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 17 α -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 17 α -hydroxylase-17,20-lyase (P450c17) after having applied two different GNRH agonist implants containing either azaglyl-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).

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 paraumbilical 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 below 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: $\text{area} = \frac{1}{2}a \times \frac{1}{2}b \times \pi$ 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).

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 (2×; 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 1 Grouping of dogs.

Group names	Abbreviations	n
GG	Dogs treated with azagly-nafarelin (Gonazon group)	3
PG	Dogs treated with buserelin acetate (Profact Depot group)	3
CG	Adult untreated control dogs	5
JG	Juvenile untreated dogs	3

Table 2 Sequences of primers for (1) RT-PCR and primers and probes for (2) RT-qPCR, accession number and amplicon length.

	Oligonucleotide sequences (5'–3')	Accession numbers	Amplicon lengths (bp)	References
AR (1)	F: CTCCTGGCAGCTCTCTTC R: CATCTCGCTTTTGACACAAC	NM_001003053	233	
GAPDH (1)	F: GCCAAGAGGGTCATCATCTC	AB038240	228	Kowalewski & Hoffmann (2008) and Gentil <i>et al.</i> (2012)
AR (2)	R: GGGGCCGTCCACGGTCTTCT F: GAGCCAGGCGTGTTGTGT R: GCTAGAGAGCAAGGCTGCAAA	NM_001003053	69	
Probe	FAM-CTGGACATGACAACAAC-CAGCCCCGA-TAMRA			
GAPDH (2)	F: GCTGCCAAATATGACGACATCA	AB038240	75	Kowalewski & Hoffmann (2008) and Gentil <i>et al.</i> (2012)
Probe	R: GTAGCCCAGGATGCCTTTGAG FAM-TCCCTCCGATGCCTGCTTCAC-TACCTT-TAMRA			

F, forward; R, reverse.

0.5 ml ice-cold protease inhibitor cocktail (Roche Diagnostics) and homogenized using an Ultraturax. 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://ddsdx.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 primary 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 Goerick-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 (μm^2) and percentage (%) of the TC, the IC and the AV of the total testicular area, area of Leydig cell (LC) nuclei (μm^2) and total number of spermatogonia (SG) and of Sertoli cells (SC) per tubule; data given as $\bar{x} \pm \text{s.d.}$

Group	Tubule area (μm^2)	TC (%)	IC (%)	AV (%)	Area LC nuclei (μm^2)	SG	SC
GG	7623.40 $\pm$ 1738.69*	76.38 $\pm$ 2.86*	20.84 $\pm$ 1.93*	2.79 $\pm$ 0.98* [†]	20.89 $\pm$ 1.10*	6.37 $\pm$ 3.54*	14.13 $\pm$ 4.55*
PG	6660.62 $\pm$ 2096.35*	75.12 $\pm$ 5.39*	21.51 $\pm$ 5.24*	3.37 $\pm$ 0.26*	20.99 $\pm$ 0.56*	4.07 $\pm$ 0.40*	12.47 $\pm$ 6.69*
CG	27 616.55 $\pm$ 3089.37 [†]	89.73 $\pm$ 1.46 [†]	9.11 $\pm$ 1.54 [†]	1.16 $\pm$ 0.13 [†]	32.23 $\pm$ 1.81 [†]	12.08 $\pm$ 3.05 [†]	15.52 $\pm$ 1.28*
JG	3253.15 $\pm$ 303.04*	57.97 $\pm$ 7.45 [†]	40.21 $\pm$ 7.57 [†]	1.82 $\pm$ 0.37*	25.76 $\pm$ 0.82 [†]	– ^a	25.10 $\pm$ 1.82 [†]
ANOVA (P value)	<0.0001	<0.0001	<0.0001	0.0005	<0.0001	0.0004	0.009

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

^aNo spermatogonia 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 horse 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 according to the manufacturer's instruction, using blocking buffer as diluent), were pipetted onto the slides and incubated for 50 min at room temperature. For staining of vimentin, 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitroblue tetrazolium (NBT) (Roche Molecular Biochemicals) was used followed by staining of the AR by AEC (AEC substrate kit for peroxidase SK4200, Vector Laboratories, distributed by LINARIS GmbH). Finally, the slides were slightly counterstained with haematoxylin and mounted in Kaiser's glycerol gelatine (Merck).

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 Glaeser, Braunschweig, Germany), dewaxed, digested with 20 $\mu\text{g}/\text{ml}$ 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.

Statistical analysis

For normally distributed data, the arithmetic mean and s.d. ($\bar{x} \pm \text{s.d.}$) are given. In case of an uneven distribution, logarithmic transformation of data was applied and data are presented as geometric mean and dispersion factor (x_g (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 ($\tilde{x}$ (Q1/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 ($\bar{x}_r$) 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

Figure 1 Tubule area (μm^2 , white bar) and area of Leydig cell (LC) nuclei (μm^2 , grey bar); data given as $\bar{x} \pm \text{s.d.}$. Different superscript letters indicate significant differences between groups ($P < 0.01$ – < 0.05).

Table 4 Relative gene expression (ratio; $\bar{x} \pm \text{s.d.}$), mean greyscale values ($\bar{x} \pm \text{s.d.}$) and % PIA (modified ± 1 s.d. range) as determined for *StAR*, *CYP11A1* and *CYP17A1*.

	Group	GG	PG	CG	JG	ANOVA (<i>P</i> value)
<i>StAR</i>	Ratio	0.52 $\pm$ 0.19*	0.64 $\pm$ 0.02*	1.07 $\pm$ 0.38*	3.98 $\pm$ 2.49 [†]	0.0115
	Mean grey scale	62.50 $\pm$ 8.38	57.51 $\pm$ 3.56	68.26 $\pm$ 2.59	63.61 $\pm$ 0.13	0.0637
	PIA	0.02 (0.00–0.07)*	0.00 (0.00–0.00)*	3.33 (1.08–6.76) [†]	0.02 (0.00–0.08)*	0.001
<i>CYP11A1</i>	Ratio	0.16 $\pm$ 0.02*	0.11 $\pm$ 0.03*	1.14 $\pm$ 0.59*	5.03 $\pm$ 4.50*	0.0438
	Mean grey scale	139.1 $\pm$ 5.35*	137.05 $\pm$ 1.20*	150.61 $\pm$ 3.94 [†]	146.03 $\pm$ 3.37* [†]	0.0062
	PIA	0.02 (0.00–0.08)*	0.00 (0.00–0.02)*	7.12 (3.70–11.56) [†]	3.87 (1.39–7.52) [†]	0.0003
<i>CYP17A1</i>	Ratio	0.47 $\pm$ 0.19*	0.37 $\pm$ 0.11*	1.14 $\pm$ 0.61*	7.01 $\pm$ 2.61 [†]	0.0001
	Mean grey scale	93.11 $\pm$ 6.17* [†]	90.51 $\pm$ 0.93 [†]	104.88 $\pm$ 7.55* ^a	95.35 $\pm$ 1.03* [†]	0.0247
	PIA	1.89 (0.83–3.38)*	2.15 (0.49–4.92)*	30.74 (20.10–42.53) ^{a,†}	11.12 (7.33–15.58)*	0.0002

*[†]Different superscript letters indicate significant differences between groups ($P < 0.01$ – < 0.05).

^a $n = 4$.

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 (μm^2) 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 JG ($P < 0.01$) (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, $39.1 \pm 7.9\%$ of the tubules showed spermatogonia and primary spermatocytes, the respective figure for PG was $35.5 \pm 28.4\%$; 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).

Figure 2 Relative gene expression (ratio; $\bar{x} \pm \text{s.d.}$) as determined for *StAR*, *CYP11A1*, *CYP17A1* and *AR*. Different superscript letters indicate significant differences between groups ($P < 0.01$ – < 0.05).

Figure 3 Immunostaining for i) StAR, ii) CYP11A1 and iii) CYP17A1 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 controls given as insets (magnification, $\times 400$).

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 higher in JG than in the other groups ($P < 0.01$), which were not different, apart from a tendency of a higher value in CG (Table 4, Fig. 2). The mean greyscale value was highest in CG and different from PG ($P < 0.05$) with GG and JG not being different from either side (Table 4, Fig. 4). PIA was significantly ($P < 0.01$) higher in CG compared with the other groups; however, there was a clear tendency for a higher PIA in JG compared with PG and GG (Table 4).

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

spermatogonia (Fig. 6) and pericytes (not shown) stained positive for the AR; identity of spermatogonia was verified by double staining (Fig. 7).

There was a significant effect of group for the three parameters presented in Table 5. 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

Figure 4 Mean greyscale values ($\bar{x} \pm \text{s.d.}$) as determined for StAR, CYP11A1 and CYP17A1. Different superscript letters indicate significant differences between groups ($P < 0.01$ – < 0.05).

Figure 5 Western blot for AR; (1) canine total testicular protein ($\sim 120 \mu\text{g}$) and (2) mouse total testicular protein ($\sim 120 \mu\text{g}$) serving as positive control. Molecular weight markers are expressed in kilodaltons. The arrows indicate AR-specific protein bands.

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% (54.5/62.5%) 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 spermatogonia 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 spermatogonia (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 (Goericke-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 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, $\times 200$).

Figure 7 Double-immunostaining for the AR and vimentin. (A) Testes of a dog treated with a GNRH agonist implant containing 18.5 mg azagly-nafarelin (GG; magnification, ×1000) and (B) negative control (magnification, ×200); Sg (–), negative spermatogonia; Sg (+), immunopositive spermatogonia; SC (+), immunopositive Sertoli cell.

greyscale values, it may further be concluded that the number of cells rather than the intensity of expression per cell are affected.

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 clearly identified by double staining. On the other side, ISH only identified Sertoli cells as clearly positive. This discrepancy may be due to lower mRNA concentrations in Leydig cells, peritubular cells and some spermatogonia when compared with Sertoli cells and the detection limit of ISH. This can be assumed for Leydig cells and peritubular cells, which are generally agreed to express the AR (Walker 2009, 2011, Wang *et al.* 2009, Fietz *et al.* 2011). However, as both positive (Galena *et al.* 1974, Grootegoed *et al.* 1977, Warikoo *et al.* 1986, Kimura *et al.* 1993, Bremner *et al.* 1994, Janssen *et al.* 1994, Vornberger *et al.* 1994, Van Rooijen *et al.* 1995, Zhou *et al.* 1996, Suarez-Quian *et al.* 1999, Pelletier *et al.* 2000, Arenas *et al.* 2001, Solakidi *et al.* 2005, Aquila *et al.* 2007, Merlet *et al.* 2007) and negative observations (Galena *et al.* 1974, Bremner *et al.* 1994, Van Rooijen *et al.* 1995, Suarez-Quian *et al.* 1999, Pelletier *et al.* 2000) were made concerning the expression of the AR in germ cells, it cannot be ruled out that staining was due to interactions of the antibody with other proteins than the AR. Thus, the further discussion on the AR only

accounts for the respective PCR data and its expression in Sertoli cells.

Clearly, expression of the AR at the mRNA level is not reduced. However, the percentage of AR-positive Sertoli cell nuclei in GG and PG is significantly reduced. This may indicate that, at least in Sertoli cells, down-regulation affects AR expression at the posttranscriptional level. The still high expression of AR mRNA points to the ‘readiness’ of Sertoli cells to a quick up-regulation of full function following abolishment of down-regulation. This is in good agreement with our earlier observations that testicular function is quickly up-regulated when the GNRH agonist implant was removed (Goericke-Pesch *et al.* 2009, Ludwig *et al.* 2009, Gentil *et al.* 2012).

Our study also clearly indicates that there is little if any expression of the AR in JG, yielding the testis insensitive to a direct effect of androgens. Clearly, this situation must be overcome during the course of puberty as was shown for other species (Bremner *et al.* 1994, McKinnell *et al.* 2001).

Conclusion

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; $x \pm s.d.$), % AR-positive Sertoli cells and % AR-positive Sertoli cells with nuclei in basal position (given as median; first and third quartile (Q1/Q3)).

Group	Ratio	% AR-positive Sertoli cells (median (Q1/Q3))	% AR-positive Sertoli cells with nuclei in basal position (median (Q1/Q3))
GG	0.95 ± 0.06*	16.71* [†] (8.36/57.38)	0.84* [†] (0.42/34.29)
PG	0.92 ± 0.06*	0.00* [†] (0.00/46.93)	0.00* [†] (0.00/9.22)
CG	1.01 ± 0.15*	99.66* (99.29/100.00)	94.71* (91.04/95.93)
JG	0.31 ± 0.16 [†]	0.00 [†] (0.00/0.00)	0.00 [†] (0.00/0.00)
Effect of group			
Type of test	ANOVA	Exact Kruskal–Wallis	Exact Kruskal–Wallis
P value	0.0001	0.0017	0.0018

*[†]Different superscript letters indicate significant differences between groups ($P < 0.01 - < 0.05$).

Figure 8 ISH for AR in (A) group CG and (B) negative control (magnification, $\times 400$).

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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References

- Aquila S, Middea E, Catalano S, Marsico S, Lanzino M, Casaburi I, Barone I, Bruno R, Zupo S & Ando S 2007 Human sperm express a functional androgen receptor: effects on PI3K/AKT pathway. *Human Reproduction* **22** 2594–2605. (doi:10.1093/humrep/dem243)

- Arenas MI, Royuela M, Lobo MV, Alfaro JM, Fraile B & Paniagua R 2001 Androgen receptor (AR), estrogen receptor- α (ER- α) and estrogen receptor- β (ER- β) expression in the testis of the newt, *Triturus marmoratus marmoratus* during the annual cycle. *Journal of Anatomy* **199** 465–472. (doi:10.1046/j.1469-7580.2001.19940465.x)
- Barth D, Giménez T, Hoffmann B & Karg H 1976 Testosteronkonzentrationen im peripheren Blut beim Rehbock (*Capreolus capreolus*). Saisonale und rhythmische Veränderungen sowie Beziehungen zu einigen Verhaltensweisen und anderen saisonabhängigen Parametern. *Zeitschrift für Jagdwissenschaft* **22** 134–148.
- Bergmann M 1987 Photoperiod and testicular function in *Phodopus sungorus*. *Advances in Anatomy, Embryology, and Cell Biology* **105** 1–76.
- Blottner S, Hingst O & Meyer HH 1996 Seasonal spermatogenesis and testosterone production in roe deer (*Capreolus capreolus*). *Journal of Reproduction and Fertility* **108** 299–305. (doi:10.1530/jrf.0.1080299)
- Bremner WJ, Millar MR, Sharpe RM & Saunders PT 1994 Immunohistochemical localization of androgen receptors in the rat testis: evidence for stage-dependent expression and regulation by androgens. *Endocrinology* **135** 1227–1234. (doi:10.1210/en.135.3.1227)
- Dube D, Assaf A, Pelletier G & Labrie F 1987a Morphological study of the effects of an GnRH agonist on the canine testis after 4 months of treatment and recovery. *Acta Endocrinologica* **116** 413–417.
- Dube D, Pelletier G & Labrie F 1987b Morphological studies of the canine pituitary luteinizing hormone (LH) cells after long-term treatment with the LH-releasing hormone (LHRH) agonist [D-Trp6]LHRH ethylamide. *Endocrinology* **120** 589–594. (doi:10.1210/endo-120-2-589)
- Fietz D, Geyer J, Kliesch S, Gromoll J & Bergmann M 2011 Evaluation of CAG repeat length of androgen receptor expressing cells in human testes showing different pictures of spermatogenic impairment. *Histochemistry and Cell Biology* **136** 689–697. (doi:10.1007/s00418-011-0871-6)
- Galena HJ, Pillai AK & Turner C 1974 Progesterone and androgen receptors in non-flagellate germ cells of the rat testis. *Journal of Endocrinology* **63** 223–237. (doi:10.1677/joe.0.0630223)
- Gentil M 2012. Wiedereinsetzen der Steroidbiosynthese nach Down-regulation der Hodenfunktion beim Rden: expression von StAR-Protein, P450scc und P450c17. Thesis, Justus-Liebig-University, Giessen.
- Gentil M, Hoffmann B, Spang A, Failing K & Goericke-Pesch S 2012 Restart of steroidogenesis in dogs during recrudescence of testicular function following downregulation with a GnRH-agonist implant. *Cell and Tissue Research* **350** 513–523. (doi:10.1007/s00441-012-1506-5)
- Goericke-Pesch S, Spang A, Schulz M, Ozalp G, Bergmann M, Ludwig C & Hoffmann B 2009 Recrudescence of spermatogenesis in the dog following downregulation using a slow release GnRH agonist implant. *Reproduction in Domestic Animals* **44** (Suppl 2) 302–308. (doi:10.1111/j.1439-0531.2009.01378.x)
- Goericke-Pesch S, Wilhelm E & Hoffmann B 2010a Hormonelle down-regulation der Hodenfunktion bei rüde und kater; eine retrospektive studie. *Der Praktische Tierarzt* **91** 563–570.
- Goericke-Pesch S, Wilhelm E, Ludwig C, Desmoulin PO, Driancourt MA & Hoffmann B 2010b Evaluation of the clinical efficacy of Gonazon implants in the treatment of reproductive pathologies, behavioral problems, and suppression of reproductive function in the male dog. *Theriogenology* **73** 920–926. (doi:10.1016/j.theriogenology.2009.11.018)
- Goeritz F, Quest M, Wagener A, Fassbender M, Broich A, Hildebrandt TB, Hofmann RR & Blottner S 2003 Seasonal timing of sperm production in roe deer: interrelationship among changes in ejaculate parameters, morphology and function of testis and accessory glands. *Theriogenology* **59** 1487–1502. (doi:10.1016/S0093-691X(02)01201-3)
- Grootegoed JA, Peters MJ, Mulder E, Rommerts FF & Van der Molen HJ 1977 Absence of a nuclear androgen receptor in isolated germinal cells of rat testis. *Molecular and Cellular Endocrinology* **9** 159–167. (doi:10.1016/0303-7207(77)90117-4)
- Janssen PJ, Brinkmann AO, Boersma WJ & Van der Kwast TH 1994 Immunohistochemical detection of the androgen receptor with monoclonal antibody F39.4 in routinely processed, paraffin-embedded human tissues after microwave pre-treatment. *Journal of Histochemistry and Cytochemistry* **42** 1169–1175. (doi:10.1177/42.8.8027537)
- Kimura N, Mizokami A, Oonuma T, Sasano H & Nagura H 1993 Immunocytochemical localization of androgen receptor with polyclonal antibody in paraffin-embedded human tissues. *Journal of Histochemistry and Cytochemistry* **41** 671–678. (doi:10.1177/41.5.8468448)

- Klonisch T, Hombach-Klonisch S, Froehlich C, Kauffold J, Steger K, Steinetz BG & Fischer B 1999 Canine preprorelaxin: nucleic acid sequence and localization within the canine placenta. *Biology of Reproduction* **60** 551–557. (doi:10.1095/biolreprod60.3.551)
- 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)
- Leidl W, Hoffmann B & Karg H 1970 Endocrine regulation and seasonal rhythm of reproduction in the male goat. *Zentralblatt für Veterinärmedizin. Reihe A* **17** 623–633. (doi:10.1111/j.1439-0442.1970.tb00817.x)
- Lewis FA & Wells M 1992. Detection of virus in infected human tissue by *in situ* hybridization. In *In Situ Hybridization – A Practical Approach*, pp 121–135. Ed. Wilkinson DG. Oxford: Oxford University Press.
- Ludwig C, Desmoulins PO, Driancourt MA, Goericke-Pesch S & Hoffmann B 2009 Reversible downregulation of endocrine and germinative testicular function (hormonal castration) in the dog with the GnRH-agonist azagly-nafarelin as a removable implant “Gonazon”; a preclinical trial. *Theriogenology* **71** 1037–1045. (doi:10.1016/j.theriogenology.2008.10.015)
- McKinnell C, Saunders PT, Fraser HM, Kelnar CJ, Morris KD & Sharpe RM 2001 Comparison of androgen receptor and oestrogen receptor β immunorepression in the testes of the common marmoset (*Callithrix jacchus*) from birth to adulthood: low androgen receptor immunorepression in Sertoli cells during the neonatal increase in testosterone concentrations. *Reproduction* **122** 419–429. (doi:10.1530/rep.0.1220419)
- Merlet J, Racine C, Moreau E, Moreno SG & Habert R 2007 Male fetal germ cells are targets for androgens that physiologically inhibit their proliferation. *PNAS* **104** 3615–3620. (doi:10.1073/pnas.0611421104)
- Pelletier G, Labrie C & Labrie F 2000 Localization of oestrogen receptor α , oestrogen receptor β and androgen receptors in the rat reproductive organs. *Journal of Endocrinology* **165** 359–370. (doi:10.1677/joe.0.1650359)
- Pfaffl MW 2001 A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29** e45. (doi:10.1093/nar/29.9.e45)
- Riesenbeck A, Klein R & Hoffmann B 2002 Downregulation, eine neue, reversible Möglichkeit zur Ausschaltung der Hodenfunktion beim Rüden. *Der Praktische Tierarzt* **83** 512–520.
- Roelants H, Schneider F, Goritz F, Streich J & Blottner S 2002 Seasonal changes of spermatogonial proliferation in roe deer, demonstrated by flow cytometric analysis of c-kit receptor, in relation to follicle-stimulating hormone, luteinizing hormone, and testosterone. *Biology of Reproduction* **66** 305–312. (doi:10.1095/biolreprod66.2.305)
- Sachs L 1982 *Applied Statistics – A Handbook of Techniques*. New York, Heidelberg, Berlin: Springer-Verlag.
- Schams D & Barth D 1982 Annual profiles of reproductive hormones in peripheral plasma of the male roe deer (*Capreolus capreolus*). *Journal of Reproduction and Fertility* **66** 463–468. (doi:10.1530/jrf.0.0660463)
- Schön J, Goritz F, Streich J & Blottner S 2004 Histological organization of roe deer testis throughout the seasonal cycle: variable and constant components of tubular and interstitial compartment. *Anatomy and Embryology* **208** 151–159. (doi:10.1007/s00429-004-0385-2)
- Sempere AJ, Mauget R & Mauget C 1998 Reproductive physiology of the roe deer. In *The European Roe Deer: The Biology of Success*, pp 161–188. Eds Andersen R, Duncan P, Linnell JDC. Oslo: Scandinavian University Press.
- Sheskin D 2007 *Handbook of Parametric and Nonparametric Statistical Procedures*, 4th edn. Boca Raton, London, New York: Chapman & Hall/CRC.
- Solakidi S, Psarra AM, Nikolopoulos S & Sekeris CE 2005 Estrogen receptors α and β (ER α and ER β) and androgen receptor (AR) in human sperm: localization of ER β and AR in mitochondria of the midpiece. *Human Reproduction* **20** 3481–3487. (doi:10.1093/humrep/dei267)
- Suarez-Quian CA, Martinez-Garcia F, Nistal M & Regadera J 1999 Androgen receptor distribution in adult human testis. *Journal of Clinical Endocrinology and Metabolism* **84** 350–358. (doi:10.1210/jc.84.1.350)
- Van Rooijen JH, Van Assen S, Van Der Kwast TH, De Rooij DG, Boersma WJ, Vreeburg JT & Weber RF 1995 Androgen receptor immunorepression in the testes of subfertile men. *Journal of Andrology* **16** 510–516. (doi:10.1002/j.1939-4640.1995.tb00571.x)
- Vickery BH, McRae GI, Briones W, Worden A, Seidenberg R, Schanbacher BD & Falvo R 1995 Effects of an LHRH agonist analog upon sexual function in male dogs. Suppression, reversibility, and effect of testosterone replacement. *Journal of Andrology* **5** 28–42. (doi:10.1002/j.1939-4640.1984.tb00774.x)
- Vornberger W, Prins G, Musto NA & Suarez-Quian CA 1994 Androgen receptor distribution in rat testis: new implications for androgen regulation of spermatogenesis. *Endocrinology* **134** 2307–2316. (doi:10.1210/en.134.5.2307)
- Walker WH 2009 Molecular mechanisms of testosterone action in spermatogenesis. *Steroids* **74** 602–607. (doi:10.1016/j.steroids.2008.11.017)
- Walker WH 2011 Testosterone signaling and the regulation of spermatogenesis. *Spermatogenesis* **1** 116–120. (doi:10.4161/spmg.1.2.16956)
- Wang RS, Yeh S, Tzeng CR & Chang C 2009 Androgen receptor roles in spermatogenesis and fertility: lessons from testicular cell-specific androgen receptor knockout mice. *Endocrine Reviews* **30** 119–132. (doi:10.1210/er.2008-0025)
- Warikoo PK, Majumdar SS, Allag IS, Das RP & Roy S 1986 Interactions among motility, fertilizing ability, and testosterone binding on spermatozoa of bonnet monkey (*Macaca radiata*). *Archives of Andrology* **16** 135–141. (doi:10.3109/01485018608986933)
- Zhou X, Kudo A, Kawakami H & Hirano H 1996 Immunohistochemical localization of androgen receptor in mouse testicular germ cells during fetal and postnatal development. *Anatomical Record* **245** 509–518. (doi:10.1002/(SICI)1097-0185(199607)245:3<509::AID-AR7>3.0.CO;2-M)

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