

Correlations among ultrasonographic and microscopic characteristics of prepubescent ram lamb testes

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Abstract

The onset of spermatogenesis during prepubertal development is accompanied by dynamic changes in testicular microstructure. Computer-assisted analysis of scrotal ultrasonograms may allow us to track these changes in a noninvasive manner; however, the echotextural characteristics of different histomorphological variables remain unclear. Hence the objective of this study was to compare echotextural and microscopic attributes of the testis over the first wave of spermatogenesis in prepubescent ram lambs. Bi-weekly ultrasound examinations and weekly testicular biopsies were carried out in 22 ram lambs from 9.5–10 weeks of age or the attainment of 15 cm³ in testicular volume, respectively, to the first detection of elongated spermatids (ESt). Testicular echogenicity was highly variable with age; however, after the alignment of data to the first detection of ESt, there was an initial increase followed by a decline, corresponding to the mitotic and postmitotic phases of spermatogenesis in prepubescent ram lambs. Testicular echotextural attributes (mean numerical pixel values and pixel heterogeneity) correlated with seminiferous tubule (ST) diameter, the number of degenerating cells/ST cross-section (XS), and the number of ubiquitin C-terminal hydrolase L-1 (a marker for prespermatogonia and undifferentiated spermatogonia) staining cells/ST XS during the mitotic and postmitotic phases. Additionally, in the postmitotic phase, significant correlations were recorded between the quantitative echotextural characteristics and ST cell density, nuclear:ST area and percentages of STs with different spermatogenic cells as the most mature germ cell type present. These results indicate that ram testes exhibit distinctive echotextural characteristics during the mitotic and postmitotic phases of germ cell differentiation. It is concluded that scrotal ultrasonography in conjunction with computerized image analysis holds potential as a non-invasive alternative to testicular biopsy in monitoring the reproductive status throughout different stages of testicular development.

Keywords: Testis, ultrasonography, echotexture, puberty, histomorphology, spermatogenesis

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Introduction

During prepubertal testicular development, there is a remarkable increase in cellular number and diversity within the seminiferous tubules (STs) owing mainly to the onset of spermatogenesis. Spermatogenic onset is initiated when prespermatogonia are converted into spermatogonial stem cells (SSCs),¹ which divide by mitosis into new SSCs and/or spermatogonia committed to the differentiation pathway. Following several further rounds of mitosis, spermatocytes are formed and undergo meiosis, involving the recombination and reduction of genetic material to form haploid spermatids. Initially round in shape, the spermatids undergo extensive cytoplasmic and nuclear remodeling, becoming elongated spermatids (ESt). Maturation of the Sertoli cells is an essential part of spermatogenic onset as it allows for the physical and metabolic support of the germ cells, as well as the formation of a fluid-filled central ST lumen,² into

which the ESts are released for passage to the rete testis. Additionally, as the Sertoli cells mature and reach the end of their mitotic lifespan,² their supportive capacity for germ cells becomes established,³ and a wave of germ cell apoptosis occurs to achieve the optimal germ to Sertoli cell ratio.⁴ How these events proceed throughout prepubertal development determines the future reproductive capability of the adult male. Therefore, it is important to closely monitor this phase of sexual development in order to be able to predict future fertility and to diagnose any reproductive complications. Testicular biopsies allow for a detailed microscopic evaluation of the testes; however, due to the expense and risk of tissue damage they are not suitable for routine clinical assessments and simply not practical to carry out in farmed livestock operations. A less invasive and more cost-effective alternative to testicular biopsies in prepubertal individuals would therefore be beneficial.

Scrotal ultrasonography is a commonly used imaging modality for examining internal macroscopic features of the testis, epididymis, and proximal genital tract. Recently however, a novel method of analyzing ultrasonograms has made the detection of microscopic changes in testicular and epididymal histomorphology a realistic possibility.^{5–13} In this procedure, brightness and uniformity of gray-scale values on the ultrasonogram are quantified using computer-assisted image analysis of minute picture elements, or pixels, comprising the image. Pixel intensity is described in terms of numerical pixel values (NPVs), which range from 0 (“absolute black”) to 255 (“absolute white”) and provide an indication of tissue echogenicity, or the ability of the tissue to reflect or scatter ultrasound waves. Pixel standard deviation (PSD) or heterogeneity of the NPVs reflects the existence of interspersed hyper- and hypoechoic areas in a tissue. Together, mean NPVs and PSD typically serve as measures of central tendency and variation, respectively, in the ultrasonographic appearance of organs and tissues.¹² Quantitative echotextural analysis in prepubescent bull calves and ram lambs has revealed an overall increase in testicular echogenicity with age^{5–7,11,13}; however, there is considerable variability, especially with more frequently performed ultrasonographic evaluations.⁵

In prepubertal bull calves and ram lambs, several significant correlations were found between ultrasonographic and microscopic attributes of the testes including outer (tubular) and inner (luminal) diameters of the STs,^{5,9} and the percentage of STs with different germ cells as the most mature cell type present.⁵ However, some of these correlations did not remain consistent in postpubertal yearling bulls.^{11,14} As the majority of postnatal microscopic changes in the testes occur during prepubertal development and there are no shifts in testicular echogenicity beyond this period,¹¹ this strongly suggests that correlations exist among echotextural variables and an array of histomorphological attributes of the developing testes.

While previous studies used castrations of age-matched groups of animals to evaluate how tissue microstructure is related to testicular echotexture, a direct comparison between two or more developmental phases necessitates multiple evaluations of the testicular tissue from the same individuals over time. Therefore, the primary objective of this study in prepubertal ram lambs was to employ computer-assisted analyses of scrotal ultrasonograms and testicular biopsies to determine the quantitative correlations among echotextural and histomorphological attributes of the testis at distinct developmental stages throughout the first wave of spermatogenesis.

Materials and methods

Animals and experimental design

Twenty-two spring-born Rideau Arcott x Polled Dorset ram lambs were housed in a field research station in Ponsonby, ON, Canada (latitude: 43°33'N) and kept under ambient light and temperature conditions in sheltered outdoor facilities. From 7 to 100 days of age, they were fed a 16% crude protein lamb grower diet (Shur-Gain Feedmills, St. Marys,

ON, Canada) *ad libitum*. Hay was offered after weaning at 50 days of age, and it was estimated that from this point until 100 days of age, daily consumption of the grower averaged 2.5 lb per lamb. After 100 days of age, rams were fed a diet of 80% whole barley and 20% whole corn with a 36% crude protein sheep supplement (Shur-Gain Feedmills) and hay, and daily consumption averaged 1 lb of grain and 0.5 lb of sheep supplement per ram. Water was always accessible to the rams. All procedures performed on live animals were approved by the Animal Care Committee at the University of Guelph according to the Canadian Council on Animal Care guidelines.

Bi-weekly ultrasound evaluations and weekly testicular biopsies commenced at 9.5–10 weeks of age or at the time of attainment of 15 cm³ in testicular volume (TV), respectively, and continued until ESTs were first detected by histological evaluation of testicular biopsies, with an additional 1–2 weeks of scanning to allow for tissue processing and histological detection of EST.

Ultrasonographic examinations

Scrotal ultrasonography utilized an Aloka SSD-900 portable ultrasound machine equipped with a 7.5-MHz linear-array transducer (Aloka Inc., Tokyo, Japan) at standardized settings for main, near, and far gains and focal points. Lubricating gel (Light Lube; Centaur VA Animal Health, Guelph, ON, Canada) was applied as a coupling material to eliminate air between the scrotum and the transducer. Left and right testes were scanned in longitudinal and transverse planes, and real-time images were recorded on a Digital Versatile Disk (DVD) recorder (Pioneer DVD Recorder DVR-510H; Pioneer Electronics of Canada Inc., Markham, ON, Canada) for later analysis. Testicular length and width were measured using the built-in electronic calipers or a caliper instrument if the entire area could not be visualized on the screen and used to calculate testicular volume (TV) with the formula $TV = 1/6\pi \times \text{length} \times \text{width}^2 \times 0.945$ developed by Wrobel¹⁵ for ruminant species.

Still images from the DVD recordings containing the largest cross-sectional surface area were captured using a computer station (Intel-Xeon Processor MP; 2.8 GHz; Intel Co., Santa Clara, CA, USA). Computer-assisted analysis of the ultrasonograms was performed using the “spot meter” technique previously described by Pierson and Adams¹⁶ with Image ProPlus® 7.0 analytical software (Media Cybernetics Inc., San Diego, CA, USA). A total of six spots in a longitudinal view and four spots in a transverse view, each measuring 60 pixels (~6 mm in diameter), were placed randomly on the “surface” of the testicular parenchyma with an equal number of the spots above and below the rete testis, excepting any image artifacts or scarring from biopsies. Mean NPVs and standard deviation (pixel heterogeneity-PSD) were determined for each animal based on the images of both left and right testes (20 spots in total), for each day of examination.

Histology and immunohistochemistry

Core needle biopsies (12, 14, or 16 gauge E-Z Core Single Action Biopsy Device; Products Group International,

Lyons, CO, USA) were taken from the left testis only. Prior to tissue collection, ram lambs were weighed and then sedated with xylazine (Rompun; Bayer, Toronto, ON, Canada; 0.2 mg/kg intramuscular). Tissue was fixed overnight in modified Davidson's solution, washed in 70% ethanol and embedded in paraffin wax blocks. Tissue was sectioned at a thickness of 5 μ m and then deparaffinized in xylene and rehydrated in a graded isopropanol-water series for staining with hematoxylin and eosin, using standard procedures, or underwent immunohistochemical staining for ubiquitin C-terminal hydrolase L-1 (UCHL-1; also referred to as protein gene product 9.5, PGP 9.5), a marker for prespermatogonia and undifferentiated spermatogonia in sheep.¹⁷

For UCHL-1 immunohistochemical staining, heat-induced antigen retrieval was performed by microwaving slides in 10-mM citrate buffer (pH 6.0) on high power for 8 min. The sections were then treated with 3% H₂O₂ in distilled H₂O for 15 min to block endogenous peroxidase activity, rinsed and incubated with 5% normal goat serum (GS; Vector Laboratories, Burlington, ON, Canada) in phosphate buffered solution (PBS; PBS-GS) for 30 min at room temperature to block nonspecific binding. Polyclonal rabbit anti-PGP 9.5 (Dako, Carpinteria, CA, USA) primary antibody (2.5 μ g/mL) in PBS-GS or PBS-GS alone was applied to test and negative control sections, respectively, and allowed to incubate overnight in a moist chamber at 4°C. The following day, sections were rinsed 3 times in PBS for 5 min each and incubated with 2.5 μ g/mL biotinylated goat anti-rabbit IgG (Invitrogen, Burlington, ON, Canada) in PBS-GS for 45 min at room temperature. After rinsing in PBS as above, the sections were exposed to avidin horseradish peroxidase R.T.U. Vectastain Elite ABC Reagent and NovaRed substrate according to the manufacturer's instructions (above from Vector Laboratories). Subsequently, the sections were counterstained in Mayer's hematoxylin:water (1:1) (Fisher Scientific, Ottawa, ON, Canada) for 30 s and mounted with Cytoseal XYL (Richard-Allen Scientific, Kalamazoo, MI, USA).

Histomorphological analysis was performed using Image ProPlus® on biopsy micrographs covering the entire tissue area taken by Q Capture (Quorum Technologies Inc., Guelph, ON, Canada) at 200 $\times$ image magnification. The following parameters were determined for all round ST cross-sections (XS): (i) the luminal (inner) and tubular (outer) diameter; (ii) the percentage of STs with prespermatogonia or spermatogonia ((pre-)Sg), spermatocytes (Sc), round or elongated spermatids (RSt and ESt, respectively) as the most mature germ cell type present (Figure 1(a) to (d)); and (iii) the number of UCHL-1⁺ cells (classified as light, medium, or dark, based on staining intensity; Figure 1(e) and (f)). Additionally, in 10 round ST XS/biopsy we determined: (iv) cell density or the number of cells based on nuclear counts per ST μ m²; (v) the ratio of average cell nuclear area to tubular ST area (nuclear:ST area); and (vi) the number of degenerating cells displaying classical morphological attributes of apoptosis (i.e. chromatin condensation and fragmentation, cytoplasmic vacuolization and eosinophilia, and cell shrinkage¹⁸).

Statistical analyses

All statistical procedures were carried out using SigmaPlot® (version 11.0; Systat Software, Inc., Richmond, CA, USA). Serial echotextural and histomorphological data were assessed using one-way repeated measures analysis of variance (ANOVA; general linear model) after alignment to both chronological age and the age of first ESt detection. The first week of ESt occurrence could not be ascertained in two ram lambs that had missed biopsies due to temporary illnesses unrelated to the experimental procedures; therefore, the data from these animals were removed from analyses with respect to the age at first ESt detection. All other statistical analyses used the data obtained from 22 ram lambs. Differences between individual means were determined using the Holm-Sidak method. A paired *t*-test was used to evaluate differences in TV between left and right testes. Correlations between echotextural and histomorphological parameters were assessed during the mitotic and postmitotic phases, with (pre-)spermatogonia as the most mature germ cell type in > or <50% of ST XS, respectively, as well as for the entire study period using the Pearson product moment. In all analyses, *P* < 0.05 was considered statistically significant and *P* $\leq$ 0.10 was considered approaching statistical significance. All results are given as mean $\pm$ standard error of mean.

Results

General results

The TV reached 15 cm³ at an average age of 10.7 $\pm$ 0.2 weeks (range: 9.5–13 weeks). (Pre-)spermatogonia had the highest prevalence in ST XS as the most mature germ cell type from 10 to 13 weeks of age or from –6 to –3 weeks relative to the first detection of ESt; therefore this period was denoted the mitotic phase and the subsequent period the postmitotic phase. The first detection of ESt occurred on average at 15.3 $\pm$ 0.4 weeks of age (range: 12–18 weeks), with a TV averaging 80.1 $\pm$ 6.5 cm³ (range: 42.1–122.4 cm³) and a body weight of 42.4 $\pm$ 1.2 kg (range: 37.5–51.5 kg).

Body weight and testicular growth

Body weight increased linearly from 11 to 16 weeks of age (*P* < 0.05) and then stayed relatively constant until 19 weeks of age (*P* > 0.05; Figure 2(a)). Average TV increased steadily from 10 to 15 weeks of age (*P* < 0.05) and then reached a plateau (Figure 2(b)). There were no differences in TV between the left and right testes except at 16 and 17 weeks of age, when the right testis was significantly larger compared to the left testis (*P* < 0.05).

Body weight increased in a curvilinear manner in the 6 weeks before ESt detection (*P* < 0.05) and then stayed relatively constant until 2 weeks after ESts were first detected (*P* > 0.05; Figure 2(c)). Average TV increased gradually from 8 until 5 weeks before the first detection of ESt (*P* < 0.05) and then rapidly increased until ESts were first detected (*P* < 0.05) before reaching a plateau in the following 2 weeks (*P* > 0.05; Figure 2(d)). No differences in TV were found between the left and right testes (*P* > 0.05) after data alignment to the time of first detection of ESt.

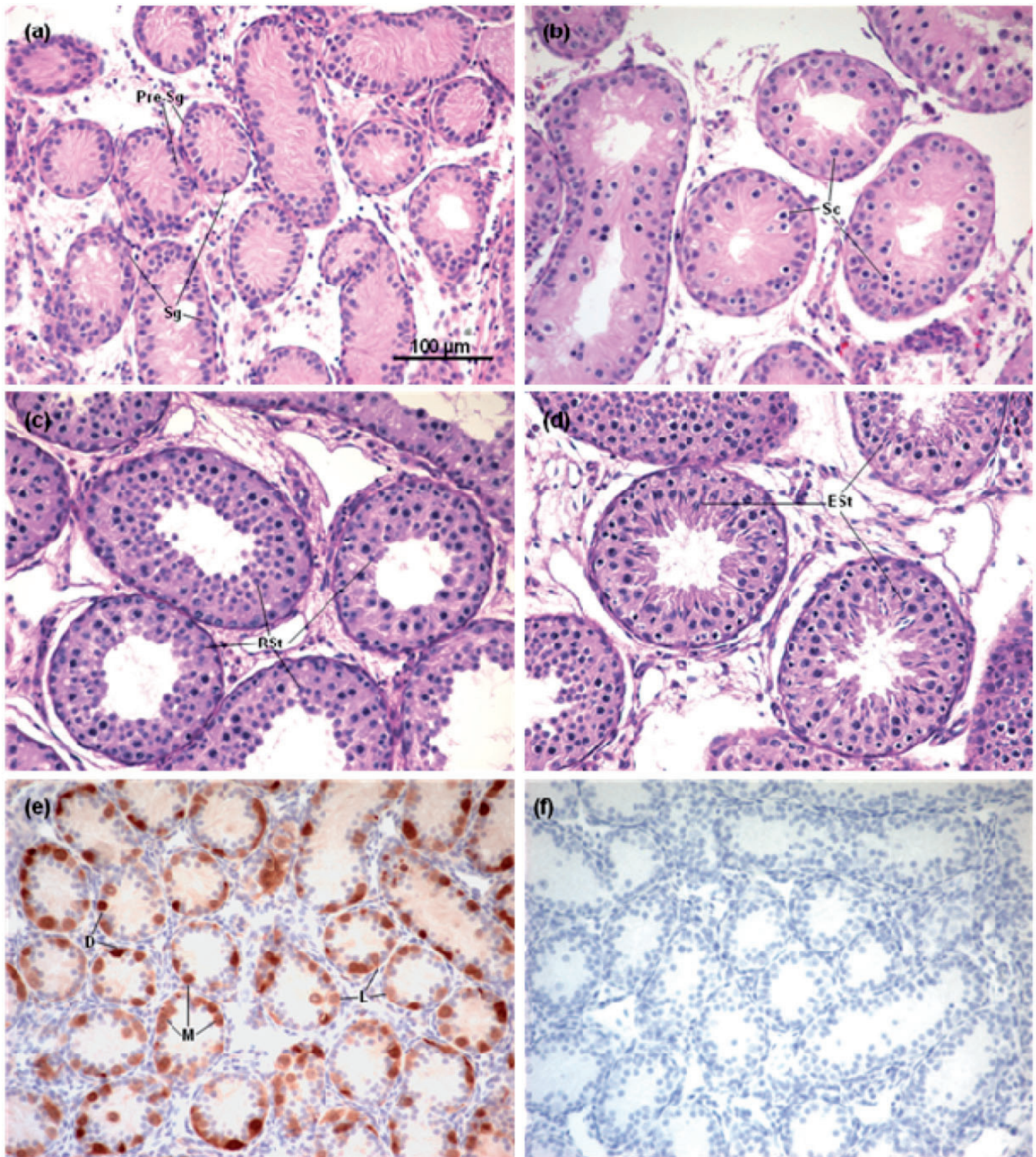


Figure 1 Prepubertal ram lamb testicular histograms taken at 200× magnification representing four different stages of germ cell development based on the most advanced germ cell type present including: (a) prespermatogonia (Pre-Sg) and/or spermatogonia (Sg), (b) spermatocytes (Sc), (c) round spermatids (RSt), and (d) elongated spermatids (ESt). Ubiquitin C-terminal hydrolase L-1 (UCHL-1) immunohistochemical staining in a test section (e) and negative control section (f) treated with or without primary antibody, respectively, with prespermatogonia as the most mature germ cell type and showing dark (D), medium (M), and light (L) staining intensity

Testicular echotexture

Temporal changes in NPVs and pixel heterogeneity (PSD) with respect to age (Figure 3(a)) and the first detection of

ESt (Figure 3(b)) displayed several differences. NPVs increased significantly from a minimum value at 10.5 weeks of age to a maximum at 13 weeks of age, decreased

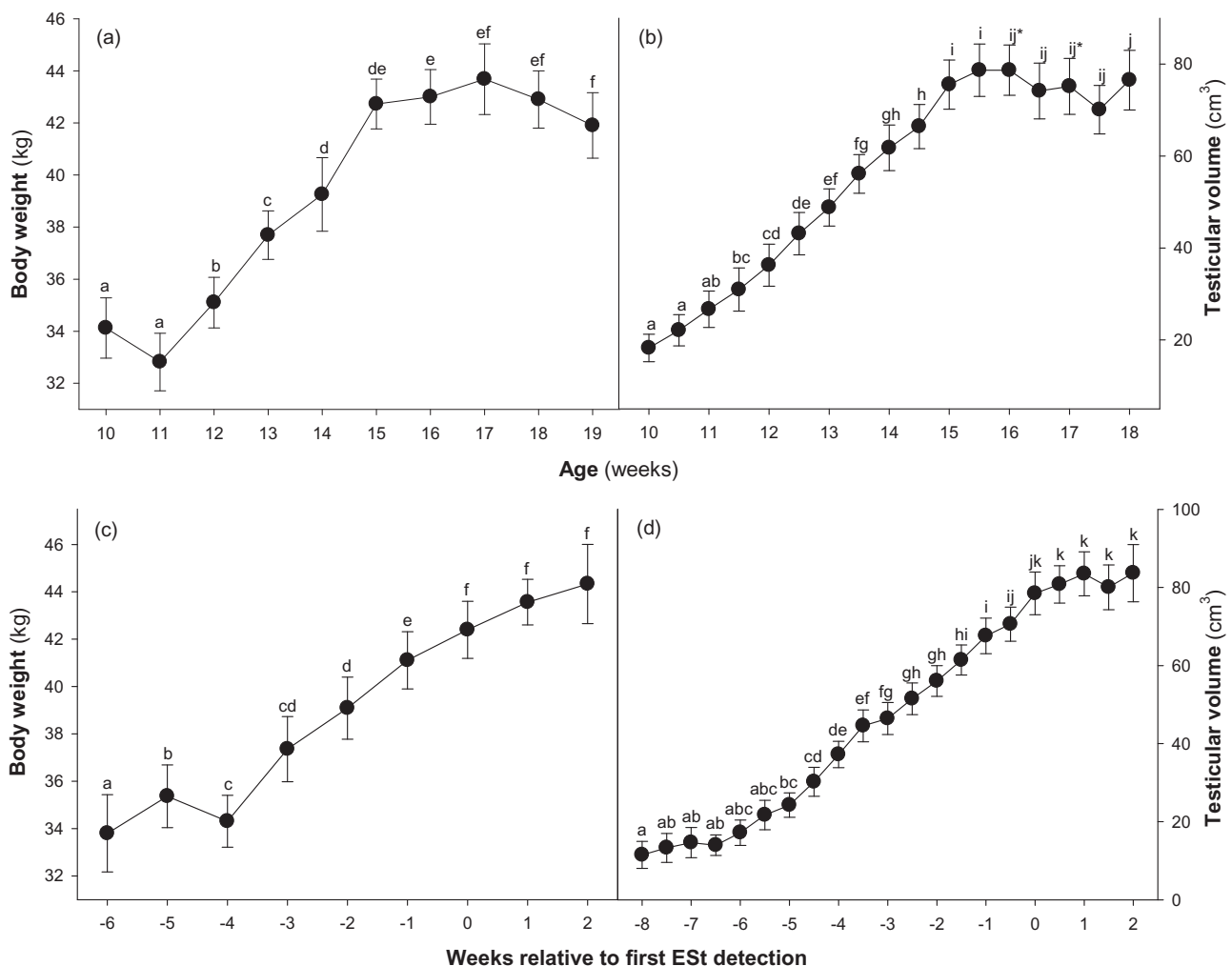


Figure 2 Mean body weight (a) and (c) and testicular volume (b) and (d) in prepubescent ram lambs with respect to weeks of age (a)–(b) and weeks relative to the first detection of elongated spermatids (ESt) (c)–(d). Different letters indicate significant differences between weeks ($P < 0.05$). Asterisks indicate that testicular volume was significantly different ($P < 0.05$) between left and right testes

to a nadir by 15.5 weeks of age ($P < 0.05$), and then continued to fluctuate ($P > 0.05$) over the next 4 weeks. The greatest increase in PSD occurred from 11 to 14 weeks of age ($P < 0.05$), after which it remained relatively constant. In contrast, NPVs rose steadily from 8 to 3 weeks before the first detection of ESt and then decreased gradually until 2 weeks after ESts were first detected ($P < 0.05$). PSD displayed a similar trend, with a significant increase from 7 to 3.5 weeks before the first detection of ESt ($P < 0.05$) followed by a numerical decrease until 2 weeks after ESts were first detected ($P > 0.05$).

Testicular histomorphology

Tubular and luminal ST diameters rose relatively linearly from 10 to 19 weeks of age ($P < 0.05$; Figure 4(a)) as did tubular ST diameter from 6 weeks before to 2 weeks after the first detection of ESt ($P < 0.05$); however, ST luminal diameter increased only from 6 weeks to 1 week before the first detection of ESt ($P < 0.05$) before reaching a plateau until 2 weeks after ESts were first detected ($P < 0.05$; Figure 5(a)). ST cell density increased exponentially from

10 to 18 weeks of age ($P < 0.05$; Figure 4(b)) or from 6 weeks before to 2 weeks after the first detection of ESt ($P < 0.05$; Figure 5(b)). With age, the nuclear:ST area decreased rapidly from 10 to 15 weeks of age ($P < 0.05$) and then stayed relatively constant until 19 weeks of age ($P > 0.05$; Figure 4(c)). Conversely, the nuclear:ST area decreased gradually from 6 weeks before to 2 weeks after ESts were first detected ($P < 0.05$; Figure 5(c)). The number of degenerating cells per ST XS increased numerically from 10 to 14 weeks of age ($P > 0.05$) or significantly from 5 to 2 weeks prior to the first ESt detection ($P < 0.05$) before numerically declining until 19 weeks of age or 2 weeks after the first detection of ESt ($P > 0.05$; Figures 4(d) and 5(d)). Following a decrease in the total number of UCHL-1 positive cells/ST XS from 10 to 11 weeks of age ($P < 0.05$), the total number of UCHL-1⁺ cells peaked numerically at 14 weeks of age ($P > 0.05$; Figure 4(e)), or at 3 to 2 weeks before ESts were first detected ($P > 0.05$; Figure 5(e)), before reaching a nadir. There were no significant changes in the numbers of different UCHL-1⁺ cell subpopulations with age; however, there was a notable decline in the number of

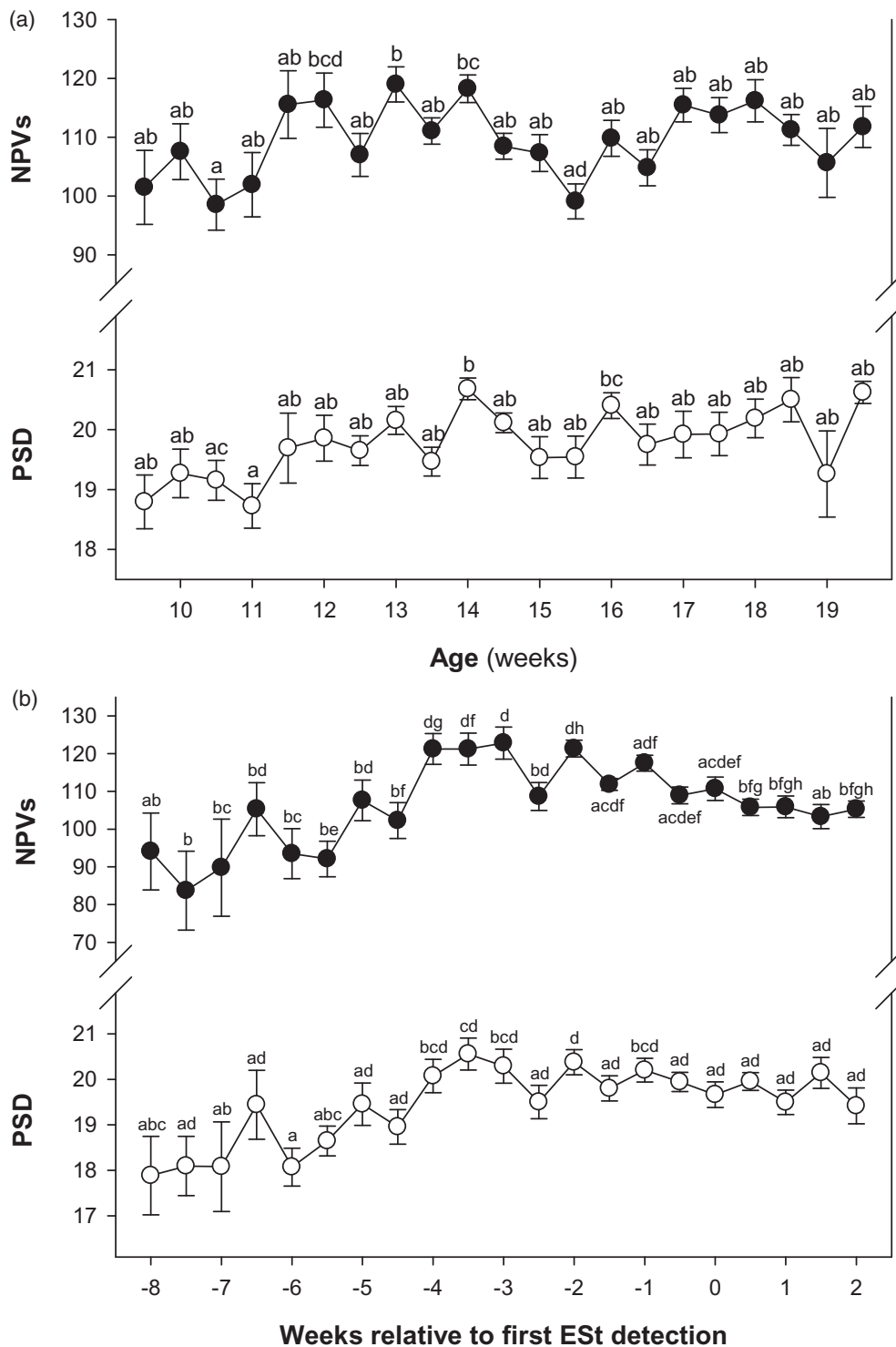


Figure 3 Numerical pixel values (NPVs) and pixel standard deviation (PSD) from testicular ultrasonograms taken at twice weekly intervals in prepubescent ram lambs and aligned to chronological age of animals (a) or weeks relative to the first detection of elongated spermatids (EST) (b). Different letters indicate significant differences between mean values ($P < 0.05$)

light-staining cells from 10 to 11 weeks of age, and a peak in the number of medium-staining cells at 14 weeks of age ($P > 0.05$; Figure 4(f)). The number of medium-staining UCHL-1⁺ cells/ST XS increased from 5 to 2 weeks before ESTs were first detected ($P < 0.05$) and numerically decreased thereafter ($P > 0.05$; Figure 5(f)).

The percentage of STs with (pre-)spermatogonia as the most advanced germ cells decreased from 10 to 15 weeks of age ($P < 0.05$) or from 6 weeks to 1 week before ESTs were first detected ($P < 0.05$), and stayed relatively constant thereafter ($P > 0.05$; Figure 6(a) and (e)). The percentage of STs with spermatocytes as the most mature germ cell type

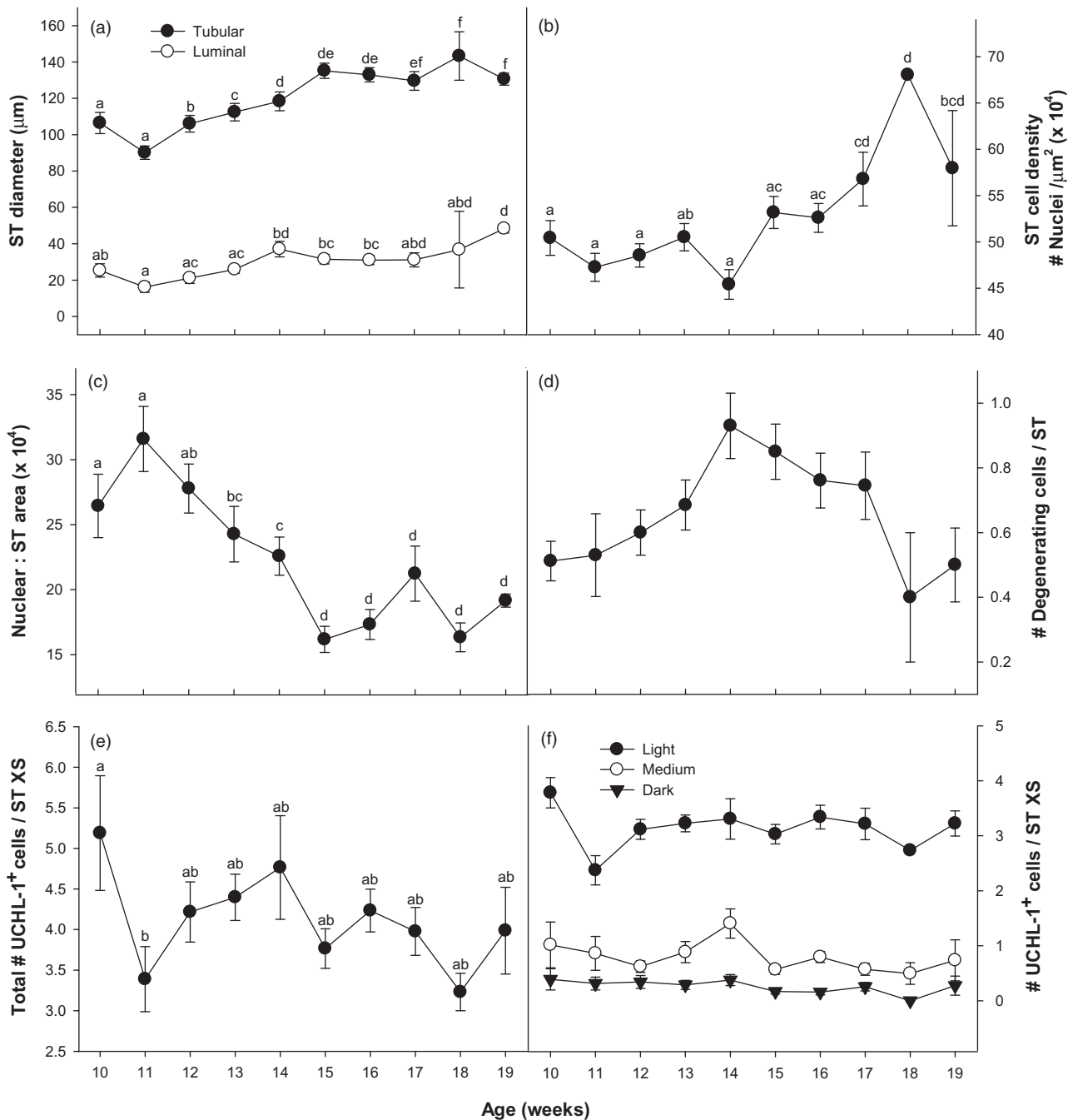


Figure 4 Mean tubular and luminal seminiferous tubule (ST) diameter (a), ST cell density (b), nuclear:ST area (c), the number of degenerating cells/ST cross-section (XS) (d), the total number of ubiquitin C-terminal hydrolase L-1 (UCHL-1) staining cells/ST XS (e), and the number of dark-, medium- and light-staining UCHL-1 cells/ST XS (f) in ram lamb testes, from 10 to 19 weeks of age. Different letters indicate significant differences between mean values ($P < 0.05$)

was highest between 12 and 17 weeks of age; however, there were no significant differences with increasing age ($P > 0.05$; Figure 6(b)). Conversely, there was a steady increase until 1 week before the first detection of ESt followed by a rapid decrease until 2 weeks after ESts were first detected ($P < 0.05$; Figure 6(f)). The percentage of STs containing round or ESt as the most mature germ cell type increased gradually starting at 15 weeks of age and then reached a peak at 18 or 19 weeks of age, respectively ($P < 0.05$; Figure 6(c) and (d)). In the weeks relative to first

ESt detection, there was a sudden increase in the percentage of STs with round and ESt as the most mature germ cell type at the time the first ESts were detected followed by a marked decrease or increase, respectively, for the following 2 weeks ($P < 0.05$; Figure 6(g) and (h)).

Correlations

Correlations between testicular echotextural and histomorphological attributes are summarized in Table 1. In the

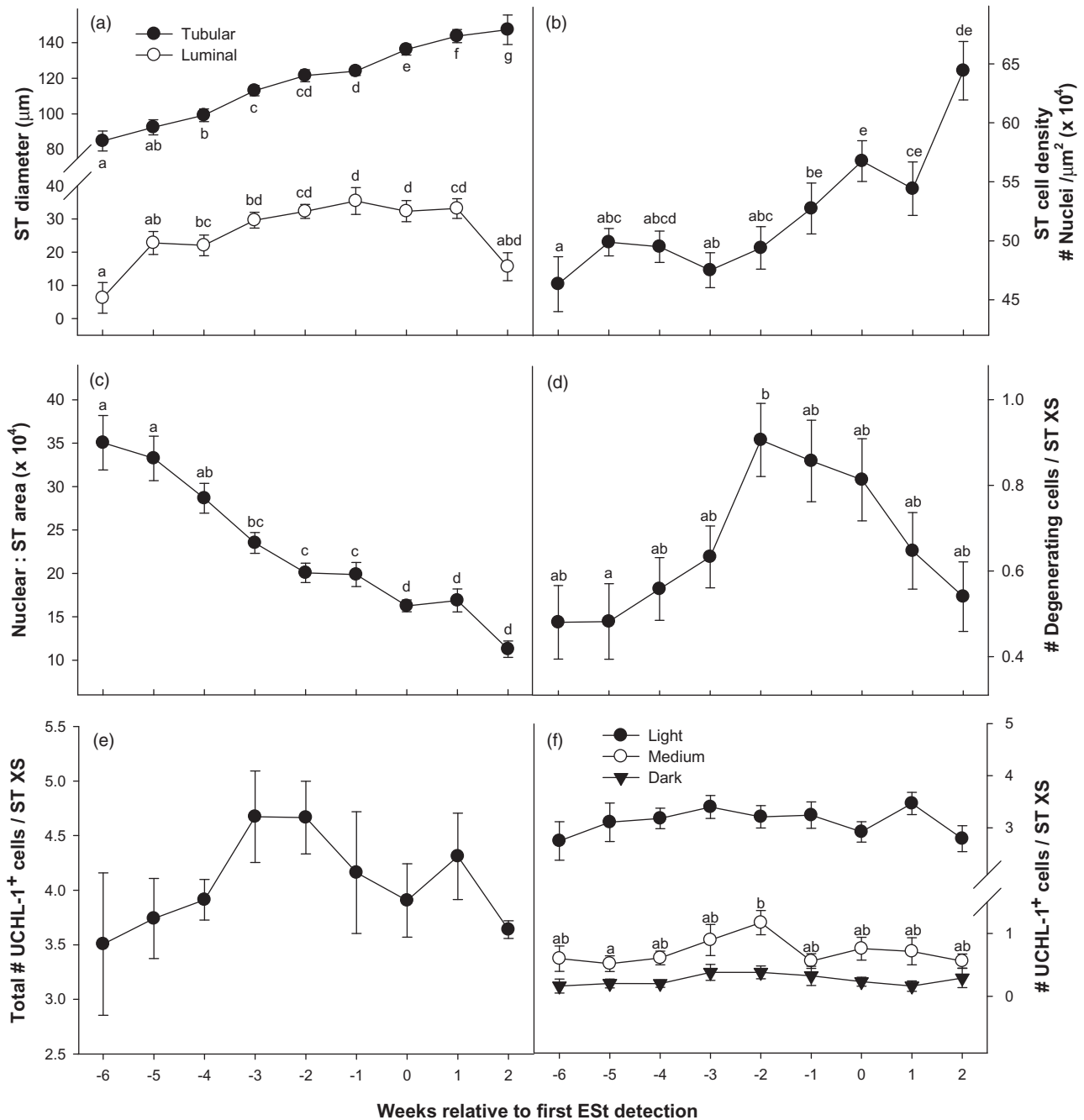


Figure 5 Mean tubular and luminal seminiferous tubule (ST) diameter (a), ST cell density (b), nuclear:ST area (c), the number of degenerating cells/ST cross-section (XS) (d), the total number of ubiquitin C-terminal hydrolase L-1 (UCHL-1)⁺ cells/ST XS (e), and the number of dark-, medium- and light-staining UCHL-1⁺ cells/ST XS (f) in ram lamb testes from -6 to 2 weeks relative to the first detection of elongated spermatids (EST). Different letters indicate significant differences between mean values ($P < 0.05$)

mitotic phase, NPVs and PSD were positively correlated with tubular and luminal diameter of the STs ($P < 0.05$), the number of degenerating cells/ST XS ($P = 0.06$ and $P < 0.05$, respectively), the number of dark- ($P = 0.08$ and $P = 0.07$, respectively) and light- ($P < 0.05$) staining UCHL-1⁺ cells/ST XS, and the total number of UCHL-1⁺ cells/ST XS ($P < 0.05$). Additionally, the number of medium-staining UCHL-1⁺ cells/ST XS was also correlated with PSD ($P = 0.06$).

In the postmitotic phase, NPVs and PSD were negatively correlated with tubular diameter ($P < 0.05$), ST cell density

($P = 0.08$ and $P = 0.07$, respectively), and percentage of STs with round ($P < 0.05$ and $P = 0.09$, respectively) and elongated ($P < 0.05$) spermatids as the most mature germ cell type. NPVs were positively correlated with the nuclear:ST area ($P < 0.05$), the number of degenerating cells/ST XS ($P = 0.07$), the numbers of dark- and medium-staining UCHL-1⁺ cells/ST XS ($P < 0.05$), the total number of UCHL-1⁺ cells/ST XS ($P = 0.07$), and the percentage of STs with (pre-)spermatogonia and spermatocytes as the most mature germ cell type ($P < 0.05$). PSD was positively correlated with the nuclear:ST area ($P < 0.05$), number of

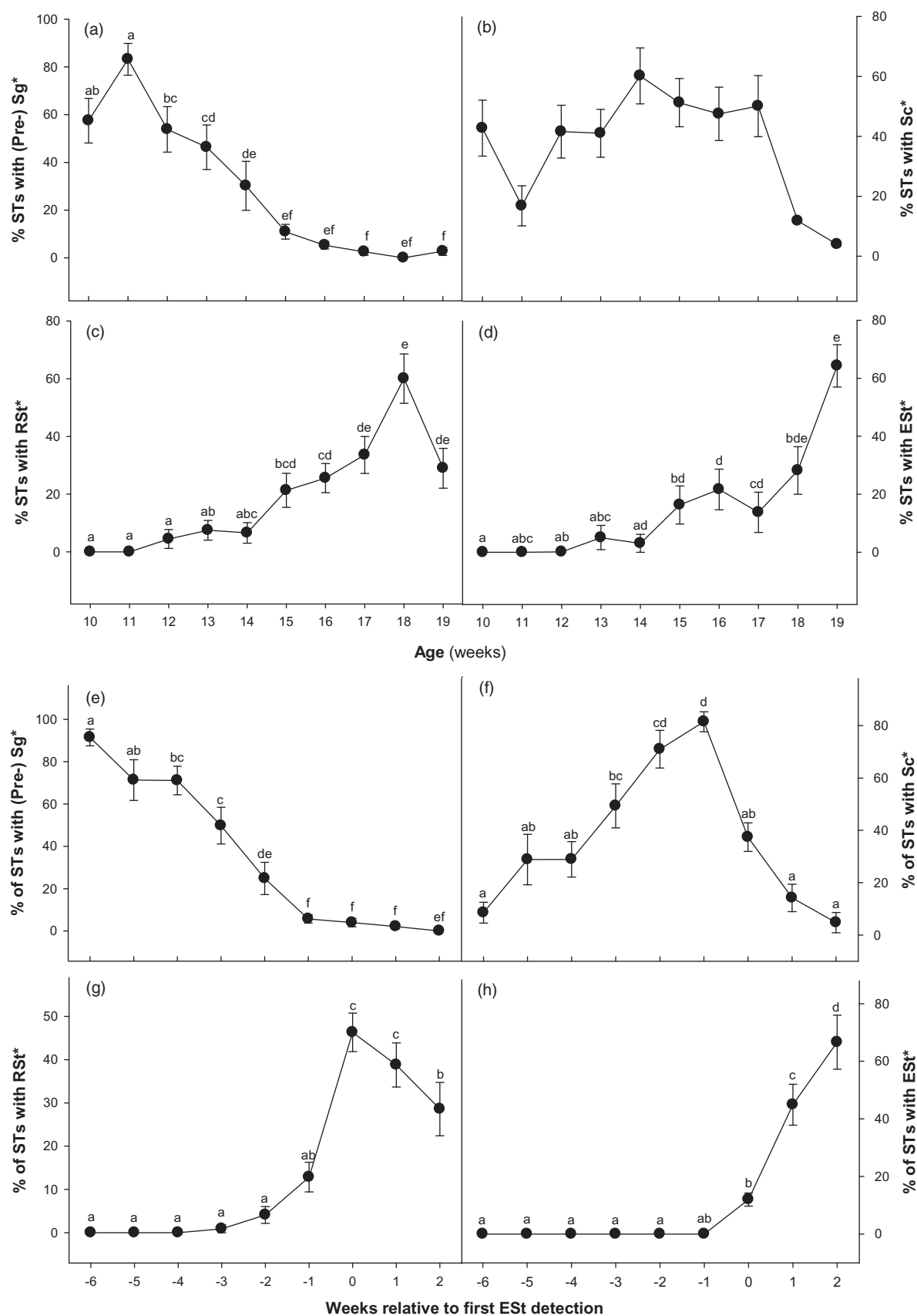


Figure 6 Mean percentages of ST cross-sections with prespermatogonia and/or spermatogonia ((Pre-)Sg (a) and (e), spermatocytes (Sc) (b) and (f), round spermatids (RSt) (c) and (g), or elongated spermatids (ES) (d) and (h) as the most mature germ cell type present in ram lamb testes from 10 to 19 weeks of age (a)–(d) or from –6 to 2 weeks relative to the first detection of ES (e)–(h). Different letters indicate significant differences between mean values ($P < 0.05$). *As the most mature germ cell type present

Table 1 A summary of Pearson product moment correlations among echotextural and histomorphological attributes in the testes of 10–19 week-old Rideau Arcott x Polled Dorset ram lambs at different phases of the first wave of spermatogenesis

Variables	Mitotic Phase		Postmitotic Phase		Overall	
	NPVs	PSD	NPVs	PSD	NPVs	PSD
ST outer diameter (tubular diameter)	0.35	0.40	−0.51	−0.36	NS	NS
ST inner diameter (luminal diameter)	0.48	0.56	NS	NS	0.19	0.15 ^a
ST Cell density	NS	NS	−0.20 ^a	−0.20 ^a	NS	NS
Nuclear:ST area	NS	NS	0.46	0.24	NS	NS
# Degenerating cells/ST XS	0.32 ^a	0.37	0.20 ^a	0.29	0.25	0.31
# Dark UCHL-1 ⁺ cells/ST XS	0.30 ^a	0.30 ^a	0.24	NS	0.25	0.18 ^a
# Medium UCHL-1 ⁺ cells/ST XS	NS	0.32 ^a	0.36	0.36	0.28	0.34
# Light UCHL-1 ⁺ cells/ST XS	0.35	0.35	NS	NS	0.23	0.21
Total # UCHL-1 ⁺ cells/ST XS	0.45	0.52	0.20 ^a	NS	0.28	0.26
% STs with (Pre-)Sg*	NS	NS	0.25	NS	NS	NS
% STs with Sc*	NS	NS	0.34	0.31	0.29	0.28
% STs with RSt*	NA	NA	−0.23	−0.20 ^a	NS	NS
% STs with ESt*	NA	NA	−0.36	−0.28	−0.19	−0.17 ^a

ESt: elongated spermatids; NA: nonapplicable; NPVs: numerical pixel values (pixel intensity); NS: nonsignificant; (Pre-)Sg: (Pre-)spermatogonia; PSD: pixel standard deviation (pixel heterogeneity); RSt: round spermatids; Sc: spermatocytes; ST: seminiferous tubule; UCHL-1⁺: ubiquitin C-terminal hydrolase L-1 positive; XS: cross-section.

*As the most mature germ cell type present.

^aApproaching to significance ($P \leq 0.10$).

degenerating cells/ST XS ($P < 0.05$), the number of medium-staining UCHL-1⁺ cells/ST XS ($P < 0.05$), and the percentage of STs with spermatocytes as the most advanced germ cell type ($P < 0.05$).

During the entire period of study, NPVs and PSD were positively correlated with luminal ST diameter ($P < 0.05$ and $P = 0.10$, respectively), the number of degenerating cells/ST XS ($P < 0.05$), the number of dark- ($P < 0.05$ and $P = 0.06$, respectively), medium- ($P < 0.05$) and light- ($P < 0.05$) staining UCHL-1⁺ cells/ST XS, the total number of UCHL-1⁺ cells/ST XS ($P < 0.05$) and the percentage of STs with spermatocytes ($P < 0.05$) as the most mature germ cell type and negatively correlated with ESt ($P < 0.05$ and $P = 0.06$) as the most mature germ cell type.

Discussion

Prepubertal testicular development is accompanied by complex changes in testicular echotexture. In agreement with previous studies,^{5–7,11,13} testicular echogenicity in the present ram lambs was highly variable with age. However, when the echotextural parameters were aligned to the first detection of ESt, they exhibited a much more linear pattern of changes, suggesting that ultrasonographic attributes are more directly associated with spermatogenic development than with chronological age of animals. Similarly, Brito *et al.*¹¹ showed that testicular echogenicity in bull calves was much less variable when aligned to the attainment of puberty as defined by semen parameters compared with an alignment to the animals' age. In the present study, there was an initial increase followed by a decrease in mean NPVs of the testicular parenchyma, which corresponded to two distinct spermatogenic periods, namely the mitotic and postmitotic phases. Clearly, testicular echotexture is affected by microstructural changes

associated with spermatogenic development during the first wave of spermatogenesis in ram lambs.

Histomorphological attributes were evaluated from unilateral testicular biopsies taken on a weekly basis. Studies of repetitive testicular biopsies performed in sexually mature llamas and prepubertal sheep have demonstrated that it is a safe procedure with maintenance of normal testicular size and function when appropriate care is taken to avoid hematoma, lesions, and infections.^{19,20} Indeed, the average TV and body weight of the ram lambs in the present study displayed normal growth trajectories for pubertal development. A period of rapid testicular growth was demonstrated in the final 5 weeks prior to ESt detection, at approximately 10–15 weeks of age, which is similar to the findings reported in Blackbelly sheep by Herrera-Alarcón *et al.*²¹ Body weight displayed a similar increase throughout pubertal development, in accordance with its positive correlation with testicular size.²² Furthermore, serial histological evaluations revealed no pathological consequences of multiple biopsies taken from the left testis in this study.

In rams, the relative ST volume increases from about 50 to 84% of the testicular parenchyma from the early prepubertal period to the adult stages of development.²³ There are two major contributing factors to ST volumetric expansion during prepubertal development: (i) the growing cell population of the seminiferous epithelium; and (ii) lumen formation and enlargement. Initially, there is a lengthening of the STs due to Sertoli cell proliferation, followed by an increase in ST diameter as the germ cell number rises with spermatogenic onset. Both the tubular and luminal ST diameter and ST cell density increased as expected throughout the present study.

The relationship between testicular NPVs and ST size in ruminants has previously been evaluated; however, the

findings have been inconsistent. A positive correlation with ST diameter was reported in bull calves during prepubertal development,⁵ while a negative correlation with ST area was reported in yearling bulls.^{11,14} Recently, the protein content of testicular parenchyma was shown to be negatively correlated with echotextural parameters in rams.²⁴ The proteome profile of a mouse testis during spermatogenic onset reveals a high level of protein expression during spermatogonial proliferation as well as during round and ESt formation, however there appears to be a reduction in the total amount of protein synthesized during the initiation of meiosis in spermatocytes.²⁵ Therefore, shifts in testicular protein content may have contributed to the switch in the direction of the correlations between ST diameter and parenchymal echogenicity in the present study. At present, it is uncertain how the level of protein expression in the testes affects the resulting testicular echogenicity. Not unlikely, specific organelles involved in protein synthesis may have distinct echotextural properties that impinge on the overall tissue echogenicity. Alternatively, protein molecules may interact with the ultrasound waves in a specific way. Tubular and luminal diameter of the STs had some of the strongest correlations with testicular echotexture; therefore, it is important to thoroughly investigate how cellular and biochemical changes in both the tubular and luminal compartments affect the resulting ultrasound image.

Throughout spermatogenesis, germ cell nuclei undergo dynamic changes in size and chromatin arrangement. In the present study, nuclear:ST area demonstrated a relatively constant decrease throughout spermatogenic onset. While there is an overall decrease in nuclear volume between spermatogonia and spermatids,²⁶ the present findings were somewhat unexpected since there is also a dramatic threefold increase in nuclear volume between early and late primary spermatocytes in sheep.²⁶ However, this discrepancy can be explained by the fact that a peak in the number of degenerating cells occurred when spermatocytes had the highest prevalence in ST XS as the most mature germ cell, in agreement with a study that reported increased apoptosis predominantly in primary (mid-pachytene) spermatocytes in rats during the first wave of spermatogenesis.²⁷ Chromatin condensation or decreased nuclear size is a hallmark morphological feature of apoptosis,¹⁸ which may have maintained the decreasing trend in nuclear:ST area. Additionally, there is a nearly twofold increase in Sertoli cell cytoplasmic volume from 70 to 100 days of age in ram lambs.²⁸ As Sertoli cells occupy approximately one-third of the ovine seminiferous epithelium²⁶ this increase in cytoplasmic volume could greatly affect the nuclear:ST area.

Recent evidence has indicated that the nucleus is likely the dominant source of subcellular ultrasound scattering and changes in nuclear configuration (i.e. mitosis, apoptosis) greatly affect tissue echogenicity.²⁹ A reduction in the nuclear:ST area was therefore hypothesized to result in a corresponding decrease in testicular echogenicity, as the ultrasound waves are less likely to be scattered by STs with cells having a smaller nuclear size. Indeed, a positive correlation was found between nuclear:ST area and testicular echotextural parameters in the postmitotic phase;

however, no correlation was found in the mitotic phase. This may be explained by the increase in the number of degenerating cells observed toward the end of the mitotic phase, which had a positive correlation with NPVs and PSD in both the mitotic and postmitotic phases. Cells undergoing apoptosis exhibit changes in nuclear morphology (i.e. condensation and fragmentation) that greatly enhance ultrasound scattering strength.²⁹ Nuclear morphology holds promise as a strong determinant of testicular echotexture; future studies on other changes in nuclear configuration during spermatogenesis such as meiosis and spermiogenesis may provide further insight into interpreting testicular echotextural fluctuations during the first wave of spermatogenesis.

SSCs are a rare germ cell population in the testis, constituting only about 10.6% of undifferentiated spermatogonia, 1.25% of all spermatogonia, or 0.03% of all spermatogenic cells in the adult mouse.³⁰ At present, there are no morphological or molecular markers that can specifically identify SSCs.³¹ UCHL-1 has been shown to be exclusively expressed in undifferentiated spermatogonia in boars, with a high level of UCHL-1 expression associated with maintenance of spermatogonia in the undifferentiated state, and a reduction of UCHL-1 expression associated with their differentiation toward meiosis.³² These findings are consistent with the heterogeneity in UCHL-1 expression found in the present study and suggest that the lower numbers of dark-staining UCHL-1⁺ cells may represent a SSC-enriched population and the higher numbers of medium- and light-staining UCHL-1⁺ cells may represent undifferentiated spermatogonia that have become committed to the differentiation pathway. The number of UCHL-1⁺ cells at different levels of intensity remained relatively stable throughout the study, consistent with the generally low rate of self-renewal described for all stem cells under steady-state conditions.³³

The total number of UCHL-1⁺ cells and the numbers of dark-, medium-, and light-staining UCHL-1⁺ cells/ST XS were all positively correlated with testicular echotextural parameters throughout spermatogenic onset. However, the numbers of dark- and medium-staining UCHL-1⁺ cells/ST XS were more strongly correlated with echotextural parameters during the postmitotic phase, whereas the number of light-staining UCHL-1⁺ cells/ST XS was correlated with NPVs and PSD only in the mitotic phase, indicating that even subtle differences in cellular histomorphology and/or function of spermatogonial populations may affect testicular echotexture. A role for UCHL-1 in promoting a wave of apoptosis during spermatogenic onset has previously been described³⁴; therefore, it is feasible that the numbers of dark- and medium-staining UCHL-1⁺ cells/ST XS are more strongly correlated with testicular echotextural parameters during the postmitotic phase due to their association with germ cell apoptosis, which was highest at the start of this period. The existence of correlations between testicular echotextural attributes and UCHL-1⁺ cells opens up several intriguing possibilities for using ultrasound as a noninvasive tool to evaluate the success of SSC transfer procedures and/or monitor the SSC population during gonadotoxic cancer treatment.

Spermatogenesis is not simply initiated at a particular age; rather, its timing is precisely determined by a complex interaction of genetic, endogenous, and environmental cues.³⁵ Indeed, even among rams born in the same season, raised in the same climate under the same feeding regimens and having a similar cross-bred genetic background, the first appearance of ESt was found to vary by 6 weeks in the current study. Furthermore, the rate of spermatogenic onset appeared to vary within ST XS of the same individual, with peaks in the percentage of STs with different germ cells as the most mature cell type present at specific times throughout prepubertal development. Spermatocytes were present in STs as the most mature germ cell type at the outset of the study at 10 weeks of age, in agreement with previous studies in ram lambs demonstrating the first appearance of primary spermatocytes at approximately 9–10 weeks of age.^{21,36} The percentage of STs with spermatocytes as the most mature germ cell type continued to increase until 1 week before the first detection of round and ESt, which occurred at 15 weeks of age, also in agreement with earlier reports.^{21,36} Therefore, the first wave of spermatogenesis proceeded gradually in the ram lambs of the present study, with differences between individuals as well as within the testes of each individual.

During spermatogenic onset, the developing germ cells undergo a number of changes in their morphological (i.e. size, geometry) and mechanical (i.e. density, compressibility) properties, which are both known to affect tissue echogenicity.³⁷ Consequently, nearly all germ cell types have been correlated with testicular echotexture.⁵ The percentages of STs with (pre-)spermatogonia and spermatocytes as the most advanced cell type were both positively correlated with testicular echogenicity; however, differing physical properties of these germ cell types may explain this occurrence. In a study that used density centrifugation to physically separate different germ cell types, spermatogonia were found to have a relatively high density, and subsequently the density level decreased until the formation of spermatocytes undergoing the first meiotic division before gradually increasing and reaching the highest level in ESt.³⁸ Therefore, the positive correlation between (pre-)spermatogonia and testicular echotextural parameters in the postmitotic phase may be explained by the high density of this germ cell population. Conversely, spermatocytes undergo a fivefold increase in cellular volume during the first meiotic prophase to become the largest members of the germ cell line²⁶ which could explain the positive correlation observed with NPVs and PSD in the postmitotic phase. The percentages of STs with round and ESt as the most mature germ cell type were both negatively correlated with testicular echotextural parameters, which is likely a consequence of the comparatively small cellular volume of both cell types, especially for ESt.²⁶ Additionally, the transition in shape from a round to an elongated cell would likely reduce cellular echogenicity further due to a dependence of ultrasound beam scattering on a perpendicular cell orientation for optimal scattering, which may have resulted in the slightly stronger negative correlation between the percentage of STs with ESt as the most mature germ cell type and testicular NPVs and PSD. Correlations

between the percentage of STs with different spermatogenic cell types as the most advanced cell present and echotextural attributes have previously been evaluated by Evans *et al.*⁵ in bull calves studied from birth to puberty. While the strength of these correlations was similar to the current findings, the directions of correlations were opposite for all but one germ cell type, namely the late (pachytene, diplotene, or secondary) spermatocytes. The reason(s) for these differences is currently unknown; however, future experimental protocols targeting different developmental stages or *in vitro* approaches may provide further insight into the echotextural characteristics of specific germ cell types. Acquiring this knowledge would be useful for potentially monitoring the stage of spermatogenesis using echotextural analysis in prepubertal individuals as well as in adults whose spermatogenesis has been interrupted by testicular recrudescence, contraceptive use, or various pathological conditions.

In summary, the results of this study provide considerable insight into how tissue microstructure affects testicular echotexture during the first wave of spermatogenesis in ram lambs. As STs are the largest morphological constituent of the testis, ST dimensions (tubular and luminal diameter) had the strongest correlations with testicular echotexture; however, these were comparable to the correlations with ST cell density, the numbers of degenerating and UCHL-1⁺ cells per ST XS, and the percentage of STs with various germ cells as the most mature type at the cellular level, as well as with the nuclear:ST area at the subcellular level. There are several avenues of research that need to be explored before computerized image analysis of ultrasonograms may be used in a clinical setting, including a more detailed evaluation of the cellular and biochemical changes in the seminiferous epithelium and lumen accompanying ST maturation and the effect of this on testicular echotexture, further investigation of how nuclear configurations associated with different spermatogenic phases and germ cell apoptosis affect ultrasonographic attributes, and a precise determination of the echotextural characteristics of SSCs and different types of germ cells. Additionally, the echotextural attributes of Sertoli cells, which are intricately involved in spermatogenic onset as well as Leydig cells, which are responsible for secreting testosterone, a hormone that has previously been correlated with testicular echogenicity,⁵ should be investigated more thoroughly. To recapitulate, our present findings could have important implications for the diagnostic and prognostic capabilities of scrotal ultrasonography including the assessment of the rate and progression of spermatogenesis as well as noninvasive evaluation of microstructural testicular anomalies in prepubertal and sexually mature individuals.

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