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REGULAR ARTICLE

Expression of prostaglandin (PG) D synthase lipocalin and hematopoietic type and PG D receptor during restart of spermatogenesis following downregulation using a slow release GnRH agonist implant in the dog

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Abstract
Prostaglandin D and the associated prostaglandin D synthases (PGDS) and receptor (DP) are considered to be involved in spermatogenesis. However, the interplay of the PGDS-DP system in male reproduction is far from being understood. The expression of PGDS lipocalin (L) and hematopoietic (H) type and DP was studied in the GnRH agonist–downregulated canine testis (week, w 0) and during recrudescence of spermatogenesis after implant removal (w 3, 6, 9, 12). H-PGDS, L-PGDS and DP were present in the adult (CG), juvenile (JG) and downregulated canine testis at the mRNA level. PGDS immunohistochemistry revealed positive staining in the cytoplasm of Leydig cells (LCs) of all samples i.e., no difference between groups. mRNA expression (ratio) of L-, H-PGDS and DP did not differ between groups w 0–12 and CG. In contrast, significant differences were found for L-PGDS ($p = 0.0388$), H-PGDS ($p < 0.001$) and DP ($p < 0.001$) for the groups at downregulation (w0, suprelorin group, SG, profact group, PRG) compared with the control groups (JG, CG). L-PGDS expression was lowest in JG, whereas H-PGDS was significantly lower in CG compared with JG and at downregulation ($p < 0.001$ to $p < 0.01$). The highest ratio for H-PGDS and DP was observed in the dogs treated with buserelin acetate (PRG). Our data show that the PGDS-DP system is expressed in juvenile and adult canine testes and that downregulation of the testicular endocrine and germinative function significantly affects *H-PGDS*, *L-PGDS* and *DP* mRNA expression indicating a role in the regulation of spermatogenesis.

Keywords L-PGDS · H-PGDS · Prostaglandin · Slow release GnRH agonist implant

Introduction

In the last years, an important role of prostaglandins (PG) and the prostaglandin endoperoxidase synthase (PTGS) 2 for testicular physiology and impaired spermatogenesis has been postulated and partly verified (Saito et al. 2002). We recently showed that PTGS2 modulates steroid biosynthesis in the canine Leydig cells, likely via regulation of StAR protein (Körber and Goericke-Pesch 2018). Not only PTGS2 has, however, been postulated/proven to be involved in the regulation of spermatogenesis and male reproduction (Frungieri et al. 2015) but also other PGs, such as PGE (Winnall et al. 2007), PGF2α (Fuchs and Chantharaksri 1981) and PGD2 (Gerena et al. 2000b).

PGD2 has been identified in the male genital tract, namely in the testis, epididymis and seminal fluid of humans (Schell et al. 2007), mice (Gerena et al. 2000a; Moniot et al. 2014), rats (Zhu et al. 2004; Sorrentino et al. 1998), goats (Jin et al. 2017), bovines (Gerena et al. 2000b), rams and stallions (Fouchécourt et al. 1999). Consequently, this prostanoid was shown to be involved not only in a wide range of physiological and pathophysiological events, such as sleep induction, body temperature regulation, nociception (Ueno et al. 1983; Urade and Hayaishi 2000a; Ujihara et al. 1988; Nagata and Urade 2012) and inflammation (Matsuoka et al. 2000) but also in male and female reproduction

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(for review: see Saito et al. 2002; Rossitto et al. 2015). Whereas older studies described the presence of one prostaglandin D2 synthase, PGDS (Baker and O'Shaughnessy 2001; O'Shaughnessy et al. 2002), it is now well known that two different types of PGDS catalyze the conversion of PGH2 into PGD2: the hematopoietic (H-) PGDS, also named spleen-type or glutathione (GSH)-requiring PGDS and the lipocalin (L-) PGDS, also known as the brain-type or GSH-independent PGDS (Urade and Eguchi 2002). Both of them have different functions and their expression in the male gonad depends on the reproductive age: H-PGDS is mainly expressed in the somatic and germ cells of the embryonic gonad of juvenile male mice (Moniot et al. 2011). In mice, H-PGDS was shown to be involved in male gonadal development by nuclear translocation of SOX9 (Moniot et al. 2011). Different to this, H-PGDS mRNA and protein expression in the adult male was only detected in mast cells of men with impaired spermatogenesis but not in healthy controls (Schell et al. 2007; Welter et al. 2011). H-PGDS is the key enzyme of PGD2 synthesis in the immune system in human and mice (Kanaoka and Urade 2003; Urade and Hayaishi 2000b). Its distribution is extremely variable among the species and tissues (Kanaoka et al. 1997; Kanaoka and Urade 2003; Kanaoka et al. 2000). Information about H-PGDS in mature animals and the knowledge of its role in male reproduction are very limited. The other PGDS, the complex L-PGDS acts as a bifunctional enzyme. In addition to the conversion of PGH2, L-PGDS binds and transports small ligands such as steroids, thyroid hormones, bilirubin and retinoids into the extracellular space (Urade and Hayaishi 2000a; Urade and Eguchi 2002). L-PGDS expression has been verified in the testis of various species (Sorrentino et al. 1998; Fouchécourt et al. 1999; Gerena et al. 2000a, b; Zhu et al. 2004; Schell et al. 2007; Moniot et al. 2014; Jin et al. 2017). In bulls, a fertility-associated seminal plasma protein was identified to be L-PGDS (Gerena et al. 1998). Correspondingly, L-PGDS seminal plasma concentrations were significantly lower in oligozoospermic compared with those in normospermic men (Tokugawa et al. 1998) possibly indicating a similar role. The addition of L-PGDS to human semen was postulated to improve progressive motility (Chen et al. 2007). The identification of L-PGDS expression at the apical ridge of the acrosome (Gerena et al. 2000b) resulted in the assumption that L-PGDS might play a role in sperm maturation and acrosome reaction by acting as a retinoic acid transporter (Gerena et al. 2000b), contributing to the blood-testis- and blood-epididymal-barrier (Tanaka et al. 1997).

However, the role of PGD2 in spermatogenesis, its synthesis by the different PGDS and its receptor DP mediating the effect, is still poorly understood and has, to the best of our knowledge, not been investigated in canine testis.

As we have previously shown that long-term gonadotropin suppression by a slow release GnRH agonist implant in the dog affects PTGS2 protein expression and thereby spermatogenesis (Körber and Goericke-Pesch 2018), we considered this model (Goericke-Pesch et al. 2009; Gentil et al. 2012; Goericke-Pesch et al. 2013) suitable to further study H-PGDS, L-PGDS and DP expression in the adult male canine testis and gain additional insights into a possible role for spermatogenesis. As the restart of spermatogenesis was associated with an upregulation of PTGS2, the inducible key enzyme for the prostaglandin biosynthetic pathway (Körber and Goericke-Pesch 2018), we hypothesized that H-PGDS, L-PGDS and DP expression in the canine testes are affected by downregulation following application of a slow release GnRH agonist implant and the subsequent restart of spermatogenesis following abolition of treatment.

Materials and methods

Canine testicular samples were collected as described in a previous study (Goericke-Pesch et al. 2009). Animal experimentation was approved by the respective authority (permit no. AZ V54-19c20/15c GI18/14, Regierungspräsidium Gießen).

Design of experiment

The underlying dog model was established by Goericke-Pesch and has been described in different publications (Goericke-Pesch et al. 2009; Goericke-Pesch et al. 2013; Gentil et al. 2012). A total of 35 sexually mature, healthy male Beagle dogs, with all parameters of semen analysis within the normal range, were included in the study. Out of the total, 30 dogs were treated with a GnRH agonist implant containing 18.5-mg azagly-nafarelin (Gonazon®, Intervet, Angers Technopole, France). Gonazon® implants were removed 5 months later at the time of downregulation with basal testosterone levels [$\overline{x}_g$ (DF)] at 0.10 (1.24) ng/mL and groups of 3–4 dogs were surgically castrated at 3-week intervals (weeks 0–24).

The testes of five adult healthy untreated male Beagle dogs (group CG) and three juvenile healthy untreated mixed breed dogs aged 2.5 months (group JG) served as untreated controls.

Two groups of adult male Beagle dogs ($n = 3$ each) with normal semen parameters were additionally treated for 5 months with either an implant containing 6.3-mg buserelin acetate (Profact® Depot; Sanofi-Aventis, Frankfurt Hoechst, Germany, group PRG) or an implant containing 7.4-mg deslorelin (Suprelorin®; Virbac, Tierarzneimittel GmbH, Germany, group SG) to decipher whether the state of downregulation differs between the three GnRH agonist slow release implants. The mean testosterone concentration at full downregulation for PRG was basal [$\overline{x}_g$ (DF)] 0.09 (1.0) ng/mL] (Goericke-Pesch et al. 2009; Goericke-Pesch et al. 2013), just as for SG [0.1 (1.18) ng/mL].

Formation of groups

The formation of groups was according to Körber and Goericke-Pesch (2018).

Samples of the different castration time points were assigned to two data sets: Data set 1 corresponded to the different states of recrudescence of spermatogenesis in comparison with the adult untreated controls (CG) (Table 1). Due to the fact that all dogs castrated at weeks 15, 18, 21 and 24 had full spermatogenesis being not different from dogs castrated at week 12 and the untreated controls, they were not further included in the statistical evaluation. In data set 2, testes with different GnRH agonist implants downregulated spermatogenesis were compared with the testes of the juvenile (JG) and adult dogs (CG) (Table 2).

Reverse transcriptase polymerase chain reaction and quantitative real-time PCR

Total RNA was isolated from three 10-μm-thick sections from paraffin-embedded tissue samples using the RNeasy ®FFPE Kit (Cat.No.73504, Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. RNA concentration and quality were assessed using a spectrophotometer (NanoDrop® ND-1000, NanoDrop Technologies, Wilmington, USA). Full-length first-strand cDNA synthesis was carried out using 200 ng/μL RNA and the RevertAid First Strand cDNA Synthesis Kit (#K1622, Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

To test for the expression of H-PGDS, L-PGDS, and DP, identical primer sets for reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative real-time PCR (RT-qPCR) were developed using known sequences available from GenBank (Table 3). RT-PCR was performed using the following PCR-cycling conditions: 95 °C for 10 min, followed by 40 cycles of 1 min at 94 °C, 2 min at 56 °C and 1`30 min at 72 °C and, finally, 72 °C for 10 min. Sterile bidest water was used instead of RNA as a no-template control. The amplification of the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control for RNA integrity. RT-qPCR was performed by adding 2-μL cDNA (dilution 1:10) to 5-μL iQTM SYBR Green Supermix (Roche Diagnostics, Basel, Switzerland), 1 μL of the forward and reverse primer (10 pmol) (Table 3) and 1-μL sterile bidest water. RT-qPCR conditions were 95 °C for 5 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s, 72 °C 20 s and melting curve with 65–97 °C. Beta-actin, 18s ribosomal RNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were tested as reference genes. GAPDH showed the slightest variations in mRNA expression and was therefore used as a reference gene for the quantitative evaluation. All samples were run in triplicate using a LightCycler®480 real-time PCR system (Software release 1.5.0, Version 1.5.0.39, Roche Diagnostics) and a non-template control was included in every assay. PCR efficiencies of target and reference genes were calculated using a relative standard curve derived from a triplet RT-qPCR run of a twofold dilution series (1:2–1:128) of pooled cDNA samples, whereas the efficiency (E) was $E = 10(-1/m)$ with m being the slope of the linear regression line (Pfaffl 2001) and calculation was carried out by LightCycler®480 software. The respective efficiencies are given in Table 3. The evaluation of the RT-qPCR results was an efficiency-corrected relative quantification according to Pfaffl (2001).

The specificity of the primers for H-PGDS, L-PGDS and DP used in RT-PCR and RT-qPCR was checked using BLAST (http://blast.ncbi.nlm.nih.gov) and the results were confirmed by sequencing of PCR products (Beckman Coulter Genomics, UK). All primers were synthesized by TAG Copenhagen A/S (Copenhagen, Denmark).

Protein extraction and Western blot analyses

Western blot analysis was performed to test for the expression of PGDS protein and thereby confirm the specificity of the PGDS antibody. Frozen canine total testicular tissue (0.6 g) was pulverized and homogenized using an Ultraturrax (IKA®, Staufen, Germany) in the presence of cold protease inhibitor cocktail (Roche Diagnostics). Afterwards, the mixture was cooked with 0.15-g SDS for 10 min and centrifuged

Table 1 Grouping of dogs during recrudescence of spermatogenesis following removal of a slow release GnRH agonist implant containing 18.5-mg azagly-nafarelin and control group

Group name	Week of castration*	Description	*n*
W0	0	No tubular lumen, most developed germ cells: primary spermatocytes	3
W3	3	Tubular lumen re-established, most developed germ cells: round and elongating spermatids	3
W6	6	Most-developed germ cells: elongating spermatids	4
W9	9	Most-developed germ cells: elongating and elongated	3
W12	12	Full spermatogenesis	3
CG	–	Adult, untreated control dogs	5

*Week of castration after implant removal

Table 2 Grouping of dogs at the state of downregulation and controls (Goericke-Pesch et al. 2013)

Group name	Abbreviation	*n*
W0	Dogs treated with azagly-nafarelin (Gonazon® group) = week 0	3
PRG	Dogs treated with buserelin acetate (Profact® Depot group)	3
SG	Dogs treated with deslorelin (Suprelorin® group)	3
CG	Adult untreated control dogs	5
JG	Juvenile, untreated dogs	3

for 10 min at 1200*g* at 4 °C. The protein concentration of the supernatant was determined using a BCA protein assay (Pierce™ BCA Protein Assay Kit, Thermo Scientific) and proteins were stored at − 80 °C.

For Western blot, approximately 150-μg protein was used following denaturation by heating for 3 min at 95 °C in the water bath. Protein separation was achieved in a 4–20% TGX gel (Mini-Protean® TGX™ Gels, Bio-Rad Laboratories, Hercules, CA, USA) and proteins were blotted on a PVDF membrane (Trans-Blot® Turbo™ Transfer Pack, Bio-Rad Laboratories) using a protein blotting system (Trans-Blot® Turbo ™ Transfer System, Bio-Rad Laboratories). To block unspecific binding sites, the membrane was incubated for 30 min at room temperature in PBS-Blotto (5-g skimmed milk powder, 1-mL thiomersal, 2%, 100-mL PBS buffer). Afterwards, the membrane was washed in 0.2% PBST (phosphate-buffered saline containing Tween 20) and incubated with the primary antibody (PGDS Polyclonal Antibody, Santa Cruz Biotechnologies, Dallas, TX, USA) diluted 1:500 (0.001 mg/mL) in PBS-Blotto for 1 h. After being washed in PBST, the membrane was incubated at room temperature for 45 min with the secondary antibody (dilution 1:200 in PBS-Blotto, biotinylated goat anti-rabbit IgG antibody, BA-1000, Vector Laboratories, Burlingame, CA, USA). Signals were visualized using Clarity™ Western ECL Blotting Substrate (Bio-Rad Laboratories) according to the manufacturer's instructions and ChemiDoc™ Imaging Systems with Image Lab™ Touch Software (Image Lab 6.0.1, Bio-Rad Laboratories). A rabbit IgG nonsense antibody (I-1000, Control Antibody, Vector Laboratories) in the same concentration as the primary antibody served as an isotype control. RAW 264.7 (IP) cell lysate (Abelson-transformed macrophage cells) (Santa Cruz) served as a positive control (Tanaka et al. 2004).

Immunohistochemistry

Sections from Bouin-fixed paraffin blocks were deparaffinized and antigens were demasked by pretreatment with cooking citrate buffer (pH = 6). Endogenous peroxidase activity was inhibited using 3% hydrogen peroxide in methanol. Goat serum (10% in ICC buffer) was used to block unspecific binding sites followed by incubation with the PGDS polyclonal antibody (Santa Cruz Biotechnology, FL-199, sc-30066, dilution 1:500 corresponding to 0.4 μg/mL) overnight at 4 °C. Each step was followed by a washing step with ICC buffer (1.2-g Na2HPO4, 0.2-g KH2PO4, 0.2-g KCl, 8.0-g NaCl, 3-mL Triton ad 1000 mL). Treatment with the secondary antibody (BA-1000, Vector Laboratories) and visualization of the signals using an immunoperoxidase system

Table 3 Sequences of primers for RT-PCR and RT-qPCR, amplicon length, efficiency and accession number

	Oligonucleotide sequence (5′-3′)	Amplicon length (bp)	Efficiency	Accession number
GAPDH		228	1.988	NM_001003142
for	GGCCAAGAGGGTCATCATCTC			
rev	GGGGCCGTCCACGGTCTTCT			
H-PGDS		143	1.975	NM_001200044.2
for	GGG CAG ATT TCT ATT GGG			
rev	GGG CCT TTG TTG TAT CC			
L-PGDS		123	2.194	NM_001003131.1_canine
for	CTGCAGCCCAACTTCCAAC			
rev	GGCCACCACTGACATACAC			
DP		52	2.049	XM_848401.2_canine
for	GGAGTGCTGGCTGTCTTTG			
rev	GGTGATGTGCCGTCTGTAG			

for, forward; *rev*, reverse

(VECTASTAIN PK-6101 Rabbit IgG Elite ABC Kit and Vector Nova-RED Substrate Kit SK-4800, Vector Laboratories) were performed according to the manufacturer's protocol. Negative controls were set up using ICC buffer and irrelevant isotype controls (I-1000, Rabbit IgG, Control Antibody, Vector Laboratories) in the respective protein concentration instead of the primary antibodies. For the descriptive evaluation of the PDGS staining, one set of slides was counterstained with hematoxylin (Mayer haemalaun solution, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) before washing and mounting with Eukitt® (Sigma-Aldrich Chemie GmbH, Munich, Germany). Additionally, another set of slides without counterstaining was used for the computer-assisted image analysis using GIMP 2.8 (https://www.gimp.org/) and ImageJ FIJI win-64 (https://imagej.net/Fiji) to quantify the percentage of the immunopositive area (PIA) and the staining intensity (mean grayscale) of the interstitial tissue as described before for ImageTool 3.0 (Gentil et al. 2012; Hoffmann et al. 2004). Immunohistochemistry of these slides was performed in one experiment so that all samples were treated the same way. The mean grayscale and PIA of the interstitial tissue were assessed in ten randomly selected images at 200-fold magnification per dog. The images were transferred into grayscale pictures; the seminiferous tubules, blood vessels and artifacts were cropped and an individual threshold was chosen subjectively. Positive signals above the threshold were used for the determination of the staining intensity (mean grayscale). For the grayscale, 0 was defined as white and 255 as black. The immunopositive area (PIA) was assessed using the binarized grayscale image. In this image, the black area represents the immunopositive-stained area (Gentil et al. 2012).

Toluidine blue staining

Toluidine blue staining was performed to investigate whether PGDS-immunopositive signals were associated with testicular mast cells (Welter et al. 2011). For the staining, 4-μm-thick tissue cuts were prepared from the formalin-fixed paraffin-embedded testicular tissue samples, deparaffinized in xylene (2 × 10 min), stepwise rehydrated in 99%, 96%, 80%, 70%, 60% and 50% ethanol (5 min each) and washed under running Aqua dest water (2 × 2 min). Subsequently, the slides were colored with 0.003% toluidine blue (Toluidine blue O, Waldeck GmbH & Co. KG, Münster, Germany) diluted in Sorensen buffer (1.2-mL solution A, 9.078-g KH_2PO_4 ad 1000 mL Aqua bidest; and 198.8-mL solution B, 2.969-g Na_2HPO_4 ad 250 mL Aqua bidest) for 15 min. Finally, the tissue slides were washed in Aqua bidest and isopropanol (10 dips each) and mounted with Eukitt® (Sigma-Aldrich Chemie Gmbh). The tissue of a canine mast cell tumor was included in the procedure as a positive control. Descriptive evaluation on all slides was performed by visual inspection using a light microscope (Olympus BX41, Shinjuku, Tokyo, Japan) at 400-fold magnification.

Statistical analysis

Assembling of groups was as follows: Data set 1 (recrudescence) consisted of the groups reflecting downregulation and restart of spermatogenesis compared with the untreated adult controls (group weeks 0, 3, 6, 9, 12 and CG). Data set 2 (downregulated testis) consisted of the groups reflecting the state of downregulation with three different GnRH agonist implants (week 0, PRG, SG) compared with juvenile (JG) and adult untreated control testes (CG).

For all tests, Graph Pad Prism7 software (GraphPad Software, Inc., La Jolla, USA) was used. Values were considered to be statistically significant at a level of $p < 0.05$. The ratio of *H-PGDS*, *L-PGDS* and *DP* as well as PIA is presented as geometric mean and dispersion factor [$\overline{x}_g$ (DF)]. Mean grayscale is presented as arithmetic mean and standard deviation [$\overline{x} \pm$ SD]. Parametric ANOVA (mean grayscale data sets 1 and 2) followed by Tukey's multiple comparison test if $p < 0.05$ or the nonparametric Kruskal-Wallis test (ratio, PIA data sets 1 and 2) was performed to identify significant differences between groups.

Previously published results on *PTGS2* mRNA expression (Körber and Goericke-Pesch 2018) were correlated with the results of *H-PGDS*, *L-PGDS* and *DP* mRNA expression (ratio) separately for both data sets using a two-tailed Spearman test for unevenly distributed data.

Results

Data set 1: Restart of spermatogenesis

H-PGDS, L-PGDS and DP mRNA expression

RT-PCR revealed a specific band for *H-PGDS*, *L-PGDS* and *DP* in all groups (not shown). mRNA expression (ratio) for *H-PGDS*, *L-PGDS* and *DP* using whole tissue homogenates did not differ significantly between the samples obtained at downregulation and the different time points of restart of spermatogenesis compared with the untreated controls (CG), mainly due to large individual variations in all groups (Table 4).

H-PGDS and *DP* mRNA expression were positively correlated with *PTGS2* expression (*H-PGDS*, $r = 0.59$, $p = 0.0048$; *DP*, $r = 0.48$, $p = 0.0275$).

PGDS protein expression

Western blot for PGDS revealed a specific immunoreactive band at approximately 28 kDa using protein homogenate from an adult dog with normal spermatogenesis (Fig. 1, lane 1). No

Table 4 *H-PGDS*, *L-PGDS* and *DP* mRNA expression (ratio) during downregulation and recrudescence of spermatogenesis (weeks 0–12) compared with healthy untreated adult controls (CG).

Group	H-PGDS	L-PGDS	DP2	PTGS2*
Week 0	2.49 (1.92)	2.67 (3.03)	4.19 (1.71)	0.77 (2.75)
Week 3	1.95 (1.78)	1.49 (1.50)	5.05 (1.22)	1.81 (1.62)
Week 6	0.30 (2.34)	2.56 (1.89)	3.18 (2.01)	0.89 (1.18)
Week 9	0.95 (2.62)	0.50 (4.52)	2.53 (2.10)	0.34 (2.57)
Week 12	0.05 (50.57)	3.64 (1.31)	0.73 (4.63)	0.18 (16.23)
CG	0.17 (3.11)	0.75 (4.53)	2.38 (1.53)	0.82 (2.18)
p value	n.s.	n.s.	n.s.	n.s.

Relative gene expression [ratio, $\overline{x}_g$ (DF)] as obtained by RT-qPCR

n.s., not significant; *Previously published (Körber and Goericke-Pesch 2018)

specific immunopositive band was visible in the isotype control (Fig. 1, lane 2).

Immunohistochemistry revealed a specific immunopositive staining against PGDS in the cytoplasm of Leydig cells in all samples (Fig. 2). Additionally, two of the dogs of the adult untreated control group showed immunopositive staining within the Sertoli cell cytoplasm mainly located close to the basal membrane (Fig. 2g). Using computer-assisted objective evaluation of PGDS protein expression in immunohistochemistry, PIA was higher in the adult control group and the late stages of recrudescence (weeks 9 and 12) (Fig. 2, Table 5) compared with the group at downregulation (week 0) and the early restart of spermatogenesis (weeks 3 and 6) with, however, no significant differences (Table 5). Also the staining intensity (mean grayscale) did not differ significantly among groups (Table 5).

Data set 2: Downregulation

H-PGDS, L-PGDS and DP mRNA expression

RT-PCR revealed a specific band for *H-PGDS*, *L-PGDS* and *DP* in all groups (not shown).

We observed significant differences in the RT-qPCR for the groups at downregulation (week 0, SG, PRG) compared with the control groups (JG, CG) for *H-PGDS* (ANOVA, $p < 0.001$), *L-PGDS* (ANOVA, $p = 0.0388$) and *DP* (ANOVA, $p < 0.001$) (Table 6). For *H-PGDS*, CG revealed significant lower ratio values compared with the downregulated testis (PRG, SG, week 0) (Tukey, $p < 0.001$ to $p = 0.004$) and JG (Tukey, $p = 0.006$). *L-PGDS* expression was lowest in JG, with significant differences compared with that in SG (Tukey, $p = 0.0361$). The highest ratio for *DP* was observed in the dogs treated with buserelin acetate (PRG), with the ratio being significantly higher than in the testes downregulated with the other GnRH agonists (Tukey, week 0, $p < 0.0001$; SG, $p = 0.0005$) and the controls (JG, CG, both $p < 0.0001$). Additionally, *DP* mRNA ratio for SG was significantly higher than in CG (Tukey, $p = 0.0393$).

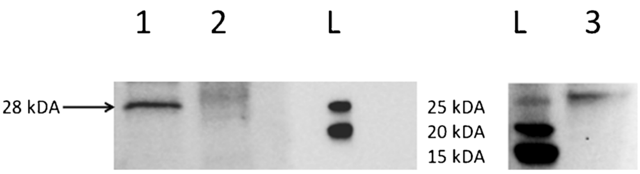

Fig. 1 Western blot analysis for PGDS. Lane 1, canine total testicular protein; lane 2, isotype control for canine protein; lane 3, RAW 264.7 (IP) cell lysate (Abelson-transformed macrophage cells) served as a positive control. Molecular weight markers are expressed in kDa; L, ladder

Spearman test revealed a trend for a correlation between *PTGS2* and *H-PGDS* ($r = 0.48$, $p = 0.0516$) but no correlation between *PTGS2* and *DP* expression.

PGDS protein expression

Positive PGDS immunostaining was localized in the cytoplasm of Leydig cells in all samples (Fig. 3). As already described for data set 1, cytoplasmatic staining within the Sertoli cell cytoplasm close to the basal membrane was identified in two of the adult untreated dogs. PIA and mean grayscale showed no significant differences between the different groups of downregulation (W0, SG, PRG) compared with the juvenile (JG) and adult (CG) control group (Table 7).

Toluidine blue staining in data sets 1 and 2

The positive control (mast cell tumor) revealed a strong purple staining of the granules in the cytoplasm of the mast cells. In the canine testicular tissue samples, there were less cytoplasmatic granules stained per identified mast cell. Mast cells were mainly located in the interstitial tissue close to the testicular capsule. Only one dog of week 6 and one dog of JG had few individual mast cells within the testicular parenchyma. In general, the total number of mast cells was low and with sporadic presence (Fig. 4). Subjectively, no differences were detected between groups and samples.

Discussion

To the best of our knowledge, we could for the first time prove the expression of *H-PGDS*, *L-PGDS* and *DP* in healthy canine juvenile and adult testis as well as during downregulation due to GnRH agonist slow release implant treatment and subsequent recrudescence of spermatogenesis on mRNA and PGDS at the protein level. Due to the lack of commercially available antibodies against DP and the lacking cross-reactivity of available DP antibodies against the respective canine proteins, the present investigations at the protein level are restricted to PGDS expression.

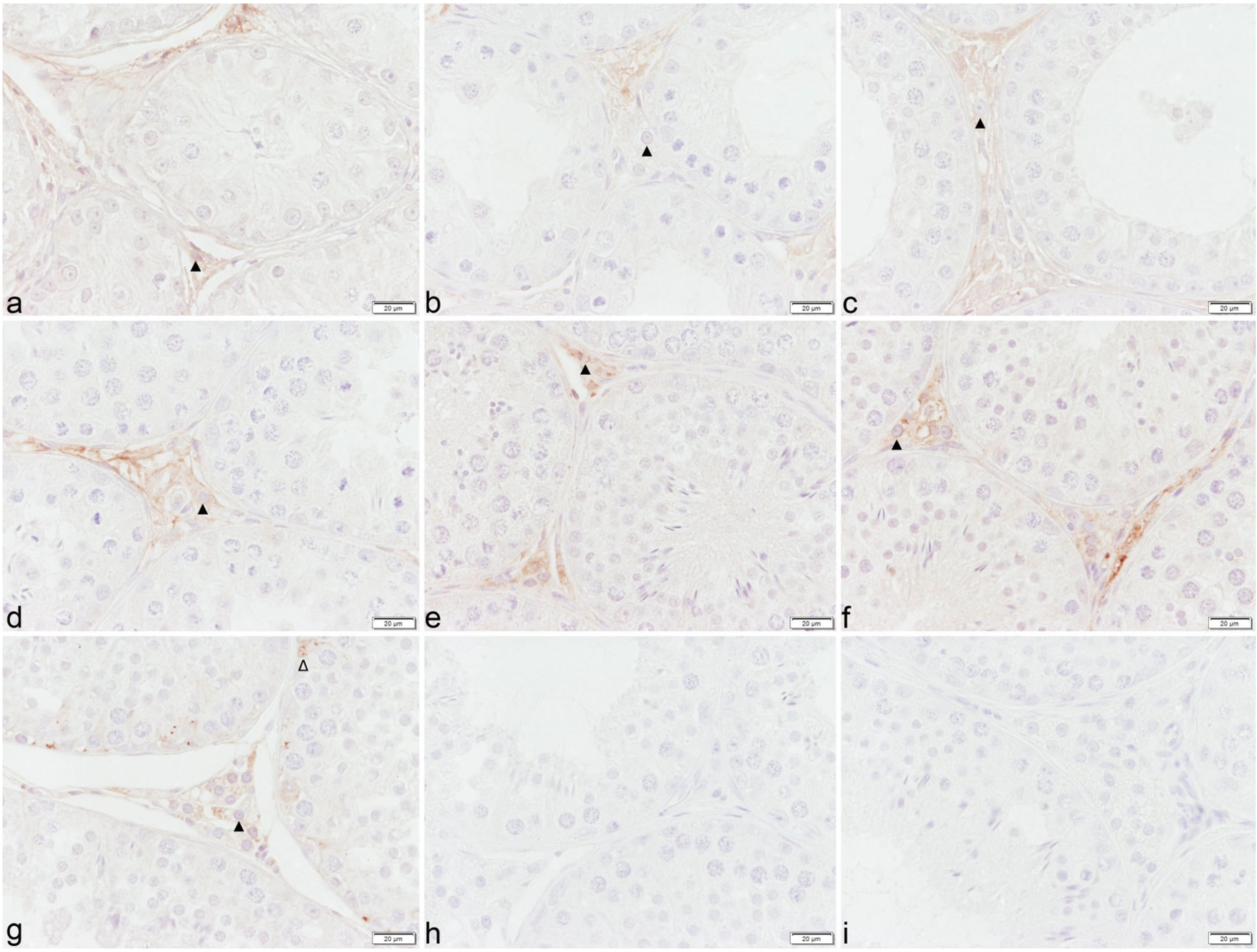

Fig. 2 Immunostaining for PGDS in dog testicular tissue (data set 1). Group week 0 (**a**); group week 3 (**b**): group week 6 (**c**); group week 9 (**d**); group week 12 (**e**); CG (control group) (**f**); CG (control group) (**g**); isotype control (**h**); negative control (**i**) (**a**–**i**, magnification × 400). Black triangle, Leydig cells; white triangle, Sertoli cells

Table 5 Results of PGDS protein expression of the interstitium during recrudescence of spermatogenesis (weeks 0–12) compared with healthy untreated adult controls (CG). Results are presented as mean grayscale values ($\bar{x} \pm SD$) and the mean percentage of the immunopositive area (PIA) [$\bar{x}_g$ (DF)]

	Interstitium	
	Mean grayscale	PIA
Week 0	77.24 ± 0.52	2.33 (1.85)
Week 3	77.62 ± 0.95	0.76 (1.53)
Week 6	76.82 ± 0.17	1.24 (2.51)
Week 9	76.95 ± 0.78	2.49 (1.61)
Week 12	77.68 ± 0.44	5.59 (2.29)
CG	77.05 ± 0.23	4.99 (1.62)
p value	n.s.	n.s.

n.s., not significant

At the mRNA level, no significant differences in *L-PGDS*, *H-PGDS* and *DP* expression were identified for data set 1, probably due to large individual variations as well as small group size. However, *L-PGDS*, *H-PGDS* and *DP* mRNA expression differed significantly in data set 2. Quantitative RT-qPCR for *L-PGDS* showed the lowest ratio for the juvenile testis and for *H-PGDS* mRNA, expression was lowest in the adult control group. The highest ratio for *H-PGDS* and *DP* was observed in the downregulated dogs treated with buserelin acetate (PRG). Baker and O'Shaughnessy (2001) described a shift in *PGDS* mRNA expression in the murine testis during the development with *PGDS* expression being restricted to the tubular compartment until day 15 and a 40-fold increase in the interstitial compartment from day 30. Moniot et al. (2009, 2014) later confirmed these findings for *L-PGDS* mRNA expression in the male gonads of mice: L-PGDS is present in prospermatogonia and SOX9-expressing Sertoli cells in the embryonic testis and shifts to the Leydig cells in the adult testis. Interestingly, in rats,

Table 6 *H-PGDS*, *L-PGDS* and *DP* mRNA expression (ratio) at downregulation of spermatogenesis (PRG, SG, week 0) compared with adult and juvenile controls (CG, JG)

Group	H-PGDS	L-PGDS	DP2	PTGS2*
Week 0	2.49 (1.92)^{a}	2.67 (3.03)^{a,b}	4.19 (1.71)^{a,c}	0.77 (2.75)
PRG	9.96 (1.02)^{a}	1.13 (1.73)^{a,b}	14.39 (1.14)^{b}	2.40 (1.51)
SG	3.34 (1.58)^{a}	4.09 (1.97)^{a}	6.33 (1.33)^{a}	0.25 (6.23)$^{\#}$
JG	2.17 (2.04)^{a}	0.01 (1.80)^{b}	2.51 (1.88)^{a,c}	0.37 (3.05)
CG	0.17 (3.11)^{b}	0.75 (4.53)^{a,b}	2.38 (1.53)^{c}	0.82 (2.18)
p value (ANOVA)	0.0001	0.0388	< 0.0001	n.s.

Relative gene expression [ratio, $\bar{x}_g$ (DF)] as obtained by RT-qPCR

Different superscripts indicate significant differences between groups within the column (Tukey-Kramer test, $p < 0.01$). Data within columns with different superscripts (a, b, c) differ with $p < 0.0001$ to $p = 0.0361$. *Previously published (Körber and Goericke-Pesch 2018). $^{\#}$ First publication

L-PGDS expression was absent in the juvenile but present in the adult testis with Sertoli and germ cells staining immunopositive (Samy et al. 2000). The results in the rat correspond well to our results with the lowest *L-PGDS* mRNA expression observed in the canine juvenile testis and a significantly higher expression in adult canine testis. Different from rats, *L-PGDS* is expressed in Leydig cells in human testis with impaired spermatogenesis (Schell et al. 2007).

Whereas *H-PGDS* expression in the adult testes has only been described in mast cells in men with mixed atrophy—positively correlated with the expression of PTGS2 (Welter et al. 2011), we identified *H-PGDS* mRNA expression in all investigated canine testes. The highest mRNA expression of *H-PGDS* but also the respective receptor, *DP*, was detected in the canine testis with arrested spermatogenesis. This observation was made independently of the GnRH agonist implant used to downregulate testicular endocrine and germinative function indicating a similar effect induced. However, interestingly and similar to previous investigations on PTGS2 (Körber and Goericke-Pesch 2018), dogs treated with buserelin acetate showed highest *H-PGDS* (tendency only) and *DP* (significantly different) mRNA expression. The histological examinations did not reveal an increased presence of testicular mast cells in downregulated canine testes as a source for PGDS staining indicating species-specific different expression patterns. It can only be speculated if the high *H-PGDS* and *DP* mRNA expression at downregulation has a functional role. The identified significant correlation (data set 1, $p = 0.0048$) and the trend (data set 2; $p = 0.0516$), respectively, between *H-PGDS* and *PTGS2* mRNA expression indicates a positive feedback of these respective enzymes of the prostaglandin pathway on each other and seems also useful as PTGS2 is required for substrate provision for PGDS.

PGDS protein expression was identified using immunohistochemistry in the interstitial compartment, namely in Leydig cells of all samples with, however, no significant differences in the percentage immunopositive area and the staining intensity for the interstitial tissue of the samples of both data sets. Although the human PGDS antibody detects H-PGDS synthase only according to the manufacturer, it remains to be clarified whether the antibody detects H-PGDS in the dog, too, or if both variants, H- or L-PGDS, were detected. Literature about PGDS expression in humans is somehow contradictory as one study identified PGDS, namely L-PGDS, expression at the protein level in human Leydig cells but only in case of impaired spermatogenesis (Schell et al. 2007). In another study, at the mRNA level, however, *H-PGDS* expression was found in testicular mast cells obtained by laser-assisted microdissection from testicular biopsies with impaired spermatogenesis (mixed atrophy) (Welter et al. 2011). Interestingly, two dogs of the adult untreated control group showed immunopositive signals in the Sertoli cell cytoplasm. Immunopositive signals in Sertoli cells have been described for L-PGDS in mice; they were stage-specific and only present shortly prior spermiation (stages VI–VIII) (Gerena et al. 2000a). L-PGDS has been identified as a retinoid transporter (Tanaka et al. 1997) and retinoid acid is known to be crucial for spermatogonial differentiation and meiosis (Griswold 2016). However, only two out of five adult untreated dogs in our study showed positive PGDS staining in the Sertoli cell cytoplasm but not germ cells; with signals not being restricted to a specific stage. Furthermore, the distribution of stages in the respective control dogs was as previously described to be normal for dogs (Goericke-Pesch et al. 2009). To further investigate the inconsistent immunopositive signal in Sertoli cells of two dogs, further immunohistochemistries were performed. Using higher dilutions resulting in lower antibody concentrations, Sertoli cell staining was omitted in the respective two dogs. Using higher antibody concentrations, Sertoli cells in all groups—except for the juvenile dogs (JG)—stained immunopositive. Immunopositive signals in dogs with normal, undisturbed spermatogenesis (week 12, adult controls, CG) were not stage-specific as reported in mice (Gerena et al. 2000a). These experiments might indicate that PGDS protein concentration in Sertoli cells is lower than in Leydig

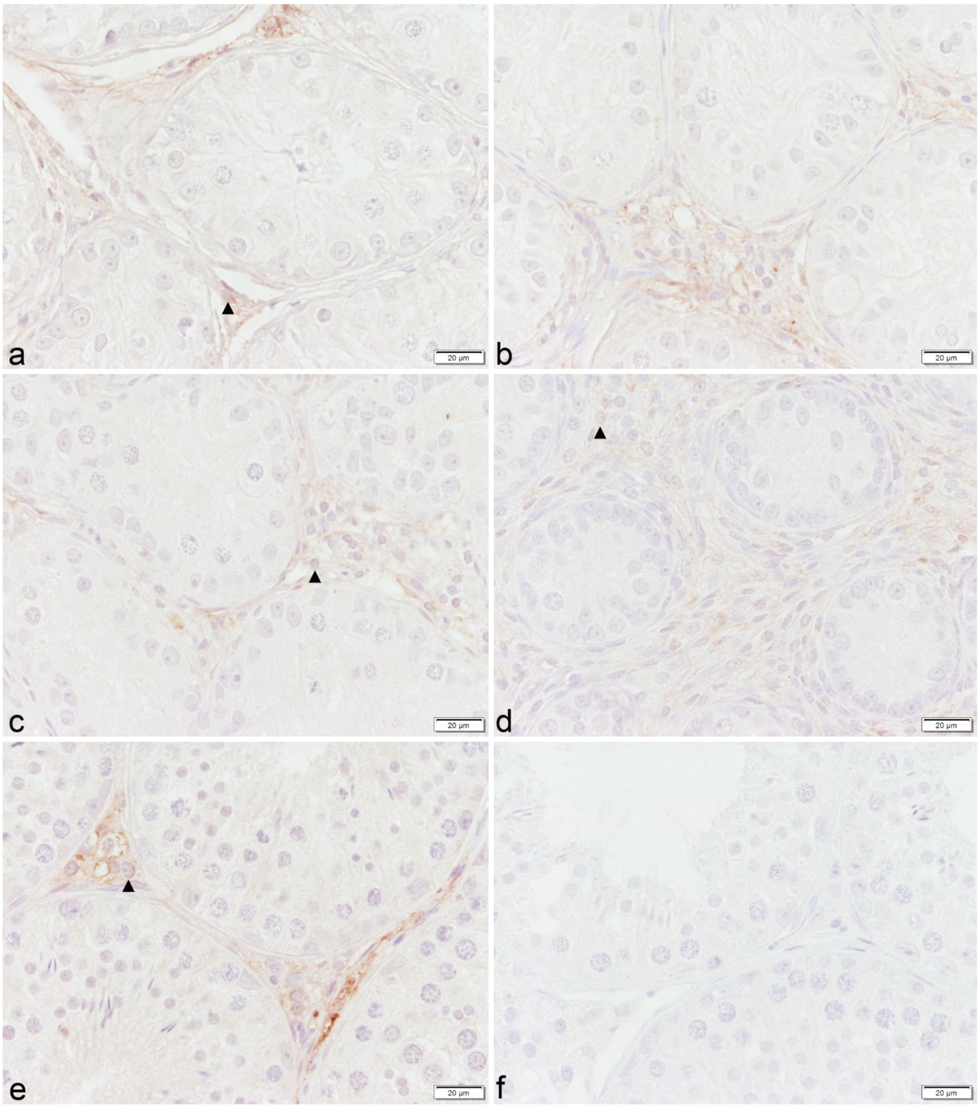

Fig. 3 Immunostaining for PGDS in dog testicular tissue (data set 2). Group week 0 (Gonazon® group) (**a**); PRG (Profact® Depot group) (b); SG (Suprelorin® group) (**c**); JG (juvenile) (**d**); CG (control group) (**e**); isotype control (**f**) (**a**–**f**, magnification × 400). Black triangle, Leydig cells

cells and why only two dogs showed inconsistent immunopositive signals in Sertoli cells in the main experiment. Consequently, it seems possible that PGDS in the dog might be involved in supporting spermatogenesis via Sertoli cell function as described earlier (Tanaka et al. 1997; Griswold 2016). Retinoic acid has also been shown to have a direct stimulatory effect on steroidogenesis in murine and rat Leydig cell culture (Chaudhary et al. 1989; Chaudhary and Stocco 1990). This might deserve further consideration when discussing the potential role of PGDS as a retinoid transporter (Tanaka et al. 1997) in general and in the dog specifically. Interestingly, however, mRNA and protein expression did not differ between groups in data set 1, although steroidogenesis is significantly upregulated during an early restart of spermatogenesis (weeks 3 and 6) (Goericke-Pesch et al. 2009; Gentil et al. 2012). Further studies with an antibody specific for L-PGDS should be conducted to clarify this issue.

Table 7 Results of PGDS protein expression of the interstitium during downregulation and compared with healthy untreated juvenile (JG) and adult (CG) controls. Results are presented as mean grayscale values ($\bar{x} \pm$ SD) and the mean percentage of the immunopositive area (PIA) [$\bar{x}_g$ (DF)]

	Interstitium	
	Mean grayscale	PIA
Week 0	77.24 ± 0.52	2.33 (1.85)
PRG	77.89 ± 0.61	3.93 (1.99)
SG	77.90 ± 0.18	1.05 (2.85)
JG	77.98 ± 2.03	1.04 (2.46)
CG	77.05 ± 0.23	4.99 (1.62)
p value	n.s.	n.s.

n.s., not significant

Unfortunately, DP protein expression could not be studied due to the lack of commercially available antibodies against

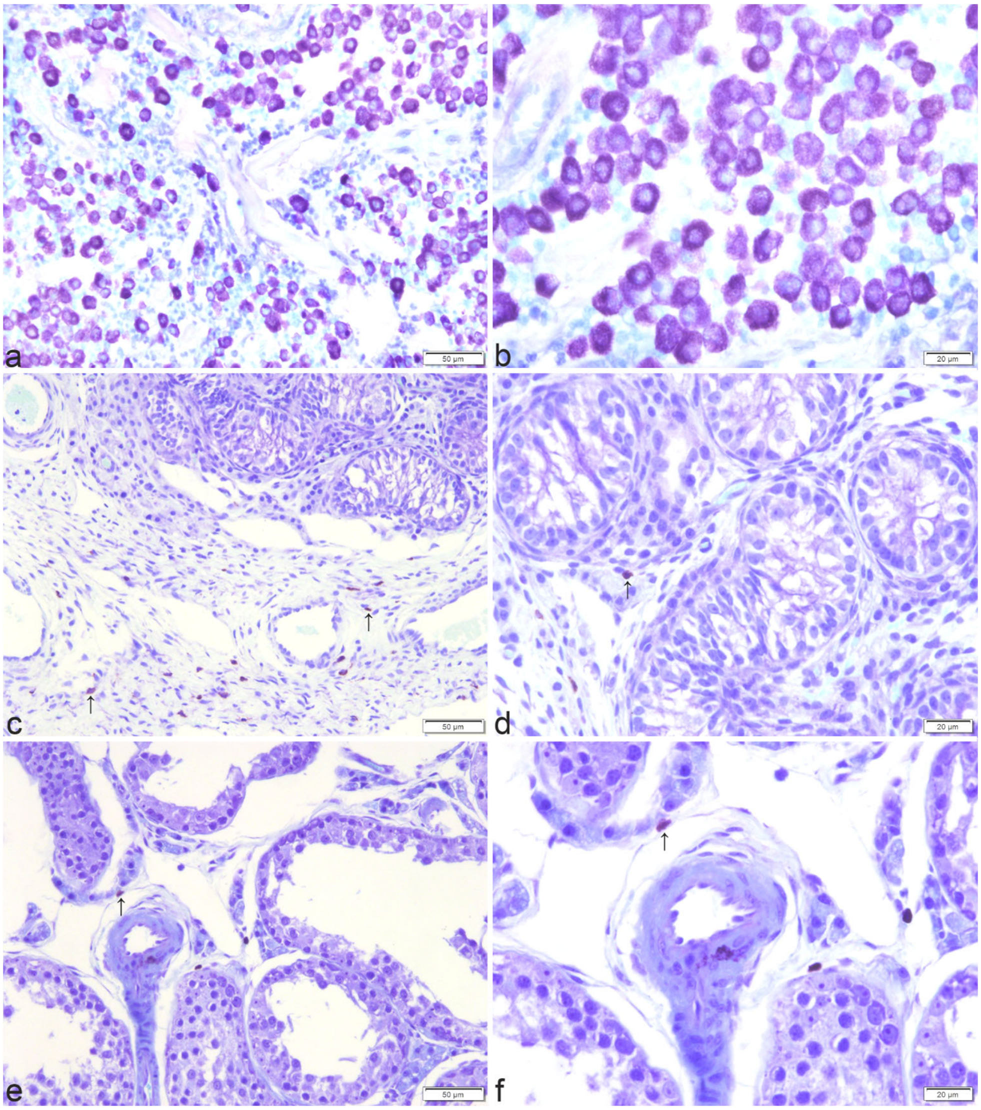

Fig. 4 Toluidine blue staining of mast cells in canine tissue. Positive control (**a**, b): canine mast cell tumor; canine testicular tissue (juvenile group) (**c**, **d**); canine testicular tissue during restart of spermatogenesis, week 6 (**e**, **f**) (**a**, **c**, **e** magnification × 200; **b**, **d**, **f** magnification × 400). Upwards arrow, mast cell

canine DP and lacking cross-reactivity, respectively. Further studies—possibly using custom-made canine-specific antibodies against DP—are required to better understand the role of PGD2 in canine spermatogenesis, specifically. Follow-up studies should also consider the use of laser-assisted cell picking to purify mRNA from pure interstitial and tubular tissue, respectively, to specify which *PGDS* variant is expressed in which compartment and to allow for the identification of possible differences in mRNA expression of the respective genes in the individual testicular compartments.

To conclude, our observations that downregulation of testicular function by application of a GnRH agonist implant and subsequent restart of spermatogenesis affects *H-PGDS*, *L-PGDS* and *DP* expression in the canine testis and that *PTGS2* expression is positively correlated with *H-PGDS* and *DP* expression during recrudescence further supports the hypothesis that the PTGS2/PG system plays an important role for autocrine and paracrine regulation of spermatogenesis. In our study, and different to men, PGDS and PTGS2 expression is not restricted to canine samples with impaired spermatogenesis indicating species-specific differences. The prostaglandin pathway—regulation of PTGS2 and the downstream products (PGD2, PGF2α, PGE2)—deserves further investigation to understand its role in canine spermatogenesis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Statement of the welfare of animals All applicable national and institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted.

Animal experimentation had been approved by the respective authority (permit no. AZ V54-19c20/15c GI18/14, Regierungspräsidium Gießen).

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