Glucose transporter 1 expression accompanies hypoxia sensing in the cyclic canine corpus luteum

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Abstract

The canine corpus luteum (CL) functions as a source of progesterone (P₄) and 17β-oestradiol (E₂); however, the transport of energy substrates to maintain its high hormonal output has not yet been characterised. This study involved the localisation and temporal distribution of the facilitative glucose transporter 1 and the quantification of the corresponding protein (GLUT1) and gene (*SLC2A1*) expression. Some GLUT1/*SLC2A1* regulatory proteins, such as hypoxia-inducible factor 1α (HIF1A) and fibroblast growth factor 2 (FGF2); mRNAs, such as *HIF1A*, *FGF2* and vascular endothelial growth factor A (*VEGFA*); and *VEGFA* receptors 1 and 2 (*FLT1* and *KDR*) were also analysed from days 10 to 70 after ovulation. Additionally, plasma P₄ and E₂ levels were assessed via chemiluminescence. Moreover, the canine KDR sequence has been cloned, thereby enabling subsequent semi-quantitative PCR analysis. Our results demonstrate time-dependent variations in the expression profile of *SLC2A1* during dioestrus, which were accompanied by highly correlated changes ($0.84 < r < 0.98$; $P < 0.03$) in the gene expression of *HIF1A*, *VEGF* and *FLT1* as well as in P₄ plasma concentrations. *FGF2* mRNA correlated with E₂ plasma concentrations ($r = 0.61$; $P = 0.01$). Our data reveal that the glucose transporter is regulated throughout the CL lifespan and suggest that CL depends on the sensing of hypoxia and the status of luteal vascularisation. Moreover, time-dependent expression of GLUT1/*SLC2A1* may lie underneath increased metabolic and energetic requirements for sustaining P₄ production.

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Introduction

The reproductive cycle of non-pregnant dogs is distinct from other species and is characterised by a long corpus luteum (CL) phase (dioestrus), which can last over 60–80 days with regression occurring despite the presence of a uterine luteolysin (Hoffmann *et al.* 1992). During this time, the CL undergoes morpho-endocrinological changes in response to gonadotrophic stimuli and local regulatory factors (Hoffmann *et al.* 2004, Kowalewski *et al.* 2008a, 2009, Papa & Hoffmann 2011). Morphological changes following ovulation until late in dioestrus (Sonnack 2009) as well as changes in steroid hormone, prostaglandin and cytokine levels have been described to act as local regulatory factors (Engel *et al.* 2005, Kowalewski *et al.* 2008a, 2008b, Papa &

Hoffmann 2011). The lifespan of the CL depends on energetic supply (Downing *et al.* 1995), which may be regulated by steroid hormones (Frolova *et al.* 2009): in endometrial cells, progesterone (P₄) directly regulates the expression of glucose transporter type 1 (*SLC2A1* gene/GLUT1 protein) through its receptor (Kim & Moley 2009), activating the signalling pathway Akt/PKA/PRKAA and increasing the expression and translocation of GLUT1 (Kim & Moley 2009), the most ubiquitously distributed facilitative glucose transporter responsible for taking up glucose at basal conditions (Palfreyman *et al.* 1992). Besides steroid hormones, the hypoxia-inducible factor 1α (HIF1A) has been implicated as a potent inducer of GLUT1 and of its gene *SLC2A1* (Takagi *et al.*

1998, Sone *et al.* 2000, Zhao & Keating 2007, Kihira *et al.* 2011, Mattmiller *et al.* 2011). HIF1A represents the inducible portion of the HIF1 protein, which is a specialised sensor for variations in oxygen tension in different tissues (Wang & Semenza 1993). The CL is a dynamic endocrine gland formed under low oxygen tension after follicular rupture (Neeman *et al.* 1997, van den Driesche *et al.* 2008), and its HIF1A content oscillates over dioestrus, mirroring that of CL- pO_2 and participating in CL development and regression, which has been shown for both the primate (Duncan *et al.* 2008) and bovine CL (Nishimura *et al.* 2008, 2010).

The formation of the CL and maintenance of its function also strongly depend on the vascular endothelial growth factor A (VEGFA) system (Ferrara *et al.* 1998, Nishimoto *et al.* 2006). The spatio-temporal expression of VEGFA and its receptors, FLT1 and KDR, in the canine CL, has been assessed during dioestrus (Mariani *et al.* 2006), and the results suggest that this angiogenic factor plays a local paracrine and autocrine role. As observed in humans, in mice, as well as in cattle, the luteal VEGFA expression also highly correlates with HIF1A expression levels, which results in the transcription of VEGF (van den Driesche *et al.* 2008, Nishimura & Okuda 2010, Zhang *et al.* 2011). Another important angiogenic factor, fibroblast growth factor 2 (FGF2), has also been shown to regulate GLUT1 expression through HIF1A. Using RNA silencing specific for *HIF1A*, decreased FGF2-induced *SCL2A1* expression was observed in 3T3-L1 adipocytes (Kihira *et al.* 2011), while the expression of FGF2 in canine ovaries, specifically during follicular development, has been reported (Songsasen *et al.* 2009); to the best of our knowledge, no time-related changes during dioestrus have been reported for canine CL. Similarly, no data have been published on the effect of pO_2 , the expression of HIF1A or its possible association with the expression of glucose transporters and angiogenic factors in the CL of non-pregnant bitches.

Therefore, we hypothesised that the expression of GLUT1 in the canine CL is associated with time-related vascular and oxygen changes during dioestrus. To test this hypothesis, the spatio-temporal expression of GLUT1, the regulatory factors HIF1A, FGF2 and VEGFA, as well as the VEGFA receptors FLT1 and KDR (VEGFA system) were investigated and the expression patterns correlated with the steroid hormone profile during dioestrus.

Materials and methods

Dogs and experimental design

Twenty-eight healthy mongrel female dogs belonging to 'Santuário' dog shelter (Sao Paulo, Brazil) were included in the study. After the onset of pro-oestrous bleeding, blood samples were collected every other day to determine the

P_4 concentrations. The day of ovulation was considered the day when P_4 plasma concentrations reached 5 ng/ml (Concannon *et al.* 1989). Seven groups ($n=4$ animals per group) were established. The CLs were harvested via ovariectomy on days 10, 20, 30, 40, 50, 60 and 70 after ovulation (p.o.), covering the entire period of dioestrus. The experimental protocol, including anaesthesia (acepromazine, 0.2 mg/kg i.m.; xylazine, 3 mg/kg and ketamine, 5–8 mg/kg i.v.) and surgery, was approved by the Committee of Ethics in the Use of Animals of the Faculty of Veterinary Medicine and Animal Science, University of Sao Paulo, Sao Paulo, Brazil (protocol number 1432/2008). After collection, the CL were dissected from the surrounding ovarian tissue and immediately frozen in liquid nitrogen for total RNA and protein extraction or fixed in 4% buffered formalin for 24 h for immunohistochemistry. The following parameters were assessed: VEGFA, FLT1, KDR, SCL2A1, HIF1A and FGF2 mRNA expression and GLUT1, HIF1A and FGF2 protein expression. Blood samples (5 ml) were collected on the day of surgery prior to anaesthesia for measurement of 17β -oestradiol (E_2) and P_4 levels.

Molecular cloning of canine KDR

As the sequence of canine KDR has not yet been reported, homology cloning was performed as described previously (Kowalewski & Hoffmann 2008), using mRNA derived from four different bitches to determine the cDNA coding of canine KDR. Primers for qualitative PCR were derived from the alignment of the predicted canine KDR sequence (GI: 73975290; www.ncbi.nlm.nih.gov/genome/guide/dog/index.html) with predicted bovine and available mouse and pig sequences (GenBank accession numbers: bovine GI: 61884824; mouse GI: 27777647 and pig GI: 7160277). A 4068-bp cDNA fragment PCR product of the predicted open reading frame (ORF) of canine KDR was amplified. Total RNA was isolated from canine CL with TRIzol Reagent according to the manufacturer's protocol (Gibco-BRL, Life Technologies). DNase treatment and RT-PCR were performed using the GeneAmp Gold RNA PCR Kit (Perkin-Elmer Applied Biosystems GmbH, Weiterstadt, Germany) as described previously (Kowalewski *et al.* 2006). The annealing temperature was 58.5 °C. PCR products were separated on a 2% ethidium bromide-stained agarose gel, purified using the Qiaex II gel extraction system (Qiagen GmbH), ligated into the pGEM-T vector (Promega), amplified in XL1 BLUE competent cells (Stratagene, La Jolla, CA, USA) and sequenced on both strands (SRD, Oberursel, Germany) with T7 and Sp6 sequencing primers. For every experiment, autoclaved water was used instead of RNA as negative control. RNA integrity and the assay procedure were tested via amplification of the reference gene glyceraldehyde-3-phosphate dehydrogenase. Finally, the cloned cDNA sequence was submitted to GenBank with access number DQ269018.1 *Canis familiaris* KDR, complete cds. The prediction of the amino acid sequence and sequence comparison was performed using ChromasPro version 1.2 oligo Software (Technelysium Pty. Ltd., Brisbane, Queensland, Australia).

RNA extraction and RT

The CLs were homogenised with TRIzol Reagent using a Bio-gen Pro 200 homogeniser (Pro Scientific, Inc., Oxford, CT, USA). This step was followed by chloroform/isopropanol/ethanol extraction, as recommended by the manufacturer (Invitrogen). RNA concentration and quality were assessed via spectrometry using a BioPhotometer (Eppendorf, Hamburg, Germany), and integrity verification was performed with a 2% agarose gel. Following the DNase treatment, 1 µg total RNA per sample was reverse transcribed using a Superscript III Kit (Invitrogen).

Real-time PCR

Real-time PCR analyses were performed using an ABI 7500 Sequence Detection System (PE Applied Biosystems) as described previously (Campos *et al.* 2010) with some modifications to match dog-specific conditions, such as the low concentrations of available cDNA. *SLC2A1*, *HIF1A*, *VEGFA*, *FLT1*, *KDR* and *FGF2* primers and probes were designed using the Assays-by-Design service from Applied Biosystems (Table 1). Cyclophilin A was used as the reference gene, as determined by NormFinder Software (Arhus, Århus, Denmark; Andersen *et al.* 2004). In 96-well plates, 125 ng cDNA diluted in 2.5 µl nuclease-free water were added to a mixture containing 6.25 µl TaqMan Universal PCR Master Mix buffer (2×, Applied Biosystems). Additionally, 0.5 µl of both forward and reverse primers (900 mM), 250 mM probe (Applied Biosystems) and 3.25 µl water were added to a final volume of 12.5 µl. All plates were sealed with MicroAmp Optical Adhesive Covers (Applied Biosystems) following the complete mixture of all reagents and subsequent centrifugation. The following cycling parameters were applied: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 15 s at 95 °C (denaturation) and 1 min at 60 °C (annealing and extension). PCR assays for target genes were conducted at least in quadruplicate for each sample, and the expression level was determined using relative quantification via linear regression of the fluorescence data. The ratios were calculated using the equation 'relative expression = N_0 (target gene)/ N_0 (cyclophilin A)' with N_0 values calculated using the LinRegPCR 7.0 (Linear Regression PCR) program (Ramakers *et al.* 2003, Roussel *et al.* 2007), followed by calculation using the $2^{-\Delta\Delta C_t}$ method (Kowalewski *et al.* 2006).

The log-linear portion of the curve used for analysis contained four to six points, with the highest R^2 value and efficiency of amplification considered as 100%.

Immunohistochemistry

Localisation of GLUT1, HIF1A and FGF2 expression was assessed via IHC on 2 µm tissue sections prepared from four CL per dog using one section per CL and four dogs per group to assure description accuracy. The antisera used were as follows: for GLUT1, a polyclonal antibody raised in goat against human (C-20, sc-1605, dilution 1:150, Santa Cruz Biotechnology); for HIF1A, a polyclonal antibody raised in rabbit against human (NB100-134, dilution 1:400, Novus Biologicals, Littleton, CO, USA); and for FGF2, a mouse MAB (clone bFM-2, dilution 1:100, Upstate, Lake Placid, NY, USA). Standard procedures for embedding and processing CL tissue have been described previously (Mariani *et al.* 2006). Negative controls were prepared using IgG isotype control antibodies (Normal rabbit IgG for HIF1A and normal goat IgG for GLUT1; Santa Cruz Biotechnologies), while positive controls were human placenta for GLUT1 (Leon-Villapalos *et al.* 2005) and HIF1A (according to the manufacturer's protocol) and bovine placenta for FGF2 (Prado *et al.* 2004).

Western blotting and quantification

Luteal homogenates were prepared with NET-2 lysis buffer (50 mM Tris-HCl (pH 7.4), 300 mM NaCl and 0.05% NP-40) containing 1 µl/ml protease inhibitor cocktail (Sigma-Aldrich) using a Polytron (Brinkmann Instruments, Westbury, NY, USA) and were centrifuged at 10 000 *g* for 10 min at 4 °C. The supernatants were collected, and the protein concentrations were determined using the Bradford method (Bradford 1976). Total protein was solubilised in sample buffer (25 mmol/l Tris-Cl, pH 6.8, 1% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue) and incubated at 95 °C for 5 min prior to loading onto the gel. Twenty-five micrograms of protein were loaded onto 8 or 12% SDS-polyacrylamide gels (Mini Protean II System, Bio-Rad Laboratories, Inc.) for the quantification of HIF1A and GLUT1 protein respectively. After electrophoresis, the proteins were transferred onto a PVDF membrane (Bio-Rad Laboratories, Inc.), and the membrane was

Table 1 List of primers for real-time (TaqMan) PCR.

Gene	Primers	Sequences	Probes	GenBank numbers
<i>SLC2A1</i>	Forward	5'-CAGCCAGAGTCCCCTGTATCTA-3'	CACCCCAGACTTCACC	XM539554
	Reverse	5'-GTCCCCACCTTCAGGTACTG-3'		
<i>HIF1A</i>	Forward	5'-GCTGCTGGAGACACAATCATATCTT-3'	CAATGACACAGAACTG	XM860420
	Reverse	5'-ACATCATTATACAACGGAACCTCCTCAAG-3'		
<i>VEGFA</i>	Forward	5'-GTGCCCACTGAGGAGTTCAAC-3'	CACCATGCAGATTATGCGGATCAAACC	AF133248
	Reverse	5'-CCCTATGTGCTGGCCTTGAT-3'		
<i>FLT1</i>	Forward	5'-TGCCTGAAACAGTGAGAAAGGA-3'	AAAGGCTGAGCATTACTAAATCTGCCT	XM534520
	Reverse	5'-TGCAGAACTGTTTGCCATTCC-3'		
<i>KDR</i>	Forward	5'-TGAGATGGCCTCGGTCATT-3'	CTACGTTCAAGATTACAGGTCTCCATT	DQ269018
	Reverse	5'-TGTTGGTCGCTAACAGAAGCA-3'		
<i>FGF2</i>	Forward	5'-CCGGTCAAGGAAATACTCCAGTTG-3'	TATGTGGCACTGAAACGA	AF060562.1
	Reverse	5'-GGTCCTGTTTGGGTCCAAGTTTAT-3'		
Cyclophilin A	Forward	ID Cf03986523_gH		XM_843327.1

incubated in blocking buffer containing 5% non-fat dry milk for 1 h at room temperature. The membrane was then probed with primary antibodies (anti-GLUT1, 1:500, C-20, sc-1605, Santa Cruz Biotechnology; anti-HIF1A, dilution 1:500, NB100-134, Novus Biologicals and anti-ACTB, Clone AC-15, dilution 1:500, A1978, Sigma-Aldrich Co., as the reference protein) overnight at 4 °C, followed by probing with Ig-HRP antibodies (anti-rabbit IgG for HIF1A and ACTB and anti-goat IgG for GLUT1; Amersham Biosciences) diluted in a 2.5% non-fat dried milk solution for 1 h at room temperature. The immunocomplexes were then visualised using ECL, as recommended by the manufacturer (Amersham Biosciences). The validation of both antibodies for canine tissue was performed using human placenta. The proteins were examined by measuring the specific bands using a ChemiDoc MP Imaging System (Bio-Rad Laboratories) and normalised to β -actin (ACTB; 42 kDa) using ImageJ Software (Bio-Rad Laboratories).

Determination of plasma P_4 and E_2 concentrations

After collection, blood samples (5 ml) were centrifuged for 10 min at 300 g, 4 °C. The serum samples were stored at -20 °C until P_4 and E_2 determination by validated chemiluminescence immunoassay. The low limit detection of the P_4 and E_2 assays was 0.03 ng/ml and 0.5 pg/ml respectively. The inter-assay coefficient of variation (CV) for P_4 and E_2 was 6.93 and 7.03% respectively. The intra-assay CV was <10% for both hormones.

Statistical analysis

All the experiments were performed at least three times with a minimum of three repeats, including western blotting analyses. Dependent variables (i.e. steroid production and gene and protein expression of SLC2A1/GLUT1, HIF1A, VEGFA, FLT1, KDR and FGF2) were analysed by Kruskal–Wallis test followed by Dunn's multiple comparison test. The correlations between hormone concentrations and gene or protein expression levels were determined using the Pearson's Correlation test. Response variables were tested according to their homogeneity and normality of variances. Data are presented as mean $\pm$ S.E.M. Statistical analyses were performed using the GraphPad Prism 4 program (GraphPad Software, Inc.). Differences with P value <0.05 were considered significant.

Results

Homology cloning of canine KDR

The available predicted sequence of canine KDR was used for the homology cloning procedure. Consequently, the canine-specific sequence was amplified, cloned and submitted to GenBank under access number GI: 82548234. This sequence comprises 4068 nucleotides of a predicted ORF of canine KDR, which encodes a 1355 amino acid protein with a deduced molecular weight of 152 kDa. BLAST analysis showed 97, 92, 90 and 85% homology with human (GI: 231570519), pig

(GI: 7160277), bovine (GI: 61884824) and mouse (GI: 27777647) KDR sequences respectively. According to the published canine genome sequence, the KDR sequence is located on chromosome 13.

Expression of SCL2A1-, HIF1A-, FGF2-, VEGFA-, FLT1- and KDR mRNA

The expression of six target genes directly involved in the response of the CL against fluctuations in substrate availability and oxygen tension in luteal cells was investigated. Dioestrus had an effect of time on the expression of *SLC2A1* ($P=0.002$), *HIF1A* ($P<0.001$), *FGF2* and the *VEGF* system ($P=0.002$; Fig. 1). *SLC2A1* (Fig. 1A) and *HIF1A* (Fig. 1B) expression levels increased on day 20 p.o., decreased at mid dioestrus (days 30 and 40 p.o.), increased again on days 50 and 60 p.o. ($P<0.05$) and were significantly down-regulated at the beginning of anoestrus (day 70 p.o.; $P<0.05$). The *FGF2* expression increased on day 30 p.o. and decreased thereafter until day 60 p.o. ($P<0.05$; Fig. 1C). *VEGFA* (Fig. 1D) and *FLT1* (Fig. 1E) expression levels were significantly elevated on days 20 and 60 p.o., while the *KDR* expression levels were elevated on days 20 and 40 p.o. ($P<0.05$; Fig. 1F).

Spatial expression of GLUT1, HIF1A, and FGF2 assessed via IHC

GLUT1 and HIF proteins were localised in the dog CL over dioestrus. Immunopositive staining could be observed restricted to the cytoplasm of luteal,

Figure 1 *SLC2A1* (A), *HIF1A* (B), *FGF2* (C), *VEGFA* (D), *FLT1* (E) and *KDR* (F) expression levels in the canine CL ($n=28$; four per group) determined via real-time (TaqMan) PCR. RGE, relative gene expression (mean $\pm$ S.E.M.) normalised to cyclophilin A. Bars with different letters represent significant differences ($P<0.05$); 10–70: days after ovulation.

endothelial and stromal cells in all phases studied (days 10–70 p.o.; Figs 2 and 3). Besides the cytoplasm staining, the nuclear staining of HIF1A from day 30 until 60 could also be observed. However, no positive staining could be observed in the negative control. Positive control (human placenta, PC; Figs 2 and 3) showed a marked positive staining.

Positive immunostaining for FGF2 was observed in the cytoplasm of luteal, endothelial and stromal cells (Fig. 4); positive staining was observed primarily in stromal cells on day 10 p.o. and in luteal cells on day 20 p.o. By day 30 p.o., luteal cells displayed marked positive immunostaining, while stromal cells were primarily negative. By days 50 and 60, FGF2 signals appeared weaker; however, at day 70, the cytoplasmic staining levels were evident again. Bovine CL served as positive control (Prado *et al.* 2004), and the negative control revealed no staining for FGF2 (Insert on Fig. 4).

Quantification of GLUT1 and HIF1A protein expression via western blotting and the correlation with the respective gene expression levels

We observed that time had an effect on the expression of both GLUT1 ($P=0.04$) and HIF1A ($P<0.0001$; Fig. 5). GLUT1 expression increased on day 20 ($P<0.05$), decreased on day 30 and showed a tendency to increase on day 40 p.o., but no further variations until day 70 p.o. (Fig. 5B; $P>0.05$) were observed. This expression pattern highly correlates with the *SCL2A1* expression during the first half of dioestrus ($r=0.83$; $P=0.009$). The HIF1A expression (Fig. 5D) showed an increase on day 30 p.o. followed by a further increase on day 60 p.o.

Figure 2 GLUT1 staining in the canine CL assessed by immunohistochemistry. Positive signals (orange–brown colour) were observed in the cytoplasm of luteal (L), endothelial (E) and stromal (S) cells at days 10, 20, 30, 40, 50, 60 and 70 after ovulation. PC, positive control (human placenta); NC, negative control. Bars=50 μ m.

Figure 3 HIF1A staining in the canine CL assessed by immunohistochemistry. Positive signals in the cytoplasm (black arrows) and the nucleus (white arrows) of luteal cells (L) were observed on days 10, 20, 30, 40, 50, 60 and 70 after ovulation. PC, positive control (human placenta); NC, negative control. Bars=50 μ m.

and a final decrease on day 70 p.o. ($P<0.05$). In contrast to GLUT1, HIF1A protein was negatively correlated with its mRNA expression in the same period ($r=-0.86$; $P=0.005$).

Plasma steroid hormone profiles

P_4 plasma concentrations were highest on day 20 and displayed a distinct and continuous decrease thereafter (Fig. 6A; $P<0.05$). The concentrations of E_2 increased from days 10 to 40 p.o. followed by a gradual decrease thereafter (Fig. 6B; $P<0.05$).

The correlation between hormone concentrations and gene expression

A significant positive correlation during the first half of dioestrus was observed between P_4 levels and the expression levels of all genes studied (Table 2), except for *FGF2*, whose expression pattern positively correlated with E_2 levels throughout the entire observation period ($r=0.61$; $P=0.01$). Positive and significant correlation was observed among the expressions of *HIF1A* and *SCL2A1*, *HIF1A* and *VEGF*, *VEGFA* and *FLT1*, *VEGF* and *SCL2A1*, and *FLT1* and *SCL2A1* (Table 2).

Correlations of hormone concentrations and protein expression assessed by western blotting

During the first half of dioestrus, the GLUT1 expression was positively correlated with P_4 plasma concentration ($r=0.86$; $P=0.005$) and negatively correlated with the expression of HIF1A ($r=-0.77$; $P=0.02$), while both

Figure 4 FGF2 staining in the canine CL assessed by immunohistochemistry. Positive signals were observed as indicated by the brown colour in the cytoplasm of luteal (L), endothelial (E) and stromal (S) cells at 10, 20, 30, 50, 60 and 70 days after ovulation. Bars = 20 µm.

proteins showed no significant correlation with the E_2 plasma concentration ($r = -0.15$; $P = 0.73$ and $r = -0.38$; $P = 0.39$ respectively).

Discussion

According to our hypothesis, the expression of HIF1A at the mRNA level, which is a factor sensitive to decreased tissue/cell O_2 concentrations, was positively correlated with the expression of *SLC2A1*, *VEGFA*, the *VEGFA* receptor *FLT1* and *FGF2* during the entire period of dioestrus. Additionally, the mRNA expression of these factors accompanied fluctuations of P_4 concentrations during the first half of dioestrus, thereby indicating the importance of these factors during CL formation.

These observations of the up-regulation of the 'glucose transport system' matching that of factors that play a role in angiogenesis raise the question of the underlying triggering mechanisms. Although hypoxia occurs during the initial phase of CL formation (Nishimura *et al.* 2010), the function of P_4 and perhaps that of E_2 should also be considered.

In addition to its function as an endocrine factor, the observations in cattle suggest that P_4 may also act as a local luteotrophic factor, capable of exerting a positive feedback loop on its receptor (Schams & Berisha 2004). Observations in dogs, in which the application of a P_4 receptor inhibitor results in preterm luteolysis, further support the hypothesis concerning a luteotrophic function of P_4 in the canine CL (Kowalewski *et al.* 2009, 2010).

It has also been reported that P_4 directly regulates *SCL2A1* expression in endometrial cells (Frolova *et al.* 2009, Kim & Moley 2009) via its own receptor (Frolova *et al.* 2009), thereby activating the intracellular signalling pathway and enhancing GLUT1 expression levels and translocation to the plasma membrane (Kim & Moley 2009). Both studies suggest an opposite function

for E_2 , i.e. the down-regulation of *SCL2A1*/GLUT1, when tested alone or in combination with P_4 . Moreover, blocking the oestrogen receptor 1 (ESR1) with ICI 182 780 restored the expression of *SCL2A1*/GLUT1 (Kim & Moley 2009). Incorporating these data into the context of the canine luteal function, it is possible that P_4 acts in the same way by enhancing *SCL2A1*/GLUT1 expression via signalling through its receptor (Hoffmann *et al.* 2004, Papa & Hoffmann 2011), which results in an increased availability of glucose, thereby reinforcing the concept that increased luteal glucose uptake (Palfreyman *et al.* 1992) corresponds to a higher steroidogenic output. In contrast to what has been observed for the dog CL in this study, Nishimoto *et al.* (2006) described, in cattle, no cycle-related *SCL2A1* expression in the early or mid luteal phase but a decrease in the late luteal phase. These differences may also rely on the local interplay between P_4 and E_2 , which reaches its highest levels in cows when the CL regresses, but not during the

Figure 5 Protein expression of GLUT1 (A and B) and HIF1A (C and D) in the canine CL during dioestrus ($n = 28$; four animals per group). Electrophoresis images are representative of three independent experiments. The total protein isolated from luteal homogenates was examined with specific antibodies and normalised to ACTB expression. The data are expressed as the mean $\pm$ S.E.M. Bars with different letters indicate a significant difference ($P < 0.05$).

Figure 6 Plasma P₄ (ng/ml; A) and E₂ (pg/ml; B) profiles during dioestrus determined via chemiluminescence ($n=28$; four animals per group). The data are presented as the mean $\pm$ S.E.M. Asterisks or different letters indicate a significant difference ($P<0.05$).

early or mid luteal phase, in which P₄ governs alone (revised by Sartori & Barros (2011)).

E₂ levels were positively correlated with the *FGF2* expression pattern, which seems to contribute to maintaining angiogenesis when VEGFA starts decreasing. Both angiogenic factors play complementary roles in CL vascularisation, as already described for cattle (Schams & Berisha 2004), and VEGFA responds to hypoxia (Neeman *et al.* 1997). Additionally, Kihira *et al.* (2011) described FGF2 as an enhancer of *SCL2A1* expression in cultured 3T3-L1 adipocytes, but further studies are necessary to characterise species and tissue specificities concerning the function of FGF2 in this respect.

The role of hypoxia during CL formation was recently addressed in cattle (Nishimura *et al.* 2010) and seemed to relate directly to the formation of the vascular bed by inducing the expression of VEGF. VEGFA protein and its receptors are expressed in the canine CL throughout dioestrus with a decrease after day 40 p.o. and unaltered receptor (FLT1 and KDR) protein expression (Mariani *et al.* 2006), which was confirmed in this study for expression at the mRNA level. In addition to its paramount role in angiogenesis, VEGFA may also be considered a luteotrophic factor, as it enhances P₄ production in bovine placental cells *in vitro* (Sousa *et al.* 2012) and induces STAR expression in bovine luteal cells (Yamashita *et al.* 2008). In this study, VEGFA expression

was highly correlated, at the mRNA level ($r=0.85$; $P=0.01$), with plasma P₄ levels, thereby suggesting the function of a luteotrophic factor in dogs. The *HIF1A* expression accompanied that of *VEGFA* and *SCL2A1* as stated above. According to our findings, the canine CL detected the first O₂ imbalance at day 30 p.o., as revealed by western blotting analysis of HIF1A, which did not decrease until day 60 p.o., when it experienced a further and abrupt increase. This corroborates with findings by Hoffmann *et al.* (2004), who observed a decreased density of luteal cells from day 30 of dioestrus onwards. Similarly, Nishimura *et al.* (2008) reported a decrease in blood perfusion to bovine CL followed first by hypoxia and then by functional and finally structural luteolysis. The presence of the HIF1A protein in the nuclei on day 50 p.o., concomitant to increased levels of *HIF1A* mRNA and subsequent to increased HIF1A protein on day 60 p.o., matches a moment in the CL lifespan, in which levels of luteal P₄ are decreasing and first signs of structural regression can be observed (Sonnack 2009). The correlation observed between the expression levels of *HIF1A*, *VEGFA* and *SCL2A1* at the mRNA level suggests a functional inter-relationship that results in the formation of GLUT1 protein. However, further evidence must be provided, and the sequence of events is unclear.

While our data concerning expression at the mRNA level seem to yield a rather clear picture, the whole situation becomes somewhat less clear when taking the protein data into account. Therefore, the concentrations of GLUT1 protein seem to be parallel to some extent to the relative gene expression of *SCL2A1*. Concerning the HIF1A protein, there was a negative correlation with its

Table 2 Correlation coefficients between steroid production and gene expression in canine CL during dioestrus.

Variables	Pearson's r	P value
P ₄ vs <i>HIF1A</i>	0.78	0.03 ^a
P ₄ vs <i>SLC2A1</i>	0.79	0.03 ^a
P ₄ vs <i>VEGFA</i>	0.85	0.01 ^a
P ₄ vs <i>FLT1</i>	0.90	0.005 ^a
P ₄ vs <i>KDR</i>	0.76	0.04 ^a
P ₄ vs <i>FGF2</i>	0.05	0.84
E ₂ vs <i>HIF1A</i>	-0.38	0.39
E ₂ vs <i>SLC2A1</i>	-0.15	0.73
E ₂ vs <i>VEGFA</i>	-0.15	0.73
E ₂ vs <i>FLT1</i>	-0.21	0.64
E ₂ vs <i>KDR</i>	0.38	0.39
E ₂ vs <i>FGF2</i>	0.61	0.01 ^b
<i>HIF1A</i> vs <i>SLC2A1</i>	0.93	0.002 ^b
<i>HIF1A</i> vs <i>VEGFA</i>	0.98	0.03 ^b
<i>HIF1A</i> vs <i>FLT1</i>	0.84	0.01 ^b
<i>HIF1A</i> vs <i>FGF2</i>	-0.37	0.18
<i>SLC2A1</i> vs <i>VEGFA</i>	0.84	0.001 ^b
<i>SLC2A1</i> vs <i>FLT1</i>	0.83	0.007 ^b
<i>SLC2A1</i> vs <i>FGF2</i>	-0.21	0.46
<i>VEGFA</i> vs <i>FLT1</i>	0.93	0.002 ^b
<i>VEGFA</i> vs <i>KDR</i>	0.53	0.21
<i>VEGFA</i> vs <i>FGF2</i>	0.17	0.55

^aCorrelated in first half of dioestrus (days 10, 20 and 30 p.o.).

^bCorrelated throughout dioestrus.

expression at the mRNA level; this observation suggests other post-translational regulatory factors, which may become activated in the ageing CL, and stresses the likely importance of P₄ concerning the up-regulation of *SCL2A1*, as was emphasised by observations on day 20, indicating that a function-associated O₂ imbalance may not occur prior to approximately day 60, which represents the time point when [Sonnack \(2009\)](#) observed the first signs of structural luteolysis.

By presenting the expression of GLUT1 and its associated regulatory proteins, our data suggest new regulatory factors possibly involved in canine CL. This concerns mostly the first half of dioestrus, on the one hand, when the canine CL is being formed and passes through its highest steroidogenic and metabolic capacity. On the other hand, for the second half of canine dioestrus ([Okkens et al. 1990](#)), prolactin appears to be the most important luteotrophic factor.

In conclusion, our data reveal a local time-dependent system comprising hypoxia sensing, induction of angiogenesis and glucose uptake, orchestrated by or influencing P₄ secretion, especially in the first half of dioestrus in the canine CL.

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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