Institute for Zoo and Wildlife Research – Berlin, Germany
"Studying stress and reproduction in reintroduced Canada lynx"

2006 University of Veterinary Medicine, Institute of Biochemistry – Vienna, Austria
"Studying stress and reproduction in reintroduced Canada lynx"

2004 Purdue University, Department of Biological Sciences – West Lafayette, Indiana
"Fluctuating Asymmetry as an Indicator of Stress"

Scientific Meetings

Invited

Fanson, K.V.P., N.C. Wielebnowski, J.R. Lucas. 2006. Determining stress and reproductive status of reintroduced Canada lynx. Iberian Lynx Ex-situ Conservation Seminar: Reproductive Physiology, Matalascañas, Spain.

Platform

Fanson, K.V.P., N.C. Wielebnowski, J.R. Lucas. 2008. Letting the cat out of the bag: Monitoring hormone expression in reintroduced Canada lynx. Felid Taxon Advisory Group's Annual Meeting, Cincinnati, Ohio.

Fanson, K.V.P., N.C. Wielebnowski, J.R. Lucas. 2007. Hormonal profiles of captive and free-ranging lynx. Canada Lynx on the Border: Biological and Political Realities for Conservation Planning, Grand Portage, Minnesota.

Fanson, K. 2007. To B.E. or not to B.E.?: The value of Behavioral Endocrinology to conservation biology. Purdue University's Annual Biology Graduate Student Retreat, West Lafayette, Indiana.

Fanson, K.V.P., N.C. Wielebnowski, J.R. Lucas. 2006. Reproductive and stress physiology of Canada lynx: Implications for reintroduction success. Felid Taxon Advisory Group's Annual Meeting, Denver, Colorado.

Fanson, K.V.P., N.C. Wielebnowski, J.R. Lucas. 2004. Assessing stress and reproductive status in reintroduced Canada lynx via fecal hormone analysis. Felid Taxon Advisory Group's Annual Meeting, Albuquerque, New Mexico.

Petterson, K.V., B.G. Fanson, and J.S. Brown. 2000. Foraging time and vigilance in rock hyrax (*Procavia capensis*), a social forager. Kalamazoo College's Annual Diebold Symposium, Kalamazoo, Michigan.

Poster

Fanson, K., N. Wielebnowski, J. Lucas. 2008. Monitoring stress physiology in reintroduced Canada lynx. First International Wildlife Reintroduction Conference, Chicago, Illinois.

Fanson, K., N. Wielebnowski, J. Lucas. 2006. Understanding the role of physiology in reintroduction success: stress and reproduction in reintroduced Canada lynx. The Wildlife Society's Annual Conference, Anchorage, Alaska.

Fanson, K., N. Wielebnowski, J. Lucas. 2006. Reproductive and stress physiology of Canada lynx: Implications for reintroduction success. Midwest Ecology and Evolution Conference, St. Louis, Missouri.

Fanson, K., J. Lucas, N. Wielebnowski. 2005. Understanding the role of physiology in reintroduction success: Stress and reproduction in reintroduced Canada lynx (*Lynx canadensis*). Forestry and Ecology Research Symposium, West Lafayette, Indiana.

Public Talks and Outreach Activities

2009 University of the Third Age – Dubbo, New South Wales, AU

2007 P.E.O. International (local chapter) – Lafayette, Indiana

2007 P.E.O. International (local chapter) – Milwaukee, Wisconsin

2007 Alaska Zoo (Tuesday Night Education Program) – Anchorage, Alaska

GRANTS & FELLOWSHIPS

2008 Purdue University – Bilsland Dissertation Fellowship

2008 Chicago Board of Trade – Endangered Species Fund ($4,500)

2006 P.E.O. International – P.E.O. Scholar Award ($10,000)

2006 Purdue University – Yeunkyeung Woo Achieve Excellence Travel Grant ($500)

2005 Chicago Board of Trade – Endangered Species Fund ($3,500)

2005 Graduate Women in Science – Sigma Delta Epsilon Fellowship ($500)

2005 Purdue University – Graduate Student Incentive Award ($750)

2004 Chicago Board of Trade – Endangered Species Fund ($3,580)

2003 Colorado Division of Wildlife – Research Grant ($6,000)

2003 Purdue University – Prospective Student Incentive Award ($2,000)

1999 Kalamazoo College Beeler Fellowship – Research Grant ($1,200)

AWARDS & HONORS

2007 Purdue University– Best oral presentation at the graduate student retreat

2006 Feline Conservation Federation – Best newsletter article

2000 Kalamazoo College – High honors for academic achievement

1996-2000 Kalamazoo College – Honors scholarship

1996-2000 Czechoslovak Society of America – Merit scholarship

PROFESSIONAL MEMBERSHIPS

2004-present Society for Behavioral Neuroendocrinology

2006-present IUCN Cat Specialist Group

2004-2007 Association of Zoos and Aquariums

2007 The Wildlife Society

2006 American Society of Mammalogists

PROFESSIONAL ACTIVITIES

2007	Felid Taxon Advisory Group In-Situ Conservation Working Group (secretary)
2004-2007	Purdue University's Ross Biological Reserve caretaker and outreach coordinator
2004-2005	Organized Ecology Journal Club meetings
2004, 2006	Assisted with prospective graduate student recruitment weekend
2004	Felid Taxon Advisory Group Ex-Situ and In-Situ Conservation Working Group
2004	Hosted guest speaker Dr. Joel S. Brown (University of Illinois, Chicago) – Biological Sciences Departmental Seminar and Eco-lunch, Purdue University