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Comparative metabolism of gestagens and estrogens in the four lynx species, the Eurasian (*Lynx lynx*), the Iberian (*L. pardinus*), the Canada lynx (*L. canadensis*) and the bobcat (*L. rufus*)

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ABSTRACT

With the increasing prevalence of faecal hormone metabolite analysis, it is important to develop a better understanding of the dynamics of faecal metabolite composition. The aim of this study was to compare the quantitative faecal gestagen and estrogen metabolite composition in the four lynx species: Eurasian lynx, Iberian lynx, Canada lynx and bobcats. Comparative HPLC immunograms were generated from faecal samples collected before, during, and after pregnancy from individual females of each lynx species. Gestagens and estrogens revealed three similar classes of immunoreactive faecal metabolites: (1) polar metabolites which were enzyme-hydrolysable and thus may be designated as conjugates, (2) non-hydrolysable polar metabolites, and (3) non-polar metabolites or free steroids. For both hormones, strong similarities in the HPLC immunograms across species suggests that steroid metabolism is relatively conserved among *Lynx* species. Gestagens were primarily excreted as polar conjugates or unknown metabolites, whereas estrogen metabolism revealed a huge proportion (~50%) consisting of 17β-estradiol and estrone. These results are consistent with patterns of steroid metabolism in other felid species. Only two minor species-specific patterns emerged. In bobcats, we observed an exceptionally high proportion of gestagen conjugates, and in Iberian lynx, there was an exceptionally high proportion of estrone. The comparison of HPLC immunograms within individuals revealed that intra-individual variations in steroid metabolite composition are considerably high. However, changes in metabolite composition did not correlate with specific reproductive stages; rather, they seemed to occur at random. We assume that these differences may reflect changes in liver metabolism and/or qualitative and quantitative variations in gut bacteria composition, resulting in differences in faecal metabolite composition.

1. Introduction

All 36 species of wild cats 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. Most felid species reproduce poorly in captivity, a problem attributed to behavioural incompatibilities, captivity stress, or inappropriate husbandry (Swanson, 2006; Swanson and Brown, 2004). Causes of female reproductive failure are challenging to diagnose because of difficulties analysing the complex endocrine interactions controlling oestrous activity, ovarian function, and conception. Therefore, understanding the endocrine principles of reproduction in felids is essential for their captive breeding management and conservation efforts.

The genus *Lynx* includes four species: Eurasian lynx (*Lynx lynx*), Canada lynx (*Lynx canadensis*), bobcat (*Lynx rufus*), and probably the most endangered felid species in the world, Iberian lynx (*Lynx pardinus*). Eurasian lynx (*L. lynx*) are one of the most widespread felid species, distributed throughout Eurasia, whereas Iberian lynx are the most restricted, with about 150 individuals on the Iberian Peninsula. The Iberian lynx population experienced a drastic decline from 100,000 to 110 individuals during the last century. In 2004, the Spanish government started a large ex-situ breeding programme for Iberian lynx to support the critically endangered wild population. Canada lynx inhabit the northern forests of North America, and bobcats range across southern North America.

All lynx species are solitary and territorial, although inter- and intra-sexual home-range overlaps have been reported. With the exception of bobcats, *Lynx* are strong seasonal breeders. Females are monoestrous, and the duration of the breeding season is only several weeks (Eurasian lynx – Henriksen et al., 2005; Jewgenow

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et al., 2006a; Naidenko and Erofeeva, 2004; Iberian lynx – Vargas et al., 2005; Canada lynx – Fanson, submitted for publication). Although bobcats still exhibit seasonal fluctuations in breeding activity, they are polyestrous and births can occur in any month of the year (Winegarner and Winegarner, 1982), giving bobcats the widest window of reproductive opportunity among *Lynx* species.

In wild felid species, patterns of reproductive hormone expression are generally similar to those described for domestic cats, characterised by several estrogen peaks during oestrus, and a progesterone increase after mating, indicating induced ovulation and corpus luteum formation (Arbeiter, 1994). As with other species, maintenance of pregnancy is dependent on continued secretion of progesterone. In domestic cats, serum progesterone concentrations are similar in pregnant and pseudo-pregnant animals for roughly the first 2–3 weeks after ovulation. After that time, progesterone concentrations decline in pseudo-pregnant cats, but remain elevated in pregnant cats. The main source of progesterone during pregnancy appears to be the corpus luteum. An ovariectomy performed at 45 days of pregnancy led to abortion preceded by a decline in plasma progesterone, suggesting that the placenta is not able to produce enough progesterone to maintain pregnancy (Verstegen et al., 1993a,b).

Conventional methods of obtaining endocrine data in domesticated animals have relied upon analysis of serially collected blood samples. In wild cats, regular blood sampling is impossible since it requires handling, restraint and anaesthesia of animals. Non-invasive methods, such as measuring hormone metabolites excreted in urine or faeces, are potentially attractive alternatives. During the last decade, longitudinal monitoring of excreted estradiol (E2) and progesterone (P4) metabolites has proven effective for characterising oestrous cycles, pregnancy, and seasonal patterns of reproduction in a host of species (see reviews: Brown, 2006; Lasley and Kirkpatrick, 1991; Lasley et al., 1991; Schwarzenberger, 2007). Steroid metabolism studies in domestic cats have demonstrated that steroid excretion occurs mainly via the faeces. The excretory products of radiolabelled estradiol (E2) and progesterone (P4) were found within 2 days in faeces (97.0% and 96.7%, respectively; Brown et al., 1994). E2 was excreted as unconjugated estradiol and estrone (40%) and as a non-enzyme-hydrolysable conjugate (60%). P4 was excreted primarily as non-enzyme-hydrolysable, conjugated metabolites (78%) and as unconjugated pregnenolone epimers (Brown et al., 1994).

This information has facilitated the development of non-invasive methods for assessing the dynamics of gonadal steroid secretion. Results from several wild cat species (e.g., leopard cat [*Prionailurus bengalensis*], cheetah [*Acinonyx jubatus*], clouded leopard [*Neofelis nebulosa*], snow leopard [*Uncia uncia*]) revealed that changes in faecal estrogen and gestagen metabolite concentrations accurately reflect ovarian activity in these species. Oestrus is indicated by a several-fold increase in E2 excretion, and ovulation is confirmed by an increase in P4 metabolite levels, though this does not necessarily indicate pregnancy. Elevated faecal P4 metabolite concentrations occur in both pregnant and pseudo-pregnant luteal phases (Brown et al., 2001). While overall concentrations of faecal gestagens are similar between pregnant and pseudo-pregnant felids, the duration of elevated faecal gestagen metabolites during pseudo-pregnancy is approximately half that observed during pregnancy (Brown et al., 1994).

In the Eurasian lynx, however, gestagen and estrogen patterns do not follow those described in other cat species. Faecal estrogens do not reflect follicular activity peaking around ovulation, and faecal progesterone metabolite profiles reveal only a tendency towards higher levels in pregnant and pseudo-pregnant females. Estrogen levels increase in both pregnant and pseudo-pregnant females with a tendency to be more elevated and prolonged in pregnant females. Surprisingly both E2 and P4 metabolites are highly correlated, showing unexpected post-partum increases. Thus it is impossible to use faecal steroid metabolites to verify pregnancy in the Eurasian lynx (Dehnhard et al., 2008).

However the results from the Eurasian lynx cannot be generalised for the other three lynx species, even if pregnancy monitoring based on faecal gestagen metabolites also failed in the other three lynx species (Iberian lynx, Pelican et al., 2006; Canada lynx, Fanson, submitted for publication; bobcats, Miller, 1995). Route of excretion and steroid metabolism can vary considerably among species, resulting in vast differences in faecal metabolite composition, even among closely related species. Additionally, excretion and metabolism may vary between steroids within the same species. As steroid metabolism mechanisms might be dissimilar among taxonomically related species we aimed to carry out a comparative study to investigate whether the particularities of the Eurasian lynx (Dehnhard et al., 2008) are also representative for the other lynx species.

Our objectives were to extend these observations in detail by (1) identifying the predominant faecal metabolites derived from progesterone (P4) and estradiol (E2) via a radiometabolism study in Eurasian lynx, (2) comparing patterns of immunoreactive gestagen and estrogen metabolites among the four *Lynx* species, and (3) examining changes in faecal metabolite composition across reproductive stages within each species.

2. Materials and methods

2.1. Animals

2.1.1. Eurasian lynx

Ten captive female lynx were housed at the scientific field station "Tchernogolovka" of the A.N. Severtzov Institute, situated 50 km Northeast from Moscow. The animals were kept within six enclosures (74 m^2) and in one large fenced enclosure (7500 m^2) that is part of the natural mixed forest providing a semi-natural environment. Each enclosure had an additional small cage (6–8 m^2 each), where some of the individuals were kept. Animals were housed separately; males and females were just combined for mating (Naidenko and Erofeeva, 2004). The animals reproduced every year with mating in March. Faecal samples were collected weekly throughout a 2-year period from individual animals within the scope of a previous study (Dehnhard et al., 2008) and stored at −20 °C. From February to April (prospective mating season) the frequency of collection was increased to 2–4 times a week.

2.1.2. Iberian lynx

Captive Iberian lynx are managed by the Iberian Lynx Captive Breeding Center (ILCBC) in Southern Spain, which was started in 2004. The captive population currently consists of over 76 animals (36 males and 40 females), divided among four facilities in Southern Spain and Portugal. Half of the ex-situ lynx population is captive born. The faecal samples were obtained from El-Acebuche breeding centre, Huelva, where animals are kept in separate enclosures (1200 m^2). All samples were taken from three prime breeding age females. Collection of faecal samples was carried out twice weekly during 2006. All females were allowed to mate. Two females gave birth; one animal did not deliver a cub, although mating was observed by a round-the-clock video vigilance system. She was considered to be pseudo-pregnant.

2.1.3. Canada lynx

Captive Canada lynx were housed at three institutions in North America: one public zoo (Zoo America, Pennsylvania, USA), and two private breeding facilities (Arkansas, USA and Alberta, CAN).

Each female was housed with a male, and enclosure size ranged from 40 to 150 m^2. Faecal samples were collected 2–3 times weekly from three captive females from February to May. All three females gave birth, but one of the litters was still-born. Dried faecal extracts (see below) were shipped to the IZW and subjected to HPLC analyses as described below.

2.1.4. Bobcat

Two female bobcats (*L. rufus*) were housed at a private breeding facility (Arkansas, USA). Bobcats were housed in male–female pairs, and enclosures (150 m^2) were located in a semi-natural wooded environment. Weekly faecal samples were collected throughout gestation (March to April 2008). Both females gave birth to healthy litters. Dried faecal extracts were shipped to IZW for analysis.

2.2. Radiometabolism study

The radiometabolism studies were performed in two 15-year-old female Eurasian lynx. Solutions (0.25 ml) containing ~250 μCi [3H]progesterone (60–110 Ci/mmol, TRK413, Amersham Bioscience, UK) and ~250 μCi [3H]estradiol (70–120 Ci/mmol, TRK322, Amersham Bioscience, UK), respectively, in ethanol were used. Sterile 0.9% NaCl solution (2.25 ml) was added to the radiolabelled solution and the total volume was injected into the cephalic vein after sedation of the animal as described in a previous study (Dehnhard et al., 2008). Those samples that contained the highest amount of radioactivity were used for HPLC analysis to characterise radiolabelled metabolites.

2.3. Processing faecal samples

Faecal samples (0.5 g) from Eurasian and Iberian lynxes were extracted for 30 min by shaking with 4.5 ml of 90% methanol. After centrifugation (15 min at 1200 g) the supernatant was transferred into a new tube.

Faecal samples (0.5 g) from the Canada lynx and bobcat were extracted overnight on a rotator with 4.5 ml of 80% ethanol and centrifuged (15 min at 1200*g*). From the supernatant, 2 ml were transferred to a separate glass tube, dried down (for shipping) and stored at −20 °C. Prior to analysis, the dried faecal extracts were reconstituted in 0.5 ml of 40% methanol.

Enzymatic hydrolysis of faecal extracts was carried out as described earlier (Jewgenow et al., 2006b).

Faecal samples from different reproductive stages of two pregnant females of each of the four lynx species as indicated in figures and tables were prepared for HPLC analyses as described below.

2.4. HPLC

Prior to HPLC analysis, faecal extracts were purified on Octadecyl C_{18} columns (0.5 ml, J.T. Baker, BAKERBOND SPE™ 7020-01). Crude faecal extracts (diluted to 15% methanol) were applied on C_{18} columns previously conditioned with 2 ml of 100% methanol followed by 2 ml 20 mM Tris-buffer, pH 8.5 + methanol (80 + 20). The columns were washed with 2 ml 20 mM Tris-buffer, pH 8.5 + methanol (80 + 20) and 2 ml water + methanol (80 + 20). Elution was carried out with 1 ml of 100% methanol. The eluates were evaporated in a heater at 55 °C under nitrogen and dissolved in 40% methanol (gestagen metabolites analyses) or 25% acetonitrile (estrogen metabolites analyses).

To characterise faecal steroid metabolites across lynx species and reproductive stages, 100 μl portions of faecal extract were used for HPLC analysis. For gestagen metabolite analysis, a reverse-phase Ultrasep ES100/RP – 18/6 μm HPLC column (4 × 250 mm, Sepserv, Berlin) was used. Metabolites were separated with a methanol + water mixture (78 + 22) at a flow rate of 1 ml/min. Fractions of 0.33 ml were collected at 20 s intervals over a period of 21 min and diluted with 1 volume of water, before 20 μl aliquots were added into the assay systems. The elution positions of authentic progesterone (4-pregnen-3,20-dione; P4), 5α-pregnan-3,20-dione (DHP), and 5α-pregnan-3β-ol-20-one (5α-P) on this column had been previously determined in separate HPLC runs.

For faecal estrogen metabolite separation, a Gemini 5 μm C6–Phenyl column (3 × 150 mm, Phenomenex, Aschaffenburg, Germany) was used. Metabolites were separated with an acetonitrile + water mixture (43 + 57) at a flow rate of 1 ml/min. Fractions of 0.33 ml were collected at 20 s intervals over a period of 19 min, the acetonitrile was removed using a lyophiliser and the fractions were reconstituted in 0.5 ml of 40% methanol before 20 μl of the fractions were added into the assay systems. The elution positions of authentic 1,3,5(10)-estratrien-3-ol-17-one (estrone), 1,3,5(10)-estratrien-3,17α-diol (17α-estradiol), and 1,3,5(10)-estratrien-3,17β-diol (17β-estradiol, all from Sigma) on this column had been determined in separate HPLC runs.

For the radiometabolism experiment, aliquots of 230 μl were used for radioactive counting.

2.5. Enzyme immunoassay (EIA)

Faecal samples were analysed for progesterone using an in-house microtitre plate enzyme immunoassay as described earlier (Göritz et al., 2001; Vogler et al., 2009). The progesterone concentration was quantified by an enzyme immunoassay (EIA) following Prakash et al. (1987) using a polyclonal antibody (rabbit) against 5α-pregnan-3β-ol-20-one-3-HS-BSA and 5α-pregnan-3β-ol-20-one-3-HS-peroxidase label. The cross-reactivities to progestagens tested by the 5α-pregnan-3β-ol-20-one EIA were as follows: 5α-pregnan-3α-ol-20-one, 650%; 5α-pregnan-3β-ol-20-one, 100%; 4-pregnen-3,20-dione (progesterone) 72%; 5α-pregnan-3,20-dione, 22%; <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-3β,20α-diol and 5α-pregnan-3α,20α-diol.

Serial dilutions of a faecal pool were parallel to the steroid standard with no significant difference in slopes ($p > 0.05$). Inter- and intra-assay coefficients of variation for two biological samples were 12.3% ($n = 11$) and 9.0% ($n = 8$), respectively.

Faecal estrogen analyses were carried out with an in-house microtitre plate enzyme immunoassay using a polyclonal antibody (rabbit) against 1,3,5(10)-estratrien-3,17β-diol-17-HS-BSA and 1,3,5(10)-estratrien-3,17β-diol-17-HS-peroxidase label (Meyer et al., 1997; Dehnhard et al., 2006). The cross-reactivities with other steroids were as follows: 1,3,5(10)-estratrien-3,17β-diol (17β-estradiol) 100%, 1,3,5(10)-estratrien-3,17-one (estrone) 100%, 1,3,5(10)-estratrien-3,17α-diol (17α-estradiol) 66%, 1,3,5(10)-estratrien-3,16α,17β-triol (estriol) 1.5%, and <0.1% for 19-nortestosterone, progesterone and testosterone.

Serial dilutions of a faecal pool were parallel to the steroid standard with no significant difference in slopes ($p > 0.05$). Inter- and intra-assay coefficients of variation for two biological samples were 11% ($n = 67$) and 5.6% ($n = 16$), respectively.

2.6. Analysis of data

Immunoreactivity is presented as % of overall eluted progesterone or estrogen activity. To analyse the metabolite composition, HPLC runs were classified into fractions according to the distribution of immunoreactivities and the positions of the steroid standards used for column calibration. Calculated p values <0.05 were considered to be significantly different. The statistical procedures were performed with the software program Instat Version 3 (Graphpad Software Inc, La Jolla, CA, USA).

3. Results

3.1. HPLC analyses of radiolabelled gestagen and estrogen metabolites

The best way to investigate the metabolism and excretion pattern of steroids is using radiolabelled hormones. The excreted metabolites of a particular steroid can be separated by reverse-phase HPLC based on differences in their polarity (Fig. 1A) with the most polar compounds eluting first and the most non-polar compounds eluting last. This approach was not feasible in every lynx species we studied, and therefore was performed only in the Eurasian lynx.

Injection of 3H (tritiated) labelled progesterone into a female Eurasian lynx revealed four major polar radiolabelled gestagen metabolites peaking in fractions 6–8, 10, 13, and 17. Only minor amounts of radiolabelled gestagen metabolites were detectable at positions of non-polar substances similar to progesterone (fraction 22–23), pregnenolone (fraction 31–32), and close to 5α-P (fraction 37–38) previously used for column calibration (data not shown, see Dehnhard et al., 2008). This suggests that in the Eurasian lynx, authentic progesterone is merely present in minor quantities in faeces.

Enzymatic hydrolysis (see Jewgenow et al., 2006b) was carried out with two faecal extracts from Eurasian lynx. This resulted in a reduction in the proportion of polar radiolabelled metabolites in fractions 7–9 by 76% and 80%, respectively, demonstrating that fractions 7–9 mainly contained hydrolysable gestagen metabolites, or conjugates. The polar metabolites in fractions 10 and 13 turned

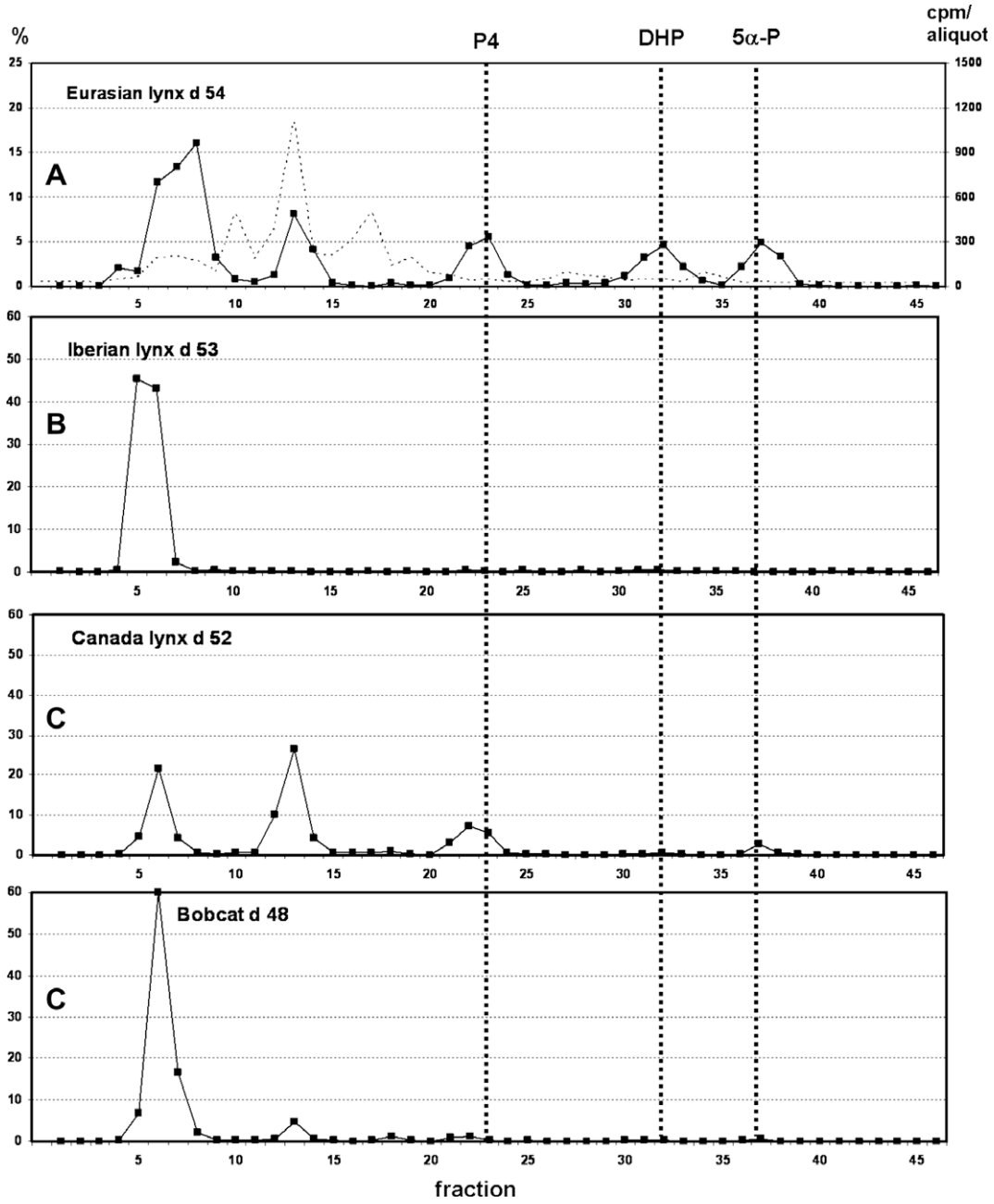

Fig. 1. Elution profile of radiolabelled gestagen metabolites in a Eurasian lynx (A, horizontal dotted line), and immunoreactive gestagen metabolites in another Eurasian lynx (A), an Iberian lynx (B), a Canada lynx (C), and a bobcat (D). Faecal extracts chosen from the second half of pregnancy were subjected to HPLC separation. Vertical dotted lines indicate the elution positions of progesterone (P4), 5α-pregnane-3,20-dione (DHP), and 5α-pregnane-3β-ol-20-one (5α-P). For evaluation of the immunograms see Table 1.

out to be non-hydrolysable (data not shown), and thus are unknown metabolites.

After injection of 3H (tritiated) labelled estradiol into a female Eurasian lynx, two major radioactive peaks of about the same size appeared. One peak consisted of a large proportion of polar metabolites between fractions 2 and 5 (most likely conjugates), and an equal radioactive peak co-eluted with 17β-estradiol in fractions 14 and 15 (Fig. 2A). Additionally, minor proportions of radiolabel appeared as shouldered peaks at fractions 7 and 10 and in fractions 20–21, 24–25 (corresponding to the elution position of estrone) and fraction 28 (Fig. 2A). These results suggest that estradiol and estrone comprised a large portion of the total estrogens excreted.

The chemical nature of both the metabolites other than estradiol and estrone and those of the conjugates remain unknown. This also applies to the gestagen metabolites between fractions 7 and 17 after 3H labelled progesterone injection (Fig. 1A).

3.2. Comparison of radiolabelled and immunoreactive metabolite HPLC profiles

Because it was not possible to conduct a radiometabolism study in each lynx species, it is important to understand how HPLC profiles differ between radiolabelled metabolites and immunoreactive metabolites. Usually the pattern of radiolabelled metabolites and immunoreactive metabolites in HPLC analysis, as measured by the appropriate antibody, do not coincide due to different immunological cross-reactivities of the antibody. Furthermore, while radiolabelled metabolites are derived from a single steroid mole-

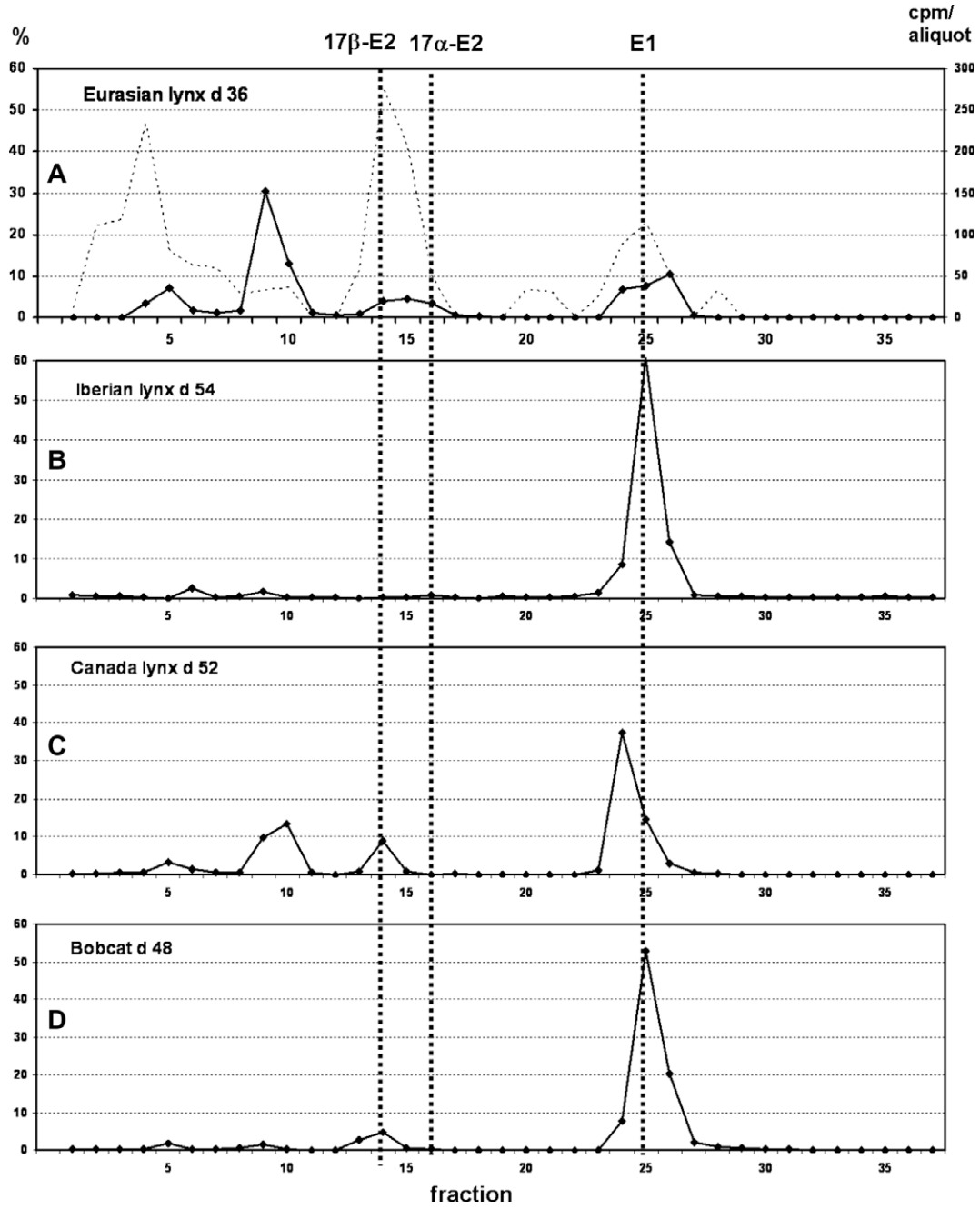

Fig. 2. Elution profile of radiolabelled estrogen metabolites in a Eurasian lynx (A, horizontal dotted line), and immunoreactive estrogen metabolites in another Eurasian lynx (A), an Iberian lynx (B), a Canada lynx (C), and a bobcat (D). Faecal extracts chosen from the second half of pregnancy were subjected to HPLC separation. Vertical dotted lines indicate the elution positions of 17β- and 17α-estradiol (17β-E2, 17α-E2) and estrone (E1). For evaluation of the immunograms see Table 2.

cule, immunoreactive metabolites may be derived from other closely related steroid molecules in circulation.

In Eurasian lynx, HPLC analysis detected four major radiolabelled gestagen metabolites, but five different immunoreactive gestagen metabolites (note that the radiometabolism data and the immunoreactivity data came from two different females; Fig. 1A). Two peaks were consistent with the two major radiolabelled metabolites in fractions 7–9 (conjugates) and fraction 13 (unknown metabolite).

Enzymatic hydrolysis reduced the proportion of the polar radiolabelled metabolites in fractions 7–9 by 80%, demonstrating that fractions 7–9 mainly contained hydrolysable gestagen metabolites, whereas the polar metabolites in fractions 10–13 turned out to be non-hydrolysable (data not shown).

For estrogen metabolites in Eurasian lynx, four metabolites were detected for both radiolabelled and immunoreactive metabolites (Fig. 2A). All immunoreactive peaks were supported by radiolabelled metabolites albeit in a deviating constellation due to their different immunological cross-reactivities towards the antibody used.

Although HPLC profiles for radiolabelled and immunoreactive metabolites were not identical, the overlap in peaks between these two profiles confirms that our EIAs were able to detect the metabolites derived from the hormones of interest (progesterone and estradiol, respectively).

3.3. Comparative HPLC analyses of immunoreactive gestagen metabolites

To compare the metabolite composition among lynx species we generated metabolite profiles from different reproductive stages for each lynx species (examples chosen from the second half of pregnancy are shown in Fig. 1A–D).

Eurasian lynx exhibited five major immunoreactive peaks: two polar metabolites and three non-polar metabolites, which co-eluted with P4, DHP and eluted close to 5α-P, respectively. Enzymatic hydrolysis (see above) reduced the proportion of the polar immunoreactive metabolites in fractions 7–9 by 95%, but had no distinct effect on the polar metabolites in fractions 10–13 (data not shown). This confirms that fractions 7–9 contained hydrolysable immunoreactive gestagen conjugates. The sample from the Iberian lynx (Fig. 1B) and the bobcat (Fig. 1D) revealed a different pattern, mainly consisting of polar conjugated gestagen metabolites. Although Canada lynx did have a higher proportion of unknown metabolites, it looks like they are similar in the placement of peaks and the height of the peaks compared to their sister taxa (Fig. 1C).

When comparing metabolite profiles within a given lynx species, distinct changes in composition could be observed throughout the reproductive stages. This could be illustrated with six samples from a Eurasian lynx collected prior to mating and after parturition

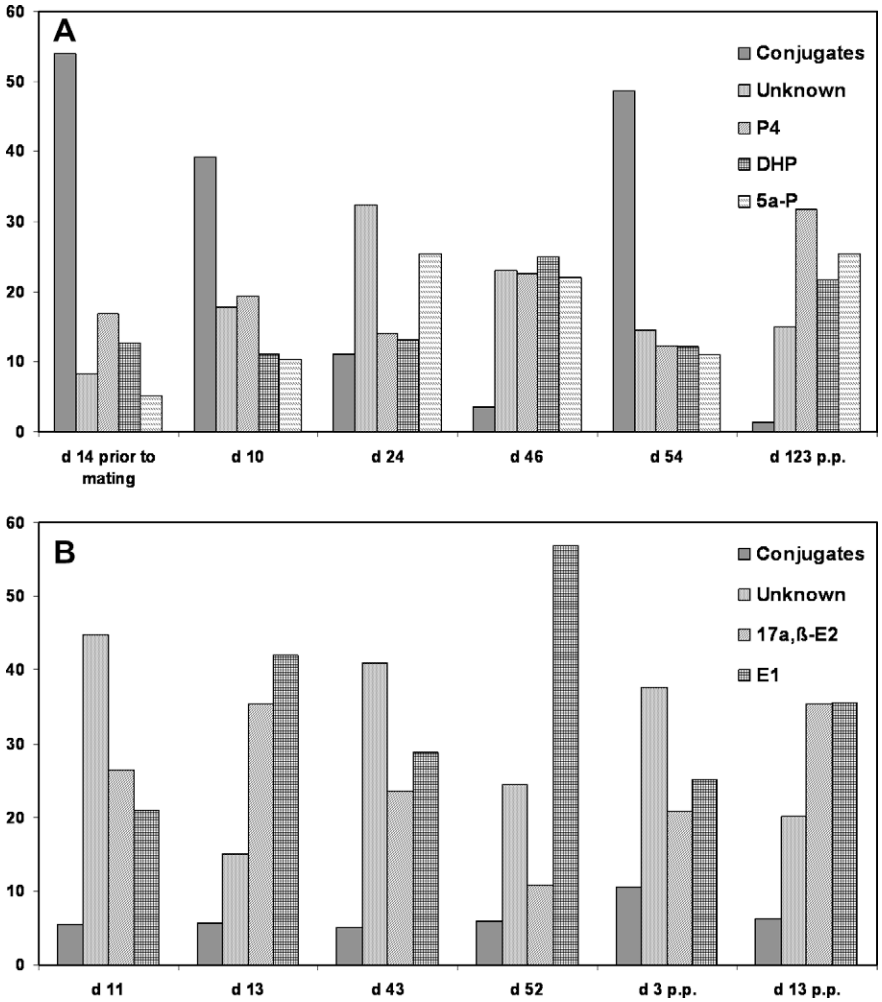

Fig. 3. Changes in faecal gestagen (A) and estrogen (B) metabolite profiles in a Eurasian and a Canada lynx, respectively, throughout pregnancy and after parturition (p.p.). Based on the peak distribution (gestagens and estrogens, see Figs. 1 and 2, respectively), immunoreactivities were grouped into different categories and expressed as % of the total immunoreactivity (see also Tables 1 and 2, gestagens and estrogens, respectively).

(Fig. 3A, corresponding data are summarised in Table 1). Notably, there was an extremely large proportion of conjugated metabolites (up to 54%) prior to and 10 days following mating and during the last days of pregnancy. Conversely, the proportion of metabolites corresponding to 5α-P increased on days 24 and 46. In general, this pattern of change in metabolites cannot be declared to be representative because in other animals, changes in profiles seemed to occur more or less at random. However, the unusually high proportion of conjugates observed at the end of pregnancy was fairly consistent across individuals and species. This final increase of conjugates was most pronounced in the Iberian lynx and the bobcat but also in the Eurasian lynx. This can be seen in Table 1 where data for one female of each species are summarised.

In comparing the dynamics of metabolite composition across *Lynx* species, we found that Eurasian lynx consistently had a relatively high proportion of metabolites corresponding to DHP and 5α-P. Conversely, comparatively low amounts of both metabolites were found in Iberian lynx, Canada lynx, and bobcats. Furthermore, Canada lynx seemed to possess a consistently high proportion of unknown metabolites (Table 1). These differences were not the result of different extraction methods (Iberian and Eurasian lynx: 90% methanol; Canada lynx and bobcat: 80% ethanol): a comparative extraction of faecal samples did not lead to differences in immunoreactive steroid metabolite compositions (data not shown).

Similar HPLC profiles were generated to investigate the estrogen metabolite composition. In the sample from the Eurasian lynx, the estrogen immunoassay demonstrated four different immunoreactive estrogens (Fig. 2A). The major proportion of immunoreactivity consisted of an unknown polar metabolite peaking in fraction 9, escorted by three minor peaks in fraction 5, 14–16, and 25 corresponding to conjugated estrogens (two estradiol isomers and estrone, respectively). A similar distribution of immunoreactivity was obtained for the Canada lynx (Fig. 2C), whereas in the Iberian lynx (Fig. 2B) and in the bobcat (Fig. 2D), the major immunoreactive metabolite corresponded to estrone based on its elution position.

To investigate changes in estrogen metabolite composition throughout pregnancy and lactation, we analysed six samples from a Canada lynx (Fig. 3B). Notably, there was a relatively low proportion of conjugated metabolites (less than 10%) and a high proportion (up to 45%) of an unknown polar metabolite. However, more than 45% of the immunoreactivity consisted of varying proportions of estrone and estradiol, with the highest amount of estrone appearing at the end of pregnancy. This metabolite composition was also confirmed in a second Canada lynx (data not shown). However, in contrast to the female in Fig. 3B, the highest proportion of estrone was observed day 6 post-partum, as opposed to day 52 of pregnancy. This suggests that the changes in metabolite composition are more related to inter- and intra-individual variations than to systematic changes in steroid metabolism during the course of pregnancy.

The comparison between the lynx species (Table 2) confirmed that during pregnancy and lactation, conjugated estrogens were of minor importance, and the proportion of unknown metabolites was highly variable. The major part of faecal estrogens consisted of estradiol and estrone promising reliable results using antibodies directed against total estrogens for their analysis.

4. Discussion

Attempts to facilitate or improve reproduction in ex-situ populations of rare felids often fail, in part because necessary basic reproductive/endocrine information is unavailable. In carnivores, early pregnancy diagnosis is complicated by the phenomenon of pseudo-pregnancy. Particularly in felids, steroid analyses do not provide an early pregnancy-specific diagnosis (Brown et al., 1994; Graham et al., 2006). Thus, our initial objective was to develop a non-invasive method for the longitudinal monitoring of ovar-

Table 1
Distribution of immunoreactive gestagen metabolites in faecal samples from four lynx species collected before, throughout pregnancy and post-partum (p.p.). Based on the peak distribution shown in Fig. 1, immunoreactivities were grouped into different categories and expressed as % of the total immunoreactivity (sum of fractions 4–40). The HPLC immunograms corresponding to the samples Eurasian lynx d 54, Iberian lynx d 53, Canada lynx d 52, and bobcat d 53 are shown in Fig. 1A, B, C, and D, respectively.

% Fraction #	Conjugates F 4–10	Unknown F 11–16	P4 F 20–25	DHP F 29–34	5α-P F 35–40
Eurasian lynx					
d 14 before mating	53.9	8.3	16.9	12.7	5.1
d 10	39.2	17.8	19.4	11.1	10.3
d 24	11.2	32.3	14.0	13.2	25.4
d 46	3.6	23.0	22.7	25.0	22.0
d 54	48.6	14.5	12.3	12.2	11.0
d 123 p.p.	1.3	14.9	31.8	21.7	25.4
Iberian lynx					
d 7 before mating	71.2	11.6	2.4	2.4	0.2
d of mating	18.0	15.3	54.7	5.3	4.2
d 14	6.9	26.0	32.7	13.9	13.4
d 53	92.5	1.2	1.3	1.9	0.8
Canada lynx					
d 11	44.2	44.5	3.3	2.2	3.1
d 13	20.8	36.4	22.8	5.6	10.4
d 43	30.8	40.9	16.2	1.6	3.7
d 52	32.2	42.8	17.0	1.7	3.9
d 3 p.p.	14.5	65.9	6.2	0.6	2.4
d 13 p.p.	5.4	68.1	14.0	0.9	2.6
Bobcat					
d 12	24.7	27.0	20.1	8.3	6.8
d 16	76.5	14.1	1.9	1.1	0.9
d 48	24.5	12.4	55.9	4.5	0.5
d 53	86.5	6.6	2.8	1.0	0.9

Table 2
Distribution of immunoreactive estrogen metabolites in faecal samples from four lynx species collected throughout pregnancy and post-partum (p.p.) Based on the peak distribution shown in Fig. 2, immunoreactivities were grouped into different categories and expressed as % of the total immunoreactivity (sum of fractions 3–37). The HPLC immunograms corresponding to the samples Eurasian lynx d 36, Iberian lynx d 54, Canada lynx d 52, and bobcat d 48 are shown in Fig. 2A, B, C, and D, respectively.

% Fraction #	Conjugates F 3–6	Unknown F 8–11	α,β-E2 F 13–17	E1 F 23–28
Eurasian lynx				
d 18	12.1	30.7	50.2	4.0
d 36	12.1	46.2	13.6	25.5
d 14 p.p.	14.1	38.5	8.8	36.0
Iberian lynx				
d 7 before mating	26.3	40.1	4.5	21.7
d of mating	8.1	7.2	3.5	77.3
d 14	1.7	2.4	12.2	81.6
d 54	3.2	2.6	1.6	86.5
Canada lynx				
d 11	5.5	44.9	26.5	20.9
d 13	5.6	15.1	35.4	42.0
d 43	5.0	40.8	23.6	28.8
d 52	5.9	24.5	10.9	56.9
d 3 p.p.	10.6	37.6	20.7	25.1
d 13 p.p.	6.2	20.1	35.3	35.5
Bobcat				
d 11	5.1	38.8	27.0	25.9
d 16	2.3	12.7	50.0	33.8
d 48	2.2	1.5	13.7	80.9
d 53	2.2	1.8	17.5	77.0

ian activity in the Eurasian lynx. However, we found that faecal metabolite analysis was ineffective for oestrous monitoring and pregnancy diagnosis in the Eurasian lynx (Dehnhard et al., 2008). First, estrogens did not reflect follicular activity peaking around ovulation (mating), and were strongly correlated to the excretion of gestagens. Second, gestagen (and estrogen) metabolite levels only increased moderately throughout pregnancy and remained elevated after parturition (Jewgenow et al., 2009). The persistent elevation of post-partum (p.p.) gestagen and estrogen metabolite levels is of particular interest because they rely on the existence of p.p. corpora lutea (Göritz et al., 2009). Such unusual endocrine profiles conflict with those reported for other felid species (Brown et al., 1994, 2001). However, similar results have also been described for the other three lynx species (Iberian lynx, Pelican et al., 2006; Canada lynx, Fanson, submitted for publication; bobcats, Miller, 1995).

The goal of this study was to describe patterns of steroid metabolism among the four *Lynx* species. To demonstrate the quantitative steroid metabolite composition, the degradation of radiolabelled progesterone and estradiol was investigated in the Eurasian lynx. To examine changes in metabolite composition between different reproductive states and species differences, comparative HPLC immunograms were generated from faecal samples collected before, throughout and after pregnancy from individual females of the four closely related lynx species.

4.1. Characterisation of faecal metabolites

The progesterone results from the radiometabolism study revealed a high proportion of polar metabolites. The broad peak of radiolabel in fractions 7–9 indicates a cluster of polar metabolites whose steroidal structures are unknown, but they were enzyme-hydrolysable and thus may be designated as conjugates. In addition, three unknown polar peaks appeared, whereas radiolabelled non-metabolized progesterone did not appear in significant amounts in this Eurasian lynx female. Our HPLC profile for radiolabelled progesterone metabolites was similar to the pattern shown for the domestic cat (Brown et al., 1994); however, the authors did not investigate which of their two polar peaks was enzyme-hydrolysable.

Principles of steroid conjugate formation are known from their urinary excretion. For most urinary steroids, the hydroxyl group is replaced by either sulphate or β-glucuronide. Progesterone is metabolised by reductions of its double bond located between C4 and C5, and of the oxo-groups at C3 and C20, leading to mono- and dihydroxylated faecal metabolites (Schwarzenberger et al., 1997) as basic sites for conjugation. However, the digestive juice of *Helix pomatia* (which is used for enzyme hydrolysis) do not contain all the sulphatases necessary for hydrolysis of all steroid sulphates (Shackleton, 1986), which might explain the proportion of non-hydrolysable P4 conjugates.

The HPLC profiles obtained for radiolabeled and immunoreactive gestagen metabolites differed somewhat. This is not surprising; usually the patterns of radiolabelled metabolites and immunoreactive hormone metabolites do not coincide due to different immunological cross-reactivities of metabolites to the antibody directed against the primary steroid. It is extremely important to consider that a peak seen in an HPLC immunogram does not reflect the quantity of faecal hormone metabolites but is a result of the affinity of the antibody for different metabolites together with the amount of metabolite in a particular HPLC fraction. The radiometabolism of progesterone suggests that the circulating hormone itself is merely present in minor quantities in faeces, implying that our P4 assay mainly depends upon cross-reactions with the excreted metabolites, which most likely show different affinities to the P4 antibody (also termed as immunoreactivity). The cross-reactivity of our antibody with conjugates may be based on its generation against 5α-pregnan-3β-ol-20-one-3-HS-BSA. This group-specific antibody mainly recognises the C20-position, whereas the C3 functional groups are not readily distinguished making the recognition of 3-hydroxy conjugates possible.

The second peak of gestagen immunoreactivity coincides with the major radiometabolite peak, which is an unknown non-hydrolysable metabolite. The finding of an exceptionally high proportion of non-hydrolysable gestagen metabolites was similar to that observed for excreted steroids in the domestic cat, leopard cat, cheetah, clouded leopard, and snow leopard (Brown et al., 1994). This phenomenon seems to be unique to felids because the majority of faecal P4 metabolites in other species are excreted in the unconjugated form [e.g., primates (Shideler et al., 1993; Wasser et al., 1994; Ziegler and Wittwer, 2005), black rhinoceros (Schwarzenberger et al., 1993), African elephant (Fiess et al., 1999), and giraffe (del Castillo et al., 2005)].

We also found significant amounts of non-polar immunoreactive metabolites whose elution positions match with those of P4, DHP and 5α-P. These unconjugated metabolites are presumably the result of bacterial hydrolysis in the gut (Adlercreutz et al., 1979; Senciall and Thomas, 1970). In the Eurasian lynx, in which the radiometabolism study was carried out, these gestagens were not supported by distinct peaks of radiolabel. Therefore the immunoreactive metabolites at the positions of P4 and DHP might indicate minor amounts of P4 and DHP which were clearly detected due to the antibody affinity towards these steroids. In addition, it might be possible that the gestagen antibody detects gestagens that had been generated from adrenal glucocorticoids by enzymatic activities of cat faecal flora. This has been shown for the conversion of desoxycorticosterone to progesterone (Winter and Bokkenheuser, 1987). Thus, changes associated with luteal activity might be masked by gestagen metabolites originating and reflecting adrenal function.

Unfortunately the radiometabolism study was performed in a different female outside the breeding season, so a direct comparison between radiolabelled and immunoreactive metabolites is not possible.

The estrogen metabolism study in the Eurasian female basically revealed similar metabolite categories as described for the gestagen metabolites. However, a greater proportion (about 50%) consisted of radiolabelled 17β-estradiol and estrone. This pattern resembles estrogen metabolite profiles reported for other felids (leopard cat, cheetah, clouded and snow leopard: Brown et al., 1994). In these species, conjugated and unconjugated estrogen metabolites were present in equal amounts, whereas estrone was present in minor quantities. This pattern also fits with the faecal cortisol metabolite composition in the domestic cat (Graham and Brown, 1996). A high proportion of conjugates (both hydrolysable and non-hydrolysable) seems to be a common feature of felid steroid metabolism. Also faecal samples from a male Eurasian lynx who received an i.v. injection of radiolabelled testosterone contained a large proportion of non-hydrolysable conjugates (Jewgenow et al., 2006b).

4.2. Inter-species comparisons

The four lynx species had very similar HPLC immunogram profiles, indicating no significant species differences in the composition of main immunoreactive gestagen and estrogen metabolites. Only two minor species-specific patterns emerged. In bobcats, we observed an exceptionally high proportion of gestagen conjugates (62.4 + 24.1%; mean + SD), and in Iberian lynx, there was an exceptionally high proportion of estrone (63.9 + 24.4%; mean + SD). However, steroid metabolism seems to be relatively conserved across *Lynx* species, as supported by the fact that across species,

gestagen metabolites are primarily excreted as polar compounds and estrogen metabolites primarily consist of 17β-estradiol and estrone. Our results contrast to experiences in the four related rhinoceros species (white [*Ceratotherium simum*], black [*Diceros bicornis*], Indian [*Rhinoceros unicornis*], and Sumatran rhinoceros [*Dicerorhinus sumatrensis*]), where faecal steroid metabolites vary considerably among these species (Schwarzenberger, 2007).

Finding similar immunoreactive gestagen and estrogen metabolite profiles of HPLC-separated faecal extracts between the four lynx species suggests that estrogen and gestagen metabolism is relatively conserved among lynx species and that our assays were suitable tools to monitor luteal function across these species. Furthermore, based on the radiolabelled metabolite profiles, our P4 and E2 EIAs were able to quantify part of these metabolites as indicated by the overlap between radiolabelled and immunoreactive metabolites. However, earlier results of longitudinal steroid analyses indicated that changes in faecal estrogen and gestagen metabolite concentrations were neither associated with ovarian follicular development, nor with luteinisation, nor with pregnancy (Dehnhard et al., 2008; Pelican et al., 2006; Fanson, submitted for publication; Miller, 1995). We suggest that these unusual longitudinal profiles might be the consequence of the prolonged presence (and function) of corpora lutea throughout most of the year (Göritz et al., 2009; Kvam, 1990). Thus, prolonged gestagen production may ensure strong seasonality in lynx, but masks any changes associated with the luteal phase of pregnancy. Therefore, due to the long lasting and unusual p.p. luteal activity in lynxes (Dehnhard et al., 2008) high gestagen metabolite levels cannot be used as an index of pregnancy.

4.3. Intra-individual comparison across reproductive stages

The comparison of HPLC immunograms generated from individuals of the four species (Tables 1 and 2, and Fig. 3) revealed high intra-individual variation in both immunoreactive gestagen and estrogen metabolites, but there was no pattern of metabolite composition that could be related to specific reproductive stages. In addition, the remarkable post-partum (p.p.) luteal activity found in the Eurasian and Iberian lynx (Dehnhard et al., 2008; Pelican et al., 2006) based on faecal progesterone metabolite analyses could not be explained by the p.p. appearance of additional metabolites of probably non-luteal origin. In three domestic cats, comparatively moderate individual differences in metabolite composition were found after injection of radiolabelled estradiol, but similar to our findings, there was no detectable effect of reproductive state on metabolite composition (Shille et al., 1984). Therefore, intra-individual fluctuations in metabolite composition seem to be dictated by random changes in steroid metabolism, and not by changes in ovarian or luteal activity associated with reproductive stages.

Steroid metabolism takes place mainly in the liver, and the metabolites are subsequently excreted as conjugates via the bile. In addition, intestinal glucuronidation of steroids has been reported to increase towards the distal end of the intestine (Czernik et al., 2000). In contrast, intestinal bacteria in the gut, whose composition may be also affected by diet, can affect the structure of these steroids by hydrolysis of biliary steroid conjugates (Palme, 2005). We assume that these intra-individual changes in faecal metabolite composition reflect qualitative and quantitative variations in gut bacteria composition. Furthermore, due to the differences in metabolite composition and presumably diverging metabolite affinities towards the antibody, it can be assumed that changes in metabolite composition might also affect quantitative metabolite analyses in faecal extracts. However, to our knowledge a study investigating intra-individual changes in steroid metabolite composition has never been conducted.

In sum, the purpose of this study was to describe the composition of estrogen and gestagen metabolites among the four lynx species across different reproductive stages. In that context, we made several important observations.

First, there was considerable variation in overall faecal estrogen and gestagen metabolite concentrations among the individuals of the four lynx species, suggesting that although steroid metabolism may be conserved among lynxes, the absolute production of steroids may be influenced by intra-individual variations.

Second, the remarkably high faecal gestagen and estrogen metabolite concentrations observed post-partum in the Eurasian, Iberian, and Canada lynx are not related to the appearance of additional metabolites.

Third, gestagen metabolite analyses are not useful as an index of pregnancy, and we are still unable to differentiate between pregnancy, pseudo-pregnancy and the post-partum period in lynx.

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