PGFM (13,14-dihydro-15-keto-PGF$_{2\alpha}$) in pregnant and pseudo-pregnant Iberian lynx: A new noninvasive pregnancy marker for felid species

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Abstract

In mammals, uterine and placental prostaglandin F$_{2\alpha}$ is involved in the regulation of reproduction-related processes such as embryonic development, initiation of parturition, and resumption of ovarian activity. Prostaglandin F$_{2\alpha}$ (PGF$_{2\alpha}$) is rapidly metabolized to its plasma metabolite PGFM (13,14-dihydro-15-keto-PGF$_{2\alpha}$), which has also been detected in urine. Therefore, the current study aimed to develop and validate an efficient, quick, and inexpensive enzyme immunoassay (EIA) for PGFM estimation in urine of the Iberian lynx (*Lynx pardinus*) for pregnancy monitoring and for differentiation between pregnancy and pseudo-pregnancy. Urine samples collected from captive Iberian lynx (11 pregnant and 4 pseudo-pregnant cycles) were subjected directly to a PGFM EIA. The assay was validated for parallelism, precision, and stability of urinary PGFM. In addition, high-performance liquid chromatography (HPLC) immunograms and liquid chromatography–mass spectrometry (LCMS) were performed to identify PGFM within urine samples. Urinary PGFM levels before mating and after parturition were about 1.5 ng/mL. After Day 20 postmating, both pregnant and pseudo-pregnant females showed slight increase of hormone levels; in pseudo-pregnant females, this elevation did not exceed 7 ng/mL. A significant increase in pregnant females was observed after Day 45 postmating; urinary PGFM increased from 10 ng/mL at Day 45 toward a peak of 46.0 ± 19.3 ng/mL around parturition. First results show that PGFM is detectable in feces as well and follows similar courses as shown for urine. In conclusion, the presented and validated PGFM assay is an easy and reliable method for noninvasive pregnancy diagnosis in the Iberian lynx (and probably other felids) if applied approximately 20 d prior parturition in pure urine or fecal extracts. High PGFM levels in urine or fecal samples may allow a pregnancy diagnosis without knowledge of mating time, making the PGFM test applicable to free-ranging animals.
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1. Introduction

The Iberian lynx (*Lynx pardinus*) is the only cat species worldwide listed as Critically Endangered in the IUCN/SSC Red List (http://www.redlist.org/). Today, the Iberian lynx remains in only two isolated populations located in the region of Andalusia and Castilla-La Mancha, Spain. The actual population size comprises approximately 200 animals in the wild. At the beginning of this century, the establishment of an ex situ conservation breeding program was recognized as an essential part of the coordinated action plan to conserve

this highly endangered species [1]. In situ efforts are mainly directed toward habitat preservation and restoration, decreasing non-natural causes of mortality and raising social awareness for the plight of the Iberian lynx. In addition, the captive breeding program plans to maintain a breeding stock of 60 lynxes—a number that will allow for the maintenance of 85% of the lynxes' genetic diversity during a 30-yr period with reintroductions commencing in 2010.

After the first litter was born in captivity in 2005, the captive population of Iberian lynx increased steadily and is 2 yr ahead of growth projections [1], with already 76 captive animals distributed between four breeding centers. Despite the recent advances toward Iberian lynx recovery, breeding Iberian lynxes in captivity is not an easy endeavor, as there are often problems related to pregnancy and parturition. In particular, primiparous females tend to abort or abandon their first litter. Therefore, a reliable pregnancy diagnosis is of particular importance as a management tool for the captive breeding program of Iberian lynx.

In wild animals, repeated blood sampling for reproductive hormone analysis is avoided because of the high risk of abortion induced by stress and/or anesthesia. Therefore, a pregnancy diagnosis that avoids these conditions by sampling urine or feces is preferred. Over the past two decades, so-called noninvasive techniques for urinary and fecal steroid analysis (estrogen, gestagen, androgen, and glucocorticoid metabolites) have been developed and have also been successfully applied for pregnancy diagnosis in several felid species [2–4]. In the lynx, however, it is impossible to use fecal and urinary gestagen and estrogen metabolite analyses as a reliable index of pregnancy or differentiation between pregnancy and pseudo-pregnancy. Both hormones rise during pregnancy, decrease toward parturition, and increase again during the lactation period [5–7]. As an alternative, the hormone relaxin was considered as a specific pregnancy-related signal [8], because relaxin is mainly produced by the cat placenta [9]. Although relaxin can be determined in the urine of pregnant domestic cats, it requires substantial sample concentration by ultrafiltration in nondomestic felids [10,11] and, in particular, Iberian lynx urine [12].

Another specific placental signal is prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). In many species, uterine and placental $PGF_{2\alpha}$ is involved in the regulation of reproductive and pregnancy-related processes such as embryonic development, initiation of parturition, and resumption of ovarian activity. In domestic ruminants, uterine tissue is a primary source of $PGF_{2\alpha}$, and secretion of uterine $PGF_{2\alpha}$ is a key regulator for the cyclical regression of the corpus luteum [13–15].

Prostaglandin $F_{2\alpha}$ is metabolized to PGFM (13,14-dihydro-15-keto-$PGF_{2\alpha}$) during the first passage through the lungs [16]. PGFM has a longer half-life in peripheral circulation than its original compound [17] and has been applied as a useful analytical marker of $PGF_{2\alpha}$ in carnivores [18,19]. Serum PGFM analyses revealed different patterns between pregnant and nonpregnant (diestrus) bitches [19]. Furthermore, Tsutsui and Stabenfeld described the $PGF_{2\alpha}$ course during pregnancy of domestic cats, although they never published the data in detail. According to them, the $PGF_{2\alpha}$ production by the fetoplacental unit and endometrium seems to begin at about Day 30 of gestation, reaches a plateau at Day 45, with a large increase observed just before delivery and subsequent abrupt postpartum fall [20].

Recently, PGFM was detected in urine of several species, although PGFM is described as a serum metabolite and undergoes further metabolism before renal clearance [21]. Administrations of tritium-labeled 8-iso-$PGF_{2\alpha}$ in rabbits revealed that about 80% of the total radioactivity was found in urine within 4 h. The major urinary metabolite was α-tetranor-15-keto-13,14-dihydro-8-iso-$PGF_{2\alpha}$ (tetranor-PGFM; Fig. 1) [22,23]. Tetranor-PGFM was also identified as the predominant urinary $PGF_{2\alpha}$ metabolite in cows; however, distinct amounts of unmetabolized $PGF_{2\alpha}$ and PGFM were also found [24].

The objective of the current study was the identification of the dominant $PGF_{2\alpha}$ metabolites in urine of the Iberian lynx and the development and validation of an enzyme immunoassay for monitoring of pregnancy- and/or parturition-related patterns of $PGF_{2\alpha}$ secretion in the Iberian lynx. Therefore, we performed high-performance liquid chromatography (HPLC) analysis to separate the prostaglandin metabolites, followed by identification of urinary PGF metabolites by means of liquid chromatography–mass spectrometry (LCMS) and an enzyme immunoassay (EIA) against PGFM.

The final goal was to establish a reliable and noninvasive method for pregnancy diagnosis including parturition monitoring in this highly endangered felid species to be used in the ex situ breeding program. An additional objective was to assess the suitability of putative fecal $PGF_{2\alpha}$ metabolites for pregnancy diagnosis, which might be applicable for free-ranging lynxes.
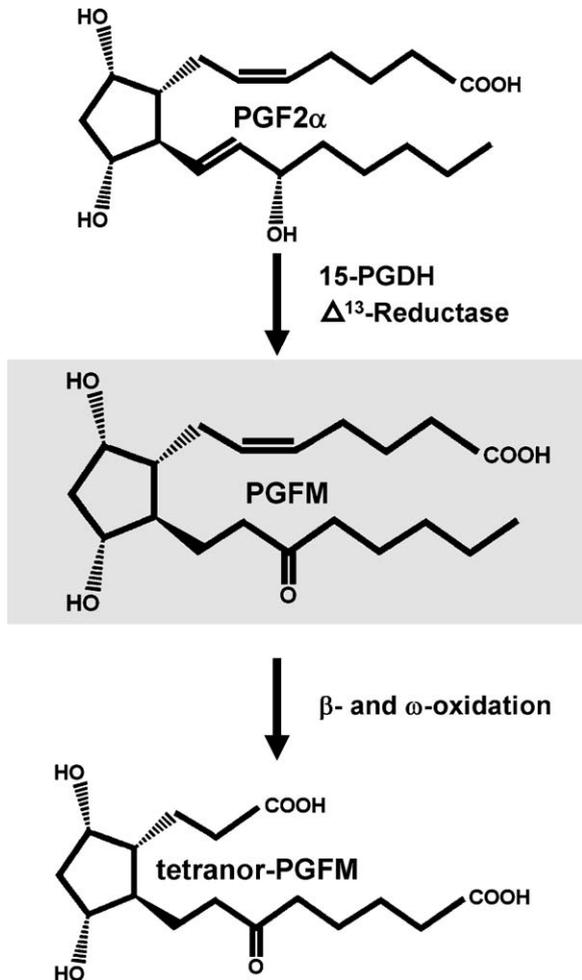
Fig. 1. Schematic diagram of PGF$_{2\alpha}$ metabolism via 15-keto-dihydro-PGF$_{2\alpha}$ (PGFM) to the urinary component tetranor-PGFM (according to Basu [21]).

2. Materials and methods

2.1. Animals

The Iberian lynx Captive Breeding Center "El Acebuche" (ILCBC), in southern Spain, manages captive Iberian lynxes. The captive population currently consists of 76 animals, more than half of them born in captivity. Animals are generally kept in separate enclosures and placed together 1 to 2 mo prior to breeding season up until 2 wk prior to the estimated whelping time. All adult females are allowed to mate, and the deliveries of cubs or abortions are ultimate indications of pregnancy. Pseudo-pregnancies occur if conception failed after successful mating.

2.2. Sampling of urine and feces

Urine was collected from captive lynxes by placing homemade collectors in their enclosures as described previously [12]. In brief, urine collectors 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 collection cup. Lynxes used these plates for urine-marking.

We used urine samples (variable volumes) that were collected throughout the breeding seasons in the years 2006 to 2009. Sampling started several days to weeks before the expected mating time and ended after parturition. Overall, urine samples from 11 pregnant and 5 pseudo-pregnant cycles were available; two pregnant females were sampled up to 5 mo after parturition. Frozen samples were shipped to the Leibniz Institute for Zoo and Wildlife Research (IZW) and stored at −20 °C until assayed. For the EIA procedure, urine was thawed, vortexed, centrifuged for 15 min at 5 °C and 3000 × g, and an aliquot of the supernatant was diluted 1:10 with assay buffer prior to analysis.

In addition, fecal samples of two female lynx (Saliega and Aura) were collected frequently over the whole breeding season 2006 until Day 80 postmating.

2.3. Sample processing

For HPLC immunograms and LCMS analyses, urine samples (0.5 mL) were acidified (pH 3) with 1 N HCL, then 2.5 mL diethylether was added and the mixture was agitated for 20 min. Subsequently, the mixture was frozen at −80 °C, the ether phase was removed, evaporated under a gentle stream of nitrogen, and the residue was dissolved in 150 μL 40% methanol.

Fecal samples were processed as described before [5]. In brief, wet fecal samples (0.5 g) were extracted with 4.5 mL 90% methanol by shaking for 30 min and centrifugation for 15 min at 3000 × g. Afterwards, the supernatant was transferred into a new tube and diluted 1:1 with water. Aliquots of the fecal extracts were subjected to the PGFM EIA (see later).

2.4. Stability of PGFM in urine and fecal samples

To investigate the stability of PGFM in lynx urine, four samples (one pure urine; three diluted 1:10 urine samples, one with the addition of 4 ng/mL PGFM) were incubated over 10 d at 37 °C. From all samples, 70-μL aliquots were taken twice daily and frozen immediately at −20 °C.

To test the stability of PGFM in feces, 0.5 g aliquots of one fecal sample were taken off and incubated over 0, 1, 2, and 3 d at 37 °C, respectively. Incubation was stopped by freezing at –20 °C. Finally, all samples were extracted as described before and analyzed within one assay.

2.5. Antibody and label

PGFM (9α,11α-dihydroxy-15-oxo-prost-5-en-1-oic acid) and tetranor-PGFM (9α,11α-dihydroxy-15-oxo-2,3,4,5-tetranor-prostan-1,20-dioic acid) were obtained from Cayman Chemicals (Cayman Europe, Tallinn, Estonia). PGF$_{2\alpha}$ (9α,11α,15S-trihydroxy-[8β]-prosta-5Z,13E-dien-1-oic acid) was from Sigma (Sigma Chemie GmbH, Deisenhofen, Germany). The assays were carried out with in-house microtiter plate EIA procedures using a PGFM antibody generated in rabbits against 9α,11α-dihydroxy-15-oxo-prost-5-en-1-oic acid-BSA as described by Schlegel et al. [25]. The cross-reactivity of the PGFM antibody against tetranor-PGFM and PGF$_{2\alpha}$ were < 0.1%.

The PGFM-HRP (horse radish peroxidase) conjugate was prepared using the mixed anhydride reaction including *N,N*-dimethylformamide (Aldrich, Tauf-kirchen, Germany) and 4-methylmorpholine (Merck, Darmstadt, Germany) according to Dawson et al. [26]. After the addition of 5 mg NaHCO$_3$, the product was dialyzed (dialysis sack; Servapor No. 44145, SERVA, Heidelberg, Germany) with chromatography buffer overnight and centrifuged at 5000 × g and 4 °C in Centricon-10 centrifugal concentrators (Amicon, Bev-erly, MA, USA). Thereafter, the tubes were washed seven times with 1 mL chromatography buffer, and the label was dissolved in 1 mL assay buffer (1% BSA). The PGFM-HRP conjugate obtained was dissolved in 1 mL assay buffer (1% BSA), immediately tested for its titer, and stored at –80 °C.

A two-dimensional titer determination for optimum dilution of each PGFM-HRP conjugate and PGFM antiserum was carried out. Antibody dilutions ranging from 1:500 to 1:32,000, and PGFM-HRP dilutions of 1:1000 to 1:23,000 were tested. The antibody titer of 1:2000 and the PGFM-HRP conjugate titer of 1:12,000 were found to be optimum in assay buffer, and an optical density at 450 nm of around 1.2 was achieved by using these titers.

The PGFM calibration standards were prepared by dilution with assay buffer and ranged from 0.4 to 200 pg per well. The sensitivity of the assay was defined as 2 standard deviations from the signal given by the zero blank. Precision and reproducibility were calculated from multiple measurements of pooled samples.

2.6. Enzyme immunoassay procedure

Microtiter plates were coated with goat anti-rabbit IgG (4 °C, overnight), blocked with BSA in assay buffer, and after 30 min at 22 °C, the excess of blocking buffer was decanted and the plates were stored at –22 °C. Prior to use, the plates were washed once with washing solution (0.05% Tween 80) using an automated microtiter plate washer (SLT 96PW; Tecan, Crailsheim, Germany). Duplicates of 20 μL urine sample or PGFM standards prepared in assay buffer ranging from 0.4 to 200 pg/20 μL were simultaneously pipetted into respective wells along with 100 μL PGFM-HRP conjugate diluted (1:12,000) in assay buffer (50 mM Na$_2$HPO$_4$/Na$_2$HPO$_4$, 0.15 M NaCl, 0.1% BSA, pH 7.4) with the aid of diluter dispenser. Then, 100 μL PGFM-specific antiserum diluted in assay buffer (1:2000) was added immediately to all wells except blank by a repeat dispenser. The plates were incubated overnight at 4 °C.

The plates were washed four times with washing solution and incubated further in the dark for 40 min after addition of 150 μL substrate solution per well (1.2 mM H$_2$O$_2$, 0.4 mM 3,3′,5,5′-tetramethylbenzidine in 10 mM sodium acetate, pH 5.5). The reaction was stopped by the addition of 50 μL 4 N H$_2$SO$_4$, and the intensity of color was measured at 450 nm with a 12-channel microtiter plate reader (Infinite M 200; Tecan), and hormone concentrations were calculated using the Magellan software (Tecan).

2.7. HPLC immunogram

A Dionex system equipped with a quaternary pump (P580), an ASI autosampler, and a column oven (STH 585) was used for chromatographic separation (Dionex GmbH, Idstein, Germany). To characterize the urinary PGF$_{2\alpha}$ metabolites, 100-μL portions of the urine extract were subjected to a reversed-phase Ultrasep ES100/RP-18/6 μm HPLC column (150 mm × 4 mm; Sepserv, Berlin, Germany). The mobile phase consisted of a mix of water:acetic acid 100:0.02 (v:v) and acetonitrile:acetic acid 100:0.02 (v:v) delivered according to the following programmed gradient: 80:20 (v:v) at 0 min, linear gradient to 70:30 (v:v) at 5 min, linear gradient to 30:70 (v:v) at 15 min, maintain this gradient until 20 min, and return to the initial condition of 80:20 (v:v) at 21 min in preparation for the next injection. The flow rate was set at 1 mL/min, and fractions of 0.33 mL were collected at 20-sec intervals over a period of 20 min using a FRAC-200 fraction collector (Pharmacia Biotech, Freiburg, Germany). The elution positions of authentic PGFM and tetranor-PGFM on this column

had been determined in separate HPLC runs after injection of 500 ng PGFM and tetranor-PGFM. Aliquots of 10 μL of each HPLC fraction were subjected to LCMS analyses and monitored for PGFM and tetranor-PGFM as described later. To generate the HPLC immunograms, the remaining HPLC fractions were lyophilized, reconstituted in 100 μL 40% methanol, and subjected to the PGFM EIA.

2.8. Liquid chromatography–mass spectrometry

A Shimadzu separation module (CBM-20A), equipped with two quaternary pumps (LC-20AD), an autosampler (SIL-20A), a degasser (DGU-20AS), and a column oven (CTO-10AS VP) was used for chromatographic separation (Shimadzu Europe, Duisburg, Germany). Separation was achieved at 30 °C on a Gemini C18 column (50 mm × 4 mm, 3 μm; Phenomenex, Torrance, CA, USA). The injection volume was 20 μL. The eluent was composed of water:acetonitrile:formic acid (80:20:0.1, v:v:v) with a flow rate set to 0.5 mL/min.

The mass spectrometry was carried out on an API3200 QTrap LC/MS/MS system with a Turbolon Spray ESI source (Applied Biosystems/MDS-SCIEX, Palo Alto, CA, USA) using multiple reaction monitoring (MRM) and equipped with a Z spray electrospray ionization (ESI) interface. A NGM-11 nitrogen generator (CMC Instruments, Eschborn, Germany) was used to generate high-purity nitrogen as nebulizer, heater, curtain, and collision gases. Source temperature was 500 °C. The electric potential applied on the capillary was –4.5 kV, and the sample cone voltage was set individually for each compound. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) experiments were performed at a pressure of 3.9×10^{-3} mbar and a collision energy setting adapted for each compound. Prostaglandins were detected using MRM of the two most abundant product ions per analyte. Time between transitions was 100 and 300 msec.

Standards of 1 or 5 ng/μL were infused (5 μL/min) into the ESI source to optimize MS/MS parameters. The product ion scan mode (range, 300 to 360) in the negative-ion mode was used for spectral analysis to select parent ions. The product ion mass spectra were obtained by choosing the molecular anions as the precursor ions and scanning MS2 (second mass spectrometer) from m/z 100 to 400 with a scan time of 1 sec. Optimization of mass spectrometer parameters was carried out by injecting 10 μL of standards (1 or 5 ng/μL) into a carrier stream of 500 μL/min acetonitrile/

water/formic acid (50/50/0.1, vol/vol/vol) generated by the HPLC pump using the automatic optimizing for the analyte (FIA) function of the instrument.

In the selected reaction monitoring mode, the instrument monitored the m/z 353.1 to m/z 195.2, m/z 353.1 to m/z 182.9, and m/z 353.1 to m/z 113.3 transitions for PGFM, m/z 311.1 to m/z 293.1, m/z 311.1 to m/z 121.3, and m/z 311.1 to m/z 109.2 transitions for tetranor-PGFM, and m/z 353.2 to m/z 309.4, m/z 353.2 to m/z 309.2, and m/z 353.2 to m/z 193.4 for $PGF_{2\alpha}$.

To confirm urinary PGFM and identify further metabolites binding to the PGFM antibody, 10 μL of the immunoreactive HPLC fractions were subjected to LCMS analysis.

2.9. Statistical analyses

Data are presented as means ± SD. Comparisons of mean values were performed by Student's paired *t*-test after testing for normality. Spearman correlation was applied to calculate correlation coefficient. 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., La Jolla, CA, USA).

3. Results

3.1. Test criteria of the PGFM assay

The detection limit of the assay was 0.4 pg per well. To demonstrate parallelism, two urine samples with medium concentration of endogenous PGFM were serially diluted with assay buffer and measured against the standard curve prepared in assay buffer. Displacement curves parallel to those of the PGFM standard were obtained (Fig. 2). The intra-assay and interassay coefficients of variation (CVs) were determined by using one urine sample (0.69 ng/mL) and buffer pools containing low (0.15 ng/mL) and high (4 ng/mL) concentrations of PGFM. The results are summarized in Table 1.

The recovery of PGFM was determined by measuring PGFM previously added to diluted lynx urine with low endogenous PGFM concentration (0.136 ng/mL). Additions of 0.3, 0.6, 1.25, 2.5, and 5.0 ng/mL PGFM were recovered as 0.33, 0.53, 1.14, 2.87, and 6.65 ng/mL, respectively. The correlation coefficient of added and measured hormone concentration was significant (r = 0.997; P < 0.001). The overall recovery of added PGFM was 119%.
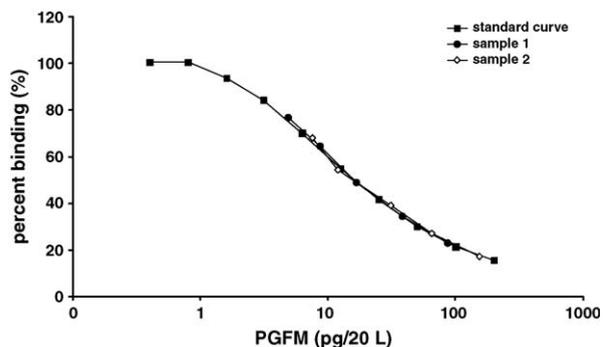
Fig. 2. Parallelism test for PGFM was performed with serially diluted Iberian lynx urine samples. Increasing volumes of urine (15, 31.3, 62.5, 125, 250 μL) were subjected directly to the PGFM EIA together with standards for PGFM ranging from 0.02 to 10 ng/mL assay buffer. The identities of curves indicate a high degree of parallelism in case of urine samples (no matrix effects).

3.2. Stability of PGFM in lynx urine

To investigate the stability of PGFM in samples of lynx urine, samples containing 1.8, 1.85, 3.3, and 3.7 ng/mL were incubated at 37 °C up to 10 d and subjected to measurements. The results revealed no significant decrease of PGFM within a 10-d storage period, indicating that PGFM in urine is stable even at elevated temperature for at least 10 d. Compared with the initial concentrations, the recoveries after 10 d of storage were 1.5 ng/mL (83%), 2.95 ng/mL (159%), 2.90 ng/mL (88%), and 4.10 ng/mL (111%). The overall recovery was 110%.

3.3. Identification of PGFM in urine of the Iberian lynx

For separation and characterization of PGFM and possible additional $PGF_{2\alpha}$ metabolites, the elution positions of prostaglandin standards were determined in separate HPLC runs (injection of 500 ng of $PGF_{2\alpha}$, PGFM, and tetranor-PGFM) followed by LCMS analysis of each HPLC fraction. The elution positions of $PGF_{2\alpha}$, PGFM, and tetranor-PGFM were in Fractions
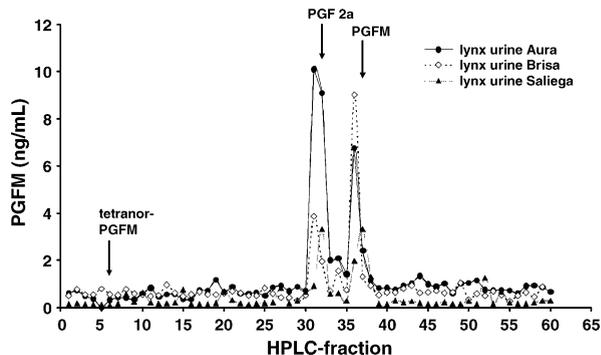

Fig. 3. HPLC immunogram of $PGF_{2\alpha}$ metabolites in urine samples from three pregnant females. Urine samples were acidified, extracted with diethylether, and the extracts were subjected to HPLC separation. PGFM immunoreactivity within the HPLC fractions was determined with the PGFM EIA. Fractions 6, 32, and 37 were used for LCMS characterization of the unknown metabolite and for PGFM identification, respectively. Arrows indicate elution position of $PGF_{2\alpha}$ (Fraction 32), PGFM (Fraction 37), and tetranor-PGFM (Fraction 6).

32, 37, and 6, respectively. The PGFM position was confirmed in Fraction 37 by applying PGFM EIA.

High-performance liquid chromatography separation of urinary extracts was followed by enzyme immune analysis of each fraction and revealed two immunoreactive peaks, one of which was coincident with PGFM elution position at Fraction 37 (Fig. 3). The identity of PGFM in Fraction 37 was confirmed in LCMS by its specific transitions m/z 353.1 to m/z 195.2, m/z 353.1 to m/z 182.9, and m/z 353.1 to m/z 113.3. In addition, LCMS analysis of Fraction 6 (elution position of tetranor-PGFM) confirmed the existence of this metabolite in lynx urine, whereas Fraction 32 did not reveal any specific $PGF_{2\alpha}$ signals. The tetranor-PGFM was clearly detectable based on its specific transitions m/z 311.1 to m/z 293.1, m/z 311.1 to m/z 121.3, and m/z 311.1 to m/z 109.2. The LCMS urine analysis was repeated in samples of three animals.

3.4. PGFM profiles in pregnancy and pseudo-pregnancy of Iberian lynxes

Urinary PGFM concentrations were estimated in samples collected from 20 d prior to mating and ending several weeks after parturition. The temporal changes in the mean levels of PGFM from 11 pregnancies are presented in Figure 4. Urinary PGFM levels (mean ± SD) were basal (1.64 ± 0.85 ng/mL) prior to mating and remained there until Day 22 postmating followed by a gradual elevation to levels of about 7 ng/mL. Day 30 marked the beginning of an exponential increase culminating in peak levels of 46.0 ± 19.3 ng/mL at Day 65 postmating. The highest concentration of
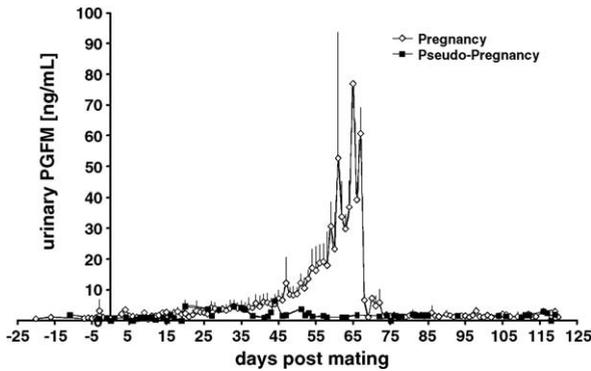
Table 1
Determination of intra-assay and inter-assay CVs of the PGFM assay by using urine samples of Iberian lynx and two buffer pools. Both buffer pools were included in every PGFM assay as controls.

Quality control	Mean, ng/mL	Intra-assay CV, % (n = 10)	Interassay CV, % (n = 45)
Urine sample	0.69	6.3	–
Buffer pool 1	0.15	10.4	14.3
Buffer pool 2	4.00	7.6	7.4

Fig. 4. Mean ($\pm$SD) $PGF_{2\alpha}$ metabolite (PGFM) concentrations in urine samples obtained from pregnant (n = 11) and pseudo-pregnant (n = 4) Iberian lynxes (data points are represented by 1 to 4 samples). A significant divergence between the hormone levels occurs between Days 45 and 67 after copulation.

100 ng/mL was measured in one female at Day 61, which contributes to the large standard deviation on that day. In this animal, a collection gap from Day 62 to 75 prevents PGFM analyses during the peripartum period. Postpartum PGFM concentrations immediately decreased to a base level (1.66 ± 0.54 ng/mL) and remained low over the rest of the sampling period (Fig. 4).

By contrast, the four pseudo-pregnant females deviated from the pregnancy-related course after Day 36, although urinary PGFM concentrations showed a transient increase to 7 ng/mL between Days 20 and 45 (Fig. 4). Due to the low sample numbers, several days were represented by just single measurements. This makes a statistical comparison with pregnancy samples impossible. After Day 43, however, the course of the

nonpregnant females separates from that of the pregnant females, when pseudo-pregnant values return to the baseline level ranges (Fig. 4).

Altogether, there is a significant increase of the urinary PGFM level in pregnancy determined between Days 45 and 67 compared with that of the period before Day 45 and after Day 67 and for all values measured in nonpregnant females.

Fig. 5 represents the urinary PGFM course from one female over a period of 4 successive years including all mating periods but with interruptions during the nonbreeding season. In 2006 this female was mated but did not deliver cubs. The following 3 yr she was pregnant and gave birth to healthy cubs. In 2008, we were unable to obtain urine samples between Days 56 and 68, thus the period of highest elevation of PGFM is missing. Interestingly, the nonpregnant cycle of 2006 was characterized by an increase of PGFM between Days 31 and 45 with values up to 10 ng/mL. It might also be possible that the pregnancy was interrupted in this case.

3.5. Detection of PGFM in feces of the Iberian lynx

Urinary and fecal PGFM concentrations determined in samples collected from the same animal are shown in Figure 6. Analyses of PGFM in both matrices of the pregnant female revealed similar courses leading to peak levels in urine and feces of 45 ng/mL and 2.77 μg/g on 63 and 64 d of pregnancy, respectively (Fig. 6A). In feces, however, an increase above baseline levels seems to occur earlier on Day 35 of pregnancy. In the nonpregnant profile, urinary and fecal PGFM showed a weak increase between Days 30 and 44 postmating with
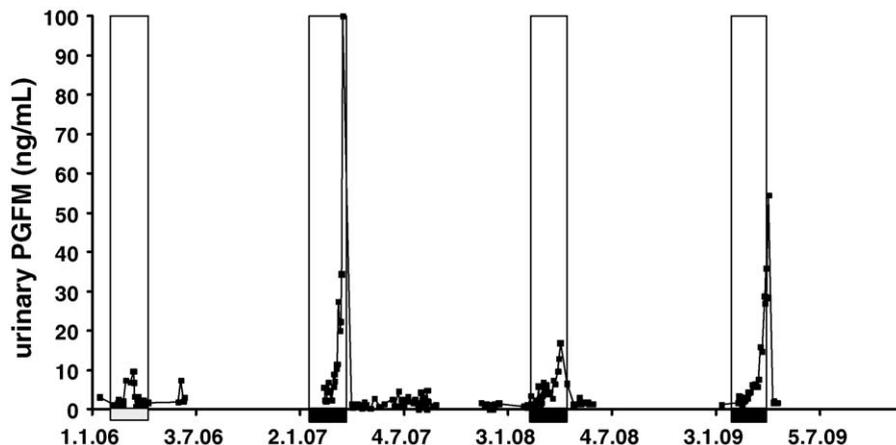

Fig. 5. Determination of PGFM in urine collected from one female Iberian lynx (Aura) during four consecutive years. The animal was mated every year. The rectangles label the breeding period from Day 1 to Day 65 postmating. In 2006, the female was mated but did not conceive (pseudo-pregnancy, gray block), whereas the animal was pregnant and delivered healthy cubs in the following years (black block).
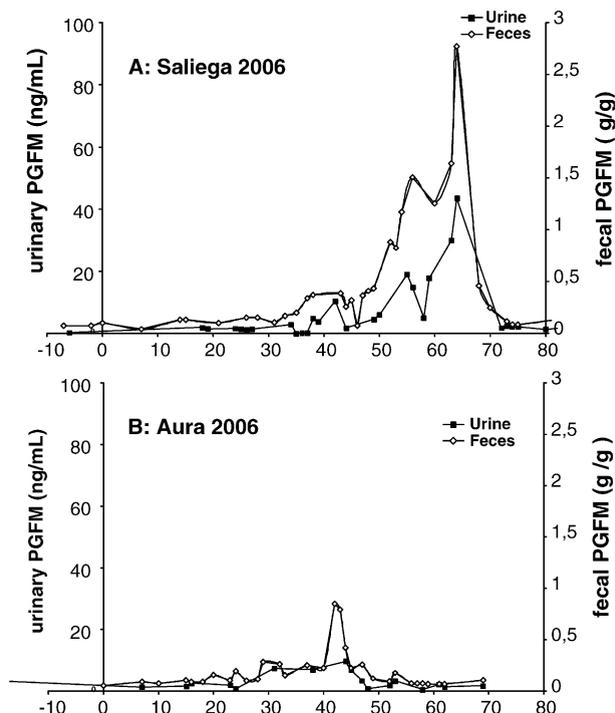
Fig. 6. Course of urinary and fecal PGFM levels in samples collected from (A) one pregnant female Iberian lynx (Saliega; 2006) and (B) one pseudo-pregnant female (Aura; 2006).

peak levels of 9.6 ng/mL urine and 0.85 µg/g feces, respectively (Fig. 6B).

For fecal and urinary samples obtained from the same day, a highly significant correlation between PGFM content in both substrates was shown ($r^2 = 0.927$, $P < 0.001$).

4. Discussion

Our results demonstrate that the $PGF_{2\alpha}$ metabolite PGFM is a reliable indicator of pregnancy in the Iberian lynx if determined by a simple EIA in native urine samples after Day 45 postmating. We were able to confirm the identity of PGFM within lynx urine by LCMS analysis and obtained the first indication that the same metabolite is present and stable in fecal samples as well.

Urinary PGFM was found at peak concentrations up to 100 ng/mL in the peripartum period. These concentrations are comparable with those that had been measured in the urine of humans (5.6 to 51.0 ng/mL) [27], whereas our basal levels between 1 and 2 ng/mL are similar to those reported for cattle [24]. PGFM analysis does not require sample pretreatment except for a simple dilution step with assay buffer. In addition, incubation of urine at 37 °C over 10 d does not affect

urinary PGFM concentrations making urine preservation and sample freezing unnecessary if contemporary analyses are intended. The high sensitivity of the PGFM EIA procedure (0.4 pg per well, which corresponds with 0.02 ng/mL when undiluted urine is used) is sufficient to measure low physiologic baseline concentrations of PGFM during the nonreproductive periods as well as distinctly higher PGFM levels during the peripartum period (when a 1:10 dilution is necessary). Those different urine dilutions did not affect measurements due to an extraordinarily high degree of parallelism excluding any effect of the urine matrix. Altogether, the method for the determination in urine was proved to have excellent precision.

The HPLC immunograms of extracted urine samples depicts PGFM in Fraction 37 (verified based on the PGFM standard when applying the same HPLC conditions) and a second immunoreactivity in Fractions 32 and 33, whose identity is still unknown. PGFM identity was further confirmed by LCMS analysis based on the specific m/z transitions of PGFM in the HPLC fractions obtained from HPLC separation as well as in the fraction containing endogenous PGFM, which was processed for LCMS identification. The substance in Fraction 32/33 elutes at the position similar to $PGF_{2\alpha}$. Even if $PGF_{2\alpha}$ has been reported to occur in human

urine [28], our PGFM antibody does not show any cross-reactivity toward $PGF_{2\alpha}$. Thus, we assume that the second immunoreactivity might belong to a PGFM isomer. For $PGF_{2\alpha}$, 10 different isomers have been described [29]. They are separable on reversed-phase columns [29], and for some isomers identical m/z transitions were shown (e.g., m/z 353/193: $PGF_{2\alpha}$ [30]; 8-epi-$PGF_{2\alpha}$ [31]; 11β-$PGF_{2\alpha}$ [32]). Therefore, the existence of PGFM isomers with significant antibody binding capacities are conceivable. By contrast, we do not expect a cross-reactivity with an eicosanoid metabolite from an eicosanoid class that deviates in 5-carbon ring structure (e.g., PGEM [33]). It has been demonstrated that antibodies generated according to the method of Schlegel et al. are very specific with no significant cross-reactivities toward other classes of eicosanoids [25,34]. Hence, our antibody was unable to trace urinary tetranor-PGFM, whereas this metabolite could be clearly detected in lynx urine based on LCMS analyses.

By all means, the EIA permits the simultaneous analyses of PGFM and its unknown isomer in lynx urine. Pregnancy and estrous cycle related serum PGFM profiles have been described before in several species such as bitch [19], buffalo [35], and rabbit [36]. Even for domestic cats, Tsutsui and Stabenfeldt [20] described quite a similar pregnancy-related course of $PGF_{2\alpha}$ as we found for urinary PGFM in the Iberian lynx. PGFM has been identified as a urinary metabolite in several species [21]. In the Iberian lynx, the pregnancy duration ranges between 64 and 65 d. Based on plasma PGFM profiles in cats and pregnant bitches, we expected an increase in PGFM concentrations prior to parturition in the last third of pregnancy [20,37].

Our analyses revealed PGFM levels of pregnant female lynxes that are characterized by an increase above 10 ng/mL after Day 45 postmating, a culmination in a distinct peak of approximately 46.0 ± 19.3 ng/mL around parturition at Day 61 to 65, and a subsequent fall to baseline after delivery. Mean baseline hormone levels before Day 20 postmating and after parturition were 1.5 and 1.66 ng/mL, respectively. Both pregnant and pseudo-pregnant females showed slight increase after Day 20 to 22 until Day 45 postmating. In pseudo-pregnant females, this elevation was not higher than 7 ng/mL and decreased subsequently to remain at baseline until the next breeding season. PGFM concentrations in pseudo-pregnant females never exceeded 7 ng/mL. The only exception was the female Aura in 2006, when she mated but no parturition was observed. According to our previous classification, Aura was categorized as pseudo-pregnant in 2006, but

her higher PGFM levels ($\sim$10 ng/mL) around Day 45 might be an indication of premature pregnancy termination. Premature birth and abortion is quite often observed in primiparous Iberian lynx females at the breeding program. Within primiparous females that copulated (n = 13), no parturition was observed in six females, and four terminated pregnancy prematurely. Witness Relaxin pregnancy test [12] was used for diagnostic purposes between Days 26 and 46 in nine of these females, and all were diagnosed pregnant, but only six (67%) delivered a litter. This provides a strong indication that an ongoing pregnancy in primiparous females is quite often terminated during the last trimester. A pregnancy diagnosis, which can be performed after Day 45 postcopulation, is very important not only as a management tool (females that have tested positive are under a very intensive care to avoid peripartum problems), but also the technique can help determine potential reasons for preterm abortions in primiparous Iberian lynx females.

The physiologic role of $PGF_{2\alpha}$ in felids (and other carnivores) remains to be elucidated. The increasing production of $PGF_{2\alpha}$ by the cat placenta might be linked to a rapid increase in fetal growth from Day 42 of gestation onwards [38]. The suggestion that prostaglandin levels in queens must reach a certain threshold level before luteolytic action occurs is supported by particularly large doses of $PGF_{2\alpha}$ (2 mg/cat) that are needed to induce abortion [39,40].

In summary, the use of both urine and fecal samples favors PGFM as a precise, practical method for its application. We obtained parallel courses when comparing urinary and fecal PGFM, and only a simple dilution of fecal extracts is necessary prior to analyses. To our knowledge, PGFM has not yet been described to occur in feces. In particular, fecal PGFM analyses may allow pregnancy diagnosis in captive and free-ranging felids. High-level PGFM in fecal samples should already be sufficient to diagnose an ongoing (last trimester) pregnancy without the knowledge of mating history. A comprehensive pregnancy detection method for felid species based on a single sample is not attainable with any available progestin or estrogen assay because of luteal activity in nonpregnant females, species-specific metabolite compositions, intraspecies variations, individual fluctuations, as well as instability of fecal steroid hormone metabolites [2,38].

Further investigations will focus on urinary and fecal PGFM determination in other felid and carnivore species, which are likely subjected to similar regulations and PGFM patterns. Comparative HPLC immunograms will be carried out to identify PGFM and putative other $PGF_{2\alpha}$

metabolites. We were already able to demonstrate PGFM in connection with pregnancies in the urine of Spectacled Bears and the Giant Panda and in feces of Eurasian lynx and the sandcat (data not shown). Thus, we speculate that PGFM courses in carnivores may follow similar patterns, as previously described for serum PGFM in dogs [19]. Altogether, this method of pregnancy diagnosis and monitoring may prove to be useful in the breeding management of felid and probably canid species and provides a foundation for future studies on pregnancy in captive exotic carnivores.

Acknowledgments

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