Genome-wide association studies for heat stress response in *Bos taurus* × *Bos indicus* crossbred cattle

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ABSTRACT

Heat stress is an important issue in the global dairy industry. In tropical areas, an alternative to overcome heat stress is the use of crossbred animals or synthetic breeds, such as the Girolando. In this study, we performed a genome-wide association study (GWAS) and post-GWAS analyses for heat stress in an experimental Gir × Holstein F₂ population. Rectal temperature (RT) was measured in heat-stressed F₂ animals, and the variation between 2 consecutive RT measurements (Δ RT) was used as the dependent variable. Illumina BovineSNP50v1 BeadChip (Illumina Inc., San Diego, CA) and single-SNP approach were used for GWAS. Post-GWAS analyses were performed by gene ontology terms enrichment and gene-transcription factor (TF) networks, generated from enriched TF. The breed origin of marker alleles in the F₂ population was assigned using the breed of origin of alleles (BOA) approach. Heritability and repeatability estimates ($\pm$ standard error) for Δ RT were 0.13 ± 0.08 and 0.29 ± 0.06 , respectively. Association analysis revealed 6 SNP significantly associated with Δ RT. Genes involved with biological processes in response to heat stress effects (*LIF*, *OSM*, *TXNRD2*, and *DGCR8*) were identified as putative candidate genes. After performing the BOA approach, the 10% of F₂ animals with the lowest breeding values for Δ RT were classified as low- Δ RT, and the 10% with the highest breeding values for Δ RT were classified as high- Δ RT. On average, 49.4% of low- Δ RT animals had 2 alleles from the Holstein breed (HH), and 39% had both alleles from the Gir breed (GG). In high- Δ RT animals, the average proportion of animals

for HH and GG were 1.4 and 50.2%, respectively. This study allowed the identification of candidate genes for Δ RT in Gir × Holstein crossbred animals. According to the BOA approach, Holstein breed alleles could be associated with better response to heat stress effects, which could be explained by the fact that Holstein animals are more affected by heat stress than Gir animals and thus require a genetic architecture to defend the body from the deleterious effects of heat stress. Future studies can provide further knowledge to uncover the genetic architecture underlying heat stress in crossbred cattle.

Key words: crossbred cattle, gene network, heat stress, post-GWAS analyses

INTRODUCTION

Heat stress is an important factor in the global dairy industry, because it is responsible for large losses in milk production, growth, and reproductive performance, and negatively affects animal health and welfare (Nardone et al., 2010). Climate change may induce increases in average temperature and decreases in rainfall, leading to worsened environmental conditions for livestock production in tropical areas. Harsh environmental conditions are common in Brazil, where two-thirds of the territory is located in tropical regions, with prevalent high temperatures due to solar radiation and grazing systems dependent on the rainy season (Cruz et al., 2011).

Briefly, when animals cannot dissipate sufficient body heat to prevent an increase in their body temperature, heat stress occurs (West, 2003). Modern high-producing dairy cows are more susceptible to the deleterious effects of heat stress (Renaudeau et al., 2012) due to their very high metabolic rate associated with milk production. *Bos indicus* animals have a greater ability to regulate their body temperature, because they

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have undergone natural selection for thousands of years under high temperatures in India. Part of this ability is the result of lower metabolic rates and higher capacity for heat loss through thermoregulatory mechanisms, which helps in heat stress prevention (Hansen, 2004). In tropical areas, an alternative to overcome heat stress in dairy production systems is the use of crossing or synthetic breeds containing some portion of *Bos indicus* genetics, such as the Girolando breed, which is derived from crossing Gir and Holstein animals.

Respiration rate (**RR**) acts as a powerful thermoregulatory mechanism that helps maintain body temperature through evaporative cooling. This physiological parameter is important in the prediction of heat stress in dairy cattle (McDowell et al., 1975). Rectal temperature (**RT**) is an indicator of thermal equilibrium; an increase in this parameter expresses failure or exhaustion of thermoregulatory mechanisms, resulting in heat stress (de Andrade Ferrazza et al., 2017).

Molecular markers can help in understanding heat stress adaptive parameters of dairy cattle that are relevant to production systems in tropical regions. This approach allows the identification of genomic regions and genes associated with heat stress response, becoming an additional attribute in the process of genetic selection of heat-tolerant animals (Dikmen et al., 2015). Dikmen et al. (2013) found QTL for RT close to genes involved in cell protection against heat stress in Holstein and concluded that some SNP may be useful in genetic selection and identification of genes controlling heat tolerance. In a subsequent study, Dikmen et al. (2015) identified specific genetic markers responsible for genetic variation of thermoregulation, showing the importance of genome-wide association studies (**GWAS**) in finding markers and genes involved in physiological response to heat stress. However, studies about genes underlying RT, via gene-transcription factor (**TF**) networks and gene ontology (**GO**) enrichment analyses, in *Bos indicus* × *Bos taurus* animals have not been reported yet.

Recently, Vandenplas et al. (2016) developed an approach that enables the assignment of breed of origin of alleles (**BOA**) in crossbred animals. Results from this approach can be used to estimate SNP effects depending on the BOA and to improve genomic prediction or GWAS in crossbred animals. According to Sevillano et al. (2018), estimated effects and explained variance of SNP strongly associated with crossbred performance are different depending upon which parental breed donated them. Therefore, including breed-specific SNP effect in the genomic evaluation model allowed, in some cases, a better prediction for crossbreed performance (Sevillano et al., 2017). Otto et al. (2018) used the BOA approach to evaluate the origin of marker alleles

of the candidate genes identified for tick resistance among the same population used in the current study. Those authors observed that most animals classified as resistant for tick infestation showed 2 alleles from the Gir breed, whereas susceptible animals presented 2 alleles from the Holstein breed.

In this study, we performed a GWAS in a Gir × Holstein F₂ experimental population to identify SNP associated with heat stress response and used gene networks to identify the most likely candidate genes. The origins of alleles of selected genes were assigned to help understand the gene mechanisms involved in heat stress response.

MATERIALS AND METHODS

Data

The data were obtained from previous Embrapa research, in which an experimental F₂ population was produced by crossing 4 Holstein bulls with 27 Gir cows to generate 150 F₁ (1/2 Gir:1/2 Holstein) animals. From this population, 65 F₁ females were mated to 4 F₁ males to generate 376 F₂ animals; the pedigree file contains a total of 476 animals. All F₂ animals were raised together in the Embrapa Dairy Cattle experimental station, located in the southeast of Brazil (Machado et al., 2010).

A total of 341 Gir × Holstein F₂ animals were subjected to a heat chamber at 42°C and 60% relative humidity (**RH**), after an adaptation period of 12 h at 22°C and 50% RH. Each evaluation included 6 animals, and during the whole period that the animals were housed in the heat chamber, water and food were not offered, aiming to reduce their effects on the animals' responses. Parental Gir, Holstein, and F₁ animals were not subjected to heat stress.

The RT and RR of the F₂ animals were evaluated in 2 replicates. The first evaluation was performed after the adaptation period, and the second evaluation 6 h after the heat chamber reached 42°C. To measure RT, a digital clinical thermometer was inserted approximately 7.5 cm into each animal's rectum. For RR measurement, flank movements were counted for 30 s. The difference between rectal temperature measurements was calculated, to estimate the variation of rectal temperature (**ΔRT**) in each animal as follows:

$$\Delta RT = RTA - RTB,$$

where *RTA* is the rectal temperature after heat stress and *RTB* is the rectal temperature before heat stress (after the adaptation period). The variation of respiration rate (**ΔRR**) was defined similarly to ΔRT. Each

Table 1. Descriptive statistics of phenotype and covariates included in the model to evaluate the variation of rectal temperature (ΔRT) in an experimental Gir $\times$ Holstein F₂ population evaluated for heat stress response

Variable	Season ¹	Parameters					
		Number	Mean	SD	CV (%)	Minimum	Maximum
ΔRT	1	314	2.65	0.66	24.78	1.00	4.45
	2	339	2.01	0.57	28.22	0.55	3.40
Coat length	1	320	1.04	0.22	21.49	0.51	1.89
	2	334	1.81	0.38	21.07	0.79	3.28
Coat thickness	1	322	3.63	0.73	20.07	1.48	6.00
	2	341	4.68	1.15	24.58	1.73	9.89
Hair density	1	320	184.91	55.70	30.12	82.00	412.00
	2	332	178.35	56.64	31.76	67.67	375.00
ΔRR ²	1	316	104.88	19.93	19.01	58.00	154.00
	2	343	88.34	28.48	32.23	12.00	160.00
BC ³	—	369	0.48	0.05	10.26	0.31	0.66

¹Season: 1 = rainy; 2 = dry.²Variation of respiration rate.³Breed composition.

animal was subjected twice to the heat chamber to perform measurement of ΔRT and ΔRR .

Animals were evaluated in contemporary groups aged from 10 and 14 mo. Additional traits that might affect heat stress evaluations were assessed during this period: coat color, coat thickness and length, and density and type of hair (Machado et al., 2010). Ancestry was estimated using Admixture software (Alexander et al., 2009) with a reduced panel (7,425 SNP), pruned by linkage disequilibrium (**LD**) between subsequent markers.

All animals of the experimental population were genotyped with the Illumina BovineSNP50 v1 Bead-Chip (Illumina, San Diego, CA), including parental Gir, Holstein, F₁, and F₂ populations. Genotype quality control was implemented using the SNPStats package in R software (Solé et al., 2006). Samples with a call rate <0.90 and heterozygosity of 3.0 standard deviations above or below the observed mean were removed. For quality control of mapped SNP, only autosomal SNP with call rate >0.90 and minor allele frequency (**MAF**) >0.03 were considered. After quality control, the genotype file contained 40,283 SNP markers.

Genome-Wide Association Analyses

A single-SNP GWAS was performed with ASReml software (Gilmour et al., 2009), applying the following model:

$$\begin{aligned}
 y_{ijklmnop} = & \mu + CG_i + CC_j + HT_k + SNP_l + S_m \\
 & + \beta_1 (CL_{ijklmnop} - \overline{CL}) + \beta_2 (CT_{ijklmnop} - \overline{CT}) \\
 & + \beta_3 (HD_{ijklmnop} - \overline{HD}) + \beta_4 (VRR_{ijklmnop} - \overline{VRR}) \\
 & + \beta_5 (BC_{ijklmnop} - \overline{BC}) + a_n + pe_o + e_{ijklmnop},
 \end{aligned}$$

where $y_{ijklmnop}$ is the variation of rectal temperature (ΔRT); μ is the general mean; CG_i is the effect accounting for contemporary groups; CC_j is the effect accounting for coat color (j ranges from 1 to 4); HT_k is the effect accounting for hair type (k ranges from 1 to 4); SNP_l is the fixed effect of SNP genotype, where l was coded as 0, 1, or 2 copies of one of the alleles; S_m is the effect accounting for season (dry or rainy season); $CL_{ijklmnop}$ is a covariate for the effect of coat length; $CT_{ijklmnop}$ is a covariate for the effect of coat thickness; $HD_{ijklmnop}$ is a covariate for the effect of hair density; $VRR_{ijklmnop}$ is a covariate for the effect of ΔRR , calculated by the difference between both measurements; $BC_{ijklmnop}$ is a covariate for the estimated indicine percent of the animal; $\beta_1, \beta_2, \beta_3, \beta_4$, and β_5 are the regression coefficients a_n is a random additive genetic effect of animal n , assuming $a \sim N(0, \mathbf{A}\sigma_a^2)$, with additive pedigree relationship matrix $\mathbf{A}$ and the additive genetic variance σ_a^2 ; pe_o is the random permanent environmental effects, assuming $pe \sim N(0, \mathbf{I}\sigma_{pe}^2)$, with identity matrix $\mathbf{I}$ and permanent variance σ_{pe}^2 ; and $e_{ijklmnop}$ is the random residual effect, assuming $e \sim N(0, \mathbf{I}\sigma_e^2)$, where σ_e^2 is the residual variance. The variance components were estimated based on same model without the SNP effect.

Although the additional evaluated traits show correlation with RT control, in this work we selected ΔRT as the main trait. For this reason, we included these additional traits only as covariates in the model to look for significant SNP that could be linked only to heat stress response, disregarding the effects of these additional traits on ΔRT . The descriptive statistics of phenotype and covariates are shown in Table 1.

After the association analysis, the genome-wide false discovery rate (**FDR**) was applied to avoid false

positives due to multiple testing. The R package qvalue (Dabney et al., 2014) was used to provide the P -values corrected for FDR (q -values) for the SNP association tests. Associations with a q -value ≤ 0.05 were considered significant.

A single-SNP GWAS for the Δ RR trait was performed with the same model used to evaluate the Δ RT trait, in which the Δ RT trait was included as covariate. Nevertheless, no significant SNP was found to be associated with Δ RR.

QTL Regions

We defined QTL regions based on the location of the significant SNP and the average LD (measured as the squared correlation coefficient, r^2) in the evaluated population. Haploview software (Barrett et al., 2005) was used to calculate the pairwise LD between markers. Because the significant SNP identified in the GWAS analysis were located on chromosome 17, we used the LD of this chromosome to fix the QTL size. In addition, the LD between significant SNP identified in GWAS (Supplemental Figures S1 and S2; <https://doi.org/10.3168/jds.2018-15305>) were also used to define QTL regions. An average $r^2 \geq 0.15$ was found for SNP within a distance of 500 kb. Thus, all significant SNP located within a region of 500 kb were considered as belonging to the same QTL region.

Gene Search and Generation of Gene Networks

Putative candidate genes within QTL regions were identified based on the UMD 3.1 assembly of the bovine genome (Zimin et al., 2009), using the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>).

The gene set was used for GO enrichment analysis via the ClueGO application in Cytoscape (Bindea et al., 2009) to generate the GO. This analysis was performed based on a unilateral hypergeometric test with Bonferroni correction and aims to analyze the gene set on the search for a functional GO term or pathway that might show correlation with selected genes.

The same gene set was used to perform the analysis of regulatory sequences. For that, promoter sequences (FASTA format) were obtained by considering 3,000 bp upstream and 300 bp downstream from genes' transcription start site in the bovine genome (Soares et al., 2017). These data were screened for the identification of enriched TF using the TFM-explorer software (<http://bioinfo.lifl.fr/TFM/TFME/>), which uses weighting matrices from the JASPAR database (<http://jaspar.binf.ku.dk/>; Sandelin et al., 2004) to detect potential transcription factor binding sites (TFBS) of a set of

gene sequences and search for over-represented TFBS. In addition, this software extracts significant groups—TFBS regions of the selected gene sequences associated with a factor—by calculating a score function threshold that was chosen to generate a P -value $\leq 10^{-3}$ for each position in each sequence, as described in Touzet and Varré (2007).

The TF list was then analyzed in Cytoscape software (Shannon et al., 2003), using the Biological Networks Gene Ontology tool (BiNGO; Maere et al., 2005) to determine significantly overrepresented functional GO terms. Based on the over-represented biological processes in the BiNGO tool and evidence from literature data related to the investigated traits, we were able to identify the TF most related to heat stress, allowing the generation of gene-TF interaction networks.

Gene-TF network analysis was performed in Cytoscape software (Shannon et al., 2003) based on the number of TFBS (connections with the selected TF) in each gene to determine the associations with Δ RT, which aided in the identification of the most likely candidate genes.

Assignment of Breed of Origin of Alleles

To assign the breed origin of marker alleles in the F_2 population, the BOA approach (Vandenplas et al., 2016) was used with the parameter settings recommended by Sevillano et al. (2016). The BOA approach consists of 3 steps. (1) The purebred and crossbred animals' genotypes were phased with AlphaPhase 1.1 software (Hickey et al., 2011). Haplotype phasing was performed using pedigree and 9 combinations of core and tail lengths (350:50, 250:100, 300:100, 350:100, 150:200, 200:200, 250:200, 300:200, and 350:200), in which each combination was run in both "Offset" and "NotOffset" modes, allowing each allele to be considered 18 times through different haplotypes of variable length. "Offset" analysis shifts the beginning of each core to halfway along the first core, creating 50% overlaps between cores (Vandenplas et al., 2016). (2) The unique haplotypes for each pure breed were determined. In this step, it is necessary to be able to observe 80% of a haplotype's copies in a specific breed, to assign its breed of origin. (3) Breed origin was assigned to each allele observed in F_2 animals based on the determined unique haplotypes.

After performing the BOA approach, 10% of F_2 animals with the lowest breeding values for Δ RT were designated as low- Δ RT, and the 10% with the highest breeding values for Δ RT were designated as high- Δ RT. Considering the LD between SNP and genes, the physical positions of candidate genes were annotated and used as coordinates for selection of adjacent SNP, which

were classified as HH (both alleles originated from the Holstein breed), GG (both alleles originated from the Gir breed), or HG/GH (one allele originated from the Holstein and one from the Gir breed), based on the results of the BOA approach. Finally, the alleles of each selected and classified SNP were quantified to evaluate the gametic segregation and check for prevalence of alleles from a certain breed when animals are classified as low ΔRT or high ΔRT . For that, a chi-squared test was used to evaluate the expected gametic segregation of 25% HH, 50% HG/GH, and 25% GG in both groups. Those SNP with a difference in prevalence of alleles from a certain breed, which did not show the expected gametic segregation, were considered significant ($P \leq 0.05$).

RESULTS AND DISCUSSION

Genetic Parameters

We found heritability estimates of 0.13 ± 0.08 and repeatability estimates of 0.29 ± 0.06 for the ΔRT trait. During the heat stress evaluation period, the RT was evaluated at 42°C and 60% RH, in which the temperature-humidity index (THI) was 97. Dikmen et al. (2012) estimated heritability of 0.17 ± 0.13 for RT, recorded between 1500 and 1700 h at different THI, in Holstein cattle during summer in north Florida. In beef cattle, heritability estimates of 0.22 for RT, recorded only during summer months when the ambient temperature was $>30^\circ\text{C}$, were found in Brahman animals, and estimates of 0.14 were found in crossbred animals showing genetic backgrounds of different tropical breeds (Porto-Neto et al., 2014).

Most studies use THI to estimate the presence or absence of the heat stress phenotype in a given environment (Biffani et al., 2016; Hagiya et al., 2017). However, evaluation of the response of each animal individually during the heat stress is relevant, as animals respond in different ways to environmental stimuli, which is accentuated in the F_2 crossbred animals used in the current study because each animal possesses a unique genetic composition.

QTL Regions

In the current study, the GWAS for ΔRT identified 6 significant SNP ($q \leq 0.05$) in the F_2 population (Figure 1; Table 2). These SNP were distributed over 3 QTL regions located on *Bos taurus* chromosome 17 (BTA17). These QTL regions were defined based on the average LD between markers at the BTA17 and the LD between significant SNP.

Several QTL for RT single records have also been reported on other chromosomes. In purebred cattle (Angus, Simmental, and Piedmontese), QTL were identified on BTA1, 8, 10, 11, 12, 20, 22, 23, 25, 26, and 27 (Howard et al., 2014). However, the same authors annotated genes related to heat stress on BTA10, 12, 22, 23, and 25 only. Porto-Neto et al. (2014) identified significant SNP associated with RT on BTA6 and 17 in a Brahman and tropical composite population. In a GWAS for RT single records evaluated at different THI in heat-stressed Holstein animals, Dikmen et al. (2013) identified QTL on BTA4, 5, 16, 24, and 26, using the single-step genomic BLUP approach. In a subsequent study, based on previously selected SNP related to thermotolerance, Dikmen et al. (2015) identified signifi-

Figure 1. Manhattan plot from the association analysis of variation of rectal temperature (ΔRT) in the experimental Gir $\times$ Holstein F_2 population. Significant SNP ($q \leq 0.05$) are shown as blue triangles, and the gray dots denote SNP that were not significant in genome-wide association studies.

cant SNP associated with RT single records on BTA4, 6, and 24. Based on selected SNP located in coding regions of genes previously associated with reproduction, production or health traits, the same authors identified significant SNP associated with RT single records on BTA6, 7, 9, 10, 11, 15, 17, 18, 22 and 25. Because the regions on BTA17 were shown to be associated with RT in the current study as well as in previous studies, and also because these regions contain candidate genes that have been shown to be involved in heat stress responses (Porto-Neto et al., 2014; Dikmen et al., 2015), it is possible that these BTA17 regions may have a role in cattle heat stress.

Candidate Genes

In the current study, 54 genes encoding proteins with different functions were annotated for ΔRT . The gene sets were grouped according to their GO terms enrichment in a GO network to better understand their functions (Figure 2). In this network, we identified genes involved in Janus kinase/signal transducers and activators of transcription (**JAK/STAT**) pathway, the interleukin 6 family cytokine (**LIF**), oncostatin M (**OSM**), the *DGCR8* microprocessor complex subunit (*DGCR8*), and a gene linked to the response to oxygen radical biological process, thioredoxin reductase 2 (*TXNRD2*). The *OSM* and *LIF* genes are cytokines that belong to the interleukin 6 (IL6) family, and they are closely related to the structures and functions of this cytokine family (Tanaka and Miyahima, 2003). Upon binding to cell surface receptors, IL6-type cytokines activate members of the JAK tyrosine kinase family, resulting in the activation of members of the STAT TF family, which are involved in the JAK/STAT pathway (Hong et al., 2013). During heat shock, this signaling pathway can be quickly activated by OSM and LIF cytokines, aiding in reduction of the effects stimulated by heat shock as well as in the regulation

of the expression of heat shock protein (**HSP**) genes *Hsp70* and *Hsp90* (Chatterjee et al., 2007; Allegra et al., 2011; Stephanou and Latchman, 2011). In particular, STAT III shows important biological functions associated with apoptosis control, cell survival, and activation and inhibition of the immune and inflammatory responses (Loor, 2010).

The *DGCR8* gene, together with nuclear RNase III enzyme Drosha, comprise the microprocessor complex, which is essential in the processing of microRNA in animals (Triboulet et al., 2009; Faller et al., 2010). According to Knuckles et al. (2017), *DGCR8* and the N6-adenosine-methyltransferase *Mettl3* are reallocated to heat-shock genes under acute temperature stress. They work in consonance to mark *Hsp70* mRNA, allowing subsequent RNA degradation to control the timing and magnitude of the heat-shock response. Previous studies described a significant increase in the protein expression levels of HSP in cattle subjected to heat stress (Belhadj Slimen et al., 2016; Hu et al., 2016). Clearing of HSP after heat stress is very important, to avoid accumulation of the proteins and presumably worse recovery after stress (Knuckles et al., 2017).

The *TXNRD2* gene, together with the thioredoxin 2 (*TRX2*) and peroxiredoxin 3 (*PRX3*) genes, comprise the mitochondrial thioredoxin system, which provides a primary line of redox regulation through high control over reactive oxygen species (**ROS**) emission from mitochondria (Stanley et al., 2011; Yoshioka, 2015), which is a central source of H_2O_2 . Animals exposed to heat stress show an increase in RR, and consequently H_2O_2 production exceeds its scavenging, leading to oxidative stress (Bernabucci et al., 2002; Srikandakumar and Johnson, 2004). Thioredoxin reductase 2 (*TRXR2*) supplies nicotinamide adenine dinucleotide phosphate (NADPH) electrons to *PRX3*, which controls H_2O_2 levels and establishes mitochondrial redox homeostasis (Stanley et al., 2011; Aon et al., 2012). Inhibition of *TRXR2* leads to impaired redox homeostasis and re-

Table 2. Significant SNP located on chromosome 17 associated with variation of rectal temperature (ΔRT) in an experimental Gir $\times$ Holstein F_2 population evaluated for heat stress response

SNP	QTL ¹	Pos ²	Effect ³	$-\log_{10}(P\text{-value})$	$q\text{-value}$ ⁴	MAF ⁵
BTA.41874.no.rs	1	71,165,342	0.15	5.45	0.05	0.33
ARS.BFGL.NGS.78729	1	71,231,159	0.15	5.43	0.05	0.34
ARS.BFGL.NGS.10248	1	71,260,851	0.15	5.43	0.05	0.34
ARS.BFGL.NGS.58770	2	74,292,319	0.18	5.75	0.01	0.24
ARS.BFGL.NGS.24012	3	74,948,921	0.17	5.44	0.03	0.27
ARS.BFGL.NGS.18349	3	74,998,349	0.17	5.44	0.03	0.27

¹QTL region where the significant SNP is located.

²Position on the chromosome (in Mbp).

³Allele substitution effect.

⁴False discovery rate-based q -value.

⁵Minor allele frequency.

Figure 2. Functional networks showing gene interactions (triangles nodes) related to variation of rectal temperature (ΔRT) and the relationship across genes and their sub-networks related to positive regulation of tyrosine phosphorylation of signal transducer and activator of transcription family 3 (STAT 3) protein in the experimental Gir $\times$ Holstein F₂ population. JAK = Janus kinase.

sults in increased levels of H_2O_2 , endangering cell functions (Prasad et al., 2014).

The same gene set was used to perform the analysis of regulatory sequences, through which, based on the biological process and literature review, we were able to identify 3 heat stress-associated TF: nuclear factor kappa B (**NF- κ B**), aryl hydrocarbon receptor nuclear translocator (ARNT), and STAT III (Table 3). A gene-TF network was generated with the 3 selected TF and

genes that show potential binding sites for these TF (Figure 3).

The most enriched TF was NF- κ B, linked to the highest number of genes. The response to heat shock has been implicated in the negative regulation of the NF- κ B signaling pathway (Yenari et al., 2005). According to Belardo et al. (2010), the effects of hyperthermic treatment are related to increase in temperature above physiological conditions and duration of exposure.

Table 3. Enriched transcription factors (TF) associated with genes identified for cattle variation of rectal temperature (ΔRT), based on gene ontology biological process and literature review; data obtained from an experimental Gir $\times$ Holstein F₂ population evaluated for heat stress response

TF ¹	Biological process	Literature review ²
ARNT	Regulation of homeostatic process	Involved in negative regulation of necrosis; can be regulated by heat shock proteins before and after hypoxia (Belenichev et al., 2011)
NF- κ B	Anti-apoptosis	Heat stress can inhibit activity of <i>NF-κB</i> and induce massive apoptosis (Belardo et al., 2010)
STAT III	Homeostatic process	Cell survival regulator (Terui et al., 2004) that participates in intracellular ROS homeostasis (He et al., 2010; Li et al., 2010)

¹ARNT = aryl hydrocarbon receptor nuclear translocator; NF- κ B = nuclear factor kappa B; STAT III = signal transducer and activator of transcription family 3.

²Cited studies are just a sample of the vast available literature.

Figure 3. Gene-transcription factor (TF) network: genes located in the QTL regions associated with variation of rectal temperature (ΔRT ; green circle nodes) and their associated TF (yellow octagon nodes). Node size corresponds to the network analyses (Cytoscape; Shannon et al., 2003) in which larger nodes denote a higher edge density associated with the number of TF binding sites. Blue square nodes show the gene ontology biological processes related to TF. Data were obtained from an experimental Gir $\times$ Holstein F_2 population evaluated for heat stress response. ARNT = aryl hydrocarbon receptor nuclear translocator; NF- κ B = nuclear factor kappa B; STAT III = signal transducer and activator of transcription family 3.

These authors observed that heat stress induced an inhibition of constitutive NF- κ B activity in HS-Sultan cells, a B-cell lymphoma directly related to temperature increase, in addition to inhibiting the activity of NF- κ B and inducing massive apoptosis in other types of aggressive B-cell neoplasms Belardo et al. (2010). In a study regarding crosstalk among cytokines and induced hyperthermia using genomic approaches, Janus et al. (2015) found a substantial overlap among the set of genes potentially regulated by heat shock protein or heat shock transcription factor 1 (HSFI) and the set of genes potentially regulated by tumor necrosis factor alpha (TNF α) or NF- κ B. This allowed the identification of predictable inhibitory effects of heat shock on the expression of classical NF- κ B target genes and new patterns of activation (or co-activation) related to responses to different types of stress and hormonal stimuli. In addition, it helped to identify repression (or co-repression) in subsets of genes associated with processes mediated by NF- κ B signaling and humoral immune responses, as well as chemokine and cytokine responses. Thus, pleiotropic effects of heat stress on the regulation of NF- κ B-dependent genes should be expected.

The ARNT TF, also known as hypoxia inducible factor 1 subunit beta (HIF1B), is a subunit of transcrip-

tion factor hypoxia-inducible factor 1 (*hif-1*; Ahn et al., 2013), a key regulator of the developmental and physiological networks required for the maintenance of O₂ homeostasis. Past studies have found that *hif-1* plays an important role in preventing overproduction of mitochondrial ROS in hypoxia conditions (Semenza, 2011). After preconditioning at intermediate temperatures, HSFI promotes survival under extreme thermal stress (Kourtis et al., 2012), and according to Horowitz and Assadi (2010) its expression increased after heat acclimation-mediated cross-tolerance. Meanwhile, ARNT is involved in the negative regulation of necrosis and can be stabilized and have its expression regulated as well as its lifetime increased by heat shock proteins before and after hypoxia (Belenichev et al., 2011).

The less enriched TF in the gene-TF network (Figure 3), STAT III regulates the expression of *Hsp70* and *Hsp90* genes, as previously described; it is also an important regulator of cellular survival after apoptotic stimuli (Terui et al., 2004) and directly protects cells from oxidative stress (Li et al., 2015). This TF is sensitive to intracellular oxidants and can be activated in response to ROS accumulation. Furthermore, it participates in intracellular ROS homeostasis and shows an inverse relationship with NF- κ B (He et al., 2010; Li et al., 2010).

From the gene-TF network, candidate genes for Δ RT were highlighted based on TFBS. The *LIF*, *DGCR8*, and *TXNRD2* genes, which also share ontology in the GO enrichment analysis, are part of the most enriched gene group in this network (Figure 3).

Breed of Origin of Alleles for Candidate Genes

Based on the GO and gene-TF networks, we were able to select 4 candidate genes to assess the origin of alleles associated with ΔRT in cattle. Considering the LD between SNP and genes, the physical positions of *LIF*, *OSM*, *TXNRD2*, and *DGCR8* genes were annotated and used as coordinates for selection of adjacent SNP. We selected a total of 7 SNP: 2 associated with the *LIF* and *OSM* genes, 3 associated with the *TXNRD2* gene, and 2 associated with the *DGCR8* gene. The animals were grouped as low ΔRT or high ΔRT based on their genomic breeding values for ΔRT , and the alleles of the selected SNP were classified and quantified according to their breed of origin (HH, GG, or HG/GH).

Based on chi-squared test results obtained from low- and high- Δ RT F₂ animals, we observed that all selected SNP for allele origin were significant ($P < 0.05$) in both evaluated groups (Table 4) and showed a deviation of the expected gametic segregation for each genotypic class: 1HH to 2HG/GH to 1GG. These results suggest a prevalence of alleles from one breed in the low- and

Table 4. Percentage of individuals classified by breed of origin of each SNP allele located in the candidate genes (*LIF*, *OSM*, *TXNRD2*, and *DGCR8*) associated with variation of rectal temperature (Δ RT) in low- and high- Δ RT animals from an experimental Gir $\times$ Holstein F₂ population evaluated for heat stress response¹

Allele	<i>LIF/OSM</i>		<i>TXNRD2</i>			<i>DGCR8</i>	
	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7
Low- Δ RT animals							
HH ²	55.3	55.3	51.4	51.4	51.4	54.5	51.4
GG ³	7.9	7.9	5.7	5.7	5.7	6.1	5.7
HG/GH ⁴	36.8	36.8	42.9	42.9	42.9	39.4	42.9
High- Δ RT animals							
HH	10.0	10.0	0.0	0.0	0.0	0.0	0.0
GG	52.5	52.5	48.1	48.1	50.0	48.1	50.0
HG/GH	37.5	37.5	51.9	51.9	50.0	51.9	50.0

¹All SNP are significant for allele origin in the chi-squared test ($P < 0.05$).

²Both alleles originated from Holstein breed.

³Both alleles originated from Gir breed.

⁴One allele originated from Holstein and one from Gir breed.

high- Δ RT groups. On average, 53% of low- Δ RT animals had 2 alleles from the Holstein breed, whereas 6.4% had 2 alleles from the Gir breed. On the other hand, in high- Δ RT animals, the average proportion of animals for HH and GG were 2.9% and 49.9%, respectively. Based on these results, we can observe that Holstein alleles are more frequent in low- Δ RT animals, and Gir alleles are more abundant in high- Δ RT animals.

It is known that *Bos indicus* animals have a greater ability to regulate their body temperature, which helps in heat stress prevention (Hansen, 2004; Cattelan and Martinez, 2013) compared with *Bos taurus* animals, which are affected by heat stress (Renaudeau et al., 2012). In the heat chamber, F₂ animals were subjected to high temperatures and RH, which may have prejudiced the evaporation mechanisms, responsible for 80% of body heat loss to the environment (Shearer and Beede, 1990; West, 2003), and favored the initiation of heat stress. Given that the genes identified in our study play important functions in controlling the effects of current heat stress, and not heat stress prevention per se, it is reasonable to assume that the significant SNP alleles were derived from the Holstein breed, which requires a more efficient genetic architecture than Gir animals to defend the body from deleterious effects of heat stress.

CONCLUSIONS

In summary, the Δ RT trait is a relevant phenotype for individual evaluation of heat stress response. A GWAS allowed the identification of significant SNP associated with Δ RT in the experimental F₂ Gir $\times$ Holstein population. Gene-TF networks and GO enrichment analyses allowed identification of candidate genes (*LIF*, *OSM*, *TXNRD2*, and *DGCR8*) that are biologically related

to heat stress response. According to BOA analysis, Holstein breed alleles could be associated with a more complex response to heat stress effects, which can be explained by the fact that Holstein animals are more affected by heat stress than Gir animals, requiring an intricate genetic architecture to defend the body from the deleterious effects of heat stress. Future studies are needed to further our understanding of the genetic architecture underlying the heat stress response in crossbred cattle.

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