PRÓ-REITORIA DE PESQUISA E PÓS-GRADUAÇÃO
DOUTORADO EM FISIOPATOLOGIA E SAÚDE ANIMAL

CAMILA DUTRA DE SOUZA FRANCISQUINI

AVALIAÇÃO SAZONAL DA QUALIDADE SEMINAL A FRESCO, REFRIGERADO E PÓS-DESCONGELAÇÃO DE TOUROS NELORE

Presidente Prudente – SP
2018

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Tese apresentada a Pró-Reitoria de Pesquisa e Pós-Graduação, Universidade do Oeste Paulista, como parte dos requisitos para obtenção do título de Doutorado.- Área de Concentração: Clínica médica e Reprodução Animal.

Orientador: Prof. Dr. Marcelo George Mungai Chacur

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Presidente Prudente, 10 de Outubro de 2018

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RESUMO

Avaliação sazonal da qualidade seminal a fresco, refrigerado e pós-congelação de touros Nelore

Em regiões de clima tropical, o acasalamento ocorre durante os meses mais quentes, os touros estão sujeitos às variações ambientais que interferem com a sua fertilidade e eficácia reprodutiva do rebanho. A utilização de algumas biotecnologias colabora para a eficiência do sistema de produção e avaliar o potencial de fertilização de uma amostra de sêmen, seja ela fresca, refrigerada ou congelada e Uma combinação de várias análises e métodos de avalição seminal oferece maior acurácia para definir uma complexa funcionalidade. Para o capítulo 1, os objetivos do estudo foram (1) avaliar o efeito das estações do ano na temperatura da superfície do escroto e do globo ocular, na qualidade do sêmen e concentração plasmática de testosterona de touros Nelore criados extensivamente, (2) verificar a composição proteica do plasma seminal em cada estação do ano e as proteínas com maior frequência e (3) investigar as relações dessas proteínas com parâmetros seminais, temperaturas da superfície do escroto e ITU. Touros Nelore (n=20) foram avaliados com duas coletas de dados com intervalo de 30 dias dentro de cada estação do ano, sendo realizados: termografia infravermelha (FLIR E40®) com análise de imagens (*software FLIR tools*) para cordão espermático (TCE), polo proximal do testículo (PPT), polo distal do testículo (PDT, cauda do epidídimo (TCEp) e gradiente de temperatura escrotal (GT). O sêmen foi coletado e analisado quanto às características quantitativas e qualitativas. Amostras de sangue foram coletadas por venopunção da jugular para obter a concentração plasmática de testosterona por radioimunoensaio (RIA). As proteínas do plasma seminal foram identificadas por eletroforese em SDS-PAGE. O índice de temperatura-umidade (ITU) por estação do ano. O GT foi maior ($P<0.05$) no outono (5ºC) e inverno (4.4ºC) comparado a primavera (3.4ºC) e verão (3.7ºC). O ITU da primavera (73.5) e verão (72) diferiram ($P<0,05$) do outono (64.5) e inverno (59.6) e houveram correlações ($P<0,01$) com TCE (0.54), TCEp (0.74), PPT (0.71), PDT (0.72) e GT (-0.35). Assim como, motilidade espermática (61.5%) e vigor (2.7) na primavera foram inferiores, em relação ao outono e inverno ($P<0.05$). A concentração plasmática de testosterona foi superior ($P<0,05$) no outono, em relação às demais estações do ano. As bandas proteicas do plasma seminal de 20, 55 e 66KDa contribuíram de forma positiva para a qualidade seminal. A concentração da banda com 50KDa foi elevada em todas as estações, sendo considerada indispensável para a fertilidade dos touros. Nas estações primavera e verão, nas quais o ITU excedeu o valor de 72, observou-se decréscimo na motilidade progressiva e vigor espermático. O verão e outono, as estações que apresentaram maiores porcentagens de defeitos espermáticos maiores e totais. As temperaturas do globo ocular e da superfície do escroto aumentaram na primavera e verão e o gradiente térmico e a concentração plasmática de testosterona diminuiu nas mesmas estações, concluindo que o estresse térmico com ITU acima de 72, nas estações primavera e verão, foi suficiente para afetar negativamente a reprodução de touros Nelore criados a pasto nos trópicos. Para o capítulo 2, Os objetivos do estudo foram: (1) avaliar a qualidade do sêmen bovino refrigerado transportado por 3 horas, nos sistemas passivos de transporte de sêmen BotuBOX® e BotuFLEX®, diluídos nos meios TRIS e BotuBOV®; e (2) avaliar a cinética espermática, estresse oxidativo, potencial

mitocondrial e integridade de membrana plasmática e acrossomal do sêmen bovino pós-descongelação, diluídos nos meios TRIS e BotuBOV® e transportados nos sistemas passivos de transporte de sêmen BotuBOX® e BotuFLEX®. O sêmen de seis touros da raça Nelore (Bos taurus indicus), refrigeradas e congeladas em 2 meios diluentes TRIS e BotuBOV® e transportados em 2 sistemas de refrigeração BotuBOX® e BotuFLEX®. Em uma amostra de sêmen refrigerado foi realizada a análise subjetiva de motilidade (MOT) e vigor (VIG) e na amostra pós-descongelação, realizada a análise computadorizada da motilidade espermática (CASA) e por citometria de fluxo, avaliação de integridade de membrana plasmática e acrossomal (MPA), produção de superóxido (O2) e células íntegras (CI). Na análise pós-refrigeração, a MOT do sêmen na associação BotuBOV® e BotuFLEX® (69.4%) e TRIS e BotuFLEX® (62.9%), foram maiores (P<0.05) quando comparado à TRIS e BotuBOX®. A maior (P<0.05) MOT (45.9%) observada na análise subjetiva, MT (47.3%) e MP (37%) no CASA, MPAI (29%), CI (19.8%) e menor produção de O2- (82%) encontrados nas amostras pós-descongelação diluídas em meio BotuBOV® e transportados no sistema BotuFLEX®. Concluímos que o sêmen diluído no meio BotuBOV® e transportado na BotuBOX® ou BotuFLEX®, e diluído em meio TRIS e transportado na BotuFLEX® mantiveram a viabilidade seminal para uso do sêmen refrigerado, bem como o emprego para a congelação. A associação entre o meio diluente BotuBOV® e o sistema de transporte refrigerado de sêmen BotuFLEX® apresentou melhor efeito sobre o sêmen congelado em relação aos parâmetros de cinética espermática, integridade de membrana plasmática e acrossomal e redução do estresse oxidativo.

Palavras-chave: termografia por infravermelho; CASA; citometria de fluxo; sêmen; bovino.

ABSTRACT

Sazonal evaluation of seminal quality to fresh, cooled and frozen-thawed of Nelore bulls

In regions of tropical climate, mating occurs during warmer months, bulls are subject to environmental variations that interfere with their fertility and reproductive efficacy of the herd. The use of some biotechnologies contributes to the efficiency of the production system and to evaluate the fertilization potential of a semen sample, be it fresh, chilled or frozen. A combination of several analyzes and seminal assay methods offers greater accuracy to define a complex functionality. For Chapter 1, the objectives of the study were: (1) to evaluate the effect of the seasons on the surface temperature of the scrotum and ocular globe, the semen quality and plasma concentration of testosterone from Nellore bulls raised extensively, (2) to verify the plasma protein composition (3) investigate the relationships of these proteins with seminal parameters, surface temperatures of the scrotum and THI. Nellore bulls (n = 20) were evaluated with two data collections with a 30-day interval within each season of the year. Infrared thermography (FLIR E40®) with image analysis (FLIR tools software) for spermatic cord (SCT), proximal pole of the testis (PPT), distal pole of the testis (DPT), tail of the epididymis (TeT) and scrotal temperature gradient (TG), and semen collected and analyzed for quantitative and qualitative characteristics. Blood samples were collected by venipuncture of the jugular to obtain the plasma concentration of testosterone by radioimmunoassay (RIA). The seminal plasma proteins were identified by electrophoresis on SDS-PAGE. The temperature-humidity index (THI) calculated per season. The TG was higher ($P < 0.05$) in autumn (5ºC) and winter (4.4ºC) compared to spring (3.4ºC) and summer (3.7ºC). The THI of spring (73.5) and summer (72) differed ($P < 0.05$) from autumn (64.5) and winter (59.6) and there were correlations ($P < 0.01$) with SCT (0.54), TeT (0.74), PPT (0.71), DPT (0.72) and TG (-0.35). Similarly, MOT (61.5%) and VIG (2.7) in spring were lower in relation to autumn and winter ($P < 0.05$). The plasma concentration of testosterone was higher ($P < 0.05$) in the autumn, in relation to the other seasons of the year. Seminal plasma proteins of 20, 55 and 66KDa contributed positively to seminal quality. The concentration of the protein with 50KDa was high in all seasons, being considered indispensable for the fertility of the bulls. In the spring and summer seasons, in which the THI exceeded the value of 72, there was a decrease in progressive motility and spermatic vigor. The summer and fall, the seasons that presented greater percentages of major and total sperm defects. Ocular globe and scrotal surface temperatures increased in spring and summer and the thermal gradient and testosterone plasma concentration decreased in the same seasons, concluding that the thermal stress with THI above 72 in the spring and summer seasons was sufficient for negatively affect the reproduction of Nelore bulls raised in pasture in the tropics. The chapter 2, the objectives of the study were: (1) assess the quality of refrigerated beef semen transported by 3 hours, passive systems of transport of semen BotuBOX® and BotuFLEX®, diluted in TRIS and BotuBOV®; and (2) evaluate the spermatic kinetics, oxidative stress, mitochondrial potential and cell membrane integrity and acrossomal bovine semen after thawing, diluted in TRIS and BotuBOV® and transported in passive systems semen transport BotuBOX® and BotuFLEX®. The six bulls semen of Nellore breed (Bos taurus indicus), cooled and frozen in 2 extenders TRIS and BotuBOV® and transported in 2 cooling systems

BotuBOX® and BotuFLEX®. In a semen sample refrigerated was made a subjective analysis of motility (MOT) and sperm vigor (VIG) and in the sample frozen-thawed, computer-assisted sperm analysis (CASA) and flow cytometry, evaluation of integrity of plasma membrane and acrossomal (IPAM), production of superoxide (O2-) and integrity of cells (IC). On after cooled analysis, the MOT of semen in the BotuBOV® and BotuFLEX® (69.4%) and TRIS and BotuFLEX® (62.9%), were higher ($P < 0.05$) when compared to the TRIS and BotuBOX®. The largest ($P < 0.05$) MOT (45.9%) observed in subjective analysis, TM (47.3%) and PM (37%) in the CASA, IPAM (29%), IC (19.8%) and lower production of the O2- (82%) found in the samples frozen-thawed diluted in BotuBOV® extender and transported in the BotuFLEX® system. We conclude that the diluted semen in the BotuBOV® and transported on BotuBOX® or BotuFLEX®, and diluted TRIS extender and transported on BotuFLEX® maintained the viability for using the cooled semen, as well as employment for freezing. The association between the BotuBOV® diluent and refrigerated transport of semen BotuFLEX® presented best effect on the frozen semen in regard to kinetics parameters, integrity of plasma and acrossomal membranes and oxidative stress reduction.

Keywords: Infrared thermography; CASA; flow cytometry; semen; bovine.

SUMÁRIO

1 OBJETIVOS DA TESE

Os objetivos da presente tese foram investigar aspectos da reprodução de touros da raça Nelore criados a pasto.

O capítulo 1 teve os objetivos de (1) avaliar o efeito das estações do ano na temperatura da superfície do escroto e do globo ocular, na qualidade do sêmen e concentração plasmática de testosterona de touros Nelore criados extensivamente, (2) verificar a composição proteica do plasma seminal em cada estação do ano e as proteínas com maior frequência e (3) investigar as relações dessas proteínas com parâmetros seminais, temperaturas da superfície do escroto e ITU.

O capítulo 2 teve os objetivos de (1) avaliar a qualidade do sêmen bovino refrigerado transportado por 3 horas, nos sistemas passivos de transporte de sêmen BotuBOX® e BotuFLEX®, diluídos nos meios TRIS e BotuBOV®; e (2) avaliar a cinética espermática, estresse oxidativo, potencial mitocondrial e integridade de membrana plasmática e acrossomal do sêmen bovino pós-descongelação, diluídos nos meios TRIS e BotuBOV® e transportados nos sistemas passivos de transporte de sêmen BotuBOX® e BotuFLEX®.

Capítulo 1

ARTIGO ENVIADO PARA INTERNATIONAL JOURNAL OF BIOMETEOROLOGY

Seasonal effects on scrotal temperature, semen characteristics, seminal plasma proteins, and testosterone plasma concentration in Nellore bulls

Abstract

The objectives of the study were (1) to evaluate the effect of seasons on the surface temperature of scrota and eyeballs, semen quality, and plasma testosterone concentration in extensively bred Nellore bulls, (2) to assess the protein composition of seminal plasma in each season and identify the most common proteins, and (3) to investigate the relationships of these proteins with semen parameters, scrotum surface temperatures, and THI. Infrared thermography (FLIR E40®) of Nellore bulls (n = 20) with image analysis for spermatic cord (SCT), proximal pole of the testis (PPT), distal pole of the testis (DPT), epididymis tail (TeT) and scrotal temperature gradient (TG), and semen collected and analyzed. Blood samples were collected to obtain the plasma concentration of testosterone by radioimmunoassay (RIA). The seminal plasma proteins were identified by SDS-PAGE. The temperature-humidity index (THI) calculated per season. The TG was higher ($P < 0.05$) in autumn (5ºC) and winter (4.4ºC). The THI of spring (73.5) and summer (72) differed ($P < 0.05$) from autumn (64.5) and winter (59.6) and there were correlations ($P < 0.01$) with SCT (0.54), TeT (0.74), PPT (0.71), DPT (0.72) and TG (-0.35). Similarly, MOT (61.5%) and VIG (2.7) in spring were lower in relation to autumn and winter ($P < 0.05$). The plasma concentration of testosterone was higher ($P < 0.05$) in the autumn. Seminal plasma proteins of 20, 55 and 66KDa contributed positively to seminal quality. The results indicates that thermal stress at a THI above 72 occurs in spring and summer and negatively affects fertility of Nellore bulls raised in the tropics.

Keywords: Infrared Thermography; Semen quality; Season; Temperature-humidity index; bovine.

Introduction

Genetic progress in domestic cattle depends on selection of sires by the breeder. Bulls are regarded as highly fertile when they are able to produce 80 calves per year or breeding season, and fertilize 80 to 85% of 30 to 50 females in estrus within the first 21 of 45 days (Galloway 1979). Subfertility may threaten herd productivity even more than infertility, if the respective animals are not restricted from breeding. Optimal bull selection will thus influence both, general genetic improvement and herd productivity (Vale Filho 2001).

Sperm function after spermatogenesis is modulated by post-translational changes of cellular proteins, therefore proteomic analyses of seminal plasma may provide important information for the understanding of mechanisms that determine the fertilizing capacity of male gametes (Moura et al. 2011). Infrared thermography is a non-invasive method that can be used without the need for capture and restraint of animals, and allows the analysis of physiological changes over time series (Redaelli et al. 2013) and responses to environmental factors (Kastelic et al. 1996a; Menegassi et al. 2015).

Ambient temperature, thermal radiation, wind speed, relative humidity, and precipitation can directly affect livestock production and resistance to diseases. Excessive heat leads to decreased food intake and protein, energy, mineral, hormone, and blood metabolism (Marai et al. 2007; Delfino et al. 2012). In tropical climates,

mating occurs during the warmer months when bulls are exposed to environmental conditions that may interfere with their fertility and, thus, with herd productivity (Berry et al. 2011; Menegassi et al. 2011).

The effect of thermal stress on bovine reproduction was previously observed in natural environment simulations using climatic chambers (Kastelic et al. 1996a) or scrotal insulation (Fernandes et al. 2008). Previous studies established species-specific thermal comfort or thermal stress, within limiting values (Bohmanova et al. 2007), using a temperature-humidity index (THI). However, few studies have assessed the effect of a seasonally high THI on semen quality in a natural environment (Ravagnolo et al. 2000; Bouraoui et al. 2002; Menegassi et al. 2015).

Semen quality depends on an animal's adaptation to its environment, including thermoregulation regarding body and scrotum. Furthermore, the protein composition of the seminal plasma is important for the protection of sperm cells against thermal stress and the production of optimal levels of testosterone for spermatogenesis. In this regard, the present study provides important information for the selection of breeding bulls.

We hypothesized that season would affect temperatures of eyeballs and scrota, quantitative and qualitative parameters of the semen, serum testosterone level, and seminal plasma proteins. We predicted that the respective variables would correlate with seasonal climate, with summer likely to exert the strongest adverse effect.

The objectives of the study were (1) to evaluate the effect of seasons on the surface temperature of scrota and eyeballs, semen quality, and plasma testosterone concentration in extensively bred Nellore bulls, (2) to assess the protein composition of seminal plasma in each season and identify the most common proteins, and (3) to investigate the relationships of these proteins with semen parameters, scrotum surface temperatures, and THI.

Materials and methods

Study animals, seasons, and site

Semen of twenty Nellore bulls aged 24 ± 1 months was collected twice in 30 days for to assess the basic semen parameters, in accordance with the standards of the Brazilian College of Animal Reproduction (CBRA, 2013). The following mean values were recorded: scrotal circumference 30.3 ± 2.15 cm; semen with progressive sperm motility (MOT) 65 ± 4.5%; sperm vigor (VIG) 2.5 ± 0.3; minor defects (MiD) 9.5 ± 4.1%; major defects (MaD) 6.6 ± 4.8%; total defects (TD) 16.2 ± 6.8%. The bulls were able to reproduce according to the aforementioned norms, and therefore, were used in this study.

The experiment was conducted in the municipality of Anaurilândia (MS, Brazil; 22°56′46″ S, 53°06′36″ W; 380 m above sea level). The climate is classified as Aw (tropical climate with summer rains), according to Köppen-Geiger (1936). The animals were kept on a natural *Urochloa decumbens* pasture, and were supplied with a mineral mix and water *ad libitum*. The starting weight of the animals was 445.6 ± 33.7 kg, the initial testicular volume 367.1 ± 68.7 cm^3 (calculated using the formula of Lunstra et al. (1988)), and the initial body mass index was 345.6 ± 82 kg/m^2 (Quetelet 1870).

Semen was collected twice during each of the four seasons on the following days: 17 November 2015 and 17 December 2015 (spring); 18 February 2016 and 19 March 2016 (summer); 17 May 2016 and 16 June

2016 (autumn); 22 August 2016 and 21 September 2016 (winter). Data were recorded between 8.00 and 11.00 am. A 30-day interval was kept between the two seasonal semen collections, which spanned the 18 days of spermiogenesis and 12 days of epididymal transit (from the end of spermatocytogenesis), according to Pineda and Faulkner (1980) and Menegassi et al. (2015). Semen was collected by electroejaculation (Autojac®, Neovet, Campinas, SP, Brazil) in automatic mode to ensure animal welfare during collection.

Temperature and humidity index (THI)

The values of ambient temperature (AT), black globe temperature (Ttg), and relative humidity (RH) were recorded every hour during the time of semen collection, using a portable black globe thermometer device (Instrutemp, Sao Paulo, SP, Brazil).

The THI of each season was estimated according to the equation described by Thom (1959):

$THI = 0.8 \times T_{tg} + RH\ (T_{tg} - 14.4) + 46.4$

where T_{tg} is the black globe temperature (ºC) and RH is relative humidity in decimal form.

Infrared thermography

Emissivity and thermal sensitivity values were assumed as constants (0.98 and 0.07 °C, respectively). The resolution gradient between the images was 19.200 (160×120) pixel. The AT and RH values of images taken on semen collection days were as follows: first spring collection 28 °C and 68%, second spring collection 31 °C and 53%; first summer collection 33 °C and 57%, second summer collection 27 °C and 50%; first autumn collection 20 °C and 58%, second autumn collection 27 °C and 52%; first winter collection 18 °C and 50%, second winter collection 25 °C and 31%.

The thermography images were captured using an infrared camera (FLIR E40 ®). For thermography of the scrotum, the camera was positioned behind the animal with one meter distance from the scrotum. For eyeball thermography, the camera was positioned at the side of the animal, at a distance of one meter from the head (Menegassi et al. 2015; Ruediger et al. 2016).

The thermograms were analyzed using FLIR Tools software version 3.2 to determine the average surface temperature of the following areas: spermatic cord (SCT), proximal pole of the testicle (PPT), distal pole of the testicle (DPT), epididymis tail (TeT), and ocular globe (OcT). The surface temperatures of the spermatic cord and tail of epididymis were measured within a circular area of the respective region; the temperatures of the proximal and distal pole of the testis were evaluated along a line around the scrotum, for each respective region; the temperature of the ocular globe was measured within a circular area comprising the eyeball surface, the skin around the eye cavity, and the lacrimal gland (Fig. 1). The thermal gradient (TG) was considered the variation between the temperatures of the two ends of the scrotum, SCT, and TeT.

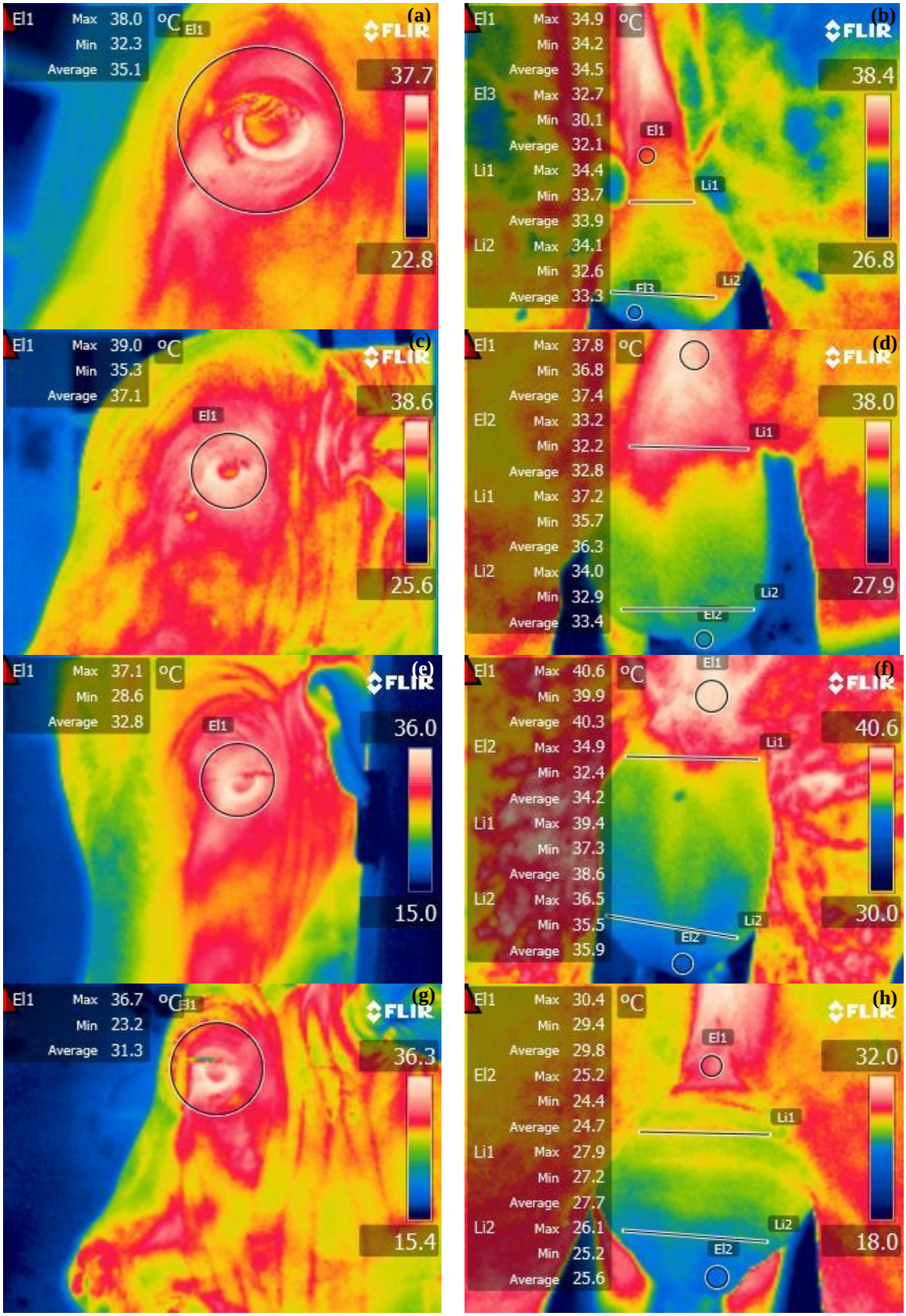

Fig. 1 Image of infrared thermography of the same animal collected during different seasons of the year. **(a)** Temperature of the eyeball in the spring. **(b)** Temperature of the spermatic cord, testicle and epididymis tail in the spring. **(c)** Temperature of the eyeball in the summer. **(d)** Temperature of the spermatic cord, testicle and epididymis tail in the summer. **(e)** Temperature of the eyeball in the autumn. **(f)** Temperature of the spermatic

cord, testicle and epididymis tail in the autumn. (**g)** Temperature of the eyeball in the winter. (**h)** Temperature of the spermatic cord, testicle and epididymis tail in winter.

Plasma testosterone concentrations

Whole blood samples were obtained by venipuncture using a vacutainer (BD Vacutainer®) system with EDTA, and plasma was used to measure testosterone concentrations with a commercial solid phase kit (RIA Testosterone, direct; Beckman Coulter). The radioimmunoassay was carried out in the laboratory of the endocrinology laboratory of the Animal Reproduction and Veterinary Radiology Department FMVZ-UNESP, Botucatu-SP. The intra-assay coefficient of variation (CV) was 12.85%. No inter-assay coefficient of variation was calculated as only a single assay was performed.

Reproductive evaluation

Several specific reproductive and semen characteristics were examined. The rectal temperature (RT) of each bull was measured with digital clinical thermometer for one minute before semen collection. Mass motion (MM), progressive sperm motility (MOT), and sperm vigor (VIG) were assessed using a light microscope (Eclipse ® 200, Nikon, Japan), according to standards of the Brazilian College of Animal Reproduction (CBRA 2013).

Morphological analysis of 200 sperm cells per ejaculate was performed in a 1:100 dilution with formol saline buffer, using phase-contrast microscopy. The following parameters were assessed: MiD: head pathology (HPMi), tail pathology (TPMi), and distal cytoplasmic droplet (DCD); MaD: head pathology (HP), acrosomal pathology (AC), proximal cytoplasmic droplet (PCD), mid-piece defect (MPD) and tail pathology (TP); and TD, according to Bath and Oko (1989).

SDS-PAGE electrophoresis

Proteins were extracted from the seminal plasma for quantitative analyses (Laemilli 1970; Bradford 1976, respectively). A sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 12% was performed at a voltage of 50 V and 400 mA for 50 minutes on the stacking gel, and at 300 V and 25 mA for 2 h. Protein bands were incubated in a 1% Coomassie brilliant blue R-250 solution over-night, and imaging of protein bands was performed using Quantum-Capt software. Molecular weight was determined based according to molecular markers with bands at 20, 27, 36, 50, 90, and 118 kDa (Prestained Protein Molecular Weight Marker, ThermoFisher Scientific).

Statistical analyses

The seasonal effect on body temperatures (SCT, TeT, PPT, DPT, TG, and OcT), seminal parameters (MM, MOT, VIG, MiD, MaD, and TD), rectal temperature, testosterone, and THI were analyzed using the statistical software SAS, version 9.3 (Statistical Analysis Software, Cary, NC). A general mixed model was fitted (MIXED procedure), with the individual as a random effect. Averages of the variation factors were compared by a Tukey's test. Statistical significance is reported at $P < 0.05$. Relations between infrared temperature, seminal parameters, testosterone concentrations, and climatic variables were analyzed using the CORRELATION procedure, and Spearman's linear correlation coefficients were calculated at $P < 0.05$. The relationship between

presence and absence of electrophoresis bands, and infrared temperature, climatic variables, and seminal parameters were examined using multiple logistic regression models.

Results

The THI was significantly higher in spring and summer compared to the other seasons, and winter produced the lowest calculated index. OcT was significantly higher in spring and summer than in the other seasons, with the lowest temperature measured in winter. TG was significantly higher in autumn than in spring and summer. PPT was significantly higher in summer than in autumn and winter. DPT showed significantly higher temperatures in summer and spring than in autumn and winter. Plasma testosterone concentrations were significantly lower in spring than in autumn. Infrared temperatures of the scrotum and ocular globe, the THI, seminal parameters, and testosterone concentrations are shown in Table 1.

Table 1: Mean and standard deviation on the seasons for temperature-humidity index (THI), infrared temperature of areas of the body, seminal parameters and plasma testosterone concentration in Nellore bulls.

Variables	Spring	Summer	Autumn	Winter
THI	73.5±1.8a	72±4.9a	64.5±3.9b	59.6±2.8c
Infrared temperatures				
OcT	36.7±0.9a	36.3±1a	34.6±1.3b	32.4±2.1c
SCT	35.9±1.3a	36.3±1.2a	36±2a	33.5±2.2b
TeT	32.5±1.2a	32.5±1.4a	31±2.8b	29.1±2.4c
PPT	34.5±1.2ab	35.1±1a	34±1.8b	31.5±2.1c
DPT	33.5±1.2a	33.6±1.2a	32.4±2.1b	30.4±2.3c
TG	3.4±1.2c	3.7±1.1bc	5±1.7a	4.4±2ab
RT	39.8±0.3a	39.7±0.4a	39.8±0.6a	39.3±0.6b
Seminal parameters				
MOT	61.5±16d	68.9±17.5bc	76.9±15.7ac	79±13.9a
VIG	2.7±0.8b	2.9±0.8ab	3.27±0.7a	3.3±0.8a
MM	1.6±1.2	1.9±1.3	1.9±1.5	1.9±1.6
MiD	8.8±4.5ab	5.5±3.9b	11.4±6.5a	9.1±7.6a
MaD	6.8±4.9c	18.3±9a	14.1±9.9ab	9.2±10bc
TD	15.6±7.6c	23.8±10.8ab	25.5±13.4a	18.4±13.1bc
Testosterone	8±3.7b	9.1±4b	13.1±2.9a	10.6±4.8ab

Inside the line, medium without equal, small letter differed ($P< 0.05$); *THI* Temperature-humidity index, *OcT* ocular globe temperature (ºC), *SCT* spermatic cord temperature (ºC), *TeT* epididymis tail temperature (ºc), *PPT* proximal pole of the testicle temperature (ºC), *DPT* distal pole of the testicle temperature (ºC), *TG* temperature gradient (°C), *RT* rectal temperature (ºC), *MOT* motility (%), *VIG* sperm vigor (0-5), *MM* mass motion (0-5), *MiD* minor defects (%), *MaD* major defects (%), *TD* total defects (%), *Testosterone* (ng/mL)

MOT was significantly higher in winter than in the other seasons, with the lowest percentage in spring. VIG was significantly lower in spring than in autumn and winter. The percentage of MaDs in summer was similar to that in autumn, and differed significantly from that in other seasons. Among the MaDs, AC was significantly higher in summer than in the other seasons, and MPD showed a significantly larger percentage in summer and autumn than in spring and winter (Table 2). The percentage of MiD was significantly lower in summer, with the highest percentage in the autumn; TPMi and HPMi were significantly higher in spring than in the other seasons (Table 2).

Table 2: Mean and standard deviation in the seasons for THI and individual sperm defects major and minor in Nellore bulls.

Variables	Spring	Summer	Autumn	Winter
THI	73.5±1.8a	72±4.9a	64.5±3.9b	59.6±2.8c
Major defects				
HP	0.6±0.9	1.2±1.5	1±1.9	1.1±1.6
AC	0.6±1.3b	5.4±5.9a	2.2±3.6b	1.3±1.5b
PCD	0.2±0.4	0.5±1.4	1.2±1.6	1.6±4.9
MPD	3.2±3.3b	8.5±4.6a	6.2±5.2a	2.4±3.2b
TP	2.2±2	2.2±2	3.2±4	1.7±1.9
Minor defects				
HPMi	3.2±2.7a	1.7±1.6b	1.8±1.6b	1.9±2.1b
TPMi	5.4±4.4b	3.6±3b	9.1±5.9a	6.9±7.4b
DCD	0.1±0.8	0.1±0.4	0.4±0.9	0.2±0.5

Inside the line, medium without equal, small letter differed ($P< 0.05$); *THI* Temperature-humidity index, MiD: *HPMi* head pathology, *TPMi* tail pathology, and *DCD* distal cytoplasmic droplet; MaD: *HP* head pathology, *AC* acrosomal pathology, *PCD* proximal cytoplasmic droplet, *MPD* mid-piece defect; *TP* tail pathology

The THI showed a significant positive correlation ($P < 0.01$) with OcT, and with the following temperatures: SCT, TeT, PPT, DPT, and RT. The THI produced a significant negative correlation with TG ($P < 0.01$). OcT was correlated positively ($P < 0.01$) with the scrotal temperatures SCT, TeT, PPT, DPT, and TR, and correlated negatively with TG ($P < 0.01$). TG correlated negatively ($P < 0.01$) with PPT, DPT, and TeT. RT showed a positive correlation ($P < 0.01$) with SCT, TeT, PPT, and DPT. The correlation coefficients of THI, infrared temperatures, and seminal parameters are presented in Table 3.

Table 3. Correlations between temperature-humidity index during spermatogenesis, infrared temperatures of the scrotum and ocular globe and seminal parameters of Nellore bulls.

Variables	THI	OcT	SCT	TeT	PPT	DPT	TG	RT	MOT	VIG	MiD	MaD
OcT	0.89**											
SCT	0.54**	0.68**										
TeT	0.74**	0.84**	0.72**									
PPT	0.71**	0.82**	0.85**	0.85**								
DPT	0.72**	0.84**	0.77**	0.95**	0.88**							
TG	-0.35*	-0.33**	0.11	-0.51**	-0.17**	-0.36**						
RT	0.55**	0.41**	0.45**	0.50**	0.40**	0.52**	-0.18*					
MOT	-0.27	-0.03	0.01	0.02	-0.01	-0.01	-0.02	-0.01				
VIG	-0.15	-0.01	0.01	0.02	-0.01	-0.02	-0.02	0.01	0.88**			
MiD	0.18	-0.28	-0.19	-0.24	-0.28	-0.22	0.13	0.09	-0.03	-0.03		
MaD	0.13	0.01	0.11	0.01	0.07	0.04	0.12	0.02	-0.06	-0.07	0.11	
TD	0.21	-0.14	-0.02	-0.12	-0.09	-0.09	0.14	0.06	-0.04	-0.06	0.60**	0.82**

THI Temperature-humidity index, *OcT* ocular globe temperature, *SCT* spermatic cord temperature, *TeT* epididymis tail temperature, *PPT* proximal pole of the testicle temperature, *DPT* distal pole of the testicle temperature, *TG* temperature gradient, *RT* rectal temperature, *MOT* motility, *VIG* sperm vigor, *MiD* minor defects, *MaD* major defects, *TD* total defects *$P<0.05$; **$P<0.01$

In total, 106 protein bands were identified following SDS-PAGE, with molecular weights between 16 and 340 kDa (fig. 2).

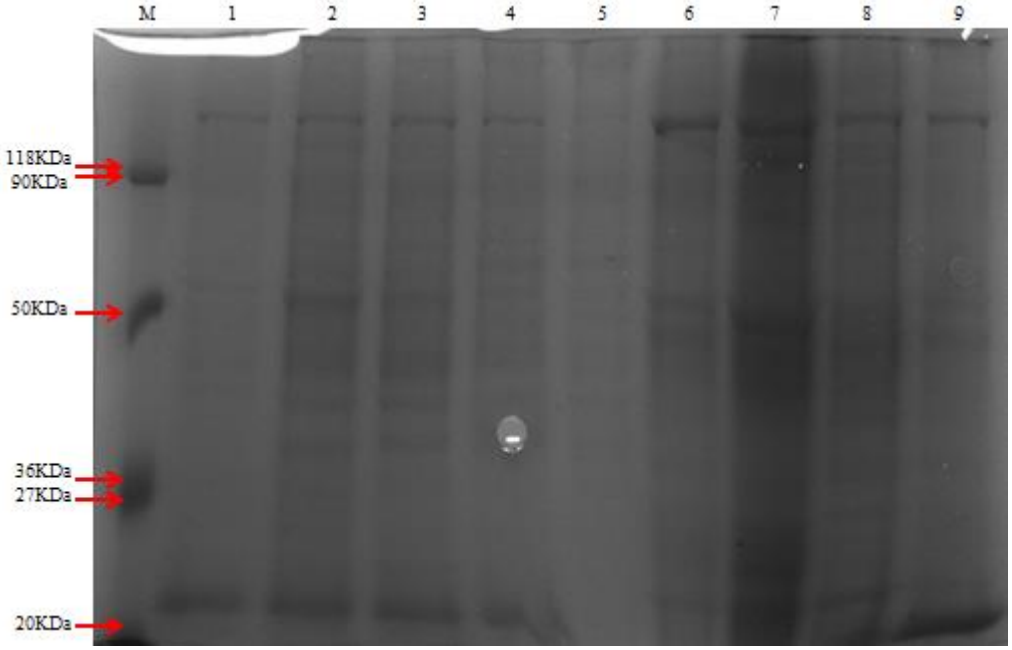

Fig. 2 The 12% polyacrylamide gels (SDS-PAGE) with proteins present in seminal plasma. Protein marker (M); Seminal plasma samples of bulls (1 to 9) in winter.

The 20 kDa protein was detected in all seasons, but was most common in winter. The 40 kDa protein was found in spring and autumn, and most often in winter. The 50 kDa protein was detected in all seasons. The 130 kDa protein was found only in winter and (more often) in autumn. The 55 kDa protein was detected in all seasons, with slightly higher frequencies in winter. The 66 kDa protein was found in all seasons, but was significantly more common in winter (fig. 3).

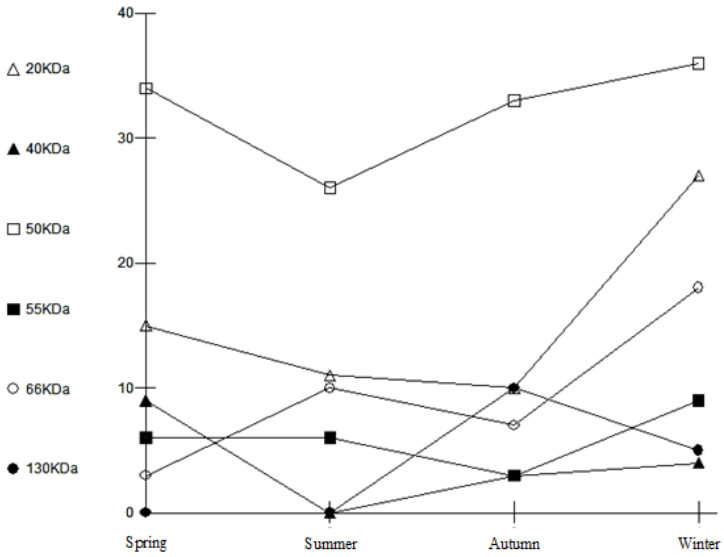

Fig. 3 Frequency of 20, 40, 50, 55, 66 and 130KDa proteins present in seminal plasma of bulls in the seasons

No significant correlation of proteins with seminal parameters, scrotal temperature and THI per season was found using a multivariate logistic regression estimation. The same relationships were analyzed by season, however, no significant relationships were observed: the 130 kDa protein and MiD (P = 0.79) and MaD (P = 0.16) in autumn; the 50 kDa protein and SCT (P = 0.88) and THI (P = 0.99) in spring, with SCT (P = 0.18) and THI (P = 0.95) in summer, with SCT (P = 0.12) and THI (P = 0.91) in autumn, and with SCT (P = 0.92) and THI (P = 0.99) in winter. The 20, 40, 55, and 66 kDa proteins were more frequent in winter, where the following regression estimations were observed: the 20 kDa protein and MaD (P = 0.16); the 40 kDa protein and MOT (P = 0.99), VIG (P = 0.50), MaD (P = 0.56), and TeT (P = 0.31); and the 55 kDa protein and TeT (P = 0.49); and the 66 kDa and MM (P = 0.15).

Thus, the regression analyses were performed using seminal parameters, scrotal temperature, and THI to identify their correlations with the 130, 66, 55, 50, 40, and 20 kDa proteins all the long year. The 130 kDa protein produced a negative regression estimation with MiD and MaD; the 66 kDa protein showed a positive regression estimation with MM. The 55 kDa protein produced a negative regression estimation with TeT, and the 50 kDa protein showed a negative regression estimation with SCT and THI. The 40 kDa protein produced significant regression estimates: negative with MOT and VIG, and positive with MaD and TeT. The 20 kDa protein produced a negative regression estimation with MaD (Table 4).

Table 4. Estimation of multivariate logistic regression model for proteins of 130, 66, 55, 50, 40, and 20KDa in of the seminal parameters, infrared temperature of the scrotum and temperature-humidity index, throughout the seasons

	130KDa		66KDa		55KDa	
	Estimate±SE	Pr>ChiSq	Estimate±SE	Pr>ChiSq	Estimate±SE	Pr>ChiSq
MOT	-0.002±0.04	0.99	-0.03±0.02	0.22	-0.05±0.03	0.10
VIG	-0.28±0.87	0.74	-0.08±0.52	0.87	0.90±0.59	0.12
MM	0.44±0.42	0.28	0.52±026	0.04*	0.05±0.30	0.85
MiD	-0.21±0.07	0.007*	-0.01±0.03	0.98	-0.01±0.03	0.67
MaD	-0.08±0.03	0.03*	-0.01±0.02	0.56	-0.01±0.02	0.76
SCT	0.18±0.29	0.54	-0.20±0.20	0.30	0.14±0.25	0.58
TeT	0.16±0.47	0.72	0.22±0.28	0.43	-0.96±0.44	0.02*
PPT	0.27±0.51	0.58	0.22±0.32	0.48	-0.46±0.38	0.22
DPT	-0.07±0.70	0.91	-0.41±0.41	0.31	1.10±0.55	0.06
THI	-1.00±1.94	0.60	-0.54±1.03	0.60	0.22±1.08	0.83
	50KDa		40KDa		20KDa	
	Estimate±SE	Pr>ChiSq	Estimate±SE	Pr>ChiSq	Estimate±SE	Pr>ChiSq
MOT	-0.01±0.03	0.84	-0.12±0.05	0.01*	0.01±0.02	0.73
VIG	-0.25±0.56	0.65	-2.89±1.10	0.001*	-0.01±0.45	0.97
MM	0.02±0.29	0.93	0.01±0.41	0.99	0.04±0.22	0.85
MiD	-0.04±0.04	0.39	-0.03±0.04	0.46	0.03±0.03	0.32
MaD	-0.01±0.02	0.98	0.13±0.06	0.04*	-0.04±0.02	0.03*
SCT	-0.64±0.32	0.04*	0.03±0.31	0.91	0.10±0.19	0.60
TeT	0.02±0.39	0.95	0.91±0.42	0.03*	0.16±0.27	0.53
PPT	0.81±0.42	0.06	0.34±0.51	0.49	-0.05±0.28	0.83
DPT	-0.51±0.51	0.31	-1.39±0.68	0.06	-0.33±0.37	0.37
THI	-3.22±1.58	0.04*	-5.25±2.76	0.06	1.04±1.74	0.55

MOT motility (%), *VIG* sperm vigor (0-5), *MM* mass motion (0-5), *MiD* minor defects (%), *MaD* major defects (%), *SCT* spermatic cord temperature (ºC), *TeT* epididymis tail temperature (ºC), *PPT* proximal pole of the testicle temperature (ºC), *DPT* distal pole of the testicle temperature (ºC), THI temperature-humidity index. *P < 0.05

248

249 **Discussion**

250 The present study shows that infrared thermography can be used as an auxiliary technique for
251 reproductive evaluation of bulls to indirectly evaluate thermal stress on reproductive parameters. The assessment
252 of the seasonal effects on scrotal and body thermoregulation and seminal parameters is crucial for the
253 identification of changes regarding health and physiology of cattle.

254 The highest THI values observed during spring and summer were consistent with the expected pattern,
255 as these two seasons are associated with high ambient temperatures and high relative humidity. Regarding the
256 critical THI value for caloric stress in animals, Bohmanova et al. (2007) reported a threshold value of 72 for
257 dairy cows. A different study (Ferraza et al. 2017) indicated that a THI below 77 was a critical limit for dry
258 cows. Menegassi et al. (2015) reported that THI values above 83 induced thermal stress in bulls of European
259 origin. Our results demonstrated that a THI value of 72 was sufficient to exceed the limit of homeothermy in the
260 study animals, causing thermal stress. Nichi et al. (2006) suggested that high ambient temperatures affect the
261 oxidative metabolism of glucose in sperm, resulting in mitochondrial dysfunctions and generation of reactive
262 oxygen species, and, subsequently, in a decrease of semen quality.

263 In the current study, a THI of 64.5 was recorded in autumn, which seemed to damage sperm
264 morphology, but did not affect sperm quantity; this may be a residual effect of the heat stress suffered during the
265 previous season. However, this residual effect was confirmed only for sperm morphology, but was not associated
266 with scrotal surface temperatures, as these were only affected by the THI of the respective current seasons. These
267 metabolic variations arise as a result of thermal stress and deplete energy reserves of the spermatozoa have to
268 adopt alternative strategies to maintain homeothermic metabolism (Baumgard and Rhoads 2013; Rhoads et al.
269 2013).

270 The increase in OcT in spring and summer and its positive correlation with the THI may be explained
271 by the high sensitivity of the ocular globe to thermal stress and its thermoregulation response (Schaefer et al.
272 2007). Thus, infrared thermography in the ocular region provides information on the animal's physiological state
273 of homeothermia and may be used as a method of daily temperature monitoring in cattle, if coupled to the water
274 fountain. In this context, the relationship between OcT, scrotal surface temperatures, and RT shows that
275 thermography in the ocular region can help to assess body temperature and scrotal temperature. OcT may thus be
276 a reliable indicator for the selection of Nellore breeding bulls in the tropics. Melero et al. (2015) also confirmed
277 the validity of OcT as a reliable measure of body temperature.

278 The THI in spring and summer was sufficient to cause an elevation of RT above the normal values (38
279 to 39.5 °C), under thermoneutral conditions (DuPreez 2000). Mota (1997) established TR as an index of
280 physiological adaptation to warm environments, as its increase suggests that the mechanisms of heat are
281 insufficient to maintain homeothermia.

282 Scrotal thermoregulation is a complex mechanism that depends on various functions allowing the
283 dissipation of heat. However, factors such as temperature and ambient humidity affect the thermoregulation
284 process (Garcia 2006). This was confirmed in the present study, regarding the relationship between THI, RT, and
285 scrotal surface temperature. Moreover, infrared thermography is an important non-invasive method and produces
286 measurements highly correlated with rectal temperatures (Stewart et al. 2005).

The highest temperatures of the spermatic cord were observed during spring and summer, and were associated with the highest THI, demonstrating the correlation of thermal stress with scrotal temperatures. In each spermatic cord, there is only one thick-walled testicular artery which is surrounded by thousands of venules that deliver venous blood from the testis. The venous return structure is referred to as the pampiniform plexus (Cook et al. 1994). The countercurrent mechanism of the pampiniform plexus can reduce arterial blood temperature at the testicles by up to 4 ºC (Kastelic et al. 2014). Thus, this mechanism did not succeed in decreasing the scrotal temperature, which subsequently led to a decrease in semen quality.

We suggest that the thermal stress experienced in spring and summer may have interfered with sperm maturation, which is associated directly with the MOT and VIG results, due to the effect of external conditions on the epididymis tail and distal testicular pole (Lunstra and Coulter 1997). Thus, the temperature of the epididymis tail was closely associated with the maintenance of the scrotal temperature, which was affected by the THI.

The surface temperatures at the proximal and distal poles of the testes were greater in spring and summer, following the increased relative humidity and ambient temperature, and hence greater value of THI. Menegassi et al. (2015) observed the same correlation at a THI of 83, which produced an increase in scrotal surface temperature of bulls. Scrotal surface temperature is strongly correlated with its internal temperature (Coulter et al. 1988), and Kastelic et al. (1996) points out that ambient temperature may affect scrotal surface and testicular temperatures. Kastelic et al. (2001) studied the scrotal insulation and concluded that even a moderate increase in testicular temperature drastically reduces sperm production, motility, and the number of viable sperm in an ejaculate, and increases the percentage of pathologically deformed sperm.

The smallest TG was observed during spring and summer, which is consistent with the expected pattern, as these seasons are associated with increased SCT and TeT. On the scrotal surface, a decrease in temperature in a dorso-ventral direction was described, which results in a positive gradient (Kastelic et al. 1995). Purohit et al. (1985) reported scrotal thermography typically shows a symmetric and steady temperature pattern, with a dorso-ventral gradient of 4 to 6 °C, to achieve good semen quality. The TG is important because of its direct relationship with scrotal thermoregulation.

The decrease in plasma testosterone concentrations was associated with the highest THI values in spring and summer. Testosterone is essential for male reproductive functions as it stimulates the final stages of spermatogenesis and prolongs the lifespan of sperm in the epididymis (Davis et al. 2014). This emphasizes the correlation of plasma testosterone concentrations and decreased semen quality during times of higher THI.

The seminal plasma contains substances that affect sperm function, and modify the fertilization potential of sperm. Previous studies reported a high correlation between protein profiles of seminal plasma and fertility of bulls (Killian et al. 1999; Chacur et al. 2006; 2010). Chacur (2012) highlights that a comparison of individual protein profile maps using SDS-PAGE with a reference map could provide useful information to relate changes in protein expression patterns to the physio-pathologic conditions that affect reproductive success.

The 20 kDa protein, known as seminal plasmin, is as assumed to be responsible for the recovery of sperm membrane permeability after being subjected to a cold shock which induces membrane rupture (Barrios et al. 2007). The 20 kDa protein also acts as an antimicrobial agent in semen (Kemme 1984). The negative relationship of this protein with MiD indicates that it is associated with high fertility due to its antimicrobial

action and protective effect on the plasma membrane following a thermal shock, which may explain its higher expression during winter.

Regarding the relationships of the 40 kDa protein, we suggest that this protein is associated with a decrease in seminal quality, as its highest concentrations were observed in spring, which corresponded to the highest THI values and thus with heat stress. A similar effect was observed occurred in a previous study on Limousin cattle, which showed presence of the 40 kDa protein in sires with low semen quality during high temperatures in summer (Chacur et al. 2006).

The tail of the epididymis is more exposed to external conditions (Lunstra and Coulter 1997), thus the 40 kDa protein produced positive relationship with the TeT, indicating that it may help to increase progressive sperm motility (Bedford 1975). Progressive motility also showed a negative relationship with the 40 kDa protein that decreased in spring.

The 55 kDa protein, termed osteopontin, was described as an indicator of fertilization capacity (Kilian et al. 1999). The functioning of the epididymis tail depends on the maintenance of low scrotal temperature, indicating that an increase in protein 55 kDa concentrations is directly linked to sperm maturation and increases fertility. The increase in osteopontin in winter, in which cold shock can affect sperm, is of paramount importance, as it helps to achieve sperm capacitation. Gerena (2000) described the function of this protein to be fundamental for the modulation of cellular functions, and modulation of the plasma membrane characteristics of sperm, fertility, in addition to participating in sperm capacitation.

The 66 kDa protein, known as albumin, can absorb lipid peroxides, and thereby exerts a protective effect on the sperm membrane, Albumin is typically correlated positively with the percentage of morphologically normal sperm in bovines (Elzanaty et al. 2007). Chacur et al. (2007) found that winter and summer influenced the protein profile of Limousin semen, with superior semen quality in the presence of the 20, 55 and 66 kDa proteins. We conclude that the 66 kDa protein has a positive effect on semen quality, as its increasing concentrations in winter may help to protect the sperm plasma membrane, and increase mass motion.

The 50 kDa protein was the most common protein in all seasons; therefore we conclude that this protein plays a crucial role for the reproduction of cattle. However, the 50 kDa protein has not yet been described in the literature, but its negative correlation with SCT and THI suggests that the presence of this protein is linked to fertility and to thermal stress adaptation, as its concentrations increased when the THI was lower, which may help to maintain the temperature within the spermatic cord.

The 130 kDa protein was associated with higher semen quality, as its increased concentrations were associated with the maintenance of sperm defects; however, it only occurred in autumn and winter which did not differ from spring regarding sperm quality. This protein was not described previously, to our knowledge.

Conclusion

In spring and summer when the THI was above 72, progressive motility and sperm vigor decreased. In summer and autumn, higher percentages of major and total sperm defects were observed. The temperatures of the ocular globe and of the scrotal surface increased in spring and summer, and the thermal gradient and the plasma testosterone concentrations decreased, which indicates that thermal stress at a THI above 72 occurs in spring and summer and negatively affects fertility of Nellore bulls raised in the tropics. Seminal plasma proteins

of 20, 55, and 66 kDa were associated with higher semen quality. The concentration of the 50 kDa protein was high in all seasons, and it is considered vital for the fertility of bulls.

All procedures performed in this experiment involving animals were in accordance with the ethical standards of the institution in which the studies were conducted, being approved by the Commission of ethics in the use of Animals (CEUA/UNOESTE) under the Protocol 3479/2015.

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Capítulo 2

ARTIGO ENVIADO PARA ANIMAL REPRODUCTION

Effect of extenders and refrigerated transport systems on kinetics, oxidative stress, and integrity of sperm membranes in cooled and frozen-thawed semen of bulls

Type Article: Biotechnology

Running title: Evaluation of semen quality frozen of bulls

Abstract

The objectives of this study were to (1) assess the quality of refrigerated bovine semen, diluted in TRIS and BotuBOV® extenders and transported for 3 h via the BotuBOX® and BotuFLEX® refrigerated transport systems, and (2) evaluate the spermatic kinetics, oxidative stress, mitochondrial potential, and cell membrane and acrosomal integrity of bovine semen after freeze-thawing. The semen of six Nellore (*Bos taurus indicus*) bulls was cooled and frozen in two extenders, TRIS and BotuBOV®, and transported via two refrigerated systems, BotuBOX® and BotuFLEX®. In the refrigerated semen sample, subjective analyses of sperm motility (MOT) and vigor were undertaken and, using the frozen-thawed sample, computer-assisted sperm analysis and flow cytometry were utilized for evaluation of the integrity of the plasma membrane and acrosomal semen (IPAM), production of superoxide (O_2^-), and integrity of cells (IC). The MOT in samples diluted in BotuBOV® extender and transported via the BotuFLEX® system (69.4%) and samples diluted in TRIS extender and transported via the BotuFLEX® system (62.9%) were higher ($P < 0.05$) than that of samples diluted in TRIS extender and transported via the BotuBOX® system. The highest ($P < 0.05$) MOT (45.9%), total sperm motility (47.3%), progressive motility (37%), IPAM (29%), and IC (19.8%), and lowest production of O_2^- (82%) were found in the frozen-thawed samples diluted in the BotuBOV® extender and transported via the BotuFLEX® system. Thus, the semen

diluted in BotuBOV® extender and transported via BotuBOX® or BotuFLEX® systems, as well as semen diluted in TRIS extender and transported via the BotuFLEX® system maintained feasibility to use refrigerated semen, as well as employment for later freezing. Use of the BotuBOV® extender and BotuFLEX® refrigerated transport system yielded optimal results for the frozen semen in terms of kinetic parameters, integrity of plasma and acrosomal membranes, and oxidative stress reduction.

Keywords: Bovine; CASA; flow cytometry; cryopreservation; extenders of semen

Introduction

Livestock has great economic importance in Brazil. Brazil had 218,230,000 head of cattle in 2016 (Instituto Brasileiro de Geografia e Estatística, 2016) and exported 1,530,000 t of meat in 2017 (ABIEC, 2017). The use of biotechnology contributes to the efficiency of the production system, including that applied to breeding. Several studies (Freitas-Dell'Aqua et al., 2011; Olaciregui et al., 2014) have aimed to evaluate the fertilization potential of a semen sample, whether fresh, cooled, or frozen. These studies specifically evaluated attributes such as sperm cell plasma membrane and acrosomal integrity; sperm motility (MOT), energy, and the ability to start sperm preparation; normal DNA; and the ability to connect to the zona pellucida (Barroso et al., 2009).

Prior assessment of the ability of sperm to fertilize is a prerequisite for reproductive biotechnology, and thus there is a need to evaluate the cells themselves, i.e., their different compartments. Computer-assisted sperm analysis (CASA) facilitates the standardization of MOT for each species, thus enabling greater objectivity and repeatability (Davis and Siemers, 1995). Farrell et al. (1996) showed the association of multiple variables using the CASA technique with a significant correlation for *in vivo* fertility compared with individual sperm kinetic parameters.

A series of sperm cell characteristics, such as plasma membrane and acrosomal integrity, feasibility, potential, and mitochondrial oxidative stress can be evaluated by flow cytometry (Freitas-Dell'aqua et al., 2009). A combination of various analyses and seminal assessment methods provides greater accuracy to determine complex

functionality, and it should not be expected that a single semen test can predict the fertility of an individual or a sample of semen (Arruda et al., 2003).

The cooled semen principle relates to the preservation of sperm viability, ensuring greater longevity when compared to fresh semen and attaining a higher rate of pregnancy when compared to frozen semen (Holt, 2000). The use of frozen semen, however, allows for rapid genetic advancement of commercial herds and is the breeding choice that best meets production needs and inheritable characteristics. Therefore, the aim of the present study was to determine which extender and refrigerated bovine semen transport system minimized the deleterious effects of semen handling during the refrigeration and freezing processes, in terms of quantitative and qualitative characteristics of sperm kinetics, plasma membrane and acrosomal integrity, oxidative stress, and mitochondrial potential.

The hypothesis of the present study was that the semen extenders TRIS and BotuBOV® and the refrigerated transport systems BotuBOX® and BotuFLEX® would influence the quality of refrigerated and frozen semen. The objectives of the study were to (1) assess the quality of refrigerated bovine semen, diluted in TRIS and BotuBOV® extenders and transported for 3 h in BotuBOX® and BotuFLEX® refrigerated transport systems, and (2) evaluate the spermatic kinetics, oxidative stress, mitochondrial potential, and cell membrane and acrosomal integrity of bovine semen after freeze-thawing.

Materials and Methods

The procedures were approved by the Commission of Ethics in the Use of Animals (CEUA/UNOESTE) under Protocol 3479.

Animals and location of the experiment

Six Nellore bulls (*Bos taurus indicus*) with an initial age of 30 ± 1 months were assessed prior to the beginning of the experiment with four harvests of semen, with a 30-day interval between each harvest. Reproductive evaluations were conducted based on the methodology described by Barth and Oko (1989) for evaluation of their inclusion in the experiment.

The animals were maintained under the same environmental conditions in pastures of *Urochloa decumbens* with mixed minerals (Fort Salt, Animal Nutrition, Brazil) and water *ad libitum*. The initial weight of the animals was 580.16 ± 8.95 kg, initial scrotal circumference was 34.5 ± 1.84 cm, initial testicular volume was 556.13 ± 69.92 cm^3 based on the formula of Lunstra et al. (1988), and initial body mass index was 311.23 ± 27.81 kg/m^2 according to formula Quetelet (1870).

The experiment was conducted at latitude 22°56′46″ S, longitude 53°06′36″ W, and 380 m altitude. The climate is classified as Aw (tropical climate with summer rains) based on the Köppen (1936) classification system.

Experimental design

The semen samples were collected by electroejaculation and frozen from each of the animals in June, August, October, and December 2016, and January 2017 between 8:00 a.m. and 9:00 a.m.

Five samples from six bulls, totaling thirty semen samples, were diluted in two extenders, TRIS and BotuBOV® (Botupharma, Botucatu, Brazil), and transported in

two refrigeration systems, BotuBOX® and BotuFLEX® (Botupharma). Thus, there was a total of 120 samples used in the frozen-thawed analysis.

Semen collection

The evaluation consisted of examining specific reproductive and semen parameters. Semen was collected via electroejaculation with an Autojac® (Neovet, Uberaba, MG, Brazil) in automatic mode, which ensured superior animal welfare during the collection of semen. The following parameters were evaluated by optical microscopy (Eclipse® 200; Nikon, Japan): mass motion, subjective MOT, and sperm vigor (VIG) based on the standards of the Brazilian College of Animal Reproduction (CBRA, 2013).

For morphological analysis of sperm, semen was diluted in formalin saline buffer (1:100) and the initial evaluation studied 200 cells using phase-contrast optical microscopy (Eclipse® 200; Nikon) to evaluate minor defects (MiD), acrosome (AC) pathology, major defects (MaD), and total defects (TD), based on the methodology described by Barth and Oko (1989).

Dilution of semen

On the rural property, each semen sample was fractionated into four equal aliquots, with two diluted in extender 1:1 in TRIS-yolk-citric acid (3.28 g TRIS, 1.78 g citric acid, 1.25 g D-fructose, 6% glycerol, 20% egg yolk, and distilled water to 100 mL of medium) produced at the Laboratory of Animal Husbandry of the Veterinary Hospital of the University of the West of São Paulo (UNOESTE) and the other two aliquots diluted in 1:1 proportion in commercial BotuBOV® extender.

126 Thus, 2 mL of semen was diluted in 2 mL TRIS and 2 mL of semen was
127 diluted in 2 mL of BotuBOV® and were then packed in falcon tubes in the BotuBOX®
128 refrigerated semen transport system. Similarly, 2 mL of semen was diluted in 2 ml of
129 TRIS and 2 mL of semen was diluted in 2 ml of BotuBOV® and placed in the
130 BotuFLEX® refrigerated semen transport system. The shipping time of the chilled
131 semen to the Animal Reproduction Laboratory at UNOESTE was 3 h. Upon arrival at
132 the laboratory, analysis of progressive MOT and VIG, as well as morphological analysis
133 was conducted, as described earlier.
134
135 *Semen cooling*
136 Semen samples were placed in 0.5 mL French reeds and diluted at a
137 concentration of 50×10^6 sperm with progressive motility (PM) by reed, laid out
138 horizontally in stainless steel trays, and placed in a commercial cooler at 5°C for 4 h.
139 There were 8 reeds/animal/extender/refrigerated semen transport system, namely, 8×6
140 $\times 2 \times 2$, totaling 192 French reeds for collection. At the end of the cooling period, one
141 reed was subjected to analysis of PM and VIG, as well as morphological analysis, as
142 described previously.
143
144 *Semen freezing*
145 Immediately at the end of the cooling period, freezing was performed in the
146 same manner for the four treatments. The reeds were placed in a liquid nitrogen (N_2)
147 for 20 min, and then directly immersed in N_2 and packed in a nitrogen canister (Papa et
148 al., 2008). Prior to computer analysis, the thawing of reeds was conducted in a water
149 bath at 37°C for 30 s and MOT, VIG, and morphological analyses were conducted.

Computer-assisted semen analysis (CASA)

For the frozen-thawed semen samples, CASA version IVOS 10 (Hamilton-Thorne Research Beverly/MA, USA) was performed. After thawing the semen doses in 37°C for 30 s in a water bath, a drop of sample was placed in a heated Makler at 38°C to analyze the sperm cells. The analyses were carried out in "*setup*" mode (Table 1), adjusted for the seminal characteristics of the cattle, and three fields were evaluated for each sample. The following sperm movement kinetic variables were analyzed: total sperm motility (TM, %), PM (%), average path velocity (VAP, µm/s), straight-line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), amplitude of lateral head displacement (ALH, µm), tail beat frequency (TBF, Hz), straightness (STR, %), linearity (LIN,%), and rapid spermatozoa (RAP, %).

Table 1. Methodology of computerized analysis (CASA) of sperm of bovine animal kinetics.

Parameters	Valores
Number of frames	30
Minimum contrast	60 pixels
Minimum size of the cell	6 pixels
Contrast to cell	60 pixels
Linearity	70%
Minimum average for VAP	< 40 µm/s
Minimum VAP for progressive cells	< 30 µm/s
Minimum VSL to slow cell	< 20 µm/s
Static head size	0.30 to 7.89
Intensity of static heads	0.41 to 1.19
Elongation of static heads	96-0
Magnification	1.95
Temperature	37°C

VAP, average path velocity; VSL, straight-line velocity.

Flow cytometry

For sperm evaluation, flow cytometry was conducted using a BD LSR Fortessa instrument (Becton Dickinson, Mountain View, CA, USA) equipped with lasers with the following excitement parameters: 488 nm blue, 100 mW, and emission filters 530/30 nm and 695/40 nm; 640 nm red, 40 mW, and 660/20 nm filter; and 405 nm violet, 100mW, and 450/50 nm filter. At least 10,000 cells per sample were analyzed and the data were evaluated by BD FACSDiva ™ software v 6.1

For the evaluation of cell membranes and acrosomal integrity, the Hoechst 33342 (H342), propidium iodide (PI), and FITC-PSA (agglutination of *Pisum sativum* conjugated to fluorescein isothiocyanate were used (Freitas-Dell'Aqua et al., 2012). The sperm were classified into five groups: damaged plasmatic membrane and integrity of acrosomal membrane (DPMIA); damaged plasma and acrosomal membrane (DPMA); integrity of plasma and acrosomal membranes (IPAM); integrity of plasmatic membrane and damaged acrosomal membrane (IPMDA); and high mitochondrial potential (HMP).

For evaluation of the mitochondrial potential and production of superoxide (O_2^-) in the mitochondrial matrix, Hoechst 33342, SYTOX Green Dead Cell Stain (markup for injured cell plasma membrane), *MitoStatus Red* (mitochondrial potential), and *MitoSOXTM* Red (superoxide anion generation in the mitochondrial matrix) were used based on the methodology described by Freitas-Dell'Aqua et al. (2016). The sperm were classified into three categories: percentage of cells without membrane permeability (IC; YOPRO-negative cells), cells with a high percentage of mitochondrial potential (HMP; *MitoStatus Red*-positive cells), and percentage of cells with oxidative stress, superoxide anion (O_2^-; *MitoSox Red*-positive cells).

Statistical analysis

The effects of the extenders and refrigerated transport systems on chilled semen characteristics (MOT, VIG, AC, MiD, MaD, and TD) post-dilution times, post-chilling, and after thawing; the sperm movement kinetics (TM, PM, VAP, VSL, VCL, ALH, TBF, STR, LIN, and RAP); and the plasma membrane and acrosomal integrities (DPMIA, DPMA, IPAM, IPMDA, and HMP), oxidative stress, and mitochondrial potential (O_2^-, IC, and HMP) in frozen-thawed semen were analyzed using the statistical software SAS® version 9.3 (Statistical Analysis Software Cary, NC). The variables were analyzed by the mixed model (MIXED procedure), considering the effects of animals as random effects and the averages of the variation factors were compared by Tukey's test ($P \leq 0.05$).

Results

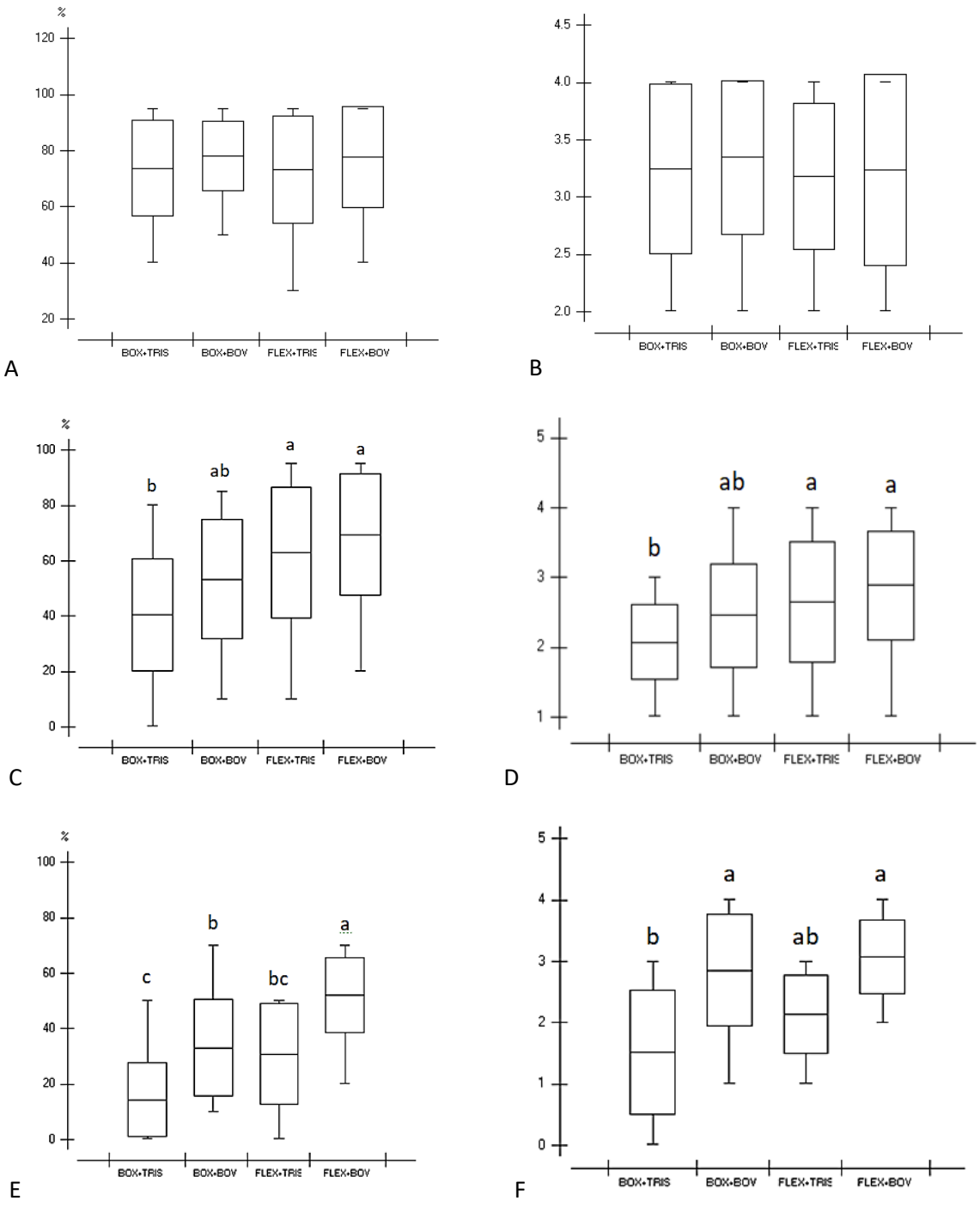
In all harvests, the samples obtained were suitable for semen processing and it was not necessary to discard any sample. Evaluation of characteristics of the fresh semen from the six Nellore bulls for the five harvests showed a volume of ejaculate of 9.1 ± 3 mL, with MOT of 79.3 ± 12%, VIG 3.6 ± 0.6%, MM 2.5 ± 1.2%, and pathologies MiD of 5.4 ± 5.9%, AC of 1.5 ± 1.6%, MaD of 7.2 ± 4.9%, and TD of 12.1 ± 8.1%. These values were considered parameters for further analysis after dilution, after refrigeration, and after freeze-thawing.

The evaluations of MOT and VIG after dilution in TRIS and BotuBOV® and transported in the refrigerated semen transport systems BotuBOX® and BotuFLEX®, after dilution, after refrigeration, and after freeze-thawing are presented in Figure 1.

There was no immediate effect of dilution on seminal characteristics. However, there was an effect of the refrigerated transport system and extender used on the MOT and VIG in the refrigerated semen, with the samples diluted in TRIS extender and transported in the BotuBOX® system showing lower values ($P < 0.05$) than those transported in the BotuFLEX® system, independent of the extender used (Figure 1).

In the analysis of the frozen-thawed semen, the superiority ($P < 0.05$) of the semen samples in the BotuBOV® extender and BotuFLEX® system compared to the other treatments was evident and the best results were obtained for MOT and VIG (Figure 1).

240



241 Figure 1. Graphics box-plots (mean ± standard deviation; maximum and minimum):
242 progressive sperm motility parameters, (A) after dilution; (C) after cooled, and (E)
243 frozen-thawed; and sperm vigor (B) after dilution; (D) after cooled, and (F) frozen-
244 thawed, bovine semen diluted in TRIS and BotuBOV®, submitted to transport prior to
245 freezing in the systems BotuBOX® and BotuFLEX® (BOX+TRIS; BOX+BOV;

246 FLEX+TRIS; FLEX+BOV). Within the graph, averages differ where small letters are
247 different ($P \leq 0.05$).

248 Sperm problems increased with increasing semen processing steps. However,
249 there was no effect of extenders (TRIS and BotuBOV®) or refrigerated semen transport
250 systems (BotuBOX® and BotuFLEX®) on the semen, i.e., there was no difference (P <
251 0.05) between the associations in any evaluation (after dilution, after refrigeration, and
252 frozen-thawed) (Table 2).

253

254 Table 2. Sperm quality parameters (mean ± SD) of bovine semen samples diluted in
255 TRIS and BotuBOV® after dilution, after cooled, and frozen-thawed, submitted to
256 transport prior to freezing using the BotuBOX ® and BotuFLEX® systems.

Variables	BotuBOX®		BotuFLEX®	
	TRIS	BotuBov®	TRIS	BotuBov®
After dilution				
AC (%)	3.2±2.7	3.8±5.3	3.5±3.5	4.1±5.6
MiD (%)	5.3±2.8	5.4±3.1	5.5±4	5.3±3.7
MaD (%)	7.6±3	9±5.2	8.7±3.8	8.1±5.4
TD (%)	12.9±4	14.3±5.6	14.7±3.7	14.3±5.6
After cooled				
AC (%)	4.7±3.5	6.1±5	6.3±2.9	7.2±5.3
MiD (%)	6.2±4.5	5.5±3.5	5.7±3.7	5.8±4.1
MaD (%)	10.8±5	12.4±6.2	11.6±4	14.6±5.1
TD (%)	17±7	17.9±6.3	17.3±5.3	20.4±5.7
Frozen-thawed				
AC (%)	10±6.6	10.4±6.8	13±8	11.1±4.7
MiD (%)	6.7±4.7	5.8±2.9	6.2±3.5	6.3±3.1
MaD (%)	16.2±6.7	17.7±7.1	20.3±7.4	18.1±5.1
TD (%)	22.9±7	23.5±7.6	26.5±6.7	24.4±5.8

257 Inside the line, medium without equal, small letter differed ($P \leq 0.05$)

258 Acrossomo (AC), Minor Defects (MiD), Major Defects (MaD), Total Defects (TD)
259

260 Regarding the seminal samples evaluated with CASA, PM and TM were larger
261 (P < 0.05) in the BotuBOV® extender and BotuFLEX® transport system treatment. The
262 combination of the TRIS extender and BotuBOX® transport system on VSL exhibited a
263 lower value (P < 0.05) than that of the other treatments (Table 3).

The BotuBOV® extender and BotuFLEX® transport system treatment yielded better results for TBF ($P < 0.05$) than the TRIS extender and BotuBOX® transport system treatment. Regarding the percentage of RAP, the BotuBOV® extender and BotuFLEX® transport system treatment did show a higher value when compared with the other treatments in the study (Table 3).

Table 3. Sperm parameters (mean ± SD) assessed by the computerized semen analysis (CASA) after thawing, bovine semen samples diluted in TRIS and BotuBOV®, submitted to transport prior to freezing by the BotuBOX ® and BotuFLEX® systems.

Variables	BotuBOX®		BotuFLEX®	
	TRIS	BotuBOV®	TRIS	BotuBOV®
TM (%)	9±10.8c	21.2±21.6bc	25±18.7b	47.3±22.9a
PM (%)	5.9±7.4c	16±15.7b	17.8±12.9b	37±18.1a
VAP (µm/s)	60.2±27	69.4±22.7	70.6±20.3	76.4±25.1
VSL (µm/s)	47.6±21b	58.5±19.3a	57.1±16.6a	65.1±21.3a
VCL (µm/s)	106.3±49.8	115.9±38.9	118.4±33.6	121.1±40.4
ALH (µm)	5.1±2.8	4.9±1.8	5.1±1.5	4.6±1.5
TBF (Hz)	21.5±10.9b	26.2±9.3ab	23±8.4ab	30±8.3a
STR (%)	69.5±29	77.3±23.8	76.8±20.3	80.5±21.1
LIN (%)	41.3±18.8	50.4±16.1	47.8±13.2	53.1±14.5
RAP (%)	6.9±9.1c	19.8±20.8b	22.9±17.2b	44.5±22.2a

Inside the line, medium without equal, small letter differed ($P \leq 0.05$)

total sperm motility (TM), progressive motility (PM), average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), tail beat frequency (TBF), straightness (STR), linearity (LIN), rapid spermatozoa (RAP)

From the oxidative stress assessment of frozen-thawed semen samples, the IC percentage was greater ($P < 0.05$) for the diluted semen in the BotuBOV® extender and BotuFLEX® transport system treatment than that with the TRIS extender and BotuBOX® transport system treatment (Table 4).

There was an effect of the TRIS extender that was independent of the refrigerated semen transport system for the production of O_2^-, which was higher ($P < 0.05$) than that of the BotuBOV® extender and BotuFLEX® transport system treatment (Table 4).

In terms of the integrity of plasma and acrosomal membranes in the frozen-thawed semen samples, diluted semen in the BotuBOV® extender, regardless of the refrigerated transport system used, contained a smaller percentage ($P < 0.05$) of DPMA than that in the TRIS extender and BotuBOX® transport system treatment, which exhibited the highest percentage (Table 4). Additionally, the percentage of IPAM suffered the effect of the BotuBOV® extender, regardless of the refrigerated transport system used and presented higher values ($P < 0.05$) than that in the TRIS extender and BotuBOX® transport system treatment, which exhibited a lower percentage (Table 4).

Table 4. Evaluation of oxidative stress, mitochondrial potential, and cell membrane and acrossomal integrity (mean ± SD) by flow cytometry of semen samples after thawing diluted in TRIS and BotuBOV®, submitted to transport prior to freezing in the systems BotuBOX® and BotuFLEX®

Variables	BotuBOX®		BotuFLEX®	
	TRIS	BotuBOV®	TRIS	BotuBOV®
Oxidative stress and mitochondrial potential				
IC (%)	8.1±9.7b	11±9.8ab	10.2±7.6ab	19.8±11a
O_2^- (%)	90±8.9a	87±10ab	90±5.7a	82±8b
HMP (%)	11.2±9.3	9±8.5	9.6±6.1	15.2±13.6
Integrity of plasma and acrossomal membranes				
DPMIA (%)	37.8±21.9	42.3±15.3	46.5±16.9	44±17
DPMA (%)	49±21.7a	34.2±15.3b	36.6±16.3ab	26.1±21.1b
IPAM (%)	12.1±12.7a	22.2±15.4a	16.3±9.5ab	29±14.4a
IPMDA (%)	1±1.2	1.3±1.4	0.6±0.8	0.9±1.2
HMP (%)	24.6±20.9	18.2±15.8	16.4±7.5	23.8±10.7

Inside the line, medium without equal, small letter differed ($P \leq 0.05$)

Percentage of cells without membrane permeability (IC), cells with high percentage of mitochondrial potential (HMP), percentage of cells with oxidative stress, superoxido anion (O_2^-), damaged plasmatic membrane and integrity acrossomal membrane (DPMIA); damaged plasma and acrossomal membrane (DPMA); integrity of plasma

and acrossomal membranes (IPAM); integrity plasmatic membrane and damaged acrossomal membrane (IPMDA); and high mitochondrial potential (HMP).

Discussion

Subjective assessments, after semen was refrigerated and frozen-thawed, and the CASA and flow cytometry analyses of frozen-thawed semen showed that the TRIS and BotuBOV® extenders and the BotuBOX® and BotuFLEX® refrigerated transport systems, when used prior to freezing, influenced the quality of the refrigerated and frozen-thawed bovine semen.

The system used for the processing of semen after harvesting was conventional and not an automated system. Despite this, the temperature outside did not interfere with the process; moreover, on subjective analysis of the cooled semen, there were no deleterious effects, exhibiting the maintenance of semen viability.

The BotuBOX® transport system used 1 ice pack and the temperature reached at least 15°C. The BotuFLEX® transport system used 2 ice packs and the temperature reached at least 5°C. The period in which the diluted semen samples were transported was 3 h, and the temperature of the semen samples transported in the BotuBOX® system was 18°C, whereas semen transported in the BotuFLEX® system had a temperature of 12.5°C. Squires et al. (1999) showed that for every 10°C that the temperature of sperm cells decreased, cell metabolism was reduced by 50%; thus, the refrigerated transport system played an important role because it minimized cell damage resulting from the metabolism of fresh semen.

Cooled semen has been shown to result in a higher pregnancy rate than that of frozen semen and has prolonged viability in relation to fresh semen; thus, it has been widely employed and studied (Holt, 2000). The use of extenders in cooled form

329 decrease sperm metabolism and retain a greater potential of the sperm fertilizing
330 population; however, this should only be used for a short period of time, mostly
331 between 24 and 72 h (Crespilho et al., 2012; Borges-Silva et al., 2015; Papa et al.,
332 2015).

333 The conditions employed in the present study, i.e. cooling for 4 h before
334 freezing, resulted in a temperature of 5°C and reduced the sperm metabolism to 10% of
335 that required for survival as compared to that at 38°C (Squires et al., 1999).

336 Despite subjective analysis after being cooled, the best results were obtained
337 for samples carried in the BotuFLEX® transport system, regardless of the extender used
338 because high cooling rates of the BotuFLEX® system enabled appropriate preservation
339 of semen with the lowering of temperature and supplanted any deficiency of the TRIS
340 extender in relation to the BotuBOV® extender. The semen in the BotuBOV® extender
341 and BotuBOX® transport system treatment, despite being statistically lower for MOT,
342 was within the value expected for a refrigerated semen sample, which was similar to the
343 experiment by Tarrago (2016), where MOT was 55.31 ± 6.47% for diluted semen in
344 BotuBOV®, with 48 h of refrigeration to 5°C, which resulted in a 48.7% pregnancy rate.
345 In this case, the BotuBOV® extender overcame the low rate of cooling of the
346 BotuBOX® transport system and allowed for the conservation of MOT.

347 The TRIS extender and BotuBOX® transport system treatment was inefficient
348 for retaining sperm viability after refrigeration, possibly because of the cooling rate of
349 the transport system. This implied that the extender failed to protect the sperm and
350 indicated the presence of thermal shock in addition to the wear and tear of metabolism.
351 Amann and Graham (1993) explained that the temperature range between 19 and 8°C is
352 the most critical phase for the occurrence of injuries and changes to sperm.

353 In addition to showing optimal results for refrigeration, the combination of the
354 BotuBOV® extender and BotuFLEX® refrigerated semen transport system was most
355 efficient in analysis after thawing. This was because the MOT was above the parameter
356 set by CBRA (2013) for frozen samples of ≥ 30%, which also occurred with the semen
357 in the BotuBOV® extender and BotuBOX® transport system treatment.

358 The highest MOT observed in the subjective analysis, and the TM and PM in
359 the CASA, agree with the results of cellular integrity (IPAM), cells without membrane
360 permeability (IC), and lower production of O_2^- in thawed samples that were diluted with
361 BotuBOV® extender and transported via the BotuFLEX® system. The interaction of the
362 sperm cells and extender, a key factor for the preservation of sperm integrity, as well as
363 the cooling rate provided by the refrigerated transport system improved the rates of
364 freezing and thawing (Manjunath et al., 2002).

365 In contrast, samples diluted in TRIS extender and transported via the
366 BotuBOX® system after thawing showed lower MOT, TM, PM, and VSL values; low
367 percentage of IC; and highest percentage of DPMA. This may be because when
368 refrigerated, the semen had already moved through the critical period, which set off the
369 abnormal movement of displacement, MOT, lesions in the membranes, metabolism, and
370 reduction of enzymes (Aurich, 2005). In this context, Nair et al. (2006), using the semen
371 of bulls, found high negative correlations between lipid peroxidation and MOT (r = -
372 0.90) and sperm viability (r = -0.93).

373 Despite the TBF, the favorable results found using the BotuBOV® extender
374 and BotuFLEX® transport system can be explained by the presence of some compound
375 in the BotuBOV® extender that stimulated the frequency of tail beating. This result
376 added to the higher VSL and greater TM and PM percentages of semen in the

BotuBOV® extender and BotuFLEX® transport system treatment. Mortimer (2000) and Verstegen et al. (2002) showed that samples with high-speed parameters values, LIN, and TBF showed better migration and penetration of the cervical mucus and showed a positive correlation with pregnancy rate.

The highest RAP percentage found in the BotuBOV® extender and BotuFLEX® transport system treatment was consistent with the highest TBF, and greater TM, PM, and VSL values; therefore, this treatment was considered optimal by the CASA when referring to sperm kinetics.

To produce O_2^-, the highest value was associated with the TRIS extender, which suggested that the manner in which the extender components interacted with the semen was not enough to protect sperm cells from peroxidative lesions. These lesions induced the generation of reactive oxygen species, which are largely responsible for damage to sperm viability and fertility (Alvarez and Moraes, 2006).

The higher percentage of IPAM and lower percentage of DPMA were associated with the BotuBOV® extender, and we suggest that this extender provided greater protection of organelles and increased plasma membrane integrity, indicating that it was favorable for sperm viability once the membranes were extremely susceptible to damage from the external environment (Holt and Medrano, 1997). Additionally, for fertilization, the acrosome must remain intact until connection with the pellucida zone. When the acrosome reacted prematurely, a decline in semen fertility index was observed (Silva and Gadella, 2006).

Based on the results of the present study, we recommend the development of semen extenders with components that protect refrigerated and frozen semen membranes against oxidative stress, as well as the establishment of adequate

refrigeration and freezing rates that allow the action of the cryoprotectant and, in the case of freezing, the translocation of water, thus reducing the negative effect of the formation of ice crystals. Extenders should be combined with the use of appropriate refrigerated semen transport systems, prior to refrigeration at 5°C or freezing.

Thus, the results clearly show the possibility of cooling for short periods in refrigerated transport systems. Semen diluted in the BotuBOV® extender and transported in the BotuBOX® or BotuFLEX® transport systems, and semen diluted in TRIS extender and transported in the BotuFLEX® transport systems maintained the feasibility to use refrigerated semen, as well as employment for later freezing. Semen diluted in the BotuBOV® extender and transported in the BotuFLEX® refrigerated system presented the best effect on semen in terms of kinetic parameters, sperm plasma membrane and acrosomal integrity, and oxidative stress reduction.

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4. CONCLUSÕES DA TESE

Conclui-se que o sêmen de touros Nelore criados a pasto nos trópicos, a fresco sofre influência das estações do ano e suas respectivas temperaturas, umidades relativas do ar e índice de temperatura e umidade. O sêmen refrigerado e congelado apresenta maior viabilidade dependendo do meio diluente e do sistema de transporte refrigerado de sêmen.

No capítulo 1,

Conclui-se que:

(1) nas estações da primavera e verão, nas quais o ITU excedeu o valor de 72, observou-se decréscimo na motilidade progressiva e vigor espermático.

(2) No verão e no outono, estações que apresentaram maiores porcentagens de defeitos espermáticos maiores e totais.

(3) As temperaturas do globo ocular e da superfície do escroto aumentaram na primavera e verão e o gradiente térmico e a concentração plasmática de testosterona diminuiu nas mesmas estações, concluindo que o estresse térmico com ITU acima de 72, nas estações primavera e verão, influenciou de forma negativa essas variáveis estudadas.

(4) As proteínas do plasma seminal de 20, 55 e 66 KDa contribuíram de forma positiva para a qualidade seminal. A concentração da proteína de 50 KDa foi elevada em todas as estações, sendo considerada importante para o desempenho dos touros.

No capítulo 2,

Conclui-se que:

(5) os resultados demonstram de maneira clara, a possibilidade do resfriamento curto em sistema de transporte refrigerado, do sêmen diluído no meio BotuBOV® e transportado na BotuBOX® ou BotuFLEX®, e diluído em meio TRIS e transportado na BotuFLEX® mantendo a viabilidade seminal para uso do sêmen refrigerado, bem como o emprego para a congelação.

(6) A associação entre o meio diluente BotuBOV® e o sistema de transporte refrigerado de sêmen BotuFLEX® apresentou melhor efeito sobre o sêmen em relação aos parâmetros de cinética espermática, integridade de membrana plasmática e acrossomal e redução do estresse oxidativo.

5. CONSIDERAÇÕES PESSOAIS

(1) A técnica de eletroforese SDS-PAGE do plasma seminal apresenta importantes resultados visto que as proteínas apresentam grande relação com a qualidade do sêmen, no entanto, a técnica demanda tempo, estrutura física, equipamentos e reagentes específicos e treinamento pessoal, limitando o seu emprego no dia-a-dia a campo. Para a pesquisa, esta técnica traz informações complementares em casos, onde, a avaliação do sêmen por si só não esclarece as causas de subfertilidade, pois as proteínas presentes no plasma seminal apresentam muitas funções, como proteção de membranas contra choque térmico entre outros;

(2) A termografia por infravermelho, apesar de ser um investimento alto custo inicialmente, é uma ferramenta que pode atuar com complementar ao exame andrológico, visto que oferece resultados de “status” térmico, pois a temperatura do globo ocular tem alta correlação com a temperatura retal e por sua vez, com alta correlação com a temperatura da superfície do escroto que está relacionada com a qualidade seminal. Sendo uma ferramenta, que contribui muito para a prática no dia-a-dia, pois além dessas vantagens citadas, é portátil, possui bateria de longa duração, as imagens são trabalhadas em software para obtenção das temperaturas, mas que imediatamente consegue-se visualizar a imagem com o gradiente de temperaturas. Necessita um treinamento rápido para utilização do equipamento e do software, sendo sua utilização indicado para pesquisadores e profissionais nas áreas de saúde e reprodução animal

(3) A técnica do CASA nas amostras de sêmen, é uma técnica sofisticada que apresenta resultados da cinética espermática, que apesar de não necessitar de reagentes e muitos equipamentos (banho-maria, placa aquecedora e câmara makler), o equipamento principal para a análise do sêmen necessita de um investimento alto e treinamento especializado para a utilização do mesmo. Para a aplicação prática no dia-a-dia de um profissional liberal, não é um equipamento que possa ser transportado à fazenda. Mas é uma técnica com aplicação consolidada na pesquisa com experimentos que tem como resultado alta correlação com a fertilidade “in vivo”;

(4) A técnica de citometria de fluxo nas amostras de sêmen é uma técnica fantástica com várias aplicações sobre a qualidade seminal, como integridade de membranas, estresse oxidativo e potencial mitocondrial. No entanto, necessita de alto investimento tanto para o equipamento quanto para as sondas, bem como, a tecnificação específica do profissional que utiliza o equipamento. Nas técnicas de CASA e citometria de fluxo, poderia ser utilizada em touros de alto interesse e alto investimento, nos quais as amostras de sêmen congelado seria levado para centros de pesquisa ou universidades para a utilização dessas técnicas.

Anexo 1

International Journal of Biometeorology

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Always use footnotes instead of endnotes.

Acknowledgments

Acknowledgments of people, grants, funds, etc. should be placed in a separate section on the title page. The names of funding organizations should be written in full.

Important notes:

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Please arrange your manuscript as follows:

- Introduction

  The introduction should state the purpose of the investigation and give a short review of the pertinent literature.

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  These should be as brief as possible. Any grant that requires acknowledgement should be mentioned. The names of funding organizations should be written in full.

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  These should only include sources that were directly cited in the manuscript, and must be listed in alphabetical order by the first author's last (family) name. These should not be numbered. Please see specific instructions on formatting below.

SCIENTIFIC STYLE

REFERENCES

Citation

Cite references in the text by name and year in parentheses. Some examples:

- Negotiation research spans many disciplines (Thompson 1990).
- This result was later contradicted by Becker and Seligman (1996).
- This effect has been widely studied (Abbott 1991; Barakat et al. 1995a, b; Kelso and Smith 1998; Medvec et al. 1999, 2000).

Reference list

The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should only be mentioned in the text. Do not use footnotes or endnotes as a substitute for a reference list.

Reference list entries should be alphabetized by the last names of the first author of each work. Order multi-author publications of the same first author alphabetically with respect to second, third, etc. author. Publications of exactly the same author(s) must be ordered chronologically.

- Journal article

  Gamelin FX, Baquet G, Berthoin S, Thevenet D, Nourry C, Nottin S, Bosquet L (2009) Effect of high intensity intermittent training on heart rate variability in prepubescent children. Eur J Appl Physiol 105:731-738. https://doi.org/10.1007/s00421-008-0955-8

  Ideally, the names of all authors should be provided, but the usage of "et al" in long author lists will also be accepted:

  Smith J, Jones M Jr, Houghton L et al (1999) Future of health insurance. N Engl J Med 965:325–329

- Article by DOI

Slifka MK, Whitton JL (2000) Clinical implications of dysregulated cytokine production. J Mol Med. https://doi.org/10.1007/s001090000086

- Book

  South J, Blass B (2001) The future of modern genomics. Blackwell, London

- Book chapter

  Brown B, Aaron M (2001) The politics of nature. In: Smith J (ed) The rise of modern genomics, 3rd edn. Wiley, New York, pp 230-257

- Online document

  Cartwright J (2007) Big stars have weather too. IOP Publishing PhysicsWeb. http://physicsweb.org/articles/news/11/6/16/1. Accessed 26 June 2007

- Dissertation

  Trent JW (1975) Experimental acute renal failure. Dissertation, University of California

Always use the standard abbreviation of a journal's name according to the ISSN List of Title Word Abbreviations, see

- ISSN LTWA

If you are unsure, please use the full journal title.

For authors using EndNote, Springer provides an output style that supports the formatting of in-text citations and reference list.

- EndNote style (zip, 2 kB)

TABLES

- All tables are to be numbered using Arabic numerals.
- Tables should always be cited in text in consecutive numerical order.
- For each table, please supply a table caption (title) explaining the components of the table.
- Identify any previously published material by giving the original source in the form of a reference at the end of the table caption.
- Footnotes to tables should be indicated by superscript lower-case letters (or asterisks for significance values and other statistical data) and included beneath the table body.

ARTWORK AND ILLUSTRATIONS GUIDELINES

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  Funding: This study was funded by X (grant number X).

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When reporting studies that involve human participants, authors should include a statement that the studies have been approved by the appropriate institutional and/or national research ethics committee and have been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

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All individuals have individual rights that are not to be infringed. Individual participants in studies have, for example, the right to decide what happens to the (identifiable) personal data gathered, to what they have said during a study or an interview, as well as to any photograph that was taken. Hence it is important that all participants gave their

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Datasets that are assigned digital object identifiers (DOIs) by a data repository may be cited in the reference list. Data citations should include the minimum information recommended by DataCite: authors, title, publisher (repository name), identifier.

- DataCite

Where a widely established research community expectation for data archiving in public repositories exists, submission to a community-endorsed, public repository is mandatory. Persistent identifiers (such as DOIs and accession numbers) for relevant datasets must be provided in the paper.

For more information:

- Research Data Policy Frequently Asked Questions

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The journal encourages authors to provide a statement of Data availability in their article. Data availability statements should include information on where data supporting the results reported in the article can be found, including, where applicable, hyperlinks to publicly archived datasets analysed or generated during the study. Data availability statements can also indicate whether data are available on request from the authors and where no data are available, if appropriate.

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Anexo 2

Animal Reproduction - Instructions to authors

The Animal Reproduction (AR) publishes original scientific papers and invited literature reviews, with a goal of contributing to a better understanding of phenomena related to animal reproduction.

All submission should be sent online system: https://mc04.manuscriptcentral.com/animalreproduction

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2. ***Manuscript Sections***
3. *Title page*
   ii. Abstract
   iii. Introduction
   iv. Materials and Methods
   v. Results
   vi. Discussion
   vii. References
   viii. Acknowledgements
   ix. List of abbreviations used
   x. Tables
   xi. Figures
3. ***Title page****: The title should be succinct but include the study design and major topics.*
4. *Title words should be in bold, with only the first letter of the first word in upper case.*
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6. ***Methods****: Should include the design of the study, type of materials involved, number of animals per group, a clear description of all methods used and/or clear references to published methods, and the type of analysis used.*

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1. *sole author: (Ginther, 1992) or Ginther (1992).*
2. *two authors: (Varley and Foxcroft, 1990) or Varley and Foxcroft (1990).*
3. *more than two authors: (Quintero et al., 2000) or Quintero et al. (2000).*
4. *more than one paper cited: (Varley and Foxcroft, 1990; Ginther, 1992; Gastal et al., 1999a; b; Quintero et al., 2000) or Varley and Foxcroft (1990); Ginther (1992); Gastal et al., (1999a; b); Quintero et al. (2000), always cited in ascending chronological order.*

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- *For PERIODICALS*

Gastal EL, Gastal MO, Ginther OJ*. 1999a. Experimental assumption of dominance by a smaller follicle and associated hormonal changes in mares. Biol Reprod, 61:724–730.*
Gastal EL, Donadeu FX, Gastal MO, Ginther OJ*. 1999b. Echotextural changes in the follicular wall during follicle deviation in mares. Theriogenology, 52:803-814.*
Hess RA, Carnes K*. 2004. The role of estrogen in testis and the male reproductive tract: a review and species comparison. Anim Reprod, 1:5-30.*
*S**artori R, Souza AH, Guenther JN, Caraviello DZ, Geiger LN, Schenk JL, Wiltbank MC****. 2004. Fertilization rate and embryo quality in superovulated Holstein heifers artificially inseminated with X-sorted or unsorted sperm. Anim Reprod, 1:86-90, 2004.*
Varley MA, Foxcroft GR*. 1990. Endocrinology of lactating and weaned sow. J Reprod Fertil Suppl, 40:47-61.*

- *For OTHER DOCUMENTS than periodicals*

Basrur PK, Yusoff RBH*. 1997. Sex anomalies in goats. In Youngquist, RS (Ed.). Current therapy in large animal theriogenology. Philadelphia, USA: WB Saunders. pp.553-290*
Ginther OJ. 1992. Reproductive biology of the mare: Basic and applied aspects. 2.ed. Cross Plains, WI, USA: Equiservices Publishing. pp.105-172.
Leal MC*. 2004. Morphometric and functional analyses of testis and spermatogenic efficiency in the marmoset (Callithrix penicillata) [in Portuguese]. Belo Horizonte, Brazil: Federal University of*

Minas Gerais. Thesis.
***Quintero B, Porter M, Sharp D, Cleaver B, Diaz T**. 2000. Effect of season on LH concentrations and LH pulse dynamics in mares located in the tropics. In Abstracts of the 14th International Congress on Animal Reproduction, 2000, Stockholm, Sweden. Stockholm: ICAR. pp .290.*

- ***For ELECTRONIC DOCUMENTS***
  CD-ROM
  ***Anderson SC, Poulsen KB**. 2002. Anderson's electronic atlas of hematology [CD-ROM]. Philadelphia: Lippincott Williams & Wilkins.*
  Journal article on the Internet
  ***Abood S**. 2002. Quality improvement initiative in nursing homes: the ANA acts in an advisory role. Am J Nurs [serial on the Internet], 102: 3pp. Available in: http://www.nursingworld.org/AJN/2002/june/Wawatch.htm. Accessed in: Aug 12th 2002.*
  Monograph on the Internet
  ***Foley KM, Gelband H**. (Eds.). 2001. Improving palliative care for cancer [monograph on the Internet]. Washington: National Academy Press. Available from: http://www.nap.edu/books/0309074029/html/. Accessed in: July 9th. 2002.*
  Homepage/Web site
  ***Cancer-Pain**.org [homepage on the Internet]. 2002. New York: Association of Cancer Online Resources, Inc. Available in: http://www.cancer-pain.org/. Accessed in: Jul 9th. 2002.*
  Part of a homepage/Web site
  ***American Medical Association** [homepage on the Internet]. 2001. Chicago: The Association. Available from: http://www.ama-assn.org/ama/pub/category/1736.html. Accessed in: Aug 12th. 2002.*
  Database on the Internet
  Open database:
  ***Who's** Certified [database on the Internet]. 2000. Evanston, IL: The American Board of Medical Specialists. Available from: http://www.abms.org/newsearch.asp. Accessed in: Mar 8th. 2001.*
  Closed database:
  ***Jablonski S**. 2001. Online Multiple Congential Anomaly/Mental Retardation (MCA/MR) syndromes [database on the Internet]. Bethesda, MD: National Library of Medicine. Available from: http://www.nlm.nih.gov/mesh/jablonski/syndrome_title.html. Accessed in: Aug 12th. 2002.*
  Part of a database on the Internet
  ***MeSH** Browser [database on the Internet]. 2002. Bethesda, MD: National Library of Medicine. Meta-analysis; unique ID: D015201; [3pp.]. Available from: http://www.nlm.nih.gov/mesh/MBrowser.html Files updated weekly. Accessed in: Jun 10th. 2003.*
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13. *There is no page charges and figures limit. Particularly in case of colorful pictures please contact the editors.*
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UNOESTE - Universidade do Oeste Paulista

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PPG - Programa de Pesquisa de Pós-Graduação
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Parecer Final

Declaramos para os devidos fins que o Projeto de Pesquisa intitulado **"TERMOGRAFIA POR INFRAVERMELHO DO ESCROTO, ELETROFORESE DO PLASMA SEMINAL E CONGELAÇÃO DO SÊMEN DE TOUROS NELORE NAS QUATRO ESTAÇÕES DO ANO"**, cadastrado na Coordenadoria de Pesquisa, Desenvolvimento e Inovação (CPDI) sob o número nº **3479** e tendo como participante(s) **MARCELO GEORGE MUNGAI CHACUR (responsável), LUCIANA MACHADO GUABERTO (docente), CAMILA DUTRA DE SOUZA (discente), ELLYN AMANDA FONSECA MARTINS (discente), FERNANDA LUIZA GUINOSSI BARBOSA DEAK (discente), GABRIELA FIGUEREDO CORNACINI (discente), GUILHERME BASTOS (discente), ISAMARA BATATA ANDRADE (discente), RODRIGO GOMES RICCI (discente), TALITA RAQUEL CAVICHIOLI SEBASTIAO (discente), WILLIAN MITUZI TATEISI (discente), EUNICE OBA (externo), LUIS ROBERTO ALMEIDA GABRIEL FILHO (externo)**, foi avaliado e **APROVADO** pelo **COMITÊ ASSESSOR DE PESQUISA INSTITUCIONAL (CAPI) e COMISSÃO DE ÉTICA USO DE ANIMAIS (CEUA)** da Universidade do Oeste Paulista - UNOESTE de Presidente Prudente/SP.

Este Projeto de Pesquisa, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica, encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de Outubro de 2008, do Decreto nº 6.899, de 15 de Julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), tendo sido APROVADO em reunião realizada em 05/10/2016.

Vigência do projeto: 01/2017 a 03/2018.

Espécie/Linhagem	Nº de Animais	Peso	Idade	Sexo	Origem
Bovinos Nelore	20	400 quilos	2 anos	M	Propriedade rural particular

Presidente Prudente, 28 de Março de 2017.

Prof. Dr. Jair Rodrigues Garcia Jr.
Coordenador Científico da CPDI

Profª Ms. Adriana Falco de Brito
Coordenadora da CEUA - UNOESTE

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