UNIVERSIDADE FEDERAL DE PELOTAS
Faculdade de Veterinária
Programa de Pós-Graduação em Veterinária

Tese

Associações de mutações genéticas com a fertilidade, produção de leite, metabolismo e saúde de vacas leiteiras

Pedro Augusto Silva Silveira

Pelotas, 2018

Pedro Augusto Silva Silveira

Associações de mutações genéticas com a fertilidade, produção de leite, metabolismo e saúde de vacas leiteiras

Tese apresentada ao Programa de Pós-Graduação em Veterinária da Faculdade de Veterinária da Universidade Federal de Pelotas, como requisito parcial à obtenção do título de Doutor em Ciências (área de concentração: Sanidade Animal).

Orientador: Augusto Schneider

Coorientador: Walter Ronald Butler

Pelotas, 2018

Universidade Federal de Pelotas / Sistema de Bibliotecas
Catalogação na Publicação

S587a Silveira, Pedro Augusto Silva

Associações de mutações genéticas com a fertilidade, produção de leite, metabolismo e saúde de vacas leiteiras / Pedro Augusto Silva Silveira ; Augusto Schneider, orientador ; Walter Ronald Butler, coorientador. — Pelotas, 2018.

97 f. : il.

Tese (Doutorado) — Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, 2018.

1. Balanço energético negativo. 2. Inflamação. 3. SNP. I. Schneider, Augusto, orient. II. Butler, Walter Ronald, coorient. III. Título.

CDD : 636.234

Elaborada por Gabriela Machado Lopes CRB: 10/1842

Pedro Augusto Silva Silveira

Associações de mutações genéticas com a fertilidade, produção de leite, metabolismo e saúde de vacas leiteiras

Tese aprovada como requisito parcial para obtenção do grau de Doutor em Ciências, Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas.

Data da Defesa: 26/02/2018

Banca examinadora:

Prof. Dr. Augusto Schneider (Orientador)
Doutor em Biotecnologia pela Universidade Federal de Pelotas

Prof. Dr^{a}. Arione Augusti Boligon
Doutora em Genética e Melhoramento Animal pela Universidade Estadual Paulista Júlio de Mesquita Filho

Prof. Dr^{a}. Jorgea Pradieé
Doutora em Veterinária pela Universidade Federal de Pelotas

Prof. Dr^{a}. Monique Tomazele Rovani
Doutora em Medicina Veterinária pela Universidade Federal de Santa Maria

Agradecimentos

Primeiramente agradeço a Universidade Federal de Pelotas, instituição da qual sou discente há doze anos, e ao Programa de Pós-Graduação em Veterinária, que tem me acolhido desde o mestrado.

Agradeço ao Instituto Federal de Educação, Ciência e Tecnologia, *Campus* Pelotas – Visconde da Graça, local onde exerço a função de médico veterinário, por incentivar a formação contínua dos servidores. E agradeço, especialmente, ao meu colega e grande amigo Lucas Hax pelo apoio em todas as horas necessárias.

Agradeço a faculdade de nutrição e aos colegas do laboratório de genética, onde realizamos parte importante deste projeto.

Agradeço ao Augusto Schneider meu orientador desde o mestrado, amigo e exemplo de pesquisador e ser humano, pela paciência e por não medir esforços em transmitir conhecimentos e guiar-me até aqui. Obrigado por permitir que eu fizesse parte deste grande projeto!

Agradeço ao professor Walter Ronald Butler, meu orientador no doutorado sanduíche, pela receptividade na Universidade de Cornell (EUA), por contribuir fundamentalmente na execução dos projetos e por tudo que me ensinou.

Agradeço aos meus colegas pós-graduandos da Universidade de Cornell pela forma como fui recebido, em especial a Susanne pelo apoio nas atividades laboratoriais.

Agradeço aos meus amigos e familiares pela ajuda nos momentos de dificuldade e compreensão nos momentos que não pude estar presente.

Por fim, agradeço aos meus pais Sadi e Sônia e a minha irmã Cynara por estarem ao meu lado em todos os momentos, pelo apoio e dedicação incondicionais, por me inspirarem a continuar e jamais desistir, por todas as conversas, conselhos e por serem grandes exemplos. Enfim, dedico este trabalho a vocês.

Resumo

SILVEIRA, Pedro Augusto Silva. **Associações de mutações genéticas com a fertilidade, produção de leite, metabolismo e saúde de vacas leiteiras.** 2018. 97f. Tese (Doutorado em Ciências) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2018.

A seleção genética de bovinos leiteiros muito tem contribuído para o aumento dos índices produtivos da pecuária leiteira no Brasil e no mundo. Por outro lado, o incremento na produção leiteira tem aumentado alguns transtornos metabólicos do periparto das vacas, reduzindo a fertilidade após o parto. Além disso, a magnitude das respostas imune e algumas citocinas inflamatórias podem impactar negativamente o retorno das vacas a ciclicidade no início da fase de lactação. Neste sentido, os polimorfismos de nucleotídeo único (SNPs) são mutações genéticas causadoras de diferenças biológicas, sendo comumente associados a alterações de interesse produtivo e econômico para o melhoramento animal. Sendo assim, buscou-se avaliar a associação de mutações genéticas com a fertilidade, produção de leite, metabolismo e saúde de vacas da raça Holandês. No primeiro estudo foi avaliada a associação de mutações no promotor do gene da Paraoxonase 1 (*PON1*) com a atividade plasmática desta enzima, fertilidade, ingestão de matéria-seca, metabolismo, produção leiteira e saúde no periparto. Em seguida, foi avaliada a associação de mutações em genes do sistema imune e ligados ao transporte de energia celular com a fertilidade, produção leiteira e ingestão alimentar, metabolismo e saúde de vacas leiteiras. Por fim, foi avaliada a associação de mutações em genes relacionados ao eixo somatotrópico com a fertilidade, produção leiteira e metabolismo pós-parto. Os SNPs no promotor do gene da *PON1* tiveram efeito sobre a atividade da enzima no plasma, assim como SNPs nos genes do receptor do hormônio do crescimento (*GHR*) e fator de crescimento semelhante a insulina (*IGF-I*) impactaram os níveis de IGF-I no sangue. Os SNPs *PON1* -221, *PON1* -392, fator de necrose tumoral alfa (*TNF-α*), receptor tipo toll 4 (*TLR-4*), coenzima 9 (*COQ9*), *IGF-I* e a interação entre GHR/IGF-I tiveram efeito no intervalo parto-concepção. Além disso os SNPs *PON1* -22, *PON1* -221 e *COQ9* tiveram efeito na contagem de células somáticas e o SNP *TNF-α* teve efeito sobre os níveis circulantes de ácidos graxos não-esterificados (NEFA). Ainda, o SNP *COQ9* foi associado com a ingestão de matéria-seca pré-parto e a mutação no *IGF-I* teve efeito nos níveis circulantes de beta-hidroxibutirato (BHBA). Portanto, as mutações nos genes *PON1*, *TNF-α*, *TLR-4*, *COQ9* e *IGF-I*, relacionadas a maior atividade da PON1, maiores níveis plasmáticos de IGF-I e menores níveis de NEFA e BHBA tiveram impacto no intervalo parto-concepção, sem afetar a produção de leite.

Palavras-chave: balanço energético negativo; inflamação; SNP

Abstract

SILVEIRA, Pedro Augusto Silva. **Association between genetic mutations and fertility, milk production, metabolism and health of dairy cows.** 2018. 97f. Thesis (Doctor degree in Sciences) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2018.

The genetic selection of dairy cattle has greatly contributed to the increase of the productive indexes of dairy cattle in Brazil and around the world. On the other hand, the increase in milk production has contributed to some metabolic disorders in peripartum of cows, reducing fertility after calving. In addition, the magnitude of immune responses and inflammatory cytokine may negatively impact the return to early cyclicity lactation of dairy cows. In this sense, single nucleotide polymorphisms (SNPs) are genetic mutations that cause biological differences of productive and economic interest for animal breeding. Thus, we aimed to evaluate the association of genetic mutations with fertility, milk production, metabolism and health of Holstein cows. In the first study, the association of mutations in the Paraoxonase 1 (*PON1*) gene promoter with the plasma activity of this enzyme, fertility, dry matter intake, metabolism, milk production and health in the peripartum were evaluated. Next, we evaluated the association of mutations in genes of the immune system and linked to the transport of cellular energy with fertility, milk production, feed intake, metabolism and health of dairy cows. Finally, we evaluated the association of mutations in genes related to the somatotropic axis with fertility, milk production and postpartum metabolism. SNPs in the *PON1* gene promoter had an effect on the activity of the enzyme in plasma, as well as SNPs in the growth hormone receptor (*GHR*) and insulin like growth factor I (*IGF-I*) genes impacted blood IGF-I levels. The SNPs *PON1* -221, *PON1*-392, tumor necrosis factor alpha (*TNF-α*), toll-like receptor 4 (*TLR-4*), coenzyme 9 (*COQ9*), *IGF-I* and the interaction between GHR/IGF-I had an effect on the calving conception interval. In addition, the SNPs *PON1* -22, *PON1* -221 and *COQ9* had an effect on milk somatic cell count and the *TNF-α* SNP had an effect on serum non-esterified fatty acids (NEFA) levels. Also, *COQ9* SNP was associated with prepartum dry matter intake and the IGF-I mutation had an effect on serum beta-hidroxibutirate (BHBA). Therefore, the mutations in the genes *PON1*, *TNF-α*, *TLR-4*, *COQ9* and *IGF-I*, related to higher PON1 activity, higher plasma levels of IGF-I and lower levels of NEFA and BHBA had an impact on the calving to conception interval, without affecting milk production.

Keywords: negative energy balance; inflammation; SNP

Lista de Figuras

Artigo 1

Artigo 2

Artigo 3

Lista de Tabelas

Artigo 1

Artigo 2

Artigo 3

Lista de Abreviaturas e Siglas

AI	Inseminação artificial
ApoAI	Apolipoproteína AI
ARMS	Amplification refractory mutation sistem
BHBA	Beta-Hidroxibutirato
bp	Pares de bases nitrogenadas
CCI	Intervalo parto-concepção
COQ9	Coenzima Q9
DIM	Dias em lactação
dL	Decilitros
DNA	Ácido desoxirribonucleico
DTO	Dias até a ovulação
EDTA	Etilenodiaminotetracético
ESR1	Receptor do estradiol
FAO	Organização das Nações Unidas para Agricultura e Alimentação
g	Gramas
GH	Hormônio do Crescimento
GHR	Receptor do hormônio do crescimento
GnRH	Hormônio liberador de gonadotrofina
h	Horas
HCl	Ácido clorídrico
HDL	Lipoproteína de alta densidade
IGF-I	Fator do crescimento semelhante a insulina
IGFIR	Receptor do IGF-I
IL	Interleucina
IVF	Fertilização in vitro

JAK	Tirosina quinases da família Janus Kinase
kg	Quilos
L	Litros
LH	Hormônio luteinizante
LPS	Lipopolissacarídeos de membrana
mg	Miligramas
min	Minutos
ml	Mililitros
mmol	Milimolar
mRNA	Ácido ribonucleico mensageiro
NEFA	Ácidos graxos não esterificados
ng	Nanogramas
NGS	Next generation system
P4	Progesterona
PCR	Reação em cadeia da polimerase
PFA	Proteína de fase aguda
PON1	Paraoxonase 1
QTL	Quantitative trait loci
RFLP	Restriction fragment length polymorphism
ROS	Espécies reativas de oxigênio
SCC	Contagem de células somáticas do leite
secs	Segundos
SEM	Erro padrão da média
SNP	Single nucleotide polymorphism
STAT5A	Signal transducers and activators of transcription 5A
TAE	Tampão tris borato EDTA
TLR-4	Receptor tipo toll 4
TNF-α	Tumor necrosis factor α
Tris	Trisaminometano
U	Unidades internacionais
umol	Micromolar
USA	Estados Unidos da América
USDA	Departamento de agricultura dos Estatos Unidos

Sumário

1 Introdução

A produção mundial de alimentos lácteos vem aumentando com o passar dos anos, acompanhando o crescimento da população mundial. O leite, além de ser considerado fonte de nutrientes essenciais para a alimentação humana, também tem impacto sobre a economia da maioria dos países, principalmente países considerados em desenvolvimento e baseados na agricultura familiar. A produção mundial de leite aumentou mais de 50% nas últimas três décadas, alcançando a marca de 831 milhões de toneladas em 2017 (FAO, 2017). No Brasil, a produção de leite esteve em sintonia com o desenvolvimento econômico do pais.

O Brasil possui o maior rebanho comercial de bovinos do mundo, contando com aproximadamente 220 milhões de animais em 2017, dos quais 23% (52 milhões) constituem o efetivo da pecuária leiteira (USDA, 2017). Cerca de 24 milhões de vacas, pertencentes a 1,4 milhão de produtores, produziram cerca de 34 bilhões de litros de leite, em 2017, colocando o país na quinta colocação no ranking mundial de produção leiteira (USDA, 2017). Contudo, a pecuária leiteira nacional ainda é caracterizada pela baixa produtividade dos rebanhos, visto que o aumento do volume de leite produzido ao longo dos anos ocorreu, em grande parte, devido ao aumento do número de vacas ordenhadas e não por melhoria de produtividade. Porém, fatores como a elevação do valor da terra, fim da fronteira agrícola e aumento nos custos de produção da pecuária leiteira nos últimos anos têm conduzido a cadeia produtiva do leite à um cenário de alternativas e novas tecnologias para o aumento da produtividade por vaca e por hectare.

A seleção genética de bovinos, que historicamente vem sendo realizada através da manutenção de indivíduos com fenótipo desejável como pais para as gerações subsequentes, cada vez mais abre espaço para novas tecnologias de seleção genética como a genotipagem, mapeamento genético e seleção assistida por marcadores. Marcadores moleculares ou genéticos são alterações do DNA que surgem com a própria evolução da espécie, como efeito de mutações, ou por introgressão de genes de outras raças ou linhagens na população, podendo acarretar mudanças de algumas de suas características fenotípicas.

Os primeiros trabalhos para desenvolver e caracterizar marcadores moleculares para espécies de interesse zootécnico datam do início dos anos 80. As primeiras publicações relatam resultados de estudos de caracterização de marcadores RFLP em suínos e bovinos (BECKMANN et al., 1986; GEORGES et al., 1987).

Nos últimos 25 anos, o estudo dos marcadores genéticos tem ajudado a mapear um grande número de lócus de características quantitativas (QTLs, do inglês *Quantitative Trait Loci*), ou seja, as regiões do genoma responsáveis por uma fração da variância genética de uma característica. Isto abre caminho para a seleção assistida por marcadores genético/moleculares. Um marcador genético é uma sequência polimórfica facilmente identificada no genoma, sendo amplamente utilizados em estudos genéticos. Recentemente, com o desenvolvimento de novos equipamentos e processos de análises, novas metodologias de alto desempenho e acurácia, e baixo custo e mão-de-obra para prospecção, caracterização e genotipagem de polimorfismos de um nucleotídeo (SNP, do inglês, Single Nucleotide Polymorphism) têm se tornado disponíveis. Essas tecnologias trouxeram novas implicações para técnicas já solidificadas, possibilitando a seleção genômica de animais mais eficientes, além de facilitar o entendimento da relação entre genética e as diferentes subáreas da produção animal. Os marcadores SNP têm como base as alterações mais elementares da molécula de DNA, ou seja, mutações em bases únicas da cadeia de bases nitrogenadas (Adenina, Citosina, Timina e Guanina). A maioria das características de interesse econômico em bovinos é controlada por vários genes. Entretanto, alguns desses genes apresentam um maior controle sobre o fenótipo expresso. A presença de SNPs nesses genes candidatos pode estar associada com fenótipos de interesse ou características indesejáveis.

Desde a conclusão do projeto genoma humano em 2003, observa-se um enorme progresso nas tecnologias de sequenciamento do genoma, levando a uma diminuição do custo por megabase e ao aumento do número e da diversidade dos genomas sequenciados. Uma complexidade surpreendente da arquitetura do genoma foi revelada, trazendo à tona novos enfoques para a predição de efeitos biológicos atrelados às diferenças individuais no DNA (GOODWIN et al., 2016). Algumas abordagens maximizam o número de bases sequenciadas na menor quantidade de tempo, gerando uma riqueza de dados que podem ser usados para entender fenômenos cada vez mais complexos. Alternativamente, algumas metodologias agora

visam o sequenciamento de partes contíguas mais longas, que são essenciais para a resolução de regiões estruturalmente complexas. Estas e outras estratégias apresentam uma variedade de ferramentas para investigar os genomas em maior profundidade, levando a uma compreensão aprimorada de como as variantes da sequência do genoma estão subjacentes ao fenótipo e às características produtivas de interesse (GOODWIN et al., 2016).

O lançamento da primeira plataforma de sequenciamento verdadeiramente de alto rendimento, conhecido como next generation system, em meados dos anos 2000, anunciou uma queda de 50.000 vezes no custo do sequenciamento do genoma humano (KIRCHER e KELSO, 2010). A partir daí, passou a ser possível a avaliação do genoma inteiro de diversas espécies. Além disto, a introdução da seleção genômica tornou viável a avaliação de animais, quanto ao seu mérito genético, logo após o nascimento. As decisões de seleção tomadas no início da vida do animal, em vez de testes de progênie prolongados, reduzem o intervalo de geração e, portanto, levam ao aumento significativo do ganho genético anual. Atualmente, é possível a genotipagem de dezenas de milhares até um milhão de SNPs em um único ensaio, levando os custos de geração de dados de US$0,10 a US$0,001 por SNP genotipado. Chips de genotipagem de alta densidade já foram gerados e validados para humanos, bovinos, ovinos, equinos, suínos e caninos, contendo até 777,000 SNPs para genotipagem de bovinos. Em algumas espécies, novos chips, contendo maiores números de SNPs já estão em desenvolvimento, refletindo o uso extenso que a tecnologia tem alcançado. Porém, a despeito de todo o avanço no potencial de avaliação do DNA, estudos que avaliem os efeitos de genes candidatos sobre características econômicas de interesse são cada vez mais necessários, assim como a identificação de SNPs com potencial de melhorar o potencial preditivo da seleção por marcadores genômicos. É possível que diferenças genéticas entre os animais interfiram na melhor adaptação ao fim da fase de gestação e início do processo de lactação, período marcado por grandes alterações metabólicas com impacto sobre a produção leiteira e reprodução de vacas leiteiras (BUTLER, 2000).

Mais do que uma fase de repouso entre lactações, o período seco nas vacas leiteiras cursa com um crescimento fetal considerável, remodelação do tecido mamário e altas demandas nutricionais. O reconhecimento da importância do período desde o final da gestação até a fase inicial da lactação levou ao desenvolvimento do conceito de período de transição, que é comumente definido como o período de três

semanas antes a três semanas após o parto (DRACKLEY, 1999). A demanda nutricional do feto atinge níveis máximos três semanas antes do parto, porém a ingestão de matéria seca diminui em 10 a 30% neste período (BELL, 1995). Dentro de três semanas após o início da lactação ocorre um rápido aumento na produção leiteira, além do aumento na produção de proteínas, gordura e lactose do leite, excedendo o aporte de nutrientes via ingestão alimentar (BERTONI et al., 2008). Além disso, a dieta da maioria das vacas leiteiras muda bruscamente no parto, sendo principalmente de dietas à base de forragem no pré-parto para dietas a base de concentrados no pós-parto. A produção de leite pós-parto e as adaptações nutricionais necessárias induzem um estado fisiológico de balanço energético negativo (BEN) (BEAM e BUTLER, 1998; BUTLER et al., 2003). Nos últimos tempos, o melhoramento genético e a melhoria da nutrição aumentaram a produção de leite por vaca. No entanto, o aumento da produção de leite foi acompanhado por uma diminuição da fertilidade em muitos países (BUTLER, 1998). As taxas de prenhez após a inseminação diminuíram de 0,45% a 1% anualmente nos rebanhos do Reino Unido e da América do Norte (BUTLER, 1998; ROYAL et al., 2000; DOBSON et al., 2007). Isto indica uma relação de antagonismo entre produção leiteira e reprodução (VANRADEN et al., 2004; CHAGAS et al., 2007; LUCY, 2007; MCCARTHY et al., 2007; MEE, 2007).

A produção de leite e desempenho reprodutivo são os barômetros econômicos da atividade leiteira. A eficiência reprodutiva determina o descarte de vacas e doenças como infecção uterina contribuem indiretamente para as elevadas taxas de descarte involuntário (GROHN et al., 2003). Compreender o papel-chave da resposta imune de vacas em transição pode ajudar a explicar os vínculos entre essas diversas condições. A nível molecular, a ativação de mecanismos locais e sistêmicos de defesa do hospedeiro induzem inflamação. Além disso, alterações significativas da expressão gênica ocorrem como uma adaptação às demandas de lactação, manutenção e involução uterina. Uma série de moléculas de sinalização são liberadas por células imunes ativadas, incluindo mediadores inflamatórios, como prostaglandinas e citocinas (Figura 1). Normalmente, as vacas demonstram sinais de alterações inflamatórias típicas antes do parto (BIONAZ et al., 2007; TREVISI et al., 2012). As citocinas desempenham um papel fundamental na estimulação das respostas inflamatórias sistêmicas, incluindo aumento da temperatura corporal e frequência cardíaca, e diminuição da ingestão alimentar (DANTZER e KELLEY, 2007). Além disso, dada a interação entre os sistemas imune, endócrino e metabólico

(STOFKOVA, 2009; PITTMAN, 2011), a diminuição da competência imune no parto aumenta a susceptibilidade ao hospedeiro em relação a infecções (TREVISI et al., 2012).

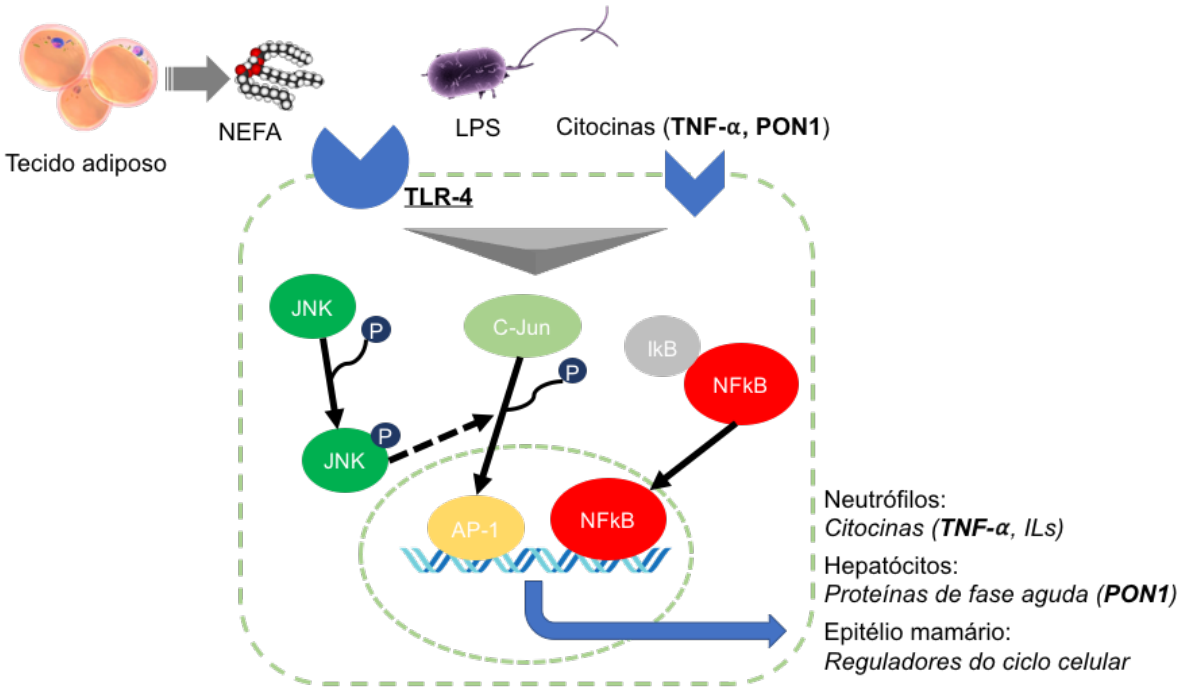

Figura 1. Ativação do sistema imune por ácidos graxos não-esterificados, lipopolissacarídeos de membrana e citocinas (adaptado de BRADFORD et al., 2015).

As condições fisiológicas das vacas ao parto associadas a um fornecimento de energia insuficiente predispõem esses animais a doenças metabólicas e microbianas, como febre do leite, endometrite, cetose, deslocamento de abomaso e retenção de placenta (DRACKLEY, 1999; DUFFIELD, 2000). O papel da resposta inflamatória no declínio da fertilidade ainda não é totalmente conhecido, dada a variedade de efeitos em vários processos fisiológicos envolvidos. Uma melhor compreensão das vias inflamatórias que desempenham um papel importante na função imune normal, metabolismo e reprodução pode melhorar a capacidade de prever e prevenir distúrbios da transição da vaca.

O processo inflamatório, iniciado através de uma agressão tecidual localizada, pode evoluir até uma resposta sistêmica mediada por citocinas como TNF-α, IL-1 e IL-6. A partir dessas mudanças, algumas proteínas de fase aguda (PFA) irão apresentar atividade aumentada enquanto outras terão sua síntese e atuação reduzidas. Devido a estes eventos ocorrerem antes mesmo da resposta específica ao

agente causador ou ao início dos sinais clínicos, as PFAs são consideradas marcadores metabólicos, auxiliando na detecção antecipada de processos patológicos e doenças subclínicas, de difícil diagnóstico. A Paraoxonase 1 (PON1), uma PFA negativa, reduz os seus níveis circulantes após danos teciduais e quebra de lipopolissacarídeos de membrana (LPS) (CAMPOS et al., 2017). O estresse oxidativo e a peroxidação lipídica também reduzem os níveis circulantes de PON1 (TURK et al., 2013). Além disto, animais com baixos níveis de PON1 no periparto apresentaram maior ocorrência de metrite e laminite e vacas com maiores níveis de PON1 nesta fase reduziram os riscos de apresentarem quadros graves de inflamação nos primeiros 30 dias em lactação (BIONAZ et al., 2007). Neste mesmo trabalho, os animais doentes já apresentavam níveis reduzidos de PON1 desde o pré-parto, o que sugere que, mais do que um marcador metabólico precoce, a menor atividade desta enzima pode contribuir no desencadeamento de distúrbios patológicos.

Vacas sofrendo de problemas de saúde são as principais candidatas a uma menor fertilidade. Claramente, vacas com uma série de problemas, incluindo parto distócico ou cesariana, manqueiras, endometrites, retenção de placenta, mastite, febre do leite, e baixos escores de condição corporal, têm maiores intervalos parto-concepção ou podem falhar em ficar prenhes (BUTLER, 2000; CHEBEL et al., 2004; BICALHO et al., 2007; DE BOER et al., 2015). Em muitos casos, esses problemas se apresentam de forma combinada. Os receptores do tipo Toll (TLRs) são responsáveis pelo reconhecimento dos patógenos e estimulação da resposta imune (KAWAI e AKIRA, 2010). Já foram identificados vários SNPs em diversos genes TLR, alguns deles sugerem um efeito sobre doenças do trato reprodutivo como metrite, endometrite clínica e endometrite citológica (PINEDO et al., 2013). Além dos efeitos diretos no útero, bactérias, produtos bacterianos ou mediadores imunológicos produzidos em resposta à infecção bacteriana, também suprimem a secreção pituitária de LH e estão associados com a inibição da foliculogênese, com a redução da esteroidogênese ovariana e com fases luteais anormais.

A recuperação da atividade ovariana após o parto apresenta um papel crítico na subsequente fertilidade da vaca (DARWASH et al., 1997). Na maioria das vacas leiteiras os folículos de tamanho médio aparecem 5 dias após o parto e os folículos grandes aparecem 10 dias após o parto (SAVIO et al., 1990). Aproximadamente metade de todas as vacas ovulam dentro de 3 semanas após o parto. Mas na outra metade, o folículo dominante da primeira onda folicular regride e a primeira ovulação

é adiada (MCDOUGALL et al., 1995). Formado pelos genes que codificam para a síntese do hormônio do crescimento (GH), fator de crescimento semelhante à insulina I (IGF-I) e seus receptores (GHR e IGF-IR), o eixo GH/IGF-I atua na regulação do metabolismo e fisiologia de mamíferos (JONES e CLEMMONS, 1995).

Logo após o parto, o aumento da utilização de glicose para a síntese de lactose na glândula mamária causa uma redução drástica nos níveis sanguíneos de insulina (BUTLER et al., 2003), com a queda nos níveis de insulina ocorre uma redução na expressão hepática dos receptores do GH (GHRs), especialmente o GHR 1A (BUTLER et al., 2003), que compreende 50% do GHR hepático (JIANG e LUCY, 2001b; JIANG e LUCY, 2001a). Isso irá causar a redução nos níveis séricos de IGF-I (FENWICK et al., 2008) e a dissociação do eixo GH/IGF-I, pois a síntese de IGF-I é dependente da ativação do GHR pelo GH (JONES e CLEMMONS, 1995). Isto leva ao aumento nos níveis circulantes de GH (BUTLER et al., 2003), uma vez que o feedback negativo exercido pelo IGF-I sobre o GH estará diminuído (MULLER et al., 1999).

Esta elevação do nível de GH é benéfica para a produção de leite, pois estimula a lipólise e aumenta a disponibilidade de glicose para a síntese de leite pela glândula mamária (BELL, 1995). Por outro lado, estas moléculas estão relacionadas à ocorrência da primeira ovulação, pois as vacas que apresentam níveis mais altos de IGF-I e níveis mais baixos de GH são as que irão ovular antecipadamente (KAWASHIMA et al., 2007). Desta forma, os genes que codificam para proteínas do eixo somatotrópico têm sido estudados como marcadores para a seleção de animais de produção (BARTKE, 2008). A maior parte do IGF-I sérico é sintetizada no fígado em resposta ao GH agindo através de seu receptor (JIANG e LUCY, 2001b). O IGF-I estimula a proliferação das células da teca e granulosa dos folículos ovarianos (ARMSTRONG e WEBB, 1997), inibindo a atresia folicular (EL-ROEIY et al., 1994). Além disso o IGF-I estimula a resposta das células foliculares à ação das gonadotrofinas (ARMSTRONG e WEBB, 1997). Assim, vacas de leite com atraso no retorno à ciclicidade possuem uma menor concentração sérica de IGF-I em comparação às vacas que ovularam mais precocemente (KAWASHIMA et al., 2007).

2 Objetivos

2.1 Objetivo Geral

Avaliar a associação de mutações genéticas com a fertilidade, produção de leite, metabolismo e saúde de vacas leiteiras.

2.2 Objetivos específicos

Avaliar a associação de mutações no promotor do gene da PON1 com a atividade sérica da PON1, fertilidade, produção leiteira, ingestão alimentar e saúde.

Avaliar a associação de mutações relacionadas ao sistema imune e ao transporte de energia celular com a fertilidade, produção leiteira e ingestão alimentar no periparto.

Avaliar a associação de mutações no eixo somatotrópico com a fertilidade, produção leiteira e metabolismo pós-parto.

3 Artigos

3.1 Artigo 1

Association of polymorphisms in the promoter region of paraoxonase 1 (*PON1*) gene with reproductive performance, health and milk production of Holstein dairy cows

Pedro Augusto Silva Silveira, Walter Ronald Butler, Sara LaCount, Tom Overton, Carlos Castilho Barros, Augusto Schneider

Submetido à revista Theriogenology

Association of polymorphisms in the promoter region of paraoxonase 1 (*PON1*) gene with reproductive performance, health and milk production of Holstein dairy cows

Running title: polymorphisms in the promoter of *PON1* gene of dairy cows

Pedro Augusto Silva Silveira [a], Walter Ronald Butler [b], Sara LaCount [b], Tom Overton [b], Carlos Castilho Barros [c], Augusto Schneider [c]*

[a] Faculdade de Veterinária, Universidade Federal de Pelotas, Campus Capão do Leão, sn, Pelotas, RS, Brazil.

[b] Department of Animal Science, Cornell University, 14853, Ithaca, NY, USA.

[c] Faculdade de Nutrição, Universidade Federal de Pelotas, Rua Gomes Carneiro, 1, Pelotas, RS, Brazil.

* Corresponding author. Tel.: +555339211270. E-mail address: augustoschneider@ufpel.edu.br (A. Schneider).

Abstract

The aim of this study was to evaluate the association of single nucleotide polymorphisms (SNPs) in the paraoxonase 1 (*PON1*) promoter region with serum PON1 activity, fertility, energy status, feed intake, occurrence of peripartum diseases and milk production of Holstein dairy cows. Eighty four Holstein cows were used in this study, blood samples were collected weekly before calving, twice a week in the first two weeks of lactation and once a week thereafter for β-hidroxybutyrate (BHBA), non-esterified fatty acids (NEFA) and serum PON1 activity analysis. Daily dietary intake of each cow was measured from 40 days prepartum up to 60 days in milk (DIM) and clinical data and milk production were evaluated up to 60 DIM. Cows were pre-synchronized with two injections of prostaglandin F2α followed by timed AI after an Ovsynch program. The pregnancy was confirmed after rectal palpation and reproductive performance data was recorded until 210 DIM. DNA was extracted from the whole blood samples for the PCR reaction and a fragment of 828 bp from the *PON1* gene promoter was sent for sequencing. Also, the SNP -221 genotyping was validated by ARMS-PCR and restriction fragment length polymorphism reaction using the *Bsl*I enzyme. Seven SNPs were identified in the promoter region of the *PON1* gene, located at positions -22, -105, -176, -221, -392, -611 and -676, considering 1 as the first nucleotide of *PON1* gene first exon, and six of them were associated with serum PON1 activity. The SNPs -221 and -392 were associated with the calving to conception interval (CCI, $P < 0.05$), and the genotypes associated to higher serum

PON1 activity were also associated with shorter CCI. Additionally, the SNPs -22 and -221 had an effect on somatic cell count (SCC) during the first six weeks of lactation ($P < 0.05$). It was possible to identify the three SNP-221 genotypes by ARMS-PCR and by digestion with the *Bsl*I enzyme. Thus, The SNPs -105, -176, -221, -392, -611 and -676 were associated with serum PON1 activity. SNPs in the -221 associated to higher serum PON1 activity, were also associated with shorter CCI and reduced milk SCC.

Keywords: fertility; inflammation; single nucleotide polymorphisms

1. Introduction

In the postpartum period, dairy cows are metabolically challenged, as energy demands exceed dietary intake and cows undergo negative energy balance (NEB) [1], resulting in several metabolic changes [2, 3]. The NEB severity and duration impairs the recovery of postpartum ovarian cyclicity and increases the calving conception interval, where cows with higher levels of non-esterified fatty acids (NEFA), β-hidroxybutyrate (BHBA) and greater loss of body condition score (BCS) have reduced fertility [2, 4]. In a scenario of increased serum levels of NEFA and BHBA from lipolysis, the hepatic metabolism is essential for adaptation to NEB [2, 5]. Intensification of the NEFA oxidation process in the liver results in increased production of reactive oxygen species (ROS) [6] leading to an inflammatory response. Therefore, the antioxidative and antiinflamatory response are essential to improved reproductive performance.

In the final weeks of gestation, the dairy cow already experiences reduced hepatic function associated with an increased inflammatory response [7, 8]. During this period, it is possible to observe an increase in serum concentrations of positive acute phase proteins, such as ceruloplasmin, serum amyloid A and haptoglobin, as well as a reduction of negative acute phase proteins, such as albumin and paraoxonase 1 (PON1) [8, 9]. PON1 is an enzyme with hydrolase activity synthesized in the liver and released into the circulation [10]. PON1 acts as an indicator of liver function, allowing the early diagnosis of several conditions that cause liver damage [10]. PON1 is also considered a negative acute phase protein, reducing circulating levels in response to cytokines released during inflammation [8]. The effective response to inflammatory processes and recovery of affected cows is dependent on the resumption of normal serum PON1 activity [11]. Several studies in early postpartum dairy cows suggest an important role of PON1 on susceptibility to postpartum disorders [8, 12-14].

The recovery of postpartum ovarian cyclicity and reproductive performance is also dependent on the magnitude of the oxidative stress and the cow response to this challenge [15].

In this sense, PON1 is transferred from serum to the ovarian dominant follicle in cattle along with HDL [16], and when added to the maturation media during *in vitro* production of bovine embryos can improve the blastocyst rates [17], suggesting its importance in the reproductive process. PON1 has cytoprotective properties reducing oxidative damage against cellular membranes [18-20]. The oxidative stress appears to be responsible for damaging the embryo, which can result in embryonic death [21, 22], therefore pointing to a role of PON1 in this process. On the other hand, a greater reduction in the prepartum levels of PON1 is predictive of higher incidence of uterine infections in the early postpartum period in dairy cows [13], which could affect ovulation, increasing the calving to conception interval. In this sense, a lower percentage of polymorphonuclear cells was observed in the uterus of cows that ovulated earlier in the postpartum period and this was associated with lower serum PON1 activity [14]. Dairy cows with low peripartum levels of PON1 had increased incidence of metritis and laminitis, while cows with higher levels of PON1 had a reduced risk of severe inflammation during the first 30 days in milk [8]. Therefore, it is hypothesized that changes in peripartum PON1 activity can reflect in the health and fertility of the dairy cow.

The *PON1* gene is located on chromosome four in the bovine and has approximately 33 kbp in length. In humans, several polymorphisms found in this gene have proved to interfere directly in the expression of the protein, and these changes, in addition to modifying PON1 activity in the circulation, are associated with a series of diseases in humans [23]. However, little is known about the interference of the genotype on PON1 activity in cows. In a recent study, we found seven single nucleotide polymorphisms (SNPs) in the promoter region of the *PON1* gene in Holstein dairy cows from Southern Brazil, and five of them were associated to changes in serum PON1 activity [24]. Among these SNPs, one located in the -221 position (A/G) is located in a transcription factor binding site linked to the acute phase response [24]. However, there are still no reports of the association of these *PON1* genetic polymorphisms with fertility, health and productive parameters in dairy cows.

2. Methods

2.1. Animals, milk collection and feed intake

All experimental procedures were approved by the Cornell University Institutional Animal Care and Use Committee. Multiparous lactating Holstein cows (n = 84 cows) were provided with *ad libitum* access to a total mixed ration fed twice daily. Weekly samples of the feed offered were composited on a monthly basis for nutrient analysis (Dairy One Cooperative,

Ithaca, NY, USA). The cows were housed in individual pens 40 days before the expected calving date and the individual daily feed intake were measured and recorded until 60 days in milk (DIM). Cows were milked twice daily and milk yield for each cow was averaged by week. Milk samples were collected twice a week in the first two weeks of lactation and once a week thereafter. Milk composition was analyzed in the using mid-NIR techniques (Barbano Lab at Cornell University [25]).

2.2 Blood analyses

Blood collections were performed by puncture of the coccygeal vein weekly before calving, twice a week in the first two weeks after calving and once a week up to 42 DIM. To measure serum PON1 activity, a 20 mM Tris/HCl buffer, pH 8.0, containing 1 mmol/L of $CaCl_2$ and 4 mmol/L phenyl acetate was used as substrate. Samples were diluted in a 20 mM Tris/HCl Buffer in the ratio 1:3. The reading was performed in a spectrophotometer, after addition of 3.3 µL of the diluted sample to 500 µL of the working solution, at 270 nm during one minute interval. BHBA was measured in whole blood samples, prior to centrifugation and plasma separation, using the NovaVet (Nova Biomedical, Billerica, MA, USA) handheld ketone meter. Plasma was separated into three aliquots for further measurement of NEFA by an autoanalyzer (Boehringer Mannheim Hitachi 104, Diagnostic Laboratory Systems, Indianapolis, IN, USA).

2.3 PON1 promoter SNPs

DNA was extracted from the whole blood samples according previous described [26], and used for the PCR reaction, using primers forward: 5'-CGGTAATCCCTGAAGAATGC-3' and reverse: 5'-GCACTTCCTACCCTGCTTTG-3' to obtain a fragment with 828 bp of the *PON1* promoter gene region. The primers were constructed using the Primer3 Plus program (http: //www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The PCR reaction was performed with a commercial kit (Roche®) and used temperatures of 94°C for 5 min, 40 cycles of 1 min at 94°C, 1 min at 57°C and 1 min at 72°C, and a final step at 72°C for 10 min. An electrophoresis with 1% agarose gel was performed, from which the DNA band was cut in the position equivalent to 828 bp. This gel fragment was purified using a commercial kit (Promega, Madison, Wisconsin, USA) and the purified samples were sent for DNA sequencing by the Sanger method (Biotechnology Resource Center, Cornell University Institute of Biotechnology). Sequences were aligned using the BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), based on the published sequence of the

bovine *PON1* gene (NCBI accession number: AC_000161.1) as the reference for alignment, and the SNPs were manually identified.

2.4 SNP PON1-221 Genotyping by ARMS-PCR

The *PON1* -221 SNP had been previously associated with serum PON1 activity and it had a stronger linkage disequilibrium with other SNPs at this region [27]. For easier identification of the *PON1*-221 genotype an amplification refractory mutation system (ARMS-PCR) containing four primers was also developed and validated against the sequencing results. Two of these primers anneal on the outside region of the PCR product (forward, CAGACGCACAGACGGGAGAA; reverse, CAGTGATGCCTCCCTGGACA), generating a 701 bp fragment (Fig. 1). The other two internal primers (forward, AACTAGCTGCCTAGAGCGAG; reverse, TGCCATTCTCCCCTTTCTGCCC), annealed inside the fragment of 701 bp, forming allele specific fragments of 516 bp (allele A) or 224 bp (allele G) (Fig. 1). For the PCR reaction using the *GOTaq* Green Master Mix (Promega, Madison, WI, USA) DNA Polymerase, the following conditions were used: 94°C for 5 min, 35 cycles of 94°C for 1 min, 66°C for 45 secs and 72°C for 1 min, and a final step at 72°C for 10 min. Electrophoresis was performed in a 1.5% agarose gel (UltraPure™ Agarose, Life Technologies), using a 100 bp DNA marker.

To validate the accuracy of genotyping by tetra-primer ARMS-PCR, after electrophoresis, nine previously known samples (three from each genotype AA, AG and GG) were submitted to sequencing. For this, a PCR for amplifying the entire promoter region was performed with specific primers (sense: 5′-CGGTAATCCCTGAAGAATGC-3′ and antisense: 5′-GCACTTCCTACCCTGCTTTG-3′; 57^{o}C annealing temperature). After the agarose gel electrophoresis, a single amplicon was observed around 828 bp, the fragment was excised from the gel, purified using a commercial kit (Bio Basic, Ludwig, Alvorada, RS, Brazil) and submitted for sequencing (HELIXXA, São Paulo, SP, Brazil). The sequences obtained were aligned using BioEdit software (Ibis Biosciences, Carlsbad, CA, USA), using the published sequence of bovine PON1 (NCBI accession number: AC_000161.1) as the reference for alignment purposes.

2.5 SNP PON1-221 Genotyping by RFLP

As another alternative genotyping method, restriction fragment length polymorphism, using the enzyme (*Bsl*I) which has a *PON1*-221 SNP compatible binding site (Webcutter, New England Biolabs, Ipswich, Massachusetts, USA) was used. The restriction fragment length

polymorphism method was used for *PON1*-221 SNP genotyping. The primers used were the same described for sequencing samples (Forward: 5'-CGGTAATCCCTGAAGAATGC-3' and Reverse: 5'-GCACTTCCTACCCTGCTTTG-3') as well as the PCR protocol. The PCR amplified DNA (828 bp) was digested with 10 U of *Bsl*I (New England) at 37°C for 2 h. Restriction fragments were separated by electrophoresis in 2.5% agarose in TAE buffer (Promega) containing 0.5 μg/mL ethidium bromide and visualized under UV light. The enzyme recognition sequence is CCNNNNN/NNGG, which is consistent with the SNP at position -221 (A or G). For the G allele, the enzyme cut the PCR fragments into 6 sites (positions 65, 171, 201, 291, 512 and 576). As for the allele A, there is no cut of the enzyme at position 291. Then, for the genotype AA six fragments with 30, 64, 65, 106, 252 and 311 bp were identified. For the GG genotype, seven fragments of 30, 64, 65, 90, 106, 221 and 252 bp and for the AG genotype eight fragments of 30, 64, 65, 90, 106, 221, 252 and 311 bp were identified.

2.6 Reproductive Management

Cows were presynchronized with two injections of prostaglandin F2α (PGF2α; 25 mg im; Lutalyse, Pfizer Animal Health, NewYork, NY, USA) given at 30 and 44 DIM. Ten days after the second injection of PGF2α, the Ovsynch program [28] was initiated in all cows. The initial GnRH dose (100 mg im; Cystorelin, Merial Ltd, Duluth, GA, USA) was followed 7 days later by an injection of PGF2α and 48 h later cows received the second dose of GnRH with timed AI 12 h thereafter. Cows that were previously inseminated but showed visual signs of estrous behavior before pregnancy diagnosis were re-inseminated. Additionally, cows not pregnant at the time of any subsequent pregnancy diagnosis (32 days post-AI) were re-enrolled in the Ovsynch program. Confirmation of pregnancy by rectal palpation of the reproductive tract was made twice at 42 and 60 days post-AI. The reproductive performance of the cows enrolled in the study was recorded until 210 DIM. The day of insemination resulting in pregnancy was used to calculate the calving to conception interval (CCI).

2.7 Clinical data

Cows were monitored for development of disease incidence or clinical signs in the postpartum period. The definitions used on farm were the following: retained placenta, retention of fetal membranes for longer than 24 h; metritis, abnormal vaginal discharge for 2 days and fever in the first 3 weeks postpartum; displaced abomasum, presence of abdominal ping requiring surgical correction; ketosis, no appetite and presence of ketone bodies in the urine;

lameness, difficulty to walk plus visual inspection; mastitis, abnormal milk, and/or high somatic cell count (SCC); and milk fever, subnormal body temperature and recumbency.

2.8 Statistical analysis

The results are presented as mean values ± standard error of the mean (SEM). All the statistical analyses were performed using SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Analyses involving repeated measures over time (e.g., plasma PON1 activity, NEFA, BHBA, milk production, and SCC) were compared by analysis of variance for repeated measures using the MIXED procedure to evaluate the main effects of time, genotype and the interaction time vs. genotype. In addition, the average PON1 activity for pre- and postpartum period was calculated and along with the CCI and feed intake were evaluated using polynomial models for the linear or quadratic effects of having none, one or two *PON1* SNPs alleles. Disease incidence and conception rate on the first postpartum AI were evaluated by Chi-square analysis. Pregnancy rate were evaluated by Kaplan-Meier survival analysis using GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA). A *P*-value lower or equal than 0.05 was considered significant and between 0.05 and 0.10 as a tendency.

3. Results

3.1 SNPs affecting serum PON1 activity

Seven SNPs were identified in the promoter region of the *PON1* gene, located at positions -22, -105, -176, -221, -392, -611 and -676, considering 1 as the first nucleotide of the first exon. The nucleotides identified at position -22 were C and G, -105 were A and G, -176 were T and G, -221 were A and G, -392 were A and C, -611 were C and T and -676 were A and T. The data of the observed genotypes and their frequency is summarized in Table 1. The serum activity of PON1 was assessed among the different genotypes found for each SNP. The SNPs found at positions -105, -176, -221, -392, -611 and -676 had a significant effect on the peripartum mean PON1 activity ($P < 0.05$) and the SNP -22 had a tendency to affect PON1 activity ($P < 0.10$) (Table 1). In addition, the SNPs -105, -176, -221, -611 and -676 were associated ($P < 0.05$) with changes in PON1 activity in the postpartum period. The SNPs -105, -176, -221 and -611 were also associated with prepartum PON1 activity ($P < 0.05$). Cows that were homozygote for adenine at positions -221 and -676, and homozygote cows for thymine at position -176 had the highest serum PON1 activity. Adenine allele and cytosine allele at

positions -105 and -611, respectively, were also associated with higher PON1 activity. Additionally, cows with at least one C allele for SNP -392 had the highest PON1 activity.

3.2 SNPs affecting fertility

The SNPs -221 and -392 were associated with the CCI ($P < 0.05$). Cows that were homozygote for guanine at position -221 and for adenine at position -392, had the lowest PON1 activity and the longest CCI ($P < 0.05$) (Table 2).

In the survival analyses, when cows having at least one A allele for the -221 SNP were grouped, an effect on the CCI was observed, with GG cows having higher CCI ($P < 0.05$) (Fig. 2). For the SNP-392 an effect in the CCI was observed, with carriers of the C alelle having a shorter CCI compared to AA cows ($P < 0.05$) (Fig. 2).

3.3 SNPs affecting feed intake, milk yield, NEFA, BHBA, diseases and SCC

There was no significant effect of the identified SNPs on feed intake, milk yield, NEFA, BHBA and occurrence of diseases. There was tendency for cows of the GG genotype for the -221 SNPs to have higher SCC during the first six weeks of lactation than cows of the AG genotype ($P = 0.057$) (GG: 6.1 ± 0.4, AG: 4.9 ± 0.3, AA: 5.2 ± 0.2 log2 cells/mL).

3.4 SNP PON1-221 Genotyping by ARMS-PCR and RFLP

Using the tested techniques it was possible to accurately identify the three SNP-221 genotypes (AA, AG and GG) by ARMS-PCR (Fig. 5) and by RFLP, using the enzyme *Bsl*I (Fig. 6). The techniques were further validated by sequencing confirming 100% of the results observed in the electrophoresis.

4. Discussion

Seven SNPs were identified in the promoter region of the *PON1* gene, located at positions -22, -105, -176, -221, -392, -611 and -676, considering the first nucleotide of the *PON1* mRNA. Previously we had found the same SNPs in a population of Holstein cows from Southern Brazil [24], which further validates the importance of our findings. In our previous study, using less than 50 cows, it was not possible to observe the presence of GG genotypes for the PON1 -22 and -176 SNPs. In this study 5 cows had the GG genotype for the -22 SNP, but no GG cows at position -176 were found. It is possible that the distribution of this genotype in the bovine populations is even rarer or deleterious. Evaluations of larger populations may confirm the existence of this genotype. Although it is not possible to calculate linkage

disequilibrium in unphased genotyping data, we could observe that all 37 cows homozygous cows for the A allele (highest PON1 activity) in the -221 position, were also homozygous for the genotypes associated with higher PON1 activity in SNPs -22, -105, -176, -611 and -676. This indicates a strong linkage between SNPs in these positions and it is expected given the close proximity. However, only 90% of the AA cows for the -221 SNP were carriers of the C allele (associated with higher PON1 activity) in the -392 position, with half of them being heterozygotes for this position. Therefore, this indicates that any these SNPs could be consistently used as marker for serum PON1 activity in dairy cows. Although the PON1 activity was slightly different from our previous study, the effects of the genotypes were consistent between studies [24].

PON1 is a negative acute phase protein and reduces its circulating levels after tissue damage and membrane lipopolysaccharide (LPS) breakdown [8, 9, 29]. Cows with lower serum PON1 activity in the peripartum have higher occurrence of metritis and laminitis, whereas cows with higher PON1 levels at this stage have a reduced risk of presenting severe inflammation during the first 30 days of lactation [8]. In the same study, sick cows already had reduced serum activity of PON1 in the prepartum period, suggesting that more than an early metabolic marker, the lower activity of this enzyme can contribute to trigger pathological conditions. In this sense, evaluation of peripartum of dairy cows showed that cows with the highest incidence of uterine infections in the postpartum period had a more drastic reduction in PON1 activity during the prepartum period [13]. Furthermore, we previously found a lower percentage of polymorphonuclear cells in the uterus of cows that ovulated earlier in the postpartum period, which was associated with a tendency of higher PON1 activity in the peripartum period [14]. These collective evidence points to PON1 as involved in the pathogenesis of earlier postpartum disorders and that is why is important to understand its genetic variability. Although most of the SNPs had an effect on serum PON1 activity, no effect of the SNPs was observed on the occurrence of diseases and this may be related to number of cows used in this study. Thirty-six cows were diagnosed with at least one disease in the study period with no significant relationship with the different genotypes. Milk production, feed intake, NEFA and BHBA also were not affected by the genotypes, further suggesting no effect of these SNPs in the health of transition dairy cows during this period.

The SNPs at positions -221 and -392 had an effect on fertility. The shortest CCI was observed for the genotypes with highest PON1 activity (SNPs -221AA and -392CC). In the transition period, high producing cows usually have higher concentrations of NEFA [30], as a result of insufficient feed intake during the beginning of lactation, increasing the risk of

metabolic and uterine diseases, and decreased reproductive performance. The acute inflammatory response from clinical and subclinical postpartum disorders reduces serum PON1 activity and may affect the expression of key enzymes involved in steroidogenesis, with a negative impact on final follicular development, especially if the inflammatory process persists for longer periods [31, 32]. As mentioned before, cows that ovulated earlier in the postpartum period tended to have higher serum PON1 activity [14]. In addition, previous studies suggest that PON1 bound to HDL is transferred from plasma to follicular fluid in bovines [16] and humans [18]. This intrafollicular PON1 may have an important role in fertility, which was confirmed as the addition of PON1 during bovine *in vitro* oocyte maturation is able to increase blastocyst development rates [17]. Moreover, women with higher serum PON1 activity produced embryos with higher number of blastomers when submiteed to IVF [18]. In this sense, the higher activity of PON1 observed in the different genotypes from this study may be quickly reflected in the composition of the follicular fluid of the preovulatory follicle affecting steroidogenesis and oocyte competence. The SNP −221 is located in a region identified as potential binding site for transcription factors that modulate the acute phase response [27, 33], therefore suggesting a link between this mutation, the inflammatory status and PON1 activity. It is important to mention that the effects of the PON1 SNPs on fertility occurred independently of any changes in DMI, milk production, NEFA or BHBA concentrations, classically know to be involved in regulation of energy balance and reproduction [2].

Dairy cows during the transition period undergo physiological changes which increase the demand for cellular oxygen and cause oxidative stress [34]. Therefore, during the early postpartum there is excessive production of ROS [8] and concomitant damage at cellular and tissue levels which are managed by cellular antioxidant defense systems, such as the HDL-ApoAI-PON1 complex [8, 35]. The excess ROS are associated with uterine diseases and can have a negative impact on fertility [36]. It was observed that infertile women do not necessarily develop a proinflammatory status, but had an increase in total serum peroxides along with a slight decrease in HDL and ApoAI concentrations [37]. PON1 activity was not affected in these women [37], suggesting a protective role for this enzyme in ovary structures against oxidative stress. In our study, cows with genotypes resulting in higher PON1 activity in the postpartum period conceived earlier postpartum than cows with lower PON1 activity. However, when evaluating postpartum beef cows with high or low serum PON1 activity, no effect on the conception rate was observed [38]. This suggests that the positive effects of PON1 on fertility observed in dairy cows, may be the result of the more challenged environment during the transition period, where PON1 can have a protective effect against the damage caused by

oxidative stress. This better recovery from the oxidative stress is directly related to the transition cow health [8]. Therefore, the observation of higher SCC in cows from the SNP -221 GG genotype, the same associated with lower serum PON1 activity, may be related to this fact. As low antioxidant status and increased oxidative stress in cows with mastitis were previously described [39]. Although, changes in acute phase proteins during clinical and subclinical mastitis seems to be highly variable [40].

Based on this study, the SNP -221 had an equilibrated distribution in the studied herd, along with a significant effect on serum PON1 activity and the calving conception interval. Additionally, as mentioned before, there was a strong alignment of the homozygous AA cows from this genotype with the genotypes for higher PON1 activity in the other positions identified. In an attempt to develop alternative rapid, simple, low cost and high throughput method, we have tested SNP-221 genotyping by ARMS-PCR and RFLP. The tetra-primer amplification refractory mutation system PCR is a fast and economical means of assaying SNPs, requiring only PCR amplification and subsequent electrophoresis for the determination of genotypes. This technique has been used in some studies to genotype several SNPs, presenting good results for the identification of mutations [41, 42]. Additionally, the availability of a variety of restriction endonuclease enzymes that cleave DNA at specific sites has made it possible to identify the presence of polymorphic regions in the isolated fragments. The RFLP technique has been used to investigate several mutations related with dairy production and reproduction [43, 44]. Using the ARMS-PCR method, it was possible to observe the difference between the AA (701 and 516 bp), AG (701, 516 and 224 bp) and GG (701 and 224 bp) genotypes for the SNP-221, after electrophoresis. Also, using the RFLP technique with the *Bsl*I enzyme, we identified cows of the genotypes AA (106, 252 and 311 bp), AG (90, 106, 221, 252 and 311 bp) and GG genotype (90, 106, 221 and 252 bp). The alternative sequencing techniques demonstrated the ability to correctly identify the different genotypes of the SNP-221 after confirmation by sequencing and may help in the use of the SNP-221 as a novel molecular marker for genetic selection in dairy cows, considering the strong linkage among SNPs in this short promoter region evaluated.

In conclusion seven SNPs were identified in the promoter region of the *PON1* gene, from which six of them affected serum PON1 activity. The genotypes associated with higher serum PON1 activity in the SNPs located at positions -221 and -392 also were associated with a reduced calving to conception interval. Using the ARMS-PCR and RFLP techniques we were able to accurately genotype the SNP at the -221 position. It is important to mention that no

effects of the genotypes were observed on milk production, dry matter intake or NEFA and BHBA concentrations.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by FAPERGS, CNPq AND CAPES.

References

[1] Ingvartsen KL, Andersen JB. Integration of metabolism and intake regulation: a review focusing on periparturient animals. J Dairy Sci. 2000;83:1573-97.

[2] Butler WR. Nutritional interactions with reproductive performance in dairy cattle. Anim Reprod Sci. 2000;60-61:449-57.

[3] Lucy MC. Mechanisms linking nutrition and reproduction in postpartum cows. Reprod Suppl. 2003;61:415-27.

[4] Beam SW, Butler WR. Effects of energy balance on follicular development and first ovulation in postpartum dairy cows. J Reprod Fertil Suppl. 1999;54:411-24.

[5] Herdt TH. Ruminant adaptation to negative energy balance. Influences on the etiology of ketosis and fatty liver. Vet Clin North Am Food Anim Pract. 2000;16:215-30, v.

[6] Mudron P, Rehage J, Qualmann K, Sallmann HP, Scholz H. A study of lipid peroxidation and vitamin E in dairy cows with hepatic insufficiency. Zentralbl Veterinarmed A. 1999;46:219-24.

[7] Grummer RR. Impact of changes in organic nutrient metabolism on feeding the transition dairy cow. J Anim Sci. 1995;73:2820-33.

[8] Bionaz M, Trevisi E, Calamari L, Librandi F, Ferrari A, Bertoni G. Plasma paraoxonase, health, inflammatory conditions, and liver function in transition dairy cows. J Dairy Sci. 2007;90:1740-50.

[9] Bertoni G, Trevisi E, Han X, Bionaz M. Effects of inflammatory conditions on liver activity in puerperium period and consequences for performance in dairy cows. J Dairy Sci. 2008;91:3300-10.

[10] Ceron JJ, Tecles F, Tvarijonaviciute A. Serum paraoxonase 1 (PON1) measurement: an update. BMC Vet Res. 2014;10:74.

[11] Bossaert P, Trevisi E, Opsomer G, Bertoni G, De Vliegher S, Leroy JL. The association between indicators of inflammation and liver variables during the transition period in high-yielding dairy cows: an observational study. Vet J. 2012;192:222-5.

[12] Pezzulo AA, Hornick EE, Rector MV, Estin M, Reisetter AC, Taft PJ, et al. Expression of human paraoxonase 1 decreases superoxide levels and alters bacterial colonization in the gut of Drosophila melanogaster. PLoS One. 2012;7:e43777.

[13] Schneider A, Correa MN, Butler WR. Short communication: acute phase proteins in Holstein cows diagnosed with uterine infection. Res Vet Sci. 2013;95:269-71.

[14] Krause AR, Pfeifer LF, Montagner P, Weschenfelder MM, Schwegler E, Lima ME, et al. Associations between resumption of postpartum ovarian activity, uterine health and concentrations of metabolites and acute phase proteins during the transition period in Holstein cows. Anim Reprod Sci. 2014;145:8-14.

[15] Al-Gubory KH, Fowler PA, Garrel C. The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. Int J Biochem Cell Biol. 2010;42:1634-50.

[16] Acosta DAV, Pfeifer LFM, Schmitt E, Schneider A, Silveira PAS, Jacometo CB, et al. Effect of prepartum somatotropin injection in late pregnant Holstein heifers with high body condition score on metabolic parameters, resumption of ovulation and milk production. Canadian Journal of Animal Science. 2013;93:287-92.

[17] Rincon J, Madeira EM, Campos FT, Mion B, Silva JF, Absalon-Medina VA, et al. Exogenous paraoxonase-1 during oocyte maturation improves bovine embryo development in vitro. Reprod Domest Anim. 2016;51:827-30.

[18] Browne RW, Shelly WB, Bloom MS, Ocque AJ, Sandler JR, Huddleston HG, et al. Distributions of high-density lipoprotein particle components in human follicular fluid and sera and their associations with embryo morphology parameters during IVF. Hum reprod. 2008;23:1884-94.

[19] Fuhrman B, Gantman A, Aviram M. Paraoxonase 1 (PON1) deficiency in mice is associated with reduced expression of macrophage SR-BI and consequently the loss of HDL cytoprotection against apoptosis. Atherosclerosis. 2010;211:61-8.

[20] Cho KH. A reconstituted high density lipoprotein containing the V156E mutant of apolipoprotein A-I exhibits anti-atherosclerotic activity in Apo-E deficient mice. J Atheroscler Thromb. 2009;16:217-29.

[21] Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. Reprod Biol Endocrinol. 2005;3:28.

[22] Guerin P, El Mouatassim S, Menezo Y. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum Reprod Update. 2001;7:175-89.

[23] Mackness B, Davies GK, Turkie W, Lee E, Roberts DH, Hill E, et al. Paraoxonase status in coronary heart disease: are activity and concentration more important than genotype? Arterioscler Thromb Vasc Biol. 2001;21:1451-7.

[24] Silveira PA, Schwegler E, Montagner P, Krause AR, Acosta DA, Halfen J, et al. Characterization of single nucleotide polymorphisms in the promoter region of the bovine paraoxonase 1 (PON1) gene affecting serum enzyme activity in dairy cows. Vet J. 2015;205:101-3.

[25] Barbano DM, Melilli C, Overton TR. Advanced use of FTIR spectra of milk for feeding and health management. Proceedings of the Cornell Nutrition Conference. Syracuse, NY2014. p. 105-13.

[26] Kanai N, Fujii T, Saito K, Tokoyama T. Rapid and simple method for preparation of genomic DNA from easily obtainable clotted blood. J Clin Pathol. 1994;47:1043-4.

[27] Silveira PA, Schwegler E, Montagner P, Krause AR, Acosta DA, Halfen J, et al. Characterization of single nucleotide polymorphisms in the promoter region of the bovine paraoxonase 1 (PON1) gene affecting serum enzyme activity in dairy cows. Vet J. 2015.

[28] Pursley JR, Mee MO, Wiltbank MC. Synchronization of ovulation in dairy cows using PGF2alpha and GnRH. Theriogenology. 1995;44:915-23.

[29] de Campos FT, Rincon JA, Acosta DA, Silveira PA, Pradiee J, Correa MN, et al. The acute effect of intravenous lipopolysaccharide injection on serum and intrafollicular HDL components and gene expression in granulosa cells of the bovine dominant follicle. Theriogenology. 2017;89:244-9.

[30] LeBlanc S. Monitoring metabolic health of dairy cattle in the transition period. J Reprod Dev. 2010;56 Suppl:S29-35.

[31] Lavon Y, Leitner G, Goshen T, Braw-Tal R, Jacoby S, Wolfenson D. Exposure to endotoxin during estrus alters the timing of ovulation and hormonal concentrations in cows. Theriogenology. 2008;70:956-67.

[32] de Campos FT, Rincon JAA, Acosta DAV, Silveira PAS, Pradiee J, Correa MN, et al. The acute effect of intravenous lipopolysaccharide injection on serum and intrafollicular HDL components and gene expression in granulosa cells of the bovine dominant follicle. Theriogenology. 2017;89:244-9.

[33] Wedel A, Ziegler-Heitbrock HW. The C/EBP family of transcription factors. Immunobiology. 1995;193:171-85.

[34] Gitto E, Reiter RJ, Karbownik M, Tan DX, Gitto P, Barberi S, et al. Causes of oxidative stress in the pre- and perinatal period. Biol Neonate. 2002;81:146-57.

[35] Agarwal A, Gupta S, Sekhon L, Shah R. Redox considerations in female reproductive function and assisted reproduction: from molecular mechanisms to health implications. Antioxid Redox Signal. 2008;10:1375-403.

[36] Abuelo A, Hernandez J, Benedito JL, Castillo C. The importance of the oxidative status of dairy cattle in the periparturient period: revisiting antioxidant supplementation. J Anim Physiol Anim Nutr (Berl). 2015;99:1003-16.

[37] Marsillach J, Checa MA, Pedro-Botet J, Carreras R, Joven J, Camps J. Paraoxonase-1 in female infertility: a possible role against oxidative stress-induced inflammation. Fertil Steril. 2010;94:1132-4.

[38] Castro NA, Pfeifer LFM, Andrade JS, Rincon JAA, Pegoraro LMC, Schneider A. Effect of serum paraoxonase-1 (PON1) activity on follicular development and pregnancy rate in cattle. Anim Reprod Sci. 2018;188:130-6.

[39] Atakisi O, Oral H, Atakisi E, Merhan O, Metin Pancarci S, Ozcan A, et al. Subclinical mastitis causes alterations in nitric oxide, total oxidant and antioxidant capacity in cow milk. Res Vet Sci. 2010;89:10-3.

[40] Turk R, Piras C, Kovacic M, Samardzija M, Ahmed H, De Canio M, et al. Proteomics of inflammatory and oxidative stress response in cows with subclinical and clinical mastitis. J Proteomics. 2012;75:4412-28.

[41] Zhang C, Liu Y, Ring BZ, Nie K, Yang M, Wang M, et al. A novel multiplex tetra-primer ARMS-PCR for the simultaneous genotyping of six single nucleotide polymorphisms associated with female cancers. PLoS One. 2013;8:e62126.

[42] Schadock I, Schneider A, Silva ED, Buchweitz MR, Correa MN, Pesquero JB, et al. Simple Method to Genotype the ACTN3 r577x Polymorphism. Genet Test Mol Biomarkers. 2015;19:253-7.

[43] Wu J, Bai JY, Li L, Huang S, Li CM, Wang GL. Genetic polymorphisms of the BMAP-28 and MASP-2 genes and their correlation with the somatic cell score in Chinese Holstein cattle. Genet Mol Res. 2015;14:1-8.

[44] Schneider A, Correa MN, Butler WR. Association between growth hormone receptor AluI polymorphism and fertility of Holstein cows. Theriogenology. 2013;80:1061-6.

TABLES

Table 1. Single nucleotide polymorphisms (SNPs) identified in the promoter region of the bovine paraoxonase 1 (*PON1*) gene and their association with serum PON1 activity* in periparturient Holstein dairy cows.

	Genotypes			***P* value**		
	CC	CG	GG	CC vs CG and GG	Linear effect	Mixed models
PON1 -22	66.7% (56/84)	27.4% (23/84)	5,9% (5/84)			
Prepartum	90.3 (±3.1)	85.5 (±4.9)	92.9 (±10.6)	0.8733	0.8151	
Postpartum	104.0 (±4.0)	86.7 (±6.2)	95.2(±13.4)	0.1242	0.5297	
Overall	99.6 (±3.1)	86.5 (±4.9)	94.5 (±10.5)			0.0861
	AA	AG	GG	AA vs AG and GG	Linear effect	Mixed models
PON1 -105	76.2% (64/84)	20.2% (17/84)	3.6% (3/84)			
Prepartum	92.8 (±2.8)	81.2 (±5.4)	54.6 (±12.9)	0.0015	0.0052	
Postpartum	102.1 (±3.7)	92.2 (±7.2)	65.1 (±17.3)	0.0231	0.0402	
Overall	99.2 (±2.8)[a]	88.8 (±5.6)[ab]	61.6 (±13.3)[b]			0.0109
	TT	GT		TT vs GT	Linear effect	Mixed models
PON1 -176	91.7% (77/84)	8.3% (7/84)				
Prepartum	90.8 (±2.6)	70.2 (±8.7)		0.0264		
Postpartum	101.7 (±3.3)	66.4 (±11.0)		0.0029		
Overall	98.3 (±2.6)[a]	68.0 (±8.6)[b]				0.0012

	AA	AG	GG	AA vs AG and GG	Linear effect	Mixed models
PON1 -221	44% (37/84)	38.1% (32/84)	17.9% (15/84)			
Prepartum	95.7 (±3.6)	89.8 (±3.9)	71.5 (±5.7)	0.0040	0.0007	
Postpartum	110.5 (±4.5)	97.6 (±4.8)	72.1 (±7.1)	0.0001	<.0001	
Overall	105.8 (±3.5)[a]	95.1 (±3.8)[b]	72.2 (±5.5)[c]			<.0001
	AA	AC	CC	AA vs AC and CC	Linear effect	Mixed models
PON1 -392	34.6% (29/84)	45.2% (38/84)	20.2% (17/84)			
Prepartum	83.7 (±4.3)	91.7 (±3.8)	92.5 (±5.7)	0.1365	0.2263	
Postpartum	87.4 (±5.5)	104.7 (±4.8)	104.8 (±7.2)	0.0155	0.0585	
Overall	86.4 (±4.3)[b]	100.5 (±3.7)[a]	101.0 (±5.6)[a]			0.0335
	CC	CT	TT	CC vs CT and TT	Linear effect	Mixed models
PON1 -611	76.2% (64/84)	20.2% (17/84)	3.6% (3/84)			
Prepartum	92.8 (±2.8)	81.2 (±5.4)	54.6 (±12.9)	0.0015	0.0052	
Postpartum	102.1 (±3.7)	92.2 (±7.2)	65.1 (±17.3)	0.0231	0.0402	
Overall	99.2 (±2.8)[a]	88.8 (±5.6)[ab]	61.6 (±13.3)[b]			0.0109
	AA	AT	TT	AA vs AT and TT	Linear effect	Mixed models
PON1 -676	61.9% (52/84)	28.6% (24/84)	9.5% (8/84)			
Prepartum	91.3 (±3.2)	87.3 (±4.8)	80.8 (±8.3)	0.2209	0.2494	
Postpartum	106.4 (±4.0)	88.2 (±5.9)	80.9 (±10.3)	0.0034	0.0247	
Overall	101.5 (±3.2)[a]	88.0 (±4.7)[b]	81.1 (±8.2)[b]			0.0127

* PON1 activity (U/mL) is expressed as the mean ± SEM in serum samples taken prepartum, postpartum or overall. $P < 0.05$ was considered to be statistically significant.

Table 2. Association of single nucleotide polymorphisms (SNPs) in the *PON1* promoter region with calving to conception interval (CCI) postpartum in Holstein dairy cows.

	Genotypes			***P* value**	
	CC	CG	GG	CC vs CG and GG	Linear effect
PON1 -22	66.7% (56/84)	27.4% (23/84)	5,9% (5/84)		
CCI (days)	95.1 (±5.9)	107.5 (±9.4)	103.5 (±20.5)	0.4208	0.6976
	AA	AG	GG	AA vs AG and GG	Linear effect
PON1 -105	76.2% (64/84)	20.2% (17/84)	3.6% (3/84)		
CCI (days)	96.1 (±5.5)	103.0 (±10.4)	143.5 (±28.7)	0.1010	0.1107
	TT	GT		TT vs GT	Linear effect
PON1 -176	91.7% (77/84)	8.3% (7/84)			
CCI (days)	97.8 (±5.1)	111.0 (±16.7)		0.4555	
	AA	AG	*GG*	AA vs AG and GG	Linear effect
PON1 -221	44% (37/84)	38.1% (32/84)	17.9% (15/84)		
CCI (days)	92.5 (±7.1)	95.0 (±7.6)	124.3 (±11.4)	0.0892	0.0218
	AA	AC	CC	AA vs AC and CC	Linear effect
PON1 -392	34.6% (29/84)	45.2% (38/84)	20.2% (17/84)		
CCI (days)	129.0 (±7.1)	86.1 (±6.0)	76.0 (±9.6)	<.0001	<.0001

	CC	CT	TT	CC vs CT and TT	Linear effect
PON1 -611	76.2% (64/84)	20.2% (17/84)	3.6% (3/84)		
CCI (days)	96.1 (±5.5)	103.0 (±10.4)	143.5 (±28.7)	0.1010	0.1107
	AA	AT	TT	AA vs AT and TT	Linear effect
PON1 -676	61.9% (52/84)	28.6% (24/84)	9.5% (8/84)		
CCI (days)	95.8 (±6.1)	99.3 (±9.3)	117.8 (±15.4)	0.2489	0.1910

$P < 0.05$ was considered to be statistically significant.

FIGURES

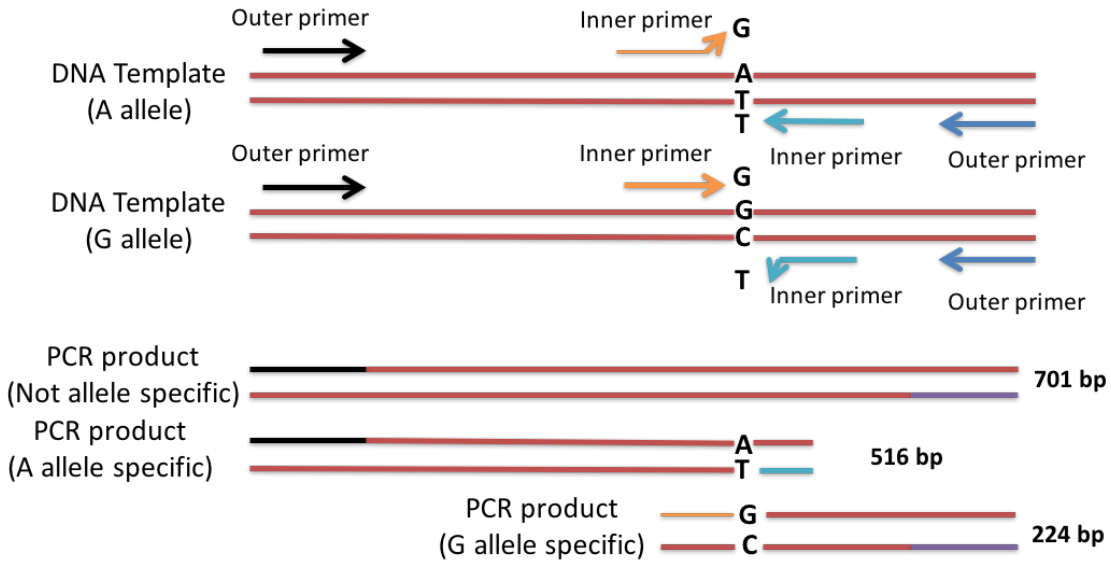

Fig. 1. Schematic presentation of the tetra-primer ARMS-PCR method.

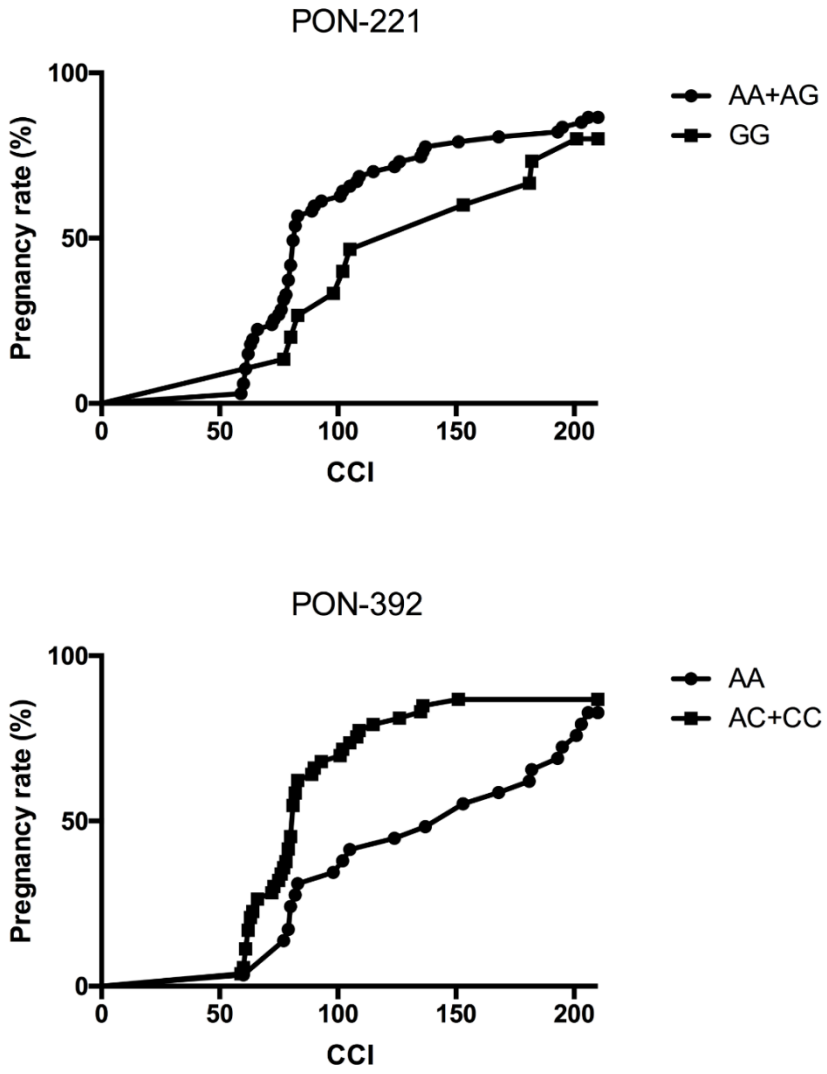

Fig. 2. Survival analysis of the effects on calving to conception interval (CCI) for the three genotypes of *PON1* -221 (A), *PON1* -221 AA + AG vs GG (B) and *PON1* -392 (C).

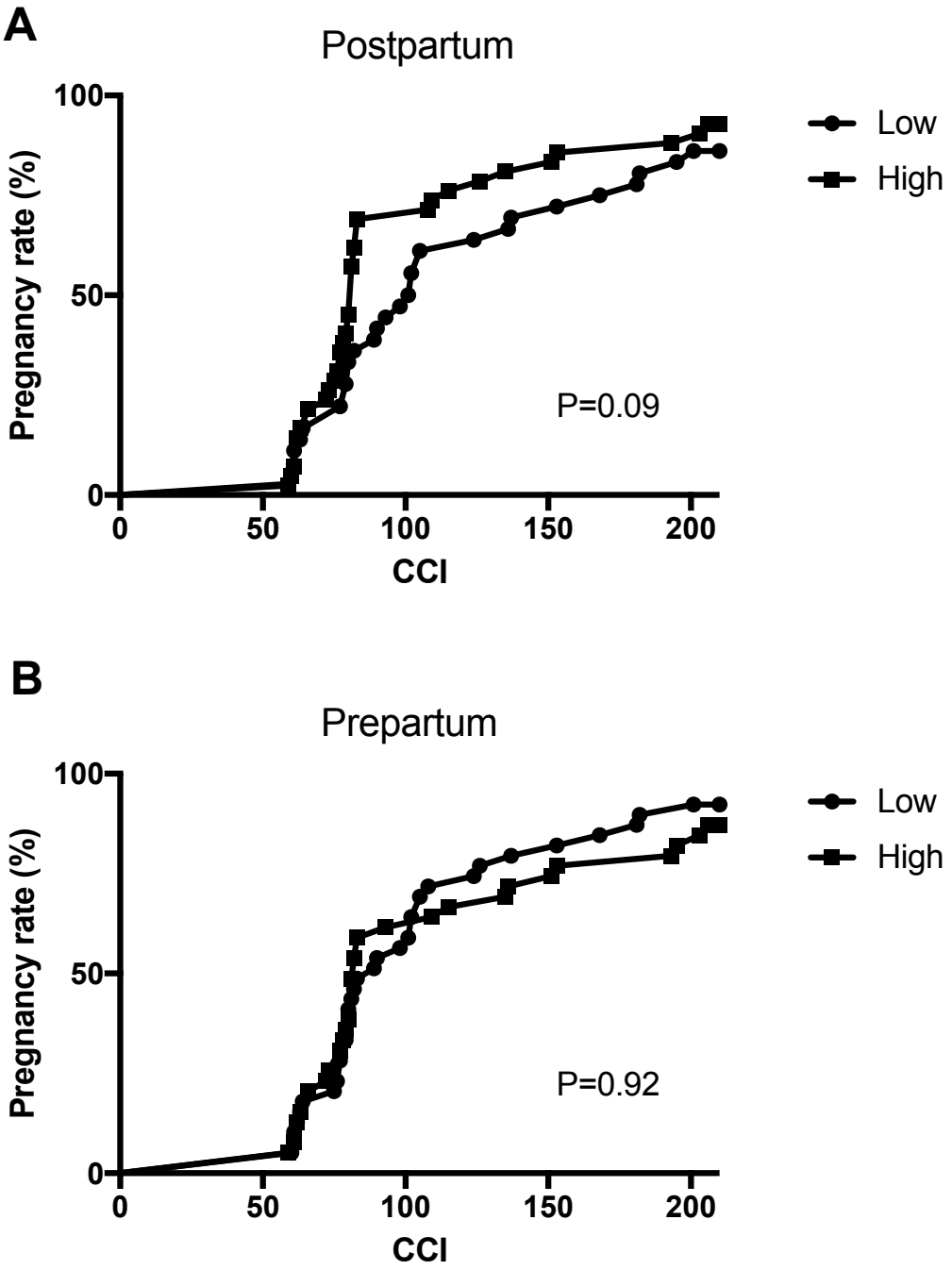

Fig. 3. Survival analysis of the effect of cows with high or low PON1 in the postpartum (A) and prepartum (B) period on calving to conception interval (CCI).

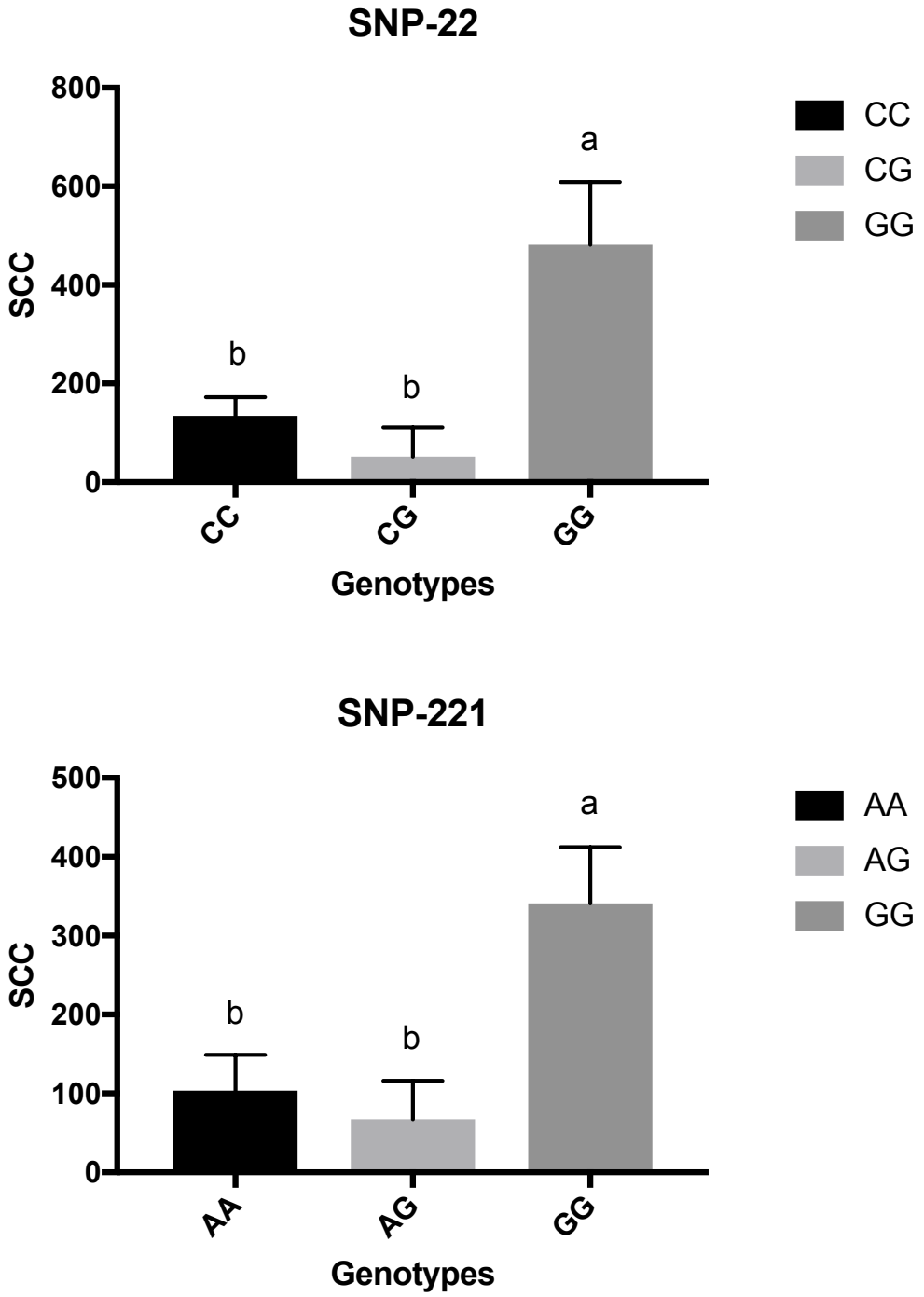

Fig. 4. Effect of *PON1* -22 and *PON1* -221 on SCC in periparturient Holstein dairy cows. [a,b]$P < 0.05$.

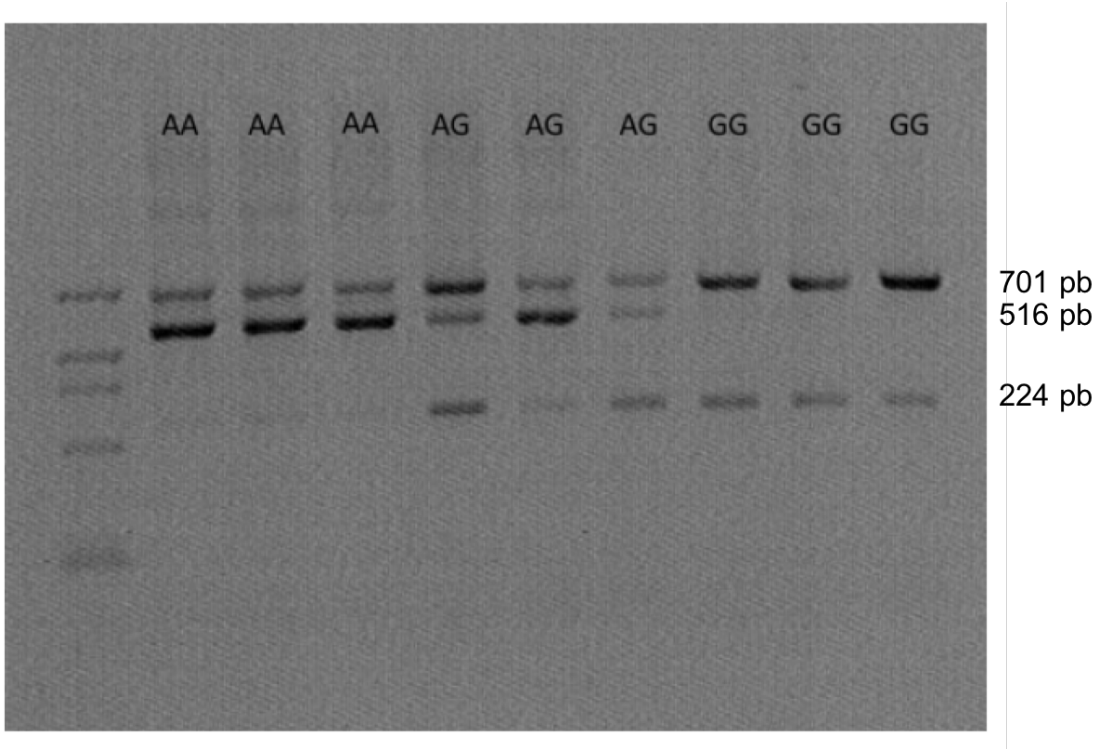

Fig. 5. SNP *PON1*-221 Genotyping by ARMS-PCR.

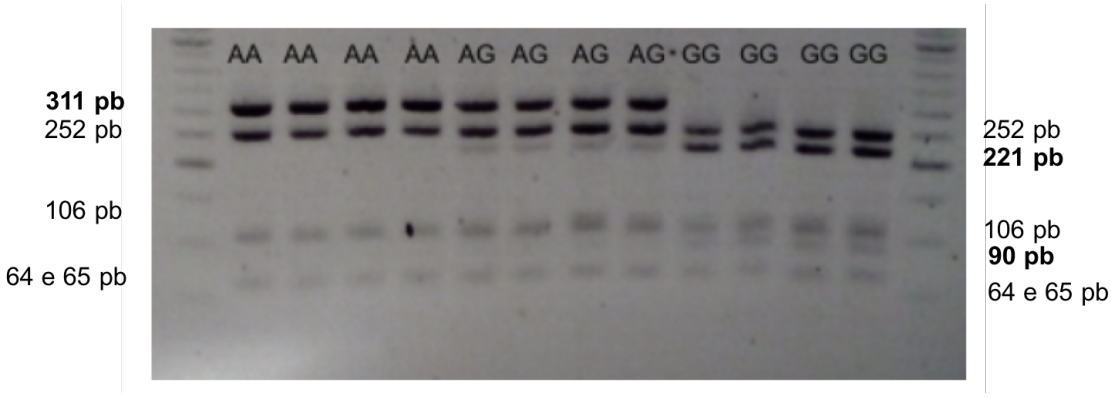

Fig. 6. SNP *PON1*-221 Genotyping by *Bsl*I.

3.2 Artigo 2

Association of polymorphisms in the *TNF-α*, *TLR-4* and *COQ9* genes with reproductive performance, feed intake, metabolism and health of Holstein dairy cows

Pedro Augusto Silva Silveira, Walter Ronald Butler, Sara LaCount, Tom Overton, Augusto Schneider

Será submetido à revista Theriogenology

Association of polymorphisms in the *TNF-α*, *TLR-4* and *COQ9* genes with reproductive performance, feed intake, metabolism and health of Holstein dairy cows

Running title: Polymorphisms in the *TNF-α*, *TLR-4* and *COQ9* genes of dairy cows

Pedro Augusto Silva Silveira [a], Walter Ronald Butler [b], Sara LaCount [b], Tom Overton [b], Augusto Schneider [c]*

[a] Faculdade de Veterinária, Universidade Federal de Pelotas, Campus Capão do Leão, sn, Pelotas, RS, Brazil.

[b] Department of Animal Science, Cornell University, 14853, Ithaca, NY, USA.

[c] Faculdade de Nutrição, Universidade Federal de Pelotas, Rua Gomes Carneiro, 1, Pelotas, RS, Brazil.

* Corresponding author. Tel.: +555339211270. E-mail address: augusto.schneider@ufpel.edu.br (A. Schneider).

Abstract

The aim of this study was to investigate the association of single nucleotide polymorphisms (SNPs) in tumor necrosis factor alpha (*TNF-α*), toll-like receptor 4 (*TLR-4*) and coenzyme 9 (*COQ9*) genes with fertility, feed intake and health of Holstein dairy cows. For this, 84 multiparous Holstein cows were used in the study. Daily dietary intake of each animal was measured from 40 days prepartum up to 60 days postpartum and clinical data was evaluated. The milk samples were collected twice a week in the first two weeks of lactation and once a week thereafter to milk composition analysis. Blood samples were collected weekly for β-hidroxybutyrate (BHBA) and non-esterified fatty acids (NEFA) measurement. DNA was extracted from the whole blood samples for the PCR reaction. Genotyping was determined by electrophoresis of the PCR product after digestion with the enzymes *Rsa* I (*TNF-α*), *Alu* I (*TLR4*) and *Tfi* I (*COQ9*). Cows were pre-synchronized with two injections of prostaglandin F2α followed by timed AI after an Ovsynch program. The pregnancy was confirmed after rectal palpation and reproductive performance data was recorded until 210 days in milk (DIM). The SNP on *TNF-α* gene had a quadratic effect on the calving to conception interval (CCI) ($P < 0.05$), with the homozygous TT and CC groups conceiving 32 days earlier than the heterozygote group ($P < 0.05$). There was an effect of the *TNF-α* gene on NEFA concentrations ($P < 0.05$). Also, there was an effect of the *TLR-4* gene on the CCI, with cows from the CC group

conceiving later compared to the TT and CT groups ($P < 0.05$) The SNP in the *COQ9* gene had a linear effect CCI, where the GG group conceived 33 days earlier than the AA group ($P < 0.05$). In addition, the SNP in the *COQ9* gene had a quadratic effect ($P < 0.05$) on prepartum feed intake, and the GG group intake was lower than the AA and AG groups during the same period. The GG genotype of the *COQ9* gene had increased milk somatic cell count ($P < 0.05$). In summary, the SNPs in the *TNF-α*, *TLR4* and *COQ9* were associated with fertility postpartum. Also, the *TNF-α* SNP was associated with NEFA and the *COQ9* SNP was associated with SCC and prepartum feed intake.

Keywords: fertility; inflammation; single nucleotide polymorphisms

1. Introduction

In recent years, dairy cows milk production has increased dramatically due to improvements in management, nutrition and genetic selection [1, 2]. Even so, the transition from gestation to lactation is a volatile period in the dairy cow health, greatly due to changes in energy demands and nutrient partitioning that occur after calving [3, 4]. In the postpartum period, there is a reduction in dry matter intake, although the energy demand increases dramatically for milk synthesis. The result is a negative energy balance (NEB) that precedes a series of health complications depending on its intensity [5]. Furthermore, an association with NEB severity and decreased immune response has been reported in cows that developed uterine disease [6, 7]. Thus, the ability of the immune system at calving and in early postpartum weeks may determine the success to prevent diseases impacting on the reproductive performance. Cytokines play a key role in stimulating systemic inflammatory responses, including increased body temperature and heart rate, and decreased feed intake [8], but its effects in the fertility of the dairy cow remains unclear.

Tumor necrosis factor (TNF-α) is a pleiotropic polypeptide cytokine produced by macrophages, neutrophils, lymphocytes, smooth muscle, fibroblasts, and endothelium [9]. TNF-α is a proinflammatory cytokine that is stimulated at the onset of inflammatory processes and when levels of non-esterified fatty acids (NEFA) and ketone bodies are elevated [10, 11]. In uterine infections, the role of TNF-α is to stimulate the expression of *IL-8*, which in turn increases phagocytosis and bacterial death [12, 13]. In dairy cows, anovulatory anoestrus, long and short luteal phases appear to be linked to uterine diseases [14, 15], therefore the duration of the inflammatory response can affect fertility. In addition, when TNF-α antiserum was injected into the intrafollicular fluid of sheep, ovulation was blocked [16], suggesting an

important role of this cytokine in the normal reproductive physiology. TNF-α is constitutively expressed in ovary follicle endothelial cells intended for ovulation and the localized release of this cytokine is a prelude to programmed cell death and follicular rupture [16]. In addition to all that, inflammatory cytokines as TNF-α may reduce feed intake and glucose production, causing hypoglycemia and increasing the mobilization of adipose tissue and metabolic disorders in the beginning of lactation [10, 17, 18], which can impair the reproductive performance. The severity of NEB [19], as well as postpartum inflammatory processes, have a negative impact on fertility [20]. In humans, single nucleotide polymorphisms (SNPs) of the *TNF* gene are associated with several diseases [21] and involved in the regulation of *TNF-α* mRNA expression [22]. In dairy cows, some studies demonstrate that SNPs in the TNF-α gene are associated with reproductive performance and immune functions [23], suggesting that *TNF-α* could be a potential genetic marker for immune response and reproductive performance in dairy cattle.

Toll-like receptors (TLRs) are transmembrane proteins that play a key role in innate immunity by recognizing pathogens and subsequently activating appropriate responses. TLR4 activation can lead to the induction of proinflammatory genes, such as the one encoding TNF-α. Recently, free fatty acids also have been identified as ligands of TLR4 [24], linking lipolysis, TLR4 and inflammation, which are recurrent events in the early postpartum dairy cow. In humans, polymorphisms in the *TLR-4* gene may reduce the efficiency of the immune response to bacterial membrane lipopolysaccharides [25]. Moreover, polymorphisms that induce the amino acid change of *TLR-4* reduce the signaling potential of the receptor, altering the resulting inflammatory response [26]. A recent study found effects of a polymorphism in the exon 3 of *TLR-4* gene in dairy cows on the number of artificial insemination (AI) per conception and days open, suggesting that this mutation may be a target factor for studying the reproductive potential of individual cows by considering immune cell activation [27].

A novel SNP in the bovine coenzyme Q9 (*COQ9*) gene has been associated with altered mitochondrial function and modulation of reproductive parameters in dairy cattle [28]. This SNP is an example of a mutation that changes the predicted protein structure. The missense mutation causes a change from G to A which induces an amino acid change from aspartic acid to asparagine at position 53 of the protein [29]. The A allele, which has a frequency of 49.1% in Holsteins, is associated with the reduced calving to conception interval (CCI) and the increased conception rate of cows, with no significant effects on milk production [30]. *COQ9*, along with other COQ proteins (COQ2-COQ8), is involved in the biosynthesis of COQ10 [31, 32], which is a component of the mitochondrial electron transport system and is required for

the synthesis of mitochondrial adenosine triphosphate [31]. Therefore, *COQ9* is critical for the metabolism of cellular energy.

Based on all these evidences, we hypothesized that mutations in genes related to the immune response, as well as genes enrolled with efficiency in cellular energy utilization, could be used as molecular markers with impact on postpartum fertility, feed intake and health. Therefore, the aim of this study was to investigate the presence of SNPs in *TNF-α*, *TLR-4* and *COQ9* genes and its association with fertility, feed intake and health of Holstein dairy cows.

2. Methods

2.1 Animals, milk collection and feed intake

All experimental procedures were approved by Cornell University Institutional Animal Care and Use Committee. A total of 84 multiparous Holstein cows were used in the study, with ad libitum access to a total mixed ration fed twice a day. Samples were collected weekly to analyze the nutritional composition of the diet (Dairy One Cooperative, Ithaca, NY, USA). The analyzes were carried out monthly using a sample of the mixture of the corresponding weekly collections. Forty days before the expected calving time the cows were housed in individual stalls to measure the daily dietary intake of each animal, performed up to 60 days in milking (DIM). The cows were milked twice a day and the milk production for each cow was calculated each week. The milk samples were collected twice a week in the first two weeks of lactation and once a week thereafter. The composition of the milk was analyzed in Barbano's laboratory, at Cornell University, using medium NIR techniques.

2.2 Blood analyses

Blood samples were collected weekly through puncture of the coccygeal vein before calving. From the beginning of lactation, the samples were collected twice a week in the first two weeks postpartum and once a week up to 42 DIM. BHBA was determined in whole blood samples prior to centrifugation and plasma separation using NovaVet portable ketone meter (Nova Biomedical, Billerica, MA, USA). Plasma was divided into three aliquots for further measurement of NEFA by an autoanalyzer (Boehringer Mannheim Hitachi 104, Diagnostic Laboratory Systems, Indianapolis, IN, USA).

2.3 Determination of genetic polymorphisms

DNA was extracted from the whole blood samples using QuickGene DNA whole blood kit S (Quick gene-810, Fujifilm), for the PCR reaction, using primers forward: 5'-GGGTGACTTGCTCTAACACTCATC -3' and reverse: 5'-AGGCCTCACTTCCCTACATCCCTA -3' to obtain a fragment with 1,233 bp of the *TNF-α* exons 2, 3 and 4 [33]. The PCR reaction, with the Roche® kit, used temperatures of 95°C for 9 min, 39 cycles of 30 secs at 95°C, 30 secs at 63°C and 90 secs at 72°C, and a final step at 72°C for 7 min. PCR-amplified DNA was digested with 10 U *Rsa* I (Biolabs, Linden, NJ, USA) at 37 °C for 2 h. Electrophoresis with 2.5% agarose gel was performed, containing 0.5 Ug/mL ethidium bromide and visualized under UV light. The expected restriction fragments in the gel were at positions 928 bp + 305 bp (TT genotype); 1,233 bp + 928bp + 305 bp (CT genotype); 1233 bp (CC genotype).

For *TLR-4* SNP genotyping, the same DNA samples were used for the PCR reaction. We used the primers forward: 5'- AGACAGCATTTCACTCCCTC -3' and reverse: 5'-ACCACCGACACACTGATGAT -3' to obtain a fragment with 382 bp of the *TNF-α* exons 3 [34]. The PCR reaction, with the Roche® kit, used temperatures of 95°C for 9 min, 35 cycles of 30 secs at 95°C, 30 secs at 55.5°C and 90 secs at 72°C, and a final step at 72°C for 7 min. PCR-amplified DNA was digested with 10 U *Alu* I (Biolabs, Linden, NJ, USA) at 37 °C for 2 h. Electrophoresis with 2.5% agarose gel was performed, containing 0.5 Ug/mL ethidium bromide and visualized under UV light. The expected restriction fragments in the gel were at positions 260 bp + 77 bp + 32 bp (not shown) + 13 (not shown) (TT genotype); 260 bp + 142 bp + 118 bp + 77 bp + 32 bp (not shown) + 13 (not shown) (CT genotype); 142 bp + 118 bp + 77 bp + 32 bp (not shown) + 13 (not shown) (CC genotype).

A similar procedure was used to genotype the *COQ9* SNP. We used the primers forward: 5'- TTGTTTGAGCCTCACAGCATTC -3' and reverse: 5'-ACAGATTCACCCAGGCACTT -3' to obtain a fragment with 556 bp from *COQ9* gene. The primers were constructed using the Primer3 Plus program (http: //www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The PCR reaction, with the Roche® kit, used temperatures of 95°C for 9 min, 35 cycles of 30 secs at 95°C, 30 secs at 56.5°C and 90 secs at 72°C, and a final step at 72°C for 7 min. PCR-amplified DNA was digested with 10 U *Tfi* I (Biolabs, Linden, NJ, USA) at 37 °C for 2 h. Electrophoresis with 2.5% agarose gel was performed, containing 0.5 Ug/mL ethidium bromide and visualized under UV light. The expected restriction fragments in the gel were at positions 407 bp + 142 bp + 7 bp (not shown) (AA genotype); 549 bp + 407 bp + 142 bp + 7 bp (not shown) (AG genotype); 549 bp + 7 bp (not shown) (GG genotype). In

order to confirm de genotyping procedure by restriction fragment length polymorphism, we sent 15 samples for sequencing, five of each genotype. A PCR was performed with the same primers and protocol as described above and an electrophoresis with 1% agarose gel, from which the DNA band was cut in the position equivalent to 556 bp. This gel aliquot was purified using a commercial kit (Promega, Madison, Wisconsin, USA). The purified samples were sent for DNA sequencing by the Sanger method (Biotechnology Resource Center, Cornell University Institute of Biotechnology). Sequences were aligned in the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), using the published sequence of bovine COQ9 (NCBI accession number: AC_000161.1) as the reference for alignment purposes, and the SNPs were manually identified.

2.4 Reproductive Management

Cows were pre-synchronized with two injections of prostaglandin F2α (PGF2α; 25 mg, Lutalyse, Pfizer Animal Health, New York, NY, USA) for the first postpartum AI, 14 days apart on days 28 and 44 after parturition. The Ovsynch program [35] was started in all cows ten days after the second injection of PGF2α. The initial GnRH dose (100 mg im; Cystorelin, Merial Ltd, Duluth, GA, USA) was followed 7 days later by an injection of PGF2α and 48 h later cows received the second dose of GnRH with timed AI 12 h thereafter. Cows that were previously inseminated but showed visual signs of estrous behavior before pregnancy diagnosis were re-inseminated. Additionally, cows not pregnant at the time of any subsequent pregnancy diagnosis (32 days post-AI) were re-enrolled in the Ovsynch program. Confirmation of pregnancy by rectal palpation of the reproductive tract was made twice at 32 and 60 days post-AI. The reproductive performance of the cows enrolled in the study was recorded until 210 DIM.

2.5 Clinical data

After calving, the cows were monitored for the development of diseases incidence or pathological clinical signs. The definitions used on farm were the following: retained placenta, retention of fetal membranes for longer than 24 h; metritis, abnormal vaginal discharge for 2 days and fever in the first 3 weeks postpartum; displaced abomasum, presence of abdominal ping requiring surgical correction; ketosis, no appetite and presence of ketone bodies in the urine; lameness, difficulty to walk plus visual inspection; mastitis, abnormal milk, and/or high somatic cell count (SCC); and milk fever, subnormal body temperature and recumbency.

2.6 Statistical analysis

The results are presented as mean values ± SEM. All the statistical analyses were performed using SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Analyses involving repeated measures over time (e.g., NEFA, BHBA, milk production, and SCC) were compared by analysis of variance for repeated measures using the MIXED procedure to evaluate the main effects of time and genotype. In addition, the CCI and feed intake were evaluated using polynomial models for the linear effects, quadratic effects of having none, one or two *TNF* C, *TRL-4* C and *COQ9* A alleles. Disease incidence was evaluated by Chi-square analysis. Pregnancy rate were evaluated by Kaplan-Meier survival analysis using GraphPad Prism 5 (GraphPad Soft- ware Inc., La Jolla, CA, USA). A probability value of $P < 0.05$ was considered significant and probability between 0.05 and 0.10 as a tendency.

3. Results

3.1 Effect of genetic polymorphisms on fertility, feed intake and milk production

The genotypic frequencies of the 84 cows used in the study on SNPs in *TNF-α*, *TLR-4* and *COQ9* genes are described in Table 1. The SNP on *TNF-α* gene showed a quadratic effect on CCI ($P < 0.05$), with the group homozygous TT getting pregnant 32 days before the heterozygote group ($P < 0.05$). There was no difference between the CC homozygous group and the TT and CT groups ($P > 0.05$). Cows in the CT group showed a tendency to have a higher feed intake in the prepartum period than cows in the both homozygous groups (quadratic effect, $P < 0.10$). There was no effect of *TNF-α* SNP on postpartum feed intake (Table 1).

The SNP in the *TLR-4* gene had no effect on CCI ($P > 0.05$). There was also no difference between feed intake in the pre-or postpartum period related to the 3 genotypes of this SNP (Table 1).

The SNP in the *COQ9* gene presented a linear effect on CCI, where the GG group with a mean CCI 33 days shorter compared to the AA group ($P < 0.05$). In addition, the SNP in the *COQ9* gene had a quadratic effect ($P<0.05$) on prepartum feed intake, and the AA group intake was lower than the AG and GG groups during the same period (Table 1). In the postpartum period, just a tendency ($P < 0.10$) of quadratic effect on feed intake was observed.

In the survival analysis, it was also possible to observe an effect of SNP on the *TNF-α* gene on postpartum fertility, with a greater CCI for cows in the CT group ($P < 0.05$) (Fig. 1). In addition, there was an effect of the SNP on the *TLR-4* gene in the CCI, with cows from the

CC group getting pregnant later compared to the TT and CT groups ($P < 0.05$) (Fig. 1). SNP in the *COQ9* gene had no effect on postpartum CCI ($P > 0.05$).

Despite the effects of SNPs on dry matter intake, there was no difference from any of the genotypes presented on milk production in this period ($P > 0.05$).

3.2 Effect of genetic polymorphisms on NEFA and BHBA

There was an effect of the SNP on *TNF-α* gene on NEFA concentrations, which were 412.7, 357.6 and 315.7 umol/L, for the CC, CT and TT groups respectively (Fig. 2). The NEFA concentration from group CC was higher than group TT ($P < 0.05$). None of other SNPs had any effect on NEFA ($P > 0.05$). Also, there was no effect from the 3 SNPs that have been evaluated on BHBA.

3.3 Effect of genetic polymorphisms on somatic cell count and health

A significant effect of the SNP on *COQ9* gene was observed on milk SCC ($P < 0.05$). GG cows presented higher SCC (294,700) when compared to AA (65,700) and AG (69,300) cows (Fig. 3). There was no statistical difference between the groups AA and AG ($P > 0.05$) regarding SCC. SNPs in the *TNF-α* and *TLR-4* genes had no effect on SCC ($P > 0.05$). Also, there was no effect of genetic polymorphisms on the occurrence of postpartum diseases ($P > 0.05$).

4. Discussion

The three SNPs had an impact on postpartum fertility. The *TNF-α* gene was associated with NEFA levels and *COQ9* gene was associated with SCC. For the SNP in the *TNF-α* gene the genotype distribution found for the CC, CT and TT genotypes was, respectively, 17.8, 41.7 and 40.5%, very different from previous studies conducted in Japan, where higher frequencies of CC and lower frequencies of TT cows were found [23, 36] compared with our study. We found a quadratic effect of this SNP on CCI, and the heterozygote group presented an impaired postpartum reproductive performance, with the highest CCI among the three genotypes. In addition, the CC group presented higher levels of NEFA compared to the TT group. Polymorphisms of immune function-related genes, especially *TNF-α*, have been associated with the first postpartum ovulation [36]. Moreover, the resumption of ovarian cyclicity prior to the first AI is one of the most important factor related to postpartum fertility, especially in high-producing cows [37]. We did not evaluate the first ovulation postpartum, but contrary to our expectation, other studies have found lower rates of the first postpartum ovulation for the TT

genotype [23, 36]. These studies did not evaluate the postpartum NEFA levels, which in our study were lower for the TT group compared to the CC group, which seems to have positively influenced the CCI in the TT homozygote group observed in our study. Despite the difference in ovulation in favor of CT and CC genotypes, there was no difference on days open [23, 36]. Cows with a more severe NEB, and consequent more dramatic loss of body condition have increased NEFA levels, during the peripartum period, having delayed resumption of ovarian activity compared with cows with less severe body condition loss [38, 39]. On the other hand, TNF-α already has an established important role in ovulation both systemically and locally in the follicle. Injections of TNF-α together with luteinizing hormone (LH) have increased LH-induced ovulation rate in rats [40], and TNF-α antiserum injection intrafollicularly in sheep has blocked ovulation [16]. Interestingly, mRNA expression of *TNF-α* in leukocytes was higher in cows of the ovulatory types compared with anovulatory types, which suggest that polymorphisms of *TNF-α* gene may affect the transcriptions levels of TNF-α and favor ovulation [36]. Furthermore, TNF-α directly regulates 66 genes associated with heifer fertility [41] and promotes cytokine reaction with systemic and local functions that are elicited in response to infection and inflammation, which are important signaling events to maintain a receptive postimplantation uterine environment. Therefore, the *TNF-α* exon mutation may impair the TNF-α functions, in animals with the T alleles, due to direct effects, but the NEFA levels seems to be higher in animals with C alleles. Together, these might explain the low reproductive performance in the heterozygotes cows (CT) observed in the current study.

For the SNP in the *TLR-4* gene the genotype frequencies observed was very similar to data found in Holstein, Sanhe and Simental cattle in a study carried out in China [34]. An effect of this SNP on postpartum fertility was observed. The CC homozygous cows had mean values for CCI 20 days longer than TT cows, which was different in the survival analysis. The *TLR-4* exon polymorphism has been associated with the number of AI and days open in dairy cows, since it modulates the apoptosis and migration of polymorphonuclear leukocytes (PMNs) and IL-1β production in the peripheral blood mononuclear cells (PBMCs) [27]. The CC homozygous cows had a higher number of AI per pregnancy and greater days open compared to CT cows [27], which agree with our current results. Ovarian function such as follicular development and corpus luteum formation are regulated by immune cells, as neutrophils, macrophages, and T-lymphocytes [42]. The inflammatory process of the reproductive tract or mammary gland with gram-negative bacteria can perturb ovarian function, follicular growth, and fecundity in cattle [43, 44], mediated by TLR4 recognition of lipopolysaccharides (LPS), even in granulosa cell [45], which will be very important in the first few weeks after calving.

LPS induces the release of cytokines such as IL-1b and TNF-α from PBMCs [46], However, LPS induce the expression of cytokines genes in PBMCs depending on the genotype, with the CT genotype possessing higher ability to release IL-1β and the migration of PMNs with the CT genotype may be enhanced [27] compared to CC. Moreover, the levels of NEFA were the same for the three genotypes and the free fatty acids can bind to TLR-4, potentiating the effects caused by inflammatory cytokines [24, 47]. So, it is possible that cows with the CC genotype presented a different response to NEFA levels, with a negative impact on postpartum fertility.

The genotype frequencies of the SNP in the *COQ9* gene found in our study were very similar to those found by other researches evaluating lactating Holstein cows in the southeastern USA [28]. For the first time this SNP on *COQ9* gene was evaluated by a restriction fragment length polymorphism using the *Tfi* I enzyme, as described in the methods. The SNP on the *COQ9* gene had an effect on postpartum period. Furthermore, there was an effect of this SNP on prepartum feed intake, with cows with the AA genotype eating less than cows with the AG and GG genotypes, until the calving. Prepartum dry matter intake is related with metabolic parameters such as liver triglycerides, NEFA, plasma insulin, postpartum dry matter intake, and metabolic disorders such as ketosis [48, 49], affecting the reproductive performance. During the last 3 weeks of pregnancy, nutrient demands by the fetus and placenta are at their greatest [50] and cows should be able to meet these requirements from the diet. In our study, the cows that presented the lowest dietary feed intake (AA) had higher CCI, even though there was no difference in postpartum feed intake, milk production and NEFA levels between the three genotypes. Despite few information about polymorphisms in the *COQ9* in dairy cows, this SNP was associated with the reproductive function in an earlier study [28]. However, the cows of genotype AA presented the best performance, contrary to our current findings. There was an additive effect of the A allele on the pregnancy rate, services per conception and days open [28]. In addition, the AA genotype required less substrate to maintain basal cellular function, and displaying a reduced leak respiration, or the respiration that is not associated with energy production [28]. In the other hand, the AG genotype showed greater expression of *COQ9* in oocytes than either homozygote, and the transcript abundance for *COQ9* was also greater for AG than other genotypes, despite this genotype being intermediate in fertility, what evidence that fertility is not determined to a large extent by amount of mRNA for *COQ9* in the oocyte [28]. We did not evaluate the effect of *COQ9* SNP in the mitochondrial function, but it was hypothesized that GG genotype improve the cellular energy metabolism, affecting the prepartum feed intake, which seemed to impact on reproductive performance. Interestingly, the *COQ9* SNP showed a significant effect on somatic cell count, with the GG group presenting

higher SCC then AA and AG. The relation between COQ9 and mammary immune function is not clear. A consequence of electron transport through mitochondrial oxidative phosphorylation complexes is the generation of reactive oxygen species (ROS) [51]. ROS have been implicated as both positive and negative modulators, acting on immune reaction [51]. Thus, the *COQ9* SNP may have an effect on ROS production, with a negative impact on SCC and an improvement on ovulation process. Also, it is possible that the SNP in *COQ9* is in linkage disequilibrium with a causative mutation located elsewhere in *COQ9* or in other nearby genes, and more studies are need to better elucidate that.

Milk production and health parameters did not present any association with these three SNPs. The transition period is recognized as the most critical time of the lactation cycle for both the production and reproduction of dairy cows [4, 52]. Genetic selection and improved nutrition increased milk yield per cow, however, increased milk production was accompanied by a decrease in fertility in Holstein cows. In this scenario, the search for genetic markers that improve postpartum reproductive performance (*TNF-α*, *TLR-4* and *COQ9*) without increasing the achievement of genetic merit for milk production has increased and may represent one benefit of the current markers presented in this study, which positively affected reproductive efficiency without reducing milk production.

In conclusion **t**he SNPs in the *TNF-α*, *TLR4* and *COQ9* were associated with postpartum fertility. Also, the *TNF-α* SNP was associated with NEFA and the *COQ9* SNP was associated with SCC and with prepartum feed intake. These results suggest the three SNPs as new targets for large studies aiming to select cows for reproductive efficiency, with no negative impact on milk production.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by FAPERGS, CNPq AND CAPES.

References

[1] Hansen LB. Consequences of selection for milk yield from a geneticist's viewpoint. J Dairy Sci. 2000;83:1145-50.

[2] Lucy MC. Reproductive loss in high-producing dairy cattle: where will it end? J Dairy Sci. 2001;84:1277-93.

[3] Bell AW. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J Anim Sci. 1995;73:2804-19.

[4] Drackley JK. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? J Dairy Sci. 1999;82:2259-73.

[5] Grummer RR, Mashek DG, Hayirli A. Dry matter intake and energy balance in the transition period. Vet Clin North Am Food Anim Pract. 2004;20:447-70.

[6] Hammon DS, Evjen IM, Dhiman TR, Goff JP, Walters JL. Neutrophil function and energy status in Holstein cows with uterine health disorders. Vet Immunol Immunopathol. 2006;113:21-9.

[7] Galvao KN, Flaminio MJ, Brittin SB, Sper R, Fraga M, Caixeta L, et al. Association between uterine disease and indicators of neutrophil and systemic energy status in lactating Holstein cows. J Dairy Sci. 2010;93:2926-37.

[8] Dantzer R, Kelley KW. Twenty years of research on cytokine-induced sickness behavior. Brain Behav Immun. 2007;21:153-60.

[9] Vilcek J, Lee TH. Tumor necrosis factor. New insights into the molecular mechanisms of its multiple actions. J Biol Chem. 1991;266:7313-6.

[10] Ohtsuka H, Koiwa M, Hatsugaya A, Kudo K, Hoshi F, Itoh N, et al. Relationship between serum TNF activity and insulin resistance in dairy cows affected with naturally occurring fatty liver. J Vet Med Sci. 2001;63:1021-5.

[11] Hoeben D, Burvenich C, Trevisi E, Bertoni G, Hamann J, Bruckmaier RM, et al. Role of endotoxin and TNF-alpha in the pathogenesis of experimentally induced coliform mastitis in periparturient cows. J Dairy Res. 2000;67:503-14.

[12] Sheldon IM, Rycroft AN, Zhou C. Association between postpartum pyrexia and uterine bacterial infection in dairy cattle. Vet Rec. 2004;154:289-93.

[13] Galvao KN, Santos NR, Galvao JS, Gilbert RO. Association between endometritis and endometrial cytokine expression in postpartum Holstein cows. Theriogenology. 2011;76:290-9.

[14] Kaneko K, Kawakami S. Influence of experimental intrauterine infusion of Arcanobacterium pyogenes solution on ovarian activity in cycling cows. J Vet Med Sci. 2008;70:77-83.

[15] Opsomer G, Grohn YT, Hertl J, Coryn M, Deluyker H, de Kruif A. Risk factors for post partum ovarian dysfunction in high producing dairy cows in Belgium: a field study. Theriogenology. 2000;53:841-57.

[16] Murdoch WJ, Colgin DC, Ellis JA. Role of tumor necrosis factor-alpha in the ovulatory mechanism of ewes. J Anim Sci. 1997;75:1601-5.

[17] Bradford BJ, Mamedova LK, Minton JE, Drouillard JS, Johnson BJ. Daily injection of tumor necrosis factor-{alpha} increases hepatic triglycerides and alters transcript abundance of metabolic genes in lactating dairy cattle. J Nutr. 2009;139:1451-6.

[18] Yuan K, Farney JK, Mamedova LK, Sordillo LM, Bradford BJ. TNFalpha altered inflammatory responses, impaired health and productivity, but did not affect glucose or lipid metabolism in early-lactation dairy cows. PLoS One. 2013;8:e80316.

[19] Beam SW, Butler WR. Effects of energy balance on follicular development and first ovulation in postpartum dairy cows. J Reprod Fertil Suppl. 1999;54:411-24.

[20] Gobikrushanth M, Salehi R, Ambrose DJ, Colazo MG. Categorization of endometritis and its association with ovarian follicular growth and ovulation, reproductive performance, dry matter intake, and milk yield in dairy cattle. Theriogenology. 2016;86:1842-9.

[21] Savva A, Kanni T, Damoraki G, Kotsaki A, Giatrakou S, Grech I, et al. Impact of Toll-like receptor-4 and tumour necrosis factor gene polymorphisms in patients with hidradenitis suppurativa. Br J Dermatol. 2013;168:311-7.

[22] Lu MC, Yang KL, Tung CH, Huang KY, Yu HC, Liu SQ, et al. Higher LPS-stimulated TNF-alpha mRNA levels in peripheral blood mononuclear cells from Chinese ankylosing spondylitis patients with -308G/A polymorphism in promoter region of tumor necrosis factor: association with distinct A33/B58/Cw10 haplotypes. Rheumatol Int. 2008;29:189-95.

[23] Kawasaki Y, Aoki Y, Magata F, Miyamoto A, Kawashima C, Hojo T, et al. The effect of single nucleotide polymorphisms in the tumor necrosis factor-alpha gene on reproductive performance and immune function in dairy cattle. J Reprod Dev. 2014;60:173-8.

[24] Schaeffler A, Gross P, Buettner R, Bollheimer C, Buechler C, Neumeier M, et al. Fatty acid-induced induction of Toll-like receptor-4/nuclear factor-kappaB pathway in adipocytes links nutritional signalling with innate immunity. Immunology. 2009;126:233-45.

[25] Omueti KO, Mazur DJ, Thompson KS, Lyle EA, Tapping RI. The polymorphism P315L of human toll-like receptor 1 impairs innate immune sensing of microbial cell wall components. J Immunol. 2007;178:6387-94.

[26] Lorenz E, Mira JP, Frees KL, Schwartz DA. Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock. Arch Intern Med. 2002;162:1028-32.

[27] Shimizu T, Kawasaki Y, Aoki Y, Magata F, Kawashima C, Miyamoto A. Effect of Single Nucleotide Polymorphisms of Toll-Like Receptor 4 (TLR 4) on Reproductive Performance and Immune Function in Dairy Cows. Biochem Genet. 2017;55:212-22.

[28] Ortega MS, Wohlgemuth S, Tribulo P, Siqueira LG, Cole JB, Hansen PJ. A single nucleotide polymorphism in COQ9 affects mitochondrial and ovarian function and fertility in Holstein cows. Biol Reprod. 2017;96:652-63.

[29] Ortega MS, Denicol AC, Cole JB, Null DJ, Hansen PJ. Use of single nucleotide polymorphisms in candidate genes associated with daughter pregnancy rate for prediction of genetic merit for reproduction in Holstein cows. Anim Genet. 2016;47:288-97.

[30] Cochran SD, Cole JB, Null DJ, Hansen PJ. Discovery of single nucleotide polymorphisms in candidate genes associated with fertility and production traits in Holstein cattle. BMC Genet. 2013;14:49.

[31] Ben-Meir A, Burstein E, Borrego-Alvarez A, Chong J, Wong E, Yavorska T, et al. Coenzyme Q10 restores oocyte mitochondrial function and fertility during reproductive aging. Aging Cell. 2015;14:887-95.

[32] Tran UC, Clarke CF. Endogenous synthesis of coenzyme Q in eukaryotes. Mitochondrion. 2007;7 Suppl:S62-71.

[33] Higuchi M, Miyashita N, Awata T. Rapid communication: a PCR-RFLP in the coding region of the bovine tumor necrosis factor-alpha locus. J Anim Sci. 1999;77:3400-1.

[34] Wang X, Xu S, Gao X, Ren H, Chen J. Genetic polymorphism of TLR4 gene and correlation with mastitis in cattle. J Genet Genomics. 2007;34:406-12.

[35] Pursley JR, Mee MO, Wiltbank MC. Synchronization of ovulation in dairy cows using PGF2alpha and GnRH. Theriogenology. 1995;44:915-23.

[36] Shirasuna K, Kawashima C, Murayama C, Aoki Y, Masuda Y, Kida K, et al. Relationships between the first ovulation postpartum and polymorphism in genes relating to function of immunity, metabolism and reproduction in high-producing dairy cows. J Reprod Dev. 2011;57:135-42.

[37] Galvao KN, Frajblat M, Butler WR, Brittin SB, Guard CL, Gilbert RO. Effect of early postpartum ovulation on fertility in dairy cows. Reprod Domest Anim. 2010;45:e207-11.

[38] Beam SW, Butler WR. Energy balance, metabolic hormones, and early postpartum follicular development in dairy cows fed prilled lipid. J Dairy Sci. 1998;81:121-31.

[39] Butler WR, Smith RD. Interrelationships between energy balance and postpartum reproductive function in dairy cattle. J Dairy Sci. 1989;72:767-83.

[40] Brannstrom M, Bonello N, Wang LJ, Norman RJ. Effects of tumour necrosis factor alpha (TNF alpha) on ovulation in the rat ovary. Reprod Fertil Dev. 1995;7:67-73.

[41] Neupane M, Geary TW, Kiser JN, Burns GW, Hansen PJ, Spencer TE, et al. Loci and pathways associated with uterine capacity for pregnancy and fertility in beef cattle. PLoS One. 2017;12:e0188997.

[42] Walusimbi SS, Pate JL. Physiology and Endocrinology Symposium: role of immune cells in the corpus luteum. J Anim Sci. 2013;91:1650-9.

[43] Sheldon IM, Cronin J, Goetze L, Donofrio G, Schuberth HJ. Defining postpartum uterine disease and the mechanisms of infection and immunity in the female reproductive tract in cattle. Biol Reprod. 2009;81:1025-32.

[44] Lavon Y, Ezra E, Leitner G, Wolfenson D. Association of conception rate with pattern and level of somatic cell count elevation relative to time of insemination in dairy cows. J Dairy Sci. 2011;94:4538-45.

[45] Bromfield JJ, Sheldon IM. Lipopolysaccharide initiates inflammation in bovine granulosa cells via the TLR4 pathway and perturbs oocyte meiotic progression in vitro. Endocrinology. 2011;152:5029-40.

[46] Conti P, Dempsey RA, Reale M, Barbacane RC, Panara MR, Bongrazio M, et al. Activation of human natural killer cells by lipopolysaccharide and generation of interleukin-1 alpha, beta, tumour necrosis factor and interleukin-6. Effect of IL-1 receptor antagonist. Immunology. 1991;73:450-6.

[47] Bradford BJ, Yuan K, Farney JK, Mamedova LK, Carpenter AJ. Invited review: Inflammation during the transition to lactation: New adventures with an old flame. J Dairy Sci. 2015;98:6631-50.

[48] Bertics SJ, Grummer RR, Cadorniga-Valino C, Stoddard EE. Effect of prepartum dry matter intake on liver triglyceride concentration and early lactation. J Dairy Sci. 1992;75:1914-22.

[49] Grummer RR. Impact of changes in organic nutrient metabolism on feeding the transition dairy cow. J Anim Sci. 1995;73:2820-33.

[50] Bell AW, Burhans WS, Overton TR. Protein nutrition in late pregnancy, maternal protein reserves and lactation performance in dairy cows. Proc Nutr Soc. 2000;59:119-26.

[51] West AP, Shadel GS, Ghosh S. Mitochondria in innate immune responses. Nat Rev Immunol. 2011;11:389-402.

TABLES

Table 1. Association of single nucleotide polymorphisms (SNPs) in *TNF-α*, *TLR-4* and *COQ9* genes with fertility postpartum and feed intake[a] in the pre-and postpartum period in Holstein dairy cows.

	Genotypes			***P* value**			
	CC	CT	TT	CC *vs* CT and TT	CT *vs* TT	Linear effect	Quadratic effect
TNF-α	17.8% (15/84)	41.7% (35/84)	40.5% (34/84)				
CCI (days)	100.7 (±10.3)	116.2 (±7.7)	84.3 (±6.9)	0.9690	**0.0033**	0.1947	**0.0205**
Intake Pre	13.9 (±0.3)	14.6 (±0.2)	14.1 (±0.4)	0.3097	0.1734	0.6858	0.0899
Intake Post	22.0 (±0.7)	22.4 (±0.4)	22.1 (±0.4)	0.8119	0.6099	0.9838	0.5827
	CC	*CT*	*TT*	CC vs CT and TT	CT vs TT	Linear effect	Quadratic effect
TLR-4	*36.9% (31/84)*	*42.9% (36/84)*	*20.2% (17/84)*				
CCI (days)	109.7 (±8.6)	95.9 (±6.9)	89.4 (±10.8)	0.2764	0.2214	0.1496	0.7157
Intake Pre	14.4 (±0.3)	14.3 (±0.2)	13.9 (±0.4)	0.3291	0.7302	0.3087	0.7570
Intake Post	21.9 (±0.4)	22.7 (±0.4)	21.6 (±0.6)	0.3752	0.2715	0.7247	0.1527
	AA	*AG*	*GG*	AA vs AG and GG	AG vs GG	Linear effect	Quadratic effect
COQ9	*17.8% (15/84)*	*53.6% (45/84)*	*28.6% (24/84)*				
CCI (days)	115.0 (±11.5)	102.0 (±6.3)	82.5 (±9.1)	0.0797	0.0862	**0.0306**	0.7441
Intake Pre	13.3 (±0.4)	14.6 (±0.2)	14.3 (±0.3)	**0.02**	0.4493	0.0897	**0.0355**
Intake Post	21.7 (±0.6)	22.7 (±0.3)	21.6 (±0.5)	0.5614	0.1028	0.9014	0.0778

[a] Feed intake (kg of dry matter/day) is expressed as the mean ± SEM considering prepartum (Intake Pre) and postpartum (Intake Post) periods. $P < 0.05$ was considered to be statistically significant.

FIGURES

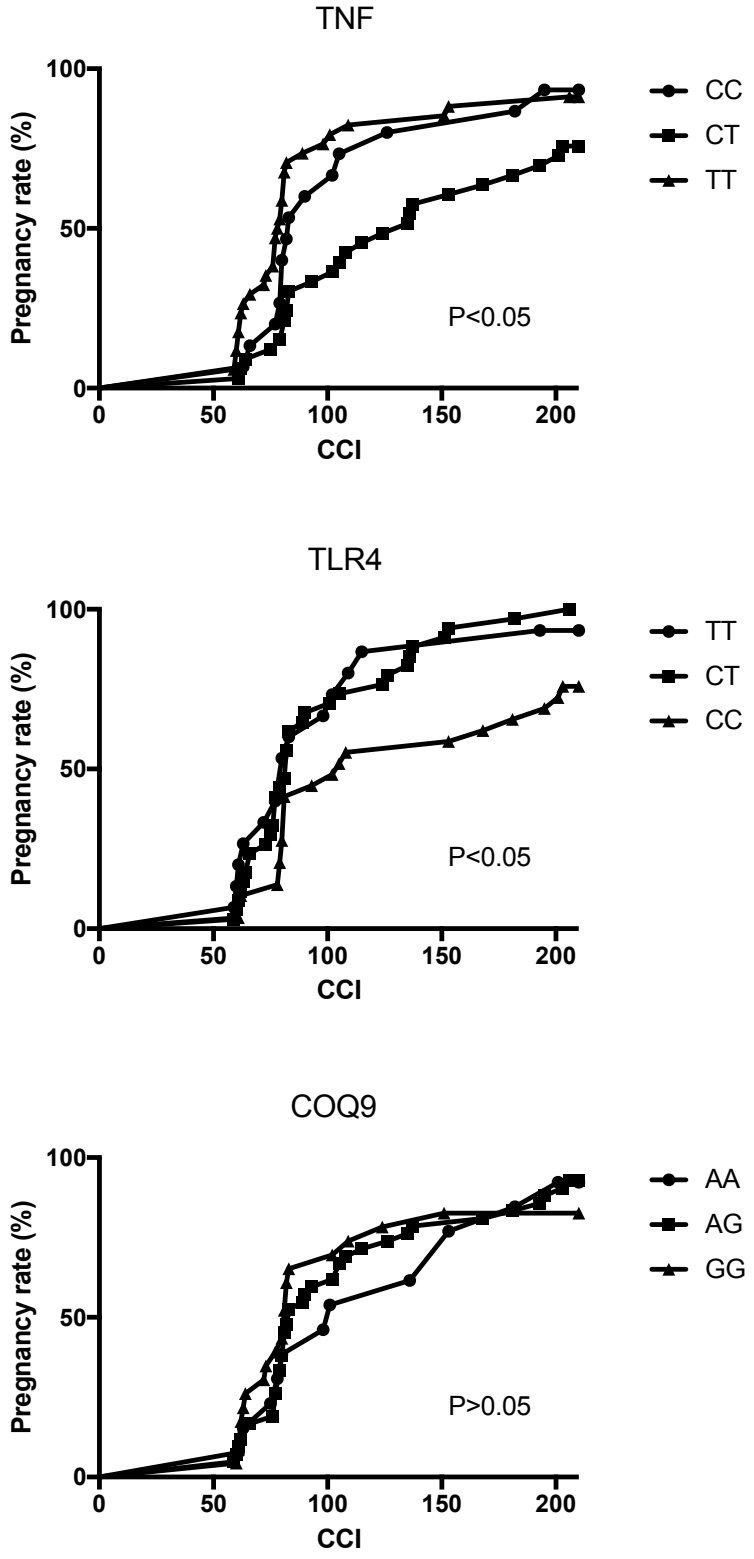

Fig. 1. Survival analysis of the effect of SNPs in *TNF-α*, *TLR-4* and *COQ9* genes on calving to conception interval (CCI).

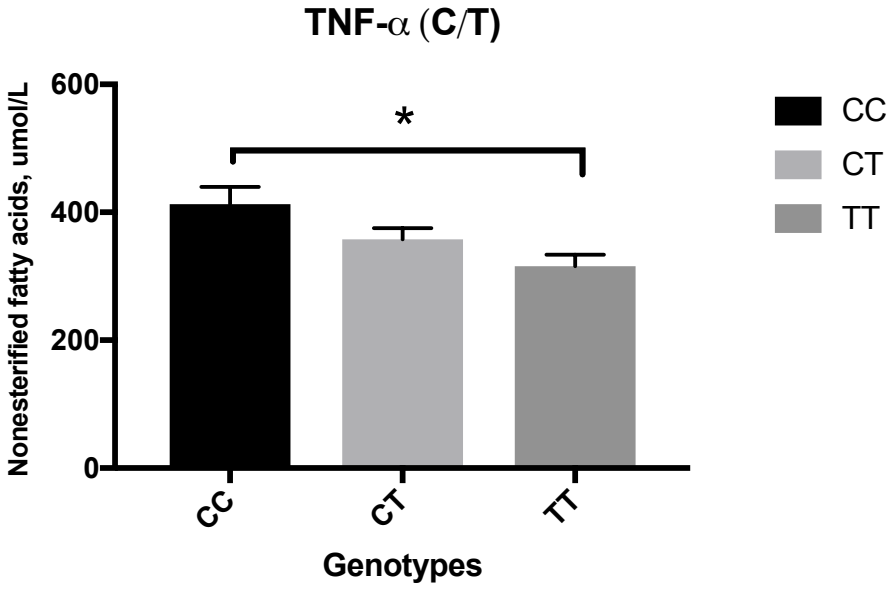

Fig. 2. Effect of *TNF-α* genotypes on NEFA in periparturient Holstein dairy cows. **P* < 0.05.

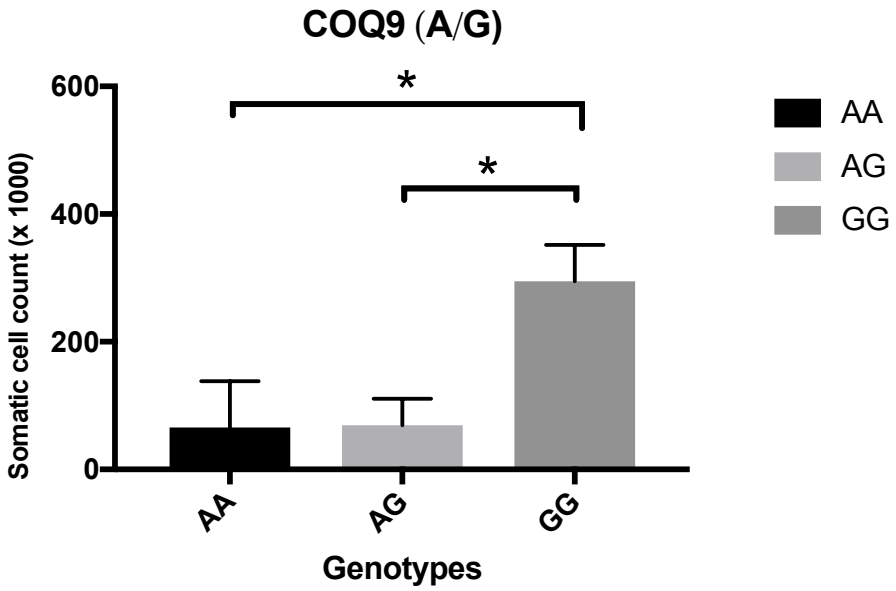

Fig. 3. Effect of *COQ9* genotypes on SCC in periparturient Holstein dairy cows. **P* < 0.05.

3.3 Artigo 3

Association of polymorphisms in the *IGF-I*, *GHR* and *STAT5A* genes with reproductive performance, metabolism and milk production of Holstein dairy cows

Pedro Augusto Silva Silveira, Walter Ronald Butler, Thaís Casarin da Silva, Carlos Castilho Barros, Marcio Nunes Corrêa, Augusto Schneider

Será submetido à revista Theriogenology

Association of polymorphisms in the *IGF-I*, *GHR* and *STAT5A* genes with reproductive performance, metabolism and milk production of Holstein dairy cows

Running title: Polymorphisms in the *IGF-I*, *GHR* and *STAT5A* genes of dairy cows

Pedro Augusto Silva Silveira [a], Walter Ronald Butler [b], Thaís Casarin da Silva [c], Carlos Castilho Barros [d], Marcio Nunes Corrêa [a], Augusto Schneider [d]*

[a] Faculdade de Veterinária, Universidade Federal de Pelotas, Campus Capão do Leão, sn, Pelotas, RS, Brazil.

[b] Department of Animal Science, Cornell University, 14853, Ithaca, NY, USA.

[c] Faculdade de Biologia, Universidade Federal de Pelotas, Campus Capão do Leão, sn, Pelotas, RS, Brazil.

[d] Faculdade de Nutrição, Universidade Federal de Pelotas, Rua Gomes Carneiro, 1, Pelotas, RS, Brazil.

* Corresponding author. Tel.: +555339211270. E-mail address: augusto.schneider@ufpel.edu.br (A. Schneider).

Abstract

The objective of this study was to evaluate the association of polymorphisms in the genes growth hormone receptor (*GHR*), insulin-like growth factor I (*IGF-I*) and signal transducer and activator of transcription 5A (*STAT5A*) with plasma concentrations of IGF-I, and the impact on reproductive performance and milk production of postpartum Holstein dairy cows. For this, 75 Holstein cows were used from 21 days prepartum up to 210 days in milk (DIM). These cows were submitted to a OvSynch-TAI protocol when they were 55 DIM. Milk samples were collected for determining ovulation. Progesterone levels above 1 ng/mL on two consecutive samples were ovulation indicators. Days from calving to first ovulation (DTO) and the calving conception interval (CCI) were evaluated. Serum concentrations of IGF-1 and β-hydroxybutyrate (BHBA) were obtained after blood collection. Genotyping was determined by electrophoresis of the PCR product after digestion with the enzyme *Alu*I (*GHR*), *Sna*BI (*IGF-I*) and *BstEII* (*STAT5A*). For the IGF-I polymorphism, eleven cows were found (14.7%) TT, 36 cows (48%) CT and 28 cows (37.3%) CC. IGF-1 levels in circulation were 79.2 ± 9.9, 66.5 ± 5.2 and 56.6 ± 5.9 ng/mL for groups TT, CT and CC, respectively, with a tendency to linear effect between genotypes (P = 0.0512). The average DTO for TT, TC and CC cows were,

respectively, 19.9 ± 4.2, 30.6 ± 2.3 and 30.4 ± 2.5 days, showing a linear effect ($P < 0.05$) between genotypes. The same linear effect ($P < 0.05$) was observed among genotypes TT, CT and CC compared to the average of the CCI, which were, respectively, 76.9 ± 12.6, 96.9 ± 6.8 and 111.7 ± 7.8. The TT cows showed serum BHBA values lower than cows TC and CC, respectively, 5.0 ± 1.4, 8.2 ± 0.7 and 8.1 ± 0.8 mg / dL ($P < 0.05$). There were no effects of STAT5A *BstE*II on plasma IGF-I or reproductive parameters ($P > 0.05$). The *GHR* AluI T allele and *IGF-I* SnaBI T had an additive effect on plasma IGF-I, number of services per conception, DTO and CCI. Milk production was not different between groups ($P > 0.05$). Thus, the IGF-1 *Sna*BI TT appears to reduce the interval from calving to first ovulation and conception of dairy cows also reducing the severity of postpartum negative energy balance (NEB) and *GHR Alu*I T and *IGF-I SnaB*I T had an additive effect regarding the reproductive performance of cows.

Keywords: fertility; somatotropic axis; single nucleotide polymorphisms

1. Introduction

Growth hormone (GH), or somatotropin, is synthesized and secreted by the anterior pituitary and stimulates the production of liver insulin-like growth factor I (IGF-I), that mediates most biological actions in target tissues [1]. The GH-IGF axis includes GH, the GH receptor, the GH binding proteins (GHBP), IGF-I, IGF-II, IGF receptors and the six IGF binding proteins (IGFBPs). The expression of *GH* and *IGF-I* receptors is observed in several tissues in bovine, with variations in the different stages of fetal development and in adult animals [2]. After binding to its receptor on the cell membrane, GH activates intracellular tyrosine kinases of the Janus Kinase (JAK) family. Then members of the signal transducer and activator of transcription (STAT) family, in particular STAT5, become phosphorylated. Phosphorylated STAT5 will promote *IGF-I* gene expression [3], and it is an important pathway in cell growth, differentiation and development in various tissues, and in the modulation of gonadotrophin actions during follicular growth in the ovary [4].

Negative energy balance, is associated with loss of postpartum body condition, a delay in the return of cows to postpartum cyclicity and reduces conception rate in the first service. These effects are proportional to the degree of loss of body condition score (BCS) at the beginning of lactation [5, 6]. Moreover, liver expression of *GHR* and *IGF-I* is reduced after calving due to the intense negative energy balance [7], accounting for low levels of serum IGF-I in the early post-partum period [8]. This reduction is associated with delayed ovulation and

return to postpartum cyclicity, increased interval-conception, and reduced embryonic development *in vitro* [9-11].

The *Alu*I and *SnaB*I polymorphisms in genes encoding *GHR* and *IGF-I*, respectively, are related to the hepatic expression of *IGF-I* mRNA and IGF-I plasma concentration in Holstein steers [12]. *GHR-Alu*I and *IGF-I Snab*I polymorphisms had no significant effect on productive parameters, although *IGF-I* genotype affected calving to first service interval in primiparous cows [13]. We have confirmed the association of the *GHR Alu*I polymorphism with a higher serum concentration of IGF-I, and in addition, with a reduction of 13 to 32 days in the conception calving interval in Holstein dairy cows [14]. Moreover, some studies have found an effect of *STAT5A* on fertilization and embryonic survival rates in cattle [15, 16].

Based on this evidence, the hypothesis of this study is that polymorphisms in the genes *GHR*, *STAT5A* and *IGF-I* are related to different plasma concentrations of IGF-I, influencing the reproductive performance, metabolism and milk production of postpartum Holstein dairy cows.

2. Methods

2.1 Animals and reproductive management

Seventy-five Holstein dairy cows were evaluated from 21 days before calving to 210 days in milk (DIM). With 55 DIM these cows were subjected to an OvSynch-IATF protocol that was repeated in cows diagnosed as non-pregnant at 30 and 60 days after AI. The number of inseminations per conception, the pregnancy rate at the first postpartum insemination and calving to conception interval (CCI) were evaluated from the calving day until 210 days in milk (DIM).

2.2 Sample collection and analyses

For determination of the genotype and biochemical/hormonal parameters, blood collection of the coccygeal vein was performed on days -21, 0, 7, 21 and 60. The samples were collected in vaccutainer tubes containing EDTA. Part of the whole blood was centrifuged and the plasma separated and frozen at -20°C for analysis of IGF-I and β-hydroxybutyrate (BHBA). Blood metabolites [nonesterified fatty acids (NEFA), beta-hydroxybutyrate (BHB), albumin, blood urea nitrogen, and aspartate aminotransferase (AST)] were analyzed by an autoanalyzer (Boehringer Mannheim Hitachi 104, Diagnostic Laboratory Systems, Indianapolis, IN, USA).

Milk samples were collected twice a week and progesterone concentrations in unextracted milk samples were assayed following published procedures [17, 18]. Progesterone levels above 1 ng/mL in two consecutive samples were indicators of ovulation and the days from calving to ovulation (DTO) were calculated.

2.3 Genotyping

DNA extraction was performed using Wizard Genomic DNA Purification Kit (Promega Corporation) and quantified by measuring the absorbance of 260 nm in a spectrophotometer. For determination of the *GHR* alleles an 836 bp fragment was amplified using the primers: forward, TGCGTGCACAGCAGCTCAACC; reverse, AGCAACCCCACTGCTGGGCAT [14] and these data have been published before [14]. For determination of the *IGF-I* alleles a 249-bp fragment was amplified using the primers: forward, ATTACAAAGCTGCCTGCCCC; reverse, ACCTTACCCGTATGAAAGGAATATACGT [13]. We determined the *STAT5A* alleles using the primers: forward, GAGAAGTTGGCGGAGATTATC; reverse, CCGTGTGTCCTCATCACCTG [15]. The annealing of the primers was performed at 66, 64 and 58°C for *GHR*, *IGF-I* and *STAT5A*, respectively. The amplified fragments were digested in a reaction containing 5 μL of PCR product and 3 U of restriction enzyme *Alu*I to *GHR*, 5 U of restriction enzyme *SnaB*I to *IGF-I* and 3 U of restriction enzyme *BstE*II to *STAT5A*. The digestion was performed in the thermocycler at 37°C for 2-3 hours. After digestion of the amplified products, the DNA fragments were separated on a 2% agarose gel. A standard molecular weight of 100-bp was used on each gel to control the size of the digested fragments. The DNA fragments were labeled with SYBR Safe (Life technologies) and visualized on the agarose gel by ultraviolet light. Gels were photographed for data analysis.

Individual genotypes were determined by analysis of the fragment size of the digestion products. The different GHR genotypes formed the following fragments: *Alu*I (AA): 602 bp, 145 bp, 75 bp; *Alu*I (TT): 747 bp, 75 bp; and *Alu*I (AT): 747 bp, 602 bp, 145 bp, 75 bp. The different IGF-I genotypes formed the following products: *SnaB*I (TT): 223 and 26 bp; *SnaB*I (CT): 249, 223 and 26 bp; *SnaB*I (CC): 246 bp (undigested). For STAT5A different genotypes we observed the following fragments: *BstE*II (GG): 676 bp; *BstE*II (CG): 820 and 676 bp; *BstE*II (CC): 820 bp.

2.4 Statistical analysis

Data from this experiment were analyzed in the SAS statistical program (SAS Institute Inc., Cary, USA). Genotypes were classified as 0, 1 and 2 for *GHR* (T), *IGF-I* (T) and *STAT5A*

(C), and 2 indicated the presence in homozygous of the favorable allele (*GHR* T, *IGF-I* T and *STAT5A* C). Besides, we combined the effect of the presence of the favorable GHR and IGF-I polymorphisms. Thus, the combined presence of the beneficial alleles in the two genes was indicated by 0, 1, 2 and 3 + 4, indicating the presence of neither or a growing combination of favorable alleles. The averages were analyzed using the GLM method, evaluating the linear, quadratic and cubic effects of the presence of the alleles. Values of $P < 0.05$ were considered significant.

3. Results

For the *IGF-I SnaB*I, 11 cows (14.7%) TT, 36 cows (48%) CT and 28 cows (37.3%) CC were found (Table 1). The IGF-I levels in the circulation were 79.2 ± 9.9, 66.5 ± 5.2 and 56.6 ± 5.9 ng/mL for the TT, CT and CC groups, respectively, with a tendency of linear effect among genotypes ($P = 0.0512$). The mean DTO for TT, CT and CC cows were, respectively, 19.9 ± 4.2, 30.6 ± 2.3 and 30.4 ± 2.5 days, presenting a linear effect ($P < 0.05$) between the genotypes. The same linear effect ($P < 0.05$) was observed between the TT, CT and CC genotypes in relation to the CCI means, which were, respectively, 76.9 ± 12.6, 96.9 ± 6.8 and 111.7 ± 7.8. It is possible to observe that TT cows anticipated the interval between calving and first ovulation around 10 days compared to CT and CC cows ($P < 0.05$). The BHBA was lower in the TT cows compared with both CT and CC ($P < 0.05$) (Table 1). In Figures 1 and 2, it can be observed that cows with the genotype TT ovulated earlier and had a shorter calving to conception interval compared to cows with the CC genotype.

No differences were observed between S*TAT5A* genotypes for the parameters evaluated (Table 2). Since the T allele for *GHR Alu*I and T allele for *IGF-I SnaB*I had the best results regarding the reproductive performance of cows, we evaluated the additive effect of the presence of these alleles on the two genes in which they appeared (Table 3).

4. Discussion

The *IGF-I* SNP and the interaction of favourable *GHR/IGF-I* SNPs were associated with plasma IGF-I and pospartum fertility, with no changes on milk production. There was a tendency of linear effect among genotypes on plasma IGF-I and animals with the TT genotype tended to have higher levels of plasma IGF-I than the other genotypes. The higher levels of IGF-I were related with shorter intervals between calving to first ovulation an calving to conception for cows of the TT genotype. In addition, this cows presented low levels of BHBA compared with both CT and CC groups, with no difference on milk production. The energy

balance is considered the main nutritional factor that regulates the reproductive function, having a greater impact on the reproductive efficiency than the ingestion of any specific class of nutrients [19, 20]. In dairy cattle, both the duration and severity of early postpartum negative energy balance (NEB) are correlated with the interval to resumption of ovulatory activity following parturition [21]. Several putative hormones, growth factors, and metabolites have been identified that are stimulatory or inhibitory to the reproductive axis. Reduced circulating concentrations of IGF-I and elevated concentrations of BHBA and NEFA are all associated with impaired reproductive performance [22]. The ability of follicles to produce sufficient estradiol for ovulation seems to depend on the availability of insulin and IGF-I, mainly at the beginning of NEB [21]. Plasma estradiol concentrations were highly correlated with plasma IGF-I levels [23]. Moreover, a bidirectional interaction between estrogen receptor α (ESR1) and the IGF-I receptor (IGFIR) signaling pathways was reported [24, 25], which support the important role of IGF-I and gonadotropins in the ovulation process.

The *IGF-I* genotype affected calving to first service interval and had an effect on BHBA. *IGF-I SnaB*I SNP occurs in the promoter region of the *IGF-I* gene [26], which suggest a difference in the transcription of *IGF-I* reflecting in the IGF-I function on reproductive performance. Cows with late resumption (>45 days postpartum) had lower levels of IGF-I compared with early or medium resumption cows [27]. In our study, cows with the TT genotype had higher IGF-I, ovulated earlier, and as result had a shorter CCI. It well established that earlier ovulation results in earlier conception [28]. However, the studies evaluating the effects of IGF *Sna*B on fertility are controversial. There are studies with no association between the genotypes of *IGF-I SnaB*I and fertility of Holstein cows [29]. Despite other studies showing an association of *STAT5A BstE*II polymorphism with fertility in Holstein cows [29], we did not find any statistical difference in our study. This can be related to the fact that *STAT* mutations did not affect serum IGF concentrations and DTO. It suggests a stronger effect of mutations direct in *IGF-I* or *GHR* genes, instead to alterations in molecules that regulate the intracellular signaling pathways.

Furthermore, the presence of the *GHR Alu*I T allele in lactating dairy cows is associated with higher circulating IGF-I concentrations and with fewer number of services per conception and a shorter calving to conception interval, in a former study conducted with the same group of samples [14]. When we combine the results obtained by the evaluation of the two SNPs, the simultaneous presence of the *GHR Alu*I T allele and *IGF-I SnaB*I T allele seems to be related to the improve reproductive performance of postpartum dairy cows. IGF-I is mainly synthesized by the liver in response to GH [1] and this relationship forms the basis of the GH–IGF axis. GH

receptors (GHRs) are found in many tissues and the liver is the site of greatest abundance [30-32]. Expression of *GHR* and *IGF-I* genes in liver is acutely responsive to nutritional status [32], and during the NEB the liver becomes refractory to GH and circulating IGF-I concentrations are dramatically reduced [33]. The GH and IGF system play an important role in the metabolic transition that favors high milk production after calving [34], and It has been well established that hepatic GHR concentration is positively correlated with plasma IGF-I and the level of nutrition [35]. Indeed, high producing dairy cows had increased GH and reduced IGF-I and insulin concentrations during early lactation [36], and this is in part due the fact that genetic selection for high milk production can decrease the reproductive efficiency [37-39]. In addition, it is well established that the resumption of postpartum cyclicity and ovulation of the first follicular wave up to three weeks postpartum are related to higher pregnancy rates in the first postpartum AI and shorter days open [19, 21, 40]. We found an effect of *IGF-I SNP* on DTO, and that decreased the calving to conception interval. In our previous study [14], GHR SNP had an effect on circulating IGF-I concentrations, in the number of services per conception and on calving to conception interval, which agree with the literature [13]. This is the first time that the additive effect of the presence of the T allele, the one related with the best results on fertility in both *IGF-I* and *GHR* SNPs, is described. In fact, animals with at least three T alleles distributed in both genes had higher levels of plasma IGF-I, a lower interval from calving to first ovulation, a fewer number of services per conception and a shorter calving to conception interval. Also, these cows had a tendency to have lower BHBA levels and better body condition score, which suggest a less intense NEB after calving. This supports the hypothesis that the mutations may decrease the IGF-I levels, through decrease the IGF-I synthesis in case of *IGF SnaB*I or decrease the GH signaling in case of *GHR Alu*I, and the presence of the allele with poor reproductive results in just one of the genes evaluated have enough power to impair the postpartum fertility.

In conclusion, the presence of *IGF-I SnaB*I T allele in lactating dairy cows is associated with higher circulating IGF-I concentrations, fewer days from calving to first ovulation, shorter calving to conception interval and lower levels of BHBA. In addition, the simultaneous presence of T allele in the GHR *Alu*I and in the *IGF-I* / *SnaB*I seems to be related to the better reproductive performance of postpartum dairy cows. Together, these results suggest that the Increasing of *IGF-I SnaB*I T and *GHR Alu*I T alleles frequency in the population may be a goal in order to increase reproductive efficiency in dairy cattle.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by FAPERGS, CNPq AND CAPES.

References

[1] Jones JI, Clemmons DR. Insulin-like growth factors and their binding proteins: biological actions. Endocr Rev. 1995;16:3-34.

[2] Mertani HC, Morel G. In situ gene expression of growth hormone (GH) receptor and GH binding protein in adult male rat tissues. Mol Cell Endocrinol. 1995;109:47-61.

[3] Argetsinger LS, Campbell GS, Yang X, Witthuhn BA, Silvennoinen O, Ihle JN, et al. Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. Cell. 1993;74:237-44.

[4] Armstrong DG, Webb R. Ovarian follicular dominance: the role of intraovarian growth factors and novel proteins. Rev Reprod. 1997;2:139-46.

[5] Garnsworthy PC, Fouladi-Nashta AA, Mann GE, Sinclair KD, Webb R. Effect of dietary-induced changes in plasma insulin concentrations during the early post partum period on pregnancy rate in dairy cows. Reproduction. 2009;137:759-68.

[6] Butler ST, Marr AL, Pelton SH, Radcliff RP, Lucy MC, Butler WR. Insulin restores GH responsiveness during lactation-induced negative energy balance in dairy cattle: effects on expression of IGF-I and GH receptor 1A. J Endocrinol. 2003;176:205-17.

[7] Radcliff RP, McCormack BL, Crooker BA, Lucy MC. Plasma hormones and expression of growth hormone receptor and insulin-like growth factor-I mRNA in hepatic tissue of periparturient dairy cows. J Dairy Sci. 2003;86:3920-6.

[8] Kobayashi Y, Boyd CK, Bracken CJ, Lamberson WR, Keisler DH, Lucy MC. Reduced growth hormone receptor (GHR) messenger ribonucleic acid in liver of periparturient cattle is caused by a specific down-regulation of GHR 1A that is associated with decreased insulin-like growth factor I. Endocrinology. 1999;140:3947-54.

[9] Butler ST, Pelton SH, Butler WR. Energy balance, metabolic status, and the first postpartum ovarian follicle wave in cows administered propylene glycol. J Dairy Sci. 2006;89:2938-51.

[10] Galvao KN, Frajblat M, Butler WR, Brittin SB, Guard CL, Gilbert RO. Effect of early postpartum ovulation on fertility in dairy cows. Reprod Domest Anim. 2010;45:e207-11.

[11] Walsh RB, Kelton DF, Duffield TF, Leslie KE, Walton JS, LeBlanc SJ. Prevalence and risk factors for postpartum anovulatory condition in dairy cows. J Dairy Sci. 2007;90:315-24.

[12] Maj A, Snochowski M, Siadkowska E, Rowinska B, Lisowski P, Robakowska-Hyzorek D, et al. Polymorphism in genes of growth hormone receptor (GHR) and insulin-like growth factor-1 (IGF1) and its association with both the IGF1 expression in liver and its level in blood in Polish Holstein-Friesian cattle. Neuro Endocrinol Lett. 2008;29:981-9.

[13] Ruprechter G, Carriquiry M, Ramos JM, Pereira I, Ana M. Metabolic and endocrine profiles and reproductive parameters in dairy cows under grazing conditions: effect of polymorphisms in somatotropic axis genes. Acta Vet Scand. 2011;53:35.

[14] Schneider A, Correa MN, Butler WR. Association between growth hormone receptor AluI polymorphism and fertility of Holstein cows. Theriogenology. 2013;80:1061-6.

[15] Khatib H, Monson RL, Schutzkus V, Kohl DM, Rosa GJ, Rutledge JJ. Mutations in the STAT5A gene are associated with embryonic survival and milk composition in cattle. J Dairy Sci. 2008;91:784-93.

[16] Khatib H, Huang W, Wang X, Tran AH, Bindrim AB, Schutzkus V, et al. Single gene and gene interaction effects on fertilization and embryonic survival rates in cattle. J Dairy Sci. 2009;92:2238-47.

[17] Waldmann A. Monoclonal antibodies to progesterone: characterization and selection for enzyme immunoassay in bovine milk. Hybridoma. 1999;18:289-96.

[18] Arnstadt KI, Grunert E, Prokopp S, Schulte B. [Further development of enzyme immunoassay (EIA) for progesterone and its application to blood, milk and saliva tests in cattle]. Zentralbl Veterinarmed A. 1982;29:387-94.

[19] Butler WR, Smith RD. Interrelationships between energy balance and postpartum reproductive function in dairy cattle. J Dairy Sci. 1989;72:767-83.

[20] Wade GN, Schneider JE, Li HY. Control of fertility by metabolic cues. Am J Physiol. 1996;270:E1-19.

[21] Beam SW, Butler WR. Effects of energy balance on follicular development and first ovulation in postpartum dairy cows. J Reprod Fertil Suppl. 1999;54:411-24.

[22] Abdelli A, Raboisson D, Kaidi R, Ibrahim B, Kalem A, Iguer-Ouada M. Elevated non-esterified fatty acid and beta-hydroxybutyrate in transition dairy cows and their

association with reproductive performance and disorders: A meta-analysis. Theriogenology. 2017;93:99-104.

[23] Beam SW, Butler WR. Energy balance, metabolic hormones, and early postpartum follicular development in dairy cows fed prilled lipid. J Dairy Sci. 1998;81:121-31.

[24] Milewicz T, Gregoraszczuk EL, Sztefko K, Augustowska K, Krzysiek J, Rys J. Lack of synergy between estrogen and progesterone on local IGF-I, IGFBP-3 and IGFBP-2 secretion by both hormone-dependent and hormone-independent breast cancer explants in vitro. Effect of tamoxifen and mifepristone (RU 486). Growth Horm IGF Res. 2005;15:140-7.

[25] Yu L, Moore AB, Castro L, Gao X, Huynh HL, Klippel M, et al. Estrogen Regulates MAPK-Related Genes through Genomic and Nongenomic Interactions between IGF-I Receptor Tyrosine Kinase and Estrogen Receptor-Alpha Signaling Pathways in Human Uterine Leiomyoma Cells. J Signal Transduct. 2012;2012:204236.

[26] Ge W, Davis ME, Hines HC, Irvin KM, Simmen RC. Association of a genetic marker with blood serum insulin-like growth factor-I concentration and growth traits in Angus cattle. J Anim Sci. 2001;79:1757-62.

[27] Pedernera M, Garcia SC, Horagadoga A, Barchia I, Fulkerson WJ. Energy balance and reproduction on dairy cows fed to achieve low or high milk production on a pasture-based system. J Dairy Sci. 2008;91:3896-907.

[28] Thatcher WW, Wilcox CJ. Postpartum estrus as an indicator of reproductive status in the dairy cow. J Dairy Sci. 1973;56:608-10.

[29] Hax LT, Schneider A, Jacometo CB, Mattei P, da Silva TC, Farina G, et al. Association between polymorphisms in somatotropic axis genes and fertility of Holstein dairy cows. Theriogenology. 2017;88:67-72.

[30] Lucy MC, Boyd CK, Koenigsfeld AT, Okamura CS. Expression of somatotropin receptor messenger ribonucleic acid in bovine tissues. J Dairy Sci. 1998;81:1889-95.

[31] Edens A, Talamantes F. Alternative processing of growth hormone receptor transcripts. Endocr Rev. 1998;19:559-82.

[32] Bornfeldt KE, Arnqvist HJ, Enberg B, Mathews LS, Norstedt G. Regulation of insulin-like growth factor-I and growth hormone receptor gene expression by diabetes and nutritional state in rat tissues. J Endocrinol. 1989;122:651-6.

[33] Vicini JL, Buonomo FC, Veenhuizen JJ, Miller MA, Clemmons DR, Collier RJ. Nutrient balance and stage of lactation affect responses of insulin, insulin-like growth

factors I and II, and insulin-like growth factor-binding protein 2 to somatotropin administration in dairy cows. J Nutr. 1991;121:1656-64.

[34] Lucy MC. Reproductive loss in high-producing dairy cattle: where will it end? J Dairy Sci. 2001;84:1277-93.

[35] Velazquez MA, Spicer LJ, Wathes DC. The role of endocrine insulin-like growth factor-I (IGF-I) in female bovine reproduction. Domest Anim Endocrinol. 2008;35:325-42.

[36] Gong JG, Lee WJ, Garnsworthy PC, Webb R. Effect of dietary-induced increases in circulating insulin concentrations during the early postpartum period on reproductive function in dairy cows. Reproduction. 2002;123:419-27.

[37] Butler WR. Review: effect of protein nutrition on ovarian and uterine physiology in dairy cattle. J Dairy Sci. 1998;81:2533-9.

[38] Royal M, Mann GE, Flint AP. Strategies for reversing the trend towards subfertility in dairy cattle. Vet J. 2000;160:53-60.

[39] Dobson H, Smith R, Royal M, Knight C, Sheldon I. The high-producing dairy cow and its reproductive performance. Reprod Domest Anim. 2007;42 Suppl 2:17-23.

[40] Butler WR. Nutritional interactions with reproductive performance in dairy cattle. Anim Reprod Sci. 2000;60-61:449-57.

TABLES

Table 1. Parameters evaluated for *IGF-I SnaB*I genotypes.

Parameter	**Genotype *IGF-I SnaB*I**			***P***	
	TT	CT	CC	Linear	TT vs CT and CC
Cows (%)	14.7 (11/75)	48 (36/75)	37.3 (28/75)	-	-
Days to 1 st ovulation	19.9 ± 4.2	30.6 ± 2.3	30.4 ± 2.5	0.03	0.02
Days to conception	76.9 ± 12.6	96.9 ± 6.8	111.7 ± 7.8	0.02	0.05
Milk (kg/day)	34.2 ± 1.8	33.7 ± 0.9	34.1 ± 1.1	0.86	0.96
IGF-I, ng/mL	79.2 ± 9.9	66.5 ± 5.2	56.6 ± 5.9	0.05	0.1
β-Hydroxybutyrate, mg/dL	5.0 ± 1.4	8.2 ± 0.7	8.1 ± 0.8	0.06	0.04

$P < 0.05$ was considered to be statistically significant.

Table 2. Parameters evaluated for *STAT5A BstE*II genotypes.

	STAT5A			***P***	
Parameter	CC	CG	GG	Linear	CC vs CG and GG
Cows (%)	26 (19/73)	43.8 (32/73)	30.2 (22/73)	-	-
Days to 1 st ovulation	28.4 ± 3.1	29.5 ± 2.5	29.4 ± 2.9	0.96	0.86
Days to 1 st AI	69.7 ± 0.3	69.7 ± 0.4	70.3 ± 0.7	0.82	0.45
Days to conception	101.3 ± 9.4	102.7 ± 7.6	93.9 ± 9.4	0.58	0.78
IGF-I, ng/mL	66.3 ± 7.2	61.8 ± 5.8	65.6 ± 6.7	0.94	0.75

$P < 0.05$ was considered to be statistically significant.

Table 3. Reproductive parameters, blood concentrations metabolites and milk production and composition for cows with combined genotypes (*GHR* and *IGF-I*). 0, 1, 2 and 3 + 4 indicate the presence of neither or a growing combination of favorable alleles for this two genes.

	GHR/IGF-I				*P*		
Parameter	0	1	2	3 + 4	Linear	Quadratic	Cubic
Cows (%)	17.4(13/75)	40 (30/75)	21.3 (16/75)	21.3 (16/75)	-	-	-
Days to 1 st ovulation	32.1 ± 3.8	28 ± 2.6	35.7 ± 3.3	20.1 ± 3.8	0.10	0.09	0.01
Days to 1 st AI	69.3 ± 0.5	69.6 ± 0.4	70.3 ± 0.5	70.2 ± 0.5	0.13	0.70	0.51
Days to conception	122 ± 11.4	106.2 ± 7.2	92.5 ± 9.3	76.2 ± 10.1	0.00	0.98	0.9
No. Of AI/conception	3.5 ± 0.4	2.9 ± 0.3	2.3 ± 0.4	1.8 ± 0.4	0.01	0.91	0.97
Pregnancy rate at 1 st AI, % (n)	23.1 (3/13)	23.3 (7/30)	25 (4/16)	56.2 (9/16)	-	-	-
IGF-I, ng/mL	77.5 ± 7.0	83.1 ± 4.7	71.6 ± 6.3	105.7 ± 6.7	0.02	0.02	0.02
Nonesterified fatty acids, mmol/L	0.45 ± 0.05	0.36 ± 0.03	0.41 ± 0.04	0.32 ± 0.04	0.12	0.99	0.11
β-Hydroxybutyrate, mg/dL	7.2 ± 0.6	6.4 ± 0.4	7.7 ± 0.6	5.1 ± 0.6	0.07	0.10	0.01
Blood urea nitrogen, mg/dL	7.2 ± 0.3	6.9 ± 0.2	7.1 ± 0.3	6.9 ± 0.3	0.72	0.87	0.48
Albumin, g/L	3.19 ± 0.7	3.16 ± 0.4	3.2 ± 0.6	3.32 ± 0.6	0.16	0.25	0.97
Aspartate aminotransferase, U/L	92.2 ± 5.4	100.7 ± 3.5	90.1 ± 4.9	84.0 ± 5.0	0.13	0.12	0.23
BCS	3.57 ± 0.05	3.52 ± 0.04	3.41 ± 0.05	3.6 ± 0.05	0.89	0.01	0.05
Milk, (kg/day)	34.4 ± 1.5	34.2 ± 1.0	33.2 ± 1.4	33.4 ± 1.5	0.55	0.86	0.72
ECM, (kg/day)	38.6 ± 1.9	39.6 ± 1.3	36.6 ± 1.7	38.1 ± 1.8	0.59	0.88	0.23
Fat, (%)	4.6 ± 0.2	4.6 ± 0.1	4.4 ± 0.2	4.5 ± 0.2	0.48	0.86	0.28
Protein, (%)	6.6 ± 0.1	6.6 ± 0.1	6.7 ± 0.1	6.9 ± 0.1	0.17	0.36	0.89
Lactose, (%)	4.69 ± 0.03	4.77 ± 0.02	4.75 ± 0.03	4.81 ± 0.03	0.02	0.8	0.12

$P < 0.05$ was considered to be statistically significant.

FIGURES

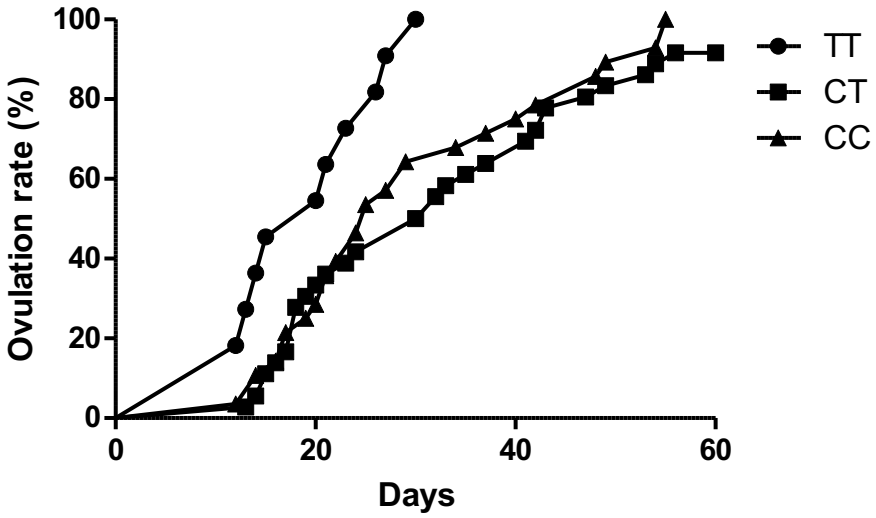

Fig. 1. Days until ovulation for *IGF-I SnaB*I.

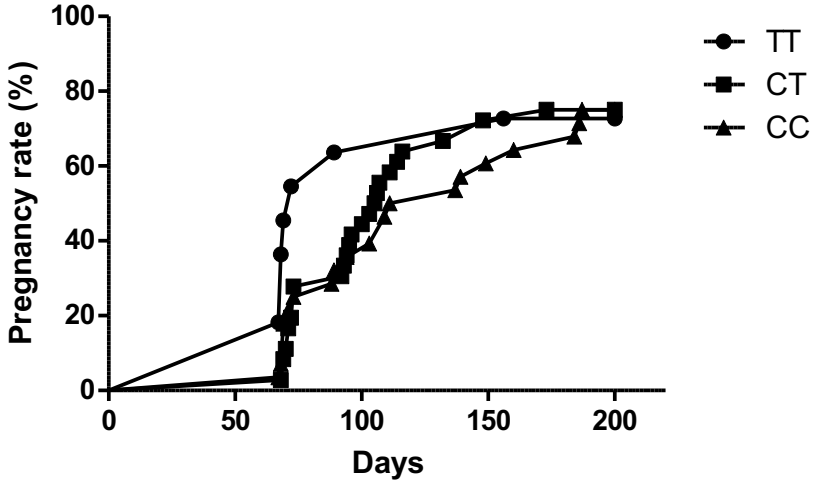

Fig. 2. Pregnancy rate for *IGF-I SnaB*I

4 Considerações Finais

Nosso trabalho avaliou os efeitos de mutações em genes ligados a resposta imune, ao transporte de energia celular e ao eixo somatotrópico sobre a fertilidade de vacas leiteiras da raça Holandês no pós-parto. Essas mutações tiveram efeito sobre a fertilidade, sendo que os SNPs no promotor do gene da *PON1* e nos genes *GHR* e *IGF-I* também impactaram, respectivamente, a atividade da PON1 e do IGF-I no plasma. Isso sustenta a hipótese de que o impacto destas mutações ocorre através de mudanças na transcrição do mRNA sintetizado nesses genes, com efeito na ação dessas proteínas na fisiologia reprodutiva das vacas.

Por outro lado, o SNP no gene do *TNF-α* teve impacto sobre os níveis de NEFA, o SNP no gene COQ9 foi associado com a ingestão de matéria-seca pré-parto e a mutação no *IGF-I* teve impacto no BHBA. Isso sugere um efeito dessas mutações sobre a adaptação das vacas ao balanço energético negativo que ocorre no periparto, podendo representar uma via indireta da ação desses SNPs com a fertilidade no pós-parto.

Além disso os SNPs -22, -221 e *COQ9* tiveram impacto na contagem de células somáticas e nenhuma mutação teve impacto na produção leiteira. Portanto, foram identificados SNPs com impacto positivo na reprodução sem reduzir o mérito genético para produção de leite. Somados, esses resultados indicam que mutações em genes ligados a resposta imune, ao transporte de energia celular e ao eixo somatotrópico podem ser utilizados como marcadores moleculares para a seleção genética de vacas leiteiras, com foco na fertilidade pós-parto.

Referências

ABDELLI, A.; RABOISSON, D.; KAIDI, R.; IBRAHIM, B.; KALEM, A.; IGUER-OUADA, M. Elevated non-esterified fatty acid and beta-hydroxybutyrate in transition dairy cows and their association with reproductive performance and disorders: A meta-analysis. **Theriogenology**, v.93, p.99-104, 2017.

ABUELO, A.; HERNANDEZ, J.; BENEDITO, J.L.; CASTILLO, C. The importance of the oxidative status of dairy cattle in the periparturient period: revisiting antioxidant supplementation. **Journal of animal physiology and animal nutrition**, v.99, n.6, p.1003-1016, 2015.

ACOSTA, D.A.V.; PFEIFER, L.F.M.; SCHMITT, E.; SCHNEIDER, A.; SILVEIRA, P.A.S.; JACOMETO, C.B.; BRAUNER, C.C.; RABASSA, V.R.; CORRÊA, M.N.; DEL PINO, F.A.B. Effect of prepartum somatotropin injection in late pregnant Holstein heifers with high body condition score on metabolic parameters, resumption of ovulation and milk production. **Canadian Journal of Animal Science**, v.93, n.2, p.287-292, 2013.

AGARWAL, A.; GUPTA, S.; SEKHON, L.; SHAH, R. Redox considerations in female reproductive function and assisted reproduction: from molecular mechanisms to health implications. **Antioxid Redox Signal**, v.10, n.8, p.1375-1403, 2008.

AGARWAL, A.; GUPTA, S.; SHARMA, R.K. Role of oxidative stress in female reproduction. **Reproductive biology and endocrinology**, v.3, p.28, 2005.

AL-GUBORY, K.H.; FOWLER, P.A.; GARREL, C. The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. **The international journal of biochemistry & cell biology**, v.42, n.10, p.1634-1650, 2010.

ARGETSINGER, L.S.; CAMPBELL, G.S.; YANG, X.; WITTHUHN, B.A.; SILVENNOINEN, O.; IHLE, J.N.; CARTER-SU, C. Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. **Cell**, v.74, n.2, p.237-244, 1993.

ARMSTRONG, D.G.; WEBB, R. Ovarian follicular dominance: the role of intraovarian growth factors and novel proteins. **Reviews of reproduction**, v.2, n.3, p.139-146, 1997.

ARNSTADT, K.I.; GRUNERT, E.; PROKOPP, S.; SCHULTE, B. Further development of enzyme immunoassay (EIA) for progesterone and its application to blood, milk and saliva tests in cattle. **Zentralbl Veterinarmed A**, v.29, n.4-5, p.387-394, 1982.

ATAKISI, O.; ORAL, H.; ATAKISI, E.; MERHAN, O.; METIN PANCARCI, S.; OZCAN, A.; MARASLI, S.; POLAT, B.; COLAK, A.; KAYA, S. Subclinical mastitis causes alterations in nitric oxide, total oxidant and antioxidant capacity in cow milk. **Research in veterinary science**, v.89, n.1, p.10-13, 2010.

BARBANO, D.M.; MELILLI, C.; OVERTON, T.R. Advanced use of FTIR spectra of milk for feeding and health management. Proceedings of the Cornell Nutrition Conference, 2014, Syracuse, NY. p.105-113.

BARTKE, A. Growth hormone and aging: a challenging controversy. **Clinical Interventions in Aging**, v.3, n.4, p.659-665, 2008.

BEAM, S.W.; BUTLER, W.R. Energy balance, metabolic hormones, and early postpartum follicular development in dairy cows fed prilled lipid. **Journal of dairy science**, v.81, n.1, p.121-131, 1998.

BEAM, S.W.; BUTLER, W.R. Effects of energy balance on follicular development and first ovulation in postpartum dairy cows. **Journal of reproduction and fertility. Supplement**, v.54, p.411-424, 1999.

BECKMANN, J.S.; KASHI, Y.; HALLERMAN, E.M.; NAVE, A.; SOLLER, M. Restriction fragment length polymorphism among Israeli Holstein-Friesian dairy bulls. **Animal Genetics**, v.17, n.1, p.25-38, 1986.

BELL, A.W. Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. **Journal of animal science**, v.73, n.9, p.2804-2819, 1995.

BELL, A.W.; BURHANS, W.S.; OVERTON, T.R. Protein nutrition in late pregnancy, maternal protein reserves and lactation performance in dairy cows. **Proceedings of the Nutrition Society** v.59, n.1, p.119-126, 2000.

BEN-MEIR, A.; BURSTEIN, E.; BORREGO-ALVAREZ, A.; CHONG, J.; WONG, E.; YAVORSKA, T.; NARANIAN, T.; CHI, M.; WANG, Y.; BENTOV, Y.; ALEXIS, J.; MERIANO, J.; SUNG, H.K.; GASSER, D.L.; MOLEY, K.H.; HEKIMI, S.; CASPER, R.F.; JURISICOVA, A. Coenzyme Q10 restores oocyte mitochondrial function and fertility during reproductive aging. **Aging Cell**, v.14, n.5, p.887-895, 2015.

BERTICS, S.J.; GRUMMER, R.R.; CADORNIGA-VALINO, C.; STODDARD, E.E. Effect of prepartum dry matter intake on liver triglyceride concentration and early lactation. **Journal of dairy science**, v.75, n.7, p.1914-1922, 1992.

BERTONI, G.; TREVISI, E.; HAN, X.; BIONAZ, M. Effects of inflammatory conditions on liver activity in puerperium period and consequences for performance in dairy cows. **Journal of dairy science**, v.91, n.9, p.3300-3310, 2008.

BICALHO, R.C.; VOKEY, F.; ERB, H.N.; GUARD, C.L. Visual locomotion scoring in the first seventy days in milk: impact on pregnancy and survival. **Journal of dairy science**, v.90, n.10, p.4586-4591, 2007.

BIONAZ, M.; TREVISI, E.; CALAMARI, L.; LIBRANDI, F.; FERRARI, A.; BERTONI, G. Plasma paraoxonase, health, inflammatory conditions, and liver function in transition dairy cows. **Journal of dairy science**, v.90, n.4, p.1740-1750, 2007.

BORNFELDT, K.E.; ARNQVIST, H.J.; ENBERG, B.; MATHEWS, L.S.; NORSTEDT, G. Regulation of insulin-like growth factor-I and growth hormone receptor gene expression by diabetes and nutritional state in rat tissues. **The Journal of endocrinology**, v.122, n.3, p.651-656, 1989.

BOSSAERT, P.; TREVISI, E.; OPSOMER, G.; BERTONI, G.; DE VLIEGHER, S.; LEROY, J.L. The association between indicators of inflammation and liver variables during the transition period in high-yielding dairy cows: an observational study. **Veterinary journal**, v.192, n.2, p.222-225, 2012.

BRADFORD, B.J.; MAMEDOVA, L.K.; MINTON, J.E.; DROUILLARD, J.S.; JOHNSON, B.J. Daily injection of tumor necrosis factor-{alpha} increases hepatic triglycerides and alters transcript abundance of metabolic genes in lactating dairy cattle. **The Journal of Nutrition** v.139, n.8, p.1451-1456, 2009.

BRADFORD, B.J.; YUAN, K.; FARNEY, J.K.; MAMEDOVA, L.K.; CARPENTER, A.J. Invited review: Inflammation during the transition to lactation: New adventures with an old flame. **Journal of dairy science**, v.98, n.10, p.6631-6650, 2015.
BRANNSTROM, M.; BONELLO, N.; WANG, L.J.; NORMAN, R.J. Effects of tumour necrosis factor alpha (TNF alpha) on ovulation in the rat ovary. **Reproduction, fertility, and development**, v.7, n.1, p.67-73, 1995.

BROMFIELD, J.J.; SHELDON, I.M. Lipopolysaccharide initiates inflammation in bovine granulosa cells via the TLR4 pathway and perturbs oocyte meiotic progression in vitro. **Endocrinology**, v.152, n.12, p.5029-5040, 2011.

BROWNE, R.W.; SHELLY, W.B.; BLOOM, M.S.; OCQUE, A.J.; SANDLER, J.R.; HUDDLESTON, H.G.; FUJIMOTO, V.Y. Distributions of high-density lipoprotein particle components in human follicular fluid and sera and their associations with embryo morphology parameters during IVF. **Human reproduction**, v.23, n.8, p.1884-1894, 2008.

BUTLER, S.T.; MARR, A.L.; PELTON, S.H.; RADCLIFF, R.P.; LUCY, M.C.; BUTLER, W.R. Insulin restores GH responsiveness during lactation-induced negative energy balance in dairy cattle: effects on expression of IGF-I and GH receptor 1A. **The Journal of endocrinology**, v.176, n.2, p.205-217, 2003.

BUTLER, S.T.; PELTON, S.H.; BUTLER, W.R. Energy balance, metabolic status, and the first postpartum ovarian follicle wave in cows administered propylene glycol. **Journal of dairy science**, v.89, n.8, p.2938-2951, 2006.

BUTLER, W.R. Review: effect of protein nutrition on ovarian and uterine physiology in dairy cattle. **Journal of dairy science**, v.81, n.9, p.2533-2539, 1998.
BUTLER, W.R. Nutritional interactions with reproductive performance in dairy cattle. **Animal reproduction science**, v.60-61, p.449-457, 2000.

BUTLER, W.R.; SMITH, R.D. Interrelationships between energy balance and postpartum reproductive function in dairy cattle. **Journal of dairy science**, v.72, n.3, p.767-783, 1989.

CAMPOS, F.T.; RINCON, J.A.A.; ACOSTA, D.A.V.; SILVEIRA, P.A.S.; PRADIEÉ, J.; CORREA, M.N.; GASPERIN, B.G.; PFEIFER, L.F.M.; BARROS, C.C.; PEGORARO, L.M.C.; SCHNEIDER, A. The acute effect of intravenous lipopolysaccharide injection on serum and intrafollicular HDL components and gene expression in granulosa cells of the bovine dominant follicle. **Theriogenology**, v.89, p.244-249, 2017.

CASTRO, N.A.; PFEIFER, L.F.M.; ANDRADE, J.S.; RINCON, J.A.A.; PEGORARO, L.M.C.; SCHNEIDER, A. Effect of serum paraoxonase-1 (PON1) activity on follicular development and pregnancy rate in cattle. **Animal reproduction science**, v.188, p.130-136, 2018.

CERON, J.J.; TECLES, F.; TVARIJONAVICIUTE, A. Serum paraoxonase 1 (PON1) measurement: an update. **BMC Veterinary Research**, v.10, p.74, 2014.

COCHRAN, S.D.; COLE, J.B.; NULL, D.J.; HANSEN, P.J. Discovery of single nucleotide polymorphisms in candidate genes associated with fertility and production traits in Holstein cattle. **BMC Genetetics**, v.14, p.49, 2013.

CONTI, P.; DEMPSEY, R.A.; REALE, M.; BARBACANE, R.C.; PANARA, M.R.; BONGRAZIO, M.; MIER, J.W. Activation of human natural killer cells by lipopolysaccharide and generation of interleukin-1 alpha, beta, tumour necrosis factor and interleukin-6. Effect of IL-1 receptor antagonist. **Immunology**, v.73, n.4, p.450-456, 1991.

CHAGAS, L.M.; BASS, J.J.; BLACHE, D.; BURKE, C.R.; KAY, J.K.; LINDSAY, D.R.; LUCY, M.C.; MARTIN, G.B.; MEIER, S.; RHODES, F.M.; ROCHE, J.R.; THATCHER, W.W.; WEBB, R. Invited review: New perspectives on the roles of nutrition and metabolic priorities in the subfertility of high-producing dairy cows. **Journal of dairy science**, v.90, n.9, p.4022-4032, 2007.

CHEBEL, R.C.; SANTOS, J.E.; REYNOLDS, J.P.; CERRI, R.L.; JUCHEM, S.O.; OVERTON, M. Factors affecting conception rate after artificial insemination and pregnancy loss in lactating dairy cows. **Animal reproduction science**, v.84, n.3-4, p.239-255, 2004.

CHO, K.H. A reconstituted high density lipoprotein containing the V156E mutant of apolipoprotein A-I exhibits anti-atherosclerotic activity in Apo-E deficient mice. **Journal of Atherosclerosis and Thrombosis**, v.16, n.3, p.217-229, 2009.

DANTZER, R.; KELLEY, K.W. Twenty years of research on cytokine-induced sickness behavior. **Brain, Behavior, and Immunity**, v.21, n.2, p.153-160, 2007.

DARWASH, A.O.; LAMMING, G.E.; WOOLLIAMS, J.A. Estimation of genetic variation in the interval from calving to postpartum ovulation of dairy cows. **Journal of dairy science**, v.80, n.6, p.1227-1234, 1997.
DE BOER, M.; BUDDLE, B.M.; HEUER, C.; HUSSEIN, H.; ZHENG, T.; LEBLANC, S.J.; MCDOUGALL, S. Associations between intrauterine bacterial infection, reproductive tract inflammation, and reproductive performance in pasture-based dairy cows. **Theriogenology**, v.83, n.9, p.1514-1524, 2015.

DE CAMPOS, F.T.; RINCON, J.A.; ACOSTA, D.A.; SILVEIRA, P.A.; PRADIEE, J.; CORREA, M.N.; GASPERIN, B.G.; PFEIFER, L.F.; BARROS, C.C.; PEGORARO, L.M.; SCHNEIDER, A. The acute effect of intravenous lipopolysaccharide injection on serum and intrafollicular HDL components and gene expression in granulosa cells of the bovine dominant follicle. **Theriogenology**, v.89, p.244-249, 2017a.

DE CAMPOS, F.T.; RINCON, J.A.A.; ACOSTA, D.A.V.; SILVEIRA, P.A.S.; PRADIEE, J.; CORREA, M.N.; GASPERIN, B.G.; PFEIFER, L.F.M.; BARROS, C.C.; PEGORARO, L.M.C.; SCHNEIDER, A. The acute effect of intravenous lipopolysaccharide injection on serum and intrafollicular HDL components and gene

expression in granulosa cells of the bovine dominant follicle. **Theriogenology**, v.89, p.244-249, 2017b.

DOBSON, H.; SMITH, R.; ROYAL, M.; KNIGHT, C.; SHELDON, I. The high-producing dairy cow and its reproductive performance. **Reproduction in domestic animals**, v.42 Suppl 2, p.17-23, 2007.

DRACKLEY, J.K. ADSA Foundation Scholar Award. Biology of dairy cows during the transition period: the final frontier? **Journal of dairy science**, v.82, n.11, p.2259-2273, 1999.

DUFFIELD, T. Subclinical ketosis in lactating dairy cattle. **The Veterinary clinics of North America. Food animal practice**, v.16, n.2, p.231-253, v, 2000.

EDENS, A.; TALAMANTES, F. Alternative processing of growth hormone receptor transcripts. **Endocrine reviews**, v.19, n.5, p.559-582, 1998.

EL-ROEIY, A.; CHEN, X.; ROBERTS, V.J.; SHIMASAKAI, S.; LING, N.; LEROITH, D.; ROBERTS, C.T., JR.; YEN, S.S. Expression of the genes encoding the insulin-like growth factors (IGF-I and II), the IGF and insulin receptors, and IGF-binding proteins-1-6 and the localization of their gene products in normal and polycystic ovary syndrome ovaries. **The Journal of clinical endocrinology and metabolism**, v.78, n.6, p.1488-1496, 1994.

FENWICK, M.A.; FITZPATRICK, R.; KENNY, D.A.; DISKIN, M.G.; PATTON, J.; MURPHY, J.J.; WATHES, D.C. Interrelationships between negative energy balance (NEB) and IGF regulation in liver of lactating dairy cows. **Domestic animal endocrinology**, v.34, n.1, p.31-44, 2008.

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS (FAO). Food Oulook: Biannual report on global food markets. 2017. Disponível em: http://www.fao.org/3/a-i7343e.pdf. Acesso em 12 jan. 2017.

FUHRMAN, B.; GANTMAN, A.; AVIRAM, M. Paraoxonase 1 (PON1) deficiency in mice is associated with reduced expression of macrophage SR-BI and consequently the loss of HDL cytoprotection against apoptosis. **Atherosclerosis**, v.211, n.1, p.61-68, 2010.

GALVAO, K.N.; FLAMINIO, M.J.; BRITTIN, S.B.; SPER, R.; FRAGA, M.; CAIXETA, L.; RICCI, A.; GUARD, C.L.; BUTLER, W.R.; GILBERT, R.O. Association between

uterine disease and indicators of neutrophil and systemic energy status in lactating Holstein cows. **Journal of dairy science**, v.93, n.7, p.2926-2937, 2010a.

GALVAO, K.N.; FRAJBLAT, M.; BUTLER, W.R.; BRITTIN, S.B.; GUARD, C.L.; GILBERT, R.O. Effect of early postpartum ovulation on fertility in dairy cows. **Reproduction in domestic animals**, v.45, n.5, p.e207-211, 2010b.

GALVAO, K.N.; SANTOS, N.R.; GALVAO, J.S.; GILBERT, R.O. Association between endometritis and endometrial cytokine expression in postpartum Holstein cows. **Theriogenology**, v.76, n.2, p.290-299, 2011.

GARNSWORTHY, P.C.; FOULADI-NASHTA, A.A.; MANN, G.E.; SINCLAIR, K.D.; WEBB, R. Effect of dietary-induced changes in plasma insulin concentrations during the early post partum period on pregnancy rate in dairy cows. **Reproduction**, v.137, n.4, p.759-768, 2009.

GE, W.; DAVIS, M.E.; HINES, H.C.; IRVIN, K.M.; SIMMEN, R.C. Association of a genetic marker with blood serum insulin-like growth factor-I concentration and growth traits in Angus cattle. **Journal of animal science**, v.79, n.7, p.1757-1762, 2001.

GEORGES, M.; LEQUARRE, A.S.; HANSET, R.; VASSART, G. Genetic variation of the bovine thyroglobulin gene studied at the DNA level. **Animal genetics**, v.18, n.1, p.41-50, 1987.
GITTO, E.; REITER, R.J.; KARBOWNIK, M.; TAN, D.X.; GITTO, P.; BARBERI, S.; BARBERI, I. Causes of oxidative stress in the pre- and perinatal period. **Biology of the Neonate**, v.81, n.3, p.146-157, 2002.

GOBIKRUSHANTH, M.; SALEHI, R.; AMBROSE, D.J.; COLAZO, M.G. Categorization of endometritis and its association with ovarian follicular growth and ovulation, reproductive performance, dry matter intake, and milk yield in dairy cattle. **Theriogenology**, v.86, n.7, p.1842-1849, 2016.

GONG, J.G.; LEE, W.J.; GARNSWORTHY, P.C.; WEBB, R. Effect of dietary-induced increases in circulating insulin concentrations during the early postpartum period on reproductive function in dairy cows. **Reproduction**, v.123, n.3, p.419-427, 2002.
GOODWIN, S.; MCPHERSON, J.D.; MCCOMBIE, W.R. Coming of age: ten years of next-generation sequencing technologies. **Nature Reviews Genetics**, v.17, n.6, p.333-351, 2016.

GROHN, Y.T.; RAJALA-SCHULTZ, P.J.; ALLORE, H.G.; DELORENZO, M.A.; HERTL, J.A.; GALLIGAN, D.T. Optimizing replacement of dairy cows: modeling the effects of diseases. **Preventive Veterinary Medicine**, v.61, n.1, p.27-43, 2003.

GRUMMER, R.R. Impact of changes in organic nutrient metabolism on feeding the transition dairy cow. **Journal of animal science**, v.73, n.9, p.2820-2833, 1995.

GRUMMER, R.R.; MASHEK, D.G.; HAYIRLI, A. Dry matter intake and energy balance in the transition period. **The Veterinary clinics of North America. Food animal practice**, v.20, n.3, p.447-470, 2004.

GUERIN, P.; EL MOUATASSIM, S.; MENEZO, Y. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. **Human reproduction update**, v.7, n.2, p.175-189, 2001.

HAMMON, D.S.; EVJEN, I.M.; DHIMAN, T.R.; GOFF, J.P.; WALTERS, J.L. Neutrophil function and energy status in Holstein cows with uterine health disorders. **Veterinary immunology and immunopathology**, v.113, n.1-2, p.21-29, 2006.

HANSEN, L.B. Consequences of selection for milk yield from a geneticist's viewpoint. **Journal of dairy science**, v.83, n.5, p.1145-1150, 2000.
HAX, L.T.; SCHNEIDER, A.; JACOMETO, C.B.; MATTEI, P.; DA SILVA, T.C.; FARINA, G.; CORREA, M.N. Association between polymorphisms in somatotropic axis genes and fertility of Holstein dairy cows. **Theriogenology**, v.88, p.67-72, 2017.

HERDT, T.H. Ruminant adaptation to negative energy balance. Influences on the etiology of ketosis and fatty liver. **The Veterinary clinics of North America. Food animal practice**, v.16, n.2, p.215-230, 2000.

HIGUCHI, M.; MIYASHITA, N.; AWATA, T. Rapid communication: a PCR-RFLP in the coding region of the bovine tumor necrosis factor-alpha locus. **Journal of animal science**, v.77, n.12, p.3400-3401, 1999.

HOEBEN, D.; BURVENICH, C.; TREVISI, E.; BERTONI, G.; HAMANN, J.; BRUCKMAIER, R.M.; BLUM, J.W. Role of endotoxin and TNF-alpha in the pathogenesis of experimentally induced coliform mastitis in periparturient cows. **Journal of Dairy Research**, v.67, n.4, p.503-514, 2000.

INGVARTSEN, K.L.; ANDERSEN, J.B. Integration of metabolism and intake regulation: a review focusing on periparturient animals. **Journal of dairy science**, v.83, n.7, p.1573-1597, 2000.

JIANG, H.; LUCY, M.C. Involvement of hepatocyte nuclear factor-4 in the expression of the growth hormone receptor 1A messenger ribonucleic acid in bovine liver. **Molecular Endocrinology**, v.15, n.6, p.1023-1034, 2001a.

JIANG, H.; LUCY, M.C. Variants of the 5'-untranslated region of the bovine growth hormone receptor mRNA: isolation, expression and effects on translational efficiency. **Gene**, v.265, n.1-2, p.45-53, 2001b.

JONES, J.I.; CLEMMONS, D.R. Insulin-like growth factors and their binding proteins: biological actions. **Endocrine reviews**, v.16, n.1, p.3-34, 1995.

KANAI, N.; FUJII, T.; SAITO, K.; TOKOYAMA, T. Rapid and simple method for preparation of genomic DNA from easily obtainable clotted blood. **Journal of Clinical Pathology**, v.47, n.11, p.1043-1044, 1994.

KANEKO, K.; KAWAKAMI, S. Influence of experimental intrauterine infusion of Arcanobacterium pyogenes solution on ovarian activity in cycling cows. **The journal Veterinary Medical Science**, v.70, n.1, p.77-83, 2008.

KAWAI, T.; AKIRA, S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. **Nature Immunology**, v.11, n.5, p.373-384, 2010.

KAWASAKI, Y.; AOKI, Y.; MAGATA, F.; MIYAMOTO, A.; KAWASHIMA, C.; HOJO, T.; OKUDA, K.; SHIRASUNA, K.; SHIMIZU, T. The effect of single nucleotide polymorphisms in the tumor necrosis factor-alpha gene on reproductive performance and immune function in dairy cattle. **Journal of Reproduction and Development**, v.60, n.3, p.173-178, 2014.

KAWASHIMA, C.; FUKIHARA, S.; MAEDA, M.; KANEKO, E.; MONTOYA, C.A.; MATSUI, M.; SHIMIZU, T.; MATSUNAGA, N.; KIDA, K.; MIYAKE, Y.; SCHAMS, D.; MIYAMOTO, A. Relationship between metabolic hormones and ovulation of dominant follicle during the first follicular wave post-partum in high-producing dairy cows. **Reproduction**, v.133, n.1, p.155-163, 2007.

KHATIB, H.; HUANG, W.; WANG, X.; TRAN, A.H.; BINDRIM, A.B.; SCHUTZKUS, V.; MONSON, R.L.; YANDELL, B.S. Single gene and gene interaction effects on fertilization and embryonic survival rates in cattle. **Journal of dairy science**, v.92, n.5, p.2238-2247, 2009.

KHATIB, H.; MONSON, R.L.; SCHUTZKUS, V.; KOHL, D.M.; ROSA, G.J.; RUTLEDGE, J.J. Mutations in the STAT5A gene are associated with embryonic survival and milk composition in cattle. **Journal of dairy science**, v.91, n.2, p.784-793, 2008.

KIRCHER, M.; KELSO, J. High-throughput DNA sequencing--concepts and limitations. **Bioessays**, v.32, n.6, p.524-536, 2010.

KOBAYASHI, Y.; BOYD, C.K.; BRACKEN, C.J.; LAMBERSON, W.R.; KEISLER, D.H.; LUCY, M.C. Reduced growth hormone receptor (GHR) messenger ribonucleic acid in liver of periparturient cattle is caused by a specific down-regulation of GHR 1A that is associated with decreased insulin-like growth factor I. **Endocrinology**, v.140, n.9, p.3947-3954, 1999.

KRAUSE, A.R.; PFEIFER, L.F.; MONTAGNER, P.; WESCHENFELDER, M.M.; SCHWEGLER, E.; LIMA, M.E.; XAVIER, E.G.; BRAUNER, C.C.; SCHMITT, E.; DEL PINO, F.A.; MARTINS, C.F.; CORREA, M.N.; SCHNEIDER, A. Associations between resumption of postpartum ovarian activity, uterine health and concentrations of metabolites and acute phase proteins during the transition period in Holstein cows. **Animal reproduction science**, v.145, n.1-2, p.8-14, 2014.

LAVON, Y.; EZRA, E.; LEITNER, G.; WOLFENSON, D. Association of conception rate with pattern and level of somatic cell count elevation relative to time of insemination in dairy cows. **Journal of dairy science**, v.94, n.9, p.4538-4545, 2011.

LAVON, Y.; LEITNER, G.; GOSHEN, T.; BRAW-TAL, R.; JACOBY, S.; WOLFENSON, D. Exposure to endotoxin during estrus alters the timing of ovulation and hormonal concentrations in cows. **Theriogenology**, v.70, n.6, p.956-967, 2008.

LEBLANC, S. Monitoring metabolic health of dairy cattle in the transition period. **Journal of Reproduction and Development**, v.56 Suppl, p.S29-35, 2010.

LORENZ, E.; MIRA, J.P.; FREES, K.L.; SCHWARTZ, D.A. Relevance of mutations in the TLR4 receptor in patients with gram-negative septic shock. **Archives of internal medicine**, v.162, n.9, p.1028-1032, 2002.

LU, M.C.; YANG, K.L.; TUNG, C.H.; HUANG, K.Y.; YU, H.C.; LIU, S.Q.; LAI, N.S. Higher LPS-stimulated TNF-alpha mRNA levels in peripheral blood mononuclear cells from Chinese ankylosing spondylitis patients with -308G/A polymorphism in promoter region of tumor necrosis factor: association with distinct A33/B58/Cw10 haplotypes. **Rheumatology International**, v.29, n.2, p.189-195, 2008.

LUCY, M.C. Reproductive loss in high-producing dairy cattle: where will it end? **Journal of dairy science**, v.84, n.6, p.1277-1293, 2001.

LUCY, M.C. Mechanisms linking nutrition and reproduction in postpartum cows. **Reproduction Supplement**, v.61, p.415-427, 2003.

LUCY, M.C. Fertility in high-producing dairy cows: reasons for decline and corrective strategies for sustainable improvement. **Society for Reproduction and Fertility**, v.64, p.237-254, 2007.
LUCY, M.C.; BOYD, C.K.; KOENIGSFELD, A.T.; OKAMURA, C.S. Expression of somatotropin receptor messenger ribonucleic acid in bovine tissues. **Journal of dairy science**, v.81, n.7, p.1889-1895, 1998.

MACKNESS, B.; DAVIES, G.K.; TURKIE, W.; LEE, E.; ROBERTS, D.H.; HILL, E.; ROBERTS, C.; DURRINGTON, P.N.; MACKNESS, M.I. Paraoxonase status in coronary heart disease: are activity and concentration more important than genotype? **Arteriosclerosis, thrombosis, and vascular biology**, v.21, n.9, p.1451-1457, 2001.

MAJ, A.; SNOCHOWSKI, M.; SIADKOWSKA, E.; ROWINSKA, B.; LISOWSKI, P.; ROBAKOWSKA-HYZOREK, D.; OPRZADEK, J.; GROCHOWSKA, R.; KOCHMAN, K.; ZWIERZCHOWSKI, L. Polymorphism in genes of growth hormone receptor (GHR) and insulin-like growth factor-1 (IGF1) and its association with both the IGF1 expression in liver and its level in blood in Polish Holstein-Friesian cattle. **Neuro Endocrinol Lett**, v.29, n.6, p.981-989, 2008.

MARSILLACH, J.; CHECA, M.A.; PEDRO-BOTET, J.; CARRERAS, R.; JOVEN, J.; CAMPS, J. Paraoxonase-1 in female infertility: a possible role against oxidative stress-induced inflammation. **Fertility and sterility**, v.94, n.3, p.1132-1134, 2010.

MCCARTHY, S.; BERRY, D.P.; DILLON, P.; RATH, M.; HORAN, B. Influence of Holstein-Friesian strain and feed system on body weight and body condition score lactation profiles. **Journal of dairy science**, v.90, n.4, p.1859-1869, 2007.

MCDOUGALL, S.; BURKE, C.R.; MACMILLAN, K.L.; WILLIAMSON, N.B. Patterns of follicular development during periods of anovulation in pasture-fed dairy cows after calving. **Research in veterinary science**, v.58, n.3, p.212-216, 1995.

MEE, J.F. The role of the veterinarian in bovine fertility management on modern dairy farms. **Theriogenology**, v.68 Suppl 1, p.S257-265, 2007.

MERTANI, H.C.; MOREL, G. In situ gene expression of growth hormone (GH) receptor and GH binding protein in adult male rat tissues. **Molecular and cellular endocrinology**, v.109, n.1, p.47-61, 1995.

MILEWICZ, T.; GREGORASZCZUK, E.L.; SZTEFKO, K.; AUGUSTOWSKA, K.; KRZYSIEK, J.; RYS, J. Lack of synergy between estrogen and progesterone on local IGF-I, IGFBP-3 and IGFBP-2 secretion by both hormone-dependent and hormone-independent breast cancer explants in vitro. Effect of tamoxifen and mifepristone (RU 486). **Growth Hormone & IGF Research**, v.15, n.2, p.140-147, 2005.

MUDRON, P.; REHAGE, J.; QUALMANN, K.; SALLMANN, H.P.; SCHOLZ, H. A study of lipid peroxidation and vitamin E in dairy cows with hepatic insufficiency. **Zentralbl Veterinarmed A**, v.46, n.4, p.219-224, 1999.

MULLER, E.E.; LOCATELLI, V.; COCCHI, D. Neuroendocrine control of growth hormone secretion. **Physiological Reviews**, v.79, n.2, p.511-607, 1999.

MURDOCH, W.J.; COLGIN, D.C.; ELLIS, J.A. Role of tumor necrosis factor-alpha in the ovulatory mechanism of ewes. **Journal of animal science**, v.75, n.6, p.1601-1605, 1997.

NEUPANE, M.; GEARY, T.W.; KISER, J.N.; BURNS, G.W.; HANSEN, P.J.; SPENCER, T.E.; NEIBERGS, H.L. Loci and pathways associated with uterine capacity for pregnancy and fertility in beef cattle. **PloS one**, v.12, n.12, p.e0188997, 2017.

OHTSUKA, H.; KOIWA, M.; HATSUGAYA, A.; KUDO, K.; HOSHI, F.; ITOH, N.; YOKOTA, H.; OKADA, H.; KAWAMURA, S. Relationship between serum TNF activity and insulin resistance in dairy cows affected with naturally occurring fatty liver. **Journal of Veterinary Medical Science**, v.63, n.9, p.1021-1025, 2001.

OMUETI, K.O.; MAZUR, D.J.; THOMPSON, K.S.; LYLE, E.A.; TAPPING, R.I. The polymorphism P315L of human toll-like receptor 1 impairs innate immune sensing of microbial cell wall components. **Journal of Immunology**, v.178, n.10, p.6387-6394, 2007.

OPSOMER, G.; GROHN, Y.T.; HERTL, J.; CORYN, M.; DELUYKER, H.; DE KRUIF, A. Risk factors for post partum ovarian dysfunction in high producing dairy cows in Belgium: a field study. **Theriogenology**, v.53, n.4, p.841-857, 2000.

ORTEGA, M.S.; DENICOL, A.C.; COLE, J.B.; NULL, D.J.; HANSEN, P.J. Use of single nucleotide polymorphisms in candidate genes associated with daughter pregnancy rate for prediction of genetic merit for reproduction in Holstein cows. **Animal Genetics**, v.47, n.3, p.288-297, 2016.

ORTEGA, M.S.; WOHLGEMUTH, S.; TRIBULO, P.; SIQUEIRA, L.G.; COLE, J.B.; HANSEN, P.J. A single nucleotide polymorphism in COQ9 affects mitochondrial and ovarian function and fertility in Holstein cows. **Biology of reproduction**, v.96, n.3, p.652-663, 2017.

PEDERNERA, M.; GARCIA, S.C.; HORAGADOGA, A.; BARCHIA, I.; FULKERSON, W.J. Energy balance and reproduction on dairy cows fed to achieve low or high milk production on a pasture-based system. **Journal of dairy science**, v.91, n.10, p.3896-3907, 2008.

PEZZULO, A.A.; HORNICK, E.E.; RECTOR, M.V.; ESTIN, M.; REISETTER, A.C.; TAFT, P.J.; BUTCHER, S.C.; CARTER, A.B.; MANAK, J.R.; STOLTZ, D.A.; ZABNER, J. Expression of human paraoxonase 1 decreases superoxide levels and alters bacterial colonization in the gut of Drosophila melanogaster. **PloS one**, v.7, n.8, p.e43777, 2012.

PINEDO, P.J.; GALVAO, K.N.; SEABURY, C.M. Innate immune gene variation and differential susceptibility to uterine diseases in Holstein cows. **Theriogenology**, v.80, n.4, p.384-390, 2013.

PITTMAN, Q.J. A neuro-endocrine-immune symphony. **Journal of Neuroendocrinology**, v.23, n.12, p.1296-1297, 2011.

PURSLEY, J.R.; MEE, M.O.; WILTBANK, M.C. Synchronization of ovulation in dairy cows using PGF2alpha and GnRH. **Theriogenology**, v.44, n.7, p.915-923, 1995.

RADCLIFF, R.P.; MCCORMACK, B.L.; CROOKER, B.A.; LUCY, M.C. Plasma hormones and expression of growth hormone receptor and insulin-like growth factor-I mRNA in hepatic tissue of periparturient dairy cows. **Journal of dairy science**, v.86, n.12, p.3920-3926, 2003.

RINCON, J.; MADEIRA, E.M.; CAMPOS, F.T.; MION, B.; SILVA, J.F.; ABSALON-MEDINA, V.A.; BUTLER, W.R.; CORREA, M.N.; PEGORARO, L.; SCHNEIDER, A. Exogenous paraoxonase-1 during oocyte maturation improves bovine embryo development in vitro. **Reproduction in domestic animals**, v.51, n.5, p.827-830, 2016.

ROYAL, M.; MANN, G.E.; FLINT, A.P. Strategies for reversing the trend towards subfertility in dairy cattle. **Veterinary journal**, v.160, n.1, p.53-60, 2000.

RUPRECHTER, G.; CARRIQUIRY, M.; RAMOS, J.M.; PEREIRA, I.; ANA, M. Metabolic and endocrine profiles and reproductive parameters in dairy cows under

grazing conditions: effect of polymorphisms in somatotropic axis genes. **Acta Veterinaria Scandinavica**, v.53, p.35, 2011.

SAVIO, J.D.; BOLAND, M.P.; ROCHE, J.F. Development of dominant follicles and length of ovarian cycles in post-partum dairy cows. **Journal of reproduction and fertility**, v.88, n.2, p.581-591, 1990.

SAVVA, A.; KANNI, T.; DAMORAKI, G.; KOTSAKI, A.; GIATRAKOU, S.; GRECH, I.; KATOULIS, A.; PAPADAVID, E.; GIAMARELLOS-BOURBOULIS, E.J. Impact of Toll-like receptor-4 and tumour necrosis factor gene polymorphisms in patients with hidradenitis suppurativa. **British Journal of Dermatology**, v.168, n.2, p.311-317, 2013.

SCHADOCK, I.; SCHNEIDER, A.; SILVA, E.D.; BUCHWEITZ, M.R.; CORREA, M.N.; PESQUERO, J.B.; PAREDES-GAMERO, E.J.; ARAUJO, R.C.; BARROS, C.C. Simple Method to Genotype the ACTN3 r577x Polymorphism. **Genetic Testing and Molecular Biomarkers**, v.19, n.5, p.253-257, 2015.

SCHAEFFLER, A.; GROSS, P.; BUETTNER, R.; BOLLHEIMER, C.; BUECHLER, C.; NEUMEIER, M.; KOPP, A.; SCHOELMERICH, J.; FALK, W. Fatty acid-induced induction of Toll-like receptor-4/nuclear factor-kappaB pathway in adipocytes links nutritional signalling with innate immunity. **Immunology**, v.126, n.2, p.233-245, 2009.

SCHNEIDER, A.; CORREA, M.N.; BUTLER, W.R. Association between growth hormone receptor AluI polymorphism and fertility of Holstein cows. **Theriogenology**, v.80, n.9, p.1061-1066, 2013a.

SCHNEIDER, A.; CORREA, M.N.; BUTLER, W.R. Short communication: acute phase proteins in Holstein cows diagnosed with uterine infection. **Research in veterinary science**, v.95, n.1, p.269-271, 2013b.

SHELDON, I.M.; CRONIN, J.; GOETZE, L.; DONOFRIO, G.; SCHUBERTH, H.J. Defining postpartum uterine disease and the mechanisms of infection and immunity in the female reproductive tract in cattle. **Biology of reproduction**, v.81, n.6, p.1025-1032, 2009.
SHELDON, I.M.; RYCROFT, A.N.; ZHOU, C. Association between postpartum pyrexia and uterine bacterial infection in dairy cattle. **The Veterinary record**, v.154, n.10, p.289-293, 2004.

SHIMIZU, T.; KAWASAKI, Y.; AOKI, Y.; MAGATA, F.; KAWASHIMA, C.; MIYAMOTO, A. Effect of Single Nucleotide Polymorphisms of Toll-Like Receptor 4

(TLR 4) on Reproductive Performance and Immune Function in Dairy Cows. **Biochemical Genetics**, v.55, n.3, p.212-222, 2017.

SHIRASUNA, K.; KAWASHIMA, C.; MURAYAMA, C.; AOKI, Y.; MASUDA, Y.; KIDA, K.; MATSUI, M.; SHIMIZU, T.; MIYAMOTO, A. Relationships between the first ovulation postpartum and polymorphism in genes relating to function of immunity, metabolism and reproduction in high-producing dairy cows. **Journal of Reproduction and Development**, v.57, n.1, p.135-142, 2011.

SILVEIRA, P.A.; SCHWEGLER, E.; MONTAGNER, P.; KRAUSE, A.R.; ACOSTA, D.A.; HALFEN, J.; GARLET, T.; BARROS, C.C.; CORREA, M.N.; SCHNEIDER, A. Characterization of single nucleotide polymorphisms in the promoter region of the bovine paraoxonase 1 (PON1) gene affecting serum enzyme activity in dairy cows. **Veterinary journal**, v.205, n.1, p.101-103, 2015a.

SILVEIRA, P.A.; SCHWEGLER, E.; MONTAGNER, P.; KRAUSE, A.R.; ACOSTA, D.A.; HALFEN, J.; GARLET, T.; BARROS, C.C.; CORREA, M.N.; SCHNEIDER, A. Characterization of single nucleotide polymorphisms in the promoter region of the bovine paraoxonase 1 (PON1) gene affecting serum enzyme activity in dairy cows. **Veterinary journal**, 2015b.

STOFKOVA, A. Leptin and adiponectin: from energy and metabolic dysbalance to inflammation and autoimmunity. **Endocrine Regulations**, v.43, n.4, p.157-168, 2009.

THATCHER, W.W.; WILCOX, C.J. Postpartum estrus as an indicator of reproductive status in the dairy cow. **Journal of dairy science**, v.56, n.5, p.608-610, 1973.
TRAN, U.C.; CLARKE, C.F. Endogenous synthesis of coenzyme Q in eukaryotes. **Mitochondrion**, v.7 Suppl, p.S62-71, 2007.

TREVISI, E.; AMADORI, M.; COGROSSI, S.; RAZZUOLI, E.; BERTONI, G. Metabolic stress and inflammatory response in high-yielding, periparturient dairy cows. **Research in veterinary science**, v.93, n.2, p.695-704, 2012.
TURK, R.; PIRAS, C.; KOVACIC, M.; SAMARDZIJA, M.; AHMED, H.; DE CANIO, M.; URBANI, A.; MESTRIC, Z.F.; SOGGIU, A.; BONIZZI, L.; RONCADA, P. Proteomics of inflammatory and oxidative stress response in cows with subclinical and clinical mastitis. **Journal of proteomics**, v.75, n.14, p.4412-4428, 2012.

TURK, R.; PODPECAN, O.; MRKUN, J.; KOSEC, M.; FLEGAR-MESTRIC, Z.; PERKOV, S.; STARIC, J.; ROBIC, M.; BELIC, M.; ZRIMSEK, P. Lipid mobilisation and oxidative stress as metabolic adaptation processes in dairy heifers during transition period. **Animal reproduction science**, v.141, n.3-4, p.109-115, 2013.

UNITED STATES DEPARTAMENT OF AGRICULTURE (USDA). Dairy: word markerts and trades. 2017. Disponivel em: https://www.fas.usda.gov/data/dairy-world-markets-and-trade. Acesso em 12 jan. 2017.

VANRADEN, P.M.; SANDERS, A.H.; TOOKER, M.E.; MILLER, R.H.; NORMAN, H.D.; KUHN, M.T.; WIGGANS, G.R. Development of a national genetic evaluation for cow fertility. **Journal of dairy science**, v.87, n.7, p.2285-2292, 2004.

VELAZQUEZ, M.A.; SPICER, L.J.; WATHES, D.C. The role of endocrine insulin-like growth factor-I (IGF-I) in female bovine reproduction. **Domestic animal endocrinology**, v.35, n.4, p.325-342, 2008.

VICINI, J.L.; BUONOMO, F.C.; VEENHUIZEN, J.J.; MILLER, M.A.; CLEMMONS, D.R.; COLLIER, R.J. Nutrient balance and stage of lactation affect responses of insulin, insulin-like growth factors I and II, and insulin-like growth factor-binding protein 2 to somatotropin administration in dairy cows. **Journal of Nutrition**, v.121, n.10, p.1656-1664, 1991.

VILCEK, J.; LEE, T.H. Tumor necrosis factor. New insights into the molecular mechanisms of its multiple actions. **The Journal of biological chemistry**, v.266, n.12, p.7313-7316, 1991.

WADE, G.N.; SCHNEIDER, J.E.; LI, H.Y. Control of fertility by metabolic cues. **American Journal of Physiology**, v.270, n.1 Pt 1, p.E1-19, 1996.

WALDMANN, A. Monoclonal antibodies to progesterone: characterization and selection for enzyme immunoassay in bovine milk. **Hybridoma**, v.18, n.3, p.289-296, 1999.

WALSH, R.B.; KELTON, D.F.; DUFFIELD, T.F.; LESLIE, K.E.; WALTON, J.S.; LEBLANC, S.J. Prevalence and risk factors for postpartum anovulatory condition in dairy cows. **Journal of dairy science**, v.90, n.1, p.315-324, 2007.

WALUSIMBI, S.S.; PATE, J.L. Physiology and Endocrinology Symposium: role of immune cells in the corpus luteum. **Journal of animal science**, v.91, n.4, p.1650-1659, 2013.

WANG, X.; XU, S.; GAO, X.; REN, H.; CHEN, J. Genetic polymorphism of TLR4 gene and correlation with mastitis in cattle. **Journal of Genetics and Genomics**, v.34, n.5, p.406-412, 2007.

WEDEL, A.; ZIEGLER-HEITBROCK, H.W. The C/EBP family of transcription factors. **Immunobiology**, v.193, n.2-4, p.171-185, 1995.

WEST, A.P.; SHADEL, G.S.; GHOSH, S. Mitochondria in innate immune responses. **Nature Reviews Immunology**, v.11, n.6, p.389-402, 2011.

WU, J.; BAI, J.Y.; LI, L.; HUANG, S.; LI, C.M.; WANG, G.L. Genetic polymorphisms of the BMAP-28 and MASP-2 genes and their correlation with the somatic cell score in Chinese Holstein cattle. **Genetics and Molecular Research**, v.14, n.1, p.1-8, 2015.

YU, L.; MOORE, A.B.; CASTRO, L.; GAO, X.; HUYNH, H.L.; KLIPPEL, M.; FLAGLER, N.D.; LU, Y.; KISSLING, G.E.; DIXON, D. Estrogen Regulates MAPK-Related Genes through Genomic and Nongenomic Interactions between IGF-I Receptor Tyrosine Kinase and Estrogen Receptor-Alpha Signaling Pathways in Human Uterine Leiomyoma Cells. **Journal of Signal Transduction**, v.2012, p.204236, 2012.

YUAN, K.; FARNEY, J.K.; MAMEDOVA, L.K.; SORDILLO, L.M.; BRADFORD, B.J. TNFalpha altered inflammatory responses, impaired health and productivity, but did not affect glucose or lipid metabolism in early-lactation dairy cows. **PloS one**, v.8, n.11, p.e80316, 2013.

ZHANG, C.; LIU, Y.; RING, B.Z.; NIE, K.; YANG, M.; WANG, M.; SHEN, H.; WU, X.; MA, X. A novel multiplex tetra-primer ARMS-PCR for the simultaneous genotyping of six single nucleotide polymorphisms associated with female cancers. **PloS one**, v.8, n.4, p.e62126, 2013.