Histological and endocrine characterisation of the annual luteal activity in Eurasian lynx (*Lynx lynx*)

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Abstract

Lynx presents a unique sexual cycle with persistent *corpora lutea* (CLs) and elevated serum progesterone (P₄) throughout parturition and lactation. In other mammals, CLs normally disintegrate after parturition, therefore the aim of our study was to characterise the annual life cycle of lynx CLs. Ovaries from Eurasian lynxes were obtained from the National Veterinary Institute in Sweden, where tissues from killed lynx were stored at -20°C . Ovaries from 66 animals were weighed; each *corpus luteum* was segmented for histology and hormone analysis. Ovary and CLs weights were constant throughout the year, peaking during pregnancy. In non-pregnant lynxes, the seasonal level of intraluteal steroids was steady for P₄ (3.2 ± 1.9 s.d. $\mu\text{g/g}$, $n=53$) and total oestrogens (18.3 ± 15.5 s.d. ng/g , $n=53$). Within histology slides, structurally intact luteal cells were found throughout the year with the highest incidence in March/April; evidence of luteal regression was predominantly found in post-breeding season. Ovaries from pregnant animals contained two types of CLs. Group A was bigger in size with large luteal cells (P₄, 72.3 ± 65.4 s.d. $\mu\text{g/g}$; oestrogen, 454.0 ± 52.4 s.d. ng/g). In contrast, group B were smaller, with greater luteal regression and lower steroid concentrations (P₄, 8.3 ± 2.9 s.d. $\mu\text{g/g}$; oestrogen, 31.5 ± 20.4 s.d. ng/g). Our results suggest that structural luteolysis proceeds throughout the year and into next breeding cycle, resulting in two CLs types on the same ovary.

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Introduction

The Eurasian lynx (*Lynx lynx*) is one of four species within the genus *Lynx*; the other three are the Canadian lynx (*Lynx canadensis*), the bobcat (*Lynx rufus*) and the critically endangered Iberian lynx (*Lynx pardinus*; Dehnhard *et al.* 2010). The *lynx* genus typically consists of strictly seasonal breeders; the exception being the bobcat, which is a non-seasonal polyoestrous breeder (Parker & Smith 1983, Kvam 1991, Hayssen *et al.* 1993).

The distribution of Eurasian lynx extends throughout Scandinavia, Russia and across China, with sparse populations in the rest of Europe and Eastern Asia (Breitenmoser *et al.* 2007). The global population is unclear, but the European range is estimated to contain ~ 7000 – 8000 (Henriksen *et al.* 2005, Breitenmoser *et al.* 2008) animals. In sharp contrast, the Iberian lynx's current population numbers at ~ 200 individuals in the wild, confined to two regions of Spain (Fernandez *et al.* 2006, Palomares *et al.* 2011); the Portuguese population is possibly extinct (Von Arx & Breitenmoser-Wüsten 2008).

The Iberian lynx suffered major population declines, mostly due to habitat loss and crippling disease within the principal prey (wild rabbit (*Orytolagus cuniculus*)) population (Von Arx & Breitenmoser-Wüsten 2008). In 2003 the Iberian Lynx *Ex Situ* Conservation Programme was formed (Calzada *et al.* 2009) and at the 'El Acebuche' breeding centre in Doñana National Park, Spain, the first litter was born in 2005 (Vargas *et al.* 2009). Since then the population has increased within five breeding centres (four in Spain; one in Portugal) to include 96 animals (49 male:47 female) of which 32 are founding animals and 64 were born within the programme (<http://www.lynxexsitu.es>). At the beginning of the programme, it was claimed that 60 individuals born in captivity would safeguard 85% of Iberian lynx genetic variability (Leus & Lacy 2009). With that goal attained, reintroductions are now a focal point for the conservation programme.

Knowledge on reproductive biology and reliable methods for reproductive monitoring are essential for the improvement of captive breeding. The reproductive

cycle of the Iberian and Eurasian lynx, however, was described as atypical for a cat species, whereby the *corpora lutea* (CLs) remain persistent and functional after parturition, lactation and into the next breeding season (Göritz *et al.* 2009). The *corpus luteum* (CL) is a temporary hormonal gland that keeps the uterine conditions optimal for pregnancy by the production of progesterone (P₄; Davis & Rueda 2002). CL formation, maintenance and regression in many species are supported by several key hormones: luteinising hormone (LH), prolactin, P₄, oestrogens and prostaglandin F_{2α} (Stocco *et al.* 2007). The process of CL formation based on cellular restructuring of the post-ovulation follicle, triggered by a surge in LH, is called luteinisation (Stocco *et al.* 2007). After ovulation, the CL helps to maintain pregnancy and then regresses during the peripartur period in almost all mammals (Stocco *et al.* 2007).

Within the Iberian lynx captive breeding programme, female lynx often breed unsuccessfully and the persistent CLs might prevent another cycle (Göritz *et al.* 2009). Thus, the reproductive potential is lost for a whole year. In the case of genetically valuable females, a hormone-induced luteolysis followed by oestrus induction would offer a second opportunity within the current season for either natural mating or artificial insemination. However very little is known about the time course of luteal function in lynx: what differences lie between pregnant and pseudo-pregnant CLs, when CLs regress functionally and structurally and what may the triggers be?

The overall aim of this study was to investigate the annual life cycle of CLs in Eurasian lynx. This builds upon previous studies (Göritz *et al.* 2009) in an attempt to confirm that the origin of elevated circulating steroid hormones outside the breeding season was from the CLs, and to find evidence of luteolysis before seasonal breeding. For this purpose ovaries from hunted or road-killed Eurasian lynx collected throughout the year were used; this tissue availability coupled with its classification as ‘Least Concern’ in the IUCN Red List of Threatened Species makes the species a parallel model for research on lynx reproductive biology (Breitenmoser *et al.* 2008). We aimed to monitor histomorphological changes and to characterise steroidogenic function of CLs based on the analyses of the luteal key hormones: P₄ and total oestrogens.

Results

Seasonal weight of CL and ovaries

When evaluating non-pregnant females, both the CLs and ovarian mass did not vary significantly throughout the year (Tables 1 and 2). In pregnant females, however, ovarian mass was significantly increased in April (Table 1), while CL mass means showed a definite increase compared with non-pregnant females, although the difference was not statistically significant due to small sample size. The CLs obtained from pregnant animals were separated into two distinct categories according to size and appearance. The larger and paler CLs were designated as group A (n=4), and the smaller, dark red CLs sometimes found within the same ovary were allocated into group B (n=12). CLs from group A were heavier (0.82±0.13 g, mean±s.d.) when compared with those of group B (0.11±0.06 g, mean±s.d.) and those from non-pregnant females (0.13±0.08 g). Total CLs mass was also greater in pregnant animals compared with non-pregnant animals (2.29±0.17 g, n=2 and 0.73±0.38 g, n=40 respectively). The increase in CLs weight was also reflected in heavier ovaries in pregnant animals (Table 1). The heaviest ovary was found in a pregnant animal (3.29 g) and the lightest ovary was found in a non-pregnant female in August (0.11 g).

The number of CLs per ovary was also recorded and the highest number per ovary was eight, and per animal was 14. The mean number of CLs per month was noted, the highest numbers were found in pregnant animals and then during January to March; the annual average was 5.9 per animal (Table 2).

Histology of CL

There was a certain level of decay present in many of the slides; thus a comprehensive comparative histological analysis was only possible in 26 of 53 animals. Nonetheless, many histology samples (n=14, Fig. 1) showed structurally intact luteal cells throughout the year with the highest incidence in March/April (five out of seven animals, Fig. 2). Before breeding season (January–February), four out of ten cases showed evidence of luteal regression, whereas after breeding season, more than two thirds of samples demonstrated

Table 1 Annual weight variation of ovaries obtained from hunted or road-killed Eurasian lynxes in Sweden. In April, four animals were recognized as pregnant and listed separately.

	Total	January	February	March	April	April Pregnant	May–September	October	November	December
n animal	66	6	7	13	4	4	12	7	7	6
n ovaries	132	12	14	26	8	8	24	14	14	12
Ovary weight (s.d.)		0.74 g (±0.41 g)	0.94 g (±0.38 g)	1.17 g (±0.39 g)	0.91 g (±0.68 g)	1.77 g ^a (±0.99 g)	0.70 g (±0.45 g)	0.87 g (±0.37 g)	1.07 g (±0.68 g)	0.77 g (±0.49 g)

^aThe ovarian weight of pregnant animals was significantly higher compared with all other periods (Kruskal–Wallis test, $\chi^2 = 15.83$, df = 8, $P = 0.04$).

Table 2 Annual weight variation of *corpus luteum* (CL) obtained from hunted or road-killed Eurasian lynxes in Sweden. Each CL was treated separately. Because of autolytic decay not all ovaries were suitable for CL dissection.

	Total	January	February	March	April	April Pregnant	May– September	October	November	December
<i>n</i> animals	53	4	8	8	3	2	7	7	8	6
<i>n</i> CL	314	29	48	55	16	16	35	38	44	33
CL per animal	5.9	7.25	6	6.8	5.3	8	5	5.4	5.5	5.5
CL weight (s.d.)		0.11 g (±0.06 g)	0.09 g (±0.06 g)	0.12 g (±0.08 g)	0.19 g (±0.19 g)	0.29 g (±0.33 g)	0.14 g (±0.07 g)	0.10 g (±0.06 g)	0.13 g (±0.07 g)	0.10 g (±0.07 g)

luteal destruction (six out of nine animals). Evidence of luteal regression was described as decreased size of luteal cells and replacement by non-granulosa luteal cells (macrophages, connective tissue and theca luteal cells), as described by Dawson (1941).

In Fig. 2 (defined cell structure only) images from February and March were considered typical examples of structurally intact luteal cells. The samples from January and December presented structurally intact luteal cells with an increased proportion of connective tissues. Smaller sized luteal cells and increased volume of connective tissue were clearly visible in the presented samples from April, September, October and November.

Although the CLs obtained from pregnant lynxes were rather decayed, both pregnant CL types could be differentiated according to structural parameters. The CLs of pregnant group A from April were composed of large luteal cells (Fig. 2). In contrast, the pregnant group B, as well as the non-pregnant April CLs samples, was characterised by an increased proportion of connective tissue and smaller luteal cells; an indicator for structural disorganisation.

Intraluteal steroid concentrations

The P_4 concentrations (Fig. 3) in CLs of non-pregnant animals remained stable throughout the year, independent of breeding (4.0 ± 1.8 µg/g February–April, $n=19$) or non-breeding season (2.6 ± 1.6 µg/g May–January, $n=32$), but increased considerably in pregnant animals (22.3 ± 8.7 µg/g April, $n=2$, Fig. 5A). The lowest concentrations of P_4 in non-pregnant animals were found between May and September (1.7 ± 0.7 µg/g, $n=7$), whereas the highest concentrations were found in March (5.2 ± 2.6 µg/g, $n=8$, Fig. 3).

Oestrogen concentrations in non-pregnant females (Fig. 4) also showed little annual fluctuation, although the mean values were higher in February to April (39.3 ± 40.9 ng/g, $n=19$) and at lower average during May–January (15.0 ± 12.8 ng/g, $n=32$). During pregnancy, however, the intraluteal oestrogen concentrations increased considerably to 181.0 ± 103.5 ng/g ($n=2$; Fig. 5B). The lowest concentrations of oestrogen in non-pregnant animals were found in December (10.7 ± 4.8 ng/g, $n=6$) and the highest in April (65.2 ± 61.8 ng/g, $n=3$). From March to October very high

variation of the intraluteal oestrogen was found, with few individual CLs in excess of 100 ng/g (Fig. 4).

There were two pregnant animals in our hormone study, confirmed by the presence of two foetuses each. The concentrations of both hormones within the CLs were considerably higher in the pregnant animals compared with non-pregnant (Fig. 5A and B). Group A contained CLs that were bigger in size and characterised by pale colour. The steroid content in group A was higher with 72.3 ± 65.4 µg/g for P_4 and 454.0 ± 52.4 ng/g for oestrogen. In contrast CLs of group B, which were markedly smaller in size and dark red in colouration, had lower concentrations of intraluteal steroids (P_4 , 8.3 ± 2.9 µg/g; oestrogen, 31.5 ± 20.4 ng/g).

Hormone values outside normal variation

Within the sample set, some CLs expressed unusually high hormone values (Figs 3 and 4). A threshold of two s.d.s from the mean of non-pregnant animals in both P_4 (9.3 µg/g) and oestrogen (66.3 ng/g) was used to classify animals as having unusually high readings. As expected, almost all CLs of the two pregnant animals fell into this

Figure 1 Histological picture of luteal tissue from a *corpus luteum* obtained from a hunted Eurasian lynx in February. Different luteal cell types are marked as followed: black arrow, structurally intact luteal granulosa cells; white arrows, luteal cells with foamy cytoplasm indicating lipid accumulation; black arrow heads, non-luteal stroma cells. Scale bar = 40 µm.

	Defined cell structure	Autolytic decayed cell structure		Defined cell structure	Autolytic decayed cell structure
January	 P ₄ : 8.72 µg/g Oestrogen: 6.95 ng/g	 P ₄ : 0.95 µg/g Oestrogen: 20.58 ng/g	April	 P ₄ : 2.62 µg/g Oestrogen: 26.80 ng/g	 P ₄ : 1.34 µg/g Oestrogen: 7.41 ng/g
February	 P ₄ : 8.34 µg/g Oestrogen: 17.45 ng/g	 P ₄ : 3.41 µg/g Oestrogen: 11.60 ng/g	May–September	 P ₄ : 2.52 µg/g Oestrogen: 10.23 ng/g	 P ₄ : 1.22 µg/g Oestrogen: 14.88 ng/g
March	 P ₄ : 3.70 µg/g Oestrogen: 5.81 ng/g	 P ₄ : 10.20 µg/g Oestrogen: 11.60 ng/g	October	 P ₄ : 5.74 µg/g Oestrogen: 8.55 ng/g	 P ₄ : 4.05 µg/g Oestrogen: 8.24 ng/g
April pregnant A	 P ₄ : 28.49 µg/g Oestrogen: 423.80 ng/g	 P ₄ : 107.67 µg/g Oestrogen: 401.55 ng/g	November	 P ₄ : 4.63 µg/g Oestrogen: 15.87 ng/g	 P ₄ : 1.59 µg/g Oestrogen: 12.00 ng/g
April pregnant B	 P ₄ : 5.69 µg/g Oestrogen: 18.25 ng/g	 P ₄ : 8.09 µg/g Oestrogen: 9.31 ng/g	December	 P ₄ : 2.19 µg/g Oestrogen: 9.09 ng/g	 P ₄ : 4.72 µg/g Oestrogen: 2.43 ng/g

Figure 2 Histological appearance of *corpora lutea* obtained from hunted or road-killed Eurasian lynx in Sweden. Transportation and freezing of whole lynx carcasses resulted in different degree of decay. For each month and two pregnant CL groups (groups A and B) a sample with good and poor histology are depicted together with the determined intraluteal steroid content. Scale bar = 20 µm. P₄, intraluteal progesterone.

region as did nine other animals. Six non-pregnant samples contained extraordinarily high oestrogen; they were found between January and July and once in October. Elevated P₄ was found in only three non-pregnant animals; these samples were from March, October and November.

Discussion

Many lynx reproductive studies in history have looked at factors such as placental scars and CL numbers (Duke 1949, Kvam 1990), but this is the first study that has incorporated CL morphology and steroid production. As expected, we found a significant increase in ovarian mass and P₄ and oestrogen production in CLs of pregnant animals, with a clear morphological and hormonal distinction between two types of CLs in one ovary. In non-pregnant animals, no significant changes were found throughout the year indicating permanent but low-level luteal functions. Our data suggest normal luteal activity during pregnancy and also support our earlier hypothesis about the persistence of CLs during

the entire year (Göritz *et al.* 2009) and into the next breeding season.

The breeding season of lynxes in Scandinavia lasts from February till early April, with mating activity peaking in March (Kvam 1991). Accordingly, highest ovarian weights were found in pregnant animals and in March. The largest CLs, however, were exclusively found in pregnant animals, although clearly two kinds of CLs were distinguished (groups A and B) suggesting that previous years CLs regress only after the formation of the current season's functional CLs. In the domestic cat, CLs reach their maximum size ~2 weeks after ovulation (Dawson 1941) independently of pregnancy or pseudo-pregnancy. The implantation of domestic cat embryos is observed at 12–14 days after ovulation (Denker *et al.* 1978, Leiser 1979), thus coinciding with CL formation.

Kvam (1991) also analysed ovaries of hunted lynxes. He already described the presence of luteal bodies throughout the entire year and differences in colour and consistency of luteal bodies from previous cycles ('1 year later'). In some lynxes the presence of luteal bodies from

Figure 3 Box-plots of the annual intraluteal progesterone (P_4) values determined in *corpora lutea* of hunted or road-killed Eurasian lynx. All animals were diagnosed non-pregnant by gross-morphological inspections of the uterus. The progesterone level did not differ between the months ($\chi^2 = 13.35$, $df = 7$, $P = 0.06$).

three cycles (3 years) was suggested; with a mean of six CLs per animal, our data corroborates this theory. In addition, the number of CLs in the ovaries was found to be much higher than the mean litter size (~ 2) described for the Eurasian lynx (Kvam 1991, Henriksen *et al.* 2005). Other possible reasons for this disparity might be a high embryonal loss or remnant CLs from previous ovulations of same breeding season. In our study, the number of foetuses in the two pregnant animals was exactly matched by the number of active (group A) CLs, suggesting that adjacent CLs may originate from a previous year's ovulations.

Although the mean number of CLs per animal did not reveal a clear pattern, higher numbers of CLs were found just before and during the breeding season (January–April, > 7) in comparison to the rest of the year (mean = 5), when young females (< 3 years old) were removed from the comparison. We suggest that this reflects a permanent turnover of CLs, with a delay of structural luteolysis of at least 1 year following ovulation.

To further characterise the function of CLs in lynx, luteal P_4 and oestrogens were determined in order to clarify the difference between the CLs that were still functional, and those which had undergone luteolysis. Both steroid hormones, P_4 and oestrogens, were still detectable in decayed samples; conversely, low steroid values were obtained from morphologically well-defined samples. Thus, the degenerative state of luteal cells seemed to have no relationship with the amount of hormones detected (Fig. 2). Data from our previous studies on serum P_4 and sono-morphology of lynx CLs during the annual cycle point towards permanent luteofunction throughout the year (Göriz *et al.* 2009). The presence of CLs from previous cycles has been verified in the mouse, rat, baboon (Davis & Rueda 2002)

and even domestic cats during lactation (Dawson 1946). In the domestic cat, however, serum P_4 decreases to the baseline level after parturition, although the structure of CLs might be conserved by the luteotrophic action of prolactin (Banks *et al.* 1983). It is impossible to differentiate between functionally active and inactive luteal cells by light microscope alone, as both types can be morphologically indistinguishable. However, in rodents, during functional luteolysis, the CL is still steroidogenically active, but instead of P_4 it continues to produce an inactive P_4 metabolite (Stocco *et al.* 2007). As our P_4 assay was validated for cross reactivity to the determined inactive metabolite (4-pregnen-20 α -ol-3-one, $< 0.01\%$) and several P_4 metabolites, it is unlikely that progestins other than P_4 were detected in our assay. Furthermore, in contrast to the domestic cat, and many other species (Stocco *et al.* 2007) with *post partum* persistence of CLs, we were also able to determine elevated serum P_4 concentrations above baseline (juvenile and aged females < 0.1 ng/ml) during pseudo-pregnancy and outside breeding season (~ 5 ng/ml, Göriz *et al.* 2009).

Swanson *et al.* (1995) analysed P_4 content in the domestic cat CLs in relation to the serum levels and revealed a strong correlation among serum P_4 , luteal mass and luteal P_4 . After CL formation (64 h *post copulation*), the P_4 level corresponded to the level which we determined within the pregnant lynx CLs, whereas 20 days after ovulation the P_4 amount in domestic cats, reported from Swanson *et al.* (1995), exceeded our P_4 content by a factor of ten (160–200 μ g/g). Irrespective of this discrepancy, our P_4 concentrations in pregnant CLs were significantly elevated in comparison to any other period of the year, including non-pregnant CLs within the breeding period (March–April). The relationship fits with serum samples for steroid

Figure 4 Box-plots of the annual intraluteal oestrogens values determined in *corpora lutea* of hunted or road-killed Eurasian lynx. All animals were diagnosed non-pregnant by gross-morphological inspections of the uterus. The total oestrogens level did not differ between the months ($\chi^2 = 12.30$, $df = 8$, $P = 0.41$).

Figure 5 Box-plots of progesterone (A) and oestrogens (B) concentration determined in CLs from two pregnant lynxes hunted in April. The pregnancies were confirmed by the presence of two foetuses in each animal. The two qualities of CLs on the ovaries were divided into group A, big size and pale colour and group B, small and dark red colour – CLs. The morphological-based categorisation was confirmed by higher P_4 and oestrogen levels within the luteal tissue.

determination we analysed during annual reproductive assessments (Göritz *et al.* 2009). Here blood serum P_4 was found to be ten times higher during pregnancy, compared with samples collected during pseudo-pregnancy, lactation, and outside the breeding period (Göritz *et al.* 2009).

Interestingly, the highest oestrogen concentrations were found in CLs of pregnant lynxes, signalling an involvement of this steroid in the maintenance of pregnancy. Non-invasive monitoring of steroids in urine and faecal samples of Eurasian and Iberian lynxes corroborates the special role of oestrogens during pregnancy in lynxes; showing elevated profiles of urinary and faecal oestrogen metabolites in pregnant animals, which were correlated to progestin metabolites (Dehnhard *et al.* 2008, Jewgenow *et al.* 2009). In contrast to P_4 , elevated oestrogens were not reflected in serum oestradiol (E_2) levels (Göritz *et al.* 2009). Limited CLs from non-pregnant lynxes contained extraordinarily high oestrogens. With one exception, these animals were within the breeding season.

Therefore, we suggest that oestrogens might precede the P_4 elevation after ovulation. A group of three non-pregnant lynxes (from March, October and November) were characterised by extraordinary high P_4 ; here we suggest either an ongoing pseudo-pregnancy or elevated P_4 levels caused by lactation (action of luteotrophic hormone, prolactin). Further investigations with a larger sample size are needed to support this hypothesis.

The mechanisms behind functional and structural luteal regression in the lynx are not yet known. In many species, the prostaglandin $PGF_{2\alpha}$ is involved in functional CL regression (Moutos *et al.* 1995, Davis & Rueda 2002, Stocco *et al.* 2007, Finkenwirth *et al.* 2010, Siemieniuch *et al.* 2010) and it seems to also play a major role in lynxes. We could recently show that PGFM, the metabolite of $PGF_{2\alpha}$, significantly increases at the beginning of last pregnancy trimester (Finkenwirth *et al.* 2010); a period in domestic cats when pregnancy is not exclusively dependant upon CLs (Tsutsui *et al.* 2009) and placental tissue might contribute towards P_4 production.

Also in the domestic cat, signs of structural luteolysis were described after day 50 of pregnancy, when CLs decrease in size and the luteal cells become highly vacuolated (Dawson 1941). The fatty infiltration of luteal tissue throughout the remainder of pregnancy and during *post partum* involution was regarded as an evidence of degeneration, marking the end of the period of functional activity of the CLs. The same evidence could be seen in lynx CLs as late as December (Fig. 2) or just before breeding season. The final stages of luteal regression were characterised by very small luteal cells and a prevalence of connective tissue as shown for CLs in April (non-pregnant or group B). This final structural luteal regression begins with programmed cell death (apoptosis) and involution of the CL, and culminates in an ovarian scar called *corpus albicans* (Davis & Rueda 2002, Luz *et al.* 2006, Stocco *et al.* 2007).

Continued post-mortem hormone analysis of CLs may provide valuable information on reproductive cyclicity in lynx. Eurasian lynxes are hunted at the onset of breeding season. In future studies, we will focus on fresh material from newly hunted lynxes to combine morphological and endocrine studies on CLs with particular emphasis on molecular determination of factors involved in the synthesis of steroids and prostaglandins, hormone reception and apoptosis.

Materials and Methods

The animals and tissue sample collection

The Eurasian lynx used in this study were from Sweden, whose population numbers are ~1300–1600 individuals (Andren *et al.* 2002, Liberg & Andren 2006); an annually reviewed legal hunting quota is permitted in order to maintain population levels which preserve the status quo between livestock holders

affected by lynx predation, hunters and government agencies (management plan for large carnivores, proposition 2008/09:210, <http://www.ud.se/sb/d/108/a/127782>, accessed 15/01/12). All lynx kills must be delivered to the National Veterinary Institute (SVA), Uppsala, Sweden, including legally hunted and road/train fatalities. At the institute the tissue was frozen soon after the animal was necropsied; samples included in this study were harvested between 2003 and 2010 and kept in frozen storage (-20°C) in a tissue-bank facility. From a total of 66 animals used in this study, all were suitable for total ovary weight analysis, 53 suitable for hormone analysis and 42 for total CLs weight data.

Ovaries and uteri were defrosted, weighed, and dissected. Each CL was weighed and cut into segments; one smaller part for histology and the remainder for determination of intraluteal steroid hormone content by enzyme immunoassay analysis. The samples were grouped per month except during May–September, when sample numbers were too low and had to be pooled. Four animals were found to be pregnant based on the presence of fetuses within the uterus.

Histology of CL

A section of CL tissue was fixed in Bouin solution, embedded in paraffin, cut into 2–3 μm slices and stained with haematoxylin–eosin (Fig. 1). Slides were examined using a light microscope with a digital camera (Olympus Soft Imaging Solutions, Münster, Germany), mounted at magnifications of $\times 20$ and $\times 40$ and processed using the cell R Software (Olympus) to ascertain the degree of autolysis, intactness of luteal cells and presence of lipid vacuoles as well as signs of structural degradation (replacement by non-luteal cells, as described by Dawson (1941)).

Intraluteal steroid hormone determination

Aliquots of CL tissue of 0.01–0.61 g were placed into 2 ml centrifuge tubes containing 28 and 14 mm ceramic balls (Precellys, Bertin Technologies, Montigny-le Bretonneux, France), to which a sample weight-specific volume of 0.09–1.12 ml PBS was added; the samples were placed in a 'Precellys 24' homogenizer (Precellys) and shaken at 7000 g for 2×15 s. An aliquot (50 μl) of the homogenate was extracted twice with 2 ml of tert-butyl methyl ether/petroleum ether (30/70, v/v) for 30 min. After freezing at -75°C , the organic phase was decanted, dried and resolved with 0.25 ml of 40% methanol and stored at -20°C until needed.

Duplicates of the extracts (20 and 10 μl respectively) were tested for both P_4 and oestrogens using in-house 96 well microtitre plate enzyme immunoassays previously validated for the Eurasian lynx (Dehnhard *et al.* 2008).

P_4 analyses were carried out as described earlier (Dehnhard *et al.* 2010) using a commercial P_4 antibody (Sigma P1922, raised in rats) and 4-pregnen-3,20-dione-3-CMO-peroxidase label. The cross reactivities to progestins were as follows: 4-pregnen-3,20-dione (P_4), 100%; 5 α -pregnan-3,20-dione, 31%; 5 α -pregnan-3 β -ol-20-one, 18%; 5-pregnen-3 β -ol-20-one, 12%; 4-pregnen-3 α -ol-20-one, 4.2%; $<0.1\%$ for 5 β -pregnan-3 α , 20 α -diol, 4-pregnen-20 α -ol-3-one, 5 β -pregnan-3 α -ol-20-one,

5 α -pregnan-20 α -ol-3-one, 5 α -pregnan-3 α ,20 α -diol, 5 α -pregnan-3 β ,20 α -diol, testosterone, E_2 , and cortisol. Intra- and inter-assay coefficients of variation (CVs) for two biological samples with low and high concentrations were 1.8 and 8.6% ($n=18$) and 2.1 and 8.5% ($n=20$) respectively.

Total oestrogen analyses were carried out using a polyclonal antibody raised in rabbits to 1,3,5(10)-estratrien-3,17 β -diol-17-HS-BSA and 1,3,5(10)-estratrien-3,17 β -diol-17-HS-peroxidase label. The cross reactivities to oestrogens were as follows: 1,3,5(10)-estratrien-3,17 β -diol (17 β - E_2), 100%; 1,3,5(10)-estratrien-3,17-one (oestrone), 100%; 1,3,5(10)-estratrien-3,17 α -diol (17 α - E_2), 66%; 1,3,5(10)-estratrien-3,16 α ,17 β -triol (oestriol), 1.5%; and $<0.1\%$ for 19-nortestosterone, P_4 and testosterone. Intra- and inter-assay CVs for two biological samples with low and high concentrations were 2.4 and 6.9% ($n=12$) and 1.3 and 4.7% ($n=18$) respectively.

In order to assess the influence of prolonged storage of CLs at ambient temperatures on intraluteal steroid concentration, some preliminary testing was carried out on CLs obtained from the domestic cat ($n=4$) after ovariectomy. CLs were kept at refrigerator temperature (4°C) and room temperature (20°C) for 0, 1, 2 and 3 days before processing. All measurements followed the same protocol as described above. Steroid hormone concentrations remained constant throughout the four days storage period at both refrigerator and room temperature.

Statistical analysis

The software package 'R version 2.14.1' (Vienna, Austria) was applied in all statistical analyses (R Development Core Team 2011). Data on ovarian and CLs weight were displayed using mean $\pm$ s.d. Hormone content was expressed as microgram P_4 or nanogram oestrogens per gram of wet CL tissue in box-plots presenting medians together with 10, 25, 75 and 90% percentiles. As the distribution of the data was not normal, non-parametric statistical analysis methods were used in the form of a Kruskal–Wallis analysis of variance generalized linear model. Means were used to make data independent. For analysis of total ovary and total CLs data, only data from animals that had either both ovaries ($n=66$) or CL present in both ovaries ($n=42$) were considered.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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