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Unusual gonadal hormone profiles in the Iberian lynx as determined by fecal monitoring

Perfiles poco habituales de hormonas gonadales en el lince ibérico según análisis de muestras fecales

Katharine M. Pelican, Teresa Abaigar, Astrid Vargas, José Manuel Rodríguez, Juana Bergara, Javier López, Anastasio Vázquez, Juan Matías Chaparro, Janine Brown and David E. Wildt

Resumen

El lince ibérico *(Lynx pardinus)* es una especie en peligro crítico que se reproduce de forma estacional en el medio silvestre, y cuyos cachorros nacen en primavera. Se dispone de muy poca información sobre la función endocrina en esta especie. El análisis de hormonas en heces ha sido adaptado con éxito en nueve especies de felinos silvestres para caracterizar su función endocrina normal y se esperaba que esta técnica se pudiera adaptar con facilidad al lince ibérico. Para ello, se trabajó con animales procedentes del centro de cría de El Acebuche, en el Parque Nacional de Doñana (España), y se analizaron muestras fecales obtenidas a diario de hembras adultas (n=4) y machos adultos (n=4) entre abril de 2004 y junio de 2006, utilizando inmunoensayos enzimáticos validados para el lince ibérico.

Todas las hembras mostraron pronunciados cambios estacionales en los niveles de estrógenos, con concentraciones superiores a los valores de referencia a partir del mes de enero y disminuciones hasta los niveles más bajos anuales entre los meses de mayo y agosto. Las hembras también mostraron un aumento en las concentraciones de estrógenos antes o durante las cópulas en seis de siete eventos reproductivos. En cambio, se observó que las fluctuaciones en las concentraciones de los metabolitos de los progestágenos no se correspondían con la temporada de cría, sino que disminuían ligeramente entre octubre y diciembre para aumentar de nuevo en enero. No se observaron diferencias en los patrones de estrógenos o progestágenos entre hembras de lince preñadas y hembras que habían copulado sin quedar preñadas. En los machos, se observó una estacionalidad moderada, con las mayores concentraciones de andrógenos en heces entre diciembre y junio, aunque los niveles eran lo suficientemente elevados en todos los meses para respaldar la posibilidad de producción de semen a lo largo de todo el año. Los resultados confirman que la estacionalidad reproductora en la hembra de lince ibérico se puede demostrar mediante la observación de cambios en la excreción de metabolitos de los estrógenos en las heces. Los machos sólo muestran una leve estacionalidad en las hormonas gonadales, lo cual parece concordar con su capacidad de engendrar progenie a lo largo de todo el año. A diferencia de los análisis de estrógenos, los progestágenos en heces no son buenos indicadores del

estado reproductivo en el lince ibérico, dado que los metabolitos 1) mantienen concentraciones altas durante más de nueve meses al año, y 2) no muestran un aumento claro durante la gestación. Por lo tanto, el análisis de hormonas en heces es menos informativo en el lince ibérico que en otros felinos ya estudiados.

Palabras clave
Hormonas fecales, estacionalidad, cría en cautividad, felino, reproducción

Abstract
The critically endangered Iberian lynx *(Lynx pardinus)* is a seasonal breeder in the wild, with cubs born in the spring. There is minimal information available on endocrine function in this species. Fecal hormone monitoring previously has been adapted successfully to nine wild felid species to characterize normative endocrine function. Our expectation was that this technique could be easily adapted to the Iberian lynx. The source of study animals was the El Acebuche breeding population within the Doñana National Park, Spain. Daily fecal samples collected from April 2004 through June 2006 from adult females (n=4) and males (n=4) were analyzed using enzyme-immunoassays validated for the Iberian lynx. All females showed marked seasonal changes in estrogen metabolites with concentrations increasing above baseline in January and declining to nadir from May through August. Females also exhibited increased estrogen concentrations before or during copulation in six of seven breeding events. In contrast, fluctuations in progestogen metabolite concentrations did not correspond to the breeding season, but rather decreased slightly from October through December before increasing again in January. There was no difference in either estrogen or progestogen patterns between pregnant lynx and females that copulated but failed to conceive. Males showed modest seasonality with the highest fecal androgen concentrations measured from December through June, although levels were sufficiently high in all months to support the possibility of year-long sperm production. Results confirm that reproductive seasonality in the female Iberian lynx can be affirmed by changes in fecal estrogen metabolite excretion. Males show only mild gonadal hormone seasonality, which appears consistent with the ability to produce offspring throughout the year. Contrary to estrogen analyses, fecal progestogen is a poor indicator of reproductive status in the Iberian lynx as metabolites 1) are sustained at high concentrations for more than nine months of the year and 2) fail to show clear elevations during pregnancy. Thus, fecal hormone monitoring is less informative in the Iberian lynx than in previously studied felids.

Keywords
Fecal hormone, seasonality, captive breeding, felid, reproduction

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Introduction

The Iberian lynx is the world's rarest felid species, being listed as critically endangered by the IUCN-World Conservation Union (IUCN, 2008). As a component of current recovery efforts, an *ex situ* management programme was established to develop a reservoir population to produce animals for eventual reintroduction. However, these animals also are invaluable for developing a base of biological information for this rare species, including in the area of reproductive physiology and endocrinology.

Detailed data on the reproductive biology of the Iberian lynx is lacking. The species is well known to be a seasonal breeder that generally produces one to three cubs from March through June after a gestation of ~60 Days (Palomares et al., 2005). A powerful tool for generating knowledge about the endocrinology of reproduction is monitoring hormonal metabolites in voided feces. This technology has revolutionized our understanding of the similarities and differences in reproductive mechanisms within the diverse species of the family Felidae (Brown, 2006; Brown et al., 2001). To date, fecal hormone monitoring techniques have been validated and proven effective for assessing reproduction and stress status in diverse felid species, ranging from the Pallas' cat (Brown et al., 2005) to the tiger (Graham et al., 2006) (see review, Brown, 2006; Brown, this book). Especially important has been understanding the fundamental reproductive biology of species to allow enhanced natural breeding management or the development of artificial insemination or *in vitro* fertilization/embryo transfer (Brown et al., 1995; Pelican et al., 2008; Swanson et al., 1996). This monitoring approach also has been applied successfully to certain free-ranging wild carnivores, including for studying how social dominance and dynamics alter stress response and survival in Kalahari meerkats (Young et al., 2007) and determining stress hormone fluctuations associated with radiocollaring African wild dogs (Creel et al., 1997).

Thus, a logical step in the recovery of the Iberian lynx was to apply this previously successful hormonal tracking technology to understand the dynamics of seasonality in both sexes and the specifics of the female's reproductive cycle and pregnant luteal phase. Our findings also complimented a parallel investigation by colleagues who compared endocrine profiles in the male Iberian lynx to those of the closely related Eurasian lynx *(Lynx lynx)* (Dehnhard et al., 2008). In that study, there was a tendency for fecal testosterone concentrations

to be slightly elevated in both species during the months of March and April, which coincided with the presumed breeding season and the peak in sperm production in the Eurasian lynx (Jewgenow et al., 2006). Given these preliminary findings, and because our laboratory had previously used non-invasive hormonal monitoring successfully in eight other felid species (Brown et al., 1994; 1995; 1996a; 1996b; 2002; Brown and Wildt, 1997; Moreira et al., 2001; Swanson et al., 1996), we proceeded to study gonadal hormone profiles in Iberian lynx at the El Acebuche Center. The goal was to document seasonal hormone patterns, identify normative estrous cycle patterns and determine if there were differences in excretion patterns between pregnant and copulating, but non-pregnant females.

Materials and methods

Animals and fecal sampling

Fresh fecal samples were collected from the ground of individual enclosures of four adult females (three to 14 years at study onset) and four adult males (1.5 to three years) from April 2004 through June 2006. Each sample was placed in a labeled plastic bag and stored at -20 ºC until analysis. All animals were wild-caught, six from the Sierra Morena Mountains and two from Doñana National Park. Diets were comprised of live rabbit (85%), quail, ungulate meat and dead rabbit. Male and female lynxes were kept in separate, but adjacent enclosures until breeding season onset. Breeding pairs were established taking into account genetic and behavioral factors (Vargas et al., this book; Fernández et al., this book). Once the selected pairs were proven to be compatible (Figure 1) they were allowed to remain together for as long as possible after copulation, but never longer than two weeks prior to the expected day of parturition. Husbandry procedures during the breeding season at El Acebuche Breeding Center are described in Vargas et al. (2005).

Fecal processing and steroid extraction

Whole fecal samples (2004-2005) or steroid hormone extracts (2005-2006) were shipped frozen from Spain to the Conservation & Research Center of the Smithsonian's National Zoological Park for endocrine evaluation. Samples in 2004-2005 were processed so that hormonal metabolites were extracted from lyophilized feces, whereas wet feces were used in 2005-2006. Steroids were extracted by following a standardized protocol that has been highly effective in previous felids studies (Brown et al., 1994). In brief, each sample was mixed thoroughly and 0.18 to 0.22 g of fecal material vortexed in 5 ml of an ethanol: water (90:10) solution for 30 min. After centrifugation (500 x g, 20 min), the supernatant was recovered and the pellet resuspended in 5 ml of

Photo: Antonio Rivas

Figure 1. One of the Iberian lynx breeding pairs featured in this study.

Figura 1. Una de las parejas reproductoras objeto del presente estudio.

90% ethanol, vortexed and re-centrifuged. Combined supernatants then were dried completely under air and re-dissolved in 1 ml methanol. Each extractant was vortexed for 1 min and sonicated for 20 min. The extract was diluted in dilution buffer and stored frozen until analyzed by enzyme immunoassay (EIA) to quantify estrogen and progesterone (female) or testosterone (male) metabolites.

Specifics for hormonal determination followed the EIA procedures of Munro et al. (1991) and previous detailed studies of felids from our laboratory. Antibodies for progesterone (monoclonal progesterone antibody CL425, 1:10,000 dilution), estrogen (polyclonal estradiol antibody R4972, 1:10,000 dilution) and testosterone (polyclonal testosterone antibody R-156/7, 1:7,500 dilutions) were provided by Coralie Munro (University of California, Davis, CA, USA). The CL425 cross-reacted with various progesterone metabolites, including 4-pregnen-3,20-dione (100%), 4-pregnen-3 α -ol-20-one (188%), 4-pregnen-3 β -ol-20-one (172%), 4-pregnen-11 α -ol-3,20-dione (147%), 5 α -pregnan-3 β -ol-20-one (94%), 5 α -pregnan-3 β ,20-dione (64%), 5 α -pregnan-3,20-dione (55%), 5 β -pregnan-3 β -ol-20-one (12.5%), 5-pregnan-3,20-dione (8.0%), 4-pregnen-11 β -ol-3,20- dione (2.7%) and 5 β -pregnan-3 α -ol-20-one (2.5%) (Graham et al., 2001). The R4972 cross-reacted with estradiol 17 β (100%) and estrone (3.3%). The R-156/7 cross-reacted with testosterone (100%) and 5 α -dihydrotestosterone (57.4%). Before analysis, fecal extracts were diluted in dilution buffer (1:10 to 1:1,400 for estrogens, 1:100 to 1:48,000 for progesterone and 1:20 for testosterone). Each enzyme-immunoassay was validated for Iberian lynx by demonstrating: 1) parallelism ($P<0.05$) between binding inhibition curves of serial dilutions of pooled fecal extracts and the appropriate steroid standard (Fig. 2); 2) accuracy of enzyme-immunoassays for fecal steroids, and 3) significant recovery of exogenous steroid added to fecal samples. Mean ± standard error of the mean extraction efficiency was 83.1 ± 0.004% as determined by recovery of ^{3}H-estradiol and ^{14}C-progesterone, or ^{3}H-testosterone added to feces before extraction. There was no difference ($P>0.05$) between extraction efficiency in wet versus dry extraction methods. Inter-assay and intra-assay variation (CV) was ≤15% and 10%, respectively. Absorbance was measured at 405 nm with an automatic microtiter plate spectrophotometer. Hormone concentrations were expressed as ng/g feces. Endocrine patterns were compared to reproductive behaviors, including onset of estrus and mating in females and subsequent parturition in pregnant individuals.

Data analysis

To determine parallelism between standards and samples, a Pearson correlation analysis was performed. To determine the breeding-associated rise in estrogen, baseline estradiol concentrations were calculated using an

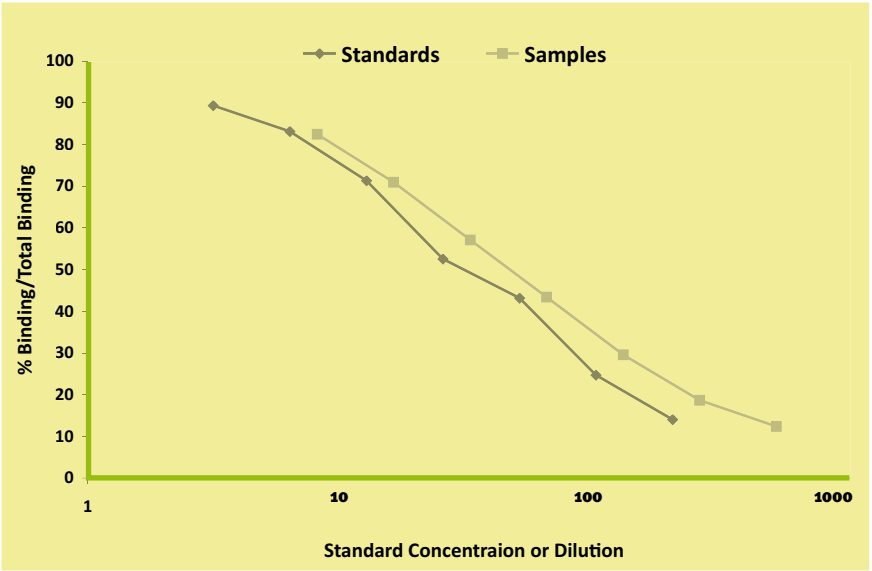

Figure 2. Parallelism ($P<0.05$) between the pregnane enzyme immuno assay and a serially diluted pooled fecal extract from female Iberian lynx.

Figura 2. Paralelismo ($P<0.05$) entre el enzimoinmunoensayo de la pregnona y una dilución seriada de un extracto conjunto de heces de hembra de lince ibérico.

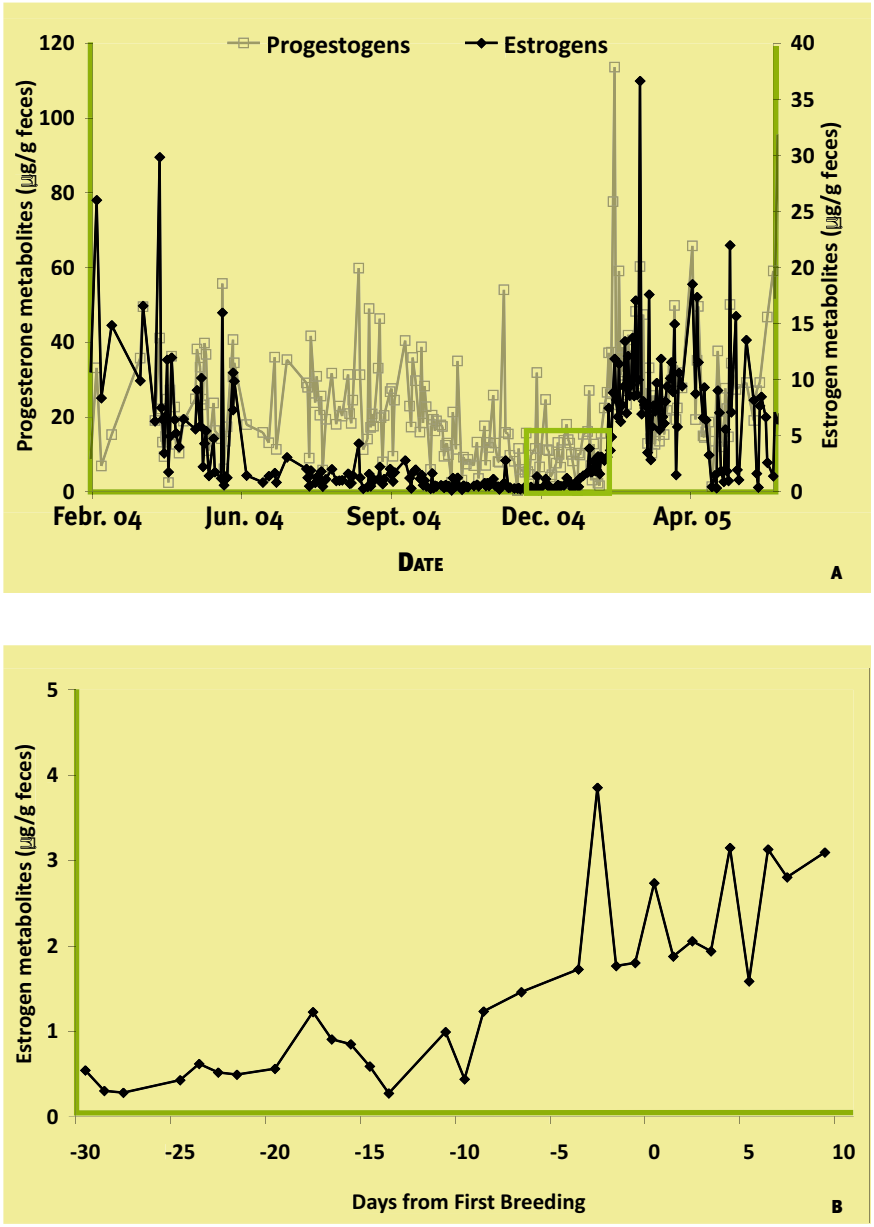

Figure 3. (A) Representative fecal hormone profiles from a female Iberian lynx, illustrating the clear and marked seasonal increase in estrogen excretion (solid diamonds). In contrast, progestogen (open squares) production generally remained elevated throughout the year, with the exception of lower concentrations during late fall and early winter. (B) A magnified view of the boxed area in profile "A" demonstrating the peri-copulatory increase in estrogen excretion in the same female in January 2005. Note the difference in scale between the two profiles. Asterisk represents the first day of estrogen metabolite elevation above baseline. Day 0 is the day of copulation.

Figura 3. (A) Perfiles representativos de hormonas fecales de una hembra de lince ibérico en los que se observa un aumento estacional claramente marcado en la excreción de estrógenos (rombos sólidos). En comparación, la producción de progestágenos (cuadrados abiertos) generalmente se mantiene en un nivel elevado durante todo el año, salvo por las concentraciones más bajas observadas a final de otoño y principios de invierno. (B) Imagen aumentada del recuadro del perfil hormonal "A" que muestra un aumento peri-copulatorio de la excreción de estrógenos en la misma hembra en enero de 2005. Obsérvese la diferencia en escala entre los dos perfiles. El asterisco representa el primer día de elevación del metabolito de estrógeno respecto de la línea basal. El día 0 corresponde al día de la copulación.

iterative process in which values that exceeded two standard deviations (SD) above the mean were excluded. The average then was recalculated and the elimination process repeated until no values exceeded the mean plus two SD (Brown et al., 1994). The average of the remaining values was considered "baseline" for that animal. Values greater than twice the SD were considered "elevated".

Results

When estrogen metabolite profiles were plotted in individual female Iberian lynx, there was a trend for increased excretion to occur during the breeding season (January through June) (Figure 3a for a representative female). Compared to other times of the year, females produced more estrogen beginning in January or February with peaks occurring in January through June followed by a return to baseline from May through August, which then remained at nadir through the end of the year (Figure 3a). During this study, the only recorded estrous behavior

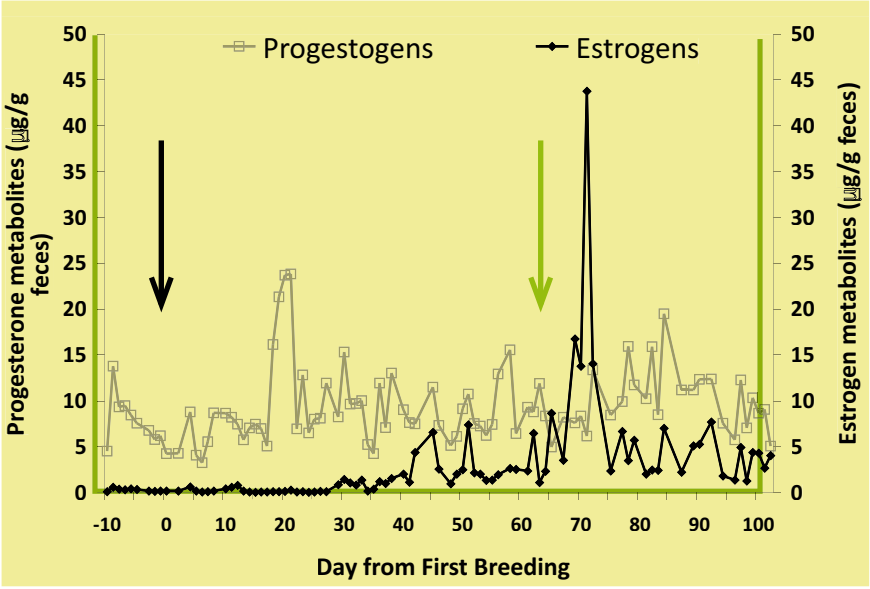

Figure 4. Representative fecal hormone profile in a pregnant Iberian lynx. Black arrow represents the day of first breeding, with a lack of a discernible estrogen surge or a sustained post-ovulatory rise in progestogens. Interestingly, a distinctive estrogen surge occurred after parturition (designated by the green arrow).

Figura 4. Perfil representativo de las hormonas fecales de una hembra gestante de lince ibérico. La flecha negra representa el primer día de apareamiento; no se observa un aumento apreciable de estrógenos ni un incremento sostenido de los progestágenos después de la ovulación. Curiosamente, se observa un aumento claro de estrógenos después del parto (señalado con la flecha verde).

(rolling, vocalization, lordosis) occurred at breeding season onset, during the first obvious and sustained rise in estrogen metabolite concentrations. During six of the seven recorded copulation events at the start of the breeding season, there was a pre-mating rise in estrogen metabolites (Figure 3b). However, this estrogen metabolite increase was modest compared to the subsequent seasonal rise (Figure 3a, b). During the latter time, estrogen metabolites increased at least 10-fold above estrual concentrations with no readily distinguishable cyclic pattern. This elevated, rather varied profile occurred in females that became pregnant (n=3) or remainded non-pregnant (n=4). Of the three monitored pregnancies, two subsequent parturitions were followed by a marked short-term increase in estrogen metabolites within 15 days (Figure 4). Although it appeared that this surge might have been associated with a post-partum, lactational estrus, no sexual behavior was observed in either individual, both of which were nursing cubs.

The progesterone assay that has been so effective in monitoring time of ovulation and the duration of the luteal phase in other felid species was essentially uninformative in the Iberian lynx. Increased excretion of progestogen metabolites occurred beginning in January and were sustained most of the year through October when there was a marginal year-end decrease before a rise coincident with next year's breeding season onset (Figure 3a). Confirmed pregnancy with the subsequent birth of cubs and lactation had no discernible impact on the trajectory of progestogen production (Figure 4). Likewise, progestogen patterns were indistinguishable between the pregnant and non-pregnant individuals.

Each adult male produced excretion profiles that reflected a trend towards slightly higher testosterone during the known breeding season (January through May) that was followed by a 10 to 50% decrease during summer and fall months (Figure 5). However, spikes in testosterone metabolite production occurred throughout the year, including during summer months.

Discussion

The Iberian lynx presents unique challenges to its conservation. Its numbers in nature have fallen precipitously due to habitat loss/fragmentation as well as decline of prey (Calzada et al., this book; Calvete, this book). The establishment of a captive breeding programme has not been without controversy, but is proving to be highly successful despite an initially small founder size and the discovery of species oddities such as siblicide (Vargas et al., 2006; 2008; this book). The uniqueness of the species now extends to its gonadal endocrinology. We anticipated a simple project that would easily characterize the extent of seasonality in the female and male

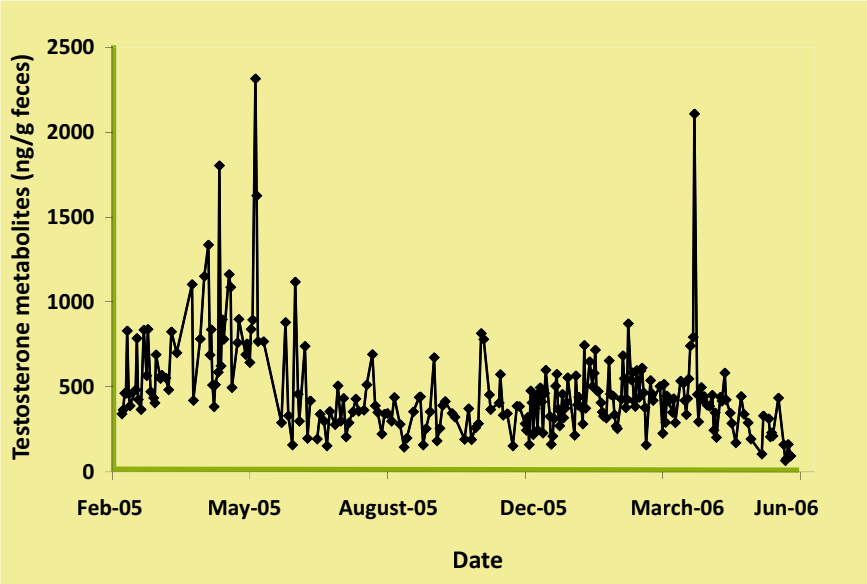

Figure 5. Representative fecal testosterone profile in an adult male Iberian lynx. Although there appeared to be a slight trend indicating a seasonal variation in androgen production in general, excretion of this gonadal hormone did not vary markedly with season.

Figura 5. Perfil representativo de testosterona fecal en un macho de lince ibérico. Aunque aparentemente se muestra una ligera tendencia que indica una variación estacional en la producción general de andrógenos, la excreción de esta hormona gonadal no varió significativamente en las distintas épocas.

Iberian lynx as well as the duration of the ovarian cycle, including tracking corpus luteum activity (the source of progesterone) post-ovulation and during pregnancy. Besides being of scholarly interest, such information has implications for management, including preparing for pairing and separating animals and assembling resources for anticipated births. For example, previous studies have discovered essentially no or marginal seasonality in the margay (Moreira et al., 2001) and tiger (Graham et al., 2006), respectively, in contrast to extremely brief annual periods of sexual activity in the Pallas' cat (Brown et al., 2002; 2006). Still other similar studies in the cheetah have identified frequent (and short) ovarian cycles interrupted inexplicably by periods of complete ovarian quiescence (Brown et al., 1996b). In essentially all cases to date, non-invasive monitoring has been effective at tracking biological activity in both of the major ovarian steroids. Thus, it was surprising to encounter challenges with the Iberian lynx. The conventional estrogen and testosterone EIAs were useful for recognizing seasonal variations in ovarian and testicular activity in this species as well as to identify elevations coincident with estrus. However, there were odd hyper-elevated estrogen patterns post-copulation that require more study. Furthermore, there was a lack of useful information on luteal steroid patterns from these traditional and previously effective assays.

Jewgenow and colleagues recently monitored fecal estrogen and progesterone metabolites in 15 pregnant and seven non-pregnant Eurasian lynx, a closely related, but distinctive species from the Iberian lynx (Dehnhard et al., 2008; Dehnhardt, this book). Eurasian lynx also failed to produce informative progesterone profiles, and metabolite patterns were indistinguishable between pre- and pregnant individuals. Additionally, estrogen profiles in pregnant Eurasian lynx were similar to non-pregnant counterparts. Interestingly, there was close correlation between the estrogen and progesterone patterns over time, with rises in the former failing to be clearly associated with estrus and breeding (Dehnhard et al., 2008). In contrast, we observed an increase in excreted estrogen in the Iberian lynx that coincided with behavioral estrus on six of seven recorded copulatory occasions.

All previous observations, including those associated with the Captive Breeding Programme, have confirmed that the female Iberian lynx is highly seasonal. Our observations indicated that increased sexual activity was associated with a detectable rise in excreted estrogen. More specifically, we observed high estrogen concentrations occurring in these captive animals from March through April, a time which coincided with previous observations of breeding in free-living lynx in the same geographic region (Palomares et al., 2005). In contrast, although there were slight trends for increased testosterone production in males during the breeding season, these elevations were not marked, suggesting that the testes were likely producing significant amounts

of androgen throughout the year. This assertion was consistent with recent observations by Jewgenow et al. (this book) who have demonstrated sperm production of Eurasian lynx via electroejaculation during the non-breeding season.

We also observed overall greater estrogen excretion in females during periods of sexual activity, including copulation, compared to the non-breeding season. With nearly daily fecal collections, it was possible to detect distinctive estrogenic (ovarian) surges at the time of copulatory events. However, these increases were dwarfed by the dramatic estrogen rise observed during the weeks following breeding. These hyper-elevations were not associated with clear cyclic patterns (as described for follicular cycles in other felids) and occurred regardless of the female being pregnant or non-pregnant. This estrogen likely was of ovarian origin as it consistently followed copulations. Although residual ovarian follicles are known to occur in certain felids during pregnancy (Schmidt et al., 1983), the extreme estrogen concentrations found in the Iberian lynx remain inexplicable and require further study. It was interesting to observe a distinctive estrogen surge in two of three nursing females, indicating early post-partum ovarian follicular activity, but in the absence of sexual behavior.

Although there are challenges in interpreting some of the estrogen data, the real enigma was the total lack of value in monitoring fecal progestogen metabolites in the lynx. All females appeared to produce elevated concentrations consistent with onset of the breeding season followed by comparatively high levels until late fall, well beyond the known breeding season.

Numerous studies in other felids have determined that progestogen metabolites are highly reflective of post-ovulatory events, rising soon after ovulation and then remaining sustained for a species-specific duration during pregnancy or, in the case of failed conception, the end of a luteal phase (Brown et al., 1995, 1996b, 2001, 2002; Moreira et al., 2001). In general, the duration of increased progesterone production during gestation is twice as long as during a "pseudopregnancy" (Brown et al., 1995, 1996b, 2001, 2002; Moreira et al., 2001). Until now the only exception has been the Eurasian lynx, a species recently shown to have equivocal fecal progestogen production in pregnant versus non-pregnant individuals (Dehnhard et al., 2008). Perhaps these two species in the lynx genus have evolved different steroid metabolism mechanisms that somehow prevent capturing the progestogen metabolites sequestered in feces that normally are quantifiable by typical EIAs in other felids. However, high performance liquid chromatography (HPLC; after infusing a Eurasian lynx with radiolabelled progesterone) has revealed metabolite profiles no different from those published for other felid species (Brown et al., 1994). Furthermore, the assay antibodies being used have been found to effectively bind to a wide range of progestogen metabolites in the Eurasian lynx (Dehnhard et al., 2008). It also is possible that biologically active progestogen metabolites also are being produced by other sources (e.g., placenta, ovaries, adrenal glands) depending on female reproductive status, and that these similarly crossreact with the EIA antibody masking biologically relevant changes. This is a valid possibility as HPLC during various reproductive stages in the Eurasian lynx have revealed changes in the ratio of polar-to-non-polar progestogen metabolites between the pregnant and lactating female (Dehnhard et al., 2008; Dehnhardt et al., this book). Additionally, ultrasonography during lactation in the Eurasian lynx has indicated the presence of corpora lutea suggestive of long-term sustainability of these structures and/or post-partum ovulation (Göritz et al., this book). The latter option does perhaps relate to our observation of an early lactational estrogen surge, which even may have resulted in spontaneous and "silent" (no sexual behavior) ovulation. The concept of protracted luteal viability or occasional spontaneous corpus luteum formation on the ovaries also would be consistent with our observations of prolonged progesterone elevations into October (Göritz et al., this book). The potential of unusual luteal physiology in both species of lynx compared to other felids warrants more investigation.

In conclusion, although there were some unexpected challenges to adapting non-invasive gonadal hormone monitoring to the Iberian lynx, it was possible to confirm that increases in estrogen metabolite content in feces was reflective of the reproductive season. Furthermore, in general, the evaluation of estrogen metabolites in daily fecal samples correlated with behavioral estrus in most, but not all copulating females. It appeared that male lynx are much less markedly variant in gonadal androgen production throughout the year, which means that reproductive seasonality in this species is more a feature of females. Most interesting is the peculiar lack of distinctive fecal progestogen patterns indicative of unique luteal activity that appears a characteristic of both the Iberian and Eurasian lynx.

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