

## Using PGFM (13,14-dihydro-15-keto-prostaglandin F2 $\alpha$ ) as a non-invasive pregnancy marker for felids

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### Abstract

Understanding the complex endocrine interactions that control reproduction in felids is essential for captive breeding management. The most important demand is a quick and reliable pregnancy diagnosis. However, the occurrence of pseudopregnancies in felids complicates matters. We investigated whether the fecal prostaglandin metabolite (PGFM) recently suggested for pregnancy diagnosis in the lynx is suitable for all felid species. We found that increased levels of PGFM during the last trimester indicate pregnancy in seven of the eight main lineages of the carnivore family Felidae. PGFM levels in a sand cat (domestic cat lineage) were basal at mating and remained so until Day 40 post-mating. Day 41 marked the beginning of a distinct increase culminating in peak levels of 6.5  $\mu\text{g/g}$  before parturition and decreasing again to baseline thereafter. Similar pregnancy profiles were obtained from the domestic cat, the leopard cat, the lynx, the ocelot and the caracal lineage, whereas in pseudopregnant individuals (sand cat, Iberian and Eurasian lynx) fecal PGFM remained at basal levels. In pregnant cheetahs (puma lineage) PGFM increased above basal following day  $\sim 48$  peaking before pregnancy but remained at baseline in pseudopregnant females. Discrepancies existed in the Panthera lineage. While Chinese leopard, Sumatran tiger, and the black panther showed marked increases of PGFM during the last weeks of pregnancy, only moderate increases in PGFM levels were found in the Indochinese tiger and the Persian leopard. Altogether, PGFM as tool for pregnancy diagnosis has been proven to be useful in breeding management of felids.

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### 1. Introduction

All 36 species of wild felids are included in Appendices I and II of CITES and tend to be one of the most endangered and vulnerable groups of mammals in the world. Twenty-three of those cat species are threatened or endangered with extirpation in at least some part of their natural range ([http://](http://www.iucnredlist.org)

[www.iucnredlist.org](http://www.iucnredlist.org)). For example, survival of the 10 non-domestic felid species endemic to Latin America is particularly jeopardized by habitat loss, human-animal conflicts, and poaching [1] whereas tiger conservation in Asia is mainly by harvest of animals for traditional medicines used by at least a quarter of the world's human population [2]. One felid species in particular—the Iberian Lynx—is critically endangered (IUCN R. List 2009) mainly due to decimation of European rabbit (*Oryctolagus cuniculus*) populations, the lynx's main prey [3].

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Because of increasing extinction risks there is a growing demand for zoos to sustain genetically healthy felids populations in case of catastrophic extinctions. Most felid species reproduce poorly in captivity, a problem attributed to behavioral incompatibilities, captivity stress, or inappropriate husbandry [1]. Causes of female reproductive failure are challenging to diagnose because of difficulties analyzing the complex endocrine milieu associated with estrous activity, ovarian function, conception, and pregnancy [4]. Therefore, understanding the endocrine principles of reproduction in felids is essential for their captive breeding management and applied conservation efforts. One of the most important demands in captive breeding programs is a quick and reliable pregnancy diagnosis, because females in captivity tend to abort or kill their offspring if management conditions are not appropriate. Thereby primiparous females have a higher rate of failure to raise their young than multiparous ones [5]. A timely installed video-surveillance system permits early detection of problems which may result in the rescue of cubs that were abandoned by their mothers for hand-raising. In this regard, non-invasive endocrine monitoring utilizing urine and fecal samples is preferred. Such techniques avoid repeated blood sampling for reproductive hormone analysis and do not represent a source of additional stress that may increase the risk of abortion in pregnant lynxes.

In several felid species, pregnancy diagnosis utilizing non-invasive fecal hormone metabolite monitoring has become a routine procedure [6]. After successful mating, progesterone level increases in blood plasma due the activity of corpora lutea. Towards the end of pregnancy, progesterone levels decrease and drop to baseline levels before parturition [6]. This plasma profile of progesterone secretion is mirrored by progesterone metabolites in feces. In many felid species, fecal progesterone (P4) metabolite concentrations increase significantly during pregnancy [7]. Pseudopregnancies (non-pregnant luteal phase) are characterized by a shorter duration of fecal progestin elevation, usually approximately one-half to two-thirds of the gestation length. For example, in the cheetah the average pseudopregnancy length is 53 d, whereas a normal full-term gestation length is 94 d [7]. In the clouded leopard average pseudopregnancy lasts 48 d, compared to a full-term gestation of 90 d [7]. The main disadvantages of fecal progestin measurements for reliable pregnancy diagnosis are the necessity of repeated (frequent) sampling as well as highly variable intra- and interspecies baseline concentrations. Furthermore, in a few felid

species fecal P4 metabolite analysis failed to demonstrate pregnancy [8–10].

As an alternative to fecal steroid analysis, urine has been utilized for tracking pregnancy specific hormones. In particular, several peptide hormones, such as luteinizing hormone (LH) and human chorionadotropin (hCG) can be detected in urine and may be related to sexual activity or pregnancy status [11,12]. Recently, it was shown that relaxin is detectable in urine of pregnant domestic cats, leopards and lynxes [10,13]. In our previous study [14] we investigated the urinary prostaglandin F<sub>2α</sub> metabolite (PGFM), which also seems to be a pregnancy related placental signal in the Iberian lynx [14]. PGF<sub>2α</sub> is a prostaglandin, that has been portrayed as a locally bioactive hormone detectable in virtually all tissues [15]. It is now widely accepted that uterine and placental prostaglandins play a key role in regulating the function and life span of corpora lutea [16] and exogenous PGF<sub>α</sub> is luteolytic in both pregnant and pseudo-pregnant bitches [17]. Serum PGFM analyses in the dog revealed different patterns between pregnant and non-pregnant (diestrus) bitches [18].

The same was obvious in the Iberian lynx. Based on the analysis of the urinary PGF<sub>2α</sub> metabolite PGFM, a clear differentiation between pregnant and pseudopregnant female lynxes was possible [14]. The PGFM patterns revealed a constant hormone increase over the last trimester (21 d) of gestation in pregnant females with peak concentrations around the time of parturition followed by a post-partum drop to baseline. In comparison, in pseudopregnant females baseline profiles were obtained during the entire period of supposed pregnancy [14]. The finding that PGFM is detectable in feces of Iberian lynxes as well and follows similar courses as shown for urine [14] encourages us to extend our investigations to other felids.

We hypothesize that the fecal PGFM detected in the lynx might be representative for other felid species and that our PGFM enzyme linked immunoassay (EIA) can be used as a simple and reliable pregnancy test. To prove this hypothesis, we collected fecal samples from pregnant and pseudopregnant females of different felid species and determined PGFM by an EIA based on a new and more sensitive PGFM-specific antibody and a PGFM peroxidase conjugate [14].

## 2. Materials and methods

### 2.1. Animals and sampling

The housing locations of study animals are shown in Table 1. Ten different zoos contributed to the

Table 1  
Species and sample origin.

Species	Lineage	Zoo	P	PP
Sand cat ( <i>Felis margarita</i> )	Domestic cat	Ebeltoft Zoo and Safari, Denmark	+	+
African wildcat ( <i>Felis silvestris lybica</i> )		Tierpark Berlin, Germany	+	
Asian leopard cat ( <i>Prionailurus bengalensis</i> )	Leopard cat	Tierpark Berlin, Germany	+	
Fishing cat ( <i>Prionailurus viverrinus</i> )		Kent Safari Park and Zoo, Port Lympne, UK	+	
Puma ( <i>Puma condolor</i> )	Puma	Tierpark Berlin, Germany	+	
Cheetah ( <i>Acinonyx jubatus</i> )		White Oak Conservation Center, Yulee, FL; Smithsonian Conservation Biology Institute, front Royal, VA, USA	+	+
Iberian lynx ( <i>Lynx pardinus</i> )	Lynx	ILCBP, Spain	+	+
Eurasian lynx ( <i>Lynx lynx</i> )		A.N. Severtzov Institute, Moscow, Russian Federation	+	+
Ocelot ( <i>Leopardus pardalis</i> )	Ocelot	Tierpark Hellabrunn, Munich, Germany	+	
Geoffroy's cat ( <i>Leopardus geoffroyi</i> )		Tierpark Berlin, Germany	+	
Oncilla ( <i>Leopardus tigrinus</i> )		Prague Zoo, Czech Republic	+	
Caracal ( <i>Caracal caracal</i> )	Caracal	Tierpark Berlin, Germany	+	
Serval ( <i>Leptailurus serval</i> )		Tierpark Berlin, Germany	+	
Black jaguar ( <i>Panthera pardus</i> )	Panthera	Tierpark Berlin, Germany	+	
North Chinese leopard ( <i>Panthera pardus japonensis</i> )		Tierpark Berlin, Germany	+	
Persian leopard ( <i>Panthera pardus ciscaucasica</i> )		Allwetterzoo Münster, Germany	+	
Sumatran tiger ( <i>Panthera tigris sumatrae</i> )		Tierpark Berlin, Germany	+	
Indochinese tiger ( <i>Panthera tigris corbetti</i> )		Tierpark Berlin, Germany	+	

P, pregnancy; PP, pseudopregnancy.

study. Fecal samples from a cheetah were obtained from the Smithsonian Conservation Biology Institute (SCBI), Front Royal, VA, USA, and the White Oak Conservation Center, Yulee, FL, USA. The study comprises 18 different felids species, representing seven of the eight main lineages of the carnivore family *Felidae* [19]. Adult females were allowed to mate and the deliveries of cubs or observed abortions were ultimate indications of pregnancy. Pseudopregnancies (PP) occurred if conception failed after appropriate mating observations. All female felids were housed under variable conditions, following the general requirements of institutional husbandry guidelines. In the case of shared enclosures housing female and male together, the keepers labeled the samples accordingly.

Unless noted, sampling commenced several days before the expected mating or immediately following observed mating and ended after parturition. The frequency of sample collection ranged from once daily to two times a week according to institutional protocols. Samples were frozen immediately and stored at  $-20^{\circ}\text{C}$  until processed.

## 2.2. Sample processing

Fecal samples were processed as described previously [14]. 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  $3000 \times g$ . The supernatant was decanted and diluted 1:1 with water followed by a dilution with 40% methanol (1:10). Aliquots of diluted extracts were then assayed for PGFM using enzyme immunoassay (EIA). For the cheetah fecal samples,  $\sim 0.2$  g of dried fecal powder were boiled in 90% ethanol: 10% distilled water [20,21]. Each sample was centrifuged at  $500 \times g$  for 20 min, the supernatant recovered and the resulting pellet redissolved in 5 ml of 90% ethanol before recentrifugation (500g, 15 min). This secondary supernatant was recovered, pooled with the first, dried under air and redissolved in 1 ml methanol (100%). Fecal extracts were vortexed, then sonicated for 15 min and stored at  $-20^{\circ}\text{C}$  until hormonal analysis.

## 2.3. Stability of PGFM in fecal samples

Repeated PGFM analyses in samples stored at  $-20^{\circ}\text{C}$  revealed stability for at least two years. To investigate the stability of PGFM at elevated temperatures, 0.5 g

aliquots of two fecal samples (both from Iberian lynx) were taken and incubated over 0, 48 and 72 hours at 37 °C, respectively. Incubation was stopped by freezing at –20 °C. Finally, all samples were extracted accordingly and analyzed within one assay.

#### 2.4. PGFM antibody

The PGFM antibody was generated in rabbits against 9 $\alpha$ ,11 $\alpha$ -dihydroxy-15-oxo-prost-5-en-1-oic acid-BSA by Sigma as described by Schlegel, et al [22]. The cross-reactivity of the PGFM antibody was tested against PGFM (9 $\alpha$ ,11 $\alpha$ -dihydroxy-15-oxo-prost-5-en-1-oic acid), tetranor-PGFM (9 $\alpha$ ,11 $\alpha$ -dihydroxy-15-oxo-2,3,4,5-tetranor-prostan-1,20-dioic acid), 11 $\beta$ -PGF<sub>2 $\alpha$</sub>  (9 $\alpha$ ,11 $\beta$ ,15S-trihydroxy-prosta-5Z,13E-dien-1-oic acid), PGF<sub>2 $\beta$</sub>  (9 $\beta$ ,11 $\alpha$ ,15S-trihydroxy-prosta-5Z,13E-dien-1-oic acid), PGE<sub>2</sub> (9-oxo,11 $\alpha$ ,15S-dihydroxy-prosta-5Z,13E-dien-1-oic acid), PGEM (9,15-dioxo-11 $\alpha$ -hydroxy-prosta-5Z-en-1-oic acid), tetranor-PGEM (9, 15-dioxo- 11 $\alpha$ -hydroxy-13, 14-dihydro- 2, 3, 4, 5-tetranor-prostan- 1, 20-dioic acid), and PGAM (9, 15-dioxo-prosta- 5Z, 10-dien- 1-oic acid); all obtained from Cayman Chemicals (Cayman Europe, Tallinn, Estonia). The PGF<sub>2 $\alpha$</sub>  (9 $\alpha$ ,11 $\alpha$ ,15S-trihydroxy-[8 $\beta$ ]-prosta-5Z,13E-dien-1-oic acid) was purchased from Sigma (Sigma Chemie, GmbH, Deisenhofen, Germany). The antibody was characterized by a high specificity towards PGFM (100%), low binding to PGEM (1.9%) and PGF<sub>2 $\alpha$</sub>  (0.5%) and negligible cross-reactivities (< 0.1%) to tetranor-PGFM, tetranor-PGEM, 11-PGF<sub>2 $\alpha$</sub> , PGF<sub>2 $\beta$</sub> , PGE and PGAM.

#### 2.5. Enzyme immunoassay procedure

The EIA was carried out as described in detail previously [14]. In brief, 96 well microtiter plates coated with affinity purified goat IgG (anti-rabbit IgG), 1  $\mu$ g/well in 100  $\mu$ l coating buffer were washed and duplicates of 20  $\mu$ l feces extract or PGFM standard were placed simultaneously with 100  $\mu$ l PGFM-HRP conjugate diluted (1:20.000) in assay buffer (50 mM Na<sub>2</sub>HPO<sub>4</sub>/Na<sub>2</sub>HPO<sub>4</sub>, 0.15 M NaCl, 0.1% BSA, pH 7.4). Thereafter, 100  $\mu$ l PGFM antiserum diluted in assay buffer (1:25.000) was added immediately to all wells except blank. The plates were incubated overnight at 4 °C. After washing, the substrate reaction was performed with 150- $\mu$ l substrate solution per well (1.2 mM H<sub>2</sub>O<sub>2</sub>, 0.4 mM 3,3',5,5'-tetramethylbenzidine in 10 mM sodium acetate, pH 5.5) and stopped with 50  $\mu$ L 4N H<sub>2</sub>SO<sub>4</sub>. The color intensity was measured at 450 nm with a 12-channel microtiter plate reader (Infinite M 200, Tecan, Crailsheim, Germany) and hormone con-

centrations were calculated according to the standard curve using the Magellan software (Tecan).

The PGFM calibration standards were prepared by dilution with 40% methanol ranging from 0.4 to 200 pg/well. Sensitivity of the assay at 90% binding was 2.3 pg/well. Serial dilutions of fecal pools from Iberian lynx proved parallelism to the standard PGFM with no differences in slopes ( $P > 0.05$ ). Precision and reproducibility were calculated from multiple measurements of pooled samples containing low and high endogenous PGFM concentrations. The inter- and intraassay coefficients of variation were 16.2 and 14.1% ( $n = 20$ ), and 7.9 and 4.2% ( $n = 8$ ), respectively.

#### 2.6. Statistical analyses

To investigate the stability of PGFM in fecal samples comparisons of mean values were performed by Student's paired  $t$  test after testing for normality using the software program InStat Version 3 (GraphPad Software, Inc., La Jolla, CA, USA).

For fecal PGFM profiles, an iterative process was used to calculate basal concentrations [23,24]. Briefly, the mean of all samples for each female was calculated and samples with concentrations greater than one standard deviation (SD) above this mean were removed. This iterative process was repeated until there were no samples with concentrations greater than the mean plus 1 SD. The mean of the remaining values was considered as the individual basal concentration. An increase above basal was defined as the day when fecal PGFM concentrations exceeded basal concentrations + 1SD for at least three consecutive samples.

### 3. Results

#### 3.1. Stability of PGFM in feces extracts

Compared with the initial PGFM concentrations of 1.5 and 0.14  $\mu$ g/g feces, the recoveries after 48 and 72 h of storage at 37 °C were 1.78 (118.6%) and 1.82 (121.3%) for the first, and 0.13 (92.8%) and 0.11 (78.6%)  $\mu$ g/g feces for the second sample, respectively. The results revealed no change ( $P > 0.05$ ; Student's paired  $t$  test) of PGFM within a 3-d storage period, indicating that PGFM in fecal samples is stable, even at elevated temperatures.

#### 3.2. PGFM profiles in pregnant female felids

Table 2 displays a summary of gestation length and PGFM levels ( $\mu$ g/g feces) of all 18 cat species examined in this study. Most species were represented by

only one individual. The interspecies comparison focused on the onset of the PGFM increase post-breeding, defined as the day PGFM levels increased above basal plus 1 SD over a period of at least three consecutive samples. For almost all felid species, it is apparent that significant PGFM elevations above baseline occurred ~3 to 4 wks before parturition, and at the beginning of the third trimester. Individual PGFM pregnancy profiles were analyzed for the different cat lineages according to the feline pedigree [19] and are presented in Table 2.

### 3.2.1. Domestic cat, leopard cat and lynx lineages

The felid species of these lineages are characterized by pregnancy lengths ranging from 65 to 70 d. Figure 1 depicts the PGFM profiles from one pregnant and one pseudopregnant sand cat (domestic cat lineage) and one pregnant fishing cat (leopard cat lineage). In the pregnant sand cat, PGFM levels were basal ( $0.07 \mu\text{g/g}$ ) following mating and remained at this level until Day 40 post-mating. Day 41 marked the beginning of a distinct increase, culminating in peak levels of  $2.1 \mu\text{g/g}$  feces 3 d before parturition. Following parturition, PGFM concentrations immediately decreased again to basal levels ( $0.1 \mu\text{g/g}$ ) and remained low over the rest of the sampling period (through Day 80). By contrast, the pseudopregnancy related fecal PGFM concentration

remained basal during the entire sampling period (Fig 1A). A comparable profile was obtained from the fishing cat (66 d pregnancy, Fig. 1B). Basal concentrations were measured until Day 30 post-mating, followed by an increase with a maximum concentration of  $7.8 \mu\text{g/g}$  feces 5 d before parturition. Following parturition, PGFM levels decreased reaching basal values within 5 d. Similar profiles were obtained from the African wild cat (domestic cat lineage) and from the Asian leopard cat (leopard cat lineage, Table 2), whereas the latter revealed the highest PGFM amplitude of all species investigated so far ( $21 \mu\text{g/g}$ ).

Figure 2 represents profiles from Iberian lynx of three different reproductive stages, including one normal pregnancy (A), one premature delivery and one aborted pregnancy (B). All three PGFM patterns proceeded similarly until day 45 post-mating. After the increase of PGFM levels during the third trimester, a shifted and a steep decrease were observed in correlation to abortion (d50), premature birth (d63) and regular termination of pregnancy (d66). The PGFM course of the pregnant female is representative for the Iberian and Eurasian species and has been used successfully for pregnancy diagnosis in support of the Iberian lynx captive breeding program (ILCBP) in 2010 and 2011.

Table 2  
Gestation length, number of pregnancies as well as baseline and maximum PGFM levels ( $\mu\text{g/g}$  feces) of 18 investigated felid species.

Species	Pregnancy n	Gestation length (days)	Baseline level ( $\mu\text{g/g}$ )	Baseline + SD ( $\mu\text{g/g}$ )	Maximum level ( $\mu\text{g/g}$ )	PGFM-increase on day
African wildcat*	1	67	0.06	0.08	1.48	n.d.
Sand cat	1	65	0.04	0.06	6.5	35
Asian leopard cat†	1	73	0.06	0.09	21.0	n.d.
Fishing cat	1	66	0.08	0.10	7.8	41
Puma	1	81	0.05	0.07	0.7	73
Cheetah‡	3	96	0.12	0.14	1.44	46
Iberian lynx‡	5	66	0.04	0.05	3.63	40
Eurasian lynx‡	3	70	0.10	0.04	1.6	45
Ocelot	1	75	0.52	0.68	8.2	58
Oncilla	2	71	0.06/0.08	0.08/0.11	1.83/3.92	24/28
Geoffry's cat	1	72	0.08	0.12	2.7	38
Caracal	1	78	0.18	0.21	9.6	47
Serval§	1	78	0.15	0.21	2.1	64
Sumatran tiger	1	103	0.06	0.10	2.0	73
North Chinese leopard	2	97–98	0.05	0.07	0.52/0.83	74/79
Black panther (jaguar)	1	110	0.04	0.04	1.38	82
Indochinese tiger	1	98	0.04	0.05	0.44	68
Persian leopard¶	1	~95	0.03	0.04	0.30	~72

\* Sampling gap between Day 44 and 59

† samples available only from Day 52

‡ mean from three and five females, respectively

§ samples available only from Day 33, and 5-days gap before Day 64

¶ mating date is missing



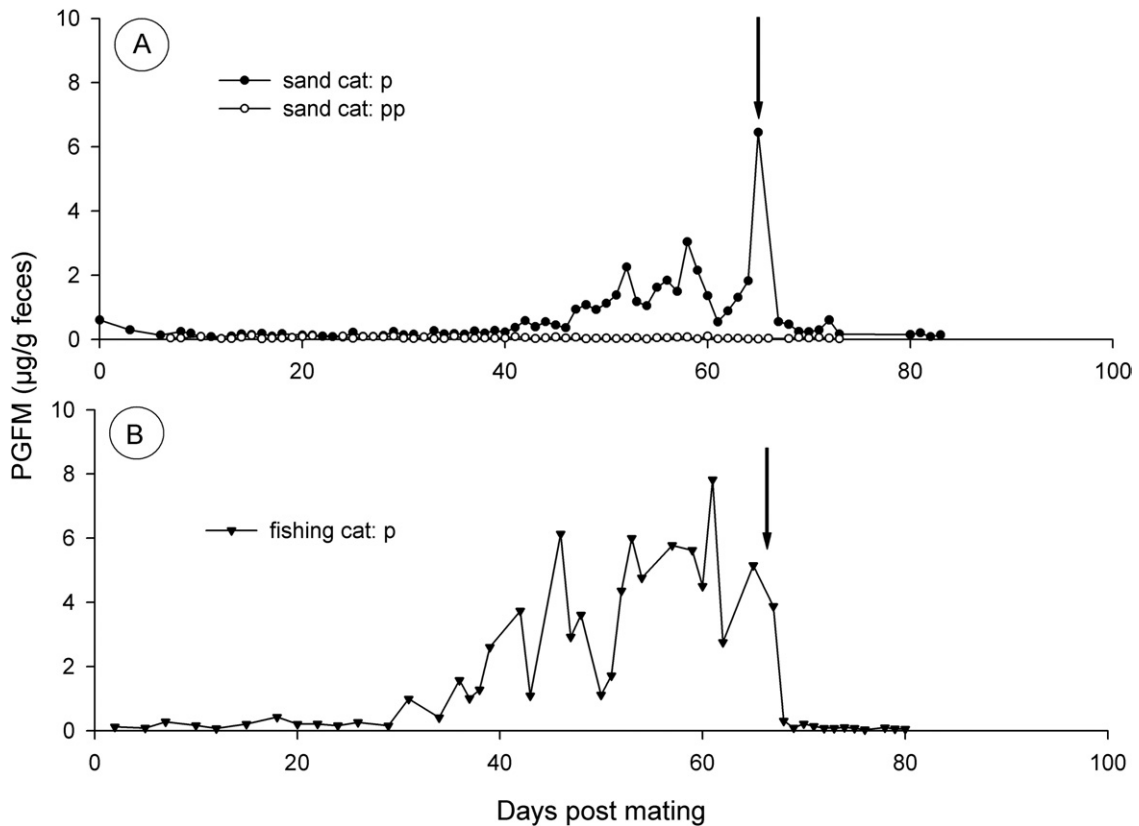


Fig. 1. Prostaglandin F<sub>2</sub>α metabolite (PGFM) concentrations obtained from a pregnant and a pseudopregnant sand cat (A. *Felis margarita*) as well as a pregnant fishing cat (B. *Prionailurus veerrinus*). A significant divergence between the sand cat profiles occurred between days 41 and 67 after copulation. Arrows indicate time point of parturition.

### 3.2.2. Puma lineage

For species of the puma lineage, we were able to analyze samples from three cheetah pregnancies ( $n = 3$ ) and five cheetah pseudopregnancies ( $n = 4$ ) and one pregnant puma (data not shown). The PGFM profile of the pregnant cheetahs (Fig. 3A) is characterized by an untypical trend compared to all other felids showing elevated PGFM concentrations around the time of mating. Thereafter, a period of relatively low PGFM concentrations ( $< 0.5 \mu\text{g/g}$ ) followed until Day 48, when PGFM levels increased above basal ( $0.22 \mu\text{g/g}$ ) again and peaked on Day 88 ( $1.60 \mu\text{g/g}$ ) in the pregnancy (pregnancy length of 96 d). Unfortunately, no samples were obtained from either the last 2 d of pregnancy or the postpartum period. The unusual trend around the time of mating is mainly caused by one female where high PGFM concentrations (up to  $1.38 \mu\text{g/g}$ ) were measured during that period, generating high SDs. A similar mating associated phenomenon was obtained in one pseudopregnant female. In the remaining two pregnancies and four pseudopregnancies, however, high

PGFM concentrations following mating were not detected. In contrast to the pregnant cycle, no PGFM elevation was observed during the pseudopregnancies. In general, for all the five pseudopregnant animals, PGFM concentration did not exceed baseline levels during the entire collection period. The mating associated elevation of PGFM seems to be untypical and has been seen so far only in the cheetah.

In the puma (pregnancy length of 81 d), the PGFM profile differed from the cheetah, as the PGFM elevation was observed only 1 wk before parturition (Table 2).

### 3.2.3. Ocelot and caracal lineages

These two feline lineages are characterized by pregnancy length of approximately 70 to 80 d. Figure 4 shows the PGFM profiles of a pregnant ocelot (ocelot lineage) and a pregnant caracal (caracal lineage). The PGFM concentrations of the ocelot increased over basal ( $0.52 \mu\text{g/g}$ ) on Day 58 of pregnancy, peaked on Day 76 ( $8.2 \mu\text{g/g}$ ) and dropped again to basal concentrations at parturition. A similar profile was observed for the

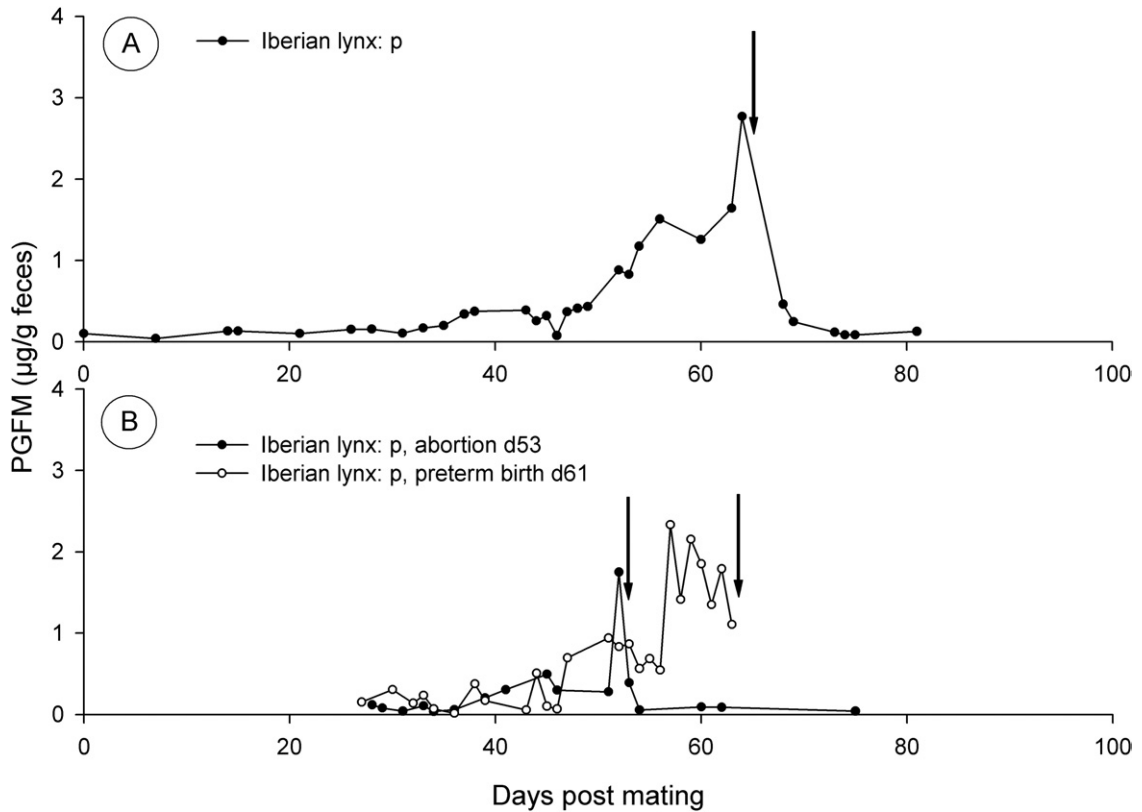


Fig. 2. PGFM concentrations in three female Iberian lynx. Female one delivered two healthy cubs on Day 66. Female two had a confirmed abortion on Day 53, whereas female three had a preterm still birth (2 cubs) on Day 63. Arrows indicate time point of parturition and abortions.

caracal, where PGFM increased on Day 47 post-breeding leading to maximum concentrations ( $\sim 10 \mu\text{g/g}$ ) at Day 61 and remained elevated prior parturition. Other representatives of the ocelot lineage (oncilla and Geoffroy's cat, Table 2) and the caracal lineage (serval, Table 2) confirmed the validity of PGFM as a pregnancy indicator and revealed similar profiles.

#### 3.2.4. *Panthera lineage*

The *Panthera* lineage was represented by one Sumatran and one Indochinese tiger (Fig. 5A), one Chinese and one Persian leopard (Fig. 5B), and a black panther (Table 2). All of these animals were naturally mated and established pregnancy. Surprisingly, the pregnancy-related PGFM profiles differed among the species. While the Chinese leopard and Sumatran tiger (Fig. 5) showed marked increases of PGFM concentrations around Day 75, the black panther is characterized by a later but distinct PGFM increase on Day 82 corresponding to 4 wks before parturition (Day 110, Table 2). Only moderate increases in PGFM levels, not exceeding  $0.4 \mu\text{g/g}$  feces, were observed in the Indochinese tiger (Fig. 5) and the Persian leopard. Compared to the

profiles of their sister taxa within the felids these values were quite low and the characteristic peak around parturition was not observed.

#### 4. Discussion

Our results demonstrate that the  $\text{PGF}_{2\alpha}$  metabolite PGFM is a reliable pregnancy indicator in several felid species. In pregnant females, fecal PGFM concentrations elevate significantly above baseline during the last trimester of pregnancy peaking towards parturition. This pattern of elevation was not observed in any pseudopregnant female. In addition, fecal PGFM concentrations also decrease drastically at time of abortion and premature birth, indicating a strong relationship of this hormone with maintenance of full-term pregnancy.

To our knowledge, this is the first report of PGFM assayed as a fecal metabolite for pregnancy determination in any species. PGFM can be determined in fecal extracts using a simple EIA. Our method does not require sample pretreatment except for a simple extraction and a dilution step with 40% methanol. In addition,

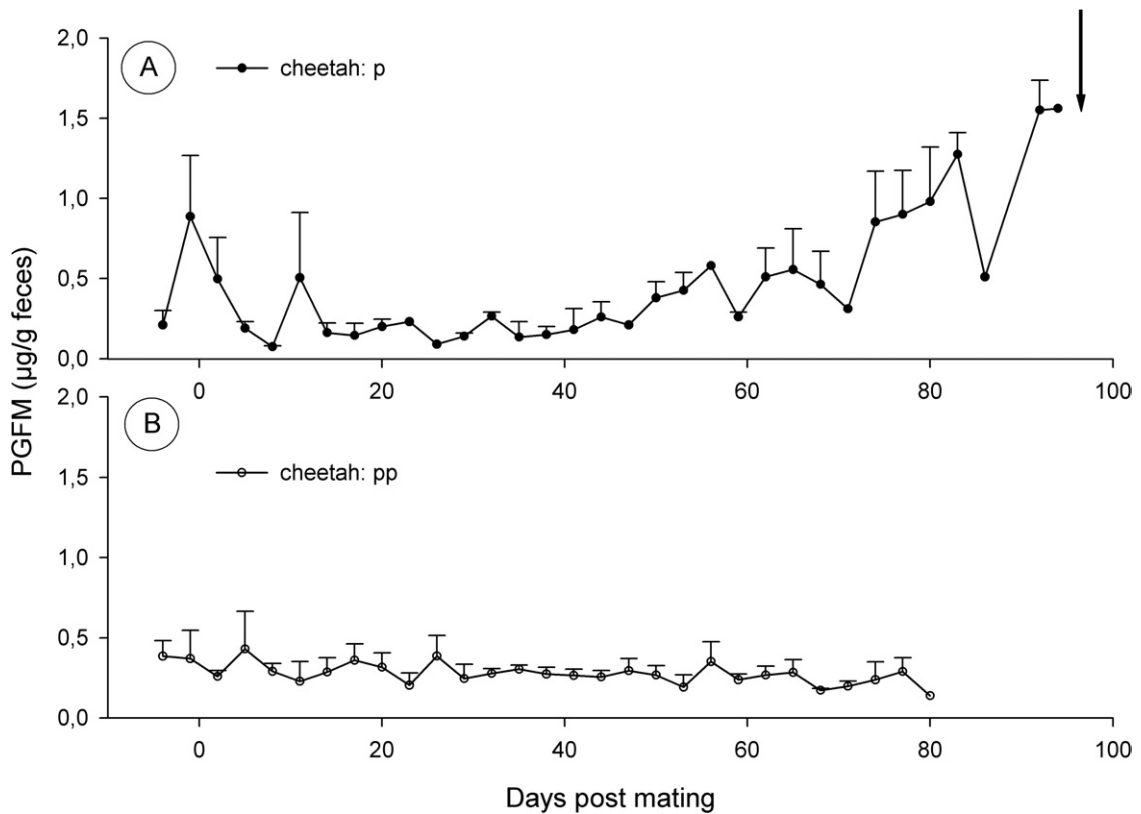


Fig. 3. Determination of PGFM in samples collected from female cheetahs (*Acinonyx jubatus*). The upper graph (4A) shows the 3-day means  $\pm$  SEM of three pregnancies, whereas the lower graph (4B) shows the 3-days means  $\pm$  SEM of five pseudopregnancies. A significant divergence between the hormone courses occurred at Day 47–49 after copulation.

incubation of fecal samples at 37 °C during 3 d does not affect fecal PGFM concentrations, which suggests a high stability of PGFM and makes feces preservation and sample deep-freezing unnecessary if contemporary analysis is intended.

The high sensitivity of the PGFM EIA method (0.4 pg/well), is sufficient to measure both low baseline concentrations (average 0.02  $\mu\text{g/g}$ ) and high PGFM levels during the peri-partum period (applying dilutions up to 1:100). The different extract dilutions did not affect measurements due to an extraordinarily high degree of parallelism excluding matrix effects (data not shown). The method for the determination of PGFM in feces proved to have very high precision.

One of our most interesting results from this present research is that the EIA allowed the simultaneous analyses of immunoreactive PGFM metabolites in fecal samples from 18 cat species of seven cat lineages. Pregnancy related fecal PGFM profiles were obtained from all species evaluated. Interestingly, peak levels in the peripartum period differed in magnitude reaching 21  $\mu\text{g/g}$  in the Asian leopard cat compared to  $< 0.5 \mu\text{g/g}$

in both the North Chinese leopard and in the puma. Our previous results from the Iberian lynx [14] as well as the sand cat and cheetah indicate that PGFM analyses may allow the differentiation between pregnancy and pseudopregnancy in captive and free-ranging felids. High-level PGFM in fecal samples are sufficient to diagnose an ongoing (last trimester) pregnancy without the knowledge of breeding date. In both the sand cat and the cheetah, deviating PGFM courses allow a clear differentiation of pregnant from pseudopregnant females beginning approximately at Days 42 and 58 post-mating, respectively.

In the four lynx species evaluated, PGFM measurements seem to be the only reliable option to diagnose pregnancy non-invasively. In contrast to other felids, steroid-based monitoring of ovarian luteal function is impossible using fecal and urinary progestagen metabolites, therefore a reliable pregnancy diagnosis method has never before been developed [8,25]. The Witness relaxin pregnancy test [13] has been used for diagnostic purposes using urine samples collected between Days 26 and 46 from pregnant Iberian lynx [10]. However,



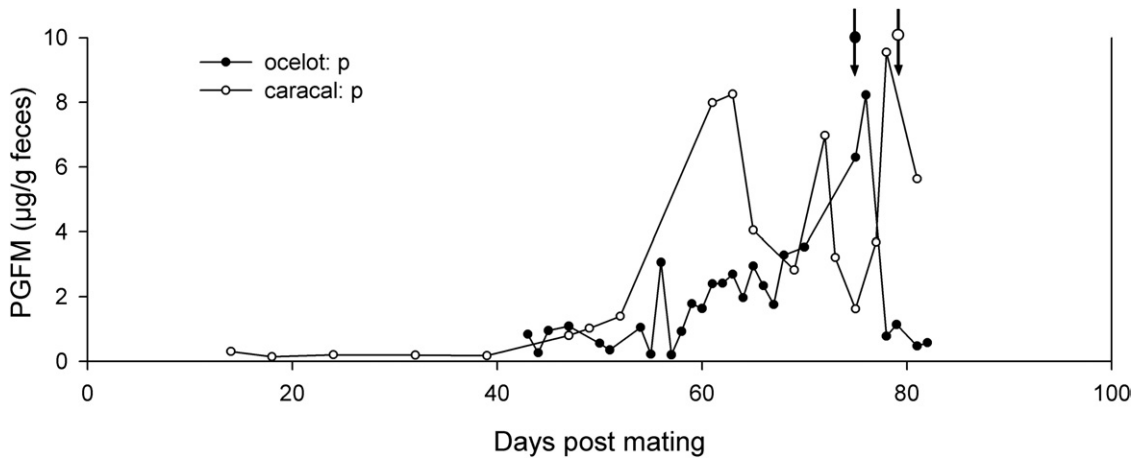


Fig. 4. PGFM concentrations in samples collected from a pregnant ocelot (*Leopardus pardalis*) and caracal (*Caracal caracal*).

not all diagnosed pregnant females delivered a litter, which indicates high rates of abortions after d46 or false positives using the relaxin test. In the Iberian lynx, PGFM analyses had been successfully used to diagnose

pregnancies in the course of the captive breeding project. During the 2010 and 2011 mating seasons, 26 of 27 pregnancies were predicted correctly, thus only one pregnancy diagnosis was uncertain. In that case, sam-

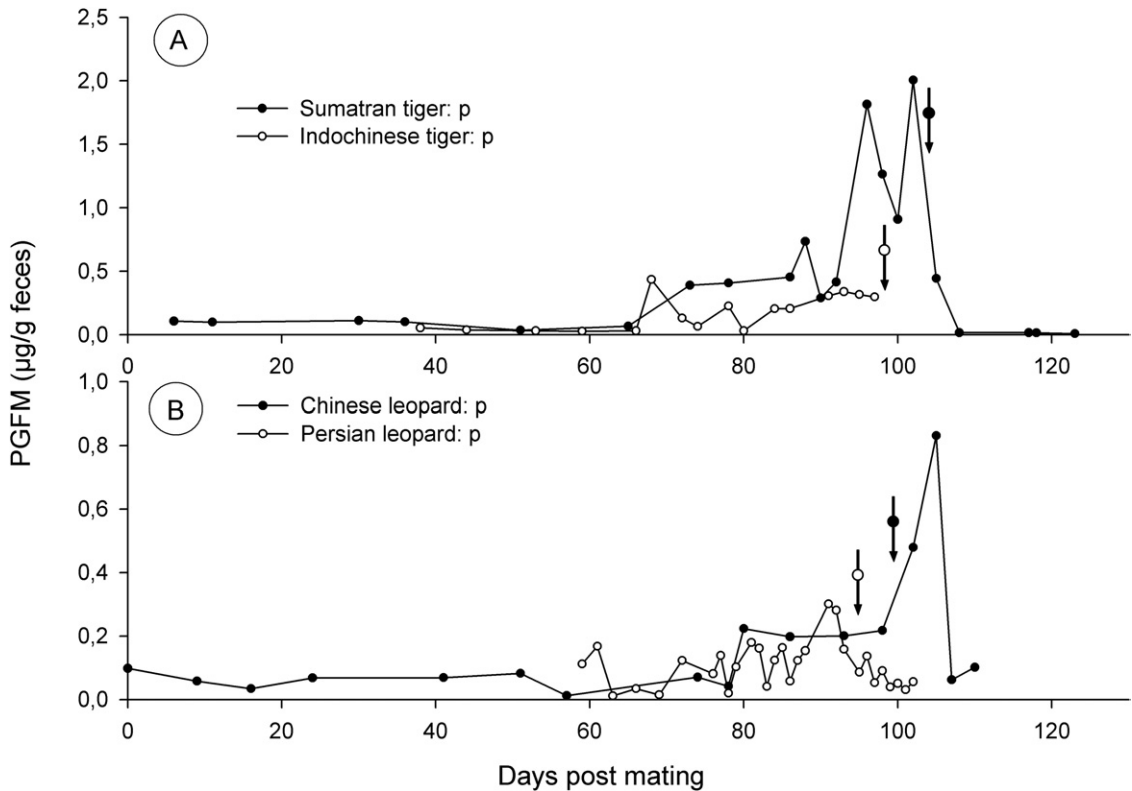


Fig. 5. PGFM concentrations in samples collected from a pregnant Sumatran (*Panthera tigris sumatrae*) and an Indochinese tiger (*Panthera tigris corbetti*) (upper graph A). The lower graph (B) presents the PGFM course in a pregnant Persian (*Panthera pardus ciscaucasica*) and a Chinese leopard (*Panthera pardus japonensis*).

ples from a male were not distinguished from the female.

Also during our pregnancy monitoring in 2011, the exact day of late abortions was determined by specific PGFM profiles in three animals (see one example in Fig. 3). Additional advantages of the PGFM assay in comparison with the relaxin test are the reduced sample processing necessary and the ability to use fecal samples. For example, urinary relaxin determination demands a concentration step by ultrafiltration before analysis. An additional carnivore comprehensive pregnancy marker is the prolactin concentrations in blood plasma, allowing the discrimination between pregnancy and pseudopregnancy via elevated values in cats and in other carnivores, such as the Japanese black bear [26], the dog [27] and the mink [28] making this hormone also an interesting target for pregnancy diagnosis if urine sample are available.

Felids express marked interspecies variations in reproductive hormone patterns. For example, the relationship between pregnant and pseudopregnant cycle length differs between tigers and Pallas cats [29]. In the tiger, the gestation length is 108 d and a pseudopregnant cycle lasts only one-third of this period (~35 d), whereas in the Pallas cat a pseudopregnancy takes about 70% (45–50 d) of pregnancy length (66 d [29]). Due to the inconsistency of steroid hormone metabolism across species, each assay detecting fecal steroid metabolites must be properly validated using both laboratory and biological tests. In contrast, our results revealed that fecal PGFM can be measured with one assay suitable for at least seven of the eight main lineages of the carnivore family Felidea.

Although most felid species we investigated were represented by only one pregnant individual, we are convinced that it is possible to generalize PGFM profiles for small felid species (e.g., sand cat, fishing cat, cheetah, lynx, ocelot, oncilla, caracal, and serval). In these species, PGFM diverges from baseline level exclusively during the last pregnancy trimester. We found only one exception from this typical “small cat” pattern in samples evaluated from the puma, where PGFM increases only 8 d before parturition. Notable inconsistency in PGFM profiles occurred within the big cats of the *Panthera* lineage, even for sister taxa of one species. For example, Indochinese and Sumatran tigers differ in maximum levels of 0.4 to 2.0  $\mu\text{g/g}$  feces PGFM, and the profiles of the Persian and Chinese leopard also varied greatly from each other (ranges of 0.3–0.8  $\mu\text{g/g}$  feces PGFM). This parallels with a comparative study in different felids using fecal P4 metabolite analyses as

an index of pregnancy. Whereas P4 metabolism mechanisms appear to be conserved (similar immunoreactive profiles) among the taxonomically related species (leopard cat, cheetah, clouded leopard, and snow leopard) peak levels varied by a factor of ~15 between the species [7]. Based on scattered samples obtained from the periparturient period of a clouded leopard (*Panthera* lineage), first results indicated that a PGFM based pregnancy diagnosis might be feasible in this species. Altogether, for some of the large felid species, more pregnant and pseudopregnant cycles must be evaluated before an overall conclusion about the suitability of the PGFM ‘pregnancy’ test can be made.

The PGFM patterns of production described here appear to be unique for felids. Our initial expectation was that PGFM might be a suitable pregnancy indicator in all carnivores. However, in most non-felid carnivore species investigated, we were able to detect a sharp PGFM peak before parturition only, indicating the luteolytic function of  $\text{PGF}_{2\alpha}$  as described in ruminants [30] and in bitches [31]. Further investigation into PGFM production in other carnivores is warranted.

The physiological role of  $\text{PGF}_{2\alpha}$  in felids remains to be elucidated. In the cat, particularly large doses of  $\text{PGF}_{2\alpha}$  (2 mg/cat) are needed to induce abortion [32,33]. Therefore, we suggest that prostaglandin levels in queens must reach an individual threshold before luteolytic action occurs. In addition, uterine  $\text{PGF}_{2\alpha}$  is known to act locally on the corpus luteum by a counter-current mechanism, but not via systemic circulation [16]. Fecal PGFM, however, reflects circulating PGFM in blood. The production and concentration of intra-ovarian  $\text{PGF}_{2\alpha}$  might be different from that in blood serum and likely increases only just before parturition as measured in the uteroovarian vein plasma of the sheep [16].

We propose that the primary source of F series prostaglandins during third trimester in felids is the utero-placenta complex. This is supported by the profiles of the two female lynx (Fig. 2) where preterm pregnancy termination was accompanied by an immediate drop of PGFM after abortion/premature birth. The increasing production of  $\text{PGF}_{2\alpha}$  during the third trimester might be linked to a rapid increase in fetal growth from Day 42 of gestation onwards [34]. This does not explain, however, why felids differ from the typical (non-felid) PGFM course, where extensive prostaglandin secretion is focused during the periparturient period. Also, it should be considered that  $\text{PGF}_{2\alpha}$  could affect steroid biosynthesis. It has been shown that  $\text{PGF}_{2\alpha}$  is involved in the up-regulation of steroid biosynthesis in

alpaca Leydig cells [35] and also inhibits the release of progesterone in caprine luteal tissues [36]. This action on luteal cell steroid production could also occur in felids, which may explain the gradual decline in peripheral progesterone concentrations in the cat during the last pregnancy weeks—a decrease which does not appear compensated by the placenta [37]. In the cat, removal of the ovaries at Day 45 induces abortion, suggesting placental steroid synthesis of minor importance to maintain pregnancy at this stage [38]. Little is known about the variation among feline placental forms, which could also be related to the differences in species-specific PGF<sub>2α</sub> patterns. We predict that PGF<sub>2α</sub> is not the key luteolytic factor in felids, considering that oxytocin of ovarian and/or neural origin also triggers luteolysis in cats [39] in addition to a complex cascade of mediators that still await elucidation.

In summary, the PGFM principle has been proved to be valid in six of the eight cat lineages, including the small cats except for the puma (puma lineage). Discrepancies existed between the big cats of the Panthera lineage exhibiting gestation lengths of 80 to 100 d. While Chinese leopard and Sumatran tiger showed marked increases of PGFM around Day 75, only moderate increases in PGFM levels not exceeding 0.4 μg/g feces were observed in the Indochinese tiger and the Persian leopard. Based on additional samples from pregnant females it has to be investigated whether the low PGFM levels might complicate pregnancy diagnosis in these species. In addition comparative HPLC immunograms will be carried out to investigate whether the composition of fecal PGF<sub>2α</sub> metabolites changes towards parturition because in the Iberian lynx PGFM itself was undetectable by our PGFM EIA one day before parturition.

Altogether, this method of pregnancy diagnosis and monitoring may prove to be useful in the breeding management of felid species and provides a foundation for future studies on pregnancy in captive exotic carnivores.

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## References

- [1] Swanson WF, Brown JL. International training programs in reproductive sciences for conservation of Latin American felids. *Anim Reprod Sci* 2004;82–83:21–34.
- [2] Still J. Use of animal products in traditional Chinese medicine: Environmental impact and health hazards. *Complement Ther Med* 2003;11:118–22.
- [3] Moreno S, Beltran JF, Cotilla I, Kuffner B, Laffite R, Jordan G, et al. Long-term decline of the European wild rabbit (*Oryctolagus cuniculus*) in south-western Spain. *Wildl Res* 2007;34:652–8.
- [4] Brown JL. Female reproductive cycles of wild female felids. *Anim Reprod Sci* 2011;124:155–62.
- [5] Wielebnowski N. Reassessing the relationship between juvenile mortality and genetic monomorphism in captive cheetahs. *Zoo Biol* 1996;15:353–69.
- [6] Brown JL. Comparative endocrinology of domestic and nondomestic felids. *Theriogenology* 2006;66:25–36.
- [7] Brown JL, Wasser SK, Wildt DE, Graham LH. Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured noninvasively in feces. *Biol Reprod* 1994;51:776–86.
- [8] Dehnhard M, Naidenko S, Frank A, Braun B, Goritz F, Jewgenow K. Non-invasive monitoring of hormones: A tool to improve reproduction in captive breeding of the Eurasian lynx. *Reprod Domest Anim* 2008;43;Suppl 2:74–82.
- [9] Jewgenow K, Goritz F, Vargas A, Dehnhard M. Seasonal profiles of ovarian activity in Iberian lynx (*Lynx pardinus*) based on urinary hormone metabolite analyses. *Reprod Domest Anim* 2009;44;Suppl 2:92–7.
- [10] Braun BC, Frank A, Dehnhard M, Voigt CC, Vargas A, Görz F, et al. Pregnancy diagnosis in urine of Iberian lynx (*Lynx pardinus*). *Theriogenology* 2009;71:754–61.
- [11] Jeffcoate IA, England GC. Urinary LH, plasma LH and progesterone and their clinical correlates in the periovarian period of domestic bitches. *J Reprod Fertil Suppl* 1997;51:267–75.
- [12] Armstrong EG, Ehrlich PH, Birken S, Schlatterer JP, Siris E, Hembree WC, et al. Use of a highly sensitive and specific immunoradiometric assay for detection of human chorionic gonadotropin in urine of normal, nonpregnant, and pregnant individuals. *J Clin Endocrinol Metab* 1984;59:867–74.
- [13] de Haas van Dorsser FJ, Swanson WF, Lasano S, Steinetz BG. Development, validation, and application of a urinary relaxin radioimmunoassay for the diagnosis and monitoring of pregnancy in felids. *Biol Reprod* 2006;74:1090–5.
- [14] Finkenwirth C, Jewgenow K, Meyer HH, Vargas A, Dehnhard M. PGFM (13,14-dihydro-15-keto-PGF(2α)) in pregnant and pseudo-pregnant Iberian lynx: A new noninvasive pregnancy marker for felid species. *Theriogenology* 2010;73:530–40.
- [15] Basu S. Novel cyclooxygenase-catalyzed bioactive prostaglandin F<sub>2α</sub> from physiology to new principles in inflammation. *Med Res Rev* 2007;27:435–68.
- [16] McCracken JA, Custer EE, Lamsa JC. Luteolysis: A neuroendocrine-mediated event. *Physiol Rev* 1999;79:263–323.
- [17] Hori T, Akikawa T, Kawakami E, Tsutsui T. Effects of administration of prostagrandin F(2)(α)-analogue fenprostalene

- on canine corpus luteum and subsequent recurrence of estrus and fecundity. *J Vet Med Sci* 2002;64:807–11.
- [18] Luz MR, Bertan CM, Binelli M, Lopes MD. Plasma concentrations of 13,14-dihydro-15-keto prostaglandin F<sub>2</sub>-alpha (PGFM), progesterone and estradiol in pregnant and nonpregnant diestrus cross-bred bitches. *Theriogenology* 2006;66:1436–41.
- [19] Johnson WE, Eizirik E, Pecon-Slattey J, Murphy WJ, Antunes A, Teeling E, et al. The late Miocene radiation of modern Felidae: A genetic assessment. *Science* 2006;311:73–7.
- [20] Crosier AE, Comizzoli P, Baker T, Davidson A, Munson L, Howard J, et al. Increasing Age Influences Uterine Integrity, but not Ovarian Function or oocyte Quality in the Cheetah (*Acinonyx jubatus*). *Biol Reprod* 2011.
- [21] Brown JL, Wildt DE, Wielebnowski N, Goodrowe KL, Graham LH, Wells S, et al. Reproductive activity in captive female cheetahs (*Acinonyx jubatus*) assessed by faecal steroids. *J Reprod Fertil* 1996;106:337–46.
- [22] Schlegel W, Urdinola J, Schneider HP. Radioimmunoassay for 13, 14-dihydro-15-ketoprostaglandin F<sub>2</sub> alpha and its application in normo- and anovulatory women. *Acta Endocrinol* 1982;100:98–104.
- [23] Herrick JR, Bond JB, Campbell M, Levens G, Moore T, Benson K, et al. Fecal endocrine profiles and ejaculate traits in black-footed cats (*Felis nigripes*) and sand cats (*Felis margarita*). *Gen Comp Endocrinol* 2010;165:204–14.
- [24] Brown JL, Graham LH, Wu JM, Collins D, Swanson WF. Reproductive endocrine responses to photoperiod and exogenous gonadotropins in the Pallas' cat (*Otocolobus manul*). *Zoo Biol* 2002;21:347–64.
- [25] Fanson KV, Wielebnowski NC, Shenk TM, Vashon JH, Squires JR, Lucas JR. Patterns of ovarian and luteal activity in captive and wild Canada lynx (*Lynx canadensis*). *Gen Comp Endocrinol* 2010;169:217–24.
- [26] Sato M, Tsubota T, Komatsu T, Watanabe G, Taya K, Murase T, et al. Changes in sex steroids, gonadotropins, prolactin, and inhibin in pregnant and nonpregnant Japanese black bears (*Ursus thibetanus japonicus*). *Biol Reprod* 2001;65:1006–13.
- [27] Günzel-Apel AR, Zabel S, Bunck CF, Dieleman SJ, Einspanier A, Hoppen HO. Concentrations of progesterone, prolactin and relaxin in the luteal phase and pregnancy in normal and short-cycling German Shepherd dogs. *Theriogenology* 2006;66:1431–5.
- [28] Tauson AH. Prolactin profiles of pregnant, lactating and nonmated female mink (*Mustela vison*). *J Reprod Fertil Suppl* 1997;51:195–201.
- [29] Brown JL. Female reproductive cycles of wild female felids. *Anim Reprod Sci* 2011;124:155–62.
- [30] Sarkar M, Das BC, Dutta Borah BK, Prakash BS. Plasma concentrations of 13, 14-dihydro-15-keto-prostaglandin F<sub>2</sub>alpha, progesterone and cortisol during periparturient period in yaks (*Poephagus grunniens*. L.). *Reprod Domest Anim* 2010;45:433–438.
- [31] Luz MR, Bertan CM, Binelli M, Lopes MD. Plasma concentrations of 13,14-dihydro-15-keto prostaglandin F<sub>2</sub>-alpha (PGFM), progesterone and estradiol in pregnant and nonpregnant diestrus cross-bred bitches. *Theriogenology* 2006;66:1436–41.
- [32] Verstegen JP, Onclin K, Silva LD, Donnay I. Abortion induction in the cat using prostaglandin F<sub>2</sub> alpha and a new anti-prolactinic agent, cabergoline. *J Reprod Fertil Suppl* 1993;47:411–7.
- [33] Wildt DE, Panko WB, Seager SW. Effect of prostaglandin F<sub>2</sub> alpha on endocrine-ovarian function in the domestic cat. *Prostaglandins* 1979;18:883–92.
- [34] Knospe C. Periods and stages of the prenatal development of the domestic cat. *Anat Histol Embryol* 2002;31:37–51.
- [35] Zerani M, Catone G, Quassinti L, Maccari E, Bramucci M, Gobbetti A, et al. In vitro effects of gonadotropin-releasing hormone (GnRH) on Leydig cells of adult alpaca (*Lama pacos*) testis: GnRH receptor immunolocalization, testosterone and prostaglandin synthesis, and cyclooxygenase activities. *Domest Anim Endocrinol* 2011;40:51–9.
- [36] Akinlosotu BA, Guraya G. Regulation of in vitro progesterone release from caprine luteal tissues by prostaglandins E<sub>2</sub> and F<sub>2a</sub>. *Theriogenology* 1992;38:63–71.
- [37] Verhage HG, Beamer NB, Brenner RM. Plasma levels of estradiol and progesterone in the cat during polyestrus, pregnancy and pseudopregnancy. *Biol Reprod* 1976;14:579–85.
- [38] Verstegen JP, Onclin K, Silva LD, Wouters-Ballman P, Delahaut P, Ectors F. Regulation of progesterone during pregnancy in the cat: Studies on the roles of corpora lutea, placenta and prolactin secretion. *J Reprod Fertil Suppl* 1993;47:165–73.
- [39] Siemienuch MJ, Mlynarczyk JJ, Skarzynski DJ, Okuda K. Possible involvement of oxytocin and its receptor in the local regulation of prostaglandin secretion in the cat endometrium. *Anim Reprod Sci* 2011;123:89–97.