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**FACULDADE DE AGRONOMIA**
**PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOTECNIA**

# Respostas biológicas de bovinos das raças Holandesa e Girolando sob estresse térmico

Marcelo Tempel Stumpf
Engenheiro Agrônomo (UFPEL)
Mestre em Zootecnia (UFRGS)

Tese apresentada como um dos requisitos à obtenção do grau de Doutor em Zootecnia
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MARCELO TEMPEL STUMPF
Engenheiro Agrônomo e
Mestre em Zootecnia

# TESE

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para obtenção do Grau de

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Faculdade de Agronomia
Universidade Federal do Rio Grande do Sul
Porto Alegre (RS), Brasil

Aprovado em: 03.07.2014
Pela Banca Examinadora

CONCEPTA M. MCMANUS PIMENTEL
PPG Zootecnia/UFRGS
Orientadora

JÚLIO OTÁVIO JARDIM BARCELLOS
PPG Zootecnia/UFRGS

MARCOS VINÍCIUS GALBERTO BARBOSA DA SILVA
EMBRAPA - CNPGL - Juiz de Fora/MG

ALEXANDRE MOSSATE GABBI
UFRGS

ANDRÉ THALER NETO
UDESC - Lages/SC

Homologado em: 09.07.2014
Por

JÚLIO OTÁVIO JARDIM BARCELLOS
Coordenador do Programa de
Pós-Graduação em Zootecnia

PEDRO ALBERTO SELBACH
Diretor da Faculdade de Agronomia

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**RESPOSTAS BIOLÓGICAS DE BOVINOS DAS RAÇAS HOLANDESA E GIROLANDO SOB ESTRESSE TÉRMICO**[1]

Autor: Marcelo Tempel Stumpf

Orientadora: Prof. Dra. Concepta Margaret McManus Pimentel

## RESUMO

Trinta e oito vacas leiteiras, 19 da raça Holandês e 19 da raça Girolando (½ e ¾), foram submetidas à elevadas temperaturas por restrição à sombra durante o período entre a ordenha da manhã e da tarde. Mensurações de temperatura retal, frequências respiratória e cardíaca e escore de ofegação, além de coletas de sangue, foram realizadas nos períodos pré ordenha. Produção de leite e características físico-químicas do mesmo foram acessadas. Durante os períodos de coleta se avaliou características ambientais com vistas a calcular um índice de temperatura e umidade (ITU). Primeiramente se buscou estabelecer diferenças em parâmetros fisiológicos, de leite e sangue de vacas com diferentes porcentagens de alelos oriundos da raça Holandesa no genoma (100, 75 e 50%) em função de aumentos no ITU. Animais puros Holandês apresentaram características que condizem com menor tolerância ao calor do que animais Girolando. Em um segundo momento se analisou o efeito do estresse térmico sobre a permeabilidade das junções firmes das células epiteliais da glândula mamária de vacas Holandês. Além disso, buscou-se perceber alterações na estabilidade do leite ao teste do álcool decorrentes de um possível aumento na permeabilidade de tais estruturas celulares. O parâmetro utilizado para aferir tal efeito foi o nível sanguíneo de lactose no plasma. Alterações lácteas foram percebidas em função de aumentos de ITU e se devem principalmente à reduções em produção de leite. Aumento nos parâmetros fisiológicos não influenciou a estabilidade do leite. Leite instável apresentou maior teor de lactose. Maior número de dias em lactação pode ser o responsável pela redução na estabilidade do leite. Percebeu-se relação inversamente proporcional entre permeabilidade das junções firmes e estabilidade do leite, porém o estado de estresse térmico, ao contrário do esperado, não apresentou influência nas células da glândula mamária.

Palavras-chave: estabilidade do leite, estresse térmico, índice de temperatura e umidade, junções firmes, tolerância ao calor

[1] Tese de Doutorado em Zootecnia – Produção Animal, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brasil. (69 p) Julho, 2014.

**BIOLOGICAL RESPONSES IN HOLSTEIN AND GIROLANDO COWS UNDER HEAT STRESS**[2]

Author: Marcelo Tempel Stumpf

Advisor: Prof. Dra. Concepta Margaret McManus Pimentel

## ABSTRACT

Thirty-eight dairy cows, 19 Holstein and 19 Girolando (½ e ¾), were submitted to elevated temperatures due to shade deprivation between morning and evening milkings. Rectal temperature, heart and respiratory rates and panting score measurements, besides blood samples collection, were performed before each milking. Milk production and its physical-chemical characteristics were studied. Climatic variables were measured during data collection to calculate a temperature-humidity index (THI). Firstly, the study evaluated changes in physiological, blood and milk parameters according to the percentage of alleles derived from the Holstein breed (100, 75 and 50%) due to increases in THI. Pure Holstein cows presented characteristics that indicate lower heat tolerance than Girolando cows. Secondly, heat stress effects on mammary gland cells tight junctions permeability were evaluated in Holstein cows. Alterations in milk stability due to changes in permeability were also assessed. Plasma lactose was the parameter used to detect changes in tight junctions permeability. Modifications in milk characteristics were probably due to reduction in milk production. Increase in physiological parameters did not affect milk stability. Unstable milk samples presented higher lactose levels. Higher days in milk might be the main responsible for reductions in milk stability. There was an inversely proportional relation between tight junctions permeability and milk stability to the ethanol test, although heat stress, contradicting the expected, did not influenced mammary gland cells.

Keywords: heat stress, heat tolerance, temperature-humidity index, tight junctions, milk stability

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[2] Doctoral Thesis in Animal Science, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. (69 p.) July, 2014.

# SUMÁRIO

| | Página |
|---|---|
| CAPÍTULO I................................................................................. | 12 |
| 1. INTRODUÇÃO GERAL............................................................... | 13 |
| 2. REVISÃO BIBLIOGRÁFICA........................................................ | 15 |
| 2.1 Homeotermia e zona termoneutra................................................ | 15 |
| 2.2 Mecanismos de dissipação de calor............................................. | 16 |
| 2.2.1 Condução.............................................................. | 16 |
| 2.2.2Convecção............................................................ | 16 |
| 2.2.3Radiação.............................................................. | 16 |
| 2.2.4 Evaporação............................................................ | 16 |
| 2.3 Tolerância ao calor.......................................................... | 17 |
| 2.4 Estresse térmico por calor................................................... | 17 |
| 2.4.1 Efeitos sobre a produção de leite..................................... | 18 |
| 2.4.2Efeitos sobre a composição do leite.................................. | 19 |
| 2.4.3 Efeitos sobre a estabilidade do leite no teste do álcool..... | 20 |
| 2.5 Junções firmes e aspectos relacionados........................................ | 20 |
| 3. HIPÓTESES E OBJETIVOS........................................................ | 22 |
| | |
| CAPÍTULO II................................................................................ | 23 |
| Temperature-humidity index elevation induces physiological, blood and milk alterations in Holstein cows in a more pronounced manner than in ½ and ¾ Holstein x Gir.............................................................. | 25 |
| | |
| CAPÍTULO III | 43 |
| Does heat stress alter mammary gland cells tight junction permeability or milk stability to the ethanol test in Holstein cows?……………...………............................................................................ | 45 |
| | |
| CAPÍTULO IV | 59 |
| 4.1. CONCLUSÕES GERAIS.......................................................... | 61 |
| 4.2. CONSIDERAÇÕES FINAIS...................................................... | 62 |
| 4.2. REFERÊNCIAS BIBLIOGRÁFICAS.............................................. | 63 |
| 4.3. VITA........................................................................... | 69 |

# LISTA DE TABELAS

Página

CAPÍTULO II:
Table 1.Climatic, physiological and milk traits of cows with different percentage of Holstein breed in their genetic configuration (100, 75 and 50% - G100, G75 and G50, respectively)…................................ 29
Table 2. Correlation analysis between percentage of Holstein (100, 75 and 50%), THI, heart rate, heat tolerance index, panting score, rectal temperature and respiratory rate with milk parameters and their corresponding significance levels...................................................... 30
Table 3. Correlation analysis between percentage of Holstein (100, 75 and 50%), THI, heart rate, heat tolerance index, panting score, rectal temperature and respiratory rate with blood parameters and their corresponding significance levels....................................................... 32
CAPÍTULO III:
Table 1. Interaction between day (1, 2 and 3) and period (morning and afternoon) for temperature-humidity index, rectal temperature, respiratory rate and milk production.................................................... 49
Table 2. Comparison between climatic, milk, plasma and physiological traits between morning and evening with corresponding significance levels.………………………………………………….....................… 49
Table 3. Comparison between climatic, milk, plasma and physiological traits and number of days in milk from cows producing stable or unstable milk with corresponding significance levels......................................................................................... 50

## RELAÇÃO DE FIGURAS

Página

CAPÍTULO I:

Figura 1. Esquema gráfico das zonas de sobrevivência, homeotermia, de conforto térmico e de moderado conforto térmico animal........................................................................................ 14

CAPÍTULO II:

Figure 1. Percentage of Holstein, temperature-humidity index, physiological and milk traits projected in principal factors 1 and 2.......................................................................................... 31

Figure 2. Percentage of Holstein, temperature-humidity index, physiological and blood traits projected in principal factors 1 and 2.……......………………....................................................................... 33

CAPÍTULO III:

Figure 1. Correspondence analysis relating high (H_PL) or low plasma lactose (L_PL) with high (H_MS) or low milk stability to the ethanol test (L_MS), high (H_ML) or low milk lactose (L_ML), high (H_DIM) or low days in milk (L_DIM), high (H_SCC) or low somatic cell count (L_SCC), high (H_MP) or low milk production (L_MP) and high (H_RT) or low rectal temperature (L_RT)............................................ 50

## LISTA DE ABREVIATURAS

| | |
|---|---|
| ˚C | Graus Celsius |
| TCS | Temperatura crítica superior |
| NRC | National Research Council |
| ITU | Índice de temperatura e umidade |
| $CO_2$:$HCO_3$ | Razão gás carbônico:bicarbonato |
| $CO_2 \leftrightarrow HCO_3$ | Reação gás carbônico:bicarbonato |
| BEN | Balanço energético negativo |
| $Na^+/K^+$ | Razão sódio/potássio |
| *B. indicus* | *Bos indicus* |
| *B. taurus* | *Bos taurus* |
| ½ | Metade |
| ¾ | Três quartos |
| G100 | Pure Holstein cows |
| G50 | ½ Holstein x Gir cows |
| G75 | ¾ Holstein x Gir cows |
| THI | Temperature-humidity index |
| MCV | Mean corpuscular volume |
| HPA | Hypothalamus-pituitary-adrenal |
| DIM | Days in milk |
| NaOH | Sodium hydroxide |
| mL | mililiters |
| ˚GL | Degrees Gay-Lussac |
| SCC | Somatic cell count |
| RR | Respiratory rate |
| RT | Rectal temperature |
| HT | Heat tolerance index |
| MCHC | Mean corpuscular hemoglobin concentration |
| RDW | Red cell distribution width |
| MPV | Mean platelets volume |
| $thou.mm^{-3}$ | thousands per milimeter in the third |
| WBGT | Wet bulb globe temperature |

| | |
|---|---|
| AT | Air temperature |
| BGT | Black globe temperature |
| RU | Relative humidity |
| SAS | Statistical analyses system |
| $L.milking^{-1}$ | Liters per milking |
| $cell.mL^{-1}$ | Cells per mililiter |
| $breaths.min^{-1}$ | Breaths per minute |
| $beats.min^{-1}$ | Beats per minute |
| ˚D | Degrees Dornic |
| ns | Non significant |
| MS | Milk stability |
| MP | Milk production |
| TA | Titratable acidity |
| MF | Milk fat |
| MPr | Milk protein |
| NDS | Nonfat dry stratum |
| % Holstein | Percentage of Holstein in genetic configuration |
| PS | Panting score |
| HR | Heart rate |
| fL | fentoliter |
| $g.dL^{-1}$ | Grams per deciliter |
| PLAT | Platelets |
| SN | Segmented neutrophils |
| EOS | Eosinophils |
| LEUK | Leukocytes |
| HZ | Holstein x Zebu |
| ANOVA | Analysis of variance |
| $Kg.day^{-1}$ | Kilograms per day |
| TJ | Tight junctions |
| cm | centimeter |
| mL | mililiter |
| µM | micrometer |

| | |
|---|---|
| L_MS | Low milk stability |
| H_MS | High milk stability |
| L_PL | Low plasma lactose |
| H_PL | High plasma lactose |
| H_DIM | High days in milking |
| L_DIM | Low days in milking |
| L_ML | Low milk lactose |
| H_ML | High milk lactose |
| L_MP | Low milk production |
| H_MP | High milk production |
| L_SCC | Low somatic cell count |
| H_SCC | High somatic cell count |
| H_HR | High heart rate |
| L_HR | Low heart rate |
| CNPq | Conselho Nacional de Desenvolvimento Científico e Tecnológico |

# LISTA DE SÍMBOLOS

| | |
|---|---|
| β | Beta |
| α | Alfa |
| $Na^+$ | Sódio |
| $K^+$ | Potássio |
| $Cl^-$ | Cloro |
| % | Por cento |

# CAPÍTULO I

# 1. INTRODUÇÃO GERAL

Cenários mais pessimistas apontam para um inequívoco aumento nas médias de temperatura na Terra em até 4 ˚C até 2100 (IPCC, 2007). Maior incidência de casos de seca, frentes de calor, precipitação intensa, entre outros eventos climáticos extremos são esperados. Os sistemas de produção animal, ao estarem inseridos e serem diretamente dependentes das condições naturais vigentes, serão afetados por tais modificações. Os animais, além de terem que lidar com as condições impostas para manter as condições fisiológicas dentro da normalidade e controlar a sua homeostase, devem produzir em qualidade e quantidade que correspondam às exigências do produtor e às demandas do mercado.

Em se tratando das previsões relacionadas ao incremento nas médias de temperatura, aumento nos danos ao sistema produtivo leiteiro são esperados. Efeitos diretos, como alterações comportamentais - aumento na ocorrência de interações agonísticas (Costa & Cromberg, 1997), maior tempo em ócio, redução no pastoreio diurno e aumento no noturno (Costa, 2000), maior ingestão de água (West, 2003) - , fisiológicas - aumento nas frequências respiratória (Mitlöhner et al., 2001) e cardíaca, escore de ofegação e temperatura retal (McManus et al., 2009) - e de redução no consumo (West, 1994) decorrem em animais expostos à altas temperaturas. Mecanismos homeorréticos são acionados de forma a tornar o animal capaz de se adaptar e se manter em um equilíbrio dinâmico com as novas contingências impostas. O animal necessita dissipar calor ao meioem busca de manter sua homeostase, ou seja, temperatura interna dentro dos níveis normais: 38 - 39,5˚C (Stober, 1993). Mecanismos de dissipação de calor requerem energia, a qual pode provir da sua partição em detrimento da glândula mamária através de alterações hormonais acionadas e controladas pelo eixo hipotálamo-hipófise-adrenal (Baumgard & Rhoads, 2013).

Efeitos indiretos também são esperados, como a aceleração no envelhecimento da biomassa vegetal e lignificação intensa dos tecidos, com a conversão de produtos da fotossíntese em componentes estruturais, reduzindo a digestibilidade e a ingestão involuntária (Renna et al., 2010). Somados os efeitos de redução de consumo, envelhecimento acelerado de tecidos vegetais e partição de energia favorecendo mecanismos de sobrevivência, têm-se a redução na produção láctea como consequência direta do estresse térmico por calor (West, 2003).

Os efeitos deletérios do calor sobre a produção leiteira passaram a ser considerados por volta de 1920, quando da tentativa de introdução e criação de raças de clima temperado em ambientes tropicais (Berman, 2012). Raças de clima temperado (*Bos taurus*) evoluíram em regiões europeias, como é o caso do gado Jersey, Guernsey, Pardo Suiço, Holandês e apresentam zonas de conforto térmico em temperaturas mais baixas; raças mais adaptadas ao clima quente (*Bos indicus*), como é o caso da raça Gir, evoluíram em regiões mais quentes da Terra e apresentam zona de conforto térmico se estendendo à temperaturas mais elevadas. Técnicas de melhoramento animal, ao cruzar bovinos leiteiros mais adaptados à regiões quentes (Gir - *Bos indicus*) com aqueles caracterizados por sua elevada aptidão leiteira

(Holandesa - *Bos taurus*), preconizam a geração de uma raça capaz de produzir altos níveis de leite e, ainda sim, ser mais tolerantes à elevadas temperaturas (raça Girolando) - (Berman, 2012). Uma vez apresentando distintas capacidades de tolerância ao calor (McManus et al., 2009), espera-se que animais puros da raça Holandesa apresentem alterações metabólicas e fisiológicas mais acentuadas do que animais cruzados Holandês x Gir em clima quente. Consequentemente, pode-se hipotetizar alterações mais significativas em suas características produtivas, tanto em volume de leite produzido como em sua composição.

# 2. REVISÃO BIBLIOGRÁFICA

## 2.1Homeotermia e Zona Termoneutra

A maioria das espécies de interesse zootécnico (mamíferos e aves) se caracteriza por possuir temperatura corpórea relativamente constante, podendo variar em alguns graus centígrados; tais animais são denominados homeotermos, de forma que o organismo, quando confrontado com variações térmicas, lança mão de mecanismos fisiológicos para lidar com tal situação e manter a temperatura do núcleo corporal dentro de limites relativamente estreitos. Os bovinos, espécie de interesse da presente tese, apresentam faixa normal de temperatura retal entre 38 e 39.5˚C (Stober, 1993), embora trabalhos mais recentes (West, 2002) considerem que temperatura retal acima de 39,2˚C possa ser indicativo de estresse por calor. A temperatura do núcleo corporal se encontra por volta de 38,5˚C (Esmay, 1969).

Todos os animais são suscetíveis a mudanças de temperatura, de forma que os mesmos possuem distintas zonas de conforto térmico, ou zona termoneutra, caracterizada como a faixa de temperatura dentro da qual os mecanismos fisiológicos necessários para que o animal lide com o ambiente são mínimos (Figura 1).

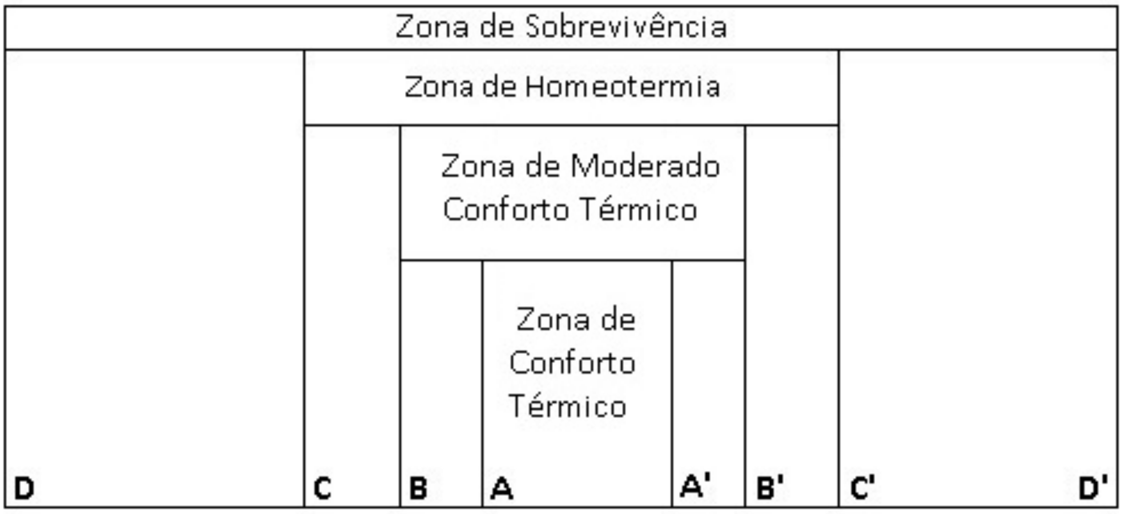


FIGURA 1: Esquema gráfico das zonas de sobrevivência, homeotermia, de conforto térmico e de moderado conforto térmico animal.

A faixa de temperatura disposta entre A e A' é denominada zona de conforto térmico; a zona compreendida entre B e B' é a zona de moderado conforto térmico ou de variação nula na produção de calor; quando entre C e C' a zona é de homeotermia e entre D e D' a zona é de sobrevivência..

O ponto B' é caracterizado como temperatura crítica superior (TCS), acima da qual os mecanismos de dissipação de calor corporal são acionados. Elevações de temperatura atingindo ou ultrapassando o ponto C', por mais que os mecanismos de resfriamento animal funcionem, não permitem a manutenção do controle homeotérmico e a temperatura corporal aumenta progressivamente. Caso a temperatura atinja o ponto D' e medidas de resfriamento animal promovidas pelo sistema produtivo não sejam tomadas o animal morre por hipertermia.

Em sentido oposto, o mesmo ocorre, com a diferença crucial de que os animais, ao invés de acionarem mecanismos de dissipação de calor, adotam aqueles que promovem aumento na produção de calor metabólico, visando lidar com as baixas temperaturas.

## 2.2 Mecanismos de dissipação de calor

Conforme mencionado no tópico 2.1, elevações de temperatura acima da TCS forçam os animais a dissiparem calor corpóreo ao meio ambiente, sempre na busca de manter a sua homeotermia. As principais formas de dissipação de calor se dão através de mecanismos sensíveis, aqueles dependentes de variações de temperatura animal-ambiente (condução, convecção e radiação), ou latente, o qual é dependente do gradiente de pressão de vapor animal-ambiente (evaporação).

### 2.2.1 Condução

A troca de calor entre o animal e o ambiente, quando embasado na condução, não envolve movimentação de moléculas, o calor flui através de moléculas adjacentes, sempre da superfície mais quente para a superfície mais fria. Assim, quando confrontado com temperaturas acima da TCS, os animais entram em contato com superfícies frias, como o solo, superfícies úmidas e até mesmo paredes das instalações.

### 2.2.2 Convecção

Ao contrário da condução, nesse caso ocorre a translocação de moléculas. Esse mecanismo se baseia na troca de calor entre o ar que circunda a superfície do animal e o ar ambiente. Em um primeiro momento o calor é transferido por condução da superfície animal ao ar; posteriormente, ocorre um fluxo convectivo ascendente ou descendente do mesmo, em razão da densidade. A perda de calor por convecção pode ser otimizada em virtude de aumentos na velocidade do ar, o qual renova o ar que circunda o animal, permitindo a troca do ar mais quente por aquele mais frio.

### 2.2.3 Radiação

Se baseia nas trocas de calor através de ondas eletromagnéticas. É o caso de animais expostos ao sol, os quais absorvem a energia solar radiante e têm sua temperatura corporal elevada. A incidência solar e a radiação podem contribuir mais com a carga térmica do que a temperatura ambiente (Silanikove, 2000).

### 2.2.4 Evaporação

A partir do momento em que as temperaturas se elevam, maior passa a ser a contribuição da evaporação nos processos de dissipação. Esse mecanismo se baseia na perda de calor pela conversão do suor secretado nas glândulas sudoríparas e da umidade proveniente do trato respiratório em vapor. Cada mL de água evaporado da superfície animal representa perdas de 2,43 J de calor (Silanikove, 2000). Altamente dependente das condições de umidade, perdas evaporativas são prejudicadas quando os animais são criados em ambientes úmidos, em virtude da redução do gradiente de pressão de vapor animal-ambiente. Assim, ambientes quentes e úmidos são mais danosos ao bem-estar animal do que ambientes quentes e secos.

## 2.3 Tolerância ao calor

Definida como a habilidade em manter estabilidade térmica em ambientes quentes, a tolerância ao calor é determinada pela relação entre dissipação de calor e produção de calor metabólico (Berman, 2012).

Diferentes raças apresentam distintas capacidades de lidar com elevadas temperaturas, sendo que tais modificações podem ser relacionadas com os locais nos quais as raças se desenvolveram. Raças europeias (*Bos taurus*) têm sua origem na Europa, enquanto raças índicas (*Bos indicus*) evoluíram na Ásia, caracterizada por elevadas temperaturas e umidade. De acordo com Roy & Collier (2012), a exposição prolongada a estressores ambientais podem fixar geneticamente ajustes metabólicos e fisiológicos, tornando o animal adaptado ao local e suas condições. Essa parece ser a explicação para maior tolerância ao calor do *Bos indicus* sobre *Bos taurus*.

*Bos indicus* produzem menos calor e apresentam maior capacidade de dissipar o mesmo ao ambiente. Menor taxa de crescimento e produção de leite resultam em menores taxas metabólicas, contribuindo para a termorregulação (Branton et al., 1966). A capacidade de dissipação de calor através de mecanismos latentes é menor afetada em animais Índicos, os quais não tiveram taxas de suor alteradas em virtude de aumento na umidade do ar (Finch, 1985). Independente da subespécie, os bovinos apresentam somente uma glândula sudorípara por folículo capilar; as raças índicas, porém, apresentam maior quantidade de folículos e possuem suas glândulas mais longas, largas, volumosas e próximas da superfície da pele (Pan, 1964), tornando-as mais eficientes quanto a dissipação de calor através do suor (Ribeiro, 2010). Além desses fatores, coloração da pele e configuração do pelame (Berman, 2004) contribuem para a dissipação e maior tolerância ao calor do que animais de origem europeia.

## 2.4Estresse térmico por calor

Animais confrontados com temperaturas acima da TCS podem se considerar como em estresse térmico, já que dependem de mecanismos de dissipação de calor, conforme explicitado acima.

A mediação de tais mecanismos se dá pelo eixo hipotálamo-hipófise-adrenal, o qual é abastecido de informações provenientes das unidades termoreceptoras e termosensitivas no sistema nervoso central (Baker, 1989). Uma das primeiras respostas do animal ao ambiente estressante é a redução no consumo de alimentos (inibição do centro lateral do apetite; Castanheira, 2009), na busca de reduzir a produção de calor proveniente de processos digestivos (calor metabólico), e maior consumo de água, para repor as perdas promovidas pela evaporação. Estima-se aumento de consumo de 1,2 Kg de água para cada 1˚C de aumento na temperatura ambiental mínima (West, 2003). Além disso, os animais aumentam suas exigências de mantença, necessárias para suportar as mudanças fisiológicas para dissipação de calor: ofegacão, suor, aumento nas reações químicas, síntese e mantença da funcionalidade das proteínas de choque térmico (Tomanek et al., 2010), aumento na atividade celular induzida pelos níveis elevados de adrenalina (Gaffin & Hubbard, 1996). De acordo com o NRC (2001), médio a severo estresse podem elevar entre 7 e 25% tais exigências, embora Fox & Tyluki (1998) sugiram aumentos de até 30%.

Ocorre vasodilatação das vias sanguíneas e aumento do fluxo de sangue para as zonas periféricas do animal, para que ocorra resfriamento do mesmo por contato com o ar mais frio. Elevação na frequência cardíaca promove aumento nesse fluxo sanguíneo, auxiliando o processo supracitado. Na busca de elevar as perdas de calor por evaporação, os animais elevam também a frequência respiratória. Segundo (Silanikove et al., 2000), tal mecanismo homeostático pode prevenir aumento da temperatura retal até índices de temperatura e umidade (ITU) de 80. O aumento nas perdas de gás carbônico em virtude do aumento da frequência respiratória promove quebra do equilíbrio $CO_2$:$HCO_3$ sanguíneo, o qual deve se encontrar por volta de 20:1 e é o principal sistema de tamponamento do sangue (Kadzere et al., 2002). Para compensar essas perdas, ácido carbônico ($H_2CO_3$) é quebrado em $CO_2$ e água. Redução nesse ácido no sangue leva a um quadro de alcalose respiratória (aumento do pH sanguíneo). Redução no teor de $CO_2$ força os rins a excretarem maior quantidade de bicarbonato (tamponante), na tentativa de manter o equilíbrio 20:1 estável (Beatty et al., 2006). Além disso, a reação CO2↔ HCO3 se desloca para a esquerda, para compensar as perdas de gás carbônico. O menor teor de bicarbonato promove uma subsequente queda no pH sanguíneo, levando ao quadro de acidose metabólica compensatória.

Percebe-se, pois, que mecanismos de sobrevivência sobrepõem aqueles relacionados à produção de leite. Somando os efeitos do menor consumo de alimentos, maiores exigências de energia para mantença, possíveis distúrbios metabólicos e maior destinação de energia à processos de perda de calor, reduções nos níveis produtivos dos animais se torna uma realidade.

### 2.4.1 Efeitos sobre produção de leite

Queda nos níveis produtivos de animais em estresse térmico severo pode chegar a 40% (West, 2003); de fato, Abreu et al. (2011) registraram diminuição de aproximadamente 38% na produção de leite de vacas sem acesso à sombra na estação quente do ano. Segundo Wheelock et al. (2010), 40 a 50% dessa redução se deve ao menor consumo de alimentos (efeito indireto), sendo o restante em virtude de outros mecanismos induzidos pelo estresse (efeitos diretos). Somado ao efeito de menor consumo, McDowell et al. (1976) atestaram, ao manterem vacas Holandês em ambiente passando de 18˚C a 30˚C, redução de até 35% na eficiência de utilização de energia para fins produtivos.

Bovinos em estresse térmico enfrentam um período de balanço energético negativo (BEN), no qual o consumo de alimentos não compensa as perdas energéticas (Drackley, 1999). Animais em balanço energético negativo decorrente de subnutrição apresentam, dentre outros sinais, redução nas concentrações de insulina (hormônio que induz a lipogênese e o acúmulo de tecidos) circulante e aumento nos níveis de somatotropinas (Bauman & Currie, 1980) - promove exportação de ácidos graxos não esterificados dos tecidos adiposos e inibe estímulo à lipogênese pela insulina (Bauman & Vernon, 1993). Assim, os animais passam a ter à sua disposição energia proveniente da lipólise (consumo das reservas de gordura corporal), sendo que os mesmos têm elevados os níveis sanguíneos de ácidos graxos não esterificados. Vacas em BEN decorrente de estresse térmico, apesar de ingerirem menos, não apresentam reduções no teor de insulina (Wheelock et al., 2010) e podem ter seus níveis de somatotropina reduzidos (Li et al., 2006). Dessa forma, as vacas se tornam metabolicamente inflexíveis, uma vez que as mesmas não podem oxidar ácidos graxos para gerar energia, pois esse processo contribui no incremento de calor metabólico (Baumgard & Rhoads, 2013). Assim, não se percebe elevações em ácidos graxos não esterificados no sangue (Shwartz et al., 2009) e os animais passam a ser dependentes de glicose como fonte de energia. Como consequência, menor aporte da mesma é provido à glândula mamária e se reduz a síntese de lactose no leite, principal regulador osmótico do volume produzido, o qual também é prejudicado.

Silanikove et al. (2009) sugerem que estresse térmico agudo promove aumento na concentração de β-caseína f (1-28) em virtude da ação de clivagem pela plasmina. Esse componente atua bloqueando os canais de potássio da membrana apical das células mamárias, promovendo despolarização potencial da membrana, reduzindo o metabolismo celular, sua atividade secretória e, por conseguinte, a produção láctea.

#### 2.4.2 Efeitos sobre a composição do leite

Reduções no teor de proteína no leite foram reportados em bovinos (Rhoads et al., 2009) e ovinos (Sevi & Caroprese, 2012). Segundo os últimos autores, exposição ao sol em altas temperaturas pode promover aumento na permeabilidade dos capilares, elevando a quantidade de enzimas proteolíticas no leite, de forma a reduzir as concentrações proteicas do mesmo. Em adição, a redução na oferta e consumo de pasto nos meses mais quentes, bem como o menor aporte de energia e nitrogênio, contribuem para os achados. A principal causa da redução nos teores de proteína em estudo conduzido por Bernabucci et al. (2002) foi a redução nos níveis de $\alpha_s$ e β-caseínas, provavelmente em virtude do menor aporte de energia e proteína. Essas caseínas são ricas em grupos fosfato e são os componentes acídicos das micelas (Schmidt, 1980). Assim, como relatado em outros trabalhos realizados durante o verão (Mariani et al., 1994; Bernabucci & Calamari, 1998; Calamari & Mariani, 1999), espera-se valores de pH mais alto e de acidez titulável mais baixos em animais em estresse térmico, embora Abreu et al. (2011) tenha obtidos resultados contrários.

O citado aumento na permeabilidade dos capilares também promove aumento nas concentrações de enzimas lipolíticas, resultando em decréscimo na síntese e alteração no perfil lipídico do leite (Sevi & Caroprese, 2012). Os mesmos autores observaram que a menor taxa de passagem no rúmen aumenta o tempo de exposição da digesta ao processo de biohidrogenação, de forma que os ácidos graxos insaturados são convertidos em saturados e têm seus teores reduzidos no leite, em detrimento de um aumento na concentração de ácidos graxos saturados. O estresse térmico e a queda na qualidade da forragem em virtude da maior atividade metabólica vegetal (envelhecimento da massa vegetal e lignificação acentuada dos tecidos) atuam em conjunto promovendo quedas no consumo (Renna et al., 2010) e na produção de ácido acético no rúmen, principal precursor da gordura láctea.

Os efeitos negativos do estresse térmico sobre os níveis de lactose no leite são decorrentes do supracitado uso da glicose como fonte de energia ao animal, reduzindo o aporte à glândula mamária e posterior síntese desse componente, além do efeito direto da queda no consumo de alimentos. Alterações na concentração de lactose, por sua vez, são mais difíceis de ocorrer, já que sua queda é acompanhada por menores níveis de produção de leite.

**2.4.3 Efeitos sobre estabilidade do leite no teste do álcool**

Os efeitos do estresse térmico sobre a estabilidade do leite no teste do álcool podem ser relacionados às quedas no consumo de alimentos dos animais. De fato, restrição de consumo resultou em redução na estabilidade do leite (Zanela et al., 2006; Abreu et al., 2011; Stumpf et al., 2013) e épocas do ano de menor oferta de alimentos promovem maior incidência de leite instável (Ponce & Hernández, 2001; Marques, 2004). Abreu et al. (2011), ao induzirem estresse térmico severo através da restrição ao uso da sombra, perceberam quedas bruscas na estabilidade do leite, porém, os mecanismos envolvidos não foram totalmente elucidados.

Ademais, distúrbios metabólicos provenientes do estresse térmico, ao alterar de forma significativa as condições fisiológicas do animal, apresentam grande potencial de reduzir a estabilidade do leite, conforme percebido por Marques et al. (2011) ao induzirem acidose metabólica através do fornecimento de sal aniônico em bovinos leiteiros. Amplas revisões bibliográficas sobre estabilidade do leite no teste do álcool e fatores relacionados foram realizadas por Stumpf (2012) e Kolling (2012).

**2.5 Junções Firmes e aspectos relacionados**

Um fator que predispõe reduções na estabilidade do leite e que passou a ser explorado recentemente é relacionado ao aumento na permeabilidade das junções firmes das células epiteliais da glândula mamária (Stumpf et al., 2013). Semipermeáveis, essas estruturas se localizam na porção apical das células epiteliais e endoteliais, circundando as mesmas (Stelwagen, 1998). Na glândula mamária, as junções firmes atuam separando o lúmen alveolar (conteúdo apical) do fluido intersticial (conteúdo basolateral) (Schneeberger & Lynch, 1992). Ao permitir essa separação, possibilitam a existência de um diferencial iônico e/ou de pequenas substâncias entre os dois lados da células (Stelwagen et al., 1998).

O funcionamento ótimo dessas estruturas (impermeabilidade) é essencial para a síntese e manutenção da qualidade láctea, pois impede a troca desregrada de componentes entre o sangue e o leite (Stelwagen et al., 1997). Por esse motivo, o parâmetro mais utilizado para determinar a permeabilidade das junções firmes é o teor de lactose sanguínea (Stelwagen et al. 1997; 2000), uma vez que esse carboidrato é sintetizado na glândula mamária e é secretado exclusivamente ao lúmen alveolar; qualquer traço desse componente no sangue indica mau funcionamento das junções. É de se esperar, pois, que aumentos no teor de lactose no sangue sejam acompanhado de quedas na concentração láctea do componente. Acompanhado dessas mudanças, podem ser percebidas redução nas concentrações de $K^+$, elevação de $Na^+$ e $Cl^-$ (Linzell et al., 1975) e alteração na relação $Na^+/K^+$(Wilde et al., 1995; Delamaire & Guinard-Flament, 2006).

Alguns dos fatores que podem alterar a permeabilidade das junções firmes são: redução na frequência de ordenhas (Sorensen et al., 2001; Delamaire & Guinard-Flament, 2006; Castillo et al., 2008); estádio lactacional, com aumento da impermeabilidade ao início da lactogênese (Neville, 1995) e redução no final da lactação, sendo um processo ligado à involução mamária (Capuco & Akers, 1999, Shamay et al., 2003); estresse, cujos efeitos não foram totalmente elucidados (Stelwagen et al., 2000, Stumpf et al., 2013), porém, no caso de estresse mecânico promovido pela menor frequência de ordenhas, o acúmulo de leite no úbere pode ativar uma via de sinalização por mecanotransdução, possivelmente afetando a permeabilidade das junções firmes (Millar et al., 1997).

## 3 HIPÓTESES E OBJETIVOS

**HIPÓTESES**

♦ Em um mesmo nível produtivo e em condições climáticas similares, vacas puras da raça Holandesa são menos tolerantes ao calor do que vacas da raça Girolando.

♦ O estresse térmico por calor em vacas Holandês eleva a permeabilidade das junções firmes das células epiteliais da glândula mamária, resultando em alterações na composição físico-química do leite e sua estabilidade ao teste do álcool.

**OBJETIVOS**

♦ Determinar a tolerância ao calor de vacas Holandesa e Girolando com níveis produtivos similares e sob condições climáticas semelhantes.

♦ Determinar se o estresse térmico por calor em vacas Holandês altera a permeabilidade das junções firmes das células epiteliais da glândula mamária e a composição físico-química do leite produzido, especialmente sua estabilidade ao teste do álcool.

## CAPÍTULO II

## Temperature-humidity index elevation induces physiological, blood and milk alterations in Holstein cows in a more pronounced manner than in ½ and ¾ Holstein x Gir[3]

[3] Artigo escrito de acordo com as normas da International Journal of Biometeorology

Temperature-humidity index elevation induces physiological, blood and milk alterations in Holstein cows in a more pronounced manner than in ½ and ¾ Holstein x Gir

**Abstract**

*Bos taurus* and *Bos indicus* cattle subspecies present different capability in coping with situations of elevated temperatures. While *B. taurus* show elevated milk production efficiency, in Brazil, *B. indicus* are characterized for their higher heat tolerance. Breeding programs crossed both subspecies to produce a high producing yet heat-tolerant breed (Girolando). Nineteen Holstein (G100) and 19 Girolando cows [(½ Holstein x Gir (G50) and ¾ Holstein x Gir (G75)] were used in a six day experiment to evaluate the consequences of heat stress due to shade deprivation on their physiological, blood and milk traits. Cows were exposed to a non-shaded environment between morning (06h00; GMT -3:00) and evening milking (14h30; GMT -3:00) with water *ad libitum*. Procedures were conducted before morning and evening milking. Physiological parameters related with mechanisms of heat dissipation were measured; milk composition, titratable acidity and ethanol stability from morning and evening milking were evaluated. Blood samples were taken in vacutainers containing EDTA and hemogram traits were evaluated. Temperature and humidity values were registered during experimental procedures and a temperature-humidity index (THI) was calculated. Statistical procedures included analysis of variance, correlation and principal factors. THI impacted on physiological, milk and blood parameters in G100, G75 and G50. Alterations in physiology, milk stability, milk (fat, lactose and protein content) and blood composition (leukocytosis, monocytes elevation, MCV reduction) were more pronounced in G100. Holstein cows presented changes in physiological parameters, as well as in some milk and blood parameters that were related with reduced capability of this breed in dealing with elevated temperatures.
**Keywords:** heat tolerance, heat stress, THI, physiology, milk, blood

**Introduction**

The deleterious effect of warm climates on animal overall performance became aware since the attempt to introduce temperate climate breeds in tropical environments during the 1920s and 1930 (Berman, 2012). Cattle subspecies such as *Bos taurus* and *Bos indicus* present differences in their ability to cope with elevated temperatures and maintain their thermal stability, a characteristic defined as heat tolerance. The first subspecies has its origin in Europe, while the second derived from subspecies that evolved in warm zones of Asia (Achilli et al. 2008). This distinction in breed evolution is responsible for the better adaptation of *B. indicus* to heat (Berman, 2012). According to Hansen (2004), *Bos indicus* cattle produce less heat and have increased capability of dissipating heat to the environment.

To expand milk production to warmer zones of the globe and still obtain satisfactory production indices, crossbreeding has been used. Its concept is based in combining high production levels of *Bos taurus* with increased heat tolerance of *Bos indicus*, developing a high producing and heat-tolerant breed. Many farmers in Brazil and throughout the world often utilize the crossing of Holstein (*B. taurus*) and Gir (*B. indicus*) cattle for such purpose (Berman, 2012).

Physiological changes like increase in respiratory rate (Eigenberg et al. 2005) and panting (Gebremedhin, 2012), as well as in heart rate (Cerutti et al. 2013), are key homeorretic mechanisms mediated by the hypothalamus-pituitary-adrenal (HPA) axis (Collier et al. 2006) that animals develop to cope with elevations in ambient temperature. Evaporative cooling by the respiratory tract can account for 15% of heat dissipation in warmer climates (Maia et al. 2005). The efficacy of heat dissipation can be attested by the magnitude of changes in rectal temperature, which is a more reliable indicator of heat stress when compared with respiration rate (Hillman et al. 2005). Alterations in blood parameters (leukocytes, red cell distribution width, mean corpuscular volume, platelets, mean corpuscular hemoglobin concentration, among others) due to hormones produced by the HPA axis (corticosteroids - glucocorticoids, mineralocorticoids, glucocorticosteroids -, epinephrine, norepinephrine) were also studied and were related with systemic stress (McManus et al. 2009) and changes in cell metabolism and metabolic heat load (Lee et al. 1976).

Along with changes in physiological and blood traits, increased temperatures may alter milk physical-chemical composition and overall production levels (West. 2003). Bernabucci et al. (2002) observed, together with lower milk yield, lower contents of crude proteins and casein in Holstein milk during the summer. This reduction was often reported during heat stress (Giustini et al. 2007; Renna et al. 2010). Decrease in fat content, although results differed among studies, was also reported (Giustini et al. 2007; Bouraoui et al. 2002). In situations of heat stress the hypothalamus stimulates the satiety center in the brain, resulting in lower feed intake and, consequently, reduced milk production (Albright and Alliston, 1972). This adaptation is carried out in order to reduce metabolic heat production due to digestion of the feed.

Heat stress has a great impact on cows' metabolism, production levels and milk characteristics, thus impairing the dairy production system as a whole. Since cows present different capabilities in coping with situations of elevated temperatures, the objectives of the present experiment were to evaluate the impact of increased THI in physiological, blood and milk traits and to compare changes in these traits in breeds with increasing percentages of Holstein in their genetic configuration: 100% (pure Holstein), 75% (¾ Holstein x Gir) and 50% (½ Holstein x Gir).

**Material and Methods**

*Local description, animals and management*

The experiment was conducted in March 2013, during the Spring, at Embrapa Gado de Leite (21°35'16"S and 43°15'56"W), in Coronel Pacheco, Minas Gerais, Brazil and lasted for six days. All experimental procedures were approved by the Research Committee and Ethical Committee for Animal Use of the Federal University of Rio Grande do Sul. Coronel Pacheco presents two well-defined seasons throughout the year: rainy, between October and April; dry: April to September. Annual rainfall is of approximately 1,440 mm. Annual temperature varies from 15.6 to 25.6 ˚C, with mean value of 20.6 ˚C.

Thirty-eight cows were used: 19 Holstein (G100) and 19 Girolando [½ Holstein-Gir (G50, n=8) and ¾ Holstein-Gir (G75, n=11)]. At the start of their respective period of analysis, Holstein cows presented average of 249.15 ± 68.19 days in milk (DIM) and 7.40 ± 2.59 $L.milking^{-1}$ milk production; G50 cows presented 95 ± 72.33 DIM and 6.2 ± 3.42 $L.milking^{-1}$ milk production; G75 showed 169.3 ± 95.85 DIM and 7.31 ± 2.95 $L.milking^{-1}$ milk production.

Breeds were analyzed in separate periods of three consecutive days each (G50 and G75 were analyzed together), but all experimental procedures were the same for both breeds. The study consisted of inducing heat stress by exposing cows to a non-shaded environment - with water *ad libitum* - between morning and evening milkings. During experimental procedures, temperature varied from 21 to 34 ˚C (average of 26.61 ˚C) and relative humidity ranged from 56 to 95% (average of 77.55 %) for G75 and G50. For G100 cows, the same parameters ranged from 22 to 35 ˚C (average of 28.3 ˚C) and from 52 to 95 % (average of 76,68%), respectively. G100 cows were housed in a free-stall, receiving total mixed ratio of maize silage and concentrate (59% corn, 35% soybean, 3.5% protein-mineral-vitamin core, 0.5% mineral salt, 1% urea and 1% bicarbonate); between milkings, cows were conducted to a *Brachiaria brizantha* pasture. G50 and G75 were conducted in *Pennisetum purpureum* pasture and fed concentrate before each milking (70% corn, 25% soybean, 3.5% protein-mineral-vitamin core, 0.5% mineral salt and 1% urea) in quantities according to milk production. Animals used belong to Embrapa, thus, housing and feeding techniques were not altered nor established by the authors, with the exception of heat stress induction.

*Milk collection and analysis*

Cows were milked twice a day, at 06h00 (GMT -3:00) and 14h30 (GMT -3:00). Milk yield was recorded individually during each milking and individual milk samples were taken to evaluate: titratable acidity, by titration with 0.1 N NaOH solution; ethanol stability of milk, by mixing 2 mL of milk with 2 mL of an alcoholic solution in ascending concentrations (ranging from 50 to 98 ˚GL) in a Petri dish - results were expressed as the minimal ethanol concentration that induced coagulation of milk proteins (clots formation). Milk samples were also collected in tubes containing Bronopol in order to evaluate concentrations of fat, protein, lactose and nonfat dry stratum by an infrared analyzer (Bentley 2000® Equipment (Chaska, Minnesota, USA)) and somatic cell count (SCC) by flow cytometry with Somacount 300® (Bentley Instruments, Chaska, Minnesota, USA).

*Physiological parameters assessment*

Before morning and evening milkings cows were hold in a pen and some physiological parameters were assessed: respiratory and heart rates trough auscultation during 30 seconds (then multiplied by two); panting score, by visual observation and in a 0 to 4 scale (Mader et al. 2006); rectal temperature with the use of a veterinarian thermometer inserted 30 cm against the rectum wall during three minutes.

Respiratory rate (RR) and rectal temperature (RT) were used to calculate the Heat Stress Index (HT), according to Benezra (1954), in which higher values are worst:

$$HT = \left(\frac{RT}{38.33}\right) + \left(\frac{RR}{23}\right)$$

*Blood collection and plasma analysis*

Before each milking, but after physiological parameters assessment, blood samples were collected via caudal venopuncture in vacutainers containing EDTA. Directly after sampling, blood was sent to laboratory in order to evaluate the percentage of leukocytes in an automatic cell counter (CC550, Cellm™). Mean corpuscular volume (MCV; fL), mean corpuscular hemoglobin concentration (MCHC; $g.dL^{-1}$), red cell distribution width (RDW; %) and mean platelet volume (MPV; fL) were determined by calculation. Platelets ($thou.mm^{-3}$), percentage of segmented neutrophils, eosinophils and monocytes were assessed by manual counting of 100 cells in Wright-stained blood smears under optical microscope.

*Climatic indices*

During experimental procedures, climatic parameters were collected hourly with the use of a black globe thermometer (Extech Instruments, Model HT30): wet bulb globe temperature (WBGT, ˚C), air temperature (AT, ˚C), black globe temperature (BGT, ˚C) and relative humidity (RU, %). Some of these values were used to calculate a temperature-humidity index (THI) through the equation (NRC, 1971):

$$THI = (1.8\,x\,AT + 32) - [(0.55 - 0.0055\,x\,RU)x\,(1.8\,X\,AT - 26.8)]$$

*Statistical analysis*

Cows were considered as the experimental unit. Mean values of all physiological, blood and milk traits were compared, including climatic indices, according to the percentage of Holstein: G100, G75 and G50. Data were analyzed with the use of the statistic program SAS 9.3 (SAS Institute, Cary, North Carolina, USA) and the procedures involved analysis of variance (PROC GLM). Principal factor analysis (PROC FACTOR) and PROC CORR analyzed the correlation between percentage of Holstein, THI and physiological parameters with blood and milk traits.

**Results**

*Climatic, physiological and milk parameters*

THI did not differ in the periods of analysis of the different breeds (Table 1). Nevertheless, changes could be observed in some physiological and milk traits according to breed configuration: G50 showed reduced respiratory and heart rates, HT and, statistically equal to G75, presented lower values of rectal temperature and panting score.

Table 1. Climatic, physiological and milk traits of cows with different percentage of Holstein breed in their genetic configuration (100, 75 and 50% - G100, G75 and G50, respectively)

| Trait | G100 | G75 | G50 | P=F |
|---|---|---|---|---|
| THI | 79.52a | 79.2a | 80.32a | 0.6356 |
| Respiratory Rate (breaths.min$^{-1}$) | 81.01a | 76.13a | 56.25b | <0.0001 |
| Heat tolerance | 4.56a | 4.33a | 3.43b | <0.0001 |
| Heart Rate (beats.min$^{-1}$) | 78.03a | 75.86a | 64.16b | 0.0018 |
| Rectal Temperature (˚C) | 39.84a | 39.34ab | 39.14b | 0.0020 |
| Panting Score | 1.54a | 1.06ab | 0.52b | 0.0003 |
| Milk production (L.milking$^{-1}$) | 6.87a | 6.80a | 6.30a | 0.5383 |
| Somatic cell count (cell.mL$^{-1}$) | 492,700a | 682,900a | 671,200a | 0.2497 |
| Milk fat (%) | 4.19a | 4.59a | 4.42a | 0.1034 |
| Milk lactose (%) | 4.40a | 4.44a | 4.36a | 0.2770 |
| Milk protein (%) | 3.39a | 3.15b | 3.11b | <0.0001 |
| Milk nonfat dry stratum (%) | 8.68a | 8.48ab | 8.34b | 0.0002 |
| Titratable acidity (˚D) | 13.98c | 14.83b | 15.70a | <0.0001 |
| Milk Stability (˚GL)[a] | 72.55c | 82.45b | 85.78a | <0.0001 |

[a] Concentration of ethanol capable of inducing clots formation in milk. Variables followed by the same letter in the row are not significantly different by Tukey test at 5% probability.

Along with milk production, SCC, milk fat and lactose were equal between the three groups. Milk protein was elevated in G100, when compared with G75 and G50. Milk nonfat dry stratum concentration was statistically equal between G75 and G100. Milk stability differed between groups, with G50 presenting elevated and G100 reduced values. This same consideration can be made for titratable acidity.

Percentage of Holstein presented low but positive correlation with milk protein and nonfat dry stratum, while it presented negative correlation with titratable acidity and milk stability to the ethanol test, being more accentuated for the latter (Table 2). Elevation in THI and in all physiological parameters was positively correlated with somatic cell count and milk fat, but all correlations were low, and were negatively correlated with titratable acidity, milk production, milk lactose and nonfat dry stratum - milk production was the most affected variable. Milk protein was negatively correlated with THI, heart and respiratory rates, but in a low manner. Milk stability, for its part, was not correlated with THI, but presented low and negative correlation with physiological parameters.

Table 2. Correlation analysis between percentage of Holstein (100, 75 and 50%), THI, heart rate, heat tolerance index, pantig score, rectal temperature and respiratory rate with milk parameters and their corresponding significance levels.

| | % Holstein | THI | HR | HT | PS | RT | RR |
|---|---|---|---|---|---|---|---|
| SCC | -0.0183$^{ns}$ | 0.2045** | 0.2116** | 0.2008** | 0.2065** | 0.2072** | 0.2085** |
| TA | -0.3662** | -0.3027** | -0.4225** | -0.4436** | -0.4169** | -0.4479** | -0.4539** |
| MP | 0.0683$^{ns}$ | -0.6199** | -0.4834** | -0.5649** | -0.5602** | -0.5680** | -0.5692** |
| MF | -0.1014$^{ns}$ | 0.3729** | 0.2496** | 0.3378** | 0.2796** | 0.3448** | 0.3319** |
| MPr | 0.3179** | -0.1735** | -0.1595* | -0.1294$^{ns}$ | -0.0879$^{ns}$ | -0.0923$^{ns}$ | -0.1343* |
| ML | 0.0396$^{ns}$ | -0.2424** | -0.1834** | -0.1756** | -0.1373* | -0.2116** | -0.1793** |
| NDS | 0.2723** | -0.2670** | -0.2328** | -0.2003** | -0.1505* | -0.1891** | -0.2068** |
| MS | -0.5814** | -0.0215$^{ns}$ | -0.1658* | -0.1821** | -0.2280** | -0.1669* | -0.1895** |

* $P<0.05$; ** $P<0.01$; ns: non significant; % Holstein: percentage of Holstein; HR: heart rate; HT: heat tolerance index; PS: panting score; RT: rectal temperature; RR: respiratory rate; SCC: somatic cell count ($cell.ml^{-1}$); TA: titratable acidity (˚D); MP: milk production ($L.milking^{-1}$); MF: milk fat (%); MPr: milk protein (%); ML: milk lactose (%); NDS: nonfat dry stratum (%); MS: milk stability to the ethanol test (˚GL).

Three principal factors were identified in milk multivariate analysis and the first two explained

46.06 and 19.06% of the total variance observed, respectively. Milk lactose MSA value was below 0.5 and was removed from this analysis (Hair et al. 2009). A group was formed (THI group) comprehending THI and physiological parameters (panting score, heart and respiratory rates, HT and rectal temperature), which indicates that these variables are correlated (Figure 1). According to Smith et al. (2002), angles of 180˚ and 0˚ indicate elevated negative and positive correlation between variables, respectively. Angle of 90˚ indicates low or null correlation. Thus, elevation in THI group values tend to reduce milk production efficiency. Titratable acidity was also negatively correlated with THI group, but in a lower extent. Null or low influence of the aforementioned group on ethanol stability, milk protein and nonfat dry stratum was observed. Due to the angle between THI group and milk fat, somatic cell count and percentage of Holstein, a certain positive relation between these variables can exist. It is expected that an increase on percentage of Holstein would induce a reducion in milk stability to the ethanol test (angle of approximately 180˚).

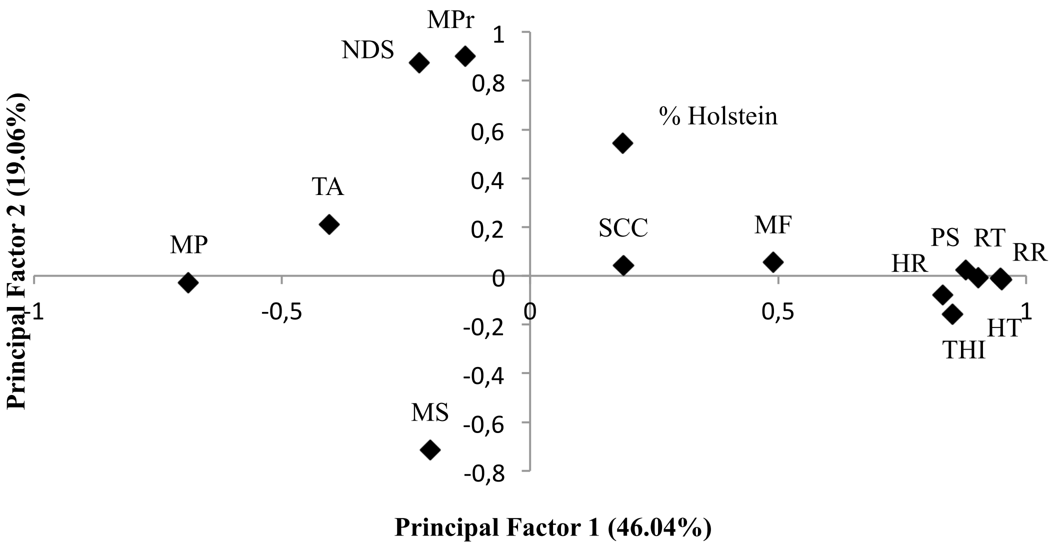


Figure 1. Percentage of Holstein, temperature-humidity index, physiological and milk traits projected in principal factors 1 and 2.

MS: milk stability; MP: milk production; TA: titratable acidity; MF: milk fat; MPr: milk protein; NDS: nonfat dry stratum; % Holstein: percentage of Holstein in genetic configuration; SCC: somatic cell count; RT: rectal temperature; PS: panting score; RR: respiratory rate; HR: heart rate; HT: heat tolerance index; THI: temperature-humidity index.

*Climatic, physiological and blood parameters*

Low and positive correlation was observed between percentage of Holstein and leukocytes, MCHC and platelets concentration in blood (Table 3). Correlations were also found between percentage of Holstein and MCV, segmented neutrophils and eosinophils, but correlations were low and negative. None of the physiological parameters and THI were correlated with concentrations of segmented neutrophils, MCHC, RDW, platelets and eosinophils. This same tendency was found for MCV, with the exception of panting score, which presented low and negative correlation with this blood parameter. Physiological traits were, although low, positively correlated with leukocyte content; THI, in turn, was not. THI presented a slight positive correlation with monocytes and MPV concentrations; this same consideration can be made for the correlation between HT and respiratory rates with MPV and monocytes. Some other positive and low correlations were found: panting score with monocytes and rectal temperature with MPV.

Table 3. Correlation analysis between percentage of Holstein (100, 75 and 50%), THI, heart rate, heat tolerance index, pantig score, rectal temperature and respiratory rate with blood parameters and their corresponding significance levels.

| | % Holstein | THI | HR | HT | PS | RT | RR |
|---|---|---|---|---|---|---|---|
| MCV | -0.1587* | 0.0229$^{ns}$ | -0.0880$^{ns}$ | -0.1032$^{ns}$ | -0.1401* | -0.0376$^{ns}$ | -0.1051$^{ns}$ |
| MCHC | 0.2554** | -0.0400$^{ns}$ | 0.0310$^{ns}$ | 0.0660$^{ns}$ | 0.0168$^{ns}$ | 0.0025$^{ns}$ | 0.0637$^{ns}$ |
| LEUK | 0.2952** | 0.0729$^{ns}$ | 0.2118** | 0.2325** | 0.1967** | 0.1707* | 0.2293** |
| RDW | 0.1335$^{ns}$ | -0.0817$^{ns}$ | 0.0206$^{ns}$ | 0.0005$^{ns}$ | 0.0234$^{ns}$ | -0.0484$^{ns}$ | 0.0036$^{ns}$ |
| MPV | 0.0704$^{ns}$ | 0.1682* | 0.1036$^{ns}$ | 0.1557* | 0.0963$^{ns}$ | 0.2085** | 0.1559* |
| PLAT | 0.2315** | -0.0697$^{ns}$ | -0.0178$^{ns}$ | -0.0412$^{ns}$ | 0.0191$^{ns}$ | -0.0039$^{ns}$ | -0.0421$^{ns}$ |
| EOS | -0.3601** | 0.1275$^{ns}$ | 0.0017$^{ns}$ | -0.0730$^{ns}$ | -0.1098$^{ns}$ | -0.0461$^{ns}$ | -0.0585$^{ns}$ |
| SN | -0.3225** | 0.1304$^{ns}$ | 0.0584$^{ns}$ | -0.0425$^{ns}$ | -0.0395$^{ns}$ | -0.0289$^{ns}$ | -0.0413$^{ns}$ |
| MONO | -0.1179$^{ns}$ | 0.1858** | 0.1182$^{ns}$ | 0.1442* | 0.1573* | 0.1116$^{ns}$ | 0.1521* |

* P<0.05; ** P<0.01; ns: non significant; % Holstein: percentage of Holstein; HR: heart rate; HT: heat tolerance index; PS: panting score; RT: rectal temperature; RR: respiratory rate; MCV: Mean corpuscular volume (fL); MCHC: Mean corpuscular hemoglobin concentration ($g.dL^{-1}$); LEUK: leukocytes (%); RDW: Red cell distribution width (%); MPV: Mean platelet volume (fL); PLAT: platelets (%); EOS: eosinophils (%); SN: segmented neutrophils (%); MONO: monocytes (%).

Four principal factors were found. The first (32.90%) and second (16.23%) principal factors were responsible for 49.13% of the total variance in the experiment. Again, THI group was formed and presented almost null correlation with MCV, MCHC, RDW, eosinophils and segmented neutrophils (Figure 2). Percentage of Holstein, segmented neutrophils, eosinophils, leukocytes, monocytes and MPV formed another group. Percentage of Holstein, leukocytes, monocytes and MPV were slightly and positively correlated with THI group due to the angle formed between them and since variables are located at the right side of the Y axis.

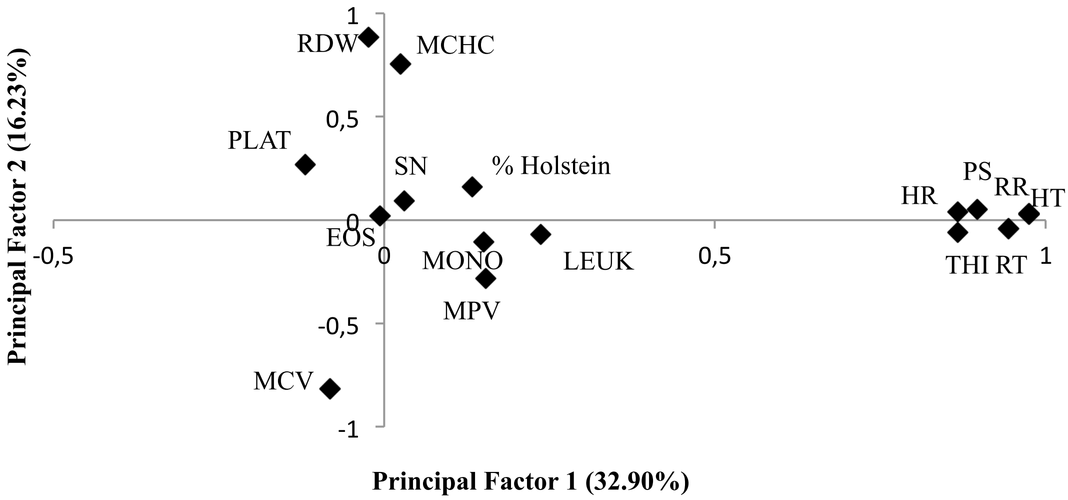


Figure 2. Percentage of Holstein, temperature-humidity index, physiological and blood traits projected in principal factors 1 and 2.

MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration; RDW: red cell distribution width; MPV: mean platelet volume; PLAT: platelets; SN: segmented neutrophils; EOS: eosinophils; % Holstein: percentage of Holstein in genetic configuration; LEUK: leukocytes; RT: rectal temperature; PS: panting score; RR: respiratory rate; HR: heart rate; HT: heat tolerance index; THI: temperature-humidity index.

**Discussion**

Despite the equality in THI to which animals from different groups were subjected, changes could be seen in physiological arrangements (Table 1). Cows from G50 had reduced respiratory rate and heart rate. Even though rectal temperature was similar to G75, when this parameter was combined with respiratory rate, it originated the heat tolerance index, which was also reduced in G50. Panting score from the latter group was not different statistically from G75, although its results were approximately 50% lower. Those results were expected, since the increase in the percentage of Holstein in the breed reduces animals' ability to cope with heat loads. Through the evaluation of rectal temperature and respiratory frequencies, de Azevedo et al. (2005) estimated the upper critical THI for ½ and ¾HZ (Holstein x Zebu) as approximately 80 and 77, respectively. Based on such results, THI was slightly above the critical for G50 (80.32), while it was 2 points higher than the critical for G75 (79.2) and, consequently, even higher for pure Holstein cattle. These physiological changes are mechanisms that animals give rise to try to maintain homeostasis.

In situations of temperatures above the upper critical level, animals' body can gain considerable amount of heat, so the capability of the animal to cope with it relies on their ability to dissipate heat trough sensible (conduction, convecction and radiation) and latent cooling (cutaneous water evaporation and water loss through respiration) mechanisms. The validity of such affirmation is confirmed by principal factor analysis, in which the elevation in THI represents the higher manifestation of mechanisms of heat dissipation. Increases in respiratory rate and panting score are therefore expected, as water loss through respiration can account for 15% of total heat loss (Maia et al. 2005) and increases in importance as ambient temperature rises (Richards, 1985). Elevated heart rates in G100 and G75 represent another way to lose heat and prevent an increase in body temperature (McManus et al. 2009). In addition, Cheung and McLellan (1998) observed that animals adapted to hot climates tend to show, along with reduction in rectal temperature, decrease in heart rate, and this adaptability of G50 might explain the difference between G100, G75 and G50 findings in the present experiment.

Rectal temperature in dairy cattle varies from 38 to 39.3˚C (Pires and Campos, 2004); thus, G100 was above (39.84 ˚C) and G75 slightly above (39.34˚C) the upper value, while G50 maintained its rectal temperature (39.14˚C) inside the normal range. Given that principal factors indicated that elevation in the percentage of Holstein results in higher rectal temperature and other physiological traits as well, Girolando cows were more efficient in coping with elevated temperatures, since increases in rectal temperature mean that heat dissipation mechanisms were not sufficient to maintain homeothermia (Mota, 1997).

Maintenance costs can increase 25% (NRC, 1989) or even 30% (Fox and Tylutki, 1998) in heat stressed cattle. Together with the elevation in energy expenditure to support mechanisms involved in heat dissipation (increased heart and respiratory rate, for instance), decrease in milk production can be expected. Although milk production was similar between groups, principal factor and correlation analysis shows the capability of elevated THI in reducing milk yield. Since ingestive parameters were not evaluated, it is not possible to infer how much of this decline in production is due to reductions in feed intake, but, according to Wheelock et al. (2010), it must be between 35 and 50%. The other 50 to 65% are due to hormonal and other physiological alterations (Baumgard and Rhoads, 2013). The present study was conducted with cows with moderate levels of production, so the negative results could be accentuated in more productive animals, since they present elevated production of metabolic heat and reduced capability to dissipate it to the environment. Cows producing 37 $Kg.day^{-1}$ produce approximately 20% more metabolic heat than cows producing 19 $Kg.day^{-1}$ (Purwanto et al. 1990); since cows were at the same production level (Table 1), physiological differences between breeds may not be attributable to milk yield.

Milk composition analysis showed that concentrations of fat from G100 (4.19%) did not differ from Girolando breeds and that protein content (3.39%) was higher than the values of G50 and G75. The angle formed between percentage of Holstein and milk protein in principal factors analysis (approximately 30˚) and the positive correlation between these variables confirm ANOVA findings. Despite that, Holstein values stayed above the normal values for the breed (fat: 3.5%; protein: 3,1% - Órdonez, 2005). Holstein cows produced 6.87 L.milking-1, below productions expected for this breed (USDA, Summary of Herd Averages (2007) and below results prior to the experiment (7.40 $L.milking^{-1}$), so the concentration effect, in which milk production reduces at a higher extent than fat (Lacy-Hulbert et al. 1999) and protein (Mackle et al. 1999) synthesis, turns these components more concentrated in the samples No correlation was found between percentage of Holstein and milk lactose content; this component's concentration in G100 milk was equal to Girolando. G100 levels stayed below the normal values for lactose (4,9% - Órdonez, 2005). Wheelock et al. (2010) indicated that heat stressed cows secrete from 200 to 400 g less milk lactose than thermal-neutral cows; elevated glucose utilization by extramammary tissues may be responsible for such findings and be related with our results. G50 and G75 presented similar chemical composition of milk. Concentrations of protein and lactose stayed close to the expected for Girolando cows: 3,2% and 4,45%, respectively. Concentrations of fat, although equal to G100 values, was above normal for Girolando, which is 3,5% (dos Reis et al. 2012). The same concentration effect might be the cause.

Milk stability from G100 was below values of Girolando (Table 1). This parameter is of great importance in developing countries such as Brazil, Uruguay, Argentina, Taiwan and Russia, since dairy industries rely on this milk trait to define its suitability for industrial purposes - milk that is unstable to a certain concentration of ethanol is rejected: in Brazil, 72 ˚GL (Ministério da Agricultura, Pecuária e Abastacimento, 2011). Negative correlations between percentage of Holstein and milk stability were also found in principal factors and correlation procedures. More important, milk stability from G100 was reduced when comparing values before and after the commencement of the experiment (75.36 ˚GL x 72.55˚GL). Abreu et al. (2011) showed that the deprivation of shade in Holstein cows reduced milk stability to the lower level evaluated in that study (68˚GL). The author attributed his results to a possible occurrence of metabolic acidosis, which, according to Marques et al. (2011), can induce milk instability. Reduction in feed intake was also found to reduce milk stability (Stumpf et al. 2013), so the probable lower levels of intake due to higher THI conditions, although not measured, might also be involved. Milk stability from G50 and G75, although statistically different, did not seem to be affected by exposure to heat stress conditions, once this parameter stayed practically constant before (86.07 and 83.26˚GL, respectively) and during the experiment (85.78 and 82.45 ˚GL, respectively). Titratable acidity differed between groups, but stayed inside preconized the range - 14 to 18˚D (Ministério da Agricultura, Pecuária e Abastecimento, 2011) - and in the same ascending tendency that was observed before the start of the trial: G100<G75<G50. Results and changes in milk physical-chemical composition shows that pure Holstein cows were more affected by exposure to the imposed adverse climatic condition.

There was a low but positive correlation between all physiological parameters and leukocytes content; this same evidence was found in principal factor analysis. According to Lassen and Swardson (1995) this increase in leukocytes (leukocytosis) is found in animals under stress due to the liberation of epinephrine and corticosteroids, hormonal changes mediated by the HPA axis. McManus et al. (2009) compared different breeds adaptation to heat and attested that Holstein cattle, which presented slight leukocytosis, was the least adapted. Neutrophilia often occurs in heat stressed cows due to mobilization of neutrophils from the bone marrow into the blood stream (Jain, 1993), but in our case the correlation between segmented neutrophils, as well as eosinophils number, with THI and physiological parameters was null. Not all types of leukocytes were evaluated, thus it is not possible to infer the main responsible for leukocytosis, but, since moderate correlation was found between monocytes, THI and some physiological parameters, this leukocyte may be involved. Glucocorticoids release by HPA axis may be responsible for monocytes elevation, as also noted by Carlson and Kaneko (1976); in contradiction, Paape et al. (1974) found no consistent changes in the number of monocytes due to glucocorticoids release.

McManus et al. (2009), in accordance with the present experiment, found no relation between rectal temperature, MCV and MCHC. Those latter variables were negatively correlated both in this and in McManus et al. (2009) study. Our experiment suggests that elevated THI do not influence MCHC levels. El-Nouty et al. (1990) also found no alteration in MCHC from Holstein cows due to increases in THI: 66.6 in winter to 81.9 in summer. Al-Haidary (2004), on the other hand, submitted Naimey sheep to heat stress and found significant reduction in MCHC. Principal factors and correlation analysis showed that pure Holstein cows present reduction in MCV when compared with Girolando. Such a reduction might be associated with depression in cellular requirements of oxygen by Holstein cows, to reduce cell metabolism and metabolic heat load (Lee et al. 1976) in an attempt to deal with elevated temperatures. Red blood cell width was unaffected by increases in THI and physiological traits, suggesting that heat did not changed this cell configuration.

Although platelets concentration showed no correlation with physiological parameters and THI, changes were found in MPV. Both in correlation and principal factor analysis, MPV presented a tendency of increase following THI and physiological parameters (with the exception of heart rate and panting score). Heat stress may induce an elevation in platelets size and in their mean volume (Keatinge et al. 1986) due to the release of platelets from the spleen and the formation of new platelets, both larger than the normal circulating population (Corash et al. 1978)

All significant correlations, positive or negative, were not high, and the distance between blood parameters and THI and physiological traits in principal factors analysis confirms this affirmation. Still, some evidences and alterations in blood parameters could be observed and attributed to differences in heat tolerance, especially between pure Holstein versus G50 and G75. THI during the entire trial ranged from 69.2 to 87.7. Even the lower THI is higher than 68, which is the upper critical value pointed by Zimbleman et al. (2009). More pronounced alterations can be found if animals were subjected to a higher range of THI, from levels below the critical until levels similar to ours.

**Conclusion**

Alterations in physiological, milk and blood parameters following increases in THI were encountered and varied according to the breed. Those changes indicated that pure Holstein cows are less capable of dealing with elevated THI. There is a possibility of more differences and evidences if cows were submitted to a larger range of THI.

**Acknolwledgements**

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## CAPÍTULO III

## Does heat stress alter mammary gland cells tight junction permeability or milk stability to the ethanol test in Holstein cows? [4]

[4] Artigo escrito de acordo com as normas da International Journal of Biometeorology

Does heat stress alter mammary gland cells tight junction permeability or milk stability to the ethanol test in Holstein cows?

**Abstract**

Nineteen Holstein cows were used in a three-day experiment to evaluate the influence of heat stress induced by shade deprivation on the stability of milk and on mammary gland cells tight junction permeability. Cows were deprived of shade between 07h00 (GMT -3:00) and 14h00 (GMT -3:00). Before each milking, rectal temperature and respiratory rate were assessed. Lactose level (indicator of tight junctions permeability) in plasma was measured. Milk somatic cell count, composition, titratable acidity and stability to the ethanol test were analyzed. Climatic variables were assessed hourly during data collection and temperature-humidity index (THI) was calculated. Analysis of variance, correspondence and logistic regression were performed.There was an interaction between days and periods for THI, rectal temperature, respiratory rate and milk production. THI increased in the afternoon, but without effects on milk stability and plasma lactose. Rectal temperature and respiratory rate were elevated during the afternoon, indicating animals' heat stress. Cows producing unstable milk presented elevated plasma lactose levels. THI did not differ in cows producing stable or unstable milk. Somatic cell count did not differ according to milk stability. The majority of milk composition alterations between morning and afternoon were due to reductions in milk production. Correspondence analysis showed that elevation in plasma lactose is followed by reduction in milk stability and vice-versa. The odds of cows producing unstable milk increased with increase in plasma lactose and days in milk. There was no influence of climatic conditions on plasma lactose and milk stability. Elevated plasma lactose (tight junctions permeability) was related with reductions in milk stability to the ethanol test, probably due to alterations in cations concentration.

**Keywords:** heat stress, Holstein cows, milk stability, tight junctions permeability

**Introduction**

Estimations predict an increase in 4.0˚C in Earth's mean temperature until 2100 (IPCC, 2007). Climate models project mean warming in South America ranging from 1-6˚C until the end of the present century, this being accentuated in the tropical zones of the continent (Yahdjian and Sala, 2008). Brazil has two-thirds of its territory located in these zones, with mean temperatures above 20˚C and temperatures predominantly higher than 30˚C in the hottest hours of the day during the greater part of the year (Baccari Jr, 2001). Therefore, dairy production systems in these areas are likely to be greatly affected, especially those based on Holstein (*Bos taurus)* cattle breeding, which presents reduced heat tolerance in comparison with *Bos indicus* (McManus et al. 2009; Berman, 2012).

Cows exposed to elevated temperatures have to increase heat dissipation to the environment to maintain homeotermia. Dissipation mechanisms demand energy and maintenance costs may be elevated by 7 to 25% (NRC, 2001) or even 47% (Baumgard and Rhoads, 2013) in heat stressed animals. Physiological changes such as increased respiratory rate and panting score, as well as cardiac rate and rectal temperatures are expected (McManus et al. 2009). The greater energy consumption by heat dissipation mechanisms combined with reduction in feed intake - mediated by the hypothalamus-pituitary-adrenal axis - promotes a status of negative energy balance in the animal, in which consumption do not meet animals requirement (Drackley, 1999). Glucose becomes preferably used in survival mechanisms not involved with milk synthesis, thus, reducing milk production (Baumgard and Rhoads, 2013). A decrease in 4.2 Kg.day$^{-1}$ milk production was reported by Klosowski et al. (2002) during the hottest months.

Protein (Terada and Shioya, 1998; Kamiya et al. 2005) and fat (Sevi et al. 2001; Giustini et al. 2007) werealso altered by cows' exposure to elevated temperatures. A recent study demonstrated reduction in milk stability due to heat stress in Holstein cows (Abreu et al. 2011). Milk stability to ethanol is a test performed by dairy industry in several countries, including Uruguay, Argentina, Russia, Taiwan and Brazil. In the latter, milk that is unstable to ethanol in the concentration of 72˚GL is considered unsuitable for industrialization and must be rejected, impairing farmers income (Ministério da Agricultura, Pecuária e Abastecimento, 2011). So, this feature of milk presents great importance in these dairy systems. Milk stability can be altered by milk composition characteristics (Horne and Parker, 1981; Chavez et al. 2004), metabolic disorders (Hernández & Ponce, 2005, Marques et al. 2011), diet related factors (Barros et al. 2001). Zanela et al. (2006) and Stumpf et al. (2013) observed reduction in milk stability following undernutrition; the latter authors attributed this reduction to a stress induced increase in the permeability of mammary gland cell tight juntions (TJ).

Tight junctions are dynamic structures located near the apical border of each epithelial cell (Nguyen and Neville, 1998) that control the flux of substances between the apical (alveolar lumen) and basolateral (blood stream) sides (Schneeberger and Lynch, 1992). When in perfect functioning, these structures are almost impermeable, controlling the paracellular movement of small substances (Stelwagen et al. 1998). The impermeability disruption promotes an uncontrolled traffic of ions and small substances between milk and blood and may be induced by longer milking interval (Castillo et al. 2008), mammary involution (Fleet and Peaker, 1978), mastitis (Symons and Wright, 1974) and stress (Stelwagen et al. 2000; Stumpf et al. 2013). The main component used to determine TJ permeability state is lactose levels in plasma (Stelwagen et al. 2000). This carbohydrate is synthesized exclusively in mammary gland (Kuhn and Linzell, 1970) and is secreted only to the alveolar lumen. Any trace of such component in blood proves that TJ are permeable and that an outflow of lactose occurred from milk to blood.

Since elevated temperatures may promote heat stress and stress may induce TJ permeability and milk stability reduction, the present experiment was developed in order to detect alterations in mammary gland cells TJ due to heat stress and perceive alteration in the stability of milk from Holstein cows.

**Material and Methods**

Nineteen pure Holstein cows were used in an experiment conducted at Embrapa Gado de Leite (21°35'16"S and 43°15'56"W), in Coronel Pacheco/MG, Brazil, from March 6$^{th}$ to 8$^{th}$of 2013, with three days duration. The Research Committee and Ethical Committee for Animal Use of the Federal University of Rio Grande do Sul approved all experimental procedures. Coronel Pacheco presents two well-defined seasons throughout the year: rainy, between October and April; dry: April to September. Annual rainfall is of approximately 1,440 mm. Annual temperature varies from 15.6 to 25.6 ˚C, with mean value of 20.6 ˚C.

Cows were housed in a free-stall receiving total mixed ration consisted of maize silage and concentrate (59% corn, 35% soybean, 3.5% protein-mineral-vitamin core, 0.5% mineral salt, 1% urea and 1% bicarbonate). Between morning (06h00; GMT -3:00) and afternoon milkings (14h30; GMT -3:00) heat stress was induced by conducting animals to a non-shaded area with*Brachiaria brizantha*pasture and water *ad libitum*. Animals used belong to Embrapa, thus, housing and feeding techniques were not altered nor established by the authors.

*Physiological parameters*

Before each milking, cows were held and were evaluated for: rectal temperature (RT): veterinary thermometer inserted 30 cm against rectum wall during three minutes; respiratory rate (RR): auscultation during 30 seconds, then multiplying values by two to assess breaths per minute.

*Blood collection and plasma analysis*

Blood samples were taken before each milking via caudal venopuncture in 10 mL heparinized vacutainers. After sampling, samples were cooled and centrifuged at 2000 x g for 15 minutes. Plasma obtained was transferred to 2 mL eppendorfs tubes and stored at -20 ˚C until analysis. Plasma lactose levels were assessed with the use of an enzimatic assay (Lactose Assay Kit – BioVision Research Products, Mountainview, CA, USA) in a microplate reader (Bio-Tek Instruments, model EL808 Micro-plate Reader (Winooski, USA)).

*Milk collection and analysis*

Together with individual milk production, individual milk samples were taken at every milking in 50 mL tubes; after sampling they were stored under refrigeration and analyzed: titratable acidity: titration with 0.1 N NaOH solution; ethanol stability of milk: 2 mL of milk and 2 mL of ethanol solution in concentrations ranging from 50 to 98 ˚GL were mixed in a Petri dish, results were expressed as the lower ethanol concentration that induced the formation of protein clots. Milk composition was evaluated from milk collected in tubes containing Bronopol: somatic cell count (SCC) by flow cytometry with Somacount 300® (Bentley Instruments, Chaska, Minnesota, USA); concentrations of fat, protein and lactose by an infrared analyzer [Bentley 2000® Equipment (Chaska, Minnesota, USA)].

*Climatic parameters*

Environment was monitored hourly with a black globe thermometer (Extech Instruments, Model HT30) during data collection period; variables included: wet bulb globe temperature (WBGT, ˚C), air temperature (AT, ˚C), black globe temperature (BGT, ˚C) and relative humidity (RU, %). Some of these values were used to calculate a temperature-humidity index (THI) through the equation (NRC, 1971):

$$THI = (1.8\, x\, AT + 32) - [(0.55 - 0.0055\, x\, RU) x\, (1.8\, X\, AT - 26.8)]$$

*Statistical procedures*

Statistical analyses were carried out with the use of SAS 9.3 (SAS Institute, Cary, North Carolina, USA). PROC MIXED was performed to evaluate the interaction between day (1, 2 and 3) and period (morning and afternoon). Comparison betweenvariables (morning x afternoon) with no interaction day x period, as well as between cows producing stable (without clots formation when mixed with ethanol in concentrations below 72 ˚GL - Ministério da Agricultura, Pecuária e Abastecimento, 2011) and unstable milk (clots formation when mixed with ethanol below or equal 72 ˚GL) were conducted by analysis of variance with the general linear model (PROC GLM). The statistical model for the comparison between cows producing stable and unstable milk was: $Y = \mu + Stability_i + e_{ij}$, where: $\mu$ = overall mean; $Stability_i$ = milk stability to the ethanol test; $e_{ij}$ = random error associated with each observation; for the comparison between morning and afternoon the model was: $Y = \mu + Period_i + e_{ij}$, in which: Period = period of the day (morning and afternoon).Correspondence analysis (PROC CORRESPOND) was performed; the threshold values to categorize observations into 'low' or 'high' categories were: 72 ˚GL for ethanol stability; 150 for days in milk (DIM); 268 μM for plasma lactose; 7.7 $L.milking^{-1}$ for milk production; 4.46 % for milk lactose; 400,000 $cell.mL^{-1}$ for somatic cell count (SCC) and 39.5 ˚C for rectal temperature. Logistic regression (PROC LOGISTIC) was performed to perceive the increase in the odds of cows producing milk with reduced stability (below 72˚GL) following elevations in plasma lactose and days in milk.

**Results**

There was an interaction between days and periods only for THI (P=0.0005), RT (P<0.0001), RR (P=0.0005) and milk production (P=0.0392). THI was elevated in the afternoon of all three days when compared with THI in the morning (P<0.0001; Table 1). Morning THI was higher in day 2 and lower in day 3; this same consideration is made for afternoon results. Rectal temperature in the morning was higher in day 3 and lower in day 2; in the afternoon, results from day 2 were increased. Results from afternoon were higher in all three days when compared with morning RT (P<0.0001). Respiratory rate in the morning of all three days was similar; afternoon results show that values of day 2 were higher. Respiratory rate in the afternoon of days 1, 2 and 3 was higher than in their respective mornings (P<0.0001). Milk production was reduced in the afternoon, when compared with morning levels (P<0.0001). Besides, this parameter was reduced in the afternoon of day 3, when compared with afternoon of days 1 and 2.

Table 1. Interaction between day (1, 2 and 3) and period (morning and afternoon) for temperature-humidity index, rectal temperature, respiratory rate and milk production.

| Temperature-humidity index | Morning | Afternoon |
|---|---|---|
| Day 1 | 74.30 Bb | 85.55 Ab |
| Day 2 | 75.36 Ba | 86.16 Aa |
| Day 3 | 72.60 Bc | 82.96 Ac |
| Rectal Temperature (˚C) | | |
| Day 1 | 38.83 Bb | 40.75 Ab |
| Day 2 | 38.57 Bc | 41.04 Aa |
| Day 3 | 39.09 Ba | 40.76 Ab |
| Respiratory Rate (breaths.min$^{-1}$) | | |
| Day 1 | 50.50 Ba | 101.60 Ab |
| Day 2 | 49.25 Ba | 126.50 Aa |
| Day 3 | 54.25 Ba | 107.38 Ab |
| Milk Production (L.milking$^{-1}$) | | |
| Day 1 | 9.78 Aa | 5.47 Ba |
| Day 2 | 9.21 Aa | 4.91 Ba |
| Day 3 | 9.61 Aa | 3.86 Bb |

Values followed by the same uppercase letter in the row and by the same lower case letter in the column are not significantly different in the Tukey test at 5% probability

There were no differences in milk stability and milk protein between morning and afternoon (Table 2). Milk fat and SCC were elevated in afternoon milk; lactose and titratable acidity, on the other hand, were reduced in this period. Plasma lactose did not differ between periods.

Table 2. Comparison between climatic, milk, plasma and physiological traits between morning and afternoon with corresponding significance levels.

| | Morning | Afternoon | CV (%) | P=F |
|---|---|---|---|---|
| Milk stability (˚GL) | 74.08a | 71.37a | 13.43 | 0.2041 |
| Titratable acidity (˚D) | 14.85a | 13.20b | 12.10 | <0.0001 |
| SCC (cell.mL$^{-1}$) | 177,400b | 286,210a | 65.59 | 0.0010 |
| Milk fat (%) | 4.09b | 4.42a | 16.57 | 0.0329 |
| Milk protein (%) | 3.39a | 3.26a | 10.03 | 0.0660 |
| Milk lactose (%) | 4.51a | 4.41b | 4.16 | 0.0182 |
| Plasma lactose (µM) | 279.45a | 257.38a | 25.89 | 0.1462 |

CV (%): coefficient of variation. Variables followed by the same letter in the row are not significantly different inthe Tukey test at 5% probability

Mean stability in unstable and stable milk was 63.28 ˚GL and 79.01 ˚GL, respectively (Table 3). Animals producing stable and unstable milk were exposed to similar levels of THI. DIM was elevated in cows producing unstable milk, but milk production was reduced in these animals. Titratable acidity and

SCC did not differ according to milk stability. Milk and plasma lactose were higher in unstable milk group, while RR and RT were similar.

Table 3. Comparison between climatic, milk, plasma and physiological traits and number of days in milk from cows producing stable or unstable milk with corresponding significance levels.

| | Unstable milk | Stable milk | CV (%) | P=F |
|---|---|---|---|---|
| Milk stability (˚GL) | 63.28b | 79.01a | 8.39 | <0.0001 |
| Temperature-humidity index | 79.94a | 78.31a | 7.07 | 0.1886 |
| Days in milk | 276.56a | 232.64b | 26.31 | 0.0031 |
| Milk production ($L.day^{-1}$) | 12.60b | 16.12a | 35.24 | 0.0029 |
| Titratable acidity (˚D) | 14.02a | 14.16a | 13.45 | 0.7373 |
| SCC ($cell.mL^{-1}$) | 213,760a | 234,130a | 69.77 | 0.5585 |
| Milk lactose (%) | 4.53a | 4.42b | 4.16 | 0.0107 |
| Plasma lactose (µM) | 292.48a | 254.84b | 25.29 | 0.0139 |
| Respiratory rate ($breaths.min^{-1}$) | 83.41a | 76.67a | 43.83 | 0.3806 |
| Rectal temperature (˚C) | 39.95a | 39.61a | 2.71 | 0.1568 |

CV (%): coefficient of variation. Variables followed by the same letter in the row are not significantly different inthe Tukey test at 5% probability

Four groups (G1, G2, G3 and G4) were identified in correspondence analysis (Figure 1). G1 comprehended: low milk stability (L_MS), high plasma lactose (H_PL), low DIM (L_DIM) and high milk lactose (H_ML); G2: high SCC (H_SCC), high milk stability (H_MS), low plasma lactose (L_PL) and low milk lactose (L_ML); G3: low milk production (L_MP) and high rectal temperature (H_RT); G4: high milk production (H_MP) and low rectal temperature (L_RT). G1 and G2, as well as G3 and G4, presented opposite patterns. G1 and G2 indicate that animals with increased concentration of plasma lactose produce milk with reduced stability and higher milk lactose and vice-versa. High SCC was also related with low plasma lactose and the other variables in G2. G3 and G4, in agreement with analyses of variance, showed that animals with elevated rectal temperature produce less milk and vice-versa. High DIM and low SCC were not related with none of the groups.

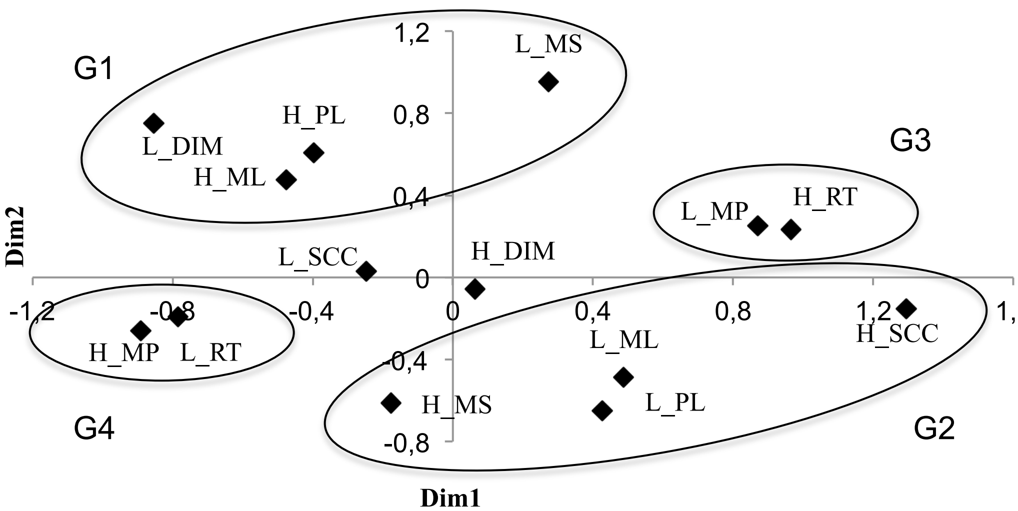


Figure 1: Correspondence analysis relating high (H_PL) or low plasma lactose (L_PL) with high (H_MS) or low milk stability to the ethanol test (L_MS), high (H_ML) or low milk lactose (L_ML), high (H_DIM) or low days in milk (L_DIM), high (H_SCC) or low somatic cell count (L_SCC), high (H_MP) or low milk production (L_MP) and high (H_RT) or low rectal temperature (L_RT).

The odds of cows producing unstable milk to 72 ˚GL increased in 1.825 units at every 50 μM increase in plasma lactose and in 1.338 units at 25 days increase in days in milk.

**Discussion**

THI in the morning was above critical (Armstrong, 1994; Zimbleman et al. 2009), but physiological parameters do not indicate a heat stress condition in this period. During the afternoon, THI was elevated to emergency levels (Hahn, 1985; DuPreez et al. 1990). In the afternoon, as expected, RT and RR, variables used to assess animal's adaptability to heat (Hemsworth et al. 1995), were increased to levels above normal (Stöber, 1993; Robinson, 1999), indicating that animals were under heat stress, specially in the afternoon of day 2, which presented the higher THI registered. To maintain heat dissipation mechanisms, the hierarchy of fuel supply is altered: energy partitioning to the mammary gland is reduced at the expense of an increase in energy utilization by extramammary tissues, such as the central nervous system and immune cells (Baumgard and Rhoads, 2013). Thus, milk production becomes physiologically deprecated and its levels are reduced, as encountered in the present experiment in the afternoon.Milk production in the afternoon of day 3 was reduced and might be related with the three days' stress length. Diminution in glucose partitioning to the mammary gland and/or lower glucose production by the liver (Baumgard and Rhoads, 2013) reduces lactose synthesis and secretion in milk (Rhoads et al. 2009), explaining afternoon results.

Higher milk fat in afternoonis associated with a reduction in milk production in a greater extend than in synthesis of fat, promoting a concentration effect (Lacy-Hulbert et al. 1999), which may have also caused the elevation in SCC (Table 2 and Figure 1). Increased SCC was alleged to elevate ionic calcium in milk (Barros et al. 1999); this ion reduces milk stability to the ethanol test by weakening the forces that prevent casein coagulation (Mikheeva et al. 2003). As in Kolling et al. (2011), the present experiment found no influence of SCC on reduction in milk stability - correspondence analysis, for example, showed that high milk stability was related with high SCC. Such lack of influence may attest the null effect of SCC on milk stability or may be because SCC, although elevated in afternoon milk, stayed at a low level and below the preconized by Ministério da Agricultura, Pecuária e Abastecimento (2011) - 400,000 cell.mL$^{-1}$. Milk protein level was unaltered by THI, but with a tendency of reduction in afternoon milk ($P<0.1$). Lower feed intake during heat stress period might have reduced energy and protein supply to the mammary gland (Lacetera et al. 1996), thus reducing protein synthesis.

Afternoon milk was more alkaline, which is in accordance with Bernabucci et al. (2002), who stated that the reduced titratable acidity during summer months was due to reduction in $\alpha_s$ and β-caseins, rich in phosphate groups and that are the two main acidic components of the casein micelles. Our experiment did not evaluate these milk traits, but such reduction may have occurred. The similarity in permeability of mammary gland cells TJ (measured by plasma lactose) between animals before and after heat stress may be the explanation for milk stability results; this relationship will be better explored later. Our results, on the contrary of other experiments relating stress induced by isolation (Stelwagen et al. 2000) and undernutrition (Stumpf et al. 2013), found no increase in TJ permeability due to stress. Acute heat stress increases glucocorticoids (cortisol) concentrations in blood after 20 minutes of exposition to elevated temperature (Christison and Johnson, 1972) and, according to Alvarez and Johnson (1973), reaches a peak after four hours. Cortisol was found to reduce tight junctions permeability (Thompson, 1996; Stelwagen et al. 1998) and may be the reason for the lack of heat stress influence on TJ opening.

Animals producing stable and unstable milk were exposed to similar THI, which explains the absence of alterations in RR and RT. These results eliminate the influences of ambient and heat dissipation mechanisms in milk parameters in Table 3 and Figure 1. Although Marques et al. (2004) observed elevated SCC in unstable milk, this was not the case in the present experiment. Titratable acidity was also unaffected and stayed at normal levels (Ministério da Agricultura, Pecuária e Abastecimento, 2011).

The relation between milk lactose and stability is not consistent. Fruscalso (2007) and Abreu (2008) found no alteration in lactose according with milk stability, while Tsioulpas et al. (2007) encountered reduced lactose as stability decreased. Our findings, contradicting the aforementioned studies, observed elevated lactose in unstable milk samples.

As in Stumpf et al. (2013), plasma lactose levels were elevated in cows producing unstable milk samples (Table 3 and Figure 1), suggesting once again that increased TJ permeability might be involved in reductions in milk stability to the ethanol test. Even not measured, elevation in milk monovalent cations ($Cl^-$ and $Na^+$) concentration and $Na^+/K^+$ ratio alteration (Wilde et al. 1995) due to increase TJ permeabiity (Linzell et al. 1975) may be responsible for the reduction in stability. Elevation in ionic strength, which is regulated by these and other ions, reduces the dielectric constant in milk, weakening the forces that prevent protein coagulation (Chavez et al. 2004).

The difference in milk stability cannot be attributed to THI and heat stress. Although low DIM and low milk stability were both in G1, Table 3 shows that elevated DIM may be responsible for reduced milk stability to the ethanol test (Barros, 1999), in our case, due to increased plasma lactose levels and tight junction permeability. These results are in agreement with estimation from logistic regression, in which a 50 µM in plasma lactose increases the odds of cows producing unstable milk samples (below 72 ˚GL) in a 1.825 unit. Also, elevation in 25 DIM increases these odds in 1.338 units.

**Conclusion**

Tight junctions permeability was not affected by heat stress, but induced reduction in milk stability, probably due to higher days in milk. We suggest the conduction of evaluations in cows with lower days in milk and exposed to a wider range of temperature-humidity index.

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# CAPÍTULO IV

## 4.1. CONCLUSÕES GERAIS

♦ Em um mesmo nível produtivo e sob condições climáticas semelhantes, vacas puras Holandesa apresentam menor tolerância ao calor do que vacas Girolando.

♦ Vacas Holandês em estresse térmico não apresentam aumento na permeabilidade das junções firmesdas células epiteliais da glândula mamária. Houve relação inversamente proporcional entre permeabilidade das junções firmes e estabilidade do leite ao teste do álcool.

## 4.2. CONSIDERAÇÕES FINAIS

O fato de todas as comparações entre os diferentes graus de sangue terem sido realizadas em animais com semelhante nível produtivo e, além disso, submetidos a condições similares de temperatura e umidade, evidenciam a menor capacidade de animais puros da raça Holandesa em tolerar calor. Dessa forma, sistemas produtivos devem enfocar no provimento de instalações preconizando o uso de mecanismos de resfriamento animal, como sombrites, ventiladores e aspersores, principalmente. Animais a pasto devem ter sombra e água à vontade em todos os locais da propriedade, sendo o uso de árvores uma opção barata, duradoura e eficiente. Nesse sentido, a utilização de sistemas de integração pecuária-floresta é alternativa interessante. A maior tolerância de bovinos Girolando ao calor não exclui a necessidade de controle das condições ambientais supracitadas. Animais mais tolerantes também apresentam sinais de estresse térmico, porém, a temperaturas mais elevadas do que animais puros da raça Holandesa.

A comparação entre os distintos graus de sangue foi realizada em condições de ITU acima do considerado crítico por alguns autores. Espera-se que, em faixas mais amplas de ITU, alterações biológicas e diferenças entre os grupos possam ser mais proeminentes e possam indicar de forma ainda mais clara a diferença em tolerância ao calor de animais puros de origem europeia e animais cruzados.

O capítulo III trouxe a confirmação da relação entre aumento na permeabilidade das junções firmes das células epiteliais da glândula mamária e redução na estabilidade do leite ao teste do álcool. Apesar disso, não se pode evidenciar a hipótese do estresse como promotor de elevação na permeabilidade dessas estruturas celulares. Essa ausência de efeito pode ser em virtude da característica do estresse, o qual, apesar de ser induzido em três dias consecutivos, durou apenas algumas horas em cada dia. Além disso, as noites não foram quentes, o que permitiu aos animais se aliviarem do estresse térmico durante o período noturno, iniciando o dia seguinte sem carga térmica acumulada. Casos de estresse crônico, em que os animais sejam expostos ao estresse de forma mais duradoura e em regiões/estações do ano com noites quentes, podem trazer resultados distintos do encontrado na presente tese. Tal hipótese se deve ao fato de o cortisol, hormônio responsável pela redução na permeabilidade das junções firmes, ser elevado em estresse agudo e reduzido em casos de estresse crônico.

Animais da raça Holandesa apresentaram menor estabilidade ao teste do álcool do que vacas Holandesa x Gir, porém, mais estudos são necessários para determinar se essa diferença se deve à tolerância ao calor ou à configurações genéticas dos animais.

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## 4.3. VITA

Nome: Marcelo Tempel Stumpf.
Filiação: Waldyr Stumpf Junior e Elisabeth Regina Tempel Stumpf.
Data de nascimento: 16/01/1986.
Local de nascimento: Porto Alegre/RS, Brasil.

Formado na escola Mário Quintana, em Pelotas, tanto no primeiro como no segundo grau. Engenheiro agrônomo formado pela Faculdade de Agronomia Eliseu Maciel da Universidade Federal de Pelotas (UFPel) no ano de 2009. Mestre em Zootecnia pela Universidade Federal do Rio Grande do Sul (2012) e ingressante no curso de doutorado do Programa de Pós Graduação em Zootecnia (UFRGS) no ano de 2012, sob orientação da Professora Doutora Concepta M. McManus Pimentel na área de nutrição e alimentação de ruminantes.