Seasonal Profiles of Ovarian Activity in Iberian lynx (*Lynx pardinus*) Based on Urinary Hormone Metabolite Analyses

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Contents

The Iberian Lynx *Ex-Situ* Conservation Programme is an essential part of a co-ordinated action plan to conserve the most endangered felid species of the world. Successful captive breeding demands reliable methods for reproduction monitoring including reliable non-invasive pregnancy diagnosis. During a 3-year study, urine samples from six captive Iberian lynx females were obtained (one non-pregnant, one pseudo-pregnant and 11 pregnant cycles). Progesterone, pregnanediol and oestradiol were determined in urinary extracts and relevant urinary oestrogen metabolites were characterized by high-performance liquid chromatography (HPLC). Urinary progestins did not follow the typical pregnancy-related course of felids. In the lynx, we failed to demonstrate an urinary progestin elevation during pregnancy. In contrast, urinary oestrogens increased from 3.8 ± 0.6 to 8.6 ± 0.5 ng/mg creatinine ($p < 0.001$) during the pregnancy. A comparison of pseudo-pregnant with pregnant cycles revealed a further increase of oestrogens caused by implantation ($p < 0.05$). In one female, which refused to mate, no difference was estimated between oestrogens levels during the breeding and non-breeding seasons. Almost 10-fold higher oestrogen concentrations were measured in urines of females that shared enclosures with males. HPLC analysis of oestrogens in urine samples collected from Iberian lynx during the pregnancy revealed that lynx urine is composed of two polar oestrogen metabolites in addition to oestrone and minor amounts of oestradiol. Oestrone was detectable in all urinary extracts (8–12% of metabolites), whereas oestradiol was elevated only during late pregnancy (18%). Thus, seasonal luteal activity in Iberian lynx can be monitored by urinary oestrogens. The increase of urinary oestradiol during late pregnancy might indicate an oestradiol secretion by the lynx placenta.

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 in the world, listed on CITES Appendix 1 and declared critically endangered by the IUCN Red list of Threatened Species, the Iberian lynx (*Lynx pardinus*).

Data gained on lynx reproduction are mostly based on skinned carcasses collected from trappers or based on reports of captive animals (Parker and Smith 1983; Kvam 1990; Henriksen et al. 2005). All the four lynx species have some general features in common, which are typical for the *Felidae*. They are solitary living with large home ranges differing in size between the sexes (Heptner and Sludskii 1980; Palomares et al. 2001; Zimmermann et al. 2006). Kvam (1990) suggested mono-oestrus and induced ovulation for Eurasian lynx based on ovarian analysis of reproductive tracts. In contrast, bobcats are poly-oestrous breeders with more

than one litter per season (Crowe 1975). Iberian lynx produce one litter per year (Fernandez et al. 2002) and express the narrowest breeding season of approximately 1 month between January and February (Palomares et al. 2005). Accordingly, parturitions are observed during March–April after approximately 65 days of pregnancy.

The Iberian Lynx *Ex-Situ* Conservation Programme is an essential part of a co-ordinated action plan to conserve this highly endangered species (Vargas et al. 2008). Today, the Iberian lynx remains in three isolated populations located in the region of Andalusia and Castilla-La Mancha, Spain. The actual population size comprises approximately 200 animals in the wild and another 55 in captivity. The captive population is planned to include 60 lynxes—a number which will allow for the maintenance of 85% of the lynxes' genetic diversity during a 30year period, and to start the re-introduction in 2010. Besides assuring the existence of the Iberian lynx in captivity, the *ex situ* programme has the benefit of allowing the study of various aspects of the species' biology and physiology that could not easily be studied in the wild. One of such aspects is the understanding of reproductive physiology and the development of methods for non-invasive monitoring of the reproductive status (Jewgenow et al. 2006; Pelican et al. 2006; Braun et al. 2009). Pregnancy diagnosis by monitoring ovarian physiology is of particular importance in this respect, especially as a management tool for the captive breeding programme. In lynx, however, faecal steroids do not follow the typical pregnancy pattern of felids (Brown et al. 1994; Pelican et al. 2006). Therefore, the aim of the present study was to test whether urine can be used as an alternative for monitoring seasonal ovarian activity.

Materials and Methods

The Iberian Lynx are kept at the El Acebuche Captive Breeding Center in Doñana's National Park in Southern Spain. The captive population consisted of 16 animals (7 males, 9 females) in 2006 and of 52 (28 males, 24 females) in 2008. The first birth in captivity was in 2005, since then 24 cubs have been raised in the breeding programme. Animals were kept in separate enclosures (550 m^2), and during mating season (January to February) all females were allowed to mate by introducing a male into the female's enclosure. Depending upon the female's behaviour, the mates were kept together with for several weeks or just for mating. All sexual interactions including mating were recorded by

remote video cameras. During the breeding season, all animals were under permanent visual observation. Mating was documented in all females, the cycle was considered to involve a pseudo-pregnancy if no parturition or abortion was observed. Delivery of cubs was the final indication of pregnancy.

Urine sampling and reproductive status

The method for urine collection from captive Iberian lynx was previously established for camera trapping of free-ranging lynxes and was performed on a regular basis within the *ex situ* breeding programme. Urine was collected from six different females by placing home-made collectors in their enclosures. The collectors were consisted of vertical stainless steel plates (60×60 cm) ending in gutters at the bottom and a slight V-shape inclination that allowed the urine to run into a collector cup. Lynxes used these plates to mark their territories. Urine samples (10–50 ml) were collected daily, stored at $-20°C$ and shipped frozen for steroid analysis. During breeding season (January to April), two to three samples per week were analysed, for the rest of the year at least one sample per month per animal was used.

During the first breeding season (2006), samples were obtained from three of the females that had mated, two of which delivered cubs. In 2007, all six females providing samples mated with their males, and became pregnant, including one female with a pregnancy resulting in a premature birth (day 62). Three of the females were continuously kept together with their mates. In 2008, samples from four females were analysed; three of them were pregnant and one was not mated (non-pregnant). This female lynx was rejected by all males (n = 3) that were put in contact with her, although the year before she successfully mated (abortion at day 42) with a male that was no longer in that breeding centre. The number of animals and urine samples for each year and reproductive stage are summarized in Table 1.

Urine samples were analysed for creatinine levels (Heistermann et al. 1997). Urinary hormone concentrations are expressed as nanogram per milligram creatinine in order to control for differences in urine concentration.

Urine aliquots of 100 μl were incubated with β-glucuronidase/sulfatase (Roche Diagnostics GmbH, Mannheim, Germany, No. 127698, 11 and 28 mUnits, respectively) at pH 4.8 for 2 h at 37°C, followed by extraction with 2.5 ml tert-butyl methyl ether/petroleum ether (30/70, v/v, twice). After freezing at $-80°C$, the organic phases were decanted, combined, evaporated at 50°C and dissolved in 0.5 ml 40% methanol. All hormone measurements were carried out in duplicate and duplicate values were used to calculate the intra-assay coefficient of variation.

Progestin determination

To characterize urinary progestins, progesterone (Göritz et al. 1997) and pregnanediol enzyme immunoassays (Meyer et al. 1997) were carried out. The progesterone (P4) assay was based on a rat antibody (Sigma-Aldrich GmbH, Steinheim, Germany, No. P1922, generated to progesterone) together with 4-pregnen-3,20-dione-3-CMO-peroxidase as label, whereas the pregnanediol antibody was generated in rabbits (to 5β-pregnane-3,20α-diol-3-gluc-BSA) and 5β-pregnane-3,20α-diol-3-gluc-peroxidase was used as label. The cross-reactivities of both antibodies and their inter- and intra-assays coefficients were as described before (Braun et al. 2009).

Oestrogen (E2) determination in urine samples

Oestrogens analyses were carried out with an in-house microtitre plate enzyme immunoassay using a polyclonal antibody (rabbit) against 1,3,5(10)-estratrien-3,17β-diol-17-HS-BSA and 1,3,5(10)-estratrien-3,17β-diol-17-HS-peroxidase label (Meyer et al. 1997). The cross-reactivity to oestrogens were as follows: 1,3,5(10)-estratrien-3,17β-diol (17β-oestradiol) 100%, 1,3,5(10)-estratrien-3,17-one (oestrone) 114%, 1,3,5(10)-estratrien-3,17α-diol (17α-oestradiol) 69.2%, 1,3,5(10)-estratrien-3-ol-17-on (estratrien) 5.9% and $<0.1%$ for 1,3,5(10)-estratrien-3,16α,17β-triol (estriol), ethylestradiol, oestronesulphate, 19-nortestosterone, progesterone, 5α-pregnandiol and testosterone. Serial dilutions of a sample pool gave parallelism to the steroid standard with no differences in slopes (p > 0.05). Intra- and inter-assay coefficients of variation for two biological samples with low (55 pg/ml) and high (107 pg/ml) concentrations were 5.8% and 12.3% (n = 10) and 9.7% and 17.0% (n = 11), respectively.

Table 1. Collection of urine samples from female lynx at the El Acebuche Iberian lynx Captive Breeding Center during breeding (January–April) and non-breeding (May–December) seasons 2006–2008

	Number of females (n = number of samples)			
Status	Non-pregnant	Pregnancy	Pseudo-pregnancy[a]	Non-breeding
Season	Breeding	Breeding	Breeding	Outside breeding season
Mating	No mating	Mating	Mating	–
Parturition	No	Cubs born	No cubs	–
2006	–	2 (n = 48)	1 (n = 18)	3 (n = 45)
2007	–	6 (n = 143)[b]	–	6 (n = 240)
2008	1 (n = 20)	3 (n = 105)	–	4 (n = 62)
Σ	1 (n = 20)	8 (n = 237)[c], 3 (n = 59)[b]	1 (n = 18)	13 (n = 347)

[a]The pseudo-pregnancy might be the result of either an infertile mating or early embryonic death.
[b]Three females were kept together with their mates during pregnancy.
[c]Number of females kept during sample collection alone (n = pure female urine samples).

HPLC analysis of oestrogen metabolites

For separation and characterization of urinary steroid metabolites, 50 μl portions of extracts were separated on an Allure Biphenyl 5-μm high-performance liquid chromatography (HPLC) column (3.2 × 150 mm; Restek, Bad Homburg, Germany) using an acetonitril : water mixture (43 : 57, v : v) at a flow rate of 1 ml/min. Fractions of 0.33 ml were collected, frozen and assayed for oestrogens. The elution positions of authentic 1,3,5(10)-estratrien-3,17-one (oestrone), 1,3,5(10)-estratrien-3,17α-diol (17α-oestradiol) and 1,3,5(10)-estratrien-3,17β-diol (17β-oestradiol) on this column had been determined in separate HPLC runs.

Statistics

The results of urinary steroid hormone determination were expressed as immunoreactive steroid in nanogram per milligram creatinine. Data presented as means ± standard errors of the mean (SEM). Comparisons of means values were performed by Welch corrected unpaired t-test. 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

Urinary P4 levels did not reveal a distinct increase during the pregnancy compared with the non-breeding season (Table 2). The same was obvious for urinary pregnanediol (PD), although there may have been a tendency towards higher concentrations during the pregnancy (Table 2). In addition, no difference was found in progestin concentrations when non-pregnant (no mating), pregnant and pseudo-pregnant lynx cycles were compared directly with the same animals (Table 3, Fig. 1). The mean progesterone concentrations were slightly lower, when no mating occurred (Table 3, Artemisa), but progesterone levels of pseudo-pregnant and pregnant cycles did not differ significantly. Thus, urinary progestins may indicate the existence of *Corpora lutea* (CL) after induced ovulation, but a reliable pregnancy diagnosis based on either progestin was unattainable. In this respect, the urinary progestins follow the pattern described for fecal progestin excretion in Iberian lynx females (Pelican et al. 2006). In contrast to many other felid species (Brown et al. 1994), pregnancies in lynx are not characterized by elevation of either fecal (Pelican et al. 2006) or urinary progestin concentration (this study). We suggest that this might be the consequence of the prolonged presence (and function) of CL throughout most of the year (Kvam 1990, Göritz et al. 2009).

Table 3. Urine oestrogens and progestins in urine samples of a non-mated (Artemisa) and pseudo-pregnant (Aura) in relation to samples collected outside breeding season and pregnancy

	Oestrogens in ng/mg Creatinine (n samples)	Progesterone in ng/mg Creatinine (n samples)
Artemisa		
Outside breeding season (May–Dec)	2.8 ± 0.2 (47)[a,b]	2.0 ± 0.2 (47)
Pregnancy 2007	46.8 ± 2.7 (15)[a,c]	1.0 ± 0.7 (16)
Breeding season 2008 (Jan–April)	0.8 ± 0.2 (20)[b,c]	1.5 ± 0.3 (20)
Aura		
Outside breeding season (May–Dec)	3.9 ± 0.3 (96)[d,e]	1.9 ± 0.1 (97)
Pseudo-pregnancy (mating, no birth)	9.2 ± 1.0 (18)[d,f]	4.4 ± 1.4 (18)
Pregnancy 2007/2008	12.1 ± 1.3 (38)[e,f]	3.0 ± 0.2 (40)

[a–f]Identical superscript alphabets indicate statistical differences between oestrogen concentrations estimated by unpaired t-test with Welch correction:
[a]$t = 16.03$, p < 0.001; [b]$t = 6.64$, p < 0.001; [c]$t = 16.7$, p < 0.001; [d]$t = 4.94$, p < 0.001; [e]$t = 6.19$, p < 0.001; [f]$t = 1.77$, p = 0.041.

Thus, a permanent progestin production may ensure the strong seasonality in lynx (but masks any changes associated with the luteal phase of pregnancy).

In most felids, increased oestradiol excretion is associated with oestrus behaviour or exogenous gonadotropin treatment (Brown et al. 1994). Unfortunately the collection of urine samples from individual females during the period of mating was impossible due to the presence of males, and urine samples collected at that time necessarily included both urine of the female and her mate (Fig. 1). Therefore, we were unable to detect a mating related urinary oestrogen peak. Urine samples collected in the presence of males were characterized by several-fold higher ($t = 35.2$, p < 0.0001) oestrogen concentrations (66.8 ± 5.7 ng E2 per mg creatinine; n = 59) in comparison to pure female urines (means of all females: 5.7 ± 0.2 ng E2 per mg creatinine, n = 611). The elevated oestrogen concentrations in mixed male and female urine were found not only during co-housing immediately before and during the mating period (and thus possibly related to oestrus of the female), but also in cases when the lynx pairs were left together during pregnancy (three females in 2007, Tables 1 and 3). It is very likely that urine from male lynx contains 10-fold higher oestrogen concentrations compared to female urine. Within the Iberian lynx captive breeding the collection of samples to monitor the onset of oestrus in the absence of males was not possible for conservation management reasons, although such information would be valuable for further characterizing and understanding the onset of the Iberian lynx breeding season.

Immune reactive steroid ng per mg creatinine (number of samples)	Outside breeding season Means (±SEM)	Pregnancy (day 1 to 64) Means (±SEM)	Unpaired t-test with Welch correction
Oestrogens (n)	3.8 ± 0.6 (342)	8.6 ± 0.5 (237)	$t = 14.28$, (p < 0.001)
Progesterone (n)	1.8 ± 0.05 (347)	2.4 ± 0.1 (237)	$t = 1.93$, (p > 0.05)
Pregnanediol (n)	1.8 ± 0.3 (26)	3.2 ± 0.4 (110)	$t = 1.61$, (p > 0.05)

Table 2. Determination of steroid concentration in urine samples collected from female Iberian lynx outside the breeding season, either before or after pregnancy, vs during pregnancy. Mean value were calculated for all samples available not discriminating between individuals
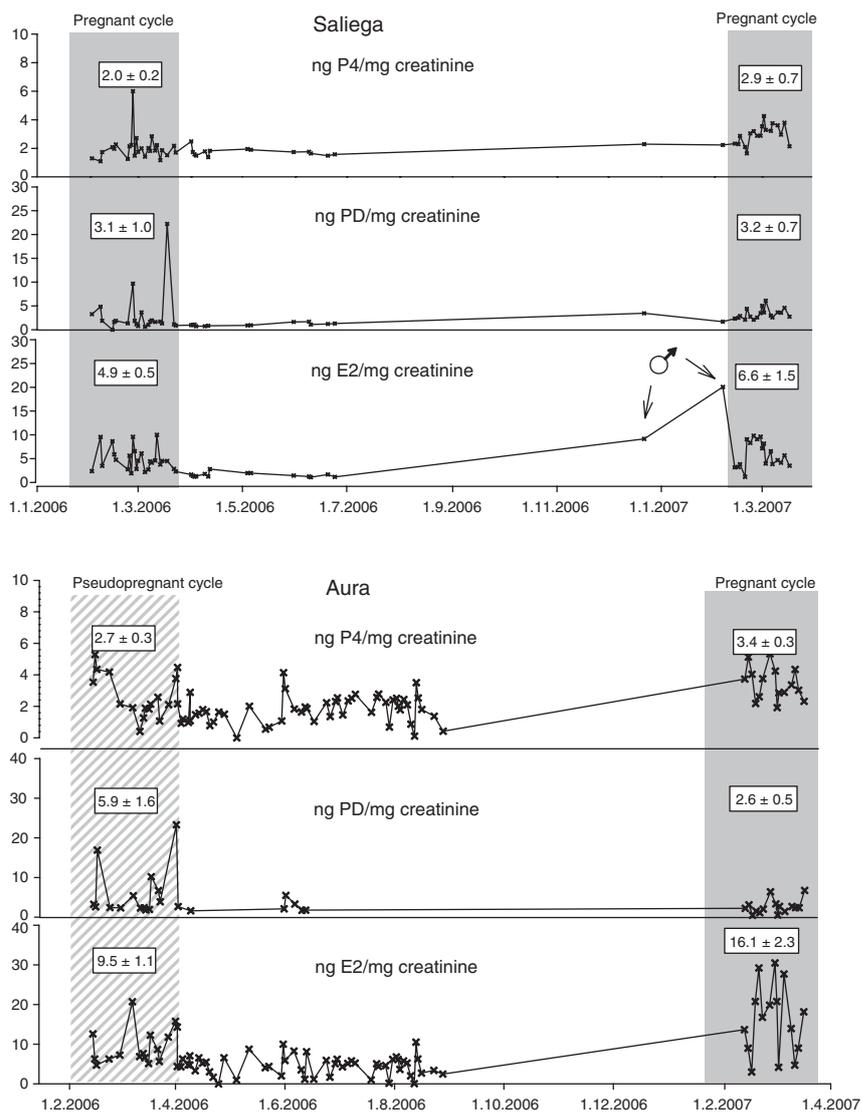
Fig. 1. Determination of progesterone (P4), pregnanediol (PD) and oestradiol (E2) in extracts of urine collected from female Iberian lynx. Exemplarily, seasonal pattern of two adult females: Saliega (upper panel) and Aura (lower panel) are presented. Saliega was pregnant (grey block) in 2006 and 2007, whereas Aura was mated in 2006, but did not conceive (pseudo-pregnancy, striated block). In 2007, she delivered three healthy cubs after 64 days of pregnancy (grey block). Arrows (♂) indicate the presence of a male

Nevertheless, in the absence of males, oestrogens were elevated ($p < 0.001$) during pregnancy (8.6 ± 0.5 ng E2 per creatinine, mean of all animals, Table 2) when compared to samples collected out of the breeding season (3.8 ± 0.2 ng E2 per mg creatinine). Figure 1 also demonstrates the considerable day-to-day variability of urinary oestradiol concentration and emphasizes the necessity of a frequent sample collection for detecting pregnancy to avoid potentially false negative results (E2 below 5 ng per mg creatinine).

In case of the female Artemisa (Table 3), a significant difference ($p < 0.001$) was estimated for urinary oestrogens between her non-breeding (2.0 ± 0.2 ng E2 per mg creatinine) and breeding (1.0 ± 0.7 ng/mg creatinine) season without mating – non-pregnant cycle. Unfortunately, this female was kept together with a male during the 2007 pregnancy and data on urinary oestrogens (46.8 ± 2.7 ng E2 per mg creatinine, Table 3) were not suitable for analysis. Although samples from only one non-mated female were available, the low oestrogens in Artemisa during the 2008 breeding season are an indication for the missing ovulation and CL formation. We conclude that Iberian lynx are induced ovulators as described for many other felid species (Brown and Wildt 1997). In contrast, the direct comparison of pregnant cycles (12.1 ± 1.3 ng E2 per mg creatinine) with a pseudo-pregnant cycle (9.2 ± 1.0 ng E2 per mg creatinine) revealed a further increase of urinary oestrogens caused by implantation (Aura, Table 3, Fig. 1). Thus, if mating occurs, CL formation is evident by elevated oestrogens and ultrasound examination (Göritz et al. 2009). The increase of oestrogens in pregnant vs pseudo-pregnant cycles, might be either explained by shorter half-life of CL in pseudo-pregnant cycle as described for domestic cats (Tsutsui and Stabenfeldt 1993), or oestrogen production by foetal structures (placenta) evident for other species, such as cattle (Hoffmann et al. 1997), pig (Knight 1994) and horse (Möstl 1994).

When urinary oestrogens sampled during the pregnancy were analysed by HPLC (Fig. 2), the results indicated that female Iberian lynx urine contains two major polar immunoreactive oestrogen metabolites eluting between 2 and 4 ml (hydrolysis resistant steroid conjugates) and reasonable amounts of oestrone at 8.6 ml. Oestradiol was eluted at 5.3 ml.
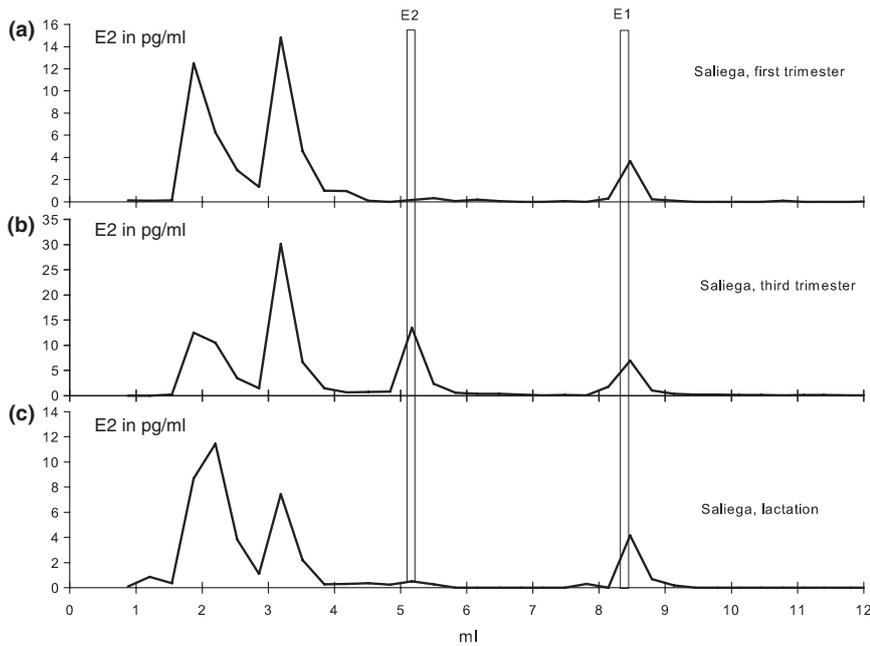
Fig. 2. HPLC separation of urinary extracts from the Iberian lynx female Saliega. The samples from Saliega were obtained during pregnancy (Panels a and b) and the lactation period (Panel c). E2 and E1 indicate the dilution fraction of authentic hormones oestradiol and oestrone

A comparison of HPLC immunogramms (Fig. 2) of urinary extract obtained during the first and third trimester of pregnancy, and lactation revealed that in all the three samples, oestrone was detectable in comparable amounts (8–12% of all immunoreactive metabolites), whereas a distinct amount of E2 (18% of immunoreactive metabolites) was measurable only during late pregnancy. We suggest that the elevation of urinary oestrogens towards the end of pregnancy and the increasing amount of authentic oestradiol are an indication for a placental oestrogen production. Although in the domestic cat a placental oestradiol synthesis was not found, there are some indications from other felids that this might be feasible. In cheetahs, Brown et al. (1994) found a three-fold faecal oestrogen elevation towards the end of pregnancy. Conversely, it is possible that any pregnancy-specific elevation in oestrogens could be the result of increased luteal steroidogenesis and luteal oestrogen secretion.

Conclusion

The present results suggest that seasonal ovarian activity in Iberian lynx can be monitored by urinary oestrogens if samples are collected frequently and in the absences of males. In contrast to urinary progestins, oestrogens reflect CL formation after ovulation. Furthermore, the observed increase of urinary E2 after mating and during late pregnancy suggest either an E2 secretion from the lynx placenta and∕or a pregnancy-specific enhanced luteal secretion of oestrogen, a point for additional study.

Author contributions

All authors contributed to the manuscript: KJ - experimental design, data analysis and manuscript writing: FG - reproductive status assessment: AV - experimental design, behavioural examinations and urine collection; MD - hormone analysis by EIA and HPLC.

Conflicts of interest

The authors have declared no conflicts of interest.

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