UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CÂMPUS DE JABOTICABAL

ESTUDO DE ASSOCIAÇÃO DE POLIMORFISMOS DE NUCLEOTÍDEO ÚNICO (SNP) COM CARACTERÍSTICAS DE TEMPERAMENTO EM BOVINOS DA RAÇA NELORE

Tiago da Silva Valente

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Orientador: Prof. Dr. Mateus José Rodrigues Paranhos da Costa
Coorientador: Prof. Dr. Fernando Sebastián Baldi Rey
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Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutor em Genética e Melhoramento Animal

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Jaboticabal, 26 de julho de 2016

DADOS CURRICULARES DO AUTOR

TIAGO DA SILVA VALENTE – Nascido em 27 de junho de 1988, na cidade de Anápolis – Goiás, filho de César Otávio Valente e Ivanilda da Silva Valente. Iniciou em março de 2006 o curso de graduação em Ciências Biológicas pela Universidade Federal de Goiás - UFG, Câmpus Samambaia, Goiânia – GO, obtendo o título de Biólogo em dezembro de 2009. A partir de março de 2010 passou a fazer parte do Grupo de Estudos e Pesquisas em Etologia e Ecologia Animal (ETCO) da Faculdade de Ciências Agrárias e Veterinárias – FCAV/UNESP – Câmpus de Jaboticabal – SP. Em 2012 concluiu o mestrado pelo Programa de Pós-Graduação em Genética e Melhoramento animal desta mesma Universidade. Neste mesmo ano iniciou o curso de Doutorado pelo mesmo Programa de Pós-Graduação, como bolsista da CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) sob orientação do Prof. Dr. Mateus José Rodrigues Paranhos da Costa e co-orientação do Prof. Dr. Fernando Sebastián Baldi Rey e Profa. Dra. Lúcia Galvão de Albuquerque. Em julho de 2015 iniciou o estágio sanduíche no exterior, permanecendo oito meses na University of Alberta – CA, orientado pelo Prof. Dr. Graham Plastow. Atualmente atua principalmente nas áreas de genética e melhoramento dos animais domésticos, etologia aplicada à produção animal, bem-estar dos animais domésticos e genética do comportamento.

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"Certamente um dos maiores estímulos na ciência é o simples prazer de descobrir coisas, de aprender algo novo sobre o mundo à nossa volta".

Hal Hellman, 1998.

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CERTIFICADO

Certificamos que o Protocolo nº 016/14 do trabalho de pesquisa intitulado **"Estudo de associação de polimorfismos de nucleotídeos de base única (SNP) com temperamento de bovinos da raça nelore",** sob a responsabilidade do Prof. Dr. Mateus José Rodrigues Paranhos da Costa está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), em reunião ordinária de 11 de março de 2014.

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ESTUDO DE ASSOCIAÇÃO DE POLIMORFISMOS DE NUCLEOTÍDEO ÚNICO (SNP) COM CARACTERÍSTICAS DE TEMPERAMENTO EM BOVINOS DA RAÇA NELORE

RESUMO - A variabilidade individual no temperamento dos bovinos é um importante fator que afeta a rentabilidade das empresas pecuárias, em função dos efeitos negativos na produção, no bem-estar animal e na segurança do trabalhador. Assim, esta característica vem despertando interesse crescente de produtores e pesquisadores, com o objetivo de entender quais os fatores genéticos e ambientais que influenciam a expressão do temperamento e os efeitos sobre o desempenho dos bovinos. Desta forma, esta tese foi desenvolvida com o objetivo de estudar a associação genômica entre polimorfismos de nucleotídeo único (SNP) com diferentes indicadores do temperamento de bovinos da raça Nelore. Os objetivos específicos foram: 1) Estudar a associação genômica entre SNP e diferentes indicadores de temperamento; 2) Avaliar a existência de regiões do genoma associadas, simultaneamente, com distintos indicadores de temperamento; 3) Identificar genes e as respectivas funções biológicas que influenciam o temperamento e; 4) Estimar parâmetros genéticos para os diferentes testes de temperamento incluindo a informação genômica. O temperamento dos bovinos foi avaliado entre 2010 e 2015, durante o manejo de pesagem aos 18 meses de idade, utilizando os seguintes indicadores: 1) escore de movimentação (MOV); 2) escore de tensão (TENS); 3) escore de agitação no tronco de contenção (CS); 4) velocidade de fuga (VF) e; 5) escore de temperamento (ET). Os animais foram genotipados utilizando painel de alta densidade com 777.962 SNPs (Illumina BovineHD Beadchip). Todas as análises para estimar os componentes de variância e parâmetros genéticos, bem como os estudos de associação genômica ampla (GWAS) foram realizadas utilizando método de passo único (*single-step* GBLUP - ssGBLUP) combinando, simultaneamente, informações de pedigree, fenótipos e genótipos, utilizando inferência Bayesiana via amostragem de Gibbs. Foi utilizado modelo animal linear para VF e modelo de limiar para os demais indicadores de temperamento (MOV, TENS, CS e ET). No primeiro estudo foram utilizados 1.384 genótipos, 16.660 animais com fenótipo para VF e 455.374 SNPs. Foram identificadas nove regiões genômicas e seis genes candidatos associados a velocidade de fuga. Entre eles, os genes *PARK2*, *GUCY1A2*, *CPE* e *DOCK1* estão relacionados com o sistema dopaminérgico; a formação da memória; a biossíntese de hormônio peptídico e neurotransmissores e, o desenvolvimento do cérebro, respectivamente. Para o segundo estudo foram utilizados, aproximadamente, 22.000 fenótipos para MOV, TENS, CS, VF e 45.000 para ET, 3.568 genótipos e 411.677 SNPs. As estimativas de herdabilidade variaram de 0,12 $\pm$ 0,03 a 0,33 $\pm$ 0,03. As estimativas a posteriori das correlações genéticas e fenotípicas entre os cinco indicadores de temperamento variaram de 0,57 $\pm$ 0,06 a 0,99 $\pm$ 0,00 e de 0,23 $\pm$ 0,01 a 0,93 $\pm$ 0,01, respectivamente. Os resultados das análises de ssGBLUP foram apresentados com base na porcentagem de variância explicada por janelas de 10 SNPs adjacentes. As 20 janelas que explicaram a maior variância genética aditiva para cada indicador foram consideradas como associadas ao temperamento. Um

total de 100 janelas, 2.033 genes conhecidos, nove QTL descritos previamente e regiões genômicas semelhantes entre TENS x CS, MOV x FS, CS x ET e FS x ET foram identificadas. Concluímos que todas os indicadores de temperamento utilizadas no presente estudo podem ser consideradas em programas de melhoramento. Com o estudo de associação genômica ampla mapeamos genes que podem influenciar o temperamento dos bovinos, demonstrando que essa é uma característica complexa e amplamente influenciados por muitas regiões/genes de pequeno efeito. As análises de enriquecimento funcional proporcionaram novas perspectivas e vias que correspondem diretamente com aspectos de comportamento emocional.

Palavras-chave: comportamento, bovinos de corte, efeitos dos SNPs, marcador molecular, reatividade

GENOME-WIDE ASSOCIATION STUDY BETWEEN SINGLE NUCLEOTIDE POLYMORPHISM (SNP) AND TEMPERAMENT TRAITS IN NELLORE CATTLE

SUMMARY - The individual variability on cattle temperament is an important factor affecting the profitability of beef cattle enterprises, due to its adverse effects in production, animal welfare and labor safety. Thus, this characteristic has increasing interest of producers and researchers, who aim to understand the genetic and environmental factors affecting the expression of temperament and its effect on cattle performance. Thus, the objective of this thesis was to study the genomic association between single nucleotide polymorphisms (SNP) and temperament indicators of Nellore cattle. The specific objectives were: 1) Study the genomic association between SNP and different indicators of temperament; 2) Assess the existence of genomic regions associated, simultaneously, with distinct temperament indicators; 3) Identify genes and their biological functions that influences temperament and; 4) Estimate genetic parameters for different temperament indicators using genomic information. The temperament was evaluated between 2010 and 2015, during the weighing handling at 18 months of age, using the following indicators: 1) movement score (MOV); 2) tension score (TENS); 3) crush score (CS); 4) flight speed (FS) and; 5) temperament score (TS). The animals were genotyped using a panel with 777,962 SNP (Illumina BovineHD Beadchip). All analyses to estimate variance components and genetic parameters, as well as the genome-wide association study (GWAS), were performed using a single-step procedure (*single-step* GBLUP - ssGBLUP) which combined, simultaneously, pedigree information, phenotyped and genotyped animals in one step, using Bayesian inference via Gibbs sampling. Was used linear animal model for FS and threshold model for other temperament indicators (MOV, TENS, CS and TS). In the first study were used 1,384 genotypes, 16,660 phenotyped animals for FS and 455,374 SNP. There were identified nine genomic regions and six candidate genes associated with flight speed. Among them *PARK2*, *GUCY1A2*, *CPE* and *DOCK1* genes which are related to dopaminergic system; memory formation; biosynthesis of peptide hormone and neurotransmitter; and brain development, respectively. For the second study were used, approximately, 22,000 phenotypes to MOV, TENS, CS, VF and 45,000 for TS, 3,568 genotypes and 411,677 SNPs. Heritability estimates for temperament indicators ranged from 0.12 $\pm$ 0.03 to 0.33 $\pm$ 0.03. The posterior means of genetic and phenotypic correlations between all five temperament indicators ranged from 0.57 $\pm$ 0.06 to 0.99 $\pm$ 0.00 and from 0.23 $\pm$ 0.01 to 0.93 $\pm$ 0.01, respectively. The results of ssGBLUP analysis were presented in based on the proportion of variance explained by 10 consecutive non-overlapping SNPs. The 20 SNP windows that explained the highest additive genetic variance for each indicator were considered as associated with temperament. Were identified A total of 100 SNP windows, 2.033 known genes, nine previously reported QTL and similar genomic regions between TENS x CS, MOV x FS, CS x TS and FS x TS. In conclusion, all temperament indicators addressed in the present study can be considered in breeding programs. With the genome-wide association study mapped genes that can influence cattle temperament, demonstrating that this is a complex trait and largely influenced by many regions/genes of small effect. Functional

enrichment analyses delivered new insights and pathways that directly match with aspects of emotional behavior.

Key words: behavior, beef cattle, SNPs effects, molecular marker, reactivity

LISTA DE ABREVIATURAS

ADG – average daily gain

BTA – *Bos taurus* autosome

CG – contemporary group

Chr – chromosomes

cM – Centimorgan

CS – crush score

DAVID – Database for annotation, visualization and integrated discovery

FS – flight speed

GWAS – genome-wide association study

HPDI – highest probability density interval

IPA – Ingenuity pathway analysis

m – meter

MAF – minor allele frequency

MOV – movement score

m/s – meters per second

NCBI – National center for biotechnology information

QC – quality control

QTL –quantitative trait loci

TENS – tension score

TS – temperament score

SD – standard deviation

SNP – single nucleotide polymorphisms

ssGBLUP – single-step genomic BLUP

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CAPÍTULO 1 - Considerações Gerais

1. INTRODUÇÃO

Diversos estudos com abordagens genômicas têm sido desenvolvidos com o objetivo de identificar os fatores genéticos que contribuem para a expressão de características importantes em humanos e animais. Especificamente na produção animal, os estudos têm focado na detecção de regiões genômicas, genes e QTL (*Quantitative Trait Loci*) associados a características que são economicamente importantes, tais como produção, reprodução, saúde e resistência a doenças (SHARMA et al., 2015; TIEZZI et al., 2015). Além disto, com o advento das metodologias de associação genômica ampla (do inglês *Genome-Wide Association Study* - GWAS), que utilizam informações de polimorfismos de nucleotídeo único (SNP) espaçados pelo genoma, vários trabalhos foram desenvolvidos para mapear a base genética de características quantitativas relacionadas a eficiência alimentar (MOORE; MUJIBI; SHERMAN, 2009; SHERMAN et al., 2009; SHERMAN; NKRUMAH; MOORE, 2010; MUJIBI et al., 2011), produção e qualidade do leite (BOLORMAA et al., 2010; JIANG et al., 2010; MAI et al., 2010; BOUWMAN et al., 2011; SCHOPEN, et al., 2011), fertilidade (SAHANA et al., 2010; HUANG et al., 2010), crescimento (SNELLING et al., 2010; BOLORMAA et al. 2011a) e qualidade de carcaça e carne (SCHENKEL et al., 2006; CORVA et al., 2007; GILL et al., 2009; BOLORMAA et al., 2011b).

De maneira semelhante, abordagens moleculares têm sido utilizadas para aprofundar o conhecimento sobre os mecanismos genéticos subjacentes à variabilidade fenotípica do comportamento animal (MORMÈDE, 2005; JENSEN et al., 2008). Para animais de fazenda esses estudos têm focado no temperamento, que é consistente ao longo do tempo (MÜLLER; VON KEYSERLINGK, 2006) e caracterizado pelo conjunto de respostas comportamentais dos animais em relação ao homem, geralmente atribuídas ao medo (FORDYCE et al., 1982). As diferenças individuais no temperamento podem apresentar implicações práticas e econômicas para os sistemas produtivos, bem como efeitos diretos em aspectos produtivos,

reprodutivos, de saúde e bem-estar animal e humano (BURDICK et al., 2011; CANÁRIO et al., 2013).

É amplamente reconhecido que o temperamento dos bovinos está sob influência direta de múltiplos genes, que interagem entre si e com os fatores ambientais. No entanto, o principal desafio para estudar a base genética do temperamento é a definição de testes padronizados que possibilitem avaliar o fenótipo comportamental em diferentes populações, raças e condições ambientais (MORMÈDE, 2005; SANT'ANNA et al., 2013), permitindo comparar os resultados obtidos nos diversos estudos. Porém, o que se observa na literatura científica relacionada ao tema é a utilização de distintas metodologias para avaliar o temperamento e, posteriormente, identificar QTL e genes candidatos (SCHMUTZ et al., 2001; HIENDLEDER et al., 2003; ESMAILIZADEH et al., 2008; GUTIERREZ-GIL et al., 2008; HULSMAN HANNA et al., 2014; KRAMER et al., 2014; LINDHOLM-PERRY et al., 2015). Neste sentido, os métodos utilizados vão desde testes comportamentais, tais como distância de fuga, teste de separação social, velocidade de fuga (GUTIERREZ-GIL et al., 2008; HULSMAN HANNA et al., 2014; LINDHOLM-PERRY et al., 2015) a escalas visuais com pontuação pré-definida (HIENDLEDER et al., 2003; KRAMER et al., 2014) e medidas automatizadas para registro do comportamento (SCHMUTZ et al., 2001).

Nos estudos citados acima foram identificados QTL e genes candidatos associados ao temperamento. No entanto, em função da natureza multidimensional e complexa dessa característica (RÉALE et al., 2007), somada à ausência de metodologia padronizada para caracterizar o fenótipo de interesse, podemos concluir que a comparação entre os resultados dos diferentes estudos é limitada, e deve ser feita de maneira cautelosa. Assim, o objetivo com este trabalho foi realizar estudos de associação entre polimorfismos de nucleotídeo único (SNP) e cinco diferentes indicadores utilizados para avaliar o temperamento de bovinos da raça Nelore. Os objetivos específicos foram: 1) Estudar a associação genômica entre polimorfismos de nucleotídeo único com diferentes métodos de avaliação do temperamento; 2) Avaliar a existência de regiões do genoma associadas, simultaneamente, com diferentes indicadores de temperamento; 3) Identificar genes e as respectivas funções biológicas que influenciam o temperamento dos bovinos; 4)

Utilizar a informação genômica nas estimativas dos componentes de (co)variância e parâmetros genéticos para diferentes indicadores de temperamento.

2. REVISÃO DE LITERATURA

2.1. IMPORTÂNCIA DO TEMPERAMENTO PARA A PECUÁRIA MODERNA

Pode-se assumir que o temperamento é o conjunto de respostas comportamentais que caracterizam um determinado indivíduo e que são consistentes ao longo do tempo e em diferentes situações (BATES, 1989). Além disto, o temperamento dos bovinos foi definido operacionalmente como o conjunto de comportamentos dos animais em relação ao homem, geralmente atribuindo essas reações a aspectos gerais de medo (FORDYCE et al., 1982; PETHERICK et al., 2002). Neste contexto, o temperamento foi um fator determinante durante o processo de domesticação das espécies (PRICE, 1999) e, permanece como tema relevante para a pecuária moderna, por ser um dos fatores que podem influenciar a eficiência dos sistemas produtivos.

As diferenças individuais observadas no temperamento podem refletir aspectos hormonais e neuroendócrinos de cada animal (BURDICK et al., 2011; BRAND et al., 2015), e estarem associadas a efeitos negativos sobre diversas funções biológicas, tais como a defesa imunológica, a reprodução, o crescimento e a qualidade do produto final (MURANI et al., 2010). Por exemplo, animais que expressam maior reatividade na presença de humanos e que são considerados como de temperamento ruim, apresentam menores ganhos de peso (VOISINET et al., 1997a; PETHERICK et al., 2002; DEL CAMPO et al., 2010; SEBASTIAN et al., 2011) e menor qualidade da carne e carcaça, com maior ocorrência de cortes escuros (VOISINET et al., 1997b; KING et al., 2006; BEHRENDS et al., 2009; DEL CAMPO et al., 2010; CAFE et al., 2011a; HALL et al., 2011). Em sistemas de cria, fêmeas mais calmas apresentaram melhores índices reprodutivos, com maior taxa de prenhez quando submetidas ao protocolo de inseminação em tempo fixo (IATF) (COOKE et al., 2009; COOKE et al., 2011; RUEDA, 2012; RUEDA et al., 2015). Somado aos efeitos em características produtivas, o temperamento também afeta

aspectos de saúde dos animais e de bem-estar durante as rotinas de manejo (FELL et al., 1999; CAFE et al., 2011a; HULBERT et al., 2011).

Além das implicações indiretas na rentabilidade dos sistemas de produção, as reações dos bovinos que caracterizam o temperamento podem apresenta valor econômico direto (BUSBY et al., 2006), gerando prejuízos e custos adicionais aos produtores. Ou seja, há redução na eficiência do trabalho quando são manejados animais de alta reatividade, demandando maior tempo e número de funcionários para realizar o manejo (PARANHOS DA COSTA, 2000), além de aumentar o risco de acidentes de trabalho e os prejuízos para o bem-estar dos animais (GRANDIN, 1993; BROUČEK et al., 2008). Desta forma, o temperamento dos bovinos pode ser considerado uma característica funcional, com implicações práticas para o sistema produtivo e reflexos diretos em questões econômicas, técnicas, éticas, e de segurança dos trabalhadores e dos animais (CURLEY et al., 2008; BURDICK et al., 2010).

Assim, aprofundar o conhecimento sobre o temperamento dos bovinos e seus efeitos para a pecuária pode ser uma alternativa para atender as exigências dos consumidores em relação à segurança alimentar, qualidade do produto, rastreabilidade, segurança do trabalhador, respeito aos recursos naturais e ao bem-estar animal (PESSUTI; MEZZADRI, 2004; HASKELL; SIMM; TURNER, 2014). Por isto, é crescente a busca por alternativas que visem melhorar o temperamento e reduzir a reatividade dos animais, seja utilizando técnicas de aprendizagem, melhorando a interação humano-animal, adequando os sistemas de criação para atender as necessidades dos animais (BOISSY; BOUISSOU, 1988; BECKER; LOBATO, 1997; SCHWARTZKOPF-GENSWEIN et al., 1997; COOKE et al., 2009; TITTO et al., 2010), ou por meio da inclusão dessa característica, como critério de seleção, nos programas de melhoramento genético (SANT'ANNA et al., 2012).

2.2. TEMPERAMENTO COMO CRITÉRIO DE SELEÇÃO

O temperamento dos bovinos é uma característica multidimensional e complexa (RÉALE et al., 2007), que sofre influência de diversos fatores ambientais, tais como idade, experiências prévias, qualidade das interações humano-animal e

sistema de produção ao qual os animais estão submetidos. Porém, é amplamente reconhecido que essa característica também é influenciada por fatores genéticos (MORMÈDE, 2005; FRIEDRICH et al., 2015), os quais interagem entre si e com o ambiente para a formação e modificação do fenótipo comportamental ao longo da vida dos animais (BURROW, 1997). Em função da complexidade intrínseca ao temperamento dos bovinos há o desafio em se definir qual o melhor método a ser utilizado para gerar informações que possam ser incluídas como critério de seleção em programas de melhoramento genético. Além disso, o que observamos na literatura é a utilização de distintas metodologias para avaliar o temperamento dos bovinos, as quais podem ser classificadas em relação à natureza dos indicadores, assim como proposto por Manteca e Deag (1993) e adaptado por Sant'Anna (2013).

Além das avaliações com base nas respostas comportamentais dos animais, alguns autores sugerem utilizar parâmetros fisiológicos (VON BORELL et al., 2007; SANCHEZ-RODRÍGUEZ et al., 2013) e características morfológicas (LANIER et al., 2001; TÕZSÉR et al., 2003) como indicadores do temperamento. Porém, essas avaliações não são práticas e dificilmente poderão ser utilizadas como critérios de seleção para temperamento. Assim, quando o objetivo é avaliar sistematicamente grande número de animais em programas de melhoramento, utiliza-se indicadores comportamentais, baseando-se no grau de movimentação, postura corporal, velocidade para percorrer determinada distância, reação dos animais após manejo específico, dentre outros.

De acordo com Sant'Anna (2013) os métodos para avaliação do temperamento podem ser classificados em quatro diferentes categorias, sendo elas: testes comportamentais, escores visuais com escala pré-definida, escala de classificação qualitativa do comportamento e métodos automatizados para avaliação do temperamento. Para aplicação dos testes comportamentais submete-se os animais a situações padronizadas, permitindo comparar as reações entre os diferentes indivíduos, com destaque para os testes de distância de fuga (FORDYCE; GODDARD; SEIFERT, 1982) e velocidade de fuga (BURROW; SEIFERT; CORBET, 1988). O teste de velocidade de fuga (ou saída) é uma das metodologias mais conhecidas e utilizadas, tendo sido validada com base em indicadores fisiológicos nas mais diversas situações de manejo e raças bovinas (BURROW, 1997; CURLEY

JR. et al., 2006; MÜLLER; VON KEYSERLINGK, 2006; CAFE et al., 2011a). Por fim, os testes de docilidade (LE NEINDRE et al., 1995; GAULY et al., 2001; PHOCAS et al., 2006), novo objeto e campo aberto (DE PASSILLÉ; RUSHEN; MARTINS, 1995; KILGOUR; MELVILLE; GREENWOOD, 2006; FORKMAN et al., 2007) também são incluídos nessa categoria, porém, apresentam limitações de ordem prática para serem utilizados como critério de seleção, tais como elevado risco de acidente para os avaliadores e tempo despendido para aplicação dos testes.

Os escores visuais com escala de pontuação pré-definida caracterizam-se pela atribuição de notas, que podem variar de 3 a 7 níveis, para quantificar a reação dos animais em determinada situação. Nessa categoria estão o escore de reatividade no tronco de contenção ou na balança, também conhecido internacionalmente como "*crush score*" ou "*chute score*" (GRANDIN, 1993; VOISINET et al., 1997a,b; BURROW e CORBET, 2000; OLMOS; TURNER, 2008; HOPPE et al., 2010, CAFE et al., 2011a,b), e o escore de temperamento em uma das divisórias do curral ou "*pen score*" (BEHRENDS et al., 2009). Esse último, também conhecido como escore de temperamento, tem sido amplamente utilizado como critério de seleção em alguns programas brasileiros de melhoramento genético (SUMÁRIO PAINT® CONSOLIDADO, 2012; SUMÁRIO DE TOUROS CONEXÃO DELTA G, 2012). No entanto, ainda não há padronização em relação à escala de notas adotada entre os diferentes programas. Além disto, não existe consenso em como deve ser considerado o fenótipo, se como critério independente de descarte ou incluído no índice de seleção do programa.

A escala de classificação qualitativa do comportamento é uma avaliação baseada na impressão do observador em relação ao estado de cada animal (MEAGHER, 2009). Nessa categoria destaca-se o método de avaliação qualitativa do comportamento, também conhecido como QBA (*Qualitative Behavior Assessment*), que foi desenvolvido como indicador de bem-estar animal (WEMELSFELDER et al., 2000) e, recentemente, validado como indicador de temperamento em bovinos da raça Nelore (SANT'ANNA; PARANHOS DA COSTA, 2013). Porém, esse método também apresenta limitações de ordem prática e, portanto, possui baixo potencial para ser adotado como critério de seleção. Os métodos automatizados para avaliação do temperamento são aqueles que registram

a reatividade dos bovinos durante o período de contenção previamente determinado, de maneira objetiva e com utilização de sensores, plataformas eletrônicas ou acelerômetros (SCHUMUTZ et al., 2001; MAFFEI et al., 2006; SCHWARTZKOPF-GENSWEIN et al., 2012).

Como revisado por Haskell; Simm; Turner. (2014) o temperamento dos bovinos apresenta variabilidade genética suficiente para ser incluído como critério de seleção em programas de melhoramento, com estimativas de herdabilidade que variam de baixa a moderada magnitude. As diferenças nos resultados encontrados na literatura se devem aos grupos genéticos avaliados (de um modo geral, maiores estimativas são reportadas para raças zebuínas em comparação com raças taurinas) e a características intrínsecas de cada metodologia utilizada para avaliação do temperamento (medidas objetivas têm maiores valores de herdabilidade do que medidas subjetivas). Além disto, os diferentes testes utilizados para avaliar o temperamento dos bovinos abordam aspectos distintos dessa característica (BURROW; DILLON, 1997; CURLEY et al., 2006; KILGOUR; MELVILLE; GREENWOOD, 2006).

Deste modo, podemos concluir que é possível promover progresso genético do temperamento e reduzir, em longo prazo, a reatividade dos animais durante as rotinas de manejo. Porém, por se tratar de uma característica complexa, informações adicionais, tais como as fornecidas pelos estudos de associação com cobertura ampla do genoma (*Genome-Wide Association Study*), são fundamentais para aprofundar nosso conhecimento sobre esse fenótipo e, principalmente, sobre os fatores genéticos subjacentes a expressão do temperamento dos bovinos.

2.3. ABORDAGEM MOLECULAR NO ESTUDO DO TEMPERAMENTO

Nas últimas três décadas diversos estudos foram realizados para investigar as bases genéticas de características relacionadas ao desempenho produtivo dos animais (como revisado por SHARMA et al., 2015). Porém, apenas no final dos anos 90 foi publicado o primeiro estudo objetivando identificar QTL associados a dezessete características não produtivas, que contribuem de maneira geral para a performance econômica de vacas das raças Holandês e Jersey (SPELMAN et al.,

1999). Esses autores avaliaram características relacionadas ao manejo, tamanho e conformação utilizando métodos subjetivos com escala de nove escores, no entanto, não identificaram nenhum QTL significativamente associado ao temperamento e a capacidade de adaptação à ordenha. De maneira semelhante, Schrooten et al. (2000) não identificaram QTL associados ao temperamento de vacas Holandesas, o qual foi avaliado utilizando a mesma metodologia de nove escores pré-definidos.

Por outro lado, Hiendleder et al. (2003) avaliaram o temperamento durante a ordenha, utilizando uma escala com escores que variam de 1 (nervosa / agressiva) a 9 (dócil), e a velocidade de ordenha (quantidade de leite produzido pelo tempo de ordenha) de vacas Holandesas. Nesse estudo foram identificados quatro QTL associados simultaneamente as duas características, os quais estão localizados nos cromossomos 5, 18, 29 e na região pseudoautossômica do cromossomo sexual (X/Y). Próximo ao QTL localizado no cromossomo 29 (20cM) foi mapeado o gene da tirosina (*TYR*), assim os autores concluíram que esse é um potencial gene candidato para temperamento e velocidade de ordenha dada a sua função no metabolismo do neurotransmissor dopamina.

Considerando o comportamento e a docilidade como características que influenciam significativamente a produtividade e a longevidade de vacas da raça Brown Swiss, Kramer et al. (2014) utilizaram painéis de SNP de alta densidade, em um estudo de GWAS para mapear genes que influenciam a expressão das características de temperamento, reatividade na ordenha e agressividade. Os autores identificaram efeito significativo de regiões nos cromossomos 4 (14 Mb), 8 (101 Mb), 14 (55 Mb) e 20 (65 Mb) sobre os fenótipos de interesse. Nestas regiões os genes conhecidos são *TCA1* no Chr4, *AKAP2*, *TXN* e *TXNDC8* no Chr8 e *ADCY2* no Chr20, porém os autores concluíram que nenhum desses genes tem papel biológico com influência descrita sobre a expressão de características comportamentais.

O primeiro estudo para detectar QTL associados ao temperamento de bovinos de corte foi realizado por Schumutz et al. (2001). Esses autores utilizaram uma plataforma eletrônica (*eletronic movement measuring device*) acoplada ao tronco de contenção para avaliar o nível de movimentação durante 60 segundos, em 130 bezerros provenientes de rebanhos canadenses (*Canadian Beef Cattle*

Reference Herd). As avaliações foram realizadas duas vezes durante o período de confinamento (primeira na desmama e a segunda entre 8 a 12 meses), com a finalidade de medir a resposta dos animais ao isolamento durante a rotina de manejo. As reações foram consideradas como reflexo do temperamento de cada animal, bem como a informação sobre a capacidade de habituação às rotinas de manejo (diferença entre a primeira e segunda avaliação). Para isto, os autores genotiparam 162 marcadores microssatélite, identificando sete QTL localizados nos cromossomos 1 (14 cM), 5 (29 cM), 9 (44 cM), 11 (57 cM), 14 (19 e 35 cM) e 15 (12 cM) que foram associados, simultaneamente, aos dois fenótipos de interesse.

Por outro lado, Gutiérrez-Gil et al. (2008) avaliaram bovinos cruzados (Charolês x Holandesa) em duas idades diferentes, utilizando os testes de distância de fuga e de separação social (atribuindo escores para o estado de alerta do animal, vocalização e grau de movimentação quando em isolamento) como indicadores do temperamento e da capacidade de habituação dos animais. Para o estudo de associação foram genotipados 165 marcadores microssatélites, sendo identificados oito QTL associados ao teste de separação social (ao nível de significância de 1%) e um QTL, localizado no cromossomo 29, associado ao teste de distância de fuga (ao nível de significância de 5%). Os autores concluíram que não houve sobreposição de QTL (pleiotropia) para os dois testes, sustentando em nível molecular a hipótese de que distintos métodos de avaliação abordam diferentes aspectos do temperamento dos bovinos, sendo esse fenótipo comportamental influenciado por diferentes fatores genéticos ou regiões genômicas.

De maneira semelhante Glenske et al. (2010 e 2011) avaliaram o temperamento de bovinos das raças Angus e Simental em três diferentes momentos (3 semanas, 5 e 8 meses de vida) com distintos testes para cada idade, atribuindo escores que variaram de 1 (calmo) a 5 (nervoso) para a reação dos bovinos. Foram identificadas deferentes regiões genômicas associadas ao fenótipo avaliado, ressaltando o gene *DRD4* (*dopamine receptor D4 gene*), localizado no cromossomo 29, como potencial candidato para temperamento. Os resultados reportados corroboram Hiendleder et al. (2003), indicando que o neurotransmissor dopamina pode influenciar a expressão do temperamento em bovinos de corte e leite.

Lourenco-Jaramillo et al. (2012) avaliaram a variação genética nos genes da tirosina hidroxilase (*TH*) e da dopamina β-hidroxilase (*DBH*) em bovinos de distintos grupos genéticos, marcadamente diferentes quanto ao seu nível de reatividade. De maneira geral as raças taurinas (Holstein, Charolês e Lidia) foram descritas como menos reativos e a raça zebuína (Brahman) como mais reativa. De acordo com os autores, não foi possível associar qualquer haplótipo a diferenças no temperamento, porém, a diversidade genética observada entre as quatro raças forneceu uma base para investigações em tais associações, sugerindo que o gene *TH* é forte candidato para explicar a variação fenotípica no temperamento dos bovinos.

Até o ano de 2015 apenas dois estudos de GWAS foram conduzidos para mapear as bases genéticas do temperamento de bovinos de corte (HULSMAN HANNA et al., 2014; LINDHOLM-PERRY et al., 2015). No entanto, os estudos utilizaram diferentes metodologias para acessar o fenótipo comportamental. Hulsman Hanna et al. (2014) avaliaram o temperamento de 769 animais cruzados (Nelore x Angus) durante o manejo de desmama, atribuindo escores de nove notas pré-definidas, em que ‘1’ representa animais calmos / dóceis e ‘9’ bezerros de temperamento ruim ou ‘selvagens’, de acordo com as reações de cada animal durante o teste de separação social. Após análise de enriquecimento funcional os autores identificaram que genes relacionados ao transporte do íon sódio, por meio da membrana plasmática dos neurônios (canais transmembrânicos de sódio), podem estar relacionados às diferentes respostas do sistema nervoso frente ao ambiente e a estímulos estressantes.

Por sua vez, Lindholm-Perry et al. (2015) avaliaram o temperamento dos bovinos de diferentes raças (Angus, Hereford, Simmental, Limousin, Charolais, Gelbvieh e Red Angus) utilizando o teste de velocidade de fuga, com o objetivo de identificar marcadores genéticos associados, simultaneamente, à eficiência alimentar (ganho médio diário e consumo médio diário) e a velocidade de fuga. Foram reportados genes localizados nos cromossomos 9 (*QKI - quaking*) e 17 (*GRIA2 - glutamate ionotropic receptor AMPA type subunit 2* e *GLRB - glycine receptor β*) associados a ambas características. Em função de seu papel biológico no sistema nervoso central e na transmissão sináptica, levantou-se a possibilidade destes genes influenciarem a expressão do temperamento na população estudada.

No entanto, após correção de Bonferroni os autores concluíram que os genes não afetam a resposta no teste de velocidade de fuga, dado que nenhum SNP foi significativo a 5% de probabilidade.

Os resultados dos diversos estudos confirmam que o temperamento é uma característica influenciada por distintos genes e regiões genômicas. Porém, além de serem utilizados diferentes desenhos experimentais, número de animais e métodos estatísticos para a identificação dos QTL ou associação com regiões genômicas, o principal problema apontado é a ausência de metodologia padronizada para avaliação e obtenção dos dados de temperamento dos bovinos, permitindo a comparação dos resultados entre as distintas pesquisas. Assim, a definição de um ou mais métodos (indicadores) padronizados poderão contribuir significativamente para aprofundar o conhecimento, em nível molecular, dos fatores genéticos que afetam a expressão dessa característica nas diferentes raças bovinas.

3. CONSIDERAÇÕES FINAIS

Diversos estudos têm focado nas diferenças individuais dos animais, associando o temperamento a importantes aspectos da produção animal, como por exemplo, ao *fitness* de cada indivíduo e a capacidade dos animais em se adaptarem aos diferentes sistemas de criação. Além disto, alguns trabalhos ressaltam a importância econômica do temperamento, como uma característica que pode contribuir para a sustentabilidade da pecuária moderna e promover melhores condições de bem-estar aos animais. Por outro lado, por se tratar de uma característica complexa e multidimensional, entendemos que é fundamental conhecer as bases genéticas subjacentes à sua expressão.

Com esta tese espera-se aprofundar o conhecimento sobre o temperamento dos bovinos, principalmente dos animais da raça Nelore, não apenas por ser uma raça extremamente importante para a pecuária brasileira, mas também por serem animais que possuem temperamento mais reativo, quando comparados com as raças taurinas. Assim, com o presente estudo buscamos identificar regiões genômicas e mapear genes que estejam associados as diferenças individuais na expressão do temperamento, bem como seus produtos gênicos e respectivos papéis

na biologia dos animais, agregando informações que possam ser relevantes para as condições brasileiras de produção animal. Além disso, esperamos que os resultados aqui obtidos possam estimular outros questionamentos e o desenvolvimento de novos estudos de associação genômica ampla para temperamento, colocando o Brasil como referência na busca por produzir produtos com qualidade ética e respeito aos animais.

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CAPÍTULO 2 - Genome-Wide Association Study between Single Nucleotide Polymorphisms and Flight Speed in Nellore Cattle[1]

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1. Abstract

Introduction

Cattle temperament is an important factor that affects the profitability of beef cattle enterprises, due to its relationship with productivity traits, animal welfare and labor safety. Temperament is a complex phenotype often assessed by measuring a series of behavioral traits, which result from the effects of multiple environmental and genetic factors, and their interactions. The aims of this study were to perform a genome-wide association study and detect genomic regions, potential candidate genes and their biological mechanisms underlying temperament, measured by flight speed (FS) test in Nellore cattle.

Materials and Methods

The genome-wide association study (GWAS) was performed using a single-step procedure (ssGBLUP) which combined simultaneously all 16,600 phenotypes from genotyped and non-genotyped animals, full pedigree information of 162,645 animals and 1,384 genotyped animals in one step. The animals were genotyped with High Density Bovine SNP BeadChip which contains 777,962 SNP markers. After quality control (QC) a total of 455,374 SNPs remained.

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Results

Heritability estimated for FS was 0.21 ± 0.02. Consecutive SNPs explaining 1% or more of the total additive genetic variance were considered as windows associated with FS. Nine candidate regions located on eight different *Bos taurus* chromosomes (BTA) (1 at 73 Mb, 2 at 65 Mb, 5 at 22 Mb and 119 Mb, 9 at 98 Mb, 11 at 67 Mb, 15 at 16 Mb, 17 at 63 Kb, and 26 at 47 Mb) were identified. The candidate genes identified in these regions were *NCKAP5* (BTA2), *PARK2* (BTA9), *ANTXR1* (BTA11), *GUCY1A2* (BTA15), *CPE* (BTA17) and *DOCK1* (BTA26). Among these genes *PARK2, GUCY1A2, CPE* and *DOCK1* are related to dopaminergic system, memory formation, biosynthesis of peptide hormone and neurotransmitter and brain development, respectively.

Conclusions

Our findings allowed us to identify nine genomic regions (SNP windows) associated with beef cattle temperament, measured by FS test. Within these windows, six promising candidate genes and their biological functions were identified. These results may contribute to a better comprehension into the genetic control of temperament expression in Nellore cattle.

Keywords

Beef cattle, Behavior, Candidate genes, Cattle temperament

2. Introduction

Cattle temperament is operationally defined as the set of an animal's behaviors in response to handling by humans, attributing these reactions to general aspects of fearfulness [1, 2]. This concept is closely related to individual differences of reactivity in stressful or challenging situations [3, 4]. There is a growing interest on cattle temperament due to its association with productivity traits, animal welfare and labor safety [5, 6], stimulating cattle producers and researchers to look for a better understanding of genetic and environmental factors that influence the expression of temperament traits, and its impacts on cattle performance and corporate profitability.

Flight speed (FS) test is among the most used behavioral methods to assess beef cattle temperament, reflecting aspects of general fear and agitation [2, 7]. This test was validated in many studies, some of them based on physiological stress indicators [8-10], besides being proved that it is reliable and repeatable in both, the short and long term [11, 12]. Additionally, this method has been described as objective, safe and simple to implement routinely on farm, besides the possibility of integrating this measurement in animal breeding programs requiring minimal additional costs [13-15].

Heritability estimates for FS are low to moderate, ranging from 0.17 ± 0.07 to 0.54 ± 0.16 for *Bos taurus* [14, 15] and *Bos indicus* breeds [16-18]. Additionally, a series of studies have shown favorable genetic correlations of FS with important economic traits, e.g. better temperament (lower FS or higher flight time) was associated with higher average daily gain (ADG) [14, 19], better reproductive performance [20, 21], higher carcass weight and yield [22], and improved meat tenderness [23]. Based on these one can assume that the selection for temperament using FS is feasible and will not produce unfavorable responses on overall performance traits.

Given its phenotypic complexity and, probably, polygenic nature, temperament could potentially be studied using genomic approaches, such as genome-wide association studies (GWAS), in order to identify genomic regions or QTL that affect the underlying genetic architecture related to individual variability [24]. In fact, there were some efforts to identify QTL that account for the variation on beef and dairy cattle temperament. These findings led to several candidate genes distributed throughout the chromosomes, which were associated with a variety of temperament indicators in *Bos taurus* cattle and their crosses, such as: milking temperament [25, 26], isolation temperament and habituation [27], docility [28], flight from feeder and social separation [29], tethering test, weighting test, separation and restraint test [30, 31].

Despite of these initiatives, the use of GWAS to identify candidate regions of the genome associated with temperament of Zebu cattle (and others tropically adapted beef breeds) is still scarce in the literature. We found only two published papers using GWAS for beef cattle temperament, one of them aimed to identify

molecular markers associated with temperament at weaning, measured by subjective social separation score in Nellore-Angus crossbred cattle [32]. In the second, a preliminary GWAS study was conducted for FS using BovineSNP50 genotypes in several European crosses (Angus, Hereford, Simmental, Limousin, Charolais, Gelbvieh and Red Angus) [33].

Zebu breeds and their crosses are commonly used for extensive cattle production in warm climates, usually with few contact with humans. Under these conditions, cattle becomes more excitable, impoverishing the animal welfare and increasing risks of labor accidents [5]. Given the importance of assessing cattle temperament and the rapid development of genomic tools, we decided to perform a genome-wide association study aiming to detect genomic regions, potential candidate genes and their biological mechanisms underlying temperament expression (measured by FS test) in Nellore cattle.

3. Materials and Methods

3.1. Animals and management

The Committee of Ethical Use of Animals from the Faculty of Agricultural and Veterinary Sciences, São Paulo State University, Jaboticabal - SP, Brazil (Certified n. 0016/14), approved this research.

Data collection was conducted in a private Nellore herd belonging to Agropecuária Jacarezinho Ltda®, which has two production units located in the southeast and the northeast regions of Brazil. The field study in both production units was permitted by Dr. Ian Hill, the Chief Executive Officer of Agropecuária Jacarezinho Ltda®. The handling procedures were similar in both units. Soon after birth, calves were assigned in handling groups according to the sex, keeping in the same group until weaning (artificially done when the calves were, approximately, 210 days old).

The first performance evaluation is conducted at weaning age, considering weight and visual scores for conformation, finishing precocity and muscling. These data are combined in an index, which is used to define the animals that would stay in

the herd after weaning or not (remaining 50% of males and 90% of females). The selected animals are relocated in new handling groups, remaining in the same group from weaning to yearling (approximately 550 days of age), when a second performance evaluation is conducted. This is done by repeating the previous measurements (weaning and scoring each animal for conformation, finishing precocity and muscling), besides measuring scrotal circumference, scoring temperament and assessing breed characteristics. Based on these measurements obtained at weaning and yearling age a new selection index is calculated, and the results are used to define the animals to be culled (50% of males and 10% of females). Breed characteristics and temperament score are not included in the index, however they are assumed as independent criteria, discarding animals with the worst grade for each trait. The pedigree data set included 162,645 animals with 49,597 dams and 1,306 sires. A summary of the data set is shown in Table 1.

Table 1. Summary of data set structure and descriptive statistics for flight speed (FS) phenotypes, genotyped animals and pedigree using single-step genomic-BLUP methodology in Nellore cattle

Number of animals in the relationship matrix	162,645
Number of sires	1,306
Number of dams	49,597
Number of phenotyped animals	16,660
Number of CG[a]	735
Number of SNPs after quality control	455,374
Number of genotyped animals remaining after quality control	1,384

[a]CG, contemporary group

3.2. Assessment of cattle temperament

Flight speed (FS) test was “devised to measure the time taken for an animal to cover a set distance after leaving a confined area” [17]. In this study the measurements were carried out when each animal left the cattle crush after weighing. The time taken by each animal to cover a known distance (ranging from 1.25 to 3.0 m, according to the facilities design) was recorded with an electronic device, comprising two pairs of photoelectric cells, a stopwatch and a processor. Time and distance were used to calculate FS phenotype, in m/s. The assumption of this test is that higher speed animals have more excitable temperament. The FS

records were taken from 16,660 animals that were born between 2008 and 2012. This test was carried out simultaneously with the performance evaluation at yearling, trying to minimize the interference in the farms handling routines. In the present dataset, FS ranged from 0.08 to 6.67 m/s, with an average (± SD) of 2.35 ± 1.04 m/s.

3.3. Genotype information and quality control

A total of 1,405 phenotyped animals were genotyped with high density bead array technology: Illumina Infinium HD Assay® and Illumina HiScan System®. The High Density Bovine SNP BeadChip contains 777,962 SNP markers spread across the genome at a mean distance of 3.43 kb between markers. Genotyped animals, born between 2008 and 2009, were randomly selected following the criteria of at least 5 animals in each contemporary group. As part of quality control (QC), the following were excluded: SNP markers with unknown genomic position, located on sex chromosomes, monomorphic and markers with minor allele frequency (MAF) below 0.05, call rate below 90% and individuals with call rate below 90%. After QC, a total of 455,374 SNPs and 1,384 genotyped animals remained.

3.4. Statistical analysis

All analyses, to estimate variance components and genetic parameters as well as the genome-wide association study (GWAS), were performed using a single-step procedure (single-step GBLUP - ssGBLUP) which combined, simultaneously, all phenotyped animals, pedigree information and genotypes in one step [34], using Bayesian inference [35] via Gibbs sampling. The animal model for FS included direct additive genetic and residual effects as random, and contemporary groups (CG: farm and year of birth, sex, farm at yearling and management groups at birth, weaning and yearling) as fixed effect. Age of animal at the time of temperament assessment was included as a covariate with linear and quadratic effects. The CG containing less than five animals and records out of the range given by the mean of the CG ± 3 SD were excluded from the analyses. To perform the statistical analyses, the GIBBS2F90 software was used [36] and the model can be represented in matrix form as:

$$\boldsymbol{y} = \boldsymbol{X\beta} + \boldsymbol{Za} + \boldsymbol{e}$$

where is $\boldsymbol{y}$ the vector of FS observations; is $\boldsymbol{\beta}$ the vector of fixed effects; $\boldsymbol{a}$ is the vector of direct additive genetic effects, assuming $\boldsymbol{a} \sim N\,(0, \boldsymbol{H}\sigma_{\mathrm{a}}^2)$, where is $\boldsymbol{H}$ the relationship coefficients matrix among animals and σ_{a}^2 genetic additive variance; $\boldsymbol{X}$ is the corresponding incidence matrix for the fixed effects; $\boldsymbol{Z}$ is the incidence matrix of the random additive direct genetic effects (associates $\boldsymbol{a}$ with vector $\boldsymbol{y}$); $\boldsymbol{e}$ is the vector of the residual effect in which $\boldsymbol{e} \sim N(0, \boldsymbol{I}\sigma_{\boldsymbol{e}}^2)$, where $\boldsymbol{I}$ is the identity matrix and $\sigma_{\boldsymbol{e}}^2$ is the residual variance. The *prior* distribution for genetic and residual variance components was an inverted Wishart and the posterior estimates were obtained using the POSTGSF90 program [37].

The inverse of $\boldsymbol{H}$ matrix was obtained as [38]:

$$\boldsymbol{H}^{-1} = \boldsymbol{A}^{-1} + \begin{bmatrix} 0 & 0 \\ 0 & \boldsymbol{G}^{-1} - \boldsymbol{A}_{22}^{-1} \end{bmatrix}$$

where $\boldsymbol{A}_{22}^{-1}$ is the inverse of numerator relationship matrix for genotyped animals and $\boldsymbol{G}^{-1}$ is the inverse of the genomic relationship matrix. The $\boldsymbol{G}$ matrix was constructed weighting each SNP effect by its expected variance in an iterative procedure, following [39]:

$$\boldsymbol{G} = \boldsymbol{ZDZ'q}$$

In the $\boldsymbol{G}$ matrix, $\boldsymbol{Z}$ is the marker incidence matrix containing genotypes (0, 1, 2 or 5) adjusted for allele frequency, $\boldsymbol{D}$ is a diagonal matrix with the inverse of expected SNP variance (initially $\boldsymbol{D} = \boldsymbol{I}$), and $\boldsymbol{q}$ is a weighting factor. The weighting factor was as in Vitezica et al. [40], ensuring that the average diagonal in $\boldsymbol{G}$ is close to that of $\boldsymbol{A}_{22}$.

Chains of 800,000 iterations were generated and the first 50,000 iterations were discarded. For parameter estimates, samples were kept each 100 cycles, generating chains with 7,950 cycles. Data convergence was checked through graphical analysis, sampled values versus rounds, and using the criteria proposed by

Geweke [41], Heidelberger and Welch [42] and, Raftery and Lewis [43] using the R software, with Bayesian Output Analysis (BOA) package from R 2.9.0 software (The R Development Core Team 2009).

The ssGBLUP method has been used to GWAS [44-46], based on the SNP effects obtained using an iterative process, which increases the weight of SNPs with large effects and reduces the weight of SNPs with small effects as presented by Wang et al. [34]:

1. First step: $\boldsymbol{D} = \boldsymbol{I}$.
2. Calculate $\boldsymbol{G}$ matrix: $\boldsymbol{G} = \boldsymbol{ZDZ'q}$.
3. Calculate GEBV for all animals in the data set using ssGBLUP.
4. Convert GEBV to SNP effects: $\hat{\boldsymbol{u}} = \frac{\sigma_u^2}{\sigma_a^2}\boldsymbol{DZ'G}^{*-1}\hat{\boldsymbol{a}}_g = \boldsymbol{DZ'}[\boldsymbol{ZDZ'}]^{-1}\hat{\boldsymbol{a}}_g$, where $\hat{\boldsymbol{u}}$ is the vector of marker effect and $\hat{\boldsymbol{a}}_g$ is the GEBV of the animals which were also genotyped.
5. Calculate the weight for each SNP marker: $d_i = \hat{\boldsymbol{u}}_i^2 2p_i(1-p_i)$ where *i* is the *i*-th SNP.
6. Normalized SNP weight to remain constant the total genetic variance.
7. Exit or Loop to step 2.

According to Habier et al. [47], the use of SNP windows captures the QTL effect better than a single SNP and is important to discriminate effects from statistical noise [48]. The iterative process was repeated three times, from step 2 to 7, and the percentage (%) of total genetic variance explained by *i*-th consecutive SNPs (SNP window) was calculated following Wang et al. [45]:

$$\frac{var_{(ai)}}{\sigma_a^2} \times 100\% = \frac{var_{\left(\sum_{j=1}^{10} \boldsymbol{z}_j \hat{u}_j\right)}}{\sigma_a^2} \times 100\%,$$

where a_i is genetic value of the *i*-th region that consist of 10 consecutive SNPs, σ_a^2 is the total genetic variance, $\boldsymbol{Z}_j$ is vector of gene content of the *j*-th SNP for all

individuals, and $\widehat{\boldsymbol{u}}_j$ is marker effect of the *i*-th SNP within the *i*-th region. A Manhattan plot was created using the R package “ggplot2”.

3.5. Gene prospection

Consecutive SNPs which explained 1% or more of the total genetic variance were considered as genomic window associated to FS. These regions were used to identify positional candidate genes based on the starting and ending coordinates of each window on *Bos taurus* genome view in the Map Viewer tool available at the National Center for Biotechnology Information (NCBI) platform (http://www.ncbi.nlm.nih.gov) UMD 3.1 version. Functional annotations were obtained using Ensembl Genome Browser (http://www.ensembl.org/index.html) and gene networks were explored by the online Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (http://david.abcc.ncifcrf.gov/). The identification of gene pathways and enrichment analysis (Gene Ontology and KEEG pathways) were performed in DAVID website from the Ensembl annotation. Human genes were used as background in pathway and gene network investigation.

4. Results and Discussion

The convergence criteria used in this study, number of cycles, fixed burn-in period and number of Markov chains were sufficient for convergence of the estimated parameter. Fifty samples were discarded as a fixed burn-in period and the 7,950 interactions remained stable allowing their convergence. Additionally, the convergence was achieved with low SD and a relatively short 95% highest posterior density interval. As a result, the additive genetic (σ^2_{a}) and residual variances (σ^2_{e}), and mean of posterior heritability (h^2) for FS were 0.17, 0.65 and 0.21 ± 0.02, respectively. Fig 1 illustrates graphically the posterior distributions of heritability for FS, showing the highest probability density interval (HPDI) that ranged from 0.15 to 0.25.

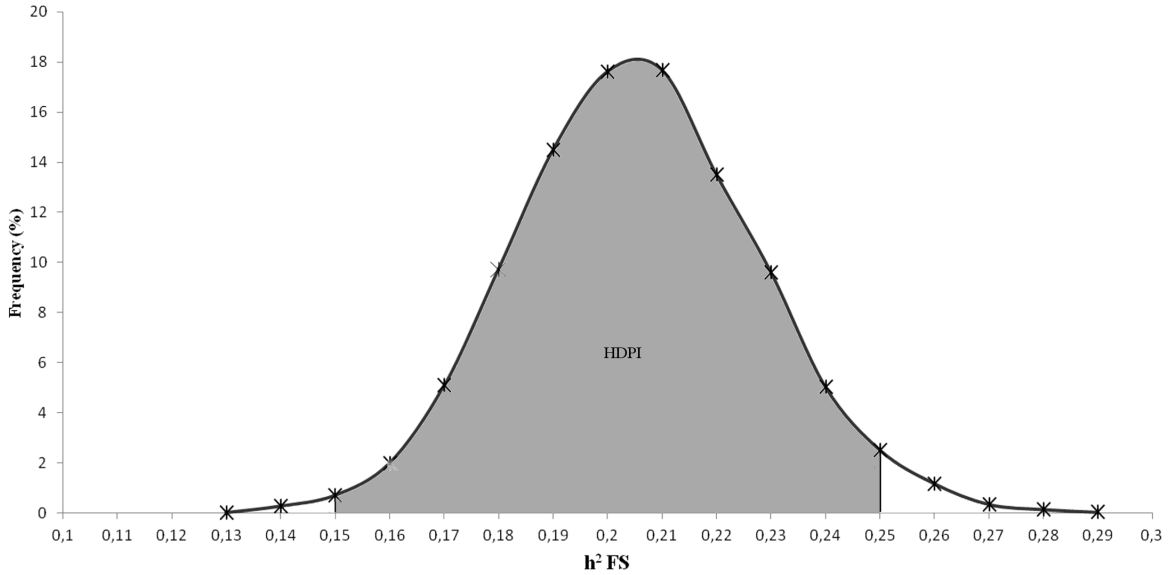

Figure 1. Estimated posterior density of flight speed (FS) heritability in Nellore cattle. The highest probability density interval (HPDI) is presented in gray region.

The heritability estimate (0.21 ± 0.02) is in accordance with the range described in the literature for Nellore and other beef breeds [14, 17, 18, 23] and was similar to that previously presented by our research group using a subset of these data, but not considering genomic information [15, 21]. The additive genetic variability presented by FS indicates the possibility of selecting to improve cattle temperament.

In recent years a large number of potential candidate genes have been associated with several traits of economic importance in farm animals [49, 50]. However, the selection of useful markers for specific traits, such as bovine temperament, should be based on knowledge of the relationship between physiological and/or biochemical process with variation of the phenotypic traits of interest [51]. The additive genetic variance explained by each 10-SNP moving windows is shown in a Manhattan plot (Fig 2). A summary of each SNP window that explained more than 1% of additive genetic variance and the candidate genes is shown in Table 2.

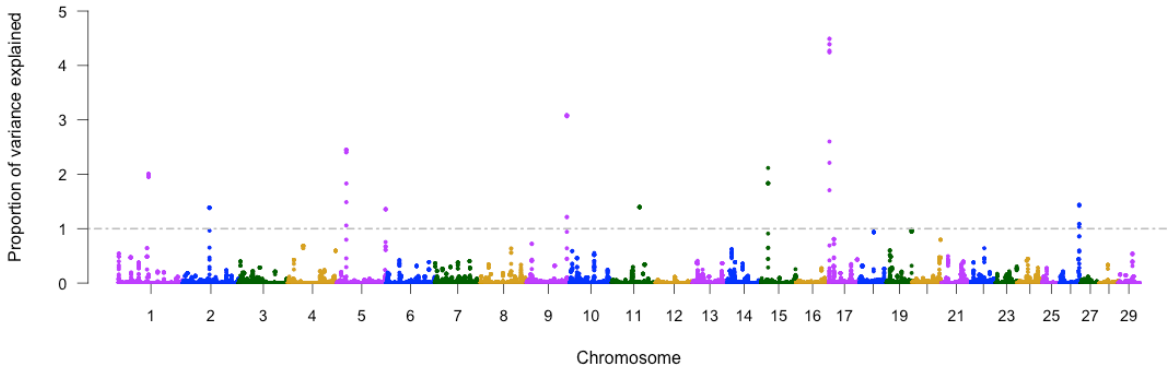

Figure 2. Manhattan plot of additive genetic variance explained by windows of 10 adjacent SNPs for flight speed (FS) in Nellore cattle.

Using the *Bos taurus* genome map, six known genes were found within the significant regions (SNP windows) identified in our study. These were *NCKAP5*, *PARK2*, *ANTXR1*, *GUCY1A2*, *CPE* and *DOCK1* located on the *Bos taurus* chromosomes (BTA) 2, 9, 11, 15, 17 and 26 respectively.

Table 2. Summary of SNP windows that explained >1% of genetic variance for flight speed (FS) in Nellore cattle, with a list of annotated genes within each window.

Chr	SNP window[a]		Var (%)[b]	Gene[c]	Gene ID	Gene Location
	Start, bp	Stop, bp				
1	73354330	73406566	2.00	---	---	---
2	65072013	65082628	1.39	*NCKAP5*	786958	64102635 - 65215462
5	22596661	22604723	2.45	---	---	---
5	119287445	119302785	1.36	---	---	---
9	98759214	98767952	3.08	*PARK2*	530858	98612089 - 99648791
11	67385287	67404876	1.40	*ANTXR1*	616010	67334564 - 67590531
15	16598639	16662233	2.12	*GUCY1A2*	613600	16604381 - 17100655
17	639678	671693	4.49	*CPE*	280753	546402 - 697925
26	47061401	47095621	1.44	*DOCK1*	537203	46735083 - 47296206

[a] *Windows with 10 consecutive SNPs based on UMD3.1.*
[b] *Percentage of additive genetic variance explained by each SNP window.*
[c] *Candidate genes associated to flight speed.*

The *CPE* (carboxypeptidase E) gene encodes a multifunctional peripheral protein that plays several non-enzymatic roles in the endocrine tissues and central nervous systems, with the highest concentration found in the brain and pituitary gland [52, 53]. Moreover, it is a prohormone-processing enzyme, which is involved in the biosynthesis of numerous peptide hormones and neurotransmitters [54, 55], including

the processing of proinsulin to insulin [56]. The enzyme carboxypeptidase E is responsible for cleaving the C-terminal amino acid residues from peptide intermediates in endocrine cells and neuropeptides in peptidergic neurons [57]. This gene is conserved in different species [53] and polymorphisms in specific regions have been associated with diabetes type 2 and obesity in humans [55, 56]; obesity and infertility in mice [53, 58] and in pigs [59]; with carcass and meat quality in different cattle breeds [51, 60]. The *CPE* expression in the brain is directly influenced by distinct physiological or environmental stress conditions to which animals are exposed, resulting in different levels of hippocampal, cortex and amygdala neuronal degeneration and neuronal survival suggesting a role in neuroprotection [61]. Additionally, this gene was linked to deficient learning and memory [62], modulation of mood and emotional responses [57] in mice.

The functions described above for *CPE* support the correlation estimates presented by Hoppe et al. [14], Sant'Anna et al. [15] and Kadel et al. [22] which reported association of FS with productive traits in beef cattle, such as growth and carcass and meat quality. Additionally, these functions corroborate the relationship of temperament with learning, memory and emotional responses in human [63, 64] and cattle [65]. Thus, *CPE* gene is a potential candidate for future studies aiming to identify specific molecular markers associated with different levels of reactivity in cattle.

The *PARK2* gene, located on BTA9 at 98 Mb, encodes the *parkin* protein, an ubiquitin-protein ligase (E3) which belongs to ubiquitin-proteasome system. According to Hase et al. [66] *PARK2* is expressed in bovine peripheral nerves and brain. These authors showed, by immunohistochemical evidence, that *parkin* is located in bovine axoplasm of myelinated nerve fibers and the Schwann cell outer membrane. The *parkin* protein works as important machinery for intracellular quality control [67-69], marking and degrading misfolded proteins. Mutations in *PARK2* gene affect mitochondrial function and apoptosis in neuronal cells [70], and have been linked with Parkinson [71-73], which is a neurodegenerative disease. Damage in the *parkin* protein function is associated with selective degeneration of dopaminergic neurons [66, 68]. The role of dopamine as a neurotransmitter in central nervous system is well known [74], where its action is mediated by specific receptors to

control locomotion, emotional behavior, cognitive functions and memory [75]. Indeed, the dopamine receptor D4 gene (*DRD4*) has been extensively linked to behavior, positive affect and reactivity in mice [76], dogs [77, 78], and horses [79, 80], as well as in humans [81-83] and cattle temperament, measured by a docility test [31].

The *GUCY1A2* gene, located on BTA15 at 16 Mb, encodes an alpha subunit of guanylate cyclase (GC), characterized as guanylate cyclase 1, soluble, alpha 2. The GC is activated by nitric oxide (NO) and catalyzes the conversion of intracellular guanosine-5'-triphosphate (GTP) to cyclic guanosine-3',5'-monophosphate (cGMP), a second messenger in signaling of intracellular transduction pathways [84, 85]. This enzyme has two forms: a membrane protein and a soluble form with specific kinetic properties and tissue distributions [86-88]. The membrane protein consists of a polypeptide chain with a transmembrane receptor which is activated by various endogenous peptides [89] and presents enzymatic activity [84]. The soluble GC (sGC) form is a heterodimeric protein consisting of α (α_1 and α_2,) and β (β_1 and β_2) subunits encoded by distinct genes [90]. The simultaneous expression of one α- with one β-subunit ($\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_1\beta_2$ and $\alpha_2\beta_2$ isoforms) is essential for enzyme catalytic activity [91, 92].

The α_2 subunit, encoded by *GUCY1A2* gene, was firstly identified in human fetal and bovine brain tissues [93]. Subsequently it was isolated in human brain, placenta, spleen, and uterus [94, 95] and in rat brain [96-98]. According to Gibb and Garthwaite [97] and Mergia et al. [98] the highest proportion of α_2 subunit is found in the mice's brain, hippocampus and cerebellum. The relevance of this subunit is related to the binding with NO to catalyze the conversion of GTP in cGMP. The signaling pathways NO/sCG/cGMP acts as a phosphodiesterase, ion-gated channel, and cGMP-dependent protein kinases to regulate vasodilation, smooth muscle relaxation, platelet aggregation and neurotransmission [88, 99-102].

The neuronal NO was associated with memory formation as a retrograde messenger. A disruption in exon two of the NO synthase gene increased the aggressive behavior in mice [103, 104]. Additionally, the increase of cGMP level in the brain, with greatest magnitude in the cerebellum, was associated with the occurrence of locomotor activity and depended on the duration of this behavior [105]. Thus, this candidate gene (*GUCY1A2*) could be related to development of the

neuronal wiring of cattle temperament. Further studies are required to identify the expression of this gene in bovine brain and the molecular basis associated to highest reactivity (locomotor activity) and poor temperament (expressed by high FS) as well as with vasorelaxation and neurotransmission.

Within the SNP window of BTA26 at 47 Mb, was identified the *DOCK1* gene (dedicator of cytokinesis 1), also known as *DOCK180*, which encodes a member of the dedicator of cytokinesis protein superfamily. In mammalian systems this family consists of eleven members named *DOCK1* to *DOCK11* and classified into four subfamilies, according to the sequence homology [106]. The structure of *DOCK1* contains an N-terminal Src homology 3 (SH3) domain, two Dock homology regions (DHR1 and DHR2 module) and a proline-rich C-terminal region. This protein acts as a guanine nucleotide exchange factor (GEF) for the Rho GTPases (a protein family of small guanosine triphosphates), by signaling the exchange of GDP (guanosine diphosphatase) for free GTP (guanosine-5' –triphosphate). The GEF activity requires binding with engulfment and cell motility 1 protein (ELMO1) [107, 108] to form a bipartite complex, which increases stability for *DOCK1* activity toward GTPase Rac1 [109] in several biological processes, such as endothelial apoptosis [108], cytoskeletal organization [110-112], cell migration and invasion [113-115], tumor cell motility and invasion [116, 117], and phagocytosis [118].

Based on the effects of Elmo1/*DOCK180*/Rac1 signaling cascade on cell migration and proliferation some attempts have been made to study the relationship of *DOCK1* with cancer [117, 119, 120]. Additionally, the effects on migration of neurons [121-123] lead to the association of *DOCK1* with brain development, playing a role in the regulation of axon guidance, dendritic spine morphogenesis in hippocampal neurons [122], axon growth [121] and axon tip motility [124]. Further investigations are important to verify the expression of *DOCK1* gene on the cattle neuronal system and synaptic connectivity and neuronal behavior related to differences in temperament.

Within BTA2 window at 65 Mb the positional known gene is *NCKAP5* (Nck – associated protein 5), which is a protein-coding gene interacting with an SH3 (Src homology region 3) domain of the adaptor protein Nck [125]. Initially, this gene was detected in human brain, leukocytes and fetal fibroblasts [125]. In recent years

NCKAP5 has been associated with human attention deficit hyperactivity disorders [126], bipolar disorders [127], schizophrenia and bipolar disorders [128], susceptibility of primary open angle glaucoma in a Japanese population [129] and increased risk of essential hypersomnia [130] by using GWAS. However, the biological function of this gene is still unclear and future research aiming to clarify the functional analysis and validation are recommended.

Considering the expression of this gene in human brain and leukocytes cells, and the strong association with emotional expression disease, our findings lead us to consider an effect of *NCKAP5* variants on temperament. Further studies are required to identify if this gene is expressed in bovine leukocyte cells. Thus, our result may contribute to elucidate the biological function of *NCKAP5*, considering the influence of brain system on temperament traits [131, 132] and the negative correlation between immune system and stable differences on temperament of mice [133], cattle [10, 134] and infant monkeys [135].

Within the SNP window of BTA11 at 67 Mb, is the *ANTXR1* gene, also known as tumor endothelial marker 8 (TEM8) [136]. This gene encodes a type I transmembrane protein that is recognized as anthrax toxin receptor 1 [137, 138]. No research was found reporting the expression of *ANTXR1* gene in cattle, however it is conserved and expressed in different tissues types of mice and humans [137, 139, 140] and dynamically expressed in chick embryogenesis [141]. The encoded transmembrane protein is a specific receptor of anthrax toxin, which is produced by opportunistic bacteria, *Bacillus anthracis* [138, 142, 143].

A nonsense mutation in *ANTXR1* gene has been associated with human recessive GAPO syndrome [144, 145] which is characterized by a loss of function of TEM8, resulting in growth retardation, alopecia, pseudoanodontia and progressive visual impairment [146]. Moreover, some researchers reported the role of *ANTXR1* in tumor vasculature formation, tumor angiogenesis [147, 148] and regulation of endothelial and fibroblastic activities [149]. *ANTXR1* is within the SNP window of BTA11 and was considered as a candidate gene influencing the expression of FS, however, a possible underlying biological relationship with cattle temperament is still unclear.

The identified regions and positional genes in our study, *NCKAP5* (BTA2), *PARK2* (BTA9), *ANTXR1* (BTA11), *GUCY1A2* (BTA15), *CPE* (BTA17) and *DOCK1* (BTA26) have not been reported in Cattle QTL database [150]. Some research has described QTL associated with several temperament traits assessed in different cattle breeds and crosses. However, each test considered distinct aspects of this complex trait and the comparison of results should be done carefully. Schmutz et al. [27] evaluated cattle behavior objectively, using an electronic movement measuring device in response to isolation during handling and habituation to the handling procedure. Considering these behavioral traits as a reflection of cattle temperament, the authors reported six associated QTL on chromosomes 1 (14 cM), 5 (29 cM), 9 (44 cM), 11 (57 cM), 14 (19 and 35 cM) and 15 (12 cM).

On the other hand, Gutierrez-Gil et al. [29] found 29 QTL distributed across 17 chromosomes affecting temperament traits, which were measured by flight from feeder and a social separation test (scoring to standing alert, vocalization and walking/running), in a Charolais × Holstein cattle population. While Hiendleder et al. [26] classifying dairy cows as ‘nervous/aggressive’ or ‘docile’ during milking, and reported QTL associated with milking temperament on BTA 5 (136 cM), 18 (105 cM), 29 (20 cM) and X/Y (9 cM).

Additionally, some candidate genes have been linked to beef cattle temperament as result of GWAS using BovineSNP50 [32, 33]. Hulsman Hanna et al. [32] reported a significant association of temperament at weaning, measured in 769 Nellore-Angus crossbred cattle by subjective social separation score, with genes related to sodium ion transport and activity, particularly voltage-gate channel activity, demonstrating individual variability in nervous system responsiveness. Specifically for FS test, Lindholm-Perry et al. [33] performed a preliminary GWAS to discover genetic markers that were associated simultaneously to the variability of feed efficiency (ADG and average daily feed intake) and temperament using 1,057 beef steers of several European crosses (Angus, Hereford, Simmental, Limousin, Charolais, Gelbvieh and Red Angus). These authors reported positional candidate genes on *Bos taurus* chromosome 9 (*quaking - QKI*) and 17 (*glutamate receptor, ionotropic, AMPA type subunit 2 - GRIA2 and glycine receptor β – GLRB*). Interestingly, the gene on BTA 9

reported by Lindholm-Perry et al. [33] is in close proximity (> 1Mb) with *PARK2* gene identified in our study.

Most of the reported chromosome regions and genes, including those identified in the present study correspond to different genome locations. According to Adamczyk et al. [151] the dispersion of bovine genome regions affecting cattle temperament and behavior occurs in function of different methods to assess these traits and specific characteristics of each breed. Furthermore, the identification of regions and positional candidate genes is more accurate using high density marker panels.

An enrichment analysis was performed based on Gene Ontology and their functional pathways to identify a network among the six genes found in our study. However, no interaction among them was identified, demonstrating that each gene is associated with a different metabolic pathway. Among the genes associated with FS expression, greater attention should be given to *CPE*, *PARK2*, *GUCY1A2* and *DOCK1* due to their biological functions related to dopaminergic system, memory formation, biosynthesis of peptide hormone and neurotransmitter and brain development, respectively. In addition, *CPE* is a promising candidate gene which can influence temperament and weight gain simultaneously in cattle due to its function on proinsulin to insulin conversion and the recognized importance of insulin as hormone which modulates feeding behavior in different species [57, 152, 153].

The next step should be the identification of candidate genes associated with other methods used to assess cattle temperament, highlighting their metabolic pathways and potential interactions. This information will provide new insights about the biological mechanisms underlying the expression of this complex phenotype and better understanding about the genetic basis of cattle temperament. Moreover, further efforts are required to validate our findings in other populations in order to generate a DNA test panel for application in marked-assisted selection of Nellore temperament.

5. Conclusion

The genome-wide association study presented here allowed us to identify nine regions associated with beef cattle temperament, measured by FS test. Among them, six known genes were identified, contributing to a better comprehension into the genetic control of FS expression in Nellore cattle. In addition, the temperament has sufficient additive genetic variability to respond to selection, being of the same magnitude as other traits traditionally used in breeding programs. Thus, information of markers should help animal selection process in order to improve Nellore cattle temperament and, consequently, the efficiency of production systems.

Abbreviations

SNP: Single nucleotide polymorphism; QTL: Quantitative trait loci; FS: Flight speed; GWAS: Genome-wide association studies; QC: quality control.

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CAPÍTULO 3 - Identification of genomic regions associated with behavioral tests traditionally used for cattle temperament evaluation

1. ABSTRACT

Background

Cattle temperament have been considered a functional trait with economic relevance and considerable effect on animal production, reproduction, health and welfare. However, the main challenge to study the genetic background is the definition of standardized tests to measure behavioral phenotypes in different animals, breeds and environmental conditions. Thus, this study was developed aiming to estimate (co)variance components, genetic parameters and to perform a genome-wide association study in order to identify genomic regions, candidate genes and the occurrence of common regions shared by movement score (MOV), tension score (TENS), crush score (CS), flight speed (FS) and temperament score (TS) assessed in Nellore cattle.

Results

Heritability estimates for MOV, TENS, CS, FS and TS were 0.17 ± 0.05, 0.15 ± 0.04, 0.12 ± 0.03, 0.20 ± 0.02 and 0.33 ± 0.03, respectively. The genetic and phenotypic correlations ranged from 0.57 ± 0.06 (MOV x FS) to 0.99 ± 0.00 (TENS x CS) and from 0.23 ± 0.01 (MOV x TS) to 0.93 ± 0.01 (TENS x CS), respectively. A total of 11, 10, 13, 6 and 6 SNP windows explained more than 1% of total genetic variance for MOV, TENS, CS, FS and TS, respectively, and the regions which captured the highest proportion of genetic variance for each temperament trait were on BTA4 at 89 Mb (5.20%), BTA1 at 150 Mb (4.15%), BTA16 at 45 Mb (4.79%), BTA17 at 594 Kb (21.04%) and BTA18 at 36 Mb (2.26%) for MOV, TENS, CS, FS and TS, respectively. Additionally, twenty SNP windows which explained the highest proportion (top 20) for each temperament trait were expanded 1 Mb in each direction, leading to 100 SNP windows and 2,033 candidate genes. Nine known QTL overlapped with ten top 20 SNP windows and the gene network demonstrated strong connections of all known genes with two categories, which match directly with the

target phenotype, such as, behavior and development and functions of nervous system. The metabolic pathways signaled for each temperament trait highlighted the biological mechanisms underlying the expression of temperament in Nellore cattle.

Conclusions

All temperament traits addressed in the present study can be considered in breeding programs aiming to achieve reactivity reduction of Nellore cattle during handling procedures. Genome-wide association analysis revealed genes that may influence the genetic architecture of different temperament traits, which are complex and largely influenced by many regions/genes of small effect. Functional enrichment analyses delivered new insights and pathways that directly match with aspects of emotional behavior.

Keywords:
Crush score, Flight speed, Genome-wide association, Nellore, Reactivity, SNP

2. INTRODUCTION

Genomic research has been developed to identify molecular markers that contribute to natural genetic variation of many phenotypic traits in human and non-human species. In livestock production, these studies focused on a wide range of economically important traits and have successfully detected quantitative trait loci (QTL) for beef and dairy cattle, pig, chicken and sheep [1-4]. For cattle, genome-wide association studies based on high throughput single nucleotide polymorphisms (SNP) have been applied to identify genomic regions and candidate genes that affect quantitative traits, such as production, reproduction, health and resistance to diseases [5, 6].

Similarly, molecular genetics and quantitative trait mapping tools have been applied to increase the comprehension about molecular mechanisms underlying genetic variability of animal behavior [7, 8]. In this way, several studies have focused on temperament of farm animals, which is consistent over time and characterized by

individual variability to cope with environmental challenges [9]. Additionally, the expression of this phenotype is influenced by genetic factors and related to differences on physiological and metabolic profiles [10]. In beef and dairy cattle, the temperament is considered a functional trait with economic relevance [11] and considerable effect on animal production, reproduction, health and welfare [12, 13].

Currently, the main challenge to study the genetic background of cattle temperament is the definition of standardized tests to measure behavioral phenotypes in different animals, breeds and environmental conditions [7]. Indeed, several methodologies were used to access temperament and then identify QTL and candidate genes associated with the target phenotype of beef and dairy cattle, and their crosses [11, 14-16]. For instance, Schmutz et al. [14] accessed the number of movements inside the squeeze chute using an electronic platform. Also working with beef cattle, Glenske et al. [17, 18] used tethering test, weighing test and separation and restraint test at 3 weeks, 5 and 8 months old, respectively, for German Simmental and Angus breeds.

In its turn, Hiendleder et al. [15] measured milking temperament ascribing scores from 1 (‘nervous / aggressive’) to 9 (‘docile’) for Holstein cows during milking. Similarly, Kramer et al. [19] used overall temperament and aggressiveness during milking for Brow Swiss dairy cattle. Furthermore, working with Charolais x Holstein crosses, Gutiérrez-Gil et al. [11] measured the temperament using flight from feeder and social separation tests. A similar social separation test within a pen was conducted in Nellore x Angus beef cattle [20] and, recently, the flight speed test was used to measure the temperament of European cross cattle [21].

In all those studies, several QTL and genes were associated with distinct temperament traits. However, given the complexity nature of behavior and the lack of standardized single methodologies, the comparison of results from different studies is limited and have to be done carefully. Besides, the use of single test should improve the genetic progress for temperament in distinct cattle breeds. Thus, the first aim of this study was to estimate (co)variance components and genetic parameters for five temperament traits of Nellore cattle combining, simultaneously, information of phenotype, genotype and pedigree in Bayesian analysis. The second objective was to perform a genome-wide association study in order to identify genomic regions,

candidate genes and the occurrence of common regions shared by movement score (MOV), tension score (TENS), crush score (CS), flight speed (FS) and temperament score (TS). Knowledge of gene networks and metabolic pathways will provide new insights for biological mechanisms underlying the expression of temperament traits in Nellore cattle.

3. METHODS

The Committee of Ethical Use of Animals from the Faculty of Agricultural and Veterinary Sciences, São Paulo State University (UNESP), Jaboticabal - SP, Brazil (Certified n. 0016/14), approved this research.

3.1. Animals and phenotype

This study was conducted in partnership with two private farms (Agropecuária Jacarezinho Ltda® and Fazenda São Marcelo®) which are specialized in beef cattle husbandry in extensive system, focusing on genetic selection of Nellore cattle under tropical conditions. The handling procedures were similar in both farms and, soon after birth, the calves were separated by sex to different handling groups. At weaning (approximately 210 days of age) visual scores (conformation, finishing precocity and muscling) and weighing were performed. These records were used in a selection index for retention in the herd, excluding 50 % of males and 10 % of females. Selected animals were relocated to new handling groups, where they remained until the yearling age (approximately 550 days). At yearling a second performance evaluation was conducted including visual scores, weight, scrotal circumference, height, breed characteristics and temperament. Based on this information a new selection index was calculated. The method of independent culling levels of selection was applied for breed characteristics and temperament, culling animals with the worst grades.

Simultaneously to the handling procedure at yearling five different tests were used to evaluate cattle temperament, from 2010 to 2015: movement score (MOV), tension score (TENS), crush score (CS), flight speed (FS) and temperament score (TS). The full description of these tests are shown in Table 1.

Table 1. Description of each method used to assess cattle temperament.

Trait	Description	Scale
Movement score (MOV)	Assessed the movement of the animals inside the squeeze chute (crush) for 4 s, just after its entrance (adapted from Grandin [22]).	1 - no movement; 2 - little movement, during less than half of the observation time; 3 - frequent movements (during half of the observation time or more), but not vigorous; 4 - constant and vigorous movements; 5 - constant and vigorous movements, animal jumps and raises its forelimbs off the ground.
Tension score (TENS)	Assessed the cattle overall body tension, and head, ear and tail movements scoring.	1 - the animal did not exhibit sudden movements of the tail, head, and neck, no muscle tremors, and white of eye was not visible; 2 - the animal exhibited few sudden movements of the tail, head, and neck, no muscle tremors, and white of eye was visible or not; 3 - the animal exhibited continuous and vigorous movements of the tail, head and neck, white of eye was visible, no muscle tremors; 4 - the animal appeared paralyzed or "freezing" reaction, muscle tremors were visible.
Crush Score (CS)	Assessed the cattle overall reactivity inside the squeeze chute (described by [23] and adapted from [24]).	1 - the animal does not offer resistance, remains with head, ears and tail relaxed; 2 - some movement, with head and ears rising up; 3 - frequent movement but not vigorous, head, ear and tail movements, sclera of the eye (eye white) may be visible; 4 - offers great resistance, abrupt and vigorous movements of the whole animal as well as the head, ear and tail, sclera of the eye visible, audible breathing and may jump or fall; 5 - the animal offers or not great resistance, sclera of the eye is always visible and has a 'freezing' reaction.
Flight speed (FS)	Measured the speed at which the animal leaves the crush after being weighed [25].	This measurement was taken using an electronic device that records the time (s) taken by each animal to cover a known distance (which ranged from 1.6 to 2.0 m, depending on the facilities), later converted to speed (m/s). The faster animals were considered as presenting more excitable temperament.
Temperament score (TS)	Assessed the reaction of the animals in a pen of the corral. To avoid the tendency of the evaluators to concentrate the grades in the intermediary level (TS = 3), the intermediate grade was removed from the scale.	1 - the animal walks slowly, allowing close proximity to the observer; 2 - trots or runs for a few seconds, allowing a moderate proximity to the observer; 4 - runs during the entire observation time, looking for an escape with constant movement of the tail, and does not allow close or moderate proximity; 5 - runs during the entire time of the assessment, jumps against fences and obstacles, and tries to attack the observer.

These tests are able to measure an overall aspect of cattle temperament [26] and a subset of the data set obtained from Agropecuária Jacarezinho Ltda® have been analyzed previously aiming to estimate the genetic and phenotypic parameters and correlations with productive and reproductive traits [23, 27-29]. Furthermore, we used a subset of this database for the first genome-wide association study of flight speed in Nellore cattle [30].

3.2. Genotypic data

A total of 3,568 animals born between 2007 and 2012 were used in the present study, being 2,280 and 1,288 samples genotyped with Illumina BovineHD and Bovine50K BeadChip Array, respectively. Genotyped animals with Bovine50K were imputed to BovineHD using the software FImpute [31]. The quality control (QC) of genotypes were performed to exclude SNP markers with unknown genomic position, located on sex chromosomes, call rate below 90%, monomorphic and markers with minor allele frequency (MAF) below 0.05. Samples with call rate below 90% were also excluded. After QC, a total of 411,677 SNPs spread across 29 *Bos taurus* autosomes chromosomes and 3,495 genotyped animals were retained for further analysis.

3.3. Statistical analysis

All analysis, to estimate (co)variance components and genetic parameters as well as the genome-wide association study (GWAS), were performed using a single-step procedure (single-step genomic BLUP - ssGBLUP) which combined, simultaneously, pedigree information, all phenotyped and genotyped animals in one step [32], using Bayesian inference [33] via Gibbs sampling. To illustrate in more detail, Table 2 shows the number of phenotyped and genotyped animals for each test used to access cattle temperament during the handling procedure at yearling. The associated pedigree data set included 239,220 animals with 75,474 dams and 1,735 sires.

Table 2. Descriptive statistics for movement score (MOV), tension score (TENS), crush score (CS), flight speed (FS) and temperament score (TS) of animals with phenotype and genotype information.

Traits	N[1]	Mean ± SD	Median	CG[2]
MOV	22,189 (1,890)	-	2	971
TENS	22,173 (1,890)	-	2	968
CS	22,160 (1,886)	-	3	966
FS (m/s)	22,045 (1,867)	2.25 ± 1.02	-	976
TS	45,010 (2,122)	-	2	1,917

[1]*Number of phenotyped (and genotyped) animals*
[2]*Number of contemporary groups (CG)*

3.3.1. (Co)variance components

The (co)variances components and genetic parameters estimation were performed applying a single- and bi-trait animal model, respectively. A linear animal model was used for FS and a threshold model when MOV, TENS, CS and TS were incorporated. The Bayesian threshold model have been considered the most suitable method for genetic analysis of categorical traits, based on the assumption that the levels of categorical variables are related to an underlying continuous scale containing the fixed and random effects [34].

For all temperament traits, the model included direct additive genetic and residual effects as random effects and contemporary groups (GC), which was a concatenation of farm and year of birth, sex, farm of yearling and management groups at birth, weaning and yearling, as fixed effect. Age of animal at the time of temperament assessment was included as a covariate with linear and quadratic effects. Only CG which contained less than five animals and records out of the range given by the mean of the CG $\pm$ 3 SD were excluded from the statistical analysis. The distribution for MOV, TENS, CS, FS and TS is presented in Figure 1.

The THRGIBBS1F90 and GIBBS2F90 software were used to perform quantitative analyses [35, 36] and the model can be represented in matrix form as:

$$\boldsymbol{y} = \boldsymbol{X\beta} + \boldsymbol{Za} + \boldsymbol{e}$$

where*:* $\mathbf{y}$ is the vector of temperament observations; $\boldsymbol{\beta}$ is the vector of fixed effects; $\boldsymbol{a}$ is the vector of direct additive genetic effects, assuming $\boldsymbol{a} \sim N\,(0, \boldsymbol{H}\sigma_{\mathrm{a}}^2)$, where is $\boldsymbol{H}$ the relationship coefficients matrix that combines all animals in the pedigree and

genomic matrix and σ_a^2 genetic additive variance; $\boldsymbol{X}$ is the corresponding incidence matrix for the fixed effects; $\boldsymbol{Z}$ is the incidence matrix of the random additive direct genetic effect (associates $\boldsymbol{a}$ with vector $\boldsymbol{y}$); $\boldsymbol{e}$ is the vector of the residual effect in which $\boldsymbol{e} \sim N(0, \boldsymbol{I}\sigma_e^2)$, where $\boldsymbol{I}$ is the identity matrix and σ_e^2 is the residual variance. The *prior* distribution for genetic and residual variance components was an inverted Wishart and the posterior estimates were obtained by using the POSTGSF90 program [36].

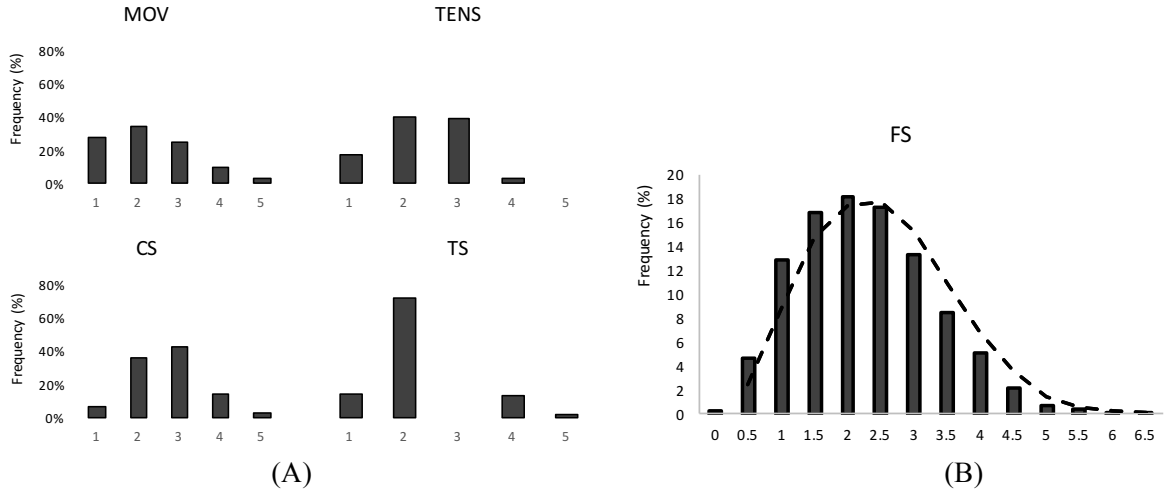


Figure 1. A) Observed frequencies for MOV = movement score; TENS = tension score; CS = crush score and; TS = temperament score. B) Distribution and trend line for FS = flight speed.

The inverse of $\boldsymbol{H}$ matrix was obtained as [37]:

$$\boldsymbol{H}^{-1} = \boldsymbol{A}^{-1} + \begin{bmatrix} 0 & 0 \\ 0 & \boldsymbol{G}^{-1} - \boldsymbol{A}_{22}^{-1} \end{bmatrix},$$

where $\boldsymbol{A}_{22}^{-1}$ is the inverse of numerator relationship matrix for genotyped animals and $\boldsymbol{G}^{-1}$ is the inverse of the genomic relationship matrix. The $\boldsymbol{G}$ matrix was constructed weighting each SNP effect by its expected variance in an iterative procedure, following [38]:

$$\boldsymbol{G} = \boldsymbol{ZDZ'q}$$

In the $\boldsymbol{G}$ matrix, $\boldsymbol{Z}$ is the marker incidence matrix containing genotypes (0, 1, 2 or 5) adjusted for allele frequency, $\boldsymbol{D}$ is a diagonal matrix with the inverse of expected SNP variance (initially $\boldsymbol{D} = \boldsymbol{I}$), and $\boldsymbol{q}$ is a weighting factor. The weighting factors were derived as in Vitezica et al. [39], by ensuring that the average diagonal in $\boldsymbol{G}$ is close to that of $\boldsymbol{A}_{22}$. To calculate the $\boldsymbol{G}$ and $\boldsymbol{H}$ matrix was used PREGSF90 software [36, 37], which provides, as default, the quality control presented above.

In the threshold model was assumed that the underlying scale to the categorical variables has a continuous normal distribution: $\boldsymbol{U} \mid \boldsymbol{\theta} \sim \boldsymbol{N}\,(\boldsymbol{W}\,\boldsymbol{\theta}, \boldsymbol{I}\boldsymbol{\sigma}_e^2)$, where: $\boldsymbol{U}$ is the vector of the underlying scale of order $\boldsymbol{r}$; $\boldsymbol{\theta} = (\boldsymbol{\beta}, \boldsymbol{\alpha})$ is the vector of location parameters of order $\boldsymbol{s}$ with $\boldsymbol{\beta}$ (as fixed effects) and order $\boldsymbol{s}$ with $\boldsymbol{\alpha}$ (as direct additive genetic effects); $\boldsymbol{W}$ is the incidence matrix known of order $\boldsymbol{r}$ by $\boldsymbol{s}$; $\boldsymbol{I}$ is the identity matrix of order $\boldsymbol{r}$ by $\boldsymbol{r}$; and $\boldsymbol{\sigma}_e^2$= 1, which aims at obtaining the identifiability in the likelihood function.

The Gibbs sampling analyses were implemented across a single chain of 800,000 iterations. The sampling interval was 100 iterations, generating chain of 8,000 samples. For parameter estimates and to compute the marginal posterior distributions the first 200 samples were discarded as fixed burn-in, remaining 7,800. Data convergence was checked through graphical analysis, sampled values versus rounds, and using the criteria proposed by Geweke [40], Heidelberger and Welch [41] and Raftery and Lewis [42] using the Bayesian Output Analysis (BOA) package from R 2.9.0 software (The R Development Core Team 2009). For each temperament trait, the mean of additive genetic and residual variance, as well as median, minimum, maximum and highest posterior density interval (HPD, containing 95% of the posterior density) were calculated based on single-trait analyses. On the other hand, the mean, standard deviation (SD), Monte Carlo error and HPD were calculated for each genetic and phenotypic correlation estimated by using a bi-trait analyses.

3.3.2. Genome-wide association analysis

The genome-wide association study (GWAS) was performed using the ssGBLUP given the advantage to work with a data set containing many phenotypes

and a few number of genotypes [43], a simple, fast and accurate methodology [37] for GWAS. The analyses were performed to calculate and, subsequently, convert the genomic estimated breeding values (GEBV) of genotyped animals to percentage of total genetic variance explained by each SNP window (SNP marker effect), which contained 10 consecutive SNPs. In order to increase the effect of important SNP windows and reduce toward zero those with low effect, the iterative process was adapted from Wang et al. [32]:

1. First step: $\boldsymbol{D} = \boldsymbol{I}$;
2. Calculate $\boldsymbol{G}$ matrix: $\boldsymbol{G} = \boldsymbol{ZDZ'q}$.
3. Calculate GEBV for all animals in the data set using ssGBLUP;
4. Convert GEBV to SNP effects: $\widehat{\boldsymbol{u}} = \frac{\sigma_u^2}{\sigma_a^2}\boldsymbol{DZ'G}^{*-1}\widehat{\boldsymbol{a}}_g = \boldsymbol{DZ'}[\boldsymbol{ZDZ'}]^{-1}\widehat{\boldsymbol{a}}_g$, where $\widehat{\boldsymbol{u}}$ is the vector of marker effect and $\widehat{\boldsymbol{a}}_g$ is the GEBV of the animals which were also genotyped;
5. Calculate the weight for each SNP marker: $d_i = \widehat{\boldsymbol{u}}_i^2 2p_i(1-p_i)$ where *i* is the *i*-th SNP;
6. Normalize SNP weight to remain constant the total genetic variance;
7. Exit or Loop to step 2.

The iterative process was repeated three times, from step 2 to 7, and the percentage of total genetic variance (%GV) explained by *i*-th consecutive SNPs was calculated following Wang et al. [43]:

$$\frac{Var(a_i)}{\sigma_a^2} \times 100\% = \frac{Var\left(\sum_{j=1}^{10} \boldsymbol{Z}_j \widehat{\boldsymbol{u}}_j\right)}{\sigma_a^2} \times 100\%,$$

where: a_i is genetic value of the *i*-th region that consist of 10 consecutive SNPs, σ_a^2 is the total genetic variance, $\boldsymbol{Z}_j$ is vector of gene content of the *j*-th SNP for all individuals, and $\widehat{\boldsymbol{u}}_j$ is marker effect of the *i*-th SNP within the *i*-th region. The GEBV was converted to SNP windows effects by using the POSTGSF90 software [36] and Manhattan plots were created using the R package *qqman*_0.1.2.

3.4. Post-GWAS in-silico analysis

The top 20 segments of 10 consecutive SNPs, which captured the highest percentage of total genetic variance for each temperament trait (MOV, TENS, CS, FS and TS) were obtained from the third iteration of ssGBLUP and considered as genomic regions associated with the target phenotype. Moreover, all top 20 SNP windows were expanded 1 Mb in each direction, aiming to increase the size of each SNP window and to map genes that could be physically close to regions considered as influencing the expression of temperament traits in Nellore cattle. The candidate gene identification of all SNP windows were obtained from *Bos taurus* genome (UMD 3.1.1 [44]) using the Map Viewer tool, available at the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov).

Metabolic pathways and gene ontology enrichment analysis (for genes located within the expanded top 20 SNP windows) were carried to aid interpretation of GWAS results. It was explored using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (http://david.abcc.ncifcrf.gov/), considering *Bos taurus* genes as background, and the online software Ingenuity Pathway Analysis (IPA) (http://www.ingenuity.com/products/ipa). For IPA, human genes were used as background in pathway and gene network investigation. The QTL mapping was realized in order to identify known QTL in cattle QTLdb database [45], that were described previously and reported for temperament or behavior traits. A total of 100 SNP windows that represents the top 20 associated with all temperament traits were consulted using the UMD 3.1 bovine genome assembly [44] as the reference to build these analyses.

4. RESULTS AND DISCUSSION

4.1. (Co)variances

The convergence criteria for all single- and bi-trait analyses were in accordance with the threshold of Geweke test ($p > 0.05$) and has been successfully achieved with the number of samples and Markov chain used. Moreover, the estimated parameters presented low SD and relatively short HPD. The additive

genetic (σ^2_a) and residual variances (σ^2_e), and posterior heritability (h^2) estimated by single-trait analyses using ssGBLUP for temperament traits (MOV, TENS, CS, FS and TS) are presented in Table 3. Heritability estimates ranged from 0.12 ± 0.03 to 0.33 ± 0.03 and are in agreement with those values previously reported by our research group for MOV, CS, FS and TS [22-25] using three-trait analyses by Bayesian inference. Additionally, the obtained results are in the same magnitude found in the literature for *Bos taurus*, *Bos indicus* and their crosses which ranged from low to moderate [46-49], suggesting that genetic additive effect to phenotypic variation is, in general, low. In spite of this, we suggest that direct selection for temperament traits should be considered in Nellore breeding programs, aiming to reduce animal reactivity during handling procedures.

Table 3. Posterior estimates of additive genetic variance (σ^2_a), residual variance (σ^2_e) and heritability (h^2) for temperament traits using ssGBLUP.

Trait		Mean ± SD	Median	Minimum	Maximum	95% HPD
MOV	σ^2_a	0.11 ± 0.02	0.11	0.07	0.19	0.08 to 0.14
	σ^2_e	0.59 ± 0.23	0.54	0.28	3.30	0.30 to 1.04
	h^2	0.17 ± 0.05	0.17	0.04	0.33	0.08 to 0.26
TENS	σ^2_a	0.05 ± 0.01	0.05	0.03	0.10	0.04 to 0.07
	σ^2_e	0.31 ± 0.08	0.29	0.17	0.95	0.19 to 0.47
	h^2	0.15 ± 0.04	0.15	0.05	0.34	0.09 to 0.22
CS	σ^2_a	0.02 ± 0.00	0.02	0.01	0.03	0.01 to 0.02
	σ^2_e	0.12 ± 0.03	0.12	0.07	0.55	0.08 to 0.19
	h^2	0.12 ± 0.03	0.12	0.03	0.24	0.07 to 0.18
FS	σ^2_a	0.16 ± 0.02	0.16	0.10	0.21	0.13 to 0.19
	σ^2_e	0.62 ± 0.01	0.62	0.56	0.68	0.59 to 0.65
	h^2	0.20 ± 0.02	0.20	0.14	0.27	0.17 to 0.24
TS	σ^2_a	0.05 ± 0.00	0.05	0.04	0.07	0.04 to 0.06
	σ^2_e	0.11 ± 0.01	0.11	0.08	0.16	0.09 to 0.13
	h^2	0.33 ± 0.03	0.33	0.23	0.44	0.28 to 0.39

SD = standard deviation; MOV = movement score; TENS = tension score; CS = crush score; FS = flight speed; TS = temperament score; HPD = highest posterior density interval containing 95% of the observations.

The posterior means of genetic and phenotypic correlations were estimated using bi-trait analyses (Table 4). In the current study, mean (± SD) values ranged from 0.57 ± 0.06 (MOV x FS) to 0.99 ± 0.00 (TENS x CS) and from 0.23 ± 0.01 (MOV x TS) to 0.93 ± 0.01 (TENS x CS) for genetic and phenotypic correlations, respectively. The present results are in agreement with those presented by

Sant'Anna et al. [23] considering only information of numerator relationship (pedigree) matrix to estimate the association between MOV, CS, FS and TS, which ranged from 0.76 ± 0.09 (MOV x TS) to 0.99 ± 0.02 (MOV x CS). Similarly, other studies were developed aiming to estimate genetic and phenotypic correlations between different tests used to measure cattle temperament of *Bos indicus*, *Bos taurus* and their crosses [46, 50-52].

The present results indicate that the tests used to access temperament inside the squeeze chute are highly associated, corroborating those reported by Benhajali et al. [50] and Schwartzkopf-Genswein et al. [51]. Thus, the use of only one indicator should be reasonable to capture the genetic variability of temperament in this particular test condition. On the other hand, the genetic correlation between FS and CS reported in the literature were low to moderate [46, 52], indicating that these tests are measuring different aspects of cattle temperament. Moreover, based on the correlation between restrained (MOV, TENS and CS) and non-restrained tests (FS and TS), we can consider that the use of both categories can access different genetic backgrounds of this complex phenotype, which is expressed in distinguished challenge situations.

Table 4. Posterior estimates (mean ± standard deviation) of genetic (above the diagonal) and phenotypic (below the diagonal) correlations between temperament traits. Highest probability density intervals containing 95% of the observations are presented within parentheses.

	MOV	TENS	CS	FS	TS
MOV	-	0.93 ± 0.03 (0.88 *to* 0.98)	0.91 ± 0.04 (0.83 *to* 0.98)	0.57 ± 0.06 (0.45 *to* 0.70)	0.68 ± 0.07 (0.53 *to* 0.81)
TENS	0.63 ± 0.02 (0.60 *to* 0.67)	-	0.99 ± 0.00 (0.99 *to* 1.00)	0.66 ± 0.07 (0.52 *to* 0.78)	0.69 ± 0.07 (0.55 *to* 0.81)
CS	0.67 ± 0.02 (0.62 *to* 0.70)	0.93 ± 0.01 (0.91 *to* 0.96)	-	0.63 ± 0.06 (0.50 *to* 0.75)	0.75 ± 0.06 (0.63 *to* 0.88)
FS	0.24 ± 0.01 (0.22 *to* 0.25)	0.25 ± 0.01 (0.23 *to* 0.26)	0.24 ± 0.01 (0.23 *to* 0.26)	-	0.73 ± 0.05 (0.64 *to* 0.82)
TS	0.23 ± 0.01 (0.21 *to* 0.25)	0.25 ± 0.01 (0.23 *to* 0.27)	0.28 ± 0.01 (0.26 *to* 0.30)	0.38 ± 0.01 (0.36 *to* 0.40)	-

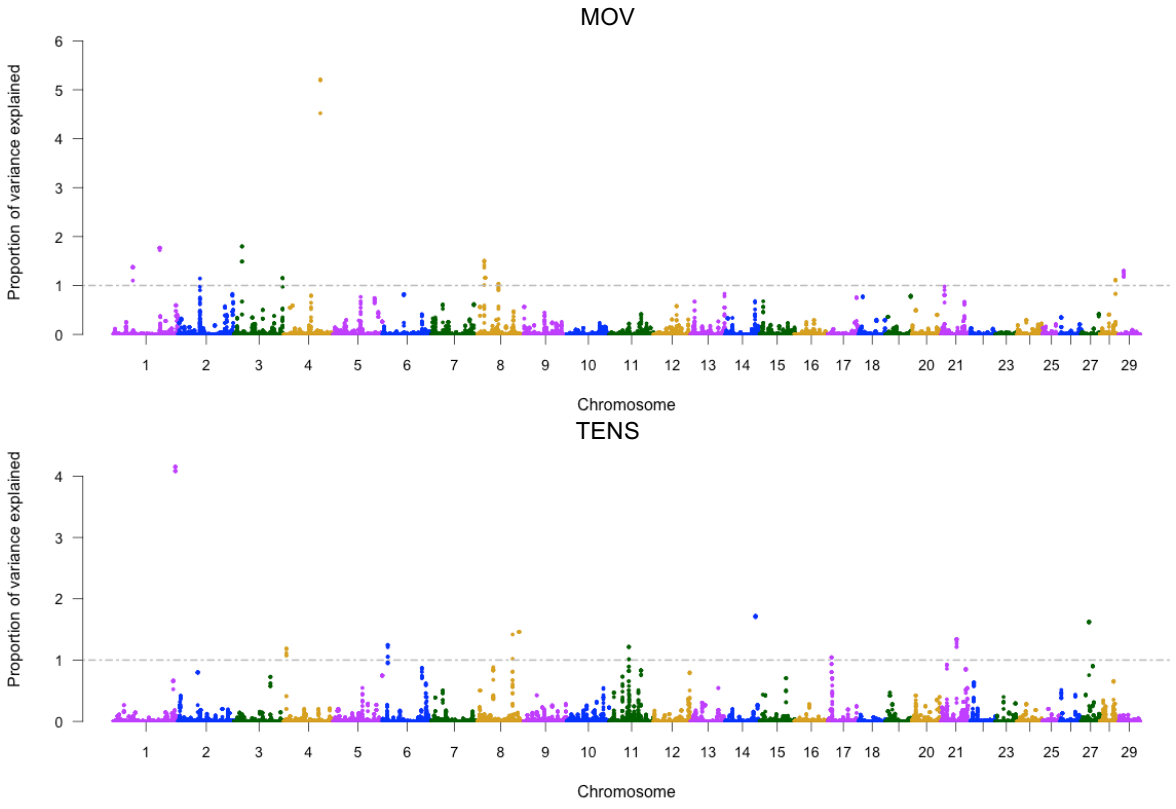
MOV = movement score; TENS = tension score; CS = crush score; FS = flight speed; TS = temperament score.

4.2. Genome-wide association study

We carried out a genome-wide association analysis aiming to identify genomic regions and candidate genes that affects the expression of temperament in Nellore

cattle. Despite being the most used breed in Brazilian farms, Nellore animals have been recognized as having worst temperament when compared with *Bos taurus* cattle [53, 54]. This is represented by the high level of reactivity during handling, which leads to a poor popular image of this breed regarding their temperament. Thus, the use of genomic approach will improve knowledge about genes influencing individual variability of beef cattle temperament.

A total of 411,677 SNPs spread across 29 *Bos taurus* autosomes chromosomes remained after quality control and were used for ssGBLUP analyses. The percentage of total additive genetic variance explained by each 10 consecutive SNPs, which correspond to the effect of these regions on the expression of the target phenotype, are shown in a Manhattan plot for each temperament trait (Figure 2). For all five tests (MOV, TENS, CS, FS and TS) were found 46 genomic regions, which explained more than 1% of total genetic variance. Additionally, were evaluated the top 20, which comprised twenty regions expanded 1 Mb in each direction, explaining the highest percentage of genetic variance. Thus, were analyzed 100 SNP windows, leading to 2,033 candidate genes (including *LOC* genes) according to NCBI Map Viewer.



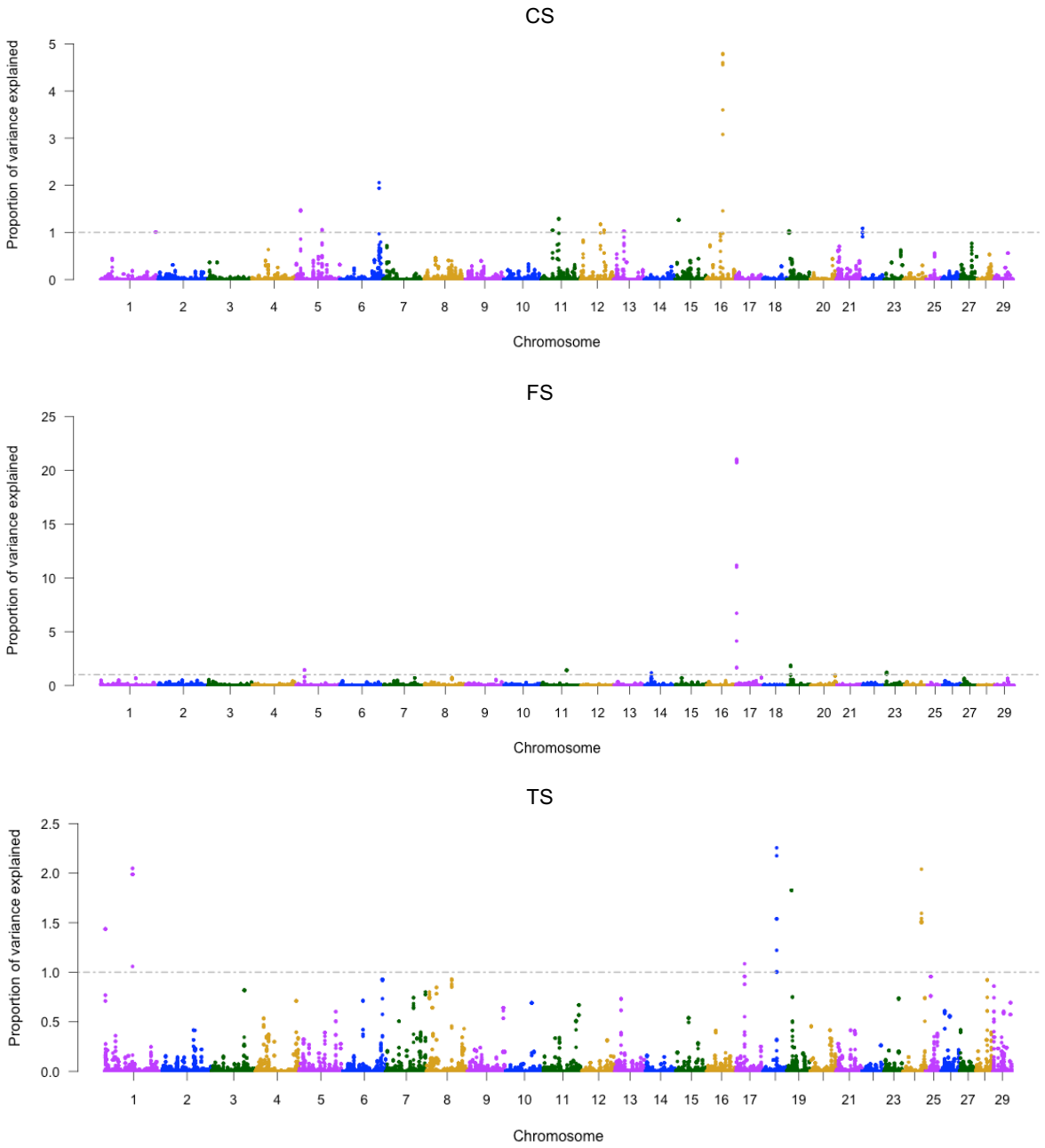


Figure 2. Manhattan plots of additive genetic variance explained by windows of 10 consecutive SNPs for movement score (MOV), tension score (TENS), crush score (CS), flight speed (FS) and temperament score (TS) in Nellore cattle.

A total of 11, 10, 13, 6 and 6 SNP windows explained more than 1% of total genetic variance for MOV, TENS, CS, FS and TS, respectively (Table 5). The most promising regions which captured the highest proportion of genetic variance for each temperament trait were on BTA4 at 89 Mb (5.20%), BTA1 at 150 Mb (4.15%), BTA16

at 45 Mb (4.79%), BTA17 at 594 Kb (21.04%) and BTA18 at 36 Mb (2.26%) for MOV, TENS, CS, FS and TS, respectively.

Table 5. Summary of SNP windows explaining more than 1% of total genetic variance (%GV) to each temperament trait.

Traits[1]	BTA	SNP Window (bp)	1st SNP	Last SNP	N[2]	%GV
MOV	1	47,008,936 – 47,050,240	rs134389342[A/C]	rs13530604[A/G]	18	1.37
	1	112,844,099 – 112,865,193	rs136122124[A/G]	rs43257268[A/G]	20	1.77
	2	52,975,117 – 52,995,056	rs42399483[A/G]	rs133446462[A/G]	12	1.14
	3	18,902,189 – 18,964,167	rs137381568[A/G]	rs134565813[A/G]	30	1.80
	3	118,230,562 – 118,258,824	rs136836342[A/G]	rs135307987[A/G]	30	1.15
	4	89,201,638 – 89,259,234	rs110084128[C/T]	rs133191793[C/T]	18	5.20
	8	16,295,352 – 16,329,935	rs137274562[C/T]	rs132667398[C/T]	22	1.51
	8	18,559,467 – 18,611,873	rs109463096[C/T]	rs109281166[A/C]	7	1.16
	8	51,117,549 – 51,130,704	rs43556216[A/G]	rs133808750[A/G]	9	1.03
	28	38,324,029 – 38,346,163	rs110853830 [C/T]	rs109652029 [A/G]	6	1.11
	29	12,425,861 – 12,450,050	rs42173015 [A/G]	rs133816711 [C/T]	21	1.30
TENS	1	150,902,701 – 150,918,711	rs137712001[C/T]	rs136578098[A/G]	30	4.15
	4	6,043,589 – 6,085,514	rs109152924[A/G]	rs108981318[A/G]	14	1.18
	6	12,281,295 – 12,324,019	rs133475522[A/G]	rs133825020[A/G]	16	1.25
	8	85,710,160 – 85,742,691	rs132803059[A/G]	rs136676168[A/G]	30	1.42
	8	101,304,601 – 101,338,353	rs43578763[C/T]	rs132723741[A/C]	25	1.46
	11	46,909,405 – 46,923,593	rs109685929[A/G]	rs109743579[C/T]	30	1.22
	14	74,135,766 – 74,162,870	rs133660328[C/T]	rs134501947[A/G]	6	1.72
	17	8,657,098 – 8,726,408	rs109995100 [A/G]	rs109590526 [A/G]	13	1.04
	21	37,514,897 – 37,553,879	rs136373783 [A/G]	rs42925195 [A/G]	8	1.34
	27	18,973,176 – 18,995,081	rs133381207 [C/T]	rs136653614 [A/G]	24	1.63
CS	1	150,903,364 – 150,920,045	rs133964621[A/G]	rs110359116[C/T]	30	1.01
	5	12,481,988 – 12,494,839	rs110177270[A/G]	rs132921603[A/C]	7	1.48
	5	71,375,395 – 71,425,699	rs42356196[C/T]	rs135157102[C/T]	28	1.06
	6	107,417,481 – 107,424,115	rs137178377[A/G]	rs109710070[A/G]	30	2.06
	11	29,740,008 – 29,750,271	rs132979785[A/G]	rs110051129[C/T]	30	1.05
	11	46,896,251 – 46,918,155	rs110428019[A/G]	rs134640008[A/G]	30	1.29
	12	54,205,110 – 54,232,971	rs133392390[C/T]	rs134076699[C/T]	15	1.17
	12	64,270,480 – 64,312,244	rs42803543[C/T]	rs133193462[C/T]	2	1.05
	13	27,840,447 – 27,856,692	rs109718193[A/G]	rs136959555[C/T]	25	1.03
	15	9,712,719 – 9,765,125	rs43766235[C/T]	rs110037438[C/T]	2	1.27
	16	45,897,787 – 45,944,191	rs137006132[C/T]	rs109283847[A/G]	28	4.79
	19	5,680,108 – 5,706,784	rs110461916 [C/T]	rs137364955 [C/T]	21	1.03
	22	525,568 – 536,876	rs133157955 [A/G]	rs110530743 [A/G]	25	1.09
FS	5	22,597,876 – 22,611,968	rs137075778[A/C]	rs110266043[A/G]	18	1.44
	11	67,369,987 – 67,394,167	rs135834212[C/T]	rs137532492[A/G]	30	1.42
	14	17,745,370 – 17,758,419	rs137759301[C/T]	rs110665019[C/T]	30	1.18
	17	594,627 – 654,461	rs136616549[A/G]	rs134770230 [A/G]	16	21.04
	19	8,121,766 – 8,163,977	rs110324799 [A/G]	rs110494846 [A/G]	28	1.89
	23	3,364,476 – 3,387,731	rs135585741 [A/G]	rs41640782 [C/T]	18	1.22
TS	1	465,262 – 487,056	rs136466556[A/G]	rs137558742[C/T]	30	1.44
	1	75,828,077 – 75,875,485	rs136527770[A/G]	rs136703568[A/G]	9	2.05
	17	23,144,388 – 23,176,735	rs110065427[A/C]	rs133503642 [A/C]	3	1.09
	18	36,829,534 – 36,899,210	rs43118103 [A/G]	rs41256107 [C/T]	30	2.26
	19	12,005,721 – 12,021,061	rs110825511 [C/T]	rs135671434 [C/T]	30	1.83
	24	50,559,249 – 50,597,089	rs109586261 [C/T]	rs137576659 [A/G]	28	2.04

[1]MOV = movement score; TENS = tension score; CS = crush score; FS = flight speed; TS = temperament score
[2]N = number of candidate genes within each SNP window

For MOV, *TMEM229A* (transmembrane protein 229A) is a candidate gene within the SNP window on BTA4. This gene has a function related to regulation of frequency or extent of cellular DNA-templated transcription in different species. In humans, this gene was associated with neonatal traits influencing the occurrence of spontaneous preterm birth [55].

The SNP window on BTA1 at 150 Mb was associated with overall aspects of body tension (TENS) and within this region the *HLCS* gene (holocarboxylase synthetase) was identified. This gene encodes an important protein with catalytic activity in the cytoplasm and mitochondria that acts in the biotin cycle for covalent binding of biotin to carboxylase and histones [56]. In humans, the biotin carboxylase enzymes affect various metabolic processes, such as metabolism of glucose (gluconeogenesis), fatty acids synthesis and branched chain amino acid catabolism [57]. On BTA16 the *SLC45A1* (solute carrier family 45 member 1) gene was associated with CS that belongs to a putative sugar transporter family and encodes a plasma membrane transporter protein for glucose and galactose. Moreover, this gene was identified in human fetal and adult brain, heart, muscle and kidney tissues [58].

For FS the SNP window on BTA17 at 594 Kb explained the highest proportion of genetic variance in which the *CPE* (carboxypeptidase E) gene was identified. This gene was previously associated with FS test in Nellore cattle using a subset of the same database composed by information of 16,660 phenotyped and 1,306 genotyped animals from only one herd [26]. The *CPE* gene encodes a peripheral membrane protein that affects the endocrine and nervous systems, biosynthesis of hormones and neurotransmitters. As reviewed by Cawley et al. [59] the polymorphic forms of encoded protein influence the occurrence of diabetes type II and obesity in humans, as well as, obesity, infertility, cognitive and emotional disorders in mice, obesity and infertility in pigs and, aspects of carcass and meat quality in beef cattle.

The SNP window on BTA18 at 36 Mb explained the highest percentage of genetic variance for TS. Within this region the *NFAT5* (nuclear factor of activated T-cells 5) gene was identified, which belongs to a family of osmo-sensitive transcription factor. The encoded protein controls the gene transcription rate during osmotic stress in mammalian cells, being associated with immune response, infection, cancer

development and cell migration [60, 61]. In summary, the biological functions of *TMEM229A*, *HLCS*, *SLC45A1* and *NFAT5* genes in cattle are still unknown and the metabolic actions described for these genes do not allow a clear association with temperament or behavioral phenotypes. Only the *CPE* gene was previously described influencing behavior and performance traits in beef cattle and other farm species, indicating the possibility to consider *CPE* as a promising candidate gene for cattle temperament.

Some studies have been conducted in order to identify genetic markers that influence the expression of temperament in different beef [11, 14, 17, 18, 20, 21] and dairy [15, 19, 62] cattle breeds. Schmutz et al. [14] used an electronic measuring device to objectively quantifying the amount of movement of 130 calves twice during the feedlot period. These authors reported seven QTL on chromosomes 1, 5, 9, 11, 14 and 15 associated with temperament and habituation. On BTA15 these authors identified the *ADCY2* gene (adenylate cyclase 2), which is similar to that found associated with milking temperament and aggressiveness in Brown Swiss cattle [19], and associated with FS on BTA20 at 65 Mb in the current study. According to Li et al. [63] this gene is expressed in different bovine tissues and encodes a membrane-associated enzyme that catalyze formation of cyclic adenosine monophosphate (cAMP) from ATP. Moreover, the *ADCY2* gene was associated with meat tenderness in different crossbred and purebred cattle [63-65].

Analyzing the structure of SNPs in myostatin gene (*MSTN*) located on BTA2 at 6 Mb, Esmailizadeh et al. [16] found a SNP that affects the expression of docility in Limousin x Jersey crosses. The same chromosome was associated with MOV, TENS and FS but in different genomic positions and thus the *MSTN* gene cannot be considered as a candidate gene influencing temperament in Nellore cattle. Similarly, Gutierrez-Gil et al. [11] reported 29 QTL and some promising candidate genes that affects temperament in different environmental challenge situations, such as active human approach and social isolation tests in Holstein x Charolais crosses. The authors highlighted the effect of *DRD4* (dopamine receptor D4) gene, which have been significantly associated with temperament and behavioral traits in humans [66], mouse [67], mice [68], birds [69], dogs [70], horses [71] and cattle [18].

According to Glenske et al. [18] the *DRD4* gene is an important genetic marker that regulates temperament, measured by three different tests, in German Angus calves. However, in the current study we detected the association of *DRD5* (dopamine receptor D5) with CS on the BTA6 at 107 Mb. These results corroborate the importance of dopamine receptor gene family in the expression of temperament in many species. Dopamine is related to learning, response to pleasant situation, motivation, memory capacity, cognitive process, fine motor skill, and modulation of neuroendocrine signaling.

Hulsman-Hanna et al. [20] performed a GWAS and identified 37 regions associated with overall temperament at weaning (measured by individual responses during social separation test) in Nellore x Angus crossbreed. The authors used 172 genes to realize enrichment analysis and reported that the variability regarding to the target phenotype is influenced by the gene network related to voltage-gate channel activity (GO:0005248, sodium ion transport and activity). Although this ontology category has not been previously reported as associated with temperament traits, the windows on BTA10 (44 Mb) and 21 (31 Mb) were 1 Mb apart of that regions presented by Gutiérrez-Gi et al. [11] and one region was similar of TS on BTA3 at 89 Mb in the current study.

Using the BovineSNP50 BeadChip array to genotype 1,057 *Bos taurus* steers from different crosses, Lindholm-Perry et al. [21] focused on three candidate genes to identify polymorphisms associated with FS test. The genes *QKI* on BTA9, *GRIA2* and *GLRB* on BTA17 play an important role on the central nervous system and synaptic transmission. However, the 12 SNPs surrounding these genes were not associated with individual variability of FS in European cattle. For dairy cattle, Kramer et al. [19] used a high density SNP BeadChip to detect genes affecting general temperament and aggressiveness in Brown Swiss cattle. The authors identified a high effect of BTA4 (14 Mb), 8 (101 Mb) and 20 (65 Mb) on the target-phenotype. Within these regions the known genes were *TAC1* on BTA4, *AKAP2*, *TXN* and *TXNDC8* on BTA8 and *ADCY2* on BTA20. They emphasize the function of *TAC1* as neurotransmitters which play a role in behavior responses, but only the candidate genes within BTA8 and 20 were similar to the present study, overlapping with TENS and FS, respectively.

The difference of genomic regions associated with temperament traits among all studies were influenced by many factors, such as sample size, cattle breeds, methodology to access temperament itself and statistical methods [72]. These aspects lead to a particular association of phenotypes and breeds with specific genetic markers. However, common genetic profiles were found among studies, with the same regions and genes influencing temperament of different breeds and behavioral responses in distinct challenge situations. These results confirm the complexity related to the genetic architecture of temperament traits, which were influenced by numerous genes, with small and large effects, displaying a polygenic structure and quantitative inheritance pattern [73]. Thus, additional genomic information highlights the similarities and differences between test conditions and behavioral phenotypes.

4.3. Identification of common SNP windows and overlapped QTL

The occurrence of genomic regions in close proximity or simultaneously associated with different temperament traits were evaluated considering the top 20 windows expanded 1 Mb in each direction. None region was similar for more than two traits and the common regions shared by different temperament traits are shown in Table 6.

Table 6. Summary of SNP windows simultaneously associated with two different temperament traits in Nellore cattle.

BTA[a]	Trait 1[b]	SNP window (bp)	Trait 2	SNP window (bp)
1	TENS	149,903,364 – 151,920,045	CS	149,902,701 – 151,918,711
11	TENS	45,743,305 – 47,783,081	CS	45,896,251 – 47,918,155
11	TENS	45,909,405 – 47,923,593	CS	45,896,251 – 47,918,155
27	TENS	27,057,988 – 29,075,265	CS	27,878,431 – 30,894,730
1	MOV	46,000,936 – 48,050,240	FS	48,079,945 – 50,108,373
17	MOV	68,709,764 – 70,746,091	FS	67,681,566 – 69,694,306
6	CS	110,716,379 – 112,765,469	TS	108,517,036 – 110,599,822
19	FS	13,622,872 – 15,644,649	TS	13,632,665 – 15,645,614

[a]BTA, *Bos taurus* autosome.
[b] MOV = movement score; TENS = tension score; CS = crush score; FS = flight speed; TS = temperament score.

Between TENS and CS similar regions were on BTA1, 11 and 27 at 150 Mb, 46 Mb and 28 Mb, respectively. Two distinct windows associated with TENS were

overlapped with CS on BTA11 at 46 Mb. MOV and FS had one similar window on BTA17 at 69 Mb, and another in close proximity on BTA1 at 49 Mb. Finally, one window was concurrently associated with FS and TS on BTA19 at 14 Mb, and one region on BTA6 at 110 Mb was in close proximity between CS and TS.

It was of interest to comprehend whether different aspects of cattle temperament were under control of common genomic regions and genes. Some highlights can arise from the present results. The first one is the existence of pleiotropic genomic regions and genes that affect distinct aspects of cattle temperament. This is the first molecular evidence reinforcing the previously reported genetic correlation estimated among different indicators used to evaluate temperament of beef cattle [74]. However, given the genetic correlation estimated in the present study, it was expected a higher number of similar SNP windows shared by them. The second one is the range of genes influencing the expression of each trait. Some regions or genes explained more of additive genetic variance for one trait and less for another, and the most important genes that affects one trait, are not necessarily, important for both. Similar results have been reported by Saatchi et al. [75] working with body weights, calving ease and carcass traits across different breeds. These results support the importance of an accurate choice of standardized methodologies to be included as selection criteria for temperament in breeding programs.

For temperament and behavioral traits were identified seventeen QTL previously reported on cattle QTLdb database [44], which are linked to different types of test, environmental situations and cattle breeds. Among them, a total of nine QTL overlapped with ten top 20 SNP windows which were associated with the temperament traits used in the present study (Table 7). Of these nine QTL, only one was common to more than one trait, which occurred within the similar SNP windows on BTA11 at 46 Mb for TENS and CS, and the other eight QTL were trait-specific. The overlap of these nine SNP windows with QTL reported in previous studies provides more reliability for the regions detected as associated with temperament traits of Nellore cattle.

Table 7. Summary of top 20 SNP windows overlapping with previously reported QTL associated with temperament tests and other behavioral traits.

Trait	BTA[1]	QTL[2]	Test	Breed	N[3]	Reference
MOV	18	7142	Social separation (vocalization)	Charolais x Holstein	30	[11]
MOV	29	1389	Milking temperament	Holstein	21	[15]
TENS	11	7138	Social separation (standing alert)	Charolais x Holstein	65	[11]
CS	11	7138	Social separation (standing alert)	Charolais x Holstein	85	[11]
CS	16	7140	Social separation (vocalization)	Charolais x Holstein	58	[11]
FS	7	7132	Social separation (vocalization)	Charolais x Holstein	20	[11]
FS	15	1498	Temperament and habituation (movement level)	Canadian Beef Cattle	18	[14]
FS	29	7153	Flight from feeder	Charolais x Holstein	7	[11]
FS	29	7154	Flight from feeder	Charolais x Holstein	7	[11]
TS	1	7127	Social separation (standing alert)	Charolais x Holstein	30	[11]

[1]BTA, *Bos taurus* autosome.
[2]QTL ID, identification based on UMD 3.1 bovine genome assembly.
[3]N = number of candidate genes within each SNP window.

4.4. Functional network and metabolic pathways

The post-GWAS analyses were performed aiming to identify connections among all genes within each SNP window that influence the variability of temperament, based on their functional pathways. Further analyses were carried focusing on the identification of specific metabolic pathways associated to each temperament trait. A functional network (Figure 3) was constructed using all known genes identified within the expanded top 20 SNP windows. Candidate genes were connected through 25 functional pathways using the IPA software ($p<0.05$). The most important network found connects two categories directly related to our target phenotypes, such as behavior and development and functions of nervous system.

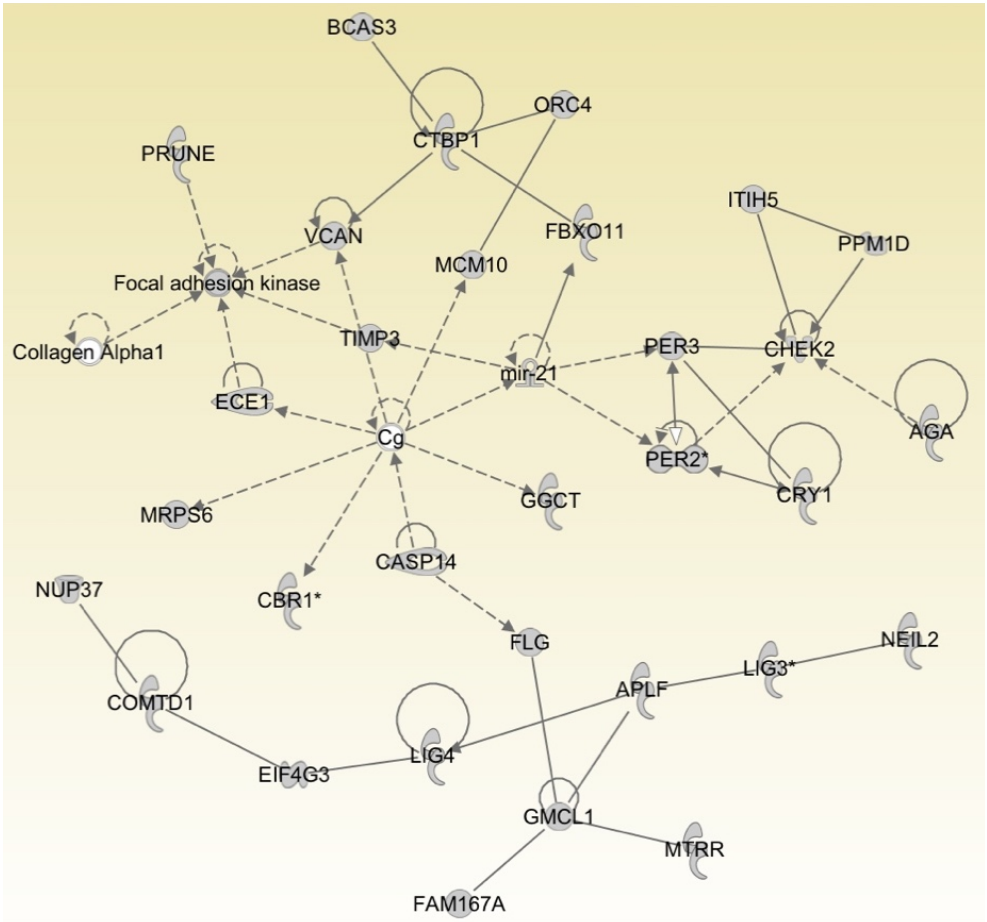


Figure 3. Gene network constructed based on the top 20 SNP windows associated with temperament traits using the IPA software ($p<0.05$).

The genes and its function annotation related to each category are shown in Table 8. The first category was composed by behavior, emotional behavior and social exploration, leading to signaled genes directly associated with behavioral activity. The second was divided into several functions related to nervous system, especially long-term depression, as well as, with nervous system activity that is the center of plasticity and physiological origin of behavior [76]. Changes in reactivity of the nervous system modulates behavior through individual experiences [77]. This ensures that the measurement methodologies used in the present study were able to capture variability of the target phenotypes. Moreover, the segregating SNPs in these populations signaled genes that allowed a better comprehension of underlying genetic architecture influencing the expression of cattle temperament likewise assist the genetic evaluation in the future.

Table 8. Summary of category, function annotation and genes related to the functional network associated with temperament traits.

Category	Function Annotation	p-value	N[1]
Behavior	behavior	0.003	85
	emotional behavior	0.004	25
	social exploration	0.005	7
Nervous System Development and Function	long term depression	0.000	15
	long term depression of spiny neurons	0.001	3
	long term depression of synapse	0.003	9
	long term depression of perirhinal cortex	0.006	2
	cell viability of granule cells	0.001	6
	cell viability of neurons	0.002	25
	formation of axon branches	0.002	2
	differentiation of astrocytes	0.003	9
	differentiation of glial progenitor cells	0.007	3
	neurogenesis of cerebral cortex	0.003	6
	neurogenesis of hippocampus	0.006	5
	neurotransmission	0.003	38
	abnormal morphology of synapse	0.003	9
	abnormal morphology of enlarged vertebral body	0.006	2
	abnormal morphology of neuromuscular synapse	0.007	6
	loss of Purkinje cells	0.004	4
	innervation of cells	0.005	11
	action potential of cerebral cortex cells	0.005	3
	activation of pyramidal neurons	0.005	3
	growth of brain	0.005	13
	migration of neuroblasts	0.005	4
	proliferation of cerebral cortex cells	0.005	4
	proliferation of pituitary cells	0.005	4
	quantity of medium spiny neurons	0.006	2
DNA Replication, Recombination, and Repair	ligation of DNA fragment	0.000	3
	ligation of DNA	0.002	4
	modification of chromatin	0.005	12
	double-stranded DNA break repair	0.005	13
	oxidation of NADPH	0.005	4
	filling of DNA	0.006	2

[1]N = number of genes within each function.

Metabolic pathway enrichment analyses were carried out separately for each temperament trait, by using the DAVID software ($p<0.05$). For MOV the identified genes were related to metabolic pathways of long-term depression, GnRH signaling (KEGG_PATHWAY) and potassium channels activity (GO: 0005267). For TENS the metabolic pathways revealed were GnRH signaling, neurotrophin signaling and genes that comprise the interleukin receptor metabolism (GO:0005149). The interleukin receptor genes were also associated with CS, corroborating the occurrence of pleiotropic genes/regions/pathways associated with both TENS and

CS. Moreover, specific genes of metabolic pathways for behavior (GO:0007610) and locomotor behavior (GO:0007626) were signalized for CS.

For FS, it was identified genes that are directly associated with behavioral responses to stimuli in the metabolic pathways for response to abiotic and biotic stress (GO:0006950), physiological defense response (GO:0006952) and response to stimulus (GO:0050896). In addition, were signaled general metabolic pathways of nervous system, such as synapse (GO:0045202), neurotransmitter receptor activity (GO:0030594), synaptic transmission (GO:0007268), neurotransmitter binding (GO:0042165) and postsynaptic membrane (GO:0045211). For TS was associated general pathways of behavior, such as Behavior (GO:0007610) and Locomotor behavior (GO:0007626).

The enrichment analyses highlighted the GnRH signaling pathway for MOV and TENS. The encoded peptide hormone is a central regulator in the hypothalamic–pituitary–gonadal (HPG) axis and plays an important role in social and sexual behavior of different species [78-80]. The HPG is inhibited by the hypothalamic-pituitary-adrenal (HPA) axis, that is associated with individual variability in response to stressful situation [12]. Furthermore, GnRH have been linked to cognitive and physiological effects [81, 82]. Although the same metabolic pathway has been identified influencing both traits (MOV and TENS), the signaling genes were different, indicating a trait-specific association.

5. CONCLUSION

In this research all temperament traits presented sufficient genetic variability to respond to direct selection and could be used in breeding programs. Genetic correlations between them were high specially among those measured inside the squeeze chute, leading to conclude that the use of only one trait in each testing condition should be enough to capture individual variability of temperament. Genome-wide association analyses revealed genes that may influence cattle temperament revealing the existence of pleiotropic regions associated with distinct traits. Additionally, were able to successfully identify previously reported QTL also associated with cattle temperament and behavior. Network analyses and metabolic

pathways highlighted the molecular mechanisms regulating the expression of cattle temperament and revealed categories that directly match with aspects of emotional behavior.

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