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# Prostaglandin $E_2$ functions as a luteotrophic factor in the dog

Mariusz P Kowalewski[1], Barbara Fox[1], Aykut Gram[1], Alois Boos[1] and Iris Reichler[2]

[1]*Institute of Veterinary Anatomy and* [2]*Section of Small Animal Reproduction, Clinic of Reproductive Medicine, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland*

*Correspondence should be addressed to M P Kowalewski; Email: kowalewski@vetanat.uzh.ch; kowalewskipl@yahoo.de*

## Abstract

**The luteal phase in dogs is governed by many poorly understood regulatory mechanisms. Functioning of the corpus luteum (CL) is unaffected by hysterectomy. Recently, the role of prostaglandins in regulating canine CL function was addressed suggesting a luteotrophic effect of prostaglandin $E_2$ ($PGE_2$) during the early luteal phase. However, compelling functional evidence was lacking. The potential of $PGE_2$ to stimulate steroidogenesis was tested in canine primary luteal cells isolated from developing CL of non-pregnant dogs. In addition, the luteal expression of prostaglandin transporter (PGT) and steroidogenic acute regulatory protein (STAR) was demonstrated and characterized in CL from non-pregnant bitches during the course of dioestrus as well as from pregnant animals during the pre-implantation, post-implantation and mid-gestation periods of pregnancy and during luteolysis; the luteal expression of $PGE_2$ receptors (EP2 and EP4) has been investigated at the protein level throughout pregnancy. Our findings show that $PGE_2$ is an activator of STAR expression in canine luteal cells from early luteal phase, significantly up-regulating STAR promoter activity and protein expression resulting in increased steroidogenesis. The 3βHSD (HSD3B2) and P450scc (CYP11A1) expression remained unaffected by $PGE_2$ treatment. The expression of PGT was confirmed in CL during both pregnancy and dioestrus and generally localized to the luteal cells. After initial up-regulation during the earlier stages of the CL phase, its expression declined towards the luteal regression. Together with the demonstration of EP2 and EP4 throughout pregnancy, and the decline in EP2 at *prepartum*, our findings further support our hypothesis that intra-luteal $PGE_2$ may play an important role in regulating progesterone secretion in the canine CL.**

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## Introduction

The domestic dog (*Canis familiaris*) is a monoestrous breeder with an oestrus cycle governed by regulatory mechanisms that distinctly differ from other domestic animal species. The length of the luteal phase is almost identical in pregnant and non-pregnant bitches until shortly before parturition and *prepartum* luteolysis (Concannon *et al*. 1989). The steep progesterone ($P_4$) decline observed in pregnant animals before parturition suggests different regulatory mechanisms for the prepartal luteolysis, contrasting with mechanisms regulating the slow luteal regression observed in non-pregnant bitches during late dioestrus. The latter appears to be a passive degenerative process in the absence of an active luteolytic agent of uterine and/or luteal origin (Hoffmann *et al*. 2004, Kowalewski *et al*. 2008*b*). In this way, the lifespan of the canine corpus luteum (CL) in non-pregnant bitches often exceeds that of pregnancy, which is a peculiarity when compared with other domestic animals and primates. Moreover, in the dog, as in primates, normal ovarian cyclicity is maintained after hysterectomy (Olson *et al*. 1984, Hoffmann *et al*. 1992). This, together with the fact that CL are the only source of $P_4$ both during the oestrus cycle and in pregnancy (Concannon *et al*. 1989), further supports the crucial role of CL-maintaining factors in regulating reproductive function in this species.

The canine luteal phase can be divided into two periods: i) the early luteal phase, lasting 2–4 weeks of steadily increasing $P_4$ concentrations, highest proliferation rate and resistance to luteolytic treatments that are effective later in the cycle (Okkens *et al*. 1986, Concannon *et al*. 1987) and ii) the mature-CL portion of phase, during which the CL is more responsive to luteal treatments. Both LH and prolactin (PRL) are luteotrophic (Concannon 1980, Onclin *et al*. 2000). For the mature CL in the second half of dioestrus, PRL becomes the main luteotrophic hormone (Concannon *et al*. 1987, Okkens *et al*. 1990), blocking LH function, however, results only in a transient $P_4$ decrease (Concannon *et al*. 1987, Onclin *et al*. 2000). Luteal regression, however, is unavoidable and occurs in spite of an increased gonadotrophic support. Recently, it was suggested that the $P_4$ production in later stages of the luteal phase could be controlled upstream of the steroidogenic machinery, due to PRL receptor-mediated effects (Kowalewski *et al*. 2011*b*). Nevertheless, the canine CL appears to possess an inherent lifespan that is likely regulated locally through

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paracrine and/or autocrine mechanisms. In this regard, the luteotrophic requirements during the first 15–25 days after ovulation appear to be especially important. This is the time when canine CL reveal their maximal $P_4$ secretion capacity and resistance to luteolytic insult. Among many local factors potentially playing a role in regulating the canine CL function are CD4 and CD8 lymphocytes and cytokines produced by them (IL8, IL10, IL12, TNF and TGFβ1), as well as trophic factors (IGF and VEGF) (Hoffmann *et al*. 2004, Engel *et al*. 2005, Mariani *et al*. 2006). Acting in an auto/paracrine manner, steroidogenic hormones, i.e. $P_4$ and oestrogens, also appear to be possible luteotrophic factors (Papa 2001, Papa & Hoffmann 2011). This idea is further supported by the observation that treatment with an antigestagen leads to preterm luteolysis (Kowalewski *et al*. 2009*a*, 2010*a*). Moreover, we recently showed that the early formation of the canine CL is characterized by an increased expression of cyclooxygenase 2 (COX2, PTGS2), the inducible key enzyme in the supply of prostaglandins, and a strongly up-regulated expression of prostaglandin $E_2$ ($PGE_2$)-synthase (PGES) (Kowalewski *et al*. 2006*b*, 2008*a*, 2009*a*). Thus, in the early canine CL, the luteotrophic effects of luteal prostaglandins, possibly due to $PGE_2$ activity, are strongly implicated as suggested in several other species, e.g. pig, sheep and human (Christenson *et al*. 1994, Weems *et al*. 1997, Harris *et al*. 2001), in which stimulatory effects of $PGE_2$ on $P_4$ production, mediated through the cAMP/PKA pathway, were reported. Therefore, and in view of observations made in other species (Christenson *et al*. 1994, Weems *et al*. 1997, Harris *et al*. 2001), together with the significantly elevated expression of COX2 and PGES during the early luteal phase in the dog (Kowalewski *et al*. 2006*b*, 2008*a*, 2009*a*), we felt prompted to investigate whether $PGE_2$ acts to increase steroidogenesis in canine luteal cells during this time.

The $PGE_2$-coupled formation of cAMP is primarily regulated through two $PGE_2$ receptors, PTGER2 and PTGER4 (also known as EP2 and EP4), the expression of which has also been reported in canine CL (Kowalewski *et al*. 2008*a*, 2009*a*). The concomitantly increased expression of 3β-hydroxysteroid dehydrogenase Δ4/5isomerase (3βHSD, HSD3B2) and steroidogenic acute regulatory protein (STAR) (Kowalewski *et al*. 2006*a*, Kowalewski & Hoffmann 2008), the two major regulators in the generation of $P_4$, in the earlier stages of the canine CL lifespan, makes the hypothesis on the luteotrophic function of the $PGE_2$ within the canine CL even more plausible. However, compelling functional evidence is still lacking. Thus, in order to address this hypothesis and to obtain the first functional insights into possible molecular mechanisms regulating canine steroidogenesis, we established a culture system for primary luteal cells and used that model to determine the potential of $PGE_2$ to induce steroidogenesis in primary luteal cells isolated from CL during the early luteal phase. We also examined the capacity of luteal cells from pregnant bitches to express $PGE_2$ receptors and STAR protein. Additionally, expression of the prostaglandin transporter (PGT) required for $PGE_2$ paracrine function (Arosh *et al*. 2004) was investigated in canine CL from non-pregnant dogs during the course of dioestrus and from pregnant bitches during pre-implantation, post-implantation and mid-gestation periods of pregnancy and during *prepartum* luteolysis.

## Results

### *$PGE_2$ enhances STAR promoter activity in canine primary luteal cells*

Primary luteal cells isolated from canine CL collected during the early luteal stage of dioestrus were stained using anti-STAR and anti-3βHSD antibodies verifying their basal steroidogenic capability (Fig. 1). The sequence of the canine *STAR* promoter has not been reported before our work. Here, the proximal *STAR* −255/−1 bp promoter fragment was isolated and characterized (Fig. 2A). To determine whether $PGE_2$ affected canine *STAR* gene expression, the promoter was subcloned into a pGL3-Basic plasmid and used for transfection of luteal cells in the luciferase assay. In order to demonstrate the functionality of the promoter and to validate the assay, cells were stimulated with increasing doses of dbcAMP. Promoter activity displayed a dose-dependent response to dbcAMP with maximal values corresponding to 2.5-fold ($P<0.05$) and 3.6-fold ($P<0.01$) induction over untreated controls reached with 0.5 and 1.0 mM dbcAMP respectively (Fig. 2B). Similarly, $PGE_2$ was able to significantly ($P<0.01$) increase *STAR* promoter activity when used at 20 μM (Fig. 2C) demonstrating that $PGE_2$ is an activator of *STAR* gene expression in canine primary luteal cells at levels that are similar to those observed with dbcAMP treatment. In order to compare the effects of $PGE_2$ on steroidogenesis in cells isolated from later stages of dioestrus, experiments were performed with cells derived from days 25 to 65 post-ovulation (p.o.), i.e. when the $P_4$ levels start to decline and the slow luteal regression is in progress. As in all our cell culture trials, the steroidogenic capacity of isolated cells was assessed by performing control experiments with dbcAMP-treated cells. However, similar to the report by Sonnack (2009), decreased viability and weak or no steroidogenic responses were observed after treatment with either stimulus, dbcAMP or $PGE_2$ (data not shown).

### *Effects of $PGE_2$ on expression of STAR, 3βHSD and P-450scc and on steroid production in canine luteal cells from the early CL phase*

After incubation of cells for 6 h in serum-free DMEM/F12 medium with either 0.5 mM dbcAMP used for the positive control or with 20 μM $PGE_2$, *STAR* mRNA expression was significantly increased by 3.75-fold



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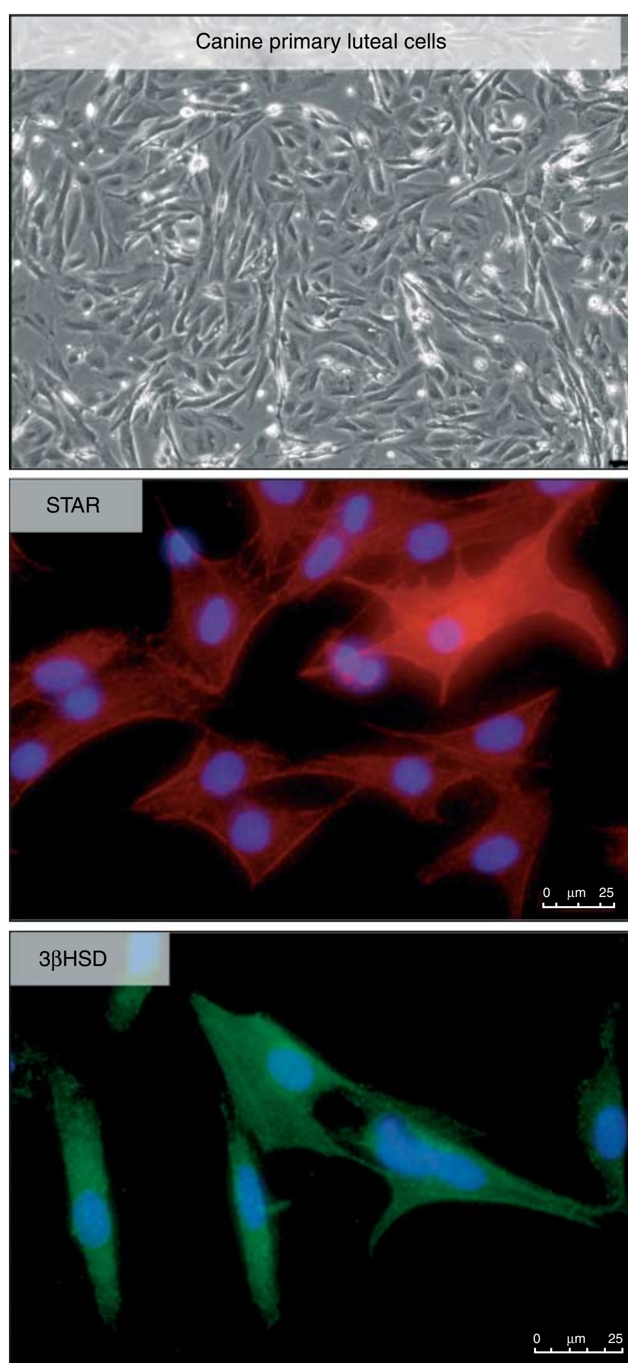


**Figure 1** Canine primary luteal cells; STAR and 3βHSD immunofluorescence staining was performed in order to confirm their steroidogenic origin and basal steroidogenic capability. Nuclear staining was achieved with DAPI, as indicated in the Materials and Methods section.

($P<0.01$) and 2.3-fold ($P<0.05$) respectively (Fig. 3A). In untreated canine luteal cells, STAR, 3βHSD and P450scc proteins could be detected by western blot analysis. When treated with $PGE_2$, STAR increased significantly (2.8-fold, $P<0.01$) and this increase closely resembled the induction level observed after stimulation with dbcAMP (Fig. 3B). The expression of 3βHSD and P450scc was not affected ($P>0.05$) by this treatment (Fig. 3B). To determine the physiological relevance of the $PGE_2$-mediated increase in STAR expression, the culture medium was collected after treatment and $P_4$ production was measured. Accumulation of $P_4$ in the medium was elevated by 2.62-fold ($P<0.05$) and 2.4-fold ($P<0.05$) by dbcAMP and $PGE_2$ respectively (Fig. 3C).

### *Luteal expression of STAR and $PGE_2$ receptors (PTGER2/EP2 and PTGER4/EP4) in early and mid-gestation and* prepartum

Luteal STAR expression revealed a highly significant effect of time ($P<0.0001$) as shown by western blot analysis. The highest STAR expression was observed at the pre-implantation stage followed by a gradual decrease towards the prepartal luteolysis; it was already significantly down-regulated at mid-gestation ($P<0.01$) and decreased further by 3.87-fold ($P<0.001$) at the time of prepartal luteolysis (Fig. 4).

As determined by western blot analysis, the expression levels of EP2 and EP4 in tissue homogenates from pregnant animals showed a significant ($P=0.0019$ and $P=0.0065$ respectively) time effect. EP2 was constantly expressed from pre-implantation until mid-gestation followed by a prepartal decrease ($P<0.05$; Fig. 4). EP4 revealed an apparent biphasic expression pattern: the lowest expression level was observed post-implantation, then it increased significantly ($P<0.01$) at mid-gestation and dropped significantly ($P<0.05$) towards prepartal luteolysis (Fig. 4). Blocking the antibodies with epitope-specific blocking peptides significantly diminished the EP2 and EP4 signals (Fig. 4).

As for EP2 and EP4, immunohistochemistry targeted the expression of both receptors to the luteal cells (Figs 5 and 6); however, in general, staining with EP4 antibody was much stronger than with anti-EP2 (Figs 5 and 6). No background staining was observed for EP4 antibody at any of the time points investigated. Signals were detected throughout gestation and tended to be stronger in the peripheral parts of the CL (Figs 5 and 6).

The weaker signals observed within the blood vessels for EP2 antibody were interpreted as background staining, as similar signals were observed when isotype control with rabbit IgG irrelevant antibodies was applied. EP2 antibody staining was negative during the prepartal luteolysis. This has been attributed to detection limits and generally lower signals observed in immunohistochemistry with this antibody.

### *Expression of PGT in corpus luteum of pregnant and non-pregnant animals*

Semi-quantitative real-time (TaqMan) PCR and immunohistochemistry were applied in order to determine the expression of *PGT* in canine luteal tissues from pregnant



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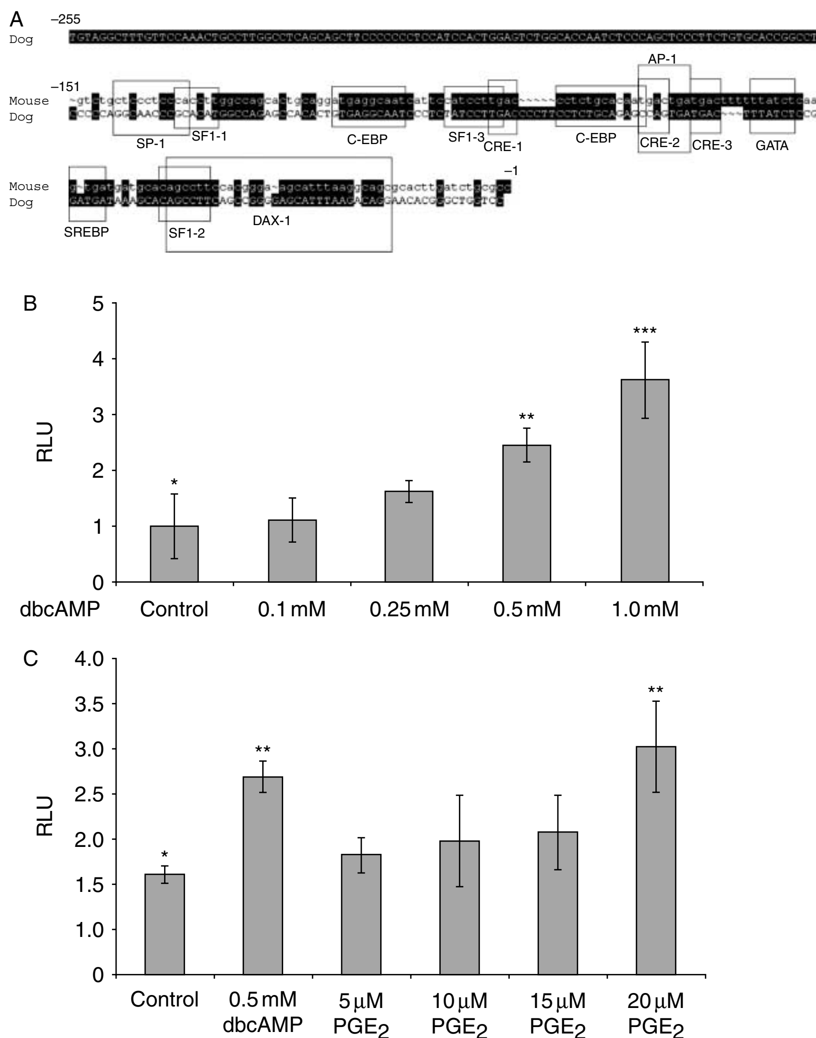


**Figure 2** Canine proximal −255/−1 *STAR* promoter. (A) Alignment with the murine proximal promoter. Shown are known transcription factor binding sites within the murine promoter; bolded are predicted binding sites within the canine promoter. (B and C) Activity of canine *STAR* promoter in canine primary luteal cells collected from non-pregnant dogs during early CL phase treated with increasing doses either of dbcAMP (B) or $PGE_2$ (C). The cell culture experiments were performed independently at least three times using cells isolated from different animals; luciferase assay experiments were conducted in duplicate. Numerical data are presented as the mean±S.D. One-way ANOVA (B, $P=0.0003$; C, $P=0.0023$) was applied followed by Dunnett's multiple comparison test; all samples were compared against the untreated control (*). **$P<0.01$ and ***$P<0.05$. RLU, relative light units.

and non-pregnant animals. The expression of mRNA and protein could be detected at all time points investigated. In both cases, a highly significant effect of time was evident ($P<0.0001$; Fig. 7A and B). In non-pregnant dogs, *PGT* was highly expressed at the beginning of dioestrus, at days 5, 15 and 25 p.o. Subsequently, a progressive decline in PGT mRNA levels towards the end of dioestrus was observed, resulting in a 3.7-fold down-regulation of its expression (Fig. 7A). Similarly, in pregnant animals, PGT mRNA was up-regulated at the beginning of the CL phase, with highest expression during post-implantation; a significant decrease was observed afterwards at mid-gestation ($P<0.01$), but no further significant changes were observed until prepartal luteolysis (Fig. 7B). At the protein level, as determined by immunohistochemistry, the expression of PGT was localized primarily to the luteal cells; the strongest signals were observed in the early CL (Fig. 8); however, the variability between individual animals was high. Some weaker signals were observed within the blood vessels throughout the luteal lifespan. This could be either due to the lower expression and hence detection limit in the vessels or to the background staining.

## Discussion

During the first third of canine luteal phase, the luteal steroidogenic machinery is strongly up-regulated, culminating in the highest $P_4$ levels observed both during pregnancy and in the non-pregnant cycle. From the expression pattern of the luteal *STAR* mRNA, which closely resembles the peripheral $P_4$ levels, it has been suggested that the generation of $P_4$ in both pregnant and non-pregnant animals might be controlled at the level of the STAR-mediated substrate supply rather than the enzymatic catalytic activity (Kowalewski & Hoffmann 2008, Kowalewski *et al*. 2009*a*). This proposition is further supported in this study. Luteal STAR protein expression was strongly time dependent, with the highest levels observed during the period of CL formation. That agrees with differences in the *STAR* mRNA levels observed previously (Kowalewski *et al*. 2009*a*).



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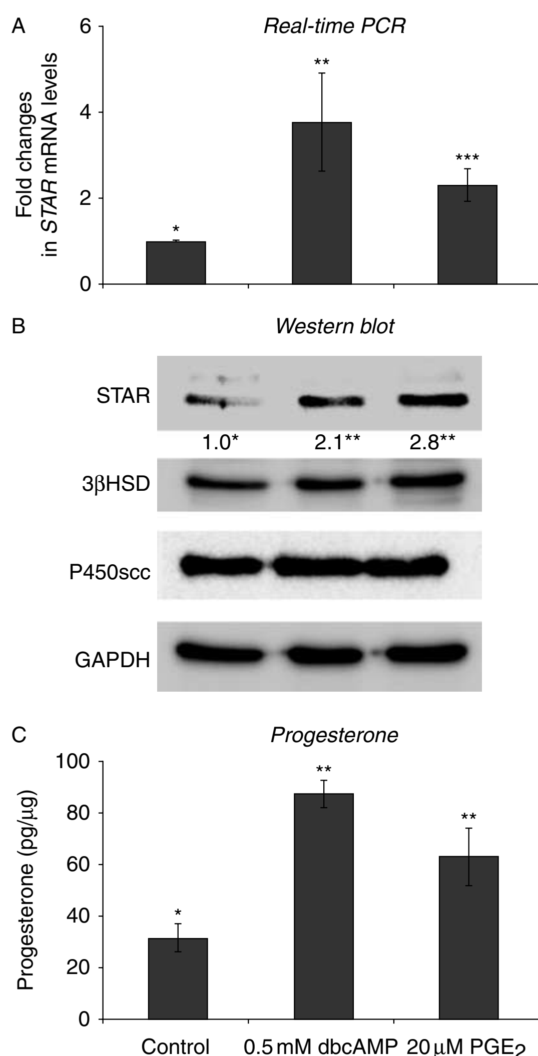


**Figure 3** $PGE_2$ increases STAR gene and protein expression and steroidogenic output in canine primary luteal cells collected from non-pregnant dogs during the early CL phase. Cells were cultured in serum-free DMEM/F12 medium with either 0.5 mM dbcAMP or 20 μm $PGE_2$. Untreated cells were used as negative control. All cell culture experiments were performed independently at least three times using cells isolated from different animals. (A) *STAR* mRNA expression as determined by real-time (TaqMan) PCR normalized against GAPDH and 18S rRNA. Numerical data are presented as the mean ± s.d. One-way ANOVA was applied ($P=0.0083$) followed by Dunnett's multiple comparison test; *untreated control, **$P<0.01$, ***$P<0.05$. (B) Cells were collected and homogenized and then 20 μg of the lysate was used in western blot analysis of STAR (30 kDa), 3βHSD (42 kDa), P450scc (45 kDa) and GAPDH (37 kDa). Protein expression was normalized against GAPDH; the average integrated optical density for STAR is shown as *n*-fold changes relative to untreated control (*). One-way ANOVA ($P=0.0001$) was applied, followed by Dunnett's multiple comparison test; **$P<0.01$. The expression of 3βHSD and P450scc protein did not differ significantly between the groups ($P>0.05$). (C) Progesterone (pg/μg) output was determined in collected culture media by RIA. One-way ANOVA ($P=0.0265$) together with Dunnett's multiple comparison test was applied; bars with different asterisks differ at $P<0.05$.

The $P_4$ production responsiveness of our primary luteal cell cultures contrasts with previous efforts to establish canine luteal cultures where steroidogenic responsiveness of the isolated luteal cells under culture condition was limited (Sonnack 2009), especially those acquired from later stages of the CL phase. In this study, isolation and culture of canine primary cells isolated from the developing CL of non-pregnant dogs permitted examination of the steroidogenic capacities of the isolated cells and their capacity for STAR and 3βHSD expression.

The demonstrated increase in STAR in response to dbcAMP was expected and used as positive control. The cAMP/PKA pathway is unquestionably the major route in trophic hormone-mediated expression and activation of STAR protein. By responding to cAMP-stimulation, STAR protein was discovered as the key steroidogenic factor and named as an acute regulator of steroidogenesis; the dynamics of its activation seem to be similar in different cell types (Manna *et al*. 2002, Dyson *et al*. 2009, Kowalewski *et al*. 2009*b*, 2010*b*). This signalling cascade initially involves transcriptional activation of the PKA type 1-mediated transcriptional promoter activity, whereas phosphorylation and hence, activation, of STAR protein is regulated by type II PKA (Dyson *et al*. 2009). STAR expression is controlled by many transcriptional factors serving both in basal and in cAMP-induced STAR promoter activity (reviewed in Manna *et al*. (2003)). Initial deletion and site-directed mutagenesis studies of murine *Star* promoter determined its −254/−113 region as essential for full activity (Caron *et al*. 1997). Further studies in several species were able to demonstrate the importance of the proximal −150/−1 fragment of the *Star* promoter, which exhibits activity equivalent to the full-length promoter (reviewed in Manna *et al*. (2003)). Canine *STAR* promoter has not been investigated before our study. In order to obtain the relevant data, here homology cloning was performed and the sequence of the canine proximal −255/−1 *STAR* promoter was elucidated. Several putative binding sites for transcriptional factors were found when *STAR* promoter was compared with its murine counterparts, e.g. C/EBP, SF-1/-2, SF-1/-3, GATA, SREBP or DAX-1. Moreover, two cAMP-responsive element half-sites (CRE-half-sites), corresponding to the known murine CRE-1 and CRE-2, were identified.

$PGE_2$ clearly stimulated STAR production in the canine luteal preparations. The results from the promoter assay, together with the data from the real-time PCR, revealed a significant increase in *STAR* promoter activity in response to treatment with $PGE_2$ that was similar to that observed with cAMP. Further studies aiming to determine the relative importance of the specific transcriptional factors in regulating canine STAR expression are planned. This is especially important in view of the impact of regulatory mechanisms mediated by STAR, but not by 3βHSD or P450scc, on canine luteal steroidogenic activity as shown in this study. At the



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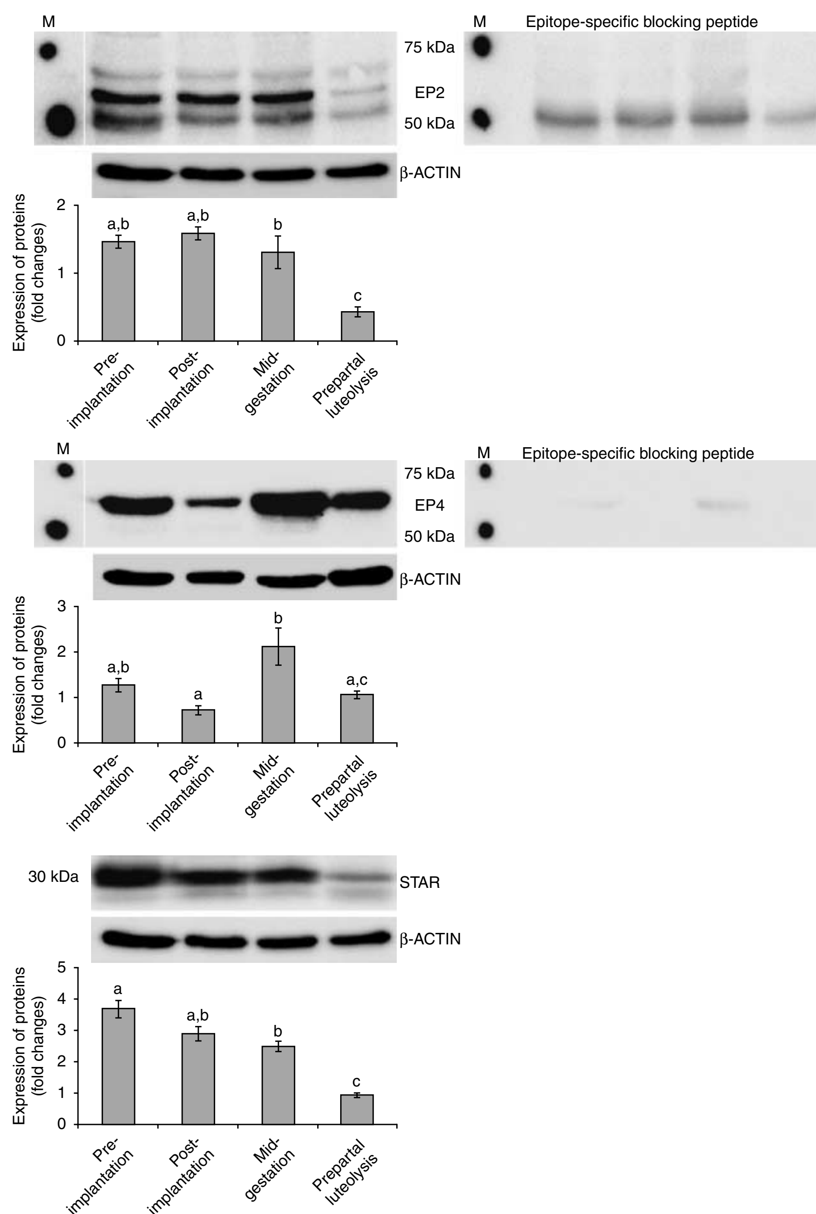


**Figure 4** Time-dependent expression of EP2, EP4 and STAR in canine CL during pregnancy. 20 μg tissue homogenates were used in western blot analysis. Representative immunoblots are shown. Lower panels represent densitometric values (integrated optical density) for EP2 (52 kDa), EP4 (55 kDa) and STAR (30 kDa) normalized against β-ACTIN (45 kDa). Available epitope-specific blocking peptides were used in order to quench the EP2- and EP4-specific signals. One-way ANOVA (EP2, $P=0.0019$; EP4, $P=0.0065$; STAR, $P<0.0001$) was applied and followed by Dunnett's post-test. Numerical data are presented as the mean ± S.D. Bars with different letters for EP2 differ at 'a,b' vs 'c', $P<0.01$, and 'b' vs 'c', $P<0.05$. For EP4 'a' vs 'b' differ at $P<0.01$, and 'b' vs 'a,c' at $P<0.05$. For STAR 'a' vs 'b' and 'b' vs 'c' differ at $P<0.01$, 'a' vs 'c' and 'a,b' vs 'c' differ at $P<0.001$. M, molecular weight marker.

concentration used $PGE_2$ appeared to stimulate STAR protein more than *STAR* mRNA when compared with the effects of the single dose of dbcAMP studied. If this reflects a biological difference and effects on STAR turnover as well as synthesis is not known. In cattle, $PGE_2$ has been demonstrated to up-regulate expression of LH receptor (Weems *et al.* 2011). Whether or not the survival of our primary cell culture would enable one to investigate such an effect *in vitro* merits investigation.

Concomitant with the up-regulated *STAR* gene expression, elevated levels of STAR protein were observed in response to $PGE_2$ treatment. The functional importance of this finding, indicating the involvement of transcriptional and post-translational regulatory factors in STAR-mediated responses, was assessed by measurement of the $P_4$ levels in the collected culture media. $PGE_2$ significantly increased the steroid release from cultured canine luteal cells. Thus, $PGE_2$ appears to be the first luteotrophic factor of the early CL phase in the dog, the functionality of which has been confirmed at the cellular level.

The impaired response to dbcAMP treatment in cells derived from the later luteal stages points towards other, as yet unknown, regulatory mechanisms involved in the endocrine and/or paracrine control of canine CL function. With regard to role of $PGE_2$ during the later stages of the CL phase, the lack of steroidogenic response suggests that there is a diminished functional luteotrophic role of $PGE_2$ during this time, in agreement with a similar conclusion based on the decreased PGES and COX2 expression (Kowalewski *et al.* 2006*b*, 2008*a*). On the other hand, however, the '*in vitro*' nature of the



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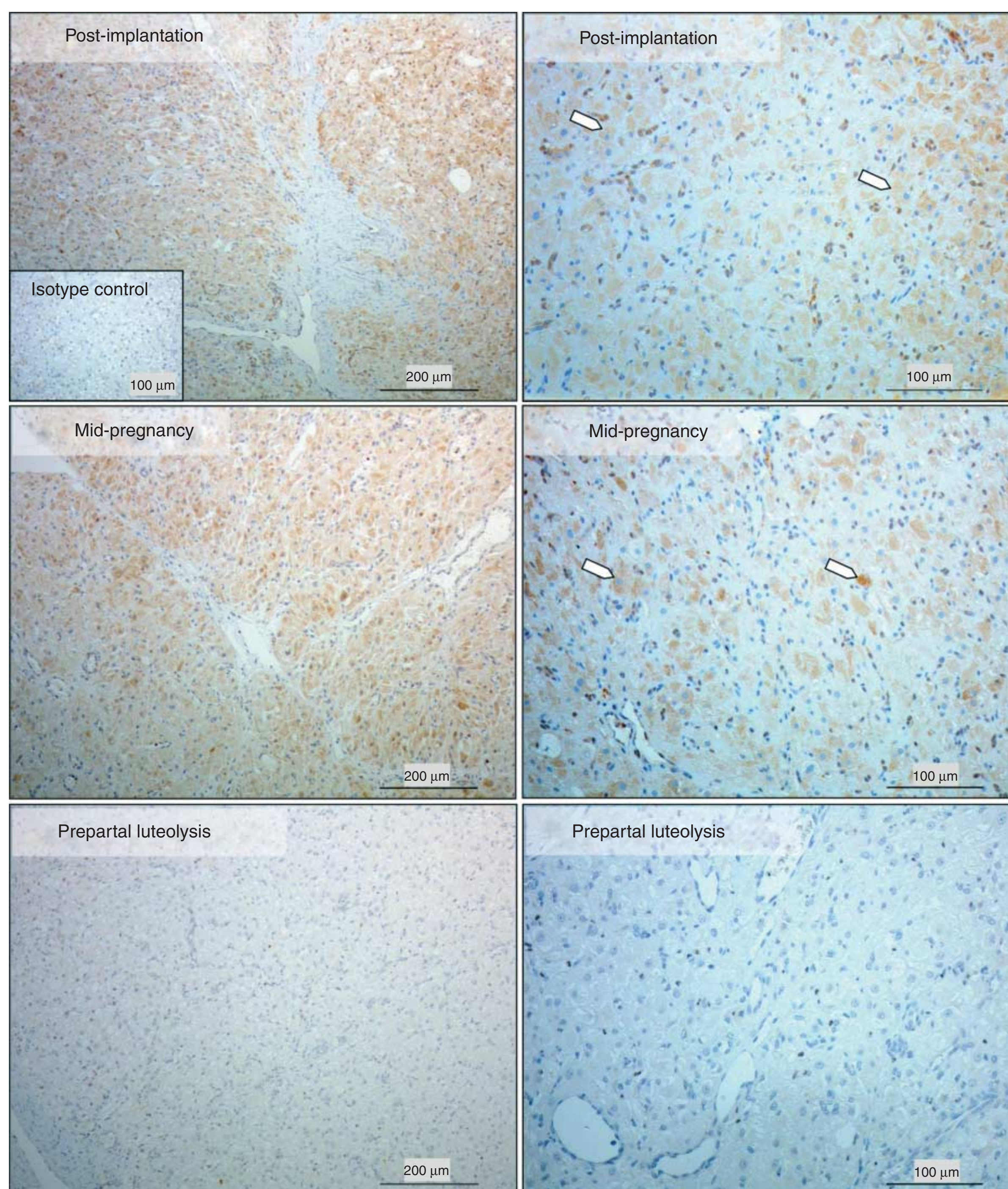


**Figure 5** Immunohistochemical localization of EP2 in canine CL. Pictures are shown at lower (left-side panels) and higher (right-side panels) magnification. Specific signals are localized in the lutein cells (open arrow).

experiments conducted in this study needs to be stressed, where the cultivated cells are obviously devoid of the additional endocrine and paracrine regulation possibly having an influence on their steroidogenic performances.

The action of $PGE_2$ in canine luteal tissue is probably mediated through the EP2 and EP4 receptors, as the expression of the other two receptors, EP1 and EP3, was either weak or absent from canine CL (Kowalewski *et al.* 2008*a*). However, possible effects via prostacyclin receptor might be considered taking into account the high concentration of $PGE_2$ needed to elicit responses in our canine luteal cell preparations. From the mRNA expression patterns of EP2 and EP4 previously observed,



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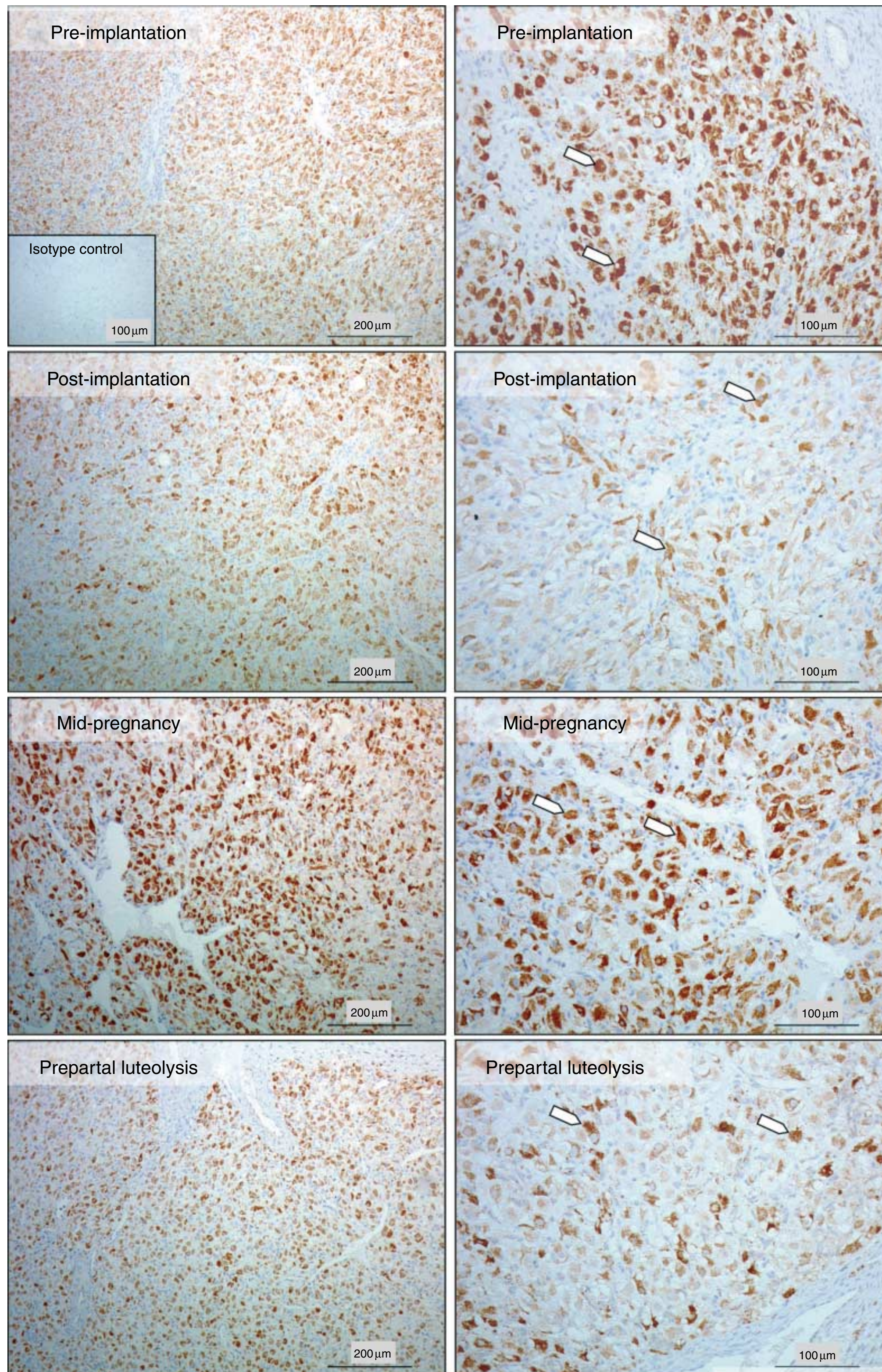


**Figure 6** Immunohistochemical localization of EP4 in canine CL. Pictures are shown at lower (left-side panels) and higher (right-side panels) magnification. Lutein cells are the only targets for EP4 expression (open arrow).



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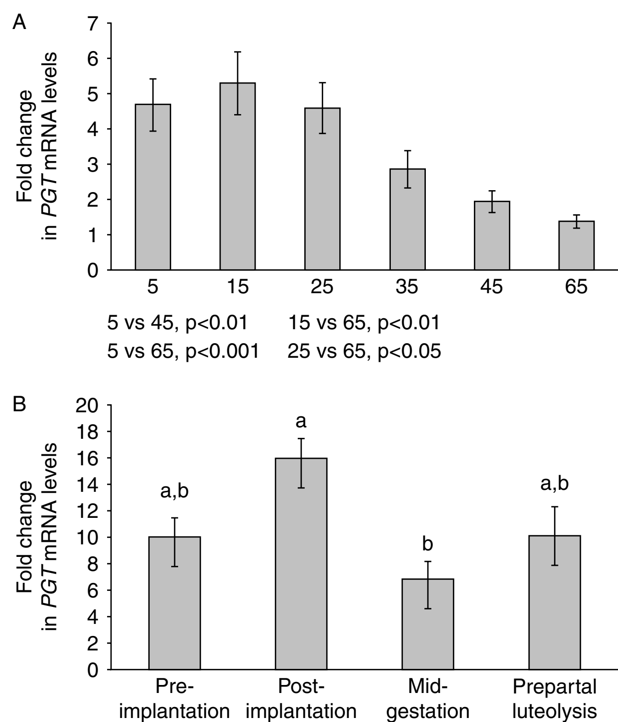


**Figure 7** Time-dependent expression of canine prostaglandin transporter (PGT) as determined by real-time (TaqMan) PCR. (A) CL from non-pregnant cycles, days 5–65 post-ovulation. (B) CL of pregnancy. Bars with different letters in (B) differ at $P<0.01$. Numerical data are presented as the mean ± S.D.

the luteotrophic function of $PGE_2$ was assumed to be mostly due to the EP2 receptor (Kowalewski *et al.* 2008*a*, 2009*a*) because the expression of EP4 at the mRNA level was not cycle related (Kowalewski *et al.* 2008*a*, 2009*a*). However, in contrast to these observations, in this study, as determined by western blot and immunohistochemistry, the protein levels of both EP4 and EP2 appeared to change over time during the luteal phase. The observed decline of EP2 during *prepartum* luteolysis raises the possibility that a corresponding reduced efficacy of $PGE_2$ might play a role in that process. The elevated expression of $PGE_2$ receptors together with increased PGES (Kowalewski *et al.* 2008*a*) and STAR protein levels during the early CL phase further suggests a functional involvement of EP2 in luteal function. The apparently up-regulated EP4-expression at mid-gestation merits further study in comparison to expression in non-pregnant bitches considering that increased PRL appears to be the basis of increased $P_4$ production during canine pregnancy. This is, however, the time when i) first signs appear of luteal degeneration at the subcellular level of smooth endoplasmatic reticulum, concomitant with the continuous $P_4$ decrease in non-pregnant bitches (Hoffmann *et al.* 2004, Sonnack 2009) and ii) the luteal regression cannot be stopped. Based on these considerations, the relative functional importance of EP2 and EP4 in regulating canine luteal function needs to be further elucidated.

The luteal PGT expression was strongly time dependent and appeared to reflect previously reported PGES levels as well as changes in luteal steroidogenic activity in both pregnant and non-pregnant dogs. This implies a role for PGT as a factor involved in the paracrine and autocrine control of luteal prostaglandin function in canines, already described in other species (Arosh *et al.* 2004). In this context, our first (and as yet unpublished) results implicate 15-hydroxyprostaglandin dehydrogenase (15PGDH) as another factor possibly involved in the local provision of luteotrophic prostaglandins in the dog.

Whether or not known luteotrophic hormones including LH and PRL or luteal oestrogens or $P_4$ can modulate luteal $PGE_2$ action also merit further study. Likewise, the possibility that mechanisms suppressing responsiveness to $PGE_2$ might be involved in parturition merits investigation.

In summary, the establishment of canine primary luteal cell culture system has provided a tool for explicating the involvement of $PGE_2$ in regulating canine luteal function. The data contribute to closing the knowledge gap in understanding the mechanisms governing reproductive function in the dog. This also provides a basis for future, more detailed investigations of etiopathogenesis of some, still not fully understood, ovarian disorders like canine luteal insufficiency, requiring the $P_4$ supplementation as a therapeutic measure for prevention of abortion.

We were able to show that the $PGE_2$-stimulated steroidogenesis is primarily regulated at the level of STAR protein expression and STAR-dependent steroid substrate transport rather than at the level of the enzymatic activity of 3βHSD and P450scc enzymes. Finally, the observed changes in PGT, EP2 and EP4 in canine luteal tissue in different stages of the luteal phase and pregnancy strengthen the evidence for paracrine and/or autocrine roles of prostaglandins in the canine CL.

## Materials and Methods

### *Tissue sampling and preservation*

Luteal tissues from pregnant and non-pregnant, clinically healthy dogs were used for this study. Animal experiments were in accordance with animal welfare legislation. Those were permit no. II 25.3-19c20-15c GI 18/14 and VIG3-19c/2015c GI, 18,14, Justus-Liebig University Giessen, Germany, and permit no. Ankara 2006/06 Faculty of Veterinary Medicine, University of Ankara, Turkey.

Groups of non-pregnant bitches ($n=5$ each) were ovariohysterectomized (OHE) on the following days post ovulation (p.o.): 5, 15, 25, 35, 45 and 65. The day of ovulation was monitored by determination of peripheral blood $P_4$ levels at 2- to 3-day intervals and was defined as the day when $P_4$ levels reached 5 ng/ml (Concannon *et al.* 1989).



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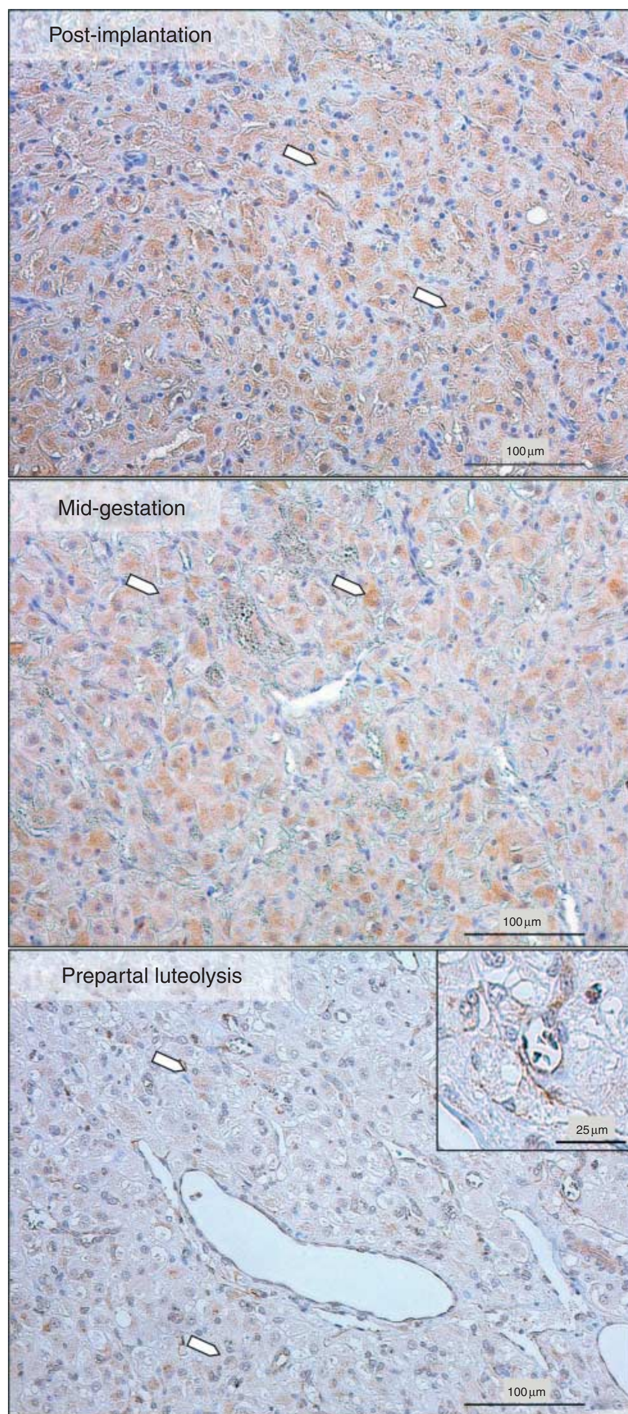


**Figure 8** Immunohistochemical localization of PGT in canine CL. Specific signals are localized predominantly to the lutein cells (open arrow); some weaker signals can be found in luteal vessels (presented in the inset to 'prepartal luteolysis').

Pregnant dogs were divided into four groups and subjected to OHE according to the following temporal classification: pre-implantation (days 8–12, $n=5$), post-implantation (days 18–25, $n=5$), mid-gestation (days 35–45, $n=5$) and prepartal luteolysis ($n=3$). As described before (Kowalewski *et al*. 2009*a*, 2010*a*, 2011*a*), the day of mating was defined as day 0 and in the pre-implantation group pregnancy was determined by detecting embryos in uterine flushings. In the prepartal group, $P_4$ was measured every 6 h from day 58 of pregnancy onwards. OHE was performed when peripheral $P_4$ levels continued to decrease below 3 ng/ml in two consecutive measurements. The corresponding mean $P_4$ concentrations from all non-pregnant and pregnant animals were reported and discussed earlier (Papa (2001), Kowalewski *et al*. (2011*b*), Kowalewski *et al*. (2010*a*) respectively).

Immediately after OHE, luteal tissue samples were washed with PBS, trimmed of surrounding connective tissue and preserved either for 24 h at 4 °C in RNA-later (Ambion Biotechnologie GmbH, Wiesbaden, Germany) and then frozen at −80 °C until further use or fixed for 24 h in 10% neutral phosphate-buffered formalin, washed with frequently replaced PBS during 1 week, dehydrated in a graded ethanol series and embedded in paraffin-equivalent Histo-Comp (Vogel, Giessen, Germany) for immunohistochemistry. No luteal homogenates were available for western blot analysis from non-pregnant dogs for this study.

### Canine primary luteal cells

Primary luteal cells were obtained from clinically healthy bitches ($n=15$) submitted to routine OHE at the Section of Small Animal Reproduction, Clinic of Reproductive Medicine, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland. OHE was performed 7–14 days after the clinical signs of heat had ceased, during the early luteal phase. CL were removed from ovaries, trimmed of connective tissue and washed in PBS. Luteal tissues from each animal were diced with a sterile scalpel blade, pooled and stirred for 2–3 h in PBS containing 0.15% Collagenase (Sigma–Aldrich Chemie GmbH) and then filtered through a 75 µm cell strainer (BD Biosciences, Basel, Switzerland) to remove undissociated tissue fragments. Subsequently, cells were washed three times in culture medium (DMEM/F12, pH 7.2–7.4, with 10% heat-inactivated FBS, 100 U/ml penicillin and 100 µg/ml streptomycin, and 1% ITS (Insulin-Transferrin-Selenium); all from Chemie Brunschwig AG (Basel, Switzerland)) with centrifugation steps in between (600 ***g***, 10 min). Finally, cells were suspended in culture medium, seeded directly into six-well plates ($\sim 3\text{–}4\times10^{-5}$ cells/well) and cultured in a humidified incubator at 37 °C under 5% $CO_2$ in air. For immunocytochemistry, sterile cover glasses were placed in wells to let the seeded cells adhere to them. Cells were grown to 70–80% confluency, which was reached after ∼48 h, and then used for experiments. The cells were not passaged (trypsinized) before the experiments: 'passage 0' cells were used. Before stimulation, cells were washed with PBS in order to remove the serum-containing medium that was replaced by serum-free medium containing N6,2-dibutyryladenosine-3,5-cyclic monophosphate (dbcAMP) or $PGE_2$ (both from Sigma–Aldrich).

In pilot experiments, the time course (1–8 h) of STAR expression, promoter activity and the ensuing steroidogenic response in canine luteal cells were determined, revealing the highest levels of STAR expression and $P_4$ output after 6 h of stimulation; this time point was hence chosen for



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all stimulation experiments. In all experiments, the basal steroidogenic capacity of freshly isolated luteal cells was tested using dbcAMP-treated cells as positive control. The cell culture medium was finally collected and stored at −20 °C for radioimmunoassay (RIA), which was performed as described previously (Hoffmann *et al*. 1973).

### Canine proximal STAR promoter and luciferase assay

Based on the high sequence homology between canine STAR promoter and its murine counterpart, a homology cloning of the −255/−1 bp region of canine proximal *STAR* promoter was performed. The genomic canine DNA isolated from CL was used in the PCR-based cloning strategy. The following primers containing the XhoI and HindIII restriction sites (underlined) respectively were used: forward 5′-TAG CTC GAG TTG TAG GCT TTG TTC CAA ACT GCC T-3′ and reverse 5′-CTA AAG CTT GGA CCA GCC CGT GTT CCT GTC TTA-3′. The PCR products were separated on a 1.5% ethidium bromide-stained gel, purified with the Qiaex II gel extraction system (Qiagen), subcloned into the pGEM-T vector (Promega) and sent for sequencing (Microsynth, Balgach, Switzerland) with T7 and SP6 sequencing primers. The canine *STAR* promoter fragment containing pGEM-T vector was digested with XhoI and HindIII (NEB GmbH, Frankfurt am Main, Germany), then fragments were purified and inserted into identically cleaved sites in the pGL3 basic vector (Promega), upstream of the *Firefly* luciferase reporter gene to yield −255/−1 canine *STAR*-pGL3. The control restriction enzyme analysis was performed in order to confirm the structure of the newly obtained recombinant plasmid.

Freshly isolated canine primary luteal cells at 70–80% confluency were used for transfection experiments that were performed as described before (Kowalewski *et al*. 2009*b*, 2010*b*). FuGENE HD transfection reagent (Roche Diagnostics Schweiz AG) was used in 1.5 ml serum-containing medium, at a ratio of 1 μg DNA to 3.5 μl reagent in complexes that were pre-incubated for at least 25 min in 100 μl serum-free medium. In each well, 0.625 μg *STAR* promoter plasmid DNA was used. The transfection efficiency was normalized by cotransfecting the cells with 20 ng pRL-SV40 plasmid (Promega) constitutively expressing *Renilla* luciferase driven by CMV immediate-early enhancer promoter. The activity of the canine *STAR* promoter construct in response to dbcAMP and $PGE_2$ treatment was determined after 6 h of incubation. Afterwards, cells were washed with ice-cold PBS and lysed with 300 μl Passive Lysis Buffer (Promega). The dual luciferase reporter system was then applied following the manufacturer's instructions (Promega). Luminescence was measured in a MLX Microplate Luminometer (Dynex Technologies, GmbH, Denkendorf, Germany). Promoter activity in the samples is reported in relative light units (RLU), reflecting the ratio of *Firefly* luciferase luminescence to that of *Renilla* luciferase.

### RNA extraction, semi-quantitative RT-PCR and data evaluation

Total RNA was isolated using TRIzol reagent (Invitrogen) and subsequently treated with RQ1 RNase–free DNase (Promega) in order to remove genomic DNA contaminants, both protocols following the manufacturers' instructions. Reagents from Applied Biosystems, were used for reverse transcription (RT); random hexamers served as primers for the cDNA synthesis. Semi-quantitative real-time (TaqMan) PCR was carried out in an automated fluorometer ABI PRISM 7500 Sequence Detection System (Applied Biosystems) according to our previously described protocol (Kowalewski *et al*. 2006*b*, 2011*a*, 2011*b*). Fast Start Universal Probe Master (ROX; Roche Diagnostics Schweiz AG) was used with cDNA corresponding to 100 ng DNase-treated total RNA for each sample; all samples were run in duplicates. For negative controls, samples lacking templates and the so-called 'RT minus' controls (samples that were not reverse transcribed) were run to rule out any contamination of reagents used for RT-PCR and to confirm the accuracy of the DNase treatment. Two different independent endogenous references (GAPDH and 18S rRNA) were included in the semi-quantitation protocol. Primers and 6-carboxyfluorescein (6-FAM) and 6-carboxytetramethylrhodamine (TAMRA)-labelled TaqMan probes were purchased from Microsynth. Sequences for primers and probes were as follows: GAPDH (forward): 5′-GCT GCC AAA TAT GAC GAC ATC A-3′, GAPDH (reverse): 5′-GTA GCC CAG GAT GCC TTT GAG-3′, GAPDH (TaqMan probe): 5′-TCC CTC CGA TGC CTG CTT CAC TAC CTT-3′ (GenBank accession number: AB028142); 18S rRNA (forward): 5′-GTC GCT CGC TCC TCT CCT ACT-3′, 18S rRNA (reverse): 5′-GGC TGA CCG GGT TGG TTT-3′, 18S rRNA (TaqMan probe): 5′-ACA TGC CGA CGG GCG CTG AC-3′ (GenBank accession number: FJ797658); STAR (forward): 5′-CGA GGC TCC ACC TGT GTG T-3′, STAR (reverse): 5′-CCT TTC TGC TCA GGC ATC TC-3′, STAR (TaqMan probe): 5′-CTG GCA TGG CCA CAC ATT TC-3′ (GenBank accession number: EF522840); PGT (forward): 5′-TGC AGC ACT AGG AAT GCT GTT C-3′, PGT (reverse): 5′-GGG CGC AGA GAA TCA TGG A-3′, PGT (TaqMan probe): 5′-TCT GCA AAC CAT TCC CCG CGT G-3′ (GenBank accession number: NM_001011558). The efficiency of PCR was measured using the *C*T slope method according to the instructions of the manufacturer of the ABI PRISM 7500 Sequence Detection System and as described previously (Kowalewski *et al*. 2011*a*), assuring ∼100% efficiency of reactions. Relative gene expression was calculated using the comparative *C*T method (ΔΔ*C*T method), expression of the target gene relative to the reference genes (*GAPDH* and *18S* rRNA), and normalized to the sample with the lowest amount of the respective target gene transcripts, which was considered as a calibrator. Data were considered valid for the ΔΔ*C*T method if the relative amounts of both reference genes for a sample were constant, and the average amount was used as the normalization factor.

The specificity of the selected PCR products was confirmed by sequencing (Microsynth). In experiments showing STAR expression in canine luteal cells after treatment with dbcAMP or $PGE_2$, the results are expressed as *n*-fold changes in gene expression over the untreated control. For the PGT, the effect of time on its luteal expression during the course of the CL phase in pregnant and non-pregnant animals was determined.



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### *Protein preparation and western blot analysis*

Tissue and cell homogenates were prepared and SDS–PAGE was performed following the previously described protocols (Kowalewski *et al.* 2009*b*, 2011*a*). NET-2 lysis buffer (50 mM Tris–HCl, pH 7.4, 300 mM NaCl and 0.05% NP-40), containing 10 µl/ml protease inhibitor cocktail (Sigma–Aldrich), was used for preparation of protein homogenates; 20 µg proteins solubilized in sample buffer (25 mmol/l Tris–Cl, pH 6.8, 1% SDS, 5% β-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue) were loaded onto 10–12% SDS–polyacrylamide gel and subsequently transferred to methanol-activated PVDF membranes (Bio-Rad Laboratories, Inc.). Non-fat dry milk 5% in PBS/0.25% Tween-20 was used as a blocking solution. Primary antibodies were incubated overnight at +4 °C. The following antibodies were used: i) polyclonal rabbit anti-STAR (a gift from Dr D M Stocco, Texas Tech University Health Sciences Center, Lubbock, TX, USA; Clark *et al.* (1994)); ii) rabbit polyclonal against 3βHSD (a gift from Dr J I Mason, University of Edinburgh, UK; Lorence *et al.* (1990)); iii) rabbit polyclonal anti-P450scc (AB1244) from Millipore (Zug, Switzerland); iv) and v) rabbit polyclonal, affinity-purified IgG against EP2 and EP4 respectively together with respective blocking peptides from Cayman Chemical Company (Ann Arbor, MI, USA). For loading controls, PVDF membranes were re-blotted with vi) mouse monoclonal antibody against GAPDH (sc47724) and vii) mouse monoclonal antibody against β-ACTIN (sc81178) from Santa Cruz Biotechnology, Inc. Donkey anti-rabbit HRP-labelled secondary IgG and SuperSignal West chemiluminescent substrate were obtained from Pierce Biotechnology (Rockford, IL, USA). Anti-mouse IgG conjugated to HRP was from Promega.

### *Immunohistochemistry and immunocytochemistry*

Luteal cross sections (2–3 µm thick) were cut and mounted on Super-Frost-Plus microscope slides (Menzel-Gläser, Braunschweig, Germany). The immunohistochemical procedure using an immunoperoxidase method was performed as previously described (Kowalewski *et al.* 2006*a*, 2006*b*). The antibodies used were as follows: rabbit polyclonal anti-EP2 and anti-EP4, the same as for western blots, purchased from Cayman Chemical Company, and goat polyclonal affinity-purified anti-PGT IgG (G-17; sc-103085) from Santa Cruz Biotechnology, Inc. The latter was tested as unsuitable for western blot analysis of canine luteal homogenates. As negative/isotype controls, rabbit IgG irrelevant antibodies I-1000 for EP2 and EP4 and, in the case of PGT, goat IgG irrelevant antibodies I-5000, both from Vector Laboratories, Inc. (Burlingame, CA, USA), were used at the same concentration as the primary antibodies. The secondary antibodies were biotinylated goat anti-rabbit IgG BA-1000 and horse anti-goat IgG BA-9500 from Vector Laboratories. The DAB substrate kit (Dako North America, Inc., CA, USA) was used to detect peroxidase activity.

Immunofluorescence was used for staining canine primary luteal cells that were seeded in six-well plates and grown on sterile cover glasses. After cells reached 70–80% confluency, formaldehyde was added at 2% final concentration and cells were incubated for 10 min at 37 °C. Afterwards, cells were washed twice with ice-cold PBST (PBS/0.25% Triton X). For antigen retrieval, 50 mM glycine (Sigma–Aldrich) in PBS was used for 5 min at room temperature followed by two washing steps in PBST (2×5 min). In order to block non-specific binding sites, cells were incubated for 30 min in a 10% solution of goat serum in PBST. Primary antibodies were anti-STAR and anti-3βHSD, the same as for western blot analysis; incubation was for 2 h at room temperature. Serum from a non-immunized rabbit, samples omitting the primary antibody and those where both the primary and secondary antibodies were omitted, served as negative controls. The latter were used for the autofluorescence control. Cells were then washed three times for 10 min with PBST and incubated with Fluorescein Goat Anti-Rabbit IgG Antibody FI-1000 (Vector Laboratories) in PBST for 1 h at room temperature. 4′,6-Diamidino-2-phenylindole (DAPI; Sigma–Aldrich) was added to the secondary antibody solution to achieve nuclear staining. After washing (3×10 min in PBST), cells were post-fixed with 2% formaldehyde for 10 min and subsequently rinsed with tap water. Finally, cover glasses with cells were mounted with Glycergel (Dako North America, Inc.) on microscope slides.

### *Statistical analysis*

All cell culture experiments were performed independently at least three times using cells isolated from different animals; luciferase assay experiments were conducted in duplicate. Representative western blots are shown. A parametric one-way ANOVA followed by Dunnett's multiple comparison post-test was applied. Statistical data are presented as the *n*-fold change relative to untreated controls.

To test for an effect of the observational group on the STAR, 3βHSD, P450scc, EP2 and EP4 protein expression and on PGT mRNA levels in luteal tissues, parametric ANOVA and the Tukey–Kramer multiple comparisons post-test were used.

All numerical data are presented as the mean±s.d. All tests were performed using the statistical software program GraphPad 3.06 (GraphPad Software, San Diego, CA, USA).

## 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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## Author contribution statement

MP Kowalewski conceived and designed the study, participated in collecting tissue samples, homology cloning procedures, statistical evaluation, interpretation of data and manuscript writing. B Fox participated in study design, collecting tissue samples and performing the laboratory part



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of the project. A Gram participated in the laboratory part of the project. A Boos was involved in knowledge transfer, critical discussion of the data and editing of the manuscript. I Reichler collected tissue samples for cell culture experiments, knowledge transfer, critical discussion of data and editing of the manuscript. All authors read and approved the final manuscript.

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## References

**Arosh JA, Banu SK, Chapdelaine P, Madore E, Sirois J & Fortier MA** 2004 Prostaglandin biosynthesis, transport, and signaling in corpus luteum: a basis for autoregulation of luteal function. *Endocrinology* **145** 2551–2560. (doi:10.1210/en.2003-1607)

**Caron KM, Ikeda Y, Soo SC, Stocco DM, Parker KL & Clark BJ** 1997 Characterization of the promoter region of the mouse gene encoding the steroidogenic acute regulatory protein. *Molecular Endocrinology* **11** 138–147. (doi:10.1210/me.11.2.138)

**Christenson LK, Farley DB, Anderson LH & Ford SP** 1994 Luteal maintenance during early pregnancy in the pig: role for prostaglandin E$_2$. *Prostaglandins* **47** 61–75.

**Clark BJ, Wells J, King SR & Stocco DM** 1994 The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). *Journal of Biological Chemistry* **269** 28314–28322.

**Concannon P** 1980 Effects of hypophysectomy and of LH administration on luteal phase plasma progesterone levels in the beagle bitch. *Journal of Reproduction and Fertility* **58** 407–410. (doi:10.1530/jrf.0.0580407)

**Concannon PW, Weinstein P, Whaley S & Frank D** 1987 Suppression of luteal function in dogs by luteinizing hormone antiserum and by bromocriptine. *Journal of Reproduction and Fertility* **81** 175–180. (doi:10.1530/jrf.0.0810175)

**Concannon PW, McCann JP & Temple M** 1989 Biology and endocrinology of ovulation, pregnancy and parturition in the dog. *Journal of Reproduction and Fertility. Supplement* **39** 3–25.

**Dyson MT, Kowalewski MP, Manna PR & Stocco DM** 2009 The differential regulation of steroidogenic acute regulatory protein-mediated steroidogenesis by type I and type II PKA in MA-10 cells. *Molecular and Cellular Endocrinology* **300** 94–103. (doi:10.1016/j.mce.2008.11.029)

**Engel E, Klein R, Baumgartner W & Hoffmann B** 2005 Investigations on the expression of cytokines in the canine corpus luteum in relation to dioestrus. *Animal Reproduction Science* **87** 163–176. (doi:10.1016/j.anireprosci.2004.10.002)

**Harris TE, Squires PE, Michael AE, Bernal AL & Abayasekara DR** 2001 Human granulosa–lutein cells express functional EP1 and EP2 prostaglandin receptors. *Biochemical and Biophysical Research Communications* **285** 1089–1094. (doi:10.1006/bbrc.2001.5301)

**Hoffmann B, Kyrein HJ & Ender ML** 1973 An efficient procedure for the determination of progesterone by radioimmunoassay applied to bovine peripheral plasma. *Hormone Research* **4** 302–310. (doi:10.1159/000178317)

**Hoffmann B, Hoveler R, Hasan SH & Failing K** 1992 Ovarian and pituitary function in dogs after hysterectomy. *Journal of Reproduction and Fertility* **96** 837–845. (doi:10.1530/jrf.0.0960837)

**Hoffmann B, Busges F, Engel E, Kowalewski MP & Papa PC** 2004 Regulation of corpus luteum-function in the bitch. *Reproduction in Domestic Animals* **39** 232–240. (doi:10.1111/j.1439-0531.2004.00508.x)

**Kowalewski MP & Hoffmann B** 2008 Molecular cloning and expression of StAR protein in the canine corpus luteum during dioestrus. *Experimental and Clinical Endocrinology & Diabetes* **116** 158–161. (doi:10.1055/s-2007-992121)

**Kowalewski MP, Mason JI, Howie AF, Morley SD, Schuler G & Hoffmann B** 2006*a* Characterization of the canine 3β-hydroxysteroid dehydrogenase and its expression in the corpus luteum during diestrus. *Journal of Steroid Biochemistry and Molecular Biology* **101** 254–262. (doi:10.1016/j.jsbmb.2006.06.029)

**Kowalewski MP, Schuler G, Taubert A, Engel E & Hoffmann B** 2006*b* Expression of cyclooxygenase 1 and 2 in the canine corpus luteum during diestrus. *Theriogenology* **66** 1423–1430. (doi:10.1016/j.theriogenology.2006.01.039)

**Kowalewski MP, Mutembei HM & Hoffmann B** 2008*a* Canine prostaglandin E$_2$ synthase (PGES) and its receptors (EP2 and EP4): expression in the corpus luteum during dioestrus. *Animal Reproduction Science* **109** 319–329. (doi:10.1016/j.anireprosci.2007.11.023)

**Kowalewski MP, Mutembei HM & Hoffmann B** 2008*b* Canine prostaglandin F2α receptor (FP) and prostaglandin F2α synthase (PGFS): molecular cloning and expression in the corpus luteum. *Animal Reproduction Science* **107** 161–175. (doi:10.1016/j.anireprosci.2007.06.026)

**Kowalewski MP, Beceriklisoy HB, Aslan S, Agaoglu AR & Hoffmann B** 2009*a* Time related changes in luteal prostaglandin synthesis and steroidogenic capacity during pregnancy, normal and antiprogestin induced luteolysis in the bitch. *Animal Reproduction Science* **116** 129–138. (doi:10.1016/j.anireprosci.2008.12.011)

**Kowalewski MP, Dyson MT, Manna PR & Stocco DM** 2009*b* Involvement of peroxisome proliferator-activated receptor γ in gonadal steroidogenesis and steroidogenic acute regulatory protein expression. *Reproduction, Fertility, and Development* **21** 909–922. (doi:10.1071/RD09027)

**Kowalewski MP, Beceriklisoy HB, Pfarrer C, Aslan S, Kindahl H, Kucukaslan I & Hoffmann B** 2010*a* Canine placenta: a source of prepartal prostaglandins during normal and antiprogestin-induced parturition. *Reproduction* **139** 655–664. (doi:10.1530/REP-09-0140)

**Kowalewski MP, Dyson MT, Boos A & Stocco DM** 2010*b* Vasoactive intestinal peptide (VIP)-mediated expression and function of steroidogenic acute regulatory protein (StAR) in granulosa cells. *Molecular and Cellular Endocrinology* **328** 93–103. (doi:10.1016/j.mce.2010.07.018)

**Kowalewski MP, Meyer A, Hoffmann B, Aslan S & Boos A** 2011*a* Expression and functional implications of peroxisome proliferator-activated receptor γ (PPARγ) in canine reproductive tissues during normal pregnancy and parturition and at antiprogestin induced abortion. *Theriogenology* **75** 877–886. (doi:10.1016/j.theriogenology.2010.10.030)

**Kowalewski MP, Michel E, Gram A, Boos A, Guscetti F, Hoffmann B, Aslan S & Reichler I** 2011*b* Luteal and placental function in the bitch: spatio-temporal changes in prolactin receptor (PRLr) expression at dioestrus, pregnancy and normal and induced parturition. *Reproductive Biology and Endocrinology* **9** 109. (doi:10.1186/1477-7827-9-109)

**Lorence MC, Murry BA, Trant JM & Mason JI** 1990 Human 3β-hydroxysteroid dehydrogenase/Δ 5-4isomerase from placenta: expression in nonsteroidogenic cells of a protein that catalyzes the dehydrogenation/isomerization of C21 and C19 steroids. *Endocrinology* **126** 2493–2498. (doi:10.1210/endo-126-5-2493)

**Manna PR, Dyson MT, Eubank DW, Clark BJ, Lalli E, Sassone-Corsi P, Zeleznik AJ & Stocco DM** 2002 Regulation of steroidogenesis and the steroidogenic acute regulatory protein by a member of the cAMP response-element binding protein family. *Molecular Endocrinology* **16** 184–199. (doi:10.1210/me.16.1.184)

**Manna PR, Wang XJ & Stocco DM** 2003 Involvement of multiple transcription factors in the regulation of steroidogenic acute regulatory protein gene expression. *Steroids* **68** 1125–1134. (doi:10.1016/j.steroids.2003.07.009)

**Mariani TC, do Prado C, Silva LG, Paarmann FA, Lima MC, Carvalho I, Campos DB, Artoni LP, Hernandez-Blazquez FJ & Papa PC** 2006 Immunohistochemical localization of VEGF and its receptors in the corpus luteum of the bitch during diestrus and anestrus. *Theriogenology* **66** 1715–1720. (doi:10.1016/j.theriogenology.2006.02.030)



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**Okkens AC, Dieleman SJ, Bevers MM, Lubberink AA & Willemse AH** 1986 Influence of hypophysectomy on the lifespan of the corpus luteum in the cyclic dog. *Journal of Reproduction and Fertility* **77** 187–192. (doi:10.1530/jrf.0.0770187)

**Okkens AC, Bevers MM, Dieleman SJ & Willemse AH** 1990 Evidence for prolactin as the main luteotrophic factor in the cyclic dog. *Veterinary Quarterly* **12** 193–201. (doi:10.1080/01652176.1990.9694266)

**Olson PN, Bowen RA, Behrendt MD, Olson JD & Nett TM** 1984 Concentrations of progesterone and luteinizing hormone in the serum of diestrous bitches before and after hysterectomy. *American Journal of Veterinary Research* **45** 149–153.

**Onclin K, Verstegen JP & Concannon PW** 2000 Time-related changes in canine luteal regulation: *in vivo* effects of LH on progesterone and prolactin during pregnancy. *Journal of Reproduction and Fertility* **118** 417–424. (doi:10.1530/reprod/118.2.417)

**Papa PC** 2001 Darstellung von Estradiol-17β- und Progesteronrezeptoren im Corpus luteum der Hündin zu definierten Zeitpunkten im Oestrus und Dioestrus. *Diss. med. vet., Justus-Liebig-University Giessen, Germany.*

**Papa PC & Hoffmann B** 2011 The corpus luteum of the dog: source and target of steroid hormones? *Reproduction in Domestic Animals* **46** 750–756. (doi:10.1111/j.1439-0531.2010.01749.x)

**Sonnack M** 2009 Untersuchungen zur Bildung, Regression und Funktionalität des Corpus luteum der nicht graviden Hündin, morphologische und biochemische Aspekte. *Diss. med. vet., Justus-Liebig-University Giessen, Germany.*

**Weems YS, Bridges PJ, Tanaka Y, Sasser RG, LeaMaster BR, Vincent DL & Weems CW** 1997 $PGE_1$ or $PGE_2$ not LH regulates secretion of progesterone *in vitro* by the 88–90 day ovine corpus luteum of pregnancy. *Prostaglandins* **53** 337–353.

**Weems YS, Arreguin-Arevalo JA, Nett TM, Vann RC, Ford SP, Bridges PJ, Welsh TH Jr, Lewis AW, Neuendorff DA, Randel RD *et al.*** 2011 *In vivo* intra-luteal implants of prostaglandin (PG) E(1) or E(2) (PGE(1), PGE(2)) prevent luteolysis in cows. I. Luteal weight, circulating progesterone, mRNA for luteal luteinizing hormone (LH) receptor, and occupied and unoccupied luteal receptors for LH. *Prostaglandins & Other Lipid Mediators* **95** 35–44. (doi:10.1016/j.prostaglandins.2011.05.002)

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