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 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-oestrus 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 30-year period, and to start the reintroduction 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 m²), 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

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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 homemade collectors in their enclosures. The collectors were consisted of vertical stainless steel plates (60 x 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 (abor- tion 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 concentra-
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Urine samples were analysed for creatinine levels (Heistermann et al. 1997). Urinary hormone concentra-
tions 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-pregnan-
3,20α-diol-3-gluc-BSA) and 5β-pregnan-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)-
estraatrien-3,17β-diol (17β-oestradiol) 100%, 1,3,5(10)-
estraatrien-3,17-one (oestrone) 114%, 1,3,5(10)-estraatrien-3,17α-diol (17α-oestradiol) 69.2%, 1,3,5(10)-estraatrien-
3-ol-17-on (estratrien) 5.9% and < 0.1% for 1,3,5(10)-
estraatrien-3,16α,17β-triol (estril), ethylestradiol, oestro-
nesulphate, 19-nortestosterone, progesterone, 5α-preg-
nandiol 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

<table>
<thead>
<tr>
<th>Status</th>
<th>Number of females (n = number of samples)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-pregnant</td>
</tr>
<tr>
<td>Mating</td>
<td>Breeding</td>
</tr>
<tr>
<td>Parturition</td>
<td>No mating</td>
</tr>
<tr>
<td>2006</td>
<td>–</td>
</tr>
<tr>
<td>2007</td>
<td>6 (n = 143)</td>
</tr>
<tr>
<td>2008</td>
<td>1 (n = 20)</td>
</tr>
<tr>
<td>Σ</td>
<td>1 (n = 20)</td>
</tr>
</tbody>
</table>

*The pseudo-pregnancy might be the result of either an infertile mating or early embryonic death.

bThree females were kept together with their mates during pregnancy.

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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 acetonitrile : 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-3,17β-diol (17β-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 pregnancy 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 pregnancy pattern described for feral 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 feral (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 through-out most of the year (Kvam 1990, Göritz et al. 2009).

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

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 values were calculated for all samples available not discriminating between individuals.
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.

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A comparison of HPLC immunograms (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 absence 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.

Acknowledgements
The authors thank all the staff (F. Martínez, T. Rivas, J. Bergara, T. Vázquez, J. Pardo, E. Vázquez, and J. López) and volunteers at the El Acebuche breeding centre for their every day engagement within the Iberian Lynx Conservation Breeding Programme. The authors also thank A. Frank, K. Paschmionka and M. Rohleder (IZW) for their excellent technical assistance. We appreciate the support of the Environmental Council of the Junta of Andalusia and the Spanish Ministry of the Environment.

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.

References

Submitted: 30 June 2008

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