Status of the down-regulated canine testis using two different GNRH agonist implants in comparison with the juvenile testis

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Abstract

Testicular function in the dog was down-regulated using two different GNRH agonist implants, with adult and juvenile testes serving as controls. Treatment resulted in an increased percentage of the interstitial area and decreased area of Leydig cell nuclei. Expression of StAR and the steroidogenic enzymes cytochrome P450 side-chain cleavage enzyme (P450scc, CYP11A1) and cytochrome P450 17α-hydroxylase-17,20-lyase (P450c17, CYP17A1) in Leydig cells was blocked at the mRNA and protein level, showing no differences between the two agonists. Staining for androgen receptor (AR) by immunohistochemistry was positive in Sertoli, Leydig and peritubular cells and some spermatogonia, with in situ hybridization confirming expression in Sertoli cells. At the mRNA level, expression of AR was not affected; however, translation was blocked (reduced percentage of AR-positive Sertoli cells), with the number of nuclei in basal position being decreased. In the juvenile testes, mRNA expression of StAR, CYP11A1 and CYP17A1 was higher compared with the other groups but distinctly lower for the AR. At the protein level, the expression was at the limit of detection for StAR; AR-positive Sertoli cells were not detected. Our observations show that the down-regulated testis is different from the juvenile one rather resembling the testicular status in seasonal breeders out of season.

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Introduction

Down-regulation of testicular function in the dog using slow-release gonadotrophin-releasing hormone (GNRH) agonist implants is now an accepted alternative approach to surgical castration. It covers all indications of surgical castration with the only difference being that down-regulation is in general a fully reversible effect (Vickery et al. 1984, Dube et al. 1987a, 1987b, Riesenbeck et al. 2002, Goericke-Pesch et al. 2009, Ludwig et al. 2009). As we have shown previously (Goericke-Pesch et al. 2009), down-regulation affects the whole steroidogenic cascade and leads to an arrest of spermatogenesis at the level of spermatogonia and primary spermatocytes (Gentil et al. 2012). Clinical observations have clearly shown that duration of testicular down-regulation may considerably vary, e.g. between 3 months and > 1 year (Goericke-Pesch et al. 2010b). Presently, it can only be speculated whether this result from the degree of down-regulation at both the pituitary and testicular level or from the individual pharmacokinetic profile of the GNRH agonist.

The aim of the present paper was to assess the status of the canine testis at full down-regulation as indicated by having reached constant testosterone concentrations at around 0.1 ng/ml. Assessment was based on morphological evaluations and determination of the expression of the StAR and the steroidogenic enzymes cytochrome P450 side-chain cleavage enzyme (P450scc) and cytochrome P450 17α-hydroxylase-17,20-lyase (P450c17) after having applied two different GNRH agonist implants containing either azagly-nafarelin (Gonazon) or buserelin acetate (Profact Depot) as the active ingredient. Additionally, the expression of the androgen receptor (AR) as an indicator for hormonal responsiveness was examined. The same parameters were assessed in testis of juvenile dogs in order to establish likely differences to the down-regulated testis.

Materials and methods

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

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Design of experiment, formation of groups

As described previously (Goericke-Pesch et al. 2009), 35 sexually mature and clinically healthy male Beagle dogs had been implanted s.c. in the parambilical area with the removable GNRH agonist implant Gonazon, containing 18.5 mg azagly-nafarelin as the active ingredient (Intervet, Angers Technopole, France). Of this group, three dogs were randomly selected for this study and were castrated 5 months later after having achieved full down-regulation (testosterone, $x^g_{DF} = 0.09 (1.0)$ ng/ml) forming group GG (Table 1). Similarly, three dogs were implanted s.c. in the neck with the non-removable GNRH agonist implant Profact Depot (Sanofi-Aventis, Frankfurt Hoechst, Germany) containing 6.3 mg buserelin acetate forming group PG and were castrated again 5 months later with testosterone concentrations being the same as in group GG. Following castration, testes were preserved for further examinations as described previously (Goericke-Pesch et al. 2009). Five adult male Beagle dogs (group CG) and three juvenile mixed breed males aged 2 months (group JG) served as untreated controls.

Tissue preservation

Collection and preservation of testicular tissue samples for immunohistochemistry (IHC) using Bouin's solution for fixation was as described previously (Goericke-Pesch et al. 2009). For the preservation of tissue for RNA and protein extraction, parenchyma samples from the area between the tunica albuginea and the mediastinum testis were immersed in seven volumes of RNA later (Ambion Biotechnologie GmbH, Wiesbaden, Germany) for at least 24 h at 4°C and then stored at −80°C.

Morphological examinations

Sections (3–4 μm) from different paraffin blocks were cut, dried, haematoxylin–eosin stained and mounted in Histokitt (Assistent, Osterode, Germany). Based on previous observations (Goericke-Pesch et al. 2009), in groups GG and PG, tubuli ($n=200$) were evaluated for the presence of spermatogonia only or spermatogonia and primary spermatocytes and the respective percentage was calculated. Area of Leydig cell nuclei was determined as described earlier (Goericke-Pesch et al. 2009).

Slides stained for AR and evaluated as described above also served to determine the tubular area and the total number of Sertoli cells and spermatogonia per tubule. For evaluation of the tubular area, Leica Image Manager IM 1000 (Leica Microsystems, version 1.20, Wetzlar, Germany) measuring tool was used. The tubular area was determined according to the equation: $area = \frac{a}{3}b \times \pi$, with $a$ and $b$ being the largest and smallest diameter in a right angle. The proportion (%) of the tubular compartment (TC), the interstitial compartment (IC) and the area of large vessels (AV) constituting the testicular parenchyma was obtained as described before when evaluating immunohistochemical staining for StAR, P450scC and P450c17 (Gentil et al. 2012).

Table 1 Grouping of dogs.

<table>
<thead>
<tr>
<th>Group names</th>
<th>Abbreviations</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>Dogs treated with azagly-nafarelin (Gonazon group)</td>
<td>3</td>
</tr>
<tr>
<td>PG</td>
<td>Dogs treated with buserelin acetate (Profact Depot group)</td>
<td>3</td>
</tr>
<tr>
<td>CG</td>
<td>Adult untreated control dogs</td>
<td>5</td>
</tr>
<tr>
<td>JG</td>
<td>Juvenile untreated dogs</td>
<td>3</td>
</tr>
</tbody>
</table>

RT-PCR and semi-quantitative real-time PCR

The procedure including primers and probes for RT-PCR and semi-quantitative real-time PCR (qPCR) for StAR, CYP11A1 (P450Scc) and CYP17A1 (P450C17) was as described previously (Gentil 2012, Gentil et al. 2012). To test for the expression of the AR, the same procedure including treatment with DNase was applied for mRNA extraction and further preservation. For PCR of the AR, primer sets were developed using known sequences available from GenBank (Table 2). PCR cycling conditions for AR were as follows: 95°C for 10 min, followed by 35 cycles of 1 min at 94°C, 2 min at 60°C, 1.5 min at 72°C and finally 72°C for 10 min. In every experiment, DEPC-treated water was used instead of RNA as no-template control. Amplification of the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as control for RNA integrity.

Primer and hydrolysis probe sequences used for qPCR of the AR as well as length of the amplicons are given in Table 2. The master mix was prepared containing 25 μl iQ supermix for qPCR (2X; Bio-Rad Laboratories), 0.75 μl of the forward and reverse primer each (20 μM) and 0.5 μl TaqMan probe (20 μM) and 40 μl with DEPC-treated water. It was mixed with 10 μl cDNA of each sample. Afterwards, 23 μl were pipetted into a well of a MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems). All samples were run in duplicates and a no-template control was included in every assay. The cycling conditions were as follows: 95°C for 3 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. GAPDH was chosen as a reference gene for all qPCRs as it was shown to be non-regulated in preliminary and main experiments (data not shown). For evaluation of the qPCR results, an efficiency-corrected relative quantification according to Pfaffl (2001) was performed as described previously in detail (Gentil et al. 2012).

The specificity of all primers used in RT-PCR and qPCR was checked using BLAST (http://blast.ncbi.nlm.nih.gov) and results were confirmed by sequencing of RT-PCR products (SRD GmbH, Bad Homburg, Germany). AR primers and probes were synthesized by Eurogentec S.A. (Seraing, Belgium); all other primers and probes were by Biomers.net GmbH (Ulm, Germany).

Protein extraction and western blot

The procedures for detection of StAR, CYP11A1 and CYP17A1 proteins have been described in detail and specificity of antisera was confirmed (Gentil et al. 2012). Similar procedures were applied to test for the expression of the AR protein: pulverized frozen tissue (~100 mg) was dispersed with
Table 2 Sequences of primers for (1) RT-PCR and primers and probes for (2) RT-qPCR, accession number and amplicon length.

<table>
<thead>
<tr>
<th>Oligonucleotide sequences (5′–3′)</th>
<th>Accession numbers</th>
<th>Amplicon lengths (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AR (1) F: CTTCTGGCACCACCTTCTTC</td>
<td>NM_001003053</td>
<td>233</td>
<td>Kowalewski &amp; Hoffmann (2008) and Gentil et al. (2012)</td>
</tr>
<tr>
<td>GAPDH (1) F: GCCAGAAGGGCTCATCCT</td>
<td>AB038240</td>
<td>228</td>
<td></td>
</tr>
<tr>
<td>AR (2) R: GGGGCCGTCCACGGGCTTCTC</td>
<td>NM_001003053</td>
<td>69</td>
<td></td>
</tr>
<tr>
<td>Probe R: GTAGCCCCAGATGCTTTCGAG</td>
<td>AB038240</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>GAPDH (1) F: GCCAAGAGGGTCATCATC</td>
<td></td>
<td>228</td>
<td></td>
</tr>
<tr>
<td>Probe R: GTACCTGGCACACTCTCTTC</td>
<td></td>
<td>233</td>
<td></td>
</tr>
</tbody>
</table>

F, forward; R, reverse.

0.5 ml ice-cold protease inhibitor cocktail (Roche Diagnostics) and homogenized using an Ultraturrax. For denaturation of proteins, the tissue–protease inhibitor mixture was cooked with 25 mg SDS for 10 min and centrifuged (10 min at 1200 g at 4 °C), and the protein concentration was determined in the supernatant at 280 nm. Following western blot procedures, an avidin–biotin blocking (Avidin/Biotin Blocking Kit SP-2001; Vector Laboratories Inc., Burlingame, CA, USA, distributed by LINARIS GmbH, Wertheim-Bettingen, Germany) was performed to block unspecific binding sites. The primary antibody used was a polyclonal antibody directed against the N-terminus of the human AR and derived from the rabbit (AR(N-20): sc–816, Santa Cruz Biotechnology, Inc., Heidelberg, Germany). It was used in a dilution of 1:200. Mouse testis protein served as an analytical control. Negative controls were set up using PBS-Blotto (5 g skimmed milk powder, 1 ml thiomersal, 2% and 100 ml with PBS buffer) only without the respective primary antibody.

**IHC and evaluation**

**STAR protein and steroidogenic enzymes**

Evaluation was performed on ten images per dog. The procedure has been described in detail elsewhere (Gentil et al. 2012); briefly, the area of the TC and the AV was evaluated and subtracted from the total area, the resulting value corresponded to the area of the IC.

Owing to the fact that all immunopositive signals were restricted to the Leydig cells, evaluation was performed via a computer-assisted image analysis using ImageTool 3.0, freeware (UTHSCSA, San Antonio, University of Texas, http://dtdsx.uthscsa.edu/dig/itdesc.html). The parameters derived were the immunopositive staining area (IPSA) and the ‘mean grey scale’ as an indicator for the intensity of immunostaining. From the IC and the IPSA, the percentage of immunopositive interstitial area (PIA) was calculated (Gentil et al. 2012). For all IHC procedures, negative controls were set up using irrelevant antibodies instead of the primary antibodies.

**Androgen receptor**

An immunoperoxidase method using the polyclonal AR antibody (AR(N-20): sc–816, Santa Cruz Biotechnology, Inc.) in a dilution of 1:10 000 was applied. A biotinylated polyclonal goat anti-rabbit Ig antibody (Vectastain ABC-Kit, Elite PK-6101 RABBIT IgG, Vector Laboratories, distributed by LINARIS GmbH) served as secondary antibody according to the manufacturer's instructions diluted in phosphate buffer with 1.4% BSA and 0.03% Triton X-100. The immunohistochemical staining procedure was according to Goericke-Pesch et al. (2009) with the following modifications: quenching of endogenous peroxidase was with 3% H2O2. Controls were incubated with the isotype-specific irrelevant serum ‘Rabbit IgG’ (Vector Laboratories, distributed by LINARIS GmbH) at an equal concentration. The colour reaction was initiated with the substrate (AEC substrate kit for peroxidase SK4200, Vector Laboratories, distributed by LINARIS GmbH) according to the manufacturer's instructions using Tris–HCl buffer instead of dest. water. Finally, the slides were washed under running tap water for 5 min, slightly counterstained with haematoxylin and mounted in Kaiser's glycerol gelatine (Merck).

In all animals, 20 seminiferous tubules were evaluated for the presence of staining and staining intensity at a 400-fold magnification. The total number of AR-positive and AR-negative Sertoli cells and spermatogonia was counted in each tubule and the location of AR-positive Sertoli cell nuclei in respect to the basal membrane was recorded. Results are expressed as % AR-positive spermatogonia and Sertoli cells, in case of the latter one making a distinction between the total percentage of positive Sertoli cells and positive Sertoli cells with nuclei in basal position. Additionally, 100 Leydig cells were checked for their staining.

**AR and vimentin double staining**

Double staining for AR and vimentin was performed to clearly differentiate between Sertoli cells and spermatogonia. The immunohistochemical procedure included antigen retrieval (washing with and cooking in citrate buffer, 3 × 5 min at 560 W in a microwave oven), blocking of endogenous peroxidases with 3% H2O2 in methanol for 30 min and blocking of unspecific binding sites (35 min) with horse serum (10%) and goat serum according to the instructions of the manufacturer (Vectastain ABC-Kit, Elite PK-6101 RABBIT IgG, Vector Laboratories, distributed by LINARIS GmbH) in phosphate buffer with 1.4% BSA and 0.03% Triton X-100 (blocking buffer). Slides
Table 3 Tubule area (µm²) and percentage (%) of the TC, the IC and the AV of the total testicular area, area of Leydig cell (LC) nuclei (µm²) and total number of spermatogonia (SG) and of Sertoli cells (SC) per tubule; data given as x±s.d.

<table>
<thead>
<tr>
<th>Group</th>
<th>Tubule area (µm²)</th>
<th>TC (%)</th>
<th>IC (%)</th>
<th>AV (%)</th>
<th>Area LC nuclei (µm²)</th>
<th>SG</th>
<th>SC</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>7623.40±1738.69*</td>
<td>76.38±2.86*</td>
<td>20.84±1.93*</td>
<td>2.79±0.98±</td>
<td>20.89±1.10*</td>
<td>6.37±3.54*</td>
<td>14.13±4.55*</td>
</tr>
<tr>
<td>PG</td>
<td>6660.62±2096.35*</td>
<td>75.12±5.39*</td>
<td>21.51±5.24*</td>
<td>3.37±0.26*</td>
<td>20.99±0.56*</td>
<td>4.07±0.40*</td>
<td>12.47±6.69*</td>
</tr>
<tr>
<td>CG</td>
<td>27 616.55±3089.37†</td>
<td>89.73±1.46‡</td>
<td>9.11±1.54‡</td>
<td>1.16±0.13‡</td>
<td>32.23±1.81‡</td>
<td>12.08±3.05‡</td>
<td>15.52±1.28‡</td>
</tr>
<tr>
<td>JG</td>
<td>3253.15±301.04*</td>
<td>57.97±7.45*</td>
<td>40.21±7.57*</td>
<td>1.82±0.37‡</td>
<td>25.76±0.82*</td>
<td>–a</td>
<td>25.10±1.82†</td>
</tr>
<tr>
<td>ANOVA</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0005</td>
<td>&lt;0.0001</td>
<td>0.0004</td>
<td>0.009</td>
</tr>
</tbody>
</table>

* † ‡ Different symbols indicate significant differences between groups (Tukey–Kramer test, P<0.01–<0.05).

No spermatogonias yet developed.

were incubated with the monoclonal mouse anti-vimentin antibody, clone V9 (Dako Cytomation Denmark A/S, Glostrup, Denmark; dilution 1:1000) or with the mouse IgG1 Ab-1 (clone NCG01, Dianova GmbH, Hamburg, Germany; dilution 1:870) as negative control for 1 h at 37°C. Afterwards, they were incubated for 20 h at 4°C with the other primary antibody (AR (N20); sc-816, Santa Cruz Biotechnology, Inc.; dilution 1:1000) or the respective rabbit IgG1 antibody as negative control, using the same protein concentration. Following another washing step using Tris–HCl buffer, both secondary antibodies, the alkaline phosphatase-conjugated goat anti-mouse IgG AP-2000 (Vector Laboratories, distributed by LINARIS GmbH; dilution 1:1000) and the HRP-conjugated goat anti-rabbit antibody (Vectastain ABC-Kit, Elite PK-6101 RABBIT IgG, Vector Laboratories, distributed by LINARIS GmbH; dilution 1:1000) or the respective rabbit IgG1 antibody as negative control, using the modified mean (x̄; s.d.) are given. In case of an uneven distribution, logarithmic transformation of data was applied and data are presented as geometric mean and dispersion factor (x̄g (DF)). In case of uneven distribution with logarithmic transformation not resulting in a normal distribution, data were presented as median and first and third quartile (x̄Q1/Q3)). As PIA was expressed in percentage with a skewed distribution to the right, data were arc sine transformed to get an approximately normal distribution. Mean values and s.d. of these arc sine transformed data were calculated and the data retransformed, yielding the modified mean (x̄g) and 1 s.d. range (Sachs 1982, Sheskin 2007).

To test for the effect of group, a one-way ANOVA was applied in case of normally distributed data, followed by the Tukey–Kramer paired comparison test in case the results of

**Statistical analysis**

For normally distributed data, the arithmetic mean and s.d. (x̄±s.d.) are given. In case of an uneven distribution, logarithmic transformation of data was applied and data are presented as geometric mean and dispersion factor (x̄g (DF)). In case of uneven distribution with logarithmic transformation not resulting in a normal distribution, data were presented as median and first and third quartile (x̄Q1/Q3)). As PIA was expressed in percentage with a skewed distribution to the right, data were arc sine transformed to get an approximately normal distribution. Mean values and s.d. of these arc sine transformed data were calculated and the data retransformed, yielding the modified mean (x̄g) and 1 s.d. range (Sachs 1982, Sheskin 2007).

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**In situ hybridization**

pGEM-T plasmid clone containing the insert for canine AR generated with the respective primers for RT-PCR was digested with the restriction enzymes NcoI (antisense cRNA) or NotI (sense cRNA) (New England Biolabs, Frankfurt, Germany) for cRNA synthesis using the Labeling Kit (SP6/T7) (Roche Diagnostics GmbH). cRNA was determined semiquantitatively by dot blot analysis of serial dilutions of DIG-labelled cRNA. In situ hybridization (ISH) was performed on paraffin-embedded sections of canine testes of all groups. Sections were mounted on Super-Frost-Plus slides (Menzel Gläser, Braunschweig, Germany), dewaxed, digested with 20 µg/ml proteinase K (Boehringer, Mannheim, Germany) and postfixed with 4% paraformaldehyde and then processed according to the procedure by Lewis & Wells (1992) and Klonisch et al. (1999). The DIG-labelled cRNA probes were detected by alkaline phosphatase-conjugated sheep anti-DIG Fab fragments (Boehringer) diluted 1:5000 in 1% ovine serum in washing buffer, as recommended by the supplier. The sections were incubated with the substrate BCIP in the presence of NBT (Roche Molecular Biochemicals) yielding a dark blue precipitate at the site of the label.

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Table 4 Relative gene expression (ratio; x±s.d.), mean greyscale values (x±s.d.) and % PIA (modified±1 s.d. range) as determined for StAR, CYP11A1 and CYP17A1.

<table>
<thead>
<tr>
<th>Group</th>
<th>GG</th>
<th>PG</th>
<th>CG</th>
<th>JG</th>
<th>ANOVA (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>StAR</td>
<td>Ratio</td>
<td>0.52±0.19*</td>
<td>0.64±0.02*</td>
<td>1.07±0.38*</td>
<td>3.98±2.49†</td>
</tr>
<tr>
<td></td>
<td>Mean grey scale</td>
<td>62.50±8.38</td>
<td>57.51±3.56</td>
<td>68.26±2.59</td>
<td>63.61±0.13</td>
</tr>
<tr>
<td></td>
<td>PIA</td>
<td>0.02 (0.00–0.07)*</td>
<td>0.00 (0.00–0.00)*</td>
<td>3.33 (1.08–7.67)*</td>
<td>0.02 (0.00–0.08)*</td>
</tr>
<tr>
<td>CYP11A1</td>
<td>Ratio</td>
<td>0.16±0.02*</td>
<td>0.11±0.03*</td>
<td>1.14±0.59*</td>
<td>5.03±4.50*</td>
</tr>
<tr>
<td></td>
<td>Mean grey scale</td>
<td>139.1±5.35*</td>
<td>137.05±1.20*</td>
<td>150.61±3.94†</td>
<td>146.03±3.37†</td>
</tr>
<tr>
<td></td>
<td>PIA</td>
<td>0.02 (0.00–0.08)*</td>
<td>0.00 (0.00–0.00)*</td>
<td>7.12 (3.70–11.56)†</td>
<td>3.87 (1.39–7.52)†</td>
</tr>
<tr>
<td>CYP17A1</td>
<td>Ratio</td>
<td>0.47±0.19*</td>
<td>0.37±0.11*</td>
<td>1.14±0.61*</td>
<td>7.01±2.61†</td>
</tr>
<tr>
<td></td>
<td>Mean grey scale</td>
<td>93.11±6.17†</td>
<td>90.51±0.93†</td>
<td>104.88±7.55†</td>
<td>95.35±1.03†</td>
</tr>
<tr>
<td></td>
<td>PIA</td>
<td>1.89 (0.83–3.38)*</td>
<td>2.15 (0.49–4.92)*</td>
<td>30.74 (20.10–42.53)*</td>
<td>11.12 (7.33–15.58)*</td>
</tr>
</tbody>
</table>

*a,b Different superscript letters indicate significant differences between groups (P<0.01–<0.05).

The ANOVA indicated a significant effect of group. In case of uneven distribution and due to small groups, exact Kruskal–Wallis test was applied followed by multiple Dunn comparisons (Bonferroni–Holm corrected), in case the results of the Kruskal–Wallis test indicated a significant effect of group. For all tests, the statistical software program package, BMDP Release 8.1 (BMDP Statistical Software, Inc., Cork, Ireland), was used. Values were considered to be statistically significant at P<0.05.

Results

Area of interstitial and TC

There was a significant effect of group for all parameters assessed (Table 3). Tubule area (μm²) was larger (P<0.01) in CG compared with GG, PG and JG, which were not different (Table 3, Fig. 1, white bar). The percent relationship between the three testicular compartments evaluated (TC, IC and AV) showed that the percentage of TC was significantly higher in GG and PG compared with JG (P<0.01) but lower than in CG (P<0.01); GG and PG were not different. The percentage of IC was higher in GG and PG compared with CG (P<0.05) but lower than in JG (P<0.01). The percentage of AV was higher in GG and PG compared with CG (P<0.01), with a higher value obtained for PG compared with CG (P<0.01) (Table 3).

Area of Leydig cell nuclei

The area of Leydig cell nuclei revealed a significant effect of group with nuclei being significantly smaller in GG and PG than in JG and CG (P<0.01) and being larger in CG than in JG (P<0.01) (Table 3, Fig. 1, grey bar).

Number of spermatogonia and Sertoli cells per tubule

There was a significant effect of group for both parameters and number of spermatogonia was lower in GG (P<0.05) and PG (P<0.01) compared with CG (Table 3). Spermatogonia had not yet developed and only gonocytes were observed in JG. Number of Sertoli cells was highest in JG (P<0.05) with no difference between GG, PG and CG.

Tubules with spermatogonia and primary spermatocytes

In group GG, 39.1±7.9% of the tubules showed spermatogonia and primary spermatocytes, the respective figure for PG was 35.5±28.4%; there was no statistically significant difference.

Expression of StAR

At the mRNA level, expression of StAR showed a significant effect of group with the ratio being significantly higher (P<0.05) in JG compared with the other groups, which were not different, apart from a tendency of a higher value in CG (Table 4, Fig. 2).

Positive immunostaining was restricted to Leydig cells (Fig. 3). There was a significant effect of group for PIA; no or only very few Leydig cells were stained in GG, PG and JG when compared with CG (Table 4, P<0.01). There was no significant effect of group concerning the mean greyscale values, although there was a tendency of a somewhat lower value in PG compared with CG (Table 4, Fig. 4).
Steroidogenic enzymes

Expression of CYP11A1

Immunostaining was restricted to Leydig cells (Fig. 3). There was a significant effect of group for all parameters assessed (Table 4, Figs 2 and 4). Expression of the mRNA (ratio) was highest in JG, followed by CG; however, a pairwise comparison yielded no differences (Table 4, Fig. 2). Mean greyscale values were higher in CG when compared with GG and PG (P<0.05) but not different to JG, which did not differ from GG and PG (Table 4, Fig. 4). Regarding PIA, almost no Leydig cells were stained in GG and PG with PIA being significantly lower compared with CG (P<0.01) and JG (P<0.05); JG and CG were not significantly different, but mean PIA of CG was about twofold higher than that of JG (Table 4).

Expression of CYP17A1

Only Leydig cells stained positive (Fig. 3). There was a significant effect of group for all parameters assessed (Table 4, Figs 2 and 4). Relative gene expression (ratio) was significantly (P<0.01) lower in JG compared with the other groups (Fig. 2). Concerning Sertoli cells, virtually all cells were stained in CG, none of them were stained in JG and in two dogs of group PG while 93.9% were stained in the third dog of this group. Also in GG, staining was highly variable (0, 16.7 and 98.1%) and consequently the distinctly lower median values were not different from PG, CG and JG. Virtually all AR-positive Sertoli cell nuclei were located at the basal membrane in CG, while in the other groups only up to 0.84% (GG) positive Sertoli cell nuclei were located at the same position; again, the differences between CG and JG were highly significant with both groups not being different from GG and PG.

As no spermatogonia were yet developed in JG, data for AR-positive spermatogonia only refer to GG, PG and CG (data given as median (first/third quartile); GG 19.33% (9.66/27.49%), PG 3.49% (1.74/12.16%) and spermatogonia (Fig. 6) and pericytes (not shown) stained positive for the AR; identity of spermatogonia was verified by double staining (Fig. 7).

Androgen receptor

Western blot showed a specific protein band at about 110 kDa in canine and murine testicular protein extracts (Fig. 5). When applying IHC, apart from Sertoli cells also Leydig cells, peritubular myoid cells, some...
CG 12.65% (5.66/14.94%). There was no significant effect of group. Similarly, no differences were observed concerning staining of Leydig cells for the AR (data given as median (first/third quartile); GG 13% (12/40.5%), PG 4% (4/33.5%), JG 56% (54.6/62.5%) and CG 69% (49/90%). When applying ISH and different to IHC, the only positive cells clearly identified were Sertoli cells (Fig. 8).

**Discussion**

Down-regulation affects the whole steroidogenic pathway including the StAR protein, at both, the mRNA and protein level leading to an arrest of spermatogenesis at the level of spermatagonia and spermatocytes as was reported earlier (Goericke-Pesch et al. 2009, Gentil et al. 2012).

The present data clearly show that basically the same state of testicular down-regulation is achieved 5 months after treatment when using GNRH agonist implants with different active ingredients (azagly-nafarelin vs buserelin acetate).

Thus, the high variation observed in the duration of clinical efficacy (Riesenbeck et al. 2002, Ludwig et al. 2009, Goericke-Pesch et al. 2010a, 2010b) not so much seems to be a matter of the degree of testicular down-regulation but rather a matter of the pharmacokinetic profile and an individual reaction towards the implant. However, as there are no data on the restoration of pituitary GNRH receptors, a protracted restoration irrespectively of a ceased GNRH release from the drug applied may also be assumed.

In the down-regulated testis and when compared with CG, the percentage of the TC area is significantly decreased while the percentage of the IC area is increased. This situation is seen in seasonal breeders like the roe deer or the Djungarian hamster where luteinizing hormone, follicle-stimulating hormone and testosterone are basal out of season (Barth et al. 1976, Schams & Barth 1982, Sempere et al. 1998, Roelants et al. 2002) with spermatogenesis being arrested at the level of spermatagonia (Bergmann 1987, Blottner et al. 1996, Goeritz et al. 2003, Schön et al. 2004) resulting in a significantly reduced tubular diameter (Schön et al. 2004) and consequently testicular mass. As previously observed (Goerick-Pesch et al. 2009), also the area of the Leydig cell nuclei is reduced. A similar observation was made in the Djungarian hamster (Bergmann 1987) and the billy goat (Leidl et al. 1970) when out of season, pointing towards a loss of or reduced steroidogenic activity. The movement of Sertoli cell nuclei from a more basal to a more luminal position in the down-regulated testis as seen in this study is characteristic for seasonal breeding animals, too (Schön et al. 2004).

As seasonal up-regulation of testicular function is a process occurring regularly in periodic intervals resulting in full fertility, it can be expected that the same situation applies to the down-regulated testis. The few observations made after repetitive treatments with a GNRH agonist implant (Riesenbeck et al. 2002, Goericke-Pesch et al. 2010a) seem to confirm this conclusion. However, further confirmation is necessary.

In the down-regulated testis, the loss of steroidogenic capacity is well demonstrated at the protein level with PIA being significantly lower in GG and PG when compared with CG. Also mRNA expression of StAR, CYP11A1 and CYP17A1 was obviously reduced in GG and PG when compared with CG, with the pairwise comparison, however, showing no statistically significant difference; yet, due to small group size, statistical analysis must be considered less strong.

Other than that in the juvenile testis, the steroidogenic apparatus (StAR protein, CYP11A1 and CYP17A1) is highly expressed at the mRNA level, even exceeding the values observed in CG. As indicated by PIA, also expression at the protein level is not different from CG for CYP11A1. Different to that, expression of StAR and CYP17A1 as indicated by PIA is distinctly lower in JG and not different from GG and PG. This observation suggests that provision of StAR seems to be regulated at the posttranscriptional level in the juvenile testis. As there were hardly any differences in mean

**Figure 5** Western blot for AR; (1) canine total testicular protein (~120 µg) and (2) mouse total testicular protein (~120 µg) serving as positive control. Molecular weight markers are expressed in kilodaltons. The arrows indicate AR-specific protein bands.

**Figure 6** Immunostaining for the AR in testes. JG, example of juvenile dogs; PG, example of dogs treated with a GNRH agonist implant containing 6.3 mg buserelin acetate; CG, example of adult untreated controls; negative control given as inset (magnification, ×200).
Concerning expression of the AR, our studies have revealed some controversial results. Thus, IHC clearly identified Sertoli cells, Leydig cells, peritubular cells and some spermatogonia as positive, the latter one only clearly identified by double staining. On the other side, ISH only confirmed expression in the Sertoli cells calling for further investigations. Treatment with a slow-release GNRH agonist implant blocked the steroidogenic apparatus showing no differences between the two agonists; mRNA expression of the AR was not affected; however, translation was blocked. Thus, in contrast to StAR, CYP11A1 and CYP17A1, which are most likely regulated at the mRNA level, expression of the AR is affected at the posttranscriptional level. In JG, mRNA expression of StAR, CYP11A1 and CYP17A1 was higher compared with the other groups but distinctly lower for the AR. Expression at the protein level was at the limit of detection for StAR; mRNA expression of the AR is regulated at the mRNA level, expression of the AR is affected at the posttranscriptional level. Thus, in the juvenile testis, the steroidogenic apparatus is highly expressed at the

In conclusion, immunostaining for StAR, CYP11A1 and CYP17A1 was restricted to Leydig cells; positive staining for the AR was observed in Sertoli cells, Leydig cells, peritubular cells and some spermatogonia. However, ISH only confirmed expression in the Sertoli cells calling for further investigations. Treatment with a slow-release GNRH agonist implant blocked the steroidogenic apparatus showing no differences between the two agonists; mRNA expression of the AR was not affected; however, translation was blocked. Thus, in contrast to StAR, CYP11A1 and CYP17A1, which are most likely regulated at the mRNA level, expression of the AR is affected at the posttranscriptional level. In JG, mRNA expression of StAR, CYP11A1 and CYP17A1 was higher compared with the other groups but distinctly lower for the AR. Expression at the protein level was at the limit of detection for StAR; AR-positive Sertoli cells were not detected in JG. Thus, in the juvenile testis, the steroidogenic apparatus is highly expressed at the

Table 5 Results of AR expression: relative gene expression (ratio; x±s.e.m.), % AR-positive Sertoli cells and % AR-positive Sertoli cells with nuclei in basal position (given as median; first and third quartile (Q1/Q3)).

<table>
<thead>
<tr>
<th>Group</th>
<th>Ratio</th>
<th>% AR-positive Sertoli cells (median (Q1/Q3))</th>
<th>% AR-positive Sertoli cells with nuclei in basal position (median (Q1/Q3))</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG</td>
<td>0.95±0.06*</td>
<td>16.71±10 (8.36/57.38)</td>
<td>0.84±1 (0.42/14.29)</td>
</tr>
<tr>
<td>PG</td>
<td>0.92±0.06*</td>
<td>99.66±1 (99.29/100.00)</td>
<td>0.00±1 (0.00/0.00)</td>
</tr>
<tr>
<td>CG</td>
<td>1.01±0.15*</td>
<td>99.66±1 (99.29/100.00)</td>
<td>94.71±1 (91.04/95.93)</td>
</tr>
<tr>
<td>JG</td>
<td>0.31±0.16†</td>
<td>0.00±1 (0.00/0.00)</td>
<td>0.00±1 (0.00/0.00)</td>
</tr>
<tr>
<td>Effect of group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type of test</td>
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<td>Exact Kruskal–Wallis</td>
</tr>
<tr>
<td>P value</td>
<td>0.0001</td>
<td></td>
<td>0.0017</td>
</tr>
</tbody>
</table>

*†Different superscript letters indicate significant differences between groups (P<0.01–<0.05).
mRNA level, but not at the protein level, again pointing to posttranscriptional regulatory mechanisms. These observations show that the down-regulated testis is different from the juvenile one rather resembling the testicular status in seasonal breeders out of season.

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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