Revised highlighted

Collection of field reproductive data from carcasses of the female Eurasian lynx *(Lynx lynx)*

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Key words. Puberty, pregnancy rates, reproductive season, corpora lutea

Abstract

Information about reproductive physiology in the Eurasian lynx (*Lynx lynx*) would generate knowledge that could be useful in the management of the Swedish lynx population based on knowledge about their reproductive potential and population development. Age related differences in ovulation and implantation rates would affect the reproductive output and the development of the population. The aims of this study were to evaluate a protocol for collection of reproductive data from carcasses by comparisons with published field data and to generate data about reproduction in the Swedish lynx. Reproductive organs from 120 females that were harvested between March 1 and April 9 from 2009 to 2011 were collected and evaluated macroscopically and for placental scars. Females had their first estrus as yearlings but did not have their first litter until the next season. Pregnancy rates were lower in 2 y old females than in females aged 3 to7 y but did not differ significantly from females aged 8 to 13 y (54.5%, 95.6 %, and 75.0% respectively). Corpora lutea (CL) from the present season were morphologically distinctly different from luteal bodies from previous cycles (LBPC). All females ≥ 3 y had macroscopically visible LBPC while only 67 % of 22-23 mo old females had 1-3 LBPC and no females < 1 y of age had LBPC. Females aged 34-35 mo had up to 8 LPBC while the highest number of LBPC counted in females ≥ 3 y of age was 11. These data would be in agreement with only one estrus per season and LBPC from at least three previous reproductive seasons in older females. The number of LBPC was significantly correlated with the weight of the ovaries r_s= 0.648, $P <0.001$) and the age of the animals (r_s = 0.572, $P < 0.001$). Uterine weight differed significantly with stage of the reproductive cycle and was highest for mature females in the luteal phase of the cycle. The estrus period, defined as occurrence of ovarian follicles lasted from March 5 to April 1 in this material. In conclusion this study confirms that useful information about lynx reproduction can be collected from reproductive organs retrieved after the death of the animals. Continuous monitoring of lynx reproductive organs would therefore make a valuable contribution to collection of field data gathering information that can be useful for the management of lynx populations and potentially for the lynx as an indicator of environmental disturbances.

1.1 Introduction

Increased knowledge about reproductive physiology in the Eurasian lynx would have several potential applications in addition to general increased knowledge. Decisions about management of the Swedish lynx population are based on knowledge about their reproductive potential and population development. The Swedish lynx is closely monitored in the field, which is the reason why there are data about field fertility and estimated population sizes [1]. Although monitoring a species in the field gives data that can be considered gold standard there are limitations in the collection of this sort of data. By studying the lynx reproductive organs in more detail additional information can be collected that would be a valuable addition to other field data.

Decreased reproduction in otters, grey seals and sea eagles was associated with high levels of organochlorine contaminants [2] indicating that the reproductive system of wild animals might be a valuable indicator of environmental disturbances. As a predator at the top of the food chain and as a strictly seasonal breeder, the lynx is likely to be sensitive to changes in the climate and the environment and therefore could be a suitable indicator for such changes. In addition inbreeding is known to affect reproduction [3,4]. Genetic variation is an important factor to consider when managing a population and restricting its growth. By continuous systematic collection of reproductive data from lynxes it might be possible to detect early indications of disturbances in the environment and the climate, or an increased level of inbreeding or the incidence of a disease in a closed/given population. In order to detect possible changes it is, however, essential to have base data for comparison and to have a thorough knowledge about the reproductive system of the specific species that is studied. When routine and validated methods exists for evaluating reproduction, continuous monitoring of reproductive organs can also be combined with measurements of concentrations of environmental contaminants [5]. The lynx is subjected to control hunting in Sweden. The hunting period coincides with the reproductive season. All lynxes that are culled or found dead in Sweden are to be sent to the Swedish National Veterinary Institute (SVA) and therefore reproductive data can be routinely collected from the organs of lynxes killed by hunting.

The litter size in the Eurasian lynx varies between 1 and 4, with most litters consisting of 2 or 3 cubs [6]. A lower field pregnancy rate in two-year old females than in older females in Norway and Sweden was reported and has also been observed in Zoos [6,7]. Pregnancy rates also varied with different geographic locations. The mean pregnancy rate varied between 22 and 74 % for 2 y old females and between 69 and 90 % for older females. Mean litter sizes varied between 1.99 and 2.34 in 61 radio-collared Eurasian lynxes in Sweden and Norway but did not differ significantly between locations and age groups [7]. It is not possible in field data to elucidate whether differences in pregnancy rates are caused by differences in implantations rates, differences in age of puberty or differences in perinatal mortality [7]. By studying reproductive organs post-mortem it is, however, possible to evaluate ovulation and implantation rates. In addition, collection of data from organs may elucidate pathological disturbances that might affect reproduction, which would be difficult to detect in field studies.

The female lynx usually have the first litter at an age of 2 y [6,8]. Rarely 1 y old females have been reported to give birth to a litter (Puschmann 1983 and Kaczensky 1991, cited by [6]). Most male lynxes are likely to be fertile the reproductive season of their second year [7]. Knowledge about age related fertility is likely to be beneficial for estimates of the development of a population as females of different ages may differ in their contribution to the population.

Kvam [10] studied reproductive organs that had been collected during 1960 to 1976 in Norway and found fresh luteal bodies and Graafian follicles in lynx females as young as 10.5 mo. Although lynx females may have their first heat as yearlings they do not seem to conceive at this heat according to the age reported when they usually have the first litter [6]. Nilsen et al. [8] concluded that lynx females that have matured at an age of 1.5 y, based on structures in the ovaries, had a significantly higher body weight than immature females. They also concluded that females that had their first litter at an age of 2 y had a lower body weight the season after than 2 y old females that had not conceived. This was interpreted as a cost in body-weight development associated with early maturation [8]. Data about puberty in relation to body condition and the possible effect of early pregnancy on body development in the lynx is, however, scarce.

A peculiarity of the lynx species is that CL remain in the ovaries as macroscopically visible structures for several years [10,11,12]. These retained ovarian luteal bodies were named luteal bodies of previous cycles or LBPC [13]. In the bobcat (*Lynx rufus*) it is possible that LBPC remain for the rest of the animal's life while it is not known for how long old LBPC remain macroscopically visible in the ovaries of the Eurasian lynx [10,12]. Compared to other felids the lynx species seems to have a prolonged luteal function with CL capable of producing low concentration of progesterone long after the end of pregnancy and maybe for several seasons in the bobcat [13,14,15,16]. Göritz et al. [15] suggested that the function of the prolonged luteal function would be to restrict the breeding season to midwinter while Woshner [13] suggested a supportive function of the corpora lutea from previous seasons for the next pregnancy. Although bobcats similar to the other lynx species have retained corpora lutea from previous ovulations they can have more than one estrus during the same reproductive season [13,17] indicating that LBPC do not inhibit estrus in this species. The exact function of retained CL from previous cycles in the lynx species is still unclear.

Mowat et al. [18] evaluated estimation of placental scars of lynxes in Canada as a method to measure pregnancy rates and found that the number of scars correlated well with birth rates. Placental scars can vary in shades but Mowat et al. [18] concluded that all shades should be counted for a correct estimation of last year's litter size. Pullainen et al. [19] used placental scars to estimate reproduction in the European lynx in Finland and found a mean litter size value and litter size distribution that is in accordance with field and Zoo data [6,7]. This indicates that evaluation of placental scars is a useful method for post-mortem retrospective evaluation of pregnancy rates and litter sizes in the Eurasian lynx.

Aims

The aims of this study were to evaluate a routine protocol for collection of reproductive data from female lynxes and if possible to collect data about reproductive season, ovulation rates, implantation rates and age of puberty as a complement to existing lynx reproductive data. In addition our aim was to present data from systematic evaluation of lynx reproductive organs as a basis for possible future continuous evaluation of lynx reproduction as a complement to the collection of field data.

2 Materials and Methods

2.1 Animals

A total of 120 female lynx that had been sent to SVA during 2009-2011 were included and evaluated later in the same season. The animals were subjected to routine necropsy at SVA, where all pathological findings were recorded. Date of death was recorded when known (n=119). The body condition score was recorded as follows; 1 = emaciated, 2 = thin, 3 = ideal, 4 = overweight and 5 = obese. Animals were aged by counting cementum annuli of a canine tooth (Matson's Laboratory, Milltown, Montana, USA). In this work, Lynx were aged according to the number of birthdays that had passed from the expected birth date, taking into consideration that Lynx birth season occurs in May [9]. Thus 0 y old females are in fact 10-11 mo, 1 y old females 22-23 mo, 2 y old females 34-35 mo old and so on.

The animals were killed or died between March 1 and April 9. Their ages varied between 10-11 mo (=0 y) and 13 y (mean 3.4 ± 3.1) and bodyweights between 9.7 and 21 kg (Table 1). For 10 females there was no record of body weight. Body condition scores ranged from 2-4. The distribution of the ages was skewed with 48.3 % of the 118 animals of known age being 2 y or less.

Lynxes were hunted in 11 different geographical regions. For analyses of possible regional effects, the different regions were categorized into 3 groups based on latitude to increase statistical power by increasing the number of animals/group (Fig 1).

2.2 Evaluation of reproductive organs

Reproductive tracts were removed from fresh or frozen-thawed lynxes at SVA and transported to SLU, which is within a 5 min walking distance from SVA. Organs were measured, weighed and inspected macroscopically. The uterine horns were opened with scissors and evaluated for the presence of placental scars. Unlike Mowat et al. [18] we did not soak the organs in water before evaluation and did not use a light source below the organs. The uterus was parted from the cranial vagina at the cervix and was weighed. Ovaries were removed, weighed and evaluated macroscopically before and after being cut longitudinally.

Animals with data for only one ovary (n=4) were excluded from evaluations about the total number of CL and total ovarian weight since both ovaries were to be considered as a physiological unit.

2.3 Statistics

Data are presented as means ± SD unless stated otherwise. The normal distribution of data or residuals was checked with the Ryan-Joiner method. Non-parametric methods were used when the data didn't fill the assumptions for parametric methods. The general linear model (GLM) and Tukey's pairwise comparisons were performed for comparisons of body weights, total ovarian weight and uterine weight between different classes of animals. Spearman's correlations were performed for evaluation of the relationships between data. Mann-Whitney's test was used to compare the number of placental scars with the number of ovulations, comparison of body weight and body conditions score for 1 y old females with and without LBPC and 2 y old females with and without placental scars, and for comparisons of pregnancy results between regions. Chi-square analysis or Fisher's exact test (depending on the expected frequency) was used for pairwise comparisons of pregnancy rates in different age groups in combination with Bonferroni's correction of P-values. Kruskall-Wallis test was used to evaluate an effect of age on the number of placental scars. P-values < 0.05 were considered to be significant. All calculations and graphs were made with Minitab 16.2.2 (Minitab Inc.).

3. Results

3.1. Evaluation of placental scars and litter sizes

Placental scars were visible as dark bands in the uterus and were usually easy to count (Fig. 2a). Sometimes patchy regions could be seen in the same uterus as the more typical placental scars (Fig 2b). These lighter regions were not counted as placental scars from the previous season. Litter sizes varied between one and four implantations with most litters (83.3 %) consisting of two or three implantations (Fig. 3). Placental scars were usually evenly distributed between the uterine horns except for two females with two scars in one uterine horn and no scars in the other.

No animals ≤ 1 y had placental scars (Fig. 2c), 12/22 females aged 2 y (34-35 months) and 55/61 females ≥ 3 y had placental scars indicating a pregnancy in the previous season (Fig. 4). Total pregnancy rate for females ≥ 2 y (n=83) was 80.7 ± 39.7 %. The difference in pregnancy rates between 2-y old females and 3-7 y old females was significant but did not differ between females 8-13 y old and the other age groups. In animals with placental scars the number ranged between one and four with a mean of 2.4 ± 0.76. In previously pregnant animals the number of placental scars did not differ significantly between the three different age groups (Table 2).

3.2. Evaluation of ovaries

Based on their macroscopical appearance corpora lutea were classified as old, from previous cycles (LBPC), or fresh, from the current season (fig. 5). Fresh CL from the current season were pink/light carmine and firm in consistency, often with an ovulation point while LBPC were brown and had a looser consistency. No effort to further differentiate LBPC into different ages was made because the differences in coloration were subtle and not consistent and sizes overlapped why an estimation of age would be highly uncertain.

3.2.1. Corpora lutea from previous seasons

Females < 1 y of age did not have any LBPC, indicating that they had not ovulated the previous season. Eight of 12 (67%) of the 1 y old (22-23 mo) females had LBPC and all females ≥ 2 y had LBPC except one 2 y old female which had small ovaries without ovarian structures (total ovarian weight 0.623 g, bw 15 kg, body condition score 3, killed March 3). The number of LBPC in 1 y old females ranged between 1 and 3 suggesting that they originated from only the previous season (under the assumption that the ovulation rate is one to four). Two y old females never had more than eight LBPC, which would be in accordance with CL remnants from up to two seasons with only one estrus/y. Females ≥ 3 y had a number of LBPC between three and 11 indicating that some of them must have had CL from at least three previous seasons (under the assumption that there is only one estrus each season). The number of LBPC always equaled or exceeded the number of placental scars. There was a significant correlation between the number of placental scars and the number of LBPC for 2 to 13 y old females ($r = 0.28$, $P = 0.012$) but when the 2 y old females were removed from the statistics the correlation lost significance ($r = -0.006$, $P = 0.96$) indicating that there is no relation between the number of LBPC and the litter size the previous season for mature females.

There was a significant positive correlation between the total ovarian weight and the age of the females (Fig. 6). The combined weight of the ovaries for each female was also significantly correlated both with the total number of LBPC ($r_s = 0.648$, $P < 0.001$) and the age of the females ($r_s = 0.572$, $P < 0.001$). The combined ovarian weight was significantly higher for females with LPBC and fresh CL than for females with LBPC but inactive ovaries (Table 3). Females with LPBC and active follicles did not, however, have a significantly different combined ovarian weight than inactive females with LPBC.

3.2.2. Active ovaries and reproductive season

One 3 y old and one 0 y old females that had one missing and one inactive ovary each were excluded from the calculations since it was not possible to know if there might have been active structures in the missing ovaries. Of the remaining 73 females that were killed before March 14 only three had active ovaries with follicles. The earliest date follicles were seen was March 5 in a 3 y old female killed in the Stockholm region. In 26 females killed between March 14 and March 24, 12 had follicles and five had fresh corpora lutea.

The earliest date fresh CL were found was March 19 in a 0 y old female killed in the Uppsala region. She was, however, one of the two females in which it was difficult to differentiate the CL as old or fresh due to decomposition of the ovaries. Based on the weight of the uterus (9.94 g) she was classified as newly ovulated. Considering her age it was also more likely that the CL were fresh than old as no other 0 y female had LBPC. Ten of the 18 females killed between March 25 and April 9 had fresh CL while five had follicles and no fresh CL (Fig. 7).

The majority of the females in this study were thus in heat between March 14 and March 24 but one female was in heat as early as March 5 and one female had mature follicles and no fresh CL as late as April 1 (Fig. 7). Although the female with the earliest occurrence of estrus and the one with the earliest occurrence of fresh CL were killed in two of the most southern regions included in the study and the one with the latest estrus was killed in one of the most northern districts, there was no obvious correlation between regions and time of heat (based on visual evaluation of graphs, no statistical calculations performed). The number of females that were killed during the period of heat may, however, have been too low to discover such possible regional differences because individual variations seem to be large.

No females killed after March 19 had inactive ovaries except two 1 y old females killed March 26 and April 9 and one 0 y old female killed March 26. The 1 y old female killed April 9 had a body weight of only 10 kg and no LBPC in her ovaries indicating that she might still be prepubertal. The other 1 y old female had LBPC, indicating that she was post-pubertal, but she had no active ovarian structures.

The weight of the uterus differed significantly with stage of the cycle (Table 3).

The number of ovulations, calculated as the number of follicles and/or fresh CL, was 2.5 ± 1.02 (range one to four, n=31) and did not differ significantly (P = 0.22) from the mean number of placental scars (n=66).

3.3. Sexual maturation

None of the 10 to 11 mo (0 y) old females had LBPC demonstrating that they had not ovulated previously (n=21) which is an expected result as they were they were likely to have been born in May the previous season. Eight of twelve 22-23 mo old females (1 y) had one to three LBPC and had thus been in heat previously, probably when they were around 12 mo old. All but one of the 2 y old females had one to eight LBPC (n = 22) indicating that usually lynx females have their first heat as yearlings or the year after. However, no females aged 22 to 23 months or younger had placental scars indicating that females that come into heat as yearlings usually do not become pregnant in the same season. Females aged 10 to11 mo had a significantly lower body weight than all other age groups demonstrating that they were physically immature at this age although a large proportion of them had their first heat around this age (Table 1).

Only six 0 y old females were killed after March 14, the date after which the majority of the females had active ovaries. Four of these young females that were killed between March 16 and March 26 had inactive ovaries, one young female killed March 30 had mature follicles (body weight 11 kg) and one young female killed March 19 (body weight 13 kg) had ovulated the same season (partly based on uterine weight because the ovaries were decayed). This is in compliance with the occurrence of LBPC in 67 % of the 1 y old females in this study.

Body weights did not differ significantly (P=0.39) between 1 y old (22-23 mo) females that had ovulated previously (n=8, median 15.5 kg) and 1 y old females without LPBC (n=4, median 14.0 kg) but there was a non-significant trend for a higher body condition score in 1 y old females that had CL from the previous season (median 2.5 *vs* 3.0; P = 0.057). Two y old (34-35 mo) females that had been pregnant the previous season (n=10, median 16.0 kg) and 2 y old females without placental scars (n=10, median 15.0 kg) had similar body weights (P=0.56).There was no significant difference in body condition score between 2 y old females with and without placental scars (P = 1.0).

3.4. Comparisons between regions

There were no differences in pregnancy rates, as evaluated by the presence of placental scars, between the three regions for 3 to 13 y old females (region 1 n=16; region 2 n= 23; region 3 n=15; P = 0.83) or 2 y old females (region 1 n=6; region 2 n=13; region 3 n=3; P = 0.65).

3.5. Other findings

There were no macroscopical signs of cystic endometrial hyperplasia or other diseases in any of the evaluated reproductive organs.

4. Discussion

The results of this study demonstrate that useful lynx reproductive data can be collected from macroscopical evaluation of organs from females killed by hunting. Data about pregnancy rates and litter sizes in the previous season, sexual maturation and reproductive season could be collected from lynxes that had been killed and sent to SVA.

As previously shown by Kvam [9] CL remain as macroscopically visible structures for more than one season in the Eurasian lynx. All females above the age of three years in this study had retained corpora lutea from previous cycles (LBPC) in their ovaries. The occurrence of LBPC indicated that the female had been in heat previously and thus was postpubertal. The number of LBPC was, however, not useful for estimation of litter size or ovulation rate since CL remain for at least three seasons according to the number of LBPC in mature females and, moreover, it is not possible to differentiate LBPC from different years macroscopically at the time of the hunting season. The combined ovarian weight was not a useful single parameter for estimation of the reproductive period (Table 3). There were, however, no females with implanted fetuses in this study and thus our data do not include pregnant animals beyond the time of the pre-implantation period. Carnaby et al. [20] found that four pregnant lynxes, in which pregnancy was confirmed by the presences of fetuses, had heavier ovaries than non-pregnant females. Since Woshner et al. [14] demonstrated that luteal tissue progesterone peaked around day 22 of pregnancy in the bobcat, it is thought that the ovaries from the luteal phase in this study may not have been collected during peak CL activity. Ovarian weight was significantly positively correlated with the number of LBPC in this present study. Both the number of LBPC and the ovarian weight were in turn significantly correlated with the age of the female, although individual variations were large. Therefore it seems likely that the number of LBPC increases with age as the female goes through more estrous periods and that the ovarian weight increases with the increasing number of LBPC. Crowe [12] also found a significant correlation between the number of CL from previous cycles and the age of the females in the bobcat. Kvam [10] used the weight of the largest ovary to estimate seasonal and age differences. Our study demonstrates, however, that ovarian weight is not a reliable indicator of cycle stage and in addition combined data of both ovaries is a better physiological measure.

The compliance between the mean litter size and the distribution of litter sizes in this study and previous field and Zoo data [6,7] confirms that the number of placental scars is a good measurement of a previous pregnancy and litter size as indicated in previous publications [18,19]. Placental scars were usually easy to count and do not seem to remain clearly visible for more than one season. Also animals with a uterus affected by the follicular or early luteal phase had clearly visible placental scars. It is not known to date how long the scars remain visible after a new ovulation. One 8 y old female killed April 9 had fresh CL but no placental scars. She had one darker area in one uterine horn that might have been an implantation but it was not possible to be certain on macroscopic evaluation due to decay. Another female killed April 1 with the highest uterine weight in the study (17.46 g), indicating a hormonal influence on the uterus, had fresh CL and 3 clearly visible placental scars. There was no evidence (studied graphically) for a lower number of females with placental scars in females with fresh CL killed late in the season. Therefore it seems likely that placental scars can be counted reliably into the early luteal phase/early pregnancy and possibly until the next implantation. No animals included in this study had visible embryos or fetuses.

The mean litter size based on the number of placental scars was 2.4 ± 0.76 with a range between 1 and 4. This is within the range of litter sizes in June reported for wild Swedish lynxes by Andrén et al. [1]. A slightly higher implantation rate than reported field litter size might be expected as some losses of embryos, fetuses and neonatal cubs is likely. Mowat et al. [18] concluded, however, that in lynxes in Canada residual scars and aborted embryos seemed to be rare, hence placental scar counts did not overestimate litter size at birth. Our data compared with reported field data suggest that the same may be the case for the Eurasian lynx in Sweden. The litter size did not differ between the different age groups in females that had been pregnant the previous season, which is in accordance with the field data reported by Nilsen et al. [7]. A lower litter size was reported in Zoos for females that were 12-15 y [6] but in this study only two females were 12 y or older, the oldest being 13 years. The lower pregnancy rate in young females previously reported in the field [7] seems to be in accordance with the data in this study, mainly due to the lower proportions of females becoming pregnant rather than a larger proportion of young females losing their litter after birth. As there was no evidence for a higher median number of ovulations than implantations, the occurrence of preimplantation embryo loss is likely to be very low.

In contrast to Nilsen et al.[7], we could not detect any regional differences in pregnancy rates. Our classification of regions was, however, not exactly the same as in the study by Nilsen et al. [7] and was based on latitude rather than the density of prey animals. In addition the number of animals per regions might have been too low for powerful statistical analyses of regional differences in our study. It is also possible that regional differences in the number of females with litters may occur after implantation and/or birth as a result of differences in the availability of prey and the loss of whole litters of fetuses and/or neonatal cubs.

Kvam [10] concluded that the majority of ovulations took place in February and March. In addition Kvam [10] reported fresh luteal bodies in 11 females killed in January (n=3) and February (n=8). In the present study, the earliest date a female with fresh luteal bodies was killed was March 19 and only three females were in heat before March 14. Based on the morphology of the CL, Kvam [10] estimated ovulation rate and could count up to 10 ovulations (based on fresh CL and follicles). It is therefore possible that LBPC were confused with fresh CL in Kvam's [10] study as the material was poorly fixed. The ovulation rate (based on the number of follicles and fresh CL) in this study was 1-4, which is in compliance with the range in litter sizes in the Eurasian lynx. According to Henriksen et al. [6] 50 % of lynx births in Zoos took place between May 19 and 31. This would correspond well with a reproductive season that usually starts in mid-March, as shown in this study, and a gestation length of approximately 70 days [10]. Andrén et al. [1] observed the earliest birth on April 28 in wild Swedish lynxes, which would correspond with a mating as early as February 15, but the mean mating dates were estimated to be March 11 in Bergslagen, and March 27 in Sarek, thus being later in the northern parts than in more southern parts. There were too few females with active ovaries in this study to draw any strong conclusions about the influence of region on the reproductive season. The female with the earliest occurrence of estrus and the one with the earliest occurrence of fresh CL in our study were killed in two of the most southern districts and the one with the latest estrus was killed in one of the most northern districts. The domestic cat is a long day breeder and estrus is stimulated by an increase in day length [20]. Therefore it is likely, as previously shown [1], that the reproductive season in the lynx would be earlier in the southern parts of Sweden. Although we found that fresh CL were distinctly different from LBPC unless the ovaries were decayed, Woshner [13] reported that even in fresh ovaries removed by laparoscopy, distinction between CL and LBPC might sometimes be impossible in the bobcat, resulting in why some caution being necessary in the interpretation of ovarian structures. A significant relation between uterine weight and the occurrence of fresh CL (Table 3) indicates that structures classified as fresh CL in this study were indeed active structures from the current season. In contrast to ovarian weight, the weight of the uterus differed significantly between females in different stages of the cycle (Table 3). Therefore the uterine weight is a useful indicator of the reproductive season. The uterine weight was useful to categorize the stage of the cycle for two of the females in this study in which classification of CL was difficult due to decay.

Our data about puberty is in compliance with previous published observations. Lynx females have their first litter the season they become 2 y old but the pregnancy rate in this age group is lower than for older females [6,7]. A significant difference in body weight between 22-23 mo old females and females ≥ 3 y of age indicates that 2 y old females might still not be fully physically mature in the season when they become 2 y old, which could be an explanation for the lower pregnancy results at this age. Although the first litter is born when the lynx female is 2 y old, 67 % of the 22-23 mo old females had been in heat the season before, as revealed by LBPC in their ovaries and 2 out of 6 10-12 mo old females killed during the reproductive period had active ovaries. There are reports that the Eurasian lynx rarely may have a first litter at the age of one year (Puschmann 1983 and Kaczensky 1991, cited by [6]). Nilsen et al. [8] found signs of ovulation in 60 % of 1.5 year old females that had been killed in Norway from January to March (with the majority killed in February) and concluded that they had matured at an age of 1.5 years based on presence of Graafian follicles or ruptured follicles. They did not, however, mention a differentiation between retained and fresh CL. Luteal bodies from previous cycles reveal retrospective events from a previous season, which explains why these females most probably had their first ovulations the previous season when they were yearlings. Our data with evidence of a first estrus in yearling females is supported by the finding of follicles or fresh CL in three females less than 1 y old in Kvam's [10] study. Considering that no females aged less than 34-35 mo had placental scars, it seems likely that females having their first heat at a young age do not become pregnant at that heat. It has not been completely elucidated if the Eurasian lynx is a strict induced ovulator or if spontaneous ovulations may occur. If they are strict induced ovulators, the occurrence of CL would be a strong indication that there has been a mating event. Some felid species, including the domestic cat, may, however, ovulate spontaneously without mating [22,23]. Spontaneous ovulation was also reported in the bobcat [13,17] and in the Canada lynx [16]. Although the domestic cat sometimes ovulates spontaneously, follicles usually undergo atresia after estrus in the absence of a mating event [24]. Follicular atresia in the absence of mating stimuli has been reported both in the bobcat and in the Eurasian lynx [13,15]. It cannot be excluded that young females without LBPC in our study may have been in heat previously without ovulating. The occurrence of ovulations one season before lynx females normally become pregnant demonstrates that the first heat and the first ovulation are not necessarily synonymous of sexual maturity. Atypical hormonal patterns have been observed at puberty in other species. The first estrus in bitches can be associated with a lack of sexual receptivity and aberrant patterns of hormone concentrations [25]. In contrast to our findings, Nilsen et al. [8] reported signs of placental scars in about 10% of the 1.5 y old females. The birth of cubs in lynx females before the season they become 2 y old is extremely rare according to previous publications [6]. Stys and Leopold [17] reported, however, that yearling bobcats conceived, as confirmed by laparoscopy but all liters were lost before parturition. They suggested that difficulties in maintaining pregnancies in yearling females might have been caused by a lack of hormonal support from retained corpora lutea from previous cycles [17]. We cannot exclude that some 1 y old females in our study may have conceived in the previous season but lost the embryos before the placentas were developed enough to leave scars that were visible the next season.

Data from Kvam [10] in the Eurasian lynx (*Lynx lynx)* and from Crowe [12] and Stys and Leopold [17] in the bobcat (*Lynx rufus*) indicate that juvenile females cycle later in the season than mature animals. Therefore it is possible that at least some of the yearlings with inactive ovaries in this study would cycle after the hunting season.

In contrast to Nilsen et al. [8] we could not detect an effect of body weight on the occurrence of the first ovulation. One year old females with LBPC had a higher mean body weight than females without. This difference was not significant, however, but the number of females in the group without retained LBPC was low giving a low power to statistical analyses. There was a non-significant tendency for higher body condition scores in 1 y old females (22-23 mo) with LBPC. The presence of LBPC is, however, a retrospective measurement while body weight and body condition scores were measured one season after the ovulations that resulted in LBPC. Comparing retrospective data with current data may thus not give a reliable result. In addition, as mentioned previously, it cannot be excluded that 1 y old females without LBPC may have been in estrus previously without ovulating as felids usually are induced ovulators and different patterns of ovulation occur in the same species [23]. The absence of LBPC may therefore not be a reliable indicator whether or not the female is prepubertal. Only two 1 y old females had active ovaries. The female with follicles weighed 11 kg, which was lower than the mean for this age group (Table 1) indicating that the determination of physical maturation as measured by body weight is uncertain as there seem to be large individual variations. In order to evaluate the effect of body weight on the age of the first heat, a larger group of individuals around 12 months of age killed during the period of heat or slightly after would be required. In contrast to Nilsen et al. [8] we did not find a significant difference in body weights between 2 y old females with and without placental scars. Neither did the body conditions scores differ significantly between previously pregnant and non-pregnant 2 y old females. Thus our data do not support the theory that females that mature early and have a litter at 2 y of age do so at the cost in body weight development. The difference between our data and those reported by Nilsen et al. [8] could arise from confounding factors between the studies such as the possible effect of regions. Another difference between the two studies is that Nilsen et al. [8] collected data from females killed between January and March with the majority being killed in February, while our data are from females killed between the beginning of March and early April with the large majority being killed in March. It is unclear if a difference of one month would affect body weights although it cannot be completely excluded for animals that are growing and perhaps recovering from rearing a litter. Nilsen et al. [8] did not detect differences in body weight depending on the month of harvest.

Estimates about the age in months of animals in this study and previous ovulations rates were based on the assumption of only one estrus per year during the usual reproductive season. Although it has been reported that occasionally Eurasian lynx females may come into estrus again after the loss of a litter with the birth of cubs as late as September being reported [6]. In rare circumstances LBPC may therefore originate from more than one estrus period during the same year and some animals may not have been born at the estimated time of year. Crowe [12] found a number of LBPC in young female bobcats that had passed one breeding season but had not entered the next that was too high to originate from only one estrus and concluded that it was likely that the bobcat can be polyestrus. This theory was later confirmed [13,17].

The ovaries of 1 y and 2 y old females in the present study contained a number of LBPC that would be consistent with only one estrus/season in the Eurasian lynx.

Although classifications of CL as old or new and counting placental scars was usually easy, in a few cases it was difficult to evaluate if CL were fresh or retained from previous cycles and if a shade in the endometrium should be counted as a placental scar or not due to decay of the organs. Sometimes very pale patchy regions were found that might represent remnants of placental scars that were older than from the previous season or that might have been remnants from embryo resorption/early abortion. We did not count them as placental scars as they differed substantially from the more typical patterns with dark bands. In order to be completely certain about the classification of these uterine shades, the history of the animals with reports of previous litter sizes would be needed. However such data were not available. The results demonstrate, however, that evaluations made on organs from carcasses largely give correct estimates of reproduction in the lynx considering the compliance of this data with field and Zoo data [6,7]. There are also sources of errors in field data, which is why a combination of different methods to study reproduction in the Eurasian lynx is likely to be beneficial.

Generally the organs of the wild lynx females included in this study were healthy with no macroscopical signs of cystic endometrial hyperplasia (CEH). This condition is common in domestic cats > 3 y of age [26] and in Zoo felids treated with Melengestrol Acetate contraceptives [27]. Cystic endometrial hyperplasia is likely to develop as a result of hormonal stimulation of the endometrium [26,28]. In contrast to domestic cats, wild lynxes usually have only one estrus/season and most of them become pregnant each season according to the high pregnancy results for the mature females in this study and in field studies [1]. The non-pregnant uteri of wild lynxes are thus not affected by repeated hormonal stimulations or exogenous gestagens in contrast to intact domestic cats or wild Zoo felids that are not regularly bred.

In conclusion, this study confirms that useful information about lynx reproduction can be collected from reproductive organs retrieved after the death of the animals. Continuous monitoring of lynx reproductive organs according to a routine protocol would therefore be a useful contribution to collection of field data to gather information for management of lynx populations and to be able to evaluate previous reproductive data retrospectively. Continuous routine collection of reproductive data could be used potentially for environmental monitoring, with the lynx as an indicator animal for changes in climate and the occurrence of environmental toxins or diseases that can affect reproduction.

Acknowledgements:

The authors would like to thank the Portuguese Science and Technology Foundation for financing Prof. Rita Payan-Carreira's sabbatical (SFRH/BSAB/938/2009) and Carl Trygger's foundation for financial support of the study.

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Revised highlighted

Tables

Table 1. Body weight in females of different ages. Mean±SD (range)

Age	10-11 mo n=22	22-23 mo n=12	34-35 mo n=20	3-13 y n=54
Body weight (kg)	11.5 ± 1.3^{a} (9.7-14.0)	14.7 ± 2.03^{b} (10.0-17.5)	15.6 ± 1.2^{bc} (12.7-17.0)	16.2 ± 1.8^{c} (12-21)

^{abc}Values with different subscripts differ significantly within rows

Table 2. Pregnancy rates and litter sizes estimated from the placental scars in females of different ages. Mean±SD (number of females)

Age	2 y	3-7 y	≥ 8 y
Number (proportion) of females with placental scars	12 (0.54) [a] (22)	43 (0.96) [b] (45)	12 (0.75) [ab] (16)
Number of placental scars in previously pregnant females	2.4 ± 0.79 (12)	2.4 ± 0.76 (42)*	2.6 ± 0.79 (12)

[ab]Values with different letters differ significantly within rows

*One female had at least one placental scar but the number could not be counted due to damage to one uterine horn, which is why she was not included in the statistical calculations on the number of placental scars.

Table 3. Combined weight of both ovaries and the uterus at different stages. Mean±SD (range), n= number of individuals

Stage	No LBPC, no active structures,	No LBPC but follicles	No LBPC but fresh CL	LBPC but no active structures	LBPC and active follicles	LBPC and fresh CL
Total ovarian weight (g)	0.43±0.14[a] (0.17-0.82) n=23	0.70[NE] (N.A) n=1	1.47±0.65[NE] (0.80-2.1) n= 3	2.26±0.78[b] (0.72-4.81) n= 59	2.36±0.58[bc] (1.30-3.34) n = 19	2.93±0.76[c] (1.50-4.24) n = 11
Uterine weight (g)	0.95 ± 0.61[a] (0.40-3.28) n = 20	3.65[NE] (N.A) n = 1	10.97 ± 1.53[NE] (9.89-12.05) n=2	4.11±1.72[b] (1.39-8.37) n=57	8.63±2.96[c] (2.54-12.7) n=19	12.65±2.95[d] (7.75-17.46) n=11

[abcd]Figures with different letters differ significantly within rows. Two females classified as having fresh CL partly based on uterine weight were removed from the comparisons. [NE]Data were not included in statistical comparisons due to a low number of animals in these groups.

Figures **Revised**

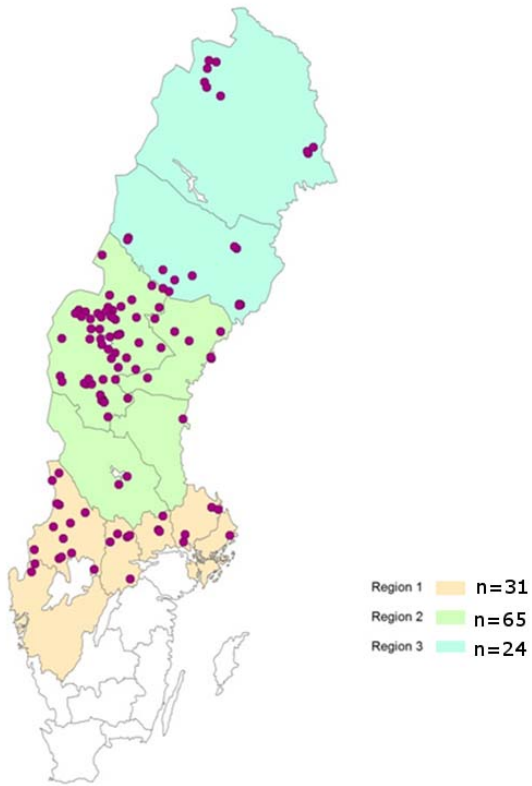

Fig 1. A map over Sweden with regions 1-3 and with location for individual lynxes marked.

Figure 2 – Internal surface of Lynx uteri. A – The placental scars appear as dark red or blackish areas at the internal surface of the uterine horns. B – Lighter patchy regions within the uterine horns were not counted as placental scars. C – A lynx uterus without placental scars.

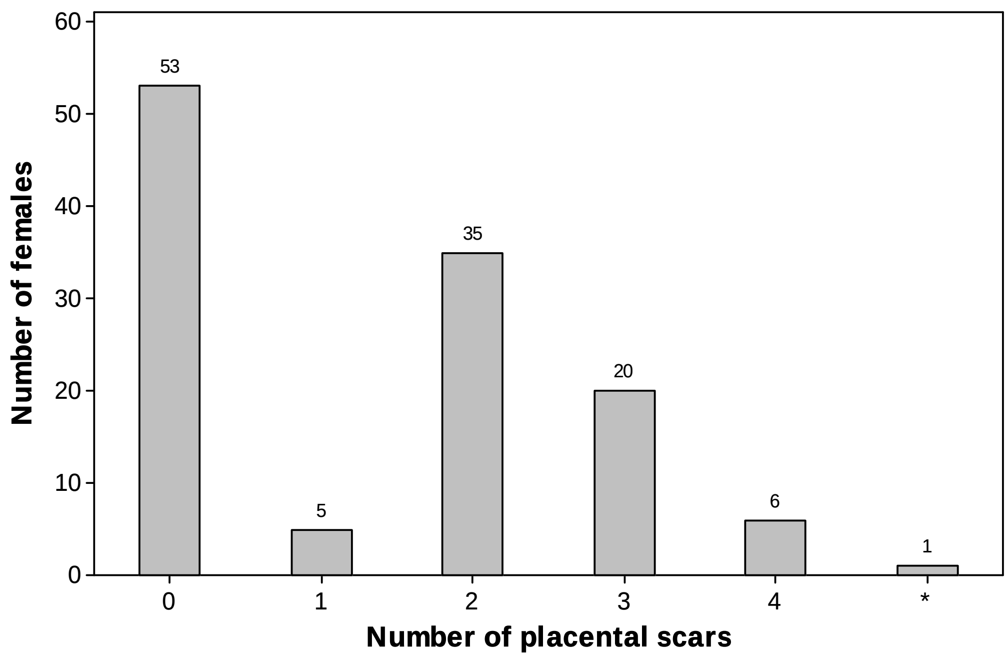

Figure 3. Distribution of the number of placental scars. * One female had placental scars but one uterine horn was damaged with the result that the exact number could not be counted.

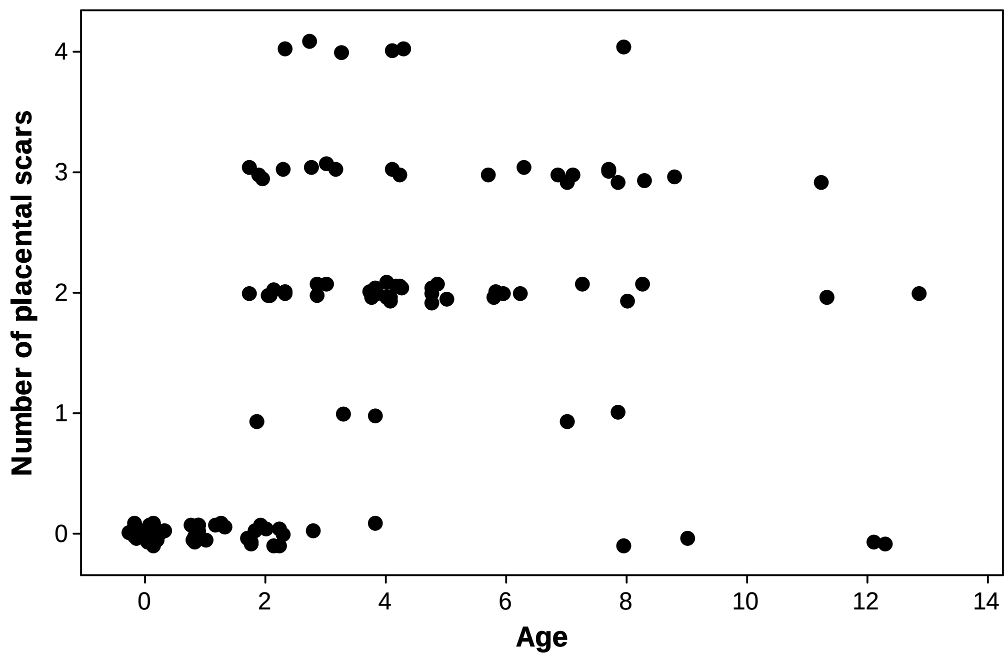

Fig 4. Scatterplot of the number of placental scars in lynx females of different ages. As the placental scars are retrospective data from the previous season they allow inferring the litter size the season prior to that of the age given on the x-axis.

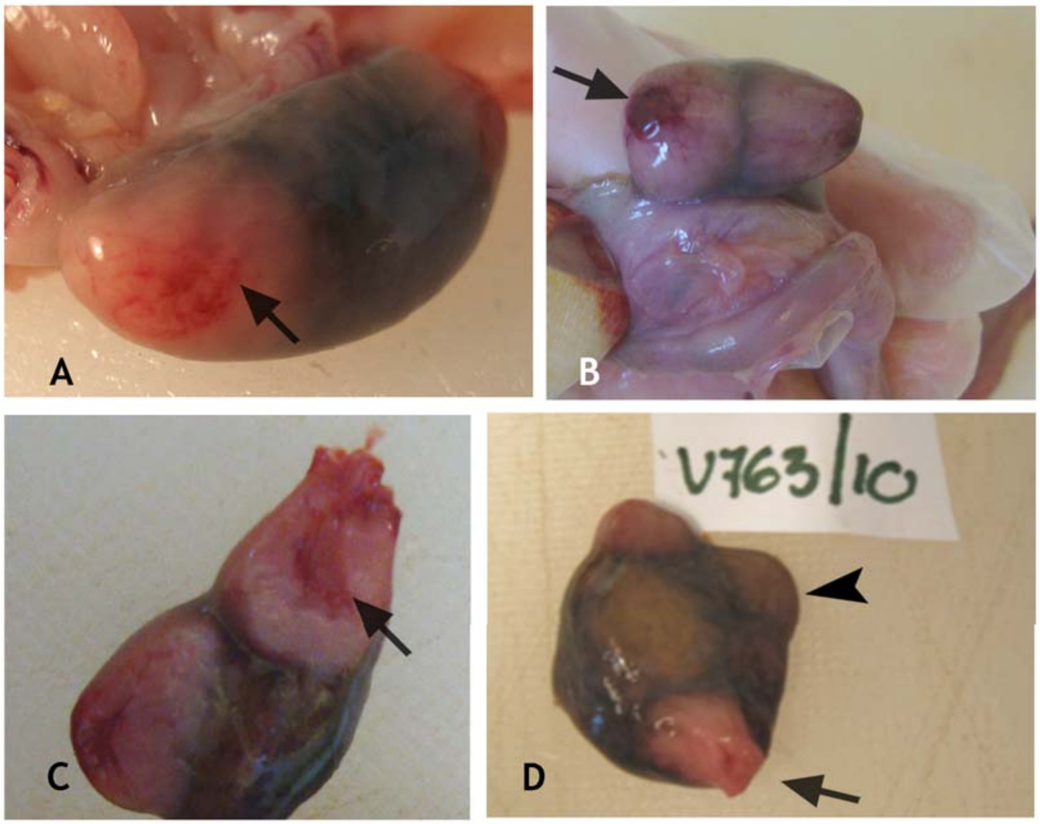

Figure 5 – Lynx ovaries showing different structures used for the classification of the ovarian activity. A – Antral follicles at the poles of this ovary; the vascularization of the follicle is clearly noticed (arrow). B – In this ovary, two young corpora lutea were observed, showing the ovulation papilla at his apex (arrow). C – A longitudinal cut of the same ovary show that those corpora lutea are still cavitary. D – A cross section of an ovary of a mature female showing the differently coloured luteal structures: the darker structures correspond to LBPC (arrowhead) while recent CL appears light carmine in colour (arrow).

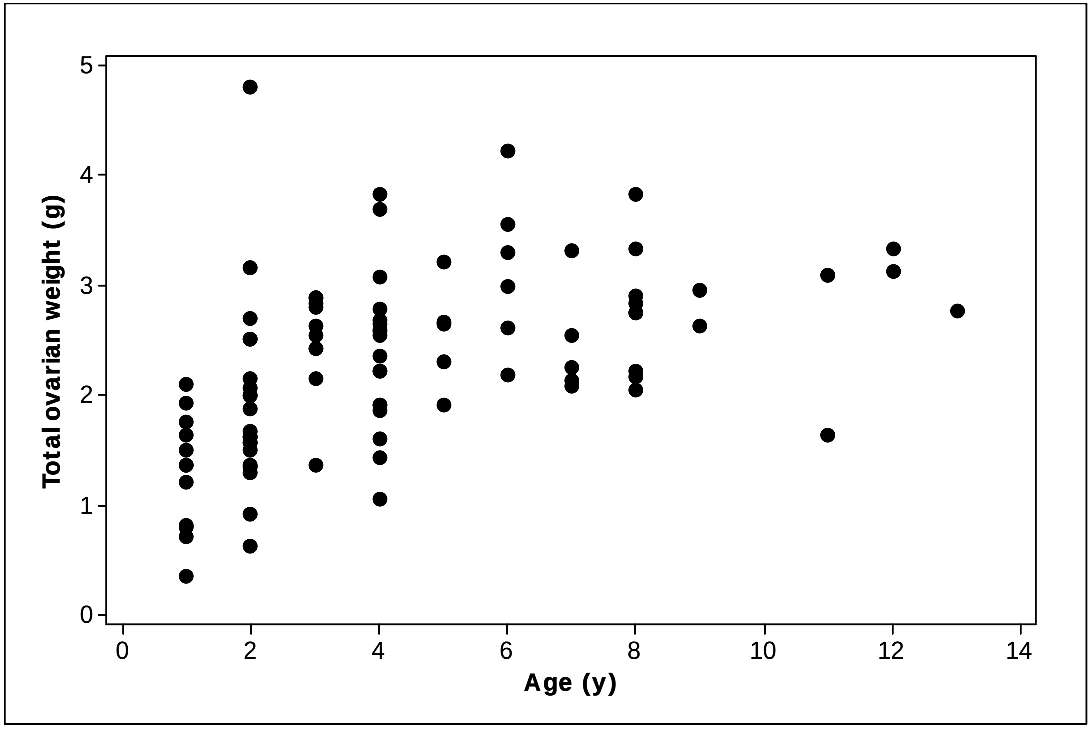

Fig 6. Total ovarian weight in animals aged 1-13 y plotted against age. r_s=0.605, P <0.001. 1 y animals are 22-23 mo, 2 y animals 34-35 mo and so on. Animals aged less than 1 y (10-11 mo) were removed from the analysis as none of them had LBPC. When only animals aged 4-13 y were included the correlation was still significant r_s=0.284, P=0.045.

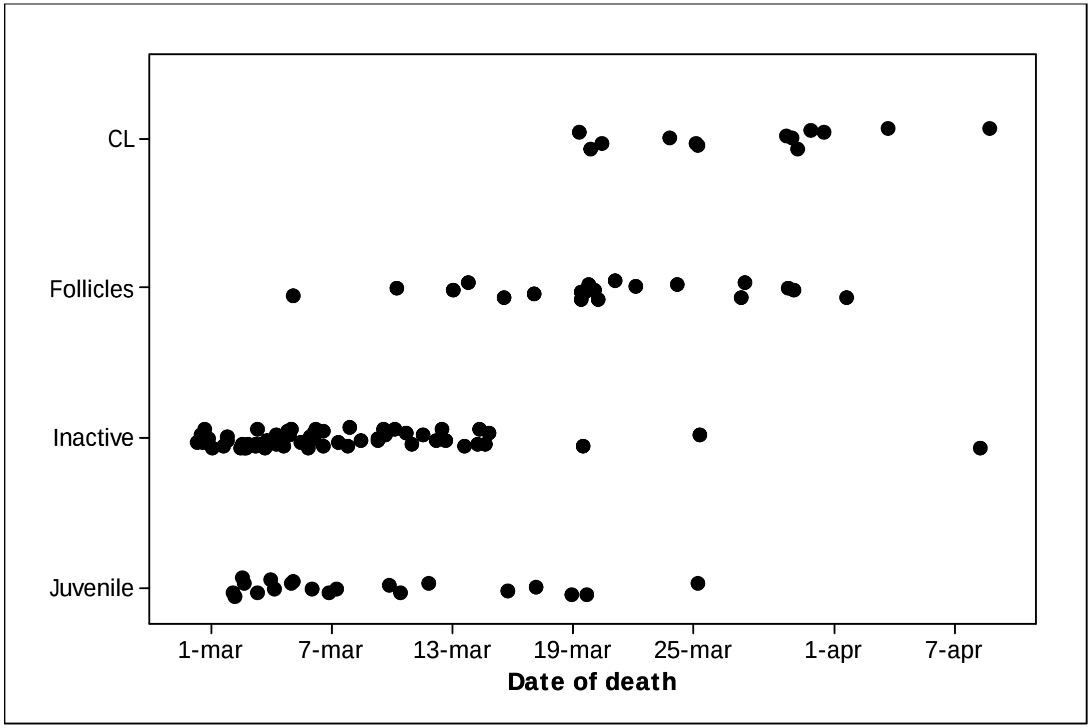

Fig 7. Stage of the reproductive cycle in relation to date of death. Juvenile - <1y old, without active ovarian structures. CL=fresh CL