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Carolina dos Santos Amaral

EFEITO DO ESTRESSE TÉRMICO EM LEUCÓCITOS BOVINOS DURANTE O RECONHECIMENTO MATERNO DA GESTAÇÃO

Santa Maria, RS

2022

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Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Medicina Veterinária, área de concentração de Sanidade e Reprodução Animal da Universidade Federal de Santa Maria (UFSM), como requisito parcial para a obtenção do título de **Doutora em Medicina Veterinária**.

Orientador: Dr. Alfredo Quites Antoniazzi

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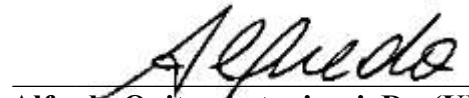

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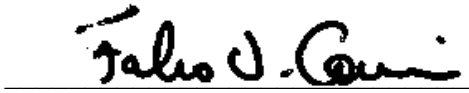

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Dedico essa tese à minha mãe, com todo meu amor e gratidão, por dedicar grande parte da sua vida à minha formação pessoal e profissional.

It is not our abilities that show what we truly are.

It is our choices.

(Albus Dumbledore)

RESUMO

EFEITO DO ESTRESSE TÉRMICO EM LEUCÓCITOS BOVINOS DURANTE O RECONHECIMENTO MATERNO DA GESTAÇÃO

AUTORA: Carolina dos Santos Amaral
ORIENTADOR: Alfredo Quites Antoniazzi

O estresse térmico representa uma das maiores causas de perdas produtivas e reprodutivas em um sistema de produção de leite. O estresse térmico induz a hipertermia, altera a foliculogênese, reduz a expressão de sinais de estro, causa diminuição da qualidade oocitária e morte ou desenvolvimento de embriões de baixa qualidade; também induz estresse oxidativo, que é um precursor do estresse de retículo endoplasmático (RE). Os efeitos do estresse térmico se tornam ainda mais acentuados nos primeiros dias de vida do embrião, comprometendo a produção de interferon tau (IFNT), proteína necessária para que ocorra o reconhecimento materno da gestação. Além do reconhecimento da gestação, o IFNT é responsável por induzir a expressão de genes estimulados por interferons (ISGs). Estudos demonstraram a expressão de ISGs em células sanguíneas logo após a sinalização por IFNT no início da gestação em ruminantes. *In vitro*, a presença de IFNT estimula a expressão de ISGs em cultivo de células mononucleares (PBMCs) e polimorfonucleares (PMNs). Além disso, sabe-se que o IFNT modula o sistema imunológico e durante o reconhecimento materno da gestação recruta células imunes para o corpo lúteo (CL), demostrando sua ação sistêmica na manutenção da gestação. Entretanto, o mecanismo pelo qual o IFNT modula a sua expressão sistêmica durante a ocorrência do estresse térmico em bovinos ainda não é completamente claro. Portanto, nossa hipótese foi que o estresse térmico altera a via dos interferons (IFNs) tipo 1 e ISGs em leucócitos de vacas leiteiras durante o período de reconhecimento materno da gestação, altera os genes da resposta imune inata em vacas leiteiras em fase inicial de gestação de um estado anti-inflamatório para um estado pró-inflamatório e induz a ocorrência do estresse oxidativo, consequentemente estresse de RE e *Heat Shock Proteins* (HSPs) no sangue periférico de vacas com gestação inicial. Primeiramente, se investigou o efeito do estresse térmico sob a modulação da via dos IFNs tipo 1 em leucócitos de vacas gestantes e parâmetros de estresse oxidativo. Os resultados demonstraram que o estresse térmico em vacas prenhes não apenas prejudica a expressão de ISGs, mas também interfere na ativação da via dos IFNs tipo 1, impedindo que ocorra a sinalização correta para o reconhecimento materno da gestação em PMNs. Além disso, o estresse térmico causa estresse oxidativo em vacas leiteiras. Posteriormente, avaliamos se o estresse térmico modula a resposta imune em vacas gestantes. Conforme os resultados, o estresse térmico modifica a expressão de genes anti e pró-inflamatórios em vacas prenhes. Por fim, verificamos os efeitos do estresse térmico sob os parâmetros de estresse de RE e HSPs durante o período de reconhecimento materno da gestação em bovinos. Os resultados desse estudo corroboraram com a hipótese de que o estresse oxidativo causado pelo estresse térmico desencadeia estresse de RE em PBMCs. A partir dos resultados obtidos foi possível sugerir que o estresse térmico influencia negativamente na sinalização endócrina em leucócitos bovinos durante o reconhecimento materno da gestação.

Palavras-chaves: estresse térmico, reconhecimento materno da gestação, resposta imune, estresse oxidativo, estresse de retículo endoplasmático.

ABSTRACT

EFFECT OF HEAT STRESS ON BOVINE LEUKOCYTES DURING MATERNAL RECOGNITION OF PREGNANCY

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ADVISOR: Alfredo Quites Antoniazzi

Heat stress represents one of the major causes of productive and reproductive losses in a milk production system. Heat stress induces hyperthermia, alters folliculogenesis, reduces the expression of estrus signals, causes decreased oocyte quality and loss or development of low-quality embryos; it also induces oxidative stress, which is a precursor of endoplasmic reticulum (ER) stress. The effects of heat stress become even more pronounced in the first days of the embryo's life, compromising the production of interferon tau (IFNT), a protein necessary for maternal recognition of pregnancy. In addition to pregnancy recognition, IFNT is responsible for inducing the expression of interferon-stimulated genes (ISGs). Studies have demonstrated the expression of ISGs in blood cells after IFNT signaling in early pregnancy in ruminants. The presence of IFNT stimulates the expression of ISGs in *in vitro* culture of mononuclear (PBMCs) and polymorphonuclear (PMNs) cells. In addition, it is known that IFNT modulates the immune system and, during maternal recognition of pregnancy, recruits immune cells to the corpus luteum (CL), demonstrating its systemic action in the maintenance of pregnancy. However, the mechanism by which IFNT modulates its systemic expression during the occurrence of heat stress in cattle is still unclear. Therefore, our hypothesis was that heat stress alters the type 1 interferons (IFNs) pathway and ISGs in leukocytes of dairy cows during the maternal recognition period of pregnancy, alters the innate immune response genes in dairy cows in early pregnancy from an anti-inflammatory state to a pro-inflammatory state and induces the occurrence of oxidative stress, consequently stress of ER and Heat Shock Proteins (HSPs) in the peripheral blood of cows with early pregnancy. First, we investigated the effect of heat stress on the modulation of the type 1 IFNs pathway in leukocytes from pregnant cows and parameters of oxidative stress. The results showed that heat stress in pregnant cows not only impairs the expression of ISGs, but also interferes with the activation of the type 1 IFN pathway, preventing the correct signaling for maternal recognition of pregnancy in PMNs. In addition, heat stress causes oxidative stress in dairy cows. Subsequently, we evaluated whether heat stress modulates the immune response in pregnant cows. According to the results, heat stress modifies the expression of anti- and pro-inflammatory genes in pregnant cows. Finally, we verified the effects of heat stress on the parameters of ER stress and HSPs during the period of maternal recognition of pregnancy in cattle. The results of this study support the hypothesis that oxidative stress caused by heat stress triggers ER stress in PBMCs. From the data obtained with this thesis, it was possible to determine that heat stress negatively influences endocrine signaling in bovine leukocytes during maternal recognition of pregnancy.

Keywords: heat stress, maternal recognition of pregnancy, immune response, oxidative stress, endoplasmic reticulum stress.

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1. INTRODUÇÃO

O estresse térmico é definido como a incapacidade do animal em dissipar calor suficientemente para manter a sua homeotermia (WEST, 1999). É também o termo utilizado para definir a condição de animais expostos a mudanças ambientais que os impossibilitam de expressar o potencial genético total (DOBSON *et al.*, 2000), sendo um problema encontrado no manejo de bovinos nos países tropicais, causando reduções na produção e reprodução (WOLFENSON; ROTH; MEIDAN, 2000). O ambiente tem grande influência sobre a fisiologia reprodutiva. Em média, apenas 30% das vacas em lactação concebem após uma única inseminação artificial, e essa taxa reduz ainda mais quando estes animais estão sob condições ambientais desfavoráveis (WILTBANK *et al.*, 2016). O decréscimo nas taxas de concepção durante os meses de calor pode estar entre 20-30% comparado aos meses de conforto térmico (DE RENSIS; SCARAMUZZI, 2003), e ainda pode ser observado durante o outono, devido a ação das altas temperaturas sobre a foliculogênese (ROTH *et al.*, 2001).

A redução da fertilidade consequente do estresse térmico é um problema multifatorial, pois acomete o funcionamento de vários tecidos e tipos celulares. Sabe-se que o estresse térmico compromete a produção de hormônios reprodutivos (DE RENSIS; SCARAMUZZI, 2003; ROTH *et al.*, 2001), desenvolvimento oocitário (AL-KATANANI; PAULA-LOPES; HANSEN, 2002) e embrionário na fase de pré-implantação (AMARAL *et al.*, 2020; SAKATANI, 2017). O ambiente uterino também sofre efeitos do estresse térmico. Em vacas submetidas ao estresse térmico há uma redução no fluxo sanguíneo uterino com diminuição da troca de calor e consequentemente aumento da temperatura interna do útero (GWAZDAUSKAS *et al.*, 1981). Essas mudanças inibem o desenvolvimento embrionário e impedem o sucesso de inseminações, além de aumentar a taxa de perda embrionária (DE RENSIS *et al.*, 2015). Embriões em fase inicial de desenvolvimento são mais susceptíveis aos efeitos do estresse térmico (DE RENSIS *et al.*, 2015) e produzem menos interferon tau (IFNT) do que embriões em conforto térmico (AMARAL *et al.*, 2020). Dessa forma, o estresse térmico impacta diretamente no período de desenvolvimento embrionário inicial e o reconhecimento materno da gestação, gerando perdas gestacionais precoces.

Perdas gestacionais acontecem nos diferentes estágios da gestação por uma variedade de causas, contudo ocorrem com maior frequência nos primeiros 30 dias (WILTBANK *et al.*, 2016). Esse período coincide com um importante evento durante a pré-implantação do embrião que é o reconhecimento materno da gestação. O reconhecimento materno da gestação em

ruminantes é o período em que o concepto sinaliza sua presença para a mãe, através da secreção de IFNT (BAZER; THATCHER, 2017). Estudos relataram que o IFNT regula a expressão de genes estimulados por interferons (ISGs) durante o início da gestação no endométrio (JOHNSON *et al.*, 1999a), células luteais (OLIVEIRA *et al.*, 2008), fígado (BOTT *et al.*, 2010), leucócitos mononucleares (PBMCs) e polimorfonucleares (PMNs) (HAQ *et al.*, 2016; KIZAKI *et al.*, 2013). Para ocorrer o reconhecimento materno da gestação em ruminantes é essencial a formação de um corpo lúteo (CL) especializado para a síntese de progesterona. O tamanho do CL é diretamente proporcional à concentração sérica de progesterona, elongação e produção de IFNT pelo embrião (MANN *et al.*, 2003; CARTER *et al.*, 2008). Essa sinalização em tecidos maternos contribui para o crescimento do concepto e modulação imune materna para prevenir a rejeição do concepto (HANSEN, 2017). Como este é o momento em que ocorre a maior mortalidade embrionária em bovinos (THATCHER *et al.*, 2001), o entendimento dos efeitos diretos e indiretos do estresse térmico no desenvolvimento embrionário inicial e sua sinalização para o reconhecimento materno da gestação adequado é fundamental para minimizar perdas econômicas e melhorar índices de produção.

Além da reprodução, o estresse térmico também afeta negativamente o sistema imunológico e a resposta inflamatória de bovinos pela redução do consumo de matéria seca (YADAV *et al.*, 2016). Durante os períodos de estresse por calor, são ativados os eixos hipotálamo-hipófise-adrenal e simpático-adrenal-medular para manter a homeostase em resposta a estímulos estressantes (BAGATH *et al.*, 2019). Com isso, ocorre a secreção crônica de cortisol e a consequente supressão imunológica (JU *et al.*, 2014; WANG *et al.*, 2011). Ambas respostas imunes inata e adaptativa atuam para tentar proteger o organismo dos danos causados pelo estresse térmico (SMITH; VALE, 2006), produzindo citocinas e fatores anti-inflamatórios, como interferons e interleucinas (LACETERA *et al.*, 2005; WEBSTER; TONELLI; STERNBERG, 2002). Contudo, essas citocinas utilizam a mesma via de sinalização intracelular que o IFNT, a via JAK/STAT (BINELLI *et al.*, 2001; LEONARD, 2001), podendo interferir na expressão de genes estimulados pelo interferon embrionário. Portanto, torna-se essencial o melhor entendimento dos efeitos do estresse térmico na sinalização endócrina do reconhecimento materno da gestação e na resposta imune, visto que esses fatores estão intimamente interligados e podem interagir entre si, modulando a resposta do embrião em leucócitos.

Ainda, o estresse térmico induz a ocorrência do estresse oxidativo (SLIMEN *et al.*, 2014). Vários estudos concluíram que a exposição ao calor aumenta a produção de ROS e induz estresse oxidativo, o que pode levar à citotoxicidade (AMARAL *et al.*, 2020; BERNABUCCI

et al., 2002). O estresse oxidativo também é um precursor e um dos principais contribuintes para a ocorrência de estresse de retículo endoplasmático (RE) (HOTAMISLIGIL, 2010). O estresse de RE é um desequilíbrio entre a capacidade de alterações conformacionais das proteínas no RE e a carga proteica, o que consequentemente leva a alterações conformacionais ou proteínas desdobradas que acabam se acumulando no lúmen do RE, prejudicando a homeostase do RE (CNOP; FOUFELLE; VELLOSO, 2012). Outro marcador de estresse térmico são as proteínas induzidas por choque térmico (*heat shock proteins*; HSPs) (ARYA; MALLIK; LAKHOTIA, 2007; SAKATANI, 2017). As HSPs são um grupo heterogêneo de proteínas denominadas chaperonas, que possuem peso molecular específico e função biológica determinada. A resposta ao choque térmico é uma resposta adaptativa celular, que ajuda a manter a homeostase celular sob estresse. Em geral, as chaperonas dão suporte a uma proteína lesada, prevenindo danos letais às células (MAYER; BUKAU, 2005).

Desta maneira, nossa hipótese é que o estresse térmico altera a via dos interferons (IFNs) tipo 1 e ISGs em leucócitos de vacas leiteiras durante o período de reconhecimento materno da gestação. Além disso, altera os genes da resposta imune inata em vacas leiteiras em fase inicial de gestação de um estado anti-inflamatório para um estado pró-inflamatório. Ainda, induz a ocorrência do estresse oxidativo, estresse de RE e HSPs no sangue periférico de vacas com gestação inicial. Portanto, os objetivos do presente trabalho foram: 1) avaliar o efeito do estresse térmico sob a modulação da via dos IFNs tipo 1 em leucócitos de vacas gestantes; 2) verificar se o estresse térmico modula a resposta imune em vacas gestantes; e 3) compreender os efeitos do estresse térmico sob os parâmetros de estresse oxidativo, estresse de RE e HSPs durante o período de reconhecimento materno da gestação em bovinos.

2. REVISÃO DE LITERATURA

2.1. IMPACTO DO ESTRESSE TÉRMICO NOS ÍNDICES REPRODUTIVOS

O estresse térmico é definido como o resultado da inabilidade do animal dissipar ou produzir calor para manter sua temperatura fisiológica (DE RENSIS; SCARAMUZZI, 2003; DOBSON *et al.*, 2000). Esta condição acontece quando o animal se encontra fora da zona de conforto térmico, consequência de temperaturas elevadas ou baixas (SLIMEN *et al.*, 2014). O estresse térmico representa uma das maiores causas de perdas produtivas e reprodutivas em um sistema de produção de leite. Vacas leiteiras possuem maiores exigências metabólicas, o que influencia na eficiência reprodutiva, ocasionando em baixos índices nos rebanhos (DE RENSIS; SCARAMUZZI, 2003).

Os prejuízos ocorridos pelo estresse térmico possuem influência das temperaturas elevadas e da umidade relativa do ar (KADOKAWA *et al.*, 2012). Em vacas de leite, a hipertermia pode ocorrer mesmo em temperaturas abaixo de 30°C, quando a umidade relativa do ar está elevada (SARTORI *et al.*, 2002). A temperatura retal de 39°C é um parâmetro classificado dentro dos limites fisiológicos para a espécie, porém já causam perdas na produção de leite e na fertilidade (SCHÜLLER *et al.*, 2014).

Dentre todas as categorias de animais em uma fazenda leiteira, vacas em produção possuem maior ingestão de matéria seca. Esses nutrientes são absorvidos, metabolizados e, consequentemente, geram calor que precisa ser dissipado. Quando o animal se encontra em uma situação de estresse térmico, torna-se desfavorável dissipar o calor produzido (KADOKAWA *et al.*, 2012). Vacas sob o efeito do estresse térmico diminuem o consumo de matéria seca (GAULY *et al.*, 2014), o que altera a atividade ruminal e provoca um balanço energético negativo. Como consequências, ocorrem distúrbios metabólicos associados como a acidose ruminal subaguda e a cetose (POLSKY; VON KEYSERLINGK, 2017). Logo, a categoria vacas em lactação é mais susceptível aos prejuízos em decorrência estresse térmico por calor (DIKMEN; HANSEN, 2009).

Estudos comprovam que vacas expostas ao estresse térmico no momento da concepção e nos 60 dias iniciais de gestação foram mais susceptíveis a perdas gestacionais quando comparadas com vacas que não sofreram estresse térmico nesse mesmo período (DE RENSIS *et al.*, 2015). O decréscimo nas taxas de concepção durante os meses de calor pode estar entre 20-30% comparado aos meses de conforto térmico (DE RENSIS; SCARAMUZZI, 2003). Essa queda na taxa na concepção ainda é observada durante o outono, onde as vacas já não se

encontram mais sob estresse térmico, devido a ação das altas temperaturas sob a foliculogênese na estação anterior (ROTH *et al.*, 2001).

O estresse em decorrência das altas temperaturas diminui os níveis circulantes de progesterona, estradiol, gonadotrofinas (DAS *et al.*, 2016) e de citocromo P450, enzima responsável pela clivagem do colesterol à pregnenolona na mitocôndria (MCCRACKEN *et al.*, 2015). Assim, ocorre diminuição das concentrações de progesterona e aumento da possibilidade de perdas durante a gestação inicial. O estresse térmico ainda pode afetar o peso da placenta e prejudicar seu o desenvolvimento vascular fisiológico (DUNLAP *et al.*, 2015).

2.1.1. Efeito do estresse térmico sobre a maturação oocitária, fertilização e mortalidade embrionária precoce

Vacas expostas ao estresse por calor têm redução da qualidade dos oócitos e esse fato afeta o desenvolvimento subsequente do embrião (SUGIYAMA *et al.*, 2003). Complexos cumulus-oócitos (CCOs) em fase de vesícula germinativa que foram expostos a temperaturas elevadas (40°C-41°C) mostram deficientes maturações citoplasmática e nuclear, além da diminuição da competência de desenvolvimento após fertilização (ROTH, 2015).

O estresse térmico de CCOs em fase de vesícula germinativa compromete as funções de oócitos, induz apoptose, interrompe os componentes do citoesqueleto (ROTH; HANSEN, 2004), e altera a transcrição materna e funções mitocondriais (ROTH, 2015). O estresse térmico muda não só a função de oócitos, mas também funções das células cumulus (RISPOLI *et al.*, 2013).

O estresse por calor excessivo também induz o estresse oxidativo levando a elevação de níveis de espécies reativas ao oxigênio (ROS) em oócitos (OZAWA *et al.*, 2002). As ROS danificam o DNA e induzem apoptose ou disfunção de organelas celulares, como a mitocôndrias (ROTH, 2015). No entanto, pesquisadores mostraram que a suplementação de antioxidantes durante a maturação *in vitro* com alta temperatura aumenta a taxa de maturação de oócitos, bem como a competência dos embriões futuros. Glutationa (GSH) é um importante componente antioxidante no oócito e atua diminuindo os níveis de ROS (NABENISHI *et al.*, 2012).

O estresse térmico está entre os fatores que acometem a eficiência dos gametas femininos e masculinos, assim como a viabilidade embrionária. Estudos *in vivo* sugerem que existe uma correlação entre a temperatura ambiente no dia da inseminação artificial e a taxa de

mortalidad de embriões (DE RENSIS *et al.*, 2015). Estudos *in vitro* também relataram que alta temperatura durante a fertilização reduziu a competência embrionária (SAKATANI, 2017).

A qualidade do oócito é reduzida pelo estresse térmico (SUGIYAMA *et al.*, 2007). Da mesma forma, a motilidade, integridade e função dos espermatozóides são diminuídas pela elevação de temperatura (SAKATANI, 2017). A incubação de espermatozoides a 40°C a 42°C por 4 horas diminui a sua motilidade e integridade (SAKATANI *et al.*, 2015). Resultados indicam que o mecanismo anti-polispermia dos oócitos é interrompido pelo estresse térmico. Além dos efeitos no mecanismo anti-polispermia, o dano aos zigotos também pode suprimir o sucesso da fertilização e competência de desenvolvimento (SAKATANI *et al.*, 2015).

Embriões em fase inicial de desenvolvimento são mais susceptíveis aos efeitos do estresse térmico, comprometendo a sua capacidade de secretar interferon tau, proteína responsável por sinalizar a gestação para a mãe. Além disso, o estresse térmico na temperatura de 40,5ºC por 6 horas durante as etapas de maturação *in vitro*, fertilização *in vitro* e o primeiro dia de cultivo *in vitro* diminui significativamente as taxas de clivagem e blastocistos (AMARAL *et al.*, 2020).

2.1.2. Resposta celular ao estresse térmico

A mitocôndria é o principal local de metabolismo do oxigênio, sendo responsável por aproximadamente 85-90% do oxigênio celular consumido. As ROS são subprodutos do metabolismo do oxigênio, portanto sua formação acontece de forma fisiológica no metabolismo orgânico (SHIGENAGA *et al.*, 1994). As ROS participam de diversas funções biológicas, como na produção de energia, na fagocitose, na regulação do crescimento celular e sinalização intracelular (DROGE, 2002; SLIMEN *et al.*, 2014).

Embora pequenas concentrações de ROS sejam benéficas, o aumento descontrolado de ROS podem causar dano celular, pela ativação das vias da apoptose e da inflamação (KHAN *et al.*, 2017). Vários tipos de estresse celular induzem a ocorrência do estresse oxidativo, incluindo o estresse térmico (SLIMEN *et al.*, 2014; ZACHUT *et al.*, 2017). Quando o embrião é submetido a altas temperaturas, ocorre a formação excessiva de ROS e o sistema antioxidante se torna incapaz de reestabelecer o equilíbrio (AMARAL *et al.*, 2020).

Para manter as concentrações de ROS balanceadas e obter um metabolismo oxidativo estável, a mitocôndria dispõe do seu próprio sistema de defesa antioxidante cuja função é controlar a produção de ROS e estabelecer o equilíbrio oxidativo. Neste sistema estão inclusos 2 principais grupos: antioxidantes enzimáticos e não enzimáticos. Antioxidantes enzimáticos

são compostos principalmente pelas enzimas superóxido dismutase (SOD), catalase (CAT) e glutationa peroxidase (GPx). Dentre os antioxidantes conhecidos como não enzimáticos estão compostos como as vitaminas A, C e E, selênio e carotenoides, entre outros (SLIMEN *et al.*, 2014). Alguns grupos de pesquisa têm desenvolvido trabalhos relacionados com a suplementação de antioxidantes não enzimáticos no meio de maturação, fertilização e cultivo de embriões, a fim de aumentar as taxas de clivagem e desenvolvimento embrionário em bovinos (ROCHA-FRIGONI *et al.*, 2016; TAKAHASHI *et al.*, 2002), suínos (LI *et al.*, 2016), ovinos (MISHRA *et al.*, 2016) e ratos (LIAN *et al.*, 2013).

O estresse oxidativo é também um precursor e um dos principais contribuintes para a ocorrência do estresse de RE (HOTAMISLIGIL, 2010). O estresse de RE é definido como um desequilíbrio entre a capacidade de alterações conformacionais das proteínas no RE e a carga proteica, o que consequentemente leva a modificações conformacionais ou proteínas malformadas que acabam se acumulando no lúmen do RE, dificultando a homeostase dessa organela (CNOP; FOUFELLE; VELLOSO, 2012). O mecanismo que medeia essa regulação da homeostase, na tentativa de inibir o estresse do RE, é a via Unfolded Protein Response (UPR) (HARDING; ZHANG; RON, 1999). Durante a ativação da via UPR, a presença de estresse é detectada por três proteínas transmembranares do RE: PERK (PKR-like RE kinase), ATF6 (Activating transcription fator 6) e IRE1 (Inositol-requiring enzyme 1). Essas proteínas se associam aos domínios luminais da chaperona HSPA5, e quando liberadas, cada uma induz um mecanismo regulatório diferente. Tem sido sugerido que a HSPA5, também conhecida como proteína regulada pela glicose 78 (GRP78) ou binding protein (BiP), é um importante marcador de estresse de RE, uma vez que é uma chaperona residente no RE e desempenha um papel vital na regulação da homeostase do RE (LIU *et al.*, 2011).

Outro marcador de estresse calórico *in vitro* e *in vivo* é a presença de HSPs, especialmente HSP70 e HSP90 (SAKATANI, 2017). Embora a síntese da maioria das proteínas seja acometida pelo estresse térmico, esse fato não se aplica às HSPs. As HSPs fazem parte de um grupo heterogêneo de proteínas denominado de chaperonas, que possuem peso molecular e função biológica específicas. De modo geral, as chaperonas auxiliam no remodelamento de uma proteína lesionada, evitando um dano celular letal (TAKENAKA; HIGHTOWER, 1992). O mecanismo de ação das HSPs inicia com a ativação do seu fator de transcrição (*heat shock transcription factors*; HSFs). Os HSFs estão presentes no citoplasma de forma inativa, onde seus monômeros sofrem o processo de fosforilação por proteínas quinases e são ativados, sendo translocados até o núcleo da célula, que se ligarão a sítios específicos para transcrever o RNAm da HSP. Embora a ação das HSPs não seja restrita ao estresse térmico, acredita-se que essas

proteínas utilizem energia da hidrólise de ATP para desnovelar proteínas danificadas ou alteradas pela hipertermia, possibilitando novo enovelamento, dessa vez na forma correta ou no lugar correto (SLIMEN *et al.*, 2014; SREEDHAR *et al.*, 2000).

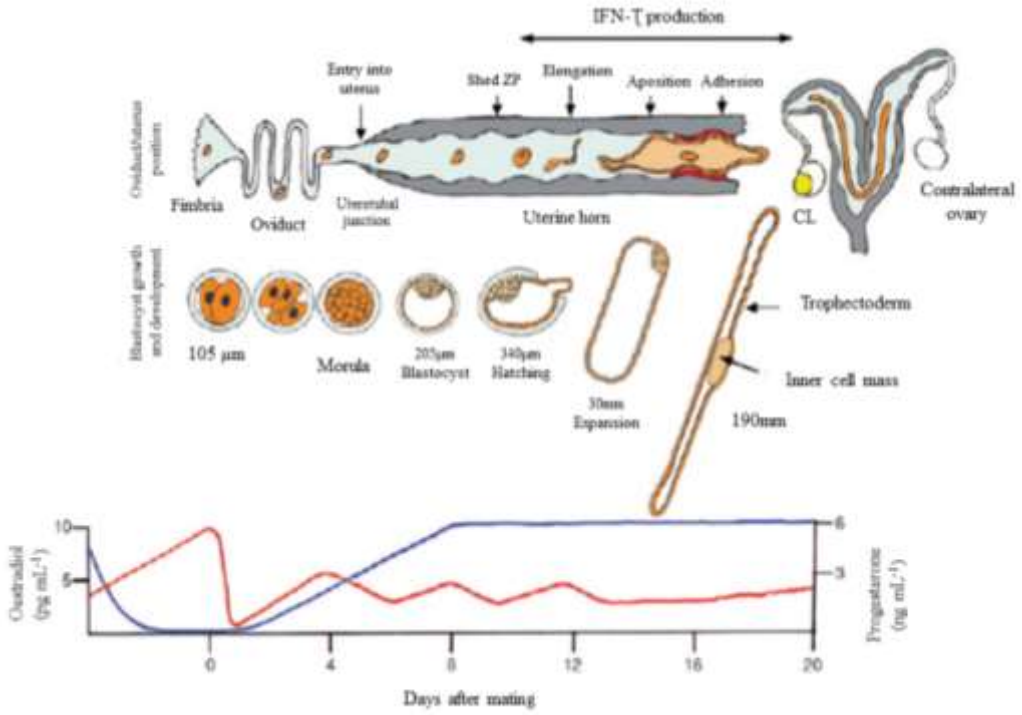
2.2. RECONHECIMENTO MATERNO DA GESTAÇÃO EM RUMINANTES

O reconhecimento materno da gestação é o período em que o concepto sinaliza sua presença para a mãe com a finalidade de aumentar a vida útil do CL e consequentemente evitar o retorno à ciclicidade (FARIN *et al.*, 1990; NISWENDER *et al.*, 2000). Em ruminantes, o período de sinalização coincide com o alongamento do embrião e a máxima produção IFNT (ANTONIAZZI *et al.*, 2010; JOHNSON *et al.*, 1999b).

Após a fecundação, inicia-se o processo de desenvolvimento embrionário. O embrião passa pelas fases de zigoto, quando ocorre o estabelecimento da singamia. Logo após sofre sucessivas mitoses, até chegar à fase de mórula. Nessa fase, que acontece de 4 a 6 dias após a fecundação, o embrião entra no útero. Em seguida, evolui para o estágio de blastocisto inicial, com a formação da blastocele e o estabelecimento de 2 tipos celulares: trofoblasto e massa celular interna (embrioblasto). O blastocisto inicial de desenvolve e expande, até o dia 8-9 quando ocorre a eclosão da zona pelúcida. Então o blastocisto passa a se alongar de forma tubular, formando um filamento capaz de preencher todo o espaço intrauterino (Figura 1). A expressão de IFNT aumenta proporcionalmente à medida que ocorre o processo de elongação (HAN *et al.*, 2006; HIRAYAMA *et al.*, 2014; SPENCER; BAZER, 1996; SPONCHIADO *et al.*, 2017).

Inicialmente, a secreção de IFNT no concepto ovino ocorre entre os dias 10 e 25, com pico de secreção entre os dias 14 e 16 da gestação (ROBERTS; EALY; EZASHI, 1999). Já em bovinos, a secreção ocorre entre os dias 12 e 26, com pico entre os dias 18 e 20 (FARIN *et al.*, 1990; HIRAYAMA *et al.*, 2014). O IFNT é a principal citocina secretada pelas células do trofoblasto embrionário, sendo responsável pela sinalização durante esse período (ROBERTS; EALY; EZASHI, 1999). O mecanismo clássico de ação do IFNT consiste no controle da transcrição de receptores de estrógenos (ESR1) e consequentemente receptores de ocitocina (OXTR) no epitélio luminal endometrial (SPENCER; BAZER, 1996). Esse controle inibe os pulsos luteolíticos de PGF, evitando o retorno à ciclicidade.

Figura 1. Desenvolvimento embrionário inicial em ruminantes.



Fonte: (SPENCER et al., 2007).

2.2.1. O interferon tau

Inicialmente, estudos na década de 1960 identificaram que existia alguma substância produzida durante a gestação inicial que adiava a manifestação do estro (MOOR; ROWSON, 1966). Já na década de 1970, foi identificado que essa substância era uma proteína produzida pelo concepto, que foi nomeada de trofoblastina (MARTAL *et al.*, 1979). Pouco tempo depois, foi comprovado que essa proteína era produzida pelas células do trofoblasto e chamou de proteína do trofoblasto ovino (ovine trophoblastic protein-1 oTP-1) (GODKIN; BAZER; ROBERTS, 1984). A oTP-1 foi sequenciada e foi identificado que sua estrutura era muito semelhante aos interferons tipo 1, e por essa razão foi renomeada para IFNT (IMAKAWA *et al.*, 1987).

Aparentemente o IFNT evoluiu do interferon ômega, essa evolução é caracterizada pela inserção de um promoter específico no troflobasto. Bovinos, ovinos e caprinos possuem diversas formas polimórficas de IFNT (EALY; WOOLDRIDGE, 2017). Fatores de transcrição que possuem papel importante na regulação da expressão do gene do IFNT. Dentre eles, os

fatores de transcrição ETS2, AP1, DLX3 e CDX2 possuem um papel fundamental na regulação da transcrição de IFNT durante o início da gestação (EZASHI; IMAKAWA, 2017).

O IFNT é uma proteína secretada em grandes quantidades pelas células do trofoblasto do embrião de ruminantes, antes da implantação (FARIN *et al.*, 1990). O RNAm começa a ser expresso a partir do quarto dia do desenvolvimento embrionário *in vitro* (TALUKDER *et al.*, 2018) e a sua sinalização já é detectada no endométrio no sétimo dia do desenvolvimento embrionário (SPONCHIADO *et al.*, 2017). O início da expressão de IFNT é programada geneticamente independente do ambiente uterino, pois ele é expresso em sistemas *in vivo* e *in vitro*. No entanto, a produção de IFNT é influenciada pelo ambiente uterino, pois a produção *in vitro* aumenta na presença de tecido uterino (KERBLER *et al.*, 1997). A expressão diminui com a implantação, pois o contato do trofoblasto com o endométrio cessa a produção de IFNT (DEMMERS; DERECKA; FLINT, 2001).

2.2.2. Receptores de interferon tipo 1 e genes estimulados por interferon tau (ISGs)

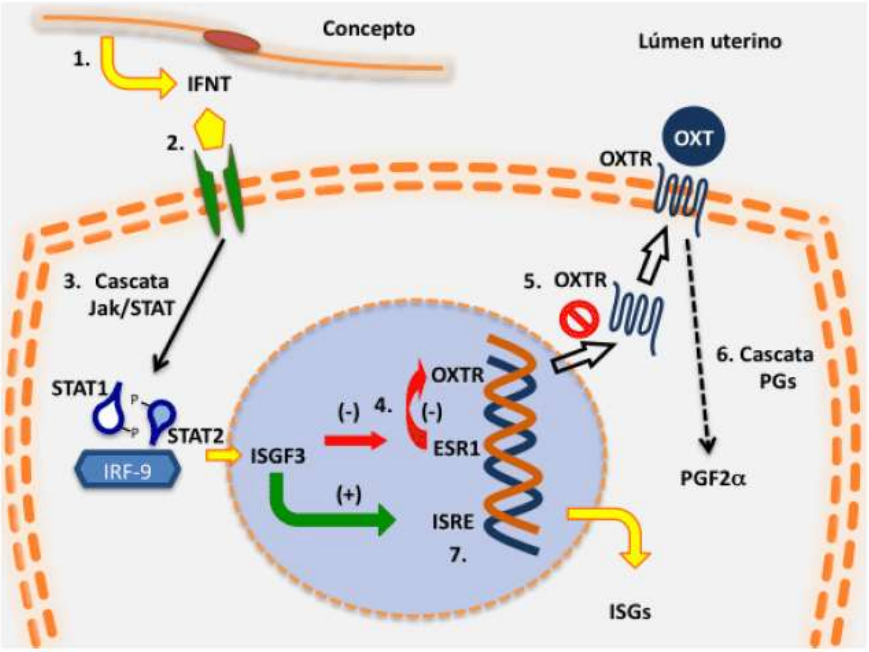
Embora o IFNT não seja induzido por vírus (CHELMONSKASOYTA, 2002), ele pertence aos IFNs do tipo 1 e usa o mesmo mecanismo de ação das respostas antivirais. O IFNT liga-se a receptores de interferon tipo 1 (IFNAR1 e IFNAR2) e induz sua resposta por meio da sinalização via JAK/STAT (BINELLI *et al.*, 2001). Os receptores IFNAR1 e IFNAR2 são expressos em todos os tecidos corporais e têm como função principal mediar respostas antivirais. Também estão localizados no útero para mediar respostas maternas em função do IFNT produzido pelo embrião (JOHNSON *et al.*, 1999a).

O IFNT se liga a esses receptores para exercer sua ação pela via de transdução de sinais JAK/STAT, onde as proteínas tirosino-quinases fosforilam proteínas STAT formando complexos multiméricos que agem como fatores de transcrição (BINELLI *et al.*, 2001). Esses complexos atuam basicamente em 2 vias: a primeira consiste no controle da transcrição de receptores de estrógenos (ESR1) e consequentemente receptores de ocitocina (OXTR) no epitélio luminal endometrial (SPENCER; BAZER, 1996); e a segunda ocorre quando os complexos se ligam a regiões definidas no DNA, chamadas de elementos responsivos à estimulação por interferons (ISREs), que regulam a expressão de ISGs (ANTONIAZZI *et al.*, 2010; JOHNSON *et al.*, 1999a).

2.2.3. Ações do interferon tau

Atualmente, sabe-se que o IFNT atua mediante 3 vias de sinalização: parácrina, endócrina e autócrina. O mecanismo de sinalização parácrino (Figura 2), também conhecido como via clássica de sinalização do reconhecimento materno da gestação em ruminantes, consiste na ligação do IFNT aos seus receptores presentes no endométrio, ativação da cascata JAK/STAT e inibição da expressão dos receptores de estrógenos (ESR1) e de ocitocina (OXTR) no epitélio luminal do endométrio. A supressão dos receptores ESR1 e OXTR evita a liberação de pulsos luteolíticos de prostaglandina F2 alfa (PGF), hormônio responsável pelo início da luteólise (MCCRACKEN; CUSTER, 1999; SPENCER; BAZER, 1996).

Figura 2. Via de sinalização parácrina do reconhecimento materno da gestação em ruminantes.



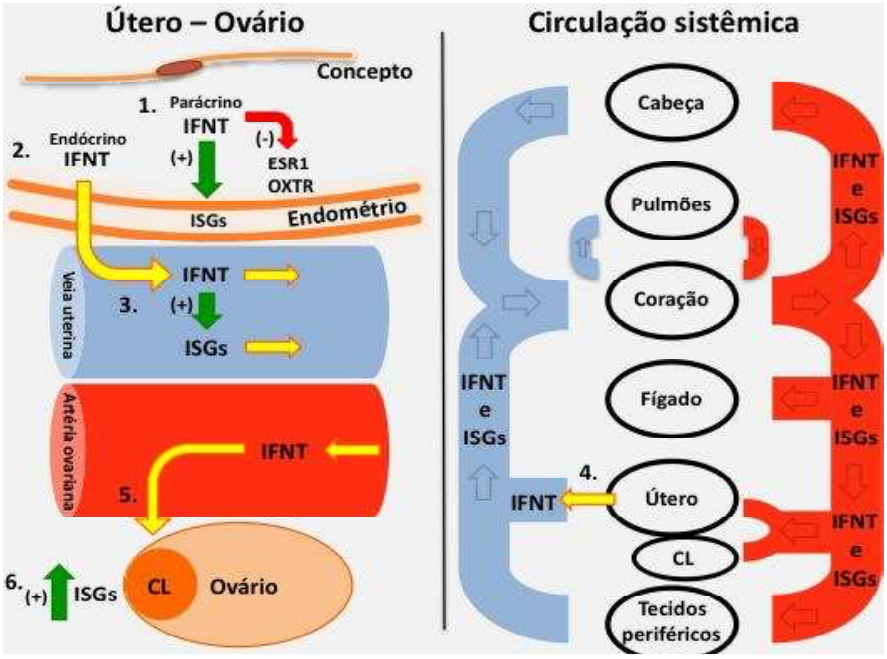
Fonte: (ANTONIAZZI *et al.*, 2010).

Além do mecanismo parácrino do reconhecimento materno da gestação em ruminantes, existe a via de sinalização endócrina (Figura 3). Foi observado que alguns animais gestantes possuíam expressão de genes ISGs na corrente circulatória, mais especificamente o gene estimulado por interferon-15 (ISG15) (HAN *et al.*, 2006). Inicialmente foi pensado na presença de um mediador da ação do IFNT, uma interferomedina (SPENCER *et al.*, 1999). Foi avaliada a expressão de ISG15 em tecidos extrauterinos durante o início da gestação em ovinos (BOTT *et al.*, 2010; OLIVEIRA *et al.*, 2008). Pelas técnicas de biologia molecular de PCR em tempo

real, western blot e imuno-histoquímica, identificou-se uma maior expressão de ISG15 em células luteais grandes no dia 15 da gestação, quando comparada com a expressão em células luteais grandes de ovelhas não prenhes (OLIVEIRA *et al.*, 2008). O ensaio antiviral mostrou maior bioatividade de interferons tipo 1 no dia 15 da gestação no sangue da veia uterina de ovelhas prenhes quando comparadas com ovelhas não prenhes (OLIVEIRA *et al.*, 2008). Assim, sugere-se que a ação dos interferons presente no soro da veia uterina de ovelhas prenhes no dia 15 da gestação é exercida pelo IFNT, pois a utilização de anticorpo específico contra IFNT bloqueia ação antiviral (BOTT *et al.*, 2010).

A partir da comprovação da ação endócrina do IFNT na veia uterina, iniciou-se a investigação de sua ação em tecidos extrauterinos que poderiam estar envolvidos com o reconhecimento materno da gestação. Um novo modelo de estudo da ação endócrina do IFNT foi desenvolvido (BOTT *et al.*, 2010). Inicialmente, foi realizada a instalação de uma bomba osmótica para infusão contínua de IFNT recombinante ovino (roIFNT) na veia uterina no dia 10 do ciclo estral, e foi verificado a expressão de ISG15 no CL. Outros experimentos foram realizados com infusão contínua de IFNT em diferentes dias e com diferentes concentrações de IFNT, e ambas foram capazes de induzir ISG15 em tecidos extrauterinos (ANTONIAZZI *et al.*, 2013; ROMERO *et al.*, 2013).

Figura 3. Mecanismo de ação endócrino do reconhecimento materno da gestação em ruminantes.



Fonte: (ANTONIAZZI *et al.*, 2010).

Foi sugerido que o IFNT possui um mecanismo de ação autócrino. Inicialmente, foi demonstrado que células do trofectoderma ovino expressavam IFNAR1, indicando a existência de alguma função do IFNT em suas próprias células produtoras (IMAKAWA et al., 2002). A partir desse resultado, outros estudos foram realizados na tentativa e elucidar essa via de sinalização. Foram adicionadas diferentes concentrações de rbIFNT no cultivo de celular de trofoblasto, e mostraram que o desenvolvimento celular e a expressão de ISGs aumentaram a medida que aumentava a concentração de rbIFNT no meio de cultivo, comprovando que o IFNT não é crucial apenas para o reconhecimento materno da gestação em ruminantes, mas também atua como um regulador autócrino de proliferação das células do trofoblasto (WANG *et al.*, 2013).

2.2.4. Uso dos ISGs como marcadores da presença embrionária

Em geral, acredita-se que as concentrações circulantes de IFNT na corrente sanguínea sejam extremamente baixas e, portanto, difíceis de detectar (BOTT *et al.*, 2010; OLIVEIRA *et al.*, 2008). No entanto, existem maneiras indiretas de detectar a ação do IFNT na corrente sanguínea. Uma delas é mensurar a expressão de ISGs em leucócitos. Existem vários estudos que correlacionam a expressão de ISGs nos leucócitos do sangue periférico (PBL) com a gestação precoce (GREEN *et al.*, 2010; HAQ *et al.*, 2016; SHEIKH *et al.*, 2018; TOJI *et al.*, 2017). Estudos analisaram a expressão gênica em frações separadas de leucócitos concluíram que a fração de PMN são mais sensíveis ao estímulo por IFNT quando comparados à fração de PBMCs. Além disso, existe uma correlação positiva entre a expressão de ISGs no PMN e a gestação precoce em bovinos (KIZAKI *et al.*, 2013; SHIRASUNA *et al.*, 2012). Dentre os ISGs que aumentam a expressão de RNAm durante o início da gestação em resposta ao IFNT, estão os genes 2',5' oligoadenilato sintetase (OAS1) (MIRANDO *et al.*, 1991; SCHMITT *et al.*, 1993), o gene de resistência ao myxovirus 1 e 2 (MX1 e MX2) (OTT *et al.*, 1998) e o gene estimulado por interferon 15 (ISG15) (AUSTIN *et al.*, 1996; ROCHA *et al.*, 2020).

2.3. IMUNOLOGIA DA GESTAÇÃO EM BOVINOS

Ao longo da evolução, os animais desenvolveram mecanismos complexos contra doenças, detectando e destruindo material biológico estranho dentro de seus organismos. Estes mecanismos necessitam ser regulados de forma precisa para desenvolver uma resposta efetiva e apropriada contra o patógeno, enquanto limita possíveis danos ao organismo do hospedeiro

(BAINBRIDGE, 2000). A exclusão de corpos estranhos foi adaptada por milhões de anos em um processo denominado de sistema imunológico (ENTRICAN, 2002).

O embrião bovino é caracterizado como semialogênico, ou seja, metade do seu material genético é herdado do pai e outra metade da mãe (BILLINGHAM *et al.*, 1953). Diante disso, o concepto possui antígenos desconhecidos pelo sistema imune materno, e mesmo assim ele não é reconhecido como estranho e rejeitado. O desenvolvimento adequado da gestação contraria o preceito imunológico e a sobrevivência embrionária e fetal ainda não é completamente conhecida do ponto de vista da imunologia. Existem teorias que tentam explicar a imunotolerância adquirida pelo sistema imune da mãe (HANSEN, 2011). Contudo, a gestação somente é possível com o desenvolvimento do padrão aloimune que protege o concepto desde as primeiras divisões mitóticas até o nascimento. Nesse período, a regulação do sistema imune é feita concomitante entre mãe e concepto, objetivando reduzir uma resposta imunológica que seja agressiva à sobrevivência e desenvolvimento do concepto (HANSEN, 2011).

O concepto possui em sua superfície moléculas do complexo de histocompatibilidade (MHC). O MHC é um lócus que codifica proteínas especializadas, cuja função é apresentar antígenos para serem reconhecidos pelos linfócitos T. Existem dois produtos gênicos principais: MHC classe 1 (MHC-I) e MHC classe 2 (MHC-II) (KELLY; TROWSDALE, 2019). Na superfície do concepto, apenas o MHC-I é expresso (DAVIES; FISHER; SCHLAFER, 2000). Durante as fases de desenvolvimento gestacional, padrões imunológicos diferentes são estabelecidos regidos por sinais do concepto para favorecer a comunicação com o sistema da mãe (MOR; CARDENAS, 2010). Esses padrões podem alternar entre um estado pró ou anti-inflamatório quando toda a gestação é analisada, e essas mudanças dependem de mediadores específicos.

No que se refere ao desenvolvimento embrionário inicial, antes da eclosão da zona pelúcida, o embrião parece imunologicamente inerte com baixa expressão de MHC, o que provavelmente protege o embrião inicial do ataque imunológico. Próximo ao período de placentação, a inércia imune do concepto é substituída por um período de ativação imunológica causada pela expressão de genes, como interferons e MHC-I. Essas mudanças desempenharão um importante papel para garantir o desenvolvimento fetal (HANSEN, 2011).

Nesse contexto, as citocinas atuam diretamente sobre a modulação da resposta imune, agindo como mediadores inflamatórios. Podem ser produzidas por macrófagos, linfócitos, células natural killers (NK) e também pelas células trofoblásticas, como é o caso do IFNT, e agem através de vias complexas de retroalimentação positiva ou negativa, controlando as células imunes efetoras (NASU *et al.*, 1999). Citocinas pró-inflamatórias podem interromper a

gestação durante seu o período inicial (CHAOUAT *et al.*, 1995). Em contraste, citocinas anti-inflamatórias auxiliam na manutenção gestacional (CHAOUAT *et al.*, 1995). O IFNT auxilia a redistribuição de células TCD4+ e TCD8+, NK uterinas, monócitos, macrófagos e células dendríticas (HANSEN, 2011).

O reconhecimento imunológico do concepto por linfócitos maternos ou células NK poderiam levar a morte embrionária (HANSEN, 2011). Contudo, não é o que acontece. Mãe e concepto agem de maneira endócrina e parácrina a fim de controlar a função imune materna durante a gravidez e evitar a morte embrionária.

2.3.1. Alterações imunológicas decorrentes do estresse térmico em bovinos

Uma das primeiras ações de um animal em condições de estresse térmico é a redução do consumo alimentar, o que consequentemente influencia na absorção de nutrientes. Em resposta a isso, gera-se um impacto negativo no sistema imunológico e na resposta inflamatória (YADAV *et al.*, 2016). Durante os períodos de estresse por calor, são ativados os eixos hipotálamo-hipófise-adrenal e simpático-adrenal-medular para manter a homeostase em resposta a estímulos estressantes (SEJIAN *et al.*, 2018). Com isso, ocorre a secreção crônica de cortisol e a consequente supressão imunológica, fazendo com que o animal se torne mais suscetível a doenças e desafios imunológicos (JU *et al.*, 2014; WANG *et al.*, 2011).

O estresse térmico limita a capacidade de vacas leiteiras produzirem resposta imune, tanto que a administração de vacinas nesses períodos parece não permitir o desenvolvimento de uma resposta imune ideal, tornando a vacina potencialmente ineficaz (HU *et al.*, 2007). Em um estudo feito em camundongos, verificou-se que o estresse crônico pelo calor afeta negativamente o sistema imunológico, alterando os níveis de linfócitos TCD4+, TCD25+, bem como FOXP3+, interleucina-10 e TGF-β (MENG *et al.*, 2013). Além disso, o NF-κB, principal regulador da sinalização inflamatória, também está aumentado em condições de estresse térmico (ABDELNOUR *et al.*, 2019). O NF-κB desempenha um papel vital na síntese de citocinas pró-inflamatórias, e o aumento destas citocinas de forma crônica pode estar relacionado à redução da produtividade em bovinos (CELI, 2011; CHAUHAN *et al.*, 2014).

Adicionalmente, o padrão das principais interleucinas (ILs) é afetado pelo calor. A expressão de fatores pró-inflamatórios, como IL1β, IL6 e também TNF-α são maiores em vacas leiteiras submetidas ao estresse térmico induzido (MIN *et al.*, 2016), enquanto fatores anti-inflamatórios como IL10 são menos expressos (SHEIKH *et al.*, 2018), sugerindo que o estado inflamatório é modulado pelo estresse térmico. As ILs utilizam a via de sinalização intracelular

JAK/STAT (LEONARD, 2001), a mesma usada pelos interferons, especialmente IFNT (BINELLI *et al.*, 2001). Em conclusão, o estresse térmico pode levar a uma resposta inflamatória em bovinos, que pode afetar a sanidade, produção e reprodução.

ARTIGO 1

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Heat stress modulates polymorphonuclear cell response in early pregnancy cows: I. interferon pathway and oxidative stress

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Heat stress modulates polymorphonuclear cell response in early pregnancy cows: I. interferon pathway and oxidative stress

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Short title: Heat stress, interferon and innate immune responses

Abstract

One of the major causes of early pregnancy loss is heat stress. In ruminants, interferon tau (IFNT) is the embryo signal to the mother. Once the interferon signaling pathway is activated, it drives gene expression for interferon-stimulated genes (ISGs) and alters neutrophils responses. The aim of the present study was to evaluate interferon (IFN) pathway, ISGs and gene expression in polymorphonuclear leukocytes (PMN) and oxidative stress in dairy cows under heat stress. Pregnant cows had their estrous cycle synchronized and randomly assigned to a comfort or heat stress group. Blood samples were collected at artificial insemination (AI) and on Days 10, 14 and 18 following AI. Pregnant cows were pregnancy checked by ultrasound on Day 30 and confirmed on Day 60 post-AI. Results are presented as mean ± SEM. The corpus luteum (CL) diameter was not different between groups of pregnant cows; concentration of progesterone of pregnant cows on Day 18 following AI was greater in comfort group compared to heat stressed group. Comfort pregnant cows had higher expression of all analyzed genes from interferon pathway, except for *IFNAR1,* on both Days 14 and 18. Conversely, heat stressed cows did not show altered expression of IFNT pathway genes and ISGs between Days 10, 14, and 18 after AI. The oxidative stress, determined as malondialdehyde (MDA) levels, was greater in heat stress group on Days 10, 14 and 18, independent of pregnancy status. Heat stress negatively influences expression of ISGs, IFN pathway gene expression in neutrophils, and oxidative stress. Our data suggest that lower conception rates in cows under heat stress are multifactorial, with the association of interferon pathway activation and the unbalanced oxidative stress being main contributing factors.

Keywords: high temperature, conceptus, ISG15, interferon, innate immune response, embryo signaling.

Introduction

Pregnancy loss is an important factor that reduces reproductive performance in dairy herds, and it occurs more frequently in the first 30 days of pregnancy [1, 2]. One of the major causes of pregnancy loss and implantation failure during early pregnancy is heat stress (HS) caused by the increase of temperatures during summer (hot seasons) [3, 4]. Effects of HS on follicular development, steroidogenesis, quality of gametes and embryos are well studied [5-9]. HS may also be an end result of an imbalance between reactive species production and antioxidant capacity, which leads to oxidative stress [10-12].

Maternal recognition of pregnancy in ruminants occurs between Days 10-20 of pregnancy, when the conceptus signals its presence in uterus [13]. Interferon tau (IFNT) is the major cytokine responsible for the embryo-maternal interaction to avoid luteolysis [14, 15] and to establish and maintain the pregnancy [16]. It is produced by embryonic trophoblast cells at pre-implantation period and acts in the endometrial luminal epithelium in a paracrine manner. IFNT inhibits endometrial estrogen and oxytocin receptors expression and prevents prostaglandin F2 alpha (PGF) luteolytic pulses [17]. IFNT also acts in extrauterine tissues (e.g. leukocytes and corpus luteum) [18, 19], protecting the corpus luteum (CL) [20, 21] and signaling the early pregnancy to peripheral blood cells via induction of interferon stimulated genes (ISGs) [22, 23].

In general, it is believed that concentrations of IFNT circulating in the bloodstream are extremely low and thus difficult to detect [19, 21]; however, there are few indirect approaches to detect IFNT action in the bloodstream. One of them is to measure ISGs expression in leukocytes. There are several studies that correlate ISGs expression in peripheral blood leukocytes (PBL) with early pregnancy [22-25]. These studies have analyzed gene expression in separate PBL fractions, especially the polymorphonuclear leukocytes (PMN), which are most sensitive to IFNT stimulation, when compared to mononuclear cells fraction (PBMC).

Additionally, there is a positive correlation between ISGs expression in PMN and early pregnancy in cattle [26, 27]. Although IFNT is not virus inducible [28], it belongs to type I IFNs and uses the same mechanism of action of antiviral response genes. IFNT binds to type I IFN receptors (IFNAR) 1 and 2 [29, 30] and activates the Janus kinase-signal transducer and activator of transcription (JAK/STAT) intracellular pathway [31]. Subsequently, phosphorylated STATs dimerize and recruit IFN-regulatory factor 9 (IRF9) to form STAT1-STAT2-IRF9 tri-complex (interferon-stimulated gene factor 3, ISGF3). This complex translocates into the nucleus to initiate transcription of ISGs [32].

Pregnancy causes an immunological challenge because a semi allogenic fetus must be supported within the pregnant female for the required gestational period. The decidua and placenta of human and mouse form key immunological barriers that sustain maternal tolerance, yet generate innate immune responses that prevent microbial infections [33]. The biology underlying the systemic crosstalk of early embryo signaling and immune system is not completely understood. Therefore, IFNT endocrine action may alter immune cells response during early pregnancy.

Considering the low pregnancy rates during warm season and the endocrine signaling of IFNT characterized by ISGs expression in extrauterine tissues, we hypothesized that oxidative stress caused by heat stress negatively impacts progesterone production and innate immune response during early pregnancy in dairy cows. The objective of our study was to evaluate relations between concentration of progesterone, oxidative stress blood markers, expression of ISGs and genes of IFN signaling pathway in neutrophils of dairy cows under comfort or heat stress environment on embryo pre-implantation period. We tested whether high temperatures during summer affect the ability of the pregnant dairy cows to signal the embryo presence and modulate IFN pathway.

Materials and methods

Chemicals

Unless otherwise indicated, chemicals and reagents were purchased from Sigma Chemical Company (Sigma-Aldrich, St. Louis, MO, USA).

Cattle and herd management

The study was approved by the Animal Care Use and Committee (CEUA-UFSM # 5728120217) of Federal University of Santa Maria and conducted on a commercial dairy farm in Southern Brazil. Thirty-two multiparous Holstein dairy cows in lactation from the same herd were included in this study. The cows were 3 to 6 years old, body condition score greater than 2.5 (1=thin and 5=obese in a scale 1 to 5), absent of any detectable reproductive and clinical disorders during the study period. Cows were milked twice a day and fed complete ration and corn silage, with *ad libitum* access to water. All sampling and data collection for this study were obtained with no additional distress.

Experimental design, synchronization protocol and artificial insemination (AI)

The experiment was conducted during two distinct seasons. The samples from comfort cows group (n=15) were collected in September (Late Winter/Early Spring), when the temperature-humidity index (THI) is approximately 65-70 in Southern Brazil. The samples from the heat stressed cows group (n=17) were collected in January (Summer), characterized by high temperatures associated with high humidity, when THI is approximately 80-85. Both groups had their estrus synchronized with the same protocol [34]. The estrous cycle synchronization protocol was initiated by the insertion of an intravaginal device (IVD)

containing 1.9g of progesterone (CIDR, Zoetis, São Paulo, Brazil), administration of 2mg (i.m.) of estradiol benzoate (Sincrodiol, Ourofino, Minas Gerais, Brazil) and 2mL (i.m.) of gonadorelin, an analogue of GnRH (Cystorelin, Boehringer Ingelheim, São Paulo, Brazil) 11 days prior to AI (Day -11). Four days before AI (Day -4) the first injection of 0.5mg (i.m.) of sodium cloprostenol, a synthetic prostaglandin F2 alpha analogue, was administered (PGF; Sincrocio, Ourofino). Two days before AI (Day -2), IVD was withdrawn and the animals received the second injection of 0.5 mg (i.m.) of PGF and 1mg (i.m.) of estradiol cypionate (ECP; E.C.P. Zoetis). Only animals that exhibited standing estrus by 48 hours after IVD withdrawal were included in the experiment (Comfort cows group n=12; Heat Stressed cows group n= 13). AI was performed 48 hours (Day 0) after IVD withdrawal, using conventional semen. The semen was obtained from ST genetics® commercial company, stored in liquid nitrogen, and thawed at 36°C for 30 seconds for subsequent AI.

Physiological parameters and environmental data

Respiratory rate (RR), heart rate (HR), and rectal temperature (RT) were evaluated at 3 p.m. on Days 10, 14 and 18 following AI. RR was expressed in breaths per minute (bpm) and was obtained using a timer to count respiratory movements for 30 seconds, multiplied by 2 to obtain the number of breaths per minute. HR was expressed in beats per minute (bpm) and was obtained using a flexible stethoscope (Standard, Bic Med, São Paulo, 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 heart beats per minute. RT was measured with a large animal clinical thermometer inserted at 3 cm depth into the rectum and held to maintain contact with the mucosa for one minute. Body condition score (BCS) was determined at the beginning of the experimental period (estrus synchronization) and weekly throughout the study. A scale of 1 (thin) to 5 (obese) in increments of 0.25 units was used, as described by

Ferguson, Galligan [35]. A single observer evaluated the BCS throughout the study to minimize variations. Ambient temperature and relative humidity (RH) were recorded at 4 p.m. on Days 0, 10, 14 and 18. The THI was calculated using the mathematical equation [36]: THI = (0.8 × Dbt) + [(RH/100) × (Dbt – 14.4)] + 46.4; where Dbt = dry bulb temperature, and RH = relative humidity.

Blood sample collection

Blood was collected from the coccygeal vein using a 21G needle coupled to a vacuum collection system (BD Vacutainer®) into 4 mL EDTA-containing tubes. The collections were performed at the time of AI (Day 0) and on Days 10, 14 and 18 following AI. Blood was obtained in two tubes of 4 mL containing EDTA for each experimental time point. The first 4 mL tube of blood was used for oxidative stress assays and the second tube for isolation of blood leukocytes and determination of blood concentration of progesterone.

Isolation of polymorphonuclear (PMN) peripheral blood cells

Isolation of PMNs was performed as follows. Briefly, after blood collection, 2 mL of whole blood was diluted in equal volume of 0.9% NaCl, followed by addition of 3mL of Ficoll-Paque PREMIUM®. Centrifugation was performed at 400xg for 15 minutes at room temperature. After centrifugation, the following layers were obtained: PBMC, Ficoll-Paque, PMN, and erythrocytes. All the upper fractions were withdrawn to collect the PMN fraction. PMNs were collected from the lower red layer. PMN samples were stored in a cryotube at -80°C for subsequent total RNA extraction. After isolation of PMN fraction, a glass-slide fraction-film was prepared to determine the purity of each fraction. Slides were stained using a rapid stain (Diff-Quik Differential Stains Set; Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendations. The cell fraction purity was accessed based

on cell morphology. PMNs are classified as neutrophils, eosinophils, and basophils. They have condensed, segmented nuclei and are identified by the staining characteristics of their secondary granules. An experienced clinical pathologist examined the slides. A differential cell count was done by identifying 100 consecutive leukocytes using a 100x objective. Samples above 95% of specific cell type (PMN) [26] were included in this study.

RNA extraction, reverse transcription, and real-time PCR

Total RNA was extracted from the PMN cells using Tri Reagent (BD), according to the manufacturer's recommendations. Quantification and estimation of RNA purity was performed using Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA; RNA concentration mean 658.17 ng/µl, SD 226.61, minimum 225.7 ng/µl and maximum 999.1 ng/µl; Absorbance 260/280 nm ratio mean 1.91, SD 0.053, minimum 1.8 and maximum 2.01). RNA was treated with DNAse Amplification Grade (Thermo Fisher, Waltham, MA, USA) for 15 minutes at 27ºC to degrade any DNA molecules. DNAse was inactivated with 1 µl EDTA for 10 minutes at 65ºC. Reverse transcription was performed using iScript cDNA synthesis Kit (BioRad, Hercules, CA, USA) for 5 minutes at 25ºC followed by 30 minutes at 42ºC and 5 minutes at 85ºC. Quantitative polymerase chain reaction (qPCR) was conducted in a thermocycler (BioRad, Hercules, CA, USA) using cDNA, forward and reverse bovine specific primers and SYBR fluorophore GoTaq® Green Master Mix (Promega Corporation, Madison, USA). The final reaction volume is 10 µl: 2 µl of cDNA and 8 µl of MIX (5 µl of SYBR, 1 µl of primer forward, 1 µl of primer reverse and 1 µl of water). Amplification was performed with initial denaturation at 95ºC for 5 minutes followed by 40 cycles of denaturation at 95ºC for 15 seconds and annealing/extension at 60ºC for 30 seconds. To optimize the RT-qPCR assays, serial dilutions of cDNA templates were used to generate a standard curve, and efficiency between 90 and 110% was considered. Samples were run in duplicate and the

194 results of expression of all analyzed genes were expressed by ΔΔCq method, having *GAPDH*
195 and *RPS18* as reference genes, as previously described [37]. The genes assessed in this study
196 are presented in Table 1.
197

198 **Table 1. Primers designed for quantitative real-time PCR analysis.**

Target	Primer sequence	GenBank
ISG15	F: GGTATCCGAGCTGAAGCAGTT R: ACCTCCCTGCTGTCAAGGT	NM_174366.1
MX1	F: GTACGAGCCGAGTTCTCCAA R: ATGTCCACAGCAGGCTCTTC	NM_173940.2
MX2	F: CTTCAGAGACGCCTCAGTCG R: TGAAGCAGCCAGGAATAGT	NM_173941.2
OAS	F: GTGGCCAAAGGTGGCTCCTA R: TGTGCCCAGATTTTGCTGAGG	NM_001040606.1
IFNAR1	F: GAATCAGCTCTACCCGCTAAT R: GCTCTGGCTTTGACACAATAC	NM_174552.2
IFNAR2	F: AGCCAGAATGTGTCAGCGAT R: AGAACAGGCGCAACATACGA	NM_174553.2
STAT1	F: CAAAGGAAGCCCCAGAGCCTA R: ACATGCCACTCTTCTGTGTTCA	NM_001077900.1
STAT2	F: CAGCCCGTTTCAGGATCAGC R: CAGTGCAGCTTTCTGCCAGT	NM_001205689.1

JAK1	F: GGGGTTAGCCGCTTAGGGAG R: CCATTCAGAGCTGAGCACTTCC	XM_024989564.1
IRF9	F: GGTTCCTGAGATCGGCCACA R: CCTGATTGAGCGGGGACAGT	NM_001024506.1
GAPDH	F: GATTGTCAGCAATGCCTCCT R: GGTCATAAGTCCCTCCACGA	NM_001034034.2
RPS18	F: CCTTCCGCGAGGATCCATTG R: CGCTCCCAAGATCCAACTAC	XM_024983403.1

F: Forward; R: Reverse.

CL diameter and progesterone analysis

Corpora lutea diameter (mm) was measured on Day 18 following AI through ovarian ultrasonography (Mindray DP10 with a 6.5 MHz linear transducer). The concentration of progesterone was determined in plasma by chemiluminescent assay kit (ADVIA Centaur, Siemens) also on Day 18 following AI. Samples were run in duplicate and were analyzed at the same plate. The intra-assay coefficient of variation was 2.0% for progesterone.

Malondialdehyde (MDA) levels

The determination of MDA concentration was performed as previously described [38]. Briefly, NaOH 3M was added to each sample, followed by incubation at 60°C for 30 minutes. After that, 6% H_3PO_4 and 0.8% thiobarbituric acid (TBA) were added to the system and the mixture was heated at 90°C for 2 hours. Then 10% SDS and n-butanol were added to extract the TBA-malondialdehyde (MDA) product, which was analyzed on Shimadzu® HPLC

equipment. The analytical column was a Phenomenex® ODS-2 C_{18}reverse-phase (250mm × 4.6mm, 5μm; 100Å, Allcrom, BR) and the mobile phase was ultrapure water and methanol (50:50; v/v). The HPLC analysis was performed under isocratic conditions at a 0.6 mL/min flow rate and UV detector set at 532 nm with a 20 μl sample volume injection. The results were expressed as nmol MDA/mg protein.

Catalase (CAT) activity

The CAT activity was spectrophotometrically assayed by monitoring the H_2O_2 consumption at 240 nm [39]. The enzymatic reaction was performed by adding the sample and the substrate (H_2O_2) at 0.3 mM in a medium containing 50 mM phosphate buffer, pH 7.0. The enzymatic activity was expressed in units (1U decomposes 1μmol of H_2O_2/min at pH 7.0 and at 25°C)/mg protein.

Superoxide dismutase (SOD) activity

The SOD activity was measured spectrophotometrically [40]. This method is based on the capacity of SOD to inhibit autoxidation of epinephrine to epinechrome. In this assay, each sample was diluted 1:10 (v/v) in phosphate saline buffer (PBS) and added in a 50 mmol/L Na_2CO_3 buffer pH 10.3 and the enzymatic reaction was initiated by adding epinephrine. The colorimetric reaction was measured at 480 nm and the results were expressed in units (1U decomposes 1μmol of epinephrine/min at pH 7 and at 25 °C)/mg protein.

Protein quantification

The protein concentration for MDA levels and CAT activity was measured by the method described by Bradford (1976). Protein concentration was determined using bovine serum albumin (BSA; 1 mg/ml) as the standard [41].

Pregnancy diagnosis by ultrasound scanning

In both groups, uterine conditions were ultrasonographically evaluated using a Mindray DP10 with a 6.5 MHz linear transducer to select only animals without any evident pathology. Pregnancy rate was determined by dividing the number of pregnant cows at the pregnancy diagnosis at 30- and 60-days following AI by the total number of cows artificially inseminated (P/AI).

Statistical analysis

Continuous data were checked for normality using Shapiro-wilk test. CL diameter, concentration of progesterone, physiological parameters, and productive performance data were analyzed by ANOVA followed by Student's *t* test. Gene expression and oxidative stress data were analyzed by repeat measures for within-group analysis and standard least squares for between-group (comfort vs. heat stress and pregnant vs. non-pregnant cows) analysis. The main effects of day, pregnancy status (PS), treatment group, day by group interaction (day*group) or day by pregnancy status interaction (day*PS) were indicated. Differences of estrus occurrence and pregnancy were evaluated through chi-squared test. All data analysis was performed using the JMP7 Software (SAS Institute Inc., Cary, NC, USA). Results are presented as mean ± standard error of the mean (SEM) and are considered different at $P<0.05$.

Results

Cows in comfort or under heat stress environment: Physiological and reproductive parameters.

In order to determine the experimental model of heat stress, THI was calculated and the indices were different during summer and late winter/early spring in the experimental period (S1 Table). Thus, cows in the summer (higher THI) were considered under HS when compared to late winter/early spring (lower THI). HS affected RT, HR, and RR in dairy cows ($P<0.05$), which were evident at all timepoints (days along the season) (S1 Fig). Effect of season on estrous occurrence and pregnancy rate were not different between groups ($P>0.05$) and are presented in Table 2. Estrous occurrence rate was 80% (12 from 15 cows) in comfort group and 76.47% (13 from 17 cows) in heat stressed group. Pregnancy rate was 50% (6 from 12 cows) in comfort group and 38.46% (5 from 13 cows) in heat stressed group. CL diameter (Fig 1A) on Day 18 following AI was significantly different ($P<0.05$) in pregnant vs non-pregnant cows, when compared within-group, with larger diameter in pregnant cows independent of season. No differences in CL diameter in pregnant cows of the two groups were found ($P>0.05$). Concentration of progesterone followed the same pattern as CL diameter, however, it was lower in heat-stressed pregnant cows when compared to pregnant cows of the comfort group ($P<0.05$). In non-pregnant cows, the CL started to regress and, therefore, the CL diameter and concentration of progesterone did not differ between groups ($P>0.05$). In relation to milk production, cows were at similar days in lactation (S2 Fig A), however, cows under heat stress had lower daily milk yield than the cows that were heat-not stressed (S2 Fig B), confirming the experimental model.

Table 2. Effect of season on estrus onset and pregnancy rate. Different letters represent significance at P<0.05 between comfort and heat stressed cows.

Variable	N	%	P-value
Estrus occurrence			
Comfort cows	12/15	80.0[a]	p> 0.05
Heat stressed cows	13/17	76.5[a]	
Pregnancy			
Comfort cows	6/12	50.0[a]	p> 0.05
Heat stressed cows	5/13	38.5[a]	

Fig 1. Ovarian corpus luteum diameter and concentration of progesterone in Day 18 post-AI pregnant cows under comfort or heat stress conditions. A) Diameter (mm) of corpus luteum. B) Concentration (ng/mL) of circulating progesterone. Values are presented as mean ± S.E.M. Different letters represent significance at P<0.05 between comfort and heat stressed cows and asterisk represents significance at P<0.05 between pregnant and non-pregnant cows.

Markers of Oxidative Stress in blood from cows in comfort or under heat stress environment.

Oxidative Stress was evaluated using MDA concentration measurement in blood from cows under comfort or heat stress environment on Days 10, 14 and 18 post AI (Fig 2). In both pregnant and non-pregnant cows, MDA concentrations were greater (P<0.05) in heat stress

297 environment on Days 10, 14 and 18. Pregnant heat stressed cows had Day 18 SOD activity
298 and Day 10 and 14 CAT activity greater than comfort pregnant cows (P<0.05). Non-pregnant
299 heat stressed cows had only Day 14 SOD activity greater than comfort non-pregnant cows
300 (P<0.05). Greater MDA levels unbalanced with antioxidant enzymes in heat stressed cows
301 indicate oxidative stress.
302
303 **Fig 2. Oxidative stress-related parameters in blood of pregnant and non-pregnant cows**
304 **in comfort or under heat stress conditions on Days 10, 14 and 18 post-AI**. A, B, and C
305 represent pregnant cows; D, E, and F represent non-pregnant cows. A and D: MDA levels. B
306 and E: SOD activity. C and F: CAT activity. Values are presented as mean ± S.E.M. The main
307 effects of day, group and day by group interaction (day*group) are indicated. Asterisk
308 represents difference at P<0.05 between comfort and heat stressed groups.
309

310 ISGs expression in PMN from cows in comfort or under heat
311 stress environment.

312 Relative mRNA expression of *ISG15, OAS, MX1* and *MX2* in PMN cells was
313 evaluated in comfort or heat stressed cows on Days 10, 14 and 18 after AI (Fig 3). The
314 expression of these genes was upregulated in Day 18 pregnant cows in comfort group when
315 compared to non-pregnant (P<0.05; Fig 3A-D). However, no difference between expression
316 of all analyzed ISGs in non-pregnant and pregnant cows was observed when the cows were
317 under heat stress (Fig 3E-H).
318
319 **Fig 3. Interferon-stimulated genes expression in polymorphonuclear cells of pregnant**
320 **and non-pregnant cows in comfort or under heat stress conditions on Days 10, 14 and 18**
321 **post-AI.** A-D represents ISGs of cows in comfort conditions; E-H represents ISGs of heat

stressed cows. A and E: *ISG15;* B and F: *OAS;* C and G: *MX1;* D and H: *MX2.* Values are presented as mean ± S.E.M. The main effects of pregnancy diagnosis (PD), day and day by pregnancy diagnosis interaction (day*PD) are indicated. Asterisk represents difference at P<0.05 between pregnant and non-pregnant cows.

IFN pathway gene expression in PMN from cows in comfort or under heat stress environment.

In order to identify IFN signaling, relative mRNA expression of *IFNAR1, IFNAR2, STAT1, STAT2, JAK1* and *IRF9* in PMN cells was evaluated in comfort or heat stressed cows on Days 10, 14 and 18 after AI (fig 4). Besides *IFNAR1*, expression of IFN pathway genes in PMN was upregulated in pregnant but not in non-pregnant comfort group cows on Days 14 and 18 (P<0.05; Fig 4A-F). However, expression of all evaluated IFN pathway genes was not different between pregnant and non-pregnant cows under heat stress (Fig 4G-L).

Fig 4. Interferon-pathway gene expression in polymorphonuclear cells of pregnant and non-pregnant cows on comfort or under heat stress conditions. A-F represents IFN-pathway components of cows in comfort conditions; G-L represents IFN-pathway components of heat stressed cows. A and G: *IFNAR1;* B and H: *IFNAR2;* C and I: *JAK1;* D and J: *STAT1;* E and K: *STAT2;* F and L: *IRF9.* Values are presented as mean ± S.E.M. The main effects of pregnancy diagnosis (PD), day and day by pregnancy diagnosis interaction (day*PD) are indicated. Asterisk represents difference at P<0.05 between pregnant and non-pregnant cows.

ISGs and IFN pathway expression in PMN of pregnant cows in comfort or under heat stress environment.

For the purpose of identifying differences in pregnant cows, relative mRNA expression of ISGs and IFN pathway genes in PMN cells was compared only in pregnant cows in comfort or heat stressed environment on Days 10, 14 and 18 after AI (Fig 5). Among the ISGs, only *OAS* (Fig 5B) was greater (P<0.05) on Day 18 in PMN of comfort cows when compared to heat-stressed pregnant cows. When IFN pathway was analyzed, only *JAK1* (Fig 5I) was greater on Days 14 and 18 and *IRF9* (Fig 5J) (P<0.05) on Day 18 was greater in comfort group pregnant animals vs heat-stressed pregnant animals. All other genes were not different between pregnant cows in comfort or heat stressed environment. The analysis performed in non-pregnant cows is shown in S3 Fig.

Fig 5. Interferon-stimulated genes and interferon-pathway gene expression in polymorphonuclear cells of pregnant cows in comfort or under heat stress conditions on Days 10, 14 and 18 post-AI. A) *ISG15;* B) *OAS*; C) *MX1;* D) *MX2;* E) *IFNAR1;* F) *IFNAR2;* G) *STAT1;* H) *STAT2;* I) *JAK1;* J) *IRF9.* Values are presented as mean ± S.E.M. The main effects of day, group and day by group interaction (day*group) are indicated. Asterisk represents difference at P<0.05 between comfort and heat stressed groups.

Discussion

In order to study the influence of heat stress on early pregnancy in cows, we designed and validated experimental model, allowing to evaluate effect of cold and warm season of the year on estrous occurrence and pregnancy rate, THI, RT, HR, RR, and daily milk yield. The experimental design allowed us to discover the following relevant findings: 1) CL diameter

did not differ between comfort and heat stressed cows; however, the progesterone production was lower in pregnant heat stressed cows; 2) MDA levels were greater in both non-pregnant and pregnant cows under heat stress, while activity of the anti-oxidant enzymes SOD and CAT did not have the proportional increase, indicating oxidative stress in cows of both groups; 3) expression of ISGs and type I IFN pathway genes in neutrophils of comfort pregnant cows was increasing over time and reached a peak on Day 18, while non-pregnant cows maintained lower expression; 4) the expression pattern of ISGs and type I IFN pathway in neutrophils from heat stressed cows did not differ between non-pregnant and pregnant cows on all days.

Pregnant cows in comfort ambient environment display increased gene expression of genes of the type I IFN signaling pathway along with the expression of ISGs in time dependent manner, indicating upregulation of the pathway, while activation of the type I IFN pathway was not detected in pregnant heat stressed cows. Neutrophils are known to be the first response in the inflammatory process; however, it has been proposed that they could respond modulating local innate and adaptative immune system [42]. IFNT regulates expression of genes of the innate immune system in the uterus and also in peripheral immune cells and other tissues throughout the body [43]. The results presented here indicate that the embryo via secretion of INT activates the neutrophils responses only in comfort pregnant cows.

The environmental conditions seem to affect the maternal recognition of pregnancy signaling. Previous *in vitro* study from our group, demonstrated the influence of heat stress on oxidative stress and IFNT production [44]. Heat stressed pregnant cows did not have the same increased expression of interferon stimulated and IFN I pathway genes on Day 18 as it was found in cows in comfort temperature. Also, our data revealed that oxidative stress may be involved in progesterone production and expression of ISGs and IFN pathway, whereas MDA

concentration was increased only in cows of heat stressed group on all experimental days. Notably, upregulation of genes directly related to maternal recognition of pregnancy was detected in PMN in dairy cows, which can provide insight into development of a new method to diagnose pregnancy.

Progesterone is the key hormone controlling early pregnancy [45] and its low concentration in early pregnancy period has been correlated to negative effects on embryo development and elongation [46]. Heat stress or increased metabolic rate reduce progesterone level in high daily milk yield cows [47-49]. Furthermore, the decrease of concentration of progesterone also can be associated with oxidative stress. It has been shown that long-term moderate oxidative stress reduces the potential for fertility. This effect may be due to poor follicular quality and consequently decreased progesterone [50]. It is reasonable to suggest that the reduction of concentration of progesterone without the decrease of CL diameter in pregnant cows under heat stress are due to oxidative stress present in these cows.

The increased SOD and CAT activities maintain low levels of MDA in pregnant cows in the comfort group; while in the heat stressed pregnant cows increased SOD and CAT activity is not able to prevent the increase of MDA level, indicating oxidative stress. It is known that exposure to heat stress results in higher mitochondrial and plasma levels of MDA, the major product of lipid peroxidation [10] and oxidative cellular stress [51, 52]. It has even been shown that MDA can be used as a blood marker for oxidative status of dairy cows during warm season [52]. Although studies show increase of antioxidant enzymes in hyperthermia situations [10], there is a study showing the decrease in SOD and CAT enzymes, which resulted in a significant reduction in thermal resistance [53]. The antioxidant enzymatic process was apparently not effective in cows under heat stress in our study. This condition seems to characterize a deficient antioxidant enzymatic system in heat stressed cows.

There are many genes upregulated by IFNT in early pregnancy and among all ISGs, we can highlight *ISG15*, *MX1, MX2* and *OAS* [18, 22] because they have greater expression in neutrophils, compared to other fractions of peripheral blood leucocytes [23]. In general, the amount of IFNT in the bloodstream is low and thus is difficult to detect, but IFNT activity can be detected in the bloodstream using radio immune assay [54] and antiviral assay [19, 21]. Another method to detect IFNT-response in the bloodstream is to identify ISGs gene expression, demonstrating the expressions of ISGs as IFNT endpoint activity. There are several studies that showed correlation between ISGs expression in peripheral blood leukocytes (PBL) during early pregnancy [22-24, 26].

Interestingly, we observed that *ISG15, OAS*, *MX1* and *MX2* genes were upregulated in PMN from pregnant cows in comfort group on Day 18 following AI, but not in heat stressed pregnant cows. One study demonstrated that heat stressed pregnant cows have greater ISGs expression [55], however, the THI in stressed cows in the study were lower than in cows in our study. The occurrence of heat stress with higher humidity, as in our study, lead to THI above 80, promoting a subtle increase in the expression of ISGs in stressed cows. The possible explanation for this observation could be that the embryonic cells that are responsible for production and secretion of IFNT at the beginning of the embryonic development [56, 57] were in oxidative stress. This is important because IFNT begins to be significantly expressed on Day 7 of development [58] and its peak production occurs between days 18 and 20 following conception [59] for the maternal recognition of pregnancy.

Based on the upregulation of ISGs by IFNT in PMN leukocytes, we investigated the type I IFN signaling pathway in PMN cells of non-pregnant and pregnant cows, in comfort or under heat stress. As expected, the *IFNAR2* receptor, *JAK1, STAT1* and *STAT2* cascade and *IRF9* regulatory factor were upregulated on Days 14 and 18 following AI in pregnant cows in comfort; however, no difference was observed in all IFN pathway genes of pregnant cows

444 under heat stress. The increase of ISGs in PMN from pregnant cows only on Days 14 and 18
445 may be explained by the fact that the embryo did not start to elongate before Day 10, and,
446 consequently, there is not enough amount of IFNT leaving the uterus at this time [60]. IFNT
447 was found to modulate IFNAR2 subunit [23], and our *in vivo* data demonstrate upregulated
448 IFNAR2 but not IFNAR1 in PMN from cows in comfort. This suggests the receptor subunit
449 controlled by IFNT is IFNAR2. Pregnant cows under heat stress conditions did not show the
450 same pattern of ISGs and IFN pathway gene expression when compared to pregnant comfort
451 cows. Although, when we compared pregnant cows in comfort to heat stressed cows, there
452 were no differences in ISGs and IFN pathway gene expression. We believe that oxidative
453 stress not only decreases concentration of progesterone, but also impairs IFN gene pathway
454 and ISGs expression, as well as activation of interferon-primed neutrophils. One study
455 characterized genes and pathways that respond to heat stress in Holstein calves, where the
456 transcriptome analysis showed that expression of genes such as IFNAR2 and STATs is
457 increased in response to heat stress [61]. Another study reported that JAKs are redox-sensitive
458 enzymes [62]. These findings support our hypothesis that cows under influence of heat and
459 oxidative stress, even if they are pregnant, have a distinct response regarding to IFNT
460 endocrine signaling in PMNs. This response makes it difficult to accurately use expression of
461 these ISGs to identify early pregnancy. In addition to the effects of HS on IFNT signaling
462 [44], IFNT has been shown to modulate local and systemic innate immune response, carried
463 out mainly by neutrophils [43, 63]. Neutrophils are essential for innate immunity and
464 resistance to pathogens, not only acting as a final effector of an acute inflammatory response,
465 but also secreting factors such as cytokines to activate cells of innate and adaptative immune
466 response. They respond to tissue- and cell-derived signal undergoing polarization [42]. This
467 led us to consider that IFNT and other embryokines may activate circulating neutrophils,
468 required in early pregnancy to protect the mother as a first defense barrier against a viral

infection, as shown by Shoemaker, Smirnova [64]; And this may be impaired in stressed (heat and/or oxidative) environments. Exactly how HS and IFNT modulate local and systemic immune response through early pregnancy remains unclear.

In conclusion, the present study observed that oxidative stress caused by heat stress decreases progesterone production and alters ISGs and IFN pathway gene expression in PMN cells of dairy cows in early pregnancy (Fig 6). The heat stress in pregnant cows not only impairs the ISGs gene expression but also interferes with IFN pathway activation, possibly modifying systemic innate immune response. Lower conception rates in cows under heat stress are influenced by several factors, and the unbalanced oxidative stress associated with impaired IFN pathway activation influencing innate immunity response could be one of the main contributing factors.

Fig 6. Schematic model illustrating the main conclusions. A) Cows in comfort environment maintains oxidative balance regardless of pregnancy status. Type I IFN pathway and ISGs genes were upregulated on Day 18 in PMN cells of pregnant cows. However, B) Heat stress conditions induce oxidative stress in pregnant and non-pregnant cows. In pregnant cows, HS decreases progesterone concentration and impairs ISGs and Type I IFN pathway gene expression in PMN cells.

Declaration of interest

The authors have nothing to declare.

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726

727 Supporting information

728 **S1 Table**. **Temperature-humidity index (THI) calculated on experimental days on two different**
729 **seasons.** The samples from cows of the comfort group were collected on late winter/early spring
730 and the samples from the cows of heat stressed group were collected on summer.

731

732 **S1 Fig. Effect of season on rectal temperature (RT), heart rate (HR) and respiratory**
733 **rate (RR) in cows on comfort or under heat stress conditions.** RT, HR and RR were

734 measured on Days 10, 14 and 18 after AI. A) RT (ºC) was measured with a large animal
735 clinical thermometer inserted to depth of 3 cm into the animal rectum and held to maintain
736 contact with the mucosa for one minute. B) HR was expressed in beats per minute (bpm) and
737 was obtained using a flexible stethoscope placed directly into the left thoracic region under
738 one of the auscultation foci for 30 seconds, multiplied by 2 to obtain the number of heart beats
739 per minute. C) RR was expressed in breaths per minute (bpm) and was obtained using a timer
740 to count the flank movements of the animal for 30 seconds, multiplied by 2 to obtain the
741 number of breaths per minute. Values are presented as mean ± S.E.M. Asterisk represent
742 significance at $p<0.05$ between comfort and heat stressed cows.

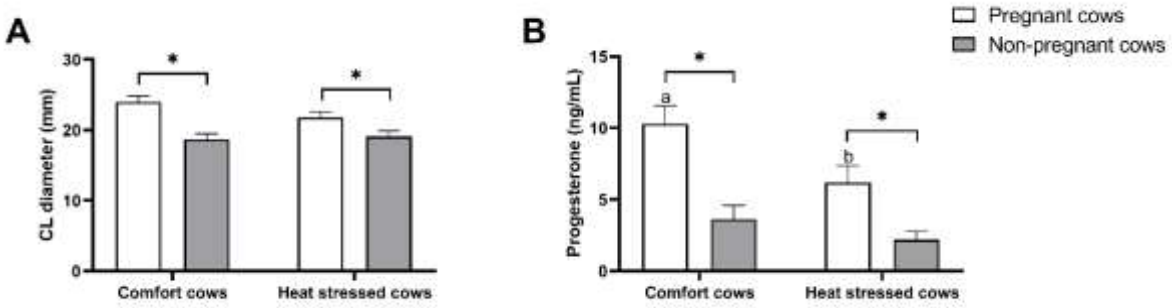
743

744 **S2 Fig. Effect of season on productive performance of dairy cows on comfort or under**
745 **heat stress conditions**. A) Daily milk yield (kg) was measured during the two milking on AI
746 day of each animal. B) Days in milk started on the last calving until AI day. Values are
747 presented as mean ± S.E.M. Asterisk represent significance at $p<0.05$ between comfort and
748 heat stressed cows groups.

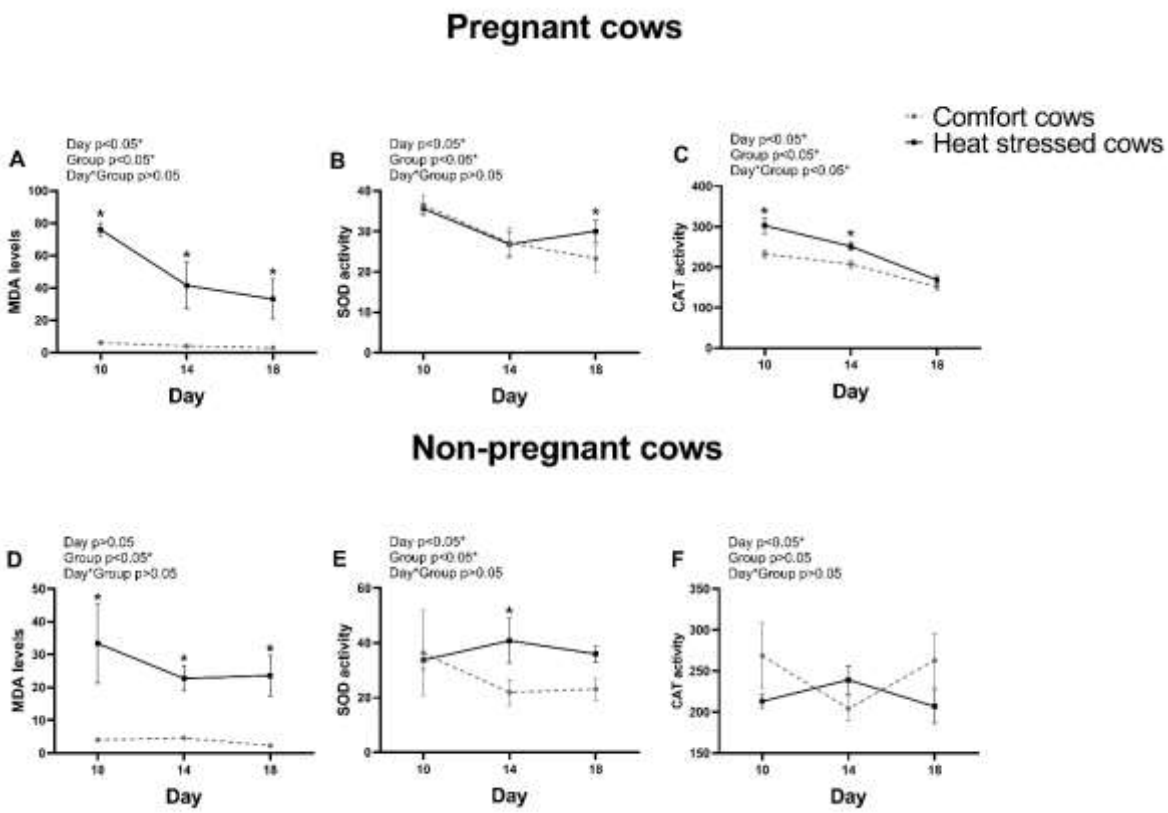
749

750 **S3 Fig. Interferon-stimulated genes and interferon-pathway gene expression in**
751 **polymorphonuclear cells on Days 10, 14 and 18 post-AI of non-pregnant cows compared**
752 **on comfort or under heat stress conditions.** A) *ISG15*. B) *OAS*. C) *MX1*. D) *MX2*. E)
753 *IFNAR1*. F) *IFNAR2*. G) *STAT1*. H) *STAT2*. I) *JAK1*. J) *IRF9*. Values are presented as mean ±
754 S.E.M. The main effects of day, group and day by group interaction (day*group) are
755 indicated. Asterisk represent difference at $p<0.05$ between comfort and heat stressed groups.

Fig 1.

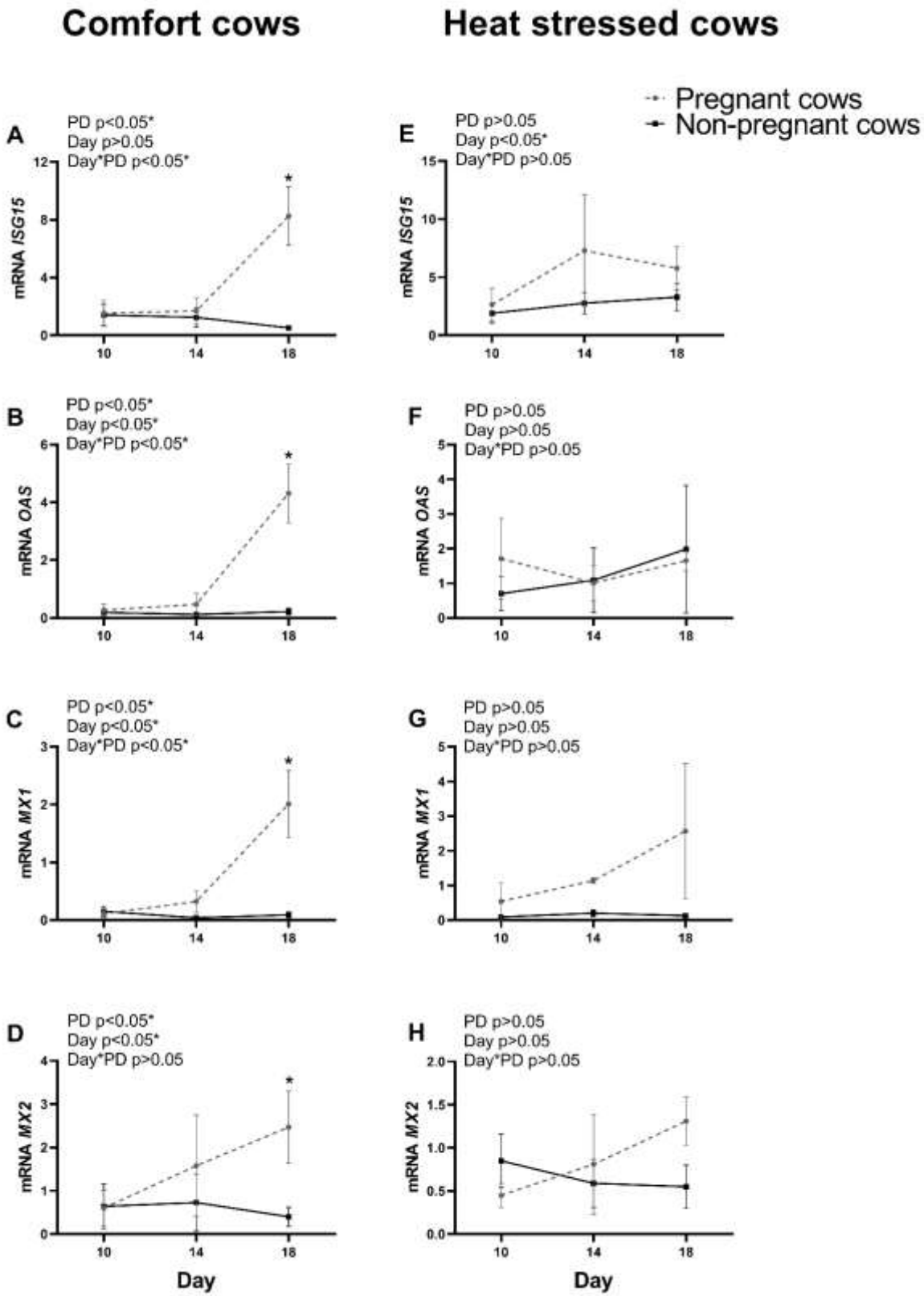

A
CL diameter (mm)
30
20
10
0
*
*
Comfort cows
Heat stressed cows
B
Progesterone (ng/mL)
15
10
5
0
a
b
*
*
Comfort cows
Heat stressed cows
Pregnant cows
Non-pregnant cows

758 **Fig 2.**


Pregnant cows
Comfort cows
Heat stressed cows
A
Day p<0.05*
Group p<0.05*
Day*Group p>0.05
MDA levels
Day
B
Day p<0.05*
Group p<0.05*
Day*Group p>0.05
SOD activity
Day
C
Day p<0.05*
Group p<0.05*
Day*Group p<0.05*
CAT activity
Day
Non-pregnant cows
D
Day p>0.05
Group p<0.05*
Day*Group p>0.05
MDA levels
Day
E
Day p<0.05*
Group p<0.05*
Day*Group p>0.05
SOD activity
Day
F
Day p<0.05*
Group p>0.05
Day*Group p>0.05
CAT activity
Day

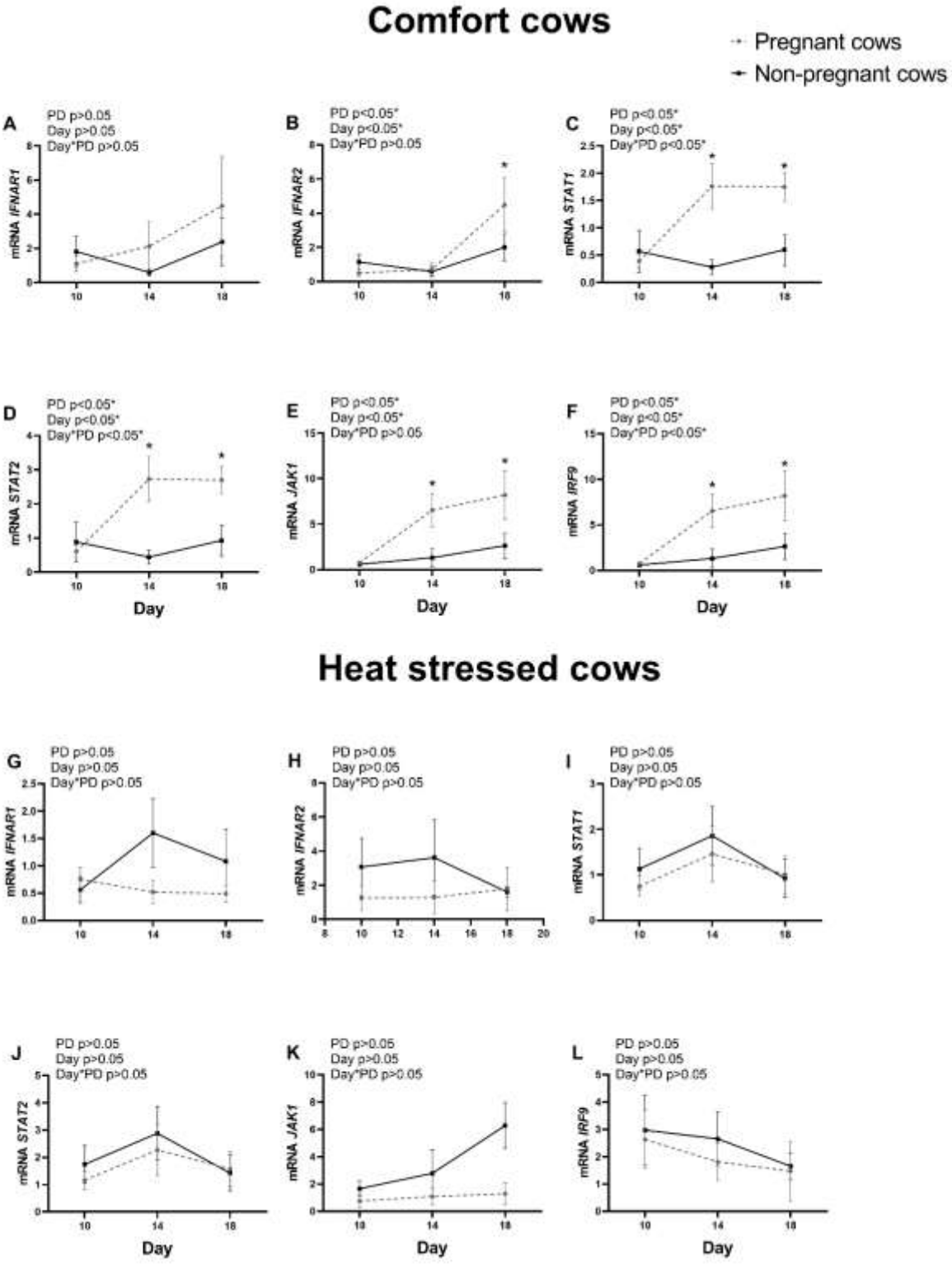
759

760 **Fig 3.**


Comfort cows
Heat stressed cows
Pregnant cows
Non-pregnant cows
A
PD p<0.05*
Day p>0.05
Day*PD p<0.05*
mRNA ISG15
E
PD p>0.05
Day p<0.05*
Day*PD p>0.05
mRNA ISG15
B
PD p<0.05*
Day p<0.05*
Day*PD p<0.05*
mRNA OAS
F
PD p>0.05
Day p>0.05
Day*PD p>0.05
mRNA OAS
C
PD p<0.05*
Day p<0.05*
Day*PD p<0.05*
mRNA MX1
G
PD p>0.05
Day p>0.05
Day*PD p>0.05
mRNA MX1
D
PD p<0.05*
Day p<0.05*
Day*PD p>0.05
mRNA MX2
H
PD p>0.05
Day p>0.05
Day*PD p>0.05
mRNA MX2
10
14
18
Day

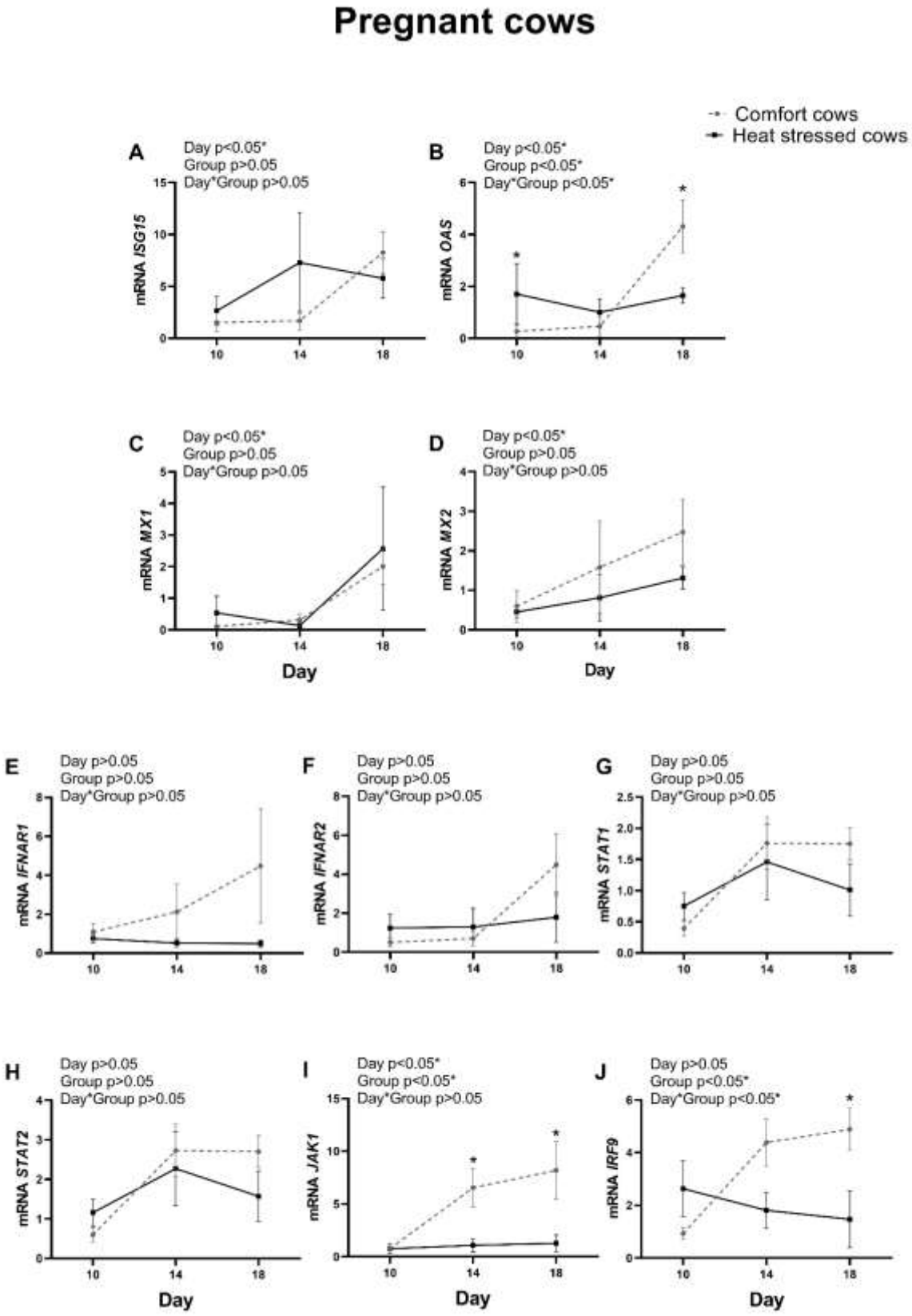
761

762 **Fig 4.**


Comfort cows
Pregnant cows
Non-pregnant cows
A
PD p>0.05
Day p>0.05
Day*PD p>0.05
mRNA IFNAR1
B
PD p<0.05*
Day p<0.05*
Day*PD p>0.05
mRNA IFNAR2
C
PD p<0.05*
Day p<0.05*
Day*PD p<0.05*
mRNA STAT1
D
PD p<0.05*
Day p<0.05*
Day*PD p<0.05*
mRNA STAT2
E
PD p<0.05*
Day p<0.05*
Day*PD p>0.05
mRNA JAK1
F
PD p<0.05*
Day p<0.05*
Day*PD p<0.05*
mRNA IRF9
Day
Heat stressed cows
G
PD p>0.05
Day p>0.05
Day*PD p>0.05
mRNA IFNAR1
H
PD p>0.05
Day p>0.05
Day*PD p>0.05
mRNA IFNAR2
I
PD p>0.05
Day p>0.05
Day*PD p>0.05
mRNA STAT1
J
PD p>0.05
Day p>0.05
Day*PD p>0.05
mRNA STAT2
K
PD p>0.05
Day p>0.05
Day*PD p>0.05
mRNA JAK1
L
PD p>0.05
Day p>0.05
Day*PD p>0.05
mRNA IRF9
Day

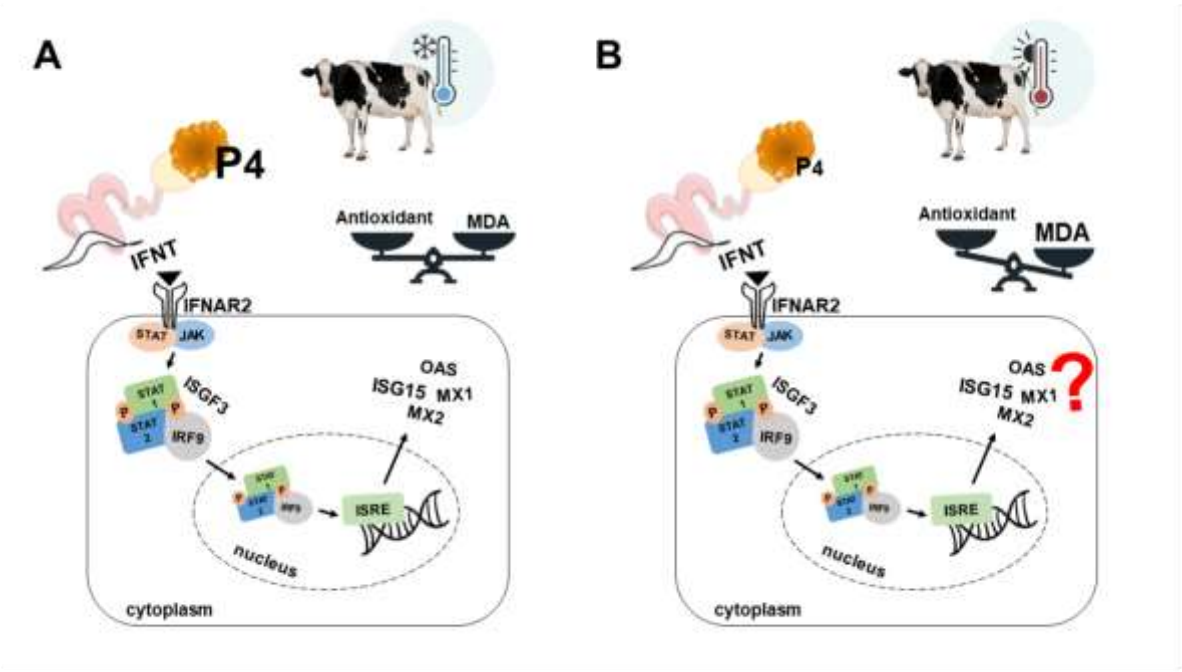
763

764 **Fig 5.**


Pregnant cows
Comfort cows
Heat stressed cows
A
Day p<0.05*
Group p>0.05
Day*Group p>0.05
mRNA ISG15
B
Day p<0.05*
Group p<0.05*
Day*Group p<0.05*
mRNA OAS
C
Day p<0.05*
Group p>0.05
Day*Group p>0.05
mRNA MX1
D
Day p<0.05*
Group p>0.05
Day*Group p>0.05
mRNA MX2
Day
E
Day p>0.05
Group p>0.05
Day*Group p>0.05
mRNA IFNAR1
F
Day p>0.05
Group p>0.05
Day*Group p>0.05
mRNA IFNAR2
G
Day p>0.05
Group p>0.05
Day*Group p>0.05
mRNA STAT1
H
Day p>0.05
Group p>0.05
Day*Group p>0.05
mRNA STAT2
I
Day p<0.05*
Group p<0.05*
Day*Group p>0.05
mRNA JAK1
J
Day p>0.05
Group p<0.05*
Day*Group p<0.05*
mRNA IRF9

765

766 **Fig 6.**


A
P4
IFNT
IFNAR2
STAT
JAK
ISGF3
IRF9
ISRE
nucleus
cytoplasm
Antioxidant
MDA
OAS
ISG15
MX1
MX2
B
P4
IFNT
IFNAR2
STAT
JAK
ISGF3
IRF9
ISRE
nucleus
cytoplasm
Antioxidant
MDA
OAS
ISG15
MX1
MX2
?

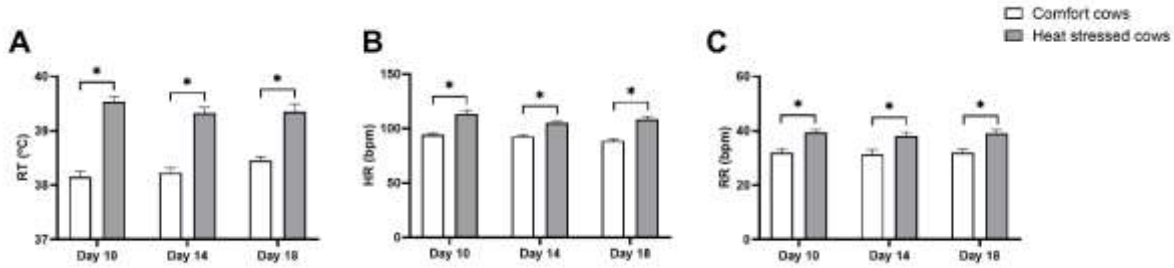
767

768 **S1 Table.**

Days	Comfort cows	Heat stressed cows
0	67.5	83.6
10	67.4	81.8
14	69.8	84.7
18	70.4	82.0

769

770 **S1 Fig.**



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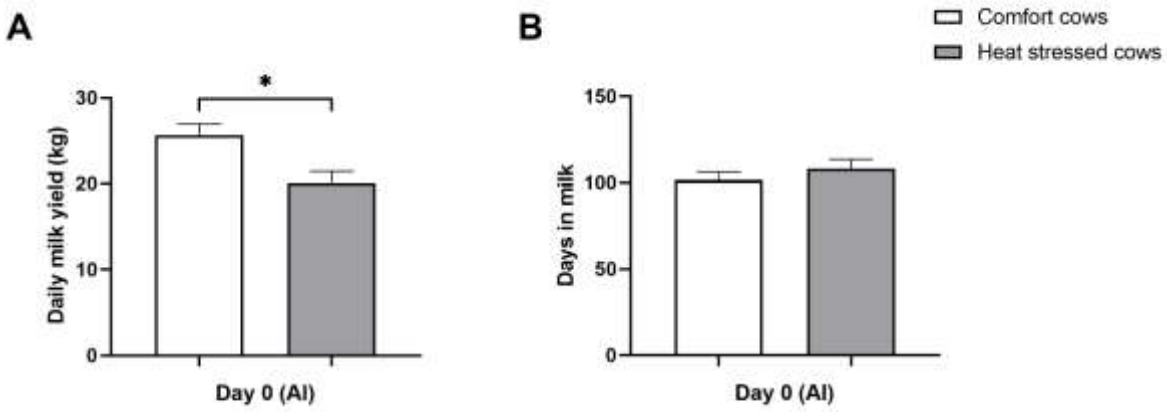

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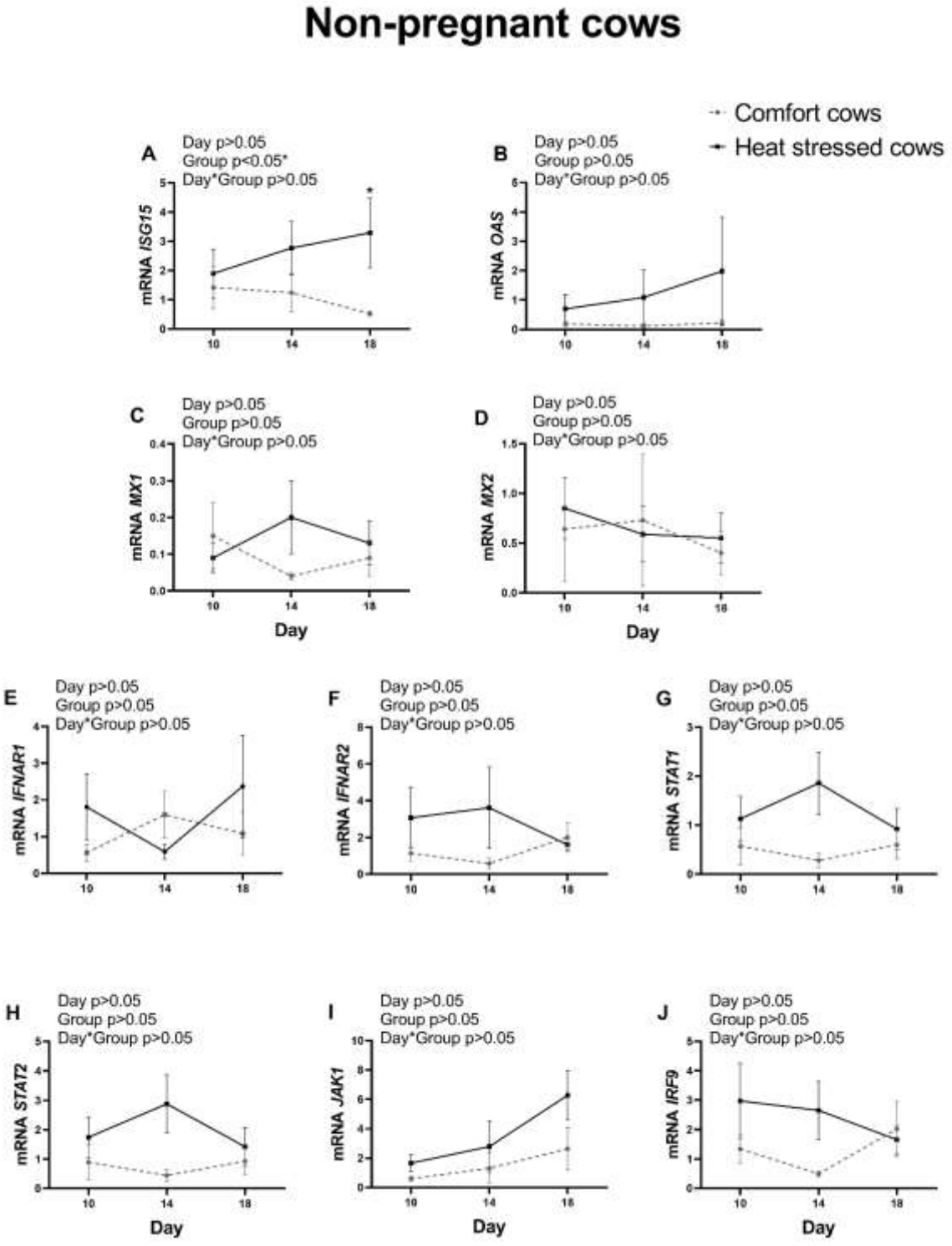

775

ARTIGO 2

TRABALHO SUBMETIDO PARA PUBLICAÇÃO

Heat stress modulates polymorphonuclear cell response in early pregnancy cows: II. pro- and anti-inflammatory markers

Carolina dos Santos Amaral, Mariani Farias Fiorenza, Valério Marques Portela, Alfredo Quites Antoniazzi

JOURNAL OF THERMAL BIOLOGY, 2022

Heat stress modulates polymorphonuclear cell response in early pregnancy cows: II. pro- and anti-inflammatory markers

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Short title: Heat stress, pro- and anti-inflammatory marker genes

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Abstract

High environmental temperature impacts immune functions, which may lead to pregnancy loss. We hypothesized the heat stress (HS) alters mRNA of pro- and anti-inflammatory genes in polymorphonuclear cells (PMNs) response towards a pro-inflammatory state in early pregnant dairy cows. In our study, PMNs were characterized during comfort or HS seasons on anti-inflammatory and pro-inflammatory markers. Cows had their estrous cycle synchronized and artificially inseminated (AI) during the comfort or HS season. Pregnancy was determined by ultrasound on Days 30 and 60 post-AI. Blood samples were collected on Days 10, 14, and 18 post-AI to analyze mRNA expression on PMNs. Our results provide evidence that HS upregulates pro-inflammatory genes (*IL17*, *NFKB* and *IL1B*) and also upregulates anti-inflammatory genes *ARG1*, *TGFB,* and *IFNA* in PMNs of early pregnancy. *IFNG* and *INOS* were regulated only by HS and *IL10* only by pregnancy. Early pregnancy establishment requires an anti-inflammatory PMNs response, and a sudden shift to a pro-inflammatory can cause pregnancy loss. Our results indicate that HS alters anti- and pro-inflammatory status in early pregnant dairy cows. The PMNs response varies from pro- and anti-inflammatory during HS, possibly to maintain inflammation while preventing extensive damage in early pregnancy.

Keywords: hyperthermia, inflammation, pregnancy, immune response

1. Introduction

Pregnancy loss in dairy cows is a factor that negatively influences reproductive performance and occurs more frequently in the first 30 days of pregnancy (Wiltbank et al., 2016). During the summer, heat stress (HS) disrupts several reproductive processes, resulting in a significant decline in dairy cows conception rate worldwide (De Rensis and Scaramuzzi, 2003). Exposure to higher temperatures suppresses the immune response in dairy cows, increases the risk of mastitis (Zhong et al., 2020a, 2020b) and somatic cell count (Bertocchi et al., 2014; Zhong et al., 2020c), impairs follicular development and steroidogenesis, reduces gametes quality, and increases to oxidative stress (Amaral et al., 2020; Roth, 2015).

The success of pregnancies depends on an immune challenge: the unresponsiveness to the semi-allogeneic fetus *in utero* and the responsiveness to pathogens (Kropf et al., 2007). In ruminants, maternal recognition of pregnancy is period the conceptus signals to the maternal system from Days 10-25 of pregnancy (Godkin et al., 1984). The major signaling molecule involved is interferon tau (IFNT) (Farin et al., 1990). IFNT acts in several tissues and cells, including immune cells (Green et al., 2010; Kizaki et al., 2013; Shirasuna et al., 2012). Immune cell's response to IFNT can be classically characterized by the expression of interferon-stimulated genes (ISGs) (Kizaki et al., 2013; Oliveira et al., 2008; Shirasuna et al., 2012), but also by the expression of pro- and anti-inflammatory genes (Talukder et al., 2019, 2018).

Immunity can be described as the mechanism by which the organism protects it host from disease (Ratcliffe, 1989). Usually, these components act in a collective and coordinated manner towards a foreign molecule generating an immune response, termed innate and adaptive immunity. Innate immunity represents the first line of defense, it is a mechanism that relies on a numbered repertoire of receptors shared by a large group of foreign antigens like microbes, injured or dead host cells (Turvey and Broide, 2010). It starts to generate a

protective inflammatory response immediately or within hours after exposure to the pathogen. Also, innate immunity has a central role in activating the adaptive immune response (Arck and Hecher, 2013; Beutler, 2004). Conversely, adaptive immunity has a repertoire of recognition for both self- and non-self-antigens (Bonilla and Oettgen, 2010). This type of response entails a closely regulated relationship between antigen-presenting cells and T and B lymphocytes, which promotes the development of immunologic memory, the activation of pathogen-specific effector pathways, and the control of host immunological homeostasis (Bonilla and Oettgen, 2010; Marshall et al., 2018; Tian, 2010).

Polymorphonuclear cells (PMNs) may be essential in the implantation process due their higher sensitivity to early embryonic signals compared to other leukocytes (Manjari et al., 2016). Pregnant cows' PMNs had greater expression of anti-inflammatory markers such as transforming growth factor-beta (TGFB), interleukin 10 (IL10), and forkhead box P3 (FOXP3) and suppressed pro-inflammatory marker tumor necrosis alpha (TNFA) (Talukder et al.,2019). The expression of pro- and anti-inflammatory genes in bovine leukocytes is also altered by heat stress (Inbaraj et al., 2016; Lacetera et al., 2005).

In dairy cows, severe HS alters peripheral mononuclear blood cells (PBMCs) proliferation capacity (Lacetera et al., 2005) and impairs phagocytosis, and leads to reactive oxygen species (ROS) production in PMNs (Amaral et al., 2021; Lecchi et al., 2016), suggesting that HS induces higher infection rates. The ROS constitute the oxidant system, which comprises indispensable molecules for embryo implantation and uterine and cervical modification during the last gestational phase. However, it is important to balance oxidative stress when it can reach high levels, such as during early and late pregnancy (Sciorsci et al., 2020).

Considering the effects of HS on innate immune response and also the effects of pregnancy on the innate immune response, we hypothesize the heat stress shifts the innate

immune response genes in early pregnant dairy cows from anti-inflammatory towards a pro-inflammatory state. Consequently, this study aims to describe PMN profile of non-pregnant and pregnant dairy cows during comfort or heat stress using anti-inflammatory (*TGFB, ARG1, IL10, VEGF,* and *IFNA*) markers that confer a more tolerant phenotype, and pro-inflammatory (*IL17, TNFA, IFNG, IL1b, NFKB, CCL2,* and *INOS)* markers that present a more suppressive phenotype.

2. Material and methods

2.1. Chemicals

Unless otherwise indicated, chemicals and reagents were purchased from Sigma Chemical Company (Sigma-Aldrich, St. Louis, MO, USA).

2.2. Cattle and herd management

Thirty-two Holstein cows in lactation from the same herd were included in this study. The multiparous cows were 3 to 6 years old, body condition score greater than 2.5 (1=thin and 5=fat on a scale of 1 to 5), milked twice a day, and fed complete ration and corn silage, with *ad libitum* access to water. Cows presented no detectable reproductive and clinical diseases during the experimental period. The study was approved by the Animal Care Use Committee (CEUA-UFSM # 5728120217) of Federal University of Santa Maria.

2.3. Experimental design, synchronization protocol, and artificial insemination

The experimental design was conducted according our previous studies (Amaral et al., 2021). Briefly, the experiment was managed during two distinct seasons on the same commercial dairy farm. Comfort cows (n=15) were collected in September (Late Winter/Early Spring) and the heat-stressed cows (n=17) were collected in January (Summer). Cows were considered at HS when the temperature and humidity index was above 74 as suggested (Armstrong, 1994). Both groups had their estrous cycle synchronized with the same protocol (Pereira et al., 2015). Only animals that exhibited standing estrus were included in the experiment (Comfort group n=12; Heat Stressed group n=13). Artificial insemination (AI) was performed using conventional semen (ST genetics®) stored in liquid nitrogen, and thawed at 36°C for 30 seconds for subsequent AI (Amaral et al., 2021).

2.4. Blood sample collection and isolation of polymorphonuclear peripheral blood cells

Blood was collected from the coccygeal vein using a 21G needle coupled to a vacuum collection system (BD Vacutainer®) in EDTA-containing tubes on Days 10, 14, and 18 following AI for isolation of polymorphonuclear cells (PMNs), as described (Amaral et al., 2021). PMN samples were stored at -80°C for subsequent total RNA extraction. After isolation of PMN fraction, a glass-slide fraction-film was prepared to determine the purity of each fraction. A differential cell count was performed by identifying 100 consecutive leukocytes using a 100x objective. Samples above 95% of specific cell type (PMN) (Kizaki et al., 2013) were included in this study.

2.5. Pregnancy diagnosis by ultrasound scanning

In both groups, pregnancy status was evaluated using a Mindray DP10 ultrasound with a 6.5 MHz linear transducer. Pregnancy rate was determined by dividing the number of pregnant cows at the pregnancy diagnosis at 30- and 60-days following AI by the total number of cows artificially inseminated (P/AI).

2.6. RNA extraction, reverse transcription, and real-time PCR

Total RNA was extracted from the samples using Tri Reagent (BD), according to the manufacturer's recommendations. Quantification and estimation of RNA purity were performed using Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA was treated with DNAse Amplification Grade (Thermo Fisher, Waltham, MA, USA) for 15 minutes at 27ºC to degrade any DNA molecules. DNAse was inactivated with 1 µl EDTA for 10 minutes at 65ºC. Reverse transcription and quantitative polymerase chain reaction (qPCR) were conducted in a thermocycler (BioRad, Hercules, CA, USA) as described method (Amaral et al., 2021). Samples were run in duplicates and the results of expression of all analyzed genes were expressed by ΔΔCq method, using *GAPDH* and *RPS18* as reference genes, as described (Pfaffl, 2001). The genes assessed in this study are presented in Table 1.

2.7. Statistical analysis

Continuous data were checked for normality using the Shapiro-Wilk test and data that did not follow a normal distribution were transformed to logarithms. mRNA expression data were analyzed by repeat measures (MANOVA) for within-group analysis and standard least squares for between-group (comfort vs. heat stress and pregnant vs. non-pregnant cows) analysis. The main effects of day, pregnancy diagnosis (PD), treatment group, day by group interaction (day*group), or day by pregnancy diagnosis interaction (day*PD) were indicated. All data analysis was performed using the JMP7 Software (SAS Institute Inc., Cary, NC,

153 USA). Results are presented as mean ± standard error of the mean (SEM), and P<0.05 was
154 considered significant.
155
156 **3. Results**

157 **3.1. Physiological and reproductive parameters of cows on comfort or under heat stress**
158 **environment**

159 It was observed that the THI was elevated during the heat stress environment,
160 compared to the comfort environment. HS affected both RT, HR, and RR of dairy cows
161 (p<0.05), and this was evident on all measurement days. In relation to milk production, heat
162 stressed cows had lower daily milk yield than the cows that were on comfort. Effect of season
163 on estrous occurrence and pregnancy rate were not different between groups (P>0.05). Estrus
164 occurrence rate was 80% in comfort group and 76.47% in heat stressed group. Pregnancy rate
165 was 50% in comfort group and 38.46% in heat stressed group (Amaral et al., 2021).
166
167 **3.2. Pro- and anti-inflammatory markers mRNA expression in PMN cells of non-**
168 **pregnant or pregnant cows in comfort**

169 Relative mRNA expression of *ARG1, TGFB, IL10, VEGF, IL17, IFNA, IFNG, TNFA,*
170 *IL1B, NFKB, CCL2,* and *INOS* was compared in PMNs of non-pregnant and pregnant cows in
171 the comfort group on Days 10, 14 and 18 after AI (Fig 1). Expression of *ARG1* (Fig 1A),
172 *VEGF* (Fig 1D), *IFNG* (Fig 1G), *TNFA* (Fig 1H), *CCL2* (Fig 1K), and *INOS* (Fig 1L) did not
173 differ (P>0.05) between non-pregnant and pregnant cows in comfort season. However, anti-
174 inflammatory markers *TGFB* (Fig 1B), *IL10* (Fig 1C), and *IFNA* (Fig 1E) were upregulated
175 on Day 18 in pregnant cows (P<0.05). Among the pro-inflammatory markers, *IL1B* (Fig 1I)

was upregulated on Days 10, 14, and 18, and *IL17* (Fig 1F) and *NFKB* (Fig 1J) were upregulated on Day 18 in pregnant cows (P<0.05).

3.3. Pro- and anti-inflammatory markers mRNA expression in PMN cells of non-pregnant or pregnant cows in heat stress

Relative mRNA expression of *ARG1, TGFB, IL10, VEGF, IL17, IFNA, IFNG, TNFA, IL1B, NFKB, CCL2,* and *INOS* was compared in PMNs of non-pregnant and pregnant cows in heat stress on Days 10, 14 and 18 after AI (Fig 2). Abundance of *VEGF* (Fig 2D), *TNFA* (Fig 2H), *IL1B* (Fig 2I), and *CCL2* mRNA (Fig 2K) did not differ (P>0.05) between non-pregnant and pregnant cows in heat stress. Among the anti-inflammatory marker genes, *ARG1* (Fig 2A), *TGFB* (Fig 2B), and *IFNA* (Fig 2E) were upregulated on Day 18 in leukocytes of pregnant cows (P<0.05); *IL10* (Fig 2C) was upregulated on Day 14 in pregnant cows (P<0.05). However, pro-inflammatory marker genes *IL17* (Fig 2F), *IFNG* (Fig 2G), and *NFKB* (Fig 2J) were upregulated on Day 18 in pregnant cows (P<0.05); and *INOS* (Fig 2L) was upregulated on Days 10, 14 and 18 in pregnant cows (P<0.05).

3.4. Pro- and anti-inflammatory markers mRNA expression in PMN cells of non-pregnant cows under comfort or heat stress

To identify differences in non-pregnant cows, relative mRNA expression of *ARG1, TGFB, IL10, VEGF, IL17, IFNA, IFNG, TNFA, IL1B, NFKB, CCL2,* and *INOS* mRNA was compared in PMNs only in non-pregnant cows in a comfort or heat stress on Days 10, 14 and 18 after AI (Fig 3). mRNA of *ARG1* (Fig 3A), *IL10* (Fig 3C), *VEGF* (Fig 3D), *TNFA* (Fig 3H), *CCL2* (Fig 3K), and *INOS* mRNA (Fig 3L) did not differ (P>0.05) in non-pregnant cows in comfort or heat stress among all days. But in heat-stressed non-pregnant cows, the anti-inflammatory marker genes *TGFB (*Fig 3B) and *IFNA* (Fig 3E) were upregulated on Day 18

after AI (P<0.05). Among pro-inflammatory marker genes, *IL17* (Fig 3F), *IFNG* (Fig 3G), and *NFKB* (Fig 3J) were upregulated on Day 18 after AI (P<0.05) in heat-stressed cows; and mRNA abundance *IL1B* (Fig 3I) was upregulated on Days 10 and 18 after AI (P<0.05) in heat-stressed cows.

3.5. Pro- and anti-inflammatory markers mRNA expression in PMN cells of pregnant cows under comfort or heat stress

Relative mRNA expression of *ARG1, TGFB, IL10, VEGF, IL17, IFNA, IFNG, TNFA, IL1B, NFKB, CCL2,* and *INOS* was compared in PMNs of pregnant cows in comfort or heat stress on Days 10, 14 and 18 after AI (Fig 4). *ARG1* (Fig 4A), *VEGF* (Fig 4D), *TNFA* (Fig 4H), *IL1B* (Fig 4I), *CCL2* (Fig 4K), and *INOS* (Fig 4L) mRNA abundance did not differ (P>0.05) in pregnant cows in comfort or heat stress among all days analyzed. Among the anti-inflammatory marker genes, *TGFB* (Fig 4B) and *IFNA* (Fig 4E) were upregulated on Day 18 of heat-stressed pregnant cows (P<0.05); *IL10* (Fig 4C) was upregulated on Days 14 and 18 of heat-stressed pregnant cows (P<0.05). Among pro-inflammatory marker genes, *IL17* (Fig 4F), *IFNG* (Fig 4G), and *NFKB* (Fig 4J) were upregulated on Day 18 after AI (P<0.05) in heat-stressed pregnant cows.

4. Discussion

This study provides an overview of the influence of heat stress on early pregnancy in cows considering genes indicators pro- and anti-inflammatory. The experimental model evaluated the effect of cold and warm seasons of the year on the estrous cycle and pregnancy rates, temperature-humidity index, rectal temperature, heart rate, and respiratory rate was published by our group (Amaral et al., 2021; Supporting Information). Our previous study demonstrated that the expression of ISGs and type I IFN pathway genes in PMNs of pregnant

cows in comfort increased in a time-dependent manner by Day 18, while non-pregnant cows kept a baseline expression. The expression of ISGs and type I IFN pathway in PMNs from heat-stressed cows did not differ between non-pregnant and pregnant cows on all days. Malondialdehyde (MDA) levels were higher in both non-pregnant and pregnant cows under heat stress. At the same time, the activity of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) did not have a proportional increase, indicating that heat stress leads to oxidative stress (Amaral et al., 2021). For this reason, the results of the study presented herein characterize the pro- and anti-inflammatory mRNA expression in PMNs in non-pregnant and pregnant cows during comfort or heat stress seasons; and verify if stressors like pregnancy and heat stress alter these indicators.

In heated-stressed pregnant cows, *ARG1* mRNA level peaked on Day 18. ARG1 is an essential regulatory enzyme for the availability of arginine (Bronte et al., 2003; Kropf et al., 2007). Arginine is a substrate for both ARG1 and INOS, distinctive anti- and pro-inflammatory markers, respectively (Maarsingh et al., 2006). To diminish the severity of HS, ARG1 can create an anti-inflammatory milieu and suppress pro-inflammatory reactions (Bronte et al., 2003; Gobert et al., 2004; Müller et al., 2009). IL4, IL10, and, mainly, TGFB can activated ARG1 (Chatterjee et al., 2006; Gobert et al., 2004). Additionally, the upregulation of ARG1 and INOS in heat-stressed pregnant cows on Day 18 may inhibit T cell activation (Müller et al., 2009). The greater level of *ARG1* mRNA was observed on Day 18 HS pregnant cows may reflect maternal immune suppression.

The anti-inflammatory marker *TGFB* was upregulated on Day 18 in comfort and heat-stressed cows, independent of pregnancy status. Changes in cytokine levels typically indicate a shift from pro- to anti-inflammatory immunity in response to HS (Kelley et al., 1982). According to several studies, TGFB may induce the expression of the heat-shock proteins HSP70 and HSP90 in embryos and pancreatic cells exposed to high temperatures (Takenaka

and Hightower, 1992; Weber et al., 2000). The TGFB mRNA abundance on this present study is comparable to those of hyperthermia-induced overexpression. Greater TGFB mRNA level may be linked to defense against the negative effects of HS (Flanders et al., 1993). Therefore, *TGFB* mRNA upregulation in response to HS might be a part of a process to generate cellular defense against stressful conditions (Weber et al., 2000). Conversely, early pregnancy may also be considered as a stressor that increases TGFB expression to generate an anti-inflammatory environment (Talukder et al., 2018). A comparison between pregnant cows in comfort or HS demonstrates that heat-stressed cows had greater *TFGB* mRNA increase on Day 18, suggesting that pregnancy-derived signals, possibly IFNT, modulate PMN response to a tolerant environment.

In the same way, *IFNA* mRNA abundance was regulated by both pregnancy and heat stress, isolated or together. IFNA is a member of Type I IFN family, which comprises cytokines important for bridging innate and adaptive defenses to shield the organism against viral infection (González-Navajas et al., 2012). IFNA has a 50% homology with IFNT, and is known to use the same signaling pathway (González-Navajas et al., 2012). It is an important cytokine for the host defense and maintenance of the immunological balance (Kopitar-Jerala, 2017; Liu et al., 2019). However, *IFNA* mRNA level was upregulated in Day 18 non-pregnant and pregnant heat-stressed cows. Type I IFN are key mediators to innate immune response with pleiotropic effects in many cell types (Pestka et al., 2004). The expression of *IFNA* mRNA during stressful situations like HS might suggest PMNs still a protective role.

In both comfort and heat stress conditions, pregnant cows expressed more *IL10* mRNA than non-pregnant cows. However, in non-pregnant cows, *IL10* mRNA abundance did not differ between comfort and heat-stressed animals, indicating that pregnancy but not HS was the source of *IL10* regulation. Coincidentally, the greater expression of *IL10* mRNA in pregnant cows was on Day 18, the same way as IFNT (Amaral et al., 2021). IL10 is related to

immune tolerance during pregnancy establishment. IL10 binds to a specific receptor complex and activates the Janus kinase (JAK/STAT) signaling pathway (Cheng and Sharma, 2016; Wu et al., 2017). IL10 and IFNA can be upregulated by the JAK/STAT signaling pathway (González-Navajas et al., 2012; Wu et al., 2017), but not only by it, as JAK/STAT pathway is upregulated also by IFNT in pregnant cows in comfort conditions, except by heat stress, as it was demonstrated in our previous paper (Amaral et al., 2021).

Among the pro-inflammatory cytokines, our data show that *IFNG* mRNA abundance is regulated only by heat stress, not by pregnancy. Interferon-gamma (IFNG) is the sole type II interferon, is structurally different from the type I IFNs, and signals through the IFNG receptor (Medzhitov, 2008). IFNG exhibits potent pro-inflammatory signaling by priming macrophages for antimicrobial actions (Kopitar-Jerala, 2017). Furthermore, it has been reported that persistent heat stress impairs the levels of IFNG, CD4+ and CD8+ cells, as well as cytotoxic T-lymphocytes (Hu et al., 2007). Our study has shown an upregulation of pro-inflammatory *INOS* mRNA on Days 10, 14, and 18 in heat-stressed pregnant cows, but no changes were reported in pregnant cows in comfort conditions, suggesting that *INOS* is upregulated by heat stress. In response to pro-inflammatory mediators such TNFA, IL1B, and IFNG immune cells and tissues express INOS mRNA (Marks-Konczalik et al., 1998). The imbalance between antioxidants and oxidants that occurs during heat stress, as shown by our group (Amaral et al., 2021), and the pro-inflammatory action of IFNG may both contribute to the upregulation of *INOS* in PMNs of cows during HS.

On all days tested, pregnancy upregulated *IL1B* mRNA expression in comfort cows. When non-pregnant cows' *IL1B* mRNA levels were compared, the heat-stressed group showed an upregulation. It means that *IL1B* may be regulated by both pregnancy and heat stress. *IL1B* is part of IL1 family of cytokines, which can play a role in innate and acquired immunity (Dinarello, 2018). In agreement, *NFKB* mRNA level was greater in pregnant cows

from comfort and heat stressed groups. The non-pregnant heat-stressed group had greater levels of *NFKB* mRNA; and when only pregnant cows were compared, the heat-stressed group similarly had greater levels of *NFKB* mRNA expression. NFKB belongs to a family of inducible transcription factors, which regulate several genes involved in different processes of the immune and inflammatory responses (Liu et al., 2017). For immune cells with pro-inflammatory phenotype, such as M1 macrophages, NFKB is a key transcription factor. It is required for the induction of various inflammatory genes, including IL1B (Wang et al., 2014). Typical cellular priming inducers include TNFA and ILIB, which are known to activate NFKB pathway (Liu et al., 2017).

Furthermore, regardless of the treatment group, Day 18 pregnant cows had greater expression of *IL17* mRNA. Also, *IL17* mRNA abundance was greater in Day 18 non-pregnant heat-stressed cows, compared with non-pregnant comfort cows. IL17 is a key cytokine linking T cell activation to neutrophil mobilization and activation. IL17 can contribute to the pathogenesis of inflammatory diseases with pro-inflammatory effects (Zenobia and Hajishengallis, 2000). Our results demonstrate that pregnancy and heat stress regulate IL1B, NFKB, and IL17, regardless of whether they occur separately or simultaneously. Pre-implantation pregnant cows usually have a more anti-inflammatory profile (Fiorenza et al., 2021; Talukder et al., 2017). However, the HS and oxidative stress that these cows are experiencing (Amaral et al., 2021) induce the expression of pro-inflammatory cytokines (Bae et al., 2017; Fonseca et al., 2016).

Moreover, in comfort, the expression of *TNFA* did not differ in cows during the first few days of pregnancy (Manjari et al., 2016; Shirasuna et al., 2012), corroborating with the presented results for comfort and heat-stressed cows. TNFA is connected to inflammatory processes that affect the success of implantation, placentation, and pregnancy. Exposure to daily heat increased *TNFA* (Hop et al., 2018), leading to complications during pregnancy

(Azizieh and Raghupathy, 2015). We believe that other cytokines, like IL17, IFNG, INOS, NFKB, and IL1B, act on PMNs to induce this response. The hypothesis that TNFA can act in other tissues of these cows, such as endometrium (Talukder et al., 2017), is not discarded.

In summary, the current study provides *in vivo* evidence that HS modulates PMN response with greater level of mRNA of the pro-inflammatory cytokines *IL17, NFKB*, and *IL1B*, and anti-inflammatory cytokines *ARG1*, *TGFB*, and *IFNA* in early pregnancy dairy cows. *IFNG* and *INOS* were regulated by HS and *IL10* by pregnancy. The presented results suggest that *ARG1, TGFB*, and *IFNA* mRNA expression modulate PMNs response under HS, offering a protective role during pregnancy, while higher expression of *IL17, NFKB*, and *IL1B* mRNA may indicate the innate response is more active and consequently induce the expression of other cytokines. Also, heat-stressed cows may suffer health consequences from *IFNG* and *INOS* (Fig 5). In conclusion, the present study demonstrated that heat stress modifies anti- and pro-inflammatory status in pregnant dairy cows. PMNs response shifts between pro- and anti-inflammatory patterns during heat stress condition.

Declaration of interest

The authors have nothing to declare.

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Authorship statement

CSA and AQA: Conceptualization. CSA and MFF: Data curation; Investigation; Methodology. VMP and CSA Formal analysis. AQA and VMP: Funding acquisition; Project administration; Resources; Software; Supervision; Validation; Visualization. CSA and MFF: Writing - original draft. CSA, MFF, VMP and AQA: Writing - review & editing.

Figure legends

Fig 1. Pro- and anti-inflammatory markers mRNA expression in polymorphonuclear cells comparing non-pregnant and pregnant cows in a comfort environment on Days 10, 14, and 18 post-AI. A) *ARG1;* B) *TGFB*; C) *IL10*; D) *VEGF*; E) *IFNA*; F) *IL17*; G) *IFNG*; H) *TNFA*; I) *IL1B*; J) *NFKB*; K) *CCL2*; L) *INOS*. Values are presented as mean ± S.E.M. The main effects of pregnancy diagnosis (PD), day and day by pregnancy diagnosis interaction (day*PD) are indicated. Asterisk represents the difference at $P<0.05$ between pregnant and non-pregnant cows.

Fig 2. Pro- and anti-inflammatory markers mRNA expression in polymorphonuclear cells comparing non-pregnant and pregnant cows in heat stress environment on Days 10,

14, and 18 post-AI. A) *ARG1;* B) *TGFB*; C) *IL10*; D) *VEGF*; E) *IFNA*; F) *IL17*; G) *IFNG*; H) *TNFA*; I) *IL1B*; J) *NFKB*; K) *CCL2*; L) *INOS*. Values are presented as mean ± S.E.M. The main effects of pregnancy diagnosis (PD), day and day by pregnancy diagnosis interaction (day*PD) are indicated. Asterisk represents the difference at P<0.05 between pregnant and non-pregnant cows.

Fig 3. Pro- and anti-inflammatory markers mRNA expression in polymorphonuclear cells of non-pregnant cows under comfort or heat stress environment on Days 10, 14, and 18 post-AI. A) *ARG1;* B) *TGFB*; C) *IL10*; D) *VEGF*; E) *IFNA*; F) *IL17*; G) *IFNG*; H) *TNFA*; I) *IL1B*; J) *NFKB*; K) *CCL2*; L) *INOS*. Values are presented as mean ± S.E.M. The main effects of day, group, and day-by-group interaction (day*group) are indicated. Asterisk represents the difference at P<0.05 between comfort and heat stressed groups.

Fig 4. Pro- and anti-inflammatory markers mRNA expression in polymorphonuclear cells of pregnant cows under comfort or heat stress environment on Days 10, 14, and 18 post-AI. A) *ARG1;* B) *TGFB*; C) *IL10*; D) *VEGF*; E) *IFNA*; F) *IL17*; G) *IFNG*; H) *TNFA*; I) *IL1B*; J) *NFKB*; K) *CCL2*; L) *INOS*. Values are presented as mean ± S.E.M. The main effects of day, group, and day-by-group interaction (day*group) are indicated. Asterisk represents the difference at P<0.05 between comfort and heat stressed groups.

Fig 5. Schematic model illustrating the experimental design and the main conclusions. A) Cows from both groups had their estrus synchronized with the same protocol. AI was considered Day 0 of experiment. Blood collections were performed on Days 10, 14, and 18

following AI for isolation of PMNs. Pregnancy diagnosis was performed on Days 30 and 60 following AI by ultrasonography to subdivide the two groups into non-pregnant and pregnant cows. B) The main results of mRNA expression: *IL10* was upregulated by pregnancy; *ARG1, TGFB, IFNA IL1B, IL17,* and *NFKB* were upregulated both by pregnancy and heat stress; and *IFNG* and *INOS* were upregulated by heat stress. *CCL2, TNFA,* and *VEGF* did not differ between groups.

400 **Tables**

401 **Table 1. Primers designed for quantitative real-time PCR analysis.**

Target	Forward and reverse primer sequence	GenBank
ARG1	F: CCAGAAGAAGTGACTCGAACAG R: GGTGGGCTAAGGTAATCAATAGG	NM_001046154.1
TGFB	F: CTGAGCCAGAGGCGGCGGACTAC R: CTGTGCGAGCTAGACTTCATTTTG	NM_001166068.1
IL10	F: GAGATGCGAGCACCCTGTCT R: GGCTGGTTGGCAAGTGGATA	NM_174088.1
VEGF	F: ATTTTCAAGCCGTCCTGTGT R: TATGTGCTGGCTTTGGTGAG	NM_001316955.1
IL17	F: CACAGCATGTGAGGGTCAAC R: GGTGGAGCGCTTGTGATAAT	NM_001008412.2
IFNA	F: TCTGCAAGAGAAGAGACACAGC R: TCTCCTGAAACTCTCCTGCAAG	NM_001017411.1
IFNG	F: TATCTCAGGGGCCAACTAGG R: GGCATCATTTCATTTATCAGCA	NM_174086.1
TNFA	F: CAAAAGCATGATCCGGGATG R: TTCTCGGAGAGCACCTCCTC	NM_173966.3
IL1B	F: GAGAGGGTTTCCATTCTGAAGT R: CATCAGCACTTCTCAAATCGAAGA	NM_174093.1
NFKB	F: CCTGCTGAATGCTCTGTCTG R: TCCTCCTTCACCTCTGTGCT	NM_001045868.1
CCL2	F: TGCAGACCCCAAGCAGAAAT R: AGAGGGCAGTTAGGGAAAGC	NM_174006.2

INOS	F: GATCCAGTGGTCGAACCTGC R: CAGTGATGGCCGACCTGATG	NM_001076799.1
GAPDH	F: GATTGTCAGCAATGCCTCCT R: GGTCATAAGTCCCTCCACGA	NM_001034034.2
RPS18	F: CCTTCCGCGAGGATCCATTG R: CGCTCCCAAGATCCAACTAC	XM_024983403.1

F: Forward; R: Reverse.

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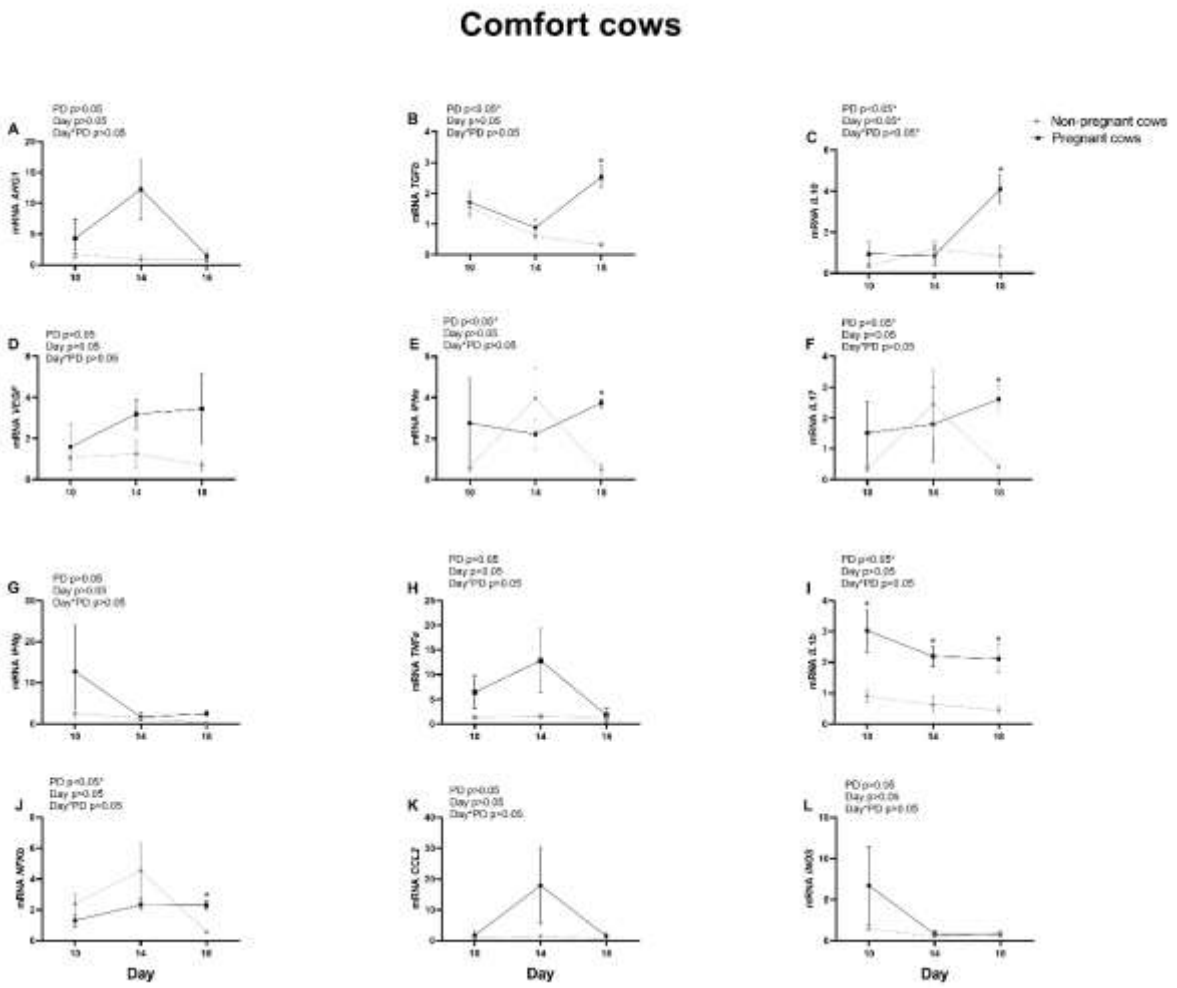
608 **Fig 1.**



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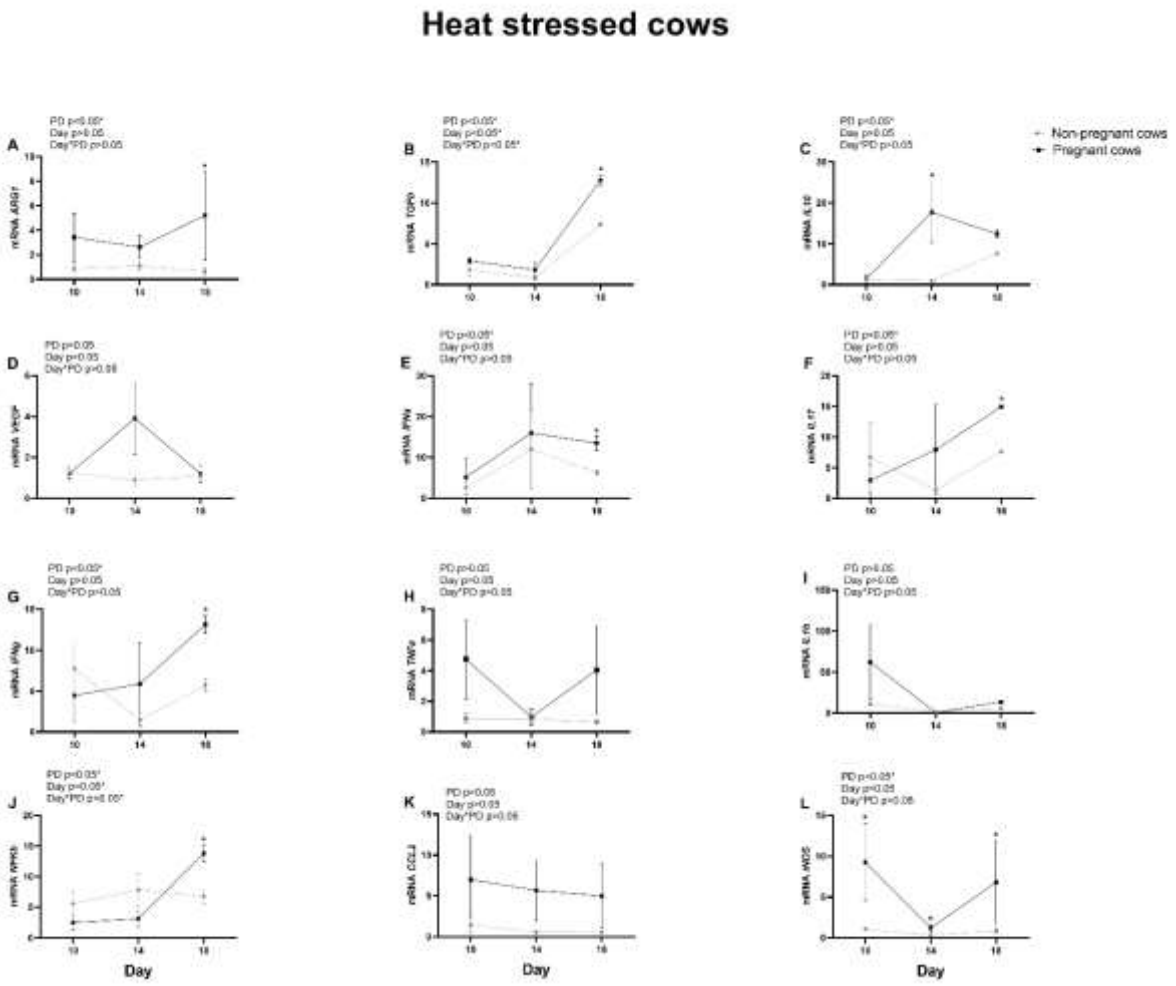
611 **Fig 2.**



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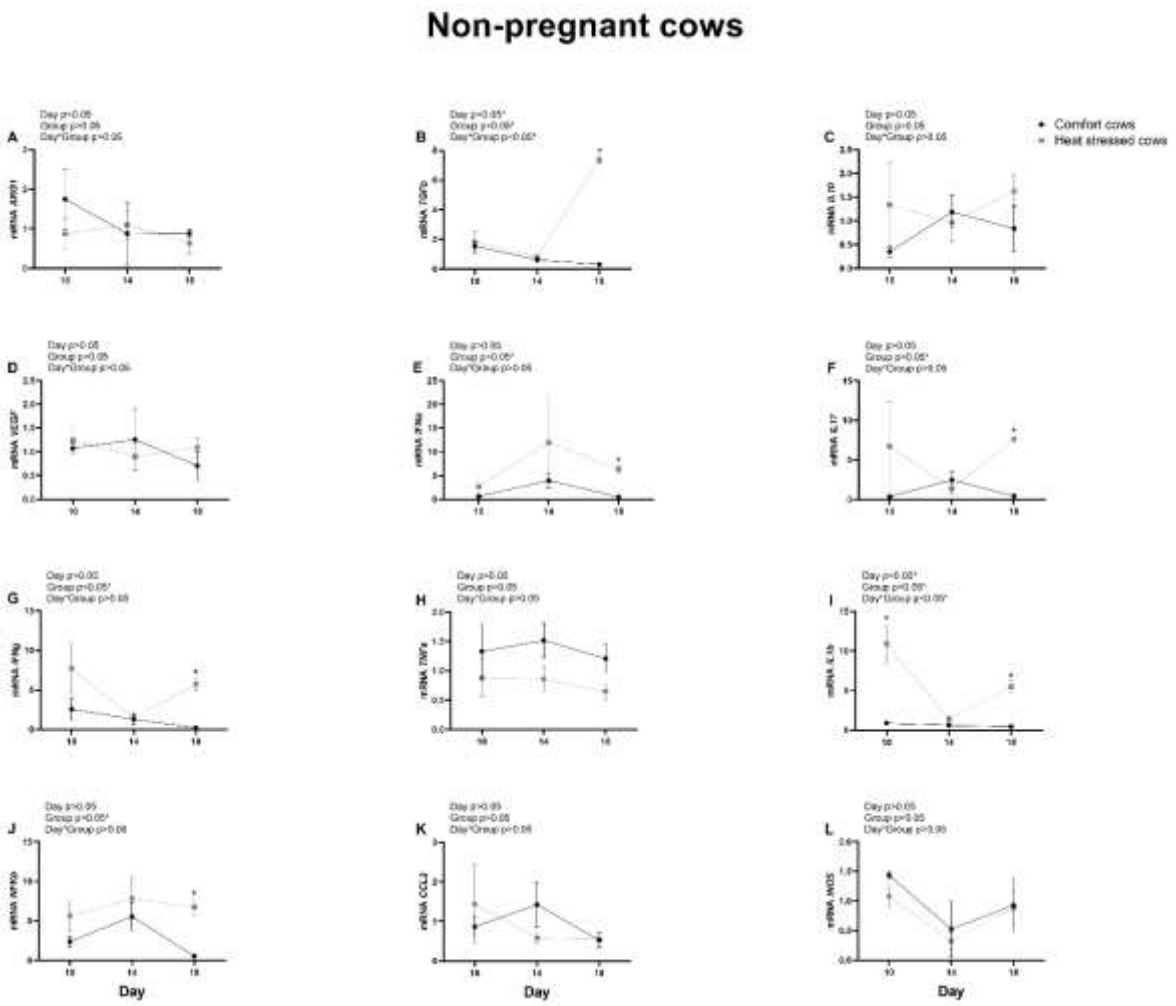
614 **Fig 3.**



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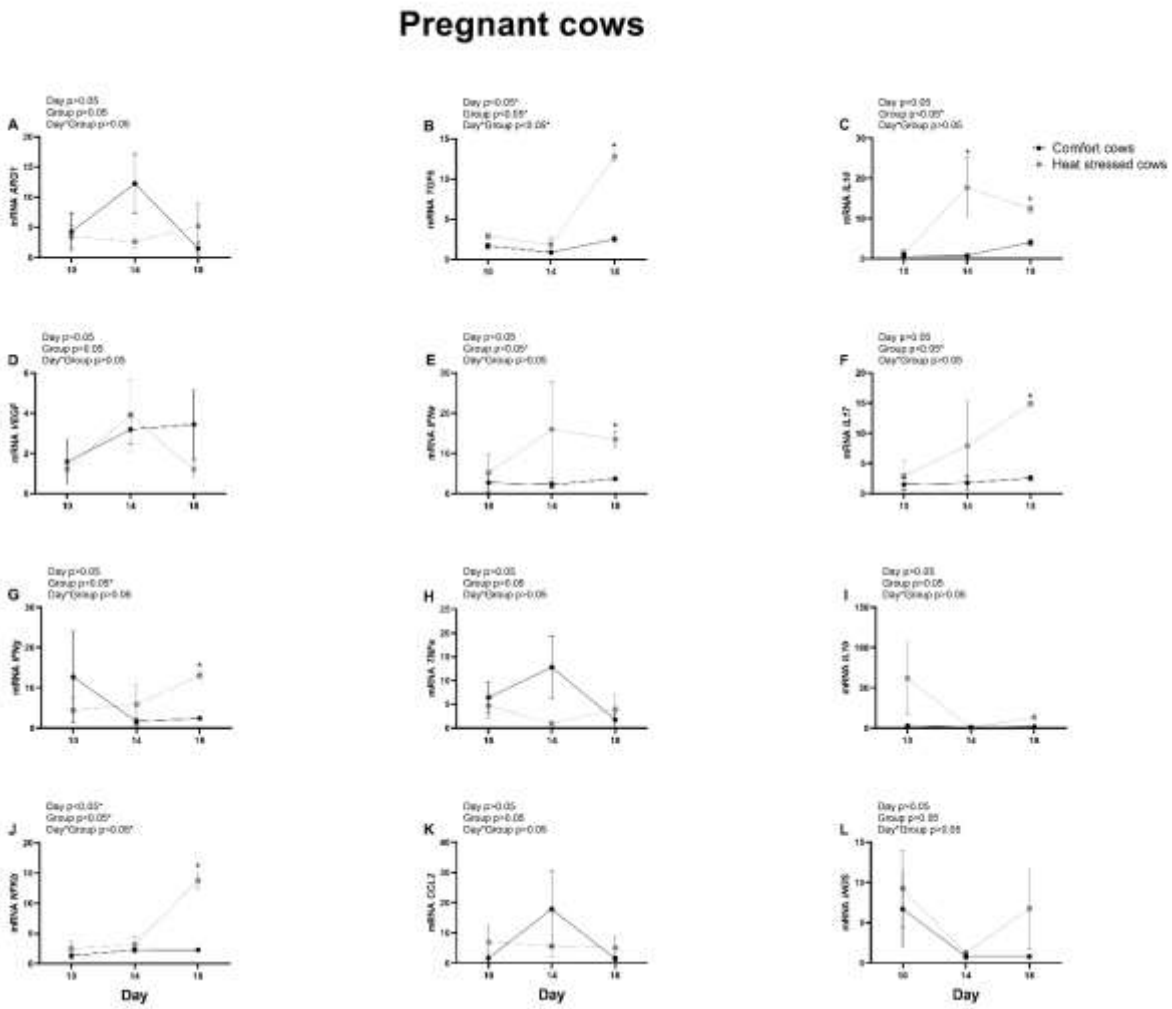
617 **Fig 4.**



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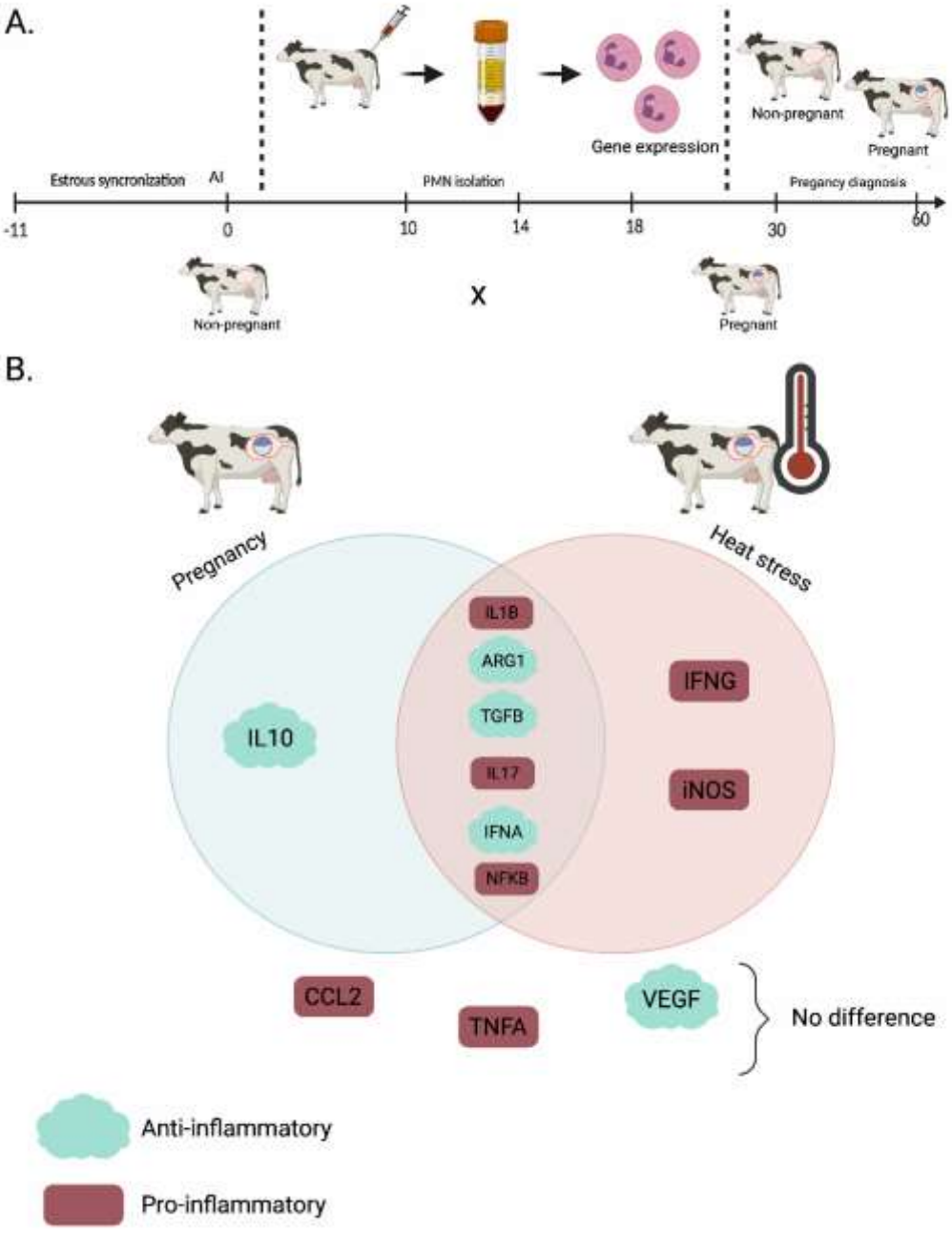
620 **Fig 5.**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Estrous syncronization
AI
PMN isolation
Gene expression
Non-pregnant
Pregnant
Pregancy diagnosis
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Non-pregnant
X
Pregnant
B.
Pregnancy
Heat stress
IL1B
ARG1
TGFB
IL17
IFNA
NFKB
IL10
IFNG
iNOS
CCL2
TNFA
VEGF
No difference
Anti-inflammatory
Pro-inflammatory

621

ARTIGO 3

TRABALHO A SER SUBMETIDO PARA PUBLICAÇÃO

Oxidative stress caused by heat stress modulates endoplasmic reticulum stress and heat shock proteins protective response in peripheral blood mononuclear cell in early pregnant cows

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Oxidative stress caused by heat stress modulates endoplasmic reticulum stress and heat shock proteins protective response in peripheral blood mononuclear cell in early pregnant cows

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Short title: ER stress and HSP response in heat-stressed cows

Abstract

We aimed verify early pregnancy modulation of ER stress and HSPs response in peripheral blood mononuclear cells (PBMCs) in cows, and whether this is affected by HS. Pregnant cows had their estrous cycle synchronized and randomly assigned to a comfort or HS group. Blood samples were collected on Days 10, 14 and 18 following artificial insemination (AI). Results are presented as mean±SEM. Under comfort conditions, there are no differences in ER stress pathway comparing pregnant and non-pregnant cows. HSP90 was greater in pregnant cows comparing with non-pregnant cows. Under HS conditions, HSPA5, uXBP1 and CHOP were upregulated on Day 10 of pregnant cows. Also, HSP70 and HSP90 were both greater in pregnant cows. Comparing only non-pregnant cows, HSPA5 and sXBP1 were upregulated on Day 10 of HS cows and there are no differences in HSPs response. However, in pregnant cows HSPA5, sXBP1, ATF6 and CHOP were upregulated on Day 10 of HS cows. HSF, HSP70 and HSP90 was upregulated in HS cows. In conclusion, oxidative stress caused by HS triggers ER stress on PBMCs.

Keywords: heat stress, dairy cows, early pregnancy loss, ER stress, heat shock proteins.

1. Introduction

Heat stress (HS) is well-defined as the result of the animal's inability to dissipate or produce heat to maintain its physiological temperature (De Rensis et al., 2017; Dobson et al., 2000; Jeelani et al., 2019). It is one of the limiting factors in dairy production and contribute to economic losses among about 60% of the world's cattle population (Edwards et al., 2009). A variety of indicators were used to estimate HS in dairy cattle. Summer heat factors generates a high temperature-humidity index (THI), has been extensively applied for assessing HS in moderate to hot conditions (Thom, 1959). High THI may not only decrease milk production but also affects the reproductive performance and immune system of lactating dairy cows (Min et al., 2016). Major physiological responses of animals to heat stress start at THI 74. THI 74 to 79 induces moderate stress and THI≥ 80 induces severe stress in the animals (Jeelani et al., 2019). It has been assumed that an increase in THI affects the body temperature of dairy cows (Armstrong, 1994), which leads to hyperthermia and impairs cellular function of several tissues including the reproductive system (Wolfenson et al., 2000).

HS induces the occurrence of oxidative stress (Slimen et al., 2014). Several studies have concluded that exposure to heat enhances ROS production and induces oxidative stress, which can lead to cytotoxicity (Amaral et al., 2020; Bernabucci et al., 2002). Oxidative stress is also a precursor and a major contributor to the occurrence of endoplasmic reticulum (ER) stress (Hotamisligil, 2010). ER stress is an imbalance between the capacity for conformational changes of proteins in the ER and the protein load, which consequently leads to conformational changes or unfolded proteins that end up accumulating in the lumen of the ER, impairs the ER homeostasis (Cnop et al., 2012). The mechanism that mediates this homeostasis regulation, trying to inhibit ER stress, is the Unfolded Protein Response (UPR) pathway (Harding et al., 1999). During UPR pathway activation, the presence of stress is detected by 3 ER transmembrane proteins: PERK (PKR-like RE kinase), ATF6 (Activating

transcription factor 6) e IRE1 (Inositol-requiring enzyme 1). These proteins associate with the HSPA5 luminal domains when released induce different regulatory mechanism. It has been suggested that HSPA5, also known as glucose regulated protein 78 (GRP78) or binding protein (BiP), is an important marker of ER stress because it is a resident chaperone in the ER and plays a vital role in regulation of ER homeostasis (Hotamisligil, 2010; Liu et al., 2011).

Another marker of HS both *in vitro* and *in vivo* is the presence of heat shock-induced proteins (HSPs), mostly HSP70 and HSP90 (Arya et al., 2007; Sakatani, 2017). HSPs are a heterogeneous group of proteins named chaperones, which have specific molecular weight and determined biological function. Heat shock response is a cellular adaptive response, which helps maintain cellular homeostasis under stress. In general, chaperones support misfolded an injured protein, preventing lethal cell damage (Mayer and Bukau, 2005). The mechanism of the HSPs starts by activation of the heat shock transcription factor (HSF). HSF is inactively present in the cytoplasm, where it is trimerized and translocated to the cell nucleus, which will bind to specific sites to transcribe HSPs message. Although the action of HSPs is not restricted to heat stress, it is believed that these proteins use energy from ATP hydrolysis to denature proteins damaged/altered by hyperthermia, enabling refolding, supporting misfolded proteins to attain or regain their native states (Mayer and Bukau, 2005; Slimen et al., 2014; Sreedhar et al., 2000).

Both ER stress and HSPs mechanisms acts on reproductive events like ovulation, oocyte maturation, fertilization, embryo development and late pregnancy (Khan et al., 2015; Lin et al., 2019; Song et al., 2012). However, it is unclear about the ER stress pathway and the protective effect of HSPs on bovine early pregnancy signaling. In the pre-implantation period occurs the major pregnancy losses (Wiltbank et al., 2016). Classically, in ruminants, the conceptus signals its presence between Days 10-20 after fertilization in the period named Maternal Recognition of Pregnancy (MRP). Trophoblast cells produces interferon tau (IFNT),

a cytokine which is responsible for the embryo-maternal interaction to avoid luteolysis (McCracken et al., 2012; Niswender et al., 2018; Oliveira et al., 2008). IFNT also signals to peripheral blood cells using type I IFN-pathway (Toji et al., 2017). Our previous data indicate that HS impairs endocrine MRP signaling in peripheral blood cells and induces oxidative stress (Amaral et al., 2021).

Considering the cascade of events generated by HS and oxidative stress on early pregnancy, we hypothesized that oxidative stress caused by heat stress negatively impacts endoplasmic reticulum, leading to ER stress and also induces heat shock proteins protective response in peripheral blood mononuclear cells (PBMC) from early pregnant cows. The aim of this study is to evaluate early pregnancy modulation of ER stress markers and HSPs response in PBMC in dairy cows, and whether this modulation is affected by heat stress occurrence.

2. Material and methods

2.1. Chemicals

Unless otherwise indicated, chemicals and reagents were purchased from Sigma Chemical Company (Sigma-Aldrich, St. Louis, MO, USA).

2.2. Cows and herd management

The study was conducted on a commercial dairy farm in Southern Brazil. Thirty-two multiparous Holstein dairy cows in lactation from the same herd were included in the study. Cows were 3 to 6 years old, body condition score greater than 2.5 (1=thin and 5=fat in a scale 1 to 5), absent of any evident reproductive and clinical disorders during the study period. Cows were milked twice a day and fed complete ration and corn silage, with access to water

106 *ad libitum*. The study was approved by the Animal Care Use and Committee (CEUA-UFSM #
107 5728120217) of Federal University of Santa Maria.
108
109 **2.3. Experimental design, synchronization protocol and artificial insemination (AI)**
110 The experiment was conducted during two distinct seasons. The comfort group
111 samples (n=15) were collected in late winter/early spring, when the temperature-humidity
112 index (THI) was approximately 65-70 in Southern Brazil. The samples from the heat stressed
113 group (n=17) were collected in summer characterized by high temperatures associated with
114 high humidity, when THI was approximately 80-85. Both groups had their estrus
115 synchronized with the same protocol (Pereira et al., 2015). Only animals that exhibited
116 standing estrus by 48 hours after intravaginal device withdrawal were included in the
117 experiment (Comfort cows group n=12; Heat Stressed cows group n= 13). AI was performed
118 48 hours (Day 0) after intravaginal device withdrawal. The semen was purchased from a
119 commercial company, assessed, stored in liquid nitrogen, and thawed at 36°C for 30 seconds
120 for subsequent AI (Amaral et al., 2021).
121
122 **2.4. Blood sample collection and isolation of peripheral blood mononuclear cells**
123 **(PBMCs)**
124 Blood was collected from the coccygeal vein using a 21G needle coupled to a vacuum
125 collection system (BD Vacutainer®) into 4 mL EDTA-containing tubes. The collections were
126 performed on Days 10, 14 and 18 following AI. Blood was obtained in two tubes of 4 mL
127 containing EDTA for each experimental time point. Isolation of PBMCs was performed as
128 follows. Briefly, after blood collection, 2 mL of whole blood was diluted in equal volume of
129 0.9% NaCl, followed by addition of 3mL of Ficoll-Paque PREMIUM®. Centrifugation was
130 performed at 400xg for 15 minutes at room temperature. After centrifugation, the following

layers were obtained: PBMC, Ficoll-Paque, PMN, and erythrocytes. PBMCs were collected from the higher white layer. PBMCs samples were stored in a cryotube at -80°C for subsequent total RNA extraction. After isolation of PBMC fraction, a glass-slide fraction-film was prepared to determine the purity of each fraction. Slides were stained using a rapid stain (Diff-Quik Differential Stains Set; Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendations. The cell fraction purity was accessed based on cell morphology. PBMCs are classified as lymphocytes and monocytes. An experienced clinical pathologist examined the slides. A differential cell count was done by identifying 100 consecutive leukocytes using a 100x objective. Samples above 95% of specific cell type (PBMC) were included in this study.

2.5. RNA extraction, reverse transcription, and real-time PCR

Total RNA was extracted from the PBMCs using Tri Reagent (BD), according to the manufacturer's recommendations. Quantification and estimation of RNA purity was performed using Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA; Absorbance 260/280 nm ratio). RNA was treated with DNAse Amplification Grade (Thermo Fisher, Waltham, MA, USA) for 15 minutes at 27ºC to degrade any DNA molecules. DNAse was inactivated with 1 µl EDTA for 10 minutes at 65ºC. Reverse transcription was performed using iScript cDNA synthesis Kit (BioRad, Hercules, CA, USA) for 5 minutes at 25ºC followed by 30 minutes at 42ºC and 5 minutes at 85ºC. Quantitative polymerase chain reaction (qPCR) was conducted in a thermocycler (BioRad, Hercules, CA, USA) using cDNA, forward and reverse bovine specific primers and GoTaq® Master Mix (Promega Corporation, Madison, USA). Amplification was performed with initial denaturation at 95ºC for 5 minutes followed by 40 cycles of denaturation at 95ºC for 15 seconds and annealing/extension at 60ºC for 30 seconds. To optimize the RT-qPCR assays, serial dilutions

of cDNA templates were used to generate a standard curve, and efficiency between 90 and 110% was considered. Samples were run in duplicate and the results of expression of all analyzed genes were expressed relative to geometric mean of *GAPDH* and *ACTB* reference genes (Pfaffl, 2001). The genes assessed in this study are presented in Table 1.

2.6. Pregnancy diagnosis by ultrasound scanning

In both groups, uteri were evaluated using a Mindray DP10 ultrasound with a 6.5 MHz linear transducer to select only animals free of any evident pathology. Pregnancy rate was determined by dividing the number of pregnant cows at the pregnancy diagnosis at 30- and 60-days following AI by the total number of cows artificially inseminated (P/AI).

2.7. Statistical analysis

Continuous data were checked for normality using Shapiro-wilk test. mRNA expression and oxidative stress data were analyzed by repeat measures for within-group analysis and standard least squares for between-group (comfort cows vs. heat stressed cows and non-pregnant vs. pregnant cows) analysis. The main effects of day, pregnancy diagnosis (PD), treatment group, day by group interaction (day*group), or day by pregnancy diagnosis interaction (day*PD) were indicated. All data analysis was performed using the JMP7 Software (SAS Institute Inc., Cary, NC, USA). Results are presented as mean ± standard error of the mean (SEM) and are considered different at $P<0.05$.

3. Results

3.1. Physiological and reproductive parameters of cows on comfort or under heat stress

It was observed that the THI was elevated during the heat stress environment, compared to the comfort environment. HS affected both RT, HR, and RR of dairy cows

(p<0.05), and this was evident on all measurement days. In relation to milk production, heat stressed cows had lower daily milk yield than the cows that were heat-not stressed. Effect of season on estrous occurrence and pregnancy rate were not different between groups (P>0.05). Estrus occurrence rate was 80% in comfort group and 76.47% in heat stressed group. Pregnancy rate was 50% in comfort group and 38.46% in heat stressed group (Amaral et al., 2021).

3.2. Effect of heat stress on endoplasmic reticulum stress marker mRNA expression in mononuclear cells from non-pregnant and pregnant cows on Days 10, 14 and 18 post-AI

Relative mRNA expression of *HSPA5*, *ATF6*, *uXBP1*, *sXBP1* and *CHOP* was evaluated in PBMCs of non-pregnant and pregnant cows on Days 10, 14 and 18 after AI of groups: comfort cows and heat-stressed cows (Fig 1). Under comfort condition (Fig 1 A-E), there is no difference between non-pregnant and pregnant cows in all genes evaluated on Days 10, 14 and 18 after AI (P>0.05). However, under heat stress condition (Fig 1 F-J), *HSPA5*, *sXBP1* and *CHOP* expression were upregulated in Day 10 in pregnant when compared to non-pregnant cows (P<0.05). There is no difference between expression of all analyzed genes on Days 14 and 18 comparing non-pregnant to pregnant cows (P>0.05). Also, *uXBP1* and *ATF6* were no different in all days (P>0.05).

Relative mRNA expression of *HSPA5*, *ATF6*, *uXBP1*, *sXBP1* and *CHOP* also was analyzed on heat stress or non-heat stress in non-pregnant and pregnant cows (Fig 2). In non-pregnant cows (Fig 2 A-E), *HSPA5* and *sXBP1* were upregulated in Day 10 in heat stressed compared to non-heat stressed cows (P<0.05), but in Days 14 and 18 there were no difference (P>0.05). Also, *ATF6*, *uXBP1* and *CHOP* expression there was no difference in all days (P>0.05). However, in pregnant cows (Fig 2 F-J), *HSPA5*, *ATF6*, *sXBP1* and *CHOP* were upregulated on Day 10 (P<0.05) in heat stressed when compared to non-heat stressed cows

206 (P<0.05), and on Days 14 and 18 there was no difference (P>0.05). *uXBP1* was not different
207 in all experimental days (P>0.05).
208
209 **3.3. Effect of heat stress on heat shock transcription factor and heat shock proteins**
210 **mRNA expression in mononuclear cells from non-pregnant and pregnant cows on Days**
211 **10, 14 and 18 post-AI**
212 Relative mRNA expression of *HSF, HSP70* and *HSP90* was evaluated in PBMCs of
213 non-pregnant and pregnant cows on Days 10, 14 and 18 after AI of comfort cows and heat
214 stressed cows (Fig 3). Cows on comfort (Fig 3 A-C), *HSP90* had greater expression on Days
215 10, 14 and 18 after AI (P<0.05). However, *HSF* and *HSP70* were not different (P>0.05); and
216 cows under heat stress condition (Fig 3 D-F), *HSF* was not different (P>0.05) and *HSP70* and
217 *HSP90* were upregulated in pregnant cows on Days 10 and 18 (P<0.05).
218 Relative mRNA expression of *HSF, HSP70* and *HSP90* also was analyzed comparing
219 the occurrence of heat stress or non-heat stress in non-pregnant and pregnant cows (Fig 4). In
220 non-pregnant cows (Fig 4 A-C), no differences in heat stress and non-heat stress were found
221 (P>0.05). However, in pregnant cows (Fig 4 D-F), *HSF* was upregulated on Day 10 after AI
222 from heat stressed cows (P<0.05). *HSP70* was upregulated on Days 14 and 18 after AI from
223 HS cows (P<0.05) and *HSP90* was upregulated on Days 10, 14 and 18 after AI from HS cows
224 (P<0.05).
225
226 **4. Discussion**
227 This article characterizes ER stress and HSPs response on PBMCs of heat-stressed
228 dairy cows on Days 10, 14 and 18 following AI, period that coincides with the endocrine
229 signaling of pregnancy. Firstly, we validated experimental model, allowing to evaluate the
230 influence of either cold or warm season of the year on temperature-humidity index (THI), and

physiological parameters rectal temperature (RT), heart rate (HR) and respiratory rate (RR). Cows naturally exposed to the heat challenge in this experiment had increased body temperature, with significant increases in RT, HR and RR and a decline in daily milk yield compared to cows in a comfort environment. Our previous studies demonstrated that the expression of type I IFN pathway and ISGs genes in PMNs of pregnant cows on comfort increased in time-dependent manner, reaching a peak on Day 18. Whilst the non-pregnant cows had no increase in the expression of the same targets. The expression of type I IFN pathway and ISGs in PMNs from heat-stressed cows did not differ between non-pregnant and pregnant cows on all days. Also, oxidative stress occurs in heat stressed cows, independent of pregnancy status. This led us to conclude that oxidative stress was induced by heat stress, but not by pregnancy (Amaral et al., 2021). For this reason, we assessed ER stress and HSPs profile in this early pregnancy *in vivo* model.

In order to measure mRNA expression responses *in vivo*, non-invasive sample collect was done to minimize the impact on experimental animals, so that the parameters evaluated were not masked by the experimental design, but that the expression of the ER stress and HSP response was reliable. Our results presented herein revealed significant findings: On comfort, there are no differences in ER stress pathway comparing non-pregnant and pregnant cows in all experimental days. Among HSP responses, *HSP90* was greater in pregnant when compared to non-pregnant cows. Under HS conditions, *HSPA5, uXBP1* and *CHOP* increased on Day 10 pregnant cows. Also, *HSP70* and *HSP90* were both upregulated in pregnant cows. Considering only non-pregnant cows, *HSPA5* and *sXBP1* increased on Day 10 of heat stressed cows and there were no differences in HSPs response. However, in pregnant cows *HSPA5, sXBP1, ATF6* and *CHOP* were upregulated on Day 10 heat stressed cows; and the HSPs responses, *HSF, HSP70* and *HSP90* were upregulated in heat stressed cows.

Early pregnancy by itself is a stressful event. Physiological, biochemical and immunological adaptations occur during this period so that the embryo can survive and develop (Hansen, 2011; Nagaoka et al., 2000; Wiltbank et al., 2016). For this reason, unbalanced oxidative stress occurs. There is some understanding of how ROS affect a variety of physiologic functions (i.e. oocyte maturation, ovarian steroidogenesis, ovulation, implantation, formation of blastocyst, luteolysis and luteal maintenance in pregnancy) (Agarwal et al., 2005). Warm conditions also seem to affect the oxidative stress in cows (Slimen et al., 2016). Heat stress impairs maternal recognition signaling and induces oxidative stress, according studies from our group (Amaral et al., 2021, 2020). Therefore, we consider that ER stress and the HSP response are induced by a consequence of oxidative stress in these cows (Gao et al., 2012).

Comfort cows (non-pregnant and pregnant) do not have modifications on ER stress pathway, suggesting no difference in all mRNA expression. Also, non-pregnant heat stressed cows had no difference on ER stress genes. In contrast, heat-stressed pregnant cows increase *HSPA5, sXBP1* and *CHOP* expression. Analyzing only non-pregnant cows, heat-stressed cows upregulate *HSPA5* and *sXBP1*. Although when we analyze only pregnant cows, *HSPA5, sXBP1, ATF6* and *CHOP* increase. It means *HSPA5* and *sXBP1* were upregulated by heat stress, independent of pregnancy status. But *ATF6* and *CHOP* were upregulated by the pregnancy and HS associated. HSPA5 chaperone have antiapoptotic property and its elevation under various stress conditions suggests your involvement in cell survival (Pfaffenbach and Lee, 2012; Wang et al., 2009). HSPA5 is also known to be an important component in modulating the UPR. Under conditions of ER stress, HSPA5 is released from the UPR sensors (ATF6, PERK, and IRE1), leading to their activation. The activated UPR relieves ER stress by decreasing protein translation and increasing the folding capacity of the ER, and consequently upregulates HSPA5 to repeat this process. Activated IRE1 encoding the XBP1

and thus through to be an important marker reflecting IRE1 signaling response (Hirota et al., 2006). It suggests that HS activate the IRE1 sensor of UPR, due to the increase in *HSPA5* and *sXBP1* expression and it is occurring due to the attempted survival and adaptation of the cell affected by heat stress. Importantly, if ER homeostasis cannot be restored, the UPR is capable of inducing apoptosis by increase of caspases and CHOP pathway (Wang et al., 2009). So, pregnancy and HS associated upregulate *ATF6* and *CHOP* expression in PBMC. Both ATF6 and IRE1 coordinate downstream components which initially promote cell-protective events; and CHOP can be mediating apoptosis in this case (Hu et al., 2019; Li et al., 2014).

Moreover, under conditions of hyperthermia, ER stress can occur simultaneously to heat shock protein (HSP) response (Xu et al., 2011). If HS exceeds the cell's ability to limit damage, the highly conserved process of apoptosis is initiated to remove damaged cells and maintain tissue function. But first, the cellular stress response function is initiated which limits damage and supports cell recovery (Jee et al., 2021). In comfort cows, *HSF* and *HSP90* were not different between groups and only *HSP90* was upregulated in pregnant cows. But in heat-stressed cows, both *HSP70* and *HSP90* were greater in pregnant cows. It suggests *HSP90* expression is controlled by pregnancy, not by HS. It was postulated HSP90 is an ATP-dependent chaperone protein involved in regulating the stability of a wide range of proteins, including the JAK/STAT pathway (Fiskus et al., 2011; Marubayashi et al., 2010). It is known that JAK/STAT pathway may be being expressed in leukocytes from pregnant cows (Amaral et al., 2021), supporting this association. Therefore, in non-pregnant cows, there was not different in all HSP gene response analyzed. However, in pregnant heat-stressed cows, *HSF*, *HSP70* and *HSP90* were greater comparing to comfort pregnant cows. In other words, what influences the HSF and HSP70 response is not only the HS, but the occurrence of the HS and pregnancy associated.

In conclusion, the findings of the present study support our hypothesis that oxidative stress caused by HS trigger ER stress on PBMCs and it is intensified with the presence of two associated stressors: early pregnancy and HS occurrence, which activates apoptosis pathway. Although the ER stress response apparently downregulated after Day 10, PBMCs continue to respond to HS and pregnancy by HSPs response, which remains high in subsequent days.

Declaration of interest

The authors have nothing to declare.

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Author Contributions

CSA and AQA designed the study. CSA did all the experimental procedures. MWM and ALP did blood samples collects. VMP and CSA performed the statistical analyzes. AQA and VMP obtained funding. CSA wrote the draft. CSA, AQA and VMP developed the final version of the manuscript. All authors read and approved the final version of the manuscript.

329 **Figure legends**

330

331 **Fig 1. Endoplasmic reticulum stress marker mRNA expression in mononuclear cells of**
332 **comfort or heat stressed cows sorted in non-pregnant and pregnant on Days 10, 14 and**
333 **18 post-AI.** A-E represents ER stress markers of cows in comfort conditions; F-J represents
334 ER stress markers of heat stressed cows. A and F: *HSPA5;* B and G: *ATF6;* C and H: *uXBP1*;
335 D and I: *sXBP1;* E and J: *CHOP*. Values are presented as mean ± S.E.M. The main effects of
336 pregnancy diagnosis (PD), day and day by pregnancy diagnosis interaction (day*PD) are
337 indicated. Asterisk represents difference at P<0.05 between non-pregnant and pregnant cows.

338

339 **Fig 2. Endoplasmic reticulum stress marker mRNA expression in mononuclear cells of**
340 **non-pregnant and pregnant cows sorted in comfort or heat stressed on Days 10, 14 and**
341 **18 post-AI.** A-E represents ER stress components of non-pregnant cows; F-J represents ER
342 stress components of pregnant cows. A and F: *HSPA5;* B and G: *ATF6;* C and H: *uXBP1*; D
343 and I: *sXBP1;* E and J: *CHOP*. Values are presented as mean ± S.E.M. The main effects of
344 day, group and day by group interaction (day*group) are indicated. Asterisk represents
345 difference at P<0.05 between comfort and heat stressed group.

346

347 **Fig 3. Heat shock transcription factor and heat shock proteins mRNA expression in**
348 **mononuclear cells of comfort or heat stressed cows sorted in non-pregnant or pregnant**
349 **on Days 10, 14 and 18 post-AI.** A-C represents HSF and HSPs of comfort cows; D-F
350 represents HSF and HSPs components of heat stressed cows. A and D: *HSF*; B and E: *HSP70*;
351 C and F: *HSP90*. Values are presented as mean ± S.E.M. The main effects of pregnancy
352 diagnosis (PD), day and day by pregnancy diagnosis interaction (day*PD) are indicated.
353 Asterisk represents difference at P<0.05 between pregnant and non-pregnant cows.

354

355 **Fig 4. Heat shock transcription factor and heat shock proteins mRNA expression in**
356 **mononuclear cells of non-pregnant or pregnant cows sorted in comfort or heat stressed**
357 **cows on Days 10, 14 and 18 post-AI.** A-C represents HSF and HSPs of non-pregnant cows;
358 D-F represents HSF and HSPs components of pregnant cows. A and D: *HSF*; B and E:
359 *HSP70*; C and F: *HSP90*. Values are presented as mean ± S.E.M. The main effects of day,
360 group and day by group interaction (day*group) are indicated. Asterisk represents difference
361 at $P<0.05$ between comfort and heat stressed group.

Tables

Table 1. Primers designed for quantitative real-time PCR analysis.

Target	Primer sequence	GenBank
HSPA5	F: CGTGCGTTTGAGAGCTCAGT R: GACAGCTTCATCTTTCCAGCG	NM_001075148.1
ATF6	F: GAACTTCGAGGATGGGTTCATAGG R: CCAGAGCACCCTGAAGAATACG	XM_024989877.1
uXBP1	F: GCAGAGACCAAGGGGAATGG R: GGGTCCAAGTTGAACAGAATGC	NM_001034727.3
sXBP1	F: AGCAGAGACCAAGGGGAATG R: TCAGAGTCCATGGGGAGATGT	NM_001271737.1
CHOP	F: GGTGCTGTCCTCAGATGAAAATCG R: GGTCCTGGCTCCTCAGTAAGC	NM_001078163.1
HSF	F: ACCTGGACAACCTGCAGACC R: GGAGCTCCTGGATCTGGCTG	NM_001076809.1
HSP70	F: CTTCAACATGAAGAGCGCCG R: TGATGGGGTTACACACCTGC	NM_203322.3
HSP90	F: GAGGAAACACTCTCGGACGG R: TCGGTCTTGCTGCTCCATAC	NM_174700.2
GAPDH	F: GCCATCAATGACCCCTTCAT R: TGCCGTGGGTGGAATCA	NM_001034034.2
ACTB	F: GGATGAGGCTCAGAGCAAGAGA R: TCGTCCCAGTTGGTGACGAT	NM_173979.3

F: Forward; R: Reverse.

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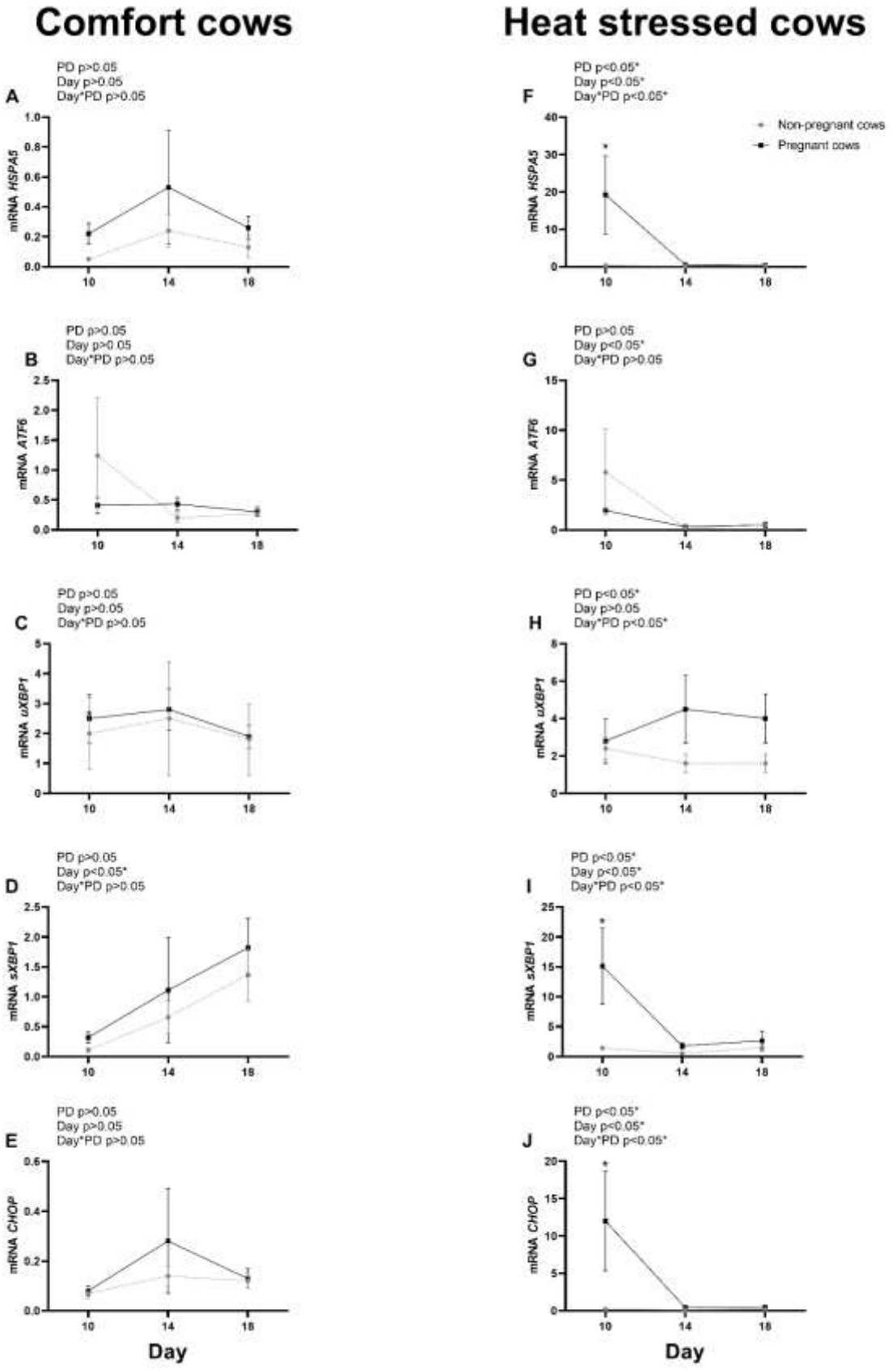
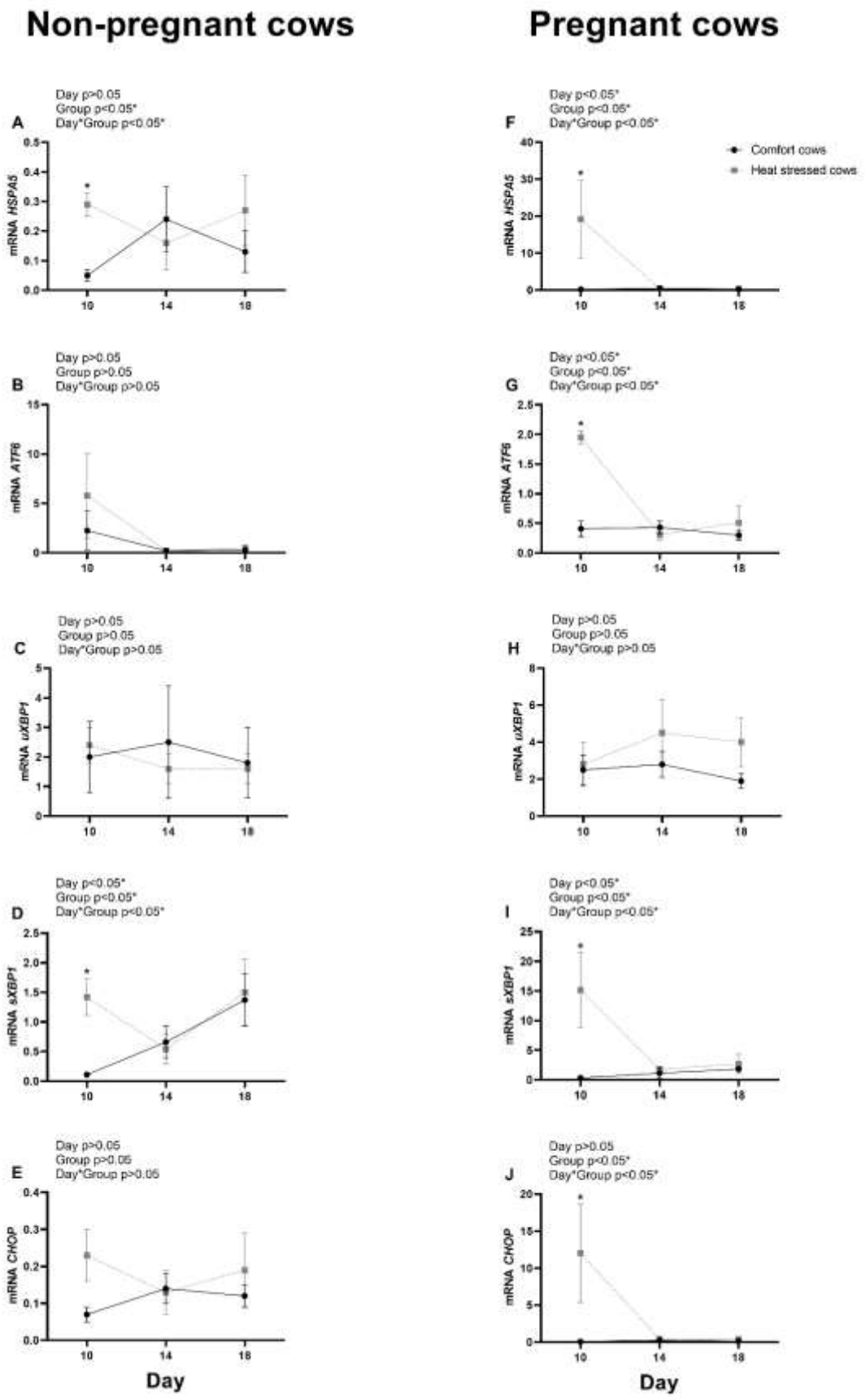
511

512 **Fig 1.**


Comfort cows
Heat stressed cows
A
PD p>0.05
Day p>0.05
Day*PD p>0.05
mRNA HSPA5
B
PD p>0.05
Day p>0.05
Day*PD p>0.05
mRNA ATF6
C
PD p>0.05
Day p>0.05
Day*PD p>0.05
mRNA sXBP1
D
PD p>0.05
Day p<0.05*
Day*PD p>0.05
mRNA sXBP1
E
PD p>0.05
Day p>0.05
Day*PD p>0.05
mRNA CHOP
F
PD p<0.05*
Day p<0.05*
Day*PD p<0.05*
Non-pregnant cows
Pregnant cows
mRNA HSPA5
G
PD p>0.05
Day p<0.05*
Day*PD p>0.05
mRNA ATF6
H
PD p<0.05*
Day p>0.05
Day*PD p<0.05*
mRNA sXBP1
I
PD p<0.05*
Day p<0.05*
Day*PD p<0.05*
mRNA sXBP1
J
PD p<0.05*
Day p<0.05*
Day*PD p<0.05*
mRNA CHOP
10
14
18
Day

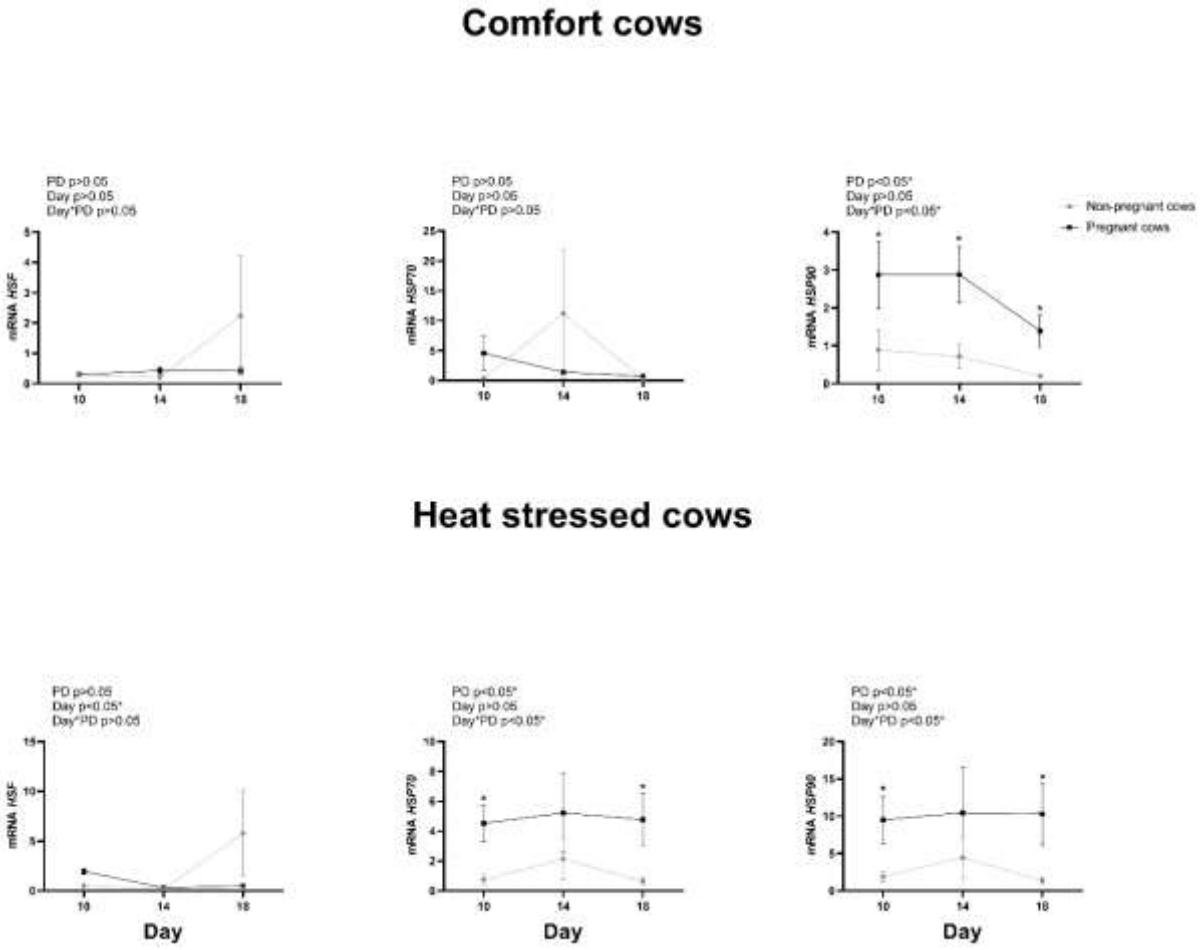
513

514 **Fig 2.**


Non-pregnant cows
Pregnant cows
A
Day p>0.05
Group p<0.05*
Day*Group p<0.05*
mRNA HSPA5
F
Day p<0.05*
Group p<0.05*
Day*Group p<0.05*
Comfort cows
Heat stressed cows
mRNA HSPA5
B
Day p>0.05
Group p>0.05
Day*Group p>0.05
mRNA ATF6
G
Day p<0.05*
Group p<0.05*
Day*Group p<0.05*
mRNA ATF6
C
Day p>0.05
Group p>0.05
Day*Group p>0.05
mRNA uXBP1
H
Day p>0.05
Group p>0.05
Day*Group p>0.05
mRNA uXBP1
D
Day p<0.05*
Group p<0.05*
Day*Group p<0.05*
mRNA sXBP1
I
Day p<0.05*
Group p<0.05*
Day*Group p<0.05*
mRNA sXBP1
E
Day p>0.05
Group p>0.05
Day*Group p>0.05
mRNA CHOP
J
Day p>0.05
Group p<0.05*
Day*Group p<0.05*
mRNA CHOP
10
14
18
Day
Day

515

516 **Fig 3.**



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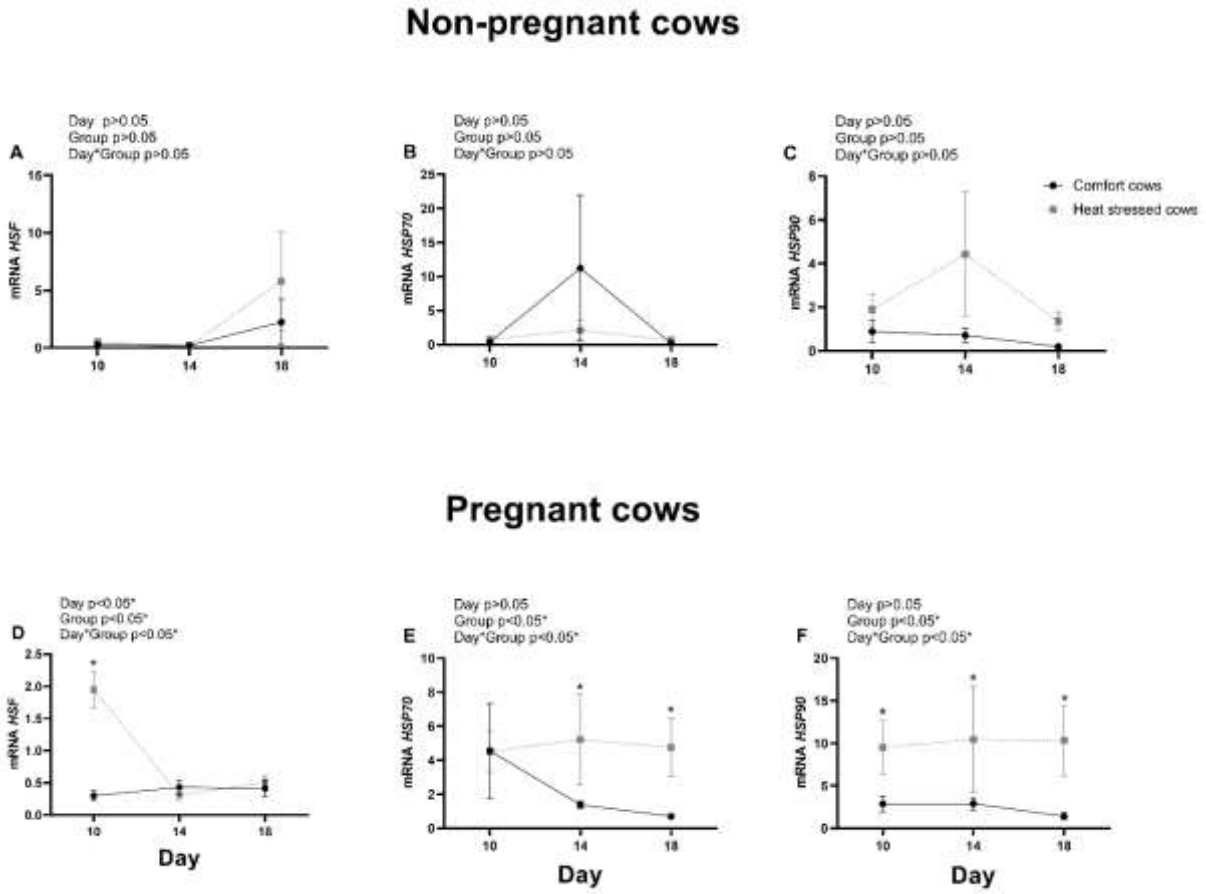
519 **Fig 4.**



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3. DISCUSSÃO

Perdas gestacionais são responsáveis por reduzir o desempenho reprodutivo em rebanhos leiteiros, ocorrendo com maior frequência nos primeiros 30 dias de gestação (AYALON, 1978; WILTBANK *et al.*, 2016). Uma das principais causas de perda embrionária e falha de implantação durante o início da prenhez é o estresse térmico causado pelo aumento das temperaturas durante as estações quentes (DE RENSIS *et al.*, 2015; WOLFENSON; ROTH; MEIDAN, 2000). Os efeitos do estresse térmico no desenvolvimento folicular, esteroidogênese e qualidade de gametas já foram demonstrados (AL-KATANANI; PAULA-LOPES; HANSEN, 2002; BRIDGES; BRUSIE; FORTUNE, 2005; LUCIO *et al.*, 2016; ROTH, 2016). Embriões bovinos produzidos *in vitro* demonstraram inibição do desenvolvimento quando expostos a altas temperaturas, reduzindo as taxas de clivagem e blastocisto (RIVERA, 2004). Nesse sentido, nosso grupo de pesquisa busca entender e caracterizar os efeitos do estresse térmico em vias de sinalização durante o reconhecimento materno da gestação.

No primeiro trabalho do nosso grupo, demonstramos os efeitos do estresse térmico em embriões produzidos *in vitro*. Nesse experimento, a temperatura de 40,5°C durante 6 horas foi estabelecida como modelo para induzir estresse térmico com base em resultados que mostram a variação de temperatura corporal de vacas em lactação durante o verão (NABENISHI *et al.*, 2012). Embriões foram submetidos ao estresse térmico nas diferentes etapas da produção *in vitro*: maturação oocitária, fertilização, primeiro dia de cultivo embrionário, e um grupo que foi estressado durante as 3 etapas consecutivas. Comparados a um grupo controle mantido a 38,5ºC, os grupos submetidos ao estresse térmico tiveram menores taxas de clivagem e blastocistos desenvolvidos no dia 7, menor expressão de RNAm e proteína de IFNT, aumento da produção de ROS, gerando estresse oxidativo (AMARAL *et al.*, 2020). A partir desse primeiro artigo que apresenta resultados *in vitro*, buscamos investigar os efeitos do estresse térmico na via de sinalização endócrina do reconhecimento materno da gestação, em leucócitos do sangue periférico em um modelo *in vivo*.

A presente tese apresenta 3 artigos desenvolvidos a partir de um experimento. O experimento foi conduzido dentro de uma mesma fazenda em duas estações do ano distintas. As vacas do grupo conforto térmico foram coletadas em setembro e as vacas em estresse térmico coletadas em janeiro, onde foi levado em consideração o índice de temperatura e umidade (*Temperature-humidity index;* THI) de cada época. Alto THI pode não apenas

diminuir a produção de leite, mas também afetar o desempenho reprodutivo e o sistema imunológico de vacas em lactação (MIN *et al.*, 2016). Portanto, o primeiro trabalho da tese buscou investigar se o estresse térmico estaria influenciando na sinalização endócrina do reconhecimento materno da gestação em células PMN do sangue periférico, pela análise da expressão de RNAm da via dos IFNs tipo 1 e ISGs. Existem estudos que comparam a expressão de ISGs nos leucócitos do sangue periférico com a gestação precoce (GREEN *et al.*, 2010; HAQ *et al.*, 2016; SHEIKH *et al.*, 2018; TOJI *et al.*, 2017). A expressão de RNAm foi analisada em frações separadas de leucócitos concluíram que a fração de células PMN são mais sensíveis ao estímulo por IFNT quando comparados à fração de PBMC (KIZAKI *et al.*, 2013). Por essa razão, no primeiro trabalho foi utilizada apenas a fração de PMNs. Os resultados revelam que vacas prenhes em ambiente de conforto possuem aumento da expressão de RNAm da via dos IFNs tipo 1 e dos ISGs de maneira tempo-dependente, exibindo um pico de expressão no dia 18 após a IA, indicando a regulação positiva da prenhez na expressão desses genes. Porém, o mesmo padrão de expressão não foi observado em vacas prenhes em condições de estresse térmico. PMNs são conhecidos por serem a primeira resposta no processo inflamatório (MANTOVANI *et al.*, 2011). Dessa forma, possivelmente essas células podem estar sendo afetadas pela ocorrência do estresse térmico.

Ainda no primeiro artigo, também investigamos se as altas temperaturas durante o verão afetam o equilíbrio oxidativo nesses animais, visto que relatamos que o estresse oxidativo ocorre em embriões submetidos a altas temperaturas *in vitro* (AMARAL *et al.*, 2020). Os resultados mostram que os níveis de malondialdeído (MDA) são maiores em vacas sob estresse térmico comparados aos níveis das vacas conforto térmico, enquanto a atividade das enzimas antioxidantes SOD e CAT não teve um aumento proporcional, indicando estresse oxidativo nas vacas em condições de estresse térmico, independente do *status* gestacional. Sabe-se que a exposição ao estresse térmico resulta em maiores níveis mitocondriais e plasmáticos de MDA, o principal produto da peroxidação lipídica (SAKATANI *et al.*, 2017; SLIMEN *et al.*, 2014). Foi demonstrado que o MDA pode ser usado como um marcador sanguíneo para o *status* oxidativo de vacas leiteiras durante períodos de calor (BERNABUCCI *et al.*, 2002). Embora estudos mostrem aumento de enzimas antioxidantes em situações de hipertermia, há um estudo mostrando a diminuição das enzimas SOD e CAT, o que resultou em uma redução na resistência térmica (SLIMEN *et al.*, 2014). Os resultados do artigo 1 demonstram que o processo enzimático antioxidante aparentemente não foi eficaz em vacas sob estresse térmico.

A modulação da resposta imune na gestação precisa encontrar um ajuste fino para não desequilibrar o *crosstalk* materno-embrionário, mas também ser capaz de oferecer proteção

contra patógenos. Vários estudos demonstraram a importância da modulação do sistema imunológico durante a gestação. As respostas imunológicas variam de anti- a pró-inflamatórias (BAUERSACHS *et al.*, 2012, 2006; FIORENZA *et al.*, 2021a; FIORENZA *et al.*, 2021b), e devem ser avaliadas de acordo com o estágio específico, por exemplo, reconhecimento materno, implantação, placentação ou gestação a termo (FIORENZA *et al.*, 2021b; TALUKDER *et al.*, 2018). Baseado nesses fatos e somados aos resultados obtidos no primeiro trabalho, nós buscamos caracterizar o perfil inflamatório de vacas gestantes submetidas ao estresse térmico. Fisiologicamente durante o período de reconhecimento materno da gestação, o IFNT modula a resposta imune materna, aumentando citocinas anti-inflamatórias, como IL10 e TGFB, e reduzindo a expressão da citocina pró-inflamatória TNFA (AZIZIEH; RAGHUPATHY, 2015; HOP *et al.*, 2018). Outro estudo mostrou que o IFNT derivado de embriões dia 7 induzem resposta anti-inflamatória e regulação positiva de ISGs em PBMC (TODER *et al.*, 2003). Portanto, sugere-se que o IFNT induz a tolerância imunológica essencial para a sobrevivência e desenvolvimento do embrião. Os resultados do artigo 2 fornecem evidências de que o estresse térmico modula a resposta inflamatória em PMNs, com o aumento de RNAm das citocinas pró-inflamatórias IL17, NFKB e IL1B e citocinas anti-inflamatórias ARG1, TGFB e IFNA em vacas no início da gestação. Além disso, IFNG e INOS foram regulados por exclusivamente pela ocorrência do estresse térmico; e IL10 foi regulado exclusivamente pela prenhez. Os resultados apresentados sugerem que ARG1, TGFB e IFNA oferecem um papel protetor durante a gestação de vacas em estresse térmico, ao mesmo tempo que IL17, NFKB e IL1B atuam induzindo uma resposta pró-inflamatória, que pode ser prejudicial à manutenção e desenvolvimento do embrião.

Posteriormente nós desenvolvemos o terceiro trabalho para avaliar se o estresse oxidativo, demonstrado no primeiro artigo, foi capaz de induzir o estresse de RE e a resposta proterora das HSPs. Para esse artigo, utilizamos a fração sanguínea de PBMCs. Já foi relatado que o estresse oxidativo é um precursor e um dos principais contribuintes para a ocorrência de estresse do RE (HOTAMISLIGIL, 2010). O estresse de RE é um desequilíbrio entre a capacidade de alterações conformacionais das proteínas no RE e a carga proteica, o que consequentemente leva a alterações conformacionais ou proteínas desdobradas que acabam se acumulando no lúmen do RE, prejudicando sua homeostase (CNOP; FOUFELLE; VELLOSO, 2012). Outra resposta à estímulos estressantes é a indução da expressão das HSPs. Essas chaperonas auxiliam no remodelamento de uma proteína lesionada, evitando um dano celular letal (TAKENAKA; HIGHTOWER, 1992). As vacas do grupo conforto térmico, independente do *status* gestacional, não apresentam modificações na via UPR do estresse de RE, sugerindo

não haver estresse de RE. Além disso, vacas não prenhes do grupo estresse térmico também não tiveram alterações na via UPR. Porém, vacas prenhes do grupo estresse térmico aumentam a expressão de RNAm de HSPA5, sXBP1 e CHOP. Analisando apenas vacas não prenhes, vacas do grupo estresse térmico regulam positivamente HSPA5 e sXBP1. Embora quando analisamos apenas vacas prenhes, HSPA5, sXBP1, ATF6 e CHOP aumentam. Isso significa que HSPA5 e sXBP1 foram regulados positivamente pelo estresse térmico, independente do estado de gestação. Mas ATF6 e CHOP foram regulados positivamente pela gestação e estresse térmico associados. CHOP é uma molécula importante na via de apoptose induzida pelo estresse de RE (HU *et al.*, 2019). Esses achados indicam que o estresse oxidativo, consequência do estresse térmico, desencadeia estresse de RE em PBMCs e é intensificado com a presença de dois estressores associados: gestação precoce e ocorrência de estresse oxidativo, que juntos ativam a via CHOP de apoptose. Além disso, sob condições de hipertermia, o estresse do RE pode ocorrer simultaneamente à resposta das HSPs (XU *et al.*, 2011). Avaliamos duas chaperonas importantes no controle ao dano do estresse térmico: HSP90 e HSP70, além do fator de transcrição HSF. Nossos resultados sugerem que HSP90 é regulada pela gestação e não pelo estresse térmico, visto que esteve elevada também no grupo de vacas prenhes em conforto térmico. A gestação por si só já é um evento estressante e que pode contribuir para o estresse celular, gerando a necessidade da ação das chaperonas (HANSEN, 2011; NAGAOKA *et al.*, 2000). Também foi demonstrado que no grupo de vacas gestantes estressadas pelo calor, HSF, HSP70 e HSP90 foram maiores em comparação com vacas gestantes de conforto. Em outras palavras, o que influencia a resposta do HSF e HSP70 não é apenas o estresse térmico, mas a ocorrência do estresse térmico e a gestação associados.

A partir dos dados demonstrados nos 3 trabalhos e apresentados nessa tese, foi possível determinar que o estresse térmico altera os padrões de sinalização endócrina da gestação em PMNs de vacas submetidas ao estresse térmico, observados na expressão de RNAm da via dos IFNs tipo 1 e ISGs, além de gerar estresse oxidativo. Ainda, modifica o perfil inflamatório em PMNs de vacas gestantes submetidas ao estresse térmico, e induz estresse de RE. Além de caracterizar diferentes rotas de sinalização durante o período de reconhecimento materno da gestação, os resultados dessa tese abrem caminho para pesquisas futuras.

4. CONCLUSÃO

O estresse térmico diminui a produção de progesterona, altera a expressão de RNAm de ISGs e da via dos IFNs tipo 1 em PMNs e causa estresse oxidativo em vacas leiteiras no início da gestação. O estresse térmico em vacas prenhes não apenas altera a expressão de ISGs, mas também interfere na ativação da via dos IFNs tipo 1, impedindo que ocorra a sinalização para o reconhecimento materno da gestação em PMNs. Nosso estudo também demonstrou que o estresse térmico modifica a expressão de RNAm de genes anti e pró-inflamatórios em vacas prenhes. A resposta dos PMNs muda entre os padrões pró e anti-inflamatórios durante a condição de estresse por calor. E, ainda, o estudo corrobora com a hipótese de que o estresse oxidativo causado pelo estresse térmico desencadeia estresse de RE em PBMCs e é intensificado com a presença de dois fatores estressantes associados: gestação inicial e estresse térmico, que juntos ativam a via de apoptose. Portanto, os resultados mostrados nessa tese fornecem novas informações sobre os efeitos do estresse térmico na gestação inicial de vacas leiteiras.

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