Synthesis and reception of prostaglandins in *corpora lutea* of domestic cat and lynx

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Abstract

Felids show different reproductive strategies related to the luteal phase. Domestic cats exhibit a seasonal polyoestrus and ovulation is followed by formation of *corpora lutea* (CL). Pregnant and non-pregnant cycles are reflected by diverging plasma progesterone (P4) profiles. Eurasian and Iberian lynxes show a seasonal monoestrus, in which physiologically persistent CL (perCL) support constantly elevated plasma P4 levels. Prostaglandins (PGs) represent key regulators of reproduction, and we aimed to characterise PG synthesis in feline CL to identify their contribution to the luteal lifespan. We assessed mRNA and protein expression of PG synthases (*PTGS2/COX2*, *PTGES*, *PGFS/AKR1C3*) and PG receptors (*PTGER2*, *PTGER4*, *PTGFR*), and intra-luteal levels of PGE₂ and PGF_{2α}. Therefore, CL of pregnant (pre-implantation, post-implantation, regression stages) and non-pregnant (formation, development/maintenance, early regression, late regression stages) domestic cats, and prooestrous Eurasian (perCL, pre-mating) and metoestrous Iberian (perCL, freshCL, post-mating) lynxes were investigated. Expression of *PTGS2/COX2*, *PTGES* and *PTGER4* was independent of the luteal stage in the investigated species. High levels of luteotrophic PGE₂ in perCL might be associated with persistence of luteal function in lynxes. Signals for *PGFS/AKR1C3* expression were weak in mid and late luteal stages of cats but were absent in lynxes, concomitant with low PGF_{2α} levels in these species. Thus, regulation of CL regression by luteal PGF_{2α} seems negligible. In contrast, expression of *PTGFR* was evident in nearly all investigated CL of cat and lynxes, implying that luteal regression, e.g. at the end of pregnancy, is triggered by extra-luteal PGF_{2α}.

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Introduction

Reproductive biology of domestic cats (*Felis silvestris f. catus*) exhibits a characteristic feature: during seasonal polyoestrus, an induced or spontaneous ovulation can be followed by either a pregnant or a shortened non-pregnant luteal phase (Wildt *et al.* 1981, Lawler *et al.* 1993). Consequently, the formation of *corpora lutea* (CL) and the functional luteal phases is reflected by different plasma progesterone (P4) profiles after days 10–12 *post coitum* (p.c.) (Paape *et al.* 1975, Verhage *et al.* 1976), coinciding with the time of blastocyst implantation (Leiser 1979). Significant differences between both profiles become visible around days 25–30 p.c., when pregnant cats have higher P4 levels (Verhage *et al.* 1976). During the non-pregnant luteal phase, plasma P4 already becomes basal at days 25–40 p.c. (Paape *et al.* 1975, Verhage *et al.* 1976). The ongoing loss of steroidogenic capacity (Siemieniuch *et al.* 2012, Zschockelt *et al.* 2014) and the concomitant structural luteal regression

(Dawson 1946, Amelkina *et al.* 2015a) proceed similarly throughout the sequential luteal stages of pregnant and non-pregnant luteal phases. However, it is assumed that the respective *corpora lutea* (CL) enter the process of luteal regression at different time points, as CL of pregnant cats show early histomorphological signs of regression from days 38–39 onwards (Amelkina *et al.* 2015a), whereas plasma P4 levels indicate that CL of non-pregnant cats begin to regress on day 21 after their formation (Paape *et al.* 1975).

Compared with other felid species, the reproduction of lynxes is rather unique (Göritz *et al.* 2009, Jewgenow *et al.* 2014). In contrast to the polyoestrous bobcat (*Lynx rufus* (Parker & Smith 1983)), the Canada (*L. canadensis* (Fanson *et al.* 2010)), Eurasian (*L. lynx* (Kvam 1991)) and Iberian lynx (*L. pardinus* (Palomares *et al.* 2005)) are strictly seasonal monoestrous. The observation that central and northern European populations of both captive and free-ranging Eurasian lynxes show the same monoestrous strategy suggests a rather conserved reproductive adaptation without plasticity regarding

this phenomenon (Painer *et al.* 2014b). Lynxes develop physiologically persistent CL (perCL) (Göritz *et al.* 2009, Fanson *et al.* 2010) present for at least two subsequent years (Painer *et al.* 2014b). Thus, on the same ovary, CL formed freshly during the breeding season (freshCL) with high steroidogenic capacity coexist with previous years' perCL (Amelkina *et al.* 2015b) of present but lower steroidogenic capacity (Carnaby *et al.* 2012, Zschockelt *et al.* 2015). However, functionality of these perCL is sufficient to support constantly elevated P4 plasma levels throughout the year, which is hypothesised to ensure a monoestrous cyclicity by preventing folliculogenesis and ovulation outside the breeding season (Göritz *et al.* 2009, Painer *et al.* 2014b). Despite constant luteal activity of structurally preserved CL, lynxes enter oestrus in springtime and increasing evidence points to a temporarily reduced function of perCL before breeding and parturition (Painer *et al.* 2014b, Zschockelt *et al.* 2015). Because there are no confounding differences regarding reproduction, the closely related Eurasian lynx is commonly used as a model species to investigate the reproductive physiology of the Iberian lynx (Göritz *et al.* 2009, Painer *et al.* 2014b, Amelkina *et al.* 2015b, Zschockelt *et al.* 2015). The latter was declared critically endangered in 2002. Due to integrated conservation efforts, the species was downgraded to endangered by the International Union for Conservation of Nature in 2015. Compilation of reproductive research on domestic cats, Eurasian lynxes and, if possible, on Iberian lynxes supports the development of assisted reproduction techniques for endangered felid species.

The autocrine/paracrine role of luteal prostaglandins (PGs) in CL function is evident in many mammalian species (Olofsson & Leung 1994, Wiltbank & Ottobre 2003). The PGs are metabolites of arachidonic acid (AA) liberated from phospholipid cell membranes by cytosolic phospholipase A₂ (cPLA₂) (Smith & Dewitt 1996). The conversion of free AA into prostaglandin G₂/H₂ (PGG₂/PGH₂) is catalysed by two isoforms of prostaglandin-endoperoxide synthase/cyclooxygenase (PTGS/COX) which differ in the regulation of their expression and tissue distribution (Wiltbank & Ottobre 2003). The common precursor PGH₂ is selectively converted to active PGs by specific terminal synthases like different prostaglandin E synthases (e.g. PTGES) for PGE₂ and several prostaglandin F synthases (PGFS; e.g. aldo-keto reductase family 1, member C3 (AKR1C3)) for PGF_{2α} (Smith & Dewitt 1996). Inactivation of plasma PGE₂ to 13,14-dihydro-15-keto-PGE₂ (PGEM) is catalysed by HPGD (hydroxyprostaglandin dehydrogenase 15-(NAD)) (Tai *et al.* 2002). Similarly, PGF_{2α} is rapidly inactivated by HPGD in the lungs to its metabolite 13,14-dihydro-15-keto-PGF_{2α} (PGFM) (Tai *et al.* 2002). Action of PGE₂ is mediated via multiple G protein-coupled membrane receptors, e.g. PTGER2 and PTGER4. After ligand binding, these subtypes mediate their effects through G proteins that stimulate adenylyl cyclase. The resulting increased

cAMP level leads to activation of the PKA-signalling pathway (Marsh & LeMaire 1974). A luteotrophic effect of PGE₂ was indicated, as it was shown to stimulate P4 secretion in bovine (Kotwica *et al.* 2003), ovine (Weems *et al.* 1997) and canine (Kowalewski *et al.* 2013) luteal cell cultures. The receptor of PGF_{2α} (PTGFR) belongs to the seven-transmembrane family of G protein-coupled receptors, generating different second messengers after ligand binding (Sugimoto *et al.* 1994). The conserved role of PGF_{2α} in mediating luteal regression is generally accepted (Niswender *et al.* 2000, Arosh *et al.* 2004). Prostaglandin F_{2α} decreases luteal synthesis of P4 in cows, ewes, humans and sows (Niswender *et al.* 1994). It affects P4 production by interfering with cholesterol transport into steroidogenic cells (McLean *et al.* 1995) or by enhancing P4 metabolism (Stocco *et al.* 2000). The cyclical regression of the CL is initiated by the secretion of PGF_{2α} from the uterus in many non-primate mammalian species (McCracken *et al.* 1999). In these species, it triggers the luteal production of PGF_{2α} (Tsai & Wiltbank 1997, Arosh *et al.* 2004), probably crucial for complete structural regression (Diaz *et al.* 2002, Hayashi *et al.* 2003). In primates, luteal regression is mediated by PGF_{2α} of luteal rather than uterine origin (Nagle *et al.* 2005).

In plasma of Eurasian lynxes (Painer *et al.* 2014b) and domestic cats (Siemieniuch *et al.* 2014), PGFM levels increase during the last trimester of gestation until a pre-partal peak. In the pregnant domestic cat, it is assumed that PGFM plasma elevations in late pregnancy mainly reflect substantial placental PGF_{2α} production (Tsutsui & Stabenfeldt 1993, Siemieniuch *et al.* 2014). This, however, does not rule out an intra-luteal source of PGF_{2α}. In cats and lynxes, the pre-partal PGFM elevation is accompanied by a decline in plasma P4 (Siemieniuch *et al.* 2012, Painer *et al.* 2014b), indicative of functional luteal regression; however, no complete structural regression, as suggested for the cat (Dawson 1946, Amelkina *et al.* 2015a), is observed in lynxes (Painer *et al.* 2014b). A smaller increase in plasma PGFM, concomitant with a P4 decrease, was shown in prooestrous lynxes (Painer *et al.* 2014b). Based on an earlier study on intra-luteal PG synthesis in lynxes (Jewgenow *et al.* 2012), it was proposed that an intra-luteal signal for the temporary functional luteal regression of perCL might occur before oestrus (Jewgenow *et al.* 2014), as a prerequisite for follicular growth and ovulation.

We assumed that the feline CL lifespan is regulated by the luteal capacity to synthesise and respond to PGs. Thus, the intra-luteal expression of PG synthases (PTGS2/COX2, PTGES, PGFS/AKR1C3) and PG receptors (PTGER2, PTGER4, PTGFR), and intra-luteal levels of PGE₂ and PGF_{2α} were assessed. We aimed to deduce potential implications of intra-luteal PGs (i) to initiate onset of luteal regression at the end of the pregnant and non-pregnant luteal phases in cats, (ii) to

maintain CL persistence in lynxes and (iii) to transiently reduce functionality of perCL before oestrus. Knowledge on the species-specific ovarian physiology and the lifespan of CL could be implemented in conservation management plans to improve the reproductive potential of endangered Iberian lynxes by assisted reproduction techniques.

Materials and methods

Animals

The methods applied, and the study design, were approved by the Internal Committee for Ethics and Animal Welfare of the Leibniz Institute for Zoo and Wildlife Research in Berlin, Germany (Permit numbers: 2010-10-01 and 2011-01-01). The tissue from domestic cats was obtained from local animal shelters and clinics after ovariectomy for the purpose of permanent contraception. This was not related to the study. The tissue from late pregnancy castration of domestic cats was obtained from the Institute of Animal Reproduction and Food Research, and all procedures were approved by the Local Animal Care and Use Committee in Olsztyn, Poland (Permit numbers: No. 41/2007/N and 61/2010/DTN). The Norwegian Experimental Animal Ethics Committee approved the collection of the ovarian tissue from Eurasian lynxes hunted legally for management purposes (Permit number: 2010/161554). Samples of Eurasian lynxes from Sweden became mainly available in the context of a legal hunting quota to maintain appropriate population levels (Swedish Government 2009, proposition 2008/09:2010, a new large

carnivore management plan). The tissue from Iberian lynxes was collected after ovariectomy for permanent contraception at breeding centres (Centro de Cría de Lince Ibérico El Acebuche, Parque Nacional de Doñana, Huelva, Spain and Centro Nacional de Reprodução do Lince Ibérico, Silves, Portugal), which was not related to the study.

Tissue collection

Ovaries and uteri of domestic cats were collected over a period of three breeding seasons (2012–2014; Table 1). Transportation was at 4°C in supplemented MEM-HEPES medium, and the CL were isolated immediately after arrival at the laboratory (2–4 h after surgery). The day of pregnancy was determined by the stage of pre-implantation embryos flushed from the oviduct (<day 5 *p.c.* (Knospe 2002); a day 10 embryo was flushed from the uterine horn), the diameter of the gestation chamber (<day 20 *p.c.* (Zambelli & Prati 2006)) or the foetal crown-rump length (>day 20 *p.c.* (Schnorr & Kressin 2006)). Accordingly, CL of pregnant cats were assigned to three stages: the pre-implantation stage (PRI) included samples from days 2–10 *p.c.*; the post-implantation stage (POI) included samples from days 14–36 *p.c.*; the regression stage (R) was represented by samples from days 38–48 *p.c.* Because the time of ovulation was unknown and embryos or foetuses were absent in the oviducts or uteri, the CL stages of the non-pregnant luteal phase were determined histomorphologically according to Amelkina and coworkers (2015a), including parameters of cell shape, type and degree of vacuolation, nucleus condition and the ratio of non-steroidogenic to luteal cells. The CL were assigned to four stages: formation (F; highly neovascularised CL contained

Table 1 Animals and tissue collection.

Species	Collaborative institution	Living condition	Collection period	Tissue preservation*	Number of animal	Number of CL**	CL per animal
<i>F. catus</i>	Germany, Poland (IZW, ZIPR); animal shelters and clinics	Feral, domestic	3 breeding seasons (2012–2014)	Ovaries: 4°C, supplemented MEM-HEPES medium CL: isolated 2–4 h after surgery – Bouin's solution; liquid nitrogen, stored at –80°C	45	45	1
<i>L. lynx</i>	Norway (SNO); legal hunting	Free-ranging	February 2011	CL: isolated immediately after dissection – Bouin's solution; RNA/later RNA Stabilization Reagent and liquid nitrogen, stored at –80°C	5	18	2 or 4
<i>L. lynx</i>	Sweden (SVA); carcass harvesting	Free-ranging	2003–2010	Ovaries: –20°C, tissue bank facility CL: isolated after thawing – Bouin's solution; directly processed for hormone extraction	30	144	1 to 14
<i>L. pardinus</i>	Portugal, Spain (ILCBP); breeding centres	Captive	February 2013	CL: isolated immediately after surgery – Bouin's solution; RNA/later RNA Stabilization Reagent and liquid nitrogen, stored at –80°C	2	22	11

*for transport to the laboratory and until processing; **number of analysed CL: due to limitation of luteal tissue histological, molecular and hormonal analyses were not always performed on the same CL resulting in alternate sample sizes for different assays; CL, corpus luteum; ILCBP, Iberian Lynx Captive Breeding Programme; IZW, Leibniz Institute for Zoo and Wildlife Research; SNO, Statens Naturoppsyn; SVA, Statens Veterinärmedicinska Anstalt; ZIPR, Institute of Animal Reproduction and Food Research.

a mixture of predominantly small transforming follicular cells with different degrees of luteinisation), development/maintenance (D/M; polyhedral luteal cells were increased in size and exhibited accumulation of numerous lipid droplets), early regression (ER; CL revealed first signs of luteal regression, large vacuoles were coarsely organised all over the cells and the ratio of non-steroidogenic to luteal cells was increased) and late regression (LR; CL showed extreme cell modifications with small, deformed luteal cells containing small condensed nuclei, vacuoles were without reaction to lipid staining). *Corpora albicantia* (CA; luteal cells with condensed nuclei were highly modified and prominently smaller luteal cells became outnumbered by non-steroidogenic cells) considered the last stage of the CL lifespan, were not assigned to either pregnancy or the non-pregnant luteal phase. The number of CL per analysed luteal stage was depicted as (n_{CL}). To achieve independent data for gene expression and hormone analyses, each animal was represented by one randomly selected CL.

Ovarian tissue from five Eurasian lynxes (EL1–EL5; Table 1) found to be in prooestrus, respectively, pre-mating (for detailed description of the reproductive stages, see [Painer and coworkers \(2014b\)](#)) was collected freshly *post-mortem* in February 2011 in Norway (Statens Naturoppsyn, SNO; Trondheim). Within the *Iberian Lynx Captive Breeding Programme* (ILCBP ([Vargas et al. 2008](#))), two metoestrous Iberian lynxes (IL1, IL2; day 7 *p.c.*, post-mating) were ovariectomised in February 2013 (Table 1). The CL were isolated immediately after surgery. The collected CL of Eurasian and Iberian lynxes were histomorphologically staged using established domestic cat CL parameters ([Amelkina et al. 2015a,b](#)). Isolated perCL of Eurasian lynxes were in the early regression stage, whereas the ovaries of both Iberian lynxes revealed coexistence of perCL (maintenance stage) and freshCL (formation stage). The number of CL per analysed luteal stage was depicted as (n_{CL}) for gene expression analysis. To achieve independent data, each luteal stage was represented by CL of individual animals. For hormone analysis, the number of CL per analysed luteal stage was depicted as (n_{animal}/n_{CL} ; data dependent).

Each feline CL was dissected and pieces were either fixed in Bouin's solution (for histology and protein analyses), placed in RNA later RNA Stabilization Reagent (for RNA isolation, Eurasian and Iberian lynx; Qiagen GmbH) or liquid nitrogen (for RNA isolation; domestic cat). For hormone analyses, the tissue was transferred to liquid nitrogen. These various solutions were applied to adequately preserve the tissues collected under different conditions.

Additional ovaries and uteri from Eurasian lynxes were obtained from the Statens Veterinärmedicinska Anstalt (SVA; Uppsala, Sweden), where all lynx specimens harvested after, e.g. legal hunting or killed by traffic accidents are delivered (Table 1). At the SVA, the tissue was frozen soon after the animal was necropsied; samples were collected between 2003 and 2010 and kept in frozen storage (-20°C) in a tissue-bank facility. In the laboratory, ovaries and uteri were defrosted and dissected. A section of CL tissue was fixed in Bouin's solution or directly processed for hormone extraction. These CL were grouped by month and assigned to different reproductive stages according to [Painer and coworkers \(2014b\)](#): prooestrus (Jan, Feb), oestrus (Mar), metoestrus (Apr) and prolonged dioestrus (Sep–Dec). The CL obtained from two pregnant

animals in April were further separated into perCL (markedly smaller in size, dark red in colouration) and freshCL (bigger in size, pale colour) according to size and appearance. These pregnancies were confirmed by the presence of two foetuses in each animal. For hormone analysis, the number of CL per analysed month was depicted as (n_{animal}/n_{CL}), representing CL obtained from multiple animals (data dependent).

Sequence analysis

Information on cat-specific sequences for *PTGS2/COX2* (GenBank accession EF036473 ([Siemieniuch et al. 2011](#))), *PTGES* (GenBank accession GU059259 ([Siemieniuch et al. 2013](#))), *PGFS/AKR1C3* (GenBank accession HM490147 ([Siemieniuch et al. 2013](#))) and the validated reference gene cyclophilin A (CYC, GenBank accession AY029366 ([Siemieniuch et al. 2012](#), [Jursza et al. 2014](#))) was published previously. Based on templates of feline luteal tissue, sequence information for *PTGER2* (GenBank accession EF177829), *PTGER4* (GenBank accession EF177830) and *PTGFR* (GenBank accession AF272340) was obtained for this study (Table 2).

Total RNA isolation from reproductive tissues of Eurasian and Iberian lynxes (innuSPEED Tissue RNA/innuPREP DNase I Digest Kit; Analytik Jena AG, Jena, Germany) and reverse transcription to cDNA (RevertAid First Strand cDNA Synthesis Kit; Life Technologies GmbH) was performed as described previously ([Zschockelt et al. 2014, 2015](#)). Lynx-specific sequences of the studied genes were not annotated in GenBank before the study. Therefore, primers for the polymerase chain reaction (PCR) were based on gene sequences of the domestic cat or on consensus sequences derived from multiple species sequence alignments (CLC Sequence Viewer 6.7; CLC bio, Aarhus, Denmark). The primers were purchased from BioTeZ Berlin Buch GmbH (Berlin, Germany; Table 2). Partial cDNA sequences were amplified from luteal, ovarian or placental tissue using the Expand High Fidelity^{PLUS} PCR System (Roche Diagnostics GmbH), as defined previously ([Braun et al. 2012](#)). The PCR reactions were conducted at 94°C for 2 min followed by 35 cycles of denaturation (94°C) for 30 s (*PTGES*, *PGFS/AKR1C3*) or 45 s (*PTGS2/COX2*, *PTGER2*, *PTGFR*), annealing for 30 s (*PTGES*, *PGFS/AKR1C3*) or 45 s (*PTGS2/COX2*, *PTGER2*, *PTGFR*; for temperatures see Table 2), elongation (72°C) for 30 s (*PTGES*) or 40 s (*PGFS/AKR1C3*) or 60 s (*PTGS2/COX2*, *PTGER2*, *PTGFR*) and a final elongation at 72°C for 7 min. For amplification of *PTGER4* cDNA, the TrueStart Taq DNA Polymerase Kit (Life Technologies GmbH) was used with $95^{\circ}\text{C}/2$ min; 35 cycles of $95^{\circ}\text{C}/45$ s, $51^{\circ}\text{C}/45$ s, $72^{\circ}\text{C}/60$ s, and $72^{\circ}\text{C}/7$ min. Purified PCR products of all genes were ligated into the pCRTM4-TOPO vector and transformed into One Shot TOP10 cells (both from Life Technologies GmbH). Sequencing of clones or purified PCR products was performed by the Services in Molecular Biology GmbH (Dr M Meixner, Brandenburg, Germany). Lynx-specific sequence information and validation of the reference genes β -actin (*BACT*, GenBank accession KM458620), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*, GenBank accession KM458621), ribosomal protein S7 (*RPS7*, GenBank accession JX993349) and TATA box-binding protein (*TBP*, GenBank accession JX993351; all *Lynx pardinus*) were presented previously ([Zschockelt et al. 2015](#)).

Table 2 Sequence analysis.

GenBank accession	Species	Primer sequence 5'–3'	T _A (°C)	Product size (bp)	Use
Genes of interest					
<i>PTGS2/COX2</i> *	<i>L. pardinus</i>	<i>PTGS2/COX2</i> Fw-1: CAC TAC AWA CTT ACC CAC TTC <i>PTGS2/COX2</i> Rv-1: AGG GGA TGC CAG TGG TAG A	55	784	a
KT119408		<i>PTGS2/COX2</i> qFw-1: CAG GAG GTC TTT GGT CTG G <i>PTGS2/COX2</i> qRv-1: CCT ATC AGT ATT AGC CTG CT	55	149	b
<i>PTGS2/COX2</i>	<i>F. catus</i>	<i>PTGS2/COX2</i> Fw-2: TCG ACC AGA GCA GAC AGA TG <i>PTGS2/COX2</i> Rv-2: CTG AAT CGA GGC AGT GTT GA	64	341	a
EF036473		<i>PTGS2/COX2</i> qFw-2: AAC AGG AGC ATC CAG AAT GG <i>PTGS2/COX2</i> qRv-2: GCA GCT CTG GGT CAA ACT TC	60	147	b
<i>PTGES</i> *	<i>L. lynx</i>	<i>PTGES</i> Fw-1: GCT GGT CAT CAA GAT GTA CG <i>PTGES</i> Rv-1: GCT TCC CAG ACG ATC TGS A	55	339	a
KM982690		<i>PTGES</i> qFw-1: TCG CTG CCT CAG AGC CCA <i>PTGES</i> qRv-1: TAG GCC ACG GTG TGC ACC	66	153	b
<i>PTGES</i>	<i>F. catus</i>	<i>PTGES</i> Fw-2: ACC ATC TAC CCC TTC CTG <i>PTGES</i> Rv-2: CAG CTT CCC AGA CGA TCT	57	214	a
GU059259		<i>PTGES</i> qFw-2: GGC CTC GTT TAC TCC TTC CT <i>PTGES</i> qRv-2: CCG AAG CTT GCC CAG ATA G	60	111	b
<i>PTGER2</i> *	<i>L. lynx</i>	<i>PTGER2</i> Fw-1: GTT CCA CGT GGT GGT GAC A <i>PTGER2</i> Rv-1: GAT GGC AAA GAC CCA AGG G	55	716	a
KM982691		<i>PTGER2</i> qFw-1: GAG GGG AAA GGC TGT CCA <i>PTGER2</i> qRv-1: GCA AAA ATT GTG AAA GGC AAG	56.5	103	b
<i>PTGER2</i> *	<i>F. catus</i>	<i>PTGER2</i> Fw-2: AAC TAC GGC CAG TAC GTC CA <i>PTGER2</i> Rv-2: CCG GAA GAC GTT TCA TTC AT	60	356	a
EF177829		<i>PTGER2</i> qFw-2: CTT CTA CCA GCG TCG TGT CA <i>PTGER2</i> qRv-2: ACT GGC CGT AGT TCA GCA AC	60	107	b
<i>PTGER4</i> *	<i>L. lynx</i>	<i>PTGER4</i> Fw-1: AGC GCT ACC TGG CCA TCA A <i>PTGER4</i> Rv-1: GAT AAG TTC AGT GTT TCA YTG G	51	830	a
KP027417		<i>PTGER4</i> qFw-1: GGG TGC CGA GAT CCA GAT <i>PTGER4</i> qRv-1: TGG TTG ATG AAC ACC CGT AC	56.5	97	b
<i>PTGER4</i> *	<i>F. catus</i>	<i>PTGER4</i> Fw-2: TTG CTG TCT ATG CGT CCA AC <i>PTGER4</i> Rv-2: GGA GGT GGC GAT GAG TAA GA	61	426	a
EF177830		<i>PTGER4</i> qFw-2: TTG CTG TCT ATG CGT CCA AC <i>PTGER4</i> qRv-2: TCC AGT CGA TGA AAC ACC AG	60	102	b
<i>PGFS/AKR1C3</i> *	<i>L. lynx</i>	<i>PGFS/AKR1C3</i> Fw-1: GAT GGC ACT GTG AAG AGA GA <i>PGFS/AKR1C3</i> Rv-1: CAT TCC TTC CCT GAG TTG GA	55	215	a
KM982692		<i>PGFS/AKR1C3</i> qFw-1: AGC CCG GCC TCA AGT ACA A <i>PGFS/AKR1C3</i> qRv-1: CAT TCC TTC CCT GAG TTG GA	61.5	145	b
<i>PGFS/AKR1C3</i>	<i>F. catus</i>	<i>PGFS/AKR1C3</i> Fw-2: CCATGGAGAAGTCAAGGATT <i>PGFS/AKR1C3</i> Rv-2: ACC TGG AAG TTC TCC CGA AT	61	400	a
HM490147		<i>PGFS/AKR1C3</i> qFw-2: TCA ACC AGA GCA AAC TGC TG <i>PGFS/AKR1C3</i> qRv-2: CAT TCC TTC CCT GAG TTG GA	60	91	b
<i>PTGFR</i> *	<i>L. lynx</i>	<i>PTGFR</i> Fw-1: CTT CAT GAC AGT GGG AAT CTT <i>PTGFR</i> Rv-1: GCT GAT GAY ATG CAC TCC AC	55	684	a
KM982687		<i>PTGFR</i> qFw-1: GCT GGA GTC CAT TTC TGG TG <i>PTGFR</i> qRv-1: CCA CGT TGC CAT TCG AAG	61	104	b
<i>PTGFR</i> *	<i>F. catus</i>	<i>PTGFR</i> Fw-2: CTC TGG TCT GTG CCC ACT TT <i>PTGFR</i> Rv-2: TGA GAC CTG CCT TGT CTG TG	62	378	a
AF272340		<i>PTGFR</i> qFw-2: AGA CTT CTT TGG CCA CCT CA <i>PTGFR</i> qRv-2: GTG GGC ACA GAC CAG AGA AT	60	104	b
Reference genes					
<i>BACT</i>	<i>L. pardinus</i>	<i>BACT</i> Fw-1: CAT CCT GAC CCT CAA GTA C <i>BACT</i> Rv-1: TCA TGA TGG AGT TGA AGG	51	625	a
KM458620		<i>BACT</i> qFw-1: GAG CAG GAG ATG GCC ACG <i>BACT</i> qRv-1: CTC GTG GAT GCC ACA GGA	62	159	b
CYC	<i>F. catus</i>	<i>CYC</i> Fw-1: CCT TCT GTA GCT CGG GTG AG <i>CYC</i> Rv-1: CTT GGA GGG GAG GTA AGG AG	56	118	a
AY029366		<i>CYC</i> qFw-1: CCT TCT GTA GCT CGG GTG AG <i>CYC</i> qRv-1: CTT GGA GGG GAG GTA AGG AG	60	118	b
<i>GAPDH</i>	<i>L. pardinus</i>	<i>GAPDH</i> Fw-1: CTG GTC ACC AGG GCT GCT <i>GAPDH</i> Rv-1: CCA TGA GGT CCA CCA CCC	53	640	a
KM458621		<i>GAPDH</i> qFw-1: AAG GGT GGG GCC AAG AGG <i>GAPDH</i> qRv-1: AGA GGG GCC AGG CAG TTG	63.5	143	b
<i>RPS7</i>	<i>L. pardinus</i>	<i>RPS7</i> Fw-1: GCC ATG TTC AGT TCG AGC G <i>RPS7</i> Rv-1: GTC TAC AAC TGA AAC TCT GGG	55	550	a
JX993349		<i>RPS7</i> qFw-1: CCT GGA GGA CTT GGT TTT CC <i>RPS7</i> qRv-1: CCT TGC CCG TGA GCT TCT	61	164	b
<i>TBP</i>	<i>L. pardinus</i>	<i>TBP</i> Fw-1: ATG GAT CAG AAC AAC AGC CTG <i>TBP</i> Rv-1: GCA GGA GTA CGT TAA CAG CC	56 – 51	970	a
JX993351		<i>TBP</i> qFw-1: AGA GAG CCC CGA ACC ACT G <i>TBP</i> qRv-1: TTC ACA TCA CAG CTC CCC AC	62.5	182	b

*gene sequence information analysed in this study; a, used for sequence analyses; b, used for expression studies; bp, base pair; Fw, forward; q, quantitative PCR; Rv, reverse; T_A, annealing temperature.

Gene expression studies by quantitative PCR (qPCR)

Quantification of qPCR results using the real-time PCR Miner method: pregnant and non-pregnant luteal phases of domestic cat

Homogenisation of domestic cat luteal tissue (up to 22 mg), total RNA extraction and reverse transcription to cDNA was done as described previously (Zschockelt *et al.* 2014). No reverse-transcription controls were included to test for genomic DNA contamination. Quality and integrity of RNA were assessed via microfluidic analysis of the respective RNA intactness using the Agilent 2100 Bioanalyzer and the Agilent RNA 6000 Nano or Pico Kits (Agilent Technologies Deutschland GmbH); RNA integrity number (RIN) values were above 7.0 (up to 9.7 and 10), representing a rather intact state of total RNA. Additionally, RNA concentration and purity were validated by the NanoDrop ND-1000 (Peglab Biotechnologie GmbH, Erlangen, Germany). The primers were either chosen based on previous studies (*PTGS2/COX2* (Siemieniuch *et al.* 2011), *PTGES* (Siemieniuch *et al.* 2013), *PGFS/AKR1C3* (Siemieniuch *et al.* 2013)) or designed with the free online primer design tool Primer3 (<http://simgene.com>; *PTGER2*, *PTGER4*, *PTGFR*). All primers were purchased from BioTez Berlin Buch GmbH (Table 2). The relative mRNA expression of target genes was determined by qPCR with focus on the overall profile course rather than the amount of mRNA copies. This strategy eliminated the necessity of cloning external recombinant plasmid DNA for calibration. The qPCR reactions were carried out in an automated fluorometer ABI PRISM 7300 Sequence Detection System (Applied Biosystems) using the SYBR Green PCR Master Mix (Applied Biosystems). The total qPCR reaction volume was 10 µL containing 3 µL cDNA (corresponding to 10 ng total RNA for genes of interest and the reference gene), 1 µL each of forward and reverse primers (500 nM) and 5 µL SYBR Green PCR Master Mix + 0.1 µL CRX. The qPCR conditions were as follows: initial denaturation (10 min at 95°C), followed by 40 cycles of denaturation (15 s at 95°C) and annealing/elongation (60 s at 60°C). After each qPCR reaction, melting curves were obtained by stepwise increases in temperature from 60 to 95°C to ensure single product amplification. Cyclophilin A was previously validated and selected among several different genes as a reference gene (Siemieniuch *et al.* 2012, Jursza *et al.* 2014). Quantification of qPCR results was performed by normalising the signals of target genes with the CYC signal using the real-time PCR Miner method, whereby calculation of reaction efficiency and the fractional cycle number at threshold (CT) were based on kinetics of individual qPCR reactions (Zhao & Fernald 2005).

Quantification of qPCR results using a calibration curve: prooestrus and metoestrus of Eurasian and Iberian lynx

Lynx luteal tissue (14–26 mg) was homogenised, total RNA was extracted and reverse transcribed to cDNA as described before (Zschockelt *et al.* 2014, 2015). No reverse-transcription controls were included and RNA concentration and purity were determined; RIN values were above 6.1 (up to 7 and 8.6), indicating that intactness of total RNA was only slightly lowered by moderate partial degradation. Intron-spanning primers were designed according to sequences identified in

the present study (Table 2). Because cloned cDNA of lynx-specific genes needed to be generated for sequence analysis (Sect. “sequence analysis”), standard dilutions for calibration of qPCR results were derived from the respective external recombinant plasmid DNA. This alternative strategy allowed for the determination of the relative amount of mRNA copies. For qPCR, diluted cDNA (corresponding to 2 or 10 ng total RNA for genes of interest; 4 ng for reference genes) was analysed with the CFX96 Real-Time PCR Detection System using the SsoFast EvaGreen Supermix (both from Bio-Rad Laboratories GmbH). Conditions for qPCR were: 98°C for 2 min and 40 cycles of denaturation at 98°C for 8 s and annealing/elongation for 8 s at different temperatures (Table 2). The qPCR products were quantified with the CFX Manager Software 1.6 (Bio-Rad Laboratories GmbH). Fragments of *BACT*, *GAPDH*, *RPS7* and *TBP* were amplified for normalisation as described previously (Zschockelt *et al.* 2015). A multiple normalisation factor based on the reference gene expression was calculated for individual CL referring to Vandesompele and coworkers (2002).

Protein expression studies by immunohistochemistry

For validation of gene expression studies, immunohistochemical analysis was performed on 3 µm sections of Bouin-fixed and paraffin-embedded CL mounted on microscope slides (Superfrost Plus; Thermo Fisher Scientific). The immunohistochemical procedure of the immunoperoxidase method for *PTGES*, *PTGER4* and *PGFS/AKR1C3* was described previously (Gram *et al.* 2013, 2014). For *PTGFR*, a protocol referring to a previous study (Braun *et al.* 2012) was applied. Briefly, slides were deparaffinised in xylol (for *PTGFR* in Roti-Histol; Carl Roth GmbH, Karlsruhe, Germany) and rehydrated in ethanol. Antigen retrieval was performed in citrate buffer (10 mM, pH 6.0). Endogenous peroxidase activity was quenched by incubation in 0.3% (for *PTGFR* in 3%) H₂O₂ with methanol. Afterwards, the slides were incubated in 10% goat serum (for *PTGFR* in 5% BSA in PBS) in order to block non-specific binding sites. Thereafter, the slides were overlaid overnight with canine-specific polyclonal antibodies: guinea pig anti-*PTGES* (1:2500 (Gram *et al.* 2014, Kautz *et al.* 2014)), anti-*PGFS/AKR1C3* (1:750 (Gram *et al.* 2013) – both custom made by Eurogentec, Seraing, Belgium) – and rabbit anti-*PTGER4* (1:750 (Gram *et al.* 2014); 101775, Cayman Chemical). For the isotype control, slides were incubated with pre-immune guinea pig serum (*PTGES*, *PGFS/AKR1C3*) or rabbit IgG irrelevant antibodies I-1000 (*PTGER4*; all from Vector Laboratories, Inc, Burlingame, CA, USA) at the same dilution as the primary antibodies. The slides were subsequently incubated with biotinylated secondary goat anti-guinea pig IgG BA-7000 antibodies at 1:100 dilution (*PTGES*, *PGFS/AKR1C3*) or goat anti-rabbit IgG BA-1000 at 1:100 dilution (*PTGER4*; all from Vector Laboratories, Burlingame, CA, USA). The signals for *PTGES*, *PTGER4* and *PGFS/AKR1C3* were enhanced with the streptavidin-avidin-peroxidase Vectastain® ABC kit (Vector Laboratories, Burlingame, CA, USA). The *PTGFR* antibody was used at a 1:100 dilution (PGF2αR (H-55), sc-67029; Santa Cruz Biotechnology). For the negative control, *PTGFR* slides were incubated with blocking solution instead of primary antibody to test for specificity of staining. Slides for *PTGFR* analysis

were subsequently incubated with goat anti-rabbit IgG-HRP (K4002; Dako Deutschland GmbH) as secondary antibody. For all slides, the peroxidase activity was finally detected using the Liquid DAB Substrate Kit (Dako North America, Inc, CA, USA). Sections were counterstained with haematoxylin, dehydrated in ethanol and covered with Histokitt (Assistant, Osterode, Germany) or Roti-Histokitt (for PTGFR; Carl Roth GmbH) and coverslips. Slides were analysed with an Axioplan microscope combined with a ProgRes C10^{plus} camera (both Carl Zeiss MicroImaging GmbH) and the Cell[^]P Soft Imaging Software (Olympus Soft Imaging Solutions GmbH, Muenster, Germany).

Intra-luteal hormone studies by enzyme-linked immunosorbent assay

The homogenisation of luteal tissue and the subsequent hormone extraction was done as described previously for domestic cat (Zschockelt et al. 2014, Amelkina et al. 2015a) and lynx (Carnaby et al. 2012, Zschockelt et al. 2015) CL. Briefly, aliquots of luteal homogenates (8–68 mg luteal tissue for cats, 9–65 mg luteal tissue for lynxes) were twice extracted with tert-butyl methyl ether (TBME)/petroleum ether (PE) and extracts were eventually dissolved in 40% methanol. Prostaglandin E₂ analyses for domestic cats and Eurasian lynxes were carried out using the commercial DetectX High Sensitivity Prostaglandin E₂ Enzyme Immunoassay Kit (K018-HX; Arbor Assays, Ann Arbor, MI, USA) according to the manufacturer's protocol. The stated cross-reactivity was as follows: 100% to PGE₂, 2% to PGF_{2α} and <0.1% to PGFM. Intra- and inter-assay coefficients of variation (CVs) for three biological samples were 7.4%

($n=20$) and 10.4% ($n=15$), respectively. During our studies, the K018-HX kit has been discontinued and replaced with a PGE₂ Multi-Format Kit (K051-H; Arbor Assays, Ann Arbor, MI, USA). Therefore, PGE₂ analyses for Iberian lynxes were carried out with the new kit. The stated cross-reactivity was, e.g. 100% to PGE₂ and 0.33% to PGF_{2α}. Intra- and inter-assay CVs for three biological samples were 7.8% ($n=20$) and 8.9% ($n=17$), respectively. The determination of intra-luteal PGF_{2α} concentrations was carried out in the same extracts, using an in-house PGF_{2α} enzyme-linked immunosorbent assay (EIA) as described earlier (Dehnhard & Jewgenow 2013). The PGF_{2α} antibody was raised in rabbits immunised against PGF_{2α}-BSA and was described to cross-react to 34% with PGF_{1α} and to less than 0.01% with PGE₂, PGFM and PGEM (Claus et al. 1990). Intra- and inter-assay CVs for two biological samples were 4.6% ($n=10$) and 7.1% ($n=10$).

Statistical analysis

Due to the inherent problems, CL of pregnant domestic cats and endangered Eurasian lynxes were obtained by chance and only few tissue samples became available. By ensuring independence of data, the respective sample sets contained not enough values to perform a normality test. Thus, no statistical analysis was applied and these results were analysed descriptively. Although the number of replicates was limited as well, statistical analysis was possible for non-pregnant domestic cats and Iberian lynxes. The R software package (R: A language and environment for statistical computing, version 2.15.2, Vienna, Austria) was applied (values of $P \leq 0.05$ were considered significant). As the

Figure 1 Intra-luteal gene expression of PGE₂ and PGF_{2α} synthases and receptors in the domestic cat. The *PTGS2/COX2* (A), *PTGES* (B), *PTGER2* (C), *PTGER4* (D), *PGFS/AKR1C3* (E) and *PTGFR* (F) relative mRNA expression [arbitrary unit] was determined by qPCR (real-time PCR Miner method), normalised with a factor calculated from qPCR results of a reference gene. The Kruskal–Wallis rank sum test was applied for the non-pregnant luteal phase; asterisks indicate statistical differences ($P_{NP} \leq 0.05$). Data were plotted per CL stage as (n_{CL}). CA, corpus albicans; CL, corpus luteum; D/M, development/maintenance; ER, early regression; F, formation; LR, late regression; NP, non-pregnant luteal phase; POI, post-implantation; PRI, pre-implantation; qPCR, quantitative polymerase chain reaction; R, regression.

distribution of data was not normal, non-parametric statistical methods were used. For non-pregnant domestic cats, the Kruskal–Wallis rank sum test was applied to determine the influence of the CL stage on the relative mRNA expression and hormone levels, followed by the Wilcoxon rank sum test for *post hoc* pairwise comparison (*P*-value adjustment: Benjamini–Hochberg). For Iberian lynxes, the Mann–Whitney *U*-test was applied to determine the influence of the CL stage on the relative amount of mRNA copies.

The intra-luteal gene expression (n_{CL}) and hormone levels (n_{CL} or n_{animal}/n_{CL}) were depicted in vertical Box Plots plotting data as median and percentiles. The $PGE_2:PGF_{2\alpha}$ ratio was depicted as (n_{CL}) or (n_{animal}/n_{CL}) in vertical bar charts with simple error bars representing standard deviations (SigmaPlot 10.0, Systat Software GmbH, Erkrath, Germany).

Results

Intra-luteal gene expression of PGE_2 and $PGF_{2\alpha}$ synthases and receptors

For the pregnant luteal phase of the domestic cat (no statistical analysis), the qPCR analyses revealed that

the medians of relative mRNA expression of *PTGS2/COX2* (Fig. 1A), *PTGES* (Fig. 1B), *PTGER2* (Fig. 1C) and *PGFS/AKR1C3* (Fig. 1E) was higher in the pre-implantation stage compared with the post-implantation and regression stages. In contrast, for *PTGER4* (Fig. 1D) and *PTGFR* (Fig. 1F), the relative mRNA expression was higher in the regression stage. During the non-pregnant luteal phase (NP; statistical analysis: Kruskal–Wallis rank sum test), the expression of *PTGS2/COX2* (Fig. 1A), *PTGES* (Fig. 1B), *PTGER2* (Fig. 1C) and *PGFS/AKR1C3* (Fig. 1E) remained unchanged ($P_{NP} > 0.05$), whereas the expression of *PTGER4* ($P_{NP}=0.05$; Fig. 1D) and *PTGFR* ($P_{NP}=0.03$; Fig. 1F) was affected by the luteal stage. In detail, the expression of *PTGER4* was lower in the development/maintenance compared with the late regression stage ($P_{NP}=0.035$). For *PTGFR*, the expression was higher in the development/maintenance stage ($P_{NP}=0.018$), early regression stage ($P_{NP}=0.048$) and late regression stage ($P_{NP}=0.018$) compared with the formation stage. The two *corpora albicantia* exhibited notably high relative mRNA expression for *PTGER2* (Fig. 1C), *PTGER4* (Fig. 1D) and *PGFS/AKR1C3* (Fig. 1E).

Figure 2 Intra-luteal gene expression of PGE_2 and $PGF_{2\alpha}$ synthases and receptors in Eurasian and Iberian lynxes. The *PTGS2/COX2* (A), *PTGES* (B), *PTGER2* (C), *PTGER4* (D), *PGFS/AKR1C3* (E) and *PTGFR* (F) relative amount of mRNA copies (per ng total RNA) are copy numbers determined by a calibration curve in qPCR that were afterwards normalised with a factor calculated from qPCR results of reference genes. Values refer to 1 ng original total RNA. The Mann–Whitney *U*-test was applied for the metoestrous Iberian lynxes; asterisks indicate statistical differences ($P_{IL1/IL2} \leq 0.05$). Data were plotted per CL stage as (n_{CL}). CL, corpus luteum; EL, Eurasian lynx; fresh, freshly formed CL; IL, Iberian lynx; per, persistent CL; qPCR, quantitative polymerase chain reaction.

For Iberian lynxes (statistical analysis: Mann–Whitney U-test), the relative amount of mRNA copies was independent of the luteal stage for *PTGS2/COX2* (Fig. 2A), *PTGES* (Fig. 2B) and *PTGER4* (Fig. 2D; for all $P_{IL1/IL2} > 0.05$). The amount of mRNA for *PTGER2* (Fig. 2C) and *PGFS/AKR1C3* (Fig. 2E) was higher in metoestrous perCL compared with freshCL in one of the two animals (for both genes: $P_{IL1} = 0.0121$; $P_{IL2} > 0.05$). Both animals revealed higher mRNA amounts of *PTGFR*

(Fig. 2F) in perCL compared with freshCL ($P_{IL1} = 0.0121$, $P_{IL2} = 0.0043$). Medians of relative mRNA amounts in perCL of prooestrous Eurasian lynxes (SNO, Norway; no statistical analysis) were equal (*PTGES*, Fig. 2B; *PGFS/AKR1C3*, Fig. 2E) or intermediate (*PTGFR*; Fig. 2F) compared with perCL and freshCL of metoestrous Iberian lynxes. For *PTGS2/COX2* (Fig. 2A), *PTGER2* (Fig. 2C) and *PTGER4* (Fig. 2D), the medians were higher in perCL of Eurasian lynxes.

Figure 3 Intra-luteal protein expression of PGE_2 and $PGF_{2\alpha}$ synthases and receptors in the domestic cat. Protein expression of *PTGES* (A), *PTGER4* (B), *PGFS/AKR1C3* (C) and *PTGFR* (D) was analysed for the non-pregnant luteal phase and in the *corpus albicans* by immunohistochemistry. Arrows indicate positive staining. Insets show isotype (*PTGES*, *PTGER4*, *PGFS/AKR1C3*) and negative controls (*PTGFR*). Scale bars represent 50 μm .

Cellular localisation and protein expression of PGE₂ and PGF_{2α} synthases and receptors

As revealed by immunohistochemistry, staining for protein expression of PG synthases and receptors was localised to luteal cells. Throughout the pregnant (data not shown) and the non-pregnant luteal phases of the domestic cat (Fig. 3), clear signals were observed for luteal PTGES (Fig. 3A) and PTGER4 (Fig. 3B). Also, the *corpus albicans* exhibited prominent staining (Fig. 3A and B). The protein expression of PGFS/AKR1C3 remained constantly low at all time points investigated during pregnancy (data not shown). In non-pregnant cats, no signals for PGFS/AKR1C3 protein expression were found in the formation stage, but weak staining was detected during the development/maintenance stage (Fig. 3C). The signals were then slightly more intense during the early regression and the late regression stages. In the *corpus albicans*, no signal was detected. The expression of PTGFR was equally strong at days 4, 24 and 48 *p.c.* in CL of pregnant cats (data not shown). During the non-pregnant phase, expression of PTGFR was weak during the early luteal phase (Fig. 3D), absent in the development/maintenance stage, but clearly evident in the early regression stage, the late regression stage and the *corpus albicans*.

At prooestrus and metoestrus of Eurasian (SNO, Norway) and Iberian lynxes, the intensity of the luteal

signal for PTGES (Fig. 4A) and PTGER4 (Fig. 4B) was equally strong between perCL and freshCL. In all analysed lynx CL, no signals for PGFS/AKR1C3 protein expression were present (Fig. 4C), but staining for PTGFR was evident (Fig. 4D). The dot-shaped staining detected in perCL of both lynx species stained with different antibodies was considered non-specific, as it was found in control slides as well.

Intra-luteal PGE₂ and PGF_{2α} concentrations

Using EIA, it was revealed that the median PGE₂ levels measured in CL of pregnant domestic cats (Fig. 5A; no statistical analysis) decreased from the pre-implantation to the regression stage. The median concentrations of PGF_{2α} were numerically higher in the post-implantation and regression stages compared with the pre-implantation stage of pregnancy (Fig. 5B). During the non-pregnant luteal phase (Fig. 5A), the PGE₂ level changed ($P_{NP}=0.038$; statistical analysis: Kruskal–Wallis rank sum test), but the *post hoc* test failed to reveal differences between specific groups. In the single *corpus albicans* sample, a moderate PGE₂ concentration was measured (196.55 ng/g). The intra-luteal PGF_{2α} level was unaffected by the luteal stage ($P_{NP}>0.05$; Fig. 5B) for non-pregnant cats. Relatively high PGF_{2α} levels were noted for the single *corpus albicans* available

Figure 4 Intra-luteal protein expression of PGE₂ and PGF_{2α} synthases and receptors in Eurasian and Iberian lynxes. Protein expression of PTGES (A), PTGER4 (B), PGFS/AKR1C3 (C) and PTGFR (D) was analysed for perCL and freshCL of prooestrus and metoestrus by immunohistochemistry. Arrows indicate positive staining. Insets show isotype (PTGES, PTGER4, PGFS/AKR1C3) and negative controls (PTGFR). Scale bars represent 50 μm. CL, *corpus luteum*; EL, Eurasian lynx; fresh, freshly formed CL; IL, Iberian lynx; per, persistent CL.

Figure 5 Intra-luteal levels of PGE₂ and PGF_{2α} in the domestic cat. The PGE₂ (A) and PGF_{2α} (B) levels [ng/g tissue] were determined by enzyme-linked immunosorbent assay. The PGE₂:PGF_{2α} ratio (C) [arbitrary unit] was calculated by division of PGE₂ values by PGF_{2α} values. The Kruskal–Wallis rank sum test was applied for the non-pregnant luteal phase; asterisks indicate statistical differences ($P_{NP} \leq 0.05$). Data were plotted per CL stage as (n_{CL}). CA, *corpus albicans*; CL, *corpus luteum*; D/M, development/maintenance; ER, early regression; F, formation; LR, late regression; NP, non-pregnant luteal phase; POI, post-implantation; PRI, pre-implantation; R, regression.

for the study (22.17 ng/g). In general, the maximum level of PGE₂ (1845.50 ng/g; formation stage) was considerably higher compared with the maximum level of PGF_{2α} (22.17 ng/g; *corpus albicans*). In cat CL, the ratio of PGE₂:PGF_{2α} was in favour of PGE₂ especially at

Figure 6 Intra-luteal levels of PGE₂ and PGF_{2α} during the annual cycle of Eurasian lynxes. The PGE₂ (A) and PGF_{2α} (B) levels [ng/g tissue] were determined by enzyme-linked immunosorbent assay. The PGE₂:PGF_{2α} ratio (C) [arbitrary unit] was calculated by division of PGE₂ by PGF_{2α} values. Data were plotted per month as (n_{animal}/n_{CL}). CL, *corpus luteum*.

pre-implantation (no statistical analysis), but was constant throughout the non-pregnant luteal phase ($P_{NP} > 0.05$; Fig. 5C).

During the annual cycle of Eurasian lynxes (SVA, Sweden; Fig. 6A, no statistical analysis), the median levels of intra-luteal PGE₂ were high in January (5334 ng/g; prooestrus) and April (4866.20 ng/g; metoestrus). Low median levels were measured in December (1666.40 ng/g; prolonged dioestrus). The intra-luteal level of PGF_{2α} was almost constant throughout the

Figure 7 Intra-luteal levels of PGE₂ and PGF_{2α} in persistent and fresh corpora lutea of metoestrous Eurasian and Iberian lynxes. The PGE₂ (A and B) and PGF_{2α} (C and D) levels [%; arbitrary unit] were determined by enzyme-linked immunosorbent assay. The hormone levels were put in relation to the respective maximum PGE₂ or PGF_{2α} value (=100%) and were depicted on a percentage basis. Presentation of arbitrary units became necessary, as different PGE₂ enzyme immunoassay kits were used for Eurasian and Iberian lynxes. Data were plotted per CL stage as ($n_{\text{animal}}/n_{\text{CL}}$). CL, corpus luteum; fresh, freshly formed CL; per, persistent CL.

year (Fig. 6B) and median levels ranged from 485 ng/g (October) to 20.35 ng/g (December). In Eurasian lynxes, the annual median levels of PGE₂ were substantially higher compared with the median levels of PGF_{2α}. Accordingly, the PGE₂:PGF_{2α} ratio revealed prevalence in the direction of PGE₂ (no statistical analysis), with higher ratios in January (prooestrus), March (oestrus) and December (prolonged dioestrus; Fig. 6C).

The level of PGE₂ and PGF_{2α} during metoestrus was further compared between perCL and freshCL of either Eurasian or Iberian lynxes (Fig. 7; no statistical analysis). For both species, the median PGE₂ level was numerically higher in perCL than in freshCL (Fig. 7A and B). The median PGF_{2α} concentration was higher in perCL compared with freshCL in both lynx species as well (Fig. 7C and D).

Discussion

This study analysed the intra-luteal PGE₂ and PGF_{2α} synthesis and reception in relation to the different reproductive strategies of the domestic cat and the two European lynx species. We demonstrated that the feline

CL is capable of synthesising PGs *de novo* during the pregnant and non-pregnant luteal phases in cats and at prooestrus and metoestrus of lynxes. While there is a distinct prevalence towards intra-luteal synthesis of PGE₂ rather than PGF_{2α}, a potential receptivity of the feline CL to intra-luteal and extra-luteal PGE₂ and PGF_{2α} signals is evident.

The CL of most mammals possesses an inherent capacity to produce PGs which is induced by specific events during the luteal phase (Olofsson & Leung 1994). The luteal expression of PTGS2/COX2 is increased at the beginning of the CL phase in dogs (Kowalewski *et al.* 2006, 2015). Likewise, luteal concentrations of PTGS2/COX2 mRNA are greater in the early compared with mid and late luteal phases in the cow (Kobayashi *et al.* 2002). In contrast, the protein content of PTGS2/COX2 is high in the mouse ovary during CL regression (Sander *et al.* 2008), and the PTGS2/COX2 expression increases from the early to the late luteal phase in human CL (Mitchell *et al.* 1991). However, our results demonstrated the steady-state expression of PTGS2/COX2 mRNA in the CL of cats and lynxes. The constant intra-luteal PTGS2/COX2 expression neither reflects the functional and structural demise of cat CL at the end of the luteal phases (Zschockelt *et al.* 2014, Amelkina *et al.* 2015a) nor the temporarily limited steroidogenic capacity of prooestrous perCL in lynxes (Zschockelt *et al.* 2015). However, it must be considered that expression on the mRNA level not necessarily reflects expression on the protein level. As no specific antibody was available, the expression pattern of PTGS2/COX2 remains subject for further studies.

The selective production of PGs is generally in favour of PGE₂ rather than PGF_{2α} during luteal development in different species (Vijayakumar & Walters 1983, Olofsson *et al.* 1992). For example, in the bovine and canine CL, PGE₂ synthesis is particularly evident during the phase of luteal formation, likely being involved in CL development and growth (Arosh *et al.* 2004, Kowalewski *et al.* 2015). Similar to other species (Vijayakumar & Walters 1983, Olofsson *et al.* 1992, Miller & Pawlak 1994), and in accordance with the confirmed presence of PTGES mRNA and protein, PGE₂ was the predominant PG measurable in feline luteal tissue, with high concentrations throughout the pregnant and non-pregnant luteal phases of the cat and during the annual cycle of Eurasian lynxes. For PGE₂, several receptors have been described with PTGER2 and PTGER4 being relevant for protecting the CL from luteolytic challenges during early pregnancy in sheep (Lee *et al.* 2012), and PTGER2 being responsible for stimulation of P4 production via regulation of luteal 11βHSD1 activity in humans (Chandras *et al.* 2007). In cattle (Arosh *et al.* 2004) and dioestrous dogs (Kowalewski *et al.* 2008a), PTGER2 mRNA exhibited highest expression in the growing CL, whereas in pregnant dogs, PTGER2 declined *pre-partum* (Kowalewski *et al.* 2013). PTGER4 showed a constant

expression pattern during the canine luteal lifespan (Kowalewski *et al.* 2008a, 2013). The results of our study imply that reception of PGE_2 in the feline CL is mainly independent of the luteal and reproductive stages, suggesting an autocrine/paracrine luteotrophic effect of PGE_2 on the CL maintenance. Sufficient prevention of CL regression by high intra-luteal PGE_2 levels would be in accordance with the resistance of early cat CL to exogenous $\text{PGF}_{2\alpha}$ given on days 4 and 5 or 12 and 13 *p.c.*, which has no effect on circulating P4 levels and the CL size (Wildt *et al.* 1979).

The confirmed high expression of PGE_2 receptors in CL of lynxes, together with substantial annual intra-luteal PGE_2 contents, might be one of the crucial intra-luteal mechanisms fulfilling the luteotrophic requirements for refractoriness of lynx CL to complete structural regression. Our remarkable finding that perCL of Eurasian and Iberian lynxes contain higher intra-luteal PGE_2 levels than freshCL might bring forth a new hypothesis on the luteotrophic role of PGE_2 in the functional and structural persistence of lynx CL. The luteotrophic action of PGE_2 might support the maintained steroidogenic activity of perCL outside the breeding season in Eurasian (Carnaby *et al.* 2012, Painer *et al.* 2014b) and Iberian lynxes (Zschockelt *et al.* 2015). This is most likely associated with elevated serum P4 levels throughout the year, which is presumed to ensure a monoestrous cycle by preventing folliculogenesis and ovulation (Göritz *et al.* 2009, Painer *et al.* 2014b). In addition, and according to our previous hypothesis on perCL as a supplemental source of P4 (Zschockelt *et al.* 2015), our present findings on high intra-luteal PGE_2 synthesis might propose the luteotrophic support of freshCL (P4 synthesis) by CL of previous years (Woshner *et al.* 2001, Jewgenow *et al.* 2014). Direct evidence for this suggested intra-ovarian and paracrine mechanism merits further investigations.

Changes in the ratio of potentially luteotrophic PGE_2 and luteolytic $\text{PGF}_{2\alpha}$ throughout the luteal phase seems to be of major functional relevance. In general, synthesis of luteolytic $\text{PGF}_{2\alpha}$ by the CL is shifted towards the late luteal phase (Wiltbank & Ottobre 2003). Luteal $\text{PGF}_{2\alpha}$ levels increase at the expense of PGE_2 during luteal regression in mice (Sander *et al.* 2008). Similarly, $\text{PGF}_{2\alpha}$ production in pigs rises from the mid to the late luteal phase (Patek & Watson 1976). Interestingly, the capacity of the feline CL to provide $\text{PGF}_{2\alpha}$ was limited and in agreement with low mRNA expression of *PGFS/AKR1C3*; only weak protein signals for *PGFS/AKR1C3* were detected at some CL stages of cats, but no signals were found in CL of lynxes. During pregnancy and dioestrus in dogs, only weak or no intra-luteal *PGFS/AKR1C3* expression was detectable as well (Kowalewski *et al.* 2008b, Gram *et al.* 2013). We conclude that the local luteal production of $\text{PGF}_{2\alpha}$ is presumably not involved in the initiation of the onset of pre-partal and non-pregnant luteal regression in felids. In lynxes, CL persist for at least two years (Painer *et al.* 2014b) and

retain their structural integrity (Jewgenow *et al.* 2012, Amelkina *et al.* 2015b). Thus, the general assumption that functional luteal regression, as initiated by extra-luteal $\text{PGF}_{2\alpha}$, precedes structural luteal regression by intra-luteal amplification of the luteolytic signal (Diaz *et al.* 2002, Hayashi *et al.* 2003), might not fully account for perCL in lynxes, in which the absence of an internal $\text{PGF}_{2\alpha}$ signal might prevent final initiation of the CL demise.

Our results imply that the capacity of feline CL to respond to $\text{PGF}_{2\alpha}$ via expression of *PTGFR* is already acquired after CL formation, but is mainly independent of the luteal stage thereafter, suggesting that luteal regression is triggered by extra-luteal $\text{PGF}_{2\alpha}$. A change in receptivity towards $\text{PGF}_{2\alpha}$ was observed only for non-pregnant cats, in which clear signals of *PTGFR* protein expression were first evident from early regression onwards. Similarly, *PTGFR* mRNA levels increase from the early to the mid luteal phase in the cow (Arosh *et al.* 2004), rabbit (Boiti *et al.* 2001), pig (Boonyaparakob *et al.* 2003) and dog (Kowalewski *et al.* 2008b, 2009). However, in non-pregnant felids, any (external) pregnancy-specific luteolytic signal is missing. For example, in lynxes, neither intra-luteal (this study), faecal and urinary (Finkenwirth *et al.* 2010, Dehnhard *et al.* 2012) nor plasma (Painer *et al.* 2014b) levels show changes in $\text{PGF}_{2\alpha}$ or PGFM profiles, suggesting that the non-pregnant luteal regression is not actively regulated by luteal or endometrial $\text{PGF}_{2\alpha}$. In contrast, for pre-term cats, the placenta appears to be one of the main sources of $\text{PGF}_{2\alpha}$, as placental $\text{PGF}_{2\alpha}$ and its plasma metabolite PGFM are elevated during the last trimester of pregnancy (Siemieniuch *et al.* 2014). Related to this, only during the late pregnancy, $\text{PGF}_{2\alpha}$ seems to play a physiological luteolytic role in felids, i.e. luteotrophic support of CL maintenance might be actively curtailed prior parturition by high expression of *PTGFR*.

In prooestrous lynxes, expression of *PTGFR* was present in luteal cells of perCL. However, $\text{PGF}_{2\alpha}$ of luteal origin is not responsible for signs of early luteal regression in these lynxes and onset of oestrus is not reflected in urinary and faecal PGFM profiles (Finkenwirth *et al.* 2010, Dehnhard *et al.* 2012). Moreover, despite artificially induced regression with cloprostenol (a $\text{PGF}_{2\alpha}$ analogue) outside the breeding season, perCL are preserved in structure for at least two subsequent years and the ovarian blood supply and steroid production reconstitute (Painer *et al.* 2014a,b). Interestingly, also during metoestrus, perCL retain their functional and structural integrity while still expressing receptors for potentially luteolytic $\text{PGF}_{2\alpha}$. It might be hypothesised that next to the lack of a substantial endogenous luteolytic $\text{PGF}_{2\alpha}$ signal, the preservation of the CL structure is further caused by an interference of anti-apoptotic (pro-survival) factors which inhibit transmission of any luteolytic signal via the apoptotic cascade (Amelkina *et al.* 2015b). Thus, despite

expression of PTGFR, the final structural CL demise is prevented and structural integrity is prolonged in lynxes, suggesting that any inherent regression programme, if existent, is not executed as proposed for the cat.

In conclusion, our data implicate a potential role of PGE₂ in maintaining CL persistence in lynxes, thus ensuring their atypical ovarian cycle compared with other felids. Factors that induce the temporal functional luteal regression prior a new breeding season are yet to be identified. To emphasise our hypotheses based on the currently available sample set, additional data are required which is complicated by the conservation status of the analysed species. The observations are therefore limited to the system of the presented animals and do not account for animal-specific individual variations. Although it is difficult to draw more solid conclusions, the present study is an essential prerequisite to develop suitable techniques of artificial ovulation induction for endangered Iberian lynxes.

Declaration of interest

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

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