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Resumen
Este estudio tiene como objetivo validar las pruebas para la detección de distintos metabolitos hormonales en heces de lince canadiense y obtener conocimientos básicos sobre su fisiología reproductiva. Mediante las técnicas de laboratorio empleadas en este estudio se consiguió validar la metodología para detectar los andrógenos fecales en los machos, y los análisis revelaron un incremento estacional claro en los niveles de andrógenos antes y durante la época de reproducción. La validación de ensayos para estrógenos y progestágenos no fue completamente convincente, aunque sí informativa. En las hembras se observó un aumento significativo en los niveles de estrógenos durante la época reproductora. La validación del ensayo no fue concluyente en parte debido a la persistencia inusualmente prolongada de cuerpos lúteos, lo que posiblemente produzca distintos perfiles hormonales en las hembras de lince comparadas con las de otros mamíferos. Asimismo, se validó una prueba para detectar glucocorticoides en heces de machos y hembras. Encontramos indicios preliminares de que las hembras tienen una respuesta fisiológica más marcada ante el estrés que los machos, aunque esto no significa necesariamente que las hembras sean más sensibles al estrés. El estrés crónico (por ejemplo, por translocación o contención) parece suprimir la expresión de andrógenos en los machos. Sería necesario llevar a cabo más estudios para obtener un conocimiento más claro sobre cómo afectan los factores estresantes presentes en el entorno a la reproducción del lince canadiense y, por ende, a su tamaño poblacional. Un mayor conocimiento sobre la fisiología reproductiva del lince podría ayudar a mejorar las prácticas de conservación.

Palabras clave
Hormonas fecales, estacionalidad, estrés
ABSTRACT
Knowledge about the reproductive physiology of lynx could help improve conservation practices. The goal of this study was to validate fecal hormone metabolite assays for Canada lynx and develop a basic understanding of their reproductive physiology. Fecal androgen assays were validated for males, and analysis revealed a clear seasonal increase in androgen expression immediately prior to and during the breeding season. The validation of fecal estrogen and progestagen assays for females was not entirely convincing, but nevertheless informative. A significant rise in fecal estrogens was observed during the breeding season for females. Assay validation may be partially confounded by the unusually long persistence of corpora lutea in Lynx species, which could produce different hormone profiles compared to other female mammals. Lastly, a fecal glucocorticoid metabolite assay was validated for both males and females. We found preliminary evidence that females may have a more pronounced physiological stress response than males, although this does not necessarily imply that females are more sensitive to stress. Chronic stress (e.g. translocation and holding) does appear to suppress androgen expression in males. Further research is needed to gain a clearer understanding of how environmental stressors may impact Canada lynx reproduction, and thereby affect their population size.

KEYWORDS
Fecal hormones, seasonality, stress
Reproductive physiology of Canada lynx (Lynx canadensis)

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INTRODUCTION

Canada lynx (Lynx canadensis) are 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, the species was listed as “threatened” 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). In response to this decline, the state of Colorado initiated a lynx reintroduction effort in 1999 (see Shenk, this book). However, poor reproductive success of reintroduced individuals has threatened the success of this effort.

There are two characteristics of lynx reproduction that may contribute to their diminishing population size and hinder reintroduction efforts. First, Canada lynx are highly seasonal breeders; they have only one chance per year to reproduce (Poole, 2003). They breed from late February to early April and 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. Although the 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 about 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). Furthermore, it is unclear whether this strong seasonality is mediated solely by females, or if both females and males experience physiological changes that may restrict their ability to mate throughout the year.

The second characteristic is that lynx recruitment fluctuates dramatically with snowshoe hare abundance, especially in the northern part of their range (Ruggiero et al., 2000). Pregnancy/conception rates, birth rates, average litter size and kitten survival, all correlate positively with prey abundance, whereas female age at sexual maturity correlates negatively (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). The reproductive physiology of lynx is therefore closely linked to the health of the snowshoe hare population, to the point that there is little to no recruitment during the low part of the cycle (Ruggiero et al., 2000; O’Donoghue et al., 2001; Poole, 2003). Consequently, lynx experience both annual and decadal restrictions to breeding.

Given these constraints to Canada lynx reproduction, it is unclear how climate change or anthropogenic activities 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. To this end, we have initiated a research project focusing on the endocrine physiology of both captive and wild Canada lynx using fecal hormone analysis. The goal of the study described here was to 1) validate assays used to quantify fecal reproductive and glucocorticoid (“stress”) hormone metabolites; 2) establish normative patterns of reproductive hormone expression in captive Canada lynx, and 3) provide a preliminary examination of the effects of stress on reproductive physiology.

Fecal samples were collected from two populations: 1) lynx in captivity and 2) lynx in holding pens. “Captive” lynx were permanently housed at captive institutions (zoos and breeding centers) and had lived in captivity most, or all, of their life. “Holding pen” lynx had been trapped in Canada or Alaska, transported to Colorado and housed in holding pens for ~2 months before they were reintroduced. Samples were collected 3-6 times per week. Hormone metabolite extraction and enzyme-immunoassay procedures have been previously described (Atsalis et al., 2004; Cavigelli et al., 2006).

**Male reproductive physiology**

Samples were collected from eight adult males housed at seven institutions. Two males, both housed alone, had been castrated. The remaining six males were all housed with intact females. Ages ranged from 4-18 years. Samples were also collected from two juvenile (<9 months) males.

**Validation** – Fecal androgen metabolites (fA) are reliable indicators of testicular activity in lynx. Both juvenile and castrated males had much lower fA concentrations than intact males (mean ± SE: 35.4 ± 30.7, 225.4 ± 63.2, 637.1 ± 116.3 ng/g feces, respectively). However, we still detected a moderate amount of fA in castrated males. There are several possible explanations for this. First, another source of androgens could contribute to circulating levels, and thereby excreted quantities, of androgens in castrated males. In humans and primates, the adrenal cortex produces a number of androgens (e.g. DHEA and DHEA-S, Rehman and Carr, 2004). Secretions of the adrenal cortex in lynx have not been studied, but could contribute to fA levels in castrated males. Second, hepatic metabolism or bacterial activity could convert other steroid molecules into androgens (Touma and Palme, 2005). A final possibility is that the antibody used cross-reacts with other steroid metabolites, because...
a non-specific, relatively broad-spectrum antibody was used. Regardless of which explanation is true, the assay is reliably detecting significant and biologically relevant signals, and thus can be successfully employed to track androgen excretion in male Canada lynx.

**Effect of Season** – Male lynx exhibit strong seasonal variation in fA expression (Figure 1). Just prior to and during the breeding season (Jan.–Mar.), fA concentrations are ~4-times higher than during the summer. Although we do not have data for the fall, we can speculate that fA concentrations (and thus testicular activity) begin to increase late in the fall (Nov. or Dec.). Living at high latitudes and/or elevations, lynx experience very harsh winters. Therefore, in order to ensure optimal kitten survival, strong selection pressure has probably shaped not only female patterns of reproduction, but has constrained male reproduction, as well.

Eurasian lynx exhibit similar patterns of seasonality. However, captive male Eurasian lynx have a second increase in testosterone expression and testicular size in May and June (Göritz et al., 2006). This may possibly be driven by the fact that if a female Eurasian lynx loses a litter, she can begin cycling again in June or July (Göritz et al., 2006). There is limited 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, although it seems very rare for Canada lynx. Given the differences in androgen expression between Eurasian and Canada lynx, we can speculate that a second estrous may be somewhat more likely to occur in Eurasian lynx.

**FEMALE REPRODUCTIVE PHYSIOLOGY**

Samples were collected from nine females housed at seven institutions. One female had been spayed and was housed alone. Six of the females were housed with intact males and given the opportunity to breed, but only three females got pregnant. The remaining two females were housed with each other. Ages ranged from 3-17 years.

**Validation** – The evidence that fecal estrogen metabolites (fE) reflect ovarian activity is somewhat equivocal. The spayed female did show significantly lower fE concentrations than intact females (mean ± SE: 324.4 ± 57.1, 802.7 ± 182.2 ng/g feces, respectively) suggesting that the assay does detect biologically relevant changes in estrogen expression. Furthermore, there are also seasonal increases in fE expression that coincide with the breeding season (Figure 1). However, we have failed to detect fE peaks associated with documented estrous behavior or known ovulations/matings. Lynx defecate about once per day, so if ovulatory estrogen spikes are very short-lived, they may be muted in the fecal sample. Therefore, it is possible that fE can be used to measure gross differences in ovarian activity, but not to monitor distinct ovarian events for this species.

A common progestogen EIA assay, which has been validated successfully for several other felid species, did not pick up relevant hormone metabolites for monitoring pregnancy in Canada lynx. We were only able to obtain data from three successful pregnancies, but the expected increase in fP concentrations could not be detected in any of the females with the initial assay (Figure 2). To further determine whether improper antibody binding (e.g. high cross-reactivity) was interfering with our ability to detect a rise in hormone concentrations generally expected during pregnancy, we then tested four other progesterone antibodies. While some antibodies indicated a slight increase in fP towards the end of the pregnancy, none show the characteristic elevation seen in some other felid species (e.g., domestic cat, tiger, cheetah, clouded leopards – J. Brown, this book).

However, while fPs could not be used to accurately detect pregnancy in Canada lynx, the measured concentrations may nevertheless accurately reflect luteal activity. There is evidence that corpora lutea (CLs) persist for extensive periods of time in Canada lynx (Nellis et al., 1972). In fact, this phenomenon is common to all Lynx species; CLs can persist for several months, and probably years (bobcats – Duke, 1949; Eurasian and Iberian lynx – Göritz et al., this book). 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. It is unclear how long the CLs remain capable of producing progesterone, but this could explain the difficulty that other Lynx researchers have also had in identifying pregnant lynx using fecal hormone analysis (Pelican et al., this book; Jewgenow et al., this book; Dehnhardt et al., this book). It is also unclear how females might accommodate this prolonged elevation of circulating progesterone while maintaining normal patterns of reproduction.
Another unexpected finding is that FE and FP show correlated patterns of expression. 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. At this point, it is unclear what is causing this trend, but there is a possibility that it is related to the unusual CL characteristics of lynx.

**Stress & Reproduction**

Normative patterns of reproductive physiology can be dramatically altered by stress. A prominent component of the physiological stress response is the hypothalamic-pituitary-adrenal (HPA) axis. Chronic up-regulation of the HPA axis can negatively affect the hypothalamic-pituitary-gonadal (HPG) axis, and thus suppress reproduction (Wingfield and Sapolsky, 2003). However, the interaction between the HPA and HPG axes is highly variable between species (Wingfield and Sapolsky, 2003). Understanding how environmental stressors may affect reproduction requires a species-specific understanding of how the HPA and HPG axes interact. Here we provide a preliminary examination of how stress might affect reproduction in lynx.

The glucocorticoid assay was validated by monitoring patterns of hormone expression during ACTH challenges (Figure 3), transfers/translocations, and exams. Results revealed that female lynx have significantly higher fecal glucocorticoid (fGC) concentrations than males. Furthermore, the relative difference in fGCs between “captive” and “holding pen” lynx (see Introduction for description) is much greater for females than males (Figure 4). Gender differences in fGC expression have been documented for a wide range of species (Touma and Palme, 2005). One explanation, which has been confirmed in some species, is that there are gender-based differences in steroid hormone production and/or metabolism (Touma and Palme, 2005). Another explanation, which is not necessarily mutually exclusive with the first, is that these differences may reflect differences in stress sensitivity. It remains unclear which is true for Canada lynx.

It is of course difficult to experimentally examine the effect of chronic stress on reproduction in a threatened species, and data collection is mostly opportunistic. However, using data collected from the reintroduced lynx population in Colorado, we have preliminary evidence that chronic elevation of the HPA axis may suppress the HPG axis, at least in males. The winter during which lynx are trapped, transported, examined, and held in holding pens, FA levels are lower and fGC levels are higher than in subsequent winters, after they have been released. We do not yet have data for females since this study is still ongoing. However, especially given the tremendous impact that hare density has on lynx reproduction, it will be key to develop a better understanding of how environmental factors and potential stressors impact reproductive physiology in this species.
CONCLUSIONS

Understanding the basic reproductive physiology of Canada lynx, including the degree of plasticity and the impact of environmental stressors, is critical for 1) developing successful captive breeding programmes; 2) designing sound conservation strategies and 3) understanding the reproductive limitations of this species in the face of environmental and climatic changes. While our current findings provide only a small start towards a better understanding, and numerous questions remain yet unanswered, continued study of these questions becomes even more urgent given the close relationship and physiological similarities of Canada lynx to other, even more threatened, Lynx species. All four Lynx species have been declining, with the most substantial decline being in the Iberian lynx population, which is now listed as “critically endangered” (IUCN, 2008). Given the biological and physiological similarities already evidenced, any information we can glean about the basic biology of one species may prove to be helpful in aiding conservation efforts for the other Lynx species as well.
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