UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA FILHO”
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA
DEPARTAMENTO DE REPRODUÇÃO ANIMAL E RADIOLOGIA VETERINÁRIA

ASPECTOS FISIOLÓGICOS E REPRODUTIVOS DA EXPOSIÇÃO DE VACAS LEITEIRAS AO ESTRESSE TÉRMICO

RODRIGO DE ANDRADE FERRAZZA

BOTUCATU – SP
DEZEMBRO/2016

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Tese apresentada junto ao Programa de Pós-Graduação em Biotecnologia Animal para obtenção do título de Doutor
Orientador: Prof. Dr. João Carlos Pinheiro Ferreira
Co-orientadora: Profa. Dra. Elizabeth Moreira dos Santos Schmidt

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EPÍGRAFE

“Que os vossos esforços desafiem as impossibilidades, lembrai-vos de que as grandes coisas do homem foram conquistadas do que parecia impossível.”

Charles Chaplin

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LISTA DE ABREVIATURAS

a.m.	*Ante meridiem*
ABNT	Associação Brasileira de Normas Técnicas
ADF	Acid detergent fiber
AGC	Automatic gain control
ANOVA	Analysis of variance
BCS	Body condition score
bpm	Breaths per minute / Beats per minute
CEUA	Comissão de Ética no Uso de Animais
CID	Collision-induced dissociation
CP	Crude protein
D	Day
Dbt	Dry bulb temperature
DevF1	Largest follicle at deviation
DevF2	Second largest follicle at deviation
DM	Dry matter
DMI	Dry matter intake
DNA	Ácido desoxirribonucleico
DTT	1,4-dithiothreitol
E2	Estradiol
EDTA	Ethylenediaminetetraacetic acid
EE	Ether extract
F1	Largest follicle
F2	Second largest follicle
FAPESP	Fundação de Amparo à Pesquisa do Estado de São Paulo
FDR	False discovery rate
FF	Fluido folicular / Follicular fluid
FMVZ	Faculdade de Medicina Veterinária e Zootecnia
FSH	Hormônio folículo estimulante
FTMS	Fourier transform mass analyzers
FXR	Farnesoid X receptor
GnRH	Hormônio liberador de gonadotropina

h	Hour
HCD	High-energy collision-induced dissociation
HR	Heart rate
HR_1	Morning heart rate
HR_2	Afternoon heart rate
HS	Heat stress
IGFBPs	Proteínas de ligação do fator de crescimento semelhante à insulina
IGFs	Fatores de crescimento semelhante à insulina
IPA	Ingenuity Pathway Analysis software
ITMS	Ion trap mass spectrometry
ITU	Índice de temperatura e umidade
LC-MS	Liquid chromatography–mass spectrometry
LH	Hormônio luteinizante
LHr	Receptores de hormônio luteinizante
LXR	Liver X receptor
MHz	Megahertz
MM	Mineral matter
MS/MS	Tandem mass spectrometry
MS2	Second stage of mass spectrometry
MW	Molecular weight
NCBI	National Center for Biotechnology Information
NCE	Normalized collision energy
NDF	Neutral detergent fiber
NFC	Nonfibrous carbohydrates
NRC	National Research Council
p.m.	*Post meridiem*
P4	Progesterone
PCA	Principal component analysis
PGF2α	Prostaglandin F2α
pI	Isoelectric point
PMSF	Phenylmethylsulfonyl fluoride
PostDev	Post deviation
PreDev	Pre-deviation

PreOv	Pre-ovulatory
PSMs	Peptide spectrum matches
r	Correlation coefficient
R^2	Coefficient of determination
RH	Relative humidity
RH_{max-p}	Maximum relative humidity of the previous day
RH_{mean}	Mean relative humidity
RH_{mean-p}	Mean relative humidity of the previous day
RH_{min-p}	Minimum relative humidity of the previous day
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RR	Respiratory rate
RR_1	Morning respiratory rate
RR_2	Afternoon respiratory rate
RXR	Retinoid X receptor
SE	Standard error
SEM	Standard error of the mean
T	Treatment
Ta	Ambient temperature
TD	Interaction between treatment and day
Th1	T helper 1
Th2	T helper 2
THI	Temperature humidity index
THI_{max}	Maximum temperature humidity index
THI_{max-p}	Maximum temperature humidity index of the previous day
THI_{mean-p}	Mean temperature humidity index of the previous day
THI_{min-p}	Minimum temperature humidity index of the previous day
T_{max-p}	Maximum temperature of the previous day
T_{mean-p}	Mean temperature of the previous day
T_{min-p}	Minimum temperature of the previous day
TMT	Tandem mass tags
TN	Thermoneutral
Tre	Rectal temperature

Tre_1	Morning rectal temperature
Tre_2	Afternoon rectal temperature
UNESP	Universidade Estadual Paulista
VT	Vaginal temperature
VT_1	Morning vaginal temperature
VT_2	Afternoon vaginal temperature

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SUMÁRIO

FERRAZZA, R. de A. **Aspectos fisiológicos e reprodutivos da exposição de vacas leiteiras ao estresse térmico.** Botucatu, 2016. 159 p. Tese (Doutorado) – Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista (UNESP), Botucatu.

RESUMO

O estresse térmico reduz a eficiência reprodutiva em vacas leiteiras. O fluido folicular (FF) contém proteínas envolvidas na atividade e diferenciação celular do folículo e maturação oocitária. A caracterização das proteínas do FF pode contribuir para melhor compreensão dos mecanismos fisiológicos envolvidos na foliculogênese e comprometimento da função reprodutiva durante o estresse térmico. No primeiro experimento avaliou-se o perfil proteomico do FF de vacas em diferentes estágios do desenvolvimento folicular (pré-desvio, desvio, dominância e pré-ovulatório) e foi testada a associação das proteínas com as concentrações intrafoliculares de hormônios esteroides. Foram encontradas 22 proteínas diferentemente expressas ($P < 0.05$) entre as categorias foliculares, sendo o desvio um momento-chave na determinação da expressão das proteínas. Além disso, três proteínas foram correlacionadas ($P < 0.05$) com as concentrações intrafoliculares de estradiol, enquanto dezesseis foram correlacionadas ($P < 0.05$) com as concentrações locais de progesterona. A análise de vias canônicas identificou a ativação/inibição das vias associadas com a resposta de fase aguda, sistema de coagulação, sistema complemento, receptor X do fígado e retinóide e produção de óxido nítrico e espécies reativas de oxigênio. No segundo experimento, testou-se o efeito do estresse térmico sobre a expressão das proteínas do FF de vacas. Na validação do modelo

experimental, foi observado que a exposição contínua ao estresse térmico afetou os mecanismos termorregulatórios, levando a um aumento pronunciado da frequência respiratória, seguido pelo aumento da temperatura retal. As variáveis ambientais do dia anterior foram mais importantes para explicar as respostas do parâmetros fisiológicos e ingestão de matéria seca, sendo a temperatura ambiente o principal fator envolvido na troca de calor. Embora a exposição prolongada ao estresse térmico indicar um ajuste adaptativo, neste estudo foi observada apenas aclimatação parcial. O crescimento folicular das vacas expostas ao estresse térmico foi afetado e pode, em parte, ser explicado pela expressão alterada das proteínas do FF. As 28 proteínas diferentemente ($P < 0.05$) expressas no FF de vacas estressadas por calor foram associadas, principalmente, às vias da função imune e cascata da coagulação. Tais achados indicam potenciais mecanismos moleculares e vias de sinalização envolvidos na foliculogênese e redução da fertilidade observada durante o verão.

Palavras-chave: Estresse térmico; Termorregulação; Foliculogênese; Proteômica; Bovino

FERRAZZA, R. de A. **Physiological and reproductive aspects of exposure of dairy cows to heat stress.** Botucatu, 2016. 159 p. Thesis (Doctorate) – School of Veterinary Medicine and Animal Science, Univ. Estadual Paulista (UNESP), Botucatu.

ABSTRACT

Heat stress reduces the reproductive efficiency in dairy cows. Follicular fluid (FF) contains proteins involved in the follicle activity and cell differentiation and oocyte maturation. The characterization of the proteome of FF may contribute to a better understanding of the physiological mechanisms involved in folliculogenesis and impairment of reproductive function during heat stress. In the first experiment, the FF proteomic profile of cows at different stages of follicle development (pre-deviation, deviation, dominance and pre-ovulatory) was evaluated and the association of proteins with intrafollicular concentrations of steroid hormones was tested. Twenty-two differentially expressed proteins ($P < 0.05$) were found among the follicular categories, with deviation being a critical time-point in the determination of protein expression. In addition, three proteins were correlated ($P < 0.05$) with the intrafollicular concentrations of estradiol, while sixteen were correlated ($P < 0.05$) with the local concentrations of progesterone. The canonical pathway analysis identified the activation/inhibition of the pathways associated with acute phase response, coagulation system, complement system, liver X receptor and retinoid, and production of nitric oxide and reactive oxygen species. In the second experiment, the effect of heat stress on the expression of cow FF proteins was tested. In the experimental model validation, we observed that the continuous exposure to heat stress affected thermoregulatory mechanisms,

leading to a marked increase in respiratory rate, followed by an increase in rectal temperature. The influence of environmental variables from the previous day on physiological parameters and dry matter intake were more important than the immediate effect and ambient temperature represented the most determinant factor for heat exchange. Although prolonged exposures to heat stress indicate an adaptive adjustment, in this study the acclimation process was only partial. The follicular growth of cows exposed to heat stress was affected and may be partly explained by the altered expression of the FF proteins. The 28 differentially ($P < 0.05$) expressed proteins in FF from heat stressed cows were closely associated to the immune function and the coagulation cascade pathways. Such findings indicate potential molecular mechanisms and signaling pathways involved in folliculogenesis and reduction fertility observed during the summer.

Keywords: Heat stress; Thermoregulation; Folliculogenesis; Proteomics; Bovine

Capítulo I

1. INTRODUÇÃO

O estresse térmico diminui a produção e a fertilidade de rebanhos leiteiros e, portanto, é um fator de significativo impacto econômico. Embora avanços nas estratégias mitigadoras de calor foram obtidos nos últimos anos, os efeitos negativos do estresse térmico na produção ainda são observados e a fertilidade continua reduzida durante o verão. Além disso, preocupações existem sobre o aquecimento global agravar ainda mais esse problema.

Os efeitos do estresse térmico sobre a fertilidade incluem aumento dos dias em aberto, diminuição da taxa de concepção e maior incidência de vacas em anestro, com efeito não só limitado ao verão, mas também nos meses subsequentes. A redução na fertilidade causada pelo estresse térmico é um problema de ordem multifatorial, pois o estresse térmico afeta as funções fisiológicas e celulares em vários tecidos. No que diz respeito à função reprodutiva, já foi demonstrado que o estresse térmico compromete o crescimento folicular (BADINGA et al., 1994; WOLFENSON et al., 1995), a secreção hormonal (WOLFENSON et al., 1995; ROTH et al., 2000), o fluxo sanguíneo para o útero (ROMAN-PONCE et al., 1978), a função do endométrio (MALAYER; HANSEN; BUHI, 1988) e do oócito (AL-KATANANI; PAULA-LOPES; HANSEN, 2002). Uma vez confirmada a gestação, o estresse térmico também pode afetar a capacidade de desenvolvimento do embrião (PAULA-LOPES; HANSEN, 2002).

Diante dos efeitos negativos do estresse térmico na reprodução, torna-se imprescindível a realização de estudos a fim de melhorar a eficiência dos sistemas de produção animal em ambientes de clima quente. Diversos estudos apontam que o estresse térmico reduz a eficiência reprodutiva em vacas leiteiras,

porém, os mecanismos moleculares que levam a infertilidade no verão são apenas parcialmente compreendidos. Dessa forma, um melhor entendimento dos mecanismos envolvidos na foliculogênese permitirá não só maior controle sobre a função reprodutiva, mas também novas abordagens no tratamento da infertilidade ocasionada pelo estresse térmico. Neste contexto, os presentes estudos têm como objetivo caracterizar o perfil proteômico do fluido folicular em diferentes estágios do desenvolvimento folicular em condições termoneutras; investigar os mecanismos de termorregulação e preditores de balanço calórico envolvidos durante o estresse térmico; e testar o efeito do estresse térmico sobre a expressão das proteínas do fluido folicular de vacas leiteiras.

2. REVISÃO DE LITERATURA

2.1. Desenvolvimento folicular

Os bovinos são animais poliéstricos anuais e apresentam um ciclo estral de 21 dias de duração, com duas a três ondas de crescimento folicular (FORTUNE; SIROIS; QUIRK, 1988). As ondas foliculares têm duração de 7 a 10 dias, desde o surgimento da onda até a emergência da próxima onda (indicando ovulação ou atresia fisiológica do folículo dominante) (GINTHER; KNOPF; KASTELIC, 1989). Em cada onda folicular, o período inicial é caracterizado por uma fase de desenvolvimento comum, em que os folículos crescem em diâmetro em resposta ao aumento dos níveis séricos do hormônio folículo estimulante (FSH). O FSH possui papel chave na regulação da emergência das ondas foliculares e manutenção do crescimento dos folículos durante o período inicial de desenvolvimento (ADAMS et al., 1992). As concentrações plasmáticas de FSH começam a diminuir nos dias dois e três após a emergência e então alguns folículos da onda cessam o seu desenvolvimento e entram em atresia, com exceção do futuro folículo dominante que adquire uma “independência” ao FSH. O desvio folicular é caracterizado pelo crescimento continuado do maior folículo ovariano e redução ou interrupção do crescimento dos folículos menores (GINTHER, 2016). O desvio folicular ocorre quando o maior folículo se encontra com um diâmetro de aproximadamente 8,5 mm em raças taurinas (KULICK et al., 2001; GINTHER, 2016) e 6,0 mm em zebuínas (GIMENES et al., 2008).

O papel da expressão de receptores de hormônio luteinizante (LH) nas células da granulosa para o estabelecimento da dominância é tema de contradição há bastante tempo. Inicialmente, acreditava-se que a independência ao FSH era determinada diretamente pela aquisição de receptores de LH nas

células da granulosa. No entanto, Evanst e Fortune (1997) demonstraram, por hibridização *in situ*, níveis indetectáveis de LHr nas células da granulosa durante a divergência folicular e caracterizaram a participação de fatores locais no suporte do crescimento folicular durante a fase de níveis séricos reduzidos de FSH (FORTUNE et al., 2001; FORTUNE; RIVERA; YANG, 2004). Dentre esses fatores, a participação do fator de crescimento semelhante a insulina (IGF), assim como de suas proteínas de ligação (IGFBPs) e proteases específicas, no crescimento folicular de bovinos, está bem estabelecida (SPICER; AAD, 2007). Esses resultados suportam a hipótese de que a seleção folicular não é mediada, e sim uma causa, da aquisição de receptores de LH na granulosa.

O IGF1 e estradiol atuam em sinergismo com o FSH na estimulação da proliferação e esteroidogênese das células da granulosa *in vitro* (MONGET et al., 2002). O IGF2 produzido pela teca é o principal ligante intrafolicular regulando o crescimento de folículos antrais bovinos via IGF1R. O nível de IGF livre depende das IGFBPs, sendo que folículos estrógeno-ativos possuem baixas concentrações das IGFBP-2, -4 e -5, enquanto que nos folículos atrésicos, apresentam-se elevadas, com consequente diminuição da biodisponibilidade de IGF (SPICER; AAD, 2007). Concentrações elevadas de IGFs livres estimulam a síntese de andrógenos, bem como a atividade da aromatase (CYP19A1) e produção de inibina (BEG et al., 2002).

Além disso, outros fatores produzidos localmente também têm sido apontados como envolvidos na seleção do folículo dominante e atresia dos subordinados. Mihm et al. (2008) caracterizaram genes diferentemente expressos entre folículos dominantes e subordinados. Esses fatores locais atuam de maneira autócrina/parácrina em funções indispensáveis para o

desenvolvimento folicular, incluindo esteroidogênese, regulação do ciclo celular, diferenciação e proteção contra apoptose.

A dominância é a fase durante a qual um único folículo selecionado suprime ativamente as concentrações de FSH e o crescimento dos demais folículos ovarianos. O folículo dominante torna-se então dependente da frequência de pulsos de LH. Na presença de progesterona elevada (fase lútea em animais cíclicos) a frequência de pulso de LH é mantida a um pulso a cada quatro horas e o folículo dominante sofre atresia. Na fase folicular (período pré-ovulatório em animais cíclicos) a frequência de pulso de LH aumenta para um pulso por hora, de modo a estimular a maturação final do folículo, o aumento das concentrações de estradiol e o *feedback* positivo sobre o hormônio liberador de gonadotropina (GnRH), em uma onda que induz a ovulação (SUNDERLAND et al., 1994).

2.2. Estresse térmico

A vaca leiteira é um animal homeotérmico e, portanto, possui zonas ótimas de temperatura, em que nenhuma energia adicional de mantença é requerida para aquecer ou resfriar o corpo. O estresse térmico pode ser definido como a tensão exercida pelos fatores climáticos sobre um organismo, determinando uma reação fisiológica proporcional à intensidade de estímulo aplicado e à capacidade de compensação do organismo (SILVA, 2000). A zona de conforto térmico para vaca leiteira foi estimada em 5°C a 25 °C (Nutrient Requirements of Dairy Cattle, 2001). Dentro desse intervalo de temperatura, as demandas fisiológicas são mínimas, a produtividade é ótima e as vacas são capazes de manter a temperatura corporal estável. Por outro lado, temperaturas

maiores do que 25 °C podem ser atingidas em regiões tropicais e subtropicais, e mesmo em regiões de clima temperado. Além da temperatura ambiente, radiação solar, umidade relativa, velocidade do vento e precipitação também contribuem para o grau de estresse térmico.

Os métodos para avaliação ou predição da sensibilidade de bovinos ao calor baseiam-se no uso de índices que levam em conta os fatores ambientais. O índice de temperatura e umidade (ITU), valor único que representa os efeitos combinados da temperatura e umidade do ar, apesar de ter sido inicialmente desenvolvido por Thom (1959) como um indicador de calor para conforto humano, é comumente usado para mensurar o nível de estresse térmico em vacas leiteiras (BIANCA, 1962; JOHNSON et al., 1962; HAHN, 1969). Um valor de ITU abaixo de 72 unidades geralmente não causa desconforto para vacas em lactação (ARMSTRONG, 1994), embora estudos mais recentes têm sugerido valores limiares menores (BOURAOUI et al., 2002).

2.3. Efeito do estresse térmico nos parâmetros fisiológicos e desempenho produtivo

A termorregulação é um processo evolutivo que permite ao animal manter o equilíbrio entre a temperatura do ambiente e a temperatura corporal. Essa habilidade é mediada por processos fisiológicos, em que os receptores periféricos enviam sinais ao cérebro, por meio do sistema nervoso, e ativam os mecanismos de termorregulação (CURTIS, 1983). Os mecanismos de termorregulação ativados durante o estresse térmico incluem a vasodilatação periférica, além do aumento da sudação e da frequência respiratória (SILVA, 2000). Com a vasodilatação há aumento do fluxo sanguíneo para regiões

periféricas na tentativa de equilibrar a temperatura corporal (MCGUIRE et al., 1989). O aumento da sudação e da frequência respiratória são as primeiras alterações visíveis. Quando esses mecanismos não conseguem regular a temperatura corporal, a soma do calor interno com o ambiental faz com que o animal apresente aumento na temperatura retal (SILVA, 2000). Paralelamente, ocorre redução no consumo de alimentos, na tentativa de reduzir o metabolismo basal e manter a temperatura constante. A magnitude da redução no consumo de alimentos é diretamente proporcional ao estresse térmico sofrido, sendo atribuída à inibição do centro do apetite no hipotálamo (BAILE; FORBES, 1974). Há também o aumento da ingestão de água para reposição das perdas sudativas e respiratórias, além de promover o resfriamento corporal, por meio do contato direto com as mucosas do trato digestivo (KHELIL-ARFA; FAVERDIN; BOUDON, 2014).

Durante o estresse térmico, a eficiência da perda de calor é essencial para manutenção da homeotermia. As alterações no processo de homeostase têm sido quantificadas por meio de medições das variáveis fisiológicas. A temperatura retal (SPIERS et al., 2004) e a frequência respiratória (GAUGHAN, 2000) têm se mostrado bons indicadores de estresse térmico para bovinos. A habilidade para predizer os efeitos climáticos sobre o rebanho é de extrema importância, não apenas para garantir o bem-estar animal, mas também com o intuito de sustentar o desempenho animal e, em última análise, a lucratividade.

2.4. Efeito do estresse térmico na função reprodutiva

Os efeitos do estresse térmico sobre a fertilidade têm sido amplamente estabelecidos no mundo todo e incluem um aumento dos dias em aberto,

redução da taxa de concepção e aumento do número de vacas em anestro. Em um estudo anterior, as taxas de concepção de vacas em lactação diminuíram de 52% para 32% quando a temperatura ambiente máxima aumentou de 23,9 °C para 32,2 °C (BADINGA et al., 1985). Similarmente, outro estudo demonstrou redução da taxa de concepção de 31% para 12% quando as vacas foram expostas ao estresse térmico 21 dias antes do dia do serviço (SCHÜLLER; BURFEIND; HEUWIESER, 2014). Al-Katanani, Webb e Hansen (1999) relataram que a taxa de não retorno ao cio 90 dias após a primeira inseminação foi menor quando a temperatura ambiente foi maior do que 20 ºC no dia -10, no dia do estro (dia 0) e no dia +10.

Os efeitos das altas temperaturas podem ser encontrados em todas as fases do desenvolvimento folicular. Um dos efeitos mais proeminentes do estresse térmico sobre o desenvolvimento folicular é a redução da diferença entre folículos dominantes e subordinados (WOLFENSON et al., 1995; GUZELOGLU et al., 2001). A diminuição do tamanho do folículo de vacas submetidas ao estresse térmico foi associada com a redução das concentrações séricas de estradiol (WOLFENSON et al., 1997), o que pode estar relacionado com falha da esteroidogênese no interior das células da teca, da granulosa ou ambas (WILSON et al., 1998b). Em condições *in vitro*, o choque térmico reduziu a produção de androstenediona e estradiol no interior dos folículos dominantes (BRIDGES; BRUSIE; FORTUNE, 2005). Uma vez que a produção de estradiol pelo folículo é prejudicada, ocorre um atraso na ovulação, além do comprometimento dos mecanismos luteolíticos (BRIDGES; BRUSIE; FORTUNE, 2005), que são dependentes do estrógeno.

A ausência de um folículo dominante durante a primeira onda folicular pode estar relacionada a diminuição das concentrações plasmáticas de inibina, fazendo com que a secreção de FSH não diminua e o crescimento de folículos subordinados continue (ROTH et al., 2000). A seleção folicular prejudicada pode levar a codominância (SARTORI et al., 2004), anovulação ou dupla ovulação (LÓPEZ-GATIUS et al., 2005). A dominância atenuada também foi associada com a emergência precoce da segunda onda folicular (WOLFENSON et al., 1995) e consequente ovulação de folículos mais velhos, os quais apresentam menor fertilidade (MIHM et al., 1994). Além disso, Shehab-El-Deen et al. (2010) relataram a diminuição na concentração de IGF1 no interior do folículo dominante, de maneira que, sem esse fator de crescimento, o folículo não se desenvolve o suficiente para impedir o crescimento dos folículos subordinados.

Outros prejuízos causados pelo estresse térmico incluem a queda na qualidade dos oócitos. Isso se deve a influência do estresse térmico na indução de alterações morfológicas no núcleo e no citoplasma do oócito ao longo do processo de maturação (ROTH; HANSEN, 2005), comprometendo a fertilização e o desenvolvimento embrionário subsequente (SARTORI et al., 2002). Outros estudos *in vitro* demonstraram que a diminuição da qualidade e da competência de desenvolvimento de oócitos expostos ao choque térmico também está relacionada à apoptose (ROTH, 2004). Além disso, a temperatura elevada pode ser responsável pela formação e acúmulo de radicais livres nas células, o qual é característico do estresse oxidativo. O efeito tóxico do estresse oxidativo nas células pode levar à peroxidação lipídica, oxidação de proteínas, danos ao DNA e indução da apoptose (CRUZ et al., 2014). Coletivamente, esses dados sugerem que a dinâmica folicular alterada, a supressão da produção de

hormônios esteróides e a baixa qualidade de oócitos contribuem para falhas reprodutivas de vacas leiteiras submetidas ao estresse térmico.

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Capítulo II

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Thermoregulatory responses of Holstein cows exposed to experimentally induced heat stress

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Abstract

Heat stress (HS) adversely influences productivity and welfare of dairy cattle. We hypothesized that the thermoregulatory mechanisms vary depending on the exposure time to HS, with a cumulative effect on the adaptive responses and thermal strain of the cow. To identify the effect of HS on adaptive thermoregulatory mechanisms and predictors of caloric balance, Holstein cows were housed in climate chambers and randomly distributed into thermoneutral (TN; n=12) or HS (n=12) treatments for 16 days. Vaginal temperature (VT), rectal temperature (Tre), respiratory rate (RR), heart rate (HR) and dry matter intake (DMI) were measured. The temperature and humidity under TN were 25.9±0.2°C and 73.0±0.8%, respectively, and under HS were 36.3±0.3°C and 60.9±0.9%, respectively. The RR of the HS cows increased immediately after exposure to heat and was higher (76.02±1.70bpm, $p<0.001$) than in the TN (39.70±0.71bpm). An increase in Tre (39.87±0.07°C in the HS vs. 38.56±0.03°C in the TN, $p<0.001$) and in VT (39.82±0.10°C in the HS vs. 38.26±0.03°C in the TN, $p<0.001$) followed the increase in RR. A decrease ($p<0.05$) in HR occurred in the HS (62.13±0.99bpm) compared with the TN (66.23±0.79bpm); however, the magnitude of the differences was not the same over time. The DMI was lower in HS cows from the third day (8.27±0.33kg d^{-1} in the HS vs. 14.03±0.29kg d^{-1} in the TN, $p<0.001$), and the reduction of DMI was strongly affected ($r=-0.65$) by changes in the temperature humidity index. The effect of environmental variables from the previous day on physiological parameters and DMI was more important than the immediate effect, and ambient temperature represented the most determinant factor for heat exchange. The difference in the responses to acute and chronic exposure to HS suggests an adaptive response. Thus, intense thermal stress strongly influence thermoregulatory mechanisms and the acclimation process depend critically on heat exposure time.

Keywords: Hyperthermia; Thermoregulation; Thermal index; Acclimation; Climate chamber; *Bos taurus*

1. Introduction

Most human and domestic animal populations are located in regions where seasonal stressors negatively influence productivity (Collier et al., 2006), and dairy herds are the most affected by heat stress (HS) among livestock industries in terms of economic losses (St. Pierre et al., 2003). The climate zones differ across the globe and, although some regions, including the Southeastern United States, are hot enough to cause HS during the summer, concerns exist that a potential global warming may worsen the problem (Nardone et al., 2010), decreasing herd productivity (West et al., 2003) and increasing mortality (Vitali et al., 2015).

Heat stress occurs when animals are exposed to temperatures that exceed their thermal comfort zone, and the rate of heat production is higher than the rate of heat dissipation (Aggarwal and Upadhyay, 2013). Several environmental factors, such as, temperature, relative humidity, wind speed, solar radiation, and precipitation may be associate with HS. The temperature humidity index (THI; Thom, 1959) combines the simultaneous effect of temperature and humidity and has been widely used to assess the degree of HS in livestock.

During HS conditions, the efficiency of heat loss is essential for the maintenance of homeothermy (Bligh, 1998). Cattle have evolved a series of physiological mechanisms for dealing with HS, including increase of core body temperature, respiratory and panting rates, sweating, and endocrine system changes (Blackshaw and Blackshaw, 1994; Sanchez et al., 1994; Gaughan et al., 1999). The ability to predict the effects of the climate on the herd is essential to ensure animal welfare and support animal performance and,

ultimately, to increase profitability. In addition, evidence exists that the effects of some environmental variables, including air temperature, do not have an instantaneous effect; instead, the effect occurs after a delay (West et al., 2003). However, the lag effects of the climate variables on physiological responses has had limited research, particularly in controlled environments.

Although many studies have evaluated the effect of HS on livestock production and unquestionably progress has been achieved in recent years with the improvement of heat mitigation techniques, many efforts still need to be made to elucidate the physiological mechanisms involved in thermoregulation of bovine species. Thus, we postulated that the activation of the thermoregulatory mechanisms vary depending on the duration of heat exposure, with a cumulative effect on the adaptive responses and thermal strain of the cow. Because the dynamic interactions among the components of the animal production system are complex, herein, we tested these hypotheses by isolating the effect of HS, which was experimentally induced in a climate chamber in a continuous and prolonged manner.

Therefore, the objectives of this study were (1) to investigate how the physiological parameters of Holstein cows are affected by HS conditions to understand the activation of adaptive mechanisms for heat dissipation, (2) to determine the best predictors of heat balance, (3) to evaluate the influence of the day shift on physiological parameters, and (4) to determine the effect of duration of heat exposure on the physiological responses.

2. Materials and Methods

The experiment was conducted at the Lageado Experimental Farm, São Paulo State University, School of Veterinary Medicine and Animal Science (Universidade

Estadual Paulista, Faculdade de Medicina Veterinária e Zootecnia - UNESP, FMVZ), Botucatu campus, State of São Paulo, Brazil. The procedures used for animal handling and care were approved by the Animal Research Ethics Committee of the UNESP under protocol No. 86/2013.

2.1. Cows and adaptation

Eighteen clinically healthy, nonlactating, pluriparous Holstein cows (*Bos taurus*) with black and white coats and between 3 and 8 years of age were used. The study was conducted between October and December 2013. Prior to the experiment, the cows were kept in a pasture-based system at the Lageado Experimental Farm. All cows underwent a pretrial period of conditioning to reduce the reactivity and improve the adjustment to the new environment and intensive management. For this purpose, the cows were maintained with halters and were managed and tied with ropes in the pens in increasing periods ranging from 15 minutes to 3 hours over 3 weeks in an area close to the climate chambers.

2.2. Experimental design and facilities

The experiment was conducted in a completely randomized design with two treatments (TN or HS) and four replicates. Six cows were used in each replicate. Therefore, at the end of the study, each treatment consisted of 12 cows. To avoid a possible carryover effect, cows subjected to HS treatment were not used in the subsequent replicates. The cows were synchronized using Ovsynch protocol (Pursley et al., 1995) added with an intravaginal progesterone device (Sincrogest, Ourofino, Brazil). Only ovulating cows were selected and housed in the climate chamber.

The climate chamber consisted of a set of two rooms (length of 4.30 m, width of 4.50 m, and height of 2.60 m), each with capacity for three cows. The cows were kept in

individual pens (3.00 x 1.10 m) with a concrete floor covered with rubber mats and had free access to food and water. The temperature control of the climate chamber was automated using a digital temperature controller located outside the chamber and a set of two sensors installed at a height of 1.70 m inside the chamber. The air was heated by oil and resistance electric heaters. The ambient temperature (Ta) was programmed for the TN and HS groups at 24 °C and 38 °C, respectively, and maintained during the 16-day experimental period. Two exhaust fans, located on the side walls of each room of the climate chamber, allowed the exchange of air with the outside. Artificial light in the climate chamber was controlled in a 12-hour regimen of fluorescent light (7:01 a.m. to 7:00 p.m.) and 12 hours of darkness (7:01 p.m. to 7:00 a.m.).

2.3. Feeding management and diet composition

All cows were subjected to the same feed management. The cows were fed individually twice a day (8 a.m. and 4 p.m.) with a total mixed ration (Table 1), and water *ad libitum*. The diet was formulated to meet or exceed the nutritional requirements of nonlactating Holstein cows according to the National Research Council recommendations (NRC, 2001). The amount of food offered to each cow was adjusted daily to yield an excess of at least 5%.

Samples of forage and concentrate ingredients were collected weekly and dried at 60 °C for 72 hours to determine moisture loss. The dried samples were ground in a Wiley mill (Arthur H. Thomas, Philadelphia, PA, USA) in a sieve with a 1 mm mesh. The variables measured were dry matter content (DM), which was dried in a forced air oven at 105 °C for 24 hours (AOAC, 1990); crude protein (CP), using the Dumas method (Wiles et al., 1998); ether extract (EE; AOAC, 1990); mineral matter (MM; AOAC, 1990); neutral detergent fiber (NDF), using sodium sulfite and α-amylase (Van Soest et

al., 1991); and acid detergent fiber (ADF; Van Soest et al., 1991). Nonfibrous carbohydrates (NFC) were calculated as follows: $100 - (CP + NDF + EE + MM)$.

2.4. Measurement of physiological variables and dry matter intake

Vaginal temperature (VT) was recorded every 15 minutes by a data-recording device (HOBO®, Onset Computer Corporation, Bourne, MA, USA) coupled to an intravaginal device without progesterone (Sincrogest®, Ourofino, Brazil). This experiment was conducted in conjunction with another unrelated experiment in which the removal of the intravaginal thermometer between days 5 and 7 was necessary to allow ovarian follicular aspiration. Respiratory rate (RR), heart rate (HR), and rectal temperature (Tre) were evaluated sequentially twice a day, at 7 a.m. and 3 p.m. Respiratory rate was expressed in breaths per minute (bpm) and was obtained using a timer to count the flank movements of the animal for 30 seconds, multiplied by 2 to obtain the number of breaths per minute. Heart rate was expressed in beats per minute (bpm) and was obtained using a flexible stethoscope (Standard, Bic Med, SP, Brazil) placed directly into the left thoracic region under one of the auscultation foci for 30 seconds, multiplied by 2 to obtain the number of beats per minute. Rectal temperature was measured with a large animal clinical thermometer (Model #5198.6, Incoterm, RS, Brazil) inserted to depth of 3 cm into the animal's rectum and held to maintain contact with the mucosa for one minute. Body condition score (BCS) was determined at the beginning of the experiment and weekly during the study period. A scale of 1 (thin) to 5 (obese) in increments of 0.25 units was used, as described by Ferguson et al. (1994). A single observer evaluated the BCS throughout the study to minimize variations.

The feed leftovers were weighed once daily before the morning feeding. Dry matter intake (DMI) was determined daily on an individual basis by subtracting the

weight of the leftovers from the total amount of feed offered each day. DMI was calculated on the basis of the feed material dried at 105 °C.

2.5. Collection of environmental data and calculation of the temperature humidity index

Ambient temperature and relative humidity (RH) were continuously recorded every 30 minutes using two sensors (HOBO U12 data logger, Onset Computer Co., Bourne, MA, USA) installed at a height of 1.70 m above the ground in each room of the climate chamber. The THI was calculated from the following mathematical equation proposed by Mader et al. (2006):

$THI = (0.8 \times Dbt) + [(RH/100) \times (Dbt - 14.4)] + 46.4$, in which Dbt = dry bulb temperature, and RH = relative humidity.

2.6. Data processing

The individual data for Ta and RH were obtained using an automatic data-logging system and were used to calculate the THI. The maximum and minimum mean of Ta, RH and THI were calculated from maximum and minimum daily values, respectively. The mean values of the environmental variables and physiological parameters in the morning (12:00 a.m. to 11:59 a.m.), afternoon (12:00 p.m. to 11:59 p.m.), and daily were calculated to correlate physiological responses and environment variables. To establish HS thresholds, Tre and RR were grouped into three THI intervals using a modified scale initially proposed by Hahn et al. (1999). In addition, to assess the effect of the duration of heat exposure, the first and last four experimental days were established for acute and chronic responses, respectively.

201 2.7. Statistical analysis

202 The parametric assumptions were tested using Levene's test (homoscedasticity)
203 and the Kolmogorov-Smirnov test (normality). The mean, minimum, and maximum
204 values of the environmental variables were compared between treatments using analysis
205 of variance (ANOVA) followed by Student's t-test. Differences were considered
206 significant when $p < 0.05$.

207 Data with a normal distribution were analyzed using PROC MIXED with repeated
208 measures over time in SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). The effects
209 of treatment, day, and their interaction were included as fixed effects. The Tukey-Kramer
210 test for the comparison of means was used in cases in which a factor or interaction was
211 significant. The statistical model used in the study was expressed by the equation:
212 $Y_{ijk} = \mu + A_i + B(A)_{ij} + C_k + AC_{ik} + e_{ijk}$, in which Y_{ijk} = dependent observation, μ
213 = overall mean, A_i = effect of treatment i, $B(A)_{ij}$ = effect of cow j under condition i, C_k
214 = effect of day k, AC_{ik} = effect of the treatment x day interaction, and e_{ijk} = residual.

215 The model was fitted to the data using the maximum likelihood method. The best
216 matrix was identified using Akaike's information criterion, so the lower the value was,
217 the better the fit of the model in question.

218 The nonparametric Wilcoxon test was applied to the BCS data, which were non-
219 normally distributed. Significant differences existed when $p < 0.05$, and trends were
220 present when $p > 0.05$ and ≤ 0.1. All results are expressed as mean and standard error of
221 the mean.

222 The association between the physiological parameters and the environmental
223 conditions was evaluated using the Pearson correlation test (PROC CORR). The results
224 are presented as correlation coefficients, and significant differences were present when p
225 < 0.05. The correlation coefficients were classified as strong ($r > 0.6$), moderate ($0.6 \leq r$

≥ 0.4), or weak (r < 0.4). Regression was performed using the REG procedure of SAS. The models were fitted according to the significance of the regression coefficients ($p < 0.05$) and according to the coefficients of determination (R^2) using the following polynomial model:

$Y_{ijk} = \alpha + Bx + Bx^2 + Bx^3 + \cdots + Bx^n$, in which Y_{ijk}= dependent observation; α = constant; and Bx, Bx^2, Bx^3, Bx^n = estimated regression coefficients.

The effect of environmental factors on the physiological parameters was estimated using multiple regression analysis with PROC REG in SAS. The dependent variables were Tre, VT, RR, HR, and DMI. The independent variables of the initial model included daily mean, minimum, and maximum values for Ta, RH, and THI. The analyses were performed using the forward, backward, and stepwise selection methods. Variables with $p < 0.05$ were retained in the model. The relative contributions of the environmental factors (independent variables) on the dependent variables were determined using partial regression coefficients.

Simple polynomial and linear regressions were constructed using PROC REG in SAS to establish the correlation between the physiological parameters and the THI for acute (0 to 3 days) and chronic periods of exposure (13 to 16 days) to HS.

3. Results

3.1. Environmental conditions

Ambient temperature, THI, and RH exhibited a treatment effect ($p < 0.0001$); Ta (36.3 ± 0.3 °C vs. 25.9 ± 0.2 °C) and THI (88.7 ± 0.4 vs. 75.4 ± 0.3) were higher in the HS group than in the TN group, whereas RH was lower in the HS group (60.9 ± 0.9% vs. 73.0 ± 0.8%). A similar result was observed for the mean minimum and maximum values of these variables, except for maximum RH, which was similar ($p > 0.05$) between the

two treatments (Table 2). The comparison of these variables in the morning and afternoon separately in each experimental environment indicated higher values in the afternoon, except for RH in the TN group, which was higher in the morning ($p < 0.01$; Table 3).

3.2. Physiological variables, BCS, and DMI

A significant interaction was present between the treatment and day for Tre ($p < 0.0001$) and VT ($p < 0.01$) from 12 hours in the climate chamber (afternoon reading), and both variables were higher ($p < 0.05$) for cows under HS treatment. The cows under TN treatment maintained normothermia (Houston and Radostits, 2000) (38.56 ± 0.03 °C and 38.26 ± 0.03 °C for Tre and VT, respectively), whereas the temperatures of cows under HS were 39.87 ± 0.07 °C and 39.82 ± 0.10 °C for Tre and VT, respectively (hyperthermia commonly experienced by dairy cows during summer; Figure 1 and Table 4). Higher means were observed ($p < 0.01$) in the afternoons than in the mornings, and this effect was always greater for the animals under HS. However, no significant difference in VT ($p > 0.05$) was observed between the morning and afternoon for cows under HS (Table 3). Thermoneutral treatment caused oscillations of 0.77 °C in Tre (between the minimum and maximum values), whereas HS caused oscillations of 1.53 °C.

The daily mean RR was 39.70 ± 0.71 and 76.02 ± 1.70 bpm for the TN and HS groups, respectively (Table 4). A significant effect existed from the interaction of the treatment and day; this effect was higher ($p < 0.0001$) in the HS condition immediately after the cows entered the climate chamber and was high throughout the experimental period (Figure 2A). Similar to Tre, the RR was higher ($p < 0.01$) in the afternoons than in the mornings. The maximum peak of the RR was 130 bpm under HS treatment and 90 bpm under TN treatment (Table 3).

There was interaction between treatment and day ($p < 0.03$) for HR, and this interaction decreased throughout the experimental period for the HS treatment. The mean value observed for HR was 66.23 ± 0.79 bpm under TN treatment and 62.13 ± 0.99 bpm under HS treatment. The cows exhibited differences between days, but the magnitude of the differences was not the same over time (Figure 2B). Heart rate was higher ($p < 0.01$) in the afternoons in both experimental treatments (Table 3).

The DMI was significantly affected by treatment ($p < 0.001$), day ($p < 0.005$), and treatment x day interaction ($p < 0.005$; Figure 3). The cows under TN increased their DMI during the experimental period, whereas those under HS decreased their DMI on the third day after entering the climate chamber. Furthermore, the DMI stabilized at approximately 4.04 kg d^{-1} starting on day 15. The mean daily DMI was higher for the TN treatment (14.03 ± 0.29 kg d^{-1}) than for the HS treatment (8.27 ± 0.33 kg d^{-1}). Despite the difference in DMI, BCS did not change significantly ($p = 0.41$) between treatments (3.19 ± 0.10 for TN and 3.07 ± 0.07 for HS).

3.3. Correlations and simple regression analysis between physiological responses

Rectal temperature was positively correlated with VT ($r = 0.71$; $p < 0.001$) and RR ($r = 0.81$; $p < 0.001$) and negatively correlated with DMI ($r = -0.62$; $p < 0.001$). Similarly, RR had a strong positive correlation with VT ($r = 0.67$; $p < 0.001$) and a negative correlation with the DMI ($r = -0.56$; $p < 0.001$). No correlation existed between HR and Tre ($r = 0.03$; $p = 0.45$) or between HR and RR ($r = 0.002$; $p = 0.95$).

The linear regression equations were better fitted and were highly significant ($p < 0.001$) between Tre and the variables VT, RR, and DMI (Figure 4). The regression equations indicated a potential increase of 0.8 ºC in VT for each increase of 1 °C in Tre.

Similarly, RR increased an average of 21.6 bpm for each increase of 1 °C in Tre. Conversely, every increase of 1 °C in Tre potentially decreased the DMI by 3.8 kg d^{-1}.

3.4. Correlations and simple regression analysis between the physiological responses and the environmental variables

The mean, minimum, and maximum temperatures were positively and strongly correlated with the physiological parameters Tre, VT, and RR and negatively and strongly correlated with the DMI. A similar correlation was observed between the THI (mean, minimum, and maximum) and the physiological parameters. However, the correlation between RH (mean, minimum, and maximum) and the physiological parameters was weak to moderate. Therefore, the climate variables Ta and THI were more strongly associated with the physiological responses. Moreover, except for maximum RH, all climate variables evaluated (mean, minimum, and maximum Ta; mean and minimum RH; and mean, minimum, and maximum THI) were strongly ($r > 0.60$) and significantly ($p < 0.001$) correlated with each other.

The second set of regression equations was generated from the THI model to determine the critical stress threshold of the cows. The THI had a strong positive correlation with Tre ($r = 0.81$), VT ($r = 0.77$) and RR ($r = 0.81$) and a negative correlation with DMI ($r = -0.65$) and HR ($r = -0.23$). The Tre predicted from the THI indicated that the upper critical temperature for adult cattle (39.2 °C; Houston and Radostits, 2000) was reached when the THI was 80. Similarly, for RR an increase of 20 bpm was observed when the THI increased from 72 to 80. The decrease in the DMI as a function of the increase in the THI was expressed by $Y = 42.519 – 0.397$ THI ($R^2 = 42.3\%$) and indicated that, in general, for each unit of increase in THI above 72, the DMI decreased by 0.40 kg per cow per day.

The effect of the shift of physiological responses and DMI on the mean daily THI during HS was assessed. A slight increase was observed in the regression coefficients of Tre, VT, RR, and DMI when the individual values were shifted forward by one day (Table 5).

3.5. Multiple regression analyses between environmental variables and physiological responses

Multiple regression equations were generated to assess the effect and relative importance of the mean, maximum, and minimum environmental variables (independent variables) throughout the experimental period on the physiological responses in the morning and afternoon (dependent variables). The minimum temperature of the previous day ($T_{min\text{-}p}$), the maximum temperature of the previous day ($T_{max\text{-}p}$), the minimum relative humidity of the previous day ($RH_{min\text{-}p}$), and the minimum THI of the previous day ($THI_{min\text{-}p}$) affected the morning Tre (Tre_1; $p < 0.0001$) and explained 56.3% of the variation. The regression coefficients indicated that the most important environmental variables were (in descending order) $T_{min\text{-}p}$, $T_{max\text{-}p}$, $THI_{min\text{-}p}$, and $RH_{min\text{-}p}$. These relations are expressed in equation 1.

$$Tre_1 = 38.5 + 0.214\ T_{min\text{-}p} + 0.048\ T_{max\text{-}p} + 0.019\ RH_{min\text{-}p} - 0.107\ THI_{min\text{-}p} \quad (1)$$

The afternoon Tre (Tre_2) was correlated ($p < 0.001$) with the mean temperature of the previous day ($T_{mean\text{-}p}$), mean relative humidity of the previous day ($RH_{mean\text{-}p}$), and $THI_{min\text{-}p}$. The regression coefficients indicated that the environmental variables $T_{mean\text{-}p}$, $THI_{min\text{-}p}$, and $RH_{min\text{-}p}$ were the most important factors (in descending order), explaining 67.2% of the variation in Tre_2 (equation 2):

$$Tre_2 = 38.0 + 0.297\ T_{mean\text{-}p} + 0.027\ RH_{mean\text{-}p} - 0.124\ THI_{min\text{-}p} \quad (2)$$

Similar to Tre_1, the morning VT (VT_1) was significantly correlated with the RH_{mean-p} but, in contrast to the Tre_1, was significantly correlated with the mean THI of the previous day (THI_{mean-p}). These variables explained 64.1% of the variation in VT_1 (equation 3):

$$VT_1 = 32.1 - 0.022\ RH_{mean-p} + 0.101\ THI_{mean-p} \quad (3)$$

The regression coefficients indicated that THI_{mean-p} was approximately 4.6 times larger than the RH_{mean-p}.

Among all the environmental variables studied, only T_{mean-p} had no effect ($p < 0.001$) on the afternoon VT (VT_2). This relation can be observed in regression equation 4, and the T_{mean-p} explained 64.4% of the variation in VT_2:

$$VT_2 = 34.2 + 0.153\ T_{mean-p} \quad (4)$$

The environmental factors that influenced ($p < 0.01$) the morning RR (RR_1) were T_{max-p}, RH_{min-p}, RH_{max-p}, and RH_{mean}. This relation can be observed in regression equation 5 ($R^2 = 61.1\%$):

Based on the regression coefficients, the relative importance of the environmental variables was (in descending order) T_{max-p}, RH_{mean}, RH_{min-p}, and RH_{max-p}.

$$RR_1 = -86.6 + 3.338\ T_{max-p} + 0.436\ RH_{min-p} - 0.406\ RH_{max-p} + 0.454\ RH_{mean} \quad (5)$$

For the afternoon RR (RR_2), THI_{max} and RH_{max-p} were significant sources of variation ($p < 0.01$; equation 6) and explained 69.5% of the variation in RR_2. Based on the regression coefficients, the importance of the environmental variables was (in descending order of importance) THI_{max} and RH_{max-p}.

$$RR_2 = -124.1 + 2.264\ THI_{max} - 0.158\ RH_{max-p} \quad (6)$$

The morning HR (HR_1) and afternoon HR (HR_2) were affected ($p < 0.001$) by the environmental variables T_{max-p}, THI_{max-p}, THI_{mean-p}, and RH_{mean-p}. These factors accounted

for 10.8% and 15.2% of the variation in HR_1 (equation 7) and HR_2 (equation 8), respectively:

$$HR_1 = 136.0 + 4.706\ T_{max\text{-}p} - 2.024\ THI_{max\text{-}p} - 1.225\ THI_{mean\text{-}p} + 0.630\ RH_{mean\text{-}p} \quad (7)$$

$$HR_2 = 144.3 + 5.393\ T_{max\text{-}p} - 1.524\ THI_{max\text{-}p} - 2.289\ THI_{mean\text{-}p} + 0.843\ RH_{mean\text{-}p} \quad (8)$$

The importance of the environmental variables, according to the regression coefficient and in descending order, was Tmax-p, THImax-p, THImean-p, and RHmean-p for HR1 and Tmax-p, THImean-p, THImax-p, and RHmean-p for HR2.

Daily DMI was influenced ($p < 0.001$) by the environmental variables $T_{min\text{-}p}$ and $THI_{min\text{-}p}$. The coefficients of the environmental variables $T_{min\text{-}p}$ and $THI_{min\text{-}p}$ had, in descending order, a more pronounced effect on the intercept and were the most important factors, explaining 46.2% of the variation in daily DMI (equation 9):

$$DMI = -2.5 - 1.510\ T_{min\text{-}p} + 0.717\ THI_{min\text{-}p} \quad (9)$$

3.6. Comparison between acute and chronic responses to heat stress

The acute and chronic thermoregulatory responses to HS (days 0-3 and 13-16, respectively) were compared via regression analysis to assess the short-term acclimation to heat (Figure 5). The results indicated a difference in the adaptive responses for the variables RR and DMI. For both variables, the slope of the regression equation for the chronic response decreased. The intercept of Tre was larger for the chronic response, and a slight change was observed in the slope between the acute and chronic responses to HS. The acute responses of VT and HR were better represented by linear and quadratic regression models, respectively, but were not significantly correlated ($p > 0.05$) with the chronic response to HS.

4. Discussion

Heat stress is responsible for a decrease in productivity and feed efficiency, and its importance in the animal industry has increased, especially in light of the future scenarios on climate change. Therefore, the development of new strategies for heat mitigation that consider the physiological and metabolic adaptations of the animal are needed. This study evaluated the effect of environmental variables and duration of heat exposure on thermoregulatory responses to elucidate the adaptive mechanisms of heat dissipation in cattle under intense thermal stress that was induced experimentally in a continuous and sustained manner.

Methodologically, this study was simulated in a climate chamber to avoid confounding effects and allow the correlation between physiological responses and the intensity and duration of HS. We used nonlactating Holstein cows, which are a more uniform and reliable experimental model for the study objectives because they are not affected by lactation. A constant temperature was used because a single temperature represents a one-dimensional treatment (constant temperature over time). In contrast, a cyclic temperature is a two-dimensional treatment (temperature change over time) that can be applied according to a variety of real situations. Much evidence exists to suggest that the severity of HS is largely dependent on the fluctuation of Ta (Maust et al., 1972, Igono et al., 1992; Muller et al., 1994; Silanikove, 2000). Despite Ta changes during the day under natural conditions, consecutive days of heat stress (heat waves) has increased and is predicted to increase further in terms of frequency, intensity, and length in the future (Nidumolu et al., 2014). Thus, in this study, the simplest experimental design assumed a constant temperature to allow the correlation between the heat status and changes in the physiological parameters over time.

Ambient temperature in the HS treatment was on average 10 °C higher than the Ta in the TN treatment. The lower humidity in the HS treatment was due to the heating

mechanism inherent to the premises of the climate chamber, where higher temperatures coincided with the lowest percentage of humidity. The THI combines the effects of temperature and humidity and has been used to predict HS levels in animals (Mader et al., 2006; Bohmanova et al., 2007). During the experimental period, the mean daily THI in the TN and HS treatments was approximately 75 and 89, respectively. The maximum mean THI of cows under HS exceeded 100, whereas the minimum mean was approximately 78 (Table 2). Therefore, the cows under HS were subjected to conditions of intense HS for cattle (THI of 90–99; Armstrong, 1994), with higher risk of mortality in dairy cows (Vitali et al., 2015). Although the cows in the TN group were subjected to mild HS conditions (THI of 72–78; Armstrong, 1994), they did not show significant signs of HS, except for a slight increase in their RR. This finding suggests that these THI ranges, although suitable for lactating dairy cows, which are known to be more susceptible to HS (Purwanto et al., 1990), do not seem appropriate for use in evaluating nonlactating dairy taurine cows.

The reference values were established at 37.8–39.2 °C for Tre, 20–30 bpm for RR, and 60–72 bpm for HR (Houston and Radostits, 2000). In the HS treatment, the mean daily Tre was higher than the reference values in 94.1% and 81.3% of the morning and afternoon periods, respectively. The RR was higher than the reference values for all day periods. The critical level of 80 bpm, limit of thermal strain from which cows can no longer maintain normal body temperature (Smith et al., 2003; Beatty et al., 2006), was reached in 81.3% of the afternoon periods and was not detected in the mornings. For these reasons, the controlled environmental conditions used herein were assumed effective in causing HS in cows during almost the entire experimental period.

Respiratory rate has been widely used as an indicator of HS in cattle (Gaughan et al., 2000). The increase in RR is an important thermoregulatory mechanism induced to

maintain constant body temperature via evaporative cooling. The total heat loss from respiratory tract accounts for approximately 15% of the total heat dissipation in cattle under heat load (McDowell et al., 1976). However, the pronounced increase and considerable variation in RR observed in this study were not sufficient to prevent hyperthermia in the cows subjected to HS. This result is consistent with other studies in continuous or cycling heat exposure (Pereira et al., 2008; Gaughan et al., 2010; Thompson et al., 2011), which correlated high RR with low thermal tolerance in *B. taurus*.

In the current study, when cows were exposed to HS, the increase in RR preceded the increase in Tre in 12 hours. Likewise, Brown-Brandl et al. (2003) using sensors to record the RR on a continuous basis also detected increase of Tre lagged compared with RR. In adult cows, the maximum mean RR is approximately 70 to 80 bpm (Stevens, 1981). This value is considered an immediate response to heat and is probably associated with a standing position (Berman, 2005). The percentage of cows in a standing position increases linearly with the increase in the THI (Anderson et al., 2013; Allen et al., 2015), which is consistent with the observation that a greater frequency of animals in the standing position is indicative of discomfort (Privolo and Riva, 2009) and laminitis (Sanders et al., 2009). Therefore, if we consider that the change in RR precedes the change in Tre, RR could be used as a predictor of the heat status. With this assumption, visual inspection of the respiratory response allows the alleviation of HS before a significant increase occurs in body temperature, interfering in normal body functions.

In this study, exposure to a heat load much higher than the thermal comfort zone in cattle (25–26 °C; Berman et al., 1985) resulted in a marked increase in all the body temperature variables measured. The use of technologies that allow the frequent and automated recording of body temperature (such as an intravaginal thermometer) provide the additional advantage of capturing diurnal changes (Vickers et al., 2010; Burdick et

al., 2012). Tre and VT were strongly correlated with each other (r = 0.71), and a diurnal pattern was observed. The means were higher in the afternoons than in the mornings, and this effect was greater in animals under HS. Effective environmental temperatures that were higher in the afternoon (particularly in the HS treatment) and associated with an elevated body temperature generated by the circadian rhythm (Shehab El-Deen et al., 2010) were the factors responsible for these effects. The range of Tre (daily maximum mean Tre – daily minimum mean Tre) of the cows under TN and HS conditions was approximately 0.8 °C and 1.5 °C, respectively. These values were similar to those obtained in the winter (0.4 ºC) and summer (1.2 ºC) for dairy cows under field conditions (Berman and Morag, 1971).

Our results indicated that HR was not influenced at the same magnitude as RR, Tre or VT. In the present study, we observed a gradual decrease in HR in the cows under HS compared with cows under TN. Conflicting results on HR have been reported in continuous heat exposure studies (Richards, 1985; Beatty et al., 2006). The decrease in HR associated with HS can be attributed to heat exchange with the environment, so the duration of exposure to high temperatures appears to be essential for adaptation in cows. In a trial of continuous exposure to HS during seven days, Cowley et al. (2015) also observed a decrease in HR both in heat stressed lactating cows as well as in thermoneutral pair-fed lactating cows. This finding is a possible explanation for our results. So it is likely that the decrease in HR is a side effect associated with DMI due to the slower metabolism, decreased visceral blood flow, and decreased peripheral vasodilatation.

Dry matter intake was strongly influenced by the experimental conditions. A linear decrease in voluntary DMI with increasing Tre was detected for cow in this study, which was proven by the moderate correlation (r = -0.59). These results are consistent with those of Bernabucci et al. (1999), in a continuous exposure of dairy heifers to HS,

497 and Brown-Brandl et al. (2003), in a cycling exposure of beef cattle to HS. The decrease
498 in DMI is followed by the decrease in the metabolic rate and a decrease in heat production,
499 which contributes to the maintenance of heat balance (Turner and Taylor, 1983). The
500 lower DMI in HS cows is associated with high-fiber diets with low degradability (Beede
501 and Collier, 1986; Fobes, 1993). The selectivity of the diet is increased, whereas the
502 forage intake in relation to the concentrate is decreased in an attempt to reduce body heat
503 derived from fermentation, digestion, and other metabolic processes (Beede and Collier,
504 1986). Additionally, food intake was not affected at temperatures between 15 °C and 25
505 °C, but the increase in Ta to values higher than 35 °C decreased food intake by 10% to
506 35% (Conrad, 1985). In the present study, the marked reduction in DMI under HS
507 conditions was greater than 30% compared with cows under TN conditions. The constant
508 temperature used in this study may have exceeded the expected response to HS. Lower
509 cyclical temperatures at night would have allowed greater food intake because the cows
510 would be able to dissipate the heat accumulated during the day (Maust et al., 1972),
511 allowing changes in some of the observed responses.

512 A considerable energetic cost is known to be necessary to dissipate stored heat,
513 with a consequent increase in maintenance requirements (Beede and Collier, 1986; NRC,
514 2001). During acute HS, the cows may be in negative energy balance (Schwartz et al.,
515 2009), compatible with high plasma concentrations of β-hydroxybutyric acid (Soriani et
516 al., 2013), so the nutrient intake cannot supply the energy requirements. Later, leveled off
517 or compensatory food consumption occurs (Brown-Brandl et al., 2003), which was
518 observed in our study only after day 15. Moreover, a redistribution of blood flow from
519 the digestive system (Hales et al., 1984) to peripheral tissues occurs in animals chronically

520 exposed to heat. This hypothesis is supported by the cardiovascular adjustments discussed
521 previously.

522 In addition to the changes observed in physiological parameters and DMI, clinical
523 signs of HS, including open-mouth panting, drooling, reluctance or inability to rise,
524 increased coat licking, and dullness, and neurological signs, such as fixed gaze and glazed
525 eyes, were also observed in the present study, as previously reported by Beatty et al.
526 (2006).

527 The correlation coefficients between Tre, VT, RR, DMI, and THI from the
528 previous day were higher than the current or following day, indicating a period of delay
529 before the response to climate variation develops fully. In fact, the delayed effect of the
530 environmental factors on DMI seems logical because of the time necessary for cows to
531 consume, digest, and metabolize nutrients. The development of HS prediction models that
532 reflect individual variations in physiological parameters on the basis of experimental
533 evidence can be useful to estimate the heat status of cattle in a wide variety of
534 environmental conditions and can allow prediction of the onset of HS. This would make
535 it possible to explore, for example, food supplementation strategies or environmental
536 changes. Therefore, these data have important implications for the prediction models that
537 typically depend on weather data measured on the same day to predict performance
538 responses (West et al., 2003).

539 Measurements of HS can help farmers keep cows at an ideal temperature and
540 prevent adverse effects caused by heat. Rectal temperature is an important indicator of
541 thermal balance. An increase in Tre indicates failure or exhaustion of the
542 thermoregulatory mechanisms. A negative correlation between Tre and DMI was
543 detected ($r = -0.59$) and was the best single predictor of HS compared with other
544 physiological parameters, in agreement with previous data on lactating cows (Spiers et

545 al., 2004). Moreover, in this study, the correlation analysis demonstrated that DMI was
546 strongly affected by environmental variables (r=-0.65). The combination of high Ta and
547 high RH increases the level of stress. Therefore, the estimated weather variables,
548 including the THI, can describe precisely the effect of environmental conditions on the
549 magnitude of the hyperthermia experienced by cows under HS. These results motivate
550 the use of the THI as an adequate variable to predict the severity of HS, with the advantage
551 that the measurement of the THI is straightforward and indirect.

552 Heat stress during the dry period may influence negatively the calf birth weight
553 and subsequent lactation (Amaral et al., 2009; Tao et al., 2011), resulting in large
554 economic losses (Ferreira et al., 2016). The definition of stress thresholds can provide
555 useful information for decision-making in the management of cows, particularly with
556 regard to thermal comfort (Mader et al., 2010). In the present study, although the upper
557 individual critical Tre established for cattle (39.2 °C; Houston and Radostits, 2000) was
558 reached with a THI of 80, the grouping of THI at intervals of severity of HS (Figure 6)
559 indicated that the adoption of a THI below 77 as a critical threshold for dry cows included
560 all animals. Despite the differences in the HS threshold values in the different regions,
561 animal categories, and methodologies for the calculation, a THI value of 72 has been
562 continuously used as a reference (Bohmanova et al., 2007; Aguilar et al., 2010). Other
563 studies have found a limit of 74 for the THI in the semi-arid regions of the United States
564 (Bohmanova et al., 2007), 69 under the climate conditions of Tunisia (Bouraoui et al.,
565 2002), and 60 for protein yield in German Holstein cows (Brügemann et al., 2011). The
566 difference between the studies provides insights into the increased production of the
567 metabolic heat generated by dairy cows, as demonstrated by Purwanto et al. (1990),
568 wherein low-yielding dairy cows (18.5 kg/day) and high-yielding dairy cows (31.6
569 kg/day) were compared to nonlactating cows and found to generate 27% and 48% more

heat, respectively. Therefore, the current values may be overestimated for the category of cows that are not lactating and suggests caution in the use of general indicators that do not include a consideration of the level of animal production.

Multiple regression analysis showed that Ta represented the most determinant factor for heat exchange, although the THI affected VT primarily in the morning and RR primarily in the afternoon. Ambient temperature and THI are closely related and the effects are difficult to separate. In other hand, the RH seemed to have a limited impact on physiological parameters and was an additional stress factor, as reported previously by Maust et al. (1972), with an increasing effect on RR, which is consistent with the fact that heat is removed from the lungs in the form of moisture (Gebremedhin et al., 2008). In addition, RR is a thermoregulatory mechanism, whereas Tre is the result of thermal balance (Berbigier, 1988). Therefore, the influence of thermal variables on RR may be mediated via different intermediate Tre mechanisms (Kabuga, 1992). In agreement with these findings, Ta had a stronger effect on physiological parameters (Legates et al., 1991; Kabuga, 1992) and milk temperature (West et al., 2003) in field conditions, although humidity seems to be a limiting factor in humid climates (Bohmanova et al., 2007).

The R^2 value for the environmental variables that influenced HR was relatively low and explained 15% or less of the total variation. This result demonstrated that, the environmental conditions had a relatively small effect on HR and suggested that other sources of variation are more important than the weather, such as food intake. In the study by Kabuga (1992), climatic variables had the greatest influence on HR and explained 25.4% and 22.1% of the variation in the morning and afternoon, respectively. However, R^2 values obtained for Tre, RR and DMI in this study were higher compared with other field studies (Maust et al., 1972, Kabuga, 1992; West et al., 2003). These differences

might be explained by the lower variation of experimental conditions in laboratory and the difference in the production level and dietary composition among the studies.

The minimum Ta and minimum THI from the previous day were more important to explain the variation in DMI than the immediate effect. The environmental factors from the current day were largely ignored. This delayed effect in the DMI and physiological responses has previously been reported in a field experiment using lactating dairy cows by West et al. (2003) and Kabuga (1992), but differs from the results of Maust et al. (1972), wherein the correlations between Tre and DMI and climate conditions were higher in the same day. Moreover, the relative importance of minimum values may be partially explained by the continuous exposure to heat, so the opportunity to dissipate heat is reduced, leading to greater DMI when Ta and THI values are lower.

The acute and chronic effects of HS were explained by the different regression lines. The RR and DMI results indicated differences between the acute and chronic responses to exposure to heat stress, providing evidence of an adaptive physiological adjustment to heat over time. However, no decrease in Tre was observed in the cows. These findings were similar to those obtained with dairy cows (Kibler et al., 1965) but differed from those obtained with heifers, in which prolonged exposure to heat caused a gradual decrease in Tre (Bernabucci et al., 1999). The difference between the results may be due to the duration of treatments, and a longer time may be necessary for the animals to balance Tre and acclimatize effectively. The decrease in RR observed in the chronic response was consistent with the results of another study under conditions of continuous and prolonged HS (Beatty et al., 2006) but different from the study conducted under controlled conditions using cyclic temperatures (Scharf et al., 2010). The fluctuation in Ta in the latter study provided an opportunity for the animals to dissipate heat and maintain homeothermy (Igono et al., 1992; Muller et al., 1994) and may explain the

discrepancy between the results obtained. Moreover, there may be an upper limit for RR (Spiers et al., 2004). Therefore, the decrease in RR is not always an indication that the animal is coping with the heat and may be due to changes in the respiratory dynamics associated with moving from a state of rapid open-mouth breathing to deeper slower-paced open mouth breathing (Gaughan et al. 2000). Dry matter intake sharply decreased after exposure to HS (acute phase). The difference between the DMI regression coefficients of the acute and chronic responses may indicate an adaptation to thermally stressful conditions and partial acclimatization after decrease in DMI over the first several days (Figure 3). The food intake in *B. taurus* heifers (Beatty et al., 2006) and in cross-bred steers (Brown-Brandl et al., 2003) significantly decreased and had a slight recovery three and seven days after continuous heat exposure, respectively. Lactating cows continuously exposed to a constant temperature of 29 ºC had stabilization of DMI four to five weeks after exposure (Kibler et al., 1965). Therefore, following the sharp decrease in DMI during the acute phase, adjustments in the nutrient requirements via reduction of the weight of visceral organs (Koong et al., 1985; Ferrell et al., 1986), changes in the expression of pre-existing characteristics coordinated by the endocrine system (Bernabucci et al., 2010), and subsequent stabilization of DMI over time seem to occur. In this study, the cows were not completely acclimatized, perhaps because of the intense HS conditions used and the need for exposure for a longer period. Thus, further studies are necessary to elucidate the period and dynamics of chronic responses to HS.

5. Conclusion

In summary, continuous exposure to intense HS influences thermoregulatory mechanisms, leading to a marked increase in RR, followed by an increase in Tre. Other responses include a decrease in DMI in an attempt to minimize total heat load and suggest

644 the induction of metabolic shifts, redistribution of blood flow, and decrease in HR as part
645 of the physiological adaptations. The influence of environmental variables from the
646 previous day on physiological parameters and DMI is more important than the immediate
647 effect and Ta represents the most determinant factor for heat exchange. Although
648 prolonged exposures to HS indicate an adaptive adjustment, in this study the acclimation
649 process was only partial.
650

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873 Kjeldahl and Dumas methods: review and interlaboratory study using dairy
874 products. J. AOAC Int., 81, 620-632.

875 Table 1. Composition of ingredients and nutrients in the diets consumed by cows housed in a
876 climate chamber

Ingredients	% of dry matter
Tifton hay	70.2
Soybean meal	2.7
Urea	0.6
Finely ground mature corn	24.8
Limestone	0.6
Premix of minerals and vitamins[1]	0.8
Salt	0.3
Calculated composition	
Crude protein (CP)	13.8
Ether extract (EE)	3.0
Neutral detergent fiber (NDF) of Tifton	48.5
Total NDF	52.2
Total acid detergent fiber (ADF)	23.1
Mineral matter (MM)	7.2
Nonfiber carbohydrates (NFC)[2]	23.8

877 [1] Premix of minerals and vitamins: each kilogram contained 52.0 g of Zn, 16.0 g of Mn, 14.0 g
878 of Cu, 900.0 mg of I, 440.0 mg of Se, 260.0 mg of Cu, 34000.0 IU of vitamin E, 5000.0 IU of
879 vitamin A, 1,400.0 g of vitamin D3, and 20.0 g of monensin. [2] Nonfibrous carbohydrates: 100
880 – (CP + NDF + Ash + EE).

881 Table 2. Mean ± SEM of the environmental conditions in the climate chamber in the
882 thermoneutral (TN) and heat stress (HS) treatments during the experimental period

Variable	Treatment	Minimum	Maximum	Mean
Temperature (°C)	TN	21.77 ± 0.41[b]	30.21 ± 0.14[b]	25.91 ± 0.22[b]
	HS	28.19 ± 0.68[a]	43.67 ± 0.12[a]	36.31 ± 0.34[a]
Humidity (%)	TN	54.52 ± 1.34[a]	85.42 ± 1.12	73.01 ± 0.79[a]
	HS	40.59 ± 1.06[b]	84.53 ± 1.89	60.90 ± 0.93[b]
THI[1]	TN	69.46 ± 0.62[b]	80.73 ± 0.31[b]	75.42 ± 0.27[b]
	HS	77.74 ± 0.74[a]	100.51 ± 0.53[a]	88.66 ± 0.36[a]

883 [1] THI = temperature humidity index. [a-b] Different letters in the same column indicate significant
884 differences ($p < 0.0001$) between the treatments.

885 Table 3. Mean ± SEM and variation of climate conditions and physiological parameters of
886 Holstein cows housed in a climate chamber according to the time of the day (morning and
887 afternoon)

Variable	Thermoneutral		Heat stress	
	Mean ± SEM	Variation	Mean ± SEM	Variation
Air temperature (°C)				
Morning	24.49[b] ± 0.05	(19.77–29.29)	35.03[b] ± 0.11	(25.23–44.23)
Afternoon	26.79[a] ± 0.05	(20.20–31.20)	36.90[a] ± 0.10	(24.03–44.57)
Relative humidity (%)				
Morning	76.07[a] ± 0.15	(58.25–97.69)	61.47[b] ± 0.29	(41.00–100.00)
Afternoon	71.54[b] ± 0.20	(44.39–91.47)	62.43[a] ± 0.28	(28.51–95.60)
THI[1]				
Morning	73.66[b] ± 0.07	(66.35–83.73)	87.01[b] ± 0.15	(73.32–101.67)
Afternoon	76.59[a] ± 0.06	(67.51–82.50)	89.87[a] ± 0.14	(72.85–04.73)
Rectal temperature (°C)				
Morning	38.42[b] ± 0.03	(37.60–39.90)	39.42[b] ± 0.06	(37.80–41.60)
Afternoon	38.67[a] ± 0.03	(37.50–40.00)	40.27[a] ± 0.05	(38.50–41.80)
Vaginal temperature (°C)				
Morning	38.30[a] ± 0.03	(36.90–39.70)	39.74 ± 0.10	(37.98–41.24)
Afternoon	38.19[b] ± 0.02	(37.40–39.50)	39.73 ± 0.10	(38.20–41.71)
Respiratory rate (bpm)				
Morning	36.97[b] ± 0.81	(20.00–66.00)	65.85[b] ± 1.33	(28.00–120.00)
Afternoon	42.06[a] ± 1.05	(16.00–90.00)	85.10[a] ± 1.47	(28.00–130.00)
Heart rate (bpm)				
Morning	64.62[b] ± 0.79	(36.00–100.00)	59.49[b] ± 0.94	(30.00–120.00)
Afternoon	67.62[a] ± 0.79	(42.00–96.00)	63.89[a] ± 0.97	(34.00–126.00)

888 [1] THI = temperature humidity index. [a-b] Different letters in the same column indicate significant
889 differences ($p < 0.01$) between the periods of the day.

890 Table 4. Daily mean, minimum, and maximum values (mean ± SEM and variation) of climate
891 conditions and physiological parameters of Holstein cows housed in a climate chamber

Variable	Thermoneutral		Heat stress	
	Mean ± SEM	Variation	Mean ± SEM	Variation
Air temperature (°C)				
Mean	25.73^{b} ± 0.19	(21.17–28.10)	36.04^{a} ± 0.41	(30.43–41.85)
Minimum	23.11^{b} ± 0.19	(19.77–25.79)	31.75^{a} ± 0.44	(24.03–38.25)
Maximum	28.65^{b} ± 0.22	(22.37–31.20)	40.62^{a} ± 0.36	(32.07–44.57)
Relative humidity (%)				
Mean	73.62^{a} ± 0.70	(60.86–84.97)	61.99^{b} ± 0.82	(47.85–76.77)
Minimum	63.61^{a} ± 1.08	(44.39–81.63)	47.52^{b} ± 0.78	(28.51–59.27)
Maximum	81.00 ± 0.68	(68.93–97.69)	77.96 ± 1.06	(56.17–100.00)
THI[1]				
Mean	75.24^{b} ± 0.26	(68.83–78.87)	88.56^{a} ± 0.46	(80.38–95.02)
Minimum	71.57^{b} ± 0.31	(66.35–76.67)	81.68^{a} ± 0.49	(72.85–88.50)
Maximum	79,15^{b} ± 0.28	(70.68–83.73)	97.53^{a} ± 0.43	(82.68–104.73)
Rectal temperature (°C)				
Mean	38.56^{b} ± 0.03	(38.02–39.15)	39.87^{a} ± 0.07	(38.33–40.90)
Minimum	38.21^{b} ± 0.03	(37.50–38.90)	39.08^{a} ± 0.09	(37.80–40.70)
Maximum	38.98^{b} ± 0.05	(38.30–40.00)	40.61^{a} ± 0.08	(38.70–41.80)
Vaginal temperature (°C)				
Mean	38.26^{b} ± 0.03	(37.92–38.92)	39.82^{a} ± 0.10	(38.32–41.41)
Minimum	37.78^{b} ± 0.04	(36.90–38.50)	38.80^{a} ± 0.13	(37.60–40.90)
Maximum	38.79^{b} ± 0.32	(38.30–39.70)	40.86^{a} ± 0.11	(39.00–42.70)
Respiratory rate (bpm)				
Mean	39.70^{b} ± 0.71	(26.33–60.67)	76.02^{a} ± 1.70	(33.33–123.00)
Minimum	28.43^{b} ± 0.82	(16.00–50.00)	57.12^{a} ± 2.12	(28.00–116.00)
Maximum	54.55^{b} ± 1.60	(32.00–90.00)	95.24^{a} ± 2.12	(44.00–130.00)
Heart rate (bpm)				
Mean	66.23^{a} ± 0.79	(52.00–80.00)	62.13^{b} ± 0.99	(49.33–102.50)
Minimum	55.95^{a} ± 0.84	(36.00–68.00)	50.79^{b} ± 0.97	(30.00–70.00)
Maximum	78.31 ± 1.10	(56.00–100.00)	75.30 ± 1.57	(54.00–126.00)

892 [1] THI = temperature humidity index. [a-b] Different letters in the same row indicate significant
893 differences ($p < 0.001$) between treatments.

894 Table 5. Effect of shift forward or backward in the dependent variables (rectal temperature,
895 vaginal temperature, respiratory rate, heart rate, and dry matter intake) in relation to temperature
896 humidity index on the coefficient of determination (R2) of the regressions

Dependent variable	Slope	SEM_{slope}	t	p value	R^2 (%)
Rectal temperature (°C)					
Previous day	32.084	0.315	101.83	<0.0001	59.53
Current day	31.609	0.285	110.83	<0.0001	65.97
Following day	31.504	0.288	109.52	<0.0001	67.81
Vaginal temperature (°C)					
Previous day	30.295	0.570	53.17	<0.0001	58.37
Current day	30.042	0.584	51.47	<0.0001	59.50
Following day	29.655	0.587	50.51	<0.0001	61.83
Respiratory rate (bpm)					
Previous day	–144.413	8.388	–17.22	<0.0001	62.70
Current day	–150.276	7.897	–19.03	<0.0001	65.53
Following day	–143.976	7.584	–18.99	<0.0001	67.48
Heart rate (bpm)					
Previous day	NS	NS	NS	NS	NS
Current day	88.807	5.528	16.06	<0.0001	5.32
Following day	NS	NS	NS	NS	NS
Dry matter intake (kg d^{-1})					
Previous day	41.854	2.167	19.32	<0.0001	39.66
Current day	42.519	2.095	20.30	<0.0001	42.29
Following day	44.068	2.139	20.60	<0.0001	44.33

897

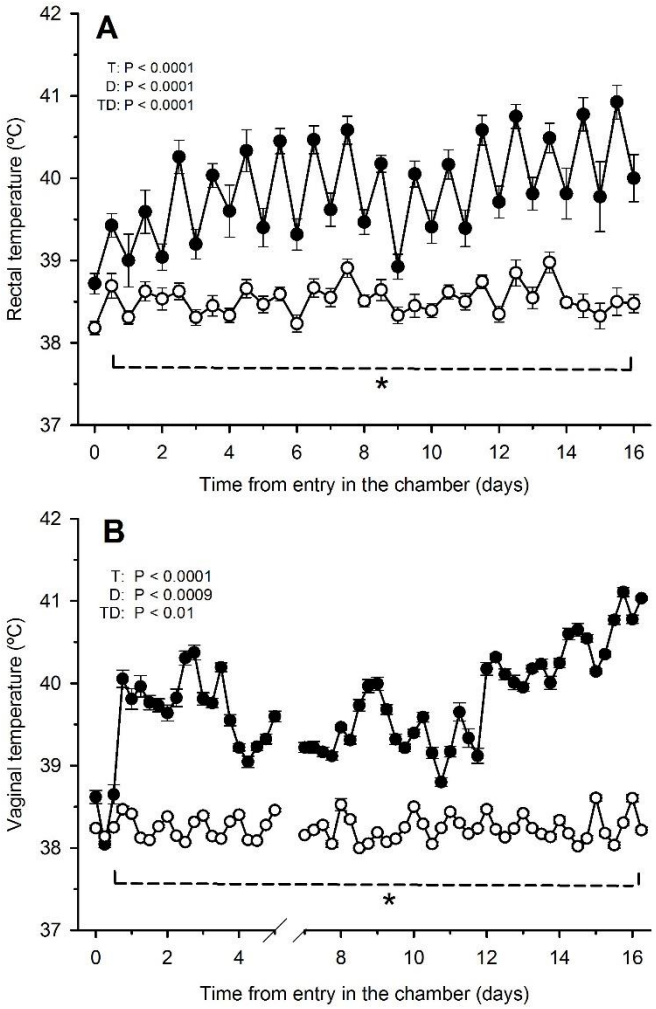


Figure 1. Mean ± SEM of rectal temperature (A) and vaginal temperature (B) of cows exposed to thermoneutral (n = 12, ○) or heat stress (n = 12, ●) conditions. Probabilities for the main effects (T, treatment; D, day) and the interaction between treatment and day (TD) are shown. The interval with a significant effect ($p < 0.05$) of the interaction between treatment and day is indicated by a dotted line with an asterisk (∗). The cut between days 5 and 7 in B represents the interval when the intravaginal thermometer was removed.

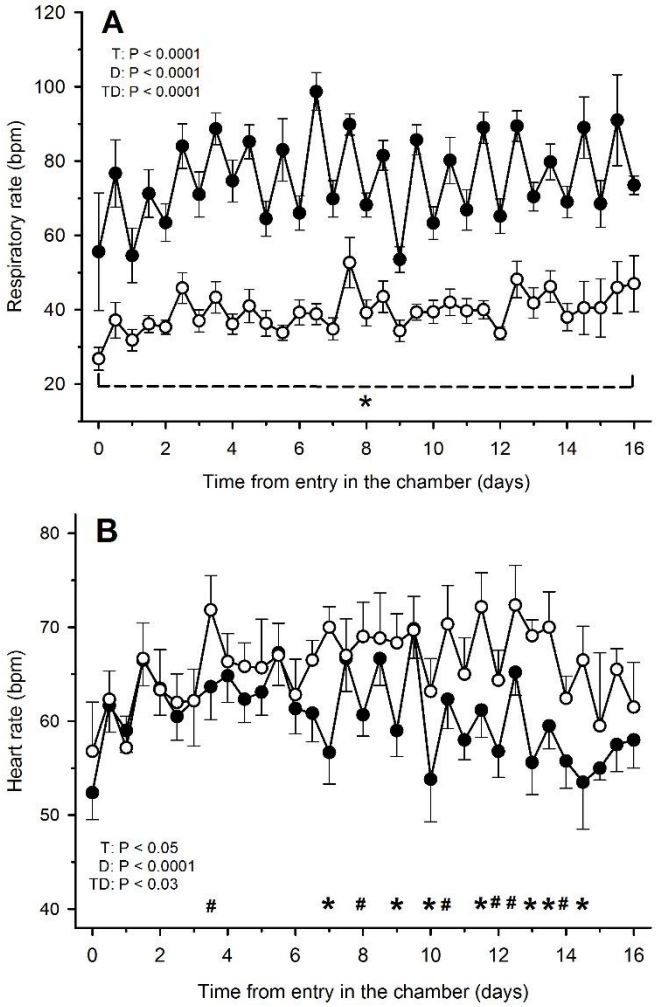
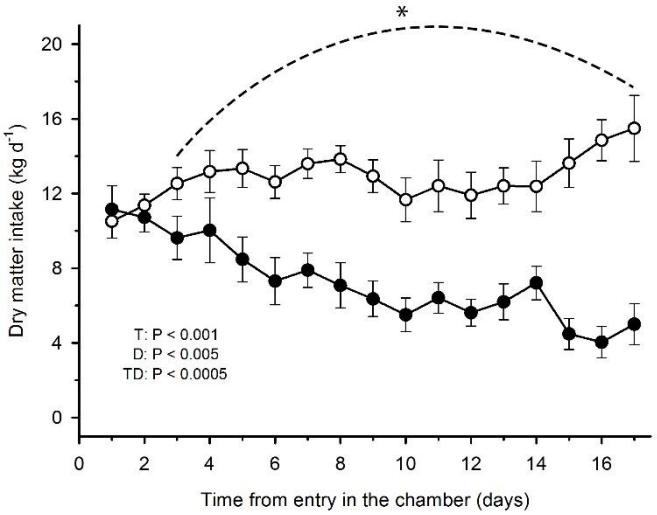


Figure 2. Mean ± SEM of respiratory rate (A) and heart rate (B) of cows exposed to thermoneutral (n = 12, ○) or heat stress (n = 12, ●) conditions. Probabilities for the main effects (T, treatment; D, day) and the interaction between treatment and day (TD) are shown. The dotted line with an asterisk (∗) indicates the interval with a significant difference ($p < 0.05$) between treatments. On the day scale, an asterisk (∗) indicates the day with a significant difference ($p < 0.05$) between treatments, and a pound sign (#) indicates a trend ($p < 0.1$).



913

914 Figure 3. Mean ± SEM of dry matter intake of cows exposed to thermoneutral (n = 12,
915 ○) or heat stress (n = 12, ●) conditions. Probabilities for the main effects (T, treatment;
916 D, day) and the interaction between treatment and day (TD) are shown. The dotted line
917 with an asterisk (∗) indicates the interval with a significant difference ($p < 0.001$) between
918 treatments.

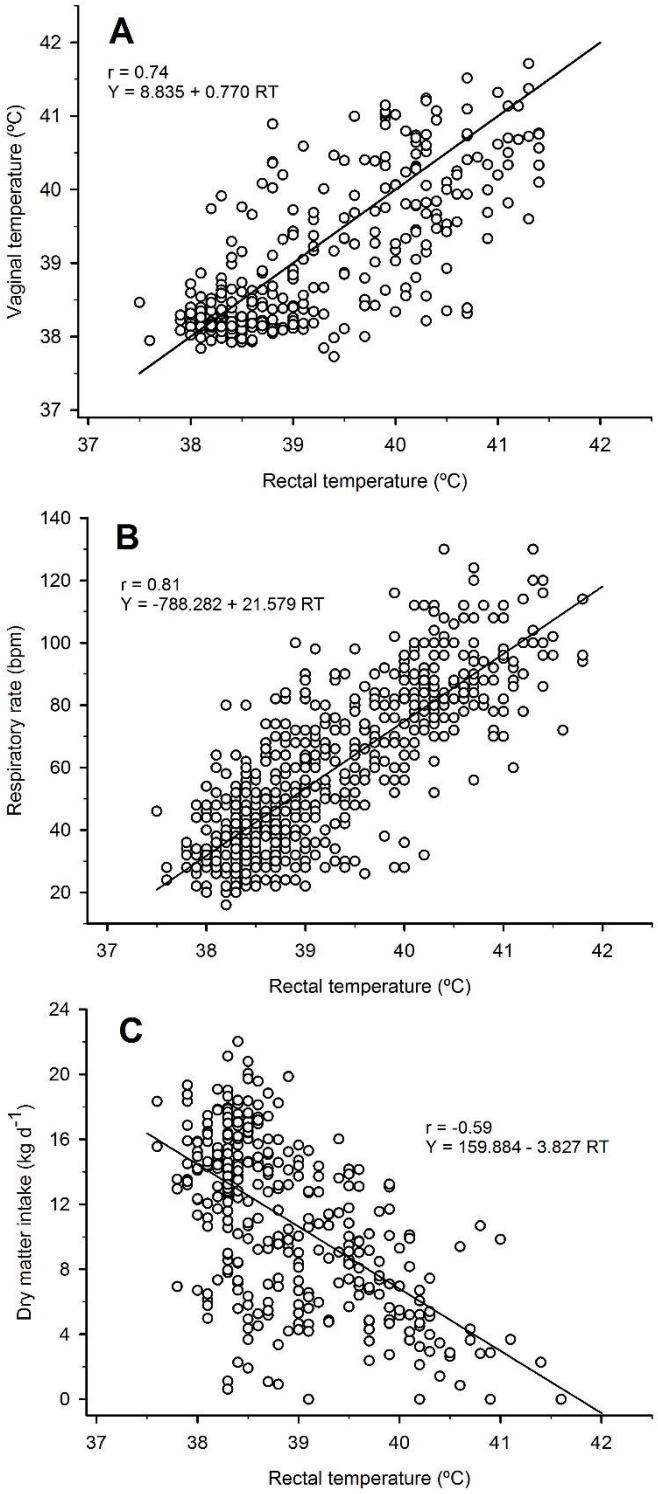
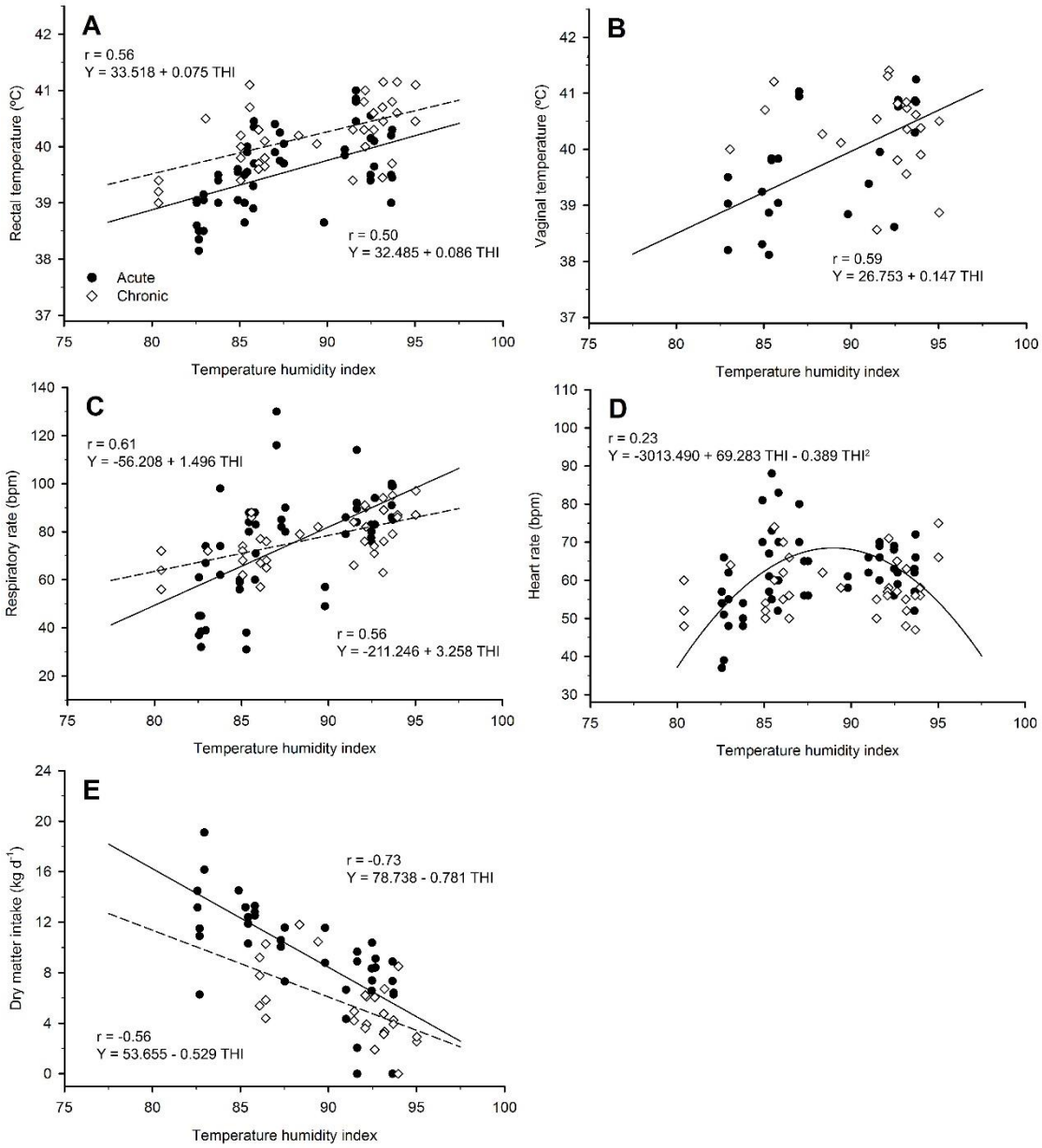


Figure 4. Linear models showing the correlation between rectal temperature and vaginal temperature (A), respiratory rate (B) and dry matter intake (C). The points represent individual observations, and the line represents the regression equation. The information on the chart refers to the linear regression at the points indicated.



924

925 Figure 5. Effect of heat stress on the acute response (●) and chronic response (◊) of the
926 rectal temperature (A), vaginal temperature (B), respiratory rate (C), heart rate (D), and
927 dry matter intake (E), expressed as Y. The points represent individual observations, the
928 solid line represents the best-fit model for the acute response, and the dotted line
929 represents the best-fit model for the chronic response to heat stress. No correlation ($p > 0.05$) was observed between vaginal temperature and heart rate for the sustained response
931 to heat stress.

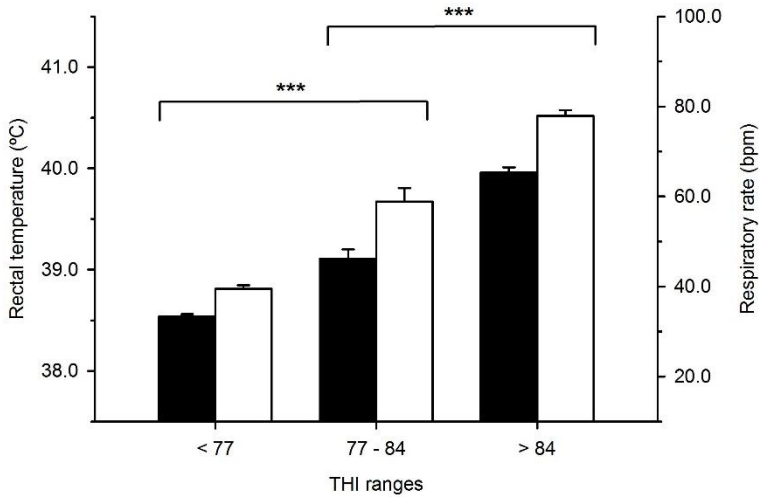


Figure 6. Mean ± SEM of rectal temperature (black bars) and respiratory rate (white bars) grouped into three intervals of daily temperature humidity index (THI). Asterisks indicate significant differences ($p < 0.001$) between the intervals for both variables.

Capítulo III

O artigo a seguir foi redigido de acordo com as normas do periódico científico 'Journal of Proteomics' (https://www.elsevier.com/journals/journal-of-proteomics/1874-3919/guide-for-authors).

Quantitative proteomic profiling of bovine follicular fluid during follicle development

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Abstract

Bovine follicular fluid (FF) constitutes the microenvironment of follicles and includes various biological active proteins that can affect follicle growth and oocyte fertilization. We performed a study involving eighteen healthy non-lactating Holstein cows to determine the protein expression profile of FF at key-stages of follicular development. Follicles were aspirated *in vivo* at pre-deviation, deviation, post deviation, and pre-ovulatory stages of follicle development, which were confirmed by measurement of estradiol and progesterone concentrations. The FF from nine cows were used for proteomic analysis. After albumin depletion, triplicates of pooled FF from three cows were reduced, alkylated, and digested with trypsin. The resulting peptides were labelled with TMTsixplex and quantified using LC-MS/MS. A total of 143 proteins was identified and assigned to a variety of biological processes, including response to stimulus and metabolic processes. Twenty-two differentially ($P < 0.05$) expressed proteins were found between stages indicating intrafollicular changes, with deviation as a critical time-point for modulation of follicular growth. For instance, up-regulation of follistatin, inhibin, serglycin, spondin-1, fibrinogen, and anti-testosterone antibody were found during early stages of follicular development. In contrast, apolipoprotein H, alpha-2-macroglobulin, plasminogen, antithrombin-III, and immunoglobulins were up-regulated after follicle deviation. Amongst the differentially abundant proteins, nineteen were found to be associated with steroidogenesis. Pathway analysis identified proteins that were mainly associated with the acute phase response signaling, coagulation system, complement system, liver/retinoid X receptor activation, and biosynthesis of nitric oxide and reactive

oxygen. In conclusion, these differentially expressed proteins provide insights into the size-dependent protein changes in the ovarian follicle microenvironment that could influence follicular function.

Keywords: Follicular development; Proteome; Mass spectrometry; Steroids; Holstein cows

1. Introduction

Follicular fluid (FF) is produced during follicular growth and provides the microenvironment for oocyte development and maturation. Main origins of FF are circulating blood, which diffuses through the follicular wall into an antrum of a follicle, and secretions by granulosa, theca cells, and oocytes [1]. The composition of FF is very complex and among other biological active molecules it contains regulatory proteins that may affect follicular growth, regulate permeability of follicular wall, follicular maturation, and ovulation.

Proteomic composition of FF can be used as an indicator of secretory activities and metabolism of follicular cells, which regulate follicle quality [2]. As proteins are vital elements in cell-function control, the alterations in protein abundance depict biological processes over the estrous cycle. A number of studies has reported proteome profiles in human FF [3–7]. However, for cows is still poorly characterized with two previous studies [8,9], addressing issues based on reproductive disorders. Therefore, the temporal characteristics of proteome profile of bovine FF could contribute to our better understanding of the highly coordinated process involved during folliculogenesis.

Due to the complex composition of FF, sensitive and specific techniques to identify the proteins in FF are essential. Quantitative proteomics can elucidate the

regulation of folliculogenesis within a time-dependent context by measuring protein abundance by high-throughput manner. Protein abundance could accurately demonstrate cellular activities than messenger RNA quantification, because messenger RNA and protein levels do not necessarily correlate [10]. Recent advances in protein quantification methods, such as isobaric labels tandem mass tags (TMT), have improved measurement precision, accuracy, and reproducibility [11], surpassing label-free quantification methods such as spectral counting [12].

In order to clarify the follicular development process, the aims of this study were to investigate the proteome profile and functional overview of bovine FF during different stages of the follicle development and to determine the association of the abundance of the identified proteins with local steroids concentrations.

2. Material and methods

2.1. Animals and experimental design

This study was approved by the Ethical Committee for the Use of Animals In Research of the FMVZ - UNESP (Permit number: 86/2013 – CEUA). The experiment was conducted at the Lageado Experimental Farm, School of Veterinary Medicine and Animal Science, Univ. Estadual Paulista, Botucatu, SP, Brazil. Eighteen nonlactating, clinical healthy, and cycling Holstein cows (3 to 8 years old) were used in this experiment. The study was conducted during the winter to avoid effects of heat stress. All cows were initially subjected to ovulation synchronization using the Ovsynch protocol [13] added with an intravaginal progesterone device (Sincrogest, Ourofino, São Paulo, Brazil). Of the 18 cows, 13 successfully responded to the estrus synchronization and were used in the experiment. From the day of ovulation, ovarian ultrasonography was performed twice daily (every 12 h) to monitor the emergence and development of a new follicular wave.

Transrectal ultrasound (MyLab30 Vet Gold with a 7.5 MHz linear-array transducer, Esaote, Genova, Italy) was used to confirm ovulation and to determine the size and location of the follicles and corpus luteum. Ultrasound measurements were used to calculate the average follicular diameter in accordance with Sartori, Rosa and Wiltbank [14]. The ovarian follicular dynamic was monitored and the FF from the largest follicle (F1) was individually aspirated at pre-deviation (F1 ~ 7.0 mm [15]). After the target follicle be aspirated, all remaining visible follicles were ablated to allow the emergence of a new follicular wave. Then, at expected deviation moment (F1 ~ 8.5 mm [16]), both the F1 and the second largest follicle (F2) were aspirated. Again, all remaining visible follicles were ablated to allow the emergence of a new follicular wave and the F1 was aspirated after acquisition of ovulatory capacity (F1 ~ 12.0 mm [17]). All the cows were subjected to a new synchronization protocol as previously reported [18]. Thirteen cows successfully responded to the estrus synchronization and, instead use the diameter of the follicle, at pre-ovulatory stage, follicular aspiration of F1 was done 24 hours after i.m. GnRH injection. Collectively, four time-points characterized the experimental groups: pre-deviation (PreDev); deviation (DevF1 and DevF2); post deviation (PostDev); and pre-ovulatory (PreOv).

2.2. *In vivo* transvaginal follicular aspiration

Caudal epidural anesthesia was performed with 5 mL of 2% lidocaine (Lidovet, Bravet, Rio de Janeiro, Brazil), the perineal area was scrubbed and the aspiration guide with the transducer was inserted via transvaginal. The follicular aspiration was performed using an ultrasound equipment (Mindray DP-3300 Vet, Mindray Bio-Medical Electronics Co. Ltd., Sheuzheu, China) equipped with a micro-convex 5 MHz transducer coupled to a needle guide system (WTA, Watanabe Applied Technology, Cravinhos, Brazil) and

connected to an aspiration line (WTA) and a 20G disposable needle (WTA). Instead of a pump, the differential pressure applied to the system to recover the FF was created using a 10 mL syringe (Descarpack, São Paulo, Brazil). Immediately after follicle collapse, the needle was removed from the ovary, the system was checked for the presence of FF and blood contamination. Each collected fluid sample was derived from a single follicle. Only the FF samples without macroscopic blood contamination were used in this study.

After collection, the FF samples were centrifuged at 2,000 g at 4 °C for 10 minutes to eliminate cells and debris, added with protease inhibitor cocktail (0.8 mmol EDTA, 1.0 µg/mL aprotinin, 1.0 µg/mL leupeptin, and 35.0 µg/mL phenylmethylsulfonyl fluoride [PMSF]) [19] and stored at -80 °C until processing for hormone dosage assays.

2.3. Hormone analysis and sample preparation

In order to confirm the follicle viability in accordance with the stage of follicle development, the concentrations of estradiol (E2) and progesterone (P4) in the FF were determined by enzyme immunoassay, as previously described by Rasmussen et al. [20] and modified for follicular fluid. If necessary, samples were diluted 1:1 to 1:1,000 to fit the standard curves. Assay sensitivity was 0.02 ng/mL and the intra-assay coefficients of variation were <10% for all assays.

Nine samples of each experimental group were used for proteomic analysis. The criteria for selecting the samples were: macroscopically blood-free fluids; intrafollicular estradiol:progesterone ratio [21] ≥ 1 for PreDev, DevF1, and PostDev groups, and < 1 for PreOv; and the inclusion of the same cows in all experimental groups. Aliquots of all selected samples (9 samples x 5 stages = 45 samples) were mixed and used as control group for relative quantification. Pooled FF samples from three cows composed each replicate. The pooled FF samples were processed using a modified trichloroacetic

150 acid/acetone precipitation method [22] to deplete albumin and thus to enhance detection
151 of low-abundance proteins from FF.
152
153 2.4. Protein in-solution digestion and peptide labeling
154 The protein concentration of the FF samples were quantified using bicinchoninic
155 acid protein assay kit (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific Inc.
156 #23227, Waltham, MA, USA) according to manufacturer's instructions. A quantity of
157 100 μg protein was reduced with 1,4-dithiothreitol (DTT) and the filter-aided sample
158 preparation method (FASP™ Protein Digestion Kit, Expedon, San Diego, CA, USA) was
159 then used [23] with a minor modification by substituting sodium chloride with acetonitrile
160 for elution of the proteins from the spin columns to avoid desalt step. Alkylation with
161 0.05 M iodoacetamide solution was performed in the dark at room temperature (23 °C)
162 for 20 minutes. Protein digestion was carried out using trypsin (Promega, Madison, WI,
163 USA) to a final enzyme:protein ratio of 1:100 at 37 °C for 16 hours.
164 The samples were labeled with TMTsixplex™ Isobaric Label Reagent Set
165 (Thermo #90062) according to the manufacturer's instructions. Briefly, TMT reagents
166 were dissolved in 41 μl of acetonitrile, added to samples and the reactions were incubated
167 for one hour at room temperature. To quench the reaction, 8 μl of 5% hydroxylamine was
168 added to the samples and incubated for 15 minutes at room temperature. The samples
169 were then combined at equal amounts, vacuum-dried and stored at -20 °C until analysis.
170 Three experimental replicates were labeled with TMT tags to maximize the accuracy of
171 quantitative proteomics.
172
173 2.5. Mass spectrometry analysis

Samples were analyzed in an LTQ Orbitrap Elite Mass Spectrometer (Thermo Scientific Inc., San Jose, CA, USA) coupled to an UltiMate 3000 RSLS nano chromatography system (Dionex, Camberley, UK). TMT-labelled peptide mixtures were reconstituted in buffer A (2% acetonitrile in 0.1 % formic acid). An amount of 3 μg was loaded on the trapping column C18 PepMap100 (5 μm, 100 A, 300 μm x 5 mm) and then separated using Acclaim PepMap RSLC C18 Nanocolumn (15 cm x 75 μm) with a linear gradient of 5-35% solvent B (80% acetonitrile/0.1% formic acid) over 135 min at a flow rate of 300 nL/min. Eluate from the column was introduced to the mass spectrometer. The ionization voltage was set to 1.7 kV and the ion transfer tube temperature to 220° C. The mass spectrometer was set up in positive ion mode using collision-induced dissociation (CID)/high-energy collision-induced dissociation (HCD) fragmentation methods for MS2. Full scan FTMS spectra were acquired in range from 380 to 1800 m/z, with resolution of 60,000. The maximum injection time for FTMS full scan was set as 200 ms reaching an AGC target value of $1x10^6$. For identification of TMT labelled peptides, the three most abundant peaks from MS spectrum were selected for each fragmentation mode. The HCD MS/MS scan was fixed to start from 100 m/z, with resolution of 15,000, using MS2 AGC target of $5x10^4$. The collision energy was set as 40% NCE. Isolation window of ± 1.5 Da was applied to isolate precursor ions with dynamic exclusion of 20 s. The ITMS CID MS/MS scan spectra were acquired with 35% NCE and an AGC target of $1x10^4$.

2.6. Database searching and bioinformatics analysis

Raw data files were loaded into Proteome Discoverer software (version 2.1, Thermo Fisher Scientific, Bremen, Germany) and searched against the *Bos taurus* NCBI protein database using both the Sequest and Mascot algorithms. The parameters used for

database searches included trypsin as protease with one missed trypsin cleavage allowed, carbamidomethylation (C) as fixed modification, oxidation (M) and TMT-6plex (Y) as variable modification. The mass tolerance of precursor ions was set at 10 ppm and 0.6 Da for fragment ion matches. Only peptides that were filtered with a confidence level of 95% and least one unique peptide was accepted. The false discovery rate was calculated using peptide validator-based on decoy database searching. Labeling efficiency was calculated as the percentage of the total number of unique peptides that were modified by TMT labeling. The peptide ratios were calculated from median peptide spectrum matches (PSMs). These peptide ratios were normalized to the total intensity and then assembled (median) to protein groups.

2.7. Statistical analysis

The normality and homoscedasticity of the data were evaluated using the Kolmogorov-Smirnov and Levene's tests, respectively. Hormone concentration data were log-transformed to attain normality. Comparisons of diameters, steroids and protein concentrations, and E2:P4 ratio between stages of follicle development were performed by PROC GLM procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC, USA) and post-hoc comparisons with Student-Newman-Keuls test. Results are reported as least square means and a P-value < 0.05 was considered as significant. For proteomic data, fold changes were calculated based on the ratio of the case versus control samples. To identify differentially expressed proteins, PROC GLM was performed in normalized relative abundances, with stage of follicle development as factor. Proteins with FDR-adjusted P-value < 0.05 were considered differentially expressed and were included in the protein lists. The potential associations between mean FF concentrations of E2 and P4 and normalized relative abundance of differentially expressed proteins among the

stages were analyzed by Pearson's correlation test and linear regression. For data exploratory analysis, hierarchical clustering using the Euclidian algorithm for dissimilarity with Ward's linkage and principal component analysis (PCA) were performed using GeneSpring software (version 13.0, Agilent Technologies Inc., Santa Clara, CA, USA).

2.8. Functional analysis

Enrichment of canonical pathways and networks of identified proteins were generated using Ingenuity Pathway Analysis software (IPA, QIAGEN Redwood City, CA, USA). The datasets contained respective gene symbols and the normalized relative abundances of proteins. Networks of these genes were generated based on information contained in the Ingenuity Knowledge Base, considering direct and indirect relationships (as general settings), experimentally observed, highly predicted interaction networks, with no restriction for tissues/cell lines or mutations. The Fisher's exact test was used by the IPA to calculate P-values and identify statistically significant pathways and networks. The direction of regulation, i.e. up- or down-regulation, was inferred from the activation z-score in the IPA.

3. Results

3.1. Morphological, hormonal, and biochemical characteristics of the bovine follicles

Mean diameters of follicles ranged between 7.2 mm in the PreDev to 14.1 mm in the PreOv and were affected ($P < 0.001$) by the stage of follicular development. Follicular fluid concentration of E2 was higher ($P < 0.001$) in the PostDev compared with the other groups. Progesterone concentration in FF was dramatically lower ($P < 0.001$) in the PreDev follicles and the increase in intrafollicular P4 concentration after the LH surge

(PreOv) was highly significant ($P < 0.001$). The estradiol:progesterone ratio in the FF, used as follicular viability criteria, indicated the PreDev, DevF1, and PostDev groups were estrogenically active. Total protein concentration was higher ($P < 0.05$) after deviation (PostDev and PreOv) and was mainly associated with E2 levels ($r = 0.67$; $P < 0.001$) (Table 1).

3.2. Proteins identified in bovine follicular fluid

We acquired a total of 50,282 MS/MS spectra and matched to 774 unique peptides, corresponding to 524 proteins. In total, 143 proteins groups were identified with high confidence and were used for the further analysis [24]. A complete list of proteins identified in FF is provided in Supplement Table S1. In the distribution of protein molecular weight (MW) and isoelectric point (pI), most MWs ranged from 10 to 80 kDa (Fig. 1A) and pI values ranged from 5 to 8 (Fig. 1B). Sequence coverage of 21% (30/143) of the proteins was below than 5% and the average sequence coverage was 16% (Fig. 1C). In addition, 56% (80/143) of the proteins had more than one unique peptide (Fig. 1D).

To explore the dataset, we used heat map to provide a first overall view of the expression of 143 proteins that were quantified from the cow reference proteome. Our results showed physiologic process involved during folliculogenesis is coordinate through relatively modest proteomics changes. Hierarchical clustering analysis using Euclidean and Ward's linkage showed two major clusters in the rows dendrogram, corresponding to different time-points of follicle development. There was a distinct change in FF proteome pre and post deviation (Fig. 2A). Principal component analysis was used to discriminate the FF proteome data set according to the stage of follicle development. The results showed a distinguished pattern for PreDev, DevF1, PostDev

and PreOv stages, while DevF2 was not clearly discriminated. Moreover, PCA revealed clustering samples by time-point (Fig. 2B). For further evaluation, we plotted the stages of follicle development according to their correlation with the principal components. A positive correlation between the PostDev and PreOv was found, with no association with samples collected at the first stages of follicle development (PreDev, DevF1 and DevF2) (Fig. 2C).

3.3. Gene Ontology analysis

The FF proteome was classified according to Gene Ontology annotations for cellular localization, biological processes, and molecular function. Classification based on cellular localization indicated 60% of proteins were extracellular (Fig. 3A). The majority of the FF proteins detected were involved in regulation of biological process (29%), response to stimulus (20%), metabolic process (14%), defense response (11%), and transport (7%) (Fig. 3B). The top molecular function categories were protein binding (38%), enzyme regulator activity (25%), metal ion binding (14%), catalytic (9%), and transporter activity (4%) (Fig. 3C).

3.4. Differentially abundant proteins in the bovine follicular fluid

Numerous proteins identified in FF are originate from bovine plasma, such as albumin, immunoglobulins, coagulation, and complement components. It was not possible to correlate a specific protein with a particular stage of follicular development. However, we found 22 differently ($P < 0.05$) expressed proteins between the stages of follicle development (Table 2). Of these proteins, follistatin, inhibin, serglycin, spondin-1, fibrinogen, and anti-testosterone antibody were found up-regulated during early stages of follicular development. On the other hand, apolipoprotein H, alpha-2-macroglobulin,

299 plasminogen, antithrombin-III, and immunoglobulins were up-regulated after follicle
300 deviation (Fig. 4).
301
302 3.5. Correlations between differentially abundant proteins and steroids content in
303 the bovine follicular fluid
304 Intrafollicular E2 concentration was positively correlated with plasminogen ($r = 0.74$; $p < 0.001$). On the other hand, a negative correlation was observed between E2 and
306 crystal structure of modified bovine fibrinogen ($r = -0.61$; $p = 0.02$) and spondin-1($r = -0.60$; $p = 0.02$) proteins (Fig. 5A). There were associations between FF concentration of
308 P4 and 16 other proteins. Of these, the six stronger correlations are presented in Figure
309 5B (additional file in Supplementary Fig. S1). Serglycin ($r = -0.88$; $P < 0.001$), follistatin
310 ($r = -0.82$; $P < 0.001$), inter-alpha trypsin inhibitor ($r = -0.81$; $p < 0.001$), and spondin-1
311 ($r = -0.73$; $P < 0.001$) were negatively correlated with P4, whereas alpha-2-macroglobulin
312 ($r = 0.84$; $P < 0.001$) and immunoglobulin M heavy chain ($r = 0.79$; $P < 0.001$) were
313 positively correlated with P4.
314
315 3.6. Pathway analysis of differentially abundant proteins in the bovine follicular
316 fluid
317 In order to determine the major pathways, biological relevance, and networks
318 related to the relative abundance of the proteins identified in bovine FF over follicle
319 development, we performed functional enrichment analysis using IPA software. The top
320 canonical pathways detected were primarily associated with acute phase response,
321 coagulation system, complement system, liver X receptor (LXR) and retinoid X receptor
322 (LXR) activation, and production of nitric oxide and reactive oxygen species (ROS).
323 Activated (orange) and inhibited (blue) pathways over different stages of follicle

development obtained from IPA are depicted on Figure 6. Notably, the most up-regulated pathway over follicle development was the acute phase response signaling, with the highest z-score at DevF1. The coagulation system pathway was down-regulated at DevF1 and PreOv. The complement system pathway was inhibited during deviation (DevF1 and DevF2) and the production of nitric oxide and ROS pathway changed from down-regulation at PreDev to up-regulation at PostDev and PreOv. The network implicated in the acute phase response signaling integrated 21 proteins, including several key regulatory molecules as Fibrinogen gamma-B chain (FGG) and complement components (Fig. 7). A second network was involved in coagulation system and is shown in Figure 8. Supplementary Table S2 summarizes the networks, genes, and proteins that correspond to the nodes of the statistically significant pathway networks.

4. Discussion

Follicular fluid contains proteins involved in follicle activity, cell differentiation, and oocyte maturation. The expression pattern and functional characterization of the FF proteome during the follicle development may contribute to further understanding of the physiological mechanisms underlying folliculogenesis. A field study was designed to determine the proteome profile of bovine FF according to the stage of follicle development and to evaluate the association of the identified proteins with follicular fluid E2 and P4 concentrations. We used a gel-free isobaric labeling proteomic technology to characterize for the first time a comprehensive over view of protein shifts during bovine follicular development. Numerous differentially ($P < 0.05$) expressed proteins involved in several essential biological processes played an important role in promoting the maturation of bovine follicles, with deviation being a critical time-point for modulation of follicular growth.

In the current study, we precisely monitored the morphologic dynamics of ovarian follicles using transrectal real-time ultrasonography and the stage of follicle development was confirmed endocrinologically by measuring the E2 and P4 concentrations in FF samples. In addition, the primary essential prerequisite for inclusion of FF samples in this study was the absence of blood contamination. All the fluids were macroscopically analyzed and thus retained blood-free. Consequently, there is an improbable correlation between the FF proteins and blood contamination of the collected follicular fluids. For proteomic analysis, our first focus was to eliminate the potential interference of high abundance proteins, which have a masking effect on less abundant proteins and hinder their identification. To overcome this limitation and for better coverage of low abundance proteins, we depleted albumin, the most abundant protein in FF [25].

Ovarian steroids hormones are major regulators of the physiology of the reproductive events. The determination of E2 and P4 concentrations in bovine FF evidenced important variations related to the stage of the follicle development. These results are largely in accord with E2 and P4 concentrations in FF previously reported in cows [26]. Indeed, in the cyclic cow, concentration of steroids in FF are higher than circulating levels [27] and reflect changes in the capacity of a follicle to become ovulatory or atretic. Follicles with an estradiol:progesterone ratio greater than 1 are estrogen-active and healthy and follicles with an estradiol:progesterone ratio less than 1 are estrogen-inactive and atretic [21]. However, near the time of ovulation, LH peak induces a switch from estradiol dominance to progesterone dominance in the follicular fluid of pre-ovulatory follicles [28], that is consistent with the short and rapid increase in progesterone receptor expression observed in the rat granulosa cells [29]. Additionally, the dynamic changes in intrafollicular total protein contents across follicle development found in this study were closely correlated with follicular functional status and could indicate a tight

protein coordination for secretory activity and metabolism of follicular cells, such as modulation of enzymatic activities in follicle development and in oocyte competence acquiring. Secretions from granulosa and theca cells and from capillary diffusion compose bovine FF. During folliculogenesis, the ovarian blood-follicle barrier plays a central function in the regulation of protein transfer [30] and becomes more permeable to plasma molecular components particularly at the terminal phase of follicle development [31]. The protein concentration present at a particular stage of follicle development can be expected to vary with the flux of water, the surface area to volume ratio of the follicle, and the permeability of the thecal blood vessels [25], although other studies have suggested that total protein do not change with follicular growth [27,32].

Gene Ontology enrichment analysis revealed that the majority of FF proteins identified in this study were classified as extracellular (60%) and belonged to a variety of biological processes and molecular functions. Intracellular proteins in the FF are related to cellular apoptosis which usually occurs during follicular development [33]. In addition, it has been reported that FF also contains proteins resulted from epithelial shedding and from extracellular vesicles [34].

Herein, we used a powerful proteomic approach to quantify changes in protein expression at key-stages of follicle development (emergence, deviation, dominance, and pre-ovulatory). The stage of follicle development affected the protein expression of the FF and the 22 differently ($P < 0.05$) expressed proteins found extend the understanding of the molecular changes accountable for remodel the follicle as well as oocyte development and maturation. To our knowledge, no studies have been published about the association between these proteins and follicular development stage in cows. Since the development of timed programs for artificial insemination [35], embryo transfer including oocyte recovery [36] and superovulation [37] are based on ovarian physiology,

399 our results potentially could assist to better explore the reproductive potential of the cow
400 and to obtain better results in the use of reproductive biotechnologies. Therefore, these
401 proteins might serve as potential biomarkers of follicle maturation and oocyte
402 competence, which is in line with the efforts in human research for noninvasive
403 assessment of oocyte quality and ovarian stimulation outcomes.

404 Most of the proteins differently expressed between the stages of follicle
405 development, including fibrinogen, A-2 macroglobulin, complement C4, serine protease
406 inhibitor, and immunoglobulins, were associated with the inflammatory response and
407 may play an essential role in bovine female reproduction. These proteins were mainly
408 related to the advanced stages of follicle maturation, prior ovulation and subsequent
409 luteinization. Previous studies also identified these proteins in human FF. For example,
410 fibrinogen is involved in maintaining early pregnancy [38] and increased after ovarian
411 hyperstimulation [39]. Therefore, the immune system plays a crucial role in follicle
412 development and supports the hypothesis that folliculogenesis and ovulation could be
413 regarded as a hormone-induced inflammatory process [40]. Moreover, during follicular
414 development, proteins associated with protease inhibition (i.e., SERPINC1 and A2M)
415 were gradually up-regulated, suggesting that protein degradation was restrained to
416 support the growth and maturation of oocytes.

417 The coagulation factors are potentially correlated to the inflammatory related
418 proteins present in FF and have relevant roles in the follicular microenvironment. Our
419 results provide compelling evidence that the proteins belonging to the coagulation
420 cascade system of FF are regulated according to the stage of follicle development.
421 Agostini et al. [41] showed an anticoagulant state during follicle growth (the follicular
422 phase) and rupture (the ovulatory phase). These authors stated that prevention of clotting
423 after ovulation ensures that FF can serve as a conduit for delivery of the oocyte to the

oviduct. In addition, thrombin was demonstrated to be an intra-ovarian signal for optimal follicular luteinization in mice [42] and decreased levels of antithrombin in the FF were associated with improved pregnancy outcome in human [43], confirming their crucial role in female reproduction.

Abundance of several proteins was found to be correlated with steroid concentration in FF, which suggests that these follicular proteins are associated with endocrine phenomena in the ovary and that these proteins modulate diverse ovarian physiological processes, including granulosa and theca cells differentiation, oocyte maturation, cumulus cell expansion, luteinization, and early embryo development. However, as pointed out by Kushnir et al. [4], it is yet unclear if associations between abundance of proteins and local concentrations of steroids are “cause and effect” relationships or if they represent covariance of unrelated events. Future studies are necessary to further evaluate these associations.

The functional enrichment analysis showed a particular balance of functions related to acute phase response signaling and coagulation system. Several proteins identified in this study were involved in both generated networks, which indicates that inflammation and coagulation cascade are active processes throughout follicular development. The effectors and inhibitors act in a control and balance time-dependent. Among these compounds, fibrinogen, complement components, and inter-alpha-trypsin inhibitor heavy chain are essentially involved in the acute phase response pathway (Fig. 7). Our comparative analysis showed coagulation system pathway is up-regulated at dominance stage and in the second largest follicle from deviation stage (Fig. 6) that suggest greater activity in large size developing follicles. The coagulation pathway was tightly regulated by three molecules (SERPINC1, PROC, and PLG), which have been previously reported as important mediators of extracellular matrix remodeling,

449 modulating follicular development, ovulation, and corpus luteum formation [6,7,44,45].
450 Furthermore, we also identified pathways related to the biosynthesis of nitric oxide and
451 ROS that could play an essential role, especially during intense metabolism in advanced
452 stages of follicular maturation (dominance and pre-ovulatory). Previous studies reported
453 increased oocyte competence [46] and improved *in vitro* fertilization outcomes [47] when
454 ROS production was elevated, although its role in female reproduction is not completely
455 understood.

456 In this study, we confirmed that temporal regulation of protein expression
457 modulates follicular development and highlight potential biomarkers for follicular
458 maturity. The understanding of ovarian folliculogenesis may have profound implications
459 in improvement of reproductive biotechnologies. Although there is much of information
460 attempting to generate specific biomarkers for follicle development, oocyte quality, and
461 clinical outcome in human, ethical concerns and scarcely available human FF [48] make
462 it difficult to perform detailed assessments of the temporal changes of the proteome
463 profile during folliculogenesis. Based on similarities in ovarian follicular dynamics and
464 endocrine control in cattle and women, the bovine model has been proposed for studying
465 reproductive events in women [49]. Therefore, our results provide an additional layer of
466 understanding on size-dependent protein dynamics in the ovarian follicle
467 microenvironment, associated with changes in local steroid concentrations.

468

469 **5. Conclusions**

470 In summary, we demonstrated numerous differentially expressed proteins in
471 bovine FF according to the stage of follicle development. The obtained data provide new
472 insights into the size-dependent protein changes in ovarian follicle microenvironment,
473 associated with changes in local steroid concentrations, which may influence follicular

function. Comparative pathways analysis highlighted the occurrence in FF of functional networks in a differential temporal balance and control, including activation/inhibition of the acute phase response, coagulation system, complement system, LXR/RXR activation, and nitric oxide and ROS production.

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661 **Table 1**

662 Morphological, hormonal, and biochemical characteristics of the *in vivo* aspirated bovine

663 follicles on different stages of follicle development

	Stage of follicle development				
Parameter[1]	PreDev	DevF1	DevF2	PostDev	PreOv
Follicle diameter (mm)	7.20 ± 0.23^{d}	8.64 ± 0.09^{c}	7.44 ± 0.15^{d}	12.13 ± 0.14^{b}	14.08 ± 0.71^{a}
Estradiol (ng/mL)	21.27 ± 5.60^{b}	26.42 ± 7.72^{b}	12.24 ± 3.03^{b}	105.15 ± 20.89^{a}	17.60 ± 3.08^{b}
Progesterone (ng/mL)	5.96 ± 1.42^{c}	13.40 ± 2.40^{b}	16.17 ± 3.27^{b}	38.35 ± 6.66^{b}	158.13 ± 51.94^{a}
E2:P4 ratio[2]	5.16 ± 2.02^{a}	1.64 ± 0.41^{bc}	0.93 ± 0.22^{c}	2.76 ± 0.46^{ab}	0.14 ± 0.04^{d}
Total protein (mg/mL)	19.41 ± 2.42^{b}	64.51 ± 24.52^{ab}	47.26 ± 11.18^{ab}	80.83 ± 9.32^{a}	71.43 ± 11.98^{a}

664 [a-d] Values with different superscripts in the same row differ significantly ($P < 0.05$).

665 [1] Refers to nine samples in each stage of follicle development that were used for proteomic

666 analysis.

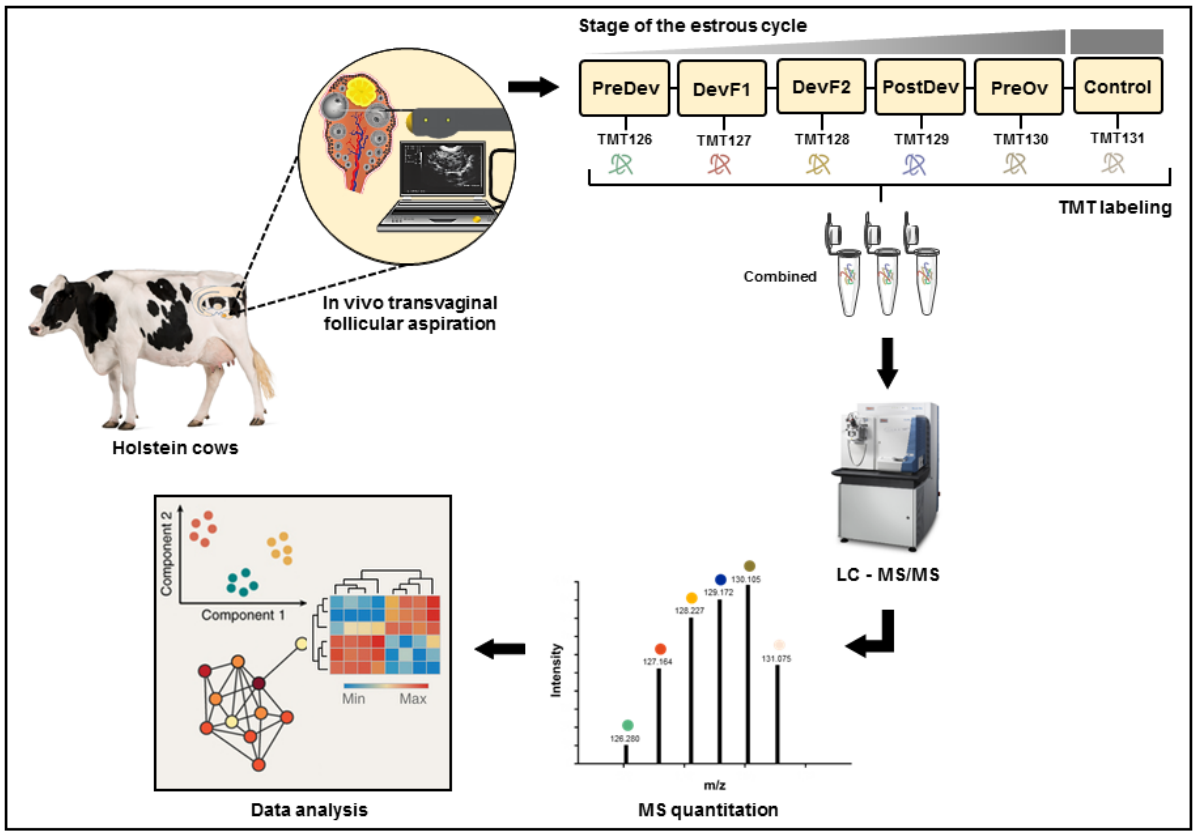
667 [2] Estradiol:progesterone concentration ratio.

668 **Table 2**

669 Differentially expressed proteins in bovine follicular fluid over follicle development

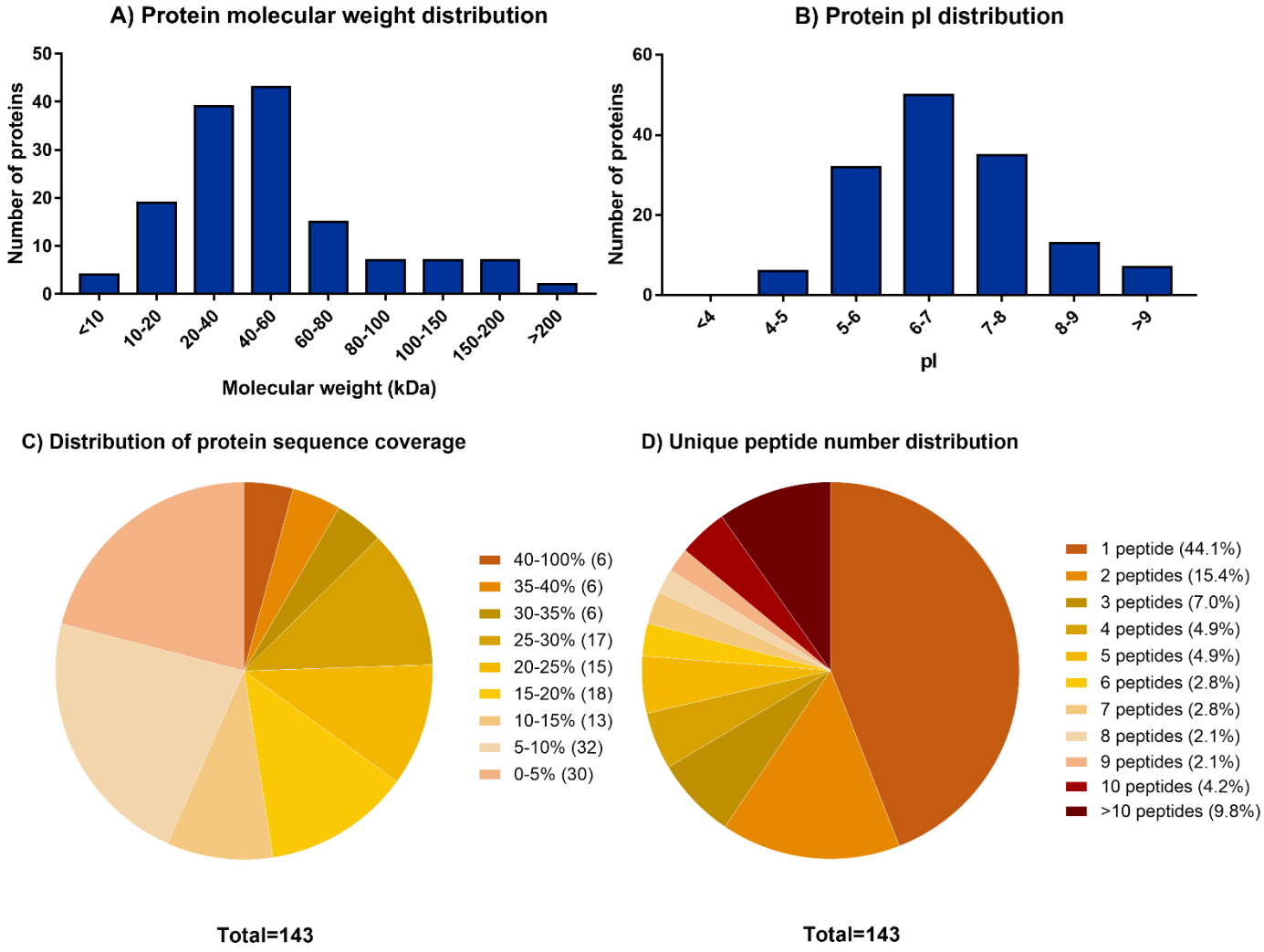
Protein name	Accession	Gene symbol	P-value
Apolipoprotein H	P17690	APOH	0.0002
Immunoglobulin M heavy chain	24496448	IGHM	0.0009
Vitamin K-dependent protein C	131065	PROC	0.0011
Anti-testosterone antibody	432627	GH1	0.0014
Immunoglobulin IgG2a(A1)	252887	IGCGAMMA	0.0018
Spondin-1	Q9GLX9	SPON1	0.0023
Serglycin	70778776	SRGN	0.0034
Alpha-2-macroglobulin	Q7SIH1	A2M	0.0035
Plasminogen	27806815	PLG	0.0038
Immunoglobulin alpha heavy chain	509264946	IGH	0.0041
Complement C4	982963988	C4A	0.0076
Follistatin	P50291	FST	0.0083
Immunoglobulin lambda-like polypeptide 1	741957421	IGLL1	0.0085
Inhibin beta A chain	27805949	INHBA	0.0154
Inter-alpha-trypsin inhibitor heavy chain H1	Q0VCM5	ITIH1	0.0166
Antithrombin-III	555957525	SERPINC1	0.0173
Immunoglobulin gamma3 heavy chain	1040566762	IGHC	0.0175
TBCC domain-containing protein 1	A4IF93	TBCCD1	0.0181
Bovine Antibody Blv1h12 With Ultralong Cdr H3	513137422	IGHD	0.0182
Protein HP-20 homolog	114051225	MGC137014	0.0211
Fibrinogen gamma-B chain	27806893	FGG	0.0336
Crystal Structure Of Modified Bovine Fibrinogen	6980814	FGB	0.0376

670



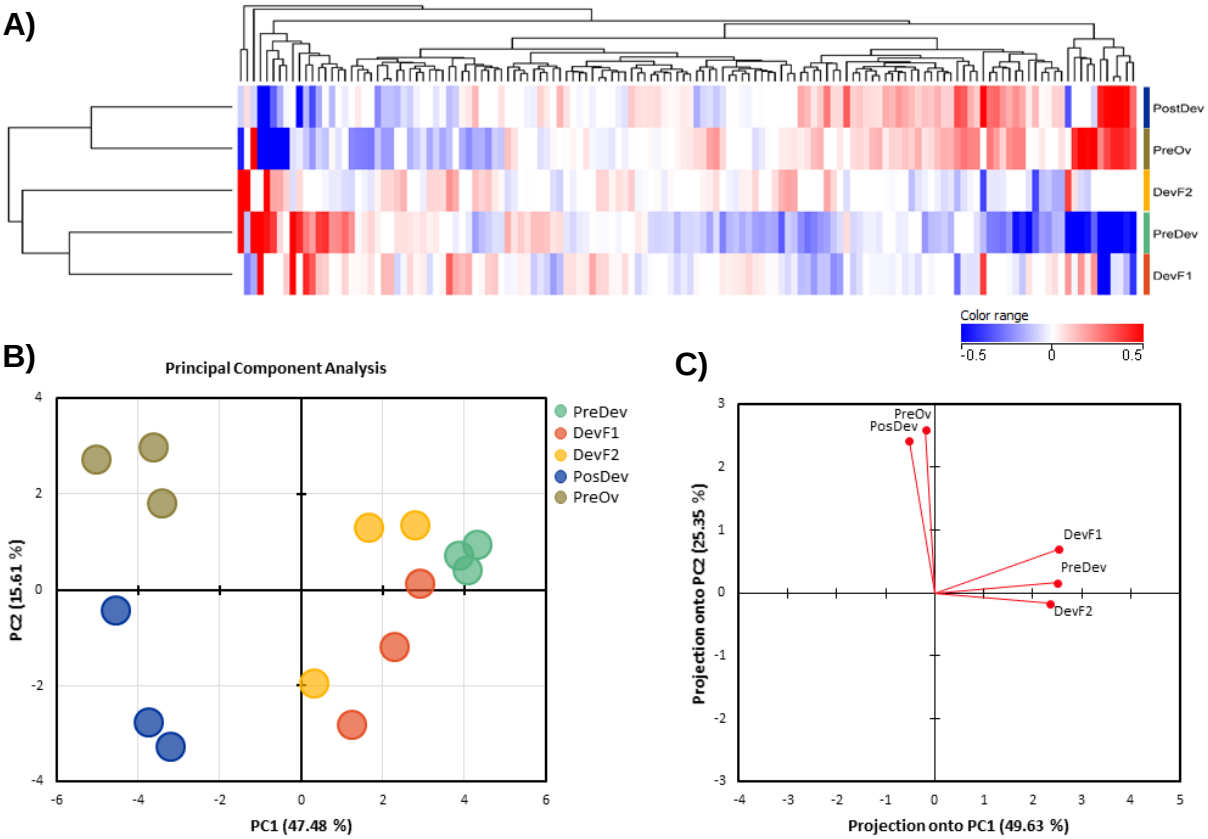
671

672 **Graphical abstract**



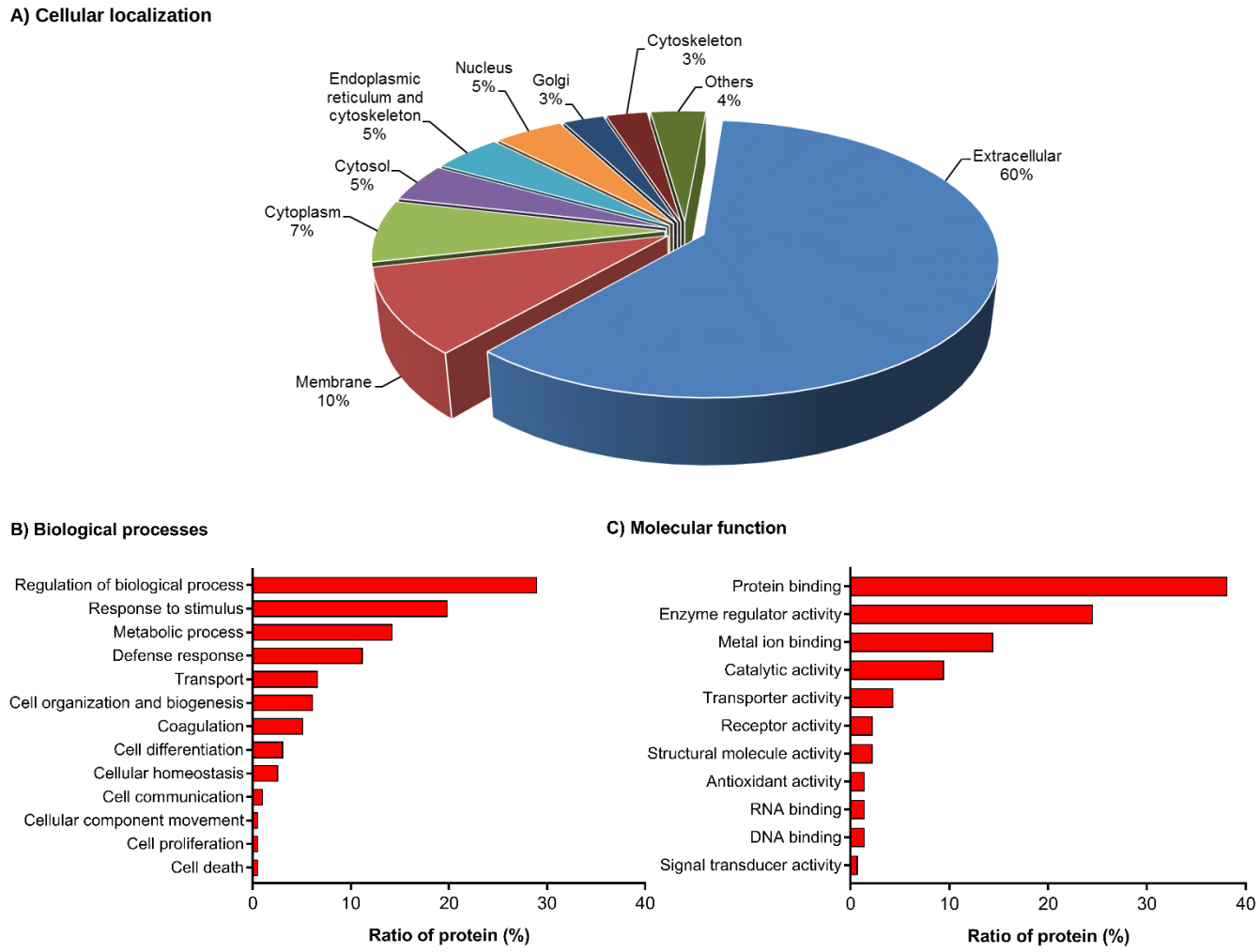
673

674 **Fig. 1.** Distribution of the protein molecular weight (A), protein isoelectric point (pI)
675 values (B), protein sequence coverage (C), and number of unique peptide (D) of the
676 identified proteins in the follicular fluid.



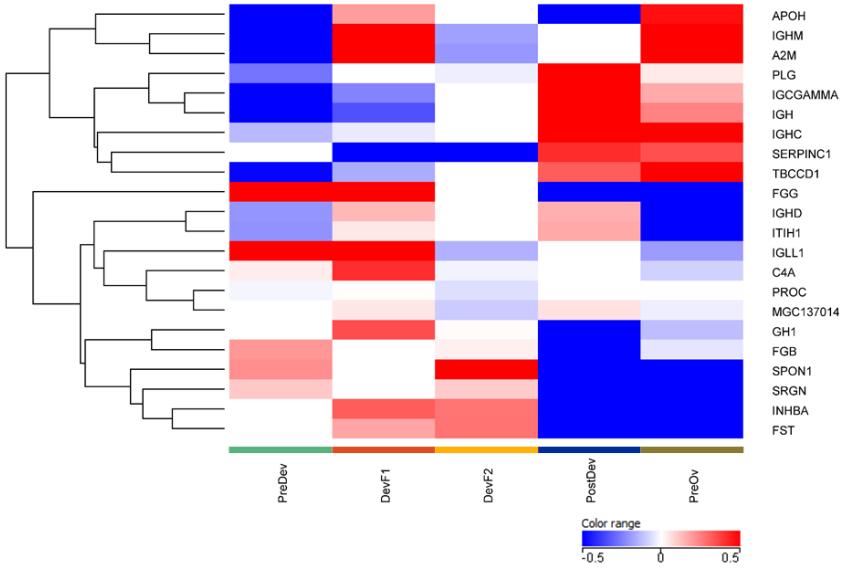
677

678 **Fig. 2.** (A) Hierarchical clustering heat map showing different expression pattern of 143
679 proteins identified by LC-MS/MS in bovine follicular fluid over follicle development.
680 The heat map indicates up-regulation (red), down-regulation (blue), and mean protein
681 expression (white). The rows represent different stages of follicular development. The
682 columns represent individual proteins. (B) PCA plots of the bovine follicular fluid
683 proteome reveal grouping according to stage of follicle development. The data points
684 refer to follicular fluid samples obtained at different stages of follicle development. Time
685 points are identified by colors shown in the legend. (C) PCA biplot with proteins plotted
686 in two dimensions using their projections onto the first two principal components, and
687 stages of follicle development plotted using their weights for the components (red points).



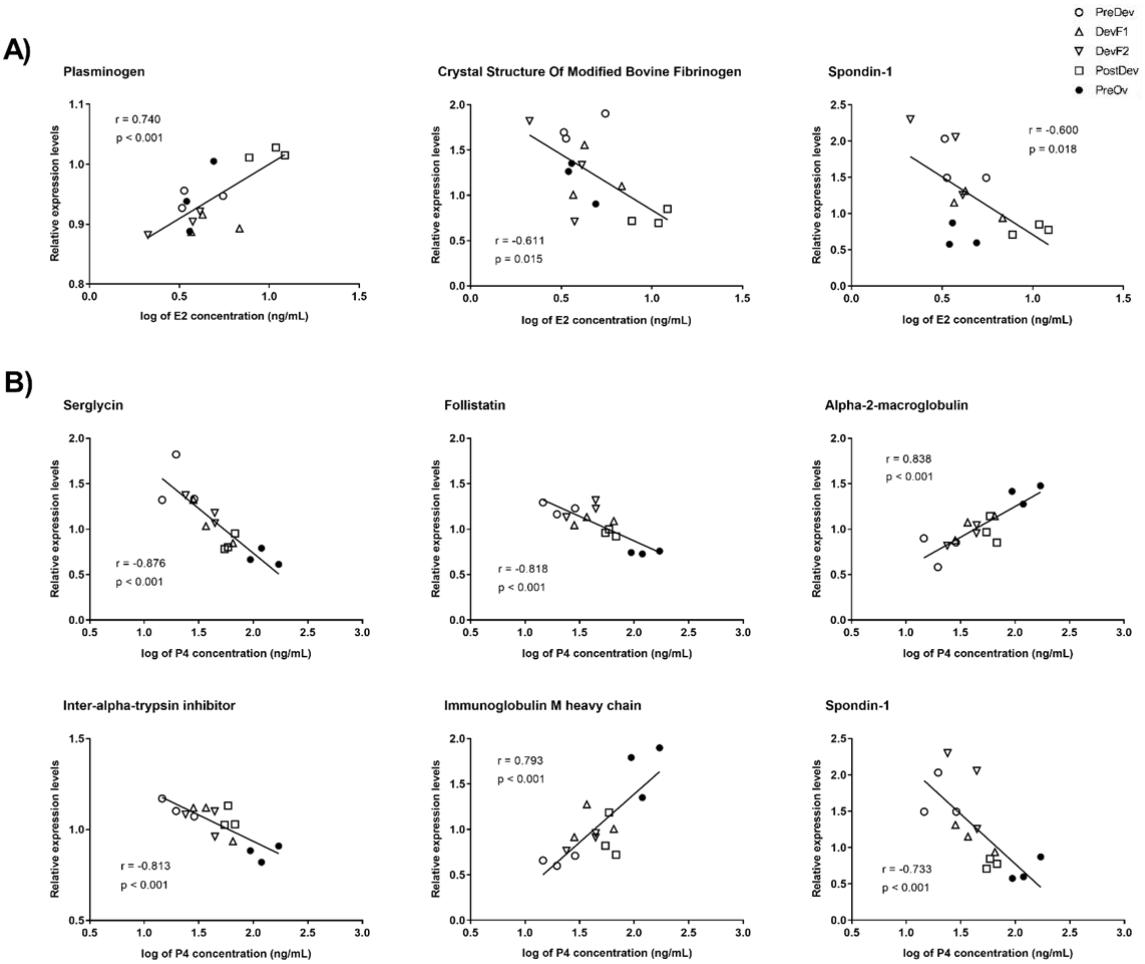
688

689 **Fig. 3.** Gene Ontology analysis of the proteins identified in bovine follicular fluid.
690 Proteins were classified according to (A) cellular localization, (B) biological processes,
691 and (C) molecular function. Results are displayed as percent of genes classified to a
692 category over the total.



693

694 **Fig. 4.** Hierarchical clustering heat map showing the effect of the stage of follicle
695 development on protein expression profiling of 22 differentially expressed proteins in
696 bovine follicular fluid. The heat map indicates up-regulation (red), down-regulation
697 (blue), and mean protein expression (white). Individual proteins are represented by a
698 single row, and each stage of follicle development is represented by a single column.



699 **Fig. 5.** Correlation between abundance of proteins and estradiol (A) and progesterone (B)
700 concentrations in bovine follicular fluid over follicle development.

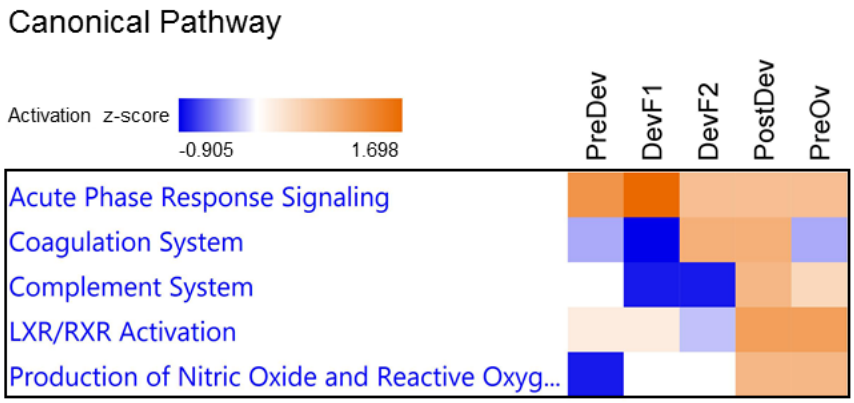
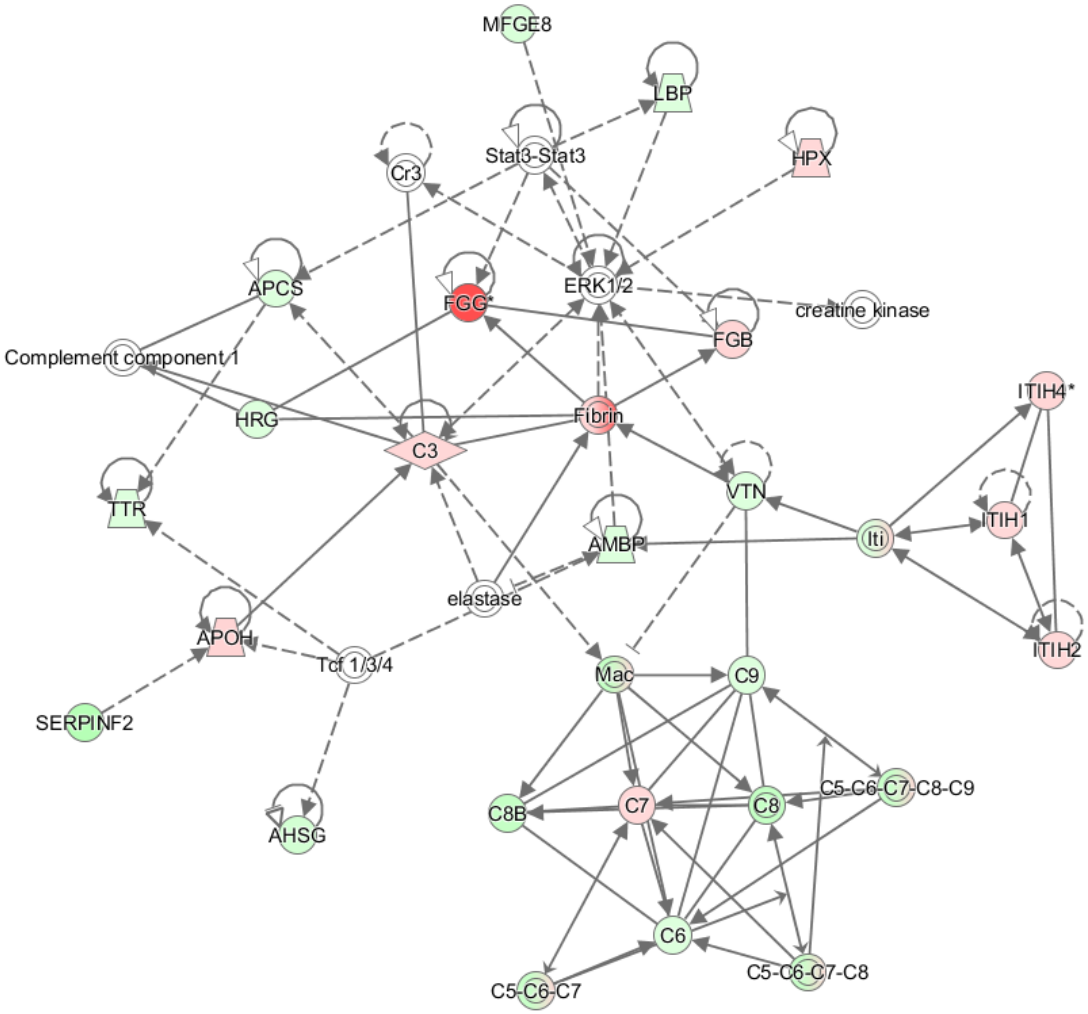
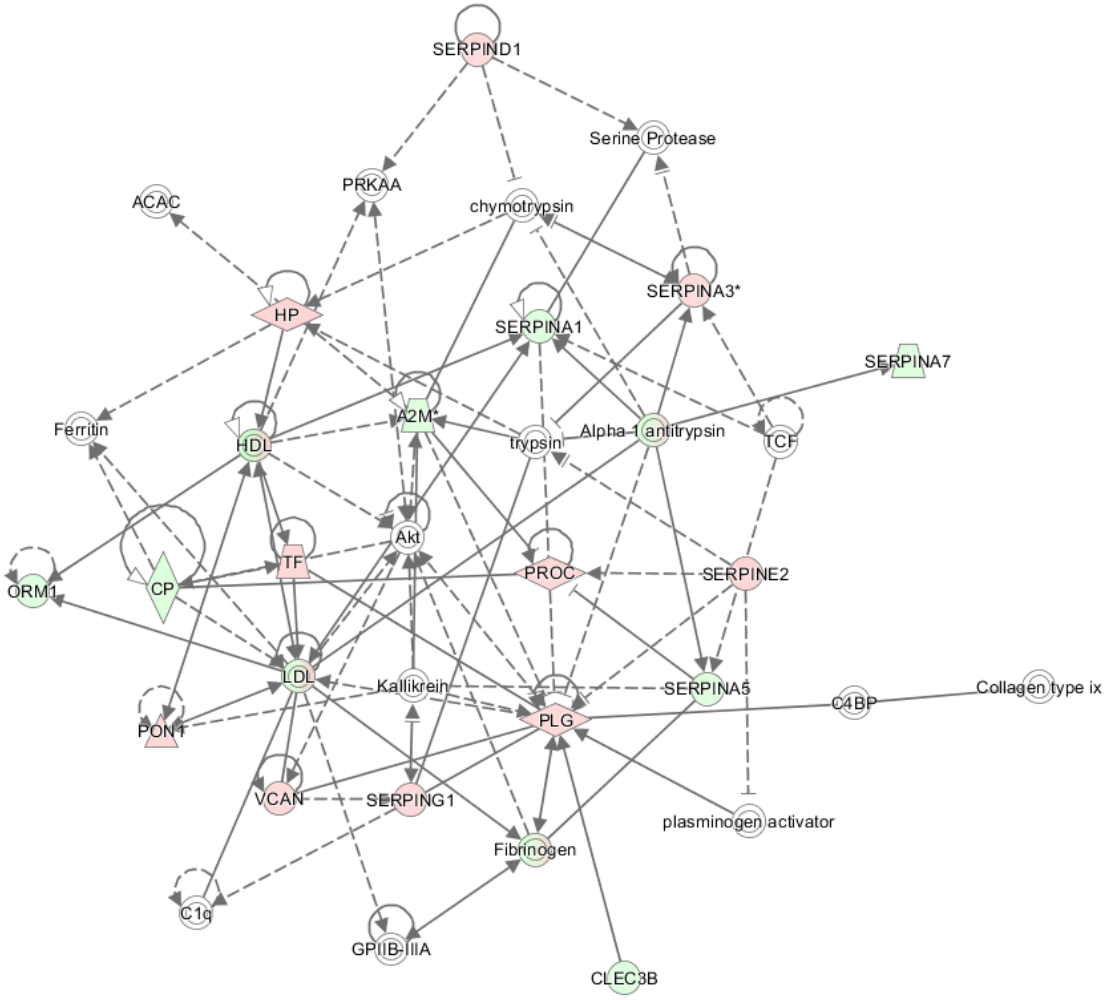


Fig. 6. List of the most significant canonical pathways and their respective scores obtained from Ingenuity Pathway Analysis software. Color intensity indicates the magnitude of regulation inferred from z-score (orange: up-regulation, blue: down-regulation, white: z-score = 0, indicating up-regulation of some proteins and down-regulation of other). LXR = liver X receptor; RXR = retinoid X receptor.



707

708 **Fig. 7.** Network of proteins involved in the acute phase response. The network was
709 generated in Ingenuity Pathway Analysis software. Entire lines represent direct and
710 evidence interactions and dash lines represent presumed interactions between proteins.
711 Colors indicate the nature of the expression: red are up-regulated and green are down-
712 regulated. Uncolored nodes mean unspecified effect in our experiment and were
713 integrated into the computationally generated network based on the evidence stored in the
714 IPA knowledge memory indicating a relevance to this network.



715

716 **Fig. 8.** Network of proteins involved in the coagulation system. The network was
717 generated in Ingenuity Pathway Analysis software. Entire lines represent direct and
718 evidence interactions and dash lines represent presumed interactions between proteins.
719 Colors indicate the nature of the expression: red are up-regulated and green are down-
720 regulated. Uncolored nodes mean unspecified effect in our experiment and were
721 integrated into the computationally generated network based on the evidence stored in the
722 IPA knowledge memory indicating a relevance to this network.

Supplementary Table S1

List of identified proteins in bovine follicular fluid

Description	Accession	Coverage (%)	# Unique Peptides	MW (kDa)	calc. pI
Adipocyte complement-related 30 kDa protein	114158576	5.0	1	26.1	5.7
AF4/FMR2 family member 4	300798451	1.1	1	127.4	9.3
Alpha-1-acid glycoprotein	Q3SZR3	39.1	10	23.2	5.9
Alpha-1-antiproteinase precursor	27806941	25.7	14	46.1	6.5
Alpha-1B-glycoprotein precursor	114053019	26.8	9	53.5	5.4
Alpha-2-antiplasmin	P28800	12.2	7	54.7	5.7
Alpha-2-HS-glycoprotein	P12763	14.2	5	38.4	5.5
Alpha-2-macroglobulin	Q7SIH1	16.7	21	167.5	6.0
Alpha-2-macroglobulin isoform X1	982926875	2.4	2	164.0	7.0
Alpha-2-macroglobulin variant 5	408689573	4.4	1	45.0	7.5
Alpha-S1-casein	P02662	7.9	2	24.5	5.0
Angiotensinogen precursor	166159174	18.4	5	45.4	9.2
Anti-respiratory syncytial virus Ig heavy chain V region	508834	7.1	1	15.1	6.7
Anti-testosterone antibody	432627	24.7	1	24.4	7.6
Antithrombin-III	555957525	37.0	16	52.4	6.8
Apolipoprotein A-I	P15497	42.6	11	30.3	6.0
Apolipoprotein R	76677514	4.1	1	21.8	6.7
Beta-2-glycoprotein 1	P17690	2.6	1	38.2	8.2
Ceruloplasmin isoform X1	528937555	17.6	14	124.2	6.1
Chain A, Crystal Structure Of Bovine Plasma Copper-Containing Amine Oxidase	61680007	16.8	11	82.7	5.9
Chain A, The Crystal Structure Of Modified Bovine Fibrinogen	6980814	45.1	1	42.7	8.0
Chain H, Crystal Structure Of Bovine Antibody Blv5b8 With Ultralong Cdr H3	513137425	13.1	2	29.1	7.8

Description	Accession	Coverage (%)	# Unique Peptides	MW (kDa)	calc. pI
Chain L, Crystal Structure Of Bovine Antibody Blv1h12 With Ultralong Cdr H3	513137422	31.5	2	22.5	6.1
Clusterin preproprotein	27806907	6.6	4	51.1	6.0
Coagulation factor IX isoform X1	529013735	3.9	2	52.0	5.9
Complement C3 isoform X1	741932316	43.1	68	187.1	6.8
Complement C3 isoform X1	528906950	2.0	3	186.2	7.0
Complement C4 precursor	262050656	13.6	10	192.7	7.4
Complement C4-A-like	982963988	12.4	4	157.7	7.8
Complement C5a anaphylatoxin precursor	262205546	4.4	8	188.7	6.6
Complement component C6 precursor	114051692	6.0	4	104.5	7.0
Complement component C7	Q29RQ1	1.1	1	93.0	7.1
Complement component C8 beta chain precursor	931717266	2.4	2	66.6	8.0
Complement component C8 gamma chain isoform X1	741944959	5.9	1	28.5	9.5
Complement component C9	Q3MHN2	10.9	6	62.0	5.9
Complement factor B	P81187	15.2	12	85.3	7.7
Complement factor H	Q28085	5.1	5	140.3	6.8
Complement factor I	296486756	4.9	3	68.9	7.7
Endopin 1b	296475221	26.3	0	46.1	6.5
Endopin 2B	38683423	24.0	5	47.0	6.2
Endopin 2C-like	296475302	28.3	6	46.9	6.0
Factor XIIa inhibitor precursor	27807349	17.5	7	51.7	6.7
Fetuin-B	Q58D62	9.0	4	42.6	5.8
Fibrinogen alpha chain	P02672	38.9	9	67.0	7.2
Fibrinogen beta chain	P02676	29.7	14	53.3	8.2
Fibrinogen gamma-B chain isoform X1	528981276	29.0	2	49.1	5.9
Fibrinogen gamma-B chain precursor	27806893	22.3	1	50.2	5.8
Fibronectin	218512156	2.1	4	272.0	5.5

Description	Accession	Coverage (%)	# Unique Peptides	MW (kDa)	calc. pI
Follistatin	P50291	7.6	2	37.9	6.1
Gelsolin	Q3SX14	20.5	13	80.7	5.8
Haptoglobin	Q2TBU0	8.2	4	44.8	7.7
Hemopexin precursor	77736171	28.1	14	52.2	7.8
Heparin cofactor 2 isoform X1	528989611	21.7	9	62.7	6.7
Histidine-rich glycoprotein precursor	27806875	16.3	8	61.9	7.7
Ig gamma-2 chain C region (clone 32.2) - bovine (fragment)	89611	29.4	3	36.0	7.8
IgG1 heavy chain constant region	7547266	29.8	3	35.8	6.5
IgG2a heavy chain constant region	1699167	26.1	2	35.8	7.6
IgG3 heavy chain constant region	1575495	19.3	1	38.7	7.6
IgG3 heavy chain constant region	1575493	20.7	1	38.6	8.0
IGK protein	115545495	21.3	6	26.6	6.5
IgM	1399521	6.2	1	15.7	8.2
IgM precursor	326937675	1.6	1	51.6	6.6
Immunoglobulin alpha heavy chain variable region	509264946	4.3	1	19.3	7.0
Immunoglobulin delta heavy chain variable region	509264562	9.9	1	32.2	6.4
Immunoglobulin delta heavy chain variable region	509264466	6.7	1	27.0	6.0
Immunoglobulin delta heavy chain variable region	509264436	3.4	1	27.9	5.6
Immunoglobulin delta heavy chain variable region	509264541	10.0	1	32.3	6.5
Immunoglobulin delta heavy chain variable region	509264513	3.9	1	19.1	7.0
Immunoglobulin epsilon heavy chain variable region	509264776	6.0	1	14.3	4.9
Immunoglobulin epsilon heavy chain variable region	509264882	6.0	1	19.3	6.0
Immunoglobulin epsilon heavy chain variable region	509264873	5.6	1	21.5	8.2
Immunoglobulin gamma 1 heavy chain constant region	91982959	27.7	3	35.9	6.9
Immunoglobulin heavy chain variable region	1293590	5.7	1	16.7	8.6
Immunoglobulin heavy chain variable region	3892122	6.4	1	13.4	9.0

Description	Accession	Coverage (%)	# Unique Peptides	MW (kDa)	calc. pI
Immunoglobulin IgG2a(A1) allotype	252887	17.2	1	6.1	7.9
Immunoglobulin lambda light chain	15088675	17.0	1	24.7	6.1
Immunoglobulin lambda light chain constant region 2 allotypic variant IGLC2a	343196996	39.6	1	11.3	9.1
Immunoglobulin lambda light chain constant region 2 allotypic variant IGLC2b	343197004	31.1	1	11.3	8.6
Immunoglobulin lambda light chain constant region 3 allotypic variant IGLC3c	343197026	42.5	5	11.2	8.3
Immunoglobulin lambda light chain variable region	1276609	6.9	1	13.5	7.9
Immunoglobulin lambda light chain variable region	1276599	6.9	1	13.6	5.1
Immunoglobulin lambda-6a light chain variable region-like	296478325	7.1	1	14.8	4.6
Immunoglobulin lambda-like polypeptide 1 isoform X1	741957421	28.4	2	24.7	8.0
Immunoglobulin light chain variable region	2555149	23.2	1	11.7	8.0
Immunoglobulin light chain variable region	2323404	30.0	1	11.3	6.5
Immunoglobulin light chain variable region	2555151	7.3	1	11.4	8.9
Immunoglobulin M heavy chain secretory form	24496448	3.8	1	65.6	6.0
Inhibin alpha chain	P07994	13.1	3	38.8	7.2
Inhibin beta A chain precursor	27805949	5.4	3	47.5	7.9
Inhibitor of carbonic anhydrase precursor	114053269	3.2	1	69.1	7.9
Inter-alpha globulin inhibitor H2 polypeptide	296481520	11.9	10	106.1	7.9
Inter-alpha-trypsin inhibitor heavy chain H1	Q0VCM5	14.8	10	101.2	7.4
Inter-alpha-trypsin inhibitor heavy chain H4 isoform X2	982961939	27.1	2	99.6	6.4
Inter-alpha-trypsin inhibitor heavy chain H4 precursor	75832116	30.1	2	101.4	6.7
Isoform LMW of Kininogen-2	P01045-2	23.5	0	48.1	6.0
Keratin, type II cytoskeletal 2 epidermal isoform X1	297474462	1.9	1	64.2	8.4
Kininogen fragment,low MW	222986	76.6	1	4.9	5.1
Kininogen-2 isoform II precursor	164450481	16.8	1	68.7	6.6
Kininogen-2 isoform X1	528936694	23.4	3	48.4	6.3
Lactadherin	Q95114-1	3.3	1	47.4	7.2

Description	Accession	Coverage (%)	# Unique Peptides	MW (kDa)	calc. pI
Leucine-rich alpha-2-glycoprotein precursor	114051379	21.7	7	38.3	7.0
Lipopolysaccharide-binding protein	Q2TBI0	2.3	1	53.7	7.1
Membrane-bound immunoglobulin epsilon heavy chain constant region	1040566768	1.8	1	55.8	6.0
Membrane-bound immunoglobulin gamma1 heavy chain constant region	1040566764	22.9	3	43.5	5.6
Membrane-bound immunoglobulin gamma2 heavy chain constant region	1040566766	19.7	2	43.7	6.6
Membrane-bound immunoglobulin gamma3 heavy chain constant region	1040566762	13.3	1	46.4	6.4
Pancreatic elastase inhibitor	861514	66.7	1	1.9	4.5
Plasma serine protease inhibitor precursor	28603766	5.4	1	45.3	9.4
Plasminogen precursor	27806815	8.1	7	91.2	7.5
Protein AMBP	P00978	5.7	2	39.2	7.6
Protein HP-20 homolog precursor	114051225	34.0	5	20.6	8.6
Protein HP-25 homolog 1	Q2KIX7	13.2	2	22.5	7.5
Protein HP-25 homolog 2	Q2KIU3	16.7	3	22.9	5.3
Prothrombin	P00735	14.1	10	70.5	6.3
Reticulocalbin-2 precursor	115497628	2.5	1	36.9	4.4
Serglycin precursor	70778776	18.7	2	16.5	4.4
Serotransferrin precursor	114326282	34.2	18	77.7	7.1
Serpin A3-1	Q9TTE1	29.7	5	46.2	6.0
Serpin A3-4	A2I7N0	25.1	2	46.3	6.3
Serpin A3-5	528922995	24.8	1	46.3	6.7
Serpin A3-8	A6QPQ2	35.2	10	46.9	6.1
Serpin peptidase inhibitor, clade E	115305397	15.9	8	43.9	9.5
Serum albumin	P02769	29.7	19	69.2	6.2
Serum albumin precursor	30794280	30.8	20	69.3	6.2
Serum amyloid P-component precursor	77735883	6.7	2	25.2	8.3
Serum paraoxonase/arylesterase 1 precursor	114053183	9.3	2	39.8	5.4

Description	Accession	Coverage (%)	# Unique Peptides	MW (kDa)	calc. pI
Sex hormone-binding globulin isoform X1	528995099	8.0	2	46.0	6.3
Spondin-1	Q9GLX9	1.0	1	90.9	6.0
TBCC domain-containing protein 1	A4IF93	2.9	1	63.4	8.9
Tetranectin	Q2KIS7	6.9	1	22.1	5.6
Thyroxine-binding globulin	Q9TT36	8.0	2	46.0	5.7
Transthyretin precursor	27806789	21.1	4	15.7	6.3
Trypsin inhibitor, TI=serpin {N-terminal, reactive-site loop}	861517	40.0	1	2.3	7.3
Ubiquitin thioesterase OTU1	Q05B57	11.5	1	38.0	5.7
Unknown (protein for MGC:159378)	151556360	21.6	1	24.7	7.6
Versican core protein	P81282	0.6	2	369.8	4.6
Vimentin	P48616	4.1	1	53.7	5.1
Vitamin D binding protein	397740864	15.2	1	53.3	5.6
Vitamin D-binding protein	Q3MHN5	15.8	1	53.3	5.5
Vitamin K-dependent protein C heavy chain	131065	1.8	1	51.4	6.1
Vitronectin precursor	78045497	15.3	6	53.5	6.3
Zinc finger CCCH domain-containing protein 15	114051974	1.9	1	48.5	5.2
Zinc-alpha-2-glycoprotein precursor	77735615	5.4	1	33.8	5.2

Supplementary Table S2

Signaling pathway networks associated with proteins identified in bovine follicular fluid

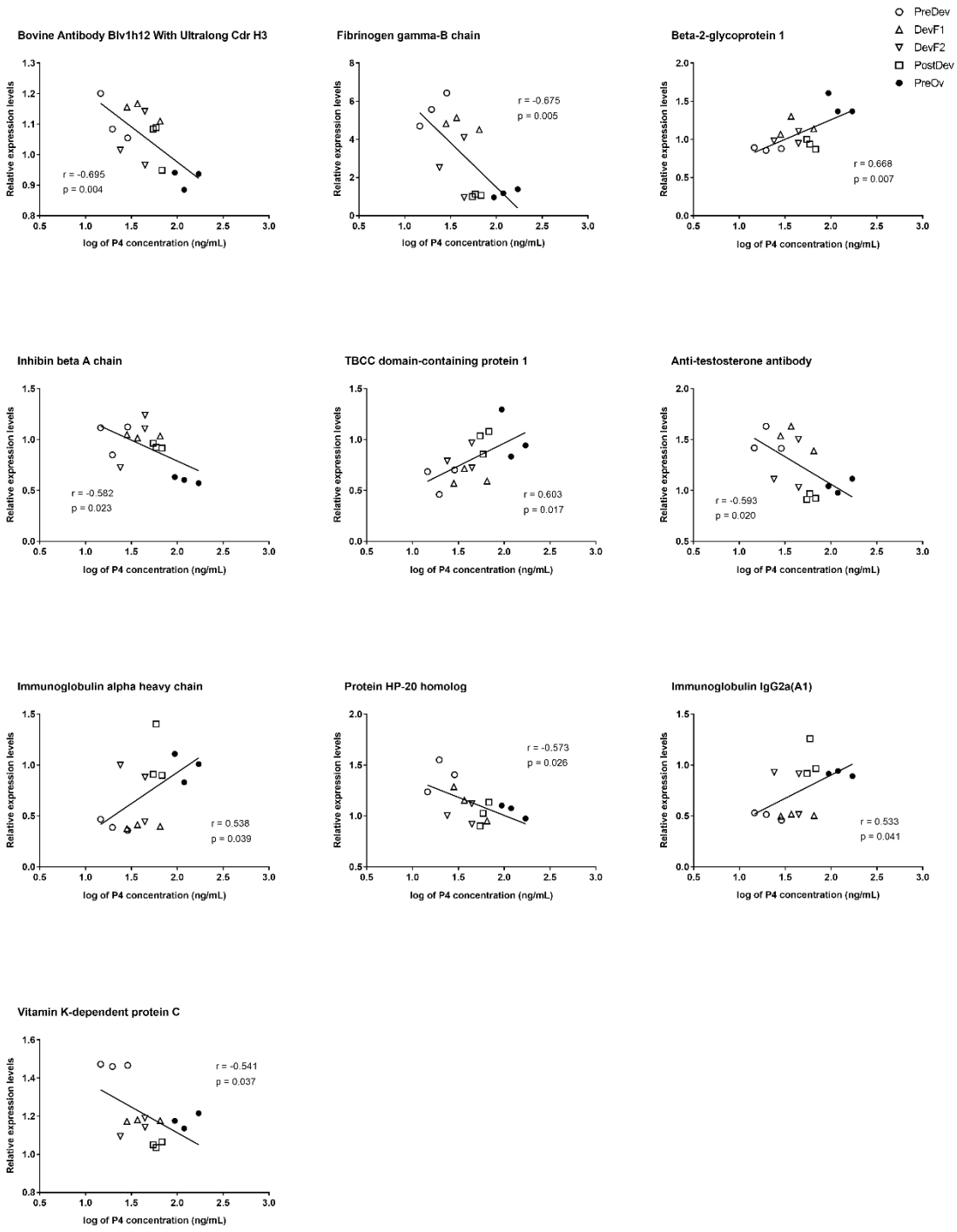
PreDev			
Network	Molecules in network	Score	Number of identified proteins
1	AHSG, AMPB, APCS, APOH, C3, C6, C7, C8, C9, C5-C6-C7, C5-C6-C7-C8, C5-C6-C7-C8-C9, C8B, Complement component 1, Cr3, creatine kinase, elastase, ERK1/2, FGB, FGG, Fibrin, HPX, HRG, Iti, ITIH1, ITIH2, ITIH4, LBP, Mac, MFGE8, SERPINF2, Stat3-Stat3, Tcf 1/3/4, TTR, VTN	45	21
2	A2M, ACAC, Akt, Alpha 1 antitrypsin, C1q, C4BP, chymotrypsin, CLEC3B, Collagen type ix, CP, Ferritin, Fibrinogen, GPIIB-IIIA, HDL, HP, Kallikrein, LDL, ORM 1, plasminogen activator, PLG, PON1, PRKAA, PROC, Serine Protease, SERPINA 1, SERPINA 3, SERPINA 5, SERPINA 7, SERPIND1, SERPINE2, SERPING1, TCF, TF, trypsin, VCAN	35	17
3	ADIPOQ, ALB, APOA1, AZGP1, C5, C3-Cfb, CFB, CFH, CF1, CLU, Collagen Alpha1, FST, GOT, Growth hormone, hemoglobin, lfn, lgG, lkb, IL1, IL12 (complex), immunoglobulin, INHA, INHBA, Inhibin, KNG1, Ldh (complex) LDL-cholesterol, Lh, mediator, NADPH oxidase, NFkB (complex), Pro-inflammatory Cytokine, SHBG, Sod, Tnf (family)	27	14
4	A1BG, ABCC6, AFF4, APA4F1, APIP, ASCC1, BLOC155, C14orf169, EED, ELAVL1, GLIPR2, HMG20B, HNF4A, HPX, INHBE, ITIH4, KLK14, MYCPKM, POLD1, PPM1F, Pzp, RCN2, RNF2, TBCCD1, THOC3, THOP1, TM9SF4, TTC27, UBE2J1, XPO1, YOD1, YTHDF1, ZC3H15	16	9
5	Actin, AMPK, calpain, Cofilin, Collagen type I, Collagen type IV, Collagen (s), Cytokeratin, ERK, F2, F Actin, FETUB, Focal adhesion kinase, GC, GSN, Integrin, KRT2, Laminin, LRG1, Mek, MIc, p70 S6k, p85 (pik3r), Pdgf (complex), PDGF BB, P13K (complex), PLA2, Pld, Rac, Rock, Sos, SPON1, SRGN, Tgf beta, VIM	15	9

DevF1			
Network	Molecules in network	Score	Number of identified proteins
1	AHSG, AMPB, APCS, APOH, C3, C6, C7, C8, C9, C5-C6-C7, C5-C6-C7-C8, C5-C6-C7-C8-C9, C8B, Complement component 1, Cr3, creatine kinase, elastase, ERK1/2, FGB, FGG, Fibrin, HPX, HRG, Iti, ITIH1, ITIH2, ITIH4, LBP, Mac, MFGE8, SERPINF2, Stat3-Stat3, Tcf 1/3/4, TTR, VTN	45	21
2	A2M, ACAC, Akt, Alpha 1 antitrypsin, C1q, C4BP, chymotrypsin, CLEC3B, Collagen type ix, CP, Ferritin, Fibrinogen, GPIIB-IIIA, HDL, HP, Kallikrein, LDL, ORM 1, plasminogen activator, PLG, PON1, PRKAA, PROC, Serine Protease, SERPINA1, SERPINA3, SERPINA5, SERPINA7, SERPIND1, SERPINE2, SERPING1, TCF, TF, trypsin, VCAN	35	17
3	ADIPOQ, ALB, APOA1, AZGP1, C5, C3-Cfb, CFB, CFH, CF1, CLU, Collagen Alpha1, FST, GOT, Growth hormone, hemoglobin, lfn, lgG, lkb, IL1, IL12 (complex), immunoglobulin, INHA, INHBA, Inhibin, KNG1, Ldh (complex) LDL-cholesterol, Lh, mediator, NADPH oxidase, NFkB (complex), Pro-inflammatory Cytokine, SHBG, Sod, Tnf (family)	27	14
4	A1BG, ABCA7, ABCC6, AFF4, ARL1, ARL8B, ASCC1, C14orf169, EED, ELAVL1, GLIPR2, HNF4A, HPX, INHBE, ITIH4, KLK14, MOCOS, MTHFD1, MYC, PIPNB, Pzp, RAB10, RCN2, RNF2, SAA2, SUCLG1, TBCCD1, THOC3, TM9SF4, TNS3, UBE2J1, XPO1, YOD1, ZC3H15	16	9
5	Actin, AMPK, calpain, Cofilin, Collagen type I, Collagen type IV, Collagen (s), Cytokeratin, ERK, F2, F Actin, FETUB, Focal adhesion kinase, GC, GSN, Integrin, KRT2, Laminin, LRG1, Mek, MIc, p70 S6k, p85 (pik3r), Pdgf (complex), PDGF BB, P13K (complex), PLA2, Pld, Rac, Rock, Sos, SPON1, SRGN, Tgf beta, VIM	15	9

DevF2			
Network	Molecules in network	Score	Number of identified proteins
1	AHSG, AMPB, APCS, APOH, C3, C6, C7, C8, C9, C5-C6-C7, C5-C6-C7-C8, C5-C6-C7-C8-C9, C8B, Complement component 1, Cr3, creatine kinase, elastase, ERK1/2, FGB, FGG, Fibrin, HPX, HRG, Iti, ITIH1, ITIH2, ITIH4, LBP, Mac, MFGE8, SERPINF2, Stat3-Stat3, Tcf 1/3/4, TTR, VTN	45	21
2	A2M, ACAC, Akt, Alpha 1 antitrypsin, C1q, C4BP, chymotrypsin, CLEC3B, Collagen type ix, CP, Ferritin, Fibrinogen, GPIIB-IIIA, HDL, HP, Kallikrein, LDL, ORM 1, plasminogen activator, PLG, PON1, PRKAA, PROC, Serine Protease, SERPINA1, SERPINA3, SERPINA5, SERPINA7, SERPIND1, SERPINE2, SERPING1, TCF, TF, trypsin, VCAN	35	17
3	ADIPOQ, ALB, APOA1, AZGP1, C5, C3-Cfb, CFB, CFH, CF1, CLU, Collagen Alpha1, FST, GOT, Growth hormone, hemoglobin, lfn, lgG, lkb, IL1, IL12 (complex), immunoglobulin, INHA, INHBA, Inhibin, KNG1, Ldh (complex) LDL-cholesterol, Lh, mediator, NADPH oxidase, NFkB (complex), Pro-inflammatory Cytokine, SHBG, Sod, Tnf (family)	27	14
4	Actin, AMPK, calpain, Cofilin, Collagen type I, Collagen type IV, Collagen (s), Cytokeratin, ERK, F2, F Actin, FETUB, Focal adhesion kinase, GC, GSN, Integrin, KRT2, Laminin, LRG1, Mek, MIc, p70 S6k, p85 (pik3r), Pdgf (complex), PDGF BB, P13K (complex), PLA2, Pid, Rac, Rock, Sos, SPON1, SRGN, Tgf beta, VIM	15	9
5	A1BG, ABCA7, AFF4, AHCYL1, ATAD3B, CCNT2, CDK12, EAF2, EED, ELAVL1, ELL, FBN2, HEXIM2, HNF4A, ICE1, INHBE, ITIH4, LARP7, MLLT3, MYC, NAP1L4, NDUFA4, NPM3, Pzp, RCN2, RNF2, SRSF6, STC2, TBCCD1, Tmsb4x (includes others), XPO1, YOD1, ZC3H15, ZFP91	14	8

PostDev			
Network	Molecules in network	Score	Number of identified proteins
1	AHSG, AMPB, APCS, APOH, C3, C6, C7, C8, C9, C5-C6-C7, C5-C6-C7-C8, C5-C6-C7-C8-C9, C8B, Complement component 1, Cr3, creatine kinase, elastase, ERK1/2, FGB, FGG, Fibrin, HPX, HRG, Iti, ITIH1, ITIH2, ITIH4, LBP, Mac, MFGE8, SERPINF2, Stat3-Stat3, Tcf 1/3/4, TTR, VTN	45	21
2	A2M, ACAC, Akt, Alpha 1 antitrypsin, C1q, C4BP, chymotrypsin, CLEC3B, Collagen type ix, CP, Ferritin, Fibrinogen, GPIIB-IIIA, HDL, HP, Kallikrein, LDL, ORM 1, plasminogen activator, PLG, PON1, PRKAA, PROC, Serine Protease, SERPINA1, SERPINA3, SERPINA5, SERPINA7, SERPIND1, SERPINE2, SERPING1, TCF, TF, trypsin, VCAN	35	17
3	ADIPOQ, ALB, APOA1, AZGP1, C5, C3-Cfb, CFB, CFH, CF1, CLU, Collagen Alpha1, FST, GOT, Growth hormone, hemoglobin, lfn, lgG, lkb, IL1, IL12 (complex), immunoglobulin, INHA, INHBA, Inhibin, KNG1, Ldh (complex) LDL-cholesterol, Lh, mediator, NADPH oxidase, NFkB (complex), Pro-inflammatory Cytokine, SHBG, Sod, Tnf (family)	27	14
4	A1BG, ABCA7, AFF4, AP4E1, ARL1, ARL8B, BLOC1S5, C14orf169, EED, ELAVL1, FOXJ3, GLIPR2, HNF4A, HPX, ITIH4, KLK14, MOCOS, MTHFD1, MYC, PIPNB, Pzp, RANBP3, RCN2, RNF2, SAA2, SUCLG1, TBCCD1, THOC3, TM9SF4, TNS3, TTC27, UBE2J1, XPO1, YOD1, ZC3H15	16	9
5	Actin, AMPK, calpain, Cofilin, Collagen type I, Collagen type IV, Collagen (s), Cytokeratin, ERK, F2, F Actin, FETUB, Focal adhesion kinase, GC, GSN, Integrin, KRT2, Laminin, LRG1, Mek, MIc, p70 S6k, p85 (pik3r), Pdgf (complex), PDGF BB, P13K (complex), PLA2, Pld, Rac, Rock, Sos, SPON1, SRGN, Tgf beta, VIM	15	9

PreOv			
Network	Molecules in network	Score	Number of identified proteins
1	AHSG, AMPB, APCS, APOH, C3, C6, C7, C8, C9, C5-C6-C7, C5-C6-C7-C8, C5-C6-C7-C8-C9, C8B, Complement component 1, Cr3, creatine kinase, elastase, ERK1/2, FGB, FGG, Fibrin, HPX, HRG, Iti, ITIH1, ITIH2, ITIH4, LBP, Mac, MFGE8, SERPINF2, Stat3-Stat3, Tcf 1/3/4, TTR, VTN	45	21
2	A2M, ACAC, Akt, Alpha 1 antitrypsin, C1q, C4BP, chymotrypsin, CLEC3B, Collagen type ix, CP, Ferritin, Fibrinogen, GPIIB-IIIA, HDL, HP, Kallikrein, LDL, ORM 1, plasminogen activator, PLG, PON1, PRKAA, PROC, Serine Protease, SERPINA1, SERPINA3, SERPINA5, SERPINA7, SERPIND1, SERPINE2, SERPING1, TCF, TF, trypsin, VCAN	35	17
3	ADIPOQ, ALB, APOA1, AZGP1, C5, C3-Cfb, CFB, CFH, CF1, CLU, Collagen Alpha1, FST, GOT, Growth hormone, hemoglobin, lfn, lgG, lkb, IL1, IL12 (complex), immunoglobulin, INHA, INHBA, Inhibin, KNG1, Ldh (complex) LDL-cholesterol, Lh, mediator, NADPH oxidase, NFkB (complex), Pro-inflammatory Cytokine, SHBG, Sod, Tnf (family)	27	14
4	Actin, AMPK, calpain, Cofilin, Collagen type I, Collagen type IV, Collagen (s), Cytokeratin, ERK, F2, F Actin, FETUB, Focal adhesion kinase, GC, GSN, Integrin, KRT2, Laminin, LRG1, Mek, MIc, p70 S6k, p85 (pik3r), Pdgf (complex), PDGF BB, P13K (complex), PLA2, Pld, Rac, Rock, Sos, SPON1, SRGN, Tgf beta, VIM	16	9
5	A1BG, ABCA7, ABCC6, AFF4, ARL1, ARL8B, BLOC1S5, C14orf169, CDK2AP2, EED, EIF2S3, GAS1, GLIPR2, HNF4A, ITIH4, KLK14, MOCOS, MTHFD1, MYC, PIPNB, Pzp, RCN2, RNF2, SAA2, SUCLG1, TBCCD1, THOC3, TM9SF4, TNS3, TTC27, UBE2J1, XPO1, YOD1, ZC3H15	14	8



Supplement Fig. S1. Correlation of abundance of proteins with progesterone concentration in bovine follicular fluid over follicle development.

Capítulo IV

O artigo a seguir foi redigido de acordo com as normas do periódico científico 'Journal of Proteomics' (https://www.elsevier.com/journals/journal-of-proteomics/1874-3919/guide-for-authors).

Heat stress induces proteomics changes in the follicular fluid of dairy cows

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Abstract

Reduced fertility associated with heat stress (HS) during summer is a complex and multi-factorial problem. We aimed in this study to investigate the effects of experimentally induced HS on follicular fluid (FF) proteome profile of cows at key stages of follicular development. Non-lactating Holstein cows were synchronized by Ovsynch protocol added with progesterone intravaginal device. From the day of ovulation, the cows were contemporaneously and randomly assigned to thermoneutral (TN; n=12) or HS (n=12) treatments. The temperature and humidity in the climate chamber were, respectively, 25.9ºC and 73.0% for TN, and 36.3ºC and 60.9% for HS. Transrectal ultrasound of all ovarian structures was performed twice a day over two follicular waves. We aspirated individually the largest follicle (F1) on day 9 of the first follicular wave and both the F1 and the second largest (F2) follicles at deviation (F1 ~ 8.5 mm) of the second follicular wave. Changes in FF proteins were analyzed using TMTsixplex labeling approach. We found 28 differentially expressed proteins in FF of cows exposed to HS versus TN. Pathways analysis revealed that the immune function and the coagulation cascades were closely associated with the HS process. These results show that HS alter protein expression of FF, which might affect follicular function as well as oocyte quality and explain in part the reproductive failure of heat stressed dairy cows.

Keywords: Heat stress; Fertility; Biomarkers; Proteomics; Bovine

1. Introduction

The increase in environment temperature during summer reduces fertility of dairy cattle. In previous studies, the decrease in conception rate of lactating cows during heat stress (HS) periods ranging between 30 and 60% compared with periods with no HS [1–

3]. The reduction in fertility associated to HS is a multifactorial problem. There is a body of evidence documenting that HS compromises embryo quality [4], oocyte development [5], endometrial function [6], blood flow to the uterus [7], hormone secretion [8,9], and follicular development. Alterations in the follicular fluid in which the oocyte grows and matures may also be involved.

Follicular fluid (FF) is produced in the growing antral follicles by secretions from granulosa and theca cells, and by the diffusion of plasma proteins (<500 kDa) through the basal membrane of the thecal vasculature [10]. Thus, the composition of FF may reflect any changes in the secretory processes of the ovarian cells and alterations in the plasma constituents due to pathological conditions. Previous studies related to FF proteome of cows focused on cystic ovarian disease [11] and less fertile cows ("repeat breeders") [12]. However, to our knowledge, no studies to date addressed the FF proteome profile in HS cows.

Productive and reproductive losses associated with HS have a significant economic impact [13]. Despite several aspects of HS have been extensively researched, the molecular mechanisms that lead to infertility in summer are only partially understood. Because gene expression change do not necessarily correlate with the abundance of the corresponding protein product, protein abundance can more accurately represent cellular activities than messenger RNA quantification. Proteomics is a well-established post-genomic tool which allows investigation of complex biological systems involved in health and disease conditions in model organisms and farm animals [14]. The advanced isobaric labeling tandem mass tags (TMT) technology has been successfully used for the quantitative analysis of a number of biological samples and conditions [15]. Here, we tested the effect of HS on bovine FF proteomics and show that HS may impair the reproductive function by changes in FF protein expression.

2. Material and methods

2.1. Animals and experimental design

This study was approved by the Ethical Committee for the Use of Animals In Research of the FMVZ – UNESP (Permit number: 86/2013 – CEUA). The experiment was conducted at the Lageado Experimental Farm, School of Veterinary Medicine and Animal Science, Univ. Estadual Paulista, Botucatu, SP, Brazil. A detailed description of the cows, feed, and management program can be found in Ferrazza et al. [16]. Briefly, eighteen nonlactating, clinical healthy, cycling Holstein cows (3 to 8 years old) were used in this experiment. The cows were initially synchronized using the Ovsynch protocol (d -9 GnRH, d -7 PGF2α, d 0 GnRH [17]) added with an intravaginal progesterone device (Sincrogest, Ourofino, São Paulo, Brazil). From the day of ovulation, the cows were contemporaneously and randomly assigned to thermoneutral (TN; n = 3) or HS (n = 3) treatments in a climate chamber. This study was conducted in a completely randomized design with four experimental replicates. As six cows were used per replicate, at the end of the study, each treatment consisted of 12 cows. To avoid a possible carryover effect, cows subjected to HS treatment were not used in the subsequent replicates.

In order to monitoring the follicular growth, transrectal ultrasound (MyLab30 Vet Gold with a 7.5 MHz linear-array transducer, Esaote, Genova, Italy) of all ovarian structures was performed twice daily (every 12 h) by a single operator to minimize variations. Ultrasound measurements of follicles were recorded and used to calculate the average follicular diameter in accordance with Sartori, Rosa and Wiltbank [18]. On day 9 of the estrous cycle (day of GnRH = day 0), the FF from the largest follicle (F1) was individually aspirated. After the target follicle be aspirated, all remaining visible follicles were ablated to allow the emergence of a new follicular wave. Concurrently the cows

received two injections of i.m. $PGF_{2\alpha}$ (0.500 mg of sodium cloprostenol, Ciosin, MSD Animal Health, SP, Brazil) administered 12 h apart to regress the corpus luteum and an intravaginal progesterone device was inserted (Sincrogest, Ourofino, São Paulo, Brazil) to ensure the emergence of the follicular wave in a high progesterone concentration. Then, at expected deviation moment (F1 ~ 8.5 mm [19]), both the F1 and the second largest follicle (F2) were individually aspirated. Therefore, two stages of follicular development characterized the experimental groups: post deviation (PostDev) and deviation (DevF1 and DevF2).

2.2. Physiological and environmental measurements

Rectal temperature (RT) and respiratory rate (RR) of the cows housed in the climate chamber were measured twice a day during the experimental period (approximately 16 days). Two data-recording devices (HOBO® U12 data logger, Onset Computer Co., Bourne, MA, USA) recorded the chamber environment temperature (AT) and relative humidity (RH) at every 30 minutes. These environmental variables were used to calculate the temperature humidity index (THI) according to the following equation [20]:

$THI = (0.8 \times Dbt) + [(RH/100) \times (Dbt - 14.4)] + 46.4$, in which Dbt = dry bulb temperature, and RH = relative humidity.

2.3. *In vivo* transvaginal follicular aspiration

Caudal epidural anesthesia was performed with 5 mL of 2% lidocaine (Lidovet, Bravet, Rio de Janeiro, Brazil), the perineal area was scrubbed and the aspiration guide with the transducer was inserted via transvaginal. The follicular aspiration was performed using an ultrasound equipment (Mindray DP-3300 Vet, Mindray Bio-Medical Electronics

Co. Ltd., Sheuzheu, China) equipped with a micro-convex 5 MHz transducer coupled to a needle guide system (WTA, Watanabe Applied Technology, Cravinhos, Brazil) and connected to an aspiration line (WTA) and a 20G disposable needle (WTA). Instead of a pump, the differential pressure applied to the system to recover the FF was created using a 10 mL syringe (Descarpack, São Paulo, Brazil). Immediately after follicle collapse, the needle was removed from the ovary, the system was checked for the presence of FF and blood contamination. Each collected fluid sample was derived from a single follicle. Only the FF samples without macroscopic blood contamination were used in this study.

Immediately after collection, the FF samples were centrifuged at 2,000 g at 4 °C for 10 minutes to eliminate cells and debris, added with protease inhibitor cocktail (0.8 mmol EDTA, 1.0 µg/mL aprotinin, 1.0 µg/mL leupeptin, and 35.0 µg/mL phenylmethylsulfonyl fluoride [PMSF]) [21] and stored at -80 °C until processing for hormone dosage assays.

2.4. Hormone assays

The concentrations of estradiol (E2) and progesterone (P4) in the FF samples were determined by enzyme immunoassay, as previously described by Rasmussen et al. [22], modified for FF. If necessary, samples were diluted 1:1 to 1:1,000 to fit the standard curves. Assay sensitivity was 0.02 ng/mL and the intra-assay coefficients of variation were <10% for all assays.

2.5. Protein in-solution digestion and peptide labeling

Individual FF samples from three biological replicates were depleted for albumin using a modified trichloroacetic acid/acetone precipitation method [23]. The protein concentrations of the FF samples were measured using the bicinchoninic acid protein

assay kit (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific Inc. #23227, Waltham, MA, USA) following the instructions of the manufacturer. An amount of 100 µg protein was reduced with 1,4-dithiothreitol (DTT) and the filter-aided sample preparation method (FASP™ Protein Digestion Kit, Expedon, San Diego, CA, USA) was then used [24] with a minor modification by substituting sodium chloride with acetonitrile for elution of the proteins from the spin columns to avoid desalt step. Alkylation with 0.05 M iodoacetamide solution was performed in the dark at room temperature (23°C) for 20 minutes. Protein digestion was carried out using trypsin (Promega, Madison, WI, USA) to a final enzyme:protein ratio of 1:100 at 37°C for 16 hours.

The samples were labeled with TMTsixplex™ Isobaric Label Reagent Set (Thermo #90062) according to the manufacturer's instructions. Briefly, TMT reagents were dissolved in 41 µl of acetonitrile, added to samples and the reactions were incubated for one hour at room temperature. To quench the reaction, 8 µl of 5% hydroxylamine was added to the samples and incubated for 15 minutes at room temperature. The samples were then combined at equal amounts, vacuum-dried and stored at -20°C until analysis.

2.6. Mass spectrometry analysis

Samples were analyzed in an LTQ Orbitrap Elite Mass Spectrometer (Thermo Scientific Inc., San Jose, CA, USA) coupled to an UltiMate 3000 RSLS nano chromatography system (Dionex, Camberley, UK). TMT-labelled peptide mixtures were reconstituted in buffer A (2% acetonitrile in 0.1 % formic acid). An amount of 3 µg was loaded on the trapping column C18 PepMap100 (5 µm, 100 A, 300 µm x 5 mm) and then separated using Acclaim PepMap RSLC C18 Nanocolumn (15 cm x 75 µm) with a linear gradient of 5-35% solvent B (80% acetonitrile/0.1% formic acid) over 135 min at a flow rate of 300 nL/min. Eluate from the column was introduced to the mass spectrometer. The

ionization voltage was set to 1.7 kV and the ion transfer tube temperature to 220 °C. The mass spectrometer was set up in positive ion mode using collision-induced dissociation (CID)/high-energy collision-induced dissociation (HCD) fragmentation methods for MS2. Full scan FTMS spectra were acquired in range from 380 to 1800 m/z, with resolution of 60,000. The maximum injection time for FTMS full scan was set as 200 ms reaching an AGC target value of $1x10^6$. For identification of TMT labelled peptides, the three most abundant peaks from MS spectrum were selected for each fragmentation mode. The HCD MS/MS scan was fixed to start from 100 m/z, with resolution of 15,000, using MS2 AGC target of $5x10^4$. The collision energy was set as 40% NCE. Isolation window of ± 1.5 Da was applied to isolate precursor ions with dynamic exclusion of 20 s. ITMS CID MS/MS scan spectra were acquired with 35% NCE and an AGC target of $1x10^4$.

2.7. Database searching and bioinformatics analysis

Raw data files were loaded into Proteome Discoverer software (version 2.1, Thermo Fisher Scientific, Bremen, Germany) and searched against the *Bos taurus* NCBI protein database using both the Sequest and Mascot algorithms. The parameters used for database searches included trypsin as protease with one missed trypsin cleavage allowed, carbamidomethylation (C) as fixed modification, oxidation (M) and TMT-6plex (Y) as variable modification. The mass tolerance of precursor ions was set at 10 ppm and 0.6 Da for fragment ion matches. Only peptides that were filtered with a confidence level of 95% and least one unique peptide were accepted. The false discovery rate was calculated using peptide validator-based on decoy database searching. The peptide ratios were calculated from median PSMs. These peptide ratios were then assembled (median) to protein groups. The protein ratios were normalized to the total intensity. Samples from cows housed in

TN environment at the second follicular wave (after acquisition of ovulatory capacity) were used as control for relative quantification.

2.8. Statistical analysis

Data on environmental and physiological measurements were normally distributed (Shapiro-Wilks) and homoscedastic (Levene's test), thus were analyzed by a paired t-test. Hormone concentration data were log-transformed to attain normality. Mean values for diameters, steroid, and protein concentrations were then compared by PROC GLM procedure of SAS (version 9.4, SAS Institute Inc., Cary, NC, USA), complemented by a Student-Newman-Keuls test. The statistical model included effects of treatment, stage of follicle development, and treatment by stage of follicle development interaction. For proteomic data, fold changes were calculated based on the ratio of the case versus control samples. The analysis of variance was used to determine significant changes in protein normalized relative abundances. Significance was set at $P < 0.05$. For data exploratory analysis, hierarchical clustering based on Euclidean distance and Ward's linkage were performed using GeneSpring software (version 13.0, Agilent Technologies Inc., Santa Clara, CA, USA) and the results were illustrated in form of heat maps.

2.9. Functional analysis

Enrichment of canonical pathways and networks of identified proteins were generated using Ingenuity Pathway Analysis software (IPA, QIAGEN Redwood City, CA, USA). The datasets contained respective gene symbols and the normalized relative abundances of proteins. The Fisher's exact test was used by the IPA to calculate P-values and to identify statistically significant pathways and networks. The direction of

225 regulation, i.e. up- or down-regulation, was inferred from the activation z-score in the
226 IPA.
227
228 **3. Results**
229 Cows exposed to HS treatment experienced higher ($P < 0.0001$) AT (36.3 ± 0.3
230 ºC) and THI (88.7 ± 0.4) than cows in TN treatment (25.9 ± 0.2 ºC and 75.4 ± 0.3,
231 respectively). Relative humidity was higher ($P < 0.0001$) for TN (73.0 ± 0.8 %) compared
232 with HS treatment (60.9 ± 0.9 %) (Table 1). The low RH for HS treatment was a result of
233 the heating mechanism inherent of the climate chamber facility, as the highest
234 temperatures agreed with lowest humidity. Rectal temperature was higher ($P < 0.001$) for
235 HS cows (39.87 ± 0.07 °C), while TN cows maintained normothermia (38.56 ± 0.03°C).
236 Respiratory rate was higher ($P < 0.001$) for HS cows (76.02 ± 1.70 bpm) than for TN
237 cows (39.70 ± 0.71 bpm) (Fig. 1).
238 In order to confirm the stage of follicle development and to evaluate the effect of
239 HS, we characterized the follicles morphologically and endocrinologically (Table 2).
240 Follicular diameter was significantly lower ($P < 0.05$) during dominance (PostDev), but
241 not at deviation (DevF1 and DevF2), for HS cows compared with TN cows. There was
242 difference ($P < 0.05$) on the intrafollicular concentration of E2, P4, and total protein across
243 the stages of follicle development, but no effect ($P > 0.05$) was detected for either
244 treatment or for interaction between stages versus treatment.
245 In the current study, we identified 158 unique proteins in the FF of dairy cows
246 (Supplementary Table S1), mostly of them plasma-matched proteins. Hierarchical
247 clustering revealed that FF proteome was markedly altered in response to HS (Fig. 2). We
248 found 28 differentially ($P < 0.05$) expressed proteins, of which seven were affected by the
249 interaction between the stage of follicle development and treatment (Table 3). To gain

250 further insight into the biological pathways related to the proteins identified in FF, we
251 performed a functional enrichment analysis using IPA software. The bar chart in Figure
252 3 shows the top ten statistically significant canonical pathways, including acute phase
253 response signaling, liver X receptor (LXR), retinoid X receptor (RXR) and farnesoid X
254 receptor (FXR) activation pathways, complement system, and coagulation system. More
255 detailed analysis were performed to identify the most up- and down-regulated canonical
256 pathways and focus genes (Fig. 4). Amongst up-regulated pathways in HS treatment were
257 those involved in the acute phase response and complement system. On the other hand,
258 the coagulation system pathway was primarily down-regulated by HS. Some other
259 specific canonical pathways such as LXR/RXR activation and biosynthesis of nitric oxide
260 and reactive oxygen species were also involved to a lesser extent.
261

262 **4. Discussion**

263 Prolonged exposure to HS reduces reproductive efficiency in cattle. Despite
264 several aspects have been extensively investigated in dairy cows affected by HS,
265 relatively little information is known about the proteomics changes that occur during heat
266 exposure. Here, for the first time, we have shown that HS could impair the reproductive
267 function by changes in FF protein expression of Holstein cows. The differentially
268 expressed proteins revealed that the immune function and coagulation cascade pathways
269 were closely associated with the HS process. Such findings shed light on potential
270 molecular mechanisms and signaling pathways involved in pathological condition
271 generated by HS.

272 The experimentally induced HS conditions used in this study strongly influenced
273 thermoregulatory mechanisms. As expected, all HS cows had higher RT and RR
274 compared to the control cows. Our results clearly indicated changes in the follicular

dynamics. Dominance was found depressed at post deviation stage and, although the largest follicle did not differ at deviation stage, the second largest follicle was larger in HS cows, demonstrating the compromising of the follicle selection mechanism [19], which ultimately can lead to follicular codominance [25] and anovulation or double ovulations [26]. Retarded follicular growth and impaired dominant follicle during HS may compromise follicle steroidogenic capacity. However, in this study, E2 and P4 concentrations in the FF were not altered by the HS treatment. Previous studies have shown an adverse effect of HS on intrafollicular concentration of E2 [27] and P4 [28]. In contrast, other studies reported equivalent intrafollicular E2 and P4 concentrations in HS cows [29,30]. The inconsistencies in steroids response to HS could be due to differences in the severity or nature (chronic versus acute) of HS and the physiological status of the cow. For example, lactating cows have higher susceptibility to HS than nonlactating cows due metabolic heat generate from milk production [31].

Application of livestock omics has great potential for understanding the physiology of the reproductive events and offer new perspectives to improve the efficiency of animal production [32]. The 28 differentially expressed proteins found in our study provide new information about the response mechanisms and biological processes shifts in HS cows. Comparative pathways analysis indicated that a large number of immunoglobulins and complement components were up-regulated in HS treatment. A previous study indicated that under stressful conditions, glucocorticoids might suppress cellular immunity and boost humoral immunity via shifts on T helper 1 (Th1)/Th2 cells and type 1/type 2 cytokine production [33]. These authors reported that the Th2 shift might have a profound effect on the susceptibility of the organism to infection and/or might influence the course of an infection, the defense mechanism primarily dependent on cellular immunity. Indeed, the occurrence of health disorders in

cows, such as mastitis [34] and lameness [35], are higher during the summer, suggesting that immune function is impaired in cows exposed to HS. Along, the complement system plays a fundamental role in the innate immunity system in addition to enhancing adaptive immune responses as it is the primary line of defense against infection [36]. Transcriptome profiling of rat ovarian follicles provided evidence that the complement system plays a role in follicle development. Moreover, in human FF, the complement system has been proposed as a requirement not only for maintaining normal pregnancy [37,38], but also for oocyte maturation [39]. Thus, an altered FF proteome profile, including increasing abundance of the complement components, constitutes further evidence of disrupted follicular milieu during HS.

In our study, the exposure of cows to HS decreased part of the components of the coagulation cascades pathway, including plasminogen, hemopexin, prothrombin, and vitronectin. Interestingly, proteins associated with blood coagulation were increased in women undergoing ovarian stimulation for *in vitro* fertilization [40,41], characterizing a state of hypercoagulability [42]. Therefore, it would be reasonable to speculate that down-regulation of the proteins involved in coagulation system of FF from HS cows have a protective effect in an estradiol-related effect. However, the precise mechanisms associated to the coagulation system profiles and HS remain undefined. Further investigations are required to determine the precise effect of these proteins on FF.

5. Conclusions

In conclusion, our findings indicate that heat stress induces proteomics changes in the follicular fluid of dairy cows. The 28 differentially abundance proteins found in this work explain in part the abnormal folliculogenesis and reveal that the immune function and the coagulation cascade pathways are closely associated with the heat stress process.

Furthermore, alterations in this follicular microenvironment might compromise the granulosa cell quality and oocyte's developmental competence and contribute to the disappointing reproductive outcome observed in heat stressed dairy cows.

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Table 1

Environmental conditions of thermoneutral and heat stress treatments (mean ± SE and variation)

Item	Thermoneutral	Heat stress
Ambient temperature (ºC)	25.9 ± 0.2[a] (24.6 – 27.7)	36.3 ± 0.3[b] (34.3 – 40.7)
Relative humidity (%)	73.0 ± 0.8[a] (64.3 – 79.0)	60.9 ± 0.9[b] (49.9 – 65.2)
THI[1]	75.4 ± 0.3[a] (73.4 – 77.1)	88.7 ± 0.4[b] (85.6 – 92.1)

[a-b] Values with different superscripts in the same row differ significantly ($P < 0.0001$).

[1] THI = Temperature Humidity Index.

480 **Table 2**

481 Effect of heat stress on follicular diameter, hormonal profile, and protein concentration

482 (mean ± SE) according to the stage of follicle development of Holstein cows

	Thermoneutral			Heat stress				P-value[1]		
Item	DevF1	DevF2	PostDev	DevF1	DevF2	PostDev	SEM	Stage	Trt	Stage x Trt
Diameter (mm)	8.67^{c}	6.57^{d}	16.10^{a}	8.67^{c}	7.57^{cd}	13.37^{b}	0.44	<0.001	0.005	0.028
Estradiol (ng/mL)	31.25	13.76	1432.72	62.46	12.07	890.79	308.33	<0.001	0.986	0.550
Progesterone (ng/mL)	7.53	2.86	79.11	15.22	5.54	97.74	6.83	<0.001	0.385	0.989
E2:P4 ratio[2]	4.42	4.74	6.50	4.59	4.98	9.16	1.77	0.161	0.491	0.730
Total protein (mg/mL)	22.07	17.11	74.33	32.39	10.91	69.07	8.73	<0.001	0.958	0.583

483 $^{a-d}$ Values within row of each variable with differing superscripts indicate statistical

484 difference ($P < 0.05$).

485 [1] Stage = effect of stage of follicle development; Trt = effect of treatment; Stage × Trt =

486 effect of the interaction between Stage and Trt.

487 [2] Estradiol:progesterone concentration ratio.

488 **Table 3**

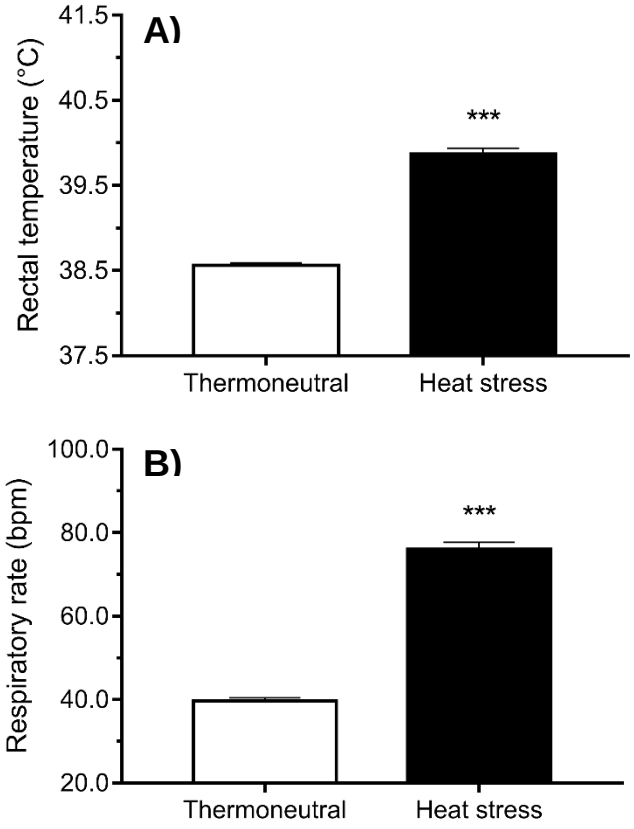
489 Differentially expressed proteins in follicular fluid from cows kept under thermoneutral

490 and heat stress treatments

Protein name	Accession	Gene symbol	P-value[1]	
			Trt	Stage x Trt
IgG light chain variable region	2323400		<0.001	0.002
Complement component C8	931717266	C8B	0.225	0.004
Plasminogen	27806815	PLG	0.246	0.009
Complement component C9	Q3MHN2	C9	0.021	0.020
Complement component C6	114051692	C6	0.974	0.021
Alpha-1-antiproteinase	27806941	SERPINA1	0.387	0.031
IgG2a heavy chain constant region	1699167	IGCGAMMA	0.708	0.049
Serpin A3-7	985701132	LOC784932	<0.001	0.075
Endopin 2B	38683423	SERPINA3-7	0.002	0.079
IgG lambda light chain	1276613		0.002	0.152
Hemopexin	77736171	HPX	0.005	0.168
Transthyretin	27806789	TTR	0.038	0.168
Inter-alpha inhibitor H4	59857769	ITIH4	0.048	0.197
IgG delta heavy chain	509264466	LOC100847540	0.014	0.313
Copper-Containing Amine Oxidase	61680007		0.044	0.355
Alpha-2-macroglobulin	Q7SIH1	A2M	0.002	0.473
IgG gamma 1 heavy chain	91982959	IGHG1	0.028	0.476
Vitronectin	78045497	VTN	0.008	0.482
IgG lambda-like polypeptide 1	741957421	IGLL1	0.026	0.578
Inhibin alpha chain	P07994	INHA	0.041	0.609
IgG n M heavy chain	24496448	IGHM	0.005	0.630
Prothrombin	P00735	F2	0.003	0.662
IgG gamma heavy chain	509264677		0.012	0.708
Complement factor B	P81187	CFB	0.005	0.724
Complement factor I	296486756	CFI	0.002	0.835
Complement component C4	983005304	LOC100852118	0.009	0.841
IgG gamma2 heavy chain	1040566766		0.005	0.846
Complement component C7	Q29RQ1	C7	0.018	0.860

491 [1] Trt = effect of treatment; Stage × Trt = effect of the interaction between the stage of

492 follicle development and Trt.



493 **Fig. 1.** Rectal temperature (A) and respiratory rate (B) of cows exposed to thermoneutral
494 and heat stress treatments.
495 *** Differences between thermoneutral and heat stress treatments ($P < 0.001$).

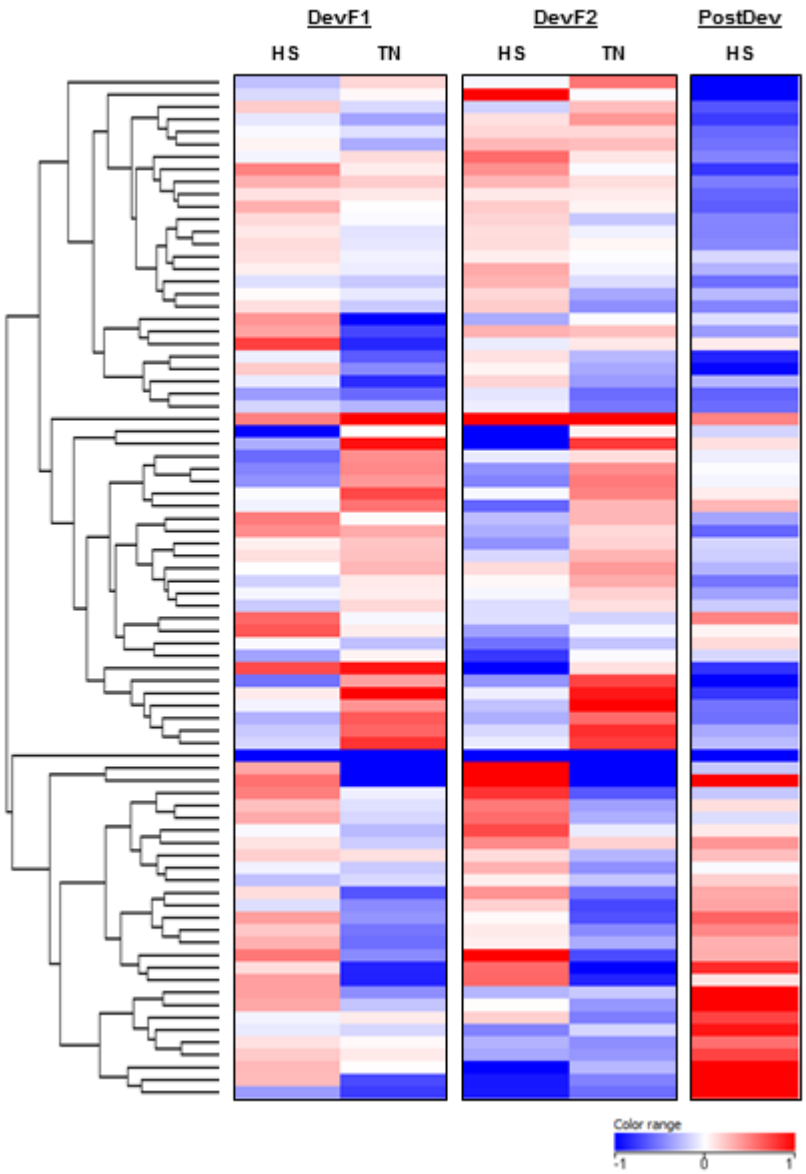
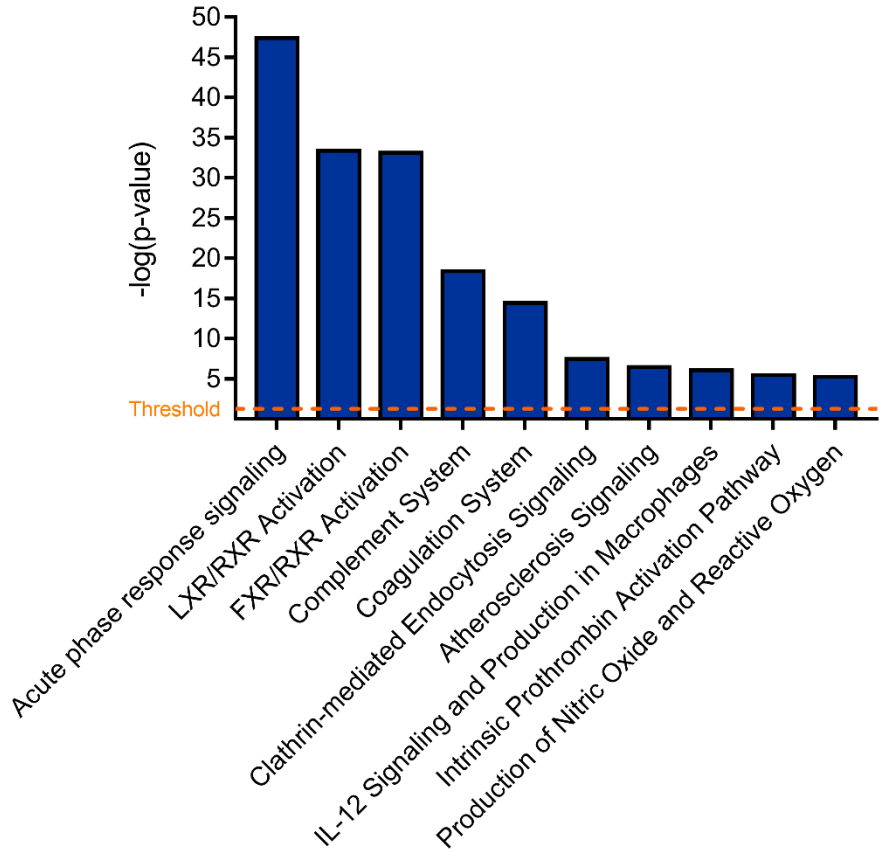


Fig. 2. Effect of heat stress on proteome profile of dairy cows. Hierarchical clustering heat map shows changes in protein abundance of follicular fluid at deviation (DevF1 and DevF2) and post deviation (PostDev) stages of follicle development. The heat map indicates up-regulation (red), down-regulation (blue), and mean protein expression (white) relative to thermoneutral cows at PostDev (control). The columns represent treatments and the rows represent individual proteins.

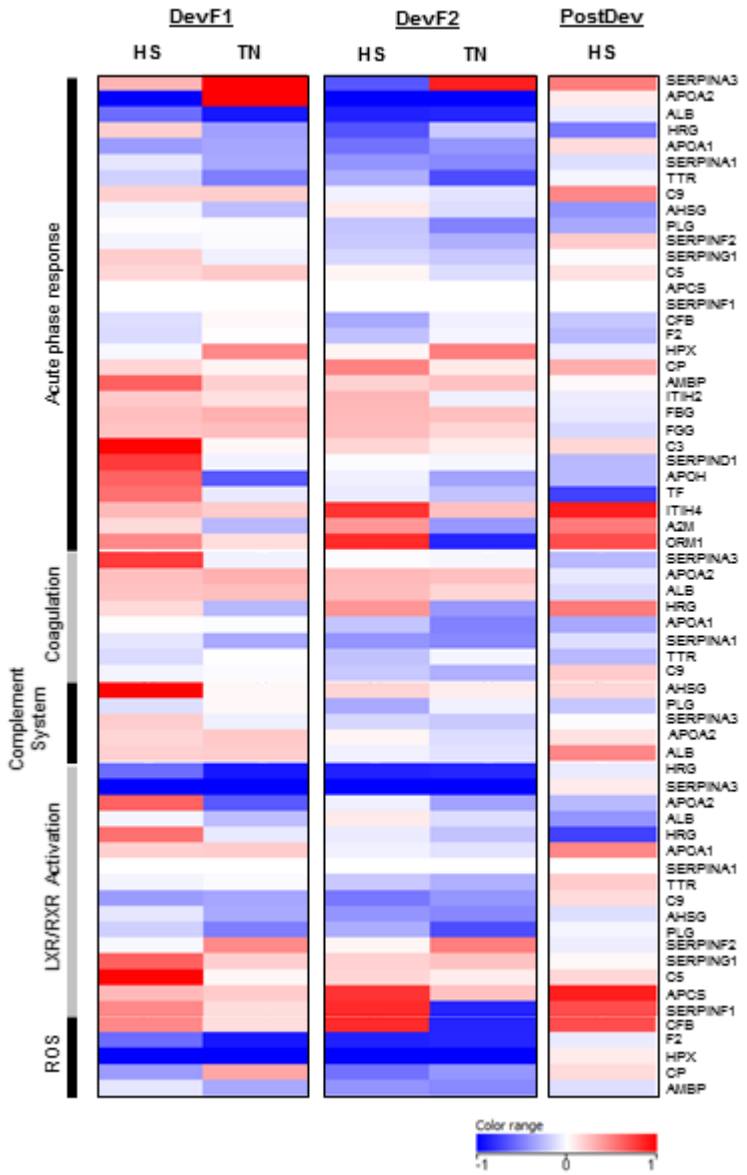


503

504 **Fig. 3.** Significant canonical pathways associated with the proteins identified in follicular

505 fluid. The significance of enrichment is indicated by negative log of the p-value obtained

506 by a Fisher's exact test in IPA.



507

508 **Fig. 4.** Heat map showing expression of the most significantly enriched genes associated
509 to respective canonical pathways at deviation (DevF1 and DevF2) and dominance
510 (PostDev). The heat map indicates up-regulation (red), down-regulation (blue), and mean
511 protein expression (white) relative to thermoneutral cows at PostDev (control). The
512 columns represent treatments and the rows represent individual proteins. HS = heat stress;
513 TN = thermoneutral; LXR = liver X receptor; RXR = retinoid X receptor; ROS =
514 production of nitric oxide and reactive oxygen species pathway.

Supplementary Table S1

List of identified proteins in bovine follicular fluid

Description	Accession	Coverage (%)	# Unique Peptides	MW (kDa)	calc. pI
Acylcholine acylhydrolase	416800	27.0	1	15.2	5.8
Albumin	229552	72.5	4	66.1	6.1
Alpha-1-acid glycoprotein	Q3SZR3	45.5	9	23.2	5.9
Alpha-1-antiproteinase	27806941	48.3	19	46.1	6.5
Alpha-1B-glycoprotein	114053019	54.1	15	53.5	5.4
Alpha-2-antiplasmin	P28800	18.9	6	54.7	5.7
Alpha-2-HS-glycoprotein	P12763	41.2	11	38.4	5.5
Alpha-2-macroglobulin [OS=Bos taurus]	Q7SIH1	41.3	12	167.5	6.0
Alpha-2-macroglobulin isoform X1	982926875	1.4	1	164.0	7.0
Alpha-2-macroglobulin variant 20	408689603	40.0	1	98.3	6.0
Alpha-2-macroglobulin variant 5	408689573	16.9	1	45.0	7.5
Alpha-S1-casein	P02662	4.7	1	24.5	5.0
Angiotensinogen	166159174	9.9	3	45.4	9.2
Anti-respiratory syncytial virus Ig heavy chain V region	508834	7.1	1	15.1	6.7
Anti-testosterone antibody	432627	43.4	1	24.4	7.6
Antithrombin-III	555957525	60.9	22	52.4	6.8
Apolipoprotein A-I	P15497	38.9	9	30.3	6.0
Apolipoprotein A-II	114052298	16.0	1	11.2	8.1
Beta-2-glycoprotein 1	P17690	21.7	4	38.2	8.2
Beta-casein	P02666	3.1	1	25.1	5.4
Carboxypeptidase N subunit 2	155372183	4.0	1	60.0	6.2
Ceruloplasmin isoform X1	528937555	42.2	29	124.2	6.1

Description	Accession	Coverage (%)	# Unique Peptides	MW (kDa)	calc. pI
Chain A, Crystal Structure Of Bovine Plasma Copper-Containing Amine Oxidase	61680007	39.3	17	82.7	5.9
Chain A, The Crystal Structure Of Modified Bovine Fibrinogen	6980814	44.6	1	42.7	8.0
Chain H, Crystal Structure Of Bovine Antibody Blv5b8 With Ultralong Cdr H3	513137425	46.2	2	29.1	7.8
Chain L, Crystal Structure Of Bovine Antibody Blv1h12 With Ultralong Cdr H3	513137422	68.5	3	22.5	6.1
Clusterin preproprotein	27806907	8.9	2	51.1	6.0
Coagulation factor IX isoform X1	529013735	6.1	1	52.0	5.9
Complement C3 isoform X1	741932316	74.5	99	187.1	6.8
Complement C4 isoform X1	983005304	20.4	9	193.4	7.4
Complement C4-A-like	982963988	15.7	3	157.7	7.8
Complement C5a anaphylatoxin	262205546	19.6	17	188.7	6.6
Complement component C6	114051692	9.8	4	104.5	7.0
Complement component C7	Q29RQ1	1.3	1	93.0	7.1
Complement component C8 alpha chain	114053319	8.1	2	66.3	6.4
Complement component C8 beta chain	931717266	11.4	3	66.6	8.0
Complement component C8 gamma chain isoform X1	741944959	6.7	1	28.5	9.5
Complement component C9	Q3MHN2	12.2	5	62.0	5.9
Complement factor B	P81187	34.3	19	85.3	7.7
Complement factor H	Q28085	9.9	10	140.3	6.8
Complement factor I	296486756	9.5	4	68.9	7.7
Corticosteroid-binding globulin	E1BF81	5.2	1	45.1	5.9
Deoxynucleotidyltransferase terminal-interacting protein 2	Q0P5H2	2.0	1	86.7	5.8
DNA topoisomerase 2-alpha isoform X1	528921315	0.5	1	174.3	8.6
Endopin 2B	38683423	37.6	7	47.0	6.2
Factor XIIa inhibitor	27807349	12.6	4	51.7	6.7
Fetuin-B	Q58D62	13.4	3	42.6	5.8
Fibrinogen alpha chain isoform X1	741955615	29.2	9	92.1	6.1

Description	Accession	Coverage (%)	# Unique Peptides	MW (kDa)	calc. pI
Fibrinogen beta chain	218931172	57.2	26	56.4	8.2
Fibrinogen gamma-B chain	27806893	49.8	1	50.2	5.8
Fibrinogen gamma-B chain isoform X1	528981276	60.2	4	49.1	5.9
Fibronectin	218512156	2.0	3	272.0	5.5
Gelsolin	Q3SX14	37.3	17	80.7	5.8
Glia-derived nexin	27807207	19.4	5	43.8	9.5
Glutamate-rich protein 3	741921205	0.5	1	165.2	5.0
Haptoglobin	Q2TBU0	2.2	1	44.8	7.7
Hemopexin	77736171	59.9	21	52.2	7.8
Heparin cofactor 2 isoform X1	528989611	26.1	10	62.7	6.7
Hepatocyte growth factor activator preproprotein	333360891	3.2	1	69.5	7.6
Histidine-rich glycoprotein	27806875	20.5	3	61.9	7.7
Histidine-rich glycoprotein	1072452	18.4	1	50.1	7.2
Ig gamma-2 chain C region (clone 32.2)	89611	62.4	3	36.0	7.8
Ig heavy chain	108750	64.7	1	50.6	6.5
IgG1 heavy chain constant region	7547266	83.6	4	35.8	6.5
IgG2a heavy chain constant region, partial	1699167	69.6	1	35.8	7.6
IgG3 heavy chain constant region	1575493	64.2	2	38.6	8.0
IgG3 heavy chain constant region	1575495	79.3	2	38.7	7.6
IGK protein	115545495	39.2	7	26.6	6.5
IgM	326937675	10.3	2	51.6	6.6
IgM	1399521	6.2	1	15.7	8.2
Immunoglobulin alpha heavy chain variable region	509264946	8.1	2	19.3	7.0
Immunoglobulin alpha heavy chain variable region	509264918	4.4	1	19.3	6.0
Immunoglobulin delta heavy chain variable region	509264531	19.1	2	31.9	6.5
Immunoglobulin delta heavy chain variable region	509264466	6.7	1	27.0	6.0

Description	Accession	Coverage (%)	# Unique Peptides	MW (kDa)	calc. pI
Immunoglobulin delta heavy chain variable region, partial	509264513	19.1	1	19.1	7.0
Immunoglobulin epsilon heavy chain variable region	509264882	20.9	2	19.3	6.0
Immunoglobulin epsilon heavy chain variable region	509264776	6.0	1	14.3	4.9
Immunoglobulin epsilon heavy chain variable region	509264854	5.6	1	17.0	7.0
Immunoglobulin gamma 1 heavy chain constant region	91982959	83.6	2	35.9	6.9
Immunoglobulin gamma heavy chain variable region	509264725	20.3	1	23.2	7.5
Immunoglobulin gamma heavy chain variable region	94961540	25.0	1	13.9	7.0
Immunoglobulin gamma heavy chain variable region, partial	509264677	22.5	2	18.8	6.5
Immunoglobulin heavy chain	1335961	18.1	1	14.6	9.4
Immunoglobulin heavy chain	1335949	21.2	1	16.4	8.4
Immunoglobulin heavy chain variable region	662033942	27.0	1	10.5	8.5
Immunoglobulin heavy chain variable region	1000348	45.8	1	7.8	9.3
Immunoglobulin IgG2a(A1) allotype	252887	89.7	2	6.1	7.9
Immunoglobulin lambda light chain	15088675	41.7	2	24.7	6.1
Immunoglobulin lambda light chain constant region 2 allotypic variant IGLC2b	343197004	74.5	2	11.3	8.6
Immunoglobulin lambda light chain constant region 2 allotypic variant IGLC2a	343196996	68.9	2	11.3	9.1
immunoglobulin lambda light chain constant region 3 allotypic variant IGLC3c	343197026	76.4	6	11.2	8.3
Immunoglobulin lambda light chain variable region	975858	44.7	2	10.7	9.1
Immunoglobulin lambda light chain variable region	975844	45.1	1	10.6	9.2
Immunoglobulin lambda light chain variable region	950177	29.0	2	13.6	7.9
Immunoglobulin lambda light chain variable region	1276613	6.9	1	13.4	5.2
Immunoglobulin lambda-6a light chain variable region-like	296478325	7.1	1	14.8	4.6
Immunoglobulin lambda-like polypeptide 1 isoform X1	741957421	55.1	1	24.7	8.0
Immunoglobulin light chain variable region	2323404	41.8	1	11.3	6.5
Immunoglobulin light chain variable region	2323378	40.5	1	11.5	7.9
Immunoglobulin light chain variable region	2323400	41.8	1	11.2	6.5

Description	Accession	Coverage (%)	# Unique Peptides	MW (kDa)	calc. pI
Immunoglobulin light chain variable region	2323390	42.3	1	11.4	4.9
Immunoglobulin light chain variable region	2555149	23.2	1	11.7	8.0
Immunoglobulin M heavy chain secretory form	24496448	9.0	2	65.6	6.0
Immunoglobulin mu heavy chain variable region	509264364	15.9	1	27.5	5.9
Immunoglobulin mu heavy chain variable region	98991264	25.8	1	13.8	8.2
Immunoglobulin variable region	2353736	18.7	1	14.7	8.3
Inhibin alpha chain	P07994	4.2	1	38.8	7.2
Inhibin beta A chain	27805949	1.9	1	47.5	7.9
Inhibitor of carbonic anhydrase	114053269	4.5	1	69.1	7.9
Insulin-like growth factor-binding protein complex acid labile subunit	115495035	3.9	1	65.9	7.2
Inter-alpha (globulin) inhibitor H4 (plasma Kallikrein-sensitive glycoprotein)	59857769	56.2	2	101.4	6.7
Inter-alpha globulin inhibitor H2 polypeptide	296481520	27.8	17	106.1	7.9
Inter-alpha-trypsin inhibitor heavy chain H1	Q0VCM5	31.2	17	101.2	7.4
Inter-alpha-trypsin inhibitor heavy chain H4 isoform X2	982961939	56.5	2	99.6	6.4
Keratin, type I cytoskeletal 17	A1L595	5.2	1	48.7	5.2
Keratin, type I cytoskeletal 24 isoform X1	741963799	3.4	1	56.1	5.0
Keratin, type II cytoskeletal 2 epidermal isoform X1	297474462	3.4	1	64.2	8.4
Keratin, type II cytoskeletal 68 kDa, component IB	358421417	3.3	1	64.7	8.1
Kininogen I	488	43.1	1	48.4	6.3
Kininogen-2 isoform I	164450479	33.0	2	68.9	6.7
Leucine-rich alpha-2-glycoprotein	114051379	28.0	7	38.3	7.0
Lipopolysaccharide-binding protein	Q2TBI0	6.4	2	53.7	7.1
Membrane-bound immunoglobulin gamma2 heavy chain constant region	1040566766	63.8	2	43.7	6.6
Membrane-bound immunoglobulin gamma3 heavy chain constant region, partial	1040566762	62.2	5	46.4	6.4
Pancreatic amylase alpha 2A	296489335	2.2	1	57.3	6.4
Pancreatic elastase inhibitor, EI=serpin {N-terminal, reactive-site loop}	861514	66.7	1	1.9	4.5

Description	Accession	Coverage (%)	# Unique Peptides	MW (kDa)	calc. pI
Pigment epithelium-derived factor	Q95121	11.8	2	46.2	7.1
Plasminogen	27806815	25.1	1	91.2	7.5
Plasminogen	296483851	28.0	1	91.2	7.6
Protein AMBP	P00978	15.1	4	39.2	7.6
Protein HP-20 homolog	114051225	46.6	8	20.6	8.6
Protein HP-25 homolog 1	Q2KIX7	25.0	3	22.5	7.5
Protein HP-25 homolog 2	Q2KIU3	38.6	7	22.9	5.3
Protein phosphatase 1 regulatory subunit 37	A7Z026	1.7	1	75.6	5.0
Prothrombin	P00735	39.4	18	70.5	6.3
Quinone oxidoreductase-like protein 1	Q59A28	3.4	1	38.8	5.7
serglycin	70778776	5.3	1	16.5	4.4
Serine/threonine-protein kinase PRP4 homolog	Q08DZ2	0.6	1	116.6	10.2
Serotransferrin	114326282	75.0	2	77.7	7.1
Serotransferrin	296490958	74.9	1	77.6	7.3
Serpin A3-1	Q9TTE1	60.8	8	46.2	6.0
Serpin A3-4	A2I7N0	48.9	3	46.3	6.3
Serpin A3-5	528922995	51.2	3	46.3	6.7
Serpin A3-7-like	985701132	26.3	5	46.7	6.4
Serpin A3-8	A6QPQ2	20.6	3	46.9	6.1
Serum albumin	30794280	79.7	11	69.3	6.2
Serum amyloid P-component	77735883	5.4	1	25.2	8.3
Serum paraoxonase/arylesterase 1	114053183	10.1	2	39.8	5.4
Sex hormone-binding globulin isoform X1	528995099	5.9	1	46.0	6.3
Tetranectin	Q2KIS7	17.3	2	22.1	5.6
Thyroxine-binding globulin	Q9TT36	2.2	1	46.0	5.7
Transthyretin	27806789	66.0	7	15.7	6.3

Description	Accession	Coverage (%)	# Unique Peptides	MW (kDa)	calc. pI
Trypsin inhibitor, TI=serpin {N-terminal, reactive-site loop}	861517	40.0	1	2.3	7.3
Ubiquitin carboxyl-terminal hydrolase 13	300796005	1.3	1	97.2	5.6
Unknown (protein for MGC:159378)	151556360	44.8	2	24.7	7.6
Vitamin D-binding protein	Q3MHN5	20.9	7	53.3	5.5
Vitronectin	78045497	22.5	7	53.5	6.3
Zinc-alpha-2-glycoprotein	77735615	14.4	3	33.8	5.2

Capítulo V

Considerações finais

O estresse térmico diminui a produção e a eficiência reprodutiva em rebanhos leiteiros, ocasionando significativo impacto econômico. O decréscimo da fertilidade associado ao estresse térmico é um problema de ordem multifatorial, pois prejudica as funções fisiológicas e celulares em vários tecidos do sistema reprodutivo, incluindo o microambiente folicular que banha os oócitos. A determinação da composição das proteínas do fluido folicular pode ser uma ferramenta útil para investigar a fisiologia ovariana, especialmente no que diz respeito ao desenvolvimento folicular e maturação oocitária. Além disso, a proteômica pode melhorar o entendimento dos mecanismos patofisiológicos que levam à infertilidade durante o verão. Neste contexto, os objetivos do presente estudo foram caracterizar o perfil proteômico do fluido folicular em momentos-chave do desenvolvimento folicular em condições termoneutras; investigar os mecanismos de termorregulação e preditores de balanço calórico envolvidos durante o estresse térmico intenso; e testar o efeito do estresse térmico sobre a expressão das proteínas do fluido folicular de vacas leiteiras.

A partir dos resultados obtidos, demonstrou-se a modulação temporal da expressão de proteínas do fluido folicular e a associação dessas proteínas com as concentrações intrafoliculares de hormônios esteróides. O estágio do desenvolvimento folicular levou a ativação/inibição das vias canônicas relacionadas com a resposta de fase aguda, sistema de coagulação, sistema complemento, receptor X do fígado e retinóide e produção de óxido nítrico e espécies reativas de oxigênio. A exposição contínua ao estresse térmico afetou os mecanismos termorregulatórios, levando a um aumento pronunciado da frequência respiratória, seguido pelo aumento da temperatura retal. As variáveis

ambientais do dia anterior foram mais importantes para explicar as respostas dos parâmetros fisiológicos e ingestão de matéria seca, sendo a temperatura ambiente o principal fator envolvido na troca de calor. Embora a exposição prolongada ao estresse térmico indique um ajuste adaptativo, neste estudo foi observada apenas aclimatação parcial. O crescimento folicular das vacas expostas ao estresse térmico também foi afetado e pode, em parte, ser explicado pela expressão alterada das proteínas do fluido folicular. As proteínas diferentemente expressas no fluido folicular revelaram que as vias da função imune e da cascata da coagulação foram intimamente associadas com a indução experimental do estresse térmico.

Coletivamente, tais achados expandem o conhecimento corrente sobre o efeito da hipertermia nos processos termorregulatórios da espécie bovina e sobre os mecanismos moleculares e vias de sinalização envolvidos na foliculogênese e na redução da fertilidade de vacas leiteiras durante o verão, oferecendo novas perspectivas para futuras pesquisas sobre o tema.