



UNIVERSIDADE DE BRASÍLIA

FACULDADE DE AGRONOMIA E MEDICINA VETERINÁRIA

Programa de Pós-Graduação em Saúde Animal

**VARIAÇÃO DA BIOMETRIA TESTICULAR DE GARANHÕES DA RAÇA
MANGALARGA MARCHADOR DURANTE UM ANO E SUA INFLUÊNCIA NA
PRODUÇÃO HORMONAL E QUALIDADE SEMINAL.**

THIAGO RODRIGUES CARDOSO BRAGA

DISSERTAÇÃO DE MESTRADO EM SAÚDE ANIMAL

BRASÍLIA - DF

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PROGRAMA DE PÓS-GRADUAÇÃO EM SAÚDE
ANIMAL, COMO PARTE DOS REQUISITOS
NECESSÁRIOS À OBTENÇÃO DO GRAU DE
MESTRE EM SAÚDE ANIMAL. O PRESENTE
TRABALHO FOI REALIZADO COM APOIO DA
COORDENAÇÃO DE APERFEIÇOAMENTO DE
PESSOAL DE NÍVEL SUPERIOR – BRASIL
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LISTA DE ABREVIATURAS E SIGLAS

APOP - Apoptosis

CASA - Computer assisted sperm analysis

CC - Capacitated cells

COR - Cortisol

FSH - Hormônio folículo estimulante

GnRH - Hormônio liberador de gonadotrofinas

HFP - High fertility potencial

IC - Intact cells

ICWAR - Intact cells without acrossomal reaction

LFP - Low fertility potential

LH - Hormônio luteinizante

LT - Left testicle

LTH - Left testicle high

LTL - Left testicle length

LTV - Left testicle volume

LTW - Left testicle width

MAD - Major defects

MID - Minor defects

PM - Progressive motility

RT - Right testicle

RTH- Right testicle high

RTL - Right testicle length

RTV- Right testicle volume

RTW - Right testicle width

TD - Total defects

TEST - Testosterone

TM - Total motility

TSP - Total sperm production

TTV - Total testicular volume

CAPÍTULO 1

1. RESUMO

O equino é uma espécie poliéstrica sazonal, fotoperiódica positiva, com efeitos da sazonalidade para as fêmeas da espécie bem descritos na literatura. Entretanto os efeitos da sazonalidade no macho são variáveis de acordo com a latitude que esses animais se encontram. Ainda é conflitante como e quais parâmetros de qualidade seminal sofrem efeito da sazonalidade, sendo descritas alterações de volume espermático, concentração, motilidade, defeitos e produção espermática e hormonal. Outro fator a apresentar alterações durante as estações é a morfometria testicular, sendo descrito principalmente alterações do volume testicular pelas estações. Assim credita-se que o principal fator a afetar tal alteração no volume testicular seja a mudança nas populações espermáticas, tais mudanças destes grupos celulares afetariam além da morfometria testicular a produção seminal e hormonal do órgão, uma vez que estas são as células responsáveis por tal função. Portanto, objetivou-se avaliar se a biometria testicular se altera entre as estações do ano, e se tais alterações implicariam em mudanças na produção hormonal e seminal de garanhões mantidos em regiões de baixa latitude.

Palavras-chave: equino, fotoperíodo, hormônio, qualidade espermática, testículo

2. ABSTRACT

The equine is a seasonal, photoperiodic species with positive effects of seasonality well described in the literature for females of the species. However, the effects of seasonality on males vary according to the latitude where these animals are located. It is still conflicting how and which parameters of seminal quality are affected by seasonality, with alterations in sperm volume, concentration, motility, defects, and spermatogenic and hormonal production being described. Another factor that undergoes changes during the seasons is testicular morphometry, with alterations in testicular volume mainly described across seasons. It is thus believed that the primary factor affecting such changes in testicular volume is the change in sperm populations; these changes in cellular groups would affect not only testicular morphometry but also the seminal and hormonal production of the organ since these are the cells responsible for such functions. Therefore, the objective was to evaluate whether testicular biometry changes between seasons and whether such alterations imply changes in hormonal and seminal production of stallions kept in low latitude regions.

Keywords: hormones, sperm quality, equine, photoperiod, semen, testes

3. INTRODUÇÃO

A espécie equina caracteriza-se como poliéstrica sazonal, fotoperiódica positiva, indicando um período de atividade reprodutiva nas estações do ano de maior incidência luminosa, e um período com menos atividade reprodutiva nas estações do ano de menor incidência luminosa (Nagy et al., 2000; Murphy, 2019). Tratando-se de uma estratégia evolutiva para que os partos aconteçam na época mais favorável ao desenvolvimento dos potros (Nagy et al., 2000).

O efeito da sazonalidade é bem descrito e estudado nas fêmeas da espécie, tanto a fim de entender tal condição, como de desenvolver técnicas para manipular o período reprodutivo, assim como para melhor atender demandas comerciais (Mc Cue et al., 2007; Murphy et al., 2014; Murphy, 2019). Entretanto ainda não há um consenso sobre o efeito exato da sazonalidade sobre o macho, sendo claro haver efeito sobre o reprodutor, porém não há unanimidade entre os trabalhos sobre quais os parâmetros reprodutivos que de fato sofrem alterações (Clay & Clay, 1992; Blanchard et al.; 2008; Dhakal et al., 2011; Leme et al., 2012; Rua et al., 2017; Ebel et al., 2020; Freitas et al., 2023).

É relatado que parâmetros como volume testicular, libido, produção, concentração e motilidade espermática, bem como concentrações plasmáticas de testosterona, hormônio folículo estimulante (FSH) e hormônio luteinizante (LH), sofrem alterações fora do período reprodutivo (Janett et al., 2003; Rua et al., 2017; Aissanou & Ayad, 2020). Acredita-se que, assim como as éguas mantidas em localidades de menor latitude tem uma tendência a apresentar um período anovulatório menor ou mesmo não o apresentar, garanhões mantidos em tais locais podem sofrer baixa ou nenhuma influência da sazonalidade nos parâmetros reprodutivos (Quintero et al., 1995; Ginther et al., 2004; Aurich, 2011; Leme et al., 2012; Cerqueira et al., 2019; Freitas et al., 2023)

Este efeito se deve pela variação de disponibilidade luminosa durante o ano em diferentes regiões do planeta. Uma vez que a sazonalidade reprodutiva se trata de um evento biológico determinado por ritmos biológicos cíclicos, que por sua vez são regulados por estímulos ambientais (Braga & Oliveira, 2023). O principal fator ambiental determinante para as funções rítmicas do corpo é a luminosidade diária percebida (Lucas et al., 2014; Dini et al., 2019; Murphy, 2019). Noites mais longas aumentam a produção de melatonina, que passa a bloquear o eixo hipotalâmico-hipofisário-gonadal, inibindo a secreção de hormônio liberador de gonadotrofinas (GnRH; Nagy et al., 2000; Aurich, 2011; Satué & Gardón, 2013).

Assim as alterações de FSH e LH circulantes influenciam na população, proporção e desenvolvimento das células testiculares durante as diferentes estações do ano. Influenciando tanto no tamanho do órgão como na sua funcionalidade, referente a produção hormonal e de gametas (Clay & Clay, 1992; Blanchard et al; 2008; Dhakalet al., 2011; Ebel et al., 2020).

Objetivou-se revisar os principais pontos a relacionados à função testicular e às alterações reprodutivas decorrentes da sazonalidade na espécie equina, a fim de contextualizar a necessidade de um maior entendimento da relação da função testicular do garanhão de acordo com as estações do ano em diferentes localidades.

4. REVISÃO BIBLIOGRÁFICA

4.1 Sazonalidade

A área da ciência destinada ao estudo dos eventos rítmicos do organismo se denomina cronobiologia. Os ritmos biológicos do corpo podem abranger fenômenos cíclicos com durações restritas a menos de um dia, ou a mudanças fisiológicas visíveis ao longo do ano (Murphy, 2019). Os ritmos biológicos tratam-se de eventos de geração

endógena, porém regulados por fatores externos, sendo o principal deles a luminosidade (Murphy, 2019). Dentro da reprodução o principal ritmo biológico em equinos trata-se da sazonalidade reprodutiva da espécie, na qual apresenta período reprodutivo em época de alta incidência luminosa e período de menor atividade reprodutiva em épocas de baixa incidência luminosa (Nagy et al., 2000; Saué & Gardón, 2013).

A regulação da sazonalidade reprodutiva se dá principalmente pelos níveis de melatonina, sendo esses responsivos aos estímulos captados pelos cones, bastonetes e células intrinsecamente fotorreceptivas da retina (Nagy et al., 2000; Murphy, 2019). Quando em dias longos o estímulo luminoso captado por tais células é transmitido à glândula pineal, que por sua vez diminui a liberação de melatonina. A melatonina atua principalmente regulando o núcleo supraquiasmático, considerado relógio central das funções rítmicas do corpo, e o hipotálamo, que por sua vez ante à diminuição dos níveis de melatonina aumenta a secreção do hormônio liberador de gonadotrofinas (GnRH) (Palmer & Guillaume, 1992; Nagy et al., 2000; Saué & Gardón, 2013; Lucas et al., 2014; Murphy et al., 2019). O GnRH estimula a hipófise, que então aumenta a produção e secreção principalmente de hormônio luteinizante (LH), uma vez que as taxas basais de hormônio folículo estimulante (FSH) tendem a se manter estáveis durante o ano (Chen et al., 1993; Gerlach & Aurich, 2000; Ginther et al., 2004; McCue et al., 2007; Williams et al., 2012)

A interpretação de um dia como sendo longo ou curto pelo organismo é determinado pelas horas de escuridão no dia ao invés de horas de luminosidade, pois a melatonina é secretada quando não há estímulo da retina. Desta forma um dia longo para a espécie equina é determinado como aquele apresentando no máximo 10 horas de escuridão em um ciclo de 24 horas (Palmer & Guillaume, 1992; Braga & Oliveira, 2023). Fator associado à denominada fase de fotossensibilidade do ciclo, período

entorno de 9 horas e meia após o escurecer no qual a produção de melatonina encontra-se mais sensível a alterações de luminosidade. Caso estimulado luminosamente até esta fase, o corpo interpreta o dia como longo, caso esta fase passe sem que haja estímulo luminoso o corpo interpreta o dia como sendo curto (Palmer & Guillaume, 1992; Nagy et al., 2000).

Mesmo que a luminosidade diária seja o principal fator a regular a sazonalidade reprodutiva, este não é o único, a temperatura, alimentação, condição corporal, idade e duração dos ciclos passados podem influenciar, mesmo que em menor escala, os ritmos biológicos (Guerin & Wang, 1994; Nagy et al., 2000; Chemineau et al., 2008; Araújo & Oliveira, 2019; Dini et al., 2019). A latitude do local na qual o animal se encontra também influencia na sazonalidade, uma vez que locais mais próximos à linha do Equador apresentam menor variação luminosa durante o ano, apresentando maiores taxas de animais que não entram em anestro reprodutivo (Quintero et al., 1995; Aurich, 2011; Cerqueira et al., 2019).

A sazonalidade reprodutiva é bem descrita na espécie equina no ciclo reprodutivo das fêmeas. Apresentando em localidades de maior latitude ausência ciclos estrais e consequentemente ovulações em períodos de menor luminosidade (inverno), e plena atividade reprodutiva em períodos de alta luminosidade (verão), com períodos transicionais entre eles (outono e primavera) (Ginther et al., 2004; Aurich, 2011). Entretanto o garanhão não apresenta uma variação tão clara dos seus parâmetros reprodutivos entre as estações, mantendo produção seminal por todo o ano (Freitas et al., 2023).

Não há um consenso sobre os parâmetros que sofrem alterações por conta da sazonalidade, os resultados até o presente momento são conflitantes, sendo descrito possíveis mudanças na biometria testicular, integridade do acrossoma, concentração,

motilidade, morfologia, viabilidade e produção espermática (Johnson & Thompson, 1983; Johnson, 1985; Clay & Clay, 1992; Blanchard et al; 2008; Gamboa et al., 2010; Dhakal et al., 2011; Ebel et al., 2020). Ainda assim é notório o efeito da sazonalidade sobre a variação hormonal, principalmente dos níveis de testosterona, LH e inibina (Gerlach & Aurich, 2000; Dhakal et al., 2011; Ball et al., 2019).

Trabalhos realizados no hemisfério sul demonstram baixa influencia da sazonalidade em qualidade e produção seminal e tamanho testicular quando comparado estação reprodutiva com estação não reprodutiva (Leme et al, 2012; Silva et al., 2021 Freitas et al., 2023). Porém faltam trabalhos que abordem dados comparando estação a estação.

4.2 Espermatogênese

Espermatogênese trata-se do processo de divisão e diferenciação celular que ocorre no testículo, na qual uma célula germinativa produzirá gametas haplóides aptos à fecundação e formação de um novo indivíduo. Tal processo é dividido em 3 etapas: espermatocitogênese, processo mitótico em que uma espermatogônia produz outras espermatogôneas (Johnson et al, 1997). Meiose, processo de divisão celular com separação do material genético, resultando em espermátides haplóides. E espermiogênese, processo de diferenciação celular onde a já haplóide espermátide altera seu formato e função originando o espermatozoide, célula responsável pela fecundação do oócito (Johnson et al, 1997).

Todos esses processos acontecem de forma cíclica no testículo dentro dos túbulos seminíferos, havendo sempre diversas células em todas as fases celulares a qualquer dado momento, tornando a produção espermática constante, ao contrário da

fêmea que apresenta ciclos com momentos férteis e não férteis (Swierstraet al., 1974). Além das células germinativas, responsáveis pela produção de gametas (espermátogoneas, espermátocitos, espermátides), o testículo também apresenta células somáticas, células de Sertoli e células de Leydig.

As células de Sertoli apresentam função estrutural fazendo parte da barreira hemato-testicular, realizando a comunicação entre as células germinativas e os componentes sanguíneos, além de produzir estrógenos, inibina e ativina (Roser, 1997; Roser, 2008). O número de células de Sertoli parece estar associado à quantidade de células germinativas e à estação do ano (Johnson et al, 1991; Johnson et al, 1997; Dhakal et al, 2011)

As células de Leydig atuam mantendo a espermatogênese através da produção de testosterona e estrógenos adjacente aos túbulos seminíferos (Roser,1997; Roser, 2008). Assim como os outros tipos celulares, a população de células de Leydig também aparentam se alterar entre as estações do ano. A atividade das células de Leydig e de Sertoli são estimuladas pela secreção de LH e FSH na corrente sanguínea, alterando sua população e produção hormonal de acordo com a variação sazonal de FSH e LH, determinada pela produção de melatonina (Roser, 2008; Dhakal et al, 2011). A regulação da produção de GnRH, LH e FSH ocorre pelos níveis séricos de hormônios e proteínas testiculares, como estrógenos e testosterona, enquanto estímulos parácrinos por meio da inibina e fator de crescimento insulínico tipo 1 regulam a função testicular (Roser, 1997; Roser, 2008; Ball et al., 2019; Dhakal et al., 2011).

4.3 Termorregulação

O testículo trata-se da gônada masculina, especializado em produção de gametas e síntese de hormônios. Para exercer sua plena função sua temperatura deve estar de 2 a

201 6 graus abaixo da temperatura corporal, por isso sua localização está fora da cavidade
202 abdominal (Kastelic, 1999; Rizzoto & Kastelic, 2020). As estruturas anatômicas que os
203 cercam também corroboram para manutenção da temperatura ideal, a pele do escroto é
204 responsável pela troca de calor do órgão interno, testículo, com o meio externo,
205 diminuindo a temperatura testicular(Kastelic, 1999). Quanto maior a superfície escrotal,
206 maior a troca de calor. Além do escroto outras estruturas anatômicas auxiliam na
207 termorregulação, como a túnica Dartos, músculo cremáster, responsáveis por,
208 respectivamente, aproximar ou afastar o testículo da cavidade abdominal e aumentar ou
209 diminuir a área da superfície escrotal (Rizzoto & Kastelic, 2020). Por fim uma das
210 principais estruturas responsáveis pela termorregulação testicular trata-se do plexo
211 pampiniforme, uma rede vascular envolvendo artéria e veia testicular, onde por meio de
212 um sistema de contracorrente o sangue arterial é esfriado pelo sangue venoso antes de
213 adentrar o testículo (Rizzoto & Kastelic, 2020).

214 Tais mecanismos são importantes para a atividade do órgão uma vez que ele
215 apresenta sensibilidade principalmente ao aumento da temperatura. Acredita-se que o
216 aumento da temperatura testicular leva ao aumento do metabolismo basal e atividade
217 celular do órgão (Kastelic, 1999; Rizzoto & Kastelic, 2020). Desta forma aumenta-se
218 também o consumo dos substratos celulares necessários para a atividade do órgão,
219 dentre eles o oxigênio. Porém devido à separação dos túbulos seminíferos da sua
220 vascularização, a barreira hemato-testicular, o aumento da demanda por oxigênio
221 tecidual não costuma ser atendido pela oferta vascular. Mesmo com um possível
222 aumento do fluxo sanguíneo no órgão, o aumento da vascularização em si também
223 levaria um aumento da temperatura transferida ao testículo (Kastelic, 1999).

224 Assim instala-se um processo de hipóxia tecidual, afetando tanto a produção
225 hormonal, quanto a espermatogênese, resultando em menor concentração, produção,

motilidade espermática, bem como aumento dos defeitos nos espermatozoides(Kastelic, 1999; Durairajanayagam et al., 2014; Lucio et al., 2016; Rizzoto et al., 2020). Caso não corrigido, o processo de hipóxia tecidual pode evoluir a um quadro de degeneração testicular (Ball, 2008; Batissaco et al., 2013). Levando a perda permanente de células germinativas, apoptose, desestruturação dos túbulos seminíferos, defeitos no DNA espermático (Turner, 2007; Durairajanayagam et al., 2014; Lucio et al., 2016).

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CAPITULO 2

ARTIGO CIENTÍFICO

Variation in stallion testicular measurements over a year and its influence on hormonal production and sperm quality

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Abstract

Although studies carried out in the Northern Hemisphere have shown variations in the stallions' sperm quality between seasons, the same result was not found in the Southern Hemisphere. This is due to some factors, mainly the latitude where the animals are. Therefore, the aim of this research was to evaluate the influence of seasons on the variation of testicular morphometric parameters and its consequent role in hormonal production (serum cortisol, and testosterone values) and seminal quality parameters in stallions kept at a low latitude. Eight stallions were followed for one year, with morphometric measurements of the testes, hormonal assays and semen collections being carried out every 15 days. Semen samples were evaluated using CASA system and flow cytometry. Testicular measurements (length, width, and height) were obtained using B-mode ultrasound, and radioimmunoassay was used for hormonal assessment. The testicular length remained stable throughout the year, while width and height showed variations, mainly in winter ($p < 0.05$). Between the hormonal levels, only testosterone had an increase in spring ($p < 0.05$). The morphometric measurement that presents the

highest correlation with seminal quality parameters was total testicular width. Although a small variation was found in the seminal quality parameters evaluated, there was no impact on seminal quality.

Keywords: hormones, sperm quality, equine, photoperiod, semen, testes

1. Introduction

Horses are seasonally breeding animals, however, stallions seems to be less affected by the photoperiodism than mares. In norther hemisphere, it has been described changes in testicular morphometric parameters, seminal quality and hormonal parameters in stallions (Johnson & Thompson, 1983; Johnson, 1985; Jasko et al., 1991; Clay & Clay, 1992; Blanchard et al., 2008; Dhakal et al., 2011; Carluccio et al., 2013; Ebel et al., 2020) in comparison with the southern hemisphere, in which only small reproductive variations have been described in the literature (Leme et al., 2012; Rua et al., 2017; Freitas et al., 2023).

Since the day-length is the main factor determining seasonality (Lucas et al., 2014; Braga and Oliveira, 2023), more detailed descriptions of seasonal reproductive changes in stallions in different countries with different latitudes are needed. There being few studies with seasonality in low latitudes places, specially in the southern hemisphere (Quintero et al., 1995, Freitas et al., 2023).

The endocrine mechanism that explains seasonality occurs through the retinal reception of changing environmental light information (24h light/dark cycles), increasing the production of melatonin, which starts to block the hypothalamic-pituitary-gonadal axis, inhibiting the secretion of gonadotropin-releasing hormone (GnRH) (Nagy et al., 2000; Aurich, 2011; Satué & Gardón, 2013; Lucas et al., 2014).

Such variations in GnRH secretion led to changes in LH and FSH secretion and consequently in spermatogenesis, steroidogenesis and physical characteristics of the testicles, mainly testicular volume (Johnson, 1985; Roser, 1997; Roser, 2008).

Under the influence of the hypothalamic-pituitary-gonadal axis, different cell groups are more stimulated in different seasons. Thereby, causing changes in testicular measurements, due to changes in the cell populations of the testicular parenchyma (Johnson, 1985; Roser, 2008). As well as changes in the hormonal testicle production rates, due to the change in the proportion of testicular cells that produce them and the change in the circulating levels of the axis' predecessor hormones, LH and FSH (Janett et al., 2003; Rua et al., 2017; Aissanou & Ayad 2020). Therefore, as the testicular size is determined mainly by its cells populations, it can be a great predictor of sperm and hormonal production (Roser, 1997; Roser, 2008; Rua et al, 2017; Silva et al., 2021).

Therefore, our aim was to evaluate whether testicular measurements vary during the seasons of the year, and if these changes would imply a variation in hormonal production and consequent seminal quality in stallions located at a low latitude.

3. Materials and methods

All procedures were approved by the Ethics Committee in Animal Experimentation (CEUA) of the Institute of Biological Sciences at the University of Brasília under protocol UnB Doc No. 45,631/2016.

3.1 Animals

It was used 8 healthy light-breed stallions (Mangalarga Marchador) aged 4 to 10 years old (average of 6 years old), weighing an average of 390kg. The stallions were housed in stalls, with access to paddocks for at least 2 hours a day, they would eat

pasture and cutted grass at will, as well as 2 kg of balanced rations. The experiment was carried out in Brasília, Brazil(15° 45' 42.5" S, 47° 52' 10.5" W). All the reproductive parameters were evaluated every 15 days for a period of one year, covering all seasons (Spring, Summer, Autumn, and Winter).

3.2 Testicular measurement by ultrasound evaluation

Testicular volume can be calculated from the combined testicular width values or from a relationship of testicular height, length, and width (Hernández-Avilés & Love, 2021). The mathematical model chosen uses width, length, and height to estimate testicular volume, as described by Love et al. (1991), and compare it with its real production accumulated over a period of 15 days, the period between the semen collections.

The left and right testicular volume (LTV and RTV) and the total testicular volume (TTV) were obtained every 15 days using an A5V ultrasound device (Sono Scape) with a 7 MHz linear probe. The length (L), width (W) and height (H) of each testicle was calculated to obtain LTV, RTV and TTV, following the previously described technique for stallion measurements (Love et al., 1991). Longitudinal and transversal plans were performed in each testicle, being the epididymis excluded from testicular US measurements. The electronic cursors were placed at the limit of tunica albuginea, and after three consecutive scans, the following parameters were obtained considering the largest measurement (cm): right and left length (L), height (H), and width (W) (cm). Right and left testicular volume (TV) were calculated using the Lambert formula, $TV = L \times W \times H \times 0.5233$, used to measure the volume of an ellipsoid (Love, 2014). Total testicular volume (TTV), which represents the sum of the right and left TV, was obtained for each stallion.

3.3 Semencollection

Semen collection was performed at each 15 days over a year. A Botucatu artificial vagina was used for it, as described by Freitas et al. (2016).

3.4 Seminal evaluation

Immediately after semen collection, volume (mL), aspect (density and colour), sperm concentration ($\times 10^6$ /mL), total sperm count in the ejaculate ($\times 10^9$), and sperm morphology (%) were assessed for fresh semen using CBRA (2013) standards. To determine concentration, sperm were counted in a haematology chamber (Neubauer) under a 400x optical microscope, after semen dilution (1:20) in buffered formalin citrate (CBRA, 2013). Sperm morphology was assessed using phase contrast microscopy in a humid chamber and was classified as major defects, minor defects, total defects, and normal sperm (CBRA, 2013).

For spermatozoon kinetic analysis, 10 μ L of semen was placed on a reading slide (Makler Counting Chamber, Sefi Medical Instruments, California, USA) and examined with a computer-assisted sperm analyser (Ivos-Ultimate 12, Hamilton Thorne Biosciences, Massachusetts, USA) adjusted for horses (SETUP 'equine species'). The parameters were as follows: 45 frames acquired at 60 frames/s; minimum contrast 70; minimum cell size four pixels; trajectory speed cut-off 20 mm/s; progressive motility cut-off 50 mm/s and linear motility 60%; linear motility cut-off 0 mm/s. Slow cells were considered static. Three random fields were selected for reading and analysis, as previously described (Freitas et al., 2016). The total motility (%; TM) and progressive motility (%; PM) were evaluated.

Flow cytometry analysis evaluation of the acrosome status, sperm capacitation, plasma membrane integrity, and mitochondrial membrane potential was performed by flow cytometry using the AMNIS FlowSight Image Cytometer (Amnis Corp., Seattle, USA) and INSPIRE V6.1 acquisition software. The fluorescent dyes were excited using a laser set at 35 mW and 488 nm. The signals emitted by the green probes were collected in channel 2 (505–560 nm), those emitted by the orange probes were collected in channel 3 (560–595 nm), and the red signals were collected in channel 4 (595–642 nm). A specific acquisition template was created to identify and acquire stallion sperm cells. Thus, 10,000 events were collected per sample or parameter. To analyse the results, dot plot graphs or histograms were created from unstained control samples, and the populations were gated based on the staining patterns. According to the manufacturer's instructions and recommendations, a compensation matrix composed of more than one fluorescent dye was used for all evaluations, and a complete calibration of the equipment was performed each time the system was powered on (Cunha et al., 2019).

3.5 Hormonal assessment

To measure the plasma testosterone and cortisol levels, blood was collected from each stallion once every 15 days via a jugular puncture in the morning between 9:00 and 11:00. After centrifugation, the serum was stored at – 20 °C. Subsequently, plasma cortisol and testosterone concentrations were determined using a solid-phase radioimmunoassay commercial kit (ImmunoChem, MP Biomedicals LCC Diagnostic Division, Solon, Ohio, USA), according to the manufacturer's instructions (Leme et al., 2012; Rua et al., 2015). The intra-assay coefficients of variation were 6.3% for cortisol and 5.4% for testosterone.

3.6 Statistical analysis

All analyses were performed using SAS general linear model procedures (SAS Institute, Wangen, Switzerland). Multiple analysis of variance (ANOVA) were performed to evaluate interactions between animals, fertility and period for all variables analysed. To determine seasonal changes in testicular morphometry parameters, semen quality and hormonal levels, four periods of three months each were defined as summer (December, January, and February), autumn (March, April, and May), winter (June, July, and August) and spring (September, October, and November). The means of seminal characteristics by season were compared using the Tukey test. Values were considered statistically significant at $p < 0.05$.

4. Results

The variations in testicular morphometric measurements during the seasons are described in Table 1 and Graph 1. The average testicular length was stable throughout the year. However, the average testicular height and width showed changes mainly between winter and the other seasons, consequently the testicular volume also showed a difference ($p < 0.05$).

Winter had the lowest values in all measurements, except for testicular length. In winter was observed a lower testicular width than summer and spring, and a lower testicular height than summer ($p < 0.05$).

Table 1. Seasonal effect over testicular biometry obtained by ultrasonography in stallions (n=8) (mean $\pm$ standard deviation).

Testicular	Summer	Autumn	Winter	Spring
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biometry

Length LT	7.5 ± 0.08	7.61 ± 0.09	7.62 ± 0.06	7.46 ± 0.07
Length RT	7.54 ± 0.12	7.59 ± 0.08	7.64 ± 0.07	7.64 ± 0.07
Width LT	4.70 ± 0.07 ^a	4.54 ± 0.06 ^b	4.22 ± 0.06 ^c	4.66 ± 0.05 ^{ab}
Width RT	4.68 ± 0.06 ^a	4.38 ± 0.06 ^{bc}	4.27 ± 0.05 ^c	4.50 ± 0.04 ^b
Height LT	4.92 ± 0.06 ^a	4.58 ± 0.07 ^b	4.62 ± 0.05 ^b	4.79 ± 0.04 ^a
Height RT	4.75 ± 0.07 ^a	4.76 ± 0.06 ^a	4.60 ± 0.04 ^b	4.72 ± 0.04 ^{ab}
TTV	184.36 ± 4.38 ^a	165.62 ± 3.87 ^b	158.77 ± 2.80 ^b	173.62 ± 2.37 ^a

LT, left testicle; RT, right testicle; TTV, total testicular volume (cm³). Length, Width, Height (cm). Different letters in the same line show statistical difference between seasons (p<0,05).

593

594 The variations in sperm production, quality, integrity, and morphology are
595 shown in Table 2.

Table 2. Seasonal effect over semen parameters in stallions (n=8) (mean ± standard deviation).

	Summer	Autumn	Winter	Spring
TSP	6,03 ± 0,36 ^{ab}	5,0 ± 0,40 ^{bc}	6,51 ± 0,34 ^a	4,86 ± 0,36 ^c
Total motility	80,17 ± 1,39 ^a	80,0 ± 1,51 ^a	77,95 ± 1,62 ^a	84,78 ± 1,51 ^b
Progressive motility	32,93 ± 1,64	36,36 ± 1,61	35,13 ± 1,65	36,53 ± 1,21
Major defects	21,52 ± 1,67	20,15 ± 1,80	20,44 ± 1,70	17,94 ± 1,39
Minor defects	22,49 ± 1,02 ^a	16,03 ± 0,96 ^b	19,30 ± 1,05 ^c	15,75 ± 0,70 ^b
Total defects	44,43 ± 1,39 ^a	36,77 ± 1,83 ^{bc}	38,03 ± 1,87 ^b	32,54 ± 1,45 ^c

TSP, total sperm production (concentration x volume). Different letters in the same line show statistical difference between seasons (p<0,05).

596 The mean values of total sperm production (TSP) were higher in winter. Total
597 motility (TM) exhibited higher mean values in spring. On the other hand, minor defects

(MID) and total defects (TD) showed higher values in summer, with minor defects having mean values in winter and lower values in autumn and spring. Meanwhile, progressive motility (PM) and major defects (MAD) were not influenced by the seasons ($p < 0.05$).

The variations in the hormonal levels during the seasons are described in Table 3.

Table 3. Seasonal effect over hormonal levels in stallions (n=8) (mean $\pm$ standard deviation).

	Summer	Autumn	Winter	Spring
Cortisol	$4,21 \pm 0,26^a$	$3,51 \pm 0,21^b$	$4,89 \pm 0,22^c$	$4,54 \pm 0,11^{ac}$
Testosterone	$0,52 \pm 0,04^a$	$0,41 \pm 0,06^a$	$0,41 \pm 0,04^a$	$0,75 \pm 0,04^b$

Different letters in the same line show statistical difference between seasons ($p < 0,05$).

The average cortisol levels were lower in the autumn compared to the other seasons ($p < 0,05$). The average testosterone levels were higher in spring ($p < 0,05$).

The variations in the cytometry sperm factor during the seasons are described in Table 4.

Table 4. Seasonal effects over sperm integrity and fecundation potential measured by cytometry in stallions (n=8) (mean $\pm$ standard deviation).

	Summer	Autumn	Winter	Spring
Intact cells	$56,72 \pm 1,52^a$	$49,99 \pm 1,06^b$	$51,48 \pm 1,18^b$	$50,57 \pm 1,29^b$
Capacitated cells	$42,5 \pm 1,53^a$	$48,91 \pm 1,07^b$	$46,63 \pm 1,18^b$	$47,08 \pm 1,35^b$
Apoptosis	$2,79 \pm 0,25^a$	$3,54 \pm 0,26^b$	$3,10 \pm 0,31^b$	$2,0 \pm 0,1^c$
ICWAR	$56,2 \pm 0,83$	$56,49 \pm 0,99$	$54,17 \pm 1,19$	$55,45 \pm 1,33$
HFP	$68,56 \pm 0,77^a$	$67,26 \pm 0,90^a$	$67,00 \pm 1,24^a$	$71,96 \pm 0,91^b$
LFP	$30,02 \pm 0,73^a$	$32,16 \pm 0,89^{ab}$	$33,20 \pm 1,23^{ab}$	$34,17 \pm 2,24^b$

ICWAR, Intact cells without acrossomal reaction; HFP, high fertility potential; LFP, low fertility potential. Different letters in the same line show statistical difference between seasons ($p<0,05$).

Summer had the highest intact cell average and the lowest capacitated cell average ($p<0,05$). The apoptosis average was lower in spring and midterm in summer ($p<0,05$). Spring had the highest high potential fertility average in the year ($p<0,05$).

The correlation between total testicular volume and total testicular width with the hormonal and seminal parameters are shown in Table 5.

Total testicular volume showed positive correlation with the intact cell average, high potential fertility average, minor defects, and testosterone levels ($p<0,05$). And a negative correlation with capacitated cell average ($p<0,05$).

	TM	PM	IC	CC	APOP	HFP	LFP	ICWAR	MAD	MID	TD	TSP	COR	TEST
TTV	0,03	-0,06	0,17*	-0,14	-0,04	0,16*	-0,13	0,12	0,04	0,21**	0,12	0,07	-0,05	0,24**
TTW	0,15*	-0,09	0,17**	-0,15*	-0,03	0,19**	-0,15*	0,14*	-0,16*	0,16*	-0,03	-0,04	-0,02	0,11

The correlation between hormonal levels and seminal parameters are described in Table 6.

Testosterone showed a negative correlation with progressive motility, capacitated cells and apoptosis ($p<0,05$). Cortisol showed a positive correlation with total sperm production ($p<0,05$).

Table 6. Correlation between cortisol and testosterone levels and seminal quality, integrity and morphology parameters in stallions.

	TM	PM	TSP	IC	CC	APOP	HPF	LPF	ICWAR
COR	0,13	0,12	0,14*	0,09	0,10	0,01	0,07	-0,04	0,00
TEST	0,05	-0,16*	0,02	0,11	-0,15*	-0,15*	0,12	0,01	0,00

TM, Total motility; PM, Progressive motility; IC, Intact cells; CC, Capacitated cells; APOP, Apoptosis;

5. Discussion

This research was conducted in Distrito Federal, central area of Brazil, 15° S latitude. This region has a tropical savana climate (Peel et al, 2007) and a small variation in daylength between the longest and the shortest day of the year of approximately 1 hour and 50 minutes (Instituto nacional de meteorologia).

Within the evaluated morphometric parameters, we observed that the total testicular volume undergoes changes throughout the seasons, contrary to what was reported by Rua et al. (2017) and Leme et al. (2012), and in agreement with the findings described by Johnson & Thompson (1983), Clay & Clay (1992), Blanchard et al. (2008), in the northern hemisphere. We suppose that one of the reasons for the difference between the results of Rua et al. (2017) and Leme et al. (2012), and ours was due to the data grouping chosen in these studies. Since they group different seasons into reproductive and non-reproductive periods, this could generate averages that are closer due to the transition periods between seasons with maximum and minimum values. While this study aimed to assess changes season by season, successfully determining that the changes occur mainly in winter in low latitude areas.

Among the morphometric measures, only height and width were responsible for variations between seasons; testicular length remained stable throughout the year. This fact can be explained by the scrotal conformation of the stallion, which, due to the horizontal positioning of the testicles (Amann, 1981; Little & Holyoak, 1992), would have more space to develop laterally and vertically. A fact not observed by Rua et al. (2017), who described changes only in testicular height. Silva et al. (2021) described

that in Mangalarga Marchador stallions, height is the morphometric testicular parameter that changes the most throughout the development of the reproductive system of stallions, being the last to stabilize within the stallion's growth curve and consequently the parameter most affecting volume variation during the animal's development.

The measurements of height, width, and testicular volume showed changes mainly between winter and other seasons or at least between winter and summer. Winter is the only season in our region with less than 12 hours of light throughout the season, and these differentiated light conditions in this season may be the likely cause of testicular morphometric changes. Raising the hypothesis that the perception of long and short days for animals kept in low latitude regions may be different from that proposed by Palmer & Guillaume (1992), so a short day would be one with less than 12 hours of sunlight. Another factor to explore would be the influence of temperature in the testicular and sperm measurements, even though in females the temperature has a low effect in the onset of reproduction, it could affect the testis thermoregulation, so changing sperm qualities.

This study did not confirm a correlation between testicular volume and sperm production, supporting the theory that testicular germinal function is less affected than endocrine function by seasonality (Hoffmann & Landeck, 1999). A small correlation of testosterone with seminal quality was observed, showing a small negative correlation with capacitated cells, apoptotic cells, and progressive motility. It is suggested that endocrine variations have a greater effect on the epididymis and accessory glands, thus affecting seminal quality more evidently than sperm production (Hoffmann & Landeck, 1999). Therefore, higher levels of testosterone would have an effect on the epididymis and accessory glands, leading to a lower number of capacitated cells and apoptotic cells in the ejaculate.

The most well-described effect of seasonality in stallions is the increase in testosterone levels during the reproductive season, consistent with the findings of this study. Testosterone showed a small correlation with testicular volume, as this organ is responsible for producing this hormone (Johnson & Thompson, 1983; Burns et al., 1984; Leme et al., 2012; Rua et al., 2017; Hind et al., 2021). LH responds to seasonal changes in GnRH, stimulating Leydig cells, and these, in greater numbers, would lead to an increase in testicular volume and an increase in testosterone production (Harris et al., 1983; Johnson & Thompson, 1983; Clay et al., 1988; Naden et al., 1990; Johnson et al., 1991; Clay & Clay, 1992; Hoffmann & Landeck, 1999; Ball et al., 2019).

The main morphometric measure showing more correlations with seminal quality and hormonal variables was the total testicular width, presenting a small positive correlation with TM, IC, CC, HFP, ICWAR, and MID,, and a small negative correlation with LFP and MAD. While total testicular volume correlated with IC, HFP, MID, and TEST.

As it was the morphometric measure with most correlations to seminal quality, we suggest that total testicular width has a greater influence on thermoregulation when compared to other testicular morphometric parameters. Due to the arrangement and conformation of the stallion's testicles, its width measurements add up to form the area of the ventral face of the scrotal sac along with the testicular length just below the testicular apex (Amann, 1981; Little & Holyoak, 1992). Thus, morphometric changes that affect the area of this ventral face would have a greater impact on testicular thermoregulation and consequently on seminal quality. Unlike what is proposed for bulls, which, due to the differentiated testicular anatomy of the stallion, the main testicular measure affecting thermoregulation would be the distance from the base to the

apex, represented by testicular height in the stallion (Kastelic et al., 1997; Kastelic, 1999).

Increased testicular temperature leads to reduced fertility, increased apoptosis rate, increased oxidative stress, sperm DNA damage, decreased epididymal function, and a decreased sperm and testosterone production (Durairajanayagam et al., 2014; Lucio et al., 2016; Rizzoto & Kastelic, 2020; Aldahhan & Stanton, 2021; Kastelic & Rizzoto, 2021). Thermoregulation is thus a process of extreme importance for maintaining testicular activity, presenting vascular and non-vascular mechanisms for temperature control. Among the non-vascular mechanisms, the main temperature control mechanisms involve an increase or optimization of the scrotal surface area for heat exchange. We suggest that the increase of testicular width provides a better surface area for heat exchange, therefore optimizing testicular function, which reflects in the observed correlation of this measurement with the seminal parameters, TM, IC, CC, HFP, ICWAR, MID.

As testicular length remained stable throughout all seasons, total testicular width would have a greater influence on changes in seminal parameters between seasons. Thus, total testicular width appears to be the most efficient morphometric measure for predicting seminal quality, even more so than total testicular volume, since a larger volume does not necessarily imply a wider ventral face, as this volume gain may originate from differences in testicular heights.

Despite seminal quality parameters not showing a clear seasonal pattern, intact cells and capacitated showed better results in summer while total defects and minor defects showed the worst results in this period, apoptosis showed better results in spring and intermediate in summer, and high fertility potencial and total motility showed better results in spring. This demonstrates that even though the testicles undergo changes in

their morphometry throughout the year, this does not have an impact on seminal quality to create a seasonal pattern, as already described by Freitas et al. (2023). Nevertheless, we were able to use the analysis of total testicular width not to evaluate seasonal impact, but to predict seminal quality in individuals or compare productive potentials between stallions.

The region where the experiment was conducted varied between a peak of 13 hours of daylight and 11 hours of darkness in summer, and 11 hours of daylight and 13 hours of darkness in winter (world data), showing low variation compared to countries with higher latitudes, reaching 8 hours of daylight and 16 hours of darkness (Palmer & Guillaume, 1992). Thus, within the context proposed by Palmer & Guillaume (1992), most of the year in low latitude countries would be interpreted as short days, less than 10 hours of darkness. This might be the reason that stallions do not undergo great changes in reproductive characteristics through the seasons in low latitudes places, in this context the main season to show some variation was the winter, the only season to be entirely under 12 hours a day of light (Instituto nacional de meteorologia).

6. Conclusion

When evaluating season by season, the results of the present study reinforce the low effects of seasonality in low latitude countries. The few changes observed, especially in morphometry, were mostly restricted to the winter, the period with the lowest light exposure. Testosterone was the only hormone to change, and only in the spring, and seminal quality parameters did not exhibit a consistent pattern across seasons. This indicates that, despite morphological changes in the testis between seasons, these alterations are not sufficient to impact seminal quality and the reproductive potential of the stallion, as already described in the literature.

Another noteworthy observation is the significance of total testicular width as a morphometric measure capable of predicting seminal quality more efficiently than total testicular volume, a measure more commonly used in previous studies.

7. Declaration of conflict of interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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