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Climatic adaptation of bovine subspecies monitored by seminal characteristics during winter months in tropical region

Adaptação climática das subespécies bovinas monitoradas pelas características seminais durante meses de inverno em região tropical

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Ana Beatriz Marques de Almeida
Doutoranda em Ciência Animal na Universidade Estadual de Londrina, PR
Instituição: Universidade Estadual de Londrina - UEL
Endereço: Av. Olávo García Ferreira da Silva, s/n - Campus Universitário, Londrina - PR, 86051-990, Brasil
E-mail: biamarquesvet30@gmail.com

Myrian Megumy Tsunokawa Hidalgo
Mestranda em Ciência Animal na Universidade Estadual de Londrina, PR
Instituição: Universidade Estadual de Londrina - UEL
Endereço: Av. Olávo García Ferreira da Silva, s/n - Campus Universitário, Londrina - PR, 86051-990, Brasil
E-mail: myrianhidalgo@hotmail.com

Fábio Morotti
Professor de Reprodução e Obstetrícia de Grandes Animais na Universidade Estadual de Londrina, PR
Instituição: Universidade Estadual de Londrina - UEL
Endereço: Av. Olávo García Ferreira da Silva, s/n - Campus Universitário, Londrina - PR, 86051-990, Brasil
E-mail: fabiomorotti@hotmail.com

Maria Isabel Mello Martins
Professora Associada de Teriogenologia de Animais de Companhia da Universidade Estadual de Londrina, PR
Instituição: Universidade Estadual de Londrina - UEL
Endereço: Av. Olávo García Ferreira da Silva, s/n - Campus Universitário, Londrina - PR, 86051-990, Brasil
E-mail: imartins@uel.br

ABCTRACT
The objective of this study was to evaluate parameters indicative of sperm quality (motility, vigor, and morphology) during the winter months in South-Mato Grosso, from *Bos taurus* and *Bos indicus* bulls, to analyze the climate influences in seminal changes. The ejaculate collects were performed on alternate days, using an artificial vagina, at a central for semen collection and bovine reproduction in the Midwest region from Brazil. In total, 122 ejaculates were evaluated, from 10 bulls, six *Bos indicus* and four *Bos taurus*. The ejaculates were submitted to evaluation of motility and vigor in phase contrast microscopy, and the sperm morphology was analyzed in a humid chamber. Samples of semen that presented 60% of motility and score 3 in vigor analysis were selected for freezing. After thawing, the motility and vigor were again evaluated. The statistical analysis was performed using a generalized linear mixed model (GLIMMIX) ($P \leq 0.05$). Fresh ($P = 0.016$) and post-thawing vigor (P

= 0.014), as well as the proportion of minor defects were influenced by the climate (P = 0.001) over the months. Among the subspecies (*indicus* x *taurus*), differences were observed only for minor morphological defects (3.27% *indicus* x 2.6% *taurus*; P = 0.009). It was concluded that, independent of the subspecies, freezing decreases the seminal parameters evaluated. In addition, indicus animals presented a lower proportion of minor defects, and taurine bulls seemed to be better adapted to the winter, although the climate did not generate significant alterations in ejaculate quality.

Key words: bulls, cryopreservation, semen, sperm motility.

RESUMO

O objetivo deste estudo foi avaliar parâmetros indicativos da qualidade espermática (motilidade, vigor e morfologia) durante os meses de inverno, de touros *Bos taurus* e *Bos indicus*, no sul do Mato Grosso, para analisar influências climáticas sobre características seminais. As coletas de ejaculados foram realizadas em dias alternados, utilizando uma vagina artificial, em uma central de coleta de sêmen e reprodução bovina na região Centro-Oeste do Brasil. No total, foram avaliados 122 ejaculados, de 10 touros, seis *Bos indicus* e quatro *Bos taurus*. Os ejaculados foram submetidos à avaliação de motilidade e vigor em microscopia de contraste de fase, e a morfologia espermática foi analisada em câmara úmida. Amostras de sêmen que atingiram 60% de motilidade e escore 3 na análise de vigor foram selecionadas para congelação. Após o descongelação, a motilidade e o vigor foram novamente avaliados. Para análise estatística utilizau-se um modelo misto linear generalizado (GLIMMIX) ($P \leq 0{,}05$). O vigor a fresco (P = 0,016) e pós-descongelação (P = 0,014), bem como a proporção de defeitos menores, foram influenciados pelo clima (P = 0,001) ao longo dos meses. Entre as subespécies (*indicus* x *taurus*), foram observadas diferenças apenas para defeitos morfológicos menores (3,27% *indicus* x 2,6% *taurus*; P = 0,009). Concluiu-se que, independente da subespécie, o processo de criopreservação prejudicou os parâmetros seminais avaliados. Além disso, animais índicos apresentaram menor proporção de defeitos menores, e os taurinos pareceram se adaptar melhor ao inverno, embora o clima não tenha gerado alterações significativas na qualidade dos ejaculados.

Palavras-chave: criopreservação, motilidade espermátic, sêmen, touros.

1 INTRODUCTION

Artificial Insemination (AI) is one of the most widely used biotechniques in breeding, representing a strategy with low cost and great potential for dissemination of genetic improvement in herds. However, the semen production from bulls with high reproductive efficiency is limited, considering the viability and sperm yield of the animals (Ram, Tiwari, Mishra, Sahasrabudhe, & Nair, 2017)

To commercialize semen, besides the genetic merit of the bull, it is necessary to guarantee high sperm quality, in such a way that the kinetic and morphological characteristics of the spermatozoa are compatible with high fertilization rates. In this context, it is essential to select breeding bulls capable of producing spermatozoa resistant to cryopreservation and maintaining high sperm viability and fertilizing capacity after thawing. However, not all bulls are considered suitable for frozen semen production, since there are innumerable intrinsic (age, bull's health, libido) and extrinsic variables (season, feeding, management) involved in this process (Queiroz, Filho, Rosa, Zúccari, & Costa e Silva, 2015; Ram et al., 2017).

For adequate spermatogenesis, the testicles should remain 2º to 6ºC below the body temperature (Barth & Bowman, 1994). Studies by Garcia (2017) have shown that thermal stress in bulls can generate testicular changes of varying levels. In addition, animals with zebu blood present more efficient testicular thermoregulation compared to taurine animals when subjected to thermal stress condition (Brito, Silva, Barbosa, Unanian, & Kastelic, 2003).

Therefore, this study aimed to evaluate the main sperm parameters of *Bos taurus* and *Bos indicus* bulls during the winter period to determine whether subspecies, climatic conditions, and their interactions may influence fertility indicators.

2 MATERIALS AND METHODS

This study was carried out according to the Ethics Committee on Animal Experimentation of the State University of Londrina based on Federal Law 11,794 of October 8, 2008.

The present study was conducted at a single farm (central for semen collection and bovine reproduction), located in the municipality of Campo Grande, Mato Grosso do Sul, Brazil (Latitude: -20.4435, Longitude: -54.6478 20 ° 26'37 ", South, 54 ° 38'52" West). The climate in the region is classified as Aw according to the Köppen (1918) scale, being considered tropical with a dry winter season. Samples of semen were collected and analyzed during winter season of South American. The average temperatures and rainfall index are described in Table 1.

Table 1. Mean temperature and rainfall index of the months of May, June, July, August, and September in Campo Grande, Mato Grosso do Sul, Brazil, during the execution of this study.

Month	Average temperature (ºC)	Rainfall index (mm)
May	20.69	183,00
June	20.99	54.20
July	20.35	119.20
August	23.53	17.20
September	25.78	65.80

Source: CEMTEC

In total, 122 ejaculates were analyzed from 10 bulls; four *Bos taurus* (two Red Angus and two Senepol) and six *Bos indicus* (four Nellore and two Gir bulls). The bulls were breeders used for semen collection and commercialization of semen doses, which had already been adopted the management conditions of the central. The animals were aged between 3 and 12 yers old, all bulls were healthy, presented good nutritional status (body score between 3 and 4), and were kept in individual open paddocks containing *Bachiaria decumbens* pasture, and received the same diet, mineral salt, and water *ad libitum*.

Semen collections occurred in July, August and September. Yet, this study considered the last autumn month (May), as well as the first winter month (June), since total spermatogenesis in bulls lasts 61 days (Staub & Johnson, 2018), besides being a period of adaptation to the climatic conditions of the season.

The semen was collected on alternate days by the artificial vagina method and, immediately afterwards, it was sent to the andrology laboratory located at the same central, where macroscopic and microscopic analyzes were performed. In the laboratory, the samples were kept in a 37°C water bath until macroscopic analysis, which was used to classify the ejaculate aspect and determine the volume.

Microscopic analysis of the semen was performed by the subjective evaluation of sperm motility and fresh vigor as recommended by the CBRA (2013). For this, a 5μL aliquot from each ejaculate was placed on a preheated slide at 37°C, covered by a cover slip, and viewed under an optical microscope (Nikon® E200) with a 40x magnification. Sperm motility was determined from an estimated proportion of mobile spermatozoa (0 to 100%) and vigor from the rectilinear and progressive movement (1 to 5 score), after evaluation of five fields, as established in the CBRA manual. Ejaculates that presented motility above 60% and a minimum vigor of 3, were selected for concentration and sperm morphology analysis.

The sperm concentration was calculated using a spectrophotometer (ACUCELL, IMV Technologies®), which also provided the number of straws to be filled, at a final concentration of $50x10^6$ spermatozoa/straw.

The sperm morphology evaluated 100 cells from each ejaculate through the cell humid chamber technique. For the morphology analysis, a phase contrast microscope (Nikon® Eclipse E400) was used at 100x magnification, using immersion oil (CBRA, 2013). Sperm defects were classified as major and minor, and only ejaculates with less than 30% total defects (CBRA, 2013) were cryopreserved.

For the cryopreservation process, the straws, which were already packaged and sealed by an automatic system, were submitted to a refrigeration curve that began in a cold room at 4ºC for 3 hours. Subsequently, the straws were subjected to liquid nitrogen vapor at -120ºC for 12 minutes, and then submerged in liquid nitrogen at -196ºC.

After submitted to refrigeration and freezing, one straw from each of the 122 ejaculates was thawed in a water bath at 37.5°C for 30 seconds for evaluation of the motility and spermatic vigor under a 40x objective optical microscope as procedures previously described. All evaluations were performed systematically by the same trained technician.

The data were analyzed using a procedure for the generalized linear mixed model (GLIMMIX) in the software MINITAB 18®, version 18.1. The model included a fixed effect of the months and subspecies and a random effect of the animals participating in the study. The Tukey test was utilized to determine the differences between groups. For descriptive analysis the data are presented as mean and standard error of the mean. To indicate an effect of the categorical variables and their interactions, $P \leq 0.05$ was considered significant.

3 RESULTS

Among the sperm parameters included in the present study, the majority of the analyzed variables (volume, motility, number of doses, and proportion of major defects) were constant ($P > 0.05$) during the winter period (Table 2), regardless of bull subspecies, or their interactions (Table 3). However, sperm vigor before and after thawing, as well as the proportion of minor defects varied ($P < 0.05$) over the period evaluated (Table 2).

Table 2. Characteristics of bovine semen of ten bulls, six *Bos indicus* and four *Bos taurus*, in the municipality of Campo Grande, Mato Grosso do Sul (Latitude: -20.4435, Longitude: -54.6478 20 ° 26 '37 ", South, 54 ° 38 '52 "West), between the months of July and September.

Sperm parameters		July	August	September	P value
Ejaculate nº (n)		37	54	31	-
Volume (mL)		6.74 ± 0.75	6.43 ± 0.37	7.06 ± 0.70	0.864
Straws nº (n)		181.0 ± 15.3	193.1 ± 14.9	258.9 ± 55.0	0.104
Motility (%)	Fresh	64.73 ± 0.82^{A}	65.27 ± 0.92^{A}	65.00 ± 0.83^{A}	0.770
	Post - thawing	33.76 ± 1.04^{B}	35.46 ± 0.62^{B}	35.16 ± 0.94^{B}	0.284
Vigor (1-5)	Fresh	3.40 ± 0.04^{Aab}	3.47 ± 0.03^{Aa}	3.30 ± 0.04^{b}	0.016
	Post - thawing	3.06 ± 0.03^{Bb}	3.19 ± 0.03^{Ba}	3.2 ± 0.04^{a}	0.014
Total defects(%)		7.62 ± 0.56	8.59 ± 0.39	8.64 ± 0.52	0.236
Mayor defects (%)		5.67 ± 0.46	5.13 ± 0.30	5.64 ± 0.35	0.517
Minor defects (%)		1.95 ± 0.20^{b}	3.46 ± 0.28^{a}	3.00 ± 0.42^{ab}	0.001

$^{A, B}$- different upper case letters for the same month and same parameter indicates statistical difference between the fresh *versus* after thawing semen.
$^{a, b}$- different lowercase letters indicate statistically significant difference between months.

In addition to the sample collection months, the minor defect rate differed ($P = 0.009$) between the subspecies of the animals, as well as which, an interaction ($P = 0.015$) was observed between the evaluation months and the subspecies (Table 3); the *Bos taurus* bulls presented a higher ($P < 0.05$) proportion of spermatic defects classified as minor during the late winter (August).

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Regardless of the evaluation months or subspecies considered, both motility and sperm vigor presented a decrease ($P < 0.05$) in the kinetics between fresh evaluation and evaluation immediately after the straws were thawed (Table 2).

Table 3. Evaluated parameters of the straws from six *Bos indicus* bulls and four *Bos taurus* animals in the municipality of Campo Grande, Mato Grosso do Sul (Latitude: -20.4435, Longitude: -54.6478 20 ° 26'37 ", South, 54 ° 38 '52 "West) in the winter months.

Effects			*Bos taurus*	*Bos indicus*	P value	Months x Subspecies interaction
Ejaculates	(n)		51	71	-	
Volume	(mL)		6.44 ± 0.20	6.86 ± 0.55	0.783	0.762
Straws(n)			231.9 ± 34.2	187.6 ± 13.0	0.409	0.276
Fresh motility	(0-100%)		63.94 ± 0.69^{A}	66.57 ± 0.73 ^{A}	0.294	0.861
Post-thaw motility	(0-100%)		34.88 ± 0.85^{B}	34.86 ± 0.57^{B}	0.812	0.922
Fresh	vigor	1-5 (Min and Max)	3.42 ± 0.03^{A}	3.40 ± 0.03^{A}	0.591	0.853
Post-thaw	vigor	1-5 (Min and Max)	3.08 ± 0.03^{B}	3.22 ± 0.03^{B}	0.191	0.543
Major defects	(%)		5.88 ± 0.37	5.09 ± 0.25	0.160	0.338
Minor defects	(%)		3.27 ± 0.30^{a}	2.60 ± 0.22^{b}	0.009	0.015
Total defects	(%)		9.16 ± 0.45^{a}	7.70 ± 0.34^{b}	0.004	0.348

[A, B]- different upper case letters for the same subspecies and same parameter, indicate a statistical difference between the fresh semen *versus* after thawing.
[a, b]- different lower case letters indicate a significant statistical difference between subspecies.

4 DISCUSSION

The data from this study show that climatic conditions during winter period did not determine variations in the majority of the sperm parameters studied in either bull subspecies. However, it was identified that the sperm vigor post thawing increased from July to September, as well as the proportion of sperm defects, which changed not only in the months, but also in the genetic group evaluated. Finally, these data provide evidence that breeders tend to increase the proportion of spermatozoa with minor defects as the warmer months approach, a condition that is more frequent in taurine than zebu animals.

During the evolution of the bovine species, zebu animals developed better tolerance to tropical climates (Hansen, 2004; Queiroz et al., 2015). This fact explains, in part, the findings from the present study, in which *Bos indicus* bulls presented better results in the sperm morphology analysis. Although the study was conducted during the winter period, the average temperature from 20.35 to 25.78 ° C may have been relatively high, so that more thermotolerant breeds were more efficient, resulting in a lower proportion of sperm defects.

The ejaculate volume did not differ during the monitored months, nor did it present differences between the subspecies. Similarly, influences of temperature and seasons were not identified in the seminal volume from crossbred Jersey bulls, however, it was identified that the management of the animals and the intervals between semen collections generated differences in the ejaculate volume (Gopinathan, Sivaselvam, Kanniappan Karthickeyan, & Venkataramanan, 2018), factors that were minimized in the present study, since all the animals had the same management and the same semen collection routine. A study conducted with buffaloes also found no difference in sperm volume in different seasons (Ram et al., 2017).

Sperm motility is considered the most reliable method for ejaculate selection, since this parameter has demonstrated a positive correlation with conception rates *in vivo* and *in vitro* (Bergstein, Weiss, & Bicudo, 2014; Zhang, Larsson, Lundeheim, & Rodriguez-Martinez, 1998). Therefore, in the present study, motility was the criterion for selecting ejaculates for freezing.

Although sperm kinetic data were similar among subspecies, there was an expectation that zebu animals would demonstrate better kinetic quality due to the question of better adaptability to the climatic conditions of the region. A study conducted with Simmental bulls (*Bos taurus*) kept in a region with temperatures above 20ºC showed a reduction in sperm quality (Igna, Moje, Mircu, Roman, & Ghiurca, 2010). In addition, Chacón et al. (2002) reported better adaptation of *Bos indicus* animals to the tropical climate in which the sperm motility of Brahman bulls did not change throughout the seasons, demonstrating the efficiency of thermal regulation in zebu animals. On the other hand, in the present study, only the morphological characteristics, followed those proposed for *Bos indicus* animals.

In a study from Koivisto et al. (2009), zebu bulls presented superior sperm motility compared to taurine bulls only in autumn and summer. Although the present study did not include all the seasons of the year, the sperm motility did not change and was not affected by the genetic grouping of the bulls during the winter. This divergence in the results can be justified by the variation in temperatures in the regions in which each experiment was executed. Koivisto et al. (2009) conducted their study in the southwestern region of Brazil, with a mean temperature in the winter of 19.9ºC, while the present work occurred in the center-west region, with a mean of 23.2ºC in the winter months; during the study the average temperature recorded was 22.47°C. In addition, in the period of highest ambient temperature (summer), it is expected that more deleterious effects of the climate on spermatic qualities would be found.

For crossbred bulls (*Bos taurus* x *Bos indicus*), the motility is higher in winter months, but with higher volume and cell production during the summer. This is possibly because hybrid bulls present good testicular thermoregulation due to the adaptability coming from heterosis effects of zebu animals (Romanello et al., 2018). Although the spermatic kinetics were similar, this fact can be confirmed by the semen morphology data, which in the present study was superior in zebu animals.

On the other hand, in both taurine and zebu animals, a decrease in sperm motility of approximately 30% was observed in the fresh semen analysis *versus* the post-thawed semen. A 50% to 60% decrease in post-thaw motility of spermatozoa is assumed (Ringwelski, Beever, & Knox, 2013). These data corroborate with the study by D'Andre et al. (2017) who detected differences in fresh and post-thaw motility among bulls of taurine and zebu breeds. In addition, the results of the present study show that the sperm cryopreservation process can cause damage to cells, and a decrease in fertilizing capacity is expected (Abavisani, Arshami, Naserian, Sheikholeslami Kandelousi, & Azizzadeh, 2013; Kaka et al., 2017).

The Brazilian College of Animal Reproduction requires minimum parameters for fresh semen to be considered suitable for freezing, such as minimum motility of 70%, vigor 3, and 70% morphologically normal sperm cells (CBRA, 2013). These requirements are necessary because the cryopreservation process is detrimental to spermatozoa, with reductions of up to 50% of mobile cells being observed, as was seen in this study, where there were differences between fresh and post-thaw motility (Celeghini et al., 2017; Fraser, Strzezek, & Kordan, 2014; Ringwelski et al., 2013).

Another factor that deserves attention is the relation of sperm motility to nutrition. For Nelore and Simmental bulls, it has been proposed that lower sperm motility may be associated with low pasture quality during the winter dry season (Chacur et al., 2012). However, in the present study, all bulls received the same diet, with the same proportion of grass, in addition to supplementation with concentrates, suggesting that this was possibly not a source of variation in the results.

Sperm vigor represents the strength /quality with which sperm move. In the present study, differences in vigor occurred during the months surveyed, but no differences were observed between taurine and zebu bulls, which is consistent with data reported by Chacur et al. (2012), who, when comparing the seminal quality of zebu and taurine bulls during the four seasons, did not identify differences in motility or vigor among subspecies.

When evaluating the interaction of the factors tested (months and subspecies), the proportion of minor defects was the only variable that demonstrated a significant change. In other words, this interaction can be interpreted as demonstrating that during the warmest months of the study (August and September) bulls presented the highest proportion of minor defects. On the other hand, zebu animals in the month of July maintained better results for sperm morphology, a fact that opposes the study by Rodrigues et al., (2002), in which *Bos taurus* obtained a lower percentage of total defects. D'Andre et al., (2017) report only one interaction between subspecies and seasons of the year for ejaculate volume and post-thaw motility.

In this context, it is known that thermal stress is detrimental to spermatogenesis and epididymal maturation (Krishnan et al., 2017), reinforced by the study in question. Some alternatives can be used to minimize thermal stress and improve sperm production, such as keeping bulls more adapted to local climatic conditions, the use of a sprinkler or irrigators, and even reducing the energetic level in the diet of the breeders (Krishnan et al., 2017).

Although the parameters of sperm kinetics did not change throughout the follow-up period or according to the bull subspecies, the proportion of sperm cells classified with minor defects during the morphological analysis was higher in the hotter months of the study and was presented more frequently in taurine bulls. Therefore, data from the present study reinforce the hypothesis that reproducers that inhabit warmer climates possibly require more testicular thermoregulatory processes same during the winter, although zebu animals may demonstrate greater adaptability under these conditions, as evidenced.

On the other hand, even though the values are within the recommendations of the CBRA, the high proportion of sperm defects found in bulls reinforces the challenge of maintaining spermatogenesis, emphasizing the need to choose bulls with good adaptation to the climatic conditions of the region, as well as the performance of periodic examinations in order to detect, in advance, situations that may compromise reproductive fertility.

It was concluded that the majority of the seminal characteristics did not present variations during the winter period or subspecies studied. However, it was found that zebu bulls, independently of the monitoring period, presented a lower proportion of minor defects in the analysis of sperm morphology, although taurine animals seemed to be better adapted to the winter. In addition, both taurine and zebu demonstrated a reduction in spermatic kinetics after the seminal freezing process.

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