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im Forschungsverbund Berlin e.V. und dem
Fachbereich Veterinärmedizin der Freien Universität Berlin

Reproduction management in female Lynx (*Lynx lynx*)

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This thesis is dedicated to my mother Eva-Maria Painer.

Thank you for all your great support!

The road not taken

Two roads diverged in a yellow wood,
And sorry I could not travel both
And be one traveler, long I stood
And looked down one as far as I could
To where I bent in the undergrowth;

Then took the other, as just as fair,
And having perhaps the better claim,
Because it was grassy and wanted wear;
Though as for that the passing there
Had worn them really about the same,

And both that morning equally lay
In leaves no step had trodden black.
Oh, I kept the first for another day!
Yet knowing how way leads on to way,
I doubted if I should ever come back.

I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, and I –
I took the one less traveled by,
And that has made all the difference.

Robert Frost (1874 – 1963) in Mountain Interval 1920.
Introduced by a gift from Prof. J. Arnemo in 2007.

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List of abbreviations

AI	artificial insemination
ART	assisted reproduction technique
BMBF	Federal ministry of education and research, Germany
BW	body weight
CEH	cystic endometrial hyperplasia
CL	*Corpus luteum, Corpora lutea*
CV	coefficient of variation
d	day
df	degree of freedom
DNA	desoxyribonucleic acid
EAZA	European Association of Zoos and Aquaria
eCG	equine chorionic gonadotropin
EDTA	Ethylenediaminetetraacetic acid
EEP	European endangered species programme
EL	Eurasian lynx (*Lynx lynx*)
ESB	European studbook
ESM	electronic supplementary material
et al.	et alii/aliae = and others
E2	oestrogen
Fig.	figure
FSH	follicle stimulating hormone
FU	Freie Universität, Berlin, Germany
GnRH	gonadotropin releasing hormone
hCG	human chorionic gonadotropin
IBL	Iberian lynx (*Lynx pardinus*)
ILCBP	Iberian lynx conservation breeding programme
IUCN	International union for the conservation of nature
IVM	in-vitro maturation
IVF	in-vitro fertilisation
IZW	Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany
kg	kilogram
LLC	large luteal cells
LH	luteinising hormone
mg	milligram
MGA	Melengestrol acetate
µg	microgram
mL	millilitre
mtDNA	mitochondrial DNA
ng	nanogram
N	number
p	statistical p-value
pFSH	porcine follicle stimulating hormone
pLH	porcine luteinising hormone
pg	picogram
PGFM	prostaglandin F2α metabolite
PGF2α	prostaglandin F2α
P4	progesterone
SLC	small steroidogenic luteinising cells
SVA	Swedish National Veterinary Institute
TAG	Taxon Advisor Group

1 Introduction

Thirty-seven wild *Felidae* (table 1) and one domestic cat species inhabit the Earth. To date, the International Union for the Conservation of Nature (IUCN) Red List of threatened species lists 11 Felidae species as *least concern*, 9 as *near threatened*, 9 as *vulnerable*, and 8 as *endangered* (Fig. 1) (IUCN, 2015). The most endangered felid species, the Iberian lynx (*Lynx pardinus*) inhabits the Iberian Peninsula, in Europe. Only recently it got downgraded from being *critically endangered* to *endangerd* (IUCN, 2015). A renowned Cat Specialist (2003, P. Jackson, Cat Specialist Group within the IUCN) claimed during a BBC interview (http://news.bbc.co.uk/2/hi/science/nature/2634853.stm), if 'the Iberian lynx gets extinct, it would be the first cat species to die out after the sabre-tooth tiger went extinct around 10.000 years ago'.

In 2003 the Iberian Lynx Conservation Breeding Programme (ILCBP) was initiated to help to maintain the genetic diversity of the species (Vargas et al., 2009). Two years later in 2005, the Leibniz Institute for Zoo and Wildlife Research (IZW) in Berlin (Germany), became "reproductive advisor" for the programme. First solutions were needed to establish a non-invasive pregnancy test to ensure a suitable handling of pregnant females (Braun et al., 2009; Dehnhard et al., 2008, 2012; Finkenwirth et al., 2010; Jewgenow et al., 2009). During basal investigations on the reproductive tract of Iberian Lynx many open questions on their cyclicity remained unclear. Female Iberian and Eurasian lynx (hereafter refered to as *Lynx*) experienced a different reproductive cycle compared to all other felids, with *corpora lutea* (CL) remaining outside breeding season (Goeritz et al., 2009a, b; Jewgenow et al., 2009). The following years the IZW established a variety of studies where the Eurasian lynx was used as model species to investigate the reproduction biology. This thesis includes results explaining the fundamental physiology of Eurasian and Iberian lynx female reproduction. An important goal was to understand the unique physiology of the CL outside breeding season, their luteogenesis and luteal regression. Furthermore, solutions were aimed for, on how the reproduction rate of *Lynx* could be increased during years where the females would not conceive during the natural cycle.

2 Review of the biology of *Lynx*

Table 1: List of *Felidae* species classified into their protection status, by the IUCN (IUCN, 2015).

IUCN red-list status	*Felidae* species
Endangered	Iberian lynx (*Lynx pardinus*), Borneo bay cat (*Catopuma badia*), Chinese desert cat (*Felis bieti*), Andean cat (*Leopardus jacobita*), tiger (*Panthera tigris*), snow leopard (*Panthera uncia*), flat-headed cat (*Prionailurus planiceps*), fishing cat (*Prionailurus viverrinus*)
Vulnerable	cheetah (*Acinonyx jubatus*), black-footed cat (*Felis nigripes*), kodkod (*Leopardus guigna*), oncilla (*Leopardus tigrinus*), sunda clouded leopard (*Neofelis diardi*), clouded leopard (*Neofelis nebulosa*), lion (*Panthera leo*), marbled cat (*Pardofelis marmorata*), rusty-spotted cat (*Prionailurus rubiginosus*)
Near threatened	African golden cat (*Caracal aurata*), Asiatic golden cat (*Catopuma temminckii*), sand cat (*Felis margarita*), pampas cat (*Leopardus colocolo*), Geoffroy's cat (*Leopardus geoffroyi*), margay (*Leopardus wiedii*), Pallas's cat (*Otocolobus manul*), jaguar (*Panthera onca*), leopard (*Panthera pardus*)
Least concern	caracal (*Caracal caracal*), jungle cat (*Felis chaus*), wild cat (*F. silvestris*), jaguarundi (*Herpailurus yagouaroundi*), ocelot (*Leopardus pardalis*), serval (*Leptailurus serval*), Canadian lynx (*Lynx canadensis*), Eurasian lynx (*L. lynx*), bobcat (*Lynx rufus*), leopard cat (*Prionailurus bengalensis*), puma (*Puma concolor*)

2.1. Classification and evolution of *Lynx*

Within the animal kingdom, the carnivora order consists of diverse species all divided into only two suborders: the dog-like (*Caniformia*) and the cat-like (*Feliformia*) suborder. The cat-like suborder splits up into 7 families: the *Felidae*, the *Hyaenidae*, the *Eupleridae*, the *Herpestidae*, the *Nandiniidae*, the *Prionodontidae* and the *Viveridae*. As mentioned above, the *Felidae* family consists of 37 wild felid

species and one domestic cat species. Currently this family is classified into 8 genetic lineages (domestic cat lineage, leopard cat l., puma l., lynx l., ocelot l., caracal l., bay cat l., panthera l.; table 2).

table 2: Current *Felidae* lineages. Modified after: Johnson et al., 2006.

Lineage	Species
domestic cat lineage	domestic cat, wild cat, Chinese desert cat, desert cat, black-footed cat, jungle cat
leopard cat lineage	Pallas's cat, rusty spotted cat, Asian leopard cat, fishing cat, flat-headed cat
puma lineage	puma, jaguarundi, cheetah
lynx lineage	Iberian lynx, Eurasian lynx, Canada lynx, bobcat
ocelot lineage	ocelot, margay, Andean mountain cat, pampas cat, Geoffroy's cat, kodkod, tigrina
caracal lineage	caracal, African golden cat, serval
bay cat lineage	bay cat, Asian golden cat, marbled cat
panthera lineage	lion, jaguar, leopard, tiger, snow leopard, clouded leopard

The genus *Lynx* consists of four species (Fig. 1): the Eurasian lynx (*Lynx lynx*, Linnaeus 1758), the Iberian lynx (*L. pardinus*, TEMMINCK 1827), the Canadian lynx (*L. canadensis*, KERR 1792) and the bobcat (*L. rufus*, SCHREBER 1777).

Fig. 1: The four *Lynx* species A: Eurasian-, B: Iberian, C: Canadian lynx, D: Bobcat. (© copyrights for images: A: J. Painer, B: Iberian lynx ex-situ conservation programme, C: P. Burwell, D: F. Spangenberg)

The present day *Lynx* (Fig. 2, 3) descended from a common ancestor which diverged from other felids around 6 million years ago (Johnson et al., 2004). Fossil records indicate that the *Lynx* group most likely originated in Africa, and thereafter dispersed into Eurasia (Werdelin, 1981). There is strong evidence that the bobcat represents the most ancestral lineage today (Johnson et al., 2004; Werdelin, 1981). The

relationships between the other three species are less well defined. They may have diverged around a similar time from a common ancestor (*L. issiodorensis*) into monophyletic lineages (Johnson et al., 2004; Werdelin, 1987) (Fig. 2, 3). Even though the Iberian and Eurasian lynx were both found all over Europe in the Pleistocene, it is likely that they never occurred together (Johnson et al., 2004; Kurten and Granqvist, 1987; Werdelin, 1981).

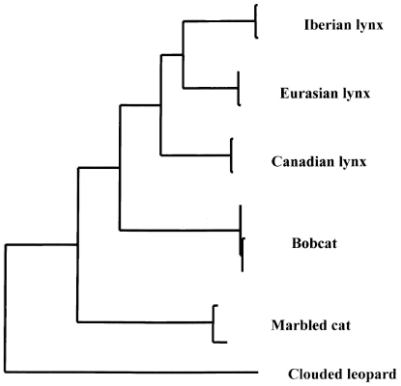

Fig. 2: Phylogenetic tree of the genus *Lynx* with a felid out-group (Marbled cat, *Pardofelis marmorata*). Modified after Johnson et al., (2004)

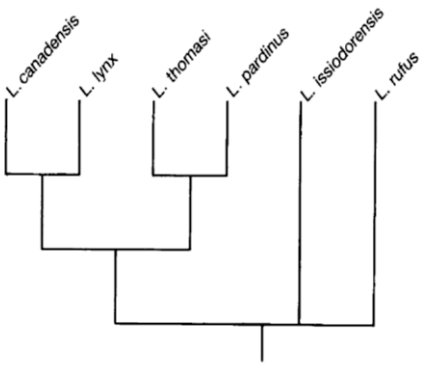

Fig. 3: Phylogenetic tree of the genus *Lynx* including their extinct ancestor (*L. issiodorensis*). Modified after Werdelin (1987).

2.2. Distribution and conservation status

2.2.1. Eurasian lynx

The Eurasian lynx is one of the most widely distributed felid in the world (Fig. 4), covering Eurasia. It was partly extirpated in some areas of Europe, reaching its nadir in the middle of the 20th century because of deforestation, increased human-lynx-conflicts and the reduction in and loss of wild ungulate prey populations (Breitenmoser, 1998; Linnell et al., 2009; Matheson, 1948). The IUCN currently (10th. September, 2015) lists it with a conservation status of *least concern* on a global scale. However, it is extinct or highly endangered in some regions, especially in central Europe. Substantial conservation efforts have been undertaken in central Europe during recent decades to protect the Eurasian lynx, its natural habitats, its prey species and to raise public awareness (Linnell et al., 2009; Schadt et al., 2002; von Arx et al., 2009). Without the intensively monitored re-introduction programmes, a natural recovery of the remaining insular populations in Scandinavia, the Carpathian mountains and the eastern Baltic would have probably not taken place (Breitenmoser and Breitenmoser-Wursten 1990; Linnell et al., 2009; von Arx et al., 2009).

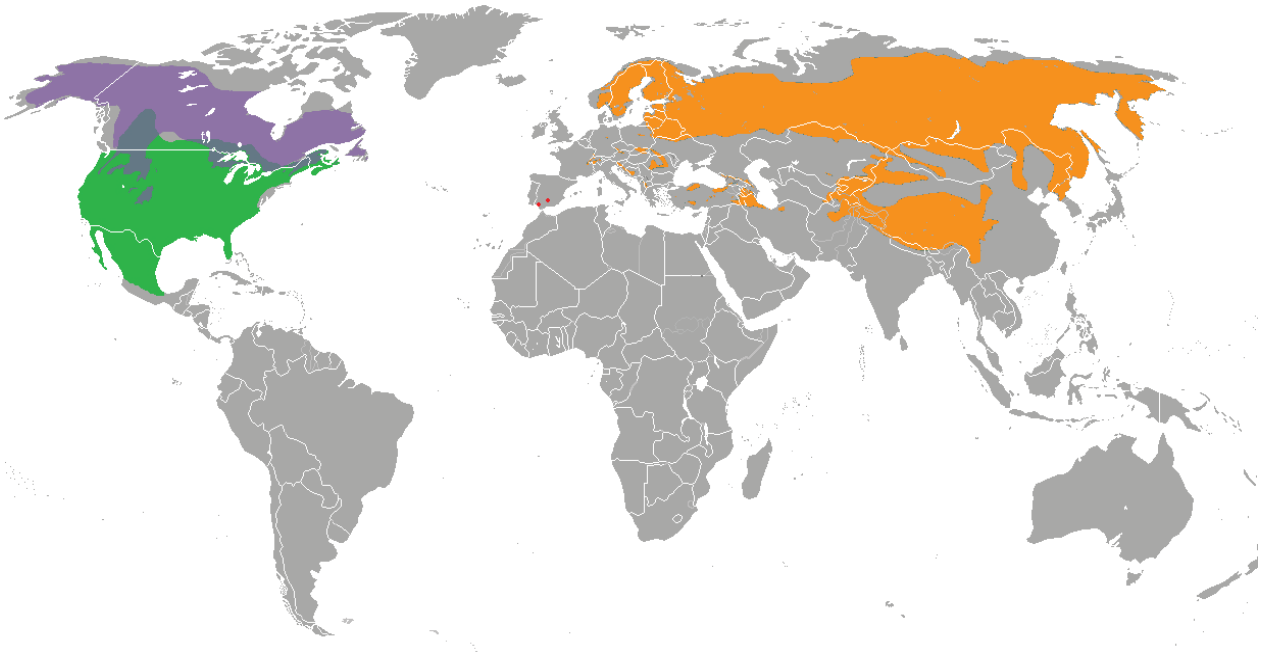

Fig. 4: Present-day distribution of *Lynx*. Orange: Eurasian lynx, red: Iberian lynx, violet: Canadian lynx, green: bobcat. Map extracted from http://commons.wikimedia.org/wiki/File:Lynx_range.png#mediaviewer/Datei:Lynx_range.png) on 10th of September 2015.

Today its habitat in central Europe is rather fragmented and effects of low gene flow and the loss of genetic variability are topics of conservation concern (Schmidt et al., 2009). Human acceptance of large predators in central Europe is unfortunately still low because of apparent competition with hunters and traditional local practices. Poaching is highest in rural areas, with protection of high numbers of free-ranging livestock and established hunting traditions as the main driving forces (Gangaas et al., 2013). In some areas, like Scandinavia, quota hunting or "harvest" of lynx was established as part of conservation management, to maintain a "balance" between prey and predators and to reduce human-predator conflict (Linnell et al., 2010; Nilsen et al., 2012a). Interestingly, this quota hunting is often sex biased, with male lynx being hunted more frequently than females (Nilsen et al., 2012a).

The Eurasian lynx's Arctic distribution over Fennoscandia and Russia is not influenced much by anthropogenic changes, and is of high conservation concern (Klein 2005). Distributions within Russia are reliably known only for some areas, indicating a population decline in some parts, but information from many regions are not updated for the current assessment of its conservation status by the IUCN (Matyushkin and Vaisfeld, 2003). In areas where the Eurasian lynx populations have recovered well, a reduction of meso-predators such as the red-fox (*Canis vulpes*) occured (Pasanen-Mortensen et al., 2013).

The evolutionary and systematic differentiation into subspecies is still debated by taxonomists. Currently, the following subspecies are widely accepted (summarised by (Versteege 2009)): the Northern lynx (*L. l. lynx*; northwest Europe to western Russia), the Carpathian lynx (*L. l. carpathicus*; Carpathian Mountains and Greece), the Caucasian lynx (*L. l. dinniki*; Caucasian Mountains, Iran and Turkey), the Turkestan lynx (*L. l. isabellinus*; Kashmir to Mongolia), the Irkutsk lynx (*L. l. kozlovi*; central Siberia), the Siberian lynx (*L. l. wrangeli*; eastern Siberia) and a third Siberian lynx (*L. l. wardii*).

In 2002, following the recommendations of the European Association for Zoos and Aquaria (EAZA) Felid Taxon Advisor Group (TAG), a European Studbook (ESB) was established to register all captive Eurasian lynx (Versteege, 2009). Almost 50% of the EAZA member zoos display Eurasian lynx in their collections. In total 342 Eurasian lynx (172 male, 166 female, 04 of unknown sex) are registered (ESB annual report 2012), but many more are expected to occur in wildlife parks which do not follow the registration recommendations. We used the Eurasian lynx as model species to gain knowledge on the reproduction biology of the e*ndangered* Iberian lynx (IUCN 2015).

2.2.2. Iberian lynx

The low genetic diversity of mitochondrial DNA (mtDNA) suggests that the current population of this species descended from a recent founder effect or a population bottleneck (Johnson et al., 2004). The population of their main prey, the European wild rabbit (*Oryctolagus cuniculus*), recently underwent a dramatic decline, mainly because of changes in agricultural and forestry management practices and outbreaks of rabbit specific viral diseases such as myxomatosis and rabbit haemorrhagic disease (Calvete, 2009; Hemmer, 1993). The lack of prey, increased human impact, habitat destruction, disease outbreaks and additional losses due to some feline viral infections such as with feline leukaemia virus (Hemmer, 1993; Lopez et al., 2009; Meli et al., 2009) led to drastic declines in the Iberian lynx population during the 20th century. A decade ago, the world population was roughly 200 individuals, with only 30 breeding females (Guzman et al., 2004). Therefore, in 2003 the Iberian Lynx Conservation Breeding Programme (ILCBP) was initiated (Vargas et al., 2009), with the goal of maintaining genetically and demographically managed captive populations and to help establish new free-ranging populations through reintroduction (Barbosa and Real 2010). The species was kept under the highest protection status '*critically endangered*'. Currently, breeding stations are situated in El Acebuche, La Olivilla, El Jerez Zoo, La Granadilla (Spain) and Los Silves (Portugal). Furthermore, a LIFE project

(Nature and Biodiversity project funded by the EU commission) was granted in 2002, to promote the recovery of the Iberian lynx in its native habitat, (Simon et al., 2009, 2012a) with currently four re-introduction sites in Spain and one in Portugal. Coordinated in-situ and ex-situ projects were designed to effectively combat the diversity of factors held responsible for the decline of the Iberian lynx population, and included, amongst others, ex-situ conservation breeding, in-situ habitat management, reintroduction of captive born healthy offspring, genetic resource banking, prey species (rabbit) management and conservation, habitat preservation, public education and awareness programmes (Roldan et al., 2009; Sarmento et al., 2012; Simon et al., 2012a, 2012b; Vargas et al., 2009). The Iberian lynx breeding programme is supervised within the European endangered species programme (EEP). Currently, there are about 404 adult free-ranging and 105 adult captive lynx on the Iberian Peninsula (pers. comm. Iberian Lynx captive breeding centre; Antonio Rivas 10th of June 2016). Until December 2015, 69 young and trained for re-introduction individuals were released to the wild, 14 individuals in captivity were taken off breeding due to suspected diseases. In 2015, the species was downgraded from being critically endangered to endangered (IUCN, 2015).

2.3. The female reproductive cycle in the domestic cat and wild felids

A variety of reproductive systems evolved in mammals. Mammals can display seasonal or aseasonal reproduction, a very narrow breeding season or a broad one (Brown, 2011; Jewgenow and Songsasen, 2014; Soulsbury 2010). Felids evolved a great variety of reproductive techniques depending on their habitat, resources or social structure they are living:

A) Their cyclicity is mostly dependent on photoperiod and ranges from being polyoestrous and seasonal (e.g. *Felis silvestris catus, Panthera tigris, Panthera uncia*) to being polyoestrous and aseasonal (e.g. *Panthera leo, Panthera pardus, Puma concolor*) (Brown, 2011). *Lynxes* are the only known monoestrus felids.

B) Ovulation can be coitus induced or spontaneous; In the past, all felids were believed to be exclusively coitus induced ovulators. However, later studies showed that even though all felid species have induced ovulations, depending on the species they also express a varying amount of spontaneous ovulation (about 60%), without a mating stimulus (Gudermuth et al., 1997; Lawler et al., 1993; Schramm et al., 1994). Most felids express a facultative spontaneous ovulation with large individual variability (Brown 2011; Wildt et al., 1981, 1999). The ovulation-pattern even seems to be responsible for some sexual

behaviour in mammals (Soulsbury, 2010; Soulsbury and Iossa, 2010). Males of induced ovulators sire a greater amount of offspring than spontaneous ovulators and they lesser perform mate-guarding (Soulsbury and Iossa, 2010). Interestingly, most mammalian species with induced ovulations live in social groups. The only gregarious large felid, the lion (*P. leo*), is also a mostly but not solely, induced ovulator (Schramm et al., 1994). Amongst felids, the ovulatory pattern is not yet known for each species. It was unknown what ovulation pattern the Iberian and Eurasian lynx followed. The bobcat was described to be able to show spontaneous ovulations (Stys and Leopold, 1993; Woshner, 1988).

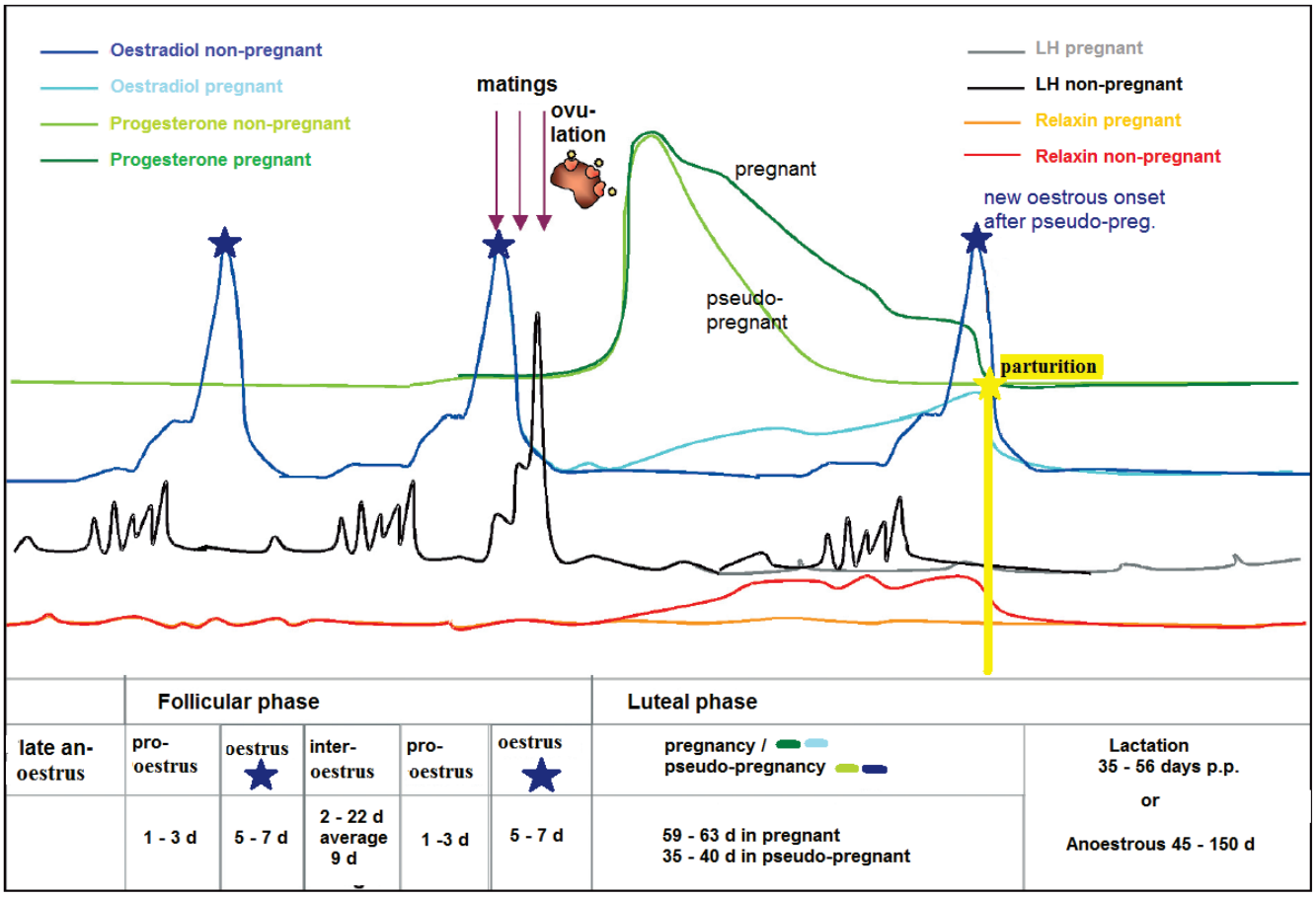

Fig. 5: Schematic diagram of the course of various reproductive serum parameters during the follicular phase and the luteal period in domestic cat (modified after © Laboklin, Germany: www.laboklin.at/pdf/de/news/la_200606_zyklus_katze.pdf, adapted after Wildt, 1988; Wildt et al., 1981; Chatdarong et al., 2002; Howard et al., 1992).

The domestic cat is the best studied feline species in reproduction physiology (Fig. 5); It expresses a seasonal polyoestrous cycle partly dependent on photoperiod, in that increasing daylight period during spring induces oestrous (Leyva et al., 1989). Here, induction of oestrous cycle is therefore dependent on latitude. Variation in cycle length and seasonality differs considerably between felid species and habitat

location (Shille et al., 1979). The domestic cat may have ovulatory or anovulatory (or non-ovulatory) cycles (Malandain et al., 2011). The anovulatory cycle only contains the follicular phase (Fig. 5), and is categorised into 1) pro-oestrus, 2) oestrus and 3a) inter-oestrus (Axner 2008, Chatdarong et al., 2002, Wildt et al., 1981, 1999). The ovulatory cycle consists of a follicular and a luteal period, and is categorised into 1) pro-oestrus, 2) oestrus, and 3b) met-oestrus, 4) di-oestrus/pseudopregnancy or pregnancy. 5) Anoestrus is the inactive stage outside breeding season.

The parameters which characterize each cycle stage are summarised as followed:

ad 1) Pro-oestrus (1-3 days (d)) is defined as the period of onset of the follicular phase, an increase of oestradiol concentrations in serum (from basal <20pg/ml serum to > 40pg/ml serum), and cornification of the vaginal cells with few erythrocytes. Behaviourally, the female rolls on her back and vocalises frequently but does not permit mounting or copulation. Basophilic basal and intermediate and a few basophilic superficial cells dominate the picture of the vaginal cytology at this stage.

ad 2) The onset of oestrus is characterised by increased behavioural signs. It can be divided into early, mid and late oestrus. Usually oestradiol (E2) concentrations in serum peak during early oestrous, and decline thereafter (mid oestrus) until they reach low basal levels again (almost 0 ng/mL, late oestrus). The whole oestrus period normally lasts 5-7 days (Chatdarong et al., 2002). Physiological opening of the cervix (cervical patency) is necessary for a successful fertilisation and is independent of ovulation and only occurs during oestrus (Chatdarong et al., 2006). In vaginal cytology mainly acidophilic, intermediate and superficial cells as well as cornified cells without nuclea are seen.

ad 3a) If no ovulation occurred, oestrus is followed by inter-oestrus, which takes on average 9 days (4 – 22d). Inter-oestrus is the period in between two oestri, it varies greatly in duration due to a variety of factors like follicular waves, but also due to not yet fully understood parameters. Non-ovulated follicles become atretic, and regress. Oestradiol and progesterone occur generally at low basal levels during inter-oestrus (Wildt et al., 1999). In vaginal cytology, intermediate cells and neutrophil granulocytes are found.

ad 3b) If intromission and copulation occurs, the vaginal neuronal stimulus can induce the secretion of luteinising hormone (LH) in the brain. This hormone increases after each mating and after its peak, which may be a different level in each individual, the follicles get the impulse to ovulate. Multiple matings are necessary in felids to stimulate the increased secretion of LH (Concannon et al. 1980; 2009). Ovulation occurs after the LH peak within 40-52 or more hours in the domestic cat (Wildt et al., 1981).

However, as described above, ovulation can also occur spontaneously without mating. The period shortly after ovulation (only the first 24 hours) is called met-oestrus. It describes the period when the follicular wall plicates and turns into luteal cells, the CL.

ad 4) After the CL are formed and the uterine mucosal cells are under the influence of P4, the cornification of superficial cells and the presence of many anucleated cells are common in vaginal cytology. After ovulation occurred two scenarios are possible in felids: i) they are either pseudopregnant in case of no fertilisation, or ii) pregnant.

ad 4i) If the follicles ovulated, spontaneously or induced, and the female did not conceive it enters the luteal period of di-oestrus or pseudopregnancy. This period is characterised by the formation of *corpora lutea* (CL) and elevated progesterone (P4) concentrations compared to oestrus or inter-oestrus period. This luteal period normally lasts 30 – 40 days in domestic cats. The CL start regressing after 10 – 35 days, progesterone concentrations decrease steadily and usually reach basal levels 40 days after ovulation (Chatdarong et al., 2005). Occasionally, the female develops an activated mammary complex during pseudopregnancy. Under the influence of increased progesterone, the endometrial gland proliferation increases.

ad 4ii) Fertilisations in cats have been observed within the first 49 hours after ovulation (Hammer et al., 1970; Howard et al., 1992) in the oviduct. The embryo enters the uterus 5.5 days post mating, on average (Swanson et al., 1994). Pregnancy is considered to last 63 – 69 days, depending on which day during the multiple matings the female ovulated.

The following hormones have an important role in reproduction management during pseudopregnancy or pregnancy:

a) P4, secreted in luteal tissue, increases in the peripheral circulation (> 20 ng/mL serum) within the first 14 days after ovulation. Afterwards P4 stays elevated, but decreases constantly until reaching a basal level of < 1ng P4/mL serum around day 60 of pregnancy. In the cat, from day 40 onwards, the placenta synthesises and secrets P4 and almost completely replaces the CL' P4 function (Braun et al., 2012b). As shown experimentally, peripheral (serum) P4 might either not be relevant for maintaining pregnancy in cats after 40 – 45 days post mating or there might be a local (placental) source of P4 which was not measured (Tsutsui et al., 2009). P4 is furthermore responsible for the development of the mammary complex. P4 is an interesting tool to measure if ovulation took place, however due to the fact that felids can be pseudopregnant, the difference between pseudopregnancy and pregnancy can not be distinguished by only testing P4.

b) E2 stays at basal levels throughout the year and only increases during the follicular phase in oestrus and prepartal to 20 – 40 pg/mL serum. It is used to determine oestrus if behavioural signs are absent.
c) Prolactin increases during the second half of pregnancy and stays elevated until the end of lactation (Banks et al, 1983; Leyva et al., 1989; Keck et al., 2000).
d) Relaxin is considered to be of placental origin and can be measured from day 21 of pregnancy onwards until about ten days prior to parturition (Braun et al., 2009; 2012a; Stewart & Stabenfeld, 1985; Tsutsui & Stabenfeldt, 1993). Therefore it is an interesting hormone to verify pregnancy.

e) Prostaglandins are described as being luteolytic in carnivores, and depending upon the stage of pregnancy, in the domestic cat they can also be luteotrophic (Shille and Stabenfeldt 1979; Weems et al., 2006). More detailed research studies on feline prostaglandin expression or receptor distribution were only initiated very recently, which verified the expression of intra-luteal prostaglandin receptors in domestic cats (Zschockelt et al., 2013). The prostaglandin F2α metabolite (PGFM) was discovered as an effective tool for pregnancy diagnosis for a variety of felids (Dehnhard et al., 2014; Finkenwirth et al., 2010).

ad 5) Anoestrus starts depending on the photoperiod. The anoestrus can last 45 – 150 days, depending on external (e.g.: climate, daylength) and breeding (e.g.: external stimulus from a male) factors (Wildt et al., 1999). Progesterone drops below 0.5 ng/mL, and E2 and LH are at minimal concentrations. In vaginal cytology basal-, parabasal, and some intermediate cells are seen. There is no serum parametrical differentiation between an anoestric and a castrated female. The initiation of anoestrus can be induced experimentally, for example, by exposing cats to 8 hours or less daylight, while follicular growth and oestrus onset can be induced by 24 hours daylight per day (Leyva et al., 1989). The same authors showed that decreased melatonin levels lead to a cycle onset in cats. Females living in natural photoperiods of temperate latitudes will therefore never experience a seasonal anoestrus (Faya et al., 2011).

Many of the above described felid-like physiological mechanism have not been examined and discussed in Lynx during past studies (Goeritz et al., 2009a; Jewgenow et al., 2009; Pelican et al., 2009). This thesis will specifically focus on the basal reproductive physiology of Eurasian and Iberian lynx.

2.4. Introduction to the reproductive biology and management of female *Lynx*

Similar to other felids, female *Lynx* indicate their interest in a potential mating partner by rolling on their back, scent-marking by spraying urine against trees and by calling. The urine spraying might help to mark their territory; it contains volatiles which may give information about the reproductive stage to potential mating partners (Dehnhard et al., 2006). Interestingly, these predator's urine scents may negatively influence movements and activity patterns of their potential prey species (Mattina et al., 1991; Sullivan and Crump, 1986). Since both above mentioned hypotheses contradict each other, we might not yet fully understand the differing meanings of scent marking. Female Eurasian lynx often prepare the lairs, usually above-ground structures where they give birth, in lowland windbreaks or in crevices in mountainous areas, where they line them with feathers, fur, moss and dry grass (Matjuschkin, 1978). The Iberian lynx females often use cork-trees, old stork nests and occasionally even holes in the ground (Valverde, 1957).

Eurasian lynx have larger litters the older they get, however their litter size is independent of their bodyweight (Henriksen et al., 2005). In general they mature at 1 ¾ years, although some mature as early as at ¾ years (Henriksen et al., 2005; Kvam 1991). The cost of maturing earlier was demonstrated in a study where females experienced weight losses in the following season compared to the late maturing members of their cohort (Nilsen et al., 2010). Young females which ovulated and mated for their first time often resorbed their first conceptus (unpublished data, Jewgenow, Goeritz and Dehnhard, 2012).

In contrast to other felids, all members of the genus Lynx, except the bobcat (Crowe, 1977), are strictly seasonal breeders (Fanson et al., 2010; Goeritz et al., 2009a; Jewgenow et al., 2009). According to occasional post mortem examinations in gross-morphological or histological analysis, and in ultrasound examinations and serum hormone analysis on alive animals, a non-cat like ovarian cycle was observed. When examining alive individuals or carcasses of female Eurasian or Iberian lynx, all authors always found CL on the ovaries, and indications of luteal activity by increased progesterone levels, even outside breeding season (Axnér et al., 2013; Braun et al., 2012a; Dehnhard et al., 2010; Goeritz et al., 2009a; Jewgenow et al.; 2009, Kvam, 1991; Pelican et al., 2009). However, it was not possible to say whether these CL from the Eurasian and Iberian lynx derived from follicles from the last oestrus or from other follicular activities. Furthermore, it could not be determined when these CL would undergo luteolysis and disappear (Goeritz et al., 2009).

Various non-invasive hormone studies were undertaken on Iberian and Eurasian lynx in recent years, in order to develop a reliable pregnancy test. Unfortunately, unlike in other felids, the faecal progesterone metabolites in *Lynx* are equally high during the entire year and do not reflect the cycle stage of the

individuals or allow a separation of pregnant and pseudopregnant females (Fig. 6) (Dehnhard et al., 2010; Dehnhard et al., 2008; Jewgenow et al., 2009; Pelican et al., 2009). The urinary profile of pregnant or pseudopregnant female *Lynx* does reflect the reproductive stage to some extent; an increase of oestrogens around implantation and in the last trimester of pregnancy is seen (Jewgenow et al., 2009), but is not sensitive enough to discriminate real pregnancy from pseudopregnancy. The placental hormone relaxin is secreted into urine and reliably detectable. Because of the elaborate procedure required (a 50 fold ultrafiltration) it is too time-intensive to use it as a simple standard pregnancy test (Braun et al., 2009). Detection of the metabolite of prostaglandin F2alpha (PGFM) is the most effective method in performing pregnancy tests in *Lynx*, because during the last trimester (average from day 45 of pregnancy onwards) an increased secretion of PGFM in urine and faeces can be detected (Dehnhard et al., 2012, Finkenwirth et al., 2010). Because prostaglandin F2alpha is metabolised during the first passage through the lungs, PGFM is also the parameter of choice in serum for pregnancy detection (Dehnhard et al., 2012; Weems et al., 2006). Urine can be collected non-invasively by installing marking traps, whereby the animals mark by spraying urine on a metal plate, which then collects into a small tube.

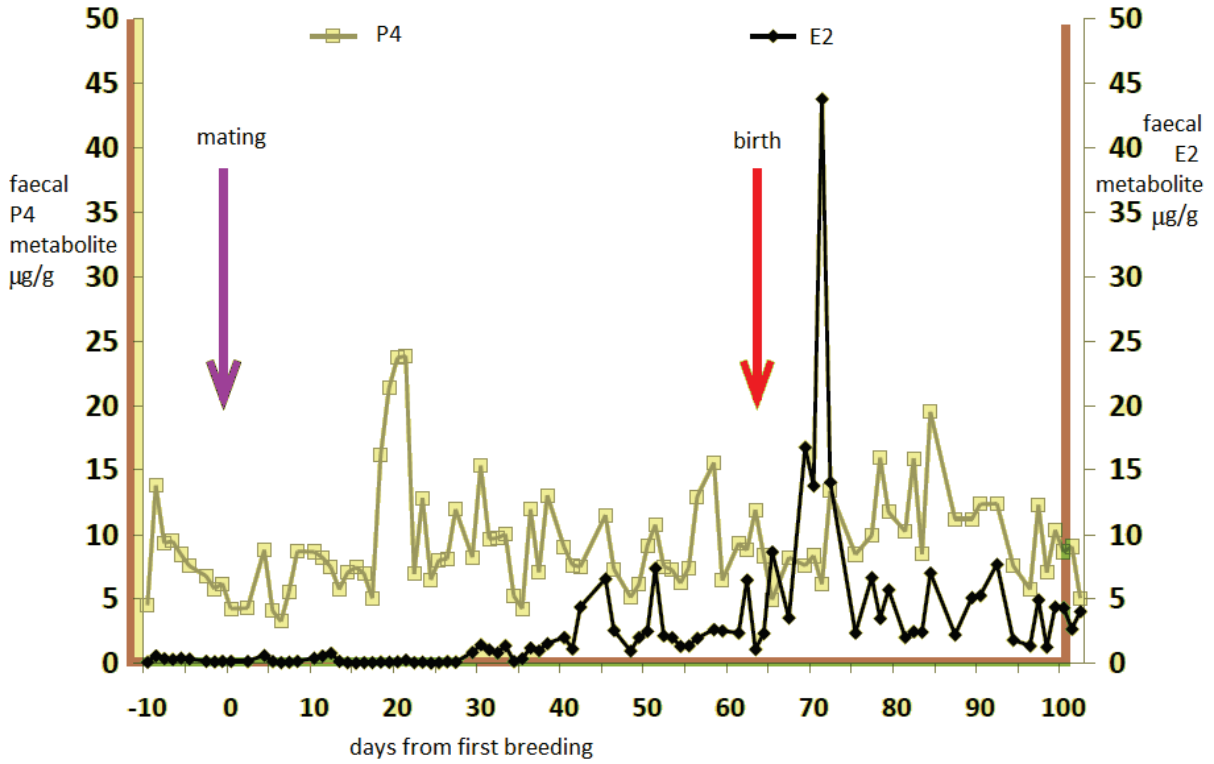

Fig. 6: Non-invasive faecal (Faeces collected in the enclosures) hormone analysis in a pregnant Iberian lynx, does not show differences in luteal activity between oestrus, pregnancy and post-partum period. First arrow indicates mating; second arrow indicates parturition (modified after Pelican et al., 2009).

2.4.1. The *corpus luteum*

What do animals need CL for? The CL is a transient hormone producing gland, mainly secreting progesterone for maintaining pregnancy (Davis and Rueda, 2002). The development of a CL and post-ovulatory progesterone secretion seem to have evolved to accommodate viviparity (and in some cases ovoviviparity), and to increase the uterine retention time for the embryo, respectively the foetus, to be able to fullfill all developmental stages within the uterus and not outside the uterus (Callard et al., 1992; Rothchild, 1980). The luteal cells derive from the conversion of a follicle, namely the follicular granulosa and theca cells, after an adequately high peak of luteinising hormone (LH). It is fascinating that the follicular cells mainly produce oestrogens before ovulation and then turn into mainly progesterone producing luteal cells afterwards (Davis and Rueda 2002). Granulosa cells develop into large steroidogenic luteal cells (LLC), whereas theca cells turn into small steroidogenic luteal cells (SLC) (Murphy 2000). Apart from steroidogenic cells there are endothelial cells, fibroblasts, pericytes and blood cells within the CL of Eurasian lynx (Manuscript 2).

Each CL found on an ovary gives indications of an ovulation. There are four types of CL in mammals, however not all mammalian species have all four types. They differ in lifespan and steroidogenic output: the CL of the a) cycle (not existing in induced ovulators), b) pseudopregnancy (not existing in primates), c) pregnancy (exists in all), and d) lactation (not seen in all species) (Stocco et al., 2007). A felid CL's life cycle during pregnancy can be divided into three phases: luteinisation, pregnancy-induced luteal maintenance or rescue (missing in pseudopregnant cycles), and functional and/or structural luteal regression (Amelkina et al., 2014; Niswender et al., 2000). Luteal cells reach their maximum expansion around days 12-14 post ovulation in the domestic cat (Amelkina et al., 2014, Dawson, 1941). Intracellular signalling pathways, cell adhesion factors, intracellular cholesterol and oxysterols are responsible for the luteinisation process, at least in humans (Murphy, 2000). Progesterone, as a paracrine or intracrine regulator, seems to be luteotrophic itself (Murphy, 2000; Rothchild, 1996). Growth hormone, prolactin and oestrogen are furthermore important for the development and maintenance of luteal cells (Niswender et al., 2000). *Rothchild* (1981) summarised that polyoestric eutherian species have short CL lifetimes, whereas monoestric species experience longer life times of CL. The difference underlies basically in postponing its intrinsic abilitiy to produce luteolytic factors. The duration of this luteolytic postponement is dependent on the duration of its progesterone secretion (Rothchild, 1981).While long-lived CL are normaly not affected by pregnancy, the lifetime of short-lived or ultrashort-lived CL is normally prolonged with pregnancy and in the later also by sterile matings (Rothchild, 1981). The domestic cat has a long-lived CL, with missing luteotropic signals (Amelkina, 2016; Zschockelt, 2016). Lynxes are monoestric species, and their CL experience a very long lifespan

with active suppression of the luteolytic processes (Amelkina, 2016). For felids the detailed processes and consequences within the CL during any of the phases of the cycle are not completely understood yet.

During pregnancy the steady progesterone secretion is essential to maintain quiescence in the myometrium, supress the maternal immune response against foetal antigens, suppress ovarian follicular activity and provide a suitable uterine environment (reviewed by (McCracken et al., 1999)). If pregnancy does not occur or fail in felids, luteal regression must occur in order to allow a new follicular phase (Bowen-Shauver and Telleria, 2003; Niswender et al., 2000). Luteolysis is defined as the structural disintegration of the CL, and regression is defined as the complex mechanism behind structural and functional losses within the CL (Bowen-Shauver and Telleria, 2003). It is postulated that luteolysis evolved in some species as a strategy to increase reproductive efficiency in biphasic cycling species (reviewed by (McCracken et al., 1999)). Consequently, it can be hypothesised that in some species such as canids and felids reproductive output per year is decreased because no active luteolysis exists. Domestic dogs will exhibit about two cycles per year and ovulate during each oestrus. Here, the regression of CL from pseudopregnancy cycles seems to be rather permissive, whereas the regression of CL from pregnancy cycles is active through structural regression (Hoffmann et al., 2004, Kowalewski et al., 2009). In cats, the mechanism underlying the regression of CL derived from pregnancy cycles or pseudopregnancy cycles still needs to be elucidated. Recent publication indicate that the shortened luteal lifespan in non pregnant cat might be due to the absence of external luteotrophic factors (Amelkina, 2016; Zschockelt, 2016). Furthermore *Amelkina* (2016) suggests, that androgens might support the physiologic persistence of *Lynx* CL by preventing apoptosis and androgens and estrogens are stimulating for the continuous P4 production.

In *Lynx*, if conception fails, a full year of reproduction is lost, which is crucial for the breeding centers. To increase the annual reproductive output in the Iberian lynx conservation breeding programme, a key question was whether artificial luteolysis of CL outside the breeding season would lead to the onset of a second cycle within the same year. Additionally, the basic knowledge required (basal knowledge about oestrus and luteal regression) to perform an artificial luteolysis was missing. Would artificial luteolysis lead to a structural and/or functional regression at all? Within this thesis, an experiment was performed to see if the CL outside breeding season would be receptive to external prostaglandin stimulation (assisted reproduction techniques) or not.

2.5. Assisted reproduction techniques in felids today

Assisted reproduction techniques (ART) include: a) hormone treatment to prepare the uterine environment and the ovaries for the onset of oestrus and a pregnancy, b) gamete collections, when semen or ova are collected, c) cryopreservation techniques, where gametes or embryos are frozen and stored until thawing for producing a conception or pregnancy, and d) fertilisation techniques, where patients are artificially inseminated and/or gametes fertilised in-vitro (IVF), matured in-vitro (IVM) or undergo intra-cellular sperm injection (ICSI), and e) embryo transfer (ET).

2.5.1. Artificial luteolysis protocols

Artificial luteolysis protocols for carnivores are mostly applied in domestic dogs, when owners want to interrupt pseudopregnancy, synchronise the cycle, time oestrus induction to increase the reproductive output, abort unwanted offspring or treat pathologies such as pathologically persisting CL or pyometra. The main luteolytic agents used in carnivores are prostaglandins, dopamine-agonists (= prolactin-antagonist), anti-gestagens or gonadotropin releasing hormone (GnRH) agonists/antagonists (Concannon et al., 2009; Kutzler, 2007; Onclin and Verstegen 1997; Pelican et al., 2006). There is no experience in the application of such drugs in *Lynx,* thus far.

3 Cumulative Doctoral Thesis

The ovarian physiology of *Lynx*, known until 2010, left the following open questions:

a) When do CL outside breeding season disappear in Eurasian and Iberian lynx?

b) Do all CL on one ovary derive from ovulations during the last oestrus cycle, or are there any other non-oestrus derived ovulations afterwards?

c) Ultrasonographic examinations could not be performed shortly before oestrus. During that period only urine and faecal samples were available. It was unknown whether elevations in P4 levels of non-invasive samples were of luteal origin.

d) Do further ovulations occur after the monoestrous cycle during breeding season, which would possibly explain the occurrence of CL long after breeding season?

e) Many (up to 18) CL of different sizes were found on ovaries outside breeding season. Are they all functionally active (i.e. secrete P4)?

f) Are these CL outside breeding season an adaptation to unsuitable husbandry in captive individuals only, or is this unknown phenomenon also found amongst free-ranging individuals? Does this phenomenon show some plasticity, if *Lynx* are examined at different latitudes in different climates and compared between captive and free-ranging living conditions?

3.1. Doctoral Thesis Objectives

At the start of this thesis it was known that *Lynx* experienced a reproductive cycle different from other felids, with CL occurring after the breeding season. It was unknown during which event these CL derived from, when they would regress and if they would still be functionally active outside the breeding season. No information was available to what extent these traits were fixed or showed any form of phenotypic (or possibly genotypic) plasticity between populations, as a function of living conditions (captivity vs free-ranging) or different latitudes, environments and climates (Fig. 7).

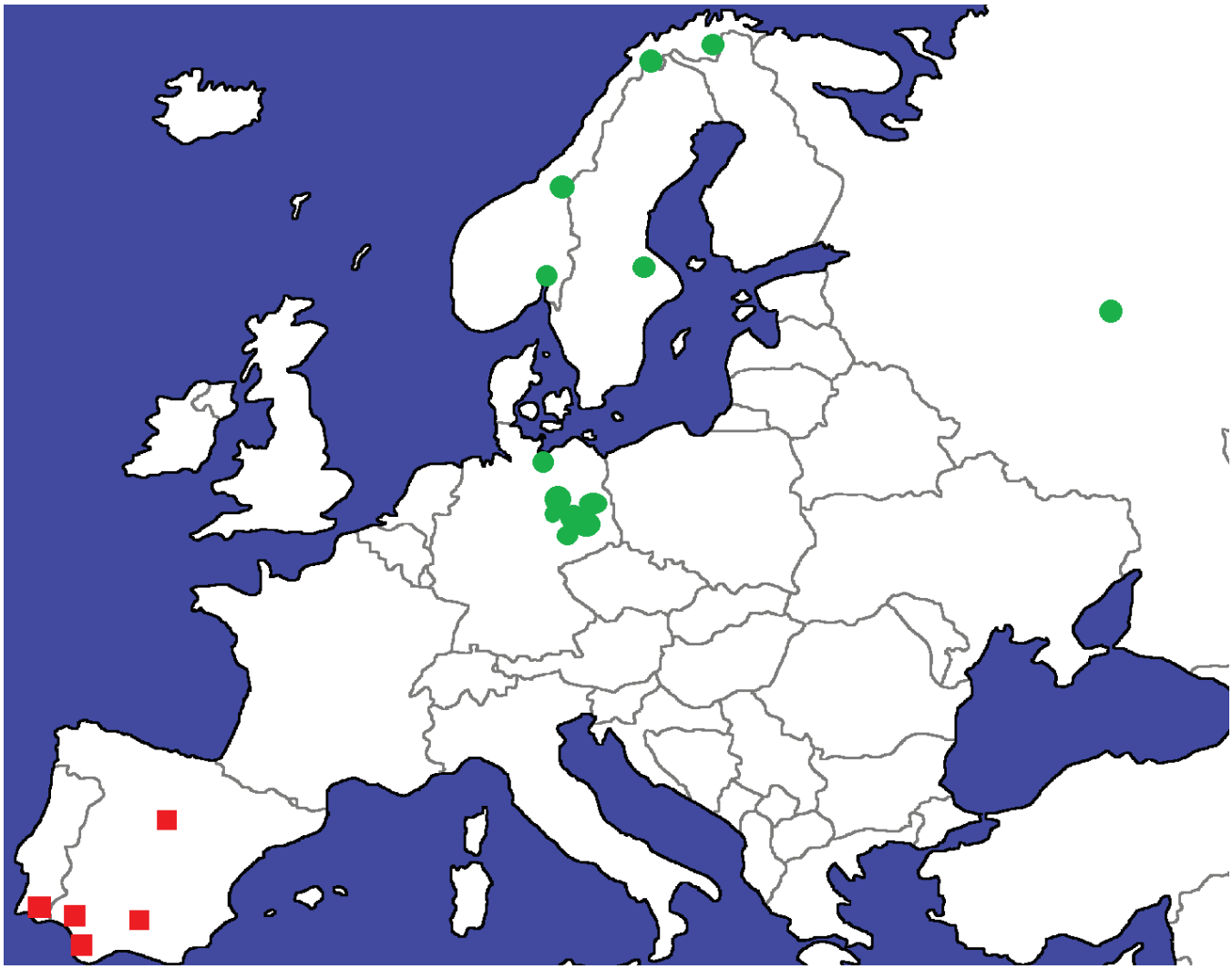

Fig. 7: Study locations of study animals used in this thesis. Samples from different origins in latitude and different environmental conditions were important for the evaluation of genotypic variation and phenotypic plasticity of the reproductive physiology of *Lynx*. Eurasian lynx (location indicated by green spots) from Germany (close to Berlin) in captivity, northern Norway (Finnmark and Tromsø) from free-ranging populations, and Russia (Tschernogolovka) in captivity were studied to describe the physiology. Samples from shot Eurasian lynx were obtained from the Agricultural University in Uppsala (SVA). Examinations of Iberian lynx (locations indicated by red squares) took place in five captive breeding centres in Spain and Portugal. (map extracted from:www.digitale-europakarte.com)

It was unknown whether luteolysis was necessary in *Lynx* before the onset of a new cycle, whether artificial luteolysis induces a second oestrus within the same year, or whether artificial luteolysis should be followed by artificial oestrus induction to induce a second cycle in *Lynx*. Furthermore, the origins of

serum P4 levels were suspected of but not demonstrated to being of luteal origin during the entire year. Finally, it was necessary to compare the efficacy of different oestrus and ovulation induction protocols for felids before applying these to the endangered Iberian lynx population.

Therefore the following projects were implemented:

1) A longitudinal study comparing the physiology of alive free-ranging and captive Eurasian lynx, using both ultrasound and endocrinological methods, in order to study the development, function and plasticity of the physiologically prolonged luteal life cycle over more than two years.

2) An analysis on a cellular basis by analysing the cell types histologically and the hormone content within the luteal tissue (intra-luteal) directly. This would complement the findings from the first study where the physiological changes were followed macroscopically via ultrasonography and the hormones generally via peripheral serum hormone analysis.

3) Development of an artificial luteolysis protocol for CL outside the breeding season in *Lynx*, to study how artificial hormones can influence the physiologically persistent CL and if a second cycle onset within the same year would be possible. It was unknown how the manipulation of physiological CL outside breeding season would conclude and whether it would cause the *Lynx'* CL to regress outside breeding season.

3.2. Published peer-reviewed manuscripts

This doctoral thesis is based on the following peer-reviewed, published manuscripts:

1) **Painer J**, Jewgenow K, Dehnhard M, Arnemo JM, Linnell JDC, Odden J, Hildebrandt TB, Goeritz F (2014). Physiologically persistent *corpora lutea* in Eurasian lynx (*Lynx lynx*) – longitudinal ultrasound and endocrine examinations intra-vitam. Plos One 9 (3): e90469.

   I conceived, designed and performed the experiments, conducted fieldwork and project logistics in Norway and Germany, analysed the data, did the statistical analysis and wrote the manuscript. All other authors contributed to

the conception and design of the study, directed or implemented some analyses, contributed to data interpretation and discussion, edited and/or reviewed the manuscript.

2) Carnaby K, **Painer J**, Soderberg A, Gavier-Widen D, Goeritz F, Dehnhard M, Jewgenow K (2012). Histological and endocrine characterisation of the annual luteal activity in Eurasian lynx (*Lynx lynx*). Reproduction 144: 477 – 484.

I partly conceived and designed the experiments, assisted when the experiments were performed, did parts of the statistical analysis, contributed to data interpretation, editing and reviewing process, and conducted the sample collection in Sweden. Ms. Carnaby performed most parts of the experiments and analysis, wrote the paper and is therefore first author of the publication. All other authors contributed to the conception and design of the study, directed or implemented some analyses, contributed to the data interpretation and discussion, edited and reviewed the manuscript.

3) **Painer J**, Goeritz F, Dehnhard M, Hildebrandt TB, Naidenko SV, Sánchez I, Quevedo Muñoz MA, Jewgenow K (2014). Hormone-induced luteolysis on physiologically persisting corpora lutea in Eurasian and Iberian lynx (*Lynx lynx* and *L. pardinus*). Theriogenology 82: 557 – 562.

I conceived, designed and performed the experiments, did the statistical analysis, wrote the paper, and conducted the fieldwork in Russia. All other authors contributed to the conception and design of the study, directed or implemented some experiments and analyses, contributed to the data interpretation and discussion, and edited and reviewed the manuscript.

3.3. Overview of the presented manuscripts

The first manuscript explains in detail the different reproductive stages of the female *Lynx* and establishes a new descriptive term and definition for the unique phase outside the breeding period when *Lynx* show physiologically persisting CL, “the prolonged di-oestrus”. The development and maintenance of the physiologically persistent CL were discovered by the application of high-resolution ultrasonography, serum hormone analysis and vaginal cytology in captive and free-ranging Eurasian lynx. For the first time, all reproductive stages of a *Lynx* ovary were registered by ultrasonography and its associated hormone profile. The main findings provided evidence that *Lynx* are monoestrus and that

this phenomenon shows little plasticity. We also show that CL of some *Lynx* species survive longer than any other active CL currently known for mammals.

To ensure that the constant P4 serum concentrations were of luteal origin, we collected frozen ovaries and uteri from an archive of free-ranging, legally hunted Eurasian lynx held at the Pathology Department of the Swedish Veterinary University in Uppsala, Sweden (SVA). The results were discussed in the second manuscript. The samples were analysed for their intra-luteal P4 and E2 content and some samples were histologically analysed, to distinguish between the different cell types. Almost all months of the year were represented by the analysed samples. The results demonstrated constant luteal activity within the CL and an absence of seasonal differences throughout the entire year when CL from pregnant animals were excluded.

In the third manuscript we report the response of *Lynx* to different hormone stimuli to induce artificial luteolysis. Study animals were exposed to artificial prostaglandins (Cloprostenol), dopamine-agonists (Cabergolin) and anti-progestins (Aglepristone) in various combinations. This experiment was performed as a prerequisite to artificial oestrus induction in *Lynx* with physiologically persisting CL. All individuals displayed signs of functional, luteal regression, noted by a reduction in blood circulation around luteal bodies and by a significantly decreased serum P4 concentration. With the artificial luteolysis we could induce a similar state which is seen as a natural trend before oestrus onset or birth; prostaglandins lead to a reduction in P4, a drop in luteal vascularisation.

3.3.1. Physiologically persistent *corpora lutea* in Eurasian lynx (*Lynx lynx*) – longitudinal ultrasound and endocrine examinations intra-vitam.

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Physiologically Persistent *Corpora lutea* in Eurasian Lynx (*Lynx lynx*) – Longitudinal Ultrasound and Endocrine Examinations Intra-Vitam

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Abstract

Felids generally follow a poly-estrous reproductive strategy. Eurasian lynx (*Lynx lynx*) display a different pattern of reproductive cyclicity where physiologically persistent *corpora lutea* (CLs) induce a mono-estrous condition which results in highly seasonal reproduction. The present study was based around a sono-morphological and endocrine study of captive Eurasian lynx, and a control-study on free-ranging lynx. We verified that CLs persist after pregnancy and pseudopregnancy for at least a two-year period. We could show that lynx are able to enter estrus in the following year, while CLs from the previous years persisted in structure and only temporarily reduced their function for the period of estrus onset or birth, which is unique among felids. The almost constant luteal progesterone secretion (average of 5 ng/ml serum) seems to prevent folliculogenesis outside the breeding season and has converted a poly-estrous general felid cycle into a mono-estrous cycle specific for lynx. The hormonal regulation mechanism which causes lynx to have the longest CL lifespan amongst mammals remains unclear. The described non-felid like ovarian physiology appears to be a remarkably non-plastic system. The lynx's reproductive ability to adapt to environmental and anthropogenic changes needs further investigation.

Citation: Painer J, Jewgenow K, Dehnhard M, Arnemo JM, Linnell JDC, et al., (2014) Physiologically Persistent *Corpora lutea* in Eurasian Lynx (*Lynx lynx*) – Longitudinal Ultrasound and Endocrine Examinations Intra-Vitam. PLoS ONE 9(3): e90469. doi:10.1371/journal.,pone.0090469

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Introduction

The Eurasian lynx (Lynx lynx, LINNAEUS 1758, hereafter called "lynx") is a large carnivore with a wide distribution in Eurasia, from the western Alps to the Russian Far East and from the southern Balkans to northern Scandinavia. In Europe they have undergone a historic period of decline, with some recovery in the most recent decades [1]. The spectrum of implied global change is raising many questions about the ability of wildlife to adapt to changes in their environment. A great deal of conservation and research focus is underway to try and understand the extent to which lynx can adapt to human modified landscapes [2] and impacts such as harvest [3]. Specific concerns regarding climate change and the way it may shape and influence seasonal patterns of resource availability and productivity are a cause for concern regarding many species. A major emerging topic is the extent to which species' phenology, especially the timing of reproduction, is able to respond to possible changes to keep pace with changes in seasonality [4,5]. However, all species clearly show some constraints in their ability to adapt to change, as traits vary hugely in their reproductive plasticity. Understanding the physiological basis of the mechanisms that control reproduction is essential to understanding the potential for a species to adapt.

Many felid species are known to express a poly-estrous reproductive pattern: being able to mate several times a year [6]. In lynx, however, a "non-felid like"ovarian cycle [7] was recently documented which includes a mono-estrous cycle (only one estrus per year); speculated to be driven by an unusual reproductive feature – persistent corpora lutea (CLs) with a constant progesterone (P4) secretion. The assumption, that this may suppress the ovarian activity, and therefore create mono-estrous reproduction, was based on histological and endocrine examinations of lynx ovaries obtained from necropsies [8,9], occasional ultrasound examinations of live animals and fecal hormone analyses [7,10,11]. To provide final proof that CLs remain active independently of pregnancy and lactation for more than one reproductive cycle, the same individuals need to be examined over a period of at least two cycles.

The ultrasound approach produces a high quality image for non-invasive soft tissue examinations and can be used to obtain clear information about the status of reproductive organs [12]. Thus, 3D ultrasound is being used more often for pregnancy

diagnostics in wildlife medicine [13] and to study ovarian topography and function in various species [12,14,15]. Topographic maps of each ovary can be generated to demonstrate the exact position of individual CLs over time. Doppler color flow has already been used to quantify ovarian blood flow in lynx ovaries [16].

The present study used detailed longitudinal data of healthy lynx females held in zoos. The study includes the evaluation of the formation of CLs after ovulation in pregnant and non-pregnant animals, the luteal function during and after pregnancy or pseudopregnancy, as well as the luteal regression before next ovulation. To exclude that the results were an artifact of working with captive animals under artificial conditions, we took advantage of access to free-ranging lynx to conduct control examinations.

Materials and Methods

(a) Ethics statement

The examinations of captive lynx were performed when the animals were immobilized for other reasons, including veterinary monitoring, minor health intervention or due to captive animal management reasons. The methods applied, and the study-design, were in agreement with the animal ethics and welfare committee at the Leibniz Institute for Zoo and Wildlife Research (IZW, Berlin, Germany. No: 2010-01-01). The study of free-ranging lynx was conducted within the frames of the Scandinavian Lynx Project, Scandlynx (http://scandlynx.nina.no/). The free-ranging lynx were being captured for ecological studies related to demography and predator prey relationships [17] totally unrelated to this study. All capture and handling procedures were approved by the Norwegian Experimental Animal Ethics Committee and followed their ethical requirements for research on wild animals (permit numbers 2012/206992 and 2010/161554). In addition, permits to capture wild animals were provided by the Norwegian Directorate for Nature Management.

(b) Animals

This study was conducted on ten captive female lynx examined 2-6 times (three animals were examined twice, one animal three times, two animals four times, three animals five times and one animal six times) each between April 2010 and July 2012. The reproductive history of each individual is listed in Table S1 in the electronic supplementary materials (ESM). The captive animals were housed in seven different zoological gardens within Germany (ESM, S1). They were all fed a standard zoo-carnivore diet. They were kept under various conditions; always solitary (N = 1), solitary for most of the year but then paired during the breeding season (N = 2), as mother-daughter groups (N = 2), as permanent female – male pairs (N = 2), or as family groups with last years' cubs and a male (N = 3). They were exposed to natural light and climates of 52°–53°N and 11°–13°E. The animals were on average 6 years old (range = 1.9–20 years) and weighed on average 17 kg (range = 12–19.5 kg). In feline species, pseudopregnancy is defined as a non-pregnant luteal phase after ovulation. Two of these animals were examined during pregnancy (days 7, 21 and 31 post copulation) and lactation (one examination each, 3 months post-delivery).

Ten free-ranging lynx were examined in February and March in northern Norway in 2011 and 2012 [17]. They were living in natural habitats with natural light and climate conditions ranging from 61°–73°N and 11°–17°E. They were on average 5 years old (range = 3–12 years) and weighed on average 17.7 kg (range = 16.8–18.4 kg). The free-ranging animals were documented to have a mean litter size of 2.2 [18].

(c) Anesthesia and sampling

The captive lynx were darted inside their enclosures using a blowpipe and a 3 mL dart syringe equipped with a 22 gauge dart-needle (1.2638 mm) (all from DanWild LLC, Dan-inject dart guns, TX, USA). An initial dose of 0.06 mg/kg Medetomidine (0.1%, Domitor, Orion Corporation, Espoo, Finland) plus 4.0 mg/kg Ketamine (Ketamine 10%; Essex GmbH, Munich, Germany) was used. During anesthesia, the animals were supplied with intranasal medical oxygen and intravenous isotonic NaCl-Infusion (0.9%, Braun, Tuttlingen, Germany). Respiration, heart rate, pulse-oxymetry (Nellcor, CA, USA), rectal temperature and eyelid-reflexes were constantly monitored.

The free-ranging lynx were darted from a helicopter and immobilized with medetomidine plus ketamine, using previously established protocols from Arnemo et al. [19,20]

Blood was withdrawn from the free-ranging and captive lynx from the vena cephalica into serum and EDTA tubes (Sarstedt, Nürnbrecht, Germany). Vaginal smears, taken from the cranial region of the vagina, were obtained using a wet cotton swap. Cells were cropped on a glass slide, and fixed with a fixation spray (Roti-Fix, Carl Roth GmbH, Karlsruhe, Germany). The slides were stained following the Papanicolaou protocol (Papanicolaou Poly-chromlösung 3b, Carl Roth GmbH, Karlsruhe, Germany) and examined under a microscope (Jenaval, Carl Zeiss, Jena, Germany). Duration of anesthesia lasted on average 50 minutes (range = 45–70) and was reversed with an intramuscular dose of 5 mg atipamezole (Antisedan, Orion Corporation, Espoo, Finland) per 1 mg of medetomidine.

(d) Ultrasound examinations of ovaries

In the captive lynx, small areas of fur were clipped in an area cranio-lateral to the second mammary complex (counted from caudal). Ethanol (70%) and ultrasound gel (Aquasonic 100, Parker Lab, NJ, USA) were poured on to achieve skin-contact for the ultrasound probe. An ultrasound laptop (Voluson i, GE-Health, Austria, Zipf) equipped with a 12 MHz linear probe (12 L – RS) and a 6–16 MHz volume probe (RSP 6 – 16 RS) were used for imaging. By applying brightness (B-) mode, the ovaries were visualized and length, width and breadth of the ovaries as well as maximum diameter of each CL were measured on screen. Volume was calculated using the mathematical formula "volume = pi * length * width * breadth / 4". The three-dimensional mode (render and tomographic mode) was used to enumerate the CLs, to obtain a topographical map of each ovary, and to verify the individual CL's development and position. With the Doppler mode, the ovarian vessels were identified and ovarian vascularization was quantified by measuring the diameter of the arteria ovarica [16]. All examination steps were stored as videos to archive the data for post examination assessments. The software package 4D View (GE-Health, Austria, Zipf) was used to analyze the dataset.

Due to the harsh climate conditions (cold temperatures) and time constraints, only 2D ultrasound and Doppler modes were applied to the free-ranging lynx. The animals were not clipped; instead the fur was combed to reveal a small skin window.

(e) Serum hormone analysis

Estrogens (E2), P4 and the prostaglandin F2a metabolite (PGFM) were analyzed in serum samples using in-house enzyme immunoassays [21,22]. These assays have been previously validated for lynx [7,21,22]. The inter-assay coefficients of variation (CVs) when measuring PGFM in plasma samples were determined by repeated analyses of two pools containing 0.94 ng/ mL and 3.3 ng/mL of PGFM and were 9.0 and 9.8%, respectively (n = 10). Intra-assay CVs are described in Finkenwirth et al., (2010)

[22] (10.4 and 7.6%). Progesterone measurements in plasma samples revealed inter-assay coefficients of variation of 12.5% (n = 11) and 14.9% (n = 10), respectively. Intra-assay CVs are described in Dehnhard et al., (2010) [21] (9.0%). Estrogen measurements revealed an inter-assay coefficient of variation of 13.3% (n = 9). Intra-assay CVs are again described in Dehnhard et al., 2010 [21] (5.6%).

(f) Statistical analysis

All tests were conducted using the software package R.2.14.1 [23]. A p-value of <0.05 was considered to be significant. We assessed correlations between different parameters (P4-, E2-, PGFM- serum hormone values, ovarian volume, CL number and size and the *arteria ovarica* diameter) using a Spearman rank correlation (rho, $N_{captive}$ = 38 examinations, $N_{free\text{-}ranging}$ = 10 examinations). In six captive lynx, structural and functional changes between reproductive stages (pro-estrus, estrus, met-estrus, prolonged di-estrus) were analyzed using a Quade-test for repeated measurements. In these animals examinations of all consecutive reproductive stages were possible. We compared differences during the months of February and March between the free-ranging population (N = 10 examinations) and the captive population (N = 10 examinations) using the Wilcoxon test (for non-normally distributed parameters) and the two samples t-test (for normally distributed parameters). For this analysis only one examination per individual was taken. All data are shared publicly in the ESM (Table S2).

Results

(a) Reproductive stages in lynx

The different reproductive stages in female lynx were classified as pro-estrus, estrus, met-estrus, pregnancy, pseudopregnancy, lactation and prolonged di-estrus (Fig. 1; Table 1). Figure 2 summarizes the luteogenesis and luteal regression in a schematic. There were no biologically relevant, and significant differences between free-ranging and captive lynx during February and March 2011 and 2012 (please also see Table S3, ESM; Wilcoxon rank test: P4: W = 30, p = 0.86; diameter *a. ovarica*: W = 105, p = 0.07; ovarian volume: W = 172, p = 0.46; CL tissue: W = 129, p = 0.06; age: W = 112.5, p = 0.05 and Two sample t - test: E2: t = 0.11, p = 0.91, df = 16; PGFM: t = −1.42, p = 0.18, df = 12; number CLs: t = −2.17, p = 0.04, df = 35.5).

One captive female was a juvenile (19 month); accordingly we observed small ovaries (0.54 cm^3 and 0.10 cm^3) without follicular or luteal activity (Figure 1A). A good differentiation between cortical and medullar parts of the ovary was detected and hormone analyses revealed low E2 (0.16 ng/mL), and non-detectable P4 values.

Pro-estrus was found in lynx examined just before the mating season in early spring. These females had already undergone at least one reproductive cycle in a previous year ($N_{captive}$ = 5, $N_{free\text{-}ranging}$ = 5). Several hypo-echoic CLs were detectable on the ovaries using ultrasound (Fig. 1B). They exhibited various sizes encompassing 0.2–0.65 cm in diameter. The number of CLs (S = 26929.36, p = 0.002, rho = 0.38) and the level of P4 (S = 27142.29, p = 0.012, rho = 0.32) increased significantly with age. Vaginal smears collected from pro-estrus stages showed a typical image of primarily round to oval shaped, basophilic basal and intermediary cells or if already under higher estrogen influence they were with enlarged basophilic or acidophilic superficial cells (ESM, Fig. S1-A; cell proportions on average ± SD: parabasal cells (pbc) = 17±16%, intermediate cells (imc) = 46±8%, superficial cells (sfc) = 35±17%, superficial anucleated cells (sac) = 2±1%). The hormones were characterized by increased PGFM and a slight decrease of P4 (Table 1).

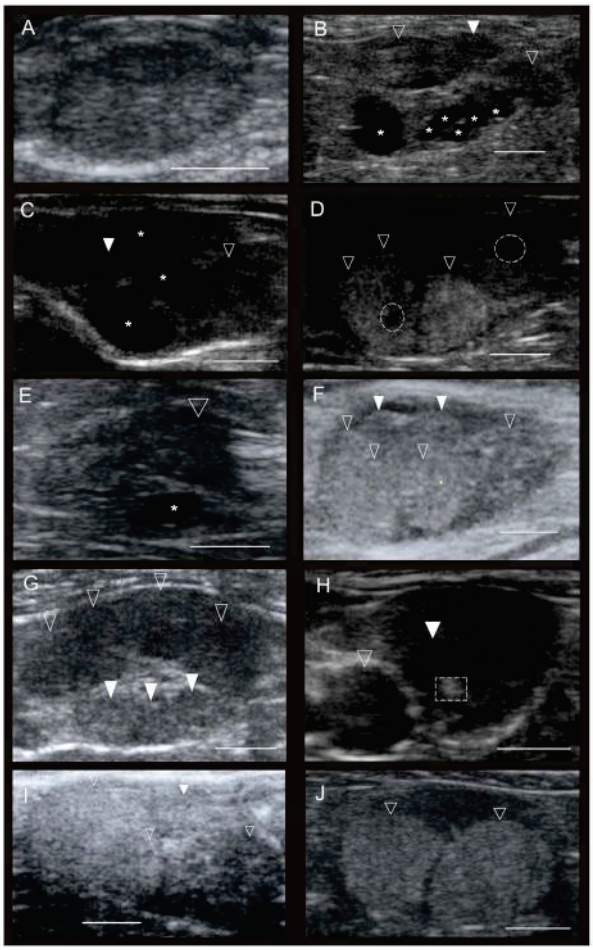

Figure 1. Ultrasonographical images of lynx ovaries during different reproductive stages. The ultrasound images (in b-mode) of lynx ovaries demonstrate different stages of ovarian activity. The white scale-bar indicates 1 cm. Asterisk indicates follicles, empty arrows show new CLs; full arrows show old *corpora lutea* (CLs) from previous years cycles. A: Juvenile lynx before puberty. Only the ovarian cortex is visible, no follicular or luteal structures; B: Pro-estrus in an adult lynx, small and medium sized, immature follicles appear next to CLs from previous years' cycles. C: Estrus in an adult lynx. Large, mature follicles and smaller immature follicles and an old CL from last year's cycle are visible; D: Met-estrus in adult lynx after recent ovulation. One old CL is visible. Two freshly ovulated, plicated follicles whose walls have begun to luteinize and formatting a new CL, centre parts are not yet fully closed (interrupted circles); E: second follicular wave, observed in only one animal during May, about 65 days after first estrus. Only one medium sized follicle visible; F: Pseudopregnancy in lynx. CLs without mating, no pregnancy, 1 month after spontaneous ovulation; G: Prolonged di-estrus during winter. Physiologically persisting CLs are hypo-echoic and still quite large; H: *Corpus albicans*. Old CL (interrupted rectangular) undergoes structural regression. CLs' tissue becomes denser and hyper echoic in ultrasound; I: Pregnancy at day 31 post copulation, CL appear hyper echoic; J: CL during lactation in lynx. Large, hyper echoic CL's.
doi:10.1371/journal.,pone.0090469.g001

In one captive female a second behavioral estrus was observed by the keepers about 2 months after her normal seasonal estrus. This female was kept without a male throughout. During

Table 1. Reproductive parameters in different reproductive stages.

cycle stage	N	E2	P4	PGFM	CL per ovary	CL tissue (cm^2)	ovarian volume	diameter. A *ovarica*	number follicles
pro-estrus	10	0.59±0.34	2.65±2.77	2.59±0.81	3.26±1.69	0.88±0.64	2.18±1.44	0.22±0.04	1.53±2.43
estrus	4	1.49±0.04	2.08±0.70	1.23±0.45	2.50±2.17	0.78±0.62	1.55±1.05	0.24±0.00	1.83±1.72
met-estrus	3	0.78±0.86	13.07±8.1	1.46±0.44	2.17±2.32	1.02±1.61	1.93±1.19	0.23±0.08	1.00±1.26
prolonged di-estrus	26	0.32±0.21	4.68±3.45	1.82±0.91	2.66±1.43	0.65±0.48	1.74±1.03	0.20±0.05	0.37±0.76
pregnancy	3	0.56±0.14	84.05 ±83.85	2.61±1.18	4.17±1.17	1.55±0.66	3.80±2.33	0.29±0.05	0.67±1.21
lactation	2	0.18–0.44	3.14–170.38	4.41–1.48	2.75±0.50	1.66±0.92	2.35±0.96	0.25±0.03	0.00±0.00

Mean values ± S.D. of various ovarian and serum parameters in free-ranging (N =10) and captive (N =38) lynx examinations during the various stages of the reproductive cycle, based on a 2 year study (2010–2012). E2 = serum estrogens in ng/mL, P4 = serum progesterone in ng/mL, PGFM = serum prostaglandin F2alpha in ng/mL, CL = *corpus luteum*, A. ovarica = *arteria ovarica*.
doi:10.1371/journal.,pone.0090469.t001

ultrasound examination one rather small follicle was seen in each of her ovaries (0.27 and 0.34 cm diameter, Fig. 1E). The corresponding hormone levels were not indicative of an estrus. Vaginal cytology showed intermediary and basophilic and acidophilic superficial cells (pbc = 27%, imc = 52%, sfc = 21%, sac = 0%). Her estrous behavior did not reach the same strength as during a proper estrus.

Estrus occurred at the end of February until mid-March for the captive lynx (N = 2) and at the end of March for the free-ranging lynx (N = 2). Behavioral signs of estrus (calling, rolling) lasted between 5 and 10 days for the captive animals observed in this study. Nevertheless, the zoo-keepers occasionally observed calling from the males or females already 3–4 weeks in advance. One captive female was in her first estrus. In this case we observed one follicle on each ovary (0.96 and 0.78 cm, Fig. 1B). The

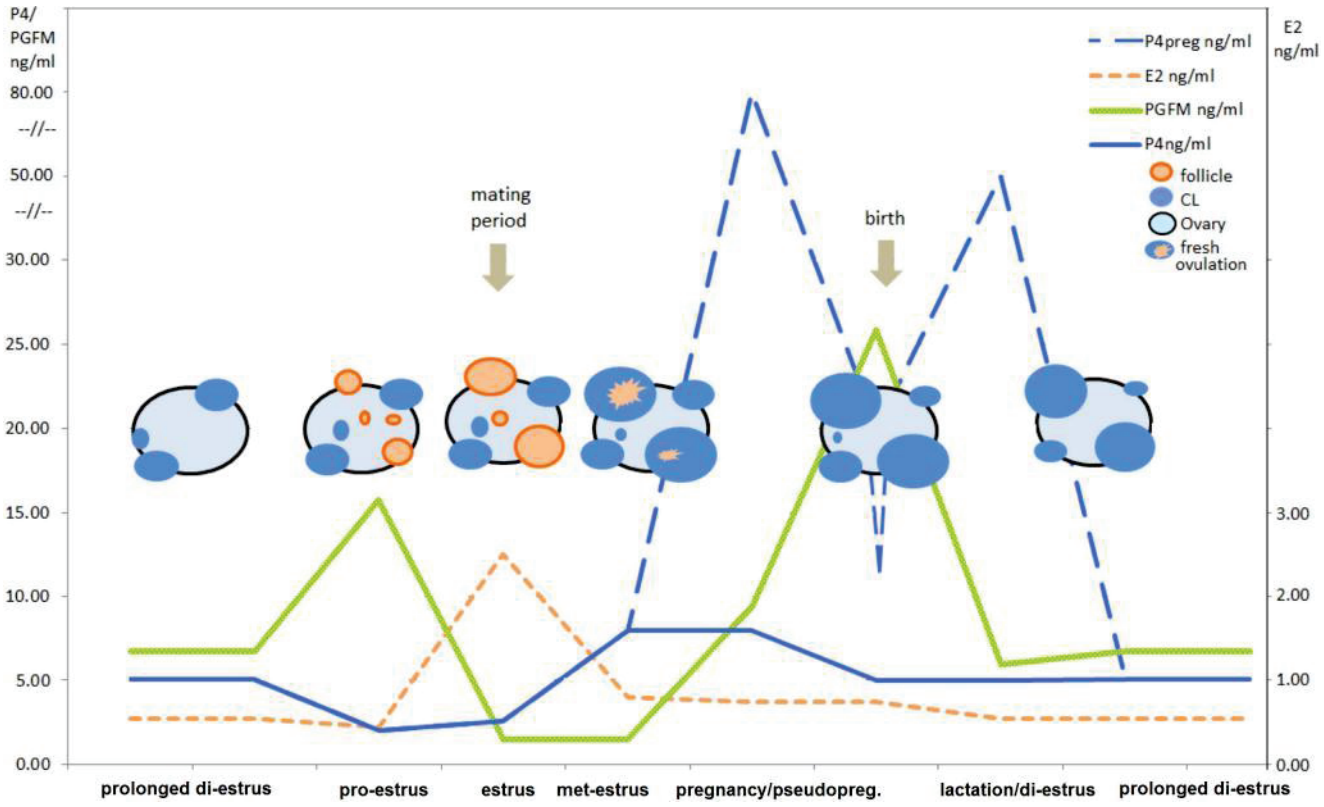

Figure 2. Schematic lynx ovarian cycle. Schematic diagram of the development of follicles and CLs within a reproductive year in pregnant and non-pregnant lynx. P4, E2, PGFM in ng/mL are shown with lines.
doi:10.1371/journal.,pone.0090469.g002

corresponding hormone concentrations were 1.58 ng/mL (E2), 1.58 ng/mL (P4), and 0.91 ng/mL PGFM. Estrous stage in older female lynx was characterized by a simultaneous appearance of follicles (0.01–0.95 cm diameter) together with hypo-echoic CL (Fig. 1C). In most cases, follicles were forming on both ovaries. Vaginal smears showed large superficial cells which were mainly acidophilic, with or without nuclei and partly cornified and folded (ESM, Fig. S1-B; pbc = 0±0%, imc = 1±2%, sfc = 49±40%, sac = 50±38%), the elevated E2 levels also pointed towards an estrous stage (Table 1).

Met-estrus, thus the period shortly after ovulation, was detected in three animals with clearly depicted ovulation scars (Fig. 1D, $N_{captive}$ = 2, $N_{free\text{-}ranging}$ = 1). Vaginal cytology showed mainly nucleus-free, acidophilic superficial cells (ESM, Fig. S1-C; pbc = 13±15%, imc = 16±6%, sfc = 29±9%, sac = 42±10%) and hormone levels indicative of luteal formation with elevated P4 levels (Table 1).

After the post-estrus luteal formation period, freshly formed fully functional CLs were judged to be a clear indication of a recent ovulation. The fresh CLs differed in their sono-morphology from the old CLs of previous cycles (Fig. 1F). They were not only bigger but also hyper-echoic. In general, the formation of new CLs was detected in both, the absence (N = 7 females) and presence (N = 3 females) of a mating partner and resulted in pseudopregnancy and pregnancy, respectively. In our study the ovulation rate was 100% each year in all females.

In pregnant females the secretion of P4 increased rapidly after ovulation. The CL tissues appeared hyper-echoic compared to pseudopregnant new CLs at the same time (Fig. 1I). These CL *graviditatis* were the same size as pseudopregnant new CLs (average 0.86 cm, range = 0.71–1.37 cm diameter) and they persisted for at least the 2 years of this study. During pregnancy P4 values were drastically elevated (>10 x), whereas in the pseudopregnant females (approx. up to 5 weeks post estrus) the P4 values were in the range obtained outside the breeding season. Five litters were born in captivity during the study period (4 triplets and 1 single kitten litters). During lactation the CLs ($N_{captive}$ = 2, Fig. 1J) appeared to be hyper-echoic compared to pseudopregnant (Fig. 1G) females at the same time. Gestation periods with observed mating and birthing dates in the captive lynx were 66–70 days, since females were often mated over 2–3 consecutive days. Vaginal smears mainly showed acidophilic superficial cells or intermediate cells (ESM, Fig. S1-D; pbc = 7±9%, imc = 31±25%, sfc = 38±21%, sac = 24±29%). Hormone levels were different between the two examined females, with one female expressing very high P4 (170 ng/mL) in contrast to 3.1 ng/mL in the other individual.,

After weaning (day 100 postpartum) the CLs appeared similar to CLs of non-pregnant females ($N_{captive}$ = 24, $N_{free\text{-}ranging}$ = 2) at the same time (Fig. 1G), and likewise hormone levels were not different. New CLs derived from this years' ovulation and old CLs derived from previous years' cycles (Table 1) were observed next to each other. They could be clearly distinguished by size (0.8–1.2 cm in diameter in new CL versus 0.2–0.65 cm for old CLs) and sono-morphologic texture (new CLs had a hyper-echoic appearance). The functional luteal activity outside the breeding season is hereafter referred to as prolonged di-estrus, or the phase of physiologically persistent CLs.

Values of P4 during prolonged di-estrus were significantly correlated with the intensity of vascular support measured using the diameter of the *A. ovarica* (Fig. 3, Spearman rank correlation, S = 10608.26, p<0.001, rho = 0.67), as well as with the number of CLs per ovary (S = 37774.55, p-value<0.001, rho = 0.39). Interestingly, no correlation was found either between month of the year and P4 concentrations in serum (S = 42362.31, p = 0.813, rho = 0.03), or to the intensity of vascular support (S = 42540.79, p = 0.164, rho = 20.18), when excluding estrus and pregnancy. Furthermore we found a positive correlation throughout the year between P4 and E2 in serum (S = 32824.11, p-value = 0.047, rho = 0.24) when excluding estrus. Vaginal cytology during prolonged di-estrus was variable; dominated either by basophilic basal cells, parabasal cells, or intermediate cells (ESM, Fig. S1-E; pbc = 25±24%, imc = 29±18%, sfc = 45±30%, sac = 1±1%). Repeated measurement within the same individual revealed a slow structural regression of CLs (diminishing diameter, decreasing echogenity) over time. A minimum life-span of two years was monitored in this study. The texture of regressing and cicatrized CLs in the final stage (*corpora albicans*) again appeared to be very hyper-echoic (Fig. 1H).

One captive animal was examined after its reproductive senescence (at 19 and 20 years of age). Steroid hormones measured were at very low levels with E2 = 0.39 ng/mL and non-detectable P4. Both ovaries appeared similar to juvenile ovaries, non-active and without functional bodies and only a minor vascularization.

(b) Repeated examination of animals

Repeated examinations were performed in six captive animals encompassing all non-pregnant cycle stages (pro-estrus, estrus, met-estrus, prolonged di-estrus). No significant differences emerged when comparing the concentrations of serum P4, E2, PGFM, ovarian volume, the intensity of vascular support (diameter A. *ovarica*) number of CL and size of CL tissue in their different reproductive stages, excluding estrus (also see table S4 in the ESM; Quade – Test, df = 22, P4: F = 0.07, p = 0.93; E2: F = 2.26, p = 0.13; PGFM: F = 0.02, p = 0.98; ovarian volume: F = 0.21, p = 0.81; diameter A. *ovarica*: F = 0.88, p = 0.43; number CL: F = 0.61, p = 0.55; size of CL: F = 0.83, p = 0.45). The number of follicles was significantly different in estrus (Quade-Test, df = 33, number follicles: F = 3.61, p = 0.02) compared to the other reproductive stages, but not significantly different within the three stages outside estrus (Quade – Test, df = 22, F = 0.08, p = 0.92). A simultaneous rise or decline of P4 and E2 was noted outside the mating season. Furthermore the number of CL, the size of CL tissue, the size of ovaries, and the position of the CLs were verified in-vivo with a sonographical 3D topography map.

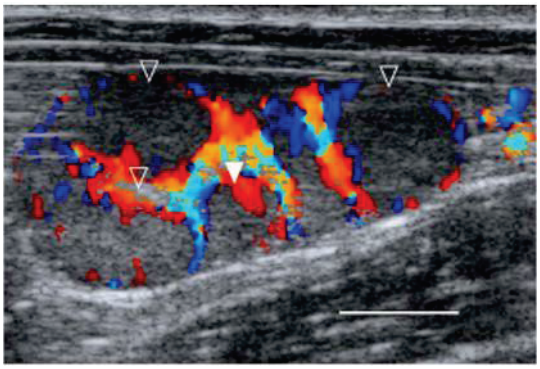

Figure 3. Persistent *corpora lutea* (CLs) of lynx in ultrasonography. Prolonged di-estrus in lynx outside breeding season in December. Empty triangle points at CLs from this years' ovarian cycle. Full triangle points out one old CL, at least two years old.
doi:10.1371/journal.,pone.0090469.g003

Discussion

By repeated ultrasound and hormonal examination in captive lynx, our study confirmed the physiological persistence of CLs derived from ovulations (Fig. 2). The results were supported by opportunistic examinations of free-ranging lynx. The life span of lynx CLs lasted for at least two years (duration of this study). In contrast to other feline species CLs in lynx did not disappear after pregnancy or the assumed luteal phase of pseudopregnancy, which is supposed to last for two thirds of the pregnancy [11]. The lynx CLs experienced only a gradual loss in size over time, while they were still expressing an active vascularization [16] and a moderate luteal P4 production [8]. During the fecund years, no typical anestrus (complete ovarian inactivity) was observed indicating that lynx have evolved a different strategy than other felids. Furthermore, the functional lifespan of lynx CLs exceeds the lifespan of elephant's CLs, which were previously reported as the longest ever documented CL lifespan among mammalian species [15].

The lynx reproductive cycle shows new and so far unknown dynamics in luteogenesis and luteal regression amongst felid species (Fig. 2). Plication of the new ovulated follicle wall and luteogenesis was observed at the start of met-estrus (Fig. 1D). Freshly formed CLs co-existed next to the CLs from previous cycles. The freshly ovulated CLs produced higher steroid amounts during met-estrus and pregnancy with approximately 10- and 100-fold higher intra-luteal P4 and E2 levels respectively [8] and 10-fold increased serum P4 levels (this study). These "new"CLs persisted after birth or pseudopregnancy without shutting down their luteal function. This was indicated by steady P4 serum levels, and the positive correlation between serum P4 and the intensity of ovarian vascularization. The physiological persistence was also confirmed by a positive correlation between the number of CLs per ovary and the animal's age, indicating a long CL lifespan and a temporary accumulation of CLs on lynx ovaries. This is very atypical for felids where the luteal activity normally starts to regress after birth in pregnant, and after the end of pseudopregnancy in non-pregnant, females. At least in the domestic cat it is known that serum P4 drops below 1 ng/mL [6] after parturition, and the CLs structurally regress to *corpora albicantia* [24]. In lynx, serum P4 levels remained elevated at average (basal) levels of around 5 ng/ mL. In contrast to P4, serum PGFM reflected different hormonal functions of persistent CLs. Serum PGFM was elevated during pro-estrus and late pregnancy (PGFM in serum free-ranging lynx, Scandlynx; mean = 18.16 ng/mL, range = 11.88–56.06, N = 6) which lead to a Prostaglandin F2a (PGF2a) mediated functional luteal regression (P4 decreased), without structural regression. Prostaglandins are known for their luteolytic effects, which decrease luteal blood supply, P4 secretion, and degrade CL structure [9,25]. The elevated serum PGFM levels were in accordance with our previous study investigating intra-luteal prostaglandin concentrations in lynx. These intra-luteal prostaglandins were only elevated prior to the breeding season [9]. However, the pro-estrus elevation of serum and intra-luteal prostaglandins could not be mirrored by the levels of PGFM determined in urine or feces of lynxes [22]. This may indicate that PGF2a is only produced at moderate levels prior to the onset of estrus (in contrast to late pregnancy) to act as an intra-ovarian signal for a transitory functional luteolysis of persistent CLs, which may serve as a necessary prerequisite for follicular growth and ovulation. Experiments in domestic cats examining the effect of PGF2a have also shown that rapid and complete recovery of luteal function is possible [26] and that PGF2a does not always initiate structural luteal regression in cats either [25].

In the past, all felids were believed to be exclusively coitus induced ovulators. However, later studies have shown that some cat species are able to ovulate spontaneously. Most felids express a combination of induced and spontaneous ovulation with individual variability [11]. Our data on captive lynx clearly indicate that lynx are able to ovulate spontaneously. As shown by the appearance of new CLs after breeding season, all captive lynx ovulated each year, either after natural mating (4 studied cycles) or spontaneously in the absence of mating (13 studied cycles), without there being a detectable difference in the lifecycle of the CLs. Felids show a broad variety of reproductive cyclicity, mostly dependent on photoperiod; ranging from being poly-estrous and seasonal (e.g. *Felis silvestris catus*, *Panthera tigris*, *Panthera uncia*) to being poly-estrous and aseasonal (e.g. *Panthera leo*, *Panthera pardus*, *Puma concolor*) [11]. In contrast, all members of the genus *Lynx*, except the bobcat [27], are strictly seasonal breeders [7,21,28].

Henriksen et al., [29] presented a mean birth date in 150 litters from captive lynx and in 23 litters from wild Scandinavian lynx: 50% of the captive litters were born within a 13 day period, and within an 8 day period in wild animals. This is corroborated by our study and also the findings of Kvam et al. [30], showing that breeding periods and therefore birthing dates are all within a very narrow window, minor latitudinal differences aside. A second estrus and later parturition seem to be very rare, but possible under extraordinary conditions (abortion, resorption, early loss of offspring) [31]. In our study, only one captive female lynx showed muted behavioral signs of a second estrus, however, minor folliculogenesis in ultrasound images and slight E2 elevations, indicated that the second "estrus"was not fully expressed.

During the lifespan of an animal there is a limited period for fecundity. This period usually starts at puberty (in female lynx mostly in their second year [18] and lasts until death. A period of reproductive senescence, as we have found in one old female lynx, usually occurs only in captivity because free-ranging lynx normally die before reaching reproductive senescence [32]. The most frequently observed physiological mechanisms to time births in mammals are either limiting the period of fertility (seasonal breeding, e.g. Pallas' cat [11]) or extending the duration of pregnancy (delayed implantations, e.g.: roe deer, some mustelidae [33,34]). Lynx seem to have developed a quite remarkable alternative strategy, where they limit the period of fertility by converting a poly-estrous cycle into a mono-estrous cycle. By doing this, they extend the duration of functional pseudopregnancy (luteal activity after ovulation without a pregnancy) for a prolonged time period to prevent follicular development before the next breeding season.

The hormonal support mechanism of persistent CLs still needs to be identified. In the domestic cat, prolactin is known to be a luteotrophic factor [35]. It might be suggested that the photoperiod response system acts via melatonin on the prolactin secretion, as shown for several seasonal breeders [36]. Up until now, only the involvement of intra-luteal (locally) produced prostaglandins can be suggested [9]. The source and neuro-endocrine regulation of prostaglandin secretion during pro-estrus in lynx remains unclear.

The evolution of a mono-estrous reproductive system in lynx could have primary (the ancestors of felids were mono-estrous) or secondary (adaptive) origins. A phylogenetic constraint, however, seems unlikely as mono-estrous breeding is a very unusual pattern among felids in general, and the most primitive member of the genus *Lynx*, the bobcat [37], is poly-estrous [27]. The alternative explanation is that the mono-estrous strategy has evolved as an adaption in order to time birth within specific times of the year. Interestingly we know from other felids that the body size, the period of parental care, and its habitat seem to be associated with

what kind of reproductive pattern they may express [38]. Smaller wild felids (Felis chaus, Felis silvestris and Felis serval) reproduce twice a year, while larger wild felids (genus Panthera) in general only reproduce every other year [39]. Most medium sized wild felids give birth to only one litter per year. Unlike the lynx, other felids are in anestrus (no hormone activity) in between their annual breeding period, which may differ in length [38]. Female lynx investing early in maturation, ovulation and placentation have been shown to be significantly lighter in the following year, indicative of high costs of reproduction [40]. Yet, the costs of reproduction are not well understood in solitary, large carnivores, like the lynx. In lynx populations that depend on ungulates like roe deer there is no obvious reason to time birth to early summer as prey is more easily located and caught in winter, and neonates do not represent a major proportion of the diet [41]. For lynx that feed on smaller prey it is unclear as to whether there is a benefit to giving birth in early summer as prey density tends to fluctuate in multi-year rather than seasonal cycles [42]. Avoiding inclement weather, detrimental for neonates left alone for prolonged periods while the mother hunts, may be a factor that would argue against extremely early births, but would not hinder later births during mid to late summer and autumn, which would be associated with poly-estrous breeding. One potential explanation could view this as an anti-infanticide strategy. Infanticide by males is very common in many felids, such as pumas [43] as loss of kittens leads to a rapid onset of a second estrus, but a second estrus is a very unlikely event in lynx. In contrast, mono-estrous breeding, especially in a species that only use one year to raise kittens [44], would effectively remove any benefits from males killing kittens. Interestingly, there is no documented case of infanticide among wild Eurasian lynx [32], to our knowledge.

The lynx reproductive cycle seems to be a rather non-plastic system. We found no difference in reproductive pattern between either wild and captive lynx or between central and northern European populations. The only anticipated difference was found concerning the timing of breeding seasons, which is most likely latitude, and therefore delayed photoperiod, dependent. However, the captive and free-ranging populations both showed the same reproductive strategy i.e. being mono-estrous due to physiologically persistent, constantly P4 secreting CLs, without plasticity regarding this phenomenon.

The main conservation implications of these results is the confirmation that lynx are indeed mono-estrous. The fact that ovulation appears spontaneously implies that pregnancy is very dependent on having access to a male during the crucial period of estrus. Anything that reduces access to males in this narrow window would result in an entire year's reproduction being lost. Many of the larger lynx populations are subject to hunter harvest in some form, and hunting is normally conducted in the late winter because of the hunter's dependence on good snow conditions [45]. Lynx hunting is male biased [18] and there is therefore a very real chance that lack of access to males could reduce population growth to a greater extent than has been appreciated. The actual impact will depend on issues such as female behavioral responses to the lack of a male and on the ability of transient (non-territorial) males to fertilize females.

A second conservation implication concerns the ability of lynx to adapt to environmental changes. The physiology described in this paper indicates that lynx will have relatively little ability to adjust birth dates as it appears to be a remarkably non-plastic system. However, the full implications of this are not clear, because we do not yet know the environmental cues for the timing of ovulation in lynx.

Supporting Information

Figure S1 Vaginal cytology in lynx. Vaginal cytology, stained with papanicolou at (A) pro-estrus, (B) estrus, (C) met-estrus, (D) pregnancy and (E) prolonged di-estrus stages. Black bar indicates 50 ㎜.
(DOC)

Table S1 Origins and reproductive status of study animals. The origins of the captive and free-ranging animals are listed below. Furthermore the reproductive period they have been examined is listed in the table. (DOC)

Table S2 Data sharing.
(DOC)

Table S3 Comparison between free-ranging and captive lynx. Comparison of various ovarian and serum parameters between single examinations of free-ranging (N = 10) and captive lynx (N = 10) during February and March 2011 and 2012. (DOCX)

Table S4 Repeated measurements from six captive Eurasian lynx throughout one reproductive year (February – February). Measurements were categorized in 3 time periods, excluding estrus (pro-estrus, pseudopregnancy, prolonged di-estrus) and tested if there are seasonal changes within an individual. (DOC)

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Author Contributions

Conceived and designed the experiments: JP KJ FG. Performed the experiments: JP FG KJ MD TBH JMA. Analyzed the data: JP. Contributed reagents/materials/analysis tools: JMA MD FG TBH KJ JDCL JO. Wrote the paper: JP KJ FG JDCL JMA. Endocrine laboratory analysis: MD KJ. Fieldwork and project logistics in Scandinavia: JMA JDCL JO JP.

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

Reproduction - October 1, 2012, vol. 144, p. 477-484

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3.3.3. Hormone-induced luteolysis on physiologically persisting *Corpora lutea* in Eurasian and Iberian Lynx (*Lynx lynx* and *L. pardinus*)

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DOI: http://dx.doi.org/10.1016/j.theriogenology.2014.05.004

4 General discussion and conclusions

The results described in manuscripts 1-3 demonstrate new and unique aspects of Eurasian and Iberian lynx (together hereafter refered to as *Lynx*) reproduction biology. The application of state-of-the-art techniques such as high-resolution ultrasonography, three-dimensional (3D) ultrasonography, Doppler ultrasonography, new endocrinological analyses in serum and tissue plus the ability to compare the data from captive with their wild conspecifics was advantageous to generate new insights. The antral follicular and basic luteal development within the female *Lynx* reproductive cycle was detected on repetitive examinations. By understanding this basic physiology better, conclusions on what kind of ART would be necessary to influence the female cycle were drawn. In the following section the most important methodological adaptations to achieve these results and conclusions on what is currently understood about the female *Lynx* cycle are discussed. Furthermore, it is discussed which kind of conservation implications can be drawn from these results.

4.1. Required methodological adaptations for wildlife examinations

4.1.1. High-resolution ultrasonography in wild felids

Ultrasound has been used for reproductive assessments of wildlife for many years. The possibility of using three-dimensional (3D) ultrasonography offered a new understanding in the development of physiological or pathological incidents during longitudinal wildlife studies (Drews et al., 2008; Goeritz et al., 2012; Hermes et al., 2006; Hildebrandt et al., 2006; Lueders et al., 2010; Roellig et al., 2011). It was evaluated to be a reliable and reproducible tool for research, if examiners are trained to work with a standardised protocol to reduce inter- and intra-examiner differences (Raine-Fenning et al., 2004; Sarris et al., 2012). Three-dimensional ultrasound offers opportunities to store the entire in-vivo configuration of an organ, rather than just a two-dimensional (2D) snap-shot. Retrospectively, the organs can all be virtually aligned to the same position and measurements can be standardised without angular displacements of their actual size (Fig. 8). Volumes can be precisely measured using special software (e.g. *4D-view*, GE health, Austria), instead of using traditional measurements of length, breadth and width, which are then combined to a volumetric measurement on the basis of an idealised three-dimensional mathematical body (Liang et al., 2009).

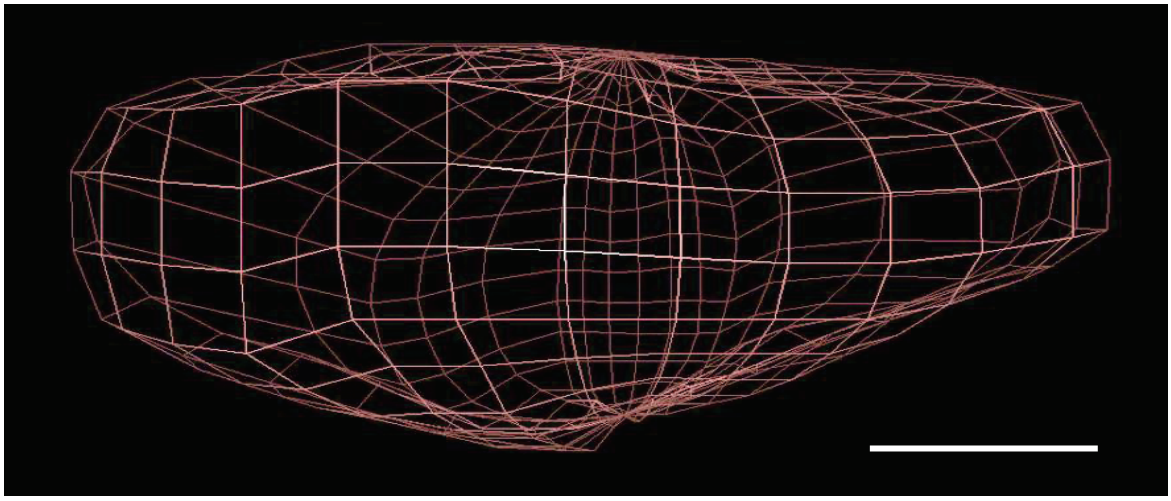


Fig. 8: A three-dimensional reconstruction of the ovarian volume in an Eurasian lynx, calculated with 4D-view software. Scale bar is 1 cm.

Within this study, 3D ultrasonography was used for topographic mapping of ovaries from repeated measurements within the physiological cycle of the same individuals, in order to demonstrate the consistency in structure and morphology of CL. The physiologically persistent CL of the *Lynx* stayed the same during the whole year and no new CL developed during the period outside the breeding season. These topographical images gave reliable evidence to document the unique luteogenesis and the longevity of individual CL, confirming that *Lynx* CL have the longest lifespan of physiologically active CL known to date in mammals. Allthough *Cetacea* and spotted hyenas (*Crocuta crocuta*) are described to have physiologically persisting CL over many years or probably a life time, it is yet unknow if they are functionally active or inactive *corpora albicantia* (Matthews, 1939; Ohsumi, 1964).

Doppler colour flow permits the measurement of floating particles (e.g. blood cells) within a stream (e.g. blood vessel), and the calculation of the velocity of flow (in m/s) and the direction of flow. Doppler colour flow is a sensitive and important tool in ultrasonography but if the practicioner is not experienced enough, it is prone to artefacts such as the aliasing effect (Nilsson et al., 1997a, b). The aliasing effect leads to a false over-interpretation of blood flow particles, a common mistake.

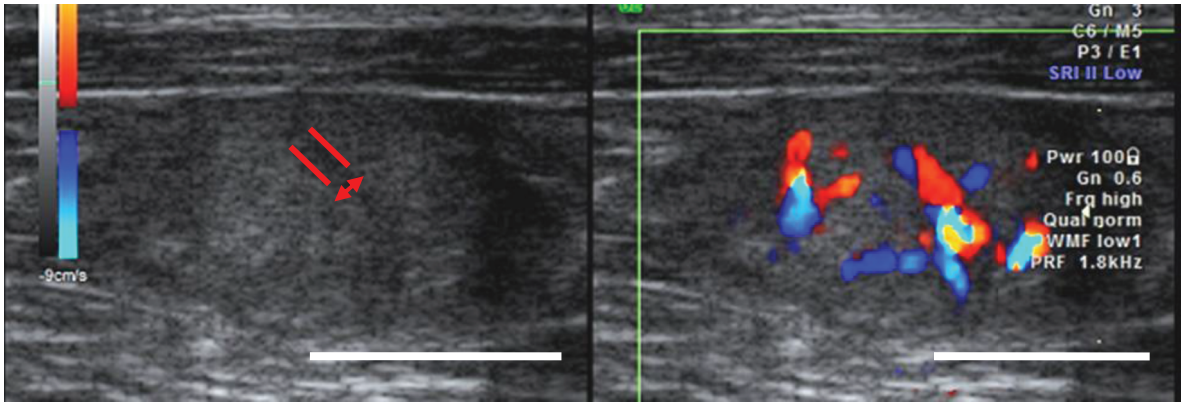


Fig. 9: Vessels can be allocated in Doppler mode (right), while accurate measurements (red lines indicate vessel intima; red arrow indicates the diameter measured) should be performed in the B-mode window (left), when using the split mode view. Scale bar is 1cm.

Measurements of vessel sizes in split mode were performed, where vessels are easily located in the Doppler window, while exact measurements from vessel intima to vessel intima (Fig. 9) can be performed in the b-mode window (Konje et al., 2001; Koster et al., 2001). Doppler colour flow is able to detect seasonal changes or therapeutic changes before effects can be seen on the organ itself, simply by detecting the intensity of vascularisation (Arduini et al., 1991; Koster et al., 2001; Manuscript 3).

3D volume scans and Doppler ultrasonography can be combined to achieve high quality images for the evaluation of vascular dynamics. Semi-quantitative evaluation of blood flow through an organ scanned with a 3D Doppler angiography (Fig. 10) provides information on physiological or pathological changes. It can be used for basic physiological research, for checks during hormone treatment designed for assisted reproduction or to examine pathological developments within a structure. The degree of vascularisation and angiogenesis is crucial for various female reproductive processes such as development of dominant follicles, formation of CL, growth of endometrium and implantation and gives a good indication of the success rate of hormone treatment and pregnancy development (Hildebrandt et al., 2009; Konje et al., 2001; Ng et al., 2007).

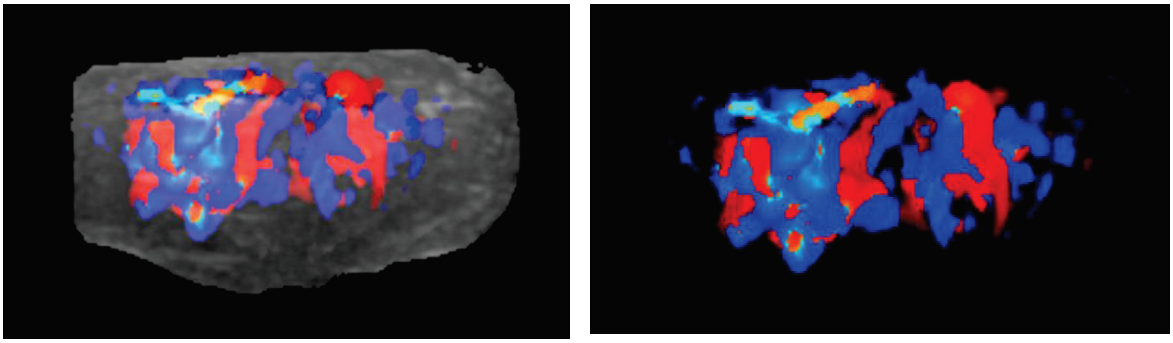


Fig. 10: The software 4D view can calculate the voxel (volume pixel) ratio of normal tissue (grey) and vessels (colour). Hereby ratios between the degree of vascularisation and the normal tissue are calculated, and compared with those in other reproductive seasons or with therapeutic success rates.

As example, a positive relationship between the diameter of the *A. ovarica* and the P4 level in serum was detected (Fig. 11; see manuscript 1 and 3). Both parameters exhibited coordinated changes throughout the season of a physiological *Lynx* cycle (manuscript 1) and mirrored the respective real-time therapeutic success while examining *Lynx* during luteolytic hormone treatment (manuscript 3). To our knowledge, comparisons between a hormone value and the diameter of ovarian vessels have not been published previously in felids. This new method might bring some advantages when patients have to be monitored closely to determine hormone treatment success rates or for basal research during physiological events. With this method, real-time evaluations can be made and give an initial indication of developments, whereas hormone measurements always take a few days before results can be evaluated. This technique, however, is only semi-quantitative and for detailed evaluations a proper endocrinological analysis has to be undertaken. A similar technique, comparing the vessel diameter with success rates of chemotherapy or IVF-embryo implantation, is already practiced in human medicine (Ng et al., 2006).

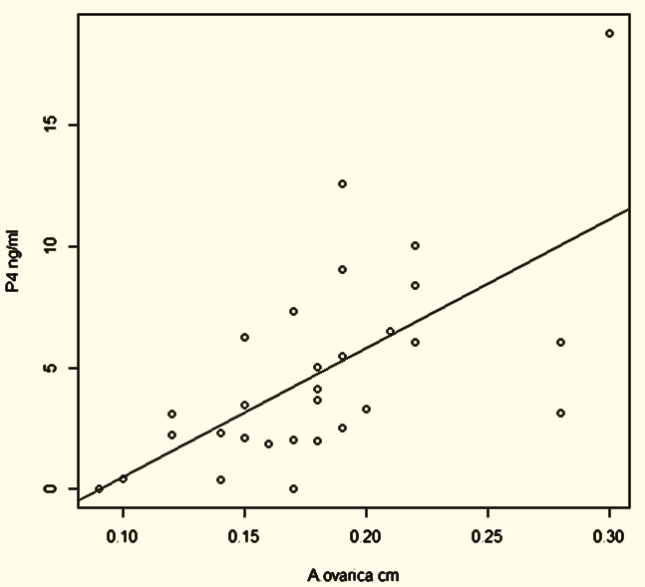


Fig. 11: Relationship between the diameter of the A. ovarica (cm) and the P4 (ng/ml) values in serum tested with (Spearman rank correlation, rho = 0.67, n = 29 , $p < 0.001$)

4.1.2. Examinations of free-ranging *Lynx* in northern Norway

For the study reported in the first manuscript, access to free-ranging Eurasian lynx was provided by the *Scandlynx* project, a research group who perform ecological studies on Eurasian lynx in Norway (Linnell et al., 2010; Mattisson et al., 2011; Nilsen et al., 2012b, see Fig. 7). The study design thus far was only performed on captive *Lynx*. The question stayed unanswered as to whether the phenomenon of physiologically persistent CL and prolonged di-oestrus was physiologic or pathologic, due to latitude or husbandry adaptations. To test the plasticity of this phenomenon, free-ranging Eurasian lynx and lynx from different latitudes needed to be included.

No significant difference in the reproductive physiology between free-ranging and captive Eurasian lynx was found (manuscript 1). Adaptive phenotypic plasticity is a phenomenon described in various species (Ghalambor et al., 2007; Scheiner and Holt, 2012). Different traits will show a different phenotype dependent on environmental influences. The physiologically persistent CL with a long lifespan of over two years, the ability to ovulate spontaneously, and the unique phenomenon of monoestrus were all of low plasticity in *Lynx*. None of these mechanisms changed with latitude or living conditions (captivity vs free-ranging conditions).

4.2. Reproduction biology in *Lynx* – what is known after the studies within this thesis

4.2.1. The *Lynx* cycle

Reproduction biology studies of wild felids are often based on random examinations performed during by-chance examination of patients. Although the *Lynx* are one of the best studied felids, most basic knowledge about reproductive parameters had previously been collected during opportunistic examinations of single individuals. Ultrasonography and hormone analysis (Goeritz et al., 2009a, b), longitudinal hormone analysis in urine and faeces (Dehnhard et al., 2010, 2012, Finkenwirth et al., 2010, Jewgenow et al., 2009, Pelican et al., 2009) and gross-morphological studies on hunted lynx (Axnér et al., 2013; Kvam, 1991) have been performed recently and through these studies, important basal knowledge could be obtained (table 3). *Lynx* exhibited CL at any time during the year. It was unclear from which cycle periods these CL could have derived from, when they would disappear and what their role would be during the year and during the next cycle onset.

Repeated examinations (N=38) from the ten captive females plus opportunistic examinations of ten free-ranging females verified the principal assumption that Eurasian lynx are indeed monoestrus. They showed physiologically persistent CL over a period of at least two years (manuscript 1). In *Lynx*, the CL did not disappear after pregnancy or the assumed luteal phase of pseudopregnancy. The CL only experienced a gradual regression over time, while they were still showing active vascularisation (Manuscript 3) and a moderate luteal P4 production (Manuscript 2). During fecund years, no typical anoestrus was observed, indicating that *Lynx* have evolved a different reproductive strategy than any other felid. Manuscript 1 shows that the functional lifespan of *Lynx* CL exceed the lifespan of any other mammalian active CL known today. The second longest lifespan of functionally active CL was recently reported for elephants (Lueders et al., 2012).

This demonstrates that currently the *Lynx* cycle has to be considered to be unique not only amongst the so far studied felids but also for mammals. New dynamics in luteogenesis and luteal regression amongst felid species were discovered (Fig. 12). The CL lifespan starts with the plication and luteogenesis of the new ovulated follicle wall at the beginning of met-oestrus, similar to other mammals. Interestingly, the freshly formed CL co-existed with the

CL from the previous or even the penultimate cycle. By ultrasound they were clearly distinguishable from the established ones through a different size.

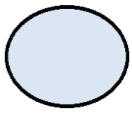
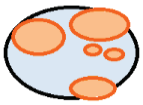
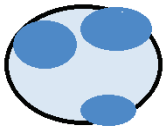
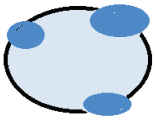
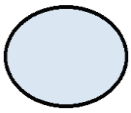
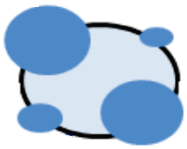
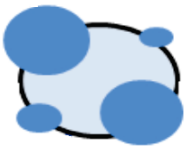
	outside breeding season	**oestrus**	**pregnancy/ pseudopregnancy**	**parturition / lactation**	**outside breeding season**
other felids	 no CL	 follicles get visible size for ultrasonography	 ovulated follicles turn into CL	 CL undergo luteolysis	 CL are gone
Lynx	 old CL from previous years' cycles	 old CL plus large follicles	 old CL plus new CL	 CL of different ages on one ovary	 CL of different ages on one ovary (eldest CL regress slowly)

Fig. 12: Scheme of the comparison between the follicular and luteal phase of CL from other felids and *Lynx* during one reproductive year (see manuscript 1).

A clear distinction between two types of CL was seen in histology and through intra-luteal hormone analysis, previous years' CL and freshly ovulated this year's CL could be well distinguished. The freshly ovulated CL produced higher steroid amounts shortly after ovulation and during pregnancy with approximately 10- and 100-fold higher intra-luteal P4 and E2 levels respectively (Fig.13, Manuscript 2) and 10-fold increased serum P4 levels (Manuscript 1).

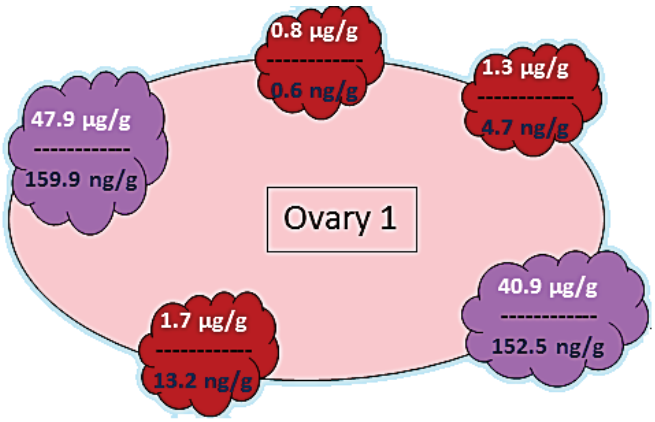


Fig. 13: Schematic drawing of one ovary of a pregnant Eurasian lynx with old CL from former cycles (red) and fresh ovulations from this pregnancy with increased E2 (black font) and P4 (white font) values (lilac) (Manuscript 2).

These "new" CL persisted physiologically after parturition or pseudopregnancy without shutting down their luteal function (manuscript 1,2). This was indicated by steady P4 serum levels. The increase of serum P4 with intensity of ovarian vascularization was already mentioned previously (Fig. 11). The physiological persistence was also confirmed by an increase of the number of CL per ovary and female age, indicating a long CL lifespan and accumulation of CL on lynx ovaries. Older animals had a maximum of up to 12 CL per ovary. This is very atypical for felids where the luteal activity normally starts to regress before parturition in pregnant females, and at the end of pseudopregnancy in non-pregnant females (Wildt et al., 1981). In *Lynx*, no difference in life-span of CL from pseudopregnant or pregnant individuals was noted.

At least for the domestic cat serum P4 drops below 1 ng/mL (Wildt et al., 1981) after parturition, and the CL structurally regress to *corpora albicantia* (Dawson, 1946). In lynx, serum P4 levels remained at average (basal) levels of around 5 ng/mL outside breeding

season. To rule out any cross-reactivity with other inactive progestin metabolites, the in-house ELISA used was validated for several progestin metabolites too (Dehnhard et al., 2010). Since there was no chance to obtain serum samples from the same individuals that supplied the post-mortem samples, we could not draw direct comparisons between serum P4 and intra-luteal P4 content. In such a study in domestic cats, serum P4, intra-luteal P4, CL mass and LH receptor density were positively associated (Swanson et al., 1995).

When do CL undergo regression? In domestic dogs, the regression of CL from pseudopregnancy cycles seem to be rather permissive whereas the regression of CL from pregnancy cylces is active through luteolysis, including structural regression (Hoffmann et al., 2004, Kowalewski et al., 2009). In domestic cats, the mechanism behind regression of pregnancy or pseudopregnancy CL is not yet fully known (Amelkina et al., 2015). In lynx, however, there seems to be no difference between the regressions of CL from pregnant or pseudopregnant cycles. In both stages, the lynx CL actively produce P4 during the whole year and only undergo a permissive regression, lasting many years (Manuscript 1, 2; Jewgenow et al.; 2012; Zschockelt et al., 2014), during which time, the CL ability to continue being functionally active in P4 secretion is unhindered. Physiologically persistent CL seem to be secured due to androgens, which impede apoptosis and promote P4 secretion (also oestrogens), and P4 itself which transduces pro-survival actions (Amelkina, 2016). Prostaglandin E2 is furthermore in discussion of being a possible luteotrophic factor (Zschockelt et al., unpublished data).

In contrast to P4, serum PGFM was elevated during pro-oestrus and late pregnancy, which resulted in a functional regression (P4 decrease), without structural regression (PGFM in serum of free-ranging Eurasian lynx, Scandlynx; mean = 18.16 ng/mL, range = 11.88 – 56.06, N = 6, unpublished data, mentioned in manuscript 1). Prostaglandins are known to decrease the luteal blood supply, P4 secretion, and degrade CL structure, all leading to functional or/and structural luteal regression (Jewgenow et al., 2012; Weems et al., 2006). The elevated serum PGFM levels were partly in accordance with our previous study investigating intra-luteal prostaglandin concentrations in *Lynx*. However, these intra-luteal prostaglandins were only elevated prior to the breeding season (Jewgenow et al., 2012). Interestingly, the pro-oestrus elevation seen in serum and intra-luteal prostaglandin analysis could not be mirrored by the levels of PGFM determined in urine or faeces of *Lynx* (Finkenwirth et al., 2010). This may indicate that PGF2α is only produced at low levels prior to the onset of oestrus (in

contrast to late pregnancy) to act as an intra-ovarian signal for a transitory functional luteolysis of physiologically persistent CL, which might not be detectable in urine and faeces. These results were also the basis of different trials to induce artificial luteolysis through prostaglandins in *Lynx* (manuscript 3). In domestic cats, a rapid and complete recovery of luteal function is possible after prostaglandin exposure (Shille and Stabenfeldt, 1979) and PGF2α does not always initiate structural luteal regression in cats either (Weems et al., 2006). Intra-luteal PGFM elevations were not found before parturition in the post-mortem samples, but in serum samples in living animals. This discrepancy might be explained with a low sample size in the post-mortem samples around the time of parturition.

Table 3: Summary of different reproductive parameters within the *Lynx* genus (modified from (Crowe, 1977; Goeritz et al., 2009a; Hayssen et al., 1993; Kvam, 1991; Manuscript 1; Sts and Leopold, 1993; Wosher, 1988)

	L. lynx	***L. pardinus***	***L. canadensis***	***L. rufus***
cycle status	1 cycle/year			1-3 cycles/year
adult bodyweight	14 – 30 kg	8 – 17 kg	9 -18 kg	10 – 14 kg
neonatal weight	250 – 360 g	150 – 220 g	200 g	112 – 226 g
litter size	2.2 (1 – 4)	3 (1 – 4)	2 (1 – 5)	3 (1-4)
first oestrus	9, 11 or 21 month			9, 11 or 21 month
sexual maturity	2 – 3 y			2 – 3 y
oestrus length	5 – 7 d			n.a.
cycle length	physiological pseudopregnancy followed by prolonged di-oestrus			44 d
gestation	65 – 69 d	63 – 66 d	60 – 65 d	64 - 70 d
lactation period (start solid food)	3 mth (6wk)	3.5 mth (5 – 6 wk)	3 mth (6wk)	3 mth (7 – 8 wk)
litter/year	1			1 (2)
breeding season	February –April	December – February	January - February	January – July
parturition time	mid May – mid June	March	May – June	April – September (July – August)

Up until now, only the involvement of intra-luteal (locally) produced prostaglandins can be suggested for the temporary functional regression (Jewgenow et al., 2012). Pre-partal prostaglandin elevations are found in many investigated species thus far and are induced by a complex synergy of local prostaglandins (Dehnhard et al., 2012; Hoffmann et al., 2004;

Jewgenow et al., 2013; Siemieniuch et al., 2011; Weems et al., 2006). Recently the expression of intra-luteal prostaglandin receptors could be verified in domestic cats (Zschockelt et al., 2013) and prostaglandins can be expected to mediate intra-luteal effects in domestic cats. In lynx, prostaglandin secretion was detected intra-luteally (Jewgenow et al., 2012), in faeces, urine (Dehnhard et al., 2012; Finkenwirth et al., 2010) and serum (Manuscript 1).

4.2.2. The fecund period of *Lynx*

In the past, all felids were believed to be exclusively coitus induced ovulators. In fact, most felids express a facultative induced/spontaneous ovulation pattern with individual variability (Brown, 2011). Manuscript 1 shows a clear indication that *Lynx* are able to ovulate spontaneously. As shown by the appearance of new CL after breeding season, all captive *Lynx* ovulated each year, either after natural mating (4 studied cycles) or spontaneously in the absence of mating (13 studied cycles). Remarkably, there was no detectable difference in the lifecycle of the CL derived from these two groups. Felids show a broad variety of reproductive cyclicity, mostly dependent on photoperiod (Brown, 2011). In contrast to a common felid cycle, the *Lynx* are monoestrus breeders (Dehnhard et al., 2010; Fanson et al., 2010; Goeritz et al., 2009a). Within a Scandinavian study, 50% of the captive litters (N = 150) were born within a 13 day period, and within an 8 day period in wild (N = 23) litters (Henriksen et al., 2005). Breeding periods and therefore birthing dates are all within a very narrow window (Kvam, 1991; Manuscript 1), minor latitudinal differences aside. A second oestrus and later parturition seem to be very rare, but were reported to have occurred under extraordinary conditions (abortion, resorption, early loss of offspring) (Kaczensky, 1991). In this study, only one captive female lynx showed muted behavioural signs of a second oestrus, however, only small follicles (less than 2 mm) in ultrasound images and only slight E2 elevations, indicated that the second "oestrus" was not fully expressed.

During the lifespan of an animal there is a limited period for fecundity, which starts in female *Lynx* mostly in their second year (Nilsen et al., 2012b) and lasts usually until death. Reproductive senescence was found in an old, captive female Eurasian lynx. Senescence is used here to refer to the decline and further cessation of fecundity with age. Senescence is seen in a variety of mammalian species (Kirkwood and Rose, 1991; Packer et al., 1998; Promislow, 1991). However, it is usually only seen in captivity, because free-ranging Eurasian lynx normally die before reproductive senescence appears (Andren et al., 2006). The

most frequently observed physiological mechanisms to time parturitions in mammals are either: restricting the period of fertility within each year (seasonal breeding, e.g. Pallas' cat (Brown, 2011)) or extending the duration of pregnancy (delayed implantations, e.g.: roe deer, some mustelids (Ferguson et al., 2006, Hermes et al., 2000)). *Lynx* limit the period of fertility in a different way. They convert a common polyoestrous felid cycle into a unique monoestrous cycle. By doing this, they extend the duration of functional pseudopregnancy for a prolonged time period to prevent oestrus onset before the next breeding season. Hence, we named this period "prolonged di-oestrus".

4.2.3. Hypothesis for the evolution of the monoestrous cycle in *Lynx*

The evolution of a monoestrus reproductive system in *Lynx* could have had ancestral (the ancestors of felids were monoestrus) or adaptive origins (manuscript 1). Monoestrous breeding is a very unusual pattern among felids in general, and the most primitive member of the genus *Lynx*, the bobcat (Johnson et al., 2004), is still showing up to 3 cycles per year (Crowe, 1977; Stys and Leopold, 1993). Hence, a phylogenetic constraint seems unlikely. The monoestrus strategy might have evolved as an adaptation in order to time parturition within specific times of the year. Mammalian offspring can be born in the most productive or the least productive time of the year, or even during hibernation (Bieber et al., 2012). Felids have a global distribution, mainly giving birth during the most productive time of the year, which can often mean giving birth all year round. To mature early was shown to result in significantly lower bodyweight in the following year, indicating the high costs of reproduction in Eurasian lynx (Nilsen et al., 2010). Yet, the costs of reproduction are not well understood in solitary, large carnivores, like the *Lynx*.

Reproductive patterns are described to be dependent on body size, the period of parental care, and the habitat (Jewgenow and Songsasen, 2014). Smaller wild felids (*Felis chaus*, *Felis silvestris* and *Felis serval*) normally reproduce twice a year, medium-sized felids give birth to only one litter per year, while larger wild felids (genus *Panthera*) often only reproduce every other year (Hayssen et al., 1993). All other felids, excluding the *Lynx*, undergo anoestrus of different length intervals in between their breeding seasons (Jewgenow and Songsasen, 2014). There is no obvious reason to time parturition to early summer for Eurasian lynx populations, which depend e.g. on roe deer. Roe deer are more easily located and caught in winter and not early summer, and their neonates (born in early summer) do not represent a major proportion

of the lynx' diet either (Mejlgaard et al., 2013). Small prey (e.g. hares, etc.) density tends to fluctuate in multi-year rather than seasonal cycles, hence it is unclear as to whether there is a benefit for Eurasian lynx to giving birth in early summer (Apps, 2000). One argument against extremely early parturitions would of course be the avoidance of inclement weather conditions, which is detrimental for neonates left alone in the den while the mother goes hunting. However, it would not hinder later parturitions during mid to late summer and autumn, which would be associated with common felid polyoestrous breeding. Within manuscript 1 an anti-infanticide strategy was hypothesized to give a potential explanation for the monoestrus in *Lynx*. In pumas, e.g., infanticide by males is very common (Logan and Sweanor, 2001), because lactating females are suppressed in their ovarian activity (due to prolactin) and a loss of all kittens would lead to a rapid onset of a second oestrus. In *Lynx*, however, a second oestrus is a very unlikely event. Monoestrous breeding, especially in a species that only uses one year to raise kittens (Samelius et al., 2012), would effectively remove any benefits from males killing kittens. To our knowledge, there is also no documented case of infanticide among wild Eurasian lynx (Andren et al., 2006), nor in captivitiy. There is one report of an infantizid in a wild Iberian lynx (Lopez et al., 2010).

4.2.4. A common lack of hormone associated pathologies in *Lynx*

Female *Lynx* are under constant luteal steroid hormone exposure during their fecund period. Side effects of long term exposure to reproductive hormones in domestic and captive, wild carnivores are a common observation by clinicians and reproductive scientists. Most common side effects from long term exposure to oestrogens (independent of age) are cystic endometrial hyperplasia and mammary gland tumours, observed in a variety of mammalian species (Bentrem et al., 2003; Misirlioglu et al., 2006). Most common side effects (independent of age) in domestic and wild carnivores from long term exposure to gestagens are cystic endometrial hyperplasia-pyometra complex, endometritis, pyometra, hydrometra/mucometra, uterine mineralization, mammary gland neoplasia, endometrial adenocarcinoma, leiomyosarcoma and indirectly, polyps and endometritis (McCain et al., 2009; Misirlioglu et al., 2006; Munson et al., 2002). Cystic endometrial hyperplasia–pyometra complex in carnivores is hormone associated; Progesterone contraceptions in carnivores often lead to cystic endometrial hyperplasia (CEH), which makes the uterus more prone to retrograde infections in domestic and exotic zoo felids (McCain et al., 2009; Munson et al.; 2002). Pyometra is normally more often found in dogs, which are spontaneous ovulators, than

in felids, which are more often induced ovulators than spontaneous (McCain et al., 2009). In domestic cat, activation of a glycoprotein (c-erbB-2) through circulating oestrogens and an over-expression of oestrogen and progesterone receptors are associated with cystic endometrial hyperplasia. Progesterone alone plays a significant role in the pathogenesis of endometrial hyperplasia and pyometra (Misirlioglu et al., 2006). Furthermore progestins (e.g. progestin contraceptive drugs like Melengestrol Acetate, MGA) promote endometrial growth by activating oestrogen receptors, entering a vicious circle. Lynx have comparatively high P4 levels (> 5 ng/ml) throughout the year, but show no pathologies associated with chronic progestin exposure. Within Manuscript 1, a positive correlation between P4 and E2 in Eurasian lynx was found. Brown (2011) found the same effect in faecal steroid metabolites of ocelot and jaguar. In *Lynx*, small follicles of less than 2mm diameter were found during ultrasonography, indicating a slight oestrogen activity. Comparable to progestin treatment (Munson et al., 2002; Stewart et al., 2010), physiologically persisting CL and chronic progestin exposure in *Lynx*, did not inhibit the follicular phase. The onset of the follicular phase (increasing E2) in lynx was always suppressed by a correlating, increasing P4 serum level, which gave an immediate negative feedback. This effect most likely suppressed further follicular maturation and probably hindered oestrogen priming. Oestrogen priming seems to be an important prerequisite for progesterone dependent physiological endometrial gland development (Boomsma and Verhage, 1982), and within the MGA-dependent cystic endometrial hyperplasia complex (Misirlioglu et al., 2006). This might be the reason why lynx are not or far less prone to progestin associated reproductive disorders. Until today, only one report of endometrial hyperplasia (Munson et al., 2002) and no report of pyometra have been documented for the *Lynx* genus or seen in over 60 Eurasian lynx and over 100 Iberian lynx examinations of any cycle stage and age. In 2016 a pyometra was found in an eleven month old female, after mating with an experienced male. Presumably, here a pyometra could develop, since the protective status of the physiologically persistent CL was yet absent (pers. comm. J. Painer, January 2016).

4.3. Assisted reproduction techniques

4.3.1. Luteolysis of physiologically persistent CL in *Lynx*

Within the third manuscript's study, a synthetic prostaglandin (Cloprostenol) was injected intramuscularly in female Eurasian lynx and resulted in a reaction similar to the natural,

physiological and functional luteal regression described above. It was similar in that during the duration of prostaglandin exposure: a) P4 decreased significantly, b) ovarian and intra-ovarian (CL) vascularisation decreased significantly, and c) the size and number of CL did not change significantly. Furthermore, sometime either after the artificial treatment or after the natural pro-oestrus or pre-natal regression,: d) the P4 level recovered to elevated basal levels – normal to *Lynx*, e) the ovarian and intra-ovarian vascularisation regained strength and volume, and f) the amount and size of CL did not differ significantly to the previous status.
Recent research shows that before the onset of *Lynx* oestrus the steroidogenic capacities are temporarily limited (Zschockelt et al., 2016). However, PGF2α associated luteolysis seems to be negligible in *Lynx*, and the temporal regression remains unclear yet (Zschockelt et al., 2016).

Artificial prostaglandins, such as Cloprostenol, are used in domestic cats and dogs for various treatments (Fransson and Ragle, 2003; Shille and Stabenfeldt; 1979; Williams et al., 1999), resulting in luteolysis with a decrease in P4, a reduction of intra-luteal vascularisation and reduction or complete regression of luteal tissue. The difference in the treatment in *Lynx* was that the physiologically persistent CL only reduced P4 and vascularisation temporarily, and no lysis of luteal tissue was observed (manuscript 3).

Prolactin is, from a phylogenetical point of view, a very old hormone with a great ability to get used for various conditions (Keck and Breckwoldt, 2000). It is an adenohypophyseal proteohormone with many diverse biological and central functions within reproduction, such as the luteotrophic effect on CL by causing the increase of progesterone receptor distribution, which has been shown in other species previously (Banks et al., 1983; Jochle and Jochle 1993; Keck and Breckwoldt 2000). Prolactin, in response to photosensitive melatonin, showed to be luteotrophic in several seasonal breeders (Banks et al., 1983; Concannon et al.; 1980; Dupre, 2011). As opposed to many other endocrine regulatory systems, the secretion of prolactin is only controlled by inhibition, through the hypothalamic neurotransmitter dopamine and not through stimulations, as for most other hormones. Dopaminergic agonists, like Cabergolin, are used in dogs for oestrus induction or to shorten the inter-oestrus or anoestrus intervals (Kutzler, 2007). In females, Cabergolin solely or in combination with PGF2α is administered for abortion induction (Erunal-Maral et al., 2004) or to treat open-cervix pyometra (Mitacek et al., 2014). In wildlife, different protocols were suggested to induce abortion in invasive species, such as the coyote and fox (DeLiberto et al., 2002,

Lengwinat et al., 2001). Its inhibition through dopamine agonists and the proceeding luteal arrest, together with prostaglandin, was documented as being successfully luteolytic in domestic cats (Jochle and Jochle, 1993; Onclin and Verstegen, 1997). Since we could only administer this protocol in two individuals, no scientifically based recommendations could be given. However in these two individuals, no side effects, but also no synergistically effects could be observed.

Luteal P4 is an autocrine luteotrophic factor (Kowalewski et al., 2009). Anti-gestagens are described for other carnivore species as being competitive with P4 receptors (Balogh et al., 2012; Goeritz et al., 2001; Jewgenow et al., 2001). Thus, blocking the P4 receptor might prevent the negative feed-back of P4 as a cause of monoestrus. *Lynx* have higher P4 levels outside breeding season than any other felid species, due to their physiologically persistent CL. In addition, P4 itself might be luteotrophic in *Lynx* as described for other species previously (Cai and Stocco, 2005; Concannon et al., 2009). Aglepristone competes with P4 on its receptors, being able to displace P4 and its supportive reactions (maintenance of pregnancy, e.g.) and is therefore causing luteolysis and/or abortion (Georgiev et al., 2008; Hoffmann et al., 2004). Its use in queens has, so far, only been reported as an abortion inducer (Georgiev et al., 2008). Conclusions as to whether or not the combination of Cloprostenol with Cabergolin and/or Aglepristone would give any synergistic effect in *Lynx*, could not be drawn from this small sample set. A larger sample set and daily blood collections would be necessary to give detailed conclusions on the effects of adding Cabergolin and/or Aglepristone to the Cloprostenol treatment; both of which we unfortunately had no access to.
Artificial luteolysis of physiologically persisting CL in *Lynx* was possible to some extent, similar to the natural regression occurring before natural oestrus and parturition; leading only to functional, but not structural regression. It should be considered whether artificial oestrus induction, with or without artificial luteolysis beforehand, would be more advantageous. As for a future protocol, treatments with and without functional artificial luteolysis, followed by oestrus and ovulation induction should be performed.

4.4. Conservation aspects

Every reproductive trait evolved over thousands of years to provide an adaptability (evolutionary adaptations) to the available resources and the environmental conditions these animals live in. It is important to understand the different phenotypes of traits. For many conservationists, the reason behind studying basal physiology is summarized in an oft-quoted statement: “you can only protect what you know”. By understanding the fundamentals of physiological processes or anatomical structures, further improvements like supporting species by knowing their specific requirements can be made. Some studies show that global changes bring new challenges to individuals, where they have to adapt to habitat destruction, climate change, increased natural catastrophic events, displacement into harsher conditions or poaching. It is therefore indispensable to understand how and if specialised evolutionary strategies, like some specific reproductive phenotypes, are able to adapt to a rapidly shifting world (Coppack and Both, 2002; Lane et al., 2012; Pau et al., 2011). Another conservation implication concerns the ability of lynx to adapt to environmental changes. Specific variations regarding climate change and the way it may shape and influence seasonal patterns of resource availability and productivity are cause of concern. A major emerging topic is the extent to which species' phenology, especially the timing of reproduction, is able to adapt to keep pace with possible changes in seasonality (Coppack and Both, 2002; Lane et al., 2012; Pau et al., 2011). However, all species clearly show some constraints in their ability to adapt to change, as traits vary hugely in their reproductive plasticity. Understanding the physiological basis of the mechanisms that control reproduction is central to understanding the potential for a species to adapt.

4.4.1. Eurasian lynx

The main conservation implications of the results from manuscript 1-3, are the confirmation that *Lynx* are monoestrus. Since female lynx can ovulate spontaneously, pregnancy is very dependent on having access to a male during the crucial period of oestrus. Anything that reduces the access to males during this very narrow window of about one week would result in an entire year’s reproduction being lost. Many of the larger lynx populations are subject to hunter harvest in some form. Eurasian lynx hunting depends upon optimal snow conditions, therefore most lynx hunting seasons are normally allocated in the late winter (Bischof et al., 2012). Unfortunately, many countries do have their lynx hunting period just before or during

breeding season. Targeting of lynx by hunters is male biased (Nilsen et al., 2012b) and there is therefore a very real chance that lack of access to males could reduce population growth to a greater extent than has been appreciated (manuscript 1). This could happen especially in an area where lynxes have to travel far distances to meet their mating partner and only have a very short window of reproduction. A great deal of conservation and research focus is underway to try and understand the extent to which lynx can live in human modified landscapes (Schadt et al., 2002) and cope with human impacts such as harvest (Linnell et al., 2010, Nilsen et al., 2012a). The actual impact on reproduction rates will therefore depend on issues such as female flexibility in behavioural responses towards transient males and on the ability of transient (non-territorial) males to fertilise females. The physiology described in this thesis indicates that lynx will have relatively little ability to adjust parturition dates, as it appears to be a remarkably non-plastic system.

4.4.2. Iberian lynx

Any circumstance hindering breeding or the success of young offspring will automatically terminate that year's reproductive output. For the conservation breeding centres in Spain and Portugal, this is of utmost importance. Currently offspring rates are high and enough cubs survive every season to make the captive breeding programme highly successful, with yearlings getting reintroduced successfully into natural habitats. However, this can change if some unwanted diseases (Jiménez et al., 2008) or other circumstances (loss of prey) impede the success of the first breeding. Hence, it is mandatory to be able to manipulate the *Lynx* cycle artificially in order to have a second oestrus and therefore a second chance at reproductive success.

5 Summary

Lynxes undergo a non-cat like ovarian cycle. Before conducting the studies contained in this thesis, hardly anything of the cycle was fully understood. Concerning conservation aspects, it was important to understand how luteogenesis functions and if it could be manipulated artificially to increase the reproductive output. Therefore, results from examinations of *Lynx* from different latitudes, from captivity and the wild, and of two different species (Iberian and Eurasian Lynx) were gathered, using high resolution ultrasonography and serum hormone analysis.

During our investigations we found out, that the persistent CL, unique to *Lynx*, are physiological and remain active over an extended period of more than two years. We established a new term for this period: the "prolonged di-oestrus". These CL seem to undergo one of the longest known lifespan of luteal tissue in mammals. After ovulation, which can be spontaneous or induced in *Lynx*, the follicular tissue undergoes a typical felid transformation into luteal tissue. Contrarily, the CL do not undergo regression after parturition or pseudopregnancy, as they do in other felids and most mammals. They continue the secretion of P4 and E2, which we could proof to be of luteal origin. Interestingly, each time E2 starts to increase (which indicates the onset of a new follicular phase), P4 increases simultaneously. This might be a negative feedback mechanism to inhibit a second oestrus within the same season and maintain the monoestrus status of the *Lynx*. All *Lynx* within a geographical region give birth approximately within the same week. This phenomenon seems to be of low plasticity, which might hinder the *Lynx* in adapting fast enough to anthropogenic or climatic changes.

Hence, it was important to discuss a variety of hormone protocols and if those were able to influence the *Lynx'* cycle. Artificial luteolysis resulted in similar reactions to a natural functional regression before oestrus onset and parturition. Naturally, PGFM elevations were detected before oestrus onset and parturition, followed by decreased luteal vascularisation and a drop in serum P4. The influence of artificial prostaglandins resulted in a significant reduction of the luteal vascularisation, as well as a drop in P4. Similarly to the natural cycle, a structural regression of the luteal tissue did not occur. Unfortunately, the hypothesised natural onset of oestrus was not achieved. The detailed molecular mechanism behind the female *Lynx* reproductive cycle remains unclear yet. The reason why an atypical monoestrous cycle evolved in *Lynx* was hypothesised to be due to nutritional or climate advantages for the birthing period, a phylogenetic constraint or an anti-infanticide strategy. The last seems to be the a more reasonable hypothesis, since an infanticide would not lead to a second oestrus onset in Lynx; however, it needs further studies to elucidate that point.

6 Zusammenfassung

Reproduktionsmanagement beim weiblichen Luchs (*Lynx lynx*)

Luchse besitzen einen einzigarten und für Katzen untypischen ovariellen Zyklus. Bevor die hier beschriebenen Studien durchgeführt wurden, waren die meisten grundlegenden Abläufe des Luchszyklus unbekannt, da die Daten vorhergehender Studien nur während einzelner, nicht wiederholter Untersuchungen und nicht während der gesamten Zyklusdauer gewonnen werden konnten. Vor allen Dingen für den Artenschutz war es wichtig zu verstehen, wie die Gelbkörperphysiologie bei Luchsen funktioniert und manipuliert werden kann. Dafür wurden Untersuchungen mittels hochauflösendem, transkutanem Abdominalultraschall und Hormonanalysen im Serum von Luchsen aus verschiedenen Breitengraden, aus der freien Wildbahn, aus menschlicher Obhut und an zwei verschiedenen Luchsspezies (Iberischer und Eurasischer Luchs) durchgeführt.

Wir konnten demonstrieren, dass die einzigartige Entstehung der persistierenden Gelbkörper (GK) beim Luchs in dieser Form physiologisch vorkommt. Die GK bleiben hormonell über einen Zeitraum von mindestens zwei Jahren (=mindestens zwei Zyklusperioden) aktiv. Für die physiologische Persistenz außerhalb der Paarungszeit haben wir den Terminus „prolonged di-oestrus“, oder „verlängerter Di-Östrus“ etabliert. Wir konnten zeigen, dass die GK der Luchse einer der längsten physiologischen Lebensdauerern eines GK unter den Säugetieren haben. Während der Studie konnten wir feststellen, dass die Ovulation bei Luchsen spontan oder durch die Paarungen induziert sein kann. Die Umwandlung des ovulatorischen Follikelgewebes zu Gelbkörpergewebe geschieht auf den ersten Blick wie bei anderen Katzenartigen. Nach der Geburt oder der Scheinträchtigkeit bildet sich der GK jedoch nicht zurück. Die GK sezernieren weiterhin P4 und E2, welche auch bestätigter Weise lutealen Ursprungs sind. Jedes Mal wenn E2 im Serum ansteigt (normalerweise der Hinweis für den Start einer neuen follikulären Phase), erhöht sich P4 ebenfalls. Es wird vermutet, dass dies ein negatives Feedback erzeugt, welche die Ausbildung eines zweiten Östrus in derselben Saison verhindert und den Luchs mono-östrisch erscheinen lässt. Alle Luchse einer Region gebären ihre Jungtiere überwiegend innerhalb einer Woche. Dieses Phänomen scheint von geringer Plastizität zu sein und könnte den Luchs daran hindern sich schnell genug an die anthropogenen und klimatischen Veränderungen zu adaptieren.

Deshalb war es wichtig verschiedene Luteolyse-Protokolle für den Luchs zu testen um zu sehen ob diese den Luchszyklus beeinflussen würden. Die künstliche Luteolyse zeigte ähnliche Resultate wie die natürliche funktionelle Regression vor dem Östrus- oder Geburtsbeginn. Natürlicherweise kann man PGFM - Erhöhungen vor Östrusbeginn oder der Geburtseinleitung feststellen. Diesen folgen typischerweise einer herabgesetzten GK-Durchblutung und einem Abfall des P4-Serumspiegels. Unter artifiziellen Bedingungen mit künstlichem Prostaglandin, konnten wir sowohl eine deutliche Erniedrigung der lutealen Durchblutung, als auch einem herabgesetzten P4-Serumspiegel feststellen. Ähnlich dem natürlichen Zyklus konnte eine strukturelle Regression des lutealen Gewebes nicht erzielt werden. Die genauen molekularen Mechanismen hinter dem Phänomen des weiblichen Luchszyklus bleiben jedoch ungeklärt. Der Grund, weshalb sich der Luchszyklus so katzenatypisch zu einem mono-östrischen Zyklus entwickelt hat, könnte von nahrungsbedingten oder klimatischen Vorteilen für die Geburtszeit sein, aufgrund von phylogenetischen Einschränkungen oder aufgrund einer Antiinfantizidstrategie sein. Letztere Hypothese erscheint eine plausible, da ein Infantizid keinen zweiten Zyklus im selben Jahr auslösen würde. Somit wären jegliche Vorteile des Infantizit für das männliche Tier hinfällig; es bedarf jedoch weiteren Studien um dies näher zu verstehen.

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9 Attachments

Attachements for manuscript 1:

Figure S1: Vaginal cytology in lynx. Vaginal cytology, stained with papanicolou at (A) pro-estrus, (B) estrus, (C) met-estrus, (D) pregnancy and (E) prolonged di-estrus stages. Black bar indicates 50µm.

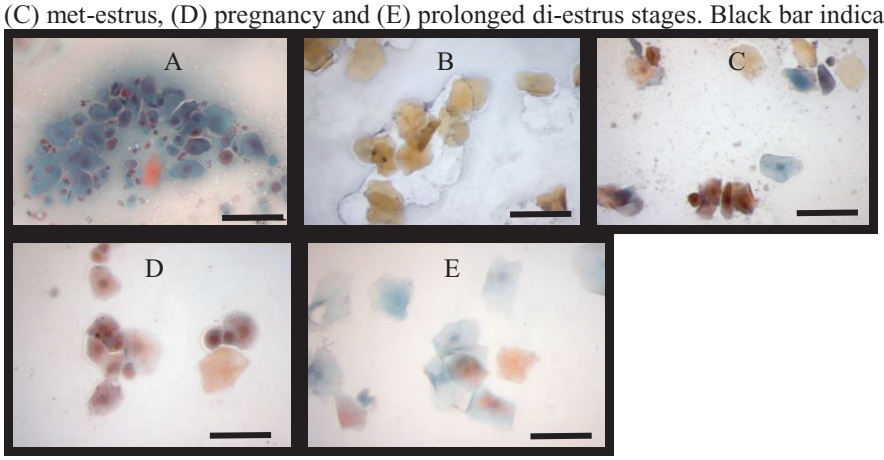

Table S1: Origins and reproductive status of study animals. The origins of the captive and free-ranging animals are listed below. Furthermore the reproductive period they have been examined is listed in the table.

ID	Origin	pro-estrus	estrus	met-estrus	pregnancy	lactation	prolonged di-estrus
1	DE/Stendal	2012			2011, 2011	2011	2010, 2010
2	DE/Thale						2010, 2010
3	DE/Johannismuehle						2010, 2010, 2011, 2012
4	DE/Stralsund	2012	2011				2010, 2010
5	DE/Bischofswerda	2012	2011				2010, 2010, 2011
6	DE/Bischofswerda	2012	2011				2010, 2010, 2011
7	DE/Essehof	2011			2012	2010	2010, 2011
8	DE/Magdeburg						2010, 2011
9	DE/Johannismuehle						2010, 2011, 2012
10	DE/Thale	2012		2012			
S1	NO/Scandlynx						2012
S2	NO/Scandlynx	2012					
S3	NO/Scandlynx						2011
S4	NO/Scandlynx	2011					
S5	NO/Scandlynx		2011				
S6	NO/Scandlynx	2012					
S7	NO/Scandlynx	2012					
S8	NO/Scandlynx			2011			
S9	NO/Scandlynx			2011			
S10	NO/Scandlynx						2011

Table S2: Data sharing

Open access download of the data sharing table from:
http://journals.plos.org/plosone/article?id=10.1371/journal.,pone.0090469#s5

ID	age	Monat	VL	intraVL	VR	intraVR
7C	6	1	0.21	0.05216667	0.18	0.112
4d	5	1	0.17	0.04	0.07	0.05
1f	11	2	0.22	0.10333333	0.22	0.076
10A	2	2	NA	NA	0.08	0.05
6c	11	3	0.31	0.11	0.26	0.112
6E	12	3	0.23	0.1	0.25	0.1075
5c	5	3	0.22	0.09	0.27	0.09666667
5E	6	3	0.24	0.108	0.24	0.06
7E	7	3	0.24	0.1225	0.3	0.124
1c	11	3	0.25	0.10166667	0.26	0.135
4c	4	3	0.14	0.05333333	NA	NA
10B	2	3	0.3	0.065	0.11	0.0525
1d	11	4	0.35	0.12166667	0.28	0.11666667
3e	9	5	0.24	0.09	0.24	0.102
9c	4	5	0.18	0.09	0.15	0.062
3A	7	6	0.23	0.08833333	0.26	0.12166667
1A	10	6	0.21	0.125	0.29	0.10428571
4A	3	6	0.17	0.05	0.14	0.05
2A	20	6	0.22	0.07	0.26	0.08
6A	10	7	0.29	0.116	0.21	0.098
5A	4	7	0.22	0.11333333	0.27	0.14
3d	11	7	0.19	0.08333333	0.13	0.08833333
9B	3	7	NA	NA	NA	NA
7A	5	8	0.28	0.1	0.21	0.074014
1e	11	8	0.26	0.11571429	0.26	0.10428571
6D	11	10	0.28	0.096	0.21	0.076
5D	6	10	0.15	0.07	0.19	0.0575
7D	6	10	0.18	NA	0.15	NA
8B	3	10	0.19	0.0975	0.21	0.086
7B	5	11	0.2	0.09333333	0.22	0.09833333
1B	10	11	0.24	0.11166667	0.27	0.09833333
6B	11	12	0.23	0.096	0.17	0.08333333
5B	4	12	0.15	0.06	0.2	0.076
3B	7	12	0.13	0.064	0.14	0.062
9A	2	12	0.19	0.06833333	0.12	0
8A	2	12	NA	NA	NA	NA
4b	3	12	0.24	0	0.18	0
2B	20	12	0.12	0	0.14	0
1	3	2	0.2	0.102	0.22	0.11
2	NA	2	0.19	0.10666667	0.18	0.12
S1w	5	2	NA	NA	NA	NA
S2w	5	2	NA	NA	NA	NA
S3w	5	2	NA	NA	NA	NA
3	NA	2	0.23	0.115	0.19	0.1075
4	NA	2	0.26	0.106	0.2	0.1
S4w	5	3	NA	NA	NA	NA
S5w	12	3	NA	NA	NA	NA
S6w	4	3	NA	NA	NA	NA

E2ng/ml	P4ng/ml	PGFMng/ml	volumeL	nfl	f1la	f2la
0.12	6.51	2.26	3.1010614	0	0	0
0.37	2.02	1.39	0.08946816	0	0	0
1.16	10.03	2.12	1.14454504	0	0	0
1.51	1.58	0.91	0.11400211	1	0.19	0
0.28	1.85	2.63	4.0698783	2	0.95	0.72
0.55	2.32	2.57	6.07595407	0	0	0
0.17	7.34	1.77	2.529202	2	0.94	0.62
0.70	2.12	0.87	0.14476459	0	0	0
0.70	180.40	1.97	1.66046445	0	0	0
0.54	27.60	1.88	1.80973408	0	0	0
0.09	0.39	2.73	2.04448959	0	0	0
1.39	18.80	1.15	0.48366782	0	0	0
0.43	44.15	3.97	5.9711152	1	NA	0
0.39	4.20	2.3	1.50295756	2	0.3	0.28
1.11	3.68	2.01	1.04362451	1	0.27	0
0.27	3.28	1.16	1.61457442	0	0	0
0.38	6.06	NA	2.13226177	0	0	0
0.09	0.00	1.24	0.41430146	2	0.01	0.11
0.36	1.99	2.05	1.93622638	0	0	0
0.19	8.41	1.41	2.95890904	0	0	0
0.20	4.11	2.05	2.51660814	1	0.16	0
0.41	2.51	1.17	2.16260955	0	0	0
0.25	3.10	1.69	1.94344576	0	0	0
0.18	3.14	4.41	3.13279619	0	0	0
0.44	170.38	1.48	2.15317535	0	0	0
0.25	6.05	0.71	2.29029566	1	0.5	0
0.25	3.48	0.91	1.52791359	1	0.62	0
0.26	5.02	2.26	2.92003183	0	0	0
0.69	12.56	NA	1.54377863	0	0	0
0.18	9.06	1.90	3.53016997	0	0	0
0.35	5.46	2.87	2.59817802	0	0	0
0.07	13.66	0.95	2.30585047	1	0.35	0
0.29	6.24	2.16	0.8322668	0	0	0
0.24	3.10	1.02	1.28963635	0	0	0
0.16	0.00	1.43	0.59304159	0	0	0
0.35	8.36	4.73	NA	0	0	0
0.12	0.35	3.69	0.64284526	0	0	0
0.39	2.21	1.05	0.16681857	0	0	0
0.80	2.3	3.95	1.59985606	0	0	0
0.08	0.37	2.63	0.73944295	0	0	0
0.85	3.97	2.13	3.87235852	5	NA	NA
0.48	0.86	2.69	2.96138304	1	NA	0
1.46	2.57	1.55	2.00393399	0	0	0
0.45	1.59	1.21	1.71469384	0	0	0
0.87	2.31	3.53	2.72187588	0	0	0
0.49	3.25	1.77	2.94336758	0	0	0
NA	NA		2.62996544	1	0.54	0
NA	NA		2.82248538	1	NA	0

f3la	f1lb	f2lb	f3lb	nclL	cl1la	cl2la
0	0	0	0	4	0.65	0.68
0	0	0	0	3	0.7	0.51
0	0	0	0	3	0.62	0.52
0	0.14	0	0	0	0	0
0	0.61	0.27	0	4	0.82	0.92
0	0	0	0	5	1.27	1.15
0	0.78	0.34	0	1	0.81	0
0	0	0	0	1	0.65	0
0	0	0	0	6	0.44	0.71
0	0	0	0	4	0.83	0.64
0	0	0	0	3	0.48	0.42
0	0	0	0	1	0.96	0
0	NA	0	0	3	0.74	1.17
0	0	0	0	3	0.43	0.6
0	0.12	0	0	2	0.7	0.54
0	0	0	0	2	0.64	0.74
0	0	0	0	2	0.69	0.81
0	0.01	0.11	0	1	0.59	0
0	0	0	0	0	0	0
0	0	0	0	3	0.7	0.6
0	0.16	0	0	2	0.74	0.68
0	0	0	0	4	0.41	0.35
0	0	0	0	2	1.09	1.28
0	0	0	0	3	1.16	0.85
0	0	0	0	3	0.81	0
0	0.32	0	0	4	0.64	0.51
0	0.3	0	0	4	0.76	0.76
0	0	0	0	4	0.72	0.87
0	0	0	0	3	0.73	0.56
0	0	0	0	4	0.73	0.63
0	0	0	0	3	0.66	0.64
0	0.21	0	0	4	0.41	0.42
0	0	0	0	2	0.42	0.63
0	0	0	0	2	0.53	0.47
0	0	0	0	NA	0	0
0	0	0	0	NA	0	0
0	0	0	0	1	0.44	0
0	0	0	0	NA	0	0
0	0	0	0	1	0.86	0
0	0	0	0	5	0.63	0.27
NA	NA	NA	NA	4	0.3	0.54
0	NA	0	0	4	0.65	0.65
0	0	0	0	4	0.69	0.46
0	0	0	0	3	0.78	0.48
0	0	0	0	5	0.8	0.48
0	0	0	0	6	0.54	0.37
0	0.75	0	0	5	0.88	0.67
0	NA	0	0	4	0.79	0.82

cl3la	cl4la	cl5la	cl6la	cl1lb	cl2lb	cl3lb
0.72	0.65	0	0	0.64	0.5	0.43
0.64	0	0	0	0.39	0.46	0.55
0.56	0	0	0	0.33	0.34	0.37
0	0	0	0	0	0	0
0.59	0.52	0	0	0.64	0.49	0.67
0.45	0.51	0.58	0	1.21	0.85	0.38
0	0	0	0	0.5	0	0
0	0	0	0	0.37	0	0
0.8	0.5	0.53	0.71	0.51	0.56	0.72
0.64	0.55	0	0	0.41	0.41	0.51
0.61	0	0	0	0.41	0.51	0.58
0	0	0	0	0.79	0	0
0.48	0	0	0	0.8	0.92	0.55
0.47				0.5	0.27	0.38
				0.51	0.46	
0	0	0	0	1	0.41	0
0.58	0.61	0.56	0	0.36	0.57	0.43
0	0	0	0	0.44	0	0
0	0	0	0	0	0	0
0.57	0	0	0	0.7	0.6	0.57
0	0	0	0	0.79	0.77	0
0.62	0.77	0	0	0.32	0.38	0.49
0	0	0	0	0.62	0.53	0
0.86	0	0	0	0.951	0.49	0.69
0	0	0	0	1.23	0	0
0.6	0.46	0	0	0.44	0.47	0.62
0.55	0.49	0	0	0.67	0.63	0.74
0.6	0.61	0	0	0.67	0.83	0.58
0.66	0	0	0	0.46	0.54	0.66
0.53	0.77	0	0	0.67	0.59	0.71
0.5	0	0	0	0.66	0.39	0.47
0.56	0.63	0.58	0	0.52	0.58	0.54
0	0	0	0	0.43	0.53	0
0	0	0	0	0.6	0.35	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0.53	0	0
0	0	0	0	0	0	0
0	0	0.00	0.00	0.65	0.00	0.00
0.41	0.39	0.65	0.00	0.41	0.25	0.39
1.07	0.65	0.00	0.00	0.39	0.54	0.70
0.55	0.64	0.00	0.00	0.65	0.82	0.48
0.47	0.76	0.00	0.00	0.64	0.42	0.45
0.67	0	0.00	0.00	0.75	0.59	0.69
0.66	0.79	0.55	0.00	0.66	0.81	0.75
0.36	0.36	0.88	1.20	0.54	0.40	0.41
0.77	0.84	0.67	0.00	0.57	0.33	0.49
0.97	0.68	0.00	0.00	0.39	0.56	0.62

cl4lb	cl5lb	cl6lb	volareaL	volumeR	nfr	f1ra
0.32	0	0	1.0002831	0.72057297	0	0
0	0	0	0.67512826	1.80164684	0	0
0	0	0	0.46228536	2.95601092	2	0.33
0	0	0	0	0.36522742	4	0.37
0.56	0	0	1.30541029	3.5023653	2	0.85
0.43	0.35	0	2.44062479	2.78950887	4	0.8
0	0	0	0.31808626	2.63120951	3	0.43
0	0	0	0.18888826	1.18531663	0	0
0.26	0.35	0.34	1.37829524	6.29123564	0	0
0.34	0	0	0.87658289	1.57699233	0	0
0	0	0	0.60067252	0.62404047	0	0
0	0	0	0.59564597	0.32656856	0	0
0	0	0	1.51770341	5.4679483	3	NA
			0.43636722	0.85809776	2	0.27
			0.47548005	1.29974364	1	0.34
0	0	0	0.74094463	1.84853197	1	0.52
0.63	0.53	0	1.28852423	2.2455476	0	0
0	0	0	0.20388936	0.27597242	0	0
0	0	0	0	3.57052529	3	0.21
0	0	0	0.9227643	4.05836594	1	0.12
0	0	0	0.87037824	1.79063241	0	0
0.66	0	0	0.8452455	1.82551823	0	0
0	0	0	1.06358619	0.13410831	0	0
0	0	0	1.65959344	1.08212944	0	0
0	0	0	0.78249219	3.05061213	0	0
0.48	0	0	0.87501209	3.46046038	3	0.54
0.41	0	0	1.25341693	2.9788935	0	0
0.67	0	0	1.54032288	1.79348498	0	0
0	0	0	0.84336055	1.1545353	0	0
0.77	0	0	1.43727864	2.26521083	0	0
0	0	0	0.72272339	1.37320978	0	0
0.61	0.51		1.13042358	3.34668917	0	0
0	0	0	0.40408736	0.92245014	0	0
0	0	0	0.37895461	0.65463565	0	0
0	0	0	0	0.13995795	0	0
0	0	0	0	NA	0	0
0	0	0	0.18315485	1.33002231	0	0
0	0	0	0	0.34636059	0	0
0.00	0.00			1.43	0.00	0.00
0.38	0.36			2.38	3.00	0.67
0.39	0.00	0.00		0.45	0.00	0.00
0.55	0.00	0.00		2.24	0.00	0.00
0.71	0.00	0.00		1.97	1.00	NA
0.00	0.00	0.00		1.35	0.00	0.00
0.43	0.81	0.00		0.68	10.00	NA
0.37	0.62	0.81		2.70	0.00	0.00
0.54	0.46	0.00		2.23	4.00	0.42
0.71	0.00	0.00		2.81	0.00	0.00

f2ra	f3ra	f4ra	f1rb	f2rb	f3rb	f4rb
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0.31	0	0	0.16	0.15	0	0
0.36	0.15	0.37	0.22	0.24	0.14	0.25
0.28	0	0	0.63	0.36	0	0
0.54	0.47	0.46	0.61	0.24	0.21	0.25
0.62	0	0	0.65	0.32	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
NA	NA	0	NA	NA	NA	0
0.42	0	0	0.1	0.12	0	0
0	0	0	0.22	0	0	0
0	0	0	0.59	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0.17	0.12	0	0.21	0.17	0.12	0
0	0	0	0.2		0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0.16	0.1	0	0.29	0.16	0.1	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0.00	0.00	0	0	0	0	0
	0.00	0	0.32		0	0
0.00	0.00	0	0	0	0	0
0.00	0.00	0	0	0	0	0
0.00	0.00	0	NA	0	0	0
0.00	0.00	0	0	0	0	0
NA	NA	NA	NA	NA	NA	NA
0.00	0.00	0	0	0	0	0
0.54	0.00	0	0.78	0.45	0	0
0.00	0.00	0	0	0	0	0

nclr	cl1ra	cl2ra	cl3rb	cl4ra	cl5ra	cl6ra
2	0.65	0.55	0	0	0	0
3	0.4	0.62	0.44	0	0	0
4	0.88	0.72	0.74	0.47	0	0
0	0	0	0	0	0	0
2	0.51	0.67	0	0	0	0
4	0.71	0.83	0.8	0.85	0	0
1	0.57	0	0	0	0	0
1	0.47	0	0	0	0	0
5	0.7	0.6	1.37	0.77	0.94	
3	1	0.7	0.94	0	0	0
NA				0	0	0
0	0	0	0	0	0	
4	0.69	1.16	0.56	0.55	0	0
2	0.73	0.72	0	0	0	0
3	0.64	0.83	0.65	0	0	0
1	0.68	0	0	0	0	0
3	0.55	0.42	0.58	0	0	0
0	0	0	0	0	0	0
1	0.67	0	0	0	0	0
3	1.05	0.65	0.59	0	0	0
3	0.5	0.58	0.78	0	0	0
3	0.57	0.46	0.64	0	0	0
0	0	0	0	0	0	0
3	0.87	1.06	0.95	0.98	0	0
2	0.77	0.98	0	0	0	0
5	0.75	0.57	0.76	0.79	0.59	0
3	0.87	0.56	0.56	0	0	0
5	0.68	0.56	0.61	0.74	0.69	0
1	0.55	0	0	0	0	0
2	0.75	0.63	0	0	0	0
3	0.82	0.62	0.64	0	0	0
6	0.43	0.25	0.38	0.54	0.59	0.54
3	0.48	0.41	0.5	0	0	0
2	0.64	0.37	0	0	0	0
NA	0	0	0	0	0	0
NA	0	0	0	0	0	0
0				0	0	0
NA	0	0	0	0	0	0
2	0.74	0.94	0	0	0	0
7	0.37	0.56	0.56	0.82	0.8	0.4
4	0.43	0.33	0.37	0.36	0	0
1	0.59	0	0	0	0	0
2	0.96	0.93	0	0	0	0
3	0.75	0.57	0.81	0	0	0
2	0.64	0.97	0	0	0	0
NA	0	0	0	0	0	0
4	0.7	0.82	0.71	0.7	0	0
6	0.75	0.94	0.65	0.92	0.69	0.91

cl6ra	cl1rb	cl2rb	cl3b	cl4rb	cl5rb	cl6rb
0	0.71	0.67	0	0	0	0
0	0.5	0.42	0.51	0	0	0
0	0.53	0.3	0.44	0.26	0	0
0	0	0	0	0	0	0
0	0.61	0.58	0	0	0	0
0	0.59	0.61	0.47	0.53	0	0
0	0.42	0	0	0	0	0
0	0.82	0	0	0	0	0
	0.62	0.41	0.89	1.37	0.64	
0	0.69	0.56	0.44	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0.6	0.83	0.51	0.57	0	0
0	0.42	0.65	0	0	0	0
0	0.42	0.58	0.57	0	0	0
0	0.59	0	0	0	0	0
0	0.4	0.45	0.51	0	0	0
0	0	0	0	0	0	0
0	0.52	0	0	0	0	0
0	0.57	0.56	0.68	0	0	0
0	0.5	0.52	0.7	0	0	0
0	0.8	0.65	0.63	0	0	0
0	0	0	0	0	0	0
0	0.99	0.89	1.27	0.74	0	0
0	1.06	0.82	0	0	0	0
0	0.32	0.42	0.52	0.47	0.5	0
0	0.73	0.68	0.57	0	0	0
0	0.6	0.53	0.8	0.58	0.6	0
0	0.55	0	0	0	0	0
0	0.6	0.42	0	0	0	0
0	0.62	0.82	0	0	0	0
0	0.41	0.42	0.47	0.61	0.4	0.49
0	0.54	0.51	0.5	0	0	0
0	0.54	0.41	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0	0	0	0	0	0
0	0.72	0.72	0	0	0	0
0.64	0.6	0.76	0.82	0.55	0.7	0.38
0	0.41	0.34	0.42	0.33	0	0
0	0.48	0	0	0	0	0
0	0.75	0.56	0	0	0	0
0	0.56	0.79	0.63	0	0	0
0	0.31	0.72	0	0	0	0
0	0	0	0	0	0	0
0	0.57	0.38	0.52	0.54	0	0
0	0.79	0.98	0.59	0.74	0.63	0.72

cl7rb	volareaR
0	0.65188048
0	0.53784066
0	0.887657
0	0
0	0.54954309
0	1.37578196
0	0.18802432
0	0.30269245
	2.79271879
0	1.17464149
0	0
0	0
0	1.55186823
0	0.60836942
0	0.88019572
0	0.31510174
0	0.55354863
0	0
0	0.27363272
0	1.07104748
0	0.86205302
0	0.90964815
0	0
0	2.9345617
0	1.27218795
0	1.21022003
0	1.04858509
0	1.59907066
0	0.23758294
0	0.56124553
0	0.79859285
0	1.01308509
0	0.5641515
0	0.39057851
0	0
0	0
0	0
0	0
0	
0.46	
0	
0	
0	
0	
0	
0	
0	
0	

Table S3: Comparison between free-ranging and captive lynx. Comparison of various ovarian and serum parameters between single examinations of free-ranging (N = 10) and captive lynx (N = 10) during February and March 2011 and 2012.

	P4	E2	PGFM	diameter *a.ovarica*	ovarian volume	number CL	CL tissue	age in years
W - value	30			105	172		129	112.5
t		0.11	-1.42			-2.17		
p-value	0.86	0.91	0.18	0.07	0.46	0.04	0.06	0.05

Table S4: Repeated measurements from six captive Eurasian lynx throughout one reproductive year (February – February). Measurements were categorized in 3 time periods, excluding estrus (pro-estrus, pseudopregnancy, prolonged di-estrus) and tested if there are seasonal changes within an individual.,

Quade test	P4	E2	PGFM	diameter A.ovarica	ovarian volume	number CL	CL tissue	amount follicles
F-value	0.07	2.26	0.02	0.88	0.21	0.61	0.83	0.08
df	22	22	22	22	22	22	22	22
p-value	0.93	0.13	0.98	0.43	0.81	0.55	0.45	0.92

10 Errata

Ad manuscript 1
The term ‘folliculogenesis’ was wrongly used. The correct term should be ‘follicular phase’.
Furthermore, in the materials and methods section “(e) serum hormone analysis”: all hormones were measured in serum and not in plasma, as written.
In the results section, under (a) reproductive stages in lynx, the sentence “One captive female was a juvenile (19 month); accordingly we observed small ovaries (0.54 cm^3 and 0.10 cm^3) without follicular or luteal activity (figure 1A).” might be misleading. Only antral follicles are visible in ultrasonography. Since no histology was performed on these ovaries, early follicular activity can not be ruled out.
In the discussion part, the bobcat is described to be polyoestrus, as mentioned by *Crowe* (1975) and *Sty* and *Leopold* (1993). In fact, precise studies are missing to clarify if the bobcat is truly polyoestrus or monoestrus.

Ad manuscript 2
Within the introduction, the bobcat is again mentioned to be polyoestrus. As explained above, this needs further studies to clarify the correct categorization of the bobcat.
In the materials and methods part, it was not mentioned how the organs were stored exactly. Within the –20°C freezer, each organ was stored separately in plastic bags without fluids. The storage was under dry, dark and airtight conditions. The longer stored organs did not differ in weight or showed more signs of weight loss due to sublimitation.

11 List of Publications

Publications (peer-reviewed, international)

1) Goettling J, Goeritz F, Jewgenow K, **Painer J** (2016): Serum chemistry and haematology for the Eurasian lynx (*Lynx lynx*). *Europ J Wildl Res*, p. 1-3.

2) Zschockelt L, Amelkina O, Koster S, **Painer J**, Okuyama M, Serra R, Vargas A, Jewgenow K, Braun BC (2015): Comparative analysis of intraluteal steroidogenic enzymes emphasizes the functionality of fresh and persistent corpora lutea during pro- and metoestrus in the lynx. *J Steroid Biochem Molec Biol*. 154, 75-84.

3) Amelkina O, Zschockelt L, **Painer J**, Serra R, Braun BC, Jewgenow K. (2015): Apoptosis related factors in the feline luteal phase and their involvement in the persistent Corpora lutea of lynxes. *Biol Reprod*. (in submission)

4) **Painer J**, Jewgenow K, Dehnhard M, Arnemo JM , Linnell JDC, Odden J, Hildebrandt TB, Goeritz F (2014) Physiologically Persistent Corpora lutea in Eurasian Lynx (*Lynx lynx*) – Longitudinal Ultrasound and Endocrine Examinations Intra-Vitam. *Plos One*. 10.1371/journal.,pone.0090469

5) **Painer J,** Jewgenow K, Dehnhard M, Hildebrandt TB, Naidenko SV, Sánchez I, Quevedo MA, Göritz F (2014). Hormone-Induced luteolysis on physiologically persisting corpora lutea in Eurasian and Iberian lynx. *Theriogenology*. 82, 4, p. 557 – 562.

6) Jewgenow K, **PainerJ**, Amelkina O, Dehnhard M, Goeritz F (2014). Lynx reproduction – Long lasting life cycle of corpora lutea in a feline species. *Reprod Biol.* 14, 2, p. 83-88.

7) Müller K, Koster S, **Painer J**, Söderberg A, Gavier-Widén D, Brunner E, Dehnhard M, Jewgenow K (2014). Testosterone production and spermatogenesis in free-ranging Eurasian lynx (*Lynx lynx*) throughout the year. *Europ J Wildl Res*. 60, 4,p. 569-577.

8) Jewgenow K, Amelkina O, **Painer J**, Goeritz F, Dehnhard M (2012). Life Cycle of Feline Corpora lutea: Histological and Intraluteal Hormone Analysis. *Repro. Dom Anim.* 47, Suppl. 6, p. 156 – 160.

9) Goeritz F, **Painer J**, Jewgenow K, Hermes R, Rasmussen K, Dehnhard M, Hildebrandt TB (2012). Embryo Retrieval after Hormonal Treatment to Control Ovarian Function and Non-surgical Artificial Insemination in African Lions (*Panthera leo*). *Reprod Dom Anim.* 47, Suppl. 6, p. 25 – 29.

10) Carnaby K, **Painer J**, Söderberg A, Gavier-Widén D, Goeritz F, Dehnhard M, Jewgenow K (2012). Histological and endocrine characterization of the annual luteal activity in Eurasian lynx (*Lynx lynx*). *Reprod***.** 144. p. 477 - 484.

11) **Painer J**, Zedrosser A, Arnemo JM, Fahlman Å, Brunberg, Segerström P, Swenson JE (2012). Effects of different doses of medetomidine and tiletamine–zolazepam on the duration of induction and immobilization in free-ranging yearling brown bears (*Ursus arctos*). *Can. J. Zoo*. 90/6, p. 753-757.

12) **Painer J**, Kaczensky P, Ganbaatar O, Huber N, Walzer C (2010). Comparative parasitological examination on sympatric equids in the Greater Gobi B, SPA, Mongolia. *European Wildl. Res*. 57/2, p 225¯232.

Publications on international conferences

1) **Painer J**, Jewgenow K, Göritz F (02/2016). Reproduction management and biology of zoo felids. *Nordic Wildl Zoo Vet Conf*. Kristiansand, Norway (talk).

2) **Painer J,** Jewgenow K, Göritz F (12/2015). Adrenal measurements in ultrasonography to evaluate chronic stress exposure of illegally kept European brown bears (Ursus arctos), *Intern Workshop rehab re-introd large carniv*, Moscow, Russia (talk)

3) **Painer J**, Jewgenow K, Göritz F (10/2015). Two-day workshop: „Wildlife Medicine in Carnivores". a) Reproduction management in Felids, b) Wildlife anaesthesia, c) Case reports on bears and tigers, d) Wildlife crime, e) diet and husbandry of brown bears, f) hand-rearing of bear cubs in captivity, g) hibernation in bears. *Vet Univ Warsaw*, Poland (invited keynote speech).

4) **Painer J,** Jewgenow K, Göritz F (09/2015). Morphometric, ultrasonographical measurements on adrenal glands as potential in-vivo indicator for chronic stress, *Proc 10th Inter Conf Beh Physiol Gen Wildl*, Berlin, Germany. (poster)

5) **Painer J**, Jewgenow K, Hildebrandt TB, Göritz F (03/2015). The Iberian lynx conservation breeding program and lynx reproduction physiology, *Annual EAZWV Hungarian sect conf*, Budapest, Hungary. (invited talk)

6) **Painer J, Goeritz F**, Hermes R, Fritsch G, Hildebrandt TB (03/2015). Bear reproduction and medical care – lessons from the field. *Carnivore workshop*. Budapest, Hungary (invited talk)

7) **Painer J, Goeritz F**, Hermes R, Jewgenow K, Hildebrandt TB (03/2015). Large felid reproduction: When things go wrong (infertility, assisted reproduction, termination of pregnancy). *Annual EAZWV Hungarian sect conf*, Budapest, Hungary (invited talk)

8) **Painer J**, Fritsch G, Hertwig C, Göritz (10/2014). Anesthesia protocol for captive brown bears (*Ursus arctos*). *Proc 23rd Intern Conf Bear Res Managem*. Thessaloniki, Greece. (poster)

9) **Painer J**, Fritsch G, Hertwig C, Göritz F (05/2014). Anaesthesia protocoll for captive brown bears (*Ursus arctos*) – quick induction, reliable maintenance and rapid recovery. Proc Intern Conf Dis Zoo Wildl Anim. *EAZWV-IZW-Zoovet Conf*. 2014, Warschau, Poland. (poster)

10) **Painer J**, Jewgenow K, Dehnhard M, Arnemo JM, Linnell JDC, Odden J, Hildebrandt TB, Göritz F (05/2014). A unique felid reproductive cycle. The longest Corpus luteum lifespan amongst mammals leads to a mono-oestrous reproduction in lynx (*Lynx lynx*). *EAZWV-IZW-Zoovet Conf*. 2014, Warschau, Poland. (poster)

11) **Painer J**, Göritz F (05/2014). Opthalmologic examination, fundoscopy, tonometry and 3D ophthalmologic ultrasound on captive brown bears. *Annual Berlin Symposium for Opthalmologists*. Berlin, Germany (invited talk)

12) **Painer J**, Jewgenow K, Goeritz (09/2013). A new approach for the evaluation of stress response - 3D adrenal ultrasound. *First Meeting on the spectacled bear alopecia syndrome*. Leipzig, Germany. (talk)

13) **Painer J**, Jewgenow K, Goncalves N, Serra R, Canales R, Perez M, Mendoza JL, Hildebrandt TB, Goeritz F (09/2013). 3D Adrenal ultrasound – a new approach for the evaluation of stress response in in the critically endangered Iberian Lynx ex-situ population. *Proc 9th Intern Conf Beh Physiol Gen Wildl*., Berlin, Germany. (poster)

14) **Painer J**, Jewgenow K, Goncalves N, Serra R, Canales R, Perez M, Mendoza JL, Hildebrandt TB, Goeritz F (05/2013). 3D Adrenal ultrasound – a new approach for the evaluation of stress in in the critically endangered Iberian Lynx ex-situ population. Proc Intern Conf Dis Zoo Wildl Anim. *EAZWV-IZW-Zoovet Conf*. 2013, Vienna, Austria, p 166. (poster)

15) **Painer J**, Jewgenow, Dehnhard M, Hildebrandt TB, Goeritz F (02/2013). Advanced 3D ultrasonography in lynx. Seasonal changes in ovarian morphology. *Proc. Ludwig Fraenkel Symposium on endocrine control of corpus luteum function*. Wroclaw, Poland. (poster)

16) **Painer J**, Jewgenow K, Hildebrandt TB, Dehnhard M, Goeritz F (11/2012). Pinseln am Ohr, kurzer Schwanz und die Hormone spielen verrückt – der Luchs und sein einzigartiger Reproduktionszyklus. *Proc 32. Arbeitstagung der Zootierärzte im deutschsprachigen Raum*, Landau, Germany, 77 – 79. (talk)

17) **Painer J**, Andrèn H, Arnemo JM, Dehnhard M, Göritz F, Linnell JDC, Odden J, Jewgenow K (10/2012). Reproductive hormones in serum samples from free ranging Eurasian lynx (*Lynx lynx*) and their value for reproductive assessments. Vet. Med. Austria (WTM), *supplement Proc. of ISWE meeting*, Vienna, Austria, 43-44. (poster)

18) **Painer J**, Jewgenow K, Dehnhard M, Hildebrandt TB, Naidenko SV, Sánchez I, Quevedo MA, Göritz F (08/2012). Hormone-Induced luteolysis on physiologically persisting corpora lutea in Eurasian and Iberian lynx – a prerequisite for artificial insemination. *Proc ISCFR 2012*, Whistler, Canada, 193. (talk)

19) **Painer J**, Hildebrandt TB, Jewgenow K, Dehnhard M, Goeritz F (05/2012). Ovarian topography and dynamics evaluated by three dimensional ultrasonography and Doppler colour flow investigations. Proc Intern Conf Dis Zoo Wildl Anim. *EAZWV-IZW-Zoovet Conf*. 2012, Bussolgengo, Italy, 63 – 65. (talk)

20) **Painer J**, Jewgenow K, Hildebrandt TB, Goeritz F (09/2011). Annual characterization of a feline ovarian cycle in the Eurasian lynx (*Lynx lynx*). Proc 8th intern Conf Beh Physiol Gen Wildl., Berlin, Germany, 130 – 131. (talk)

21) **Painer J**, Goeritz F, Jewgenow K (09/2011). Unique annual cycle of ovarian activity in Eurasian lynx – the statistical approach (2011). *Proc 2nd PhD Student Symposium*, Berlin, Germany, 26. (talk)

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13 Selbständigkeitserklärung/Declaration of originality

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

Declaration of originality:

I herewith confirm that the present work was authonomously prepared. I assure that I only used cited sources or acknowledged help.

Berlin, Johanna Painer