doi: 10.17221/147/2016-VETMED

Expression profile of the *SOX9* gene in the testes of sexually immature and mature male goats (*Capra hircus*), and its potential influence on postnatal testis development

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ABSTRACT: The aim of this study was to compare the expression levels of the *SOX9* (SRY-box 9) gene in the testes of 18 White improved male goats (*Capra hircus*) divided into three age groups (one, 10 and 15 months of age with seven, eight and three individuals per group, respectively). Abnormalities in testis development were observed in three individuals from the group of 10-month-olds. Additionally, differences in *SOX9* expression that may affect the process of testis maturation and testicular spermatogenic activity were investigated among individuals. The expression of the *SOX9* gene in testicular tissues was analysed using qPCR. Maximal *SOX9* expression was observed in 10-month-old males with normal testes, while expression was significantly reduced in the same age group of males with abnormal testes. Abnormalities in testis development were associated with low parameters of semen quality. The lowest expression levels of *SOX9* were observed in 15-month-old goats. The results of the present study indicate that *SOX9* expression changes significantly during the development of male goats.

Keywords: gene expression; goat; gonads; testis development; sexual maturation; SOX9; SRY

The molecular mechanism of testis development and differentiation in the prenatal period, involving genes such as *SRY* (Sex Determining Region Y gene), *SOX9* (SRY-box 9 gene), *WT1* (Wilms Tumor 1 gene), *SF1* (Splicing Factor 1 gene), *GATA4* (GATA Binding Protein 4 gene) and *AMH* (Anti-Mullerian Hormone gene), is well documented (Kanai et al. 2005; Plotton et al. 2012). However, the effects of these gene products on postnatal gonadal maturation, how their expression is regulated at different developmental stages, as well as their relationships with other factors are poorly understood. It is known that *SOX9* plays a key role in the maintenance of testicular status in mice (Barrionuevo et al. 2009; Barrionuevo et al. 2012). A deficiency in *SOX9* expression in adult life may lead to various abnormalities, from gonadal dysgenesis to transdifferentiation into an ovary (Barrionuevo et al. 2009; Barrionuevo et al. 2012; Plotton et al. 2012). Experiments on *SOX9* conditional null mutant mice (Barrionuevo et al. 2009) confirmed that SOX9 plays a crucial role in embryonic sex determination. Also, SOX9 together with SOX8 (SRY-box 8 gene) is essential for spermatic cord formation during early testis development. The formation of the spermatic cord is fundamental to the maintenance of spermatogenesis in the adult testis, and its absence leads to infertility in adult males (Barrionuevo et al. 2009; Jiang et al. 2013).

As described above, it is known that *SOX9* is critical for proper development of gonads in mammals such as mice. Thus, we hypothesised that atypical expression of the *SOX9* gene might be linked to abnormalities in testis development and to poor se-

Supported by the Polish Ministry of Science and Higher Education, Poland (Grant No. N N311 266835).

doi: 10.17221/147/2016-VETMED

men parameters in goats. The first aim of the present study was to compare the expression levels of the *SOX9* gene in the testes of sexually immature and mature male goats. The second goal was to correlate *SOX9* expression in individual goats with testis maturation and testicular spermatogenic activity.

MATERIAL AND METHODS

The study was approved by the Local Ethics Committee for Animal Research in Szczecin, and complied with the Polish regulations and guidelines for experiments on animals. A total of 18 White improved male goats (*Capra hircus*) were divided into three age groups (one, 10 and 15 months of age with seven**,** eight and three individuals per group, respectively). In order to exclude potential female-to-male sex-reversed goats, the sex was confirmed by the presence of the *SRY* gene using PCR. The primer sequences used for this purpose are given in Table 1.

Testicular tissues collected *post mortem* into test tubes containing RNAlater® (Sigma Aldrich) were used for the *SOX9* expression analysis. Total RNA was isolated using the Total RNA kit (A&A Biotechnology, Gdynia, Poland), quality was assessed by 1% agarose gel electrophoresis and the RNA was quantified fluorometrically using a Qubit fluorimeter and the Quant-iT™ RNA BR Assay Kit (Invitrogen GmbH, Germany). Approximately 1 µg of total RNA was processed for reverse transcription using a First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Lafayette, USA). The relative quantity of *SOX9* was assessed using the KAPA™ SYBR® qPCR Kit (Kapa Biosystems, USA) in the Rotor-Gene instrument (Qiagen GmbH, Germany). The primer pairs used for qPCR are given in Table 1. The real-time PCRs were performed with single-stranded cDNA (40 ng) and all samples were run in triplicate in reactions containing 12.5 µl of SybrGreen PCR Master Mix, 200nM of each primer and H_2O, up to a final volume of 15 µl. Prior to the analysis of *SOX9* expression levels, the expression stability of the two reference genes (*ACTB* – Actin Beta gene and *UXT* – Ubiquitously-Expressed, Prefoldin-Like Chaperone gene) for testicular tissue was determined using geNorm v. 3.5 software. *SOX9* expression levels were normalised to the *UXT* gene. For relative transcript quantification, standard curves for the reference gene (*UXT*) and the gene of interest (*SOX9*) were generated using a four-fold serial dilution of cDNA. The amplification efficiency for *UXT* and *SOX9* was 104% and 102%, respectively. The relative expression of the gene of interest was analysed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001). The relative fold change in *SOX9* expression among age groups was tested with the Kruskal-Wallis non-parametric ANOVA.

Testicular tissue sections collected *post mortem* were fixed in Bouin's liquid and embedded in paraffin blocks. Serial sections stained with the Periodic acid-Schiff stain (PAS) were used for histological evaluation of the testes.

At the age of seven months, all eight bucks from the group of 10-month-olds were subjected to semen evaluation. Semen was collected by electroejaculation during the short photoperiod (mid-September to mid-December). The analysis was carried out four times at 3-week intervals. The electroejaculated semen was evaluated immediately after collection and the following parameters were determined according to routine methods: ejaculate volume (within an accuracy of 0.1 cm^3), percentage progressive motility (within an accuracy of 5%), sperm concentration (in Burker's chamber), the percentage of spermatozoa with primary and secondary

Table 1. Primer sequences used for PCR and qPCR

Gene	Reference[1]	Primer sequences	T_a[2] (°C)	Product size (bp)
SRY	Z30646	F:5'-TGAATAGAACGGTGCAATCG-3' R:5'-CAGCTGCTTGCTGATCTCTG-3'	60	295
SOX9	EF203710	F:5'-CTCAAGGGCTACGACTGGAC-3' R:5'-CCCAGAGTCTTGCTGAG-3'	60	179
UXT	JN711468.1	F:5'-TGGACCATCGTGACAAGGTA-3' R:5'-TGAAGTGTCTGGGACCACTG-3'	62	155
ACTB	HQ993072.1	F:5'-CTCTTCCAGCCTTCCTTCCT-3' R:5'-TAAAGGTCCTTGCGGATGTC-3'	60	190

[1]GenBank accession number

[2]Annealing temperature

doi: 10.17221/147/2016-VETMED

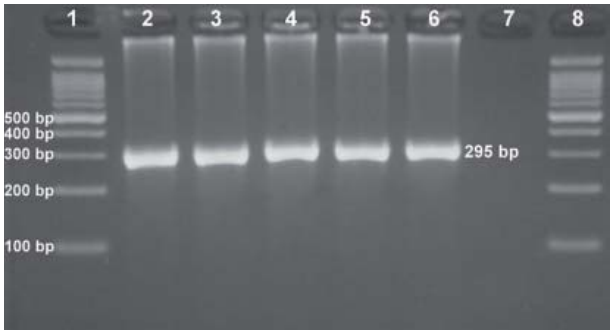


Figure 1. Confirmation of Y chromosome presence by electrophoretic separation of the PCR products amplified using primers for the SRY gene; lanes 1 and 8 (DNA ladder), lanes 2–6 (males), lane 7 (blank sample)

morphological abnormalities (Blom 1981) and the percentage of spermatozoa with intact acrosomes (Saacke and White 1972). Morphological abnormalities and acrosome integrity were assessed using

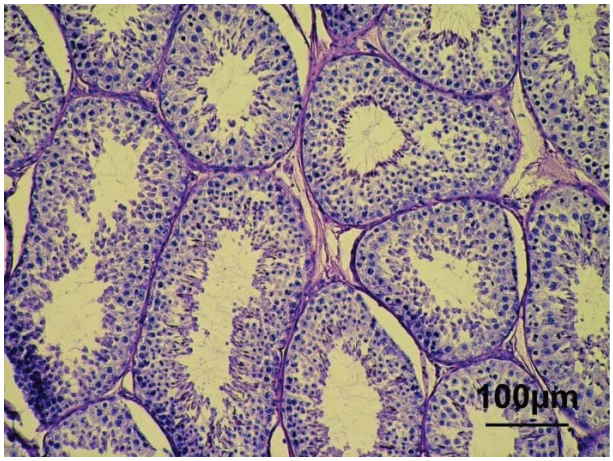
Table 2. Relative quantity of *SOX9* gene expression in the testicles of White improved goats of different ages

Age (months)	Individual	ΔCt	SD	RQ
1	A1	5.76	0.32	1.16
	B1	5.06	0.14	1.89
	C1	3.54	0.14	5.43
	D1	4.33	0.36	3.14
	E1	6.07	0.00	0.94
	F1	6.06	0.20	0.95
	G1	7.33	0.27	0.39
	$\bar{x}$	5.45	0.21	1.44
10	A10	0.91	0.34	33.59
	B10	2.40	0.28	11.96
	C10	0.49	0.14	44.94
	D10	0.19	0.38	55.33
	E10	0.76	0.14	37.27
	$\bar{x}$	0.95	0.27	32.67
	F10[1]	5.11	0.17	1.83
	G10[1]	4.97	0.41	2.01
	H10[1]	4.33	0.37	3.14
	$\bar{x}$	4.80	0.33	2.26
15	A15[2]	5.98	0.08	1.00
	B15	5.61	0.33	1.29
	C15	5.89	0.09	1.06
	$\bar{x}$	5.83	0.20	1.11

Ct = threshold cycle, RQ = relative quantity of gene expression, SD = standard deviation

[1]Individuals with abnormally developed testicles

[2]Calibrator sample



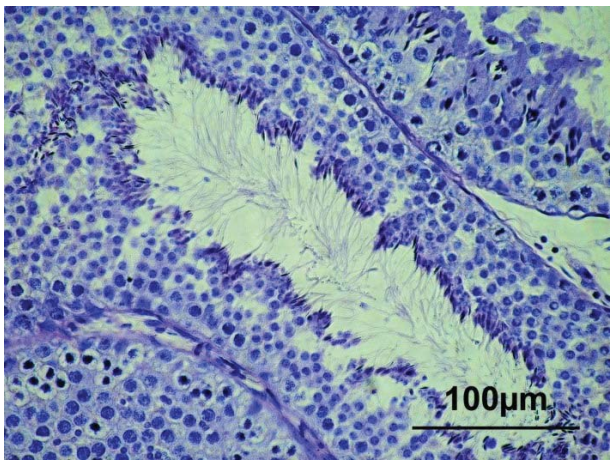


Figure 2. The picture of complete spermatogenesis in the seminiferous tubules. A PAS-positive basal lamina of the seminiferous tubule is clearly visible

10% nigrosin staining. Testis size was expressed in terms of scrotal circumference (measured with a tape measure to the nearest 0.5 cm) and testicular volume calculated from the measurements of length, width and thickness (using a calliper accurate to 0.1 cm). These calculations were performed after deducting scrotal skin-fold thickness (Thibier and Colchen-Bourlaud 1972).

RESULTS

The presence of the *SRY* gene was confirmed in all individuals (Figure 1). The data on the relative quantification of *SOX9* gene expression in the studied individuals are reported in Table 2. Statistical analysis revealed a significant relationship between age and the relative level of *SOX9* expression (P = 0.0092). The relative quantity of *SOX9* gene expression in the group of 1-month-old bucks was

doi: 10.17221/147/2016-VETMED

22.7-fold lower than that in the group of 10-month-olds ($P = 0.0137$) and almost equal to that observed in the group of 15-month-old bucks ($P = 1$).

The mean relative quantity of *SOX9* gene expression in 10-month-old goats was estimated from individuals with normal testis morphology, typical of bucks at the post-pubertal stage. In five individuals, the normal structure of seminiferous tubules (characteristic of the functionally active testes) with complete spermatogenesis was observed in the his-

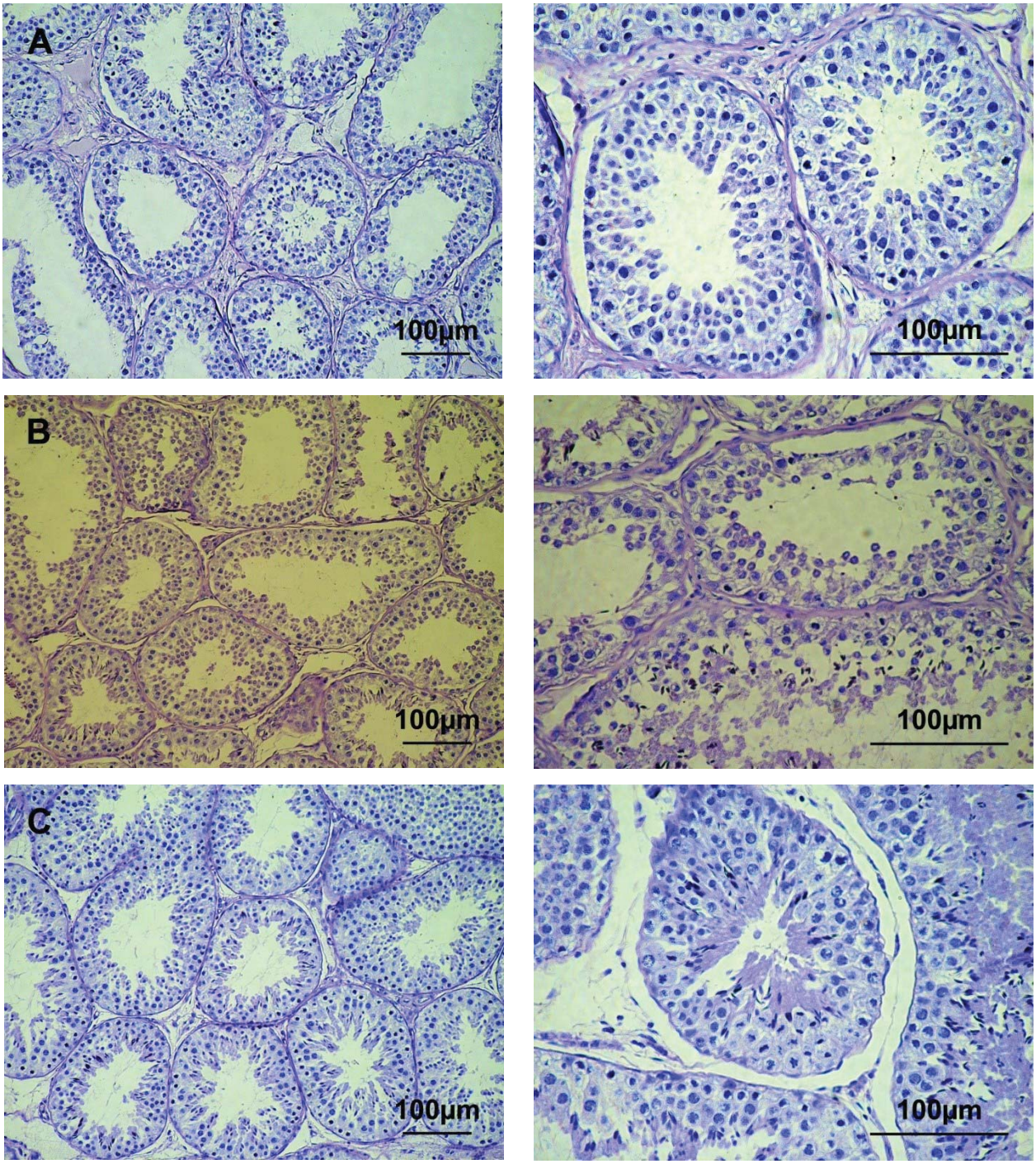


Figure 3. Abnormalities in the testes and signs of reduced or improper spermatogenesis observed in three 10-month-old bucks (F10, G10 and H10). (**A**) The divisions of spermatogonia are visible in one seminiferous tubule. (**B**) Spermatogenesis is visible in the seminiferous tubules, however, with a few spermatozoa (oligospermia) that are not completely differentiated. (**C**) Spermatozoa that are not completely differentiated are present in the seminiferous tubules

doi: 10.17221/147/2016-VETMED

tological sections (Figure 2). In three goats (animals No. F10, G10 and H10), abnormalities of the spermatogenic epithelium with undifferentiated cells lacking nuclei and that were desquamating into the lumen were found in the seminiferous tubules (Figure 3). Moreover, morphological alterations indicating oligospermia or complete absence of the final differentiation of spermatozoa were visible. In these three bucks, the expression level of the *SOX9* gene was considerably lower as compared to the normal bucks (Table 2).

DISCUSSION

The present study shows a relatively low level of *SOX9* gene expression in 1-month-old bucks. At this age, the gonads are undergoing the onset of the intensive division of gonocytes and differentiation into prespermatogonia. According to Montazer-Torbati et al. (2010), the highest expression of *SOX9* in goats is found during the perinatal period, after which it decreases drastically. It is likely that this is caused by the activation of oestrogen receptor alpha, which negatively regulates *SOX9* expression (Kao et al. 2012). Thus, it can be concluded that age differences in the group of 1-month-old bucks (30 ± 3 days) could be the main reason for the inter-individual variations in *SOX9* expression levels. Moreover, no histopathological alterations of the testes were observed, which could have been associated with *SOX9* expression. The testicular parenchyma in all bucks from this age group contained sex cords, formed by immature sustentacular cells (Sertoli cells) and gonocytes. The results of experiments performed on conditional null mutant mice (Barrionuevo et al. 2009) showed that inactivation of the *SOX9* gene after the stage of sex determination neither disturbs the process of testis differentiation or sex cord formation during the embryonic period, nor induces abnormalities in testis morphology or their initial spermatogenic activity within the first two months of the postnatal period. Based on the study of XY(*Sry*$^-$),*Ods*/+ mice, Qin and Bishop (2005) concluded that *SOX9* is sufficient for normal testis development during the embryonic and early postnatal periods.

The highest levels of *SOX9* expression were observed in the normally developed 10-month-old males (Table 2). In these bucks, a distinct lumen in the seminiferous tubules and all generations of germinal cells, including spermatozoa, were found in the seminiferous epithelium. Sustentacular cells were fully differentiated, whereas interstitial cells (Leydig cells) were found in the form of clusters in the interstitial tissue (Figure 2). On the other hand, in the abnormally developed testes of 10-month-old males, dystrophy of spermatogenic epithelial cells, oligospermia, developmentally arrested spermatocytes, and damaged spermatogenic epithelium with undifferentiated cells without nuclei which were desquamating into the lumen, were observed. These changes were associated with low values of measured parameters of the collected semen (Table 3) and a considerably lower level of *SOX9* gene expression as compared to the males with normally developed testes (Table 2). A similar post-pubertal arrest of testes development was observed in *SOX9* conditional null mice, in which the spermatogenic epithelium showed reduced cellularity with a decreased number of spermatids. In addition, approximately two-thirds of the tubules contained spermatogonia and early spermatocytes, while the remaining tubules were completely devoid of these (Barrionuevo et al. 2009). A very similar phenotype was described for the XY(*Sry*$^-$),*Ods*/+ mice, in which the testicular level of SOX9 protein was reduced by about 75% (Qin and Bishop 2005). These authors proposed that the aforementioned

Table 3. Comparison of semen parameters between bucks with normal and abnormal testis development in the group of 10-month-old animals

Parameter	Group[1]		*P*-value[2]
	normal (n = 5)	abnormal (n = 3)	
Ejaculate volume (cm^3)	0.66 ± 0.21	0.55 ± 0.07	0.3711
Concentration ($\times 10^9/cm^3$)	1.30 ± 0.42	0.10 ± 0.03	0.0369
Progressive motility (%)	52.77 ± 19.73	4.00 ± 0.87	0.0347
Primary abnormalities (%)	3.30 ± 2.76	12.50 ± 10.76	0.2217
Secondary abnormalities (%)	9.41 ± 2.76	27.17 ± 22.15	0.5510
Intact acrosome (%)	76.18 ± 2.51	31.67 ± 14.15	0.0369

[1]Individuals were classified into two groups based on the histopathological picture of testis tissue sections collected *post mortem* after semen evaluation

[2]*P*-values for the Mann-Whitney *U*-test for group comparison

doi: 10.17221/147/2016-VETMED

phenotype could result from the lack of SOX9 in adult sustentacular cells. This can lead to the aberrant expression of genes and proteins important for the structural integrity of testicular tubules such as extracellular matrix components and molecules involved in the formation of sustentacular-sustentacular or sustentacular-germ cell contacts, as well as junctional molecules. Thus, the lack of SOX9 in adult sustentacular cells impairs their functional interaction with germ cells and causes dysregulation of the spermatogenic cycle leading to sterility (Barrionuevo et al. 2009).

The group of mature 15-month-old bucks was characterised by the lowest level of *SOX9* gene expression among the three age groups investigated. The inter-individual differences were negligible in this group, indicating that the expression of the *SOX9* gene was quite stable in sexually mature male goats, which is in accordance with the results of Montazer-Torbati et al. (2010).

The *SOX9* gene belongs to the SOX (SRY-related HMG box) family of transcription factors. The SOX9 protein contains several conserved structural elements including a C-terminal transactivation domain, a dimerization domain, and two nuclear export signals. The HMG domain binds to consensus sites in the regulatory regions of its target genes, and thus is the most important with respect to target gene expression. SOX9 is a factor with multiple functions in organogenesis (Bhandari et al. 2012), among which testis induction and formation are crucial for interpreting the results of the present study. A discussion regarding the transcriptional activity of this determinant of the male developmental pathway should focus on two important aspects. The first concerns target gene activation, involved in testis initiation, differentiation and development. The second one is linked to how testis-specific expression of SOX9 is itself regulated. Both problems seem to be equally complex. The initiation and regulation of SOX9 expression, restricted in the testis to precursor and, subsequently, differentiating and differentiated sustentacular cells, occur via a testis-specific and very long testis-specific enhancer of Sox9 (TES) enhancer, well described in mice and humans (Sekido and Lovell-Badge 2008; Benko et al. 2011). In its central part, a highly-conserved region of 1.4 kb (a core region – TESCO) was identified, which contains consensus sites for multiple transcription factors. They can be classified as positive and negative regulators (SRY, SF1, FGF9, FGFR2, GATA4, FOXL2 and ER alpha), and the list is regularly updated (Barrionuevo et al. 2012). An analogous situation is observed in the case of potential target genes of SOX9. Although it is accepted that its major target is the anti-Mullerian hormone (AMH) promoter, the latest research conducted on rats has shown that there are over 100 genes with consensus binding sites for SOX9 (Bhandari et al. 2012).

The main regulator initiating SOX9 transcriptional activity via TESCO in the primitive male gonads is SRY. Its major role is SOX9 activation (together with SF1), which once initiated, is maintained throughout pre- and postnatal life. It is accepted that SRY is only transiently expressed during the determination of the bipotential gonad. Therefore, other factors must be involved in maintaining SOX9 expression. A recent study by Montazer-Torbati et al. (2010) directly challenges this model and shows that, in goats, SRY transcript levels, which peak at 36 days post-coitum (dpc), are maintained until about the first month after birth before drastically decreasing in sexually mature individuals. Consequently, it can also activate SOX9 transcription in the first weeks of postnatal life. At 3–4 months of age, the developmental stage of the gonads of male goats is associated, first and foremost, with the onset of intensive gonocyte divisions and their further differentiation into prespermatogonia. The high AMH levels (induced by *SOX9*) maintained at this time inhibit their entry into meiotic divisions, simultaneously inhibiting the proliferation of interstitial cells (Montazer-Torbati et al. 2010). Immature sustentacular cells also proliferate, but the intensity of this process depends, among other things, on the reduction in endogenous oestrogens elicited by the irreversible transformation of androgens by a microsomal enzymatic complex named aromatase (Kao et al. 2012). Its activity in many mammalian species is mainly localised to immature sustentacular cells (Carreau et al. 2007), like that of oestrogen alpha receptors. Therefore, the lower amounts of *SOX9* transcripts (albeit sufficient for maintaining an appropriate level of AMH expression) in the group of the youngest individuals, could have been caused by a high activity of oestrogen alpha receptors (unpublished data) capable of binding regulatory elements in TESCO *SOX9* and inhibiting its transcription (Jakob and Lovell-Badge 2011).

The highest levels of *SOX9* transcription were observed at 10 months of age (puberty), while lev-

doi: 10.17221/147/2016-VETMED

els were drastically decreased in sexually mature individuals (15 months of age). As the amount of *SRY* transcripts is dramatically reduced at this stage (Montazer-Torbati et al. 2010), this elevated expression of *SOX9* would appear to be SRY-independent. Nevertheless, the extended regulatory element TESCO, as already mentioned, may be involved in maintaining an appropriate level of SOX9 transcripts. However, the role of SOX9 in this period is puzzling. It is known that at this stage AMH levels (upregulated by SOX9) are considerably decreased. At this stage of testicular development, AMH is strongly inhibited by an increasing concentration of intra-testicular androgens acting via androgen receptors (Grinspon and Rey 2010). Sexually mature males from the control group (sexual maturity period) were characterised by the lowest testicular levels of *SOX9* mRNA transcripts, as also previously reported by Montazer-Torbati et al. (2010). However, whether this is a permanent decrease or only transcriptional silencing during the spring-summer season (when reduced sexual activity in seasonal breeds is observed) remains unclear. Certainly, this is an issue that should be further investigated.

The transcriptional pattern of *SOX9* was significantly altered in males with abnormal testicular development. It should be determined whether the low level of *SOX9* expression in these individuals was the cause or result of these abnormalities. On the other hand, the high expression levels of *SOX9* in the cryptorchid may be associated with the atrophy of the seminiferous epithelial cells and excessive proliferation of sustentacular cells (Hutson et al. 1996), in which this transcription factor is active. This type of atrophy has been described in such cases.

In conclusion, the results of the present study showed that the expression level of *SOX9* changes significantly during the development of male goats.

Acknowledgement

We would like to thank Prof. Dr. Maria Katkiewicz for her help with the description and interpretation of histological results.

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Received: October 13, 2016
Accepted after corrections: March 24, 2017