

Testicular Morphological and Ultrasonographic Characterization of Male Gray Brocket Deers (*Mazama gouazoubira*) in Different Reproductive Status*

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ABSTRACT

Background: Gray brocket deer (*Mazama gouazoubira*) populations have been declining due to human intervention. Yet, only a few studies have assessed ultrasonographic testicular characteristics in cervids. Considering the relevance of monitoring testicular size, blood flow, and parenchyma, the present study aims to establish baseline information on scrotal circumference, testicular volume, and spectral Doppler parameters, to describe differences among adult male gray brocket deer in different reproductive status, and to correlate ultrasound parameters with testes size measurements.

Materials, Methods & Results: Six adult male gray brocket deers were used in the study. Scrotal circumference and testicular volume were measured. B mode ultrasound images of testes (longitudinal and cross-sectional views) and epididymes were subjected to computer-assisted analysis, obtaining the numerical pixel values (NPV) and pixel standard deviation (PSD). Using spectral Doppler, suprastesticular artery blood flow velocities (peak systolic velocity - PSV, end diastolic velocity - EDV, time-average maximum velocity - TAMAX, resistivity - RI and pulsatility indices - PI) were obtained. Semen was analyzed through total motility, vigor, and concentration tests. Three animals were normospermic (F+ group) and three were oligo/azoospermic (F- group). Groups were compared using a one-way ANOVA or Kruskal-Wallis followed by Student-Newman-Keuls (SNK) test. Ultrasound parameters were correlated to testes size parameters using Pearson's correlation for parametric variables and Spearman's correlation for non-parametric variables. F+ group presented significantly higher scrotal circumference (14.57 ± 1.19 cm), testicular volume (26.18 ± 4.94 cm³), and testes cross-sectional NPV (69.88 ± 24.00) and PSD (10.78 ± 3.42) than group F- (NPV: 28.26 ± 13.75 , PSD: 6.70 ± 1.84). No significant differences were observed between the groups regarding the spectral Doppler ultrasound parameters. Significant correlations were observed between scrotal circumference and longitudinal ($r = 0.76$) and cross-sectional testes NPV ($r = 0.89$), and testicular volume was correlated with longitudinal ($r = 0.78$) and cross-sectional testes NPV ($r = 0.91$) and with cross-sectional testes PSD ($\rho = 0.82$).

Discussion: Increased testicular echogenicity (higher NPV) has been positively associated with improved testicular growth, cell population expansion, inner and outer seminiferous tubules diameter, spermatids percentages and testis weight. In addition, more heterogenous testes (higher PSD) were associated with higher sperm output. It was suggested that the animals in group F- had compromised testicular development and spermatogenesis. The correlation observed between testes NPV and scrotal circumference was proposed to be associated with seminiferous tubules impairment. The F- group showed lower testicular volume, NPVs and PSDs in cross-sectional testicular images, suggesting higher protein levels and lower lipid contents were present in their parenchyma, influencing in testicular echogenicity and echotexture. No differences in spectral Doppler parameters were observed between the two groups. Also observed in stallions. However, PSV, EDV, TAMAX could be potential infertility indicators in other mammalians. These different results may be due to different locations of the evaluated vessel, species and techniques, age, ambient temperature, pathological conditions, and anaesthesia. Thus, it is suggested that scrotal circumference, testicular volume, and testes NPV are good indicators of male reproductive health in gray brocket deer and may help with better male selection in the species.

Keywords: Cervidae, scrotal circumference, pixel intensity, dopplervelocimetry.

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INTRODUCTION

Although *Mazama gouazoubira* is globally classified as of “least concern” by the International Union for Conservation of Nature, its populations are decreasing due to the extension of human communities [10]. Assisted reproduction programs are important tools for species conservation [36], thus improved diagnostic techniques may help with male selection, increasing conservation programs efficiency [46].

Ultrasonography has proven to be a valuable, and non-invasive diagnostic technique for assessing genital macroscopic morphology, pathology and testicular blood flow in several mammalian species [3,29,39,41]. Testes pixel intensity has been used to objectively identify differences in testicular parenchyma during sexual development [6], and after scrotal insulation [4]. Also, the Doppler technique detected blood flow changes in testicular artery in different seasons [27], and in males with fertility disorders [29,34]. Yet, few studies have investigated male deer reproductive system [23,46].

Moreover, estimating scrotal circumference and testicular volume is a cheap, non-invasive method, widely used in several deer species [13,15,24,31,32], essential to breeding soundness examination in domestic ruminants [1,5] and associated with fertility in rams [19] and in bulls [42].

Therefore, considering the poor literature about ultrasound and testis size description in gray brocket deer, the aims of this study were to establish baseline information on scrotal circumference, testicular volume, and spectral Doppler parameters for the species; to describe differences found between deer in different reproductive status; and to correlate ultrasound parameters with testes size measurements.

MATERIALS AND METHODS

Animals

Six adult male gray brocket deer (*M. gouazoubira*) were used. The evaluations were performed from January to December 2018. Two evaluations were completed on each male, except on male 6, which was evaluated once ($n = 11$). The males were housed in private institutions: two were at Aba-Yby Conservation Institute - Ecopoint Environmental Education Ltd. (Fortaleza, CE, Brazil) and four were at Claro Commercial Wild Animal Breeding Farm (Caucaia, CE, Brazil). The males were subjected to the usual management of each institution above. At Claro Farm, the males were fed equine food, grass (*Cymbodon dactylon* or *Brachiaria* sp.) and roots (e.g. carrots and yuca) daily. At Aba-Yby Institute, males were offered ovine food, fruits, and grass daily. At both institutions, the males received water *ad libitum*. More information concerning the animals is provided in Table 1.

Table 1. All data about the six gray brocket deer (*Mazama gouazoubira*) used in the present study.

Male	City - State	Age	Weight (kg)*	Proved fertility	Housed with female	Origin/born
Male 1	Fortaleza, CE	3 years	14	No	No	Captive
Male 2	Caucaia, CE	5 years	15	Yes	Yes	Captive
Male 3	Caucaia, CE	11 years	15	Yes	Yes	Captive
Male 4	Fortaleza, CE	3 years	15.6	No	Yes	Captive
Male 5	Caucaia, CE	1 year	15	No	Yes	Captive
Male 6	Caucaia, CE	3 years	20	No	Yes	Captive

*approximately

Physical evaluation

Firstly, all males were sedated using 5 to 10 mg/kg ketamine hydrochloride (Cetamin®)¹ and 0.5 to 1.5 mg/kg xylazine hydrochloride (Xylazine 10%®)², both injected intramuscularly [16,18]. Once the anesthesia took effect, a physical exam was per-

formed. The testicular consistency was assessed by palpation. Scrotal circumference (cm) was obtained using a measuring tape and testicular volume (cm³) was obtained using a pachymeter and following the formula: $4/3 \times \pi \times ABC$, where A = width/2, B = height/2, C = length/2 [15].

Semen collection and general assessment

Males were placed in a lateral decubitus position. The penis and prepuce were cleaned with saline solution (sodium chloride 0.9%) and feces excess were removed from the rectum before introducing the probe with the electrodes positioned towards the prostatic surface. Semen was collected using an electroejaculator (Neovet Autoejac v2®)³. The procedure consisted of three sets of stimuli, with ten stimuli for each voltage: the first series starting with 4 V, 5 V, and 6 V; the second with 5 V, 6 V, and 7 V; and the third with 6 V, 7 V, and 8 V. Each series was separated by a 5 min interval rest [32]. The semen was collected in a 50 mL conical tube.

The microscopic parameters of total motility (0 - 100%) and vigor (score 0 to 5) were assessed using a light microscope at 400x magnification. A 10 µL fresh semen aliquot was added to a solution containing 2 mL 1 % formaldehyde-saline (0.9 % sodium chloride solution). The concentration of sperm was quantified by counting the cells in five fields in a Neubauer chamber with a light microscope at 400x magnification [40]. Males 1, 2, and 4 were normospermic (high fertility potential; group F+). Male 3 appeared oligospermic in the first collection, but azospermic in the second collection; and males 5 and 6 were azospermic on both analyses (low or no fertility potential; group F-).

Ultrasound examination

After the physical exam, the ultrasound assessment was performed using Mindray Z5Vet⁴. Males 1 to 5 were evaluated twice using the B mode technique (n = 10). Longitudinal and cross-sectional sections of testis and the epididymal tail were evaluated with a convex transducer under the following parameters: 5 MHz, gain of 64, depth of 16.6 (only on animal 1's first evaluation, depth was 9.2), frame rate of 56, B dynamic range of 110. Two images of each section were taken and saved. Afterwards, they were submitted to a computerized analysis (spot-meter technique) using the software ImageJ⁵. Six 3 mm × 3 mm square regions of interest (ROI) in testis longitudinal view, four in testis cross-sectional views, and two in epididymal images were selected (Figure 1). The mean pixel intensity (numerical pixel values, or NPV) and the pixel heterogeneity (pixel standard deviation, or PSD) were calculated inside those ROI in each image [37]. NPV were defined as gray-scale values of individual picture elements ranging from 0 (absolute black) to 255 (absolute white).

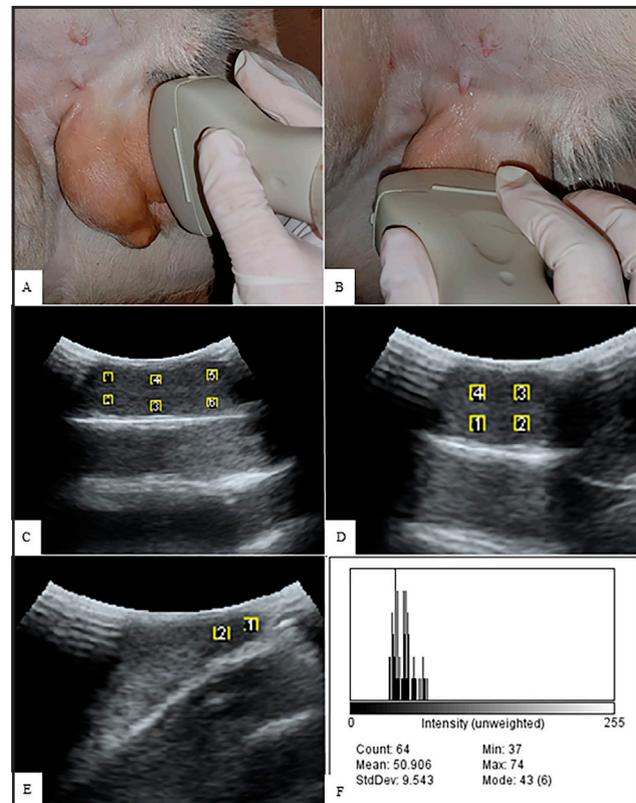


Figure 1. Computer-assisted analysis of the ultrasonographic images of gray brocket deer testes. A- Position of the transducer for visualizing a longitudinal section of the testis. B- Position of the transducer for visualizing a cross-sectional section of the testis. C- Spot-meter technique of pixel analysis: six open squares, 3×3 mm² in size, are placed on a longitudinal section of the ultrasonographic image of the testis. D- Four open squares, 3×3 mm² in size, are placed on a cross-sectional section of the ultrasonographic image of the testis. E- Two open squares, 3×3 mm² in size, are placed on a longitudinal section of the ultrasonographic image of the epididymis tail. F- Total pixel number (Count), mean NPV (Mean), standard deviation of NPV (StdDev), minimum value of NPV (Min), and maximum value of NPV (Max), most frequently occurring NPV within the selection (Mode) shown on a histogram.

Moreover, the spectral Doppler evaluation was performed on animals 1 to 6 (n = 11) using a linear transducer at 10 MHz. The angle of insonation used was set at 0°. At the region of the spermatic cord, the suprastesticular artery flow was detected with color Doppler, the gate was positioned within the lumen of the vessel, and the equipment's algorithm package was used to calculate peak systolic velocity (PSV), end diastolic velocity (EDV), time-average maximum velocity (TAMAX), resistive index (RI) and pulsatility index (PI) during three waves of a cardiac cycle [34]. The same operator performed all examinations.

Statistical analysis

The data was analyzed using the statistical software R-project⁶, being submitted to the Cramer-Von

Mises normality test and the Box-Cox homoscedasticity test. For comparison of means between groups F+ and F-, the homogenous and normally distributed parameters (PSV, EDV, TAMAX, RI, NPVs of testes longitudinal and cross-sectional views, NPVs and PSDs of epididymis, PSDs of testes cross-sectional view, scrotal circumference, testicular volume) were compared using a one-way ANOVA followed by Student-Newman-Keuls (SNK) test. Logarithmic (Ln) transformation was performed on EDV, TAMAX, PSDs of testes cross-sectional view and epididymal PSDs. The non-parametric data (PI, PSDs of testes longitudinal view) were compared using a Kruskal-Wallis test followed by SNK test.

Ultrasound parameters were correlated to testes size parameters using Pearson's correlation for parametric variables (PSV, RI, NPVs of testes longitudinal and cross-sectional views, epididymal NPVs, scrotal circumference, and testicular volume) and Spearman's correlation for non-parametric variables (PI, EDV, TAMAX, PSDs of testes longitudinal and cross-sectional view, and epididymal PSDs). The results are expressed as mean ± SD. Significance was set at $P < 0.05$.

RESULTS

Semen evaluation

Males 1, 2 and 4 ejaculated in both collections and presented good quality semen (motility: $96.83 \pm 3.71\%$; vigor: 4.41 ± 0.38 ; concentration: $94.08 \pm 65.04 \times 10^6$ sperm/mL). Male 3 ejaculated a low count sperm (4×10^6 sperm/mL) with poor motility (5%) and vigor (1) on its first collection. On the second collection, it presented azoospermia. Males 5 and 6 were azoospermic in all collections. Male 6 presented intense germ cell epithelial degeneration in the histopathological exam which was compatible to testicular degeneration.

Physical examination

During physical examination, males 1, 2, 4, and 5 had normal consistence regarding their testes, but soft ones were identified in males 3 and 6. F+ group showed significant wider scrotal circumference (14.57 ± 1.19 cm; $P < 0.05$) and larger testis volume (26.18 ± 4.94 cm³; $P < 0.05$) when compared to group F- (Scrotal circumference: 12.06 ± 0.62 cm; 14.99 ± 2.66 cm³; $P < 0.05$).

Ultrasound evaluation

During the subjective B mode evaluation, it was observed that testes and epididymis were in

the normal position, inside the scrotum. No abnormalities were noticed in the testicular and epididymal parenchyma. Testicular parenchyma had a smooth echotexture. The mediastinum was observed as a hyperechoic line (longitudinal view) or point (cross-sectional view) in the center. The *tunica albuginea* was observed as a hyperechoic line surrounding the parenchyma and enclosing it and, the scrotum skin, as thicker hyperechoic line. The epididymis tail was slightly hypoechoic, comparing to testicular echogenicity, and it is also surrounded by tunica albuginea and the skin (Figure 2).

The waveform originated from suprastesticular artery blood flow is shown in Figure 3. As shown in Table 2, no significant differences were observed between the groups regarding the spectral Doppler ultrasound parameters: PSV (F+: 10.66 ± 5.60 cm/s, F-: 9.77 ± 4.71 cm/s), EDV (F+: 5.59 ± 2.17 cm/s, F-: 5.67 ± 2.13 cm/s), TAMAX (F+: 0.38 ± 2.62 cm/s, F-: 7.14 ± 2.77 cm/s), PI (F+: 0.89 ± 0.75 , F-: 0.53 ± 0.12), and RI (F+: 0.40 ± 0.10 , F-: 0.38 ± 0.06). However, NPVs and PSDs of cross-sectional testes images were higher on F+ group (NPV: 69.88 ± 24.00 , PSD: 10.78 ± 3.42) than F- group (NPV: 28.26 ± 13.75 , PSD: 6.70 ± 1.84). Longitudinal testis (F+: 66.16 ± 30.66 , F-: 29.42 ± 12.42) and epididymal images NPVs (F+: 42.28 ± 14.32 , F-: 24.95 ± 6.78) were not significantly different between the groups. Likewise, longitudinal testes (F+: 13.92 ± 10.47 , F-: 7.71 ± 3.41) and epididymal images PSDs (F+: 12.74 ± 6.59 , F-: 10.77 ± 8.31) showed no significant differences.

Table 2. B mode and spectral Doppler ultrasound parameters of normospermic (F+) and oligo/azoospermic (F-) gray brocket deer (*Mazama gouazoubira*).

Parameter	F+	F-
PSV (cm/s)	10.66 ± 5.60^a	9.77 ± 4.71^a
EDV (cm/s)	5.59 ± 2.17^a	5.67 ± 2.13^a
TAMAX (cm/s)	6.38 ± 2.62^a	7.14 ± 2.77^a
PI	0.89 ± 0.75^a	0.53 ± 0.12^a
RI	0.40 ± 0.10^a	0.38 ± 0.06^a
NPV (longitudinal testes)	66.16 ± 30.66^a	29.42 ± 12.42^a
NPV (cross-sectional testes)	69.88 ± 24.00^a	28.26 ± 13.75^b
NPV (epididymis)	42.28 ± 14.32^a	24.95 ± 6.78^a
PSD (longitudinal testes)	13.92 ± 10.47^a	7.71 ± 3.41^a
PSD (cross-sectional testes)	10.78 ± 3.42^a	6.70 ± 1.84^b
PSD (epididymis)	12.74 ± 6.59^a	10.77 ± 8.31^a

^{a,b}Different letters within a row show significant difference between groups ($P < 0.05$). PSV= peak systolic velocity; EDV= end diastolic velocity; TAMAX= time-average maximum velocity; PI= pulsatility index; RI= resistivity index; NPV= numerical pixel values; PSD= pixel standard deviation. Values (Mean ± SD).

Correlations between ultrasound and testis size parameters

Scrotal circumference was strongly correlated to testicular volume ($r = 0.88, P = 0.0003$). There was a moderate correlation between longitudinal testes NPVs and testicular size parameters such as scrotal circumference ($r = 0.76, P = 0.01$) and testicular volume ($r = 0.78, P = 0.008$). Cross-sectional testes NPVs

were strongly correlated to scrotal circumference ($r = 0.89, P = 0.0006$) and testicular volume ($r = 0.91, P = 0.0003$). Cross-sectional testes PSDs were strongly correlated to testicular volume ($r = 0.82, P = 0.004$), while the other ultrasound parameters showed no significant correlations with testicular size parameters, as displayed on Table 3.

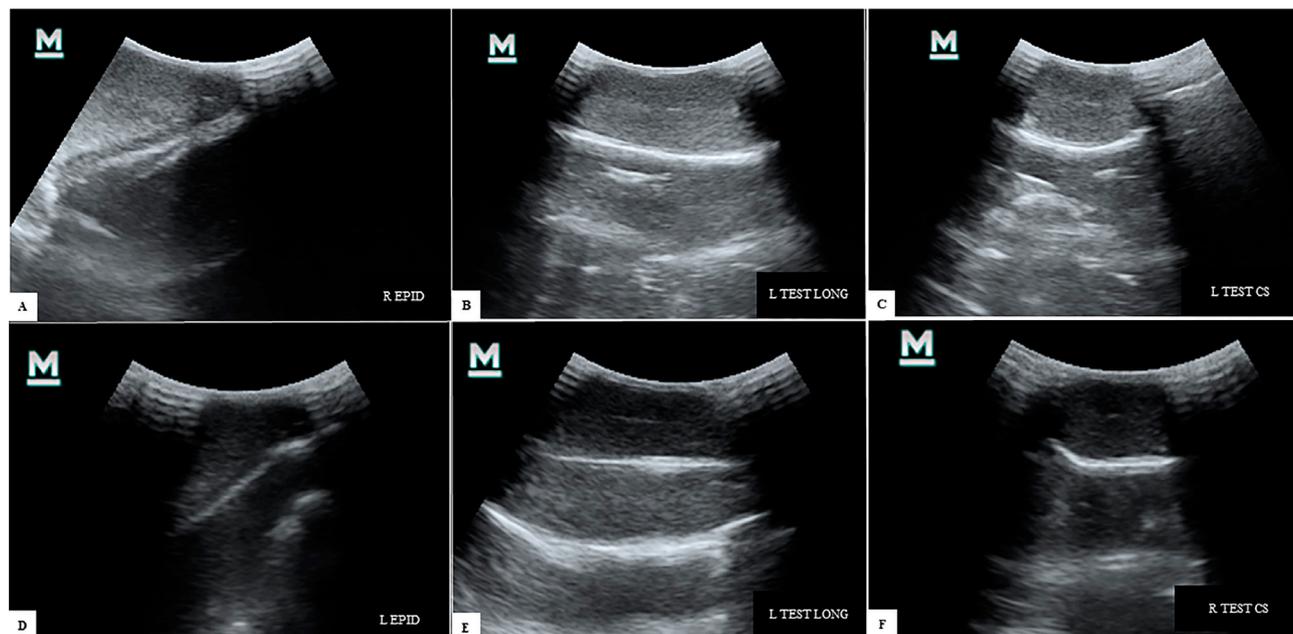


Figure 2. Mode B ultrasonographic images of right (R) or left (L) epididymis (EPID) and testes (TEST) on longitudinal (LONG) or cross-sectional (CS) views. A-C: Images of F+ males. D-F: Images of F- males.

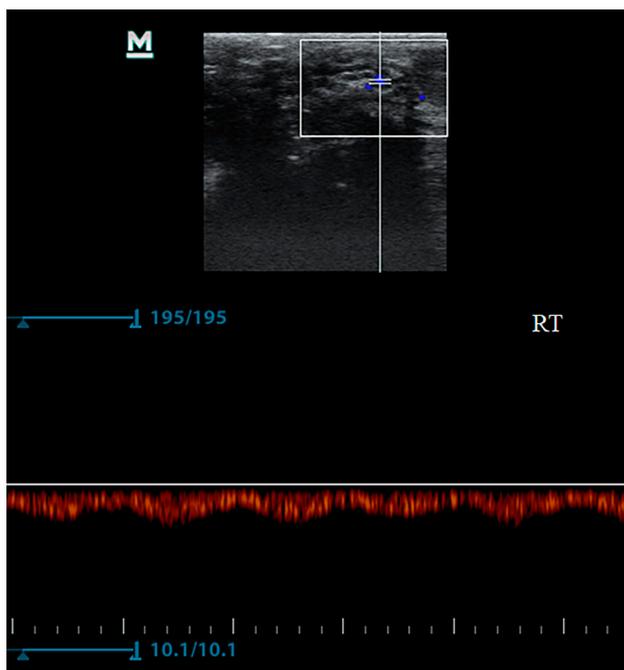


Figure 3. Waveform of suprastesticular artery of gray brocket deer (*Mazama gouazoubira*) using spectral Doppler technique.

Table 3. Correlations between the ultrasound parameters, scrotal circumference, and testicular volume in gray brocket deer (*Mazama gouazoubira*).

Parameter	Scrotal circumference	Testicular volume
PSV (cm/s)	r = 0.13, P = 0.71	r = 0.33, P = 0.32
EDV (cm/s)	ρ = -0.17, P = 0.61	ρ = 0.16, P = 0.63
TAMAX (cm/s)	ρ = -0.20, P = 0.56	ρ = 0.19, P = 0.57
PI	ρ = 0.22, P = 0.51	ρ = 0.37, P = 0.26
RI	r = 0.44, P = 0.18	r = 0.46, P = 0.15
NPV (longitudinal testes)	r = 0.76, P = 0.01*	r = 0.78, P = 0.008*
NPV (cross-sectional testes)	r = 0.89, P = 0.0006*	r = 0.91, P = 0.0003*
NPV (epididymis)	r = 0.50, P = 0.14	r = 0.40, P = 0.26
PSD (longitudinal testes)	ρ = 0.44, P = 0.21	ρ = 0.59, P = 0.07
PSD (cross-sectional testes)	ρ = 0.80, P = 0.0052	ρ = 0.82, P = 0.004*
PSD (epididymis)	ρ = 0.14, P = 0.70	ρ = 0.33, P = 0.35

*Shows significant correlations ($P < 0.05$). r = Pearson's correlation coefficient; ρ = Spearman's correlation coefficient; PSV= peak systolic velocity; EDV= end diastolic velocity; TAMAX= time-average maximum velocity; PI= pulsatility index; RI= resistivity index; NPV= numerical pixel values; PSD= pixel standard deviation.

DISCUSSION

The present results give basal information on testes and epididymis sonographic appearance, testes perfusion, and testicular size in healthy male gray brocket deers as well as pathological changes associated with azoospermia. To the best of our knowledge, this study is the first to report changes in testicular echogenicity, scrotal circumference, and testicular volume in normospermic and oligo/azoospermic gray brocket deer adult males and to find correlation between these variables.

Scrotal circumference is a simple repeatable method of measuring testicular size which is strongly correlated with testicular weight [35], semen production [17,45] and with fertility [35]. Testicular volume is also a simple method of measuring testes and is also correlated to scrotal circumference [45]. It is positively correlated to sperm motility and morphology and negatively correlated to immature germ cells [14]. In the present study, scrotal circumference and testicular volume measurements were different between normospermic and azoospermic deers. The source of these deer's poor semen quality and decreased testes size is possibly testicular degeneration [11,28] which can be caused by multiple factors such as advanced age, heat, medication (nitrofurans and benzimidazoles), nutritional deficiencies, stress, trauma or corticosteroid therapy [21]. The cause for sperm disturbances was confirmed only in male 6, which presented intense germ cell epithelial degeneration compatible to testicular degeneration in the histopathological analysis.

Unlike what might happen in rams [7], testicular degeneration may not cause noticeable alterations in deer's testes parenchyma using ultrasound subjective evaluation. Similarly, no ultrasound subjective alterations were noted in bulls after scrotal insulation. However, lower mean pixel intensity was also observed in bulls' testes after scrotal insulation, suggesting the occurrence of reduced echogenicity during a degenerative process. Thus, computerized analysis of B mode images may help providing detailed and objective information about subtle changes and have an important role in reproductive assessment [4].

Some factors can influence testicular pixel intensity (NPV), such as age [12,37] and genotype. In bulls, there was an increase in testicular echogenicity (testicular pixel intensity) during sexual development, which is associated with improved testicular growth and cell population expansion [12]. Also, testes NPV were moderately and positively correlated with inner and outer seminiferous tubules diameter in bulls [20], and moderate to strong positive correlations were observed between testes NPV and seminiferous tubules area and lumen in stallions [37]. Thus, it is suggested that the animals in group F- had compromised testicular development and spermatogenesis due to their lower testicular NPV than the ones in group F+. The deer in F+ in the present study presented good seminal concentration and higher values for scrotal circumference and testicular volume. This can also be linked with observations of a previous study in bulls in which pixel intensity showed moderate positive correlations to spermatids percent-

ages and testis weight [20], meaning that the higher the NPV, the more spermatids are observed and the larger are testes (in a physiological setting).

The F- group had lower testicular volume, NPVs and PSDs in cross-sectional testicular images, suggesting higher protein levels and lower lipid contents were present in their parenchyma and those components account for echogenicity and echotexture of the testicular parenchyma [2].

About pixel heterogeneity, group F+ presented more heterogeneous testes (in cross-sectional view), so higher PSD is associated to a better spermatogenic function. In dogs, more heterogeneous testes were associated with higher sperm output [33] and, in stallions, scrotal testes from young males were more heterogeneous than retained testes [37]. In addition, testes PSD (obtained through ultrasound evaluation in direct contact with tunica albuginea) was positively correlated to lumen area of seminiferous tubules in rams [22] and stallions [37]. In animals with spermatogenesis imbalance, the cell population is reduced inside seminiferous tubules [11], and their area is decreased too [37]. Hence, it is suggested that a decrease in variation between anechoic and echogenic parenchyma is observed in those males due to decrease in density of seminiferous tubules and consequent cell population reduction inside these tubules.

Scrotal circumference can be moderately associated with testes cell population parameters. In bulls, it was positively correlated to daily sperm production [35] and negatively correlated to the degree of germinal epithelial loss [30], suggesting that changes on seminiferous tubules can affect scrotal circumference measurements and testes echogenicity and echotexture which was corroborated with observations from this study, in which moderate to strong correlations were observed between testes NPV and scrotal circumference.

Scarce literature describes spectral Doppler parameters of testicular artery. RI and PI of testicular artery have been described in domestic animals, such as rams [7], stallions [38] and dogs [43] and in wild animals, such as llamas and alpacas [29]. Discrepancies in RI and PI may be observed amongst males, suggesting spectral Doppler indices are highly variable [7]. Those parameters can be associated with semen quality [9,27]. Thus, describing spectral Doppler parameters in gray brocket deer may contribute to establishing reference values for healthy males and aiding in diagnosing vascular disturbs involving testes [25,34] or evaluating if a pre-existing pathology is affecting testicular blood supply [26,34].

In this study, no differences in spectral Doppler parameters were observed between the two groups. Similarly, in fertile and subfertile stallions, there was no difference among blood flow parameters in the suprastesticular artery [34,38]. On the contrary, in dogs with and without testicular tumor, differences in PSV and TAMAX were observed [26] and, in fertile and infertile dogs, PSV and EDV were different [43]. In camelids, RI showed no difference between fertile and infertile males, but PSV and EDV were higher for fertile males [29]. These different results may be due to different locations of the evaluated vessel, species and techniques. It is known that many factors can influence vascular changes, including age [38], ambient temperature, seasonality [41,44], pathological conditions [34,43], and anaesthesia [8]. Thus, they may have influenced the spectral Doppler parameter's variability in the suprastesticular artery of gray brocket deer.

CONCLUSIONS

In conclusion, changes in testicular tissue in deer with different reproductive status were detectable using testes NPV, scrotal circumference, and testicular volume measurements where lower values were observed in deer with reproductive disruptions. Thus, these techniques can be feasible procedures and good indicators of male reproductive health in gray brocket deer, supporting in male selection for assisted reproduction programs. The correlations between testicular size and testes NPV and PSD can be associated with germ cell population and spermatogenesis; however, further studies are required to confirm this association in the species.

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Declaration of interest. The authors declare no conflict of interest. The authors alone are responsible for the content and writing of paper.

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