Available online at www.sciencedirect.com
ScienceDirect
Theriogenology 71 (2009) 754–761
Theriogenology
www.theriojournal.com

Pregnancy diagnosis in urine of Iberian lynx (*Lynx pardinus*)

B.C. Braun [a,*], A. Frank [a], M. Dehnhard [a], C.C. Voigt [a], A. Vargas [b], F. Göritz [a], K. Jewgenow [a]

[a] *Leibniz-Institute for Zoo and Wildlife Research, PF601103, D-10252 Berlin, Germany*
[b] *El Acebuche Iberian Lynx Captive Breeding Center, ILCBC, Doñana, Matalascañas, Huelva 21760, Spain*

Received 23 January 2008; received in revised form 23 September 2008; accepted 27 September 2008

Abstract

Diagnosis of pregnancies is an important management tool for the Iberian lynx Conservation Breeding Program, a program geared to recover the world's most endangered felid. Non-invasive methods such as fecal hormone analyses are not applicable to the lynx, since fecal progestin does not follow the typical pregnancy pattern of felids. Therefore, we aimed to test whether urine can be used as an alternative substance for pregnancy diagnosis in the Iberian lynx.

Progesterone immunoreactive metabolites were determined in urine samples of pregnant and non-pregnant females before and during breeding season. Additionally, we used the Witness® Relaxin test to determine relaxin in blood and urine. No differences were found in progestin concentrations determined in urine samples collected from pregnant and non-pregnant animals between day 1 and 65 following mating. Although the Witness® Relaxin test was positive in serum samples collected from animals between day 32 and 56 of pregnancy, it failed in both fresh and frozen urine samples collected from the same stage of pregnancy. A weak relaxin reaction in urine samples collected from animals between day 29 and 46 of pregnancy was detectable after urines were concentrated by ultrafiltration (>50×). Concentrated samples obtained from non-pregnant and early pregnant animals yielded negative test results. In conclusion, the Witness® Relaxin test can be applied for pregnancy diagnosis in Iberian lynx in both serum and concentrated urine samples obtained during the second half of pregnancy. A positive relaxin test indicates an ongoing pregnancy, whereas negative tests must be judged carefully as hormone concentrations might be below detection thresholds.

Keywords: Relaxin; Lynx; Pregnancy diagnosis; Urine

1. Introduction

The Iberian lynx is the world's most endangered wild felid [1]. Today, this felid species survives in three isolated populations located in the region of Andalusia ($n = 2$) and Castilla-La Mancha ($n = 1$), Spain. The actual population size consists of approximately 200 animals in the wild and another 55 in captivity. The Iberian lynx Conservation Breeding Program pursues two goals: (1) to maintain a hedge population of 60 lynxes, which will secure the maintenance of 85% of the lynxes' genetic diversity during a 30-year period and (2) to commence with a reintroduction program in 2010 [2,3]. Besides assuring the continuous existence of the Iberian lynx in captivity, the ex-situ program facilitates the study of various aspects of the species' biology and physiology that could not easily be studied in free-ranging animals. For example, it is possible to shed light on reproductive physiology of this highly endangered felid with a special focus on the development of methods that would allow the non-invasive monitoring

* Corresponding author. Tel.: +49 30 5168 616; fax: +49 30 5126 104.
E-mail address: braun@izw-berlin.de (B.C. Braun).

0093-691X/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2008.09.054

of the reproductive status [4]. Pregnancy diagnosis is a particularly important management tool for the captive breeding program.

In several felid species, pregnancy diagnosis based on fecal hormone metabolites analyses has become an almost routine procedure. After successful mating, progesterone concentration increases in the circulation due to the activity of the *corpora lutea*. Towards the end of pregnancy progesterone concentrations decline and drop to baseline at or shortly after parturition [5,6]. This plasma profile of progesterone secretion is mimicked by immunoreactive progesterone metabolites (progestin) in the feces of a variety of cat species [7]. Pseudo-pregnancies (non-pregnant luteal phase) are characterized by a shorter elevation of fecal progestin for about one-half to two-thirds of the gestation length. In tigers, for example fecal progestin concentrations increased after mating, which supports the view that tigers are primarily induced ovulators [8]. The non-pregnant luteal phase was determined to last 34.50 ± 1.85 days in tigers. During pregnancy, fecal progestin concentrations remained elevated for 108 days until parturition. Thus, pregnancy diagnosis in tigers is possibly based on elevated fecal progestin after approximately day 35 of gestation [8].

In contrast, progestin secretion in Iberian lynx feces did not follow the typical pregnancy pattern of felids and made pregnancy diagnosis by fecal progestin irrelevant [9]. There are some indications that the Eurasian [10] and Canada lynx species [11] are characterized by the same patterns, although these two species are not studied as intensively as the Iberian lynx.

Sexual hormone metabolites in urine samples can be used as an alternative to fecal hormone metabolites for tracking reproductive patterns [12]. Steroid hormones are secreted via the kidney into the urine, mainly conjugated as sulphates or glucuronides. Those products are more water-soluble than the parent steroids [13]. Additionally, several peptide hormones like LH, hCG and relaxin have been detected in urine and, thus, can be related to sexual activity or status of pregnancy [14–17]. Recently, de Haas van Dorsser et al. [18] have shown that relaxin is detectable in urine of pregnant domestic cats and leopards (*Panthera pardus*). In domestic cats, the placenta is the major source of relaxin [19]. Serum [20] as well as urinary relaxin [18] increases at the beginning of the second trimester (days 20–25) reaching a plateau and a subsequent decrease 10–15 days before parturition. During the period of elevated urinary relaxin concentrations a bench top kit (Witness® Relaxin) was also successfully applied for pregnancy detection in domestic cats [21].

The aim of the present study was to establish a reliable and non-disturbing pregnancy diagnosis to be applied within the Iberian lynx Conservation Breeding Program. Since the urine collection from captive Iberian lynx was established for camera trapping of free-ranging lynxes and is performed on a regular basis, urinary progestin and relaxin were considered to be used for non-invasive pregnancy diagnosis. Additionally, serum samples collected by blood-sucking bugs [22] from potentially pregnant Iberian lynxes were analyzed for relaxin.

2. Materials and methods

The Iberian lynx Captive Breeding Center ''El Acebuche'' (ILCBC) in Southern Spain manages captive Iberian lynxes. The captive population consisted of 16 animals (7.9) in 2006 and of 29 (11.18) in 2007. The first birth in captivity was achieved in 2005. By 2008, 15 litters had been born, totalling 24 (15.9) surviving cubs and 26 lynxes had been caught in the wild (11.15). Altogether, these 50 animals represent the current Iberian lynx captive population. The animals are kept in separate enclosures (550 m^2).

The investigations presented in this paper based on samples taken at the El Acebuche Captive Breeding Center in Doñana's National Park on females at prime breeding age (more than 2 years old). All females were allowed to mate; the deliveries of cubs or abortions were final indications of pregnancy. Pseudo-pregnancies occur if fertilization failed after successful mating.

2.1. Blood collection and analysis

Blood samples were obtained by applying larval instars of blood-sucking bugs, *Dipetalogaster maximus* (Reduviidae, Heteroptera) [22], in March 2007 and 2008. Five (2007), respectively seven (2008), animals were sampled once. The bugs were placed into containers covered with mesh, which were hidden in cork plates. The lynxes were trained to use cork plates as resting sites. Since all enclosures were equipped with cameras, the time entire span each individual rested on the cork plate was continuously monitored (Fig. 1). After 30–45 min of uninterrupted resting on the plates the bugs were removed. The ingested blood was withdrawn from the bugs as described in Voigt et al. [22] and centrifuged. The serum was subjected to the Witness® Relaxin test following the manufacturer's instructions (see below).

Fig. 1. Iberian lynxes were monitored with cameras during the "Bug Sampling" procedure. On the computer monitor four different houses of the enclosures are visible. The upper panels show resting sites equipped with cork plates. Arrows indicate the wholes for the bug containers. The lower panels show females on the cork plates. The left one is relaxing. The right female is released to the outside enclosure after 30 min move less resting.

2.2. Urine sampling

Urine was collected from captive lynxes by placing homemade collectors in their enclosures at El Acebuche Breeding Centre, Doñana, Spain. Urine collectors consisted of vertical stainless steel plates (60 cm × 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 were collected on a nearly daily basis in 2006 and 2007 and immediately frozen. Frozen samples were shipped to Leibniz-Institute for Zoo and Wildlife Research in Berlin. During the first breeding season, we obtained samples only from three females of which all had mated before and two of them later delivered cubs. In 2007, seven lynxes had mated, of which five became pregnant and one of them gave birth prematurely. Overall, five pregnant, three non-pregnant and one abortive cycle were available for progestin analysis.

For relaxin determination, single fresh and frozen urine samples were obtained in February and March of 2007/2008.

2.3. Progestin determination in urine samples

Urine aliquots of 100 μL were incubated with β-glucuronidase/sulfatase (Roche No. 127698, 11 and 28 mUnits, respectively) at pH 4.8 for 2 h at 37 °C. Following hydrolysis, the samples were extracted twice with 2.5 mL tert-butyl methyl ether/petroleum ether (30/70, v/v). 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 duplicates to assess a coefficient of variation.

To characterize the major urinary progestin metabolites, a 50-μL portion of the urine extract was subjected to a reversed-phase Ultrasep ES100/RP-18/6 μm HPLC column (4 mm × 250 mm, Sepserv, Berlin). Metabolites were separated with a methanol + water mixture (82 + 18) and fractions of 0.33 mL were collected at 20-s intervals and subjected to both, a progesterone [23] and pregnanediol enzyme immunoassay [24].

Both assays were carried out with in-house microtitre plate enzyme immunoassay procedures using

either a commercial progesterone antibody (Sigma P1922, generated in rats to progesterone) and a 4-pregnen-3,20-dione-3-CMO-peroxidase as label or using a pregnanediol antibody generated in raised rabbits to 5β-pregnane-3,20α-diol-3-gluc-BSA and 5β-pregnane-3,20α-diol-3-gluc-peroxidase as label. The cross-reactivity's of the progesterone antibody were as follows: 4-pregnen-3,20-dione (progesterone), 100%; 5α-pregnan-3,20-dione, 31%; 5α-pregnan-3β-ol-20-one, 18%; 5-pregnen-3β-ol-20-one, 12%; 4-pregnen-3α-ol-20-one, 4.2%; <0.1% for 5β-pregnan-3α,20α-diol, 4-pregnen-20α-ol-3-one, 5β-pregnan-3α-ol-20-one, 5α-pregnan-20α-ol-3-one, 5α-pregnan-3α,20α-diol, 5α-pregnan-3β,20α-diol, testosterone, estradiol, and cortisol. Serial dilutions of urinary extracts showed 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 and high concentrations were 5.0 and 5.1% ($n = 10$) and 13.7 and 22.8% ($n = 10$), respectively. The cross-reactivity's of the pregnanediol antibody were as follows: 4-pregnen-20α-ol-3-one, 211%; 5α-pregnane-20α-ol-3-one, 230%; 5α-pregnane-3β,20α-diol, 243%; 5α-pregnane-3α,20α-diol, 100%; <0.1% for 5α-pregnane-3β-ol-20-one, 5β-pregnane-3α-ol-20-one, progesterone, pregnane-3,20-dion,5α-pregnane-3α-ol-20-one. Again serial dilutions of urinary extracts showed parallelism to the steroid standard with no differences in slopes ($p > 0.05$).

Urine samples were analysed for concentrations of creatinine (Cr) and urinary hormone concentrations are expressed as ng/mg creatinine in order to control for differences in urine concentration [25]. In brief, 100 μL of a 1:1 mixture of 0.04 M picric acid and 0.75 M NaOH were added to 50 μL centrifuged urine in microtitre plates. The plates were incubated for 15 min in darkness and measured at 492 nm.

2.4. Statistics

The results of urinary steroid hormone determination were expressed as immunoreactive progesterone in ng per mg creatinine. Data presented as means ± standard deviation (S.D.). It was only possible for one animal to assess the difference between progestin concentrations during and after pregnancy, and also between pregnant and pseudo-pregnant cycles by one-way ANOVA followed by a post hoc Bonferroni test. All statistical tests were based on a 5% level of significance. The statistical procedures were performed with the software program InStat Version 3.0 (GraphPad Software Inc., LaJolla, USA).

2.5. Ultrafiltration of urine samples

Ultrafiltration of urine samples was necessary to concentrate urinary proteins between 3 and 50 kDa, as no relaxin was detected in freshly collected urine samples. In order to concentrate those proteins, 4 mL of a urine sample (stored frozen) was subjected to a microcon YM50 ultrafiltration unit (Millipore, USA) to separate high molecular proteins by centrifugation (14000 × g, 4 °C). According to the manufacturer's manual proteins of high molecular weight (above 50 kDa) are retained by the membrane, whereas proteins of low molecular weight, e.g. relaxin, pass through. The flow through of about 3.8 mL was subjected to a second ultrafiltration step with a centricon YM3 unit (Millipore) separating proteins larger then 3 kDa molecular weight. This centrifugation was performed at 7500 × g, 4 °C until a concentrate of approximately 100–200 μL was achieved. At this stage, relaxin and other 3–50 kDa proteins are supposed to be retained on the membrane. After addition of 1 mL PBS (adjusting neutral pH), the sample was subjected to further centrifugation steps (7500 × g, 4 °C, 30 min), until almost no further volume decrease of the membrane fraction was achieved. The volume of this membrane fraction, which we called concentrated urine, was measured to identify the concentration factor. The initial volumes of the urine samples were reduced 50–100-fold. An aliquot of the concentrate was subjected to the Witness®Relaxin test (see below). Alternatively to microcon YM50 and centricon YM3 units, Amicon Ultra-4 50 K respectively 3 K units (Millipore, USA) can be applied for ultrafiltration.

2.6. Test with Witness®Relaxin (Synbiotics Corporation)

Blood serum samples drawn from bugs were directly subjected to the Witness®Relaxin test according to the manufacturer's manual. In brief, two serum drops were transferred to the sample well followed by two drops of the provided buffer. The test results were read off from the test kits after approximately 15 min. The appearance of a specific relaxin and a control band was judged as an indication of relaxin in blood serum. A missing relaxin band after a prolonged time (1 h) was an indication of a negative test result.

Freshly collected urine samples were processed as described by de Haas van Dorsser et al. [21]. Thus, urine was mixed with an equal amount of lynx blood serum (serum from non-pregnant female Iberian lynx obtained during immobilization in November 2006) before it was

subjected to the Witness®Relaxin Kit. Concentrated urine samples were diluted 1:1 (v/v) with blood serum from a non-pregnant Eurasian lynx or Iberian lynx and 64 μL of the urine-serum mixture (estimated volume of two normal blood drops) were added to the sample well followed by two drops of provided buffer. Positive relaxin signals were normally observed not earlier than 20 min after application; control bands were visible in all tests.

3. Results

Following HPLC metabolite separation of an urine extract, the progesterone assay demonstrated four different immunoreactive progestin metabolites. Three of them were consistent with the elution positions of progesterone, DHP and 5α-P, respectively, with the major proportion (33.5%) corresponding to authentic progesterone. By contrast, the pregnanediol assay revealed a pattern of five different metabolites including a minor proportion (3.1%) of authentic pregnanediol. Therefore all further urine progestin determinations were performed with the progesterone assay.

The progestin concentration in Iberian lynx urine samples was estimated to range between 0.25 and 6 ng progesterone/mg creatinine (Fig. 2). We found a tendency for higher progestin concentrations per mg creatinine during the time of pregnancy (2.8 ± 1.75 ng, $n = 105$) in comparison to before (2.11 ± 0.98 ng, $n = 19$) and after pregnancy (1.96 ± 0.65 ng, $n = 22$), although no significant difference was estimated for individual animals (d.f. = 178, $F = 4.719$, $p > 0.05$). The urinary progestin concentrations varied largely for pregnant and pseudo-pregnant individuals (pregnant: 2.8 ± 1.75 ng, pseudo-pregnant: 2.07 ± 1.23 ng/mg creatinine). Thus, also no difference was found between pregnant and pseudo-pregnant lynx cycles (time interval of 1–65 days after mating). The direct statistical comparison of a pregnant and pseudo-pregnant cycle was possible in only one Iberian lynx (Aura; d.f. = 36; $F = 1.93$, $p > 0.05$). All other animals were either pregnant or non-pregnant during the 2-year study period.

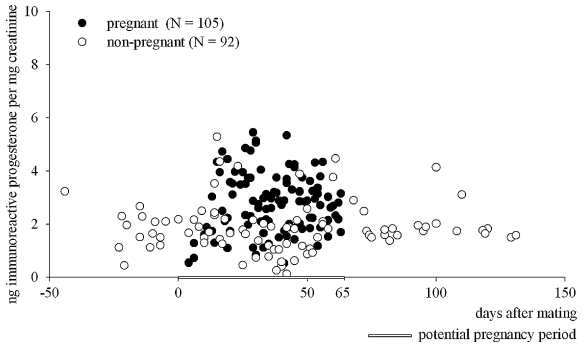

Fig. 2. Determination of immunoreactive progesterone metabolites in urine of Iberian lynx. Before mating and after birth, the progestin concentrations in urine of Iberian lynxes were 2.11 ± 0.98 ng, respectively, 1.96 ± 0.65 ng/mg creatinine. Progestin concentrations tended to increase during the course of pregnancy (1–65 days after mating). Progestin concentrations in sample from pregnant and non-pregnant animals varied largely (pregnant: 2.8 ± 1.75 ng, non-pregnant: 2.07 ± 1.23 ng/mg creatinine).

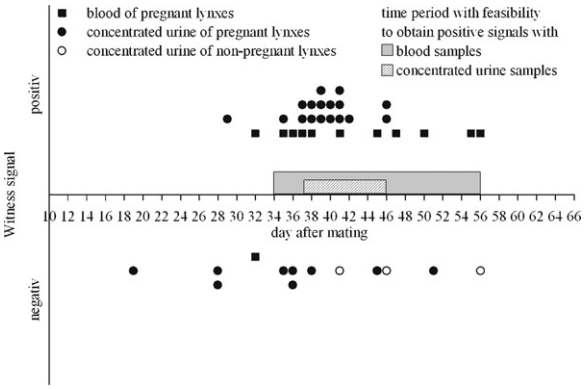

Fig. 3. Witness®Relaxin test results of urine and blood samples of pregnant and non-pregnant Iberian lynxes obtained during second and third trimester of the potential 65-day pregnancy period. Pregnancy of lynxes was diagnosed with Witness®Relaxin test based on blood samples between day 32 and 56 after mating. Positive test results were obtained with concentrated urine samples (>50×) collected from pregnant females between day 29 and 46 post-mating. All urine samples from non-pregnant lynxes were tested negative in this period.

The Witness®Relaxin test (Fig. 3) yielded positive results in serum samples of four out of five animals in 2007 and of all animals tested in 2008. The positively tested animals were all between day 32 and 56 of an ongoing pregnancy. The animal tested negative with the Witness®Relaxin test was at day 32 after her first mating.

Witness®Relaxin test failed in both fresh and frozen urine samples of pregnant animals. Only when urines were concentrated by ultrafiltration at least 50× a weak Relaxin positive reaction was observed between day 29 and 46 of the pregnancy ($n = 17$). No relaxin signal was detected in some occurrences ($n = 5$); these urines were collected on days 35, 36, 38 and 45 from pregnant animals. All concentrated urine samples from non-pregnant and early (before day 29) and late (after day 50) pregnant animals were tested negative (Fig. 3).

4. Discussion

A precise diagnosis of pregnancy will enhance the outcome of the Iberian lynx Conservation Breeding

Program, since an intensive management during birth time is necessary to reduce perinatal losses. Even primiparous, inexperienced Iberian lynx females, experienced a great risk of infanticide and/or rejection of cubs exists.

During the period of pregnancy, anesthesia for blood sampling or ultrasound examination is suspended because of the high risk of abortion. Furthermore, part of the operational procedure aims as much as possible to minimize potential human contact with the lynxes, particularly as the final goal of the Iberian lynx Conservation Breeding Program is to reintroduce lynxes back into the wild. Therefore, pregnancy diagnosis, which avoids any excitements of the potential pregnant lynxes, is necessary. Methods of choice are the measurement of steroid hormones in fecal or urine samples, or applying a non-disturbing blood sampling method via bugs.

In many felids, immunoreactive progesterone can be applied for pregnancy diagnosis, if determined after the second trimester [7]. In the Iberian lynx, however, both fecal progestin [9] and urinary progesterone metabolites [our results] failed to identify a pregnant cycle, although a tendency towards higher progestin concentration was found during pregnancy. Fig. 2 clearly demonstrates that several individual urine sample points were as low as before mating (under 3 ng/mg creatinine). It might be suggested that longitudinal sampling and continuously determination of progestin concentration might allow pregnancy estimation, however this is both time consuming and ultimately not very reliable. More importantly, in the distinction between a pregnant and a non-pregnant luteal cycle, urinary progestin failed. In pseudo-pregnant females mating did not result in a pregnancy, although the *corpora lutea* are fully functional as a result of induced ovulation. *Corpora lutea* produce progesterone, metabolites of which are measurable in urine and not different from a normal pregnant cycle.

The lifetime of pseudo-pregnant *corpora lutea* seems to be longer in lynxes than in other felids. In domestic cats, the luteal activity within pseudo-pregnant cycles drops down at the beginning of the third trimester, making a differentiation possible in this period [5,6]. This was also shown for fecal progestin and in other wild felids [8]. Since progesterone elevation after mating of Iberian lynxes is not connected with the survival of a fetus, a more specific signal, like placental relaxin is necessary for a reliable pregnancy diagnosis.

Placental relaxin is used in domestic cats for pregnancy diagnosis in both blood serum and urine samples [18,19,21]. This peptide hormone serves different functions during pregnancy; e.g. relaxin promotes the softening of the cervix. Relaxin consists of two peptide chains that arise from prohormone processing and are connected by disulfide bridges [26,27]. The relaxin profiles during pregnancy are species-specific [14,20,27–29]. In Iberian lynx the median pregnancy duration ranges between 64 and 65 days [30], which is almost the same duration as in domestic cats [5]. Therefore, and because lynxes are a felid species, we expected a relaxin peak to occur within the same time period in Iberian lynxes as in domestic cats, with highest concentrations between day 30 and 55 [18].

This assumption was confirmed on serum samples taken from the lynxes by blood-sucking *Triatomine* bugs. This minimally invasive approach for blood collection made it possible to diagnose the pregnancy related hormone relaxin with the Witness®Relaxin bench top test very quickly and reliably, if it was used during days 34–56 post-copulation. Despite the fact that blood sampling by bugs was possible in only a few occasions ($n = 12$) our data showed that the relaxin profile during pregnancy of lynxes might be similar to that of domestic cats.

Our tests with serum demonstrated that the antibody of the Witness®Relaxin test cross-reacts with lynx relaxin. Furthermore, the lynx relaxin seemed not to be degraded in the main by the bug ingesta, since it still reacted with the antibody of the test kit.

Applying fresh and frozen urine samples of pregnant Iberian lynxes to the Witness®Relaxin test, however, did not result in a pregnancy diagnosis, as previously shown by de Haas van Dorsser et al. [21] for domestic cats. We speculate that these negative results are attributed to the lower specificity and sensitivity of the Witness®Relaxin antibody for lynx relaxin than for domestic cat relaxin, since the test kit was originally directed at canine relaxin. De Hass van Dorsser et al. also described that the test is much less sensitive with urine than serum samples [21]. In case of lynx urine, the sensitivity of the test kit was reduced to such an extent that a positive immune reaction only registered after a 50-fold concentration by ultrafiltration. In general, the domestic cat and canine relaxin sequences share only 58% identity, but the antibody against canine relaxin also recognizes domestic cat relaxin. The expected close relationship between domestic cats and lynxes allows us to anticipate a similar reaction towards the canine antibody of both feline relaxin hormones. Yet, the requirement of having to concentrate lynx urine shows that either the relaxin concentration in lynx is lower than it is in cats or that the epitope region of the antibody differs more between lynxes and dogs than between the feline and the canine version.

In case of pregnant domestic cats, the Witness®Relaxin test was positively reliable on day 28 [21]. In contrast, the time period for pregnancy detection in lynxes was restricted to days 37–46 post-mating, as between day 29 and 37 both possible test results, positive and negative, were achieved. Additionally, 2 out of 15 samples (Esperanza d38, Brisa d45) failed in the Witness®Relaxin test within the suggested time frame of days 37–46. Samples of Esperanza (2007) gave the first positive signal at day 42 following the lynx's mating, much later than in other pregnant lynxes. Also, this particular animal was tested negative on day 32 in serum collected with bugs. We suggest that Esperanza was not impregnated during first day of mating. Since she copulated over a time period of 2.5 days, day 32 post-mating lies at the border at which Witness®Relaxin tests provide reliable results in lynx. The other outlier – negative Witness®Relaxin at day 45 in a pregnant female (Brisa 2008) – was tested positive on days 41 and 42 after mating. Overall, our results indicate the variability of the range limit between detectable and non-detectable amounts of relaxin for the Witness®Relaxin test and that negative test results have to be judged carefully. At least two concentrated urine samples have to be tested.

Apart from this, a positive relaxin test in samples from Iberian lynx indicates an ongoing pregnancy, whereas negative tests must not be considered as a proof for a missing pregnancy, since the measurable relaxin concentrations might be below the detection threshold of the test kit. Furthermore our results demonstrate that relaxin can be detected with the Witness®Relaxin bench top test within a certain period during pregnancy, which ranges between day 34 and 56 for blood serum and day 37 and 46 for concentrated urine samples, respectively. Although the bug serum sampling seems to be the best and so far most reliable method for pregnancy diagnosis in Iberian lynx, it offers some drawbacks. The increased management efforts it demands as well as animals' cautious acceptability of manipulated resting places allow only occasional sampling. Thus, measurement methods based on urinary relaxin should be developed with the aim to increase the sensitivity. This would make pregnancy diagnosis easier to use at the network of facilities within the Iberian lynx Conservation Breeding Program.

Acknowledgements

We thank all the staff (Fernando Martínez, Toñe Rivas, Juana Bergara, Tasio Vázquez, Juan Pardo, Eva Vázquez, and Javier López) and volunteers at the El Acebuche breeding centre for their every day engagement within the Iberian lynx Conservation Breeding Program. Also we would like to acknowledge Alexandra Geißer and Katrin Paschmionka (IZW) for their excellent technical assistance, Doris Fichte for her help in maintaining the breeding colony of bugs at the IZW, and Prof. Dr. Almuth Einspanier (University Leipzig) for her helpful discussion on relaxin based pregnancy diagnosis in carnivores. We appreciate the support of the Environmental Council of the Junta of Andalusia and the Spanish Ministry of the Environment for their support. We thank Till Schröder for his assistance with the manuscript.

References

[1] Cat Specialist Group. *Lynx pardinus*. In IUCN 2007. 2007 IUCN red list of threatened species. www.iucnredlist.org; 2002.
[2] Vargas A.In: Iberian Lynx ex-situ conservation seminar series: book of proceedings; 2006.p. 5–11.
[3] Vargas A, Sánchez I, Martínez F, Rivas A, Godey JA, Roldán E, et al. The Iberian lynx *Lynx pardinus* conservation breeding program. Int Zoo Yearbook 2008;42(1):190–8.
[4] Jewgenow K, Naidenko SV, Goeritz F, Vargas A, Dehnhard M. Monitoring testicular activity of male Eurasian (*Lynx lynx*) and Iberian (*Lynx pardinus*) lynx by fecal testosterone metabolite measurement. Gen Comp Endocrinol 2006;149(2):151–8.
[5] Schmidt PM, Chakraborty PK, Wildt DE. Ovarian activity, circulating hormones and sexual behavior in the cat. II. Relationships during pregnancy, parturition, lactation and the postpartum estrus. Biol Reprod 1983;28(3):657–71.
[6] Concannon PW, Lein DH. Feline reproduction. In: Kirk RW, editor. Current veterinary therapy VII. Philadelphia/London/Toronto: W.B. Saunders; 1983. p. 932–5.
[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(4):776–86.
[8] Graham LH, Byers AP, Armstrong DL, Loskutoff NM, Swanson WF, Wildt DE, et al. Natural and gonadotropin-induced ovarian activity in tigers (*Panthera tigris*) assessed by fecal steroid analyses. Gen Comp Endocrinol 2006;147(3):362–70.
[9] Pelican K, Abaigar T, Vargas A, Rodriguez JM, Bergara J, Riva A, et al. In: Iberian lynx ex-situ conservation seminar series: book of proceedings; 2006.p. 113–5.
[10] Jewgenow K, Frank A, Göritz F, Vargas A, Naidenko S, Dehnhard M. In: Iberian lynx ex-situ conservation seminar series: book of proceedings; 2006.p. 116–7.
[11] Fanson K.In: Iberian lynx ex-situ conservation seminar series: book of proceedings; 2006.p. 118–9.
[12] Dehnhard M, Hildebrandt TB, Knauf T, Jewgenow K, Kolter L, Göritz F. Comparative endocrine investigations in three bear species based on urinary steroid metabolites and volatiles. Theriogenology 2006;66(6–7):1755–61.
[13] Taylor W. The excretion of steroid hormone metabolites in bile and feces. Vitam Horm 1971;29:201–85.
[14] Steinetz BG, Brown JL, Roth TL, Czekala N. Relaxin concentrations in serum and urine of endangered species: correlations

with physiologic events and use as a marker of pregnancy. Ann N Y Acad Sci 2005;1041:367–78.
[15] Jeffcoate IA, England GC. Urinary LH, plasma LH and progesterone and their clinical correlates in the periovulatory period of domestic bitches. J Reprod Fertil Suppl 1997;51:267–75.
[16] Canfield RE, O'Connor JF, Birken S, Krichevsky A, Wilcox AJ. Development of an assay for a biomarker of pregnancy and early fetal loss. Environ Health Perspect 1987;74:57–66.
[17] Cole LA. Immunoassay of human chorionic gonadotropin, its free subunits, and metabolites. Clin Chem 1997;43(12):2233–43.
[18] 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(6):1090–5.
[19] Addiego LA, Tsutsui T, Stewart DR, Stabenfeldt GH. Determination of the source of immunoreactive relaxin in the cat. Biol Reprod 1987;37(5):1165–9.
[20] Stewart DR, Stabenfeldt GH. Relaxin activity in the pregnant cat. Biol Reprod 1985;32(4):848–54.
[21] de Haas van Dorsser FJ, Lasano S, Steinetz BG. Pregnancy diagnosis in cats using a rapid, bench-top kit to detect relaxin in urine. Reprod Domest Anim 2007;42(1):111–2.
[22] Voigt CC, Fassbender M, Dehnhard M, Wibbelt G, Jewgenow K, Hofer H, et al. Validation of a minimally invasive blood-sampling technique for the analysis of hormones in domestic rabbits, *Oryctolagus cuniculus* (Lagomorpha). Gen Comp Endocrinol 2004;135(1):100–7.
[23] Göritz F, Hildebrandt T, Jewgenow K, Wagner N, Hermes R, Strauß G, et al. Transrectal ultrasonographic examination of the female urogenital tract in nonpregnant and pregnant captive bears (Ursidae). J Reprod Fertil Suppl 1997;51: 303–12.
[24] Meyer HHD, Rohleder M, Streich WJ, Goltenboth R, Ochs A. Sexual steroid profiles and ovarian activities of the female panda YAN YAN in the Zoo of Berlin. Berl Munch Ticrarztl Wschr 1997;110:143–7.
[25] Heistermann M, Trohorsch B, Hodges JK. Assessment of ovarian function in the African elephant (*Loxodonta africana*) by measurement of 5a-reduced progesterone metabolites in serum and urine. Zoo Biol 1997;16:273–84.
[26] Bryant-Greenwood GD, Schwabe C. Human relaxins: chemistry and biology. Endocr Rev 1994;15(1):5–26.
[27] Sherwood OD. Relaxin's physiological roles and other diverse actions. Endocr Rev 2004;25(2):205–34.
[28] Einspanier A, Nubbemeyer R, Schlote S, Schumacher M, Ivell R, Fuhrmann K, et al. Relaxin in the marmoset monkey: secretion pattern in the ovarian cycle and early pregnancy. Biol Reprod 1999;61(2):512–20.
[29] Steinetz BG, Büllesbach EE, Goldsmith LT, Schwabe C, Lust G. Use of synthetic canine relaxin to develop a rapid homologous radioimmunoassay. Biol Reprod 1996;54(6):1252–60.
[30] Vargas A, Martinez F, Bergara J, Rodriguez JM, Rivas A, Rodriguez D, et al. In: Iberian lynx ex-situ conservation seminar series: book of proceedings; 2006.p. 75–88.