Research Report

Evaluating the use of ultrasonography with Doppler to assess peripubertal development and predict breeding soundness examination classifications in ram lambs

Jamie Stewart,[a] Olivia Reiff,[a] Alyssa Helms,[a] Sherrie Clark,[a] Scott Greiner,[b] Kevin Pelzer[a]
[a]Department of Large Animal Clinical Sciences, Virginia-Maryland College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA
[b]School of Animal and Poultry Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA

Abstract

The objectives were to evaluate changes in testicular characteristics of peripubertal rams and determine their association with breeding soundness examination (BSE) classifications. We hypothesized that testicular echotexture and blood flow assessed via ultrasonography with Doppler would predict subsequent BSE classifications. Ram lambs (n = 40) were examined on intake for performance testing in May. In May and June, scrotal circumference (SC) and scrotal skin temperature measurements were obtained. Additionally, ultrasonography with Doppler was used to calculate total testicular volume (TTV), assess pixel intensity, and measure resistive (RI) and pulsatility (PI) indices of the right testicular artery. Rams underwent BSE ~ 10 weeks after the initial evaluation and either passed or failed based on SC and sperm quality. SC, TTV, and pixel intensity increased between May and June, whereas RI and PI decreased ($p < 0.01$). SC and scrotal skin temperature were greater ($p = 0.03$) for rams with an 'excellent' BSE classification than those that failed. SC was also positively correlated ($r = 0.53$; $p < 0.01$) with percent normal sperm at BSE. Testicular pixel intensity, RI, and PI did not differ ($p \geq 0.28$) based on BSE classification. Decreased ($p = 0.01$) heart rates on intake in May were reported in rams with 'excellent' BSE classifications and associated with greater percent normal sperm ($r = 0.42$; $p = 0.01$) at BSE. In conclusion, ultrasonography with Doppler can be useful for assessing peripubertal development in ram lambs but provided limited capability in predicting BSE classification.

Keywords: Ram lambs, testicular volume, testicular pixel intensity, heart rate

Introduction

Although the number of sheep operations in US has declined during the past 40 years, size of US sheep industry has recently stabilized with potential for growth in the foreseeable future.[1] To improve profitability within the sheep industry, producers will rely heavily on the development of new technologies that allow for effective dissemination of valuable genetics. Specifically, there is a need to select for animals that are less prone to developing diseases following exposure to certain pathogens and parasites, due to growing concerns with drug resistance. The Virginia Cooperative Extension reported on the increasingly widespread resistance of parasites to commercial dewormers on Virginia sheep farms.[2] The barber pole worm, *Haemonchus contortus*, is the most detrimental of these parasites with the ability to cause severe anemia and death in affected small ruminant hosts. The use of terminal sire crossbreeding systems has the potential to not only improve growth performance in lambs, but also promote parasite resistance.[3]

Early selection of reproductively sound rams is the most efficient way to improve genetics within a flock. The Virginia Tech Southwest Ag Research and Extension Center Ram Test was initiated in 2012 to help quantify growth and parasite resistance in rams. Although programs such as these have proven to be useful for selecting genetically valuable sires, the submission of peripubertal ram lambs precludes our ability to predict their reproductive soundness until after the test is completed. As a result, producers may submit ram lambs that perform well in growth performance and parasite resistance testing, but still fail the breeding soundness examination. Unfortunately, this practice may discourage producers from

CONTACT Jamie Stewart jlstewart13@vt.edu

Citation: Clinical Theriogenology 2024, 16, 10416, http://dx.doi.org/10.58292/CT.v16.10416

submitting ram lambs for testing due to the expense and uncertainty as to whether they will actually be useable for breeding programs. There is, therefore, a need to develop technologies that may predict future reproductive soundness in ram lambs before they are subjected to performance testing.

Evaluation of breeding soundness in rams includes an assessment of overall health, reproductive organs, and semen evaluation, resulting in a satisfactory, questionable, or unsatisfactory classification.[4] Ram lambs subjected to performance testing are typically peripubertal, making it difficult to assess semen quality since spermatogenesis is just beginning. The length of sperm transit in the ram is ~ 42 days (6 weeks), so semen evaluation after performance testing is usually suitable for predicting reproductive soundness prior to sale. However, evaluation of testicular architecture at spermatogenic onset may predict subsequent semen quality and aid in earlier ram selection. Histological evaluation of testicular biopsy samples is one method to evaluate spermatogenic function with no detectable detriment to testicular development in rams.[5] However, its usefulness is severely limited by the amount of time and money required to process and analyze samples. Scrotal ultrasonography may be a noninvasive alternative to help predict future fertility in rams. Testicular echotexture on ultrasonography was significantly correlated with changes in testicular microstructure, including seminiferous tubule diameter and presence of mature germ cells in ram lambs.[6] Similarly, testicular pixel intensity in bulls was a useful indicator for pubertal and mature status and was associated with sperm production, sperm morphology, and seminiferous tubule area.[7] Additionally, blood indices via Doppler of internal iliac artery that supplies blood to accessory sex glands, predicted sperm motility and vigor in Dorper rams.[8] The objective was to determine if testicular ultrasonography with Doppler evaluation of the testicular artery may provide veterinarians with an effective means for predicting breeding soundness examination classifications in ram lambs submitted for performance testing.

Materials and methods

Procedures used in this study were approved by the Institutional Animal Care and Use Program at Virginia Tech (Protocol # 19-075). Enrolled ram lambs (n = 40) were randomly selected from those housed at the Shenandoah Valley Agricultural Experiment Station in Raphine, VA for routine performance testing. Ram lambs ranging from 2 to 7 months were classified as either fall-born (average age: 5.9 ± 0.3 months) or winter-born (average age: 3.9 ± 0.2 months). Represented breeds included Suffolks (n = 19), Dorpers (n = 11), and Katahdins (n = 10). On arrival to the facility in late April, ram lambs received oral doses of fenbendazole (10 mg/kg; Panacur, Merck Animal Health, Rahway, NJ, USA), levamisole (8 mg/kg; Prohibit, Agri Laboratories, St. Joseph, MO, USA), and moxidectin (0.2 mg/kg, Cydectin, Elanco, Greenfield, IN, USA) anthelmintics and were vaccinated for respiratory pathogens (1 ml intranasal; Inforce 3, Zoetis, Parsippany, NJ, USA), Clostridium perfringens type C and D and tetani (2 ml; Barvac CD&T ovine, Boehringer Ingelheim Animal Health, Duluth, GA, USA), and contagious ovine ecthyma (Ovine ecthyma vaccine live virus, Colorado Serum Company, Denver, CO, USA). Rams were examined on intake by a veterinarian to detect any signs of illness or testicular and epididymal abnormalities.

Evaluation of ram lambs

Enrolled ram lambs were examined at 1 week after intake (May) and again ~ 7 weeks later (June). At first examination in May, heart and respiratory rates, body condition score (BCS) on a 1-5 scale,[9] and FAMACHA score (1-5)[10] were determined. In both May and June, scrotal circumference (SC) was measured with a flexible tape by securing it snugly against the greatest horizontal diameter.[11] A small strip of hair/wool was shaved on each testis as needed to facilitate scrotal skin temperature measurements and testicular ultrasonography. Rectal temperature was measured using a digital thermometer, and scrotal skin temperature was measured at the proximal, middle, and distal end of the scrotum using a handheld infrared thermometer held ~ 2 inches from the scrotum (Figure 1A). The difference between the proximal and distal end of the scrotum was calculated and recorded as the scrotal skin thermal gradient, whereas the middle temperature was recorded as the scrotal skin temperature. The mean scrotal skin temperature and scrotal skin thermal gradient between 2 testes was recorded and used for analyses.

Ultrasonography was used to measure the length (L) and width (W) of testis to calculate the estimated testicular volume ($TV = 0.5236 \times L \times W^2$) and ratio (ratio = W/L). Total testicular volume (TTV) for each ram was calculated by adding the TV for the left and right testis. The average testicular ratio between 2 testes in each ram was calculated for analyses. Images obtained via B-mode ultrasonography, where the mediastinum testes in a vertical plane was evident, were saved and used for pixel intensity measurements (Figure 1B). Computer analysis of each image was performed using image analysis software (Image J, US National Institutes of Health, MD, USA), as described.[12] Briefly, measurements were taken on each image in 4, ~ 10 mm circles within 10 mm of the mediastinum testis, where the parenchyma appeared homogenous. Inside the circles, pixel intensity was measured according to a shade on a 1 to 255 gray-scale (1 = black; 255 = white). The average pixel intensity between 2 testes in each ram was calculated for analysis. Testicular blood flow was also assessed using ultrasonography equipped with color Doppler (EXAPad Mini, IMV Imaging). Pulsed wave spectral Doppler was performed on the right testis testicular artery to measure pulsatility index (PI) and resistive index (RI) (Figure 1C).

Ram lambs underwent a routine breeding soundness examination (BSE) ~ 10 weeks after the initial evaluation (3 weeks after the second evaluation) in July. In addition to a focused reproductive examination, semen was collected using a hand-held ram ejaculator (Lane Manufacturing) and evaluated by an experienced veterinarian for sperm motility and morphology. Total sperm motility was estimated by immediately evaluating a drop of semen at low power under light microscopy. Concentrated samples were diluted with prewarmed saline to facilitate motility assessments. Sperm morphology was assessed by staining a small drop of semen with eosin-nigrosin stain and counting 100 sperm under light microscopy with oil immersion at 1,000 x magnification. Each sperm was classified as morphologically normal or abnormal, with abnormalities further classified into primary or secondary defects, as described.[13,14] Based on modified parameters,[15] Rams were allocated into 4 BSE classifications: 1. 'excellent'- rams with a minimum SC of 31 cm (fall-born) or 30 cm (winter-born) with normal sperm morphology ≥ 90% and sperm motility ≥ 50%; 2. 'satisfactory' - rams with a minimum SC of 31 cm (fall-born) or 30 cm (winter-born) with normal sperm morphology ≥ 70% and sperm motility ≥ 30%; 3. 'questionable' - rams with a SC

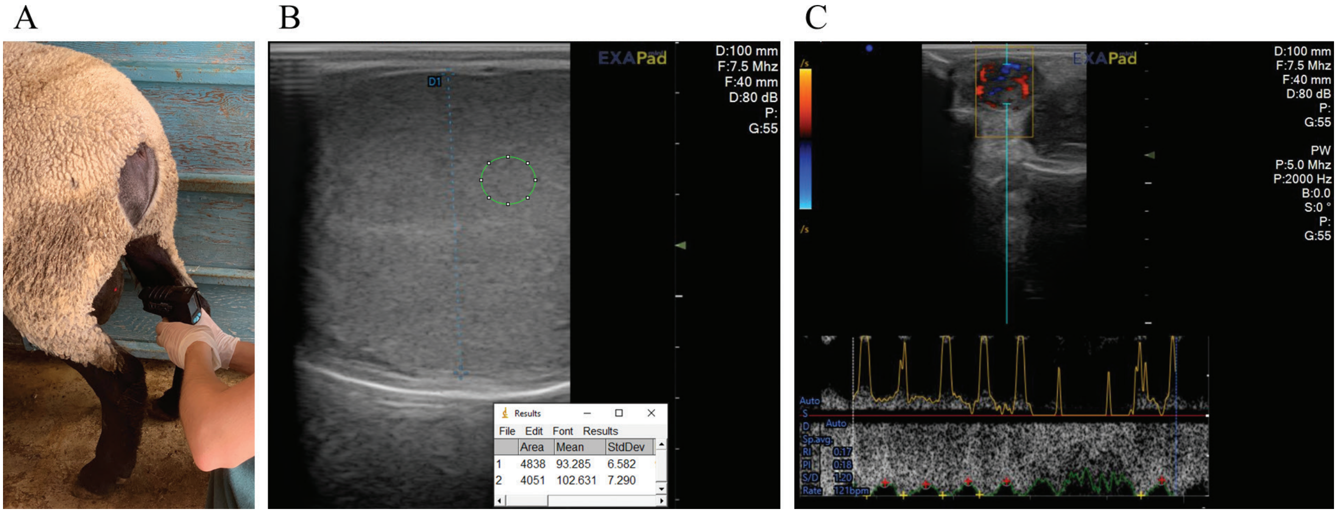

Figure 1. A. Scrotal skin temperature measurements were acquired using an infrared thermometer, held ~ 2 inches from the scrotum. The middle-most part of the testis was used for the scrotal skin temperature, whereas the difference between measurements acquired at the proximal and distal-most aspects of the testis were used to calculate scrotal skin thermal gradient. B. To determine testicular pixel intensity, 4 ~ 10 mm spots were measured around the mediastinum using ImageJ software and averaged for each testis. C. To measure testicular blood flow, a linear probe was positioned over the scrotal neck of the right testis using B-mode ultrasonography until the testicular vascular cone was visualized. Pulsed wave spectral Doppler was then used to measure the resistive index and pulsatility index of the right testicular artery.

of 28-31 cm (fall-born) or 28-30 cm (winter-born) with normal sperm morphology 30-70% and sperm motility ≥ 30%; and 4. 'fail' - rams with SC < 28 cm with normal sperm morphology < 30% and/or sperm motility < 30%.

Data analyses

Analyses were performed using R (https://www.r-project.org/). Testicular measurements (SC, TTV, ratio, pixel intensity, RI, PI, scrotal skin temperature, scrotal skin thermal gradient) were analyzed by ANOVA to assess differences based on fixed effects of BSE classification and month of evaluation using ram ID as a random variable and breed and age category (fall versus winter born) as independent covariates. For scrotal skin temperature and thermal gradient, rectal temperature was also included as an independent covariate. Physical examination parameters at first evaluation (May) were also analyzed by ANOVA to assess differences based on the fixed effect of BSE classification, using ram ID as a random variable and breed and age category as independent covariates. A Pearson's product-moment correlation test was used to identify associations between testicular/physical examination measurements obtained in May or June and percent normal or motile sperm at the final BSE in July. A correlation test was also performed to determine associations between testicular size measurements (SC versus TTV) among months. Strong correlations were designated as those with an $r > 0.40$, with moderate correlations at $r = 0.3$-0.40, weak correlations at $r = 0.25$-0.30, and no correlation at $r < 0.25$. Significance was declared at $p \leq 0.05$ with tendencies discussed between $p = 0.06$ and 0.09.

Results

Testicular size and shape

The SC measurements obtained in May, June, and July were all positively and significantly correlated with one another, as were TTV measurements (Table 1). Not surprisingly, the strongest correlations were between TTV and SC in May ($p < 0.01$) and between TTV and SC in June ($p < 0.01$). Percent normal sperm in July was moderately and positively correlated with SC in May ($p = 0.01$) and strongly correlated with TTV in May ($p < 0.01$; Table 2). There was no association between June or July SC or June TTV with percent normal sperm in July. There was also no association between testicular ratio in May or June and percent normal sperm. In addition, percent motile sperm did not have any associations with SC, TTV, or testicular ratio at any month. The effects of BSE classification and month on SC, TTV, and testicular ratio are summarized (Table 3). There were main effects of age category ($p < 0.01$), breed ($p = 0.03$), month ($p < 0.01$), and BSE classification ($p = 0.03$) on SC measurements, but no BSE classification by month interaction ($p = 0.65$). Rams that failed BSE in July had smaller ($p = 0.03$) SC measurements than those that were classified as 'excellent'. SC measurements were smaller ($p < 0.01$) in May (25 ± 0.6 cm) than in June (30 ± 0.4 cm), and the older fall-born lambs had greater ($p < 0.01$) SC (30 ± 0.6 cm) than the younger winter-born lambs (26 ± 0.6 cm). Suffolk ram lambs tended to have smaller ($p = 0.09$) SC measurements (26 ± 0.7 cm) than Katahdins (29 ± 1.0 cm); but neither differed ($p < 0.01$) from the Dorpers (28 ± 0.9 cm). It is worth noting that the Dorper ram lambs were, on average, older (6 ± 0.3 months) than the Katahdins (4.4 ± 0.4 months; $p < 0.01$) and the Suffolks (3.9 ± 0.2 months; $p < 0.01$); however, the Katahdins and Suffolks did not differ ($p = 0.54$) in age from each other. Similarly, there were main effects of age category ($p < 0.01$), month ($p < 0.01$), and BSE classification ($p = 0.02$) on TTV measurements, but no effect of breed ($p = 0.17$) or BSE classification by month interaction ($p = 0.28$). Rams classified as 'excellent' tended to have greater TTV measurements than those classified as 'questionable' ($p = 0.06$) or that failed ($p = 0.07$). TTV measurements in May (110 ± 10 cm^3) were smaller ($p < 0.01$) than in June (217 ± 11 cm^3; $p < 0.01$), and the older fall-born lambs had greater ($p < 0.01$) TTV (204 ± 13 cm^3) than the younger winter-born lambs (142 ± 12 cm^3). There were main effects of month ($p < 0.01$)

Table 1. Correlation coefficients between scrotal circumference measurements of peripubertal ram lambs obtained in May, June, and July and total testicular volume measurements obtained in May and June

Testicular parameters	SC May	SC June	SC July	TTV May	TTV June
SC May		0.74***	0.41***	0.90***	0.55***
SC June	0.74***		0.58***	0.65***	0.82***
SC July	0.41***	0.58***		0.27*	0.64***
TTV May	0.90***	0.65***	0.27*		0.44***
TTV June	0.55***	0.82***	0.64***	0.44***	

***p < 0.01, **p = 0.01-0.05, *p > 0.05.

Table 2. Correlation coefficients between testicular parameters of peripubertal ram lambs measured in May or June and percent morphologically normal sperm or percent motile sperm collecting during a routine breeding soundness examination in July

Testicular parameters	Month	Percent normal sperm	Percent motile sperm
Scrotal circumference	May	0.39**	0.16
	June	0.15	0.07
	July	−0.007	0.12
Total testicular volume	May	0.53***	0.21
	June	0.04	−0.12
Testicular ratio	May	−0.17	−0.2
	June	−0.1	−0.008
Testicular pixel intensity	May	0.07	−0.1
	June	−0.17	−0.22
Resistive index	May	0.11	−0.006
	June	−0.01	−0.09
Pulsatility index	May	−0.009	−0.05
	June	0.08	−0.04
Scrotal skin temperature	May	0.24	0.17
	June	0.17	0.25
Scrotal skin thermal gradient	May	0.06	−0.05
	June	0.21	0.1
Rectal temperature	May	0.02	0.23
	June	−0.02	−0.12
Body condition score	May	0.1	0.12
Heart rate	May	−0.42**	−0.24
Respiratory rate	May	0.08	0.08
FAMACHA	May	−0.20	−0.23

***p < 0.01, **p = 0.01-0.05.

and age category (p = 0.05) on testicular ratio, but no effect of breed (p = 0.23), BSE classification (p = 0.88) or BSE classification by month (p = 0.68) interaction. Testicular ratio measurements in May (0.70 ± 0.02) were smaller (p < 0.01) than those in June (0.77 ± 0.01).

Testicular echotexture, blood flow, and temperature

Testicular pixel intensity in May and June had no association with percent normal or motile sperm at BSE in July (Table 2). Likewise, there was no main effect of BSE classification (p = 0.28) or BSE classification by month interaction (p = 0.42) on pixel intensity in ram lambs (Table 3), but there were main effects of month (p < 0.01), breed (p < 0.01), and age category (p < 0.01). Testicular pixel intensity was greater (p < 0.01) in June (78 ± 3.1) than in May (59 ± 2.4) and was greater (p = 0.02) in the younger winter-born ram lambs (70 ± 3.1) than older fall-born ram lambs (65 ± 2.7). Interestingly, pixel intensity was greater in Suffolk ram lambs (76 ± 3.2) than Dorpers (60 ± 3.9; p < 0.01) or Katahdins (59 ± 3.4; p < 0.01); however, the Katahdins and Dorpers did not differ (p = 0.93).

Table 3. A summary of testicular measurements obtained in May or June based on breeding soundness examination (BSE) classification assigned to a ram lamb in July

Month	BSE classification	SC (cm)	TTV (cm^3)	Testicular ratio	Pixel intensity	RI	PI	SST (°C)	Gradient (°C)
May	Excellent	25.4 ± 0.9^{a}	130 ± 17^{a}	0.70 ± 0.03^{a}	57 ± 4.7^{a}	0.56 ± 0.05^{a}	0.99 ± 0.17^{a}	31.1 ± 0.4^{a}	2.7 ± 0.3
	Satisfactory	25.6 ± 1.6^{ab}	120 ± 26^{ab}	0.71 ± 0.06^{a}	59 ± 6.5^{a}	0.56 ± 0.09^{a}	1.16 ± 0.37^{a}	30.2 ± 0.7^{ab}	2.1 ± 0.3
	Questionable	24.6 ± 1.1^{ab}	109 ± 21^{b}	0.69 ± 0.05^{a}	61 ± 4.5^{a}	0.48 ± 0.07^{a}	0.76 ± 0.13^{a}	29.4 ± 0.5^{b}	2.7 ± 0.6
	Fail	22.9 ± 1.4^{b}	73 ± 17^{b}	0.72 ± 0.04^{a}	59 ± 3.6^{a}	0.50 ± 0.06^{a}	0.99 ± 0.20^{a}	29.7 ± 0.4^{b}	2.2 ± 0.4
June	Excellent	31.0 ± 0.6^{c}	235 ± 16^{c}	0.76 ± 0.02^{b}	73 ± 5.4^{b}	0.44 ± 0.06^{b}	0.77 ± 0.16^{b}	30.8 ± 0.4^{a}	1.9 ± 0.2
	Satisfactory	29.6 ± 1.1^{cd}	220 ± 24^{cd}	0.74 ± 0.03^{b}	75 ± 6.9^{b}	0.36 ± 0.06^{b}	0.52 ± 0.11^{b}	30.5 ± 0.5^{ab}	2.4 ± 0.4
	Questionable	28.9 ± 0.7^{cd}	175 ± 16^{d}	0.80 ± 0.03^{b}	83 ± 8.5^{b}	0.42 ± 0.08^{b}	0.56 ± 0.12^{b}	30.4 ± 0.4^{b}	1.8 ± 0.4
	Fail	29.4 ± 1.0^{d}	214 ± 28^{d}	0.78 ± 0.01^{b}	85 ± 4.5^{b}	0.45 ± 0.08^{b}	0.66 ± 0.13^{b}	30.5 ± 0.4^{b}	2.0 ± 0.3

$^{a\text{-}d}$Within a column, means without a common superscript differed ($p < 0.05$).

Neither RI nor PI in May or June had an association with percent normal or motile sperm at BSE in July (Table 2). There was a main effect of month on RI (p = 0.03) and PI (p = 0.02), but no effects of breed (p ≥ 0.30), age category (p ≥ 0.50), BSE classification (p ≥ 0.69), or BSE classification by month interaction (p ≥ 0.75) on either. In May, RI (0.53 ± 0.03) and PI (0.97 ± 0.1) were greater than in June (RI: 0.43 ± 0.03; PI: 0.66 ± 0.08).

Neither scrotal skin temperature nor scrotal skin thermal gradient in May or June had an association with percent normal or motile sperm at BSE in July (Table 2). There was a main effect of BSE classification (p < 0.01) and a tendency for a BSE by month interaction (p = 0.07) on scrotal skin temperature, as well as interactions with rectal temperature (p < 0.01; Table 3) and age category (p = 0.03), but not month (p = 0.16) or breed (p = 0.12). Ram lambs that were classified as 'excellent' had greater scrotal skin temperatures than those classified as 'questionable' (p < 0.01) or that failed (p = 0.03). There were no effects of BSE classification (p = 0.94), rectal temperature (p = 0.60), breed (p = 0.82), age category (p = 0.58), or BSE classification by month (p = 0.60) interactions on scrotal skin temperature gradient, but there was a tendency (p = 0.09) for interaction with month (Table 3).

Physical examination parameters

Interestingly, there was a moderate and negative correlation (p = 0.01) between the heart rate measured in May and the percent normal sperm at BSE in July (Table 2). There was no association between heart rate and percent motile sperm. Body condition score, rectal temperature, respiratory rate, and FAMACHA score also did not have any association with percent normal or motile sperm at BSE in July (Table 2). There was a main effect (p = 0.03) of BSE classification on heart rates of ram lambs on intake in May, but no interactions with breed (p = 0.26) or age category (p = 0.99). Heart rates of rams on intake in May were lower (p = 0.05) in rams classified as 'excellent' than those that failed BSE (Figure 2A). There were no differences in respiratory rate (p = 0.91), rectal temperature (p = 0.87), or BCS (p = 0.14) measured in May based on BSE classification in July (Figures 2A and 2B). There were also no effects of breed (p = 0.93) or age category (p = 0.65) on respiratory rates; a main effect of breed (p = 0.04), but not age category (p = 0.60) on rectal temperature; and a main effect of age category (p < 0.01), but not breed (p = 0.76) on BCS. There was a main effect of BSE classification (p = 0.02) and a tendency for age category (p = 0.07) to affect FAMACHA score, but no interaction of breed (p = 0.85). Ram lambs that failed BSE had higher (p = 0.02) FAMACHA scores than those classified as 'questionable' (Figure 2B).

Discussion

The results presented herein demonstrated that ultrasonography with Doppler to assess testicular blood flow and echotexture can be used to assess peripubertal development in ram lambs, but is not predictive of sperm motility, morphology, or BSE classification. Not surprisingly, rams that failed BSE in July had smaller SC measurements in May and June compared to those classified as 'excellent.' It is worth noting that the Suffolk rams were the youngest breed group represented (average 3.9 months), but their SC did not differ in size from the Dorper rams (average: 6 months). Though the Suffolk rams did not differ significantly in age from the Katahdin rams (average: 4.4 months), they tended to have smaller SC. These findings highlighted important breed characteristics that need to be considered when measuring SC and determining an ideal minimum size in small ruminants. There were also moderate to strong correlations between both SC and TTV measurements obtained in May and the percent of normal sperm measured at the July BSE, but no association between the percent normal sperm and the June SC/TTV measurements. The association with testicular size in May could be indicative of rams entering puberty at an earlier age that would account for those with better sperm morphology. It is possible that a reevaluation of those that did not pass BSE in July may have yielded a passing score at a later date as the rams progressed further into puberty. Seasonality may also have a role in the minimal differences observed, as the performance test is performed during the summer, when daylength is longest. The breeding season subsequently commences in august as the daylength begins declining. Suffolk sheep are a strongly seasonal breed, so it would make sense that their testis size would not be as big in size until the daylength begins decreasing in August compared to Katahdin and Dorper sheep that are less influenced by photoperiod.

An interesting, yet unexpected, finding was that the rams that were classified as 'excellent' during their BSE in July had, on average, lower heart rates upon intake in May compared to those that failed BSE. Correspondingly, there was a moderate

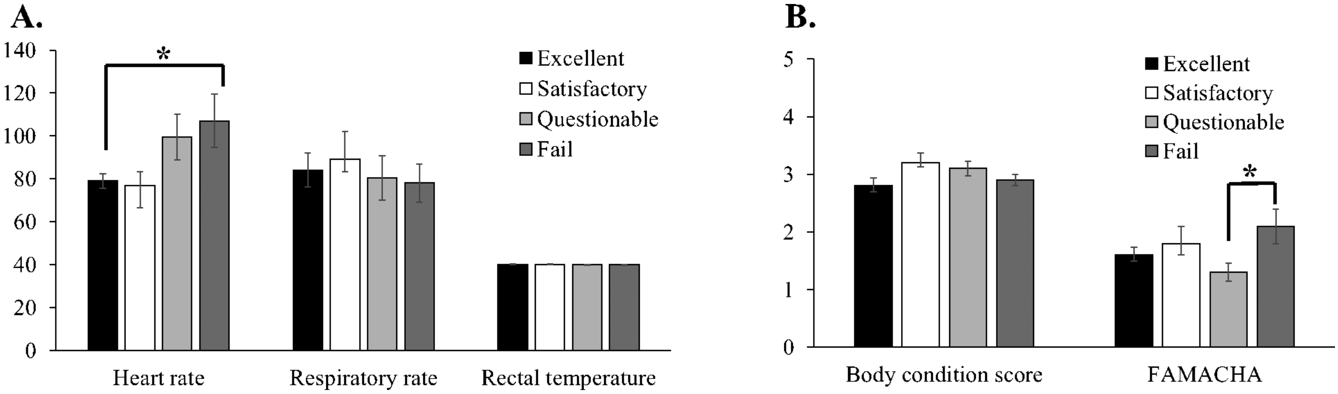

Figure 2. A summary of physical examination parameters measured in May based on breeding soundness examination (BSE) classification assigned to a ram lamb in July. A. *Heart rate was lower ($p = 0.03$) in rams that were classified as 'excellent' in July than those failed. B. *FAMACHA score was greater ($p = 0.02$) in rams that failed July BSE than those that were classified as 'questionable'. There were no differences in respiratory rate, rectal temperature, or body condition score based on BSE classifications.

and negative correlation between the May heart rate measurement and percent of normal sperm observed at the July BSE. Ram temperament may be one explanation for this finding, as it seems feasible that calmer rams would be more likely to have a decreased heart rate upon intake. There are a limited number of studies, however, that have reported minimal to no association between male temperament and semen quality in bulls[16,17] and rams.[18] Another factor to consider would be individual animal susceptibility to heat stress, as the heart rate increases with elevated ambient temperatures.[19] One might expect the opposite effect, however, with rams experiencing elevated heart rate being better able to thermoregulate testicular temperature due to increased efficiency of sensible heat exchange through conduction.[20] Interestingly, scrotal skin temperature was also greater in rams that classified as 'excellent' versus those that classified as 'questionable' or failed, and there was a weak and negative correlation ($r = -0.28$, $p = 0.01$) between mean scrotal skin temperature and heart rate. These findings may represent a link between heart rate and scrotal skin temperature that results in decreased susceptibility to the negative effects of heat stress on spermatogenesis. Despite these findings, there were no notable changes in the rectal temperature or respiratory rate based on the BSE classification, nor was there an effect of ram age or breed. Further work is needed to better understand the connection between heart rate and scrotal skin temperature and its associated effect on sperm quality.

The scrotal skin thermal gradient measured herein (1.85-2.50 °C) was greater than those reported for haired rams previously (0.5-0.9 °C),[21] but more in line with those measured in bulls (1.6 °C)[22] and cross-bred wooled rams (2.8 °C).[23] Interestingly, the mean scrotal skin temperature measured in the current study was lesser (29.5-30.7 °C) than those reported in haired rams (32-34 °C)[21] and more similar to those reported in bulls (~ 29.9 °C).[22] Environmental factors may have contributed to these differences as our study was performed during the summer months in southeast US, whereas the previous ram study was performed in the summer months in Brazil,[21] and the bull study was performed during summer months in Canada.[22] Interestingly, a study in cross-bred wooled rams determined that the scrotal skin temperature measurements differed based on ambient temperatures of 10 °C (26.1-28.9 °C) versus 25 °C (27.6-30.5 °C), but the gradient remained the same (2.8 °C).[23] On the contrary, haired sheep experienced an increase in the temperature gradient in the summer months versus winter.[20] Based on these findings, there appears to not only be differences in scrotal skin temperature gradients based on ram breed, but also potential interactions between breed and ambient temperature and humidity indices. Although we identified no effect of breed in our statistical model for the temperature gradient, we did have a combination of haired and wooled sheep in our study, and the breed interaction with environmental temperature fluctuations warrants further investigation. Regardless, the scrotal skin thermal gradient did not differ based on BSE classification or have any correlation with sperm quality in the current study. Further studies that investigate substructural sperm characteristics, such as plasma membrane integrity, DNA fragmentation index, or presence of reactive oxygen species may provide more insight into the relevance of any potential seasonal and breed differences.

Similar to the changes in testicular size, testicular ratio also increased from May to June, but did not differ based on BSE classification. The ratios measured in May were consistent with a long/ovoid shape (testicular ratio of 0.626-0.750) versus those measured in June that were more consistent with an ovoid/spherical shape (testicular ratio of 0.751-0.875).[24–26] In contrast to our findings, authors of another study reported that the testes of Dorper rams raised in a subtropical climate became longer and less spherical (increased length, decreased depth) once they reached sexual maturity versus peripuberty.[27] Since we did not assess the rams in the current study past the peripubertal stage, it is still possible that the testicular shape would continue to change throughout the year. Interestingly, bulls with longer shaped testes produced more sperm per day than those with more spherical shaped testes, despite the latter having an equal or larger SC.[24] Further investigations into the relationship between puberty, testicular shape, and semen quality in rams may be of benefit for clinicians and researchers to better understand its potential usefulness for determining or predicting breeding potential.

Testicular pixel intensity of ram lambs increased between May and June. These findings are consistent with bulls, where testicular echogenicity increased during sexual development.[7] Specifically, it was noted that echogenicity began to increase at 16-12 weeks before puberty and reached maximum values 4-0 weeks before puberty.[7] Ram lambs in the current study were 2-7 months of age at entry into the test station, and, on average, will undergo puberty between 5 and 7 months of age.[28] Consistently, pixel intensity measurements were greater in peripubertal Dorper rams compared to postpubertal rams.[27] We identified that the younger, winter-born ram lambs had greater pixel intensity overall compared to the older, fall-born ram lambs that may suggest that the greater pixel intensity observed in June may correspond with near peak values at puberty, and future studies with data expanding into the post-pubertal stages could likely find similar patterns as those reported previously. We also identified some differences between breeds in the current study, with the less seasonal breeds (Dorpers and Katahdins) having decreased pixel intensity measurements than the more seasonally influenced Suffolks. Differences in ages may have influenced these breed differences, with Suffolks being significantly younger than the Dorpers. Still, further studies are needed to better evaluate the associations between breeds, seasonality, and testicular pixel intensity to better understand their connection to spermatogenesis.

It has been reported that pixel intensity is significantly correlated with seminiferous tubule cell density, the presence of more pre and undifferentiated spermatogonia, and the mitotic and postmitotic phases of spermatogenesis in the peripubertal ram lamb.[6,29] Therefore, we were surprised that testicular pixel intensity did not differ in May or June between rams who passed or failed BSE in July; nor was there any correlation with sperm motility or morphology. These findings are consistent with those reported in bulls, where there was no biological correlation between pixel intensity measurements and semen quality.[12] Interestingly, pixel intensity measurements in bulls were useful for identifying animals suffering from testicular fibrosis.[12] There is, therefore, still a need to investigate its use in diagnosing subclinical testis pathologies in rams.

A recent study that also utilized testicular ultrasonography to predict fertility in rams similarly identified no correlation between pixel intensity measurements (measured separately as the number of black versus white versus grey pixels) and sperm motility or morphology.[30] Interestingly, using ultrasonography video clips and a software program called ECOTEXT®, researchers were able to measure the density of tubules, the percentage of the total area occupied by the lumen of the tubules in the parenchyma, and the mean diameter of the lumen of the seminiferous tubules at high resolution.[30] The authors of the article reported a positive correlation between tubular density and both progressive motility and normal sperm morphology.[30] In contrast to our study, researchers of that study also reported a small, positive correlation between PI and total sperm motility ($r = 0.203$), but no correlation with RI ($r = 0.186$). It is worth noting that semen was collected from each ram monthly over the course of a year in that study.[30] In another study, it was determined that rams undergoing a 'standard frequency' for semen collection (2 consecutive ejaculates per day and 2 collection days per week) had decreased tubular area and diameter and increased pixel intensity (greater number of grey and white pixels, but lesser number of black pixels) and PI than those that were abstained from collection for a month.[31] Therefore, the contradictory findings in our study can be related to the fact that the rams were not collected as frequently or as a result of us evaluating only peripubertal ram lambs versus sexually mature rams.

In peripubertal ram lambs, we identified that blood flow increased from May to June (as evidenced by decreased PI and RI). In a previous study, there were no differences in Doppler measurements of the testis artery reported between peripubertal and sexually mature Dorper rams.[27] Therefore, it would be interesting to know if testis blood flow follows a similar pattern as pixel intensity where it increases in the peripubertal stages and reaches maximum values at puberty. It was also reported that the volume of ejaculate, but no other semen characteristics, was inversely related to the PI of the testicular arteries in Dorper rams.[27] These findings are consistent with ours in that we identified no significant correlation between Doppler measurements in May or June and sperm motility or morphology in July. Neither semen volume nor concentration were assessed in the current study, as these can fluctuate greatly when collection is performed by electroejaculation. In mature, fat-tailed rams (2-4 years), marked increases in testicular blood flow were observed within the breeding season, with the least blood flow (highest PI and RI values) measured in June/July.[32] Interestingly, both PI and RI were significantly correlated with sperm concentration and progressive motility (both assessed on the same day) in that study.[33] Similar testicular blood flow patterns and correlation with sperm quality were reported in Polish Heath[33] and Chois breed[34] rams. In the current study, neither RI nor PI (measured in May or June) differed based on ram BSE classification in July, nor was there any correlation with sperm quality. We elected to measure these classifications several weeks before the BSE to evaluate the effects on spermatogenesis (~ 42 days in rams) rather than on the same day as in previous studies. Additionally, since testing was performed outside the natural breeding season (when blood flow would be decreased), it would be interesting to know if a correlation existed if these rams were evaluated into the subsequent breeding season. There is, therefore, a need to further assess the relationship between testicular blood flow and sperm quality amongst different age groups and breeds of sheep both within and outside of the natural breeding season.

In conclusion, the results of this study supported the use of ultrasonography with Doppler for evaluation of peripubertal ram lambs. Although the information provided herein is limited in its ability to predict BSE classifications of ram lambs, it does provide a baseline for future studies that should account for factors, such as breed, age, ambient temperature, and season. Additionally, the use of advanced technologies such as computer automated sperm analyzers and flow cytometry may be beneficial in demonstrating a deeper connection between testicular blood flow, echotexture, sperm movement patterns, and substructural sperm characteristics.

Acknowledgement

The authors of this paper thank Gabe Pent and the staff at the Steele's Tavern Ram Test Station for allowing us to use rams housed at the test station for this study. Additionally, we thank the clinical year veterinary students at the Virginia-Maryland College of Veterinary Medicine who provided technical assistance throughout this project. This project was supported using funding generously provided by the Virginia Agricultural Council.

Conflict of interest

None to declare.

References

1. Lupton CJ: Impacts of animal science research on United States sheep production and predictions for the future. J Anim Sci 2008;86:3252-3274. doi: 10.2527/jas.2008-1148
2. O'Brien D, Schoenian S, Whitley N: Dewormer resistance on Virginia sheep farms 2017. Available from: https://vtechworks.lib.vt.edu/handle/10919/5523 [cited 16 October 2023].
3. Weaver AR: Evaluation of terminal sire breeds for hair sheep production systems. Master of Science. Virginia Polytechnic Institute and State University; 2017.
4. Ott RS, Memon MA: Breeding soundness examinations of rams and bucks, a review. Theriogenology 1980;13:155-164. doi: 10.1016/0093-691X(80)90124-7
5. Lunstra DD, Echternkamp SE: Repetitive testicular biopsy in the ram during pubertal development. Theriogenology 1988;29:803-810. doi: 10.1016/0093-691X(88)90217-8
6. Giffin JL, Bartlewski PM, Hahnel AC: Correlations among ultrasonographic and microscopic characteristics of prepubescent ram lamb testes. Exp Biol Med 2014;239:1606-1618. doi: 10.1177/1535370214543063
7. Brito LFC, Barth AD, Wilde RE, et al: Testicular ultrasonogram pixel intensity during sexual development and its relationship with semen quality, sperm production, and quantitative testicular histology in beef bulls. Theriogenology 2012;78:69-76. doi: 10.1016/J.Theriogenology.2012.01.022
8. Camela ESC, Nociti RP, Santos VJC, et al: Ultrasonographic characteristics of accessory sex glands and spectral Doppler indices of the internal iliac arteries in peri- and post-pubertal Dorper rams raised in a subtropical climate. Anim Reprod Sci 2017;184:29-35. doi: 10.1016/j.anireprosci.2017.06.010
9. Kenyon PR, Maloney SK, Blache D: Review of sheep body condition score in relation to production characteristics. New Zeal J Agric Res 2014;57:38-64. doi: 10.1080/00288233.2013.857698
10. Burke JM, Kaplan RM, Miller JE, et al: Accuracy of the FAMACHA system for on-farm use by sheep and goat producers in the southeastern United States. Vet Parasitol 2007;147:89-95. doi: 10.1016/J.Vetpar.2007.03.033
11. Ley WB, Sprecher DJ, Lessard P, et al: Scrotal circumference measurements in purebred Dorset, Hampshire and Suffolk lamb and yearling rams. Theriogenology 1990;34:913-925. doi: 10.1016/0093-691X(90)90561-7
12. Tomlinson M, Jennings A, Macrae A, et al: The value of trans-scrotal ultrasonography at bull breeding soundness evaluation (BBSE): the relationship between testicular parenchymal pixel intensity and semen quality. Theriogenology 2017;89:169-717. doi: 10.1016/J.Theriogenology.2016.10.020
13. Chenoweth PJ, Hopkins FM, Spitzer JC, et al: Guidelines for using the bull breedings soundness evaluation form. Clinical Theriogenology 2010;2:43-50.
14. Kuster CE, Singer RS, Althouse GC: Determining sample size for the morphological assessment of sperm. Theriogenology 2004; 61:691-703. doi: 10.1016/S0093-691X(03)00240-1
15. Tibary A, Boukhliq R, El Allali K: Ram and buck breeding soundness examination. Rev Mar Sci Agron Vét 2018;6: 241-255.
16. Adamczyk K, Makowska A, Jedraszczyk J, et al: Effect of behaviour of Holstein-Friesian and Simmental bulls on semen quality. J Cent Eur Agric 2013;14:22-34. doi: 10.5513/JCEA.V14I2.2159
17. Braz KMG, Monteiro FM, Fernandes LG, et al: Does bull temperament impact growth performance and semen quality? Livest Sci 2020;236:104038. doi: 10.1016/J.Livesci.2020.104038
18. Martin GB, Jorre de St Jorre T, Al Mohsen FA, et al: Modification of spermatozoa quality in mature small ruminants. Reprod Fertil Dev 2011;24:13-18. doi: 10.1071/RD11902
19. Marai IFM, El-Darawany AA, Fadiel A, et al: Physiological traits as affected by heat stress in sheep—A review. Small Rumin Res 2007;71:1-12. doi: 10.1016/J.Smallrumres.2006.10.003
20. Kahwage PR, Esteves SN, Jacinto MAC, et al: Assessment of body and scrotal thermoregulation and semen quality of hair sheep rams throughout the year in a tropical environment. Small Rumin Res 2018;160:72-80. doi: 10.1016/j.smallrumres.2018.01.015
21. Kahwage PR, Esteves SN, Jacinto MAC, et al: High systemic and testicular thermolytic efficiency during heat tolerance test reflects better semen quality in rams of tropical breeds. Int J Biometeorol 2017;61:1819-1829. doi: 10.1007/S00484-017-1367-4/TABLES/5
22. Kastelic JP, Coulter GH, Cook RB: Scrotal surface, subcutaneous, intratesticular, and intraepididymal temperatures in bulls. Theriogenology 1995;44:147-152. doi: 10.1016/0093-691X(95)00155-2
23. Kastelic JP, Cook RB, Coulter GH: Effects of ambient temperature and scrotal fleece cover on scrotal and testicular temperatures in rams. Can J Vet Res1999;63:157.
24. Bailey TL, Monke D, Hudson RS, et al: Testicular shape and its relationship to sperm production in mature Holstein bulls. Theriogenology 1996;46:881-887. doi: 10.1016/S0093-691X(96)00245-2
25. Claus LAM, Barca Junior FA, Koetz Junior C, et al: Scrotal skin thickness, testicular shape and vascular perfusion using Doppler ultrasonography in bulls. Livest Sci 2019;226:61-65. doi: 10.1016/J.Livesci.2019.06.005
26. Bailey TL, Hudson RS, Powe TA, et al: Caliper and ultrasonographic measurements of bovine testicles and a mathematical formula for determining testicular volume and weight in vivo. Theriogenology 1998;49:581-594. doi: 10.1016/S0093-691X(98)00009-0
27. Camela ESC, Nociti RP, Santos VJC, et al: Changes in testicular size, echotexture, and arterial blood flow associated with the attainment of puberty in Dorper rams raised in a subtropical climate. Reprod Domest Anim 2019;54:131-137. doi: 10.1111/RDA.13213
28. Maquivar MG, Smith SM, Busboom JR: Reproductive management of rams and ram Lambs during the pre-breeding season in US sheep farms. Animals 2021;11:2503. doi: 10.3390/ANI11092503
29. Giffin JL, Franks SE, Rodriguez-Sosa JR, et al: A study of morphological and haemodynamic determinants of testicular echotexture characteristics in the ram. Exp Biol Med 2009;234:794-801. doi: 10.3181/0812-RM-364/ASSET/IMAGES/LARGE/10.3181_0812-RM-364-FIG4.JPEG
30. Carvajal-Serna M, Miguel-Jiménez S, Pérez-Pe R, et al: Testicular ultrasound analysis as a predictive tool of ram sperm quality. Biology 2022;11:261. doi: 10.3390/BIOLOGY11020261/S1
31. Montes-Garrido R, Riesco MF, Anel-Lopez L, et al: Application of ultrasound technique to evaluate the testicular function and its

correlation to the sperm quality after different collection frequency in rams. Front Vet Sci 2022;9:1035036. doi: 10.3389/Fvets.2022.1035036/Bibtex
32. Hedia MG, El-Belely MS, Ismail ST, et al: Monthly changes in testicular blood flow dynamics and their association with testicular volume, plasma steroid hormones profile and semen characteristics in rams. Theriogenology 2019;123:68-73. doi: 10.1016/J.Theriogenology.2018.09.032
33. Kozłowska N, Faundez R, Borzyszkowski K, et al: The relationship between the testicular blood flow and the semen parameters of rams during the selected periods of the breeding and non-breeding seasons. Animals 2022;12:760. doi: 10.3390/ANI12060760
34. Ntemka A, Kiossis E, Boscos C, et al: Effects of testicular hemodynamic and echogenicity changes on ram semen characteristics. Reprod Domest Anim 2018;53:50-55. doi: 10.1111/RDA.13279