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Ultrasonographic evaluation of testicular and Pampiniform plexus characteristics in young bulls under different microclimatic conditions in a tropical environment

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This study aimed to evaluate the ultrasonographic characteristics of the testicles and pampiniform plexus of young bulls from 8 to 19 months of age under different microclimatic conditions. For this, 46 animals (8.0 ± 0.5 months and 211.0 ± 5.9 kg) were raised in two intensive rotational systems for 12 months, in which 24 animals (Nelore, n = 12; Canchim, n = 12) in a silvopastoral system and 22 animals (Nelore, n = 10; Canchim, n = 12) in a non-shaded pastoral system. Testicular biometric measurements, B-mode ultrasound assessments (echogenicity and heterogeneity of the testicular parenchyma and mediastinum), color Doppler (blood perfusion of the testicular parenchyma and pampiniform plexus), spectral Doppler of the supratesticular artery (pulsatility and resistance index), and blood collection from the jugular to determine testosterone concentration were performed monthly. Data were analyzed using the PROC MIXED procedure of SAS ($p < 0.05$), and possible interactions between production systems and animal age are presented. There was no difference in testicular volume variables ($p = 0.4$) between production systems over time. For parenchymal echogenicity ($p = 0.3$), a progressive increase was observed over time, with a maximum peak at 12 months of age, but without difference between systems. For the relative Doppler area of the testicular parenchyma ($p = 0.4$) and plexus ($p = 0.1$), values were decreased at 16 months of age, but without a difference between systems. For the velocimetric indices of the supratesticular artery, there were decreases in the pulsatility index ($p = 0.3$) and resistance index ($p = 0.04$), with no difference between the production systems. In conclusion, the variations observed in the present study are more related to age. Thus, there are no differences in the characteristics observed by ultrasound in young bulls kept in different microclimatic conditions under the circumstances studied.

Keywords Animal andrology, Beef cattle, Testicular hemodynamics, Production systems, Microclimate, Color doppler, Spectral doppler

The implementation of sustainable production systems has been proposed as an alternative to associate and optimize different productive activities in the same space[1,2]. For example, silvopastoral system (SPS) has been used in cattle production because it promotes environmental enrichment through the implementation of trees, consequently improving animal welfare by creating a milder microclimate, thus promoting climatic comfort[3,4]. This demand for sustainable production alternatives has arisen because heat stress is one of the most common

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stressors in production systems[5]. In this way, agroforestry systems can help to mitigate the effects of the tropical climate and, more recently, extreme weather events[6].

The environment is critical for the productive and reproductive development of animals[7]. Animals that are exposed to improper environmental and thermal conditions are more susceptible to developing harmful changes in their physiological functions[8]. Specifically in bulls, rising environmental temperatures can cause testicular damage[5,9] and directly affect the gonadal blood supply, compromising the testicular function and leading to changes in the seminiferous tubules. These deleterious effects occur due to failures in testicular thermoregulation, as there is an increase in the metabolism of gonadal tissue and a reduction in the local blood supply. Thus, the condition of testicular degeneration can be established[10,11]. In this sense, some studies indicate a direct effect of increased temperature on reproductive organs[12], especially on testicular cells, causing specific responses of testicular tissue to thermal stress[13,14].

In this sense, testicular ultrasonography (US) has been used as an auxiliary tool in evaluating male cattle. Romanello et al.[15] reported echogenic differences between bulls raised in different microclimates. Rodrigues et al.[16] reported its applicability in evaluating animals of different ages. Furthermore, there is the possibility of studying the vascular architecture and hemodynamic characteristics of the blood vessels of the organs, since it is known that higher environmental temperatures significantly increase testicular blood flow, an effect observed through spectral Doppler ultrasonography in adult bulls and rams[17,18]. However, although cattle raised in tropical environments are known to be thermotolerant and highly adaptable animals[19–21], detailed information on the gonadal response assessed by ultrasound in young animals in different environmental conditions still needs to be completely available.

Based on what has already been reported in adult cattle, it has been hypothesized that an inadequate environment may promote testicular changes in young bulls; however, this information is poorly understood. This information gap provides an opportunity to perform studies with diagnostic techniques in bovine andrology to identify possible changes related to the thermal challenge caused by heat. Therefore, the present study aimed to evaluate gonadal development and testicular ultrasonographic characteristics, including B-mode, color Doppler and spectral Doppler, in young bulls (from 8 to 19 months of age) raised in shaded and non-shaded areas, focusing on the effects of production systems on testicular parenchyma echogenicity, blood perfusion, supratesticular artery hemodynamics and serum testosterone concentrations in these animals during the development phase.

Results

Climate variables

The monthly microclimatic characterization of the production systems throughout the experimental period is presented in Fig. 1. However, air temperature (Fig. 1A) reached its highest values between September and December, with no difference between the production systems ($p = 1.0$). The temperature of the black globe (Fig. 1B) showed differences between treatments in all months evaluated ($p = 0.02$), with the NS system superior to the SPS group. Relative humidity (Fig. 1C) showed fluctuations during the experimental period, but no difference between systems was observed throughout the months evaluated ($p = 1.0$). For wind speed (Fig. 1D), there was a discrepancy between the systems in all assessed months, with less intense gusts in the SPS Group ($p = 0.008$). The radiant heat load (Fig. 1E; $p < 0.0001$) and the black globe temperature and humidity index (Fig. 1F; $p = 0.006$) showed differences between the systems in all months evaluated, with greater intensity in the NS Group. The averages of the microclimatic characterization of the production systems during the period in which the ultrasound analyses were performed are presented in Table 1. The WS was higher for the ICLF group than the NS group ($p < 0.0001$). For the other variables analyzed, the NS group was superior to the ICLF group ($p < 0.0001$).

Biometric variables

Possible interactions between systems and age for testicular biometric characteristics and testicular consistency are shown in Fig. 2. The body weight of the animals differed over time regardless of the system ($p = 0.0007$), with a notable gradual increase (Fig. 2A). For the characteristics of scrotal circumference (Fig. 2B), testicular consistency (Fig. 2C), testicular length (Fig. 2D), testicular width (Fig. 2E), and testicular volume (Fig. 2F) there were no differences in the evaluation of interaction between systems and age ($p > 0.05$). When comparing the means of biometric characteristics and testicular consistency between the NS and SPS systems (Table S1), no differences were observed for any of the variables ($p > 0.05$). When comparing the ages regardless of treatment (Fig. S1), differences were observed for all testicular biometric variables ($p < 0.0001$), with a progressive increase over time. A reduction was observed in testicular consistency from 15 to 19 months ($p < 0.0001$).

Ultrasound variables in B-mode

For possible interactions between systems and age for the variables evaluated in B-mode ultrasound images (Fig. 3), the echogenic values of the testicular parenchyma (parenchymal echogenicity; Fig. 3A and parenchymal heterogeneity; Fig. 3B), testicular mediastinum (mediastinal echogenicity; Fig. 3C and mediastinal heterogeneity; Fig. 3D), vessel width of plexus (Fig. 3E), and mediastinal width (Fig. 3F) no interactions were observed between production systems and age ($p > 0.05$). When comparing the means of characteristics in B-mode ultrasound between the NS and SPS systems (Table S2), no differences were observed for any variable ($p > 0.05$). For the variables comparing the ages independent of the treatment (Fig. S2), differences were observed in all parameters in B-mode ($p < 0.0001$), with increasing values accompanying animal growth.

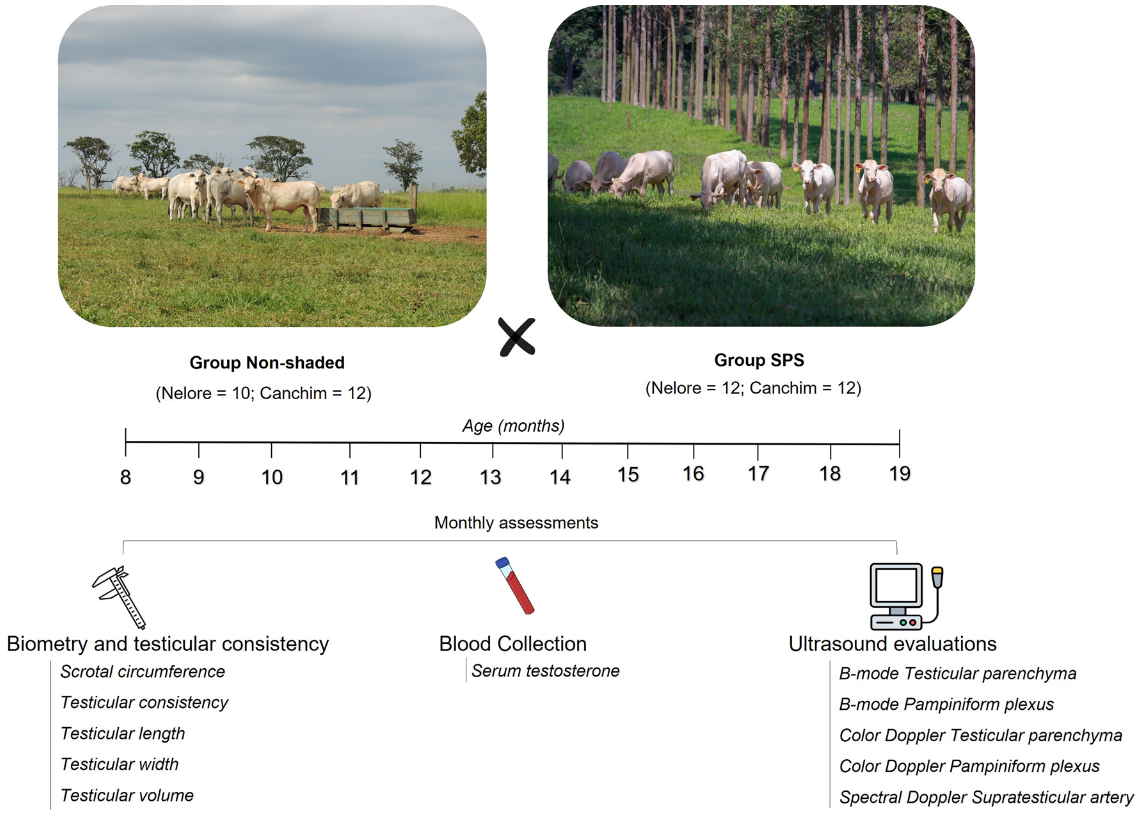

Fig. 1. Monthly averages of (**A**) air temperature (AT; °C), (**B**) black globe temperature (BGT; °C), (**C**) relative humidity (RH; %), (**D**) wind speed (WS; m/s), (**E**) radiant heat load (RHL; W/m^2) and (**F**) black globe temperature and humidity index (BGHI) over the experimental period in system non-shaded (NS) and silvopastoral system (SPS) between the 8:00 a.m. and 6:00 p.m. Superscript asterisks indicate differences between treatments for the times indicated ($p < 0.05$).

	Production systems		
Variables	**NS**	**SPS**	***p*-value**
AT (°C)	26.00 ± 3.96^{a}	25.80 ± 4.04^{b}	0.002
BGT (°C)	36.02 ± 5.93^{a}	31.73 ± 5.94^{b}	< 0.0001
RH (%)	53.66 ± 16.65^{b}	55.94 ± 16.02^{a}	< 0.0001
WS (m/s)	2.15 ± 0.79^{a}	1.63 ± 0.68^{b}	< 0.0001
RHL (W/m^2)	720.10 ± 90.44^{a}	594.5 ± 89.89^{b}	< 0.0001
BGHI	83.01 ± 6.29^{a}	78.90 ± 6.22^{b}	< 0.0001

Table 1. Mean ± standard deviation for air temperature (AT, °C), black globe temperature (BGT, °C), relative humidity (RH, %), wind speed (WS, m/s), radiant heat load (RHL, W/m^2) and black globe temperature and humidity index (BGHI) of the systems non-shaded (NS) and silvopastoral (SPS) from 10:00 a.m. to 2:00 p.m, period of ultrasound evaluations. [a,b]Means followed by distinct lowercase letters on the same row differ significantly ($p < 0.05$).

Ultrasound variables in color doppler mode

The variables analyzed in color Doppler mode of the testicular parenchyma and pampiniform plexus and their possible interactions between systems and age are presented in Fig. 4. For RDA parenchyma (Fig. 4A; $p = 0.3$), DA parenchyma (Fig. 4B; $p = 0.6$), and LSP parenchyma (Fig. 4D; $p = 0.5$), there was no interaction between systems and age. For HSP parenchyma (Fig. 4C; $p = 0.02$), there was an interaction, in which it is highlighted that at 15 months, the SPS group ($0.12 \pm 0.01\%^{a}$) presented greater values than at 16 and 19 months (0.02 ± 0.01^{b} and $0.02 \pm 0.01\%^{b}$) of the same treatment, however, these values similar to those of the NS group throughout the months. For pampiniform plexus perfusion, there was also interaction only for HSP plexus (Fig. 4G; $p = 0.02$), in which between nine and 14 months there was a gradual increase without marked differences between treatment systems, with a higher peak for the SPS group at 14 ($11.94 \pm 1.06\%^{ab}$) month-old and a decrease at 16 ($8.42 \pm 1.12\%^{abcde}$) month-old, but with no differences about the NS group. For the other variables, there were no

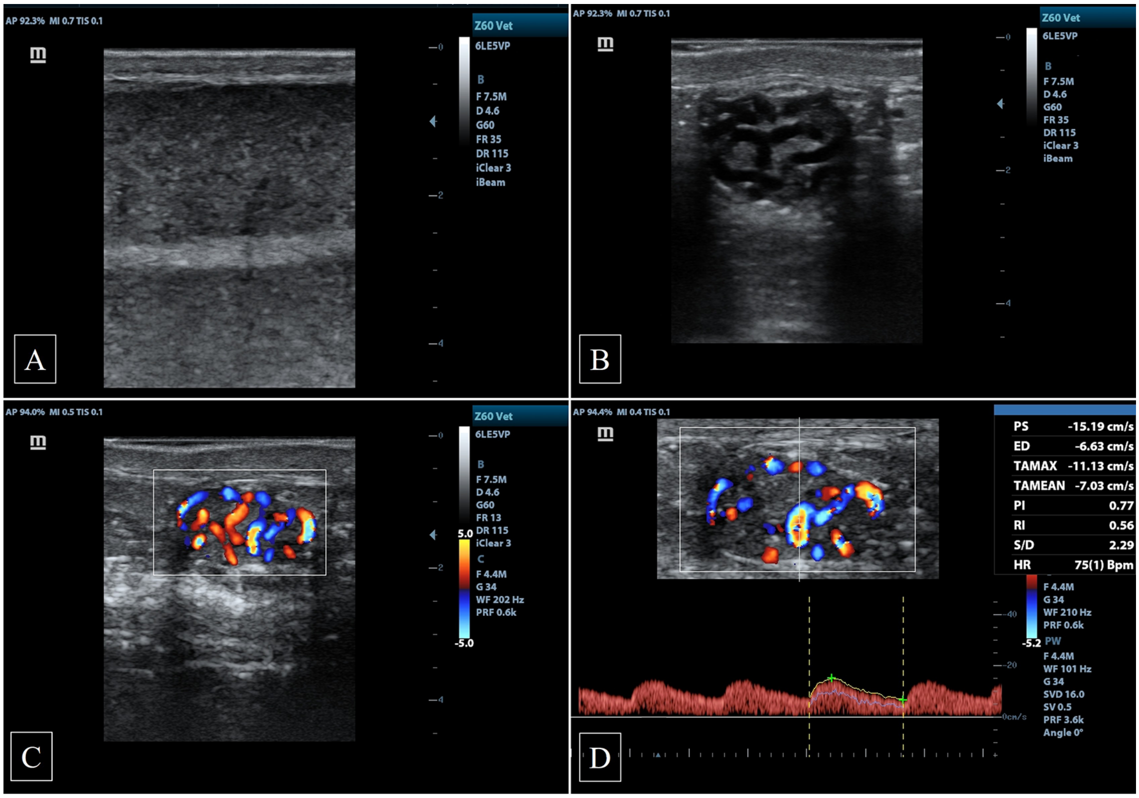

Fig. 2. Mean ± standard error of body weight, scrotal/testicular biometrics and testicular consistency of young bulls kept in non-shaded (NS) or silvopastoral (SPS) systems. (**A**) body weight (kg), (**B**) scrotal circumference (SC, cm), (**C**) testicular consistency (TS, 1–5), (**D**) testicular width (TW, cm), (**E**) testicular height (TH, cm) and (**F**) testicular volume (TV, cm^3). Trat = NS or ILCF; age = from eight to 19 months.

interactions ($p > 0.05$). When comparing the means of the characteristics in color Doppler mode between the NS and SPS systems (Table S3), no differences were observed for any variable ($p > 0.05$). For comparisons between ages regardless of treatment (Fig. S3), the variables RDA parenchyma, DA parenchyma, HSP parenchyma, and LSP parenchyma demonstrated that their values reached their maximum peak at 15 months ($p < 0.005$). A more progressive increase was observed over age ($p < 0.0001$) for RDA plexus, HSP plexus, and LSP plexus.

Ultrasound variables in spectral Doppler mode and serum testosterone concentrations

The spectral Doppler mode variables and serum testosterone concentrations are presented in Fig. 5. There was no interaction between system and age for PI (Fig. 5A; $p = 0.3$), but there was an interaction for RI (Fig. 5B; $p = 0.04$), in which there was a decrease in the variable over the ages in both production systems. For serum testosterone (Fig. 5C), no interaction between the production system and age was observed ($p > 0.2$). When comparing the means of the spectral Doppler mode variables and serum testosterone characteristics between the NS and SPS systems (Table S2), no differences were observed for any variable ($p > 0.05$). For age comparison independent of treatment in the spectral Doppler and serum testosterone parameters (Fig. S4), PI showed differences ($p < 0.0001$), with decreasing values over time. There were differences in serum testosterone concentrations when compared between eight and 12 months of age ($p = 0.03$).

Discussion

Research on scrotal thermoregulation and the environment's impact on possible dysfunctions in beef cattle has been studied[22]. However, little is known about the effects of production systems on testicular ultrasound characteristics of young bulls kept in different microclimates from 8 to 19 months of age.

During the experimental period, the system without shading demonstrated that the climatic variables BGT, WS, RHL and BGHI presented higher averages than those of the SPS system, throughout the experimental period (Fig. 3). These more intense climate variables in the unshaded system were expected, and are due to the low density of trees, since in the case of SPS, the favorable effect of the tree component reduces the direct incidence of solar radiation[3,4]. In addition, the microenvironment can affect wind speed, where the SPS system's high tree density reduces wind speed due to the physical barrier created by the trunks and canopy[15]. Regarding ITGU, both production systems reached averages above 76 points between September and April, in which Bâeta and Souza[23] established BGHI equal to 74 as the threshold for environmental thermal comfort for cattle. The means of the variables presented during the testicular ultrasound evaluations (Table 1) are generally higher when compared to the means given in most months (Fig. 1). The monthly means take into account the entire

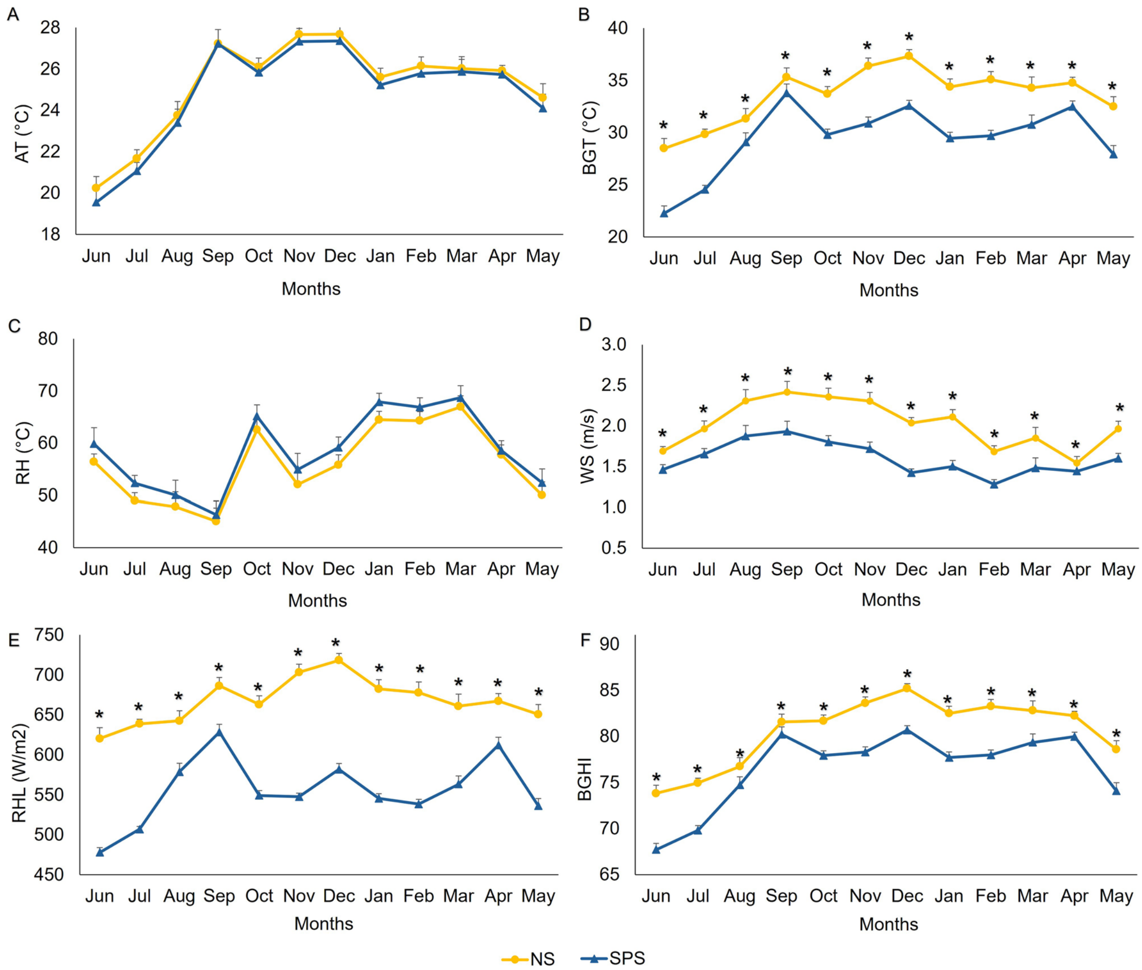

Fig. 3. Mean ± error of the B-mode ultrasound variables of the testicular parenchyma and pampiniform plexus of young bulls kept in non-shaded (NS) or silvopastoral (SPS) systems. (**A**) parenchyma echogenicity (0–255), (**B**) parenchyma heterogeneity, (**C**) mediastinal echogenicity (0–255), (**D**) mediastinal heterogeneity, (**E**) vessel width of plexus (mm) and (**F**) mediastinal width (mm). Trat = NS or ILCF; age = from eight to 19 months. Superscript asterisks indicate differences between treatments for the ages indicated ($p < 0.05$).

diurnal period. In contrast, the results presented during the ultrasound evaluations were obtained during a more significant thermal challenge period.

Given these climatic variables presented, it is known that when adult bulls are kept in an environment with high temperatures, effective thermoregulation may not occur, which may consequently cause testicular dysfunction[24]. However, these responses are poorly known in young bulls. In adult males, one of the dysfunctions caused by testicular hyperthermia is the degeneration of parenchymal tissue, causing a decrease in the size of the testicle[25] and, consequently, a reduction in testosterone concentrations[14]. However, these dysfunctions were not observed in the present study. This experiment was conducted using young animals, in which the testes are anatomically positioned closer to the abdominal cavity. Thus, it can be hypothesized that these organs were exposed to minimal or no direct radiation. As a result, the production system had no observable effect on them.

Therefore, the responses on the parameters related to the testicular biometrics show that the differences were quite evident concerning the age of the animals and less dependent on the production systems. Over time, testicular growth is a result of an increase in the diameter and length of the seminiferous tubules[26], in which these processes are related to the bodily development and sexual initiation of animals. Although it was not evaluated in the present study, apparently, the age and body weight of the animals have a positive correlation with testicular dimensions[27,28].

Regarding the ultrasound characteristics, specifically in B-mode, no differences were observed when related to the production systems, but rather to the ages. A significant increase in the echogenicity and heterogeneity of the testicular parenchyma was observed in both systems over time. These increases in echogenic values were expected since there is an increase in echogenicity due to the increasing diameter of the seminiferous tubules and the differentiation of the morphology and reorganization of the Sertoli cells as the animals age[16]. This increase in echogenicity over time was also observed in the testicles of adult Nelore and Canchim bulls[15], and when evaluating the testicles of adult Nelore and Caracu bulls[16]. In our study, a sharp drop in echogenicity was observed from 16 months onwards, and a tendency for the drop to continue in the following months of age in

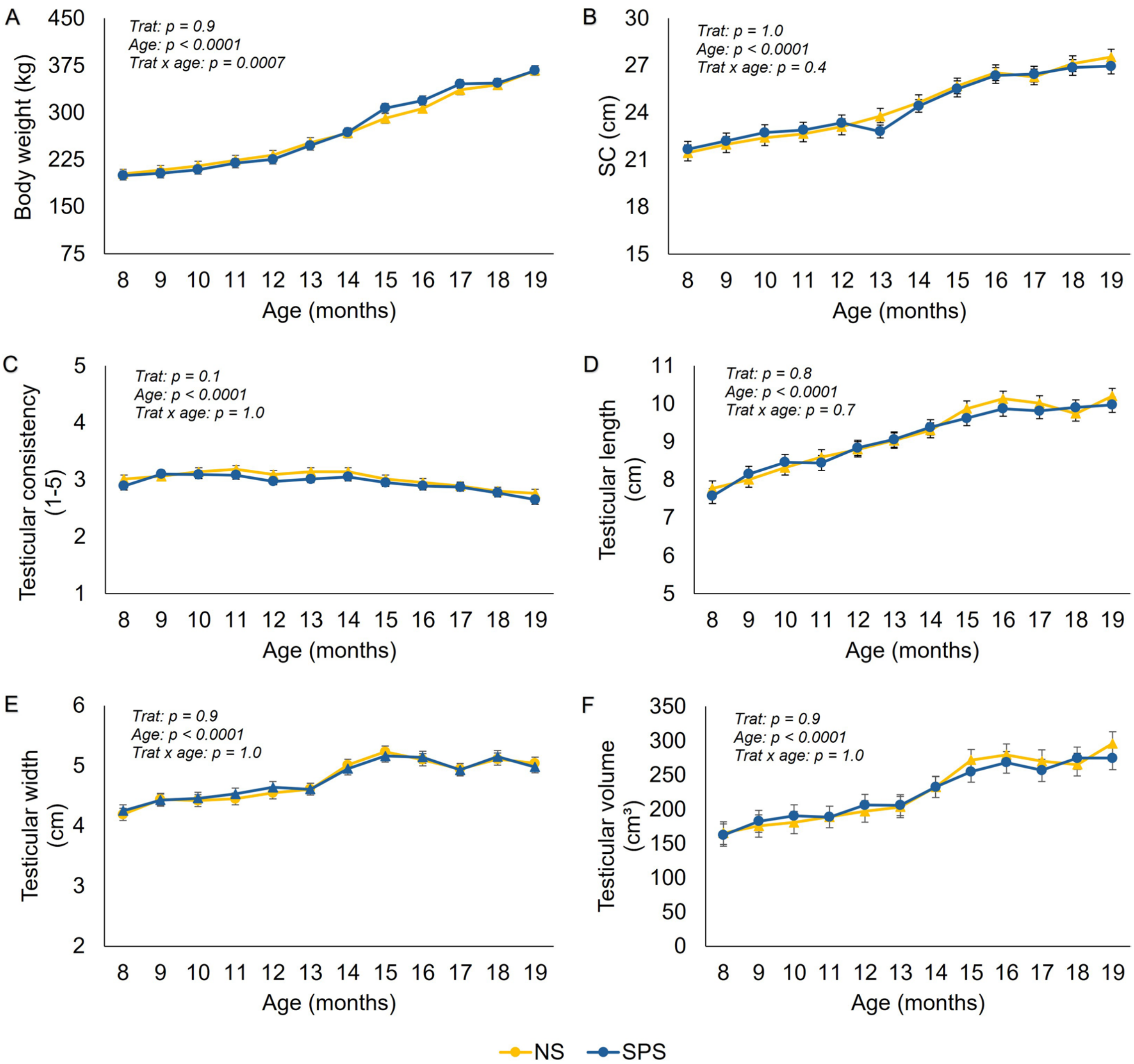

Fig. 4. Mean ± error of the color Doppler mode ultrasound variables of the testicular parenchyma and pampiniform plexus of young bulls kept in non-shaded (NS) Mean ± error of the B-mode ultrasound variables of the testicular parenchyma and pampiniform plexus of young bulls kept in non-shaded (NS) or silvopastoral (SPS) systems. (**A**) relative area of color Doppler of parenchyma (RDA parenchyma, %), (**B**) color Doppler area of parenchyma (DA parenchyma, mm^2), (**C**) relative to the high-speed Doppler pixels of parenchyma (HSP parenchyma, %), (**D**) relative of low-speed Doppler pixels of parenchyma (LSP parenchyma, %), (**E**) relative area of color Doppler of plexus (RDA plexus, %), (**F**) color Doppler area of plexus (DA plexus, mm^2), (**G**) relative to the high-speed Doppler pixels of plexus (HSP plexus, %) and (H) relative of low-speed Doppler pixels of plexus (LSP plexus, %). Trat = NS or ILCF; age = from eight to 19 months. Superscript asterisks indicate differences between treatments for the ages indicated ($p < 0.05$).

animals in both systems. This fall may be related to the pre-puberty process or the onset of puberty[29,30], in which there is an accumulation of fluid in the seminiferous tubules due to the increase of testicular activity, which consequently reduces the echogenicity of the parenchyma assessed by B-mode ultrasound[31]. Furthermore, the echogenic characteristics presented in the present study agree with other previously published studies[16,32,33]. Increased echogenicity and mediastinal width with age were also reported in *Bos indicus*[34] and in *Bos taurus* bulls[33]. This increase in the mediastinum is related to changes in the *rete testis*, as the seminiferous tubules open into the mediastinum, and as they age, these tubules become more elongated, consequently increasing their dimensions[35,36]. These increases in size over time were also observed in the vessels of the pampiniform plexus in animals from both systems. These increases can be explained by aging because, with age, thermoregulatory structures such as the pampiniform plexus adapt to the needs of testicular thermoregulation[37].

The more significant number of high-speed pixels over time in both the pampiniform plexus and the testicular parenchyma may be related to decreased vascular resistance, causing blood to reach the organ faster. We infer that this may occur because younger animals may have an increased scrotal temperature due to the testicles being closer to the abdominal cavity and, consequently, an increased metabolism. In this context, it is possible to

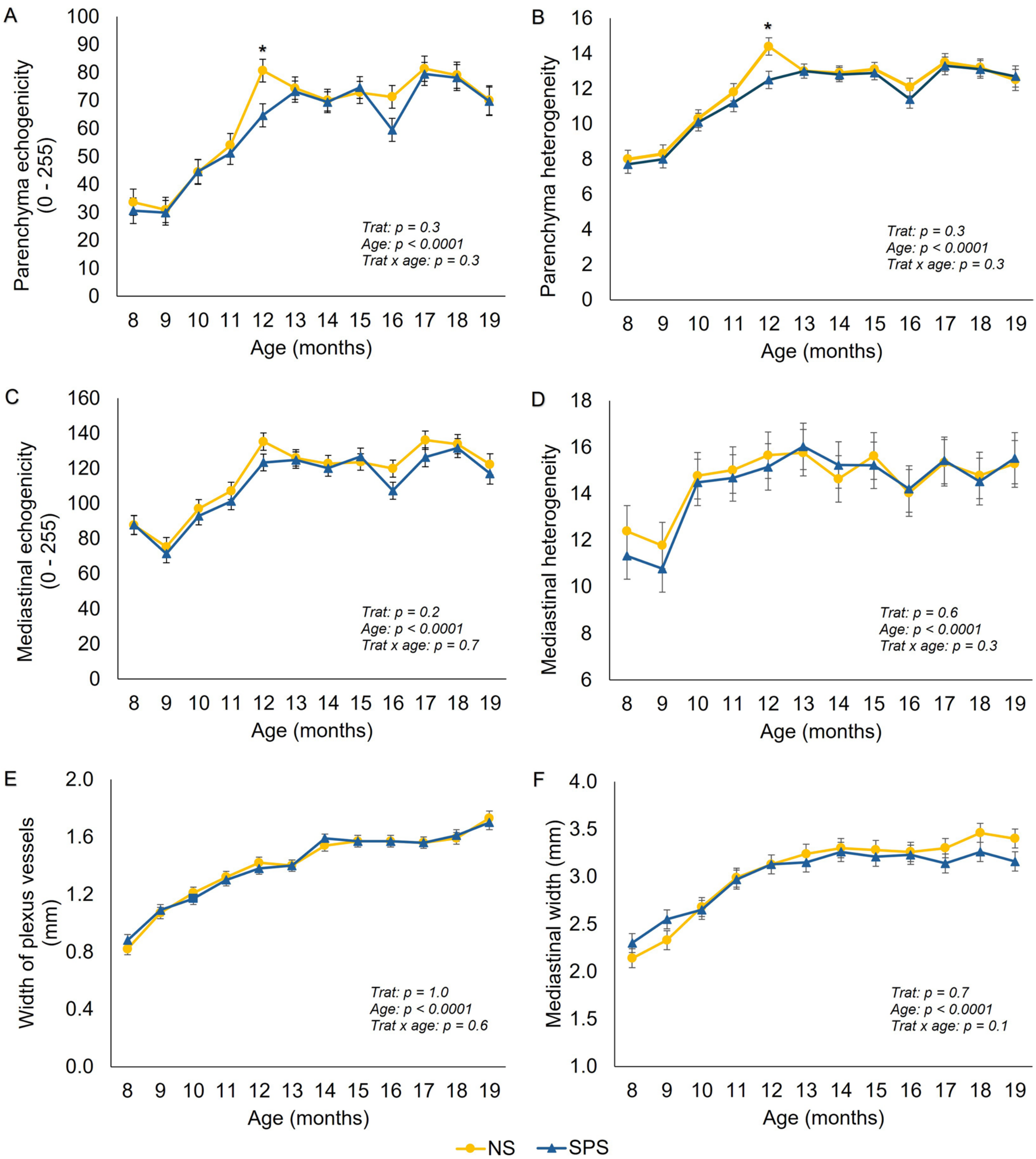

Fig. 5. Mean ± error of the velocimetric variables of the supratesticular artery and hormonal concentrations of young bulls kept in non-shaded (SN) or Mean ± error of the B-mode ultrasound variables of the testicular parenchyma and pampiniform plexus of young bulls kept in non-shaded (NS) or silvopastoral (SPS) systems. (**A**) pulsatility index (PI), (**B**) resistance index (RI), and (**C**) serum testosterone (ng/mL). Different lowercase letters differ significantly ($p < 0.05$). Trat = NS or ILCF; age = from eight to 19 months. Superscript asterisks indicate differences between treatments for the ages indicated ($p < 0.05$).

hypothesize that the animals did not suffer harmful effects from the high temperatures in the non-shaded system detected by ultrasound, possibly due to the adaptation of the testes to higher temperatures in the young phase.

The pulsatility and resistance indices of the supratesticular arteries did not show any effects related to the production systems. However, they were associated with the age of the animals, with a decrease in the indices being observed over time. The PI and RI are related to testicular perfusion, and, typically, low resistance indices occur in vessels with high blood flow[38]. Thus, these indices are potentially associated with the functional characteristics of the testes. Furthermore, our results corroborate previous results from Claus et al.[37], where younger animals (12 months) presented higher PI (0.49 ± 0.02 vs. 0.32 ± 0.16) and RI (0.51 ± 0.20 vs. 0.40 ± 0.15) than older animals (24 months), respectively. Therefore, the diagnostic procedures used in the present study allowed the characterization of a blood flow pattern in the supratesticular artery in young bulls throughout growth, regardless of the production systems in which they were raised.

Regarding serum testosterone, no interaction between system and age was observed, nor was there a growth pattern over time, even though the concentrations were within the range of previous studies[39]. However, some studies indicate that testosterone concentrations in bulls exhibit a distinct pattern of increase as they mature[40]. The variations recorded over the ages for serum testosterone concentrations in young bulls in the present study may have been significantly influenced by several factors, including circadian secretion patterns, body weight, and maturation of sexual organs[41,42]. According to Moura et al.[43], the relationship between body weight and testosterone levels is well established, in which physical development directly influences hormonal profiles and vice versa. Serum testosterone elevations occur before rapid testicular growth; therefore, this elevation may indicate that physical and testicular growth is approaching[43–45]. Thus, the increase in serum testosterone occurs to prepare the reproductive tract for rapid anatomical development[42]. Therefore, these factors mentioned may have overshadowed any differences caused by the production systems.

In general, microclimates had no effects on the testicular ultrasound differences evaluated in the present study. However, it is worth noting that the region in which the experiment was carried out had an average air temperature of 24.9 °C during the experimental period. However, in most areas of Brazil where beef cattle farming is extensive, average temperatures can reach high values (34.65 °C; [46]) depending on the season. Therefore, the use of integrated systems such as SPS may be more efficient in regions with high temperatures. In addition, the breeds used in this study may have an efficient thermoregulatory response. Nelore animals are already known for their resistance and thermotolerance in high-temperature environments. In the case of Canchim animals, although they are a composite breed (5/8 *Bos taurus* × 3/8 *Bos indicus*), their zebu composition may provide them with traits that allow them to better adapt to the tropical climate.

Conclusion

Under the conditions evaluated in this study, no differences were observed in the testicular ultrasonographic characteristics of young bulls aged 8–19 months raised in distinct microclimatic environments. The variations detected in these characteristics were primarily associated with age-related developmental progression rather than environmental factors.

Methods

Ethics

All experimental procedures were approved by the Animal Use Ethics Committee of Embrapa Pecuária Sudeste (São Carlos, SP, Brazil; Protocol CEUA PRT02/2023). The experiment was conducted in compliance with Brazilian laws and reported according to the precepts of the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

Location

The present study was carried out from May 2023 to June 2024 at Embrapa Pecuária Sudeste, São Carlos, SP, Brazil (21º57′42" S, 47º50′28" W and 860 m a.s.l.). The region has a tropical altitude climate type (Cwa), according to Köppen-Geiger's climate classification. During the experimental period, the climate was monitored using automatic weather stations installed within the production systems. Each weather station was equipped with thermohygrometer sensors (HC2-S3, Rotronic, Bassersdorf, Switzerland—Accuracy: ± 0.8% RH, ± 0.1 °C AT), ultrasonic anemometer (WindSonic, Gill, Lymington, United Kingdom—Accuracy: ± 2%) and copper-constantan thermocouple (Accuracy: ± 0.4%) inserted in a black metallic globe. The sensors were installed 1.5 m above the ground surface and were connected to a datalogger (CR3000, Campbell Scientific, Logan, USA). The monthly averages of climate variables comprised the air temperature (AT; °C), black globe temperature (BGT; °C), relative humidity (RH; %), wind speed (WS; m/s), radiant heat load (RHL; W/m^2). Subsequently, the black globe temperature and humidity index (BGHI) was calculated according to Buffington et al.[47], and used as an indicator of animal thermal comfort:

$$\mathrm{BGHI} = \mathrm{BGT} + 0.36\,(\mathrm{DPT}) + 41.5$$

where BGT = black globe temperature (°C) and DPT = dew point temperature (°C).

Production systems

The experimental area comprised two production systems corresponding to the NS and SPS treatments. I) Non-shaded system (NS Group): system with an area of 12 ha, with four subsystems, established for intensive rotational grazing of *Urochloa brizantha* (cv BRS Piatã), composed of pastures with minimal natural shade, resulting from the presence of remaining native trees, which determine a useful shade area of 1.02%; and II) Silvopastoral system (SPS Group): shaded pasture system, in silvopastoral system, with an area of 12 ha, with four subsystems, established for intensive rotational grazing of *Urochloa brizantha* (cv Piatã), with eucalyptus trees (*Eucalyptus urograndis*, clone GG100). The trees were arranged in single rows, in an east–west direction, with a spacing of 30 m between rows and four meters between plants, characterizing a density of 84 trees/ha. The trees were, on average, 31 m tall and 33 cm in diameter at 1.30 m above the ground, determining a useful shaded area of 30%. In both systems, the animals followed the same feeding management scheme, with a rotation of animals in the paddocks every 6 days, with a rest period of 30 days on the pastures, totaling grazing cycles of 36 days.

Animals

Forty-six Nelore (n = 22) (*Bos indicus*) and Canchim (n = 24) (*5/8 Bos taurus* × *3/8 Bos indicus*) young bulls with known genealogy, health, and zootechnical records were used. The animals began the experiment at

8.0 ± 0.1 months, 5.0 ± 0.0 body condition score (BCS; [48]), and 200.5 ± 3.3 kg body weight (BW). The animals were pre-selected from a herd of 160 contemporary bulls to ensure the homogeneity of the experimental batches. The animals were managed under equal health and nutritional conditions (intensive rotational system with *Urochloa brizantha*, cv BRS Piatã), with ad libitum access to water and mineral mix supplementation (Guabiphos 60S; Guabi Nutrição e Saúde Animal Ltda, Sales de Oliveira, SP, Brazil), presenting the following levels per kilogram of product: calcium (150–170 g), phosphorus (60 g), sodium (110 g), sulfur (10 g), fluorine (600 mg), magnesium (5000 mg), cobalt (64 mg), copper (950 mg), iodine (64 mg), manganese (950 mg), selenium (12 mg) and zinc (3170 mg). The animals were divided into two groups: the non-shaded (NS) group consisted of 10 Nelore (8.0 ± 0.2 months, 5.0 ± 0.0 BCS, and 211.0 ± 5.9 kg BW) and 12 Canchim bulls (8.0 ± 0.1 months, 5.0 ± 0.0 BCS and 195.0 ± 8.5 kg BW), which were allocated to non-shaded pastures. The silvopastoral system (SPS) group was formed by 12 Nelore bulls (8.0 ± 0.2 months, 5.0 ± 0.0 BCS, and 200.0 ± 6.4 kg) and 12 Canchim young bulls (8.0 ± 0.1 months, 5.0 ± 0.0 BCS and 194.0 ± 6.3 kg BW), allocated in shaded pasture areas, with random distributions. For the parameters evaluated in this study, the animals were always handled in batches of six animals, which were conducted in alternating order on each date established for the experimental procedures. This criterion was adopted to prevent a fixed order of evaluation of the animals throughout the experiment and to avoid biases in the responses due to differences in data collection times. The illustration of the experimental design is shown in Fig. 6.

Testicular biometry, consistency, volume, and body weight

Once monthly for 12 months, the following parameters were evaluated. The scrotal circumference was measured using a flexible tape graduated (mm) in the region of the largest circumference of the scrotum[49]. Testicular consistency was assessed by bilateral palpation, and testicular tone was classified from 1 to 5, where 1 = flaccid, 2 = elastic-soft tense, 3 = elastic tense, 4 = elastic firm, and 5 = hard[50]. To determine the testicular volume (cm^3), the length and width of the right and left testicles were measured using a digital metal caliper (mm). Subsequently, the testicular volume was calculated through the spheroid prolate equation, where volume $= 2 \times \left[\frac{4}{3} \times \pi \times \left(\frac{\text{width}}{2}\right)^2 \times \left(\frac{\text{length}}{2}\right)\right]$, according to Bailey et al.[51]. The animals were individually weighed on a digital scale in the handling pen every 30 days.

Ultrasonographic assessments

Echogenicity and heterogeneity of the testicular parenchyma and mediastinum

Ultrasound assessments were performed monthly using a Z60 Vet® device (Mindray Bio-Medical Electronics Co, Shenzhen, China) coupled to a 5.0–8.0 MHz multifrequency linear transducer (52 mm; model 6LE5VP) (Fig. 7). Echogenic values were obtained by B-mode ultrasonographic evaluation of the right and left testicular parenchyma at a frequency of 7.5 MHz (overall gain: 60%; depth 6.2 cm and dynamic range, DR: 70 dB), with images obtained in longitudinal sections with the animals properly restrained in a chute. Testicular scanning was performed in the middle third of the organ, prioritizing the sagittal region. The definition of the region to be analyzed was adopted to follow a pattern of evaluations in the same testicular region of all animals and consequently in all months of the assessment. From the sequence of recorded ultrasound images, three frames were selected and analyzed in Image Pro Plus 7.0™ software (Media Cybernetics, Inc., San Diego, CA, USA) using grayscale pixel numerical values ranging from 0 (absolute black) to 255 (absolute white). Echogenicity (mean pixel value) and pixel heterogeneity (standard deviation of the mean pixel value) were obtained from six ellipses determined in the longitudinal section. Each ellipse was 60 mm wide and 60 mm high and distributed in regions of interest throughout the parenchyma of each testicle[16]. Three ellipses were created for the mediastinum, each measuring 20 mm wide and 20 mm high, distributed throughout the structure.

Width of the mediastinum and vessels of the pampiniform plexus

For measurements, the ImageJ® software (National Institutes of Health, Bethesda, MD, USA) was used. The mediastinal width was measured in frames selected in B-mode ultrasound images with the largest area of the structure presented, in which a line was drawn perpendicular to the structure. To assess the diameter of the pampiniform plexus vessels, three frames demonstrating the largest portions of the vessels were selected, and thus, measurements of the diameter of three portions of the structure were taken, and the averages were subsequently extracted.

Blood perfusion of the testicular parenchyma and pampiniform plexus

Using the color Doppler mode, ultrasound scans of the right and left testicles were performed in cross-section, in the middle third of the organ, prioritizing the sagittal region and the pampiniform plexus in a region of wide observation of the structure. The color Doppler mode settings were also the same during all evaluations (pulse repetition frequency (PRF) of 0.6 kHz; gain of 40%; depth of 8 cm; frequency of 4.4 MHz and wall filter (WF) of 202 Hz). Three consecutive frames from each organ in which the largest area of color Doppler signals was detected were selected, and the region of interest (ROI) for the evaluation was extracted using Adobe Fireworks® software (Adobe Systems Incorporated, San Jose, CA, USA). Subsequently, the extracted ROI was input into a color Doppler image analysis algorithm (PerfUS—Algorithm for ultrasound-based blood perfusion measurements), and the following variables were obtained: Relative area of color Doppler of parenchyma (RDA parenchyma, %); Color Doppler area of parenchyma (DA parenchyma, mm^2); Relative of high-speed Doppler pixels of parenchyma (HSP parenchyma, %); Relative of low-speed Doppler pixels of parenchyma (LSP parenchyma, %), Relative area of color Doppler of plexus (RDA plexus, %); Color Doppler area of plexus (DA plexus, mm^2); Relative of high-speed Doppler pixels of plexus (HSP plexus, %); Relative of low-speed Doppler pixels of plexus (LSP plexus, %).

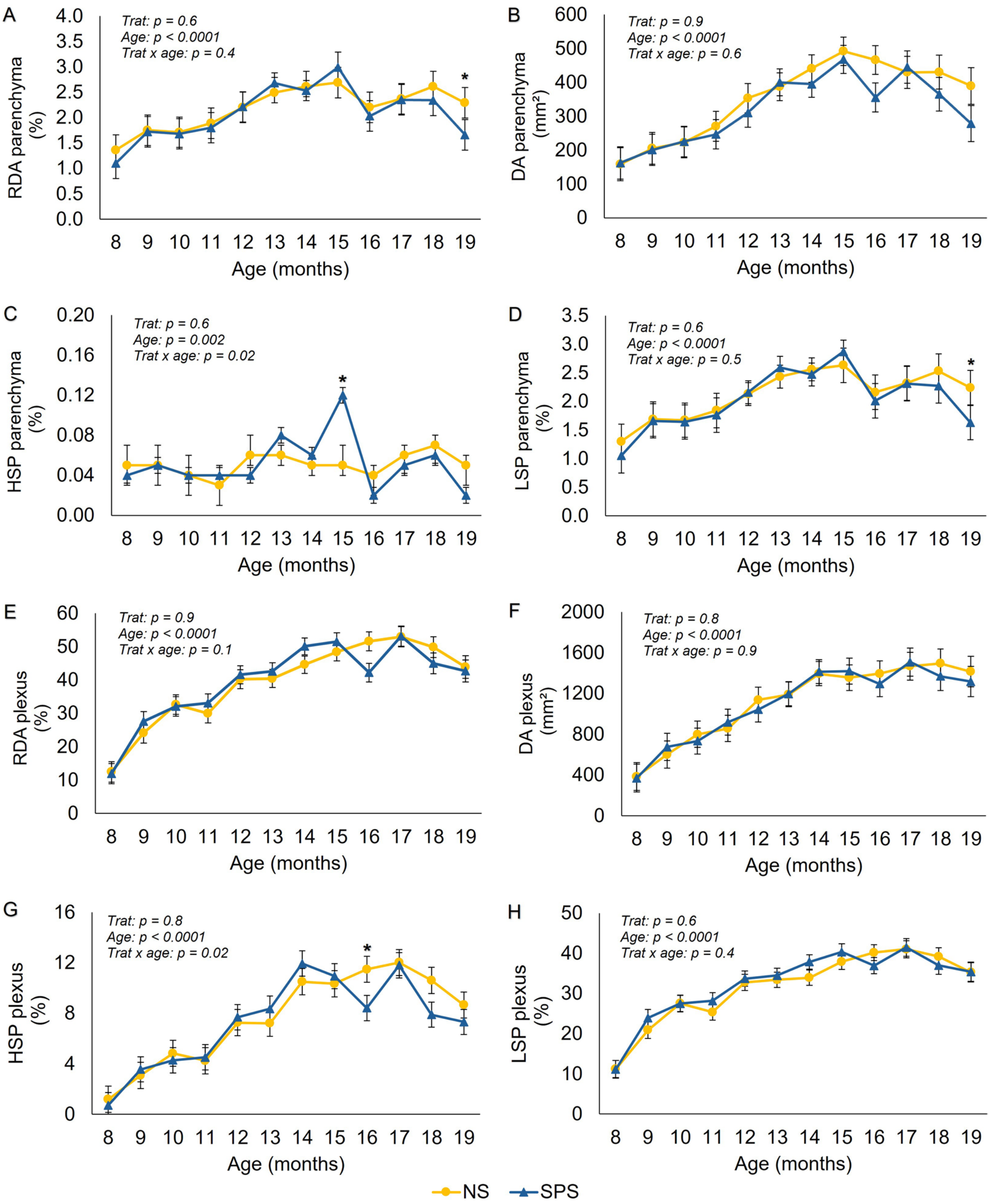

Fig. 6. Experimental design of the study to evaluate the effect of production systems on testicular ultrasound characteristics and serum testosterone concentrations of young bulls. The animals were evaluated for 12 months in non-shaded (NS) or silvopastoral (SPS) systems. The present evaluations were performed once a month, measuring biometric variables (scrotal circumference, width, height and testicular volume) and testicular consistency. Blood samples were collected for serum testosterone levels. For ultrasonography, the following variables were evaluated: B-mode (echogenic values of the testicular parenchyma and mediastinum, width of the mediastinal vessels and plexus), color Doppler (blood perfusion and flow velocity of the testicular parenchyma and pampiniform plexus) and spectral Doppler (pulsatility and resistance indices of the supratesticular artery).

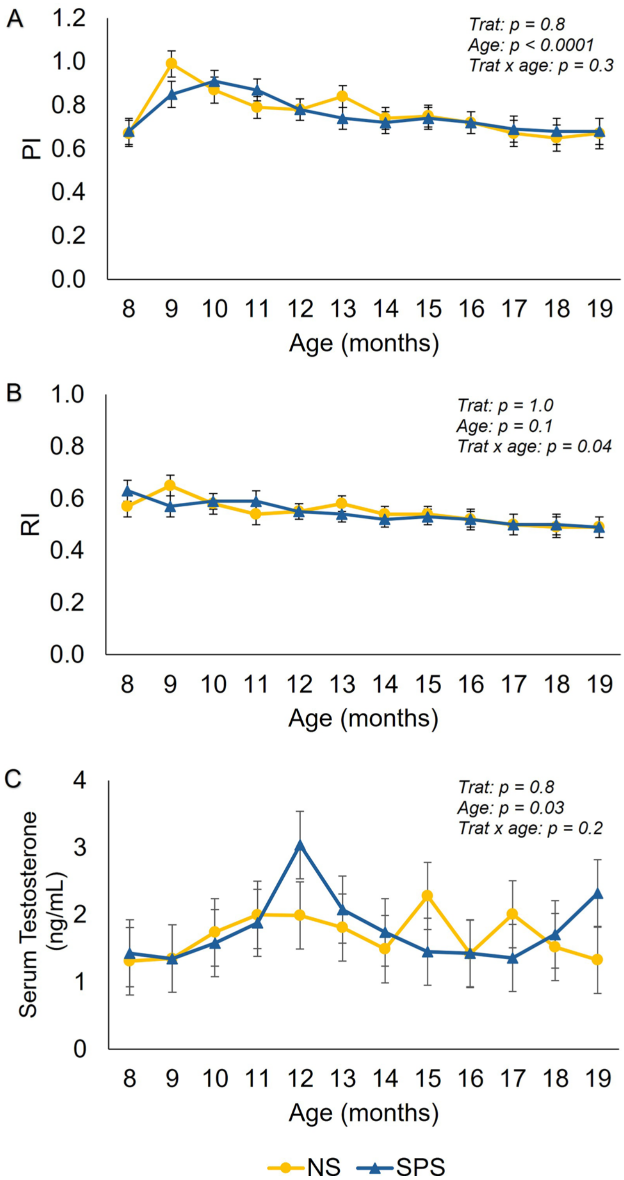

Fig. 7. Illustrative images of the ultrasound evaluation of the testicular parenchyma and pampiniform plexus of young bulls in non-shaded (NS) or silvopastoral (SPS) systems. (**A**) B-mode image of the testicular parenchyma, (**B**) B-mode image of the pampiniform plexus, (**C**) color Doppler mode image of the pampiniform plexus, (**D**) spectral Doppler mode image of the supratesticular artery.

Hemodynamics of the supratesticular artery
The hemodynamics of the pampiniform plexus were assessed by ultrasonography in spectral Doppler (pulsed) mode. The transducer was positioned over the spermatic cord, an average of two to four centimeters above the testicle, to allow a perpendicular approach to the supratesticular artery. The scan was initiated in B-mode, and once the structure was identified, the color Doppler and spectral Doppler modes were consecutively activated to identify the vessels and record the blood flow characteristics detected in at least three cardiac cycles. After image acquisition, one of the cycles was measured to obtain the parameters of interest. In all evaluations, the insonation angle relative to the vessel's longitudinal axis was always less than 60° to avoid underestimation of the systolic peak[16]. The spectral indices were obtained by manual tracing using proprietary software embedded in the Z60 Vet® equipment (Shenzhen Mindray Bio-Medical Electronics Co., China). The following indices were evaluated: resistance index (RI = [PS–EDV]/PSV) and pulsatility index (PI = [PSV–EDV]/mean velocity), where PSV = peak systolic velocity (cm/s) and EDV = end-diastolic velocity (cm/s). All evaluations were performed with the following settings: pulse repetition frequency (PRF) of 3.6 kHz; gain of 40%; depth of 8 cm; frequency of 4.4 MHz and wall filter (WF) of 101 Hz.

Serum testosterone concentrations

Blood samples were collected monthly from all bulls for hormonal measurements. For serum testosterone, blood samples were collected by jugular venipuncture at a predefined time (8:00–10:00 a.m.) in 10 mL siliconized vacuum tubes without anticoagulant. Blood samples were centrifuged (Centrifuge KC4, Kindly Ind. Com. Equip. Médicos Ltda, São Caetano do Sul, SP, Brazil) at 4000 revolutions per minute (RPM) for 15 min to separate the serum, which was fractionated into aliquots and stored in polypropylene microtubes at − 20 °C for later analysis. Serum testosterone was measured using the commercial ImmuChem TM Testosterone Double Antibody kit (MP Biomedicals Diagnostics Division, Solon, OH, USA). The dosages were determined by radioimmunoassay with assay sensitivity and intra-assay coefficient of 0.08 ng/mL and 7%, respectively.

Variables and statistical analysis

Climatic variables (AT, BGT, RH, WS, RHL, and BGHI) were recorded throughout the experiment, and monthly averages were presented between 08:00 a.m. and 06:00 p.m. or between the ultrasound evaluation interval (10:00 a.m. to 2:00 p.m.). The values of the variables were recorded in monthly collections over 12 consecutive months. Subsequently, the collection dates were replaced by the average age of the animals in each month, allowing the association of the collected data with the respective ages of the animals at the time of the evaluation (8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18 and 19 months). The following variables were collected: biometric, B-mode ultrasound, color Doppler, spectral Doppler, and hormone concentrations. For the echogenic variables, blood perfusion and width of the pampiniform plexus vessels, three measurements were performed per testis (left and right), and subsequently, the averages per animal were generated. To evaluate the hemodynamic indices of the supratesticular artery (right and left) and the width of the testicle mediastinum (left and right), averages were taken per animal. Statistical analysis of the left and right organs was performed. However, no statistical differences were found, so it was decided to conduct the analyses using the averages.

In general, no triple interactions were observed between production systems, breeds and age. Thus, the data presented for all variables in this study are assessments of possible interactions between production systems (NS × SPS) and age (8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, and 19 months old). The model included the fixed effects of treatment and age class, the interaction between these two effects, the use of body weight as a covariate, and batch and BCS as random effects. Data were analyzed by the PROC MIXED with a repeated statement to evaluate the interaction between sequential measures. Climatic variables were compared between groups by one-way ANOVA, and a post-hoc comparison was performed using Tukey's test. Mean differences were considered when $p < 0.05$. Analyses were performed using Statistical Analysis System (SAS) software. Results in the text, tables, and figures are represented as LS means ± standard error of the mean (SEM).

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Declarations

Competing interests
The authors declare no competing interests.

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