UNIVERSIDADE DE BRASÍLIA

FACULDADE DE AGRONOMIA E MEDICINA VETERINÁRIA

RELAÇÕES ENTRE CARACTERÍSTICAS ADAPTATIVAS, QUALIDADE ESPERMÁTICA E PERFIL PROTEICO DO PLASMA SEMINAL DE TOUROS ADAPTADOS À REGIÃO SUBTROPICAL

PAULA LORENA GRANGEIRA SOUTO

TESE DE DOUTORADO EM CIÊNCIAS ANIMAIS

BRASÍLIA/DF

FEVEREIRO DE 2018

UNIVERSIDADE DE BRASÍLIA
FACULDADE DE AGRONOMIA E MEDICINA VETERINÁRIA

RELAÇÕES ENTRE CARACTERÍSTICAS ADAPTATIVAS, QUALIDADE ESPERMÁTICA E PERFIL PROTEICO DO PLASMA SEMINAL DE TOUROS ADAPTADOS À REGIÃO SUBTROPICAL

PAULA LORENA GRANGEIRA SOUTO

ORIENTADOR: ALEXANDRE FLORIANI RAMOS

CO-ORIENTADORA: CONCEPTA MARGARET MCMANUS PIMENTEL

TESE DE DOUTORADO EM CIÊNCIAS ANIMAIS

PUBLICAÇÃO: 202D/2018

BRASÍLIA/DF
FEVEREIRO DE 2018

UNIVERSIDADE DE BRASÍLIA
FACULDADE DE AGRONOMIA E MEDICINA VETERINÁRIA

RELAÇÕES ENTRE CARACTERÍSTICAS ADAPTATIVAS, QUALIDADE ESPERMÁTICA E PERFIL PROTEICO DO PLASMA SEMINAL DE TOUROS ADAPTADOS À REGIÃO SUBTROPICAL

PAULA LORENA GRANGEIRA SOUTO

TESE DE DOUTORADO SUBMETIDA AO PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS ANIMAIS, COMO PARTE DOS REQUISITOS NECESSÁRIOS À OBTENÇÃO DO GRAU DE DOUTOR EM CIÊNCIAS ANIMAIS.

APROVADA POR:

_____
ALEXANDRE FLORIANI RAMOS, Doutor (EMBRAPA) (ORIENTADOR)

_____
IVO PIVATO, Doutor, UnB (EXAMINADOR INTERNO)

_____
MARÍLIA VIVIANE SNEL DE OLIVEIRA, Doutora, UPIS (SUPLENTE)

_____
LUCIANA KEIKO HATAMOTO-ZERVOUDAKIS, Doutora, UFMT (EXAMINADOR EXTERNO)

_____
OCTAVIO LUIZ FRANCO, Doutor, UCB (EXAMINADOR EXTERNO)

BRASÍLIA/DF, 27 de fevereiro de 2018.

REFERÊNCIA BIBLIOGRÁFICA E CATALOGAÇÃO

SOUTO, P.L.G. **Relações entre características adaptativas, qualidade espermática e perfil proteico do plasma seminal de touros adaptados à região subtropical.** Brasília: Faculdade de Agronomia e Medicina Veterinária, Universidade de Brasília, 2018, 113p. Tese de doutorado.

Ficha catalográfica elaborada automaticamente,
com os dados fornecidos pelo(a) autor(a)

Souto, Paula Lorena Grangeira

SSO728r Relações entre características adaptativas, qualidade espermática e perfil proteico do plasma seminal de touros adaptados à região subtropical / Paula Lorena Grangeira Souto; orientador Alexandre Floriani Ramos; co-orientador Concepta Margaret Mcmanus Pimentel. -- Brasília, 2018.

113 p.

Tese (Doutorado - Doutorado em Ciências Animais) -- Universidade de Brasília, 2018.

1. Reprodução animal. 2. Andrologia. 3. Adaptação climática. 4. Recursos genéticos. 5. Proteômica. I. Ramos, Alexandre Floriani, orient. II. Pimentel, Concepta Margaret Mcmanus , co-orient. III. Título.

AGRADECIMENTOS

Ao CNPq, à CAPES e à Universidade Federal de Mato Grosso pelo apoio financeiro para a execução do experimento. Além disso, agradeço a CAPES pela bolsa concedida no país e também durante o período sanduíche no exterior. Agradeço também ao Scotland's Rural College – SRUC, em especial à Dra. Joanne Conington, por me receber e contribuir com o desenvolvimento deste trabalho.

À Universidade de Brasília (UnB) e ao Programa de Pós-Graduação em Ciências Animais pelo suporte acadêmico.

Ao professor Ernandes Alencar pelo auxílio na análise de coloração do pelame.

Aos pesquisadores e demais colaboradores do Setor Campo Experimental Fazenda Sucupira, da Embrapa Cenargen, e da Fazenda Nhumirim, da Embrapa Pantanal, pelo apoio laboratorial, logístico e de trabalho de campo, durante o período experimental.

Ao Dr. Edson e Dra. Vera Martins, agradeço pela dedicação, por fornecer os touros e a infraestrutura da Fazenda Bom Jesus do Herval. Além de dados experimentais, trouxe comigo também uma parte da história e da rica cultura local. Obrigada pela amizade!

Aos colegas de iniciação científica, de mestrado e de doutorado, agradeço pelas trocas de conhecimento ao longo do curso e pela companhia nos momentos bons e ruins. Desejo sucesso a todos!

À minha coorientadora Dra. Concepta McManus, agradeço pela dedicação, por todo o apoio e incentivo acadêmico, especialmente nos meus estudos em bioestatística, por me mostrar o caminho sempre que precisei recorrer à sua sabedoria, me inspirando a buscar cada vez mais conhecimento. Minha admiração e respeito!

Ao meu orientador Dr. Alexandre Floriani Ramos, agradeço por todas as oportunidades confiadas a mim ao longo destes anos, pelo suporte acadêmico e pelos ensinamentos que contribuíram para o avanço de mais um importante passo na minha formação pessoal e profissional. Minha gratidão e reconhecimento!

ÍNDICE

RESUMO

RELAÇÕES ENTRE CARACTERÍSTICAS ADAPTATIVAS, QUALIDADE ESPERMÁTICA E PERFIL PROTEICO DO PLASMA SEMINAL DE TOUROS ADAPTADOS À REGIÃO SUBTROPICAL

Paula Lorena Grangeira Souto[1], Concepta Margaret McManus Pimentel[1], Alexandre Floriani Ramos[1,2]

[1]Faculdade de Agronomia e Veterinária - UnB, Brasília-DF, [2]Embrapa Recursos Genéticos e Biotecnologia, Brasília-DF

O objetivo deste estudo foi comparar a qualidade seminal de touros localmente adaptados com touros de raça comercial criados na região do Planalto Sul Catarinense e analisar as relações com a adaptação climática. Características da morfologia externa dos touros, respostas fisiológicas e proteínas do plasma seminal foram investigadas no verão e no inverno. Em cada estação houve três coletas de sêmen por touro e as amostras foram avaliadas para motilidade total e vigor, concentração espermática, viabilidade das membranas e defeitos da célula. O plasma seminal foi obtido por centrifugação e submetido à proteômica por *shotgun* para identificação das proteínas. Dados ambientais como temperatura e umidade relativa do ar, temperatura do globo negro (BGT), índice de temperatura e umidade (ITU) e os índices de conforto térmico foram obtidos. As condições climáticas diferiram significativamente entre as estações. No Capítulo 2, concluiu-se que touros Crioulo Lageano e Angus apresentaram qualidade seminal similar e satisfatória ao longo do ano. Temperatura do ar, BGT e ITU foram os principais fatores que potencialmente afetariam as características espermáticas. No Capítulo 3, foi possível concluir que os touros experimentaram estresse térmico moderado no verão, o que levou a mudanças fisiológicas significativas, porém a

normotermia foi mantida o que mostrou boa adaptação às condições climáticas da região estudada. Características de morfologia externa do animal foram as mais importantes para explicar as respostas fisiológicas, enquanto que no inverno prevaleceram as características climáticas. No Capítulo 4, determinou-se que houve efeito de raça e de estação sobre a abundância de proteínas no plasma seminal. Sugere-se que as proteínas diferencialmente abundantes fazem parte dos mecanismos envolvidos com a adaptação climática e manutenção da homeostase dos touros. Este estudo contribui para o conhecimento sobre a fisiologia do trato reprodutivo de touros de raças localmente adaptadas e argumentam em favor da utilização das mesmas na cadeia pecuária.

Palavras-chave: bovino, conforto térmico, criopreservação, estresse térmico, mudanças climáticas, proteômica, sêmen.

ABSTRACT

RELATIONSHIP BETWEEN ADAPTABILITY, SPERM QUALITY AND PROTEIN PROFILE OF THE SEMINAL PLASMA OF BULLS ADAPTED TO SUBTROPICAL REGION

Paula Lorena Grangeira Souto[1], Concepta Margaret McManus Pimentel[1], Alexandre Floriani Ramos[1,2]

[1]College of Agronomy and Veterinary Medicine - University of Brasilia, DF, [2]Embrapa Genetic Resources and Biotechnology, Brasilia, DF

This study aimed to compare the semen quality of locally adapted bulls with bulls of a commercial breed raised at Southern Highlands of Santa Catarina, as well as, to analyse the relationships with climate adaptation. Characteristics of the external morphology of the animals, physiological responses and proteins in the seminal plasma were investigated in summer and in winter. Semen samples were obtained three times in each season, from all bulls, and analysed for sperm motility, vigor, number of sperm per millilitre, sperm membrane status and sperm defects. Seminal plasma was obtained by centrifugation and the proteins identified by shotgun proteomics. Environmental data, such as air temperature, air humidity, black globe temperature (BGT), temperature humidity index (THI) and thermal comfort indexes were obtained. Climate conditions differed significantly between seasons. Chapter 2 unveiled that the semen quality of Crioulo Lageano and Angus bulls was similar and satisfactory across the year. Air temperature, BGT and THI were considered the most important factors that could affect seminal traits. In Chapter 3, it was possible to conclude that the bulls experienced moderate heat stress in summer, which led to significant physiological responses, but they were able to maintain normothermia, showing good adaptation to the climate

conditions at the region studied. External morphological characteristics were highly important to explain physiological responses in summer, whereas in winter the climatic features prevailed. In Chapter 4, it was determined that season and breed influenced on the abundance of the proteins in seminal plasma. We suggest that the differentially abundant proteins are part of a range of mechanisms that participate in climate adaptation and homeostasis maintenance of the sires. This study contributes to the knowledge about the male physiology in locally adapted cattle and argues in favour of the use of them in the livestock breeding systems.

Keywords: bovine, climate change, cryopreservation, proteomics, semen, thermal comfort, thermal stress.

LISTA DE FIGURAS

LISTA DE TABELAS

Capítulo 4

LISTA DE SÍMBOLOS E ABREVIAÇÕES

µL	Microlitre
ACM	Atividade citoquímica mitocondrial
ADH	Alcohol dehydrogenase
ALB	Serum albumin
ALH	Amplitude of lateral head displacement
am	Latin 'ante meridiem', which means 'before noon'
AO	Acridine orange
ATA	Mean air temperature
BCF	Beat cross frequency
BCS	Body condition score
BGT	Black globe temperature
BGTM	Black-globe temperature in the morning
BL	Body length
BLAST	Basic local alignment search tool
BSP1	Seminal plasma protein PDC-109
BSP3	Seminal plasma protein A3
BW	Body weight
C3	Complement C3
CASA	Computer-assisted sperm analysis
CAT	Catalase
CCL2	C-C motif chemokine 2
CD	Chest depth
CFAH	Complement factor H
CFDA	6-carboxyfluoresceindiacetate
CLU	Clusterin
cm	Centimetre
cm²	Square centimetre
cm³	Cubic centimetre
CNP	C-type natriuretic peptide
Den DF	Number of degrees of freedom associated with the model errors
DF	Number of degrees of freedom in the model
DNA	Ácido desoxirribonucleico / deoxyribonucleic acid
DTA	Minimum air temperature in the afternoon
DTM	Minimum air temperature in the morning
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EROs	Espécies reativas de oxigênio
FAO	Organização das Nações Unidas para Alimentação e Agricultura / Food and
FITC-	Fluorescein isothiocyanate-conjugated peanut agglutinin
GPx	Glutathione peroxidase
GSA	Gradient temperature from the top to the bottom of the scrotum in the afternoon

GSH-Px	Enzimas glutationa peroxidase
GSM	Gradient temperature from the top to the bottom of the scrotum in the morning
GSS	Glutathione synthetase
GSTA5	Glutathione S-transferase A5
GSTT1	Glutathione S-transferase theta-1
GSTT3	Glutathione S-transferase theta-3
H2O2	Peróxido de hidrogênio
HAW	Height at withers
HB	Haemoglobin
HD	Hair coat density
HG	Heart girth
HH	Hip height
HL	Hair coat length
HT	Haematocrit
IBGE	Instituto Brasileiro de Geografia e Estatística
IM	Intact plasma membrane
IMIA	Intact plasma membrane and acrosome
INMET	National Institute of Meteorology
IPSP	Plasma serine protease inhibitor
ISA	Spermatozoa with intact plasma membrane and acrosome
ITU	Índice de temperatura e umidade
JC-1	5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide
JC-1a	Intact acrosome, intact plasma membrane and high mitochondrial membrane
JC-1b	Damaged acrosome, damaged plasma membrane and low mitochondrial
JC-1c	Intact acrosome, damaged plasma membrane and low mitochondrial membrane
JC-1e	Intact acrosome, damaged plasma membrane and high mitochondrial
JC-1f	Damaged acrosome, damaged plasma membrane and high mitochondrial
kDa	Kilodalton
Km/h	Kilometres per hour
LIN	Linearity
M	Molar
m	Metres
MANOVA	Multivariate analysis of variance
MCT	Mean compensated temperature of the day
MCV	Mean corpuscular volume
mg	Milligram
mL	Milliliter
mM	Nanomolar
MMPs	Metalloproteinases
MS:	Mass spectrometry
N/L	Neutrophil: lymphocyte ratio
Na^+/K^+	Sódio-Potássio adenosina trifosfatase / Sodium-potassium adenosine
NC	Normal cells
NCBI	National Center for Biotechnology Information

NETs	Neutrophil extracellular traps
nm	Nanometre
NO2	Óxido nítrico
NRC	National research council
O2-	Ânion superóxido
ºC	Graus/degrees Celsius
OH-	Radical hidroxila
OH•	Hydroxyl radical
PBS	Phosphate buffered saline
PI	Propidium iodide
PIB	Produto interno bruto
PLGS	Proteinlynx Global Server™
PLS	Partial least squares
PLT	Platelets
pm	Latin 'post meridiem', which means 'after noon'
PPIB	Peptidyl-prolyl cis-trans isomerase B
PS	Panting score
PUFA	Ácidos graxos poliinsaturados
R²	Coefficient of determination
RBC	Erythrocytes
RH	Air relative humidity
RHM	Mean relative humidity
ROS	Reactive oxygen species
rpm	Revolutions per minute
RR	Respiratory rate per minute
RT	Rectal temperature
SC	Scrotal circumference
SDS-	Sodium Dodecyl Sulfate polyacrylamide Gel Electrophoresis
SkinCl	Skin colour
SOD	Superóxido dismutase
sptz	Spermatozoa
ST	Skin thickness of the body
StdDev	Standard deviation
STR	Straightness
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
STT	Skin thickness of the testes
T12	Air temperature at 12 o'clock noon
T24	Air temperature at midnight
T3	Triiodotironina
T4	Tiroxina
Ta	Air temperature
TALP	Tyrode's albumin lactate pyruvate
TCI	Temperatura crítica inferior
TCS	Temperatura crítica superior

TD	Total sperm defects
TFA	Trifluoroacetic acid
THI:	Temperature and humidity index
TIMP2	Inibidor tecidual de metaloproteinase-2 / Metalloproteinase inhibitor 2
TL	Testicular length
Tn	Minimum air temperature of the day
TV	Testicular volume
TV	Testicular volume
TW	Testicular width
Tx	Maximum air temperature of the day
UPLC	Ultra-performance liquid chromatography
UTA	Maximum air temperature
UTM	Maximum air temperature in the morning
VAP	Average pathway velocity
VCL	Curvilinear velocity
VIP	Variable Importance for Projection
VSL	Straight-line velocity
WBC	White blood cell count
WCI	Wind chill index
WS	Wind speed

CAPÍTULO 1

INTRODUÇÃO E REVISÃO DE LITERATURA

1. INTRODUÇÃO

Na última década o rebanho bovino brasileiro cresceu aproximadamente 6% (IBGE, 2018) e mesmo com o delicado cenário político-econômico pelo qual o país vem passando nos últimos anos o crescimento acumulado no setor agropecuário em 2017 foi de 14,5%, com participação de 23% a 24% do PIB, sendo um dos poucos setores que fecharam o ano com crescimento positivo (IBGE, 2017). Porém, o potencial de expansão e fortalecimento da pecuária brasileira é significativo, visto que os números de hoje refletem um processo relativamente recente de melhoramento genético animal.

Desde a introdução dos primeiros bovinos no país, na época do descobrimento, passaram-se séculos em que animais de raças da península ibérica foram se desenvolvendo exclusivamente por seleção natural em determinados momentos e ambientes ao ponto de desenvolver características específicas de adaptação às condições aqui encontradas (Mariante et al., 2009). As raças aqui formadas passaram a ser conhecidas como “crioulas”, “locais” ou “localmente adaptadas”, dentre as quais podemos destacar: Caracu, Mocho Nacional, Curraleiro Pé-duro, Pantaneiro e Crioulo Lageano (Egito et al., 2014). No início do século 19, iniciou-se uma movimentação internacional de recursos genéticos animais, viabilizada por novos conceitos acerca de melhoramento genético e pela invenção do navio a vapor, o que propiciou o envio de reprodutores de alto valor genético para outras partes do mundo. Mas foi somente em 1962 que vieram reprodutores Nelore pertencentes às linhagens que mais influenciaram geneticamente o rebanho brasileiro (FAO, 2010a; Oliveira et al., 2002). Inicialmente, os resultados da heterose obtidos pelos acasalamentos de animais das raças exóticas

com os das raças localmente adaptadas foram positivos para a pecuária, porém, a realização indiscriminada destes cruzamentos levou várias raças ao risco de extinção enquanto outras já se perderam (Egito et al., 2002; FAO, 2010a; Mariante & Cavalcante, 2006; Teixeira et al., 2011).

Ademais, ao longo dos anos houve uma utilização intensiva de poucos reprodutores Nelore considerados de alto valor genético a fim de se obter o maior número possível de progênie por touro, o que levou ao afunilamento da base genética da raça acarretando perda progressiva da variabilidade genética (Carneiro et al., 2007; Henrique et al., 2009; Paulo et al., 2017). Este estreitamento genético diminui a possibilidade de adaptação das raças às adversidades ambientais, como novas enfermidades, mudanças no clima e nos sistemas de produção. Essa situação somada à crescente demanda mundial de alimentos, devido ao crescimento demográfico e até mesmo as mudanças nas preferências dos consumidores, ameaça a segurança alimentar da população humana (FAO, 2010a). Assim sendo, o estudo e o fomento de raças localmente adaptadas são importantes e estratégicos, visto que a distância e a variabilidade genética do rebanho podem auxiliar a manter ou atingir o *status* de ótima produção animal (Carvalho et al., 2015). Por isso, ações para a identificação e a estimulação da utilização dessas raças são necessárias, seja através de seus produtos ou de seus genes, a fim de atender às características de interesse comercial, uma vez que estes recursos genéticos podem ser úteis no futuro (FAO, 2010b).

A informação disponível sobre as raças localmente adaptadas ainda é limitada, principalmente no tocante à fisiologia das mesmas, e este conhecimento é essencial para que se compreendam os mecanismos adaptativos desses animais e a partir daí se desenvolvam biotécnicas para otimizar o uso dos destes recursos genéticos em benefício do desenvolvimento da cadeia produtiva.

A criopreservação de gametas é uma importante ferramenta estratégica que permite preservar o material genético para posterior utilização, sendo um método de conservação de espécies amplamente adotado. Ao longo dos anos diversas técnicas de preservação de células espermáticas têm sido pesquisadas no intuito de mitigar os efeitos deletérios causados durante o processo e, assim, melhorar a qualidade do sêmen criopreservado (Asadpour et al., 2011; Mariante & Ramos, 2011; Uysal et al., 2007). Contudo, existe uma variação na qualidade do sêmen entre indivíduos da mesma espécie e até mesmo dentro de raça. Em geral, alterações na capacidade funcional da célula durante o processamento reduzem a motilidade e viabilidade espermática. Mesmo

com a melhoria das técnicas de criopreservação ainda há perdas em torno de 20 a 50% de espermatozoides viáveis nas melhores condições (Abdulkareem, 2014; Bucak et al., 2015; Madeira et al., 2013) e acredita-se que parte das mudanças na qualidade do sêmen pode ser atribuída à dinâmica da composição proteica do plasma seminal (Chacur et al., 2011; Queiroz et al., 2015; Sharma et al., 2014). Porém, pouco se conhece sobre as formas de proteção conferidas por essas proteínas ao espermatozoide ao longo das estações do ano. Além disso, no caso de um país de clima predominantemente tropical como o Brasil, os reprodutores são submetidos a variações ambientais importantes ao longo das estações do ano e a ocorrência de estresse térmico é comum em várias regiões. Isto reforça a importância da realização de estudos sobre a influência do ambiente térmico sobre a fisiologia da reprodução de bovinos.

2. OBJETIVOS

2.1 Objetivo geral

Avaliar a qualidade seminal de touros da raça localmente adaptada Crioulo Lageano criados *in situ* em comparação com touros da raça comercial Angus, em duas épocas do ano, e analisar as relações entre características da fisiologia reprodutiva e de adaptação climática.

2.2 Objetivos específicos

- Avaliar a qualidade espermática pré e pós-criopreservação de touros Crioulo Lageano criados *in situ* em comparação com touros da raça comercial Angus, durante o verão e o inverno.
- Relacionar as características de morfologia corporal e testicular com as características climáticas e seminais de touros Crioulo Lageano criados *in situ* em comparação com touros da raça comercial Angus, durante o verão e o inverno.

- Avaliar a abundância de proteínas no plasma seminal de touros Crioulo Lageano criados *in situ* em comparação com touros da raça comercial Angus e identificar potenciais marcadores relacionados com a fertilidade do macho, durante o verão e o inverno.
- Analisar as relações entre as proteínas diferenciais ($p<0,05$) no plasma seminal de touros Crioulo Lageano criados *in situ* em comparação com touros da raça comercial Angus com as características seminais e climáticas, durante o verão e o inverno.
- Investigar o efeito sazonal do clima e da morfologia externa dos animais no conforto térmico e nas respostas fisiológicas de touros Crioulo Lageano criados *in situ* em comparação com touros da raça comercial Angus, durante o verão e o inverno.

3. REVISÃO DE LITERATURA

3.1 Termorregulação

Entende-se por termorregulação (Figura 1) o processo de regulação da temperatura em um sistema físico qualquer. Esse controle se dá pelo equilíbrio entre a energia térmica produzida pelo organismo animal e as perdas, ganhos e trocas dessa energia com o meio ambiente. Ou seja, ambiente e animal formam um sistema equilibrado (Souza & Batista, 2012). A energia ganha tem origem química, através dos processos metabólicos; origem mecânica, que corresponde à força física do organismo; e origem térmica, pela troca de energia com o ambiente (Silva, 2000; Takahashi et al., 2009). Quando um animal se encontra em um ambiente com temperaturas acima ou abaixo daquelas nas quais o organismo melhor opera, há uma condição conhecida como estresse térmico. Ao mesmo tempo o animal pode estar em estresse térmico e não haver tensão, ou seja, se o há mecanismos suficientes para compensar a ação das forças exercidas pelo ambiente térmico e vencer o estresse, então não haverá tensão (Silva, 2000).

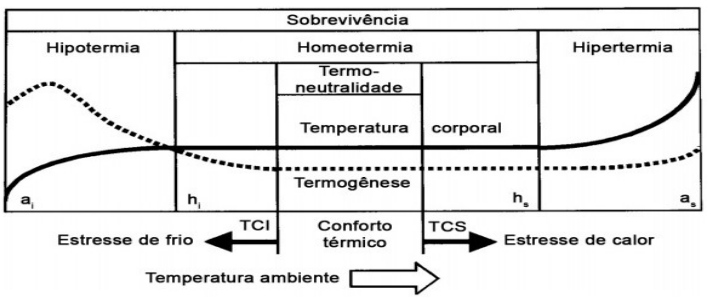

Figura 1: Representação esquermática simplificada do processo de termorregulação. Temperatura crítica inferior (TCI) e superior (TCS). Fonte: Livro "Introdução à bioclimatologia animal", p.123, Silva, R.G. (2000).

Os animais podem ser classificados de acordo com os mecanismos utilizados para a manutenção da temperatura corporal em homeotérmicos e pecilotérmicos. Os animais homeotérmicos são capazes de manter estável a temperatura interna do corpo independente das variações ambientais, enquanto os pecilotérmicos possuem a temperatura do corpo variável, consoante com a temperatura ambiental, pois não contam com um sistema termoreguratório interno como os homeotérmicos (Silva, 2000; Souza & Batista, 2012).

Dentro dos limites da homeotermia, há uma faixa menor chamada de zona termoneutra, que consiste em uma amplitude de temperatura ambiente na qual o metabolismo animal é mínimo, configurando a faixa do conforto térmico. Acima da temperatura da zona de conforto térmico, diz-se que há o estresse pelo calor e abaixo desta, o estresse pelo frio. O desafio está no fato de que o desempenho máximo do animal é obtido justamente na zona de termoneutralidade (Barbosa et al., 2014; Silva, 2000; Takahashi et al., 2009).

Para mensurar a adaptação e o conforto térmico dos animais são usados índices existentes na bioclimatologia e são considerados fatores ambientais, características do pelame, características corporais e respostas fisiológicas. Existem dois tipos de índices: índices baseados em medidas ambientais, tais como a temperatura operativa, o índice de estresse térmico de Givoni, o índice de temperatura e umidade (ITU) e o índice de globo negro e umidade; e índices baseados em medidas nos animais, como os índices Benezra e de Ibéria para tolerância ao calor, o índice de tolerância ao calor de Rauschenbach-Yerokhin e o índice de seleção para adaptação de gado de corte (Silva, 2000).

A temperatura corporal depende da quantidade de energia que o organismo possui em reserva por unidade de massa, sendo que todos os animais necessitam mantê-la em uma faixa considerada ótima para cada espécie. Para garantir a homeotermia, o organismo precisa tanto aumentar a quantidade de energia, através da termogênese, quanto diminuí-la através da termólise. Estes dois processos regulatórios contemplam três tipos de mecanismos: comportamentais, autônomos e adaptativos. Esses mecanismos são regulados por termorreceptores sensíveis ao frio ou ao calor localizados nos centros hipotalâmicos e na periferia corporal, formando um complexo sistema de controle retroalimentado da temperatura corporal. O sistema central detecta

alterações no limiar da temperatura e aciona respostas termorreguladoras autônomas para manter a homeotermia (Silva, 2000; Souza & Batista, 2012). Os mecanismos comportamentais para a regulação da temperatura corporal compreendem principalmente as alterações no deslocamento dos animais, aumentando ou diminuindo a exposição térmica. Alguns dos mecanismos autônomos compreendem o controle de funções orgânicas como a ingestão de água e alimentos, as alterações na circulação sanguínea e no funcionamento das glândulas sudoríparas, o controle da respiração e a variação na posição dos pelos. E por fim, os mecanismos adaptativos configuram alterações de médio ou longo prazo em características dos animais tais como o tipo e a cor do pelame e da pele, alterações hormonais, dentre outras (Silva, 2000).

A capa externa do corpo dos bovinos, o pelame, tem um importante papel nas trocas térmicas com o ambiente. A condutância térmica da capa tem variação circadiana e auxilia no processo de termólise e de termoconservação, quando os animais estão em repouso (Silva, 2000). Neste âmbito, os pelos opacos da raça Aberdeen Angus refletem 93 das 1000 unidades de energia na forma de radiação que recebem. Em contrapartida, os pelos brilhantes do Zebu refletem 198 unidades das 1000 que recebem. Outro fator que os protege da radiação ultravioleta é a pigmentação da epiderme. E o fato de que zebuínos apresentam barbela e cupim faz com que a superfície para a dissipação do calor seja maior, o que pode lhes render certa vantagem no conforto térmico (Takahashi et al., 2009).

3.2 Tolerância térmica

Nas últimas décadas a produção pecuária mundial tem aumentado, principalmente nas regiões de clima tropical. Isto se deve a vários fatores, dentre eles, o crescimento da população global, o aumento do poder de compra do consumidor final e mudanças nas preferências alimentares. No entanto, o clima vem se tornando um fator importante que pode trazer problemas significativos à produção pecuária em regiões tropicais e subtropicais (FAO, 2015).

Há uma estimativa de que até o final de 2100, a temperatura média da superfície da Terra poderá se elevar entre 1,8°C e 4°C (FAO, 2009). Nesse momento, somente os indivíduos dentro de cada espécie que melhor se adaptarem às novas condições sobreviverão, inclusive o Homem. Ademais, a perda da diversidade genética

ameaça a segurança alimentar no futuro, pois diminui a possibilidade de adaptação às adversidades ambientais tais como, novas enfermidades, mudanças no clima e nos sistemas de produção, conforme os alertas emitidos pela Organização das Nações Unidas para Alimentação e Agricultura – FAO. Diante disso, o estudo de raças localmente adaptadas torna-se fundamental, pois essas podem desempenhar papel fundamental em programas de melhoramento genético animal contribuindo com diversidade genética. Embora não existam dúvidas quanto à necessidade de se conservar a variabilidade genética animal, sabe-se que a manutenção das raças localmente adaptadas depende de inserção das mesmas nos sistemas de produção existentes e no mercado consumidor, seja através de seus produtos ou de seus genes a fim de atender às demandas de interesse comercial (Felix et al., 2013).

As raças zebuínas são adaptadas aos trópicos, e ainda que sejam exóticas ao Brasil, adquiriram genes para termotolerância durante o processo de evolução. Os zebuínos apresentam taxa metabólica mais baixa, devido a fatores como uma menor exigência quanto à lactação e taxa de crescimento, órgãos internos proporcionalmente menores, além de propriedades físicas relacionadas à pele e aos tecidos que facilitam a dispersão do calor em detrimento do que ocorre em raças taurinas. Essas diferenças genéticas se estendem para o nível celular, sob o qual já foram observados menores efeitos deletérios da alta temperatura na função celular em bovinos da raça Brahman do que em bovinos da raça Angus e Holandesa, cujos linfócitos são menos resistentes ao estresse térmico (Hansen, 2004; Paula-Lopes et al., 2012). Comparando as características fisiológicas ligadas ao estresse térmico de bovinos Pantaneiros, raça localmente adaptada, e de Nelores, raça especializada, foi observado que ambas apresentaram similar tolerância ao calor (Barbosa et al., 2014).

Porém, mesmo as raças selecionadas naturalmente para o clima tropical podem perecer ao calor excessivo (Hansen, 2009; Matsuzuka et al., 2005; Silva, 2000; Takahashi et al., 2009). Esses efeitos também ocorrem em outras espécies, como suínos (Sirotkin, 2010) e camundongos (Matsuzuka et al., 2005), além de outros mamíferos (Hansen, 2009). Contudo, a utilização de genes zebuínos no cruzamento com raças não adaptadas pode imprimir também genes de características indesejáveis como pior qualidade de carne, baixa produção de leite, baixa persistência na lactação, puberdade tardia, estro curto e problemas de temperamento. Isto ocorre, por que mesmo nessas raças onde os estudos estão mais avançados poucos esforços foram despendidos para identificar marcadores específicos para termotolerância (Hansen, 2004).

Em raças bovinas localmente adaptadas o conhecimento sobre os mecanismos relacionados à termotolerância ainda é escasso, principalmente nos ramos da biologia molecular e da genética. Dentro dos estudos existentes sobre ruminantes na literatura, já foram avaliados parâmetros fisiológicos como taxas cardíaca e respiratória, padrões hematológicos, taxa de sudação, tipo de pele e de pelame, demonstrando a capacidade adaptativa dessas raças (Barbosa et al., 2014; Barros et al., 2015; McManus et al., 2009; 2011). Em um experimento realizado no Pantanal, houve interação significativa entre raça e temperatura ambiente e, também, entre raça e temperatura da superfície da pele de vacas e bezerros Pantaneiros, Nelores e mestiços (Santos et al., 2005). Em estudos feitos no Brasil Central, a raça bovina Curraleiro Pé-Duro se mostrou entre as raças que apresentam melhores características para tolerância ao calor (Bianchini et al., 2006).

3.3 O estresse térmico na reprodução animal

Todas as espécies animais podem ser afetadas pelo estresse térmico causado pelo calor e também pelo frio, mas na pecuária se destacam os estudos acerca de bovinos e ovinos (Silva, 2000). Considerando que o estresse térmico é um fator que pode afetar as funções reprodutivas, as variações no clima encontradas no Brasil podem estar relacionadas com a queda na qualidade seminal de reprodutores observada em certos períodos do ano (Gabaldi & Wolf, 2002; Souza & Batista, 2012; Vianna et al., 2013).

A formação dos gametas masculinos, conhecida como espermatogênese, tem duração aproximada de 60 dias no touro. É composta de diversas fases em que as espermatogônias dividem-se até formar os espermatócitos e estes, por sua vez, têm seu número de cromossomos diminuídos pela metade após uma série de divisões meióticas, num processo chamado de espermatocitogênese. A célula haploide resultante é chamada de espermátide, que sofre uma sequência de transformações até se tornar espermatozoide, fase conhecida como espermiogênese. Finalmente, os espermatozoides formados são liberados no lúmen dos túbulos seminíferos, na fase de espermiação (Hafez & Hafez, 2000).

A formação das células espermáticas pode ser afetada pelo aumento da temperatura testicular (Gabaldi & Wolf, 2002). Considerando que o estresse térmico

pode afetar a espermatogênese, visto que pode levar ao aparecimento de alterações no epitélio seminífero dos testículos, o clima tropical característico do Brasil pode estar relacionado com a queda na qualidade seminal de touros (Gabaldi & Wolf, 2002).

A qualidade do sêmen congelado em ruminantes também pode ser influenciada pela época do ano, dependendo das particularidades climáticas de cada região, podendo ocorrer principalmente elevação no percentual de espermatozoides anormais e variação na concentração espermática (Chacur et al., 2012; Freneau, 2011; Silva et al., 2005; Teixeira et al., 2011). Avaliando reprodutores bovinos da raça Curraleiro Pé-duro, adaptada ao bioma Cerrado, verificou-se que o sêmen fresco manteve características adequadas durante todo o ano e que a qualidade do sêmen congelado é melhor entre os meses de junho a setembro (Teixeira et al., 2011).

Em touros de origem taurina criados em regiões tropicais, geralmente o declínio na qualidade do sêmen é mais pronunciado quando há estresse calórico. Isso parece ocorrer não somente por possuírem um sistema de termorregulação menos adaptado ao calor, mas também pela maior quantidade de ácidos graxos poliinsaturados presentes na membrana da célula espermática, o que predispõe a um estresse oxidativo mais intenso (Rodrigues et al., 2015).

Estudando bovinos submetidos à insulação escrotal por um período de 48 horas, Rahman et al. (2011) observaram que o aumento da temperatura testicular afeta principalmente as células que estão nas fases da meiose e da espermiogênese. Em um estudo com touros taurinos, zebuínos e mestiços, a qualidade do sêmen nos taurinos e mestiços foi pior que nos zebuínos quando expostos a ambientes com altas temperaturas (Brito et al., 2004).

Mudanças sazonais na proteômica do plasma seminal foram notadas em touros Tabapuã criados no estado de São Paulo, Brasil, e observou-se que a qualidade espermática foi melhor no inverno em comparação ao verão (Chacur et al., 2012). Em outro experimento no mesmo estado, a influência da estação do ano sobre as características do sêmen foi avaliada por Chacur et al. (2012) que verificaram que enquanto touros Nelore mantiveram as características seminais estáveis ao longo do ano, sendo outono o mês de menor estresse, touros da raça Simental apresentaram melhor qualidade do sêmen apenas em duas estações. Contrariando outros estudos, em um experimento com bovinos Brangus (5/8 Angus × 3/8 Nelore) no Rio Grande do Sul, não foram encontradas mudanças significativas na qualidade do sêmen ao longo das estações do ano, mesmo quando o índice de temperatura e umidade (ITU) esteve entre

88 e 93, faixa do índice que é classificada como alto desconforto térmico (Menegassi et al., 2016). Em bovinos da raça Braford (5/8 Hereford × 3/8 Nellore), quando o ITU atingiu 83 pontos não houve alterações de morfologia espermática, mas houve de motilidade, movimento de massa e vigor. Os autores concluem que apesar dos animais estarem sob estresse, este não foi suficiente para causar danos significativos (Menegassi et al., 2015).

A probabilidade de ocorrência de estresse térmico pelo calor é maior em ambientes quentes e com alta umidade, devido á redução da taxa de perda de calor evaporativa pela superfície corporal (Takahashi et al., 2009). Em resposta a esses eventos, ocorre o aumento da temperatura corporal e a glândula tireoide, que é responsável pela termogênese, diminui a liberação dos hormônios triiodotironina (T3) e tiroxina (T4), ocasionando um declínio da taxa metabólica e todo o eixo hipotálamo-hipófise-gônada é afetado. Essas mudanças metabólicas parecem estar intimamente ligadas aos efeitos negativos na reprodução durante o estresse térmico. Por fim, as adaptações fisiológicas ao ambiente térmico podem fazer com que a termorregulação testicular, além da liberação de hormônios necessários para a espermatogênese, seja prejudicada podendo resultar em uma espermatogênese imperfeita (Queiroz et al., 2015).

O espermatozoide entra em contato com as substâncias do plasma seminal em todas as fases de sua formação. No epidídimo a célula espermática encontra altas concentrações de carnitina, glicerilfosforilcolina, ácido salicílico, sódio e potássio. As ampolas secretam frutose e citrato e as vesículas seminais contêm a maior concentração de proteínas, altas concentrações de frutose e a maior parte das prostaglandinas presente no plasma seminal (Guasti et al., 2012). Dentre as funções do plasma seminal destacam-se as modificações promovidas nas membranas espermáticas, que previnem processos oxidativos e imunológicos, além da influência exercida sobre o metabolismo espermático (Muinõ-Blanco et al., 2008).

Sabe-se que a sazonalidade influencia diretamente na presença ou ausência de proteínas no plasma seminal e essas mudanças são atribuídas à variação dos níveis de gonadotrofinas e dos respectivos receptores testiculares de acordo com a estação do ano, o que provavelmente afeta a função endócrina das gônadas e a secreção de plasma seminal pelas glândulas acessórias e epidídimo (Cardozo et al., 2006; Cavalcante et al., 2013; Chacur et al., 2011; Queiroz et al., 2015). Algumas dessas proteínas estão relacionadas com a capacitação espermática, a motilidade espermática, e

outras também estão envolvidas no processo de reação de acrossoma, fertilização e desenvolvimento embrionário (Moura et al., 2011), demonstrando assim a importância da influência dessas proteínas nas características espermáticas e qualidade seminal. Em touros Holandeses foram encontradas proteínas espermáticas de 25 kDa, identificadas como inibidor tecidual de metaloproteinase-2 (TIMP2), e bandas proteicas de 110 kDa, identificadas como enzima conversora de angiotensina, Hexoquinase-1 e uma isoforma testículo-específica de subunidade α-4 da enzima Na^+/K^+ ATPase, que tiveram uma baixa expressão antes do período de estresse térmico pelo calor, aumentaram durante o momento de estresse e voltaram ao patamar inicial após o fim do estresse. De acordo com Newton et al. (2009) essas proteínas podem ser utilizadas como marcadores moleculares para a função espermática e para a fertilidade.

Segundo a Organização das Nações Unidas para a Alimentação e Agricultura - FAO, a conservação *ex-situ* contempla a criopreservação de sêmen, embriões, células somáticas e DNA. Particularmente, a criopreservação de sêmen é uma ferramenta fundamental na cadeia pecuária, mas apesar dos avanços obtidos nesta biotecnologia, nota-se variação quanto à capacidade de congelamento de cada indivíduo. O fato de que machos de várias espécies podem ser classificados como sendo de pior ou de melhor congelabilidade pode estar ligado a fatores determinados geneticamente, como por exemplo, características estruturais da membrana espermática (Apu et al., 2012; Kuisma et al., 2006; Perumal et al., 2012).

Influências extrínsecas, como o efeito de estação do ano na congelabilidade do sêmen relatada por diversos autores. Pesquisadores observaram que ejaculados de touros Holandeses coletados no verão eram mais sensíveis à criopreservação que aqueles coletados no inverno (Orgal et al., 2012). Em outro experimento realizado no Centro-Oeste brasileiro avaliou-se o sêmen criopreservado de touros taurinos e zebuínos, nas estações de verão e inverno. Dentro da mesma raça houve diferença significativa na motilidade espermática e na atividade citoquímica mitocondrial (ACM) somente em touros zebuínos, que estiveram mais elevadas no inverno. Enquanto que o sêmen dos taurinos não sofreu variações em relação à época do ano. A única diferença entre as raças foi encontrada nos valores de ACM no inverno que foram significativamente maiores nos touros zebuínos (Rodrigues et al., 2015). Contudo, esses resultados devem ser analisados com cautela, visto que, em ambas as raças o sêmen criopreservado não apresentou parâmetros aceitáveis de acordo com o Colégio Brasileiro de Reprodução Animal para a qualidade espermática.

Outros fatores como as espécies reativas de oxigênio (EROs) ou radicais livres, também podem interferir na qualidade seminal. Embora em bovinos a peroxidação lipídica da membrana espermática no sêmen fresco não seja tão importante, as membranas do espermatozoide se tornam mais sensíveis durante a criopreservação, o que pode afetar a sobrevivência da célula. Os metabólitos do oxigênio são altamente instáveis e também podem ser formados a partir de fontes ambientais como o calor e a radiação. Além disso, a destruição dos antioxidantes endógenos pode ser potencializada pelo estresse térmico (Borges et al., 2011). Este conjunto de acontecimentos pode favorecer ainda mais a peroxidação lipídica da membrana espermática pelas EROs prejudicando a qualidade do sêmen.

Durante a criopreservação o dilema está no fato de que embora os ácidos graxos poliinsaturados (PUFA) melhorem a fluidez da membrana devido às insaturações (ligações duplas) características desses lipídeos, fazendo com que haja menor grau de crioinjúrias na fase de transição lipídica do estado líquido-cristalino para o estado de gel, também tornam os espermatozoides mais sensíveis à lipoperoxidação ou à criocapacitação (FAO, 2012; Nichi et al., 2007). A intensidade da produção de EROs pode variar de acordo com a raça e a época do ano, sendo que os metabólito mais importantes na reprodução animal são o radical hidroxila (OH-), o ânion superóxido (O_2-), o peróxido de hidrogênio (H_2O_2) e o óxido nítrico (NO_2) (Queiroz et al., 2015). O plasma seminal é rico em antioxidantes naturais como as enzimas glutationa peroxidase (GSH-Px) e a superóxido dismutase (SOD), o ácido úrico e a vitamina E. Porém durante a manipulação do sêmen para a criopreservação ou durante o processo de inseminação essas substâncias são diluídas ou até mesmo retiradas dependendo da técnica utilizada. Uma forma para contornar este problema seria a adição de enzimas antioxidantes aos meios de congelamento (Asadpour et al., 2011).

As trocas de calor por radiação entre os animais e o meio ambiente são bastante relevantes em se tratando de regiões de clima tropical (Figura 2). Para tentar minimizar ou evitar os problemas advindos do estresse térmico, existem estratégias que abrangem intervenções ambientais, na fisiologia animal e soluções para as técnicas de biotecnologia da reprodução. Há décadas, estudos vêm relatando a importância das modificações físicas no ambiente, como mudanças nas instalações e sombreamento, além da seleção genética de indivíduos mais tolerantes ao calor para reduzir os efeitos adversos supracitados em diversas espécies (Renaudeau et al., 2012; Roth, 2008).

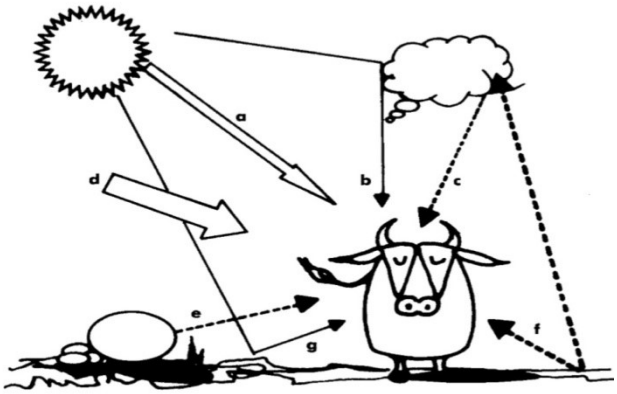

Figura 2. Fontes de radiação térmica sobre um animal. Radiação solar de ondas curtas (a) direta e (b) refletidas pelas nuvens; (c) ondas longas emitidas pelo Sol e refletidas nas nuvens; (d) radiação celeste de ondas curtas; (e) radiação de ondas longas emitidas por corpos e objetos e (f) pelo solo; e de ondas curtas refletidas na superfície do solo (g). Fonte: Livro "Introdução à bioclimatologia animal", p.123, Silva, R.G. (2000).

Dentre as recomendações básicas estão: evitar o manejo dos animais nos períodos mais quentes do dia e realizar a adequação das instalações zootécnicas, quando necessário. Na criação extensiva, o sombreamento natural e/ou artificial é um dos métodos com melhor custo-benefício, sendo que o natural tem a vantagem da evaporação das folhas. Além disso, a cobertura vegetal proporciona um isolamento térmico ao solo, o que evita aquecimento excessivo pela radiação solar e influencia na reflexão dessa radiação sobre os animais. Métodos para aumentar as perdas de calor pelo animal, como promover a ventilação natural nas instalações e o resfriamento dos animais por meio de aspersão de água podem ser necessários, quando se tratar de animais não adaptados e de alta produção (Renaudeau et al., 2012). Estas práticas são, na maioria dos casos, onerosas e não economicamente e/ou tecnicamente viáveis em países tropicais em desenvolvimento. Por isso, o melhoramento genético para tolerância ao calor continua sendo a abordagem mais efetiva frente aos desafios climáticos, visto que é esperado que raças localmente adaptadas ao ambiente ao qual evoluíram lidem melhor com os efeitos das mudanças climáticas que as raças exóticas especializadas (FAO, 2015; Silva, 2000).

Em um estudo sobre adaptação climática, pesquisadores avaliaram o efeito do estresse térmico sobre os fibroblastos da pele de bovinos zebuínos e mestiços. A pele constitui a interface mais importante do animal com o ambiente. Concluiu-se que

os animais zebuínos são mais adaptados a condições de clima tropical que os mestiços (Singh et al., 2014).

Estudando a termoregulação testicular de taurinos, zebuínos e mestiços (5/8 ou 5/16 Charolês x Zebu), Brito et al. (2004) observaram que taurinos apresentam menor comprimento e volume da artéria testicular proporcionalmente ao volume do tecido testicular quando comparados com zebuínos e mestiços. Isto sugere que o aporte de sangue para os testículos pode ser mais limitado em taurinos, contribuindo para a maior suscetibilidade desta subespécie a altas temperaturas ambientes. Além disso, a artéria testicular de bovinos taurinos e mestiços tem um menor número de ramos que penetram no parênquima testicular quando comparado com zebuínos, o que também pode contribuir negativamente para a termorregulação testicular. Estudos nesse sentido ainda precisam ser realizados com as raças localmente adaptadas aos biomas brasileiros.

4. REFERÊNCIAS BIBLIOGRÁFICAS

ABDULKAREEM, T. A. Influence of thawing period on some post-thaw semen characteristics of Holstein bulls following catalase addition to Tris extender. **Al-Anbar J. Vet. Sci.**, v. 7, n. 1, p. 22–28, 2014.

APU A. S. et al. Comparative study of fresh and frozen-thawed semen quality in relation to fertility of Black Bengal goats. **Iran. J. Appl. Anim. Sci.**, v. 2, n. 2, 157–161, 2012.

ASADPOUR, R.; JAFARI, R.; TAYEFI-NASRABADI, H. Effect of various levels of catalase antioxidant in semen extenders on lipid peroxidation and semen quality after the freeze-thawing bull semen. **Veterinary Research Forum**, v. 2, n. 4, p. 218–221, 2011.

BARBOSA, B. R. P. et al. Tolerância ao calor em bovinos das raças Nelore branco, Nelore vermelho e Pantaneira. **Rev. bras. saúde prod. anim.**, v. 15, n. 4, p. 854–865, 2014.

BARROS, D. V. et al. Evaluation of thermal comfort, physiological, hematological, and seminal features of buffalo bulls in an artificial insemination station in a tropical environment. **Trop. Anim. Health. Prod.**, v. 47, n. 5, p. 805–813, 2015.

BIANCHINI, E. et al. Características corporais associadas com a adaptação ao calor em bovinos naturalizados brasileiros. **Pesq. Agropec. Bras.,** v. 41, n. 9, p. 1443–1448, 2006.

BORGES, J. C. et al. Membrana plasmática de espermatozoides bovinos: efeito de metabólitos do oxigênio, antioxidantes e criopreservação. **Rev. Bras. Reprod. Anim.**, v. 35, n. 3, p. 303–314, 2011.

BRITO, L. F. C. et al. Testicular thermoregulation in *Bos indicus*, crossbred and *Bos taurus* bulls: Relationship with scrotal, testicular vascular cone and testicular morphology, and effects on semen quality and sperm production. **Theriogenology**, v. 61, n. 2–3, p. 511–528, 2004.

BUCAK, M. N. et al. Lycopene and resveratrol improve post-thaw bull sperm parameters: Sperm motility, mitochondrial activity and DNA integrity. **Andrologia**, v. 47, n. 5, p. 545–552, 2015.

CARDOZO, J. A. et al. Monthly variations in ovine seminal plasma proteins analyzed by two-dimensional polyacrylamide gel electrophoresis. **Theriogenology**, v. 66, n. 4, p. 841–850, 1 set. 2006.

CARNEIRO, T. et al. Diversidade genética e eficiência de DNA microssatélites para o controle genealógico da raça Nelore. **Arq. Bras. Med. Vet. Zootec**, v. 59, n. 5, p. 1257–1262, 2007.

CARVALHO, G. M. C. et al. Genetic structure of goat breeds from Brazil and the United States: Implications for conservation and breeding programs. **J. Anim. Sci.**, v. 93, p. 4629–4636, 2015.

CAVALCANTE, J. M. M. et al. Plasma seminal ovino e sua aplicação na biotecnologia reprodutiva. **Arq. Bras. Med. Vet. Zootec.,** v. 37, n. 3, p. 254–259, 2013.

CHACUR, M. G. M. et al. Influência da estação sobre as proteínas do plasma seminal na raça Tabapuã (*Bos taurus indicus*). **Arch. Zootec.,** v. 60, n. 230, p. 301–304, 2011.

CHACUR, M. G. M. et al. Influência da estação do ano nas características do sêmen e na concentração de hormônios em touros Nelore e Simental. **Arq. Bras. Med. Vet. Zootec.**, v. 64, n. 3, p. 540–546, 2012.

EGITO, A. A.; MARIANTE, A. S.; ALBUQUERQUE, M. D. S. M. Programa Brasileiro de Conservaçao de Recursos Genéticos Animais. **Arch. Zootec.**, v. 51, p. 39–52, 2002.

EGITO A.A., et al. Estrutura populacional e diversidade genética de raças bovinas brasileiras localmente adaptadas. **Actas Iberoam. Conserv. Anim.,** v. 4, p. 16–18, 2014.

FAO. **Cryoconservation of animal genetic resources.** No. 12 ed. Rome: FAO Animal Production and Health Guidelines, 2012.

FAO. **Coping with climate change - The roles of genetic resources for food and agriculture**. Rome: 2015.

FAO. **How to feed the world in 2050.** High-level expert forum. Anais...Rome: Food and Agriculture Organization of the United Nations, 2009.

FAO. **Situação mundial dos recursos genéticos animais para a agricultura e alimentação - versão resumida**. Brasília: 2010a.

FAO. **Plano de ação mundial para os recursos genéticos animais e declaração de Interlaken.** Brasília: Embrapa Recursos Genéticos e Biotecnologia, 2010b.

FELIX G. A. et al. Potencial de uso de raças bovinas locais brasileiras: Curraleiro Pé-duro e Pantaneiro. **Encicl. Biosf.**, v. 9, n. 16, 1715–1741, 2013.

FRENEAU, G. E. Aspectos da morfologia espermática em touros. **Rev. Bras. Reprod. Anim.**, v. 35, n. 2, p. 160–170, 2011.

GABALDI, S. H.; WOLF, A. A importância da termorregulação testicular na qualidade do sêmen em touros. **Ciên. Agr. Saúde.** FEA, Andradina, v. 2, n. 2, p. 66–70, 2002.

GUASTI, P. N.; MONTEIRO, G. A.; PAPA, F. O. Componentes do plasma seminal e sua influência sobre a criopreservação e fertilidade de espermatozoides equinos. **Vet. Zootec.,** v. 19, n. 2, p. 169–180, 2012.

HAFEZ B.; HAFEZ E. S. **Reproduction in Farm Animals.** 7. Ed. Baltimore, Maryland, USA: Lippincott Williams & Wilkins, 2000.

HANSEN, P. J. Physiological and cellular adaptations of zebu cattle to thermal stress. **Anim. Reprod. Sci.**, v. 82–83, p. 349–360, 2004.

HANSEN, P. J. Effects of heat stress on mammalian reproduction. **Philos. Trans. R Soc. Lond. B Biol. Sci**, v. 364, n. 1534, p. 3341–3350, 2009.

HENRIQUE, C. et al. Histórico genético e populacional do rebanho Nelore Puro de Origem no Sertão Nordestino. **Pesq. Agropec. Bras.**, v. 44, n. 7, p. 713–718, 2009.

IBGE. PIB varia 0,1% em relação ao 2° tri e chega a R$ 1,641 trilhão. **Agência IBGE notícias.** Ed. Estatísticas econômicas, Brasil, 01 de dezembro de 2017. Disponível em: <https://agenciadenoticias.ibge.gov.br/agencia-noticias/2013-agencia-de-noticias/releases/18458-pib-varia-0-1-em-relacao-ao-2-tri-e-chega-a-r-1-641-trilhao.html>. Acesso em: 10 fev. 2018.

IBGE. Efetivo dos rebanhos, por tipo de rebanho. **Sistema IBGE de Recuperação Automática – SIDRA**, 2018. Disponível em: <https://sidra.ibge.gov.br/tabela/3939>. Acesso em: 10 fev. 2018.

KUISMA P. et al. Fertility of frozen-thawed stallion semen cannot be predicted by the currently used laboratory methods. **Acta Vet. Scand.**, v. 48, n. 14, 1–8, 2006.

MADEIRA, E. M. et al. Avaliação de diferentes crioprotetores intra e extracelulares na criopreservação de sêmen de touros. **Arq. Bras. Med. Vet. Zootec.**, v. 65, n. 2, p. 415–420, 2013.

MARIANTE, A. S.; CAVALCANTE, N. **Animais do Descobrimento: raças domésticas da história do Brasil**. 2.ed. Brasília: Embrapa Recursos Genéticos e Biotecnologia, 2006.

MARIANTE, A. S.; RAMOS, A. F. Criopreservação de recursos genéticos animais brasileiros. **Rev. Bras. Reprod. Anim.**, v. 35, n. 2, p. 64–68, 2011.

MARIANTE, A. D. S. et al. Present status of the conservation of livestock genetic resources in Brazil. **Livest. Sci.**, v. 120, n. 3, p. 204–212, fev. 2009.

MATSUZUKA, T. et al. Effects of heat stress on the redox status in the oviduct and early embryonic development in mice. **J Reprod. Dev.**, v. 51, n. 2, p. 281–7, 2005.

MCMANUS, C. et al. Heat tolerance in naturalized Brazilian cattle breeds **Livest. Sci.**, v. 120, n. 3, p. 256–264, 2009.

MCMANUS, C. et al. Skin and coat traits in sheep in Brazil and their relation with heat tolerance. **Trop. Anim. Health Prod.**, v. 43, n. 1, p. 121–126, 2011.

MENEGASSI, S. R. O. et al. Scrotal infrared digital thermography as a predictor of seasonal effects on sperm traits in Braford bulls. **Int. J Biometeorol.**, v. 59, n. 3, p. 357–364, 2015.

MENEGASSI, S. R. O. et al. The uses of infrared thermography to evaluate the effects of climatic variables in bull's reproduction. **Int. J Biometeorol.**, v. 60, n. 1, p. 151–157, 2016.

MOURA, A. A.; ANDRADE, C. R.; OLIVEIRA, R. V. Proteínas do plasma seminal, funções espermáticas e marcadores moleculares da fertilidade. **Rev. Bras. Reprod. Anim.,** v.35, n. 2, p. 139–144, 2011.

MUINÕ-BLANCO, T.; PEEZ-PEÁND, R.; CEBRIA-PEEZ, J. Seminal Plasma Proteins and Sperm Resistance to Stress. **Reprod. Dom. Anim**., v. 43, n. 4, p. 18–31, 2008.

NEWTON, L. D. et al. Elevated testicular temperature modulates expression patterns of sperm proteins in Holstein bulls. **Mol. Reprod. Dev.**, v. 76, n. 1, p. 109–118, 2009.

NICHI, M. et al. Roles of lipid peroxidation and cytoplasmic droplets on in vitro fertilization capacity of sperm collected from bovine epididymides stored at 4 and 34°C. **Theriogenology**, v. 67, n. 2, p. 334–340, 2007.

OLIVEIRA, J. H. F.; MAGNABOSCO, C. U.; BORGES, A. M. S. M. **Nelore: base genética e evolução seletiva no Brasil**, Embrapa Cerrados, 2002. Disponível em: <http://www.infoteca.cnptia.embrapa.br/bitstream/doc/566499/1/doc49.pdf>

ORGAL, S. et al. Season-induced changes in bovine sperm motility following a freeze-thaw procedure. **J Reprod. Dev.**, v. 58, n. 2, p. 212–218, 2012.

PAULA-LOPES, F. F. et al. Heat stress induced alteration in bovine oocytes : functional and cellular aspects. **Anim. Reprod.**, v. 9, n. 3, p. 395–403, 2012.

PAULO, M. et al. Estrutura populacional do rebanho Nelore criado no semi-árido do Nordeste brasileiro. **Ciênc. Anim. Bras.,** v. 18, p. 1–14, 2017.

PERUMAL P. et al. Reduced glutathione and cysteine hydrochloride on sperm motility and velocity parameters of poor crossbred bull semen. **International Journal of Bio-resource and Stress Management**, v. 3, n. 2, 145–151, 2012.

QUEIROZ, V. L. D. et al. Sazonalidade na congelabilidade de sêmen bovino. **Arq. Ciênc. Vet. Zool.**, v. 18, n. 1, p. 39–47, 2015.

RAHMAN, M. B. et al. Scrotal insulation and its relationship to abnormal morphology, chromatin protamination and nuclear shape of spermatozoa in Holstein-Friesian and Belgian Blue bulls. **Theriogenology**, v. 76, p. 1246–1257, 2011.

RENAUDEAU, D. et al. Adaptation to hot climate and strategies to alleviate heat stress in livestock production. **Animal**, v. 6, n. 5, p. 707–728, 2012.

RODRIGUES, M. P. et al. Perfil oxidativo e funcional de sêmen bovino criopreservado em diferentes estações do ano. **Braz. J Vet. Res. Anim. Sci.,** v. 52, n. 2, p. 134–140, 2015.

ROTH, Z. Heat stress, the follicle, and its enclosed oocyte: mechanisms and potential strategies to improve fertility in dairy cows. **Reprod. Domest. Anim.**, v. 43, n. 2, p. 238–244, 2008.

SANTOS, S. A. et al. Variações da temperatura corporal e da pele de vacas e bezerros das raças pantaneira e nelore no pantanal. **Arch. Zootec.**, v. 54, n. 206–207, p. 237–243, 2005.

SHARMA, L. et al. Seasonal variations in seminal plasma proteins of buffalo. **Reprod. Domest. Anim.**, v. 49, n. 3, p. 387–391, 2014.

SILVA, G. D. A. et al. Efeito das épocas do ano e de turno sobre os parâmetros fisiológicos e seminais de caprinos no semi-árido paraibano. **ACSA**, v. 1, p. 7–14, 2005.

SILVA, R. G. **Introdução à bioclimatologia animal**. São Paulo: FAPESP, 2000.

SINGH, A. K. et al. Effect of thermal stress on HSP70 expression in dermal fibroblast of zebu (Tharparkar) and crossbred (Karan-Fries) cattle. **J Therm. Biol.**, v. 43, n. 1, p. 46–53, 2014.

SIROTKIN, A. V. Effect of two types of stress (heat shock/high temperature and malnutrition/serum deprivation) on porcine ovarian cell functions and their response to hormones. **J. Exp. Biol.**, v. 213, n. 12, p. 2125–2130, 2010.

SOUZA, B. B.; BATISTA, N. L. Os efeitos do estresse térmico sobre a fisiologia animal. **ACSA**, v. 8, n. 3, p. 6–10, 2012.

TAKAHASHI, L. S. et al. **Bioclimatologia Zootécnica**. p. 91, 2009.

TEIXEIRA, H. C. A. et al. Seasonal influence on semen traits and freezability from locally adapted Curraleiro bulls. **Anim. Reprod. Sci.**, v. 125, n. 1–4, p. 56–61, 2011.

UYSAL, O. et al. Effect of various antioxidants on the quality of frozen-thawed bull semen. **J Anim. Vet. Adv.**, v. 6, n. 12, p. 1362–1366, 2007.

VIANNA, E. et al. Estresse por calor: alterações na fisiologia reprodutiva e estratégias para amenizar seus efeitos negativos na fertilidade de fêmeas bovinas. **Arq. Ciênc. Vet. Zool.**, v. 16, n. 1, p. 79–85, 2013.

CAPÍTULO 2

SEASONAL SEMEN QUALITY OF LOCALLY ADAPTED BULLS RAISED IN A SUBTROPICAL CLIMATE: RELATIONSHIP WITH EXTERNAL MORPHOLOGY OF THE ANIMALS AND CLIMATE

ABSTRACT

Livestock production is considered an important factor for global warming, however, climate change has also been considered one of the major factors affecting livestock production and reproduction. Based on these concerns, there is a need to study the consequences of the environment on livestock. This study was carried out in the Southern region of Brazil and aimed to assess the seasonal influence of climatic factors and external morphology of the animals on semen quality of adult bulls. Semen samples were obtained three times in summer and three times in winter from bulls from a locally adapted (Crioulo Lageano) and exotic commercial breed (Angus), both *Bos taurus*. The percent of motile cells, sperm vigor, number of sperm per mililiter, sperm membrane status and abnormalities of spermatozoa were analysed on fresh semen. Those traits, as well as the sperm kinetics using CASA, were also assessed on post-thawed semen. Environmental data such as air temperature, air relative humidity, black globe temperature (BGT) and the temperature humidity index (THI) were measured. Further, body and testicular metrics, as well as hair coat characteristics, were recorded in each season on all bulls. Environmental traits differed significantly between seasons. Results of the partial least squares regression and Spearman correlations confirmed a negative relationship between climatic variables and sperm traits in both seasons. Semen quality of the bulls was similar and satisfactory across the year and heat stress was not sufficient to impair spermatozoa viability. Moreover, external morphological traits were considered non-important in the final model. In conclusion, air temperature, BGT and THI were considered to be the most important factors that could affect seminal characteristics.

Keywords: adaptation, global warming, livestock, reproduction, sperm, testicles, THI.

1. INTRODUCTION

Approximatelly, one out of five bulls is considered unfit for use in breeding programs due to poor semen quality (Barth & Waldner, 2002). The reproductive process depends on a complex series of events that may be influenced by many factors including both genetic and environment. “Environment” can be defined as all external factors, such as climate, soil, and also living things, surrounding an organism and ultimately determine its form and survival (Merriam-Webster, 2017).

There is significant genetic variation in bull fertility with differences between individuals (Barth & Waldner, 2002; Han & Peñagaricano, 2016). In turn, environmental effects, such as thermal stress, could impact substantially on the bulls’ reproductive efficiency. With the current environmental issues surrounding cattle production and climate change, the topic of livestock adaptation is particularly important given their role in the production of greenhouse gas emissions and contribution to global warming. On the other hand, livestock production is also affected by climate change and even the most adapted breeds can succumb to thermal stress (FAO, 2007).

The climate in Brazil is classified mostly as being in a large tropical zone, representing 81.4% of the territory, but there is also a smaller dry zone and, in the Southern region, a humid subtropical zone (Alvares et al., 2013). The main cattle breeds raised in the Southern region are of the taurine subspecie. It is well known that taurine breeds are more vulnerable to heat stress, given their origin in the Northern Hemisphere. Even the most resilient animal can still suffer from heat stress when subjected to substantial adverse environmental conditions, with an inability to dissipate heat despite the provision of shade and other techniques to alleviate thermal stress. However, locally

adapted cattle are expected to cope better with the climatic challenges than their exotic counterparts (FAO, 2015).

Studies reporting thermal stress in cattle reproduction are mainly focused on females, whilst there are fewer studies reported about sires (Bishop-Williams et al., 2015; Silva et al., 2007; Dalcin et al., 2016; Mcgee et al., 2008; Roth, 2008). This fact highlights the importance of performing more studies to understand how males are affected by thermal stress.

For optimal spermatogenesis, bovine intratesticular temperature must be around 4 to 5ºC lower than body-core temperature (Hafez & Hafez, 2000; Kastelic, 2013). In general, mammals expend around 85% of the energy obtained from food just to maintain homeostasis (Reece, 2015). Testicular thermoregulation is maintained by several complex factors including phenotypic features, such as testicular vascular cone anatomy, morphology of the testes and scrotal skin thickness, as well as, physiological mechanisms, such as the regulation of testicular blood flow, testicular vascular permeability and the tonic control of the testicular muscles (Brito et al., 2004; Gabaldi e Wolf, 2002; Kastelic et al., 1997). Thermal comfort can influence the optimal thermoregulation and the capability of the animals to respond to external factors.

It is well known that a single laboratory assay is not enough to determine true semen quality (Amann, 1989; Kaya & Memili, 2016; Vasan, 2011) and it is important to determine which semen traits are more influenced by environmental factors and bull phenotype. It is possible to assess the contribution of each factor to semen quality and this is especially useful in the case of bulls kept in a bovine reproduction centre.

The aims of the present study were to investigate the seasonal influence of environment, focusing in the climate traits and phenotypic traits related to climate adaptation on semen quality of bulls of a locally adapted breed in comparison with an exotic commercial breed, both raised in a subtropical region.

2. MATERIAL AND METHODS

The experiment followed the bioethics norms in animal experimentation and was previously approved by the Internal Technical Committee of Brazilian Agricultural Research Corporation – Embrapa Genetic Resources and Biotechnology / 006-2013.

2.1 Local and animals

The study was carried out in "Bom Jesus do Herval" Farm located in the Southern Brazil (27º 48' 58" S, 50º 19' 34" W) during the months of February/2016, summer, and July/2016, winter. Pasture at this region consists mainly of native grass species such as *Andropogon lateralis Nees* and *Schizachyrium tenerum*.

The bulls of this study, four Angus and four Crioulo Lageano, aged from 2 to 6 years and reproductively active, were judged as sound for breeding by previous breeding soundness evaluation. They were born and raised in the same region with similar environmental conditions, such as available naturally shaded areas, water *ad libitum* and mineral supplements.

2.2 Semen collection and processing

During each season studied, semen was collected from all bulls, three times in two weeks, by electroejaculation (Duboi® electroejaculator, Campo Grande, MS, Brazil). This method is commonly used for untrained bulls and has been

succesfully adopted in many others studies (Freneau et al., 2006; Menegassi et al., 2015; Rego et al., 2015; Reis et al., 2016). Electrical stimuli were applied in a continuous rhythm and collection of semen samples started when the pre-ejaculatory fluids became opaque and once the fluids began turning clear again the collection finished.The semen samples were kept at 37ºC for the next steps.

For semen freezing, samples were added to egg-yolk based extender containing also tris, fructose, lactose, citric acid, streptomycin, penicillin and glycerol, to give a concentration of 40 million of sperm per 0.5 mL straw, following the method used by Teixeira et al. (2011). Straws were kept in liquid nitrogen until the post-thaw analyses began.

2.3 Semen assessments

In this study, the characteristics evaluated in the fresh semen were sperm motility (%), vigor (0-5), semen concentration ($\times 10^9$ sptz/mL), sperm membrane status (%) and abnormalities of spermatozoa (%), according to methods used in previous studies (Souto et al., 2017; Teixeira et al., 2011). Abnormalities were split into tail defects, mid-piece defects, head defects and classified in either minor or major defects following the recommendations of the Brazilian College of Animal Reproduction (CBRA, 2013). Sperm membrane status was evaluated by Trypan-blue and Giemsa staining and, then, cells were classified as being ‘alive or dead’, according to plasma membrane status, and ‘intact or damaged’ acrosome, according to acrosome status (Moreira et al., 2016).

Post-thawed semen was evaluated in triplicate for sperm kinematics and abnormalities, as mentioned above for fresh semen, for membranes integrity and DNA denaturation. Percentage of motile and progressive cells, average pathway velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN), percentage of slow, rapid and static cells were assessed using a CASA system (Sperm analysis System, Ivos-Ultimate 12’s, Hamilton Thorne Biosciences, Beverly, MA, USA). For this, 10 µL of sperm were placed in pre-warmed glass-slide (Makler® counting chamber, Sefi Medical Instruments, Haifa, Israel, 10 µm). At least three fields were selected for reading and analysis.

For membrane evaluations, two dual-staining protocols were used and one triple-staining protocol. The fluorescent probes used were fluorescein isothiocyanate-conjugated peanut agglutinin - FITC-PNA (Sigma-Aldrich®, L7381), propidium iodide – PI (Molecular Probes®, P1304), 6-Carboxyfluorescein diacetate - CFDA (Sigma-Aldrich®, C-5041) and JC-1 (5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide, Molecular Probes®, T-3168).

For the assessment of acrosome integrity, a combination of FITC-PNA with propidium iodide was used, as described by Nagy et al. (2003). The working solution was composed of 20 µL FITC-PNA (1 mg/mL in phosphate buffered saline-PBS solution), 10 µL PI (0.5 mg/mL in 0.9% saline solution), 10 µL formal saline solution (96 mL of 0.9% saline solution and 4 mL of 40% formal) and 960 µL 3% sodium citrate. An aliquot of 10 µL of sperm was mixture into a 40 µL of the working solution and incubated at room temperature for at least 10 minutes in the dark. A total of 200 cells were counted by epifluorescent microscopy (B-2A fluorescence filter cube, Nikon®, excitation filter: 450-490 nm; barrier filter: 520 nm; dicroic mirror: 505 nm). Sperm that were not stained were considered to have an intact plasma membrane. Reacted acrosomes displayed a green fluorescence whereas in intact acrosomes no fluorescence was visible.

Plasma membrane status was evaluated using CFDA with propidium iodide as described by Harrison & Vickers (1990). The stain solution was prepared with 20 µL CFDA (0.46 mg/mL in dimethyl sulphoxide), 10 µL PI (0.5 mg/mL in 0.9% saline solution), 10 µL 1:80 formalin solution and 960 µL 3% sodium citrate. The sample was incubated and examined using the same technique described above. Cells stained green were classified in cells with intact plasma membrane, whereas those stained red were deemed to have a damaged plasma membrane.

The method for the simultaneous evaluation of sperm membranes developed by Celeghini et al. (2010) was adjusted in order to use FITC-PNA instead of FITC-PSA. The working solutions of PI (0.5 mg/ml in 0.9% saline solution), FITC-PNA (1 mg/ml in phosphate buffered saline-PBS solution) and JC-1 (153 µM in dimethylsulfoxide) were used. An aliquot of 100 µl of post-thaw semen was added in 300 µl of TALP medium and kept at 37ºC for five minutes, then, 40 µl of this mixture was poured into a tube containing 1 µl FITC-PNA, 2 µl PI and 5 µl JC -1 and incubated for 8 minutes at 37ºC in the dark. An aliquot of 7 µl was put on a glass slide with a cover slip over it and immediately examined by epifluorescence microscopy (B-2A

fluorescence filter cube, Nikon®, excitation filter: 450-490 nm; barrier filter: 520 nm; dichroic mirror: 505 nm) (Souto et al., Submitted).

The susceptibility of sperm nuclear DNA to acid-induced denaturation was assessed using the acridine orange assay (AO) as described by Unanian (2000). In summary, a smear was prepared using a drop of post-thawed semen, the slide was dried at room temperature and fixed overnight into a fresh Carnoy's solution (3 volumes of methanol and 1 volume of glacial acetic acid). Slides were stained with 10 mL of 10 mg/ml AO solution, 40 mL of 0.1 M citric acid, and 2.5 mL of 0.3 M $Na_2HPO_4.7H_2O$, the final pH was 2.8. After five minutes the slides were rinsed gently with type 2 water and observed by epifluorescence microscopy.

2.4 Environmental data

Meteorological data started to be recorded from the day prior to first semen collection until the last day of the experiment. Maximum, minimum and mean values for air temperature (°C) and relative humidity (%) were obtained by an automated meteorological station of the National Institute of Meteorology (INMET). Mean radiant temperature of the environment, which is another tool used to estimate the thermal comfort, was measured using a black globe thermometer (Black globe temperature). The globe used consists of a matt black painted hollow copper sphere of approximated diameter 15 cm, containing a thermometer bulb fixed at the center of the sphere, without source of heat.

The discomfort index, represented by temperature humidity index was calculated in the morning and in the afternoon following Thom's (1959) formula:

$$THI = (0.8 \times Ta) + (RH/100) \times (Ta - 14.4) + 46.4$$

Where THI is temperature humidity index, Ta is air temperature (°C) and RH is the air relative humidity (%). Another formula was also used to calculate the temperature and humidity index according to National Research Council's formula:

$$THI_{NRC} = (1.8 \times Ta + 32) - (0.55 - 0.0055 \times RH) \times (1.8 \times Ta - 26)$$

Where THI_{NRC} is temperature humidity index, Ta is air temperature (°C) and RH is relative humidity (%). The mean compensated-temperature of the day (MCT), which is another method to calculate the average day air temperature, was calculated by

following formula (INMET, 2009; National Research Council, 1971; Varejão–Silva, 2006):

$$MCT = \frac{T12 + Tx + Tn + (2 \times T24)}{5}$$

Where T12 is the air temperature at 12 o'clock noon, *Tx* is the maximum air temperature of the day, *Tn* is the minimum air temperature of the day and *T24* is the air temperature at midnight.

2.5 Physical data

Morphological traits included height at withers, hip height, body length, heart girth, chest depth and cannon bone girth, that were measured by a hipometer and a tape, and body weight. Testes traits such as scrotal circumference, testicular volume and skin thickness of the scrotum were measured by a tape and a caliper. The formula used for calculate the testicular volume was: $TV = 2 \times [(TW/2)^2 \times 3.14 \times (TL)]$, where *TV* is the testicular volume (cm^3), *TW* is the testicular width (cm) and *TL* is the testicular length (cm).

Skin thickness of the body was measured by an adipometer and skin colour was visually determined using a standard scale for bovine skin pigmentation according method describe by Silva (2000). Hair coat colour was measured using the CIELAB colour system (ColorQuest XE Spectrophotometer (HunterLab, Reston, USA), which determines the coordinates: *L (brightness), *a (red colour intensity) and *b (yellow colour intensity), and three measurements were obtained for each bull. To determine hair coat density and hair coat length, hair samples were taken from the flank area, 30 cm below the spinal column, using adapted pliers and all hairs were counted to give a numbers of hairs per cm^2. The longest ten hairs were measured in each sample using graph paper (Silva, 2000).

2.6 Statistical analysis

Statistical analyses were performed using the Statistical Analysis System® package (v.9.3, SAS Inc., Cary, NC, USA). Analysis of variance (PROC GLM/GLIMMIX) was carried out to evaluate the effect of the weather and thermal comfort indexes (19 variables), body, testicular and hair coat measurements (27

variables), breed and season and their interactions on semen quality (39 variables). Spearman correlations (PROC CORR) were used to determine the relationships among all variables. The variables that were not statistically associated with each other were excluded from the data set. After this first reduction, the model remained with a larger number of variables than the number of observations recorded. For this scenario a Partial least squares regression - PLS (PROC PLS) was performed separately for each season. The criteria used for dropping the variables from the PLS model were based on the Variable Importance for Projection (VIP) statistic of Wold (1994), on the examination of the variable patterns in of the Correlation loading plot. A VIP value less than 0.8 is considered to be "small". A small VIP and small absolute coefficients suggest that the variable should be removed from the model (SAS, 2015). Further, when two variables, had shown a high correlation coefficient ($\rho \geq 0.70$) with each other, one of them was considered to be excluded from the model. After these analyses, 7 physical, 7 seminal and 5 meteorological traits remained in the final models.

3. RESULTS AND DISCUSSION

During the study, the air temperature in summer ranged from 17°C to 30.2°C and in winter ranged from -1.6°C to 18.7°C. Air humidity in summer ranged from 31% to 97% and in winter ranged from 33% to 100% (Figure 1). The black globe mean temperature in summer was 29.2±6.6 with minimum value recorded of 21.1°C and maximum of 41.3°C, whilst in winter the mean was 12.9±9.0 with lowest and highest temperatures of 0.6°C and 28.1°C, respectively. The minimum and maximum value for temperature and humidity index (THI) in summer was 63 and 79, respectively, and in winter was 31 and 65, respectively (Figure 2). In summer, THI indicated that the bulls were experiencing heat stress in the afternoon, but in the morning this index was considered to be normal. THI_{NRC} values followed the same pattern displayed by THI, where in summer the calculated values were higher than those ones in winter, 69.65±3.78 and 51.53±9.65, respectively. However, regardless of either season or period of the day, THI_{NRC} were classified as being normal according to NOAA's National Weather Service (1971). Air temperature, black globe temperature and thermal comfort indexes revealed a significant difference between summer and winter. Nevertheless, the relative humidity did not change between seasons.

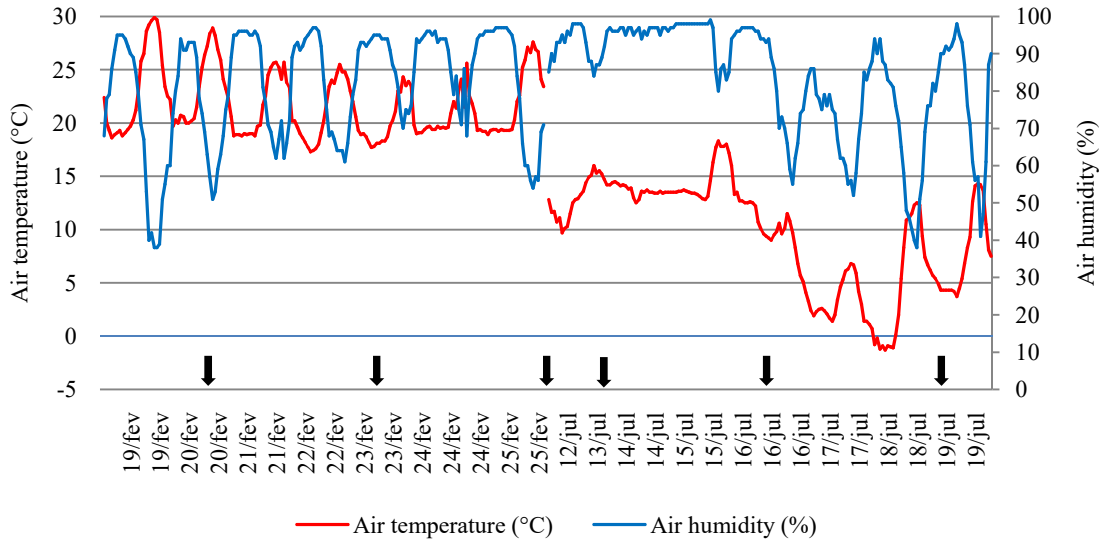

Figure 1: Daily mean air temperature (°C), air relative humidity (%) during the experimental period. Black arrows indicate the days of semen collection.

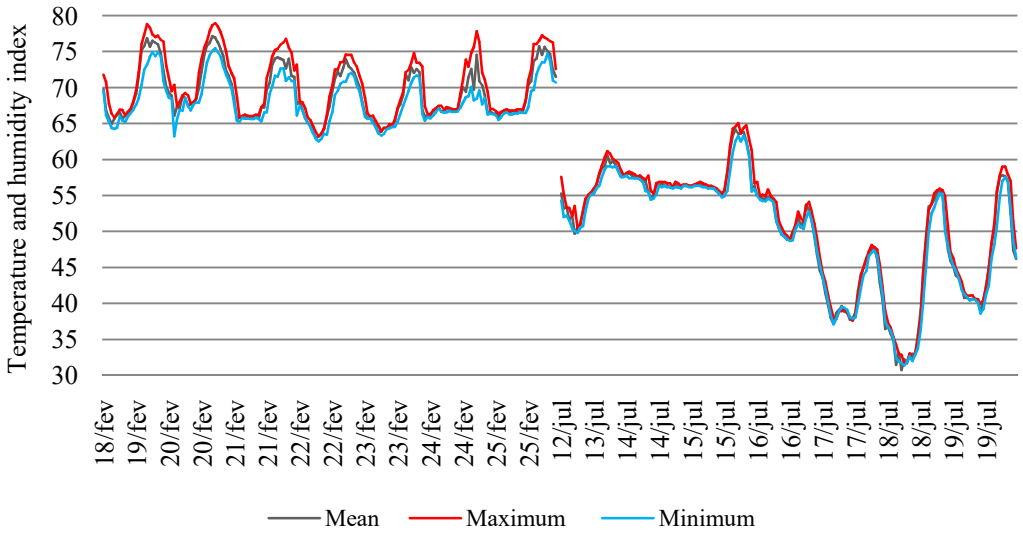

Figure 2: Daily temperature and humidity index - THI during the experimental period.

The morphological traits are showed in Table 1. In general, Angus bulls were heavier and larger, but shorter than Crioulo Lageano bulls. Looking at the hair coat and skin traits it is noted that the difference in exterior morphology between breeds is also well characterized. Angus bulls have darker and longer hair coat than Crioulo Lageano bulls in both seasons. Hair coat density was similar between breeds in summer but in winter it was higher in Angus bulls.

Table 1. Means±standard deviation of morphological traits in Angus and Crioulo Lageano bulls in summer and winter

	Summer		Winter	
	Angus	Crioulo Lageano	Angus	Crioulo Lageano
BW (kg)	862.67±214.97a	673.67±137.42c	714.5±177.98b	645.92±155.50d
HAW (cm)	135.83±7.58	141.00±9.92	137.67±8.85	143.58±7.70
HH (cm)	136.83±5.41c	143.50±7.91ab	138.83±3.79bc	145.33±5.12a
BL (cm)	166.25±16.89a	154.58±10.12b	173.33±8.03a	164.67±5.57ab
HG (cm)	224.33±22.07a	207.00±15.87bc	213.67±20.23ab	198.92±13.30c
CD (cm)	78.21±5.94	73.54±3.59	75.88±2.22	73.42±3.53
SC (cm)	40.83±5.02	37.5±3.55	40.67±4.64	37.92±3.96
TV (cm^3)	1471.22±393.80a	1161.82±281.07c	1221.74±168.77b	1109.10±419.83d
STT (mm)	6.58±1.38	6.50±1.17	7.83±1.34	7.58±1.68
SkinCL (%)	81.25±24.23ab	74.58±12.87a	85.42±26.41b	78.75±20.68a
ST (mm)	11.88±1.38b	13.88±1.59a	13.44±1.02b	16.75±2.26a
CIE*L	31.51±7.31b	49.65±8.20a	32.50±8.34b	49.51±11.66a
CIE*a	2.96±2.18b	6.78±1.69a	1.77±2.65b	6.06±1.19a
CIE*b	3.92±2.82b	14.61±1.10a	1.67±2.49c	12.91±0.86a
HD ($x10^3$)	1019.50±211.47a	1056.00±240.87a	1225.75±170.82a	610.50±183.05b
HL (mm)	13.10±0.92b	6.55±0.84c	31.65±4.85a	14.30±3.06b

BW: body weight, HAW: height at withers, HH: hip height, BL: body length, HG: heart girth, CD: chest depth, SC: scrotal circumference, TV: testicular volume, STT: skin thickness of the testes, ST: skin thickness of the body, SkinCl: skin colour, CIE*L: brightness, CIE*a: red color intensity, CIE*b: yellow colour intensity, HD: hair coat density, HL: hair coat length. Means with different subscripts in the row are significantly different at $P<0.05$.

Similarly to our results (Table 2), Friesian bulls in Lybia, also a tropical country, presented better results for semen quality during the winter (Alragubi, 2015). The author suggests that it may be explained by the general conditions during the winter, which favour testosterone activity, spermatogenesis and seminal fluids secretion, as well as, an increase of feed intake due the higher thyroxin concentration in comparison with hot seasons.

Table 2. Means±standard deviations for fresh semen traits of Angus and Crioulo Lageano bulls in summer and in winter

	Summer		Winter	
	Angus	Crioulo Lageano	Angus	Crioulo Lageano
Motile (%)	85.42±6.20	84.17±10.19	89.50±4.38	86.43±8.99
Major defects (%)	18.00±13.85	10.83±5.17	10.08±4.17	9.29±4.23
Minor defects (%)	5.21±3.46	5.00±3.86	5.83±4.59	3.14±3.13
Total defects (%)	23.21±13.16	15.83±6.41	15.92±3.63	12.43±4.24
ISA (%)	90.50±4.45	90.28±6.54	94.17±3.21	94.86±3.39

ISA: Spermatozoa with intact plasma membrane and acrosome. P>0.05.

The results for sperm kinematics (Table 3) are in accordance with what had been found in the literature (Islam et al., 2017). As mentioned above, THI indicated that there was moderated heat stress in one period of the day. Nonetheless, it was not enough to impact upon the bulls' semen quality, since the only differences observed in post-thawing semen was in regard to sperm velocity and these isolated changes in sperm kinetics do not mean a better semen condition.

There were no differences within and between seasons for sperm chromatin integrity in post-thawed semen. The percent of cells with intact DNA in summer was 97.3±2.1 for Angus and 97.7±1.8 for Crioulo Lageano and in winter was 98.6±1.0 for Angus and 97.3±3.2 for Crioulo Lageano. In agreement with our results, others authors also found a few cells with abnormal chromatin in bulls (Januskauskas et al., 2003), with averages around 3.5% (Roy et al., 2012). In this study, it was observed that the individual effect of the bull seems to be more relevant for chromatin fragmentation than season or age. In a study with tropical bulls, Fortes et al. (2012) observed no difference in percent of chromatin integrity between young and mature bulls. A mild heat stress was able to impair significantly the sperm chromatin integrity in men (Ahmad et al., 2012). Human semen has a higher percent of DNA denaturation, which can exceed 30%, compared to bulls and this could be due the differences in the ratio of protamines surrounding sperm chromatin gives to bull sperm more resistence than human sperm (Rex et al., 2017). Boe-Hansen et al. (2008) observed that when the DNA fragmentation was above 2.1% in boar semen, the total number of piglets born was smaller. This findings highlight that is importance to include the sperm chromatin integrity assay in the breeding soundness evaluation as it can identify some individuals with abnormal DNA and hence, low fertility.

Table 3. CASA-based sperm kinematics and morphological traits of post-thaw semen in Angus and Crioulo Lageano bulls in summer and in winter

	Summer		Winter	
	Angus	Crioulo Lageano	Angus	Crioulo Lageano
VAP (μm/s)	69.22±9.19b	79.71±16.87a	71.10±7.90ab	78.13±7.60ab
VSL (μm/s)	55.64±6.35b	64.08±15.48a	59.31±7.31ab	64.20±7.47a
VCL (μm/s)	117.51±18.32b	138.22±25.86a	115.85±10.38b	130.79±9.78a
ALH (μm)	0.06±0.01	0.07±0.01	0.05±0.00	0.06±0.01
BCF (Hz)	28.52±3.33	31.10±5.33	30.65±3.38	30.07±2.46
Straightness (%)	81.27±3.26	80.63±3.13	83.13±2.94	82.29±2.87
Linearity (%)	50.39±38.34	49.38±38.32	53.21±3.39	52.00±4.12
Motile (%)	50.23±19.84	45.54±22.85	51.70±10.04	51.71±16.78
Progressive (%)	29.73±12.42	28.58±17.71	32.08±8.55	32.43±14.66
Rapid (%)	37.65±15.82	36.13±21.70	38.50±9.65	40.00±16.57
Static (%)	31.85±21.50	37.17±28.26	29.92±10.72	32.00±19.50
Major defects (%)	23.79±8.57	21.22±7.85	24.83±0.11	22.64±12.94
Minor defects (%)	2.42±1.35	3.53±2.35	4.65±3.03	6.23±5.00
Total defects (%)	25.92±8.85	24.75±8.92	29.50±10.66	28.44±11.68

VAP: average path velocity, VSL: straight-line velocity, VCL: curvilinear velocity, ALH: amplitude of lateral head displacement, BCF: beat cross frequency. Means with different subscripts in the row are significantly different at $P<0.05$.

Results for fluorescent probes association are shown in Table 4. Three cell categories, JC1d, JC1g and JC1h, had zero frequences and are not presented here. Means for intact cells with intact acrosome were similar among the three probes associations. Based on the triple-staining results, it is notable that the sum of the categories of sperm with damaged plasma membrane and high mitochondrial membrane potential was superior in summer. Elevated environmental temperatures are likely to impair the plasma membrane and the mitochondrial function of post-thawed sperm, but there is a lack of consensus among the studies. Seasonal alterations of membrane lipids composition are believed to be due environmental stress, such as high temperatures (Argov-Argaman et al., 2013).

A study conducted by Malama et al. (2017) in Friesian bulls raised in a region of Mediterranean climate showed that the effect of heat stress during the hot season on sperm quality was variable among bulls, as some bulls presented fewer cells with intact membranes and high mitochondrial membrane potential in summer, whereas others did it in winter. Another study conducted by Valeanu et al. (2015) found no

significant seasonal differences in mitochondrial membrane potential in taurine bulls raised in a temperate climate. Moreover, changings in mitochondrial membrane potential can be attributed to other factors. For instance, Reis et al. (2014) suggested that the mitochondrial membrane potential is dependent on the manganese concentration in the diet, which is directly associated with pasture quality throughout the seasons as well as to mineral supplementation.

Table 4. Viability of post-thaw sperm assessed in summer and in winter by dual- and triple-staining in Angus and Crioulo Lageano bulls

	Summer		Winter	
	Angus	Crioulo Lageano	Angus	Crioulo Lageano
IMIA (%)	39.00±16.39	37.83±17.12	37.58±13.91	38.00±11.80
IM (%)	40.42±11.37	45.75±20.74	39.75±11.91	38.57±15.54
JC1a (%)	41.67±16.18	41.42±19.07	42.33±12.31	41.43±11.39
JC1b (%)	8.50±16.10	6.83±9.81	17.50±11.6	10.29±8.20
JC1c (%)	19.25±14.85	15.00±8.68	19.67±7.92	18.00±11.94
JC1e (%)	25.83±12.8a	24.50±13.89a	16.00±12.00b	24.71±12.43a
JC1f (%)	4.75±4.67b	12.17±16.71a	4.33b±4.58b	5.57±5.71b
Intact chromatin (%)	97.25±2.14	97.67±1.83	98.58±1.00	97.33±3.23

IMIA: Intact plasma membrane and acrosome; IM: Intact plasma membrane; JC-1a: Intact acrosome, intact plasma membrane and high mitochondrial membrane potential. JC-1b: Damaged acrosome, damaged plasma membrane and low mitochondrial membrane potential. JC-1c: Intact acrosome, damaged plasma membrane and low mitochondrial membrane potential. JC-1e: Intact acrosome, damaged plasma membrane and high mitochondrial membrane potential. JC-1f: Damaged acrosome, damaged plasma membrane and high mitochondrial membrane potential. Means with different subscripts in the row are significantly different at $P<0.05$.

Correlations among traits in summer (Table 5) suggest that smaller bulls with lighter coat tend to be less affected by heat stress, consequently, presenting better results in post-thaw semen characteristics.

The percentage of normal cells had a positive correlation with hair coat density, whilst total sperm defects were in the opposite direction. The coat develops an important role in animals' thermoregulation working as an external insulation against solar radiation (Lacetera, 2003).

Table 5. Spearman correlations among semen characteristics (column variables) with external morphology of the animals and environmental features (row variables) in summer.

	Morphological characteristics								Environment		
	BW	HG	SC	TV	STT	SkCl	*a	*b	HD	THI	BGT
VAP	-0.24	-0.28	**-0.43**	**-0.42**	-0.28	**-0.45**	**0.57**	**0.45**	0.40	-0.38	-0.34
VSL	-0.24	-0.24	**-0.42**	-0.37	-0.25	-0.28	**0.40**	0.29	0.30	-0.32	-0.24
VCL	-0.21	-0.23	-0.38	-0.37	-0.27	**-0.49**	**0.62**	**0.53**	0.39	**-0.44**	**-0.41**
Motile	**-0.43**	-0.39	**-0.49**	**-0.50**	**-0.42**	-0.28	0.18	-0.02	0.17	-0.11	-0.05
Rapid	**-0.45**	**-0.44**	**-0.53**	**-0.55**	-0.38	-0.29	0.28	0.08	0.23	-0.19	-0.11
Static	**0.41**	0.33	**0.45**	**0.47**	0.37	0.27	-0.15	0.03	-0.12	0.09	0.06
TD	0.05	-0.07	0.05	-0.02	0.03	0.14	-0.04	-0.06	**-0.43**	0.06	0.03
NC	-0.03	0.08	-0.04	0.04	-0.01	-0.13	0.03	0.04	**0.42**	-0.07	-0.03
IMIA	-0.39	-0.36	-0.45	**-0.44**	-0.18	-0.27	0.15	0.03	-0.02	-0.10	-0.05
IM	**-0.43**	**-0.41**	**-0.51**	**-0.50**	-0.07	-0.21	0.31	0.19	0.12	0.00	0.06
JC1a	-0.39	-0.35	**-0.42**	-0.41	-0.15	-0.16	0.10	-0.01	-0.03	-0.01	0.04

VAP: average path velocity; VSL: straight-line velocity; VCL: curvilinear velocity; TD: Total sperm defects; NC: Normal cells; IMIA: Intact plasma membrane and acrosome; IM: Intact plasma membrane; JC1a: Intact acrosome, intact plasma membrane and high mitochondrial membrane potential. BW: bodyweight, HG: heart girth, SC: scrotal circumference, TV: testicular volume, STT: skin thickness of the testes, SkCl: skin colour, *a: red color intensity (CIELAB), *b: yellow colour intensity (CIELAB), HD: hair coat density. THI: temperature and humidity index; BGT: black globe temperature. Bold text indicates a significant correlation ($P<0.05$).

As mentioned above, smaller sires would perform better in a hot environment, which is due to the greater body surface area for heat loss they have compared with larger sires. Conversely, a larger body size in winter helps to conserve body heat, as the heat loss is slower due the smaller surface area. Therefore, the positive relationship between body measurements with sperm kinetics (Table 6) suggests that larger sires would perform better than smaller ones under coolest conditions. Once the environmental temperatures are below optimum, but still within the thermoneutral zone, there is a cool zone (National Research Council, 1981). The black globe temperature was negatively correlated with the percentage of static cells. In other words, this means that when the thermal sensation is becoming colder more sperm cells tend to be static, but the reason is unclear.

Relative humidity in winter showed a positive correlation with normal cells and was negatively correlated with total sperm defects. Bhakat et al. (2014) found that osmolarity in the seminal plasma and total abnormalities were lowest during the cold-humid season in crossbred bulls. They speculated that variations in osmolality in seminal plasma may be due to fluctuation in body core temperature depending on

environmental temperature. However, it is noteworthy to mention that the relationship between relative humidity and sperm morphology observed herein is likely to be a trend since the effect of season on sperm characteristics could not be seen in this study, besides the fact that air humidity was maintained constant across the seasons.

Table 6. Spearman correlations among semen characteristics (column variables) with external morphology of the animals and environmental features (row variables) in winter.

	Morphological characteristics						Environment			
	BW	HH	BL	HG	*L	*a	ATA	UTA	RHM	BGT
VAP	0.34	**0.66**	**0.43**	0.35	-0.04	-0.04	0.12	0.11	0.12	-0.10
VSL	0.28	**0.64**	0.43	0.29	-0.02	-0.14	-0.01	0.12	0.12	-0.10
VCL	0.33	**0.75**	0.34	0.27	0.13	0.03	0.11	0.12	0.04	-0.16
Motile	0.37	0.29	0.44	**0.43**	-0.43	0.05	-0.04	0.04	0.25	0.34
Rapid	0.36	0.31	**0.51**	**0.43**	**-0.48**	-0.05	0.00	0.08	0.26	0.24
Static	-0.36	-0.22	-0.34	-0.42	0.43	-0.20	-0.08	-0.07	-0.18	**-0.44**
TD	-0.11	0.01	-0.14	-0.11	0.41	-0.22	-0.19	-0.11	**-0.47**	-0.35
NC	0.11	-0.01	0.14	0.11	-0.41	0.22	0.19	0.11	**0.47**	0.35
IMIA	0.02	0.05	0.10	0.10	-0.31	0.16	0.20	0.08	0.20	0.40
IM	0.17	-0.01	0.24	0.25	-0.42	0.20	-0.01	0.03	0.21	0.41
JC1a	-0.04	-0.10	0.07	0.04	-0.33	0.16	-0.01	0.14	0.08	0.41

VAP: average path velocity; VSL: straight-line velocity; VCL: curvilinear velocity; Minor: minor sperm defects; TD: Total sperm defects; NC: Normal cells; IMIA: Intact plasma membrane and acrosome; IM: Intact plasma membrane; JC-1a: Intact acrosome, intact plasma membrane and high mitochondrial membrane potential. BW: bodyweight, HH: hip height, BL: body length, HG: heart girth; *L: brightness, *a: red color intensity, ATA: mean air temperature, UTA: maximum air temperature, RHM: mean relative humidity, BGT: black globe temperature. Bold text indicates a significant correlation ($P<0.05$).

A partial least squares (PLS) regression was performed to reduce the number of dependent and independent variables into a few factors. In summer, the first five factors explained 85.8% of the variance in explanatory variables and 70.8% of the variance in dependent variables, whilst in winter the same number of factors explains 95.4% and 54.0%, respectively.

Analysis of the Correlation loading plot (Figure 3) revealed that, in summer, body length, testicular volume and scrotal circumference were positively correlated with each other and negatively correlated with sperm cell velocity. Temperature humidity index and relative humidity were also in the negative direction, but less correlated than physical measurements. Contrary to expectations, hair coat traits were considered non-important variables in the final model. The positive relationship between body and

testicular measurements was also found in other species (Luz et al., 2013; Islam et al., 2017; Perumal, 2014; Turri et al., 2016).

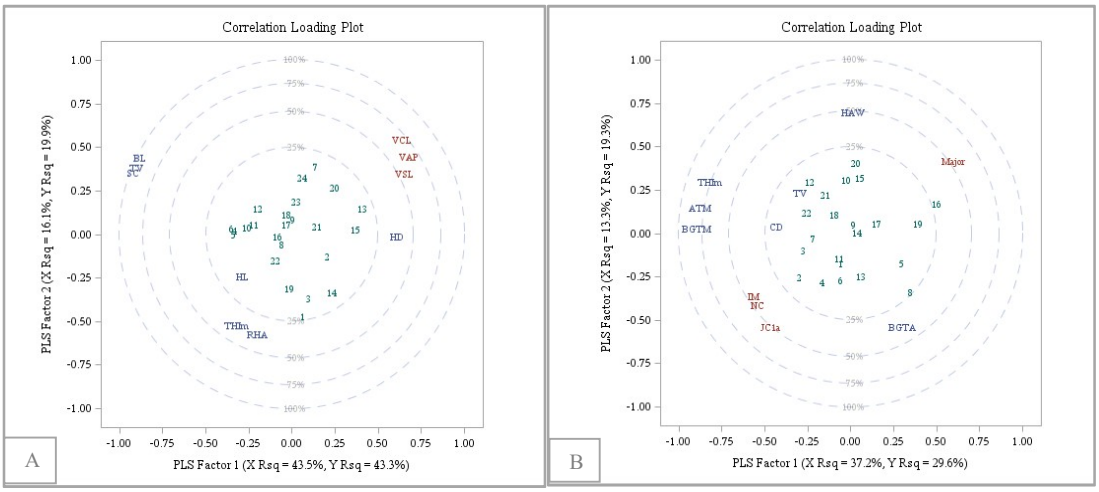

Figure 3: Correlation loading plot for semen traits and environmental traits in summer (A) and in winter (B). VAP: average path velocity; VSL: straight-line velocity; VCL: curvilinear velocity; Major: Major sperm defects; NC: Normal cells; IM: Intact plasma membrane; JC-1a: Intact acrosome, intact plasma membrane and high mitochondrial membrane potential. BL: body length, SC: scrotal circumference, TV: testicular volume; HD: hair coat density; HL: hair coat length; THIm: temperature and humidity index; RHA: relative humidity; ATM: mean air temperature; BGTM: black globe temperature in the morning; BGTA: black globe temperature in the afternoon; CD: chest depth; HAW: height at withers.

The negative relationship found in summer between body and testicular traits with sperm traits is not in agreement with the study conducted by in Brahman crossbreed bulls by Islam et al. (2017) and in buffalo bulls by Kumar & Srivastava (2017). Pastore et al. (2008) found a positive, but very low correlation (r=0.17) between scrotal circumference and sperm motility in Nelore bulls and Pinho et al. (2012) observed no difference in body weight between bulls grouped as sound and unsound for breeding. Another study concluded that body measurements can be used to estimate semen quality in goats (Ambali et al., 2013).

In winter, body measurements did not influence semen traits as they did in summer. Normal cells and percentage of intact membranes were negatively correlated with major sperm defects, which was expected, and they were in the same direction as air temperature, temperature and humidity index and black globe temperature in the morning. The relation among variables in winter must be interpreted differently than in summer. The negative relationship between major defects and the meteorological characteristics during the winter suggests that lower temperatures may be also detrimental to spermatogenesis. In other words, the percentage of sperm defects tend to increases when the environment is getting cooler. Alterations in sperm morphology,

especially the increasing of bent tail defect, are often found after periods of colder weather. Morphology defects appear to be caused by the contact of spermatozoa with abnormal secretions in the epididymis, which suffer changes in its secretion composition influenced by testosterone and any stressors such as weather (Barth & Waldner, 2002; Jelinski et al., 2002; Rojas-Downing et al., 2017). However, except for this alteration, in this study, the coldest temperatures were not sufficient to cause more severe changes in the sperm quality of the bulls.

4. CONCLUSION

Taken together, our results outline that the semen quality of Crioulo Lageano and Angus bulls was similar and satisfactory across the year. In conclusion, meteorological characteristics related to thermal comfort, such as air temperature, black globe temperature and temperature and humidity index (THI) were considered to be the most important in regards to the bulls' seminal condition rather than exterior morphological measurements, i.e., the size of the bulls.

5. REFERENCES

AHMAD, G. et al. Mild induced testicular and epididymal hyperthermia alters sperm chromatin integrity in men. **Fertil. Steril.** v. 97, p. 546–553, 2012.

ALRAGUBI, S. Factors affecting on semen production in Friesian bulls in Libya**. Int. J. Biopharm.** v. 6, p. 32–36, 2015.

ALVARES, C. A. et al. Köppen's climate classification map for Brazil. **Meteorol. Zeitschrif**., v. 22, p. 711–728, 2013.

AMANN, R.P. Can the fertility potential of a seminal sample be predicted accurately**? J. Androl.**, v. 10, p. 89–98, 1989.

AMBALI, A. L.; AKPA, G. N.; SULEIMAN, I. O. Relationships between sperm morphology, semen characteristics, testicular measurements and body conformation traits in red sokoto goat. **I.J.A.B.R.,** v. 3, n. 3, p. 348–353, 2013.

ARGOV-ARGAMAN N. et al. Season-induced variation in lipid composition is associated with semen quality in Holstein bulls. **Reproduction**, v. 145, n. 5, p. 479–489, 2013.

BARTH, A. D.; WALDNER, C. L. Factors affecting breeding soundness classification of beef bulls examined at the Western College of Veterinary Medicine. **Can. Vet. J.,** v. 43, p. 274–84, 2002.

BHAKAT, M. et al. Effect of season on semen quality of crossbred (Karan Fries) bulls. **Adv. Anim. Vet. Sci.**, v. 2, p. 632–637, 2014.

BISHOP-WILLIAMS, K. E. et al. Heat stress related dairy cow mortality during heat waves and control periods in rural Southern Ontario from 2010-2012. **BMC Vet. Res.**, v. 11, p. 291, 2015.

BOE-HANSEN, G. B. et al. Sperm chromatin structure integrity in liquid stored boar semen and its relationships with field fertility. **Theriogenology,** v. 69, p. 728–736, 2008.

BRITO, L. F. C. et al. Testicular thermoregulation in *Bos indicus*, crossbred and *Bos taurus* bulls: relationship with scrotal, testicular vascular cone and testicular morphology, and effects on semen quality. **Theriogenology**, v. 61, p. 511–528, 2004.

CBRA. **Manual para exame andrológico e avaliação de sêmen animal**, 3ª Ed ed., Belo Horizonte: Colégio Brasileiro de Reprodução Animal (CBRA), 2013.

DALCIN, V. C. et al. Physiological parameters for thermal stress in dairy cattle. **Rev. Bras. Zootec.** v. 45, p. 458–465, 2016.

FAO. **The state of the world's animal genetic resources for food and agriculture.** Edited by Barbara Rischkowsky & Dafydd Pilling, Rome, 2007.

FORTES, M. R. S., et al. The integrity of sperm chromatin in young tropical composite bulls. **Theriogenology**, v. 78, p. 326–333, 2012.

FRENEAU, G. E., et al. Puberdade em touros Nelore criados em pasto no Brasil: características corporais, testiculares e seminais e de índice de capacidade andrológica por pontos. **Arq. Bras. Med. Vet. Zootec.**, v. 58, p. 1107–1115, 2006.

GABALDI, S. H.; WOLF, A., 2002. A importância da termorregulação testicular na qualidade do sêmen em touros. **Ciên. Agr. Saúde**, v. 2, n. 2, p. 66–70.

HAFEZ, B., HAFEZ, E. S. **Reproduction in Farm Animals**, 7th Ed. Lippincott Williams & Wilkins, Baltimore, Maryland, USA, 2000.

HAN, Y., PEÑAGARICANO, F.. Unravelling the genomic architecture of bull fertility in Holstein cattle. **BMC Genet.** v. 17, n. 143, p. 1–11, 2016.

INMET. **Metodologia**, in: Andrea Malheiros Ramos, Luiz André Rodrigues dos Santos, L.T.G.F. (Ed.), Normais Climatológicas do Brasil 1961-1990. Instituto Nacional de Meteorologia, Brasília, p. 465, 2009.

ISLAM, M. R. et al. Computer assisted sperm analysis of Brahman crossbred breeding bull semen. **Bang. J Anim. Sci.** v. 46, p. 1–9, 2017.

JANUSKAUSKAS, A.; JOHANNISSON, A.; RODRIGUEZ-MARTINEZ, H. Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure, and field fertility. **Theriogenology**, v. 60, p. 743–758, 2003.

JELINSKI, M. et al. **Understanding bull breeding soundness evaluations**. Vet. Agri-Health Serv, 2002.

KASTELIC, J. P. **Thermoregulation of the testes,** in: Hopper, R.M., editor. Bovine Reproduction. Hoboken: Wiley-Blackwell. p. 26–29, 2013.

KASTELIC, J.P., BYRNE COOK, R., COULTER, G.H. Scrotal/testicular thermoregulation and the effects of increased testicular temperature in the bull. **Vet. Clin. North Am. Food Anim. Pract.** v. 13, p. 271–282, 1997.

KAYA, A., MEMILI, E., 2016. Sperm macromolecules associated with bull fertility. **Anim. Reprod. Sci.**, v. 169, p. 88–94.

KUMAR, S., SRIVASTAVA, S. Testicular biometry and its correlation with body weight and semen output in Murrah bull. **Buffalo Bulletin**. v. 36, n.1, 2017.

LACETERA, N. Interactions between climate and animal production. **Wageningen Academic Publishers**, 2003.

LUZ, P.A.C. et al. The correlation between age, body weight and testicular parameters in Murrah buffalo bulls raised in Brazil. **J. Reprod. Dev.** 59, p. 14–7, 2013.

MALAMA, E. et al. Use of computer-assisted sperm analysis and flow cytometry to detect seasonal variations of bovine semen quality. **Theriogenology,** v. 87, p. 79–90, 2017.

MCGEE, D. A. et al. Effects of summer climatic conditions on body temperature in beef cows. **Nebraska Beef Cattle Reports.** v. 5, 2008.

MENEGASSI, S. R. O. et al. Scrotal infrared digital thermography as a predictor of seasonal effects on sperm traits in Braford bulls. **Int. J. Biometeorol.** v. 59, p. 357–364, 2015.

MERRIAM-WEBSTER. **Environment**. [https://www.merriam-webster.com/dictionary/environment], 2017.

MOREIRA, N. H. et al. Influência sazonal sobre o perfil seminal e congelabilidade do sêmen de touros pantaneiros. **Ciênc. Anim. Bras.**, v. 17, p. 60–69, 2016.

NATIONAL RESEARCH COUNCIL (U.S.). **Committee on Physiological Effects of Environmental Factors on Animals**: **A guide to environmental research on animals**. National Academy of Sciences, 1971.

NATIONAL RESEARCH COUNCIL (U.S.). **Effect of environment on nutrient requirements of domestic animals**. National Academies Press (US), Washington (DC), 1981.

NOAA's NATIONAL WEATHER SERVICE. **Livestock Weather Safety Index**, 1971.

PASTORE, A. A. et al. Características biométricas, testiculares, seminais e parâmetros genéticos de touros pertencentes ao programa de melhoramento genético da raça Nelore. **ARS Vet.,** v. 24, p. 134–141, 2008.

PERUMAL, P. Scrotal circumference and its relationship with testicular growth, age , and body weight in Tho Tho (*Bos indicus*) bulls. **International Scholarly Research Notices**, p.6, 2014.

PINHO, R.O., et al. Testicular echotexture and seminal quality of young Montana Tropical Compound bulls classified as sound and unsound for breeding**. Rev. Bras. Zootec.,** v. 41, p. 1961–1965, 2012.

REECE, W.O. **Body temperature and its regulation,** in: Reece, W.O., Erickson, H.H., Goff, J.P., Uemura, E.E. (Eds.), Dukes' Physiology of Domestic Animals. Wiley-Blackwell, p. 962–973, 2015.

Rego, J.P.A., et al. Seminal plasma protein profiles of ejaculates obtained by internal artificial vagina and electroejaculation in Brahman bulls. **Anim. Reprod. Sci.,** v. 160, p. 126–137, 2015.

REIS, L. S. L. S. et al. Effect of manganese supplementation on the membrane integrity and the mitochondrial potential of the sperm of grazing Nelore bulls. **Anim. Reprod. Sci.,** v. 150, p. 1–6, 2014.

REIS, L. S. L. S., et al. Integrity of the plasma membrane, the acrosomal membrane, and the mitochondrial membrane potential of sperm in Nelore bulls from puberty to sexual maturity**. Arq. Bras. Med. Veterinária e Zootec.,** v. 68, p. 620–628, 2016.

REX, A.S., AAGAARD, J., FEDDER, J. DNA fragmentation in spermatozoa: a historical review. **Andrology**, v. 5, p. 622–630, 2017.

ROJAS-DOWNING, M.M., et al. Climate change and livestock: impacts, adaptation, and mitigation. **Clim. Risk Manag**., v. 16, p. 145–163, 2017.

ROTH, Z. Heat Stress, the follicle, and its enclosed oocyte: mechanisms and potential strategies to improve fertility in dairy cows. **Reprod. Domest. Anim.,** v. 43, p. 238–244., 2008.

ROY, R. et al. Is sperm DNA fragmentation a good marker for fi eld AI bull fertility? **J. Anim. Sci.,** v. 90, n. 1, p. 2437–2449, 2012.

SAS. **SAS/STAT, 14.1 User's Guide the PLS procedure**. 2015.

SILVA, R. G. **Introdução à bioclimatologia animal.** FAPESP, São Paulo, 2000.

SILVA, R. G., MORAIS, D. A. E. F., GUILHERMINO, M. M. Evaluation of thermal stress indexes for dairy cows in tropical regions. **Rev. Bras. Zootec.,** v. 36, p. 1192–1198, 2007.

SOUTO, P. L. G. et al. Reproductive characteristics of Crioulo Lageano breed bulls (*Bos taurus*) at puberty. **Anim. Reprod.,** v. 14, p. 1034–1042, 2017.

SOUTO, P. L. G., SILVA, F.I.C., RAMOS, A.F. Alternative fluorophore combination protocol for simultaneous assessment of integrity of sperm cell membranes and mitochondrial function in bulls. (**Submitted**).

TEIXEIRA, H.C.A. et al. Seasonal influence on semen traits and freezability from locally adapted Curraleiro bulls. **Anim. Reprod. Sci.**, v. 125, 56–61, 2011.

THOM, E.C. The Discomfort Index. **Weatherwise**, v. 12, 57–61, 1959.

TURRI, F. et al. Relationship between body weight, sexual secondary traits and epididymal semen quality in the Alpine goat. **Small. Rumin. Res.,** v. 135, p. 81–84, 2016.

UNANIAN, M. M. **Integridade da cromatina: método complementar para avaliação da qualidade do sêmen bovino**. Brasília: Embrapa Recursos Genéticos e Biotecnologia, (Documentos, 56), v. 21, 2000.

VALEANU, S. et al. Seasonal variation in sperm quality parameters in Swedish red dairy bulls used for artificial insemination. **Livest. Sci.,** v. 173, p. 111–118, 2015.

VAREJÃO–SILVA, M. A. **Meteorologia e climatologia**, 2nd ed. Recife, Brazil, 2006.

VASAN, S. S. Semen analysis and sperm function tests: how much to test? **Indian J. Urol.**, v. 27, p. 41–8, 2011.

WOLD, S. PLS for Multivariate Linear Modeling. **QSAR: Chemometric Methods in Molecular Design. Methods and Principles in Medicinal Chemistry**, 1994.

CAPÍTULO 3

INFLUENCE OF SEASON AND EXTERNAL MORPHOLOGY OF THE ANIMALS ON THERMAL COMFORT AND PHYSIOLOGICAL RESPONSES IN BULLS OF TWO BREEDS ADAPTED TO SUBTROPICAL CLIMATE

ABSTRACT

Homoeothermic animals activate physiological and behavioural responses in an attempt to maintain normothermia and to allow themselves to adapt to changes in environmental conditions. Although thermal discomfort is likely to be more severe in hot climates, in subtropical regions animals are also exposed to periods of thermal stress. The aim of this study was to assess whether season affects thermal comfort and physiological responses of bulls in a subtropical climate. Four Angus and four Crioulo Lageano bulls were used in this study. Measurements of body and testicles metrics, skin pigmentation and thickness, hair number, length and pigmentation, respiratory rate, panting score, rectal temperature and haematological profile were recorded on three occasions in summer and in winter, morning and afternoon. Surface temperature of the flank, eye and scrotum was obtained by infrared thermography. Hair coat colour was determined by CIELAB method and hair density and length were measured manually. Thermal comfort indexes were calculated. Meteorological data, including air temperature, relative humidity and wind speed, were recorded by an automated meteorological station. Statistical analysis included analyses of variance, correlations and a partial least squares regression to determine which traits were the most important in thermal adaptability. Characteristics of the external morphology of the bulls were important for explaining physiological changes in both seasons, but their contribution was greater in summer. Bulls experienced moderate heat stress in summer, which led to significant physiological responses, which were more pronounced in Angus bulls. The main differences in thermal adaptation found between breeds were the hair coat characteristics and respiratory rate. Despite using different mechanisms to cope with environmental challenges, all bulls were able to maintain optimal testicular

thermoregulation as well as systemic normothermia throughout the seasons, showing good adaptation to the climate conditions during the experiment.

Keywords: conservation, reproduction, stress, thermography, thermoregulation

1. INTRODUCTION

The main challenges to be faced in world crop and livestock production by the end of 2050 involve climate and social-economic issues. One hand, it will be necessary an increase in food production by 70% to feed the world, wich is predicted to be one third more populated compared to the present. On the other hand, climate change is likely to be one of the major risks for long-term food security, as it can also affect the production and survival of animals and crops around the world hence jeopardizing biodiversity (FAO, 2009; Hansen et al., 2012). More extreme weather and climate events, such as the unusually hot summers and occurrence of heat waves above three standard deviations of the historical average are being seen, causing a significant number of in human and in livestock deaths in some regions (Azhar et al., 2014; Bishop-Williams et al., 2015; IPCC, 2014; Trenberth et al., 2012). Usually, there are options available to mitigate thermal discomfort in farming systems but they are quite expensive, especially in continent-sized countries, such as Brazil (FAO, 2009; McManus et al., 2016; Rojas-Downing et al., 2017).

Livestock products provide approximately 33% of the protein consumed globally and climate change may also reduce the average carcass weight. This could contribute to changes in the criteria for animal selection as part of the breeding strategies in the future, as environmental conditions in the world are changing faster than species can adapt by natural selection. Thus, the inclusion of individuals which are well adapted to the climate in genetic improvement programs should be considered in order to guarantee sustainability of livestock systems (FAO, 2007; Nardone et al., 2010; Sodhi & Ehrlich, 2010; Thornton, 2010). There are several benefits that come from animal adaptation, among them animal well-being improvement, as individuals are able to cope better with stressful factors around them; reduction of costs in livestock chain that comes from the

need to modify the environment to manage thermal stress; enhancement of forage resource utilization and land conservation, as adapted breeds are more adapted to grazing low quality and/or native forage than the highly productive breeds, hence contributing to environment conservation; and, the assurance of food security, as well-adapted animals are resilient to environment variations (Hohenboken et al., 2005). Nonetheless, choosing well-adapted animals means taking into account some lack of productivity (Sejian et al., 2015). Furthermore, finding a good equilibrium between adaptability and productivity is complex. Thermoregulatory mechanisms, for instance, are likely to be different between breeds due the presence of genes for many adaptive features found in certain breeds to the detriment of others (Hansen, 2004; Olson et al., 2003; Pereira et al., 2008).

Homeothermic animals have a thermoneutral zone, which is a range where the minimum metabolic rate is maintained and heat production is relatively constant to regulate the core body temperature (Renaudeau et al., 2012). When the environmental conditions reach values that are outside of the limit of the thermoneutral zone of the animal a condition known as thermal stress occurs, which in turn prompts behavioural and physiological responses in an attempt to preserve homeostasis (Bernabucci et al., 2010). Ruminant physiology is undoubtedly affected by thermal stress and its magnitude can be quantified through formulating discomfort indexes and by measuring physiological traits. Studies about the effects of the climate on physiological responses in cattle have been reported (Cardoso et al., 2015; Dalcin et al., 2016) but few information on locally adapted cattle and how they perform in comparison with commercial exotic breeds are available (Cardoso et al., 2016; McManus, Castanheira, et al., 2011). Further, most of the locally adapted breeds are endangered however they are important genetic resources as they have a range of unique characteristics and also could contribute with their genes for maintenance of the biodiversity in the livestock sector (FAO, 2007).

The aim of this study was to investigate the seasonal effect of climate and morphology of the animals on thermal comfort and physiological responses in bulls from a locally adapted cattle breed, Crioulo Lageano, in comparison with bulls from an exotic commercial breed, Angus, both raised in southern Brazil.

2. MATERIAL AND METHODS

The experiment followed the bioethics norms in animal experimentation and was approved by the Internal Technical Committee of Brazilian Agricultural Research Corporation – Embrapa Genetic Resources and Biotechnology/006-2013.

2.1 Experimental site and animals

The experiment was conducted during the summer and winter of 2016 in the Southern region of Brazil (27º 48' 58" S, 50º 19' 34" W), where the mean elevation is 884 m above sea level. Eight clinically healthy bulls, four Angus and four Crioulo Lageano, aged from 2 to 6 years and reproductively active, were judged as sound for breeding by previous breeding soundness evaluation. Bulls were raised in a bovine reproduction centre, under the same environmental conditions and handling.

Crioulo Lageano is a horned breed and evolved to thrive in Southern grassland biomes, known as Pampas, a subtropical region in Brazil, whereas Angus is a polled breed evolved in Northeastern Scotland, a temperate region. All bulls in this study were born and raised in the same region where the experiment was performed.

2.2 Meteorological traits

Average, maximum and minimum values for wind speed (km/h), air temperature (°C) and relative humidity (%) were recorded by an automated

meteorological station of the National Institute of Meteorology - INMET in summer and in winter. Mean radiant temperature of the environment was measure using a black globe, which consists of a matt black painted hollow copper sphere of approximated diameter 15 cm, containing a thermometer bulb fixed at the centre of the sphere, without source of heat. The measurements were recorded three times in different days in each season. The temperature humidity index was calculated in the morning and in the afternoon following Thom's formula:

$$THI = (0.8 \times Ta) + (RH/100) \times (Ta - 14.4) + 46.4$$

Where THI is temperature humidity index, Ta is air temperature (°C) and RH is the air relative humidity (%). Another formula was also used to calculate the temperature and humidity index according to National Research Council's formula:

$$THI_{NRC} = (1.8 \times Ta + 32) - (0.55 - 0.0055 \times RH) \times (1.8 \times Ta - 26)$$

Where THI_{NRC} is the NRC temperature humidity index, Ta is air temperature (°C) and RH is relative humidity (%).The Livestock Weather Safety Index uses THI to classify stress level into three categories, as follows: THI ≤ 78 are considered as "no stress", THI from 79 to 83 are considered as "danger stress level" and THI ≥ 84 are considered as "emergency stress level" (LCI, 1970).

2.3 Physiological traits and thermal comfort indexes

Bulls were led to a squeeze chute at 8:00 am and at 15:00 pm, on three different days in summer and in winter, to measure the physiological traits and thermal comfort indexes. Respiratory rate was measured by counting the thoracoabdominal movements per minute. Rectal temperature (°C) was measured using a veterinary clinical thermometer and panting score (0-4) was assessed following the scoring system demonstrated in Table 1 and used in previous studies (Mader et al., 2006; Mcgee et al., 2008).

Table 1. Cattle panting scoring system, adapted from Kerr (2015)

PS	RR	Description
0	<40	Normal, no panting. Hard to see chest moving.
1	40–70	Slight panting, mouth closed, easy to see chest moving.
2	70–120	Fast panting with drooling or foam from mouth.
2.5	70–120	Same as 2 with occasional open-mouth panting.
3	120–160	Open-mouth panting, some drooling. Extended neck, head up. Threshold for concern if lasts more than two hours.
3.5	120–160	Same as 3 with tongue somewhat extended, significant drooling.
4	>160	Open mouth with tongue fully extended for prolonged periods, significant excessive drooling. Extended neck, head
4.5	Very low to very high	Same as 4 but head down, +/- drooling. Labored abdominal breathing.

PS: panting score, RR: respiratory rate per minute.

Blood samples were collected through jugular venipuncture into vacuum tubes containing 5% EDTA. The samples were cooled and taken to the laboratory for processing in a Cell-Dyn® 3700 automated hematology analyzer. The blood traits analysed included red blood cell count, platelets, haematocrit, haemoglobin, white blood cells counting, lymphocytes, neutrophils, bands, eosinophils, monocytes, basophils, neutrophil:lymphocyte ratio and haematimetric indices of mean corpuscular volume, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration.

The adaptability to thermal environment in animals is estimated mainly by measuring of the respiratory rate and body temperature. However, the need to handling the animals to record those data can prompt to physiological responses to stress and the interpretation of the results may be impaired. Alternatively, the body surface temperature measured by infrared thermography can be useful as it avoid the handling of the animals (Cardoso et al., 2016). Surface temperatures of the bull's flank, eye and of scrotum (the difference between the line 1 and 2, Figure 1-C, produced the top-to-bottom temperature gradient of the testicle) were measured by infrared thermography (FLIR T420-Series® system) and analyzed using FLIR Tools® software (version 5.6.16078.1002). The calibration parameters used was the emissivity and reflected temperature, morning and afternoon. The setting for emissivity was 0.98 for all the images. The reflector method used to measure the reflected temperature was a

crumpled and re-flattened piece of aluminum foil, photographed by the infrared camera as described by Usamentiaga et al. (2014).

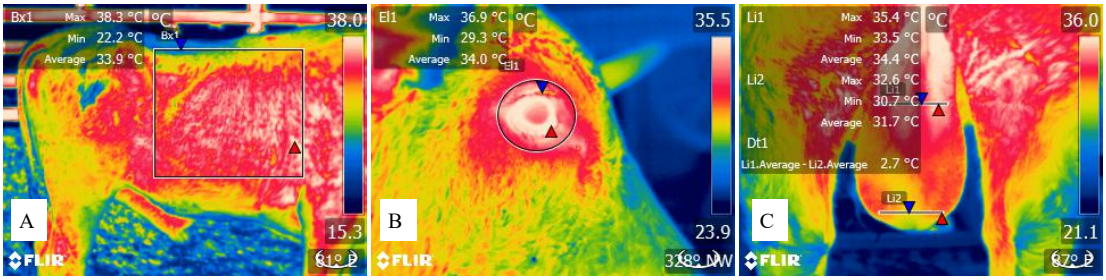

Figure 1. Analysis of surface temperature of the body, in flank region (A), eye (B) and testes (C) by infrared thermography.

Thermal comfort indexes were calculated using the following formulas:

$$Iberia's\ index = 100 - [18(RT - 38.33)]$$

Where, 100 = maximum efficiency in maintaining body temperature below 38.33°C; 18 = constant; RT = mean final rectal temperature; 38.3°C = normal mean rectal temperature for cattle. Value closer to 100 indicates better adaptation.

$$Benezra's\ index = RT/38.33 + (RR/23)$$

Where, RT = rectal temperature (°C); RR = respiratory rate per minute; 38.33°C = normal mean rectal temperature for cattle; 23 = normal mean respiratory rate for cattle. The values being close to two signify that animals are better adapted.

Despite Brazil being a tropical country, in the Southern region where this study was held the winter is usually colder than other regions. Indices for heat stress have been the subject of more studies than those for cold stress. To better understand whether the bulls had experienced cold stress we used the wind chill index (WCI), which is an index that helps to evaluate cold stress occurrence and intensity. Wind chill represent the 'feels like' temperature and is expressed in temperature-like units. WCI formula was adapted by Tucker et al. (2007) to be used in cold stress research in cattle, as follows:

$$WCI = 13.12 + 0.62 \times Ta - 13.17 \times (WS)\text{^}0.16 + 0.40 \times Ta \times (WS)\text{^}0.16$$

Where Ta is the air temperature (°C) and WS is the wind speed (km/h).

2.4 Morphological traits

Bulls were morphologically characterized by measuring body, testes and coat traits. The body traits were the height at withers, hip height, body length, heart girth, chest depth and cannon bone girth, measured by a hipometer and a tape, and body weight.

The formula $TV = 2 \times \left[\left({}^{TW}/_{2}\right)^{2} \times 3.14 \times (TL)\right]$ was used for calculate the testicular volume, where, TV is the testicular volume, TW is the testicular width (cm) and TL is the testicular length (cm). Scrotal circumference was measured by a tape whereas TW, TL and skin thickness of the scrotum were measured by a caliper.

Skin thickness of the body was measured by an adipometer and skin colour was visually determined using a standard scale for bovine skin pigmentation according method describe by Silva (2000). Hair coat colour was measured in triplicate by a spectrophotometer using the CIELAB colour system, which determines the coordinates: *L (brightness), *a (red colour intensity) and *b (yellow colour intensity).

To determine the hair coat density and hair coat length, hair samples were taken from the flank area, 30 cm below the spinal column, using adapted pliers and all hair were counted to give a numbers of hairs per cm^2. The longest ten hairs were measured in each sample using graph paper (Silva, 2000).

2.5 Statistical analyses

Statistical analyses were performed using the Statistical Analysis System® package (v.9.3, SAS Inc., Cary, NC, USA). Multivariate analysis of variance - MANOVA was carried out to evaluate the effect of season and breed on thermal comfort indexes, body and testicular morphology, on hair coat measurements and on the physiological responses. Spearman correlations (PROC CORR) were estimated among all variables. Variables that did not present significant correlations with any other traits were removed from the data set and the original number of variables was reduced. After this first reduction, the model remained with a higher number of variables than the number of observations recorded and because a standard multivariate analysis does not fit well in this context, a partial least squares regression model (PLS) was chosen due to its capability to maximize explanation of both *x* and *y* variables, even with a small

sample size and non-normal data, which are characteristics of this data set (Hair Jr et al., 2014). A PLS (PROC PLS) was used to study the relationships between one or several response variables (*y*) (physiological data) with independent variables (*x*) (climatic data and morphologic measurements) by creating latent variables, also known as factors. This technique solves problems related to high dimensionality of the model and to multicollinearity, i.e., when independent variables are highly correlated with each other (Vinzi et al., 2010), as is the case in this study. An initial model including season and breed effects was tested as it was supposed to give more power to the model, however, the effect of breed became noise into the model and season effect did not add any useful information to the analysis.

The criteria used for dropping the variables from the PLS model were based on the examination of Variable Importance for Projection (VIP) and on the patterns and positioning of the variables in the Correlation loading plot. The VIP in PLS procedure is displayed as a variable importance plot that illustrates the contribution of each variable in fitting the PLS model. According Wold's criterion a value less than 0.8 is considered to be "small", which means that the variable is largely uncorrelated with the *y* response, and indicates that the variable should be removed from the model (SAS, 2015). Further, another strategy used to optimize the model was checking the squared correlation coefficient, R^2, among variables. When variables had a very high correlation with another and if they supply redundant information based on their biological function, one of them was removed (Todeschini & Consonni, 2000; Varmuza et al., 2013). After these analysis 14 physical traits, 6 physiological traits and 5 meteorological traits remained in the final models.

3. RESULTS AND DISCUSSION

Regardless of the season, all values in the afternoon were higher than in the morning, which was expected. Except for relative humidity, all traits changed significantly between seasons (Table 2). The THI values in summer indicated that there was moderate heat stress, achieving the 'danger zone' in some periods, but in winter the index was classified as being normal, according Livestock Weather Safety Index (LCI, 1970). Wind speed changed significantly between summer (mean±standard deviation of 9.54±4.83; ranging from 0.72 to 24.12 km/h) and winter (mean±standard deviation of 12.95±7.88; ranging from 1.44 to 33.48 km/h). As expected, there was no cold stress in winter considering the threshold of temperatures below -10°C for beef cattle, according the EFSA Panel on Animal Health and Welfare (AHAW, 2012). In this study the weather conditions between seasons were quite distinct ($P<0.05$) with periods of thermal discomfort in summer and some days of frost in winter.

Despite Brazil being mainly a tropical country, in the Southern region where this study was held, the winter usually is colder than in other regions. Indices for heat stress have been the subject of more studies than those for cold stress. To better understand if the bulls had experienced cold stress, we used the wind chill index (WCI), which is an index that helps to evaluate cold stress occurrence and intensity. The wind chill index (WCI) in winter was 5.38±6.44, with a minimum value of -7.97 and a maximum of 16.15. In humans this index is used to estimate the risk of frostbite and hypothermia (Environment Canada, 2014), but for cattle no risk classes in terms of WCI have been validated. Based on the Environment Canada's WCI risk classes, the WCI

observed in this study is considered to be low for the risk of frostbite, but an increase of thermal discomfort could be expected.

Table 2. Meteorological features and temperature and humidity index in the morning and in afternoon during the experimental periods

	Summer		Winter		
Morning	Mean	StdDev	Mean	StdDev	P-value
Air temperature (°C)					
Average mean	19.48	0.88	8.79	5.76	<.0001
Average high	20.27	1.17	8.20	5.83	<.0001
Average low	19.50	0.90	7.41	5.89	<.0001
Relative humidity (%)					
Average mean	92.63	1.82	91.68	5.14	0.4008
Average high	89.92	2.24	89.42	6.32	0.7164
Average low	93.13	0.92	93.08	4.61	0.9655
Black globe temperature	23.58	2.35	5.26	2.53	<.0001
THI	72.15	2.24	55.64	7.45	<.0001
Afternoon	Mean	StdDev	Mean	StdDev	P-value
Air temperature (°C)					
Average mean	24.36	2.14	13.83	4.57	<.0001
Average high	26.45	2.13	14.15	4.34	<.0001
Average low	24.83	2.02	12.49	4.46	<.0001
Relative humidity (%)					
Average mean	72.03	8.71	66.89	15.97	0.1727
Average high	71.71	7.05	71.01	14.18	0.8309
Average low	61.92	8.62	62.18	18.15	0.9490
Black globe temperature	34.81	4.28	20.04	6.00	<.0001
THI	81.81	4.79	66.86	5.50	<.0001

*StdDev: standard deviation; THI: Temperature and humidity index following Thom's formula.

Significant differences were detected for both effects of breed, season and their interaction on morphological traits (Table 3). The bulls in this study had a body condition score (BCS) of 4.13±0.69. In general, Angus bulls were heavier, but shorter than Crioulo Lageano bulls. There were no differences in heart girth-body weight ratio between breeds neither summer (Angus, 0.27±0.06; Crioulo Lageano, 0.32±0.04) nor winter (Angus, 0.31±0.04; Crioulo Lageano, 0.32±0.06), which means that the animals had similar body surface area. Lighter animals usually have larger

relative body surface area (Maia et al., 2008), but probably due to the difference in height between breeds they were similar in this measurement.

Angus bulls had a significantly darker and longer hair coat, with lower reflectance than Crioulo Lageano bulls in both seasons. Studies have reported the existence of a gene that produces a very short, sleek hair coat in *Bos taurus* cattle and indicated that the gene locus is most likely on bovine chromosome 20 (Mariasegaram et al., 2007; Olson et al., 2003). The authors stated that slicked-haired animals have greater heat tolerance. In this study, only Crioulo Lageano bulls presented this type of hair, although, in winter their hair was longer than in summer, but still significantly shorter than Angus hair.

Hair coat density was similar between breeds in summer but in winter it was higher in Angus bulls. Both breeds have dark skin, with Angus being slightly darker than Crioulo Lageano. Heat exchange between the animal and environment depends upon body surface area, as well as the hair coat and skin properties. Animals with a small number of hair fibres per unit area are more likely to exchange thermal energy with the environment than those that have high-density hair coats (Bianchini et al., 2006; Maia et al., 2008; Silva & Maia, 2011).

The higher coat reflectance, lighter coloured and shorter hair found in Crioulo Lageano bulls certainly favour heat losses by conduction, convection and radiation and these could be important thermoregulatory mechanisms for this breed. However, despite that morphological difference between breeds, no differences were recorded neither in skin surface temperatures, as found in sheep by McManus et al. (2011), nor in core body temperatures, which indicate that Angus bulls are likely to use other mechanisms to maintain homeostasis, such as evaporative losses by respiration and sweating. Animals that have dark skin and/or coat tend to have greater heat absorption, and may be more susceptible to heat discomfort, but has being stated that this is compensated by an increased heat loss by cutaneous evaporation (Silva & Maia, 2011). Campos Maia et al. (2008) found that the main way of latent heat dissipation in Holstein cows in a tropical environment is the cutaneous evaporation and they suggested that as much larger is the relative body surface area, larger is the heat losses by evaporation.

Table 3. Multivariate Analysis of Variance - MANOVA Test Criteria and Exact F Statistics for the Hypothesis of No Overall season, breed and season*breed Effects for morphological traits of Crioulo Lageano and Angus bulls raised in Southern of Brazil

Effect	Statistic	Value	F Value	DF	Den DF	Pr > F
Season	Wilks' Lambda	0.13	14.83	13	30	<.0001
	Pillai's Trace	0.87	14.83	13	30	<.0001
	Hotelling-Lawley Trace	6.42	14.83	13	30	<.0001
	Roy's Greatest Root	6.42	14.83	13	30	<.0001
Breed	Wilks' Lambda	0.10	21.85	13	30	<.0001
	Pillai's Trace	0.90	21.85	13	30	<.0001
	Hotelling-Lawley Trace	9.47	21.85	13	30	<.0001
	Roy's Greatest Root	9.47	21.85	13	30	<.0001
Season*Breed	Wilks' Lambda	0.27	6.11	13	30	<.0001
	Pillai's Trace	0.73	6.11	13	30	<.0001
	Hotelling-Lawley Trace	2.65	6.11	13	30	<.0001
	Roy's Greatest Root	2.65	6.11	13	30	<.0001

F Value: F statistic for the given predictor and test statistic; DF: number of degrees of freedom in the model; Den DF: number of degrees of freedom associated with the model errors; Pr> F: p-value associated with the F statistic.

In general, the physiological responses of the bulls were similar (Table 4). Increasing respiratory rate and hence panting in summer were expected as evaporative cooling, by evaporation of water from the mucous membranes of the respiratory tissues, is one of the first mechanisms to dissipation of heat (Horowitz, 2001). In cattle, panting score and salivary secretion increase together in order to promote cooling by evaporation (Reece, 2015). In this study, it was noticed that salivation was more intense in Angus bulls, especially in summer afternoon than in Crioulo Lageano bulls. Nonetheless, with regard to the Benezra index, no differences were found between breeds and the highest values were found in summer afternoon. The Iberia index was significantly different in summer morning and winter afternoon, but the differences were only found in one of the periods of the day and considering that this index did not show strong correlations with the physiological measurements, lead us to assume that Iberia index was not a good adaptability indicator to be used in this study. A Benezra index above 2 during summer indicated that bulls experienced some thermal discomfort and had a low degree of adaptation to heat stress (Benezra, 1954). In buffalo bulls, on the contrary, the general average for Benezra's index throughout the year was 2.06±0.15 which indicates good adaptation to the environment (Barros et al., 2015) .

Table 4. Mean±standard deviation of physiological traits and thermal comfort indexes per breed in the morning and in the afternoon during the experimental periods

	Summer		Winter	
Variable	Angus	Crioulo Lageano	Angus	Crioulo Lageano
		Morning		
Rectal temperature (ºC)	38.49±0.62	38.44±0.27	38.14±0.52	37.17±0.81
Panting score (0-4)	1.92a±0.67a	1.08±0.29ab	0.67±0.65bc	0.08±0.29c
Respiratory rate	49.67±5.77a	30.00±6.27b	9.17±2.69c	5.75±2.45d
Benezra' index	3.16±0.25a	2.31±0.27ab	1.39±0.12b	1.22±0.12b
Body ST (ºC)	35.01±2.67a	33.83±1.09a	27.77±5.85b	27.40±5.87b
Eye ST (ºC)	34.58±1.50a	33.84±1.33a	31.99±1.49b	30.92±1.76b
Scrotal ST (ºC)	32.36±0.84a	32.08±1.36a	28.99±1.1ab	27.54±3.09b
Iberia's index	85.69±12.19c	94.54±6.79b	100.24±7.71ab	105.64±15.73a
GSM (ºC)	1.71±1.21c	2.30±0.94c	7.24±3.22a	3.92±1.47b
		Afternoon		
Rectal temperature (ºC)	39.13±0.68	38.63±0.38	38.32±0.43	38.02±0.87
Panting score (0-4)	2.33±0.78a	1.5±0.52a	0.50±0.67b	0.08±0.29b
Respiratory rate	66.00±19.63a	33.33±8.41b	9.58±3.03c	5.92±2.64d
Benezra's index	3.89±0.86a	2.46±0.36ab	1.42±0.14bc	1.25±0.13c
Body ST (ºC)	36.17±2.78a	35.94±2.35a	26.53±6.05b	27.72±5.64b
Eye ST (ºC)	36.10±1.21a	35.85±1.24a	33.07±1.9b	32.10±1.64b
Scrotal ST (ºC)	33.45±1.04	33.38±1.60	30.51±1.26	30.05±2.72
Iberia' index	97.09±11.11b	97.99±4.94b	103.39±9.45b	120.94±14.57a
GSA (ºC)	1.08±1.07c	0.97±0.70c	5.88±1.98a	3.02±1.67b

GSM: Gradient temperature from the top to the bottom of the scrotum in the morning. GSA: Gradient temperature from the top to the bottom of the scrotum in the afternoon. ST: surface temperature. Means with different subscripts in the row are significantly different at $P<0.05$.

Body surface temperature and eye surface temperature were similar for both breeds within the same season, but they were significantly lower in winter. These seasonal differences were also found by Menegassi et al. (2015) in a study with Bradford bulls. When the surface temperature exceeds 35ºC it is more difficult for cows to dissipate heat and maintain homeostasis (Bernabucci et al., 2010). In summer, the body surface temperature reached that threshold, but the core body temperature remained constant throughout the year for all bulls. Body surface temperature directly influences heat flow and the closer it is to the core body temperature the faster tends to be the heat loss from the core to the extremities (Randall et al., 2000). Angus presented higher respiratory rates, panting scores, and salivation than Crioulo Lageano bulls. In 14% of the collections, respiratory rate in Angus bulls was above the reference value (Reece et al., 2015) and sometimes open-mouth panting was observed, whereas in

Crioulo Lageano this did not occur at any time. However, despite these differences both breeds were able to maintain a normal rectal temperature even during the hottest periods of the day. This indicates that the breeds possibly use different mechanisms to increase thermolysis.

As well as physiological mechanisms, mammals also use behavioural and morphological changes to either accumulate or dissipate heat. The fact that Crioulo Lageano is a horned breed give some information about the lower values found in the respiratory measurements in comparison with Angus bulls. In horned species, the horn's vascularized bony core also contributes to the heat loss by vasodilatation elicited by heat exposure which leads to an evaporative heat loss. But in winter, this characteristic may represent an important expenditure of energy (Picard et al., 1994). Taking this into account, apparently the Crioulo Lageano bulls did not need to increase heat loss by respiration as much as Angus needed and the dissipation of heat through the horn´s surface is likely to have importance for their thermoregulation, as recognised in other horned ruminants (Picard et al., 1996; Taylor, 1966).

Testicular thermoregulation is regulated by a complex of processes independently of core body temperature regulation (Kastelic et al., 1997). However, extreme environmental temperatures may affect proper thermoregulation and lead to a decrease in semen quality and even to testicular degeneration (Alragubi, 2015; Newton et al., 2009). Scrotal surface temperatures in this study were constant across seasons in the afternoon. In the morning, a significant difference was only found in the Crioulo Lageano bulls, but this was within the physiological range. Moreover, the top-to-bottom temperature gradient of the scrotum was positive in both periods of the day and across the year, with the highest values in winter. This indicates that testicular thermoregulation of the bulls occurred normally in both seasons. This positive top-to-bottom gradients are expected under physiological conditions due the temperature on the top of the scrotum, the closest point of the scrotum in relation to the body, must be higher than in the bottom of the scrotum. Statistical differences between breeds in the top-to-bottom gradients were only observed in winter, whilst in summer it remained unchanged. Nevertheless, the variation in Angus bulls was greater than in Crioulo Lageano bulls and is likely that for this reason no significant differences were found. Brito et al. (2004) also found a positive top-to-bottom gradient in a study that included *Bos indicus*, crossbred and *Bos taurus* bulls, and the temperatures recorded from the top, middle and bottom of the scrotum differed among these three genetic groups.

Significant differences were only seen in blood traits for bands, haemoglobin (HB) and haematocrit (HT) (Table 5). Angus bulls presented higher values for bands in summer in comparison with winter, whilst for Crioulo Lageano bulls it did not change significantly. HB and HT were significantly different in winter and the higher values were found in the Crioulo Lageano bulls. Despite this, they were within the reference values for the species (Doornenbal et al., 1988; Jackson & Cockcroft, 2002). Significant increases in HB and HT concentrations occur when the white blood cell count (WBC) and neutrophil count increase by two to three-fold, which characterizes a stress leukogram. In this study, the WBC remained within the normal values in both seasons. Another situation in that HT can raise is by splenic contraction and from RBC swelling that occurs after the sample is collected, but both cases are artifacts or errors (Weiss & Wardrop, 2010).

Table 5. Complete blood count in Crioulo Lageano and Angus bulls in summer and in winter

	Summer		Winter	
	Angus	Crioulo Lageano	Angus	Crioulo Lageano
WBC ($\times10^3/\mu L$)	10.27±2.97	8.31±1.91	9.80±1.21	9.40±2.49
Bands ($\times10^3/\mu L$)	0.36±0.21a	0.26±0.12ab	0.08±0.07c	0.14±0.11bc
Neut ($\times10^3/\mu L$)	3.22±0.88	3.21±0.82	2.59±0.40	2.77±0.85
Eosi ($\times10^3/\mu L$)	0.99±0.51	0.68±0.36	1.25±0.47	0.82±0.32
Lymph	4.89±2.54	3.59±1.15	5.24±1.11	4.99±2.06
Mono	0.81±0.36	0.58±0.20	0.65±0.20	0.68±0.30
RBC ($\times10^6/\mu L$)	7.27±0.75	7.98±0.62	7.25±0.53	8.00±0.75
HB (g/dL)	12.77±1.31ab	13.24±0.97ab	11.83±1.96b	13.86±0.68a
HT (%)	36.90±3.80ab	38.59±2.88a	33.48±5.48b	39.55±2.02a
MCV (fL)	51.01±4.92	48.59±4.77	46.23±6.97	49.63±2.78
PLT ($\times10^6/\mu L$)	0.26±0.12	0.24±0.14	0.30±0.13	0.22±0.12
N/L	0.75±0.28	0.96±0.29	0.51±0.1	0.54±0.18

WBC: white blood cell count, Neut: neutrophils, Eosi: eosinophils, Lymph: lymphocytes, Mono: monocytes, RBC: erythrocytes, HB: haemoglobin, HT: haematocrit, MCV: mean corpuscular volume, PLT: platelets, N/L: neutrophil: lymphocyte ratio.

In the white blood cell count, only bands increased significantly in summer. The absolute values of neutrophils and N/L were higher in summer than in winter, but without significant differences. These numeric changes were noted in all leukocyte values, but they were within the normal range for the species. The increase in

RT accompanied of an increase in white blood cells may be explained because of the role of the immune system in protecting against environmental stressors. This relationship was also observed in sheep by McManus et al. (2009). When atmospheric air becomes warmer, rectal temperature tends to increase and this induces physiological responses including secretion of catecholamines in an attempt to maintain core body temperature (Brenner et al., 1998). These hormones are released in the face of excitement, fear or any other stressful factor and incite demargination of neutrophils from the marginal pool into the circulating pool, which boosts the white blood cell count. In general, in an excitement leukogram, i.e., a physiological leukogram, mature neutrophils are the main cells observed and usually the counting return to normal values in up to 30 minutes after the stimuli. A different profile is observed in a stress leukogram, which is characterized by an increase of neutrophils, decrease of lymphocytes and eosinophils. In this experiment, the leukograms were similar to excitement rather than stress, but the mean values of the cells categories were maintained within the reference values. Further, in bovines, a marked increase in the N/L ratio, often greater than 1.0, is observed in stress conditions (Weiss & Wardrop, 2010). In this study, some values for N/L ratio were above 1.0 during summer, whilst in winter this did not occur. Nonetheless, the moderate stress in summer did was not enough to produce a true stress leukogram, i.e, with neutrophila, eosinopenia, lymphopenia.

Only variables that presented significant correlations and a coefficient magnitude above 0.3 will be discussed here. All environmental traits, except air humidity, presented high positive correlations with haematological traits (bands, neutrophils, mean corpuscular volume (MCV) and neutrophil-lymphocyte ratio), physiological responses (rectal temperature, respiratory rate and panting score), Benezra heat tolerance index and surface temperatures recorded by thermography (eye, body and scrotum). The positive correlation between rectal temperature (RT) and temperature and humidity index (THI) means that the bulls may not be able to maintain core body temperature due to increase on environmental temperature and humidity. In a tropical country, no or negative correlation between RT and THI would be desirable to select animals for breeding programs, especially bulls that are exposed to harsh conditions during the breeding season. As well in this study, a positive correlation was observed in Holstein cows (Dikmen & Hansen, 2009; Rejeb et al., 2009) raised in a temperate region and even in the tropically adapted breeds Curraleiro/Pé-duro, Pantaneiro and

Nelore cattle (Cardoso et al., 2016). These results lead us to assume to find a negative correlation between RT and THI is rare, at least in cattle.

Rectal temperature showed positive correlation with bands, neutrophils and neutrophil-lymphocyte ratio (N/L) and was negatively correlated with haematocrit and haemoglobin. The increase of RT starts thermolysis mechanisms, such as evaporation ones, resulting in loss of body water. Depending on how much water is lost, changes in the haematocrit can be seen due the haemoconcentration by dehydration, which was not the case herein (Thrall et al., 2007).

The increase in the rectal temperature also led to an increase in the respiratory rate, panting score, surface temperatures of the body, eye and scrotum, as well as in Benezra index. A positive correlation between rectal temperature and respiratory rate was also observed in hair sheep by Correa et al. (2013). It is stated that when bovines are in heat stress, respiration corresponds to up to 30% of the evaporative losses; the other 70% are due to cutaneous evaporation (Silva, 2000). In agreement with the present study, Daltro et al. (2017) found the rectal temperature positively correlated with the thermographic measurements in dairy cows. The peripheral vasodilatation is one of the key biophysical thermoregulatory mechanisms that transfer thermal energy from the internal organs to the skin in order to dissipate heat. This increases the surface temperature and, hence augments heat loss by conduction and convection (Silva, 2000).

In addition to the correlation with rectal temperature, there were also a positive correlation between Benezra index with respiratory rate, panting score, surface temperatures measured by infrared thermography, bands, neutrophils, mean corpuscular volume (MCV) of erythrocytes, body weight, heart girth, chest deep and testicular volume. As discussed above, a Benezra index above 2 means heat discomfort (Benezra, 1954) and in these circumstances, thermolysis mechanisms are readily activated. The positive relationship between the heat tolerance index and morphometric measurements was also reported in hair sheep in an experiment held in the Central-West Region of Brazil (Seixas et al., 2017). These correlations indicate that larger animals have poor adaptation to hot environments. Size of the animal is an important factor in heat flow, as larger animals have a relatively lower surface area to dissipate heat (McManus, Castanheira, et al., 2011).

MCV showed a positive correlation with air temperature in both periods of the day. An adjustment in cell size is observed in response to environmental challenges, such as hypoxia or an increase in temperature. In this latter case, the oxygen

demand increases due to the greater metabolic activity observed at higher temperatures which prompts an increase in the total volume of red blood cells to augment the delivery of oxygen to the tissues (Gillooly et al., 2014).

Body and testicular measurements were positively correlated with MCV and negatively correlated with red blood cells counting (RBC). In other words, larger animals tend to have larger MCV and, due larger cells naturally occupy more space, consequently RBC will be lower. A moderated negative correlation between MCV and morphological measurements was also observed in a study with Brazilian cattle by McManus et al. (2011a). The size of blood cells may depend on body mass in mammals, but this is not the rule. Empirical knowledge for mammals support the assumption of invariance of capillary size and flow rate, as well as, of the size of erythrocytes in relation of body mass (Savage et al., 2007). However, there is a lack of consensus about whether the number, size, and metabolic rate of cells change according to body size and the reason for this may be because measuring cell size is notably laborious (Kozłowski et al., 2010). Larger animals have relatively lower average of oxygen consumption than smaller animals, meaning that first ones do not need a higher rate of oxygen supply, thus the diffusion gradient through the capillary in theory may be slower (Schmidt-Neilsen & Larimer, 1958).

A partial least squares regression (PLS) was used in order to explore the influence of weather conditions and morphological traits on the physiological responses. After the PLS procedure was repeated several times and checking both VIP, coefficient value, and the *x/y*-scores plot, the variables that were considered to be more important in explaining the physiological responses of the animals in summer were the following: body weight, height at withers, heart girth, chest depth, testicular volume, skin thickness of the body, coat brightness, coat red color intensity, yellow colour intensity of the hair and hair coat length; and in winter were the following: body length, heart girth, skin thickness of the body, hair coat density, hair coat length, maximum and minimum air temperature. The cut-off value in Figure 2 represents the Variable Importance for Projection (VIP) statistic of Wold (1994) which considers 0.8 as a minimum value for VIP, meaning that variables above this cut-off are those that most contribute in explaining the model.

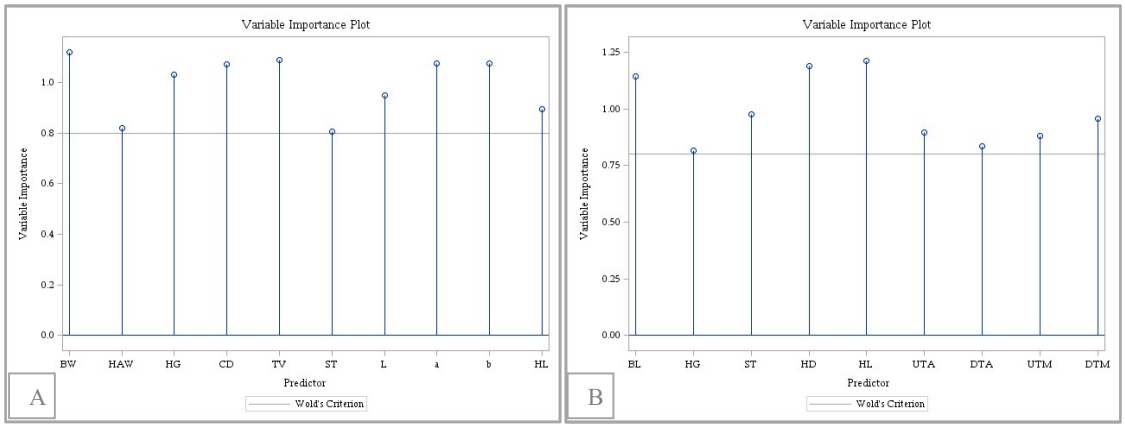

Figure 2. Variable importance plot obtained at the end of partial least squares procedure in summer (A) and in winter (B). BW: body weight, HAW: height at withers, HH: hip height, BL: body length, HG: heart girth, CD: chest depth, TV: testicular volume, ST: skin thickness of the body, L: coat brightness, a: coat red color intensity, b: coat yellow colour intensity, HD: hair coat density, HL: hair coat length, UTM: maximum air temperature in the morning, DTM: minimum air temperature in the morning, UTA: maximum air temperature in the afternoon, DTA: minimum air temperature in the afternoon.

For a good model, the first few factors must present a high correlation between the *x*- and *y*-scores. Figure 3 shows the plot of *x*-scores versus *y*-scores obtained in this study, where a high correlation between the *x*- and *y*-scores for the first and second factors can be seen and somewhat lower correlation for the third factor.

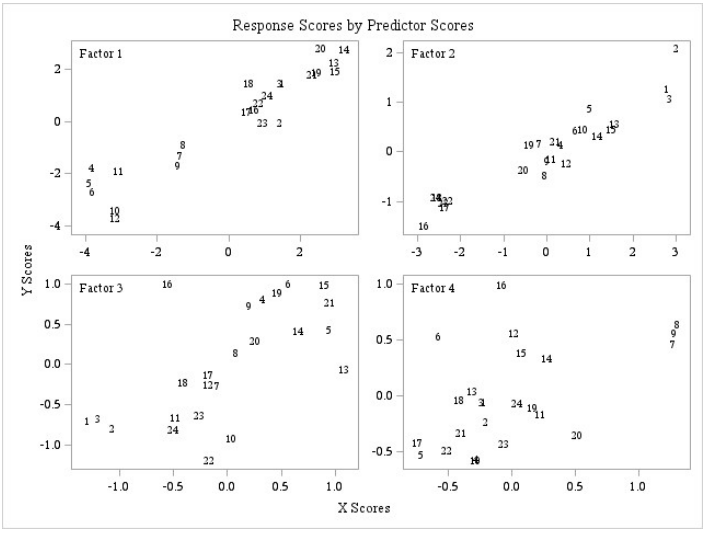

Figure 3. Plot of *x*-scores against *y*-scores obtained by partial least squares procedure

The first three factors explained the majority of the variance in summer and in winter (Table 6 and 7). In summer, these factors explained 94.58% of the variance in explanatory variables and 72.02% of the variance in dependent variables, whilst in winter was 79.89% and 71.35%, respectively.

Table 6. Variation Accounted by Partial Least Squares Factors for summer

Number of extracted factors	Model effects		Dependent variables	
	Current	Total	Current	Total
1	59.22	59.2	57.94	57.94
2	30.85	90.0	10.73	68.68
3	4.52	94.5	3.34	72.02
4	3.37	97.9	0.92	72.94
5	0.72	98.6	3.71	76.65
6	1.11	99.7	1.54	78.18
7	0.12	99.9	4.39	82.57
8	0.08	99.9	0.62	83.19
9	0.01	99.9	1.36	84.55
10	0.01	100	0.69	85.24

Table 7. Variation Accounted by Partial Least Squares Factors for winter

Number of extracted factors	Model effects		Dependent variables	
	Current	Total	Current	Total
1	43.45	43.45	63.96	63.96
2	29.42	72.87	3.62	67.58
3	7.02	79.89	3.77	71.35
4	12.58	92.46	0.89	72.24
5	4.12	96.58	0.66	72.90
6	1.09	97.67	0.27	73.17
7	2.32	99.98	0.03	73.20
8	0.01	99.99	3.33	76.52
9	0.01	100	0.22	76.75
-	-	-	-	-

In summer, there was a high positive relationship among body and testicular measurements with MCV, hair length, Benezra index and panting scores, whereas all these traits were negatively correlated with RBC, hair coat colour and somewhat lower correlated with skin thickness. All bulls presented highest Benezra index in summer differing significantly from the index in winter. A high Benezra index and fast panting indicate that an animal is less adapted (Benezra, 1954; Silva, 2000). In winter, coat colour was not important unlike in summer, the hair coat density and hair length were highly positive associated with each other and both were in the same direction than the cooler air temperatures recorded in the morning. The results for coat traits in winter are in agreement with others studies in cattle that found that coat and skin traits explained little variation in physiological parameters (Cardoso et al., 2016; McManus, Castanheira, et al., 2011), but this is not true for the summer in this study.

The positive relationship between physical traits and MCV found in summer could be explained by the following cascade of events. As mentioned above, larger animals are more vulnerable to heat stress, which causes hyperventilation – observed in this study – following by alkalosis respiratory status and an increase of stress hormones in circulation. Stress hormones, such as cortisol, stimulates reticulocytes releasing from the bone marrow reflecting on a high MCV, as these cells are larger than mature erythrocytes (Cockcroft, 2015; Dalcin et al., 2016; Mairbäurl, 2013; Weiss & Wardrop, 2010).

In summary, on the one hand, in summer, a smaller animal, with light and short hair is better adapted to the region studied. It is known that body size is

related to adaptation in different environments and can affect physiological traits and heat tolerance (Barendse, 2017; Cardoso et al., 2015; Rojas-Downing et al., 2017). On the other hand, an animal with dense and long hair coat appear to be more desirable for the winter. In terms of coat characteristics, the Crioulo Lageano bulls were seem to be better adapted to warm conditions whilst the Angus bulls are designed for cooler temperatures. Nevertheless, both breeds were able to maintain the body core temperature across the seasons, even experiencing moderate heat stress in summer.

4. CONCLUSION

In conclusion, characteristics of the external morphology of the bulls were important for explaining physiological changes in both seasons, but their contribution was greater in summer than in winter.

The main differences in thermal adaptation found between Angus and Crioulo Lageano bulls were in the hair coat characteristics, which favoured Crioulo Lageano in summer; and in the respiratory rate, which was likely to play an important role for the thermoregulation in Angus. Our results suggest that the bulls temporarily experienced moderate heat stress that prompted significant physiological changes, but in Crioulo Lageano they were less pronounced. Despite using different mechanisms for heat loss, it was clear that all bulls were able to maintain optimal testicular thermoregulation as well as the body core temperature throughout the seasons, showing good adaptation to the weather conditions in the Southern Brazil where the study was held.

5. REFERENCES

AHAW. Scientific opinion on the welfare of cattle kept for beef production and the welfare in intensive calf farming systems. **The EFSA Journal,** v. 10, n. 5, p. 1–166, 2012.

ALRAGUBI, S. Factors affecting on semen production in Friesian bulls in Libya. **Int. J Biopharm**, v. 6, n. 1, p. 32–36, 2015.

AZHAR, G. S. et al. Heat-related mortality in India: excess all-cause mortality associated with the 2010 Ahmedabad heat wave. **PLoS ONE,** v. 9, n. 9, e109457v, 2014.

BARENDSE, W. Climate adaptation of tropical cattle. **Annu. Rev. Anim. Biosci.,** v. 5, n. 1, p. 133–150, 2017.

BARROS, D. V. et al. Evaluation of thermal comfort, physiological, hematological, and seminal features of buffalo bulls in an artificial insemination station in a tropical environment. **Trop. Anim. Health. Prod.,** v. 47, n. 5, p. 805–813, 2015.

BENEZRA, M. A new index for measuring the adaptability of cattle to tropical conditions. **J Anim. Sci.**, 1954.

BERNABUCCI, U. et al. Metabolic and hormonal acclimation to heat stress in domesticated ruminants. **Animal**, v. 4, n. 7, p. 1167–1183, 2010.

BIANCHINI, E. et al. Características corporais associadas com a adaptação ao calor em bovinos naturalizados brasileiros. **Pesq. Agropec. Bras.**, v. 41, n. 9, p. 1443–1448, 2006.

BISHOP-WILLIAMS, K. E. et al. Heat stress related dairy cow mortality during heat waves and control periods in rural Southern Ontario from 2010-2012. **BMC Vet. Res.,** v. 11, p. 291, 2015.

BRENNER, I. et al. Stress Hormones and the Immunological Responses to Heat and Exercise. **Int. J Sports Med.**, v. 19, n. 2, p. 130–143, 1998.

BRITO, L. F. C. et al. Testicular thermoregulation in Bos indicus, crossbred and Bos taurus bulls: Relationship with scrotal, testicular vascular cone and testicular morphology, and effects on semen quality and sperm production. **Theriogenology**, v. 61, n. 2–3, p. 511–528, 2004.

CARDOSO, C. C. et al. Physiological and thermographic response to heat stress in zebu cattle. **Livest Sci.** v. 182, p. 83–92, 2015.

CARDOSO, C. C. et al. Heat tolerance in curraleiro pe-duro, pantaneiro and nelore cattle using thermographic images. **Animals**, v. 6, n. 2, p. 1–11, 2016.

COCKCROFT, P. D. **Bovine medicine.** 3ed. Chichester, UK: John Wiley & Sons, 2015.

CORREA, M. P. C. et al. Multivariate analysis of heat tolerance characteristics in Santa Inês and crossbred lambs in the Federal District of Brazil. **Trop. Anim. Health Prod.**, v. 45, n. 6, p. 1407–1414, 2013.

DALCIN, V. C. et al. Physiological parameters for thermal stress in dairy cattle. **R Bras. Zootec.**, v. 45, n. 8, p. 458–465, 2016.

DALTRO, D. S. et al. Infrared thermography as a method for evaluating the heat tolerance in dairy cows. **R Bras. Zootec.,** v. 46, n. 5, p. 374–383, 2017.

DIKMEN, S.; HANSEN, P. J. Is the temperature-humidity index the best indicator of heat stress in lactating dairy cows in a subtropical environment? **J Dairy Sci.**, v. 92, n. 1, p. 109–116, 2009.

DOORNENBAL, H.; TONG, A. K. W.; MURRAY, N. L. Reference values of blood parameters in beef cattle of different ages and stages of lactation. **Can. J Vet. Res.,** v. 52, p. 99–105, 1988.

FAO. **How to feed the world in 2050. High-level expert forum.** Anais. Rome: Food and Agriculture Organisation of the United Nations, 2009.

FAO. **The state of the world's Animal Genetic Resources for Food and Agriculture**. Rome: Edited by Barbara Rischkowsky & Dafydd Pilling, 2007.

GORDON, I**. Reproductive technologies in farm animals.** Wallingford, Oxfordshire, UK, CABI, 2004.

HANSEN, J.; SATO, M.; RUEDY, R. Perception of climate change. **PNAS,** v. 109, n. 37, p. E2415–E2423, 2012.

HANSEN, P. J. Physiological and cellular adaptations of zebu cattle to thermal stress. **Anim. Reprod. Sci**., v. 82–83, p. 349–360, 2004.

HOHENBOKEN, W. et al. Genetic improvement of beef cattle adaptation in America. Proceedings of the Beef Improvement Federation's 37th annual research symposium and annual meeting. v. 37. **Anais...**Billings, MT: Beef Improvement Federation, 2005.

HOROWITZ, M. Heat acclimation: phenotypic plasticity and cues to the underlying molecular mechanisms. **J Therm. Biol.**, v. 26, p. 357–363, 2001.

IPCC. **Climate Change 2014: Synthesis Report.** Contribution of Working Groups I, II and III to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (R.K. Pachauri, L.A. Meyer, Eds.). Geneva, Switzerland: Core Writing Team, 2014.

JACKSON, P. G. G.; COCKCROFT, P. D. **Appendix 2: Laboratory Reference Values: Haematology.** In: Clinical Examination of Farm Animals. Oxford, UK: Blackwell Science, p. 302–302, 2002.

KASTELIC, J. P.; BYRNE COOK, R.; COULTER, G. H. Scrotal/testicular thermoregulation and the effects of increased testicular temperature in the bull. **Vet. Clin. North Am. Food Anim. Pract.,** v. 13, n. 2, p. 271–282, 1997.

KERR, S. **Livestock heat stress: recognition, response, and prevention**. Washington State University. Extension Fact Sheet FS157E, Washington, US Pullman, 2015.

KOZŁOWSKI, J. et al. Cell size is positively correlated between different tissues in passerine birds and amphibians, but not necessarily in mammals. **Biol. Lett.,** v. 6, p. 792–796, 2010.

LCI. **Livestock weather safety index chart.** 1970.

MADER, T. L.; DAVIS, M. S.; BROWN-BRANDL, T. Environmental factors influencing heat stress in feedlot cattle. **J Anim. Sci.,** v. 84, n. 3, p. 712–719, 2006.

MAIA, A. S. C.; SILVA, R. G.; LOUREIRO, C. M. B. Latent heat loss of Holstein cows in a tropical environment: a prediction model. **Rev. Bras. Zootec.,** v. 37, n. 10, p. 1837–1843, 2008.

MAIRBÄURL, H. Red blood cells in sports: effects of exercise and training on oxygen supply by red blood cells. **Front. Physiol.,** v. 4, p. 332, 2013.

MARIASEGARAM, M. et al. The slick hair coat locus maps to chromosome 20 in Senepol-derived cattle. **Anim. Genet.**, v. 38, n. 1, p. 54–59, 2007.

MCGEE, D. A. et al. Effects of summer climatic conditions on body temperature in beef cows. **Nebraska Beef Cattle Reports,** v. 5, 2008.

MCMANUS, C. et al. Heat tolerance in Brazilian sheep: Physiological and blood parameters. **Trop. Anim. Health Prod.,** v. 41, n. 1, p. 95–101, 2009.

MCMANUS, C. et al. Skin and coat traits in sheep in Brazil and their relation with heat tolerance. **Trop. Anim. Health Prod.**, v. 43, n. 1, p. 121–126, 2011a.

MCMANUS, C. et al. Use of multivariate analyses for determining heat tolerance in Brazilian cattle. **Trop. Anim. Health Prod.**, v. 43, n. 3, p. 623–630, 2011b.

MCMANUS, C. et al. Dynamics of Cattle Production in Brazil. **PLOS ONE,** v. 11, n. 1, p. e0147138, 2016.

MENEGASSI, S. R. O. et al. Scrotal infrared digital thermography as a predictor of seasonal effects on sperm traits in Braford bulls. **Int. J Biometeorol.,** v. 59, n. 3, p. 357–364, 2015.

NARDONE, A. et al. Effects of climate changes on animal production and sustainability of livestock systems. **Livest. Sci.** v. 130, n. 1–3, p. 57–69, 2010.

NEWTON, L. D. et al. Elevated testicular temperature modulates expression patterns of sperm proteins in Holstein bulls. **Mol. Reprod. Dev.,** v. 76, n. 1, p. 109–118, 2009.

OLSON, T. A. et al. Evidence of a major gene influencing hair length and heat tolerance in *Bos taurus* cattle. **J Anim. Sci.,** v. 81, p. 80–90, 2003.

PEREIRA, A. M. F. et al. Effect of thermal stress on physiological parameters, feed intake and plasma thyroid hormones concentration in Alentejana, Mertolenga, Frisian and Limousine cattle breeds. **Int. J Biometeorol**, v. 52, p. 199–208, 2008.

PICARD, K. et al. Bovid Horns: An important site for heat loss during winter? **J Mammal.,** v. 75, n. 3, p. 710–713, 1994.

PICARD, K.; FESTA-BIANCHET, M.; THOMAS, D. The cost of horniness: Heat loss may counter sexual selection for large horns in temperate bovids. **Écoscience**, v. 3, n. 3, p. 280–284, 1996.

RANDALL, D. et al. **Usando a energia: enfrentando desafios ambientais.** In: Eckert fisiologia animal mecanismos e adaptações. 4ed. Rio de Janeiro: Guanabara Koogan, p. 619–673, 2000.

REECE, W. O. **Body temperature and its regulation**. In: REECE, W. O. et al. (Eds.). Dukes' Physiology of Domestic Animals. 13 Ed., Wiley-Blackwell, p. 962–973, 2015.

REECE, W. O. et al. **Dukes' physiology of domestic animals**. 13 Ed. Oxford, UK: Wiley-Blackwell, 2015.

REJEB, M. et al. A Complex Interrelationship between Rectal Temperature and Dairy Cows' Performance under Heat Stress Conditions. **Open J Anim. Sci.,** v. 6, p. 24–30, 2009.

RENAUDEAU, D. et al. Adaptation to hot climate and strategies to alleviate heat stress in livestock production. **Animal**, v. 6, n. 5, p. 707–728, 2012.

ROJAS-DOWNING, M. M. et al. Climate change and livestock: Impacts, adaptation, and mitigation. **Clim. Risk Manag.,** v. 16, p. 145–163, 2017.

SAS. **SAS/STAT 14.1 User's Guide The PLS Procedure**. Cary, NCSAS Institute Inc., 2015.

SAVAGE, V. M. et al. Scaling of number, size, and metabolic rate of cells with body size in mammals. **Proc. Natl. Acad. Sci.,** v. 104, n. 11, p. 4718–4723, 2007.

SCHMIDT-NEILSEN, K.; LARIMER, J. L. Oxygen dissociation curves of mammalian blood in relation to body size. **Am. J Physiol.,** v. 195, p. 424–428, 1958.

SEIXAS, L. et al. Study on environmental indices and heat tolerance tests in hair sheep. **Trop. Anim. Health Prod.,** v. 49, n. 5, p. 975–982, 2017.

SEJIAN, V. et al. **Climate change impact on livestock: adaptation and mitigation.** New Delhi: Springer, 2015.

SILVA, R. G. **Introdução à bioclimatologia animal**. São Paulo: FAPESP, 2000.

SILVA, R. G.; MAIA, A. S. C. Evaporative cooling and cutaneous surface temperature of Holstein cows in tropical conditions. **R Bras. Zootec.,** v. 40, n. 5, p. 1143–1147, 2011.

SODHI, N. S.; EHRLICH, P. R. **Conservation biology for all.** Oxford: Oxford University Press, 2010.

TAYLOR, C. R. The vascularity and possible thermoregulatory function of the horns in goats. **Physiol. Zool.** v. 39, n. 2, p. 127–139, 1966.

THORNTON, P. K. Livestock production: recent trends, future prospects. **Phil. Trans. R Soc. B.,** v. 365, p. 2853–2867, 2010.

THRALL, M. A. et al. **Hematologia e bioquímica clínica veterinária.** 1ed. São Paulo: Roca, 2007.

TODESCHINI, R.; CONSONNI, V. **Handbook of molecular descriptors**. Wiley-VCH, 2000.

TRENBERTH, K. et al. **Heat waves and climate change: A science update from climate communication**. Report. 2012.

TUCKER, C. B. et al. Effects of shelter and body condition on the behaviour and physiology of dairy cattle in winter. **Appl. Anim. Behav. Sci.,** v. 105, p. 1–13, 2007.

USAMENTIAGA, R. et al. Infrared thermography for temperature measurement and non-destructive testing. **Sensors (Basel),** v. 14, n. 7, p. 12305–48, 2014.

VARMUZA, K.; FILZMOSER, P.; DEHMER, M. Multivariate linear QSPR/QSAR models: Rigorous evaluation of variable selection for PLS. **Comput. Struct. Biotechnol. J.,** v.5, n.6, e201302007, 2013.

VINZI, V. E. et al. **Handbook of partial least squares: concepts, methods and applications.** Berlin, Heidelberg: Springer Handbooks of Computational Statistics, 2010.

WEISS, D. J.; WARDROP, K. J. **Schalm's veterinary hematology.** 6 Ed. Ames, Iowa, USA: Wiley-Blackwell, 2010.

WOLD, S. PLS for Multivariate Linear Modeling. **QSAR: Chemometric Methods in Molecular Design. Methods and Principles in Medicinal Chemistry**, 1994.

CAPÍTULO 4

SEASONAL DIFFERENCES IN SEMINAL PLASMA PROTEINS FROM TWO BOVINE BREEDS ADAPTED TO A SUBTROPICAL CLIMATE

ABSTRACT

This study was designed to evaluate the seasonal expression of seminal plasma proteins from two bovine breeds adapted to a subtropical climate and to investigate the interrelations between these proteins and their associations with the post-thawing sperm characteristics and environment features. Semen samples were obtained three times in summer and three times in winter from four Crioulo Lageano and four Angus bulls. The seminal plasma was obtained by centrifugation and the other portion of the semen was cryopreserved. Seminal plasma proteins were identified by NanoUPLC-MS. Post-thawing assessments of sperm kinetics, morphology and membranes integrity were performed. Environmental data such as air temperature, air humidity and black globe temperature (BGT) were recorded and the temperature humidity index (THI) was calculated in summer and in winter. Results showed that climate data changed significantly between seasons. Although no statistical differences could be observed in semen quality between breeds, it was interesting to note that the protein profiles varied within and between seasons. We concluded that the most important proteins in summer affecting sperm characteristics were TIMP-2, DNase, Clusterin, CFAH and GPx6. TIMP-2 and DNase were increased in Crioulo Lageano in comparison with Angus, whilst Clusterin, CFAH and GPx6 were decreased. To the best of our knowledge, this is the first report of a recently evolved type of glutathione peroxidase, GPx6, in seminal plasma of bovine. In winter, six proteins were considered to be more important, as follows, BSP1, BSP3, CCL2, Sulfhydryl oxidase and TIMP-2. BSP1 and TIMP-2 were decreased whilst BSP3, CCL2 and Sulfhydryl oxidase were found increased in this season in Crioulo Lageano in comparison with Angus. These results contribute to the

knowledge about the physiology of reproductive tract in locally adapted cattle breeds in comparison with commercial breeds.

Keywords: Bull, Gelsolin, Glutathione peroxidase, peptide, proteomics, semen, sperm.

1. INTRODUCTION

Genetic diversity is essential to improve livestock breeds to changing environments and changing demands. Locally adapted breeds are recognized by the Food and Agriculture Organisation of the United Nations - FAO as unique genetic resources, which have adaptive characteristics that meet challenges related to climate change, for instance. However, it is estimated that at least one livestock breed per month is being extinct (FAO, 2007). The existing knowledge about these breeds is still very limited when compared with commercial breeds and the lack of value for the genetic resources is one of the reasons to explain that fact. Crioulo Lageano cattle is a locally adapted breed evolved exclusively by natural selection through the last 400 years in Southern Brazil (Bett et al., 2013), whereas Angus cattle was developed originally during the 19th century in the temperate and oceanic climates of Scotland (RBST, 2015).

Research about reproductive biology is essential to enhance the livestock chain. Performing a large-scale study, Menegassi et al. (2012) found that semen quality and sexual behaviour problems are among the main causes of rejections of beef bulls in breeding soundness evaluation at Southern Brazil. Seasonal variation in bull fertility and semen quality, especially the post-thawing semen, are due to many intrinsic and extrinsic factors in regard to the bulls' organism. Unfavourable environmental conditions, such as heat stress, are important factors involved in this seasonality (Britt, 1988; Hansen, 2004; Orgal et al., 2012; Teixeira et al., 2011).

The male reproductive tract produces a complex secretion called seminal plasma which is the fluid portion of the semen. Besides helping in the spermatozoa transport, it is known that seminal plasma plays a large range of vital roles for

maintenance of the sperm cell functions and metabolism such as motility, capacitation, nutrition and membrane stability (Juyena & Stelletta, 2012; Tvrdá et al., 2013).

Seminal plasma contains minerals, sugars, organic salts, lipids, prostaglandins, proteins and other components produced by the testes, epididymides and accessory sex glands (Druart et al., 2013). Killian et al. (1993) found fertility-associated report, seminal plasma proteins have received each time more attention in studies about bull fertility and semen freezability (Fraser, 2017; Rodríguez-Martínez et al., 2011; Roncoletta et al., 2013; Souto, 2013). Nevertheless, in comparison with the proteome of human seminal plasma, the number of proteins that have been identified in domestic mammalians is little, as well as the knowledge about their role (Druart et al., 2013; Juyena & Stelletta, 2012).

Seminal plasma proteomics is dynamic and varies not only among breeds but also within them and even between semen collections. Period of the year can also influences on proteomic profile of seminal fluid and this is likely to influence on semen quality as well (Chacur, 2012; Krishnan et al., 2017). However, a largely matter of speculation on both inhibitory and stimulatory protein effects remains unknown.

Proteomic studies of semen serve as an important line of inquiry that allows the uncovering of fundamental knowledge about the molecular intricacies of the male reproductive tract, which may be useful for the development of new biotechnologies to be used in animal reproduction. These new tools could improve the quality of stored semen in germplasm banks as well as to optimize the use of sires in reproduction centres, but the first step in this process is the identification and validation of semen quality biomarkers.

Thus, the aim of this study was to analyse the seasonal abundance of proteins in seminal plasma of Crioulo Lageano bulls in comparison with seminal plasma of Angus bulls, as well as, to investigate the interrelations between these proteins and their associations with the post-thawing sperm characteristics and the climate features in summer and in winter.

2. MATERIAL AND METHODS

Procedures herein were approved by the Internal Technical Committee of Brazilian Agricultural Research Corporation – Embrapa Genetic Resources and Biotechnology / 006-2013.

2.1 Animals, semen collection and processing

The experiment was conducted during summer and winter of 2016, in the Southern region of Brazil, Lages-SC, located below the Capricorn Tropic (27º 48' 58" S, 50º 19' 34" W), thus characterized by a humid subtropical climate. The experimental animals, eight clinically healthy bulls, four Angus and four Crioulo Lageano, aged from 2 to 6 years and reproductively active, were born and raised in the same region of the study.

Semen samples were collected in summer and in winter, by electroejaculation, in three different days in each season. Each ejaculated was split into two aliquots, one for obtaining seminal plasma samples (centrifugation, 700 g for 10 min) and the other one for cryopreservation following the method described by (Teixeira et al., 2011). The three seminal plasma samples formed a ‘pool’ for each biological replicate for each bull, giving a total of four biological replicates per breed in each season. Biological replicates were centrifuged at 12000 rpm for 1 hour at 4°C, in order to remove any somatic cell or debris from seminal plasma, and were frozen in liquid nitrogen until further analysis. Post-thawing semen was evaluated in triplicate for sperm motility, average pathway velocity (VAP), straight-line velocity (VSL),

7curvilinear velocity (VCL), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN), percentage of rapid and static cells by Computer Assisted Sperm Analysis (CASA; HTM-IVOS, Hamilton Thorne Research, Beverly, MA, USA); for sperm abnormalities by phase contrast microscopy, following the classification of the Brazilian College of Animal Reproduction (CBRA, 2013) for sperm morphological defects; and for membrane status by fluorescence dye. Two fluorescent probe solutions were prepared daily, one for acrososome assessment (20 µL fluorescein isothiocyanate-conjugated peanut agglutinin-FITC-PNA, 1mg/mL, Sigma-Aldrich®) and another for plasma membrane assessment (20 µL 6-Carboxyfluoresceindiacetate-CFDA, 0.46 mg/mL, Sigma-Aldrich®). The fluorescent probe solutions were prepared separately and added to 10 µL of propideum iodide (0.5 mg/mL, Molecular Probes®), 10 µL of formalin solution (1:80) and 960 µL of 3% sodium citrate. Then, 40 µL of the probe solution were added to 10 µL of semen, incubated for at least 10 minutes in the dark and 200 cells were counted by epifluorescent microscopy (B-2A fluorescence filter cube, Nikon®). Further, a simultaneous evaluation of sperm membranes was performed using 0.5 mg/ml propideum iodide, 1 mg/ml FITC-PNA and 153 µM JC-1 (5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide) according to the method described by (Souto et al., Submitted).

2.2 Environmental data

Throughout the experimental period held in each season, air temperature (°C) and relative humidity (%) were recorded by an automated meteorological station of the National Institute of Meteorology (INMET). The black globe temperature or Mean radiant temperature of the environment, which simulates animals' thermal sensation as it takes radiation into account, was measured at the same environment where the animals were. Further, to estimate the animals' thermal discomfort, the temperature humidity index was calculated following Thom's formula: THI= (0.8Ta) + (RH/100) x (Ta-14.4) + 46.4, where THI is temperature humidity index, Ta is air temperature (°C) and RH is the air relative humidity (%).

2.3 Protein extraction and sample preparation for NanoUPLC-MS^E

Protein extraction from seminal plasma was performed according to Carmo et al. (2017) with modifications. In summary, 750 µL extraction buffer (0.7 M sucrose, 0.5 M Tris-HCl, 30 mM HCl, 50 mM EDTA, 0.1 M KCl, and 40 mM DTT) was added to the same volume of phenol (pH 8.0, equilibrated, Molecular Biology Grade, Ultrapure, Affymetrix/USB) and poured into a tube containing an aliquot of 100 µl of seminal plasma. Afterward, samples were shaken for 15 min at room temperature and centrifuged for 3 min at 13.400 ×g to separate insoluble material for aqueous phase. The aqueous phase, which is the phenolic phase, was carefully recovered, poured into a new tube and precipitation solution (0.1 M ammonium acetate in methanol 100%) was added. The tubes were shaken, incubated overnight at -20ºC and then centrifuged (15 minutes, 12000 rpm, 4ºC) to form the protein pellet. Pellets were washed (3 minutes of centrifugation, 12000 rpm, 4ºC) with 80% cooled acetone, dried for 10 to 20 min at room temperature and prepared for nanoUPLC-MS^E acquisition according to the method describe by Murad & Rech (2012) with modifications.

The pellet was suspended in 60 µL of 50 mM ammonium bicarbonate (NH_4HCO_3) and 25 µL of RapiGEST^{TM} (Waters, USA; 0.2% v/v) was added. The sample was incubated in a digital block heater set at 80°C, under continuous shaking (500 rpm) for 15 min. After a brief centrifugation, 3 µL of 100 mM DTT was added to reduce and prevent the disulfide bonds. The tube was gently homogenised and incubated in the block heater set at 60°C, under continuous shaking (500 rpm) for 30 min, and then, centrifuged again. To alkylate cysteine residues, iodoacetamide (3 µL of a 300 mM solution) was added, the tube was mixed and incubated in the dark at room temperature for 30 min. After that, 2 µg of trypsin (diluted in 10 µL of 50 mM of NH_4HCO_3) was added, the sample was mixed and incubated at 37°C for 19 hours for peptide digestion. Afterward, 10 µL of a 5% TFA solution was added in order to cleave and precipitate the RapiGEST^{TM}, the sample was homogenised, incubated for further 90 min at 37°C and centrifuged at 14000×g at 6°C for 30 min. The supernatant was recovered and the protein content was quantified using a NanoDrop™ spectrophotometer (Thermo Scientific, Waltham, MA, USA). Subsequently, the sample was dried in SpeedVac^{TM} concentrator and suspended in a solution containing 10 µL of 1 pmol/µL ADH (MassPREP^{TM} Digestion Standard Yeast Alcohol Dehydrogenase, ~1

nmol/vial, Waters, USA) and 190 µL of a 200 mM ammonium formate solution. The final volume of 200 µL was transferred to a Waters Total Recovery vial (Waters, USA).

2.4 NanoUPLC-MS^{E} proteomics and bioinformatics analysis

NanoUPLC-MS^{E} (Nano Ultra-performance Liquid Chromatography coupled with mass spectrometry) proteomics was performed using a nanoACQUITY^{TM} system (Waters Corp., USA) equipped with a Symmetry C18 5 µm, 5 mm x 300 µm precolumn and a nanoEase^{TM} BEH130 C18 1.7 µm, 100 µm x 100 mm analytical reversed-phase column (Waters, USA). Each biological sample was run in triplicate following the methods described before (Carmo et al., 2017; Murad & Rech, 2012). The data were analysed using a Mass-Informatics™ platform (ProteinLynx Global Server™ – PLGS, version 2.5, Waters, MS Technologies) and the processed spectra were searched against the non-redundant database of proteins of *Bos taurus* from Uniprot (http://www.uniprot.org/proteomes/UP000009136). Only proteins present in at least seven technical replicates were used, which allowed a final list with proteins present in at least 3 out of 4 biological replicates. Furthermore, results were filtered by PLGS statistics in order to select proteins with a confidence level greater than 95%. Peptides with uncharacterized amino acid sequence were identified using the Basic Local Alignment Search Tool (BLAST) against the knowledge database for *Bos taurus* in UniProt (http://www.uniprot.org/blast/) and in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Functional classifications based on gene ontology were performed using UniProt protein knowledge base and the software Blast2GO (www.blast2go.com) from which suitable graphs were produced. In addition, Microsoft Excel (Microsoft, USA) was used for management of the data.

Protein-protein interactions were predicted using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database version 10.5, using default settings of minimum required interaction score (medium confidence of 0.40) and active interaction sources based on criteria of text mining, experiments, databases, co-expression, neighbourhood, gene fusion and co-occurrence (Szklarczyk et al., 2017).

2.5 Statistical analyses

Statistical analyses were performed using the Statistical Analysis System® package (v.9.3, SAS Inc., Cary, NC, USA). Analysis of variance (PROC GLM, PROC GLIMMIX) was carried out to evaluate the effect of the climate features, breed and season on the sperm characteristics. Exploring the dataset using schematic box plot (PROC BOXPLOT), outlier observations were excluded from the modeling. Spearman correlations (PROC CORR) were carried among all variables. Only variables that had shown significant correlations were maintained on the data set, thus the original number of variables becomes smaller. After this first reduction, the model remained with a larger number of variables than the number of observations recorded. In this scenario an ordinary multivariate analysis could not be applied and that was one of the reasons why a Partial least squares regression model - PLS (PROC PLS) was performed for each season. The model included sperm characteristics, as dependent variables or 'x', and the differentially abundant proteins and climate data, as the explanatory variables or 'y'. Initially, breed effect was considered in the model but it did not remain significant after repeating the analysis. The criteria used for dropping the variables from the PLS model were based on the Variable Importance for Projection (VIP) statistic of Wold (1994), on the examination of the variable patterns in the Correlation loading plot. The VIP is displayed as a variable importance plot for the contribution of each variable in fitting the PLS model and a value less than 0.8 is considered to be "small". Further, when two variables showed a high correlation with each other, one of them was considered to be excluded from the model. PLS regression creates factors combining *'x'* and 'y' variables and allows determining which one explains most of the variance in the model.

3. RESULTS AND DISCUSSION

Air temperature (°C) during experimental period in summer was 21.71±3.14 and in winter was 9.63±5.05, differing significantly between seasons. Air humidity (%) was unchanged (P>0.05), been 81.08±15.29 in summer and 83.92±15.33 in winter. Average temperature and humidity index (THI) in summer was 69.24±3.68, occurring moderate heat stress in the afternoon, whereas in winter the THI was very low, 50.27±8.19.

Despite occurrence of heat stress in summer, its level was not enough to impact upon the post-thawing semen quality of the bulls, which remained unchanged throughout the year for both breeds. Sperm motility (%) for Angus was 50.23±19.84 and 45.54±22.85, whereas for Crioulo Lageano it was 51.70±10.04 and 51.71±16.78, in summer and in winter, respectively. Significant differences were observed only in the average path velocity (VAP), straight-line velocity (VSL) and curvilinear velocity (VCL) in summer (Figure 1). During the winter, only VCL changed significantly, being 115.85±10.38 for Angus and 130.79±9.78 for Crioulo Lageano.

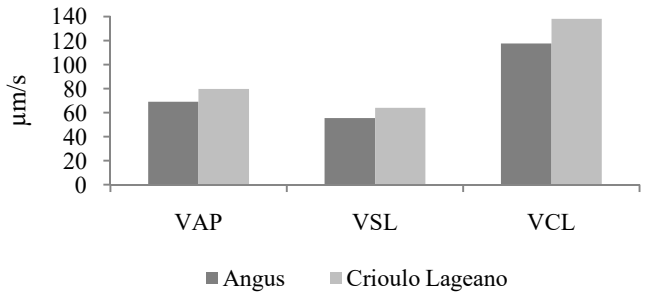

Figure 1: Path velocity (VAP), straight-line velocity (VSL) and curvilinear velocity (VCL) of post-thawing sperm of Angus and Crioulo Lageano bulls in summer. P<0.05

The shotgun proteome analysis performed by nanoUPLC-MS revealed 471 different proteins accession numbers. Only proteins observed in at least 3 biological replicates were considered in order to achieve reliable results, which provided a final list with a total of 49 unique proteins. Differentially abundant proteins were determined by comparison of seminal samples of Crioulo Lageano bulls with those of Angus bulls. As shown in Table 1, in summer, 18 proteins were significantly increased, 17 were decreased and 12 were unchanged, whereas in winter, 15 proteins were increased, 11 were decreased and 22 remained unchanged.

Druart et al. (2013) compared the seminal plasma proteomes of several species by shotgun proteomic analysis and identified 160 proteins in goat buck, 109 proteins in ram, 82 proteins in boar, 59 proteins in stallion, 21 proteins in camel, 10 proteins in alpaca and 89 proteins in bull, which is higher than the number found in this study. Kelly et al. (2006) identified a total of 96 proteins using the two dimensional liquid chromatography proteomic approach and others 55 using SDS-Polyacrylamide gel electrophoresis proteomics.

It was interesting to observe that the proteins Gelsolin and VIP peptides were found exclusively in one of the breeds. Gelsolin was exclusively found in samples of Angus bulls and the Vasoactive Intestinal Polypeptide (VIP), also known as VIP peptides, was observed only in Crioulo Lageano bulls.

Pieces of evidence indicate that gelsolin participates in acquiring of fertilization competence of the spermatozoa. Before sperm capacitation the amount of actin in sperm head is low, increased in capacitated cells, and reduced again after acrosome reaction. Because gelsolin is a calcium-dependent protein, when the calcium concentration elevates during capacitation it is activated, prompting to actin network disruption and the occurrence of acrosome reaction (Cabello-Agüeros et al., 2003; Finkelstein et al., 2010).

Gelsolin was also identified in the cauda epididymal fluid from Holstein bulls (Moura et al., 2010) and in the seminal plasma of Santa Inês rams (Souza et al., 2012). Gelsolin could protect spermatozoa against cytotoxic effects of actin leaked from damaged and dead sperm. Actin is an intracellular protein and when it is released into the extracellular space it polymerizes forming long filaments. Gelsolin is able to shorten actin filaments and to enhances its clearance (Harris & Weeds, 1984; Kevin Li-Chun et al., 2015; Yin & Stossel, 1979).

Table 1. Differentially abundant proteins in seminal plasma samples of Crioulo Lageano bulls when compared to Angus bulls in summer and in winter

UniProt accession	Protein name	Gene ID	Chr	Score	Abundance		Gene ontology
					Summer	Winter	
ANG1_BOVIN	Angiogenin 1	ANG1	10	2296.48	Decreased	Unchanged	Developmental protein Ⓕ
E1BN79_BOVIN	Carboxylic ester hydrolase	CES5A	18	3202.65	Decreased	Unchanged	Hydrolase activity Ⓕ
CLUS_BOVIN	Clusterin	CLU	8	56562.57	Decreased	Unchanged	Chaperone Ⓕ
CFAH_BOVIN	Complement factor H	CFH	N/A	14440.03	Decreased	Decreased	Complement activation Ⓟ
F1MCF5_BOVIN	Glutathione peroxidase	GPX6	1	5110.78	Decreased	Unchanged	Response to oxidative stress Ⓟ
E1BI74_BOVIN	NAD(P)(+)--arginine ADP-ribosyltransferase	LOC507756	15	3074.78	Decreased	Unchanged	Protein ADP-ribosylation Ⓟ
F1MI46_BOVIN	Osteopontin	SPP1	6	6956.43	Decreased	Increased	Androgen catabolic process Ⓟ
Q3T010_BOVIN	Phosphatidylethanolamine binding protein 4	PEBP4	8	4222.16	Decreased	Decreased	N/A
IPSP_BOVIN	Plasma serine protease inhibitor	SERPINA5	21	40934.04	Decreased	Decreased	Heparin-binding Ⓕ
Q58DP6_BOVIN	Ribonuclease 4	RNASE4	10	11314.21	Decreased	Unchanged	Endonuclease activity Ⓕ
SFP4_BOVIN	Seminal plasma protein BSP 30 kDa	BSP5	18	74231.52	Decreased	Increased	Heparin-binding Ⓕ
ALBU_BOVIN	Serum albumin	ALB	6	6059.63	Decreased	Decreased	Lipid-binding Ⓕ
SPAD1_BOVIN	Spermadhesin 1	SPADH1	26	348438.2	Decreased	Increased	Single fertilization Ⓟ
Q4R0H2_BOVIN	Spermadhesin 2	SPADH2	26	83217.68	Decreased	Increased	Single fertilization Ⓟ
G3N0V0_BOVIN	Immunoglobulin gamma heavy chain	N/A	20	844.38	Decreased	Decreased	Antigen binding Ⓕ
G3MWX7_BOVIN	Predicted: epididymal-specific lipocalin-5	LOC100295548	11	16578.42	Decreased	Unchanged	N/A
E1B9P4_BOVIN	Epididymal sperm binding protein 1	ELSPBP1	18	1158.78	Decreased	Unchanged	Sperm capacitation Ⓟ

F1MJH1_BOVIN	Gelsolin	GSN	8	1144.39	Ae	Ae	Actin filament binding Ⓕ
VIP_BOVIN	VIP peptides	VIP	9	1968.71	CLe	-	Hormone activity Ⓕ
EFNA1_BOVIN	Ephrin A1	EFNA1	3	17343.96	Unchanged	Decreased	Ephrin receptor binding Ⓕ
TRFL_BOVIN	Lactotransferrin	LTF	22	6993.28	Unchanged	Increased	Antibacterial humoral response Ⓟ
PAFA_BOVIN	Platelet activating factor acetylhydrolase	PLA2G7	23	11222.45	Unchanged	Increased	Lipid degradation Ⓟ
Z13_BOVIN	Spermadhesin Z13	SPADH2	26	73949.23	Unchanged	Increased	Single fertilization Ⓟ
F1N3U5_BOVIN	Vanin 2	VNN2	9	6274.14	Unchanged	Increased	Pantetheine hydrolase activity Ⓕ
NGF_BOVIN	Beta nerve growth factor	NGF	3	8567.96	Increased	Increased	Metalloprotease inhibitor Ⓕ
CCL2_BOVIN	C-C motif chemokine 2	CCL2	19	21445.91	Increased	Increased	Chemotaxis Ⓟ
ANFC_BOVIN	C-type natriuretic peptide	NPPC	2	77602.31	Increased	Unchanged	Reproductive process Ⓟ
PYY2_BOVIN	Caltrin	PYY2	19	15643.09	Increased	Decreased	Calmodulin binding Ⓕ
CATL1_BOVIN	Cathepsin L1	CTSL	8	1024	Increased	Increased	Protease Ⓕ
F1MGQ1_BOVIN	Deoxyribonuclease gamma	DNASE1L3	22	16249.78	Increased	Unchanged	Endonuclease Ⓕ
F1N430_BOVIN	Metalloproteinase inhibitor 2	TIMP2	19	68521.05	Increased	Decreased	Metalloendopeptidase inhibitor activity Ⓕ
NUCB1_BOVIN	Nucleobindin 1	NUCB1	18	7778.24	Increased	Unchanged	Calcium ion binding Ⓕ
Q0IIH5_BOVIN	Nucleobindin 2	NUCB2	15	1495.03	Increased	Increased	Calcium ion binding Ⓕ
PPIB_BOVIN	Peptidyl-prolyl cis-trans isomerase B	PPIB	10	11381.2	Increased	Unchanged	Chaperone-mediated *ptn folding Ⓟ
SFP3_BOVIN	Seminal plasma protein A3	BSP3	18	5764.99	Increased	Increased	Fertilization Ⓟ
SFP1_BOVIN	Seminal plasma protein PDC 109	BSP1	18	223501.5	Increased	Decreased	Fertilization Ⓟ

RNS_BOVIN	Seminal ribonuclease	SRN	10	27516.01	Increased	Unchanged	Endonuclease Ⓕ
F1MM32_BOVIN	Sulfhydryl oxidase	QSOX1	16	1139.57	Increased	Increased	Flavin-linked sulfhydryl oxidase activity Ⓕ
TFPI2_BOVIN	Tissue factor pathway inhibitor 2	TFPI2	4	13467.3	Increased	Unchanged	Protease inhibitor Ⓕ
E1BLI4_BOVIN	PREDICTED: carbohydrate-binding protein AQN-1 isoform X1	LOC100295703	26	5165.05	Increased	Increased	N/A
F1MZX2_BOVIN	Serpin family E member 2	SERPINE2	2	11413.26	Increased	Unchanged	Seminal vesicle epithelium development Ⓟ
Q3T0Z0_BOVIN	WAP four-disulfide core domain protein 2	WFDC2	13	57812.71	Increased	Decreased	Peptidase inhibitor activity Ⓕ

Ⓕ Molecular function. Ⓟ Biological process. Chr: Chromosome location. CLe: Crioulo Lageano exclusive. Ae: Angus exclusive. *ptn: protein. N/A: not annotated

The organisation of actin filaments at the Sertoli cell-spermatid interface is critical for spermatid transport during spermiation and a mild heat stress can cause disorganisation of the actin cytoskeleton of germ cells, however they could recover and continue to develop (Qian et al., 2014; Rivera et al., 2004). These facts support the idea that Gelsolin found in Angus samples is part of a range of the physiological mechanisms used to maintain a normal spermatogenesis.

Vasoactive intestinal peptide (VIP) identified in Crioulo Lageano samples is a pleiotropic neuropeptide that was found in rats, human and a specie of reptile in the nerves supplying the seminal vesicles and testis, as well as the presence of its receptors was reported in normal prostate of rats, spermatozoa, Leydig cells, spermatids and spermatocytes (Kepper & Keast, 1997; Rosati et al., 2017). In humans VIP participates to penile erection (Hammond, 1960) and stimulates sperm motility (SIOW et al., 1999). Moreover, VIP was reported in cholinergic nerve fibres supplying accessory genital glands in pigs and the authors suggest that it may play a role in the regulation of the secretory function of prostate epithelial cells (Kaleczyc et al., 1999; Klimczuk et al., 2005). Another possible function in which VIP peptides is likely to be involved is in the testosterone production (Rosati et al., 2017).

It is noteworthy to mention that although the differences observed between the two breeds may not necessarily be linked to neither high nor low semen quality, some proteins showed to be more important than others in influencing sperm characteristics in face of environmental challenges. Additionally, some proteins were found to be potentially involved in the response to thermal stress, either to avoid cell damage or as a product of cell injury.

A correlation analysis was performed per season among seminal plasma proteins abundance, sperm characteristics and the climatic data. The results which shown significant and strong correlations were highlighted below and summarized in Tables 2 and 3.

Interesting associations were found with climate data, such as the one in which Gelsolin, exclusively found in Angus, was positively correlated with air temperature, air humidity and THI in winter, besides being negatively correlated with intact membranes (IMIA). In summer, Caltrin showed a negative significant relationship with air temperature and with the discomfort index THI, whislt the protein Ribonuclease 4 was positively correlated with BGTM, variable that represents the thermal sensation experienced by the animals.

Twelve proteins in summer and eight proteins in winter had shown strong and significant correlations with sperm kinetics. Only Serum albumin, Cathepsin L1and Sulfhydryl oxidase presented significant correlations with sperm defects. Ten proteins were correlated significantly with sperm membranes viability in summer whereas in winter this kind of relationship was only detected with Gelsolin.

Initially, some correlations appear to be intriguing because some proteins were positively related to semen quality in one season and the opposite happened in another. However, these results must be interpreted carefully and the fact the protein abundance was different in each season must be taken into account. Moreover, the results of the correlation analysis constituted the basis for the subsequent analysis and the possible reasons for these findings will be discussed below.

Table 2: Main Spearman correlations among seminal plasma proteins, sperm characteristics and environmental data in summer

	Sperm kinetics					Sperm defects	Membranes viability			Environmental data		
	VAP	VSL	VCL	Motile	Rapid	Major	IMIA	IM	JC1a	AT	THI	BGTM
Serum albumin	0.12	-0.17	0.21	-0.48	-0.48	**0.71**	-0.43	-0.38	-0.48	0.43	0.43	-0.04
Cathepsin L1	-0.02	-0.43	0.07	-0.69	-0.60	**0.71**	**-0.90**	-0.57	**-0.81**	-0.12	-0.12	0.23
CFAH	**-0.74**	**-0.86**	-0.60	-0.52	-0.52	0.50	-0.50	-0.67	-0.52	0.12	0.12	0.49
Clusterin	-0.57	**-0.81**	-0.43	-0.69	-0.62	0.64	**-0.71**	**-0.81**	**-0.76**	0.21	0.21	0.46
Glutathione peroxidase	-0.62	**-0.83**	-0.50	**-0.74**	-0.67	0.38	-0.62	**-0.81**	**-0.71**	0.02	0.02	0.41
Deoxyribonuclease	-0.50	**-0.81**	-0.45	**-0.95**	**-0.95**	0.36	**-0.79**	**-0.90**	**-0.86**	-0.02	-0.02	0.49
TIMP 2	-0.40	**-0.71**	-0.36	**-0.86**	**-0.76**	0.36	**-0.90**	**-0.79**	**-0.88**	-0.33	-0.33	0.49
IPSP	**-0.71**	-0.69	-0.64	-0.60	-0.60	0.52	-0.40	-0.67	-0.52	0.29	0.29	0.58
PPIB	-0.17	-0.55	-0.14	**-0.86**	**-0.74**	0.10	**-0.79**	**-0.74**	**-0.83**	-0.36	-0.36	0.22
Caltrin	-0.26	-0.40	-0.29	-0.62	-0.48	-0.05	-0.62	-0.45	-0.60	**-0.71**	**-0.71**	0.35
WAP four-disulfide	-0.33	-0.67	-0.24	**-0.79**	-0.67	0.64	**-0.95**	**-0.76**	**-0.90**	-0.12	-0.12	0.46
Spermadhesin 2	-0.57	**-0.74**	-0.55	**-0.76**	**-0.71**	0.36	**-0.81**	**-0.71**	**-0.76**	-0.33	-0.33	0.67
Ribonuclease 4	**-0.81**	-0.69	**-0.74**	-0.36	-0.36	0.57	-0.43	-0.50	-0.40	0.12	0.12	**0.75**
PDC 109	-0.60	**-0.83**	-0.57	**-0.76**	**-0.76**	0.19	**-0.74**	**-0.86**	**-0.76**	-0.02	-0.02	0.62
Spermadhesin 1	-0.62	**-0.74**	-0.52	-0.62	-0.57	0.64	**-0.74**	-0.62	-0.67	-0.17	-0.17	0.63

VAP: average path velocity, VSL: straight-line velocity, VCL: curvilinear velocity, Motile: percentage of motile sperm, Rapid: percentage of rapid sperm, Major: percentage of major sperm defects, IMIA: percentage of sperm cells with intact plasma membrane and intact acrosome, IM: percentage of sperm cells with intact plasma membrane, JC1a: Intact acrosome, intact plasma membrane and high mitochondrial membrane potential, AT: average air temperature, THI: average temperature and humidity index, BGTM: black-globe temperature in the morning, WAP four-disulfide: WAP four-disulfide core domain protein 2 precursor, PDC 109: Seminal plasma protein PDC 109, PPIB: Peptidyl-prolyl cis-trans isomerase B, TIMP 2: Metalloproteinase inhibitor 2, IPSP: Plasma serine protease inhibitor, CFAH: Complement factor H. Correlation coefficients in bold are significant at 95% of confidence level.

Table 3: Main Spearman correlations among seminal plasma proteins, sperm characteristics and environmental data in winter

	Sperm kinetics				Sperm defects	Membranes viability	Environmental data		
	VAP	VCL	Motile	Rapid	TD	IMIA	AT	AH	THI
PDC 109	0.50	-0.11	0.71	**0.86**	-0.32	0.11	-0.14	0.39	-0.14
Lactotransferrin	0.34	0.38	**0.77**	0.54	-0.67	-0.07	0.13	0.00	0.13
TIMP 2	0.39	-0.21	0.57	**0.82**	-0.29	0.04	-0.07	0.54	-0.07
Sulfhydryl oxidase	0.07	0.14	0.61	0.32	**-0.93**	0.46	-0.46	-0.39	-0.46
Seminal plasma protein A3 (BSP3)	**0.79**	**0.89**	0.61	0.39	-0.18	0.29	-0.54	-0.29	-0.54
C-C motif chemokine 2	**0.79**	0.54	**0.96**	**0.79**	-0.50	0.29	-0.36	-0.07	-0.36
Complement factor H (CFAH)	0.39	-0.25	0.43	**0.79**	-0.04	-0.18	0.21	0.75	0.21
Cathepsin L1	-0.70	**-0.93**	-0.37	-0.22	-0.11	0.00	0.30	0.15	0.30
Gelsolin	-0.27	-0.49	-0.09	0.22	0.18	**-0.80**	**0.76**	**0.80**	**0.76**
Vanin 2	**0.82**	0.54	**0.96**	**0.82**	-0.57	0.39	-0.29	-0.04	-0.29

VAP: average path velocity, VCL: curvilinear velocity, Motile: percentage of motile sperm, Rapid: percentage of rapid sperm, TD: percentage of total sperm defects, IMIA: percentage of sperm cells with intact plasma membrane and intact acrosome, AT: average air temperature, AH: relative air humidity, THI: average temperature and humidity index, PDC 109: Seminal plasma protein PDC 109, TIMP 2: Metalloproteinase inhibitor 2. Correlation coefficients in bold are significant at 95% of confidence level.

Only proteins showing significant correlation coefficients in the correlation analysis further confirmed by multivariate analysis were considered to be the most important to explain the variance in post-thawing sperm characteristics. These proteins will be discussed here.

In summer, the Partial least squares regression (PLS) model explained 96.1% and 92.2% of the variation in the *'x'* and 'y' variables, respectively. In summer, the proteins identified as Clusterin, Complement factor H and Glutathione peroxidase 6 were grouped, as well as, Metalloproteinase inhibitor 2 (TIMP-2) and Deoxyribonuclease gamma showed high correlation between each other. These five proteins showed strong negative relationship with sperm motility, rapid sperm and the category of intact membranes with high mitochondrial potential (JC1a), and also with other variables related to sperm kinetics and viability observed in Spearman correlations (Table 2). In winter, the PLS model explained 95.9% and 84.2% of the variation in the *'x'* and 'y' variables, respectively. In this cooler season, the proteins identified as Seminal plasma protein A3, Seminal plasma protein PDC 109, C-C motif chemokine 2, Metalloproteinase inhibitor 2 (TIMP-2) and Sulfhydryl oxidase showed high positive correlation with sperm kinetics and, additionally, Sulfhydryl oxidase showed a high negative correlation coefficient with total sperm defects. The literature about expression of Sulfhydryl oxidase in the male reproductive tract is very limited. The protein was identified in mice and rat male reproductive tract in high concentrations (Colin Thorpe et al., 2002). In hamster, it was suggested that it may have protective functions against the harmful actions of sulfhydryl groups to the spermatozoa (Chang & Morton, 1975). Bovine studies mentioning this protein in seminal plasma could not be found in the literature used to produce this discussion. The findings of this study suggest that higher concentrations of Sulfhydryl oxidase may be beneficial for sperm function.

Gene ontology of the differentially abundant proteins revealed that most of them were involved in regulatory processes. The biological process named 'single-organism process', accounted for 76.32% in summer and 77.78% in winter, followed by 'metabolic process' (52.63%) and 'regulation of biological process' (47.37%) in summer; and by 'regulation of biological process' (55.56%) and 'multi-organism process' (48.15%) in winter (Figure 2). These proteins were found to be mainly involved in molecular functions such as protein-binding, enzyme regulator activity, ion binding and hydrolase activity, in summer, and in receptor-binding, protein binding and ion binding in winter. The biological process of 'single-organism process' can be any

process linked up to the functioning of integrated living units, such as cells, tissues and organisms, and this term in gene ontology is used for annotation of proteins whose biological process is unknown (EMBL-EBI, 2018). Often, proteins found in seminal plasma have a clear function in other tissues where they also are found, however, their specific role in the seminal fluid remains obscure.

According to gene ontology analysis the protein C-type natriuretic peptide (CNP), which was found increased in summer and unchanged in winter in Crioulo Lageano bulls compared with Angus bulls, is involved in the biological process of ‘reproductive process’. Recent studies had shown that CNP can induce a significant dose-dependent increase in sperm motility and acrosome reaction in human semen (Xia et al., 2016) and induces sperm attraction to oocytes in mouse (Kong et al., 2017). In bovine, a study published in The Annual Scientific Meeting of the Endocrine Society of Australia and the Society for Reproductive Biology suggested that CNP might contribute with sperm motility. Nevertheless, in this study no significant correlation was found with the sperm traits.

A deep look into the gene ontology showed that in the biological process named ‘immune system process’, three proteins (Complement factor H, Angiogenin-1 and Clusterin) were decreased and one (C-C motif chemokine 2) was increased in summer, accounting for 10.53% of the significant proteins, whereas in winter the opposite happened, one protein (Complement factor H) was decreased and three (Platelet activating factor acetylhydrolase, Lactotransferrin and C-C motif chemokine 2) were increased, accounting for 14.81% of the significant proteins. This means that, on the one hand, the defense response in seminal plasma was greater in Crioulo Lageano bulls in winter, but on the other hand, it was more expressive in Angus in summer. It is important to highlight that despite these differences the bulls' semen quality remained the same regardless of the season.

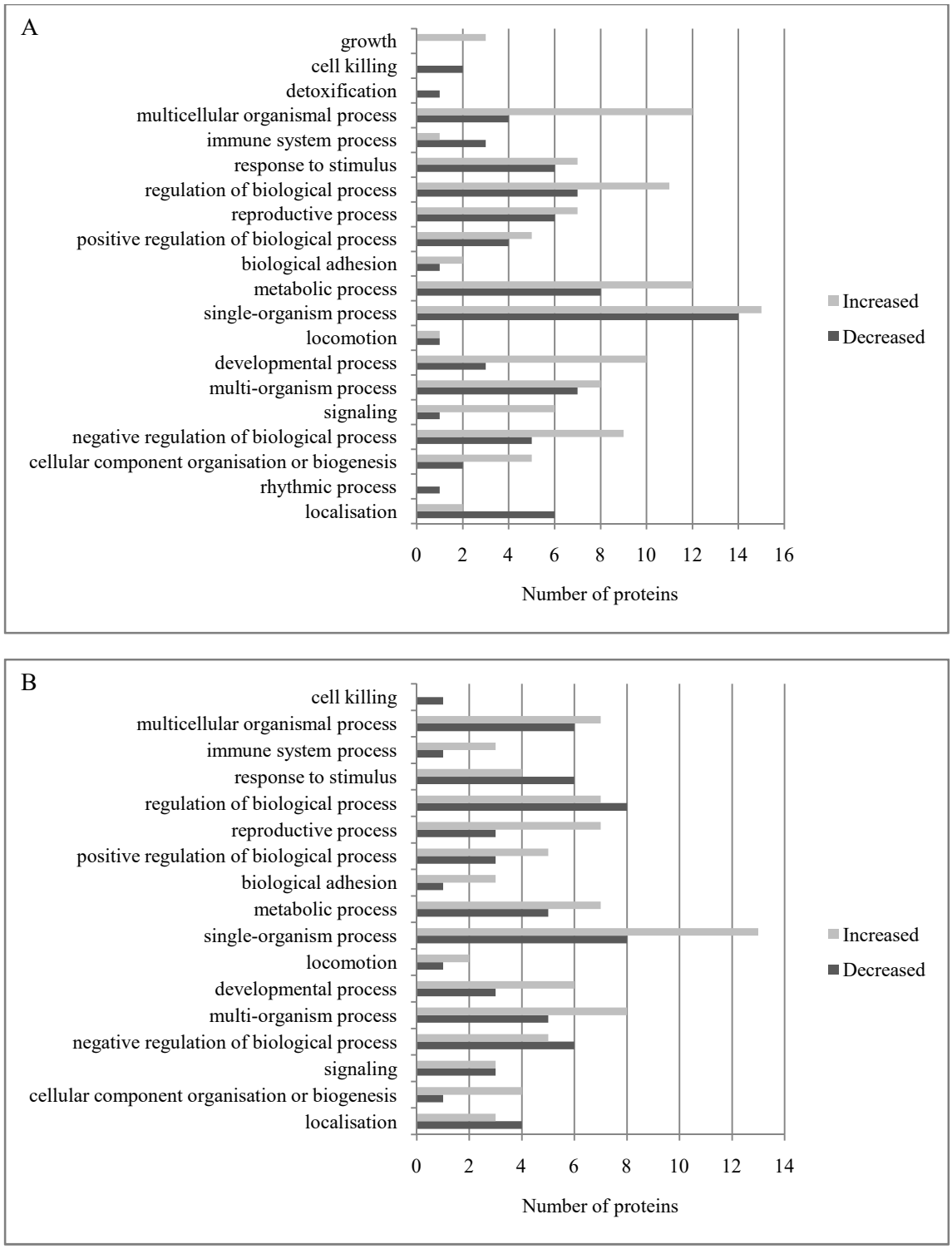

Figure 2: Functional enrichment analysis for "molecular function" of proteins identified in seminal plasma of Crioulo Lageano bulls in comparison with Angus bulls in summer (A) and in winter (B).

Clusterin is one of the main proteins secreted by Sertoli cells and its general functions are related to extracellular chaperone that prevents aggregation of nonnative proteins, maintenance of partially unfolded proteins in a state appropriate for subsequent refolding by other chaperones, such as heat shock protein HSPA8/HSC70

(UniProtKB, 2018). Accumulation of Clusterin is related to poor semen quality due to the protein binding to lipids that are exposed in dead sperm, which in turn prevents oxidative injuries to the other cells (Fallis, 2013; Kumar et al., 2012). Ibrahim et al. (2000) found that all clusterin-positive spermatozoa were abnormal but that not all abnormal spermatozoa were clusterin positive in bull's semen. Interestingly, the negative correlation found in this study between clusterin and intact cells is in accordance with these facts, but there was no difference between breeds in the number of sperm defects even if clusterin was increased in Angus bulls in summer. Although the correlations with sperm defects were not significant at 5%, probably because of the limited number of animals, the positive relationship cited by other studies was observed (Boe-Hansen et al., 2015). Ultimately, in this study clusterin was negatively correlated ($P<0.05$) with sperm kinetics and intact membranes, which was afterward confirmed by the multivariate analysis, and this protein was decreased in summer in Crioulo Lageano bulls whereas in Angus it remained unchanged throughout the seasons. The functional enrichment analysis of this complex protein revealed that it participates in the biological process, such as, 'positive regulation of biological process', 'response to stimulus', 'response to stress' an 'negative regulation of apoptotic process'.

Complement system activation is involved in the selection of the sperm cells in the epididymis, acting in opposition to dead and dying sperm cells, and the system is regulated by proteins that are bound to sperm surface membranes, such as CFAH (Westfalewicz et al., 2017). When CFAH is increased in seminal plasma this is likely to be because there is a high complement activity, either due to the existence of a larger number of damaged sperm or due to a bacterial infection. On the other hand, if CFAH is decreased this may indicate that plenty of cells are in good status and there is no demand for an over-activity of the complement system. Thus, finding this protein either decreased or unchanged in the seminal plasma may be a desirable condition. The results of this study reflect exactly this hypothesis, as CFAH was found decreased in the seminal plasma in both seasons and the semen quality was considered to be satisfactory. Studies in other species also support this reasoning. Sakaue et al. (2010) found that complement activity in female genital tract was 6 to 10-fold stronger than the activity measured in seminal plasma. They also found CFAH in ejaculated sperm membranes in boar, concluding that a higher quantity of CFAH is needed to bind the sperm to protect them against greater complement attack into female ducts, facilitating sperm transport and fertilization (Perez-Patiño et al., 2016; Sakaue et al., 2010).

Although the oxygen is essential to cell metabolism, the products generated by its consumption, called reactive oxygen species (ROS), could be also harmful for the cell itself. Accumulation of ROS such as hydrogen peroxide (H_2O_2) or hydroxyl radical (OH•) will produce serious cell damage, attacking first the lipids and even causing cell death. But under physiological concentrations, it was found that ROS develops key roles in cell responses working as messengers and signaling pathways in response to stress. It is known that the caput epididymis produces ROS in order to complete sperm DNA maturation. Another possible function of ROS in the epididymis is to disrupt any bacterial proliferation there. Glutathione peroxidase is one of the main enzymes that are part of cell mechanisms to keep the balance of ROS by catalysing the conversion of H_2O_2 into a molecule of water (Drevet, 2006). Mammals have eight different types of glutathione peroxidases (GPx) and they can be blocked in three evolutionary groups as GPx1/GPx2, GPx3/GPx5/GPx6 and GPx4/GPx7/GPx8. GPx4 seems to be the most ancient and GPx5/GPx6, the most recently evolved GPx. But, from these eight forms of GPx, five (GPx1-4, GPx6) are characterized as selenoproteins, i.e., they contain selenium in the form of the amino acid selenocysteine and, hence, they have antioxidant properties. Thus, in theory the presence of a GPx in seminal plasma tend to be beneficial to the sperm. Nonetheless, in this study, glutathione peroxidase (GPx6) showed a negative correlation with motile sperm, VSL, IM and JC1a, and was found decreased in summer in Crioulo Lageano compared to Angus bulls. Interestingly, the fact is that the sperm parameters were within the acceptable values required for cryopreserved semen in bulls, according to the Brazilian College of Animal Reproduction (CBRA, 2013).

Several members of the GPx family have been related to playing an important role in spermatogenesis, but the knowledge about the recently evolved GPx6 remains very limited. To the best of our knowledge, GPx6 was not found in bovine seminal plasma before our study.

Considering that GPx5 and GPx6 are part of the same evolutionary group is feasible to compare the results found about GPx5 to better understand the role of GPx6. In boar semen, GPx5 was found negatively correlated with the percentage of sperm membrane integrity and sperm motility (Vilagran et al., 2016). The authors also observed that the low sperm quality group of boars presented higher concentrations of this GPx in seminal plasma than the high sperm quality group. They suggested that lower sperm quality could be more susceptible to damage caused by reactive oxygen

species (ROS) and a higher concentration of GPx could be part of the physiological mechanisms to compensate this. It is important to note that the efficient action of the antioxidants sometimes can be overcome by excessive production of ROS, leading to an inevitable damage of the cell (Beer-Ljubić et al., 2009). A study with younger Simmental bulls found that the oxidative stress in seminal plasma was higher in summer, nevertheless, the content of GPx in the seminal fluid was unchanged among seasons (Majić Balić et al., 2012). Another study identified an epididymal secretory glutathione peroxidase precursor (GPx5) which was increased in morphologically abnormal bovine sperm and related to cellular stress and apoptosis (Shojaei Saadi et al., 2013). In men, GPx *in vitro* activity was negatively correlated with sperm morphology and motility but GPx expression in seminal plasma had no correlation with the sperm traits (Macanovic et al., 2015).

Taking all of this mentioned into account and assuming that glutathione activation is the first cell response to variations in H_2O_2 balance, we hypothesize that the reason why GPx6 was found to be decreased in seminal plasma in summer may be due to the dynamic of ROS production and efficacy to recycle it. Even in the hotter season, when higher production of ROS is expected, the bulls' semen quality did not change. Angus bulls samples were more abundant in GPx6 in comparison with Crioulo Lageano bulls, but they also managed to maintain equal sperm quality. Thus, either the ROS concentration was within the normal / basal physiological boundaries or it was not high enough to disturb homeostasis, the fact is that an increase of GPx was not needed.

Seminal plasma has several types of proteases and their inhibitors that have been described, but the precise roles of these proteins remain unknown (Gurupriya et al., 2014). Metalloproteinase inhibitor 2 (TIMP-2) is a heparin-binding protein associated with process of the ovulation, fertilization and embryonic development (McCauley et al., 2001). That protein was first reported in bull seminal plasma by Calvete et al. (1996) and after by Mortarino et al. (1998). More recently, some authors have stated that metalloproteases such as MMP14, MMP2, MMP9, and their inhibitors may play an important role in spermatogenesis (Ayvazova et al., 2016; Gurupriya, 2014; Newton et al., 2009). Metalloproteinases inhibitors are found in several sites along the male reproductive tract of human (Ayvazova et al., 2016), bovine (McCauley et al., 2001), ram (Souza et al., 2012), among others. Although it is known that TIMP-2 modulates metalloproteinases activities, which are mainly linked with important biological process for the cell, the precise functions and mechanism of action of them in

seminal plasma are not fully understood, and this could be due the fact that metalloproteinases inhibitors are just one part of the complex chain of events regulating these enzymes activities (Kumar et al., 2012; Tentes et al., 2007).

In the present study, TIMP-2 was increased in summer and decreased in winter. TIMP-2 identified in this study share 99.09% of identity of the metalloproteinase inhibitor-2 precursor found in seminal plasma of Brahman bulls by Boe-Hansen et al. (2015). In their study the protein was negatively related with percentage of morphologically normal sperm, which in certain way agree with our results. The functional enrichment analysis reveals that this protein is associated with the biological process of 'metal ion binding', which is because TIMP-2 binds to metalloproteinases (MMPs), which are zinc-dependent enzymes, and inhibits them irreversibly. It worth pointing out that, in general, zinc-binding proteins are responsible for regulation of chromatin condensation, sperm motility and acrosome reaction, hence affecting directly the fertilization process (Mogielnicka-Brzozowska et al., 2011). Interestingly, in summer when TIMP-2 was increased there was a negative correlation with sperm kinetics, whereas in winter, when the protein was decreased it was positively associated with sperm kinetics. Thus, it may suggest that an excess of TIMP-2 expression could be harmful to the sperm cell, however is necessary to confirm this in another study. For a long time, the only thing known about MMPs was that they degrade extracellular matrix (Calvete et al., 1996). MMPs are likely to be involved in cell remodelling processes during spermatogenesis, being important in the sperm morphology, for instance, and during sperm capacitation (Kim et al., 2013). Nevertheless, most of the functions of these proteolytic enzymes in reproduction are currently under investigation.

Deoxyribonuclease or just DNase (DNASE1L3) was increased in summer and also had a negative relationship with variables of good semen quality in this study. DNase activity in semen is reported in several species including chicken (Sato et al., 2003), human (Singer et al., 1983), fish (Lanes et al., 2009), rabbit (Takeshita et al., 1994), mouse (Carballada & Esponda, 2001), bovine (McCauley et al., 1999), among others. Kelly et al. (2006) identified a 84% identical to the N-terminal sequence of a DNase I-like protein in bovine seminal plasma reported by McCauley et al. (1999). McCauley et al. (1999) found a DNase in bovine seminal vesicle and prostate glands similar to a human DNase I-like protein, and they suggest that the protein may play a role in sperm capacitation and/or cell degeneration and could modify the fertility

potential. In human the protein is believed to be produced in epididymis and may participates in the disintegration of DNA of dead cells (Singer et al., 1983). Alghamdi & Foster (2005) suggest that during insemination neutrophils are activated by the presence of sperm, which lead them to extrusion of their nuclear DNA and histones to form neutrophil extracellular traps (NETs). NETs difficult sperm motility and then, seminal DNase degrades those NETs releasing entangled sperm. Nevertheless, there is little knowledge about the correlations between DNase in seminal plasma and sperm measurements.

3.1 Protein-protein interaction analysis

Protein network analysis was also performed (Figure 3) and confirmed that TIMP-2 interacts with MMPs 2, 9 and 14, BSP1 and BSP3. Besides these three MMPs, BSP1 and BSP3, Menezes et al. (2017) also found MMP 16 and BSP5 interacting with TIMP-2, in a study with Curraleiro Pé-duro bulls that used gel-based approach to identify proteins by mass spectrometry (ESI-Q-ToF). MMP 9 also interact with C-C motif chemokine 2 (CCL2) and both are involved in immune system processes. This could affect in certain way the relation between MMP9 and its inhibitor, TIMP-2.

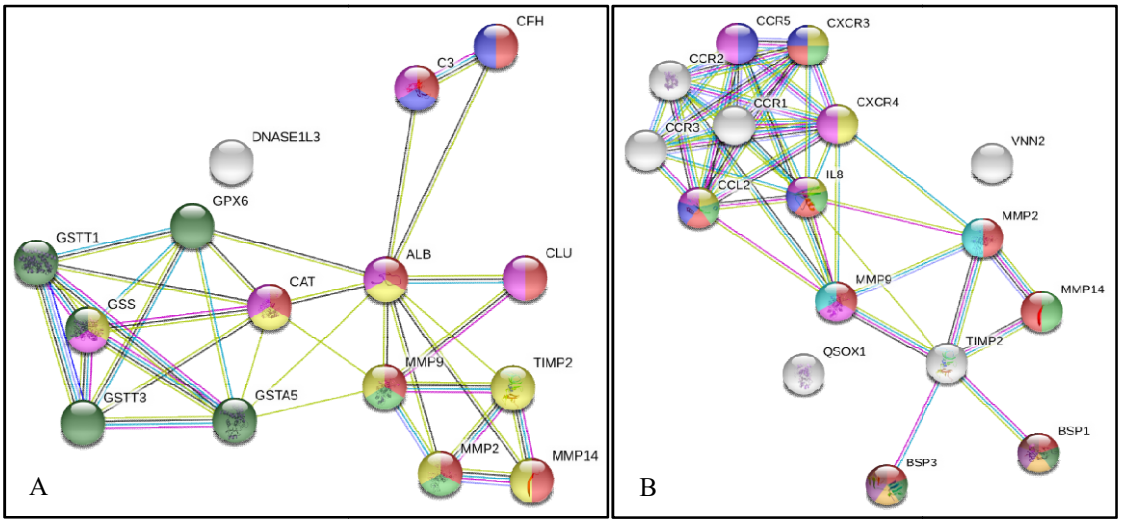

Figure 3: Protein-protein interaction network showing the most important proteins in summer (A) and in winter (B) and their predicted associations. The interaction map was generated using default settings (Medium confidence of 0.40 and the following active interaction sources: neighbourhood, gene fusion, co-occurrence, co-expression, experimental evidences, existing databases and text mining). STRING, 10.5. Important terms to describe the proteins and the colours codes used in these networks are listed in Table 4.

Table 4: Colours of the pathways and proteins used in the network analysis in figure 3

Summer		
Pathway description	Node colour	Matching proteins in the network
Glutathione metabolism		**GPX6**,GSS,GSTA5,GSTT1,GSTT3
Complement activation		C3, **CFH**
Staphylococcus aureus infection		C3, **CFH**
Complement cascades		C3, **CFH**
Collagen catabolic process		MMP2,MMP9
Protein binding		ALB,C3,CAT,**CLU**,GSS
Protein metabolic process		ALB,C3,CAT,**CFH,CLU**,MMP14,MMP2,MMP9
Metal ion binding		ALB,CAT,GSS,MMP14,MMP2,MMP9,**TIMP2**

GPX6: Glutathione peroxidase 6, GSS: Glutathione synthetase, GSTA5: Glutathione S-transferase A5, GSTT1: Glutathione S-transferase theta-1, GSTT3: Glutathione S-transferase theta-3, C3: Complement C3, CFH: Complement factor H, MMP2: 72 kDa type IV collagenase, MMP9: Matrix metalloproteinase-9, MMP14: Matrix metalloproteinase-14, ALB: Serum albumin, CAT: Catalase, CLU: Clusterin, TIMP2: Metalloproteinase inhibitor 2.

Winter		
Regulation of chemotaxis		**CCL2**,CXCR3,CXCR4,IL8
Positive regulation of locomotion		**CCL2**,CXCR3,IL8,MMP14
Multicellular organismal process		**BSP1,BSP3,CCL2**,IL8,MMP14,MMP2,MMP9
Inflammatory response		**CCL2**,CCR5,CXCR3,IL8
Immune system process		**CCL2**,CCR5,CXCR4,IL8,MMP9
Sperm capacitation		**BSP1,BSP3**
Collagen catabolic process		MMP2,MMP9
Spermatid development		**BSP1,BSP3**
Spermatid differentiation		**BSP1,BSP3**
Single fertilization		**BSP1,BSP3**

CCL2: C-C motif chemokine 2, CXCR3/CXCR4: C-X-C chemokine receptor type 3/4, IL8: Interleukin-8, MMP2,MMP9,MMP14: Matrix metalloproteinases-2,9 and 14, BSP1: Seminal plasma protein PDC-109, BSP3: Seminal plasma protein A3, CCR5: C-C chemokine receptor type 5

In winter, BSP3 and CCL2 were increased whilst BSP1 was decreased and these three proteins had strong positive correlations with sperm kinetics. In Curraleiro bulls the intensity of a protein spot identified as BSP3 was also positively associated with sperm motility and intact cells. BSPs in non-capacitated sperm bind to

an oviduct epithelial trisaccharide to form a sperm reservoir, hence they are essential to maintain sperm motility and viability during storage (Ashrafzadeh et al., 2013). Interestingly, BSP1 and BSP3 were associated to the pathways of ‘spermatid development’ and ‘spermatid differentiation’. Information available in the Protein knowledgebase of UniProt database describe that BSP3 is able to either stimulate or inhibit the release of pituitary gonadotropins, but its exact function still not understood.

Ultimately, these results revealed that some proteins were significantly associated with environmental data, but with low intensity, which probably was due to the mild climate with some occasional thermal stress at that subtropical zone. Seasonal changings in bovine semen status and/or its proteins are due to different situations that animals experience in each period of the year, such as, nutrition, climate, parasites, hormones, reproductive season, among others. The precise reasons why some proteins are increased in one season and decreased in another remain inconclusive, but interesting outcomes in this study were found and they enrich the knowledge about seminal plasma proteins in bovine.

The potential of bull’s fertility depends upon the proper functioning of a dynamic universe of biological processes which involves intrinsic and extrinsic factors. Among these factors, are not only the wide range of seminal plasma proteins but also of hormones, immune system components and environmental factors, such as thermal stress, for instance. The present study contemplated some of these aspects and the results underline the complexity of the male reproductive physiology. Additionally, it is important to investigate these proteins by using others OMICS approaches, besides proteomics, as there is a huge amount of information to be understood about their functions.

4. CONCLUSION

In summary, semen quality of Crioulo Lageano and Angus bulls was equal and did not change between summer and winter. Nevertheless, it was evident that protein expression varied significantly when seminal plasma samples of Crioulo Lageano bulls were compared with Angus bulls' samples, as well as, through seasons. In other words, the maintenance of semen quality across the seasons suggests that those differentially abundant proteins found in seminal plasma are likely to be part of a range of mechanisms and biological processes used by each bull to cope with thermal challenges and hence keep homeostasis.

Our results showed that the most important proteins in summer affecting sperm characteristics were TIMP-2, DNase, Clusterin, CFAH and GPx6. To the best of our knowledge, this seems to be the first report of a recently evolved type of glutathione peroxidase, GPx6, in seminal plasma of bovine. In winter, six proteins were considered to be more important, as follows, BSP1, BSP3, CCL2, Sulfhydryl oxidase and TIMP-2.

These proteins must be further investigated to better understand the molecular factors involved in their functions in seminal plasma, as unveiling this knowledge could be useful on the development of new reproductive biotechnologies, such as, the use of these proteins in the development of additives in semen extenders to improve the sperm cryopreservation protocols, which therefore could increase the fertility rate and optimize the use of sires in bovine reproduction centres.

5. REFERENCES

ALGHAMDI, A. S.; FOSTER, D. N. Seminal DNase Frees Spermatozoa Entangled in Neutrophil Extracellular Traps. **Biol. Reprod.,** v. 73, n. 6, p. 1174–1181, 2005.

ASHRAFZADEH, A.; KARSANI, S. A.; NATHAN, S. Mammalian Sperm Fertility Related Proteins. **Int. J Med. Sci,** v. 10, n. 12, p. 1649–1657, 2013.

AYVAZOVA, N. P. et al. Matrix metalloproteinase mmp-2 and mmp-9 activities in seminal plasma. **J Biomed. Clin. Res**., v. 9, n. 2, 2016.

BALIĆ, I. M. et al. Effect of age and environmental factors on semen quality, glutathione peroxidase activity and oxidative parameters in Simmental bulls. **Theriogenology**, v. 78, n. 2, p. 423–31, 2012.

BEER-LJUBIĆ, B. et al. Cholesterol concentration in seminal plasma as a predictive tool for quality semen evaluation. **Theriogenology**, v. 72, n. 8, p. 1132–40, 2009.

BETT, R. et al. Cattle Breeds: Extinction or Quasi-Extant? **Resources**, v. 2, n. 3, p. 335–357, 2013.

BOE-HANSEN, G. B. et al. Seminal plasma proteins and their relationship with percentage of morphologically normal sperm in 2-year-old Brahman (*Bos indicus*) bulls. **Anim. Reprod. Sci.,** v. 162, p. 20–30, 2015.

CABELLO-AGÜEROS, J.F., HERNÁNDEZ-GONZÁLEZ, E.O., MÚJICA, A., 2003. The role of F-actin cytoskeleton-associated gelsolin in the guinea pig capacitation and acrosome reaction. **Cell Motil. Cytoskeleton**, v. 56, p. 94–108.

CALVETE, J. J. et al. A procedure for the large-scale isolation of major bovine seminal plasma proteins. **Protein Expr. Purif**., v. 8, p. 48–56, 1996.

CARBALLADA, R.; ESPONDA, P. Regulation of foreign DNA uptake by mouse spermatozoa. **Exp. Cell Res.,** v. 262, n. 2, p. 104–113, 2001.

CARMO, L. S. T. et al. Plant responses to tomato chlorotic mottle virus: Proteomic view of the resistance mechanisms to a bipartite begomovirus in tomato. **J Proteomics**., v. 151, p. 284–292, 2017.

CBRA. **Manual para exame andrológico e avaliação de sêmen animal.** 3 Ed. Belo Horizonte: Colégio Brasileiro de Reprodução Animal (CBRA), 2013.

CHACUR, M. G. M. Seminal plasma proteins as potential markers of relative fertility in Zebu bulls (*Bos taurus indicus*). **Electrophoresis**, v. 31, n. 2, p. 188–200, 2012.

CHANG, T. S. K.; MORTON, B. Epididymal sulfhydryl oxidase: a sperm-protective enzyme from the male reproductive tract. **Biochem. Biophys. Res. Commun.,** v. 66, n. 1, 1975.

COLIN THORPE et al. Sulfhydryl oxidases: emerging catalysts of protein disulfide bond formation in eukaryotes. **Arch. Biochem. Biophys.**, v. 405, p. 1–12, 2002.

DAVIS, M. T. et al. Automated LC-LC-MS-MS platform using binary ion-exchange and gradient reversed-phase chromatography for improved proteomic analyses. **J Chromatogr B.,** v. 752, n. 2, p. 281–291, 2001.

DOMON, B.; AEBERSOLD, R. Mass spectrometry and protein analysis. **Science**. v. 312, n. 5771, p. 212–217, 2006.

DREVET, J. R. The antioxidant glutathione peroxidase family and spermatozoa: A complex story. **Mol. Cell Endocrinol.**, v. 250, n. 1–2, p. 70–79, 2006.

DRUART, X. et al. Proteomic characterization and cross species comparison of mammalian seminal plasma. **J Proteomics,** v. 91, p. 13–22, 2013.

EMBL-EBI. QuickGO::**Term GO:0008150**. Disponível em: <https://www.ebi.ac.uk/QuickGO/term/GO:0008150>. Acesso em: 17 jan. 2018.

FALLIS, A. **The sperm cell: production, maturation, fertilization, regeneration.** edited by Christopher De Jonge and Christopher Barratt. 359 p., UK: Cambridge University Press; 2006.

FAO. **The state of the world's animal genetic resources for food and agriculture**. Rome: Edited by Barbara Rischkowsky & Dafydd Pilling, 2007.

FINKELSTEIN, M., ETKOVITZ, N., BREITBART, H. Role and regulation of sperm gelsolin prior to fertilization. **J Biol. Chem.,** v. 285, p. 39702–39709, 2010.

FRASER, L. Markers for sperm freezability and relevance of transcriptome studies in semen cryopreservation: a review. CARREIRA, R. P. (Ed.). **Theriogenology**. InTech, p. 47–62, 2017.

GEVAERT, K. et al. Exploring proteomes and analyzing protein processing by mass spectrometric identification of sorted N-terminal peptides. **Nat. Biotechnol.** v. 21, n. 5, p. 566-569. 2003.

GURUPRIYA, V. S. et al. Proteases and Proteases Inhibitors of Semen – A Review. **Advances in Animal and Veterinary Sciences,** v. 2, n. 8, p. 447–456, 2014.

HAMMOND, J. **Physiology of reproduction.** Nature, 1960.

HARRIS, H. E., WEEDS, A. G. Plasma gelsolin caps and severs actin filaments. **FEBS Lett.,** v. 177, p. 184–188, 1984.

IBRAHIM, N. M. et al. Correlation between clusterin-positive spermatozoa determined by flow cytometry in bull semen and fertility. **J Androl.,** v. 21, n. 6, p. 887–894, 2000.

JAYASRI, K.; PADMAJA, K.; PRASAD, P. E. Proteomics in Animal Health and Production. **IOSR-JAVS**, v. 7, n. 4, p. 50–56, 2014.

JUYENA, N. S.; STELLETTA, C. Seminal Plasma: An Essential Attribute to Spermatozoa. **J Androl.,** v. 33, n. 4, p. 536–551, 2012.

KALECZYC, J. et al. Immunohistochemical properties of nerve fibres supplying accessory male genital glands in the pig. A colocalisation study. **Histochem. Cell Biol.**, v. 111, p. 217–228, 1999.

KELLY, V. C. et al. Characterization of bovine seminal plasma by proteomics. **Proteomics**, v. 6, n. 21, p. 5826–5833, 2006.

KEPPER, M. E. KEAST, J. R. 1997. Location, immunohistochemical features, and spinal connections of autonomic neurons innervating the rat seminal vesicles. **Biol. Reprod.**, v. 57, p. 1164–1174.

KEVIN LI-CHUN, H. et al. Gelsolin decreases actin toxicity and inflammation in murine multiple sclerosis. **J Neuroimmunol.,** v. 287, p. 36–42, 2015.

KILLIAN, G. J.; CHAPMAN, D. A; ROGOWSKI, L. A. Fertility-associated proteins in Holstein bull seminal plasma. **Biol. Reprod.,** v. 49, n. 6, p. 1202–1207, 1993.

KIM, S. H. et al. Effects of hormones on the expression of matrix metalloproteinases and their inhibitors in bovine spermatozoa. **Asian-Australas J Anim. Sci.,** v. 26, n. 3, p. 334–342, 2013.

KLIMCZUK, M. et al. Immunohistochemical characterisation of cholinergic nerve fibres supplying accessory genital glands in the pig. **Vet. Med. Czech.**, v. 50, p. 119–130, 2005.

KONG, N. et al. Natriuretic peptide type C induces sperm attraction for fertilization in mouse. **Scientific Reports**, v. 7, n. 39711, p. 12, 2017.

KRISHNAN, G. et al. Mitigation of the Heat Stress Impact in Livestock Reproduction. In: CARREIRA, R. P. (Ed.). **Theriogenology**. InTech, p. 63–86, 2017.

KUMAR, P. et al. Seminal Plasma Proteome: Promising Biomarkers for Bull Fertility. **Agric. Res.**, v. 1, n. 1, p. 78–86, 2012.

LANES, C. F. C.; SAMPAIO, L. A.; MARINS, L. F. Evaluation of DNase activity in seminal plasma and uptake of exogenous DNA by spermatozoa of the Brazilian flounder Paralichthys orbignyanus. **Theriogenology**, v. 71, n. 3, p. 525–533, 2009.

MACANOVIC, B. et al. Correlation between Sperm Parameters and Protein Expression of Antioxidative Defense Enzymes in Seminal Plasma: A Pilot Study. **Dis Markers**, p. 5, 2015.

MCCAULEY, T. C. et al. Purification and characterization of fertility-associated antigen (FAA) in bovine seminal fluid. **Mol. Reprod. Dev.**, v. 54, n. 2, p. 145–153, 1999.

MCCAULEY, T. C. et al. Identification of a heparin-binding protein in bovine seminal fluid as tissue inhibitor of metalloproteinases-2. **Mol. Reprod. Dev.**, v. 58, n. 3, p. 336–341, 2001.

MENEGASSI, S. R. O. et al. Causes of rejection of beef bulls in breeding soundness evaluation. **R Bras. Zootec.,** v. 42, n. 7, p. 1648–1653, 2012.

MENEZES, E. B. et al. Proteomic analysis of seminal plasma from locally-adapted "Curraleiro Pé-Duro bulls" (*Bos taurus*): identifying biomarkers involved in sperm physiology in endangered animals for conservation of biodiversity. **Anim. Reprod. Sci.,** v. 183, p. 86–101, 2017.

MOGIELNICKA-BRZOZOWSKA, M., KORDAN, W. Characteristics of selected seminal plasma proteins and their application in the improvement of the reproductive processes in mammals. **Pol. J Vet. Sci.,** v. 14, p. 489–499, 2011.

MORTARINO, M. et al. Two-dimensional polyacrylamide gel electrophoresis map of bull seminal plasma proteins. **Electrophoresis**, v. 19, n. 5, p. 797–801, 1998.

Moura, A.A. et al. Proteomics of cauda epididymal fluid from mature Holstein bulls. **J. Proteomics**, v. 73, p. 2006–2020, 2010.

MURAD, A. M.; RECH, E. L. NanoUPLC-MSE proteomic data assessment of soybean seeds using the Uniprot database. **BMC Biotechnol.**, v. 12, n. 1, p. 82, 2012.

NEWTON, L. D. et al. Elevated testicular temperature modulates expression patterns of sperm proteins in Holstein bulls. **Mol. Reprod. Dev.,** v. 76, n. 1, p. 109–118, 2009.

PEDDINTI, D. et al. Comprehensive proteomic analysis of bovine spermatozoa of varying fertility rates and identification of biomarkers associated with fertility. **BMC Syst. Biol.,** v. 2, n. 19, 2008.

PEREZ-PATIÑO, C. et al. Characterization of the porcine seminal plasma proteome comparing ejaculate portions. **J Proteomics,** v. 142, p. 15–23, 2016.

QIAN, X. et al. Actin binding proteins, spermatid transport and spermiation. **Semin. Cell. Dev. Biol.,** p. 75–85, 2014.

RBST. **Aberdeen Angus (Native) - RBST**. Available: <https://www.rbst.org.uk/Rare-and-Native-Breeds/Cattle/Aberdeen-Angus-Native> Acessed: 22 jan. 2018.

RIVERA, R.M. et al. Reorganization of microfilaments and microtubules by thermal stress in two-cell bovine embryos. **Biol. Reprod.,** v. 70, p. 1852–1862, 2004.

RODRÍGUEZ-MARTÍNEZ, H. et al. Seminal plasma proteins: What role do they play? **Am. J Reprod. Immunol.,** v. 66, n. 1, p. 11–22, 2011.

RONCOLETTA, M. et al. Proteômica para investigar a congelabilidade do semen bovino. **Braz. J Vet. Res. Anim. Sci.,** v. 50, n. 1, p. 43–49, 2013.

ROSATI, L., ANDREUCCETTI, P., PRISCO, M., 2017. Vasoactive intestinal peptide (VIP) localization in the epididymis of two vertebrate species. **C. R. Biologies**., v. 340, p. 379–385.

SAKAUE, T. et al. Factor H in porcine seminal plasma protects sperm against complement attack in genital tracts. **J Biol Chem.,** v. 285, n. 3, p. 2184–2192, 2010.

SATO, F. et al. Evaluation of deoxyribonuclease activity in seminal plasma of ejaculated chicken semen. **Asian J Androl.,** v. 5, n. 3, p. 213–216, 2003.

SHOJAEI SAADI, H. A. et al. Proteins associated with critical sperm functions and sperm head shape are differentially expressed in morphologically abnormal bovine sperm induced by scrotal insulation. **J Proteomics**, v. 82, p. 64–80, 2013.

SINGER, R. et al. Deoxyribonuclease Activity in Human Seminal Fluid. **Arch. Androl.** v. 10, n. 2, p. 169–172, 1983.

SIOW, Y. et al. Effects of vasoactive intestinal peptide on human sperm motility. **Arch. Androl.,** v. 43, p. 67–71, 1999.

SOUTO, P. L. G. **Perfil proteico do plasma seminal de touros Pantaneiros e sua relação com a congelabilidade do sêmen.** Brasília: Faculdade de Agronomia e Medicina Veterinária, Universidade de Brasília, 112p. Dissertation. 2013.

SOUTO, P. L. G., SILVA, F. I. C., RAMOS, A. F. Alternative fluorophore combination protocol for simultaneous assessment of integrity of sperm cell membranes and mitochondrial function in bulls. (**Submitted**).

SOUZA, C. E. A. et al. Proteomic analysis of the reproductive tract fluids from tropically-adapted Santa Ines rams. **J Proteomics**, v. 75, n. 14, p. 4436–4456, 2012.

SZKLARCZYK, D. et al. The STRING database in 2017: quality-controlled protein–protein association networks, made broadly accessible. **Nucleic Acids Res.**, v. 45, 2017.

TAKESHITA, H. et al. Detection of deoxyribonucleases I and II (dnases I and II) activities in reproductive organs of male rabbits. **Int. J Biochem.,** v. 26, n. 8, p. 1025–1031, 1994.

TEIXEIRA, H. C. A. et al. Seasonal influence on semen traits and freezability from locally adapted Curraleiro bulls. **Anim. Reprod. Sci**., v. 125, n. 1–4, p. 56–61, 2011.

TENTES, I. et al. Matrix metalloproteinase (MMP)-2 and MMP-9 in seminal plasma. **J Assist. Reprod. Genet.**, v. 24, n. 7, p. 278–281, 2007.

TVRDÁ, E. et al. Mineral nutrients and male fertility. **J Microbiol. Biotechnol. Food. Sci**., v. 3, n. 1, p. 1–14, 2013.

UNIPROTKB. **CLU - Clusterin precursor - Bos taurus (Bovine) - CLU gene**. Available <https://www.uniprot.org/uniprot/P17697>. Accessed: 19 jan. 2018.

VILAGRAN, I. et al. Triosephosphate isomerase (TPI) and epididymal secretory glutathione peroxidase (GPX5) are markers for boar sperm quality. **Anim. Reprod. Sci.** v. 165, p. 22–30, fev. 2016.

WESTFALEWICZ, B. et al. Analysis of bull (*Bos taurus*) seminal vesicle fluid proteome in relation to seminal plasma proteome. **J Dairy Sci.** v. 100, n. 3, p. 2282–2298, 2017.

WOLD, S. PLS for Multivariate Linear Modeling. **QSAR: Chemometric Methods in Molecular Design. Methods and Principles in Medicinal Chemistry**, 1994.

XIA, H. et al. Role of C-type natriuretic peptide in the function of normal human sperm. **Asian J Androl.** v. 18, p. 80–84, 2016.

YIN, H.L., STOSSEL, T.P. Control of cytoplasmic actin gel-sol transformation by gelsolin, a calcium-dependent regulatory protein. **Nature**, v. 281, p. 583–586, 1979.

ZHU, W.; SMITH, J. W.; HUANG, C. M. Mass spectrometry-based label-free quantitative proteomics. **J Biomed. Biotechnol.,** v. 2010, 2010.

CONSIDERAÇÕES FINAIS

Os resultados deste estudo permitem concluir que a ocorrência de estresse pelo calor de intensidade moderada durante o verão ocasionou respostas fisiológicas significativas, porém não foi suficiente para prejudicar a qualidade do sêmen de touros das raças Crioulo Lageano e Angus, a qual se manteve constante e satisfatória ao longo das estações. Características relacionadas com o conforto térmico animal, como a temperatura do ar, a temperatura de globo negro e o índice de temperatura e humidade foram consideradas as mais importantes em relação à condição seminal, em detrimento das características de morfologia externa dos animais.

As principais diferenças entre as raças no tocante à adaptabilidade ao ambiente térmico foram observadas em características de pelame, mostrando que touros Crioulo Lageano possuem uma adaptação um pouco melhor para condições climáticas mais quentes, enquanto que touros Angus se encontram em conforto térmico durante períodos de temperaturas mais frias, e na frequência respiratória que, por sua vez, parece ser um dos mecanismos mais importantes para a termoregulação em Angus. Embora tenha sido observado que os touros usaram diferentes mecanismos para a perda de calor, os mesmos foram capazes de manter ótima termoregulação testicular e corporal ao longo das estações, mesmo com os desafios ambientais que cada indivíduo enfrentou, o que demonstra boa adaptação à região onde foram estudados.

A morfologia externa dos animais foi mais importante que as outras características medidas para explicar as mudanças fisiológicas ocorridas durante o verão, porém no inverno as características climáticas foram as que prevaleceram.

A análise das proteínas diferenciais presentes no plasma seminal revelou associações significantivas com os dados ambientais, mas com uma intensidade baixa. Isto provavelmente ocorreu devido ao clima ameno encontrado nesta região sub-tropical. Apesar da qualidade seminal entre raças não ter sido diferente, a abundância das proteínas do plasma seminal variou significativamente entre elas, o que nos leva a crer que estas proteínas fazem parte de uma gama de mecanismos e processos biológicos que são ativados para lidar com as mudanças no ambiente térmico e garantir a manutenção da homeostase. As proteínas que mostraram maior portencial para afetar as características espermáticas no verão foram TIMP-2, DNase, Clusterina, CFAH e GPx6. Este parece ser o primeiro relato deste recente tipo evolutivo de glutationa peroxidase, GPx6, no plasma seminal bovino. No inverno, as proteínas BSP1, BSP3, CCL2, Sulfhydryl oxidase e TIMP-2 se destacaram.

Sugere-se que estas proteínas sejam investigadas mais profundamente para um melhor entendimento de suas funções no plasma seminal. Esses esclarecimentos podem ser úteis no desenvolvimento de novas biotecnologias reprodutivas, como a utilização de peptídeos candidatos como aditivos em meios de conservação de sêmen, o que implicaria na qualidade do material criopreservado e no seu potencial fertilizante, assim como, poderia otimizar o uso de reprodutores em centros de rerprodução de bovinos.