

A Study of Morphological and Haemodynamic Determinants of Testicular Echotexture Characteristics in the Ram

JENNIFER L. GIFFIN,* SARAH E. FRANKS,* JOSE RAFAEL RODRIGUEZ-SOSA,† ANN HAHNEL,*
AND PAWEL M. BARTLEWSKI*,¹

**Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada N1G 2W1; and †Center for Animal Transgenesis and Germ Cell Research, New Bolton Center, School of Veterinary Medicine, University of Pennsylvania, Kennett Square, Pennsylvania 19104*

The ultrasonographic image of an organ is a product of scattering and reflection of high-frequency ultrasound beams by discrete units of tissue. The number of acoustic tissue interfaces and vascularity affects the quantitative characteristics of grey-scale ultrasonographic images. This study was undertaken to examine the influences of scrotal/testicular integument and blood flow on testicular echotexture parameters in the ram. Serial ultrasonographic images were obtained during surgical castration of 7 Rideau Arcott rams aged 20–22 weeks. The first 2 sets of images were taken through the scrotum, prior to and after induction of anaesthesia. The third set was taken through the tunica vaginalis, the fourth set was obtained through the tunica albuginea, the fifth set was taken when the testicular cord and internal blood vessels were clamped, and the final set of images was recorded after allowing the blood to drain from dissected testicles (5 min). All images were then subjected to computerized image analyses and the testicles were processed for histology. The removal of the scrotal skin and tunica vaginalis both resulted in significant ($P < 0.05$) increments in numerical pixel values (NPVs) and pixel heterogeneity (standard deviation of pixel values) of the testicular parenchyma. There were no differences ($P > 0.05$) in testicular echotexture between images taken just before or after clamping the testicular cord vessels, or after draining. At all stages, NPVs were correlated ($P \leq 0.10$) to the seminiferous tubule (ST) area and the ST lumen area, except for NPVs and the ST lumen area in images obtained

through the tunica albuginea ($P = 0.20$). We concluded that: 1) attenuation of ultrasound waves by the scrotal skin and tunica vaginalis significantly altered testicular echotexture characteristics; 2) vascular blood flow did not affect the echotextural attributes of the rams' testes; and 3) NPVs were a good indicator of ST microstructure *in situ* and *ex vivo*. *Exp Biol Med* 234:794–801, 2009

Key words: ultrasonography; computer-assisted image analysis; testis; seminiferous tubules; ram

Introduction

Scrotal ultrasonography is frequently performed to monitor male reproductive health. The use of this technique in both clinical and research settings offers many benefits as it allows for repeated and non-invasive visualization of the scrotal content in the same individuals (1–3). Recent studies in pre- and peripubertal bulls and rams have demonstrated that changes in ultrasonographic attributes of the testicular parenchyma are closely related to the histomorphological characteristics of seminiferous tubules (1) and the onset and efficiency of spermatogenesis (1, 2, 4–6).

The use of grey-scale ultrasonographic imaging for microstructural and functional assessment of reproductive organs has greatly improved our understanding of the dynamic changes in testicular histophysiology (1, 5). Quantitative ultrasonogram analysis methods have greatly facilitated interpretation of ultrasonographic findings (7). The computer-assisted estimation of numerical pixel values (NPVs), expressed on the scale of 0 (“absolute” black) to 255 (“absolute” white), increases both the precision of results and the range of perceivable intensity variations that can be detected in the structures examined. NPVs are a measure of the echotexture or echogenicity of a tissue, which depends on its density and water/macromolecular content (7, 8).

The ultrasonographic display of the tissue is a product of reflection and scattering of high-frequency ultrasound

This study was funded by the Ontario Ministry of Agriculture, Food and Rural Affairs (PMB and AH), and the Natural Sciences and Engineering Research Council of Canada (AH).

¹ To whom correspondence should be addressed at the Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, ON, Canada N1G 2W1. E-mail: pmbart@uoguelph.ca

Received December 9, 2008.

Accepted March 27, 2009.

DOI: 10.3181/0812-RM-364

1535-3702/09/2347-0794\$15.00

Copyright © 2009 by the Society for Experimental Biology and Medicine

beams by discrete units of tissue (i.e., acoustic tissue interfaces). During the ultrasound imaging procedure, the proportions of sound beams that are transmitted or reflected are affected by different conditions, placing limitations on the accuracy of image interpretation and pixel analyses. One such condition is the various ultrasound settings (i.e., main, near and far gain, focal points, etc.). These settings, however, are under the direct control of the operator and can be manipulated or kept constant, as required. The interaction of the sound beams at different tissue interfaces is dependent upon biophysical principles, which may result in decreased signal strength with increasing depth of structures monitored. The number of acoustic tissue interfaces and vascularity may both affect the quantitative echotextural attributes of ultrasonographic images. Therefore, the objective of the present experiment in ram lambs was to examine the influences of the scrotal/testicular tissue layers and blood flow on the testicular echotexture parameters and to correlate the echotextural parameters with histological characteristics of the seminiferous tubules (STs).

Materials and Methods

The Animal Care Committee at the University of Guelph approved all procedures described in this section and performed on live sheep.

Serial ultrasonographic images were obtained during surgical castration of 7 Rideau Arcott rams aged 20–22 weeks (July) that were housed in a field research station in Ponsonby, ON, Canada (latitude: 43°33'N). Animals were kept in outdoor pens, under ambient light and temperature conditions, with easy access to indoor facilities. All lambs were weaned at 50 days of age. Starting at 14 days of age and until 50 days after weaning, the lambs were fed a 16% protein lamb grower (Floradale Seeds, Floradale, ON, Canada) *ad libitum*.

Surgery was done under general anaesthesia induced with xylazine (Rompun®, Bayer, Toronto, ON, Canada; 0.2 mg/kg i.m.) and maintained by ketamine (Ketalean, Animal Health Inc., Cambridge, ON, Canada; 2 mg/kg IV) and diazepam (Diazepam, Sabex, Boucherville, PQ, Canada; 0.2 mg/kg IV) administration. Ultrasonography utilized an Aloka SSD-900 echo camera equipped with a 7.5-MHz linear array probe (Aloka Inc., Tokyo, Japan) placed in a plastic bag filled with lubricant gel (Light Lube, Centaur VA Animal Health, Guelph, ON, Canada), for easier cleaning, and disinfected with isopropyl ethanol between sequential examinations.

The first set of images was taken in animals examined in a standing position prior to Rompun® injections. From that point on until the removal of testicles, the images were taken in animals placed in dorsal recumbency. The second set of images was taken through the scrotum just prior to the surgical procedure, the third set was taken through the tunica vaginalis, the fourth set was obtained through the

tunica albuginea, and the fifth set was taken after the testicular cord and internal blood vessels had been clamped off with forceps. The final set of images was recorded after allowing the blood to drain from dissected testicles for 5 min.

All images were obtained at constant settings of the ultrasound scanner for overall gain, near and far gains and focal points, and were recorded on a DVD recorder (Pioneer® DVD Recorder DVR-510H, Pioneer Electronics of Canada Inc., Markham, ON, Canada). Still images containing the largest cross-sectional area in a longitudinal-plane view of each testicle were selected for computerized analysis using commercially available image analytical software (Image ProPlus®; Media Cybernetics Inc., San Diego, CA). Figure 1A depicts the general appearance of ultrasonograms that were analyzed. The “spot meter” technique previously described by Pierson and Adams (7) was employed for analysis of pixel intensity values of the testicular parenchyma. Six spots of 60 pixels in diameter (approx. 6 mm) were placed on the image of the testicular parenchyma (Fig. 1A); 3 computer-generated spots were placed above and 3 were placed below the central rete testis, excepting the rete testis itself and any obstructive artifacts. The mean numerical pixel values (NPVs) and standard deviations of NPVs (pixel heterogeneity-PSD) in each spot were recorded.

Testicular tissue samples (Fig. 1B) were fixed overnight in modified Davidson's fluid (9), processed into paraffin using standard techniques, sectioned at a thickness of 5 µm, and stained with hematoxylin and eosin. Digital images of the histological slides (1 image per tissue sample, 3 images per testicle) were taken under ×200 magnification using the computer program Q Capture® (Quorum Technologies Inc., Guelph, ON, Canada). All morphometric analyses were done using the ImagePro®Plus analytical software. The outer seminiferous tubule (ST) circumference was outlined and used to calculate the relative ST area (i.e., proportion of the total area occupied by STs). Similarly, the luminal area was computed from a digital outline of the inner seminiferous tubule circumference. Three individuals to whom identities of rams were not known performed all echotextural and morphometric analyses.

All data sets were initially screened for outliers using Grubb's test (the maximum normed residual test) (<http://www.graphpad.com/www/grubbs.htm>). Differences in mean NPVs, as well as pixel heterogeneity (PSD) determined by spot analyses, were assessed using the PROC MIXED procedure on SAS (SAS Institute Inc., Cary, NC) to determine the linear model that included the main effects of the individual performing the analyses (3 individuals), testicle examined (left vs. right), spot location (3 spots above vs. 3 spots below the rete testis), set of images obtained, and the interactions between those factors. The relationships between echotextural and histological parameters were studied by simple linear regression (SigmaStat® 3.0 for Windows®, 2003, Systat Software, Inc., Richmond, CA);

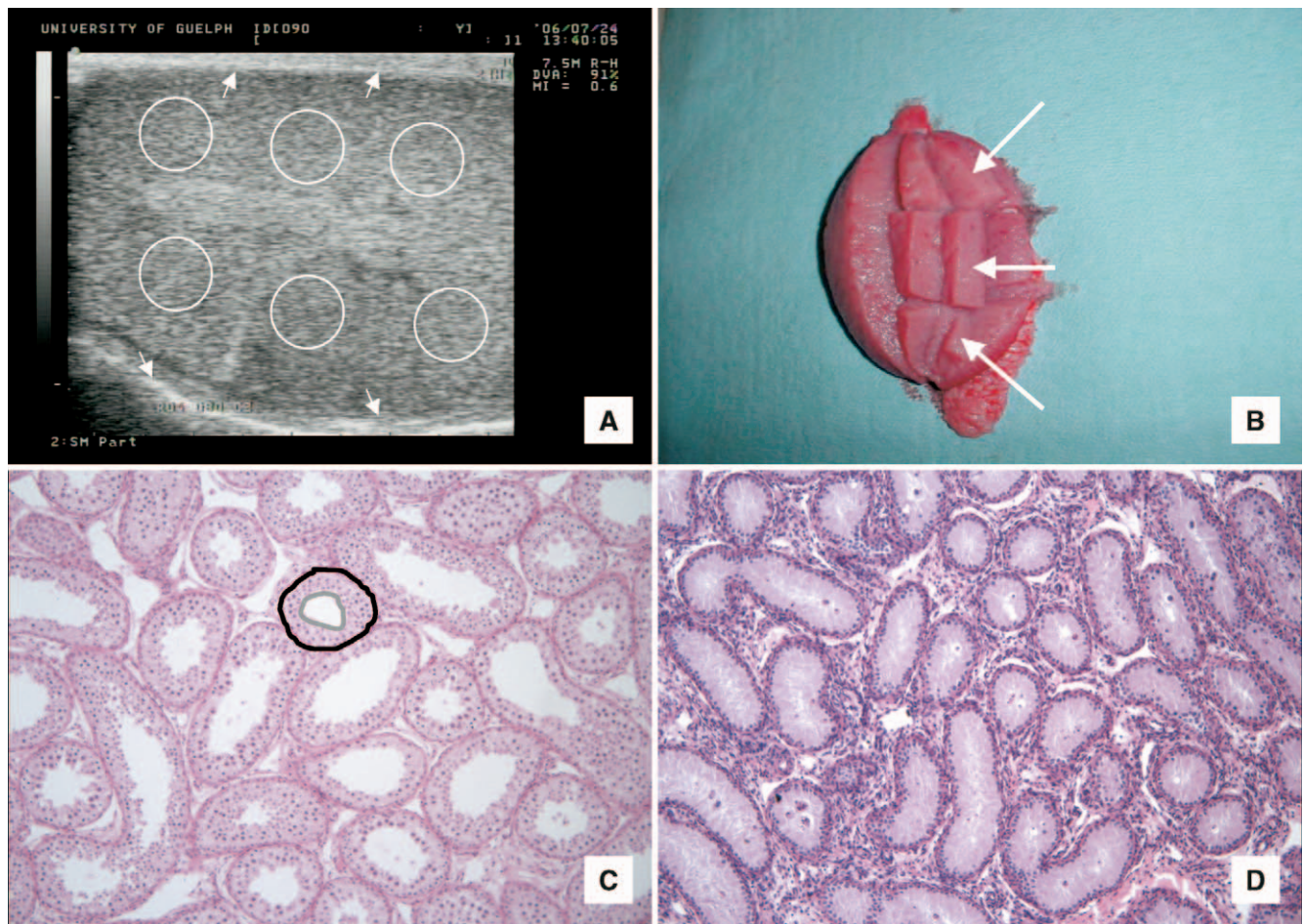


Figure 1. (A) A digital ultrasonographic image of the ram's testis, in longitudinal section, used for computer-assisted echotextural analysis. Arrows delineate the skin of the scrotum (top) and the scrotal septum (bottom). The rete testis is the large elongated area in the center (hyperechoic). Circles represent potential placement areas for computer-generated spot meters used for the assessment of numerical pixel values (NPVs) and pixel heterogeneity (standard deviation of pixel values-PSD). (B) One half of a longitudinally dissected testis. Arrows point to the middle section from which samples were taken for histological examinations. (C) All outer seminiferous tubule (ST) circumferences (black) were manually outlined and used to calculate the relative area of STs. Similarly, the luminal area was computed from digital outlines of the inner seminiferous tubule circumferences (dark grey). (D) Histological appearance of testicular tissue samples with the lowest relative volume densities of STs identified by the Grubb's test; the ultrasonographic and histological data for this testis were consequently withdrawn from all statistical analyses. A color version of this figure is available in the online journal.

the independent variables were the relative (%) areas of STs and ST lumen, and the dependent variables were mean NPVs and PSD values determined using the ImagePro®Plus software in the upper 3 spot meters (i.e., spots encompassing the regions of testicles from which tissue samples were dissected for histological examinations). In all analyses, a P value < 0.05 was considered statistically significant. All results are given as mean $\pm$ SEM.

Results

The Grubb's test revealed the existence of a single consistent outlier in the data set, denoted "SL" (Figs. 3 and 4). Inspection of histological slides revealed small size of STs in the 3 tissue samples obtained from this testicle (Fig. 1D). The data for this testicle were removed from all statistical analyses.

There were no differences between echotextural

parameters determined by the 3 individuals, and no differences between values recorded in the spot meters placed above or below the rete testis ($P > 0.05$). Therefore, the 18 values for each variable were averaged for each testis (6 spots per image, 3 individuals/measurements per testicle). The removal of the scrotal skin and tunica vaginalis both resulted in significant ($P < 0.05$) increments in NPVs of testicular parenchyma (Fig. 2A). Removal of the scrotal skin also resulted in increased ($P < 0.05$) pixel heterogeneity (PSD) values (Fig. 2B). There were no differences ($P > 0.05$) in either echotextural parameter between images taken just prior to or after clamping the testicular cord vessels and blood draining. No differences in echotextural parameters were found between the left and the right testis of the rams of the present study ($P > 0.05$).

At all stages, average NPVs for the upper 3 spot meters (placed above the rete testis) were significantly correlated to

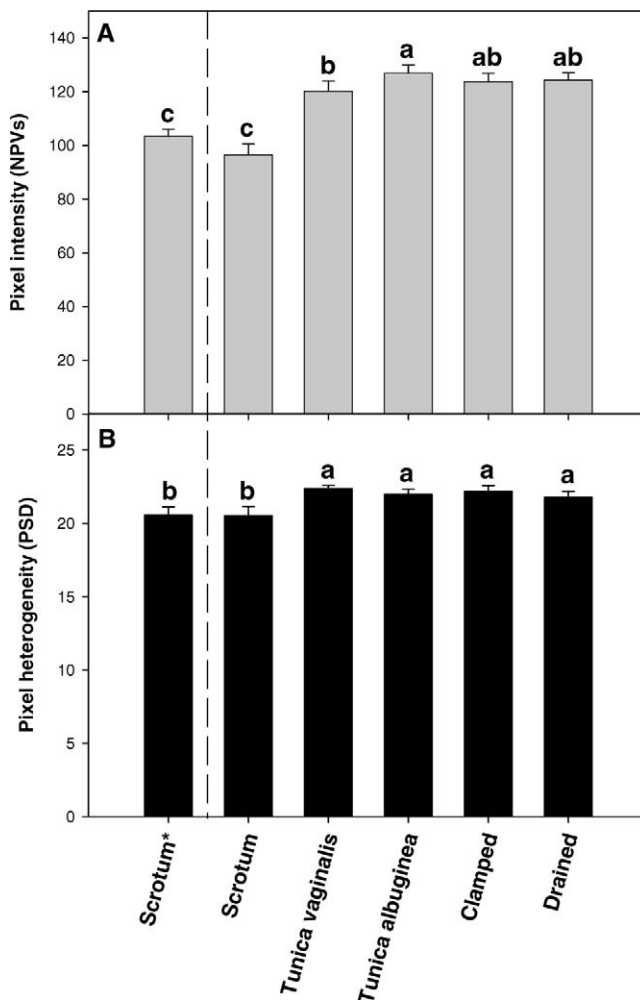


Figure 2. Mean ($\pm$ SE) numerical pixel values (NPVs-upper panel) and pixel heterogeneity (PSD-lower panel) for the testicular parenchyma determined by computer-assisted image analyses of testicular ultrasonograms obtained during castration in 7 Rideau Arcott rams. "Scrotum*" refers to testicles scanned before sedation and placing the transducer in a lubricant-filled plastic bag; "Scrotum" refers to testicular images obtained through the intact scrotal skin; "Tunica vaginalis" and "Tunica albuginea" refer to testes with scrotum removed and images taken through tunica vaginalis and tunica albuginea, respectively; "Clamped" refers to testes after the testicular cord and internal blood vessels were clamped with forceps; and "Drained" denotes testes after allowing for the blood to drain from for 5 min. Means with different letters are significantly different ($P < 0.05$).

the ST area as well as the ST lumen area ($P < 0.05$), except for images obtained through the tunica albuginea, in which the correlation between NPVs and the ST lumen area was not significant ($P = 0.20$; Fig. 3H), and the correlations between the ST area and NPVs for testicular images recorded through the tunica albuginea and after draining (Fig. 3G and 3K) approached significance ($P = 0.06$; $P = 0.10$). Correlations between the ST or ST lumen areas and PSDs were not significant ($P > 0.05$), except for the correlation between PSD values obtained from images recorded through the tunica albuginea and the ST lumen area (Fig. 4H), and PSD values determined after clamping

the testicular blood vessels and the ST area (Fig. 4I). The correlations between PSDs and the ST lumen area in animals examined before surgery approached significance ($P = 0.07$; Fig. 4B).

Discussion

Computerized analysis of digital ultrasonographic images provides a sensitive and objective means of quantifying tissue echogenicity, with the ability to detect changes in structures previously only visualized by histology. In this study, it was determined that both the scrotum and the tunica vaginalis had a significant effect on the overall testicular echotexture. The removal of these 2 tissue layers increased the mean testicular pixel intensity by $\sim 25\%$ and the pixel heterogeneity by $\sim 9\%$. These changes in echogenicity are likely due to scattering of the ultrasound beams caused by the 2 relatively thick superficial layers, resulting in an overall loss, or attrition, of sound waves reflecting off the more internal testicular parenchyma. The increase in pixel heterogeneity, generated by tissue dissimilarity, is further evidence of the sound wave interference by the scrotal skin.

Differences in the extent of ultrasound beam attrition from non-parenchymal tissue layers enveloping the testis are expected to exist under differing individual conditions. For example, the thickness of the tunica albuginea in men has been shown to increase with age (10), and it also varies across the surface of the testis, depending on smooth muscle fibre content and deposition (11) and its proximity to the rete testis (12). In addition, there are differences in testicular capsule morphology across species (13). In this study, for the purpose of obtaining a set of standardized testicular echotexture values, the rams were age- and weight-matched, the images were obtained in a consistent manner (i.e., in 1 plane of view), and the data for a testicle with histological and echotextural deviations were withdrawn from statistical analyses. Alterations in any of these factors as well as a pathological condition, such as scarring or inflammation that would cause a thickening of the scrotal layers, may cause an even greater dispersion of ultrasound waves, resulting in overall lower pixel intensity values and heterogeneity. Further studies are required to examine the extent of scrotal and testicular capsule interference on testicular parenchyma echotexture under normal and pathological conditions and to assess their impact on the accuracy of detecting alterations in testicular macro- and micromorphology.

The presence of blood and vascular blood flow at the time of the ultrasound procedure did not appear to impinge on the overall echotextural attributes of the testicular tissue in the ram lambs of the present study. Ultrasonographic data compiled from images taken when blood was flowing, pooled in the testicle, and drained were not significantly different from one another. This leads us to suggest that differences in ultrasonographic characteristics of the testes are due mainly to the histomorphology of the STs. Positive

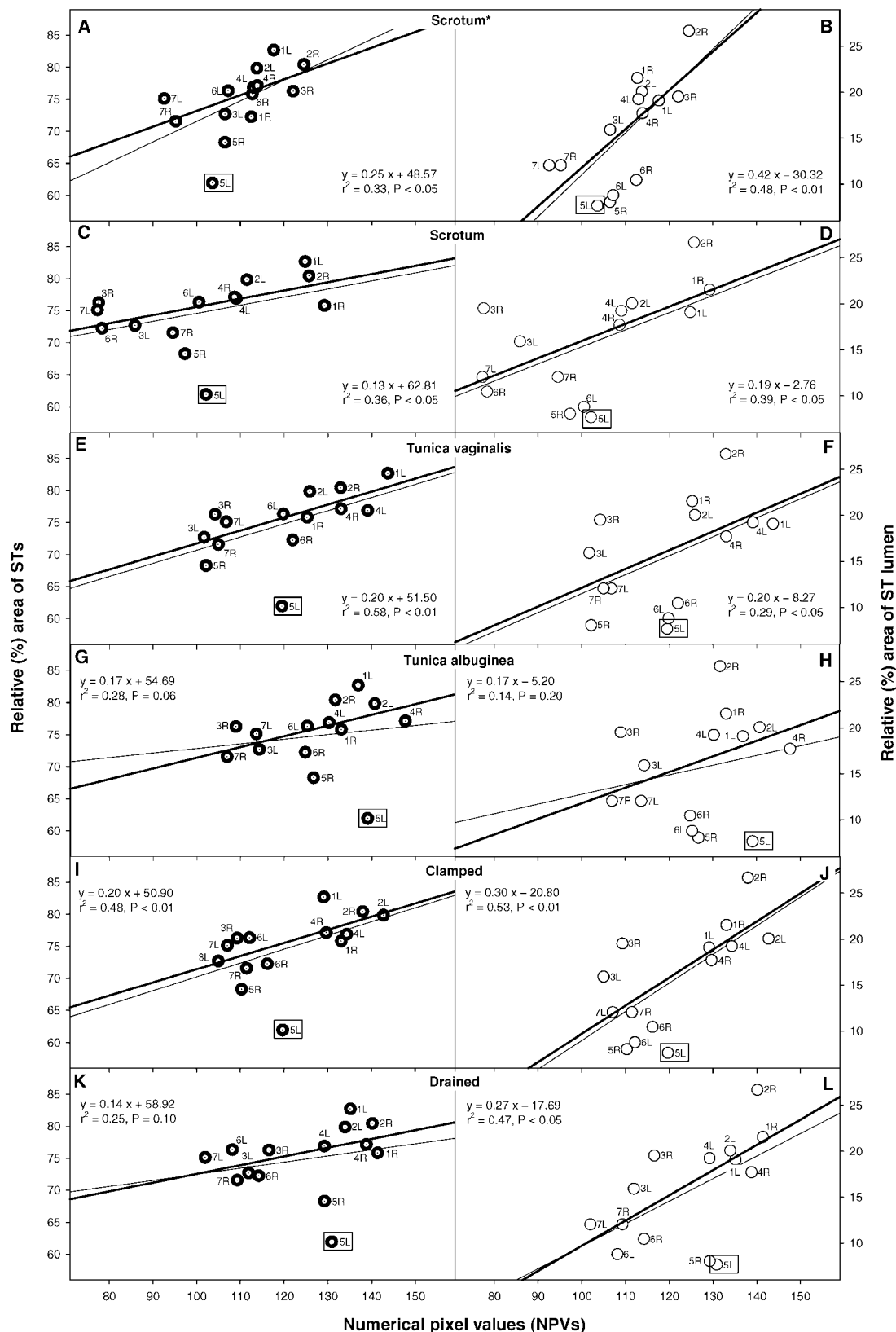


Figure 3. Scatter plots, regression lines and equations, and the squares of the coefficients of correlation (r^2) for the correlations between numerical pixel values (NPVs; input variable) and the ST area (left) or the ST lumen area (right; dependent variable) of testicles scanned at various time points during surgical castration of 7 Rideau Arcott ram lambs. Thin and thick lines represent the regression lines determined before or after removal of the data for "5L" (ram #5, left testicle; values identified by the outlier test), respectively, and regression equations and r^2 values are for the thick lines. 1–7: animal numbers. L, R denote left and right testicle.

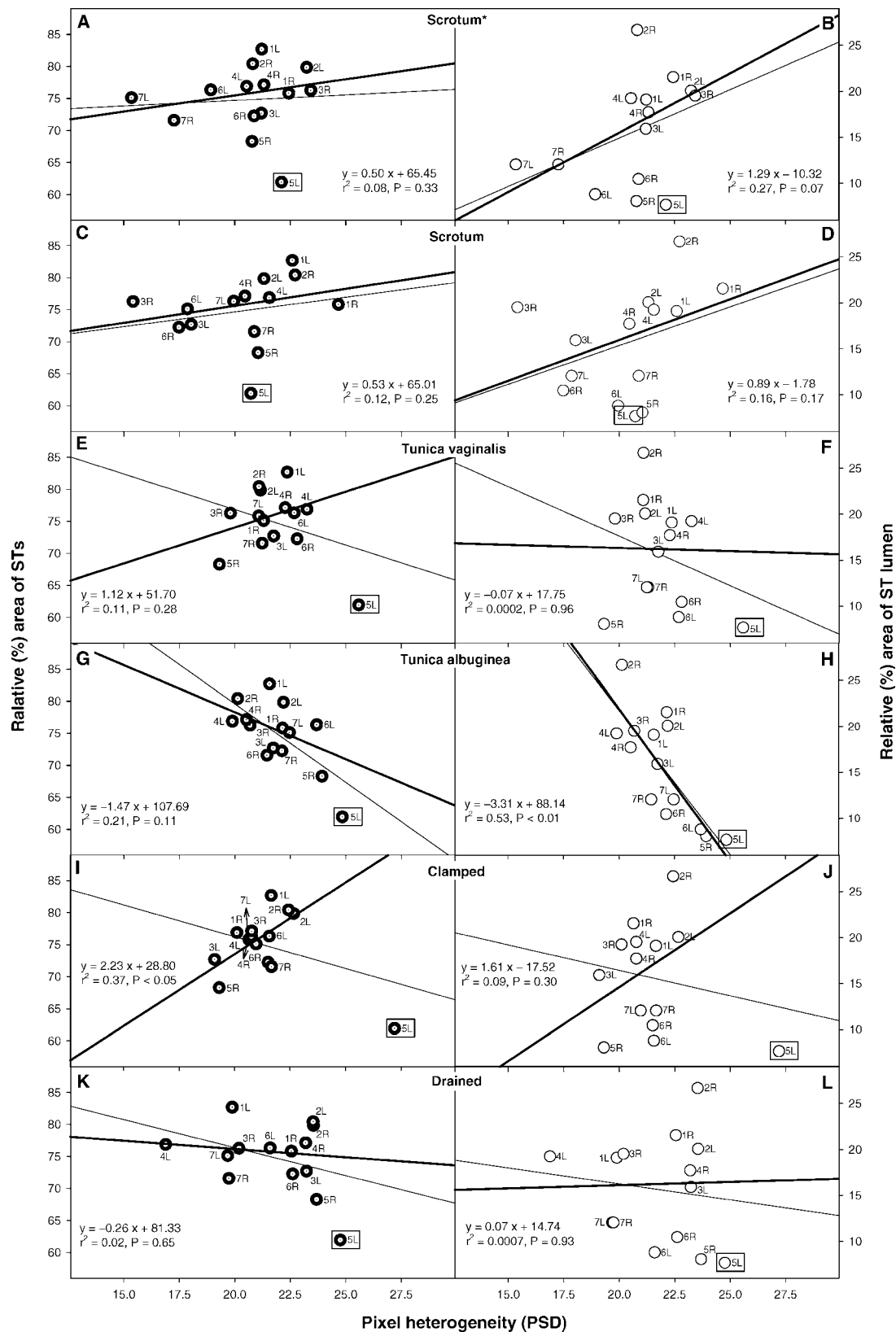


Figure 4. Scatter plots, regression lines and equations, and the squares of the coefficients of correlation (r^2) for the correlations between pixel heterogeneity values (PSD or standard deviation of numerical pixel values; input variable) and the ST area (left) or the ST lumen area (right; dependent variable) of testicles scanned at various time points during surgical castration of 7 Rideau Arcott ram lambs. Thin and thick lines represent the regression lines determined before or after removal of the data for “5L” (ram #5, left testicle; values identified by the outlier test), respectively, and regression equations and r^2 values are for the thick lines. 1–7: animal numbers. L, R denote left and right testicle.

correlations were demonstrated between both the ST area and the ST lumen area, and NPVs. Previously, the size of the seminiferous tubules (i.e., outer and inner diameters) has been shown to be positively and significantly correlated with mean pixel values in pre- and peripubertal bull calves (1). Indeed, the size of the STs, which were seen to occupy 67%–83% of the rams' testes (Figs. 3 and 4), may be primarily responsible for changes in testicular echogenicity. These changes may be due to the presence of echoic protein-rich fluid in the lumen and/or the presence and accumulation of sperm cells in both the tubular and luminal compartments. In addition, the changes in testicular echogenicity may also be a consequence of histophysiological changes in the interstitial tissue, which contains abundant testosterone-producing Leydig cells. The reason for a lack of consistent significant correlations after the removal of the tunica vaginalis (Figs. 3G, 3H, and 3K) is difficult to explain, but could be due to the interruption of ultrasonic wave projection by the high degree of vascularization of the tunica albuginea (14). Further examinations of the dynamic histomorphological changes in STs and Leydig cells during castration may be necessary to clarify their relative influence on ultrasonographic image attributes.

The results of correlation analyses among the ST area, the ST lumen area, and pixel heterogeneity were less conclusive than for the NPVs. The ultrasonographic scanning preceding the removal of the tunica vaginalis revealed a moderate (approaching significance) positive correlation between pixel heterogeneity and the proportion of testicular parenchyma occupied by ST lumen (Fig. 4B). This supports, at least partially, the existence of associations between ST size and echotextural parameters. However, the pixel heterogeneity values measured subsequently in images obtained through the tunica albuginea showed a negative correlation that was significant for the luminal area (Fig. 4H). Once the blood flow was stopped and the blood allowed to pool in the testis, this relationship changed again to a positive correlation that was significant for the STs (Fig. 4I). Finally, after the testes were dissected and the blood drained, the linear relationship between pixel heterogeneity and histological variables almost completely disappeared (Figs. 4K and 4L). In essence, the fluctuations in pixel heterogeneity estimates of the testicular ultrasonograms indicate that the testicular parenchyma exhibits a highly diverse combination of cellular arrangements and vascularization, each with their respective echogenicity (4, 5). Therefore, the reasons for a drastic switch in the correlations prior to and after clamping the testicular blood vessels, together with the finding that virtually no correlations were seen after draining, are puzzling. Not unlikely, these variable results were due to events associated with the castration process itself, such as disruption of autonomic nervous system stimulation and testicular blood flow and/or contractions of the testicular capsule (15).

In summary, the complex structural organization of the testis, with densely packed STs and other parenchymal

components surrounded by the testicular capsule and the scrotum, presents a unique challenge to the interpretation of ultrasonographic findings that were intended to reveal the changes in testicular microstructure. Attenuation of the ultrasound waves by the scrotal layer and tunica vaginalis did not seem to affect the correlations between NPVs and testicular histomorphological characteristics in healthy ram lambs. However, various pathological changes in the testicular integument, the plane of view, and positioning of an ultrasound probe could all be important factors in determining precise correlations between testicular echotexture and histomorphology. Lastly, although the presence of blood and vascular blood flow in the testis do not appear to affect the overall testicular echogenicity, changes in vasculature within the tunica albuginea may affect the strength of the associations among quantitative ultrasonographic and histological characteristics.

We would like to thank Ms. Sabrina Sangupta, Ms. Stephanie Wilson, Mr. Sean Kamani, and Mr. Karan Dhir for their efforts and contributions in data collection, analysis, and interpretation, and the Ponsonby Station staff (Ms. Pam Hasson and Mr. Jeff McFarlane) for the care and management of experimental animals.

1. Evans ACO, Pierson RA, Garcia A, McDougall LM, Hrudka F, Rawlings NC. Changes in circulating hormone concentrations, testes histology and testes ultrasonography during sexual maturation in beef bulls. *Theriogenology* 46:345–357, 1996.
2. Arteaga AA, Barth AD, Brito LFC. Relationship between semen quality and pixel-intensity of testicular ultrasonograms after scrotal insulation in beef bulls. *Theriogenology* 64:408–415, 2005.
3. Rubenstein RA, Dogra VS, Seftel AD, Resnick MI. Benign intrascrotal lesions. *J Urol* 171:1765–1772, 2004.
4. Chandolia RK, Bartlewski PM, Omeke BC, Beard AP, Rawlings NC, Pierson RA. Ultrasonography of the developing reproductive tract in ram lambs: effects of a GnRH agonist. *Theriogenology* 48:99–117, 1997.
5. Chandolia RK, Honaramooz A, Omeke BC, Pierson R, Beard AP, Rawlings NC. Assessment of development of the testes and accessory glands by ultrasonography in bull calves and associated endocrine changes. *Theriogenology* 48:119–132, 1997.
6. Aravindakshan JP, Honaramooz A, Bartlewski PM, Beard AP, Pierson RA, Rawlings NC. Pattern of gonadotropin secretion and ultrasonographic evaluation of developmental changes in the testis of early and late maturing bull calves. *Theriogenology* 54:339–354, 2000.
7. Pierson RA, Adams GP. Computer-assisted image analysis, diagnostic ultrasonography and ovulation induction: strange bedfellows. *Theriogenology* 43:105–112, 1995.
8. Gabor G, Sasser RG, Kastelic JP, Mezes M, Falkay G, Bozo S, Volgyi Csik J, Barany I, Hidas A, Szasz F Jr., Boros G. Computer analysis of video and ultrasonographic images for evaluation of bull testes. *Theriogenology* 50:223–228, 1998.
9. Latendresse JR, Warbritton AR, Jonassen H, Creasy DM. Fixation of testes and eyes using a modified Davidson's fluid: comparison with Bouin's fluid and conventional Davidson's fluid. *Toxicol Pathol* 30: 524–533, 2002.
10. Arenas MI, Betencourt FR, Fraile B, Panaigua R. Immunocytochemical and quantitative study of the tunica albuginea testis in young and aging men. *Histochem Cell Biol* 107:469–477, 1997.
11. Leeson TS, Cookson FB. The mammalian testicular capsule and its muscle elements. *J Morphol* 144:237–254, 2005.

12. Davis JR, Langford GA, Kirby PJ. The testicular capsule. In: Johnson AD, Gomes WR, VanDemark NL, Eds. *The Testis*. New York: Academic Press, pp282–337, 1970.
13. Aire TA, Ozegbe PC. The testicular capsule and peritubular tissue of birds: morphometry, histology, ultrastructure, and immunohistochemistry. *J Anat* 210:731–740, 2007.
14. Wrobel KH. Male reproductive system. In: Dellmann HD, Eurell J, Eds. *Textbook of Veterinary Histology*. Baltimore, Maryland: Lippincott Williams & Wilkins, pp226–246, 1998.
15. Mitchell MA, Seeley RR. Autorhythmic contractions of the postnatal rabbit testicular capsule and its response to prostaglandins, epinephrine and acetylcholine. *Biol Reprod* 17:591–598, 1977.