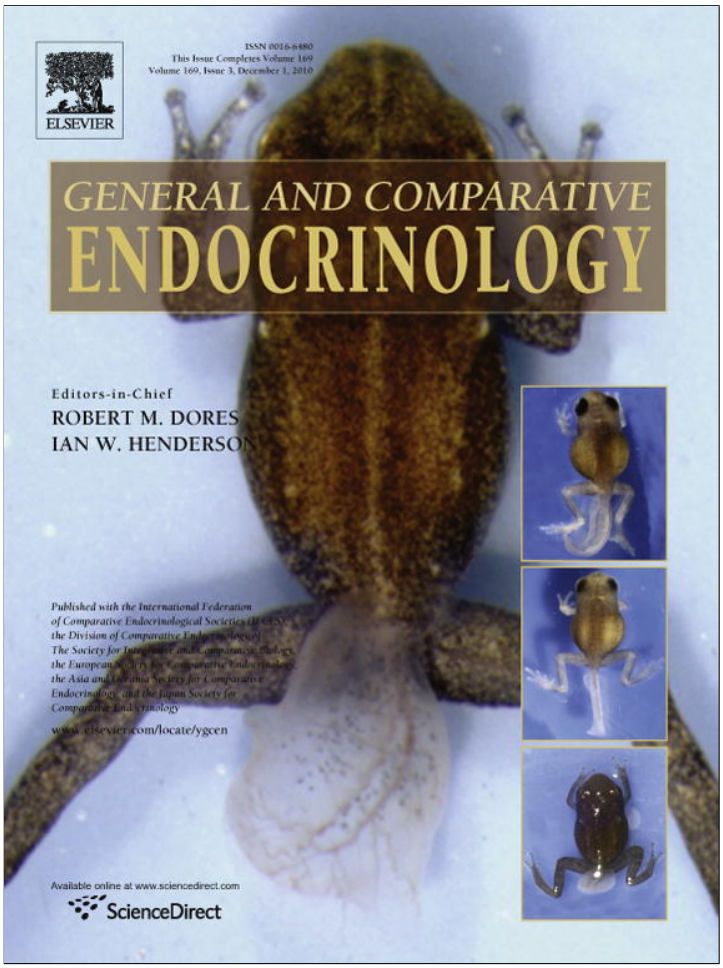

General and Comparative Endocrinology 169 (2010) 217–224

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Patterns of ovarian and luteal activity in captive and wild Canada lynx (*Lynx canadensis*)

Kerry V. Fanson [a,b,f,*], Nadja C. Wielebnowski [b], Tanya M. Shenk [c], Jennifer H. Vashon [d], John R. Squires [e], Jeffrey R. Lucas [f]

[a] Department of Brain, Behaviour, and Evolution, Macquarie University, 209 Culloden Road, Marsfield, NSW 2122, Australia
[b] Department of Conservation Science, Chicago Zoological Society, 3300 Golf Road, Brookfield, IL 60513, USA
[c] Mammals Research, Colorado Division of Wildlife, 317 West Prospect, Fort Collins, CO 80526, USA
[d] Maine Department of Inland Fisheries and Wildlife, 650 State Street, Bangor, ME 04401, USA
[e] Rocky Mountain Research Station, Forestry Sciences Laboratory, 800 E. Beckwith Avenue, Missoula, MT 59808, USA
[f] Department of Biological Sciences, Purdue University, 915 W. State Street, West Lafayette, IN 47907, USA

ARTICLE INFO

Article history:
Received 4 May 2010
Revised 29 August 2010
Accepted 9 September 2010
Available online 17 September 2010

Keywords:
Estrogen
Fecal metabolites
Lynx
Non-invasive
Progesterone
Seasonality

ABSTRACT

Canada lynx face some unique breeding restrictions, which may have implications for population viability and captive management. The goal of this study was to improve our understanding of basic reproductive physiology in Canada lynx. Using fecal hormone metabolite analysis, we established normative patterns of fecal estrogen (fE) and progestagen (fP) expression in captive and wild female Canada lynx. Our results indicate that Canada lynx have persistent corpora lutea, which underlie their uncharacteristic fP profiles compared to other felids. Thus, fP are not useful for diagnosing pregnancy in Canada lynx. We also found that Canada lynx are capable of ovulating spontaneously. Captive females had higher concentrations of fE and fP than wild females. Both populations exhibit a seasonal increase in ovarian activity (as measured by fE) between February and April. Finally, there was evidence of ovarian suppression when females were housed together.

1. Introduction

Canada lynx (*Lynx canadensis*) are a prominent mesocarnivore inhabiting North America's boreal forest, but are listed as a threatened species (USFWS, 2000). Relatively little is known about the basic physiology of lynx, particularly reproductive physiology. Compared to other felids, Canada lynx experience two unique constraints on reproduction, which may impact population dynamics, captive breeding efforts, and conservation programs for this species.

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 with most kittens born in May. 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 1 week or less. Little is known about estrus in wild lynx, but behavioral indicators suggest that wild females also have a restricted window of receptivity. 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 population recruitment fluctuates dramatically with snowshoe hare abundance, especially in the northern part of their range (Ruggiero et al., 2000). Hare abundance correlates positively with conception rate, birth rate, average litter size, and kitten survival; it correlates negatively with female age at sexual maturity (Poole, 2003; Ruggiero et al., 2000). Biologists have 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.

Physiological adaptations to these annual and decadal breeding restrictions may impact the success of captive breeding and conservation programs. It is often 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. From 2000 to 2008, there has only been one successful lynx birth (defined as survival of kittens beyond 2 weeks) at an institution accredited by the American Association of Zoos and Aquariums

* Corresponding author at: Department of Brain, Behaviour, and Evolution, Macquarie University, 209 Culloden Road, Marsfield, NSW 2122, Australia. Fax: +61 2 9850 4299.
E-mail address: kerryfanson@gmail.com (K.V. Fanson).

0016-6480/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2010.09.003

(Goff, 2008). Additionally, the number of private lynx breeders has been declining recently due to erratic breeding results (L. Culver, personal communication). 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 provide 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 progestagen (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 progestagen 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 progestagen 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.

2. Methods

2.1. Animals and fecal sample collection

2.1.1. Captive

This study included 26 captive Canada lynx females from 17 institutions (9 spayed females, and 19 intact females). [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 conducted during 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, and seed beads) was delivered to each individual in a food treat and used to distinguish between feces.

2.1.2. Wild

Fecal samples were collected from wild females in Colorado (56 individual lynx, 601 samples), Maine (8 lynx, 45 samples), and Montana (10 lynx, 52 samples). This dataset includes samples collected during 18 known 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. Samples were generally collected within 24 h after defecation, and evidence suggests that in winter field conditions, fE and fP remain stable for at least 4 days (Fanson, 2009). Therefore, we are confident that we were able to obtain meaningful results from samples collected in the field.

For most analyses involving reintroduced lynx, we only included samples that had been collected at least 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 in Colorado prior to release (6 non-pregnant and 6 pregnant females, as 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.

2.2. Steroid extraction and analysis

All samples were stored in zip-lock bags at −20 °C, and were shipped on ice to the Brookfield Zoo for analysis. We extracted steroid metabolites by adding 5 ml of 80% ethanol 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 milliliter of supernatant was transferred to a new polypropylene tube and diluted with 1 ml assay buffer (0.1 M phosphate-buffered saline (PBS) containing 1% BSA, pH 7.0). Extracts were stored at −20 °C.

Single-antibody enzyme-immunoassays (EIAs) were used to quantify fecal estrogen (fE) and progesterone (fP) metabolites. Both antibodies and corresponding horseradish peroxidase (HRP) conjugates were obtained from C. Munro (University of California, Davis, CA). Assay procedures were similar to those previously described (Atsalis et al., 2004; deCatanzaro et al., 2003; Graham et al., 2001). Briefly, 96-well microtitre plates (Nunc maxisorp) were coated with 50 μl antibody solution and incubated overnight at 4 °C. Plates were washed to remove unbound antibody. Immediately after being washed, standards, controls and diluted fecal extracts were added to each well (20 μl/well for the estradiol assay; 50 μl/well for the progesterone assay), along with 50 μl of HRP conjugate. After incubating for 2 h at room temperature, plates were washed and 100 μl of substrate solution (1.6 mM hydrogen peroxide, 0.4 mM azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) in 0.05 M citrate buffer, pH 4.0) was added to each well. When the absorbance of the zero-concentration wells was approximately 1.0, plates were read with a single filter at 405 nm using an optical density plate reader (Dynex MRX Revelation, Dynex Technologies, Chantilly, VA). All samples were assayed in duplicate, and data are expressed as ng/g wet fecal weight. 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.

2.3. Statistical analysis

All data were analyzed using SAS 9.1 (Cary, NC), and fE and fP were modeled 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 the text and figures are least-squares means that have been back-transformed. One captive female (F15) had unusually low fE and fP values (see Section 4). 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.

2.3.1. 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. 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 collected from 12 females (6 pregnant and 6 non-pregnant) while they were in holding pens, so we controlled for location (wild or holding pen) in this model. 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 to 18 years old, holding pen females ranged from 1 to 11 years old, and wild females ranged from 1 to 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 Section 4). 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 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). Since all samples came from radio-collared lynx, age was estimated when the radio-collar was put on the female. In some cases, the lynx was trapped as a kitten so the year of birth is known, and in other cases, a more precise age estimate was obtained from tooth enamel (collected post-mortem). 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).

2.3.2. 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–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 Section 3.5). 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).

2.3.3. 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.

2.3.4. 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 2 years, but has not successfully given birth), or unsure (not housed with suitable mate for at least 2 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.

3. Results

3.1. Status

In captive females, status had a significant effect on fE ($F_{3,21} = 10.59$, $P < 0.001$; Fig. 1A) and fP concentrations ($F_{3,19} = 97.53$, $P < 0.001$; Fig. 1B). For both fE and fP, spayed females had the lowest concentrations, and pregnant and pseudo-pregnant females had the highest concentrations. These trends were similar for both wild and penned lynx (fE: $F_{1,84} = 10.51$, $P = 0.002$; fP: $F_{1,84} = 4.07$, $P = 0.047$; Fig. 1). However, females in holding pens had higher fE and fP concentrations than wild females (fE: $F_{1,84} = 124.09$, $P < 0.001$; fP: $F_{1,84} = 22.94$, $P < 0.001$).

For the two lynx that were spayed during the study, both fE and fP concentrations decreased after they were spayed. This difference was significant for fP (intact mean = 1451 ng/g, spayed mean = 32 ng/g, $F_{1,1} = 1809.88$, $P = 0.02$). However, it was not significant for fE (intact mean = 220 ng/g, spayed mean = 49 ng/g, $F_{1,1} = 20.36$, $P = 0.14$).

Longitudinal fP profiles for pregnant lynx were variable (Fig. 2). 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. The youngest female (F4), who was also an inexperienced breeder, showed the most obvious

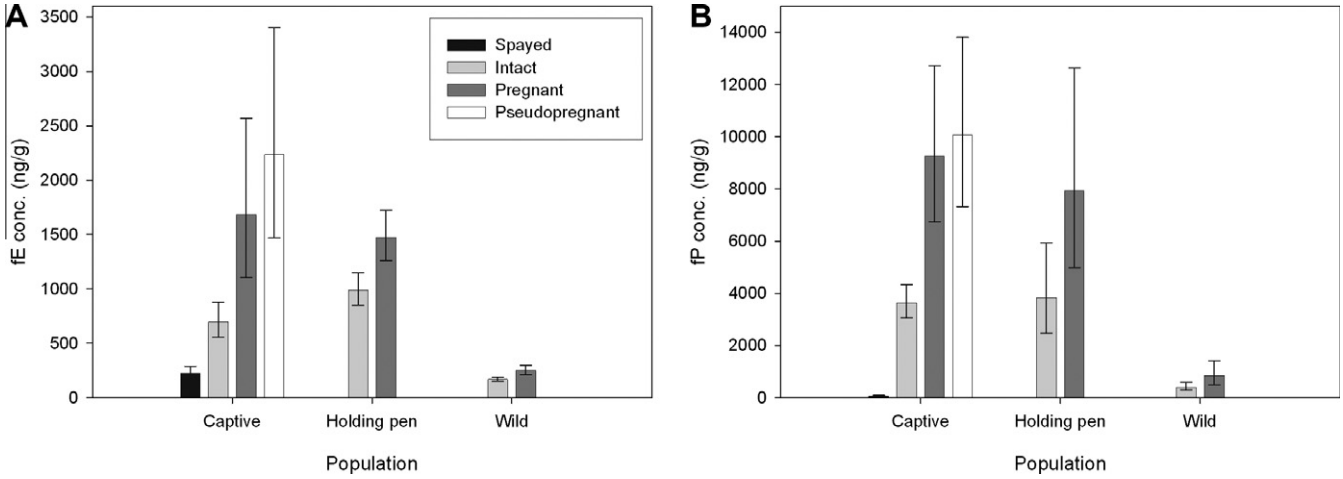

Fig. 1. Effect of female reproductive status on concentrations of (A) fE and (B) fP in captive, temporarily penned, and wild Canada lynx populations (back-transformed LS mean + SE).

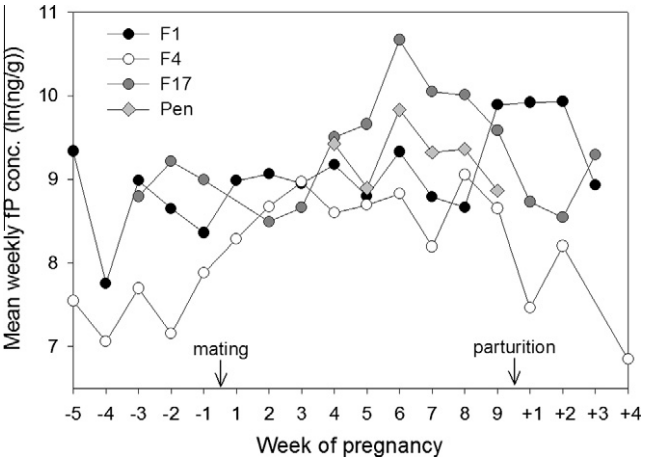

Fig. 2. Longitudinal fP profiles during pregnancy for the three captive lynx and one reintroduced lynx while in a holding pen (representative of all pregnant females in holding pens). The female in the holding pen conceived in the wild, so date of conception is estimated based on radiograph data. Note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

increase in fP throughout her pregnancy, although it was still not nearly as pronounced as pregnancy profiles published for other felid species (Brown, 2006; Graham et al., 1995). For pregnant females in holding pens, fP profiles were quite variable, but patterns were generally similar to those of pregnant females in captivity (Fig. 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.

3.2. Seasonality

Month had a significant effect on fE and fP in both populations (fE: captive, $F_{11,116} = 4.14$, $P < 0.001$, wild, $F_{4,184} = 4.20$, $P = 0.003$; fP: captive, $F_{11,108} = 3.46$, $P < 0.001$, wild, $F_{4,192} = 8.59$, $P < 0.001$; Fig. 3). Year also had a significant effect on fE and fP in captive females (fE: $F_{3,127} = 8.97$, $P < 0.001$; fP: $F_{3,118} = 7.22$, $P < 0.001$), but not in wild females.

Captive females had significantly higher fE values during breeding months than non-breeding months (breeding – March/April: mean = 690 ng/g, 95% CI = 542–878; non-breeding – June–September: mean = 354 ng/g, 95% CI = 282–446; $t_{119} = 5.27$, $P < 0.001$). Interestingly, fP concentrations were significantly lower during breeding months than later in the year (breeding mean = 1588 ng/g, 95% CI = 1096–2300; non-breeding mean = 3193 ng/g, 95% CI = 2240–4550; $t_{112} = 2.35$, $P = 0.02$).

For the seven females with sufficient data across the breeding season, latitude had a significant effect on timing of peak fE values ($\beta = 0.21 \pm 0.08$ 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).

3.3. Population

Population had a significant effect on both fE and fP levels (fE: $F_{3,88} = 25.06$, $P < 0.001$; fP: $F_{3,88} = 8.19$, $P < 0.001$; Fig. 1). Pairwise comparisons revealed that captive lynx had higher concentrations of fE and fP than all three wild populations (fE: captive vs. CO, $t_{88} = 8.09$, $P < 0.001$; vs. ME, $t_{88} = 3.51$, $P = 0.004$; vs. MT, $t_{88} = 6.79$, $P < 0.001$; fP: captive vs. CO, $t_{88} = 3.95$, $P < 0.001$; vs. ME, $t_{88} = 4.18$, $P < 0.001$; vs. MT, $t_{88} = 3.21$, $P = 0.01$). Mean fE values were 2- to 3-fold greater in captive females (captive = 505 ng/g, CO = 151 ng/g, ME = 200 ng/g, MT = 113 ng/g). Mean fP values were at least 4-fold greater (captive = 2284 ng/g, CO = 594 ng/g, ME = 184 ng/g, MT = 456 ng/g). None of the wild populations were significantly different from each other ($P > 0.1$).

3.4. Age

Age did not have a significant effect on fE or fP levels for captive females >2 years old (fE: $\beta = 0.04 \pm 0.04$ ln(ng/g)/year, $t_{15} = 0.96$, $P = 0.35$; fP: $\beta = 0.01 \pm 0.05$ ln(ng/g)/year, $t_{13} = 1.38$, $P = 0.19$), nor did it have a significant effect for wild females of all ages (fE: $\beta = -0.03 \pm 0.02$ ln(ng/g)/year, $t_{84} = 1.38$, $P = 0.17$; fP: $\beta = 0.06 \pm 0.07$ ln(ng/g)/year, $t_{84} = 0.85$, $P = 0.40$; Fig. 4). At the individual level for the wild females, the random coefficients model indicated that neither fE nor fP changed significantly with age ($P > 0.5$, $n = 22$ lynx).

3.5. Housing situation

The sex of a female's cage-mate had a significant effect on fE concentrations ($F_{2,11} = 8.65$, $P = 0.006$) but not on fP concentrations. Mean fE concentrations were significantly lower for females housed with another female (340 ng/g) than for females housed

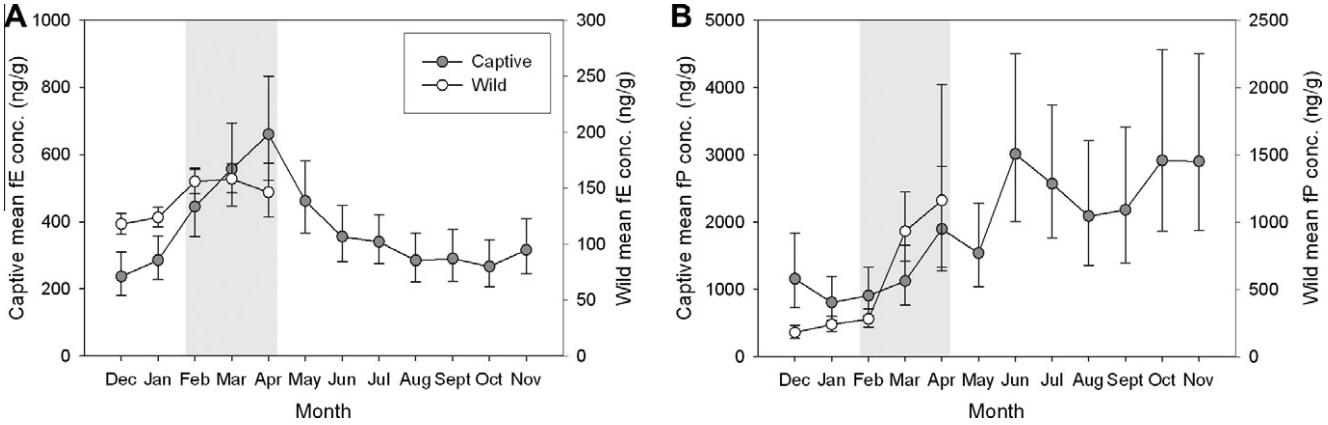

Fig. 3. Seasonal patterns of (A) fE and (B) fP expression in female Canada lynx (back-transformed LS mean ± SE). Only intact females were included in this analysis. The shaded area represents the breeding season. Note that different *y*-axis scales are used for wild and captive populations.

with a male (996 ng/g; $t_{11} = 3.46$, $P = 0.02$). The number of cagemates a female was housed with did not have a significant effect on fE or fP concentrations ($P > 0.4$).

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. 5).

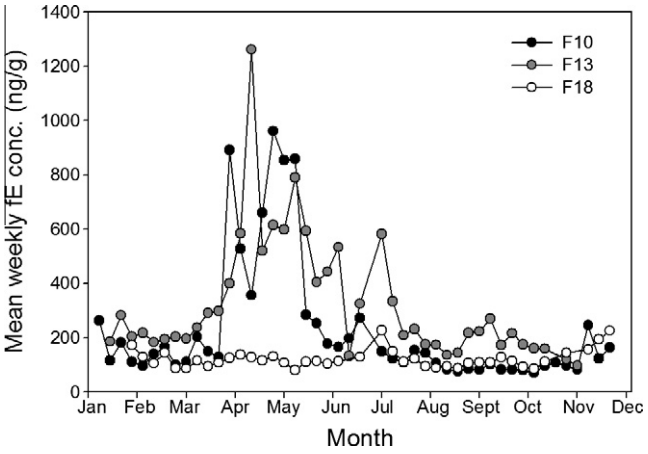

Fig. 5. Longitudinal fE profiles for three female Canada lynx housed together. Two females show seasonal ovarian activity, but the ovarian activity of the third female appears suppressed.

4. Discussion

Understanding the reproductive physiology of a species can be valuable to captive management and conservation efforts. In this study, we employ the non-invasive technique of fecal hormone metabolite analysis to provide the first description of reproductive endocrinology in captive and wild female Canada lynx, a threatened species.

The ability of the chosen estradiol assay to detect biologically relevant changes in ovarian activity was confirmed by the fact that spayed females had significantly lower fE concentrations than intact females. Furthermore, the majority of fE peaks occurred during March or April, which coincides with the breeding season and the general timeframe of 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.

The progesterone assay detected significantly higher fP concentrations in pregnant females than non-pregnant females, which may suggest that this assay measures biologically relevant changes

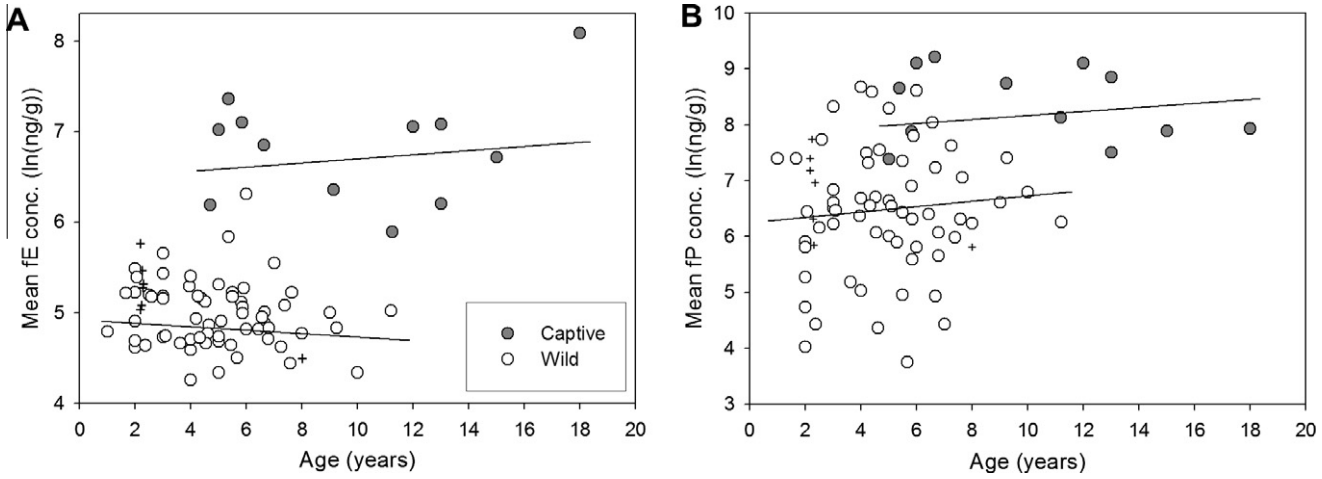

Fig. 4. Effect of age on concentrations of (A) fE and (B) fP in captive and wild female Canada lynx. " + " represents points that were excluded from analysis (2-year old females and F15, see text for Section 4). Note that the *y*-axis is in log-scale.

in hormone expression. However, we expected the magnitude of this difference to be much greater. In other felids, fP values increase 5- to 30-fold during pregnancy (Graham et al., 1995), whereas in Canada lynx, fP values only increased 2-fold (Fig. 1). Interestingly, fP are not useful for identifying pregnancy in the other three *Lynx* species. In Iberian lynx, neither fecal nor urinary progestagens increase significantly during pregnancy (Braun et al., 2009; Pelican et al., 2006). Eurasian lynx exhibit an increase in fP during pregnancy, but the increase is small, and fP concentrations do not decrease at parturition (Dehnhard et al., 2008; Jewgenow et al., 2006). In bobcats, spikes in fP values occur prior to mid-gestation, but concentrations also return to baseline (Miller, 1995). Therefore, fP do not appear to be a reliable method of identifying pregnancy in *Lynx* species.

Although the progesterone assay did not detect the *expected* increase in fP in pregnant lynx, several lines of evidence suggest that the progesterone assay does accurately reflect luteal activity. First, biochemical evidence in lynx indicates that fP are immunoreactive with the progesterone antibody. A radiometabolism study with Eurasian lynx revealed that steroid metabolites are primarily excreted in the feces (K. Jewgenow, personal communication), as is the case for most felids (Palme et al., 2005). Progesterone was heavily metabolized and excreted almost entirely as polar conjugates (Dehnhard et al., 2008, 2010). Again, this is similar to many other species for which fP assays are well established; very little parent hormone is excreted (Brown et al., 1994; Palme et al., 2005). Subsequent immunoassays revealed that the progesterone antibody reacted with several fP (Dehnhard et al., 2008, 2010), and was appropriate for assessing patterns of circulating progesterone expression. A comparative HPLC study revealed that metabolism of progestagens is conserved among the four *Lynx* species (Dehnhard et al., 2010). Thus, the biochemical evidence suggests that progesterone assays can detect relevant metabolites of progesterone in feces.

Second, there is growing evidence that lynx exhibit uncharacteristic patterns of luteal activity compared to other felids. Corpora lutea (CLs) are the primary source of circulating progesterone in most female mammals. In felids, CLs persist for the duration of the pregnancy (or for one-third to one-half the length of gestation during pseudo-pregnancies) and then regress (Brown, 2006). However, in all four *Lynx* species, it appears that CLs persist well beyond pregnancy [Canada lynx (Nellis et al., 1972); Eurasian lynx (Dehnhard et al., 2008; Göritz et al., 2009; Kvam, 1990); bobcats (Duke, 1949; Johnson and Holloran, 1985; Miller, 1995; Pollack, 1950); Iberian lynx (Göritz et al., 2009)]. In bobcats, luteal bodies are retained between cycles, perhaps for life, and these CLs are capable of producing progesterone (Duke, 1949; Woshner et al., 2001). In Eurasian and Iberian lynx, ultrasonography revealed the presence of CLs as late as November (~6 months after the breeding season), and serum progesterone levels correspond with these persistent CLs (Göritz et al., 2009). In Canada lynx, fP concentrations increase during the breeding season and do not decline until December. 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 unclear, 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 luteal bodies from previous cycles actually facilitate successful reproduction in bobcats (Miller, 1995). Persistent CLs might help regulate seasonal changes in reproductive physiology and maintain the strong seasonality observed in lynx (Göritz et al., 2009). 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. Clearly, the basic reproductive endocrinology of *Lynx* and the role of persistent CLs merit much more attention.

Previously, it was unknown whether Canada lynx ovulate spontaneously or reflexively (Nowak, 1999). Our evidence indicates that Canada lynx can ovulate spontaneously, at least on occasion. We found that fP concentrations increased in unmated females (housed alone or with another female) during the breeding season. Additionally, virgin females exhibited a dramatic decline in fP when their ovaries (and consequently any CLs) were removed. 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 that some species can ovulate spontaneously, and some individuals may alternate between the two strategies. 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 of induced vs. spontaneous ovulation, but we observed at least five clear cases of spontaneous ovulation based on fP profiles.

Both fE and fP exhibit seasonal fluctuations in captive and wild lynx. Mean fE concentrations increase during the breeding season. In wild lynx, the highest fE values occur in February/March, which coincides with peak fecal androgens in wild males. However, in captive females, the highest fE values occur in March/April (one month later than peak androgens in captive males). There is quite a bit of variability in the timing and duration of the seasonal increase in fE among females, which is at least partially due to latitudinal variation. We found that seasonal increases in fE occur later as latitude increases. In other felid species that exhibit some degree of reproductive seasonality, photoperiod appears to be the primary mediator of seasonality (Brown, 2006; Brown et al., 2002).

For fP, the seasonal trend is much different. In captive lynx, fP concentrations are lowest prior to the breeding season, increase in April, and remain elevated through December. This is consistent with the occurrence of spontaneous ovulation (as some females were unmated) and the persistence of CLs. Wild females also exhibit a pronounced increase in fP concentrations during the breeding season, but due to sampling limitations, we do not know the trajectory of fP expression beyond April. Iberian lynx also exhibit a prolonged elevation of fP following the breeding season (Pelican et al., 2006). However, in Iberian lynx, fP values decrease around October and remain low until the following breeding season. We did not observe a decrease in fP concentrations for Canada lynx until January.

A unique aspect of this study is our ability to compare fecal hormone concentrations between wild and captive populations. We found that captive females have notably higher fE and fP levels than wild females. There are very few studies that compare fecal hormone concentrations between wild and captive populations, and results are variable between studies (Fujita et al., 2001; Terio et al., 2004; Ziegler et al., 1997). Similar to our findings, Fujita et al. (2001) found that fecal progestagens and estrogens were both higher in captive than in wild Japanese macaque females. 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 wild samples might have been from males).

We also found that fecal androgens are significantly higher in captive male lynx (Fanson et al., this issue), and fecal glucocorticoids are higher for both sexes in captivity (Fanson, 2009). Interestingly, wild lynx held in pens for 21–128 days have fE and fP concentrations similar to captive lynx (Fig. 1), but hormones return to 'wild' levels after they are released. One captive female (F15) had very low steroid metabolite concentrations compared to other captive females, but similar to wild females (Fig. 4). Her male

cage-mate also had unusually low fecal androgen metabolites compared to other captive males (see Fanson et al., this issue). This pair was housed in Newfoundland in one of the largest enclosures in the study (4600 m^2, as compared to 500 m^2 for the second largest enclosure). These data suggest that the mechanism underlying the observed population difference is neither hormone nor gender specific, and that hormone expression may change fairly rapidly in response to captivity.

One possible explanation is that the observed population differences in fE and fP concentrations may be linked to energy regulation. In humans, it is well documented that diet, metabolism, and body condition can affect dynamics of steroid endocrinology (Hajamor et al., 2003; Tchernof et al., 1996). Generally, both captive lynx and wild lynx temporarily housed in holding pens have more regular access to food and limited potential for physical activity. Captive lynx also generally have greater mass than wild lynx (11–20 kg for captive lynx vs. 7–11 kg for wild lynx). One exception is F15 (the excluded captive female), but given her enclosure size and the climatic conditions, her activity levels and energetic demands may be more comparable to wild lynx.

A second, though not mutually exclusive explanation, is that elevated fE and fP in captive lynx may be due to increased adrenal activity caused by physiological or psychological stressors associated with captivity. Indeed, we observed higher fecal glucocorticoid levels in captive lynx. Adrenal glands are capable of producing other steroids, and there is evidence in Canada lynx that some 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. In humans, the adrenal cortex is known to produce androstenedione which is then converted to estrone by the liver (Lievertz, 1987). Steroid over-production is associated with metabolic dysfunction and other pathologies. Particularly in light of the recent poor breeding success in captivity, it is important to develop a better understanding of the significance and potential implications of elevated steroid hormone levels in captive lynx.

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 is either some turn-over in luteal bodies between breeding seasons, or the production of progesterone by CLs changes over time. In bobcats, older CLs produce lower levels of progesterone than fresh CLs (Woshner et al., 2001).

In captive 2-year old females, fE and fP were significantly lower than in older females. Captive Iberian lynx show a similar trend: 2-year old females had significantly lower fE and fP values (Pelican et al., 2006). We did not observe a similar result in wild females, and that population included even younger individuals. Canada lynx are generally reproductively mature by 9 months of age, although when prey density is low, they may not mature until their second winter (Ruggiero et al., 2000). All captive females in this study should have been reproductively mature. However, all of the 2-year old females in our study were housed in "female only" groups, and this social environment may affect ovarian function.

Females 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. In female groups, one female had much lower fE concentrations and failed to exhibit a seasonal increase in fE (Fig. 5). Interestingly, the female that exhibited this ovarian suppression was also the subordinate female, as ranked by keepers (K. Fanson, unpublished data). It is well-known that social structure can impact reproductive function, and social suppression has been documented in a wide variety of species (Mellen, 1991; Wasser and Barash, 1983; Wielebnowski et al., 2002). 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 (1991) found that cats maintained in groups other than single male–female pairs were less likely to reproduce and rarely succeeded in rearing any offspring. 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.

This study provides the first longitudinal investigation of female reproductive hormones in captive and wild Canada lynx. Our key findings include: (1) CLs seem to persist for an unusually long time compared to other felids, (2) Canada lynx can ovulate spontaneously, although the frequency is unknown, (3) captive females show much higher fE and fP levels than wild females, which may be due to differences in energy regulation or adrenal activity, and (4) female group housing may contribute to ovarian suppression and/or delayed reproductive maturity. Understanding the basic reproductive physiology of a species is valuable for improving captive breeding programs and conservation efforts.

Acknowledgments

We greatly appreciate the hard work of everyone involved in collecting fecal samples and the assistance provided by participating institutions (*Captive*: Alaska Zoo, Assiniboine Park Zoo, Big Cat Rescue, Brec's Baton Rouge Zoo, Buttonwood Park Zoo, Dirt Willy's Game Bird Farm, N.O.A.H. Feline Refuge Center, Pueblo Zoo, Salmonier Nature Park, Scovill Zoo, The Wildcat Sanctuary, Toronto Zoo, Walk on the Wildside Feline Refuge, Wild Trax Feline Refuge, Wildlife Science Center, Zoo America, and Zoo Sauvage de St. Félicien; *Wild*: Colorado Division of Wildlife, Maine Department of Inland Fisheries and Wildlife, USFS Rocky Mountain Research Station). Thanks to Astrid Bellem and Jocelyn Bryant for technical support. Funding for this project was provided by the Chicago Zoological Society/Chicago Board of Trade, SDE Graduate Women in Science, Purdue University, and PEO International.

References

Atsalis, S., Margulis, S.W., Bellem, A., Wielebnowski, N., 2004. Sexual behavior and hormonal estrus cycles in captive aged lowland gorillas (*Gorilla gorilla*). Am. J. Primatol. 62, 123–132.

Braun, B.C., Frank, A., Dehnhard, M., Voigt, C.C., Vargas, A., Göritz, F., Jewgenow, K., 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., Wasser, S.K., Wildt, D.E., Graham, L.H., 1994. Comparative aspects of steroid-hormone metabolism and ovarian activity in felids, measured noninvasively in feces. Biol. Reprod. 51, 776–786.

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

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

Dehnhard, M., Naidenko, S., Frank, A., Braun, B., Göritz, F., Jewgenow, K., 2008. Non-invasive monitoring of hormones: a tool to improve reproduction in captive breeding of the Eurasian lynx. Reprod. Domest. Anim. 43, 74–82.

Dehnhard, M., Fanson, K., Göritz, F., Naidenko, S., Vargas, A., Jewgenow, K., 2010. 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*). Gen. Comp. Endocrinol. 167, 287–296.

Duke, K.L., 1949. Some notes on the histology of the ovary of the bobcat (*Lynx*) with special reference to the corpora lutea. Anat. Rec. 103, 111–131.

Fanson, K.V., 2009. Stress and Reproductive Physiology in Canada Lynx (*Lynx canadensis*): Implications for *In-situ* and *Ex-situ* Conservation. PhD Dissertation, Purdue University, West Lafayette, Indiana.

Fanson, K.V., Wielebnowski, N.C., Shenk, T., Jakubas, W., Squires, J.R., Lucas, J.R, this issue. Patterns of Testicular Activity in Captive and Wild Canada Lynx (*Lynx canadensis*).

Fujita, S., Mitsunaga, F., Sugiura, H., Shimizu, K., 2001. Measurement of urinary and fecal steroid metabolites during the ovarian cycle in captive and wild Japanese macaques, *Macaca fuscata*. Am. J. Primatol. 53, 167–176.

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

Göritz, F., Dehnhard, M., Hildebrandt, T.B., Naidenko, S.V., Vargas, A., Martinez, F., Lopez-Bao, J.V., Palomares, F., Jewgenow, K., 2009. Non cat-like ovarian cycle in the Eurasian and the Iberian lynx – ultrasonographical and endocrinological analysis. Reprod. Domest. Anim. 44, 87–91.

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

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

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

Jewgenow, K., Frank, A., Göritz, F., Vargas, A., Naidenko, S., Dehnhard, M., 2006. A Comparative Analysis of the Endocrine Patterns of the Eurasian and the Iberian Lynx in Captivity. Iberian Lynx Ex-situ Conservation Seminar Series, Doñana, Spain.

Johnson, N.F., Holloran, D.J., 1985. Reproductive activity of Kansas bobcats. J. Wildl. Manage. 49, 42–46.

Kvam, T., 1990. Ovulation rates in European lynx, *Lynx lynx*, from Norway. Z. Saugetierkd. 55, 315–320.

Lievertz, R.W., 1987. Pharmacology and pharmacokinetics of estrogens. Am. J. Obstet. Gynecol. 156, 1289–1293.

Littell, R.C., Milliken, G.A., Stroup, W.W., Wolfinger, R.D., Schabenberger, O., 2006. SAS for Mixed Models, second 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 Biol. 10, 95–110.

Miller, D.L., 1995. Feasibility of Embryo Transfer Using the Bobcat (*Felis rufus*). Masters Thesis, Mississippi State University, Starkville, Mississippi.

Nellis, C.H., Wetmore, S.P., Keith, L.B., 1972. Lynx-prey interactions in central Alberta. J. Wildl. Manage. 36, 320–329.

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

Palme, R., Rettenbacher, S., Touma, C., El-Bahr, S.M., Mostl, E., 2005. Stress hormones in mammals and birds – comparative aspects regarding metabolism, excretion, and noninvasive measurement in fecal samples. Ann. N. Y. Acad. Sci. 1040, 162–171.

Pelican, K., Abaigar, T., Vargas, A., Rodriguez, J.M., Bergara, J., Rivas, A., Martinez, F., Rodriguez, D., Brown, J.L., Wildt, D.E., 2006. Hormone Profiles in Iberian Lynx: Application to *In situ* and *Ex situ* Conservation. Iberian Lynx Ex-situ Conservation Seminar Series, Doñana, Spain.

Pollack, E.M., 1950. Breeding habits of the bobcat in northeastern United States. J. Mammal. 31, 327–330.

Poole, K.G., 2003. A review of the Canada lynx, *Lynx canadensis*, in Canada. Can. Field Nat. 117, 360–376.

Ruggiero, L.F., Aubry, K.B., Buskirk, S.W., Koehler, G.M., Krebs, C.T., McKelvey, K.S., Squires, J.R., 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. Annual Report to US Fish and Wildlife Service. USFWS.

Tchernof, A., Labrie, F., Belanger, A., Despres, J.P., 1996. Obesity and Metabolic Complications: Contribution of Dehydroepiandrosterone and Other Steroid Hormones. International Symposium on DHEA Transformation into Androgens and Estrogens in Target Tissues – Intracrinology, Quebec, Canada.

Terio, K.A., Marker, L., Munson, L., 2004. Evidence for chronic stress in captive but not free-ranging cheetahs (*Acinonyx jubatus*) based on adrenal morphology and function. J. Wildl. Dis. 40, 259–266.

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.

Wasser, S.K., Barash, D.P., 1983. Reproductive suppression among female mammals – implications for biomedicine and sexual selection theory. Q. Rev. Biol. 58, 513–538.

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

Woshner, V.M., Miller, D.L., Waldhalm, S.J., Cox, N.M., Jacobson, H.A., Leopold, B.D., 2001. Progesterone in Luteal Bodies of Bobcats. In: 55th Annual Conference of the Southeastern Association of Fish and Wildlife Agencies, Louisville, Kentucky.

Ziegler, T.E., Santos, C.V., Pissinatti, A., Strier, K.B., 1997. Steroid excretion during the ovarian cycle in captive and wild muriquis, *Brachyteles arachnoides*. Am. J. Primatol. 42, 311–321.