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Non Cat-Like Ovarian Cycle in the Eurasian and the Iberian Lynx – Ultrasonographical and Endocrinological Analysis

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Contents

The Iberian lynx is considered the most endangered felid species. Therefore, an *ex situ* conservation program was initiated to protect this species from extinction. Additional knowledge on lynx reproduction biology and reliable methods for reproductive monitoring are important for developing a captive breeding program. The aim of this study in lynx was to implement transrectal ultrasonography to visualize ovarian structures (follicles, corpora lutea) and to assess ovarian activity in addition to analysis of serum progesterone and oestradiol. Because of limited access to Iberian lynxes, the less-endangered Eurasian lynx and bobcat were also studied in this comparative study. Recent endocrinological studies based on faecal and urinary progesterone and oestrogen metabolites revealed that steroid profiles in both these species were alike and did not follow the typical pattern of other felids. Pregnancy diagnosis was not possible, since progesterone concentrations did not differ between pregnant and pseudo-pregnant animals. Progesterone was also detected after parturition as well as after weaning until the onset of a new oestrous cycle. In the present study, the presence of corpora lutea during the non-breeding season was confirmed by ultrasonography and by elevated serum levels of progesterone averaging 3.56 ± 1.3 ng/ml in Eurasian and 6.1 ± 0.26 ng/ml in Iberian lynx, respectively. The ultrasonographical findings on the ovarian structures suggest strongly that corpora lutea developed after ovulation stay active until November and regress before the onset of the next oestrus.

Introduction

The genus Lynx includes four species: the Eurasian lynx (*Lynx lynx*), the Canada lynx (*Lynx canadensis*), the Bobcat (*Lynx rufus*) and the most endangered felid species of the world listed on CITES Appendix 1, the Iberian lynx (*Lynx pardinus*). General reproductive parameters do not differ between the four species (Table 1, modified after Parker and Smith 1983; Kvam 1991; Hayssen et al. 1993). All except the Bobcat (BC) are strict seasonal, monoestrous breeders. The Iberian lynx (IL) has the narrowest breeding season of approximately 1 month (January to February) with births in March to April (Palomares et al. 2005); the three other species are less restricted. The BC is understood to be polyoestric till July. Male lynxes reach sexual maturity within 3 years. In recent studies, in the Eurasian lynx (EL), we have shown that male reproduction is seasonal corresponding to the female oestrus (Jewgenow et al. 2006a). Faecal testosterone concentration, volume of ejaculates, percentages of motile spermatozoa and intact sperm were maximized during breeding season in February/March (Goeritz et al. 2006).

The IL *ex situ* conservation program is an essential part of a coordinated action plan to conserve this highly endangered species (Vargas et al. 2008). Knowledge of reproductive biology and reliable methods for reproductive monitoring are essential for improvement of captive breeding. In several felid species, pregnancy diagnosis based on faecal hormone metabolites became almost a routine procedure. However, it has been shown that progesterone metabolites in lynxes did not follow the typical pregnancy pattern and made pregnancy diagnosis unattainable by faecal (Jewgenow et al. 2006b; Pelican et al. 2006) as well as by urinary progesterone (Dehnhard et al. 2008; Jewgenow et al. 2009). Surprisingly, progesterone measurement suggestive of luteal activity was also detected after weaning of the cubs. This does not occur in domestic cats (Tsutsui and Stabenfeldt 1993).

Because information on female reproductive physiology in lynxes is limited, the aim of this study was to use transrectal ultrasonography of ovarian follicles and corpora lutea (Cll) and assays of progesterone and oestradiol in serum to assess ovarian activity outside the breeding season. As a result of limited access to ILs, the less-endangered EL and the BC were also used in this comparative study.

Materials and Methods

Iberian lynxes in captivity were managed by the IL *ex situ* conservation program and housed at three different locations in Southern Spain (El Acebuche Captive Breeding Center in Doñana's National Park, Jerez Zoo and La Aliceda Captive Breeding Centre). Almost all females of the captive populations were examined in late October 2006 (n = 10) and late November 2007 (n = 18). In December 2006, additionally, six female IL were captured for reproductive assessment from the wild population in the Doñana's National Park. Eurasian lynxes were kept in a field research station situated 50 km northeast of Moscow, surrounded by the natural vegetation of the Russian South Taiga forests. Females were examined by ultrasound in June 2002/2005 (n = 11), July (n = 7) and November 2003 (n = 3). The BCs examined were kept in Russia at the same station (n = 1 female, June 2007) and at the Jerez Zoo in Spain (n = 3 females, November 2007). All females were kept in separate enclosures. During breeding season, adult females were allowed to mate. Mating was observed, either by direct observation

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Table 1. General reproductive parameters of the four lynx species: the Eurasian lynx (*Lynx lynx*), the Canada lynx (*Lynx canadensis*), the Bobcat (*Lynx rufus*) and the most endangered felid species of the world listed on CITES Appendix 1, the Iberian lynx (*Lynx pardinus*)

Reproductive parameter	*L. lynx*	*L. pardinus*	*L. canadensis*	*L. rufus*
Body length (cm)	80–130	75–100	90–100	72–98
Weight (kg)	18–35	8–17	9–18	11–14
Neonatal weight (g)	250–360	130–150	200	112–226
Litter size	2–3 (1–5)	2–3 (1–4)	2 (1–4)	3.5 (1–6)
Age of sexual maturity (years)	2–3	3	2	?
Oestrous length (days)	2–7	2–5	–	2
Cycle length (days)	–	–	–	44
Gestation (days)	68–72	63–65	60–65	50–60
Lactation (months)[a]	3	3–4	–	3
Solid food (weeks)[b]	6	8–9	–	7–8
Litter/year	1	1	1	>1
Breeding season	Jan–Apr	Jan–Feb	Jan–Feb	Jan–July

[a]Lactation duration as time from parturition to weaning.
[b]Age of cubs when supplemental intake of solid food started.

(in Russia) or by 24 h surveillance using remote controlled camera systems (in Spain). After mating, the males were usually removed from the enclosures. Pregnancy was defined based on the delivery of cubs and/or hormone assessment (Braun et al. 2009). Pseudopregnancy was defined as evidence of luteal function without detection of parturition or abortion after mating. For data evaluation, the animals were grouped according to age and breeding status: (i) juvenile; (ii) adult, no mating; (iii) adult, mating, no birth (pseudopregnant); and (iv) adult, mating, birth.

Ultrasonography

The animals were immobilized by IM administration (via blow pipe) of 3.5–4.0 mg/kg xylazine hydrochloride (Rompun™ 10%; Bayer, Leverkusen, Germany) and 3.0–3.5 mg/kg ketamine hydrochloride (Ketamine 10%™; Essex, Munich, Germany). After examination and sample collection, anaesthesia was reversed with 0.2 mg/kg atipamezol hydrochloride (Antisedan™; Pfizer, Karlsruhe, Germany). The entire genital tract of each female was examined from caudad to craniad by transrectal ultrasonography in lateral recumbency (Goeritz et al. 1997; Hildebrandt et al. 2000). A real-time, B-mode ultrasound scanning system (CS 9100 Oculus, Picker International GmbH, Espelkamp, Germany) equipped with a 7.5-MHz curved linear transducer (EUP-F 334) was used. The transducer was fitted with two specific adaptors (15 and 25 cm in lengths, A. Schnorrenberg Chirurgiemechanik, Schönwalde, Germany) and introduced into the rectum using ultrasound gel. The dimensions of the ovaries were measured and their volume calculated at that of a simple spheroid (Goeritz et al. 2003). Corpora lutea were counted. The diameter of the smallest and largest corpus luteum (Cl) was measured and the mean volume of total luteal tissue (mm^3) was calculated per animal ($V = 4/3 \times \pi \times$ mean Cl radius$^3 \times$ number of Cll). For follicle counts, follicles were defined as anechoic structures >1 mm in diameter.

Endocrinology

Blood samples were taken during each ultrasound investigation by venepuncture. Additionally, blood was collected using the same method during pregnancy from one BC (in June) and two EL (in May). Serum was separated after centrifugation. In the highly endangered IL, blood samples were obtained in March (n = 14), non-invasively using blood-suckling bugs (*Dipetalogaster maxima*) as described and validated earlier (Voigt et al. 2004). The bugs were placed into cork plates, which were installed at the lynxes' preferable resting sites (Braun et al. 2009). The ingested blood was withdrawn from bugs and centrifuged.

The obtained serum (syringe collection during anaesthesia) and plasma (bug ingested blood) samples were kept frozen until hormone assessment. Progesterone and oestradiol were measured in the blood serum and plasma by enzyme immunoassays with double antibody technique (Meyer et al. 1997). Briefly, 0.1 ml serum/plasma was extracted with 2 ml butyl *tert*-methyl ether/petroleum ether 30/70 (v/v) for 30 min. After freezing at -80°C, the fluid petroleum ether phase was decanted and evaporated at 55°C. The steroids in the residues were reconstituted in 1 ml 40% methanol (v/v) and duplicates of 20 μl were analyzed.

The progesterone (P4) assay was based on a rat antibody (Sigma P1922, generated to progesterone) together with 4-pregnen-3,20-dione-3-CMO-peroxidase as label as described and validated previously for lynx (Goeritz et al. 1997). The oestrogen assay was an in-house microtitre plate enzyme immunoassay previously validated for lynx (Dehnhard et al. 2008; Jewgenow et al. 2009).

Statistics

Data are presented as means ± SEM. Comparisons of mean values were performed by Welch corrected unpaired *t*-test, when the number of examinations or samples exceeded n = 6. Relationship between volume of luteal tissue and progesterone concentration in blood serum was determined by Spearman correlation. All statistical tests were based on a 5% level of significance. The statistical procedures were performed with the software program INSTAT Version 3 (Graphpad Software Inc, San Diego, CA, USA).

Results and Discussion

In contrast to many other felid species (Brown et al. 1994), pregnancy diagnosis in lynx cannot be made based on elevation of faecal (Pelican et al. 2006) or urinary progesterone metabolite concentration (Dehnhard et al. 2008; Jewgenow et al. 2009). In both species luteal activity was measurable not only during pregnancy but also in mated non-pregnant and lactating females. In the present study, luteal activity after parturition was confirmed by ultrasonographical detection of Cll (Fig. 1) and by elevated serum levels of progesterone (Table 2) in EL and IL, in June/July and November/December, respectively. These findings are in

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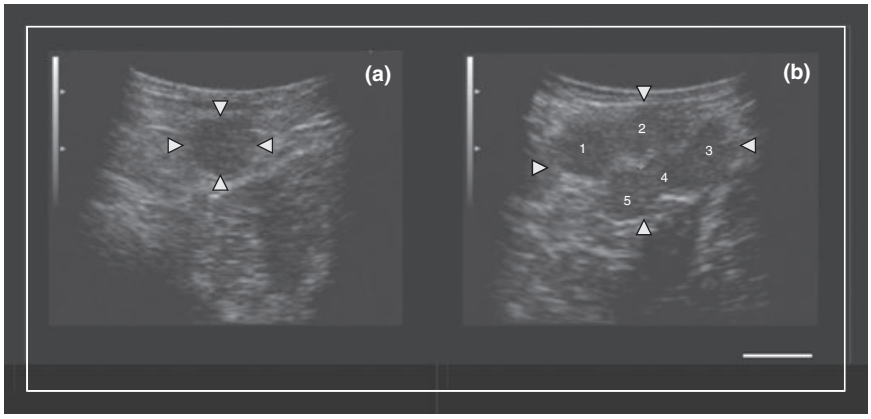

Fig. 1. Sonograms of ovaries of two different adult Iberian lynxes out of breeding season in late November. The ovary of an animal in which no mating was observed during breeding season appears inactive (a). The homogeneous parenchyma does not show any Cll. The ovary of an animal in which mating, birth and lactation was observed (b) is characterized by multiple Cll. They appear as spherical moderate echoic structures. Ovarian border and Cll are indicated by arrowheads and numbers, respectively. The scale bar represents 1 cm

agreement with our recent results on steroid hormone metabolite concentrations measured in faeces and urine (Jewgenow et al. 2006b, 2009; Dehnhard et al. 2008). A typical faecal ovarian hormone profile in EL included an absence of a significant oestrogen or progesterone elevation before mating, a positive correlation between faecal progesterone and oestradiol metabolites, increases in both hormones during pregnancy, decreases towards parturition, and increased concentrations again during lactation period. To our knowledge, the existence of elevated progesterone during lactation and presumably active Cll throughout much of the year is unique for felid species. A moderate serum progesterone concentration of approximately 5 ng/ml was measured out of breeding season (Table 2) in the present study, although values are approximately 4–10 times higher during pregnancy (EL = 61.1 ± 13.3 ng/ml; IL = 17.0 ± 10.1 ng/ml; BC = 28.4 ng/ml). The progesterone detected is assumed to be of luteal origin because of the existence of multiple large Cll detected ultrasonographically and reflected by an increased volume of luteal tissue after mating (Table 2). Although mean progesterone concentration was directly correlated to the mean volume of luteal tissue (Spearman correlation $r = 0.927$, $p < 0.01$), additional study is required to confirm that the source is ovarian and not adrenal. The low but steady progesterone concentration may function to induce a negative feed back to inactivate folliculogenesis. That would represent a mechanism to turn a normally polyoestric cycle seen in most felids into in a monoestric cycle in the lynx and thus contribute to the seasonality of its breeding pattern so as to match the seasonal sperm production in the males (Goeritz et al. 2006; Jewgenow et al. 2006a). It is known that long-term application of exogenous progesterone derivatives, frequently used in cats for contraception (Concannon and Lein 1983; Concannon and Meyers-Wallen 1991) suppress ovarian function, although they can also induce uterine pathology (Munson 1993). The apparent correlation between oestrogen and progesterone secretion suggested by results to date may reflect an important role of oestrogen, perhaps in maintaining progesterone receptors and sensitivity of progesterone.

Juvenile animals had small ovaries (mean ovarian volume in mm^3: 110.4 ± 70.5 in BC; 160.7 ± 91.3 in IL; 288 in EL) without Cll and very low (< 0.5 ng/ml) progesterone concentrations (Table 2). Adult lynxes without observed mating also had no Cll indicating that the normal mechanism to likely be one of induced (reflex) ovulation. Frequent mating in captive breeding programs is likely important as it increased the number of ovulations and the number of cubs born per female (Naidenko et al. 2007). Mean number of Cll detected ultrasonographically after birth was 3.0 and 5.5 in EL and IL, respectively (Table 2). While in EL number of Cll was very often consistent with the number of cubs born, there were always more Cll detected than cubs born in IL (mean litter size in EL = 2.6, and in IL = 2.1). Whether this is caused by absence of fertilization of oocytes after ovulation, or by early embryonic resorption, or possibly by a fresh ovulation and development of accessory Cll is uncertain. For IL and EL, the volume of total luteal tissue (expressed as mm^3 per animal) and progesterone concentrations were directly correlated (Spearman correlation $r = 0.927$, $p < 0.01$). Therefore, the luteal volume calculated by summarizing the spherical volume of all Cll may be an additional parameter to describe individual luteal activity.

Conclusions

The ultasonographical findings strongly suggest that Cll developed from ovulations in mid-winter and stay active until at least late November. Their functional role in lynx reproduction is still unknown, but we hypothesize the associated elevated progesterone may support lactation, prevent a new oestrous cycle and thus restrict the breeding season to midwinter (as opposed to the situation in the polyoestric BC). Further comparative longitudinal ultrasound and hormone evaluations on all the lynx family species are needed to further elucidate their unique reproductive patterns.

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Table 2. Individual morphological and endocrinological parameters (Mean ± SD) describing the ovarian activity of the bobcat, the Eurasian and the Iberian lynx at the time of ultrasound examinations conducted out of breeding season (from June to November)

Species	Age class (no. of animals)	Recent breeding history*	Mean (±SD) ovarian volume per animal (mm^3)	Mean (±SD) no. of follicles per animal	Mean (±SD) no. of Cll and (cubs born) per animal	Mean (±SD) volume of luteal tissue (mm^3) (no. of animals)§	Mean (±SD) P4 (ng/ml) (no. of samples)	Mean (±SD) E2 (pg/ml) (no. of samples)
Bobcat	Juvenile (n = 1)		110.4 ± 70.5	0	0	0	0.4	0.1
	Adult (n = 2)	No mating	739.0	0	0	0	4.4 ± 2.5 (2)	1.7 ± 1.7 (2)
	Adult (n = 1)	Mating, birth	2477.8 ± 2108	0	1.5 ± 0.7 (1.5 ± 0.7)	109.2 (1)	6.3	1.8
	Adult (n = 1)	Pregnancy† (3rd week, June)	n.d.	n.d.	n.d.	n.d.	28.4	1.15
European lynx	Juvenile (n = 1)		288	0	0	0	n.d.	n.d.
	Adult (n = 2)	No mating	1703.7 ± 1216.7	0	0	0	n.d.	n.d.
	Adult (n = 1)	Mating, no birth	2843.8 ± 1637	2 ± 1.4	4 ± 1.4	1342.5 ± 286.6 (2)	3.9 ± 0.9 (2)	1.2 ± 0.1 (2)
	Adult (n = 17)	Mating, birth	2315.8 ± 1131.1	0.5 ± 1.1	3.3 ± 0.8 (2.6 ± 1.2)	2067.3 ± 743.4 (8)	4.9 ± 2.3 (16)	0.8 ± 0.4 (10)
	Adult (n = 2)	Pregnancy† (3rd week, May)	n.d.	n.d.	n.d.	n.d.	61.1 ± 13.3 (2)	n.d.
Iberian lynx	Juvenile (n = 4)		160.7 ± 91.3	0	0	0	0.5 ± 1.0[a] (15)	0.6 ± 0.6 (13)
	Adult (n = 3)	No mating	221.3 ± 167.7	0.3 ± 0.6	0	0	2.3 ± 0.2 (2)	n.d.
	Adult (n = 9)	Mating, no birth	994.9 ± 929	0	3.8 ± 1.2[a]	1082.0 ± 584.5 (6)	4.3 ± 2.1[b] (9)	3.7 ± 6.4 (8)
	Adult (n = 19)	Mating, birth	1319.7 ± 825.4	0.7 ± 0.5	5.3 ± 1.2[b] (2.1 ± 0.3)	1441.8 ± 581.8 (14)	4.3 ± 1.4[b] (19)	2.8 ± 3.5 (14)
	Adult (n = 14)	Pregnancy† (3rd–8th week, March)‡	n.d	n.d.	n.d.	n.d.	17.0 ± 10.1[c] (14)	n.d.

*Breeding history in the year of examination; no ultrasound examination was conducted during pregnancy.
†Blood collection by venepuncture under general anaesthesia.
‡Non-invasive blood collection with blood-suckling bugs (Braun et al. 2008).
§Volume of luteal tissue calculated for each animal ($V = 4/3 \times \pi \times$ mean Cl radius$^3 \times$ no. of Cll); Cl = corpus luteum, Cll = corpora lutea.
n.d., not determined.
a,b,c different superscripts indicate significant differences between the means estimated by Welch unpaired *t*-test corrected.

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Author contributions

All authors contributed to the manuscript: FG - ultrasonography, data analysis and manuscript writing; MD-hormone analysis; TBH - ultrasonography for reproductive assessment; SVN, AV-behavioural observation, data analysis, sample collection, FM-anaesthesia and health assessment of Iberian lynx; JVLB, FP - capture of wild Iberian lynx, KJ - data analysis and manuscript writing.

Conflicts of interest

The authors have declared no conflicts of interest.

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