

Article

Thermotolerance-Related SNPs and Heat Stress Effects on Productive Performance and Gene Expression in Holstein Cows

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Abstract

Heat stress (HS) is a major issue affecting productivity and physiological responses in dairy cattle. Advances in gene function research have made it possible to identify favorable genotypes for HS tolerance. This study evaluates the combined effects of temperature–humidity index (THI) and genomic load of favorable SNPs in the genes *GRM8*, *SMAD3*, and *TLR4* on daily milk yield (DMY), rectal temperature (RT), and gene expression in Holstein cows ($n = 160$). Environmental conditions ranged from thermoneutral to severe HS, allowing the assessment of genotype by environment interactions. A subset of cows ($n = 40$) was selected based on genomic load to evaluate gene expression under opposite thermal conditions. Results showed that increasing THI was associated with reduced DMY and increased RT, with more pronounced effects in cows carrying fewer favorable genotypes. Slope analyses revealed that cows with higher genomic load exhibited a moderate decline in DMY and lower increase in RT, indicating enhanced resilience to HS. Significant THI $\times$ genomic load interactions were detected above a threshold of 79 units ($p < 0.05$). Gene expression analyses supported these findings, showing differential expressions of *GRM8*, *SMAD3*, and *TLR4* under HS conditions. The functional and predictive value of these genes as markers of thermotolerance could help producers by supporting their application in genetic selection programs, thus providing another tool to combat HS and improve resilience and productivity in dairy systems.

Keywords: dairy cattle; heat stress; genomic load; SNPs; thermo-tolerance



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1. Introduction

Heat stress (HS) in dairy cattle is a physiological condition that occurs when cows are unable to dissipate excess body heat under high environmental temperature and humidity, resulting in the disruption of thermoregulatory balance [1,2]. When cows are exposed to thermally challenging climates, several animal body responses can indicate the onset of HS, including increased heart rate, elevated respiratory rate, and higher body temperature [3]. HS also induces a reduction in feed intake as a physiological response to decrease metabolic heat production [4]. However, this metabolic suppression negatively affects milk yield and composition, growth, feed efficiency, fertility, and animal welfare [5,6], resulting in

substantial economic losses [7]. Consequently, HS represents one of the most significant challenges limiting dairy cattle production worldwide.

Climate change has emerged as a major threat to the sustainability of livestock production systems, particularly in subtropical and arid regions [8,9]. In the state of Sonora, a warm and semiarid region from Northern Mexico, environmental conditions have shown a sustained increase in the frequency of heat days and heat waves, driven by rising temperatures and reduced precipitation. These trends are projected to persist through 2060, with implications for agriculture, animal production, and public health [10]. In addition, limited water availability and constrained access to reliable electrical power in this region restrict the implementation of conventional heat abatement strategies such as evaporative cooling and ventilation systems, increasing the vulnerability of dairy systems to HS.

In dairy cows, HS is typically quantified using the THI, which integrates environmental temperature and humidity [11]. Although dairy cows can maintain productivity within a certain THI range, once a critical threshold is surpassed, their ability to regulate body temperature becomes compromised [12]. Significant reductions in milk yield have been reported when cows are exposed to elevated THI values, demonstrating the high sensitivity of dairy cattle to HS conditions [13–15]. However, individual animals differ in their response to HS, suggesting the existence of a genetic component underlying thermotolerance. In this regard, single nucleotide polymorphisms (SNPs) associated with productive and physiological responses to HS in dairy cattle have been detected [16,17]. These findings support the use of molecular markers as a potential tool for selecting animals with enhanced capacity to cope with challenging thermal environments [18]. Therefore, studying the genetic basis of heat tolerance can support the selection of dairy cattle capable of maintaining high productivity under HS conditions [16].

Several studies have identified genes associated with thermotolerance and productive responses in Holstein cattle, reinforcing the importance of genetic factors in adaptation to HS conditions. Fang et al. [19] reported significant associations of *TRH*, *PRL*, and *PRLR* genotypes with milk yield, reproductive performance, and HS resilience. Similarly, Shi et al. [20] identified *HCRTR1*, *AGRP*, *PC*, and *GUCY1B1* as candidate genes associated with thermotolerance through genotype-by-environment interactions. In addition, Xu et al. [21] and Sigdel et al. [16] reported differential expression of genes related to cellular stress response and milk production under HS conditions, supporting the potential use of molecular markers for the genetic improvement of heat tolerance in dairy cattle. Together, these studies support the concept that functional genetic variation contributes to physiological and productive adaptation to HS. Nevertheless, the biological mechanisms involved in thermotolerance and the cumulative effects of favorable alleles remain incompletely understood.

In a previous study from our group, genome-wide association analyses (GWAS) combined with associative studies of marker SNPs at specific genes allowed to identify three candidate SNPs associated with adjusted 305-day milk yield in Holstein cows exposed to HS [22]. Such SNP markers were rs43410971, rs382039214, and rs8193046, which were located within the genes *GRM8* (glutamate metabotropic receptor 8), *SMAD3* (SMAD family member 3), and *TLR4* (toll-like receptor 4), respectively. Building on those findings, the present study focused on novel analyses that have not been previously published. Specifically, we evaluated the slopes of change in DMY and RT under HS, as well as differential gene expression according to the load of favorable SNP alleles. These analyses allowed us to explore how the cumulative genetic load influences physiological and productive responses to HS, providing additional insights on gene functionality beyond the predictive associations previously reported.

Therefore, the objective of this study was to evaluate the combined effects of temperature–humidity index (THI) and cumulative genomic load of selected SNPs in the genes *GRM8*, *SMAD3*, and *TLR4* on daily milk yield, rectal temperature and gene expression in Holstein cows under HS environmental conditions.

2. Materials and Methods

The Institutional Animal Care and Use Committee of the Instituto Tecnológico de Sonora approved all procedures performed on animals (approval code 2024-06).

2.1. Location and Experimental Design

The present study was performed during summer (11 June through 12 August) in the Academic Research Unit in Dairy Production of the Instituto Tecnológico de Sonora (ITSON), located in Block 910 of the Yaqui Valley, Sonora in northwest Mexico. The geographical coordinates are 27°21'49.0" North and 109°54'47.4" West. Climatic conditions showed variations throughout the year in temperature (−2.5 to 45.9 °C), relative humidity (25 to 90%), and solar radiation (0.05 to 761.7 W/m²). The site has an altitude of 46 m above sea level and average annual precipitation of 371.6 mm ranging from 3 to 90 mm on rainy days.

The study included 160 lactating Holstein cows with 94 ± 6.1 days in milk, 3 to 6 years old, average body weight of 667.5 ± 39.8 kg, and average body condition of 3.5 ± 0.01 points (where 0 = emaciated and 5 = obese). Cows were housed in shaded facilities with unrestricted access to a water source and a commercially available trace mineral supplement. Flooring and shading provisions were implemented according to the specific requirements of Holstein cows. Cows were provided with a mixed ration twice daily, formulated based on an average body weight of 650 kg and an expected milk yield of 30 kg per day, with an average composition of 3.5% fat and 3.2% true protein.

2.2. Phenotypic Traits

Milk production was measured during milking twice a day (0500 h and 1700 h), using an electronic weighing system with a scale from 0 to 40 kg (Metatron 21, Westfalia Surge, México, S.A. de C.V., Aguascalientes, Mexico) integrated into the milking parlor. Daily milk yield (DMY; kg) was calculated from the production recorded at each milking; two milk production records per cow per day were obtained and used to determine daily production values during the study period.

Rectal temperature (RT; °C) was recorded twice a week at 0700 h and 1700 h using a digital thermometer (TES-1310®, TES Electrical Electronic Corp., Taiwan, China) equipped with a type K contact probe (9 cm length), which was inserted to ensure contact with the rectal mucosa. The dataset included a total of 19,840 records from DMY (2 daily records $\times$ 62 days $\times$ 160 cows) plus 5760 physiological records (4 weekly records $\times$ 9 weeks $\times$ 160 cows).

2.3. Climatic Data and Temperature–Humidity Index (THI)

Ambient temperature (AT; °C) and relative humidity (RH; %) were recorded using weather meters (DROP D2AG—Livestock Heat Stress Monitor; Kestrelmeters, 21 Creek Circle, Boothwyn, PA, USA), which were placed on a support column for the roof of the shade at a height of 2.73 m. The sensors were programmed to collect data every 10 min and were installed on hooks away from the metal column to prevent the sensor from touching the metal column and ceiling directly. The sensor has an accuracy of ± 0.5 °C and $\pm 2\%$ RH with resolutions of 0.1 °C and 0.1% RH.

THI was calculated using the formula $THI = (1.8T + 32) - [(0.55 - 0.0055RH)(1.8T - 26)]$ [23]. THI values reported in the results represent the mean daily (24 h)

thermal load experienced by the animals. The degree of existing HS was classified as follows: absence (<68), threshold (68 to 72), moderate (73 to 79), and severe (>79 units) using the scale established by Zimbelman et al. [24].

2.4. SNP Marker Selection

As mentioned earlier, the SNPs rs43410971, rs382039214, and rs8193046 were previously reported by Zamorano-Algandar et al. [22] as predictors for milk production. In addition, these markers were also associated with physiological traits such as rectal temperature and respiratory rate, which are supposed to be traits indicative of thermo-tolerance because the cows were exposed to summer HS during middle lactation. Therefore, in order to confirm their functional ability as thermotolerance markers in Holstein cows, the SNPs rs43410971, rs382039214, and rs8193046 in the genes *GRM8*, *SMAD3* and *TLR4*, were evaluated in cows exposed to increasingly stressful environmental conditions observed during the summer period.

2.5. SNP Genotyping Assay

A vacutainer tube with anticoagulant EDTA (EDTA-Na₂; Venoject[®], Terumo, Lakewood, CA, USA) was used to collect blood samples (3 mL) from each cow by puncturing the jugular vein. The samples were transported to laboratory facilities at ITSON and centrifuged at 3500 rpm for 30 min. An electronic pipet was used to remove 200 µL of the leukoplatelet layer, which were refrigerated at −20 °C. Then, a commercial kit (DNeasy Blood & Tissue Kits; QIAGEN, Hilden, Germany) was used for DNA extraction according to manufacturer's instructions. A NanoDrop automated spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure both concentration and purity of the DNA.

The TaqMan method for allelic discrimination and RT-qPCR (StepOne[™], Applied Biosystems, Foster City, CA, USA) were performed to genotype the SNPs rs43410971, rs382039214, and rs8193046 following the procedures described by Castillo-Salas et al. [25]. The PCR was carried out using the StepOne Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Genotype calling and analysis of PCR data were performed using the StepOne software v2.3 (Life Technologies Corporation, Carlsbad, CA, USA).

2.6. Slope Prediction for DMY and RT

A mixed effects model with repeated measures was used to predict DMY and RT as a function of the THI, in order to calculate the slopes of change under HS conditions according to the genomic load. The mixed model equation was:

$$Y_{ijklmn} = \mu + A_i + \beta_0 B_j + \beta_1 (B_j \times A_i) + C_k + D_l + E_m + F_n + e_{ijklmn}, \quad (1)$$

where Y_{ijklmn} was the response variable (i.e., DMY, RT), μ was the overall mean, A_i was the fixed effect of the genomic load group (cumulative number of favorable SNP genotypes), B_j was the continuous covariate of THI, and $(A_i \times B_j)$ represented the interaction term. The model also included the fixed effects of body condition score (C_k), parity (D_l), and time (E_m) to account for summer progression, as well as the random effect to account for within-cow correlation (F_n), and e_{ijklmn} as the residual error. Also, β_0 was the intercept coefficient, and β_1 was the regression coefficient. Repeated observations over time were modeled using an autoregressive covariance structure (AR(1)).

2.7. Gene Expression (mRNA) Assay

A subset of 40 cows was sampled for gene expression analysis. Total RNA was extracted from peripheral blood leukocytes obtained from whole blood samples. These

samples were selected for their minimally invasive collection and their ability to reflect dynamic transcriptional responses to environmental challenges. Blood samples were collected at 1500 h to capture the peak effect of HS, after approximately 10 h of exposure to high ambient temperatures, ensuring that gene expression reflected sustained rather than transient responses. Sampling included thermoneutral conditions at baseline (no HS) at the beginning of the study period (11 June) and moderate to severe HS conditions during the middle of the experimental period (12 July).

Cows were selected based on the number of favorable genotypes they carried (0, 1, 2, or 3), with 10 animals per category to ensure balanced representation. Then, cows carrying one or more favorable SNP genotypes ($n = 30$) were classified as the favorable group, while cows without favorable SNP genotypes ($n = 10$) were considered the unfavorable group. To minimize confounding effects on gene expression, animals were selected to be as similar as possible in terms of days in milk, parity, and management conditions. Additionally, samples were processed in a randomized manner to control for potential batch and plate effects during qPCR analysis.

The identified three SNPs, as well as the recognized thermo-tolerance gene *HSP70*, were further validated at the gene expression level using quantitative polymerase chain reaction (qPCR). Oligonucleotide primers were designed based on the *Bos taurus* genome using Primer-BLAST v2.7.0, and optimal annealing temperatures and primer efficiencies were established through PCR optimization. Primer specificity was verified by sequencing PCR amplicons cloned into the PCR 2.1-TOPO vector (Thermo Fisher Scientific Life Sciences, Waltham, MA, USA). Complementary DNA (cDNA) was synthesized from 0.4 µg of total RNA per reaction using SuperScript III (Thermo Fisher). Quantitative PCR was conducted using SYBR Green chemistry (Qiagen, Valencia, CA, USA) on an iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Gene expression levels were normalized to the geometric mean of the ribosomal protein S15 reference gene. Relative expression was calculated using the comparative Ct approach, and fold changes were calculated using the $2^{-\Delta\Delta C_t}$ method. All reactions were performed in triplicate to ensure measurement reliability.

2.8. Statistical Analysis

All statistical procedures were performed using the SAS software (Version 9.4; SAS Inst. Inc., Cary, NC, USA). The UNIVARIATE and GLM procedures were used to evaluate the assumptions of normality and homogeneity of variances, respectively. Average mean values for phenotypic traits were calculated with PROC MEANS.

Both DMY and RT were analyzed using a mixed linear model. The statistical model was $Y_{ijklm} = \mu + A_i + B_j + C_k + D_l + E_m + e_{ijklm}$, which included the traits DMY and RT as response variables (Y_{ijklm}). The target fixed effect was the genetic load (A_i), defined as the effect of specific combinations of favorable SNP genotypes across the studied genes (*GRM8*, *SMAD3*, *TLR4*). The model also included the fixed effects of body condition score (B_j), and parity (C_k), the linear covariate of days in milk (D_l), the cow as a random effect (E_m), as well as the residual error term (e_{ijklm}). If the genetic load was confirmed as significant source of variation ($p < 0.05$), then the LSMEANS procedure was performed to compare the average phenotypic values among gene combinations.

A similar mixed-effects model, $Y_{ijklm} = \mu + A_i + B_j + C_k + D_l + E_m + e_{ijklm}$, was used to evaluate gene expression under two thermal scenarios (thermoneutral and heat stress) as main effect (A_i). Body condition score (B_j) and parity (C_k) were incorporated as fixed effects, and days in milk as a linear covariate (D_l), the cow as a random effect (E_m), and the residual error term (e_{ijklm}). Additionally, a Pearson correlation analyses was performed to assess the degree of association between gene expression and the productive

and physiological traits DMY and RT under both thermal scenarios. Statistical differences were declared significant at $p < 0.05$, and highly significant at $p < 0.01$.

3. Results

3.1. Climate and Cow Performance

Environmental conditions and productive performance indicators were monitored throughout the study period to characterize the progression of thermal stress and its physiological impact on the cows. In this regard, Table 1 summarizes the climatic data, as well as the productive and physiological traits recorded during the study. THI increased from 69.4 to 81.7 units, confirming that thermal conditions progressed from thermoneutrality to moderate and severe heat stress (HS).

Table 1. Mean, standard error (SE), minimum (Min), maximum (Max), and quartiles (25 Pctl, 50 Pctl, and 75 Pctl) values for environmental variables, daily milk yield, and rectal temperature in lactating Holstein cows managed during the summer.

Variable	Mean $\pm$ SE	Min	Max	25 Pctl	50 Pctl	75 Pctl
Ambient temperature ($^{\circ}\text{C}$)	27.81 $\pm$ 0.30	23.7	31.6	25.2	28.6	30.0
Relative humidity (%)	54.05 $\pm$ 1.24	34.5	76.1	47.9	56.7	59.4
THI (units)	76.02 $\pm$ 0.27	69.4	81.7	72.0	77.1	79.0
Daily milk yield (kg)	20.91 $\pm$ 0.26	12.0	27.0	18.0	21.3	23.5
Rectal temperature ($^{\circ}\text{C}$)	38.28 $\pm$ 0.05	36.8	39.7	37.6	38.4	38.8

3.2. Combined Effect of THI and Genotype on Daily Milk Yield (DMY)

As summer progressed, the increasing THI led to a rapid transition from moderate to severe heat stress conditions, which appeared to be associated with a steady decline in DMY (Figure 1).

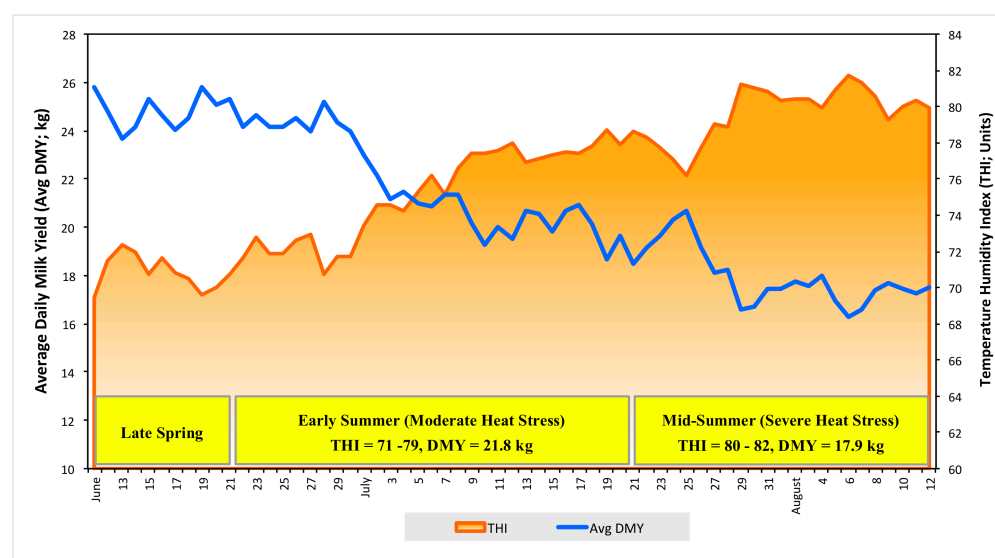


Figure 1. Average values for daily milk yield and temperature–humidity index during the experimental period (11 June through 12 August).

The decline in DMY was more pronounced as the number of SNP markers with favorable genotype decrease, and this relationship between the genotype and the surrounding environment was more evident once the THI reached and surpassed 79 units. Above this THI threshold, an interaction effect between the THI and the number of favorable SNP genotypes on DMY was detected ($p < 0.05$; Figure 2).

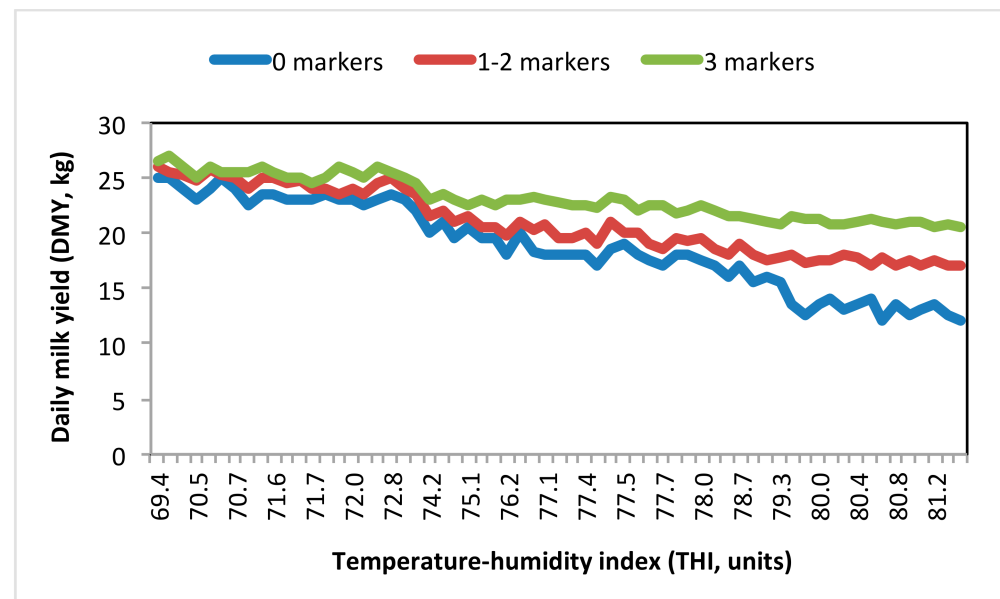


Figure 2. Daily milk yield according to the THI during the experimental period for Holstein cows with 0, 1–2 or 3 favorable SNP markers from the genes *GRM8*, *SMAD3*, and *TLR4*.

3.3. Combined Effect of THI and Genotype on Rectal Temperature (RT)

In contrast to DMY, RT showed a steady increase as summer progressed. As the THI intensified, HS increased from moderate to severe (Figure 3), which was also reflected in a progressive elevation of RT.

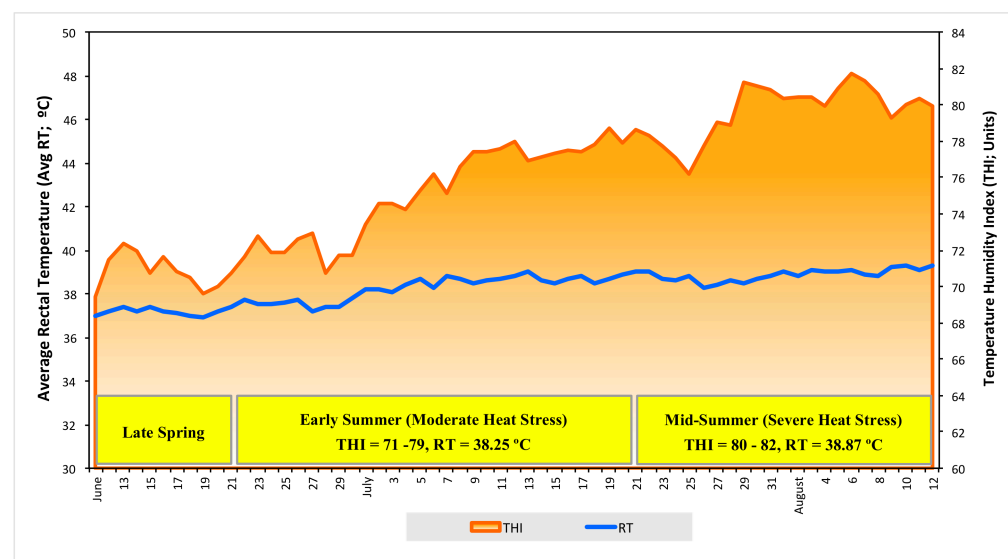


Figure 3. Average values for daily rectal temperature and temperature–humidity index during the experimental period (11 June through 12 August).

The increase in RT was less pronounced as the number of favorable SNP genotypes increased, suggesting a moderate physiological response in animals with greater genetic advantage. This genotype–environment relationship became slightly more evident once THI exceeded 79 units. Above this threshold, an interaction between THI and the number of favorable SNP genotypes on RT was observed ($p < 0.05$) (Figure 4).

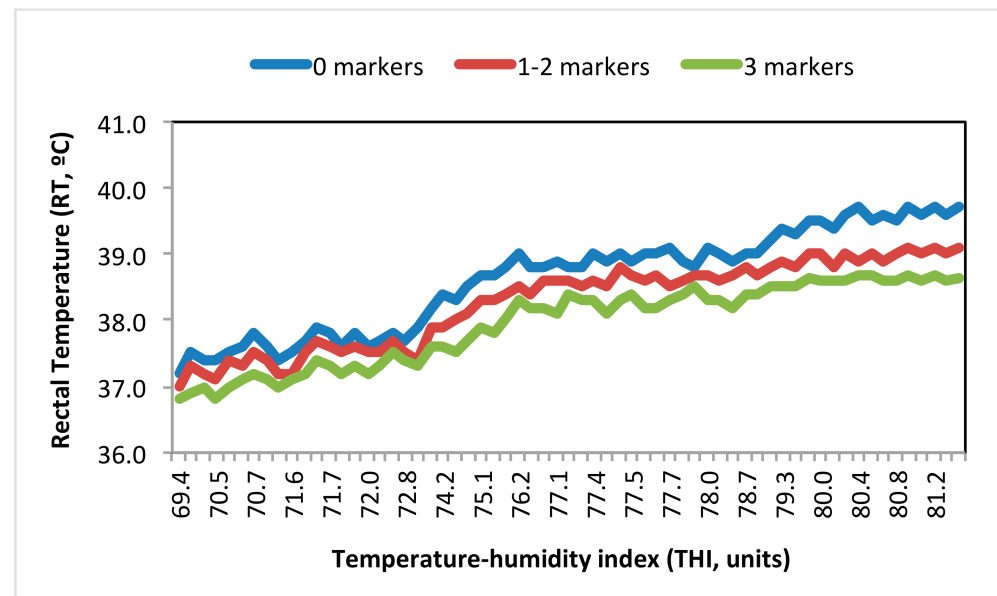


Figure 4. Rectal temperature according to the THI during the experimental period for Holstein cows with 0, 1–2 or 3 favorable SNP markers from the genes *GRM8*, *SMAD3*, and *TLR4*.

3.4. Predictive Analyses of SNP Contribution

Slopes' prediction for daily milk yield and rectal temperature according to genomic load was performed when THI was ≤ 79 and when THI exceeded 79 units (Table 2). The slopes for DMY were significantly lower as genomic load decreased ($p < 0.01$). Notably, when THI exceeded 79 units, the slope for cows lacking favorable genotypes declined even further, indicating a more pronounced reduction in milk production under severe HS conditions. In contrast, the decline in slopes observed in cows carrying one or more favorable genotypes was markedly attenuated, suggesting a greater resilience to increasing thermal load.

Table 2. Slope analysis for DMY and RT according to genetic load (i.e., number of SNPs from the genes *GRM8*, *SMAD3*, and *TLR4* with the favorable genotype) in Holstein cows exposed to HS under two thermal scenarios.

Trait	NFG	THI ≤ 79		THI > 79	
		Slope (β_1)	p -Value	Slope (β_1)	p -Value
DMY	0	−0.913	<0.01	−1.184	<0.01
	1–2	−0.826	<0.01	−0.215	<0.01
	3	−0.509	<0.01	−0.166	<0.01
RT	0	0.203	<0.01	0.207	<0.01
	1–2	0.188	<0.01	0.099	<0.01
	3	0.177	<0.01	0.057	<0.01

NFG = number of favorable SNP genotypes; β_1 = slope of the regression line; p -value = statistical significance.

A similar trend was observed for rectal temperature, as slopes decreased with increasing genomic load. However, when THI exceeded 79 units, the slope increased slowly in cows lacking favorable genotypes, whereas it decreased markedly as the number of favorable genotypes increased.

The 3 genes included in the current study showed a beneficial contribution to the phenotypic traits DMY and RT, with both traits improving as genomic load increased; a more pronounced effect was observed when the *TLR4* gene was present (Table 3).

Table 3. Least square means for productive and physiological performance according to the number of SNPs from the genes *GRM8*, *SMAD3*, and *TLR4* with the favorable genotype in Holstein cows exposed to HS.

NFG	Genes	Daily Milk Yield		Rectal Temperature	
		<i>n</i>	Mean $\pm$ SE	<i>n</i>	Mean $\pm$ SE
0		20	17.01 $\pm$ 0.75 ^a	20	39.12 $\pm$ 0.10 ^a
1	<i>GRM8</i>	20	17.29 $\pm$ 0.63 ^b	20	38.94 $\pm$ 0.08 ^a
1	<i>SMAD3</i>	20	17.52 $\pm$ 0.60 ^b	20	38.96 $\pm$ 0.08 ^a
1	<i>TLR4</i>	20	19.27 $\pm$ 0.22 ^c	20	38.63 $\pm$ 0.09 ^b
2	<i>GRM8</i> + <i>SMAD3</i>	20	21.41 $\pm$ 0.55 ^c	20	38.45 $\pm$ 0.07 ^b
2	<i>GRM8</i> + <i>TLR4</i>	20	22.78 $\pm$ 0.29 ^d	20	38.06 $\pm$ 0.07 ^c
2	<i>SMAD3</i> + <i>TLR4</i>	20	22.90 $\pm$ 0.30 ^d	20	38.09 $\pm$ 0.07 ^c
3	<i>GRM8</i> + <i>SMAD3</i> + <i>TLR4</i>	20	23.42 $\pm$ 0.30 ^d	20	37.91 $\pm$ 0.09 ^c

NFG = number of favorable SNP genotypes; ^{a,b,c,d} indicate statistical difference between genetic load groups at $p < 0.05$.

3.5. Quantitative RT-PCR Validation of Gene Expression

Descriptive statistics for Holstein cows included in the gene expression study are presented in Table 4. Statistical values observed in this subset were comparable to those previously reported for the original population of 160 cows from which the subset was derived, confirming the overall homogeneity of the sampled animals.

Table 4. Descriptive statistics (N, mean, standard error) for lactating Holstein cows managed during the summer and included in the gene expression study.

Variable	N	Mean	Standard Error
Daily milk yield (kg)	40	21.32	0.37
Parity	40	2.86	0.24
Weight (kg)	40	658.27	42.51
Body condition score	40	3.26	0.46

The expression patterns of the genes *GRM8*, *SMAD3*, *TLR4*, and *HSP70* differed significantly ($p < 0.05$) in Holstein cows carrying favorable SNP genotypes sampled under thermoneutral and HS conditions (Figure 5). The *HSP70* gene was included as a control due to its well-established sensitivity to thermal stress, allowing us to assess the consistency of expression trends observed in the three candidate genes evaluated in this study. Notably, the expression profiles of the three studied genes showed patterns comparable to that of *HSP70* under HS conditions.

In cows lacking favorable genotypes, no differences were observed in the expression patterns of *GRM8*, *SMAD3*, and *TLR4* between thermoneutral and HS conditions (Figure 6). However, the expression pattern of *HSP70* did differ significantly ($p < 0.05$), showing values very similar to those observed in cows carrying favorable genotypes. These findings suggest that, although the cows were able to establish a general HS response, the absence of differential expression in the candidate genes may reflect a reduced capacity to activate specific mechanisms associated with thermotolerance.

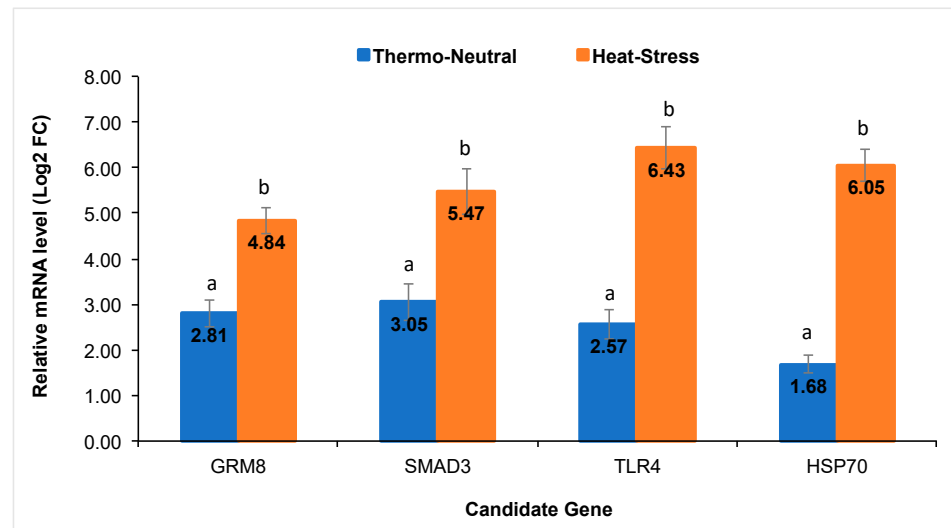


Figure 5. Quantitative real-time PCR (RT-PCR) validation of gene expression (i.e., *GRM8*, *SMAD3*, *TLR4*, and *HSP70*) in cows with favorable SNP genotypes ($n = 1, 2, 3$). Blue bars correspond to the means of mRNA expression calculated as fold change (Log2FC) for cows in thermo-neutral conditions, and brown bars are the means for cows under heat stress. ^{a,b} Indicate statistical difference between groups at $p < 0.05$.

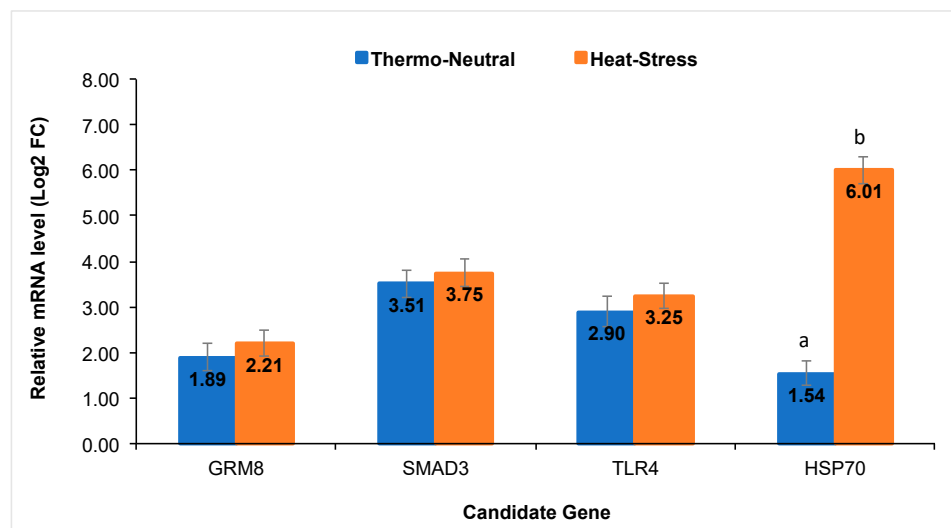


Figure 6. Quantitative real-time PCR (RT-PCR) validation of gene expression (i.e., *GRM8*, *SMAD3*, *TLR4*, and *HSP70*) in cows without favorable SNP genotypes ($n = 0$). Blue bars correspond to the means of mRNA expression calculated as fold change (Log2FC) for cows in thermo-neutral conditions and brown bars are the means for cows under heat stress. ^{a,b} Indicate statistical difference between groups at $p < 0.05$.

3.6. Relationship Between Phenotypic Traits and Gene Expression

Gene expression was significantly associated with DMV ($p < 0.05$) under HS conditions, whereas a significant inverse relationship was observed between gene expression patterns and RT. Notably, *HSP70* exhibited the strongest association among the genes evaluated, followed by *TLR4*. However, under thermoneutral conditions, the strength of these associations decreased, and only *HSP70* and *TLR4* remained significantly associated with DMV and RT (Table 5).

Table 5. Pearson correlation analysis between gene expression patterns with productive and physiological traits in Holstein cows under two thermal scenarios.

Genes	Daily Milk Yield		Rectal Temperature	
	Thermo-Neutral	Heat Stress	Thermo-Neutral	Heat Stress
<i>GRM8</i>	0.198	0.536 *	−0.277	−0.462 *
<i>SMAD3</i>	0.223	0.419 *	−0.218	−0.395 *
<i>TLR4</i>	0.475 *	0.587 *	−0.480 *	−0.514 *
<i>HSP70</i>	0.362 *	0.724 **	−0.409 *	−0.672 **

** values are highly significant at $p < 0.01$; * values are significant at $p < 0.05$.

4. Discussion

Northwest Mexico is a semiarid region characterized by hot summers, prolonged drought periods, and elevated ambient temperature and relative humidity, conditions that favor the onset of HS in cattle. This environment provides a valuable natural setting to investigate the genetic mechanisms underlying HS tolerance in lactating Holstein cows. Furthermore, the observed variability in the response to thermal stress among Holstein cows suggests the existence of a genetic component associated with this trait, highlighting the importance of thermotolerance research. Some previous studies have reported the individual contribution of genes to milk production in Holstein cows managed under HS [19,21,22]. In this context, the present study provides consistent evidence that the combined effect of three genetic markers, together with their expression profiles under HS conditions, supports their functional relevance in thermotolerance.

The descriptive THI values observed in this study confirmed that the cows were exposed to environmental conditions ranging from thermoneutrality to moderate and severe HS throughout the study period. The distribution of THI values, as reflected by intermediate percentiles, indicates that most observations occurred under moderate HS conditions, with a substantial proportion of data points approaching or exceeding the threshold associated with severe HS. The distribution of DMY and RT further reflects the variability in animal responses to HS. For DMY, percentiles suggested that a subset of cows experienced a marked reduction in milk production, whereas most animals maintained production within a narrower range. In contrast, RT percentiles showed a progressive increase in body temperature, with a proportion of cows reaching values consistent with HS. Together, these distributions highlighted substantial inter-individual variation in both productive and physiological responses, supporting the presence of differential resilience to HS and reinforcing the relevance of evaluating genetic factors associated with thermotolerance.

It has been reported that a genetic component influences milk production in dairy cows exposed to HS, suggesting the presence of genes related to thermotolerance [16]. The response or sensitivity to thermal stress in dairy cows appears to be associated with pronounced genetic variations, indicating that specific genomic regions and major genes likely underlie the response to HS [26]. Accordingly, the identification of molecular markers or SNPs associated with thermotolerance has been proposed as an important strategy to sustain productivity in livestock exposed to HS [27]. Marker-assisted selection studies allow the validation of genes using independent populations [28] to identify individuals with favorable alleles at a single marker or genomic regions associated with economically relevant traits such as milk production [22,29,30] and thermotolerance [31–33] in dairy cows exposed to HS conditions. Additionally, a better understanding of the functional impact of candidate genes on physiological responses and productive performance in Holstein cows under HS conditions would be highly valuable.

As expected, increasing THI was associated with a decline in DMY, the most relevant consequence of HS, driven primarily by reduced feed intake and metabolic adjustments

aimed at minimizing internal heat production. The magnitude of this decline was influenced by genomic load, as cows carrying a higher number of favorable SNP genotypes in the genes *GRM8*, *SMAD3*, and *TLR4* exhibited a markedly attenuated reduction in milk production. This pattern suggests that these genes contribute positively to maintaining productive efficiency under thermal stress, potentially through mechanisms related to metabolic regulation, cellular resilience, and immune function. Notably, the interaction detected above the THI threshold of 79 units indicates that the beneficial effects of these genes become more pronounced as environmental stress intensifies.

In contrast to production traits, RT was positively correlated with THI. However, cows with a higher genomic load exhibited a lower slope of RT with THI, indicating a more efficient thermoregulatory capacity under elevated thermal conditions. This response suggests that animals carrying favorable SNP genotypes in the genes *GRM8*, *SMAD3*, and *TLR4* are better able to dissipate heat or mitigate its physiological impact, thereby maintaining thermal homeostasis during HS. The genotype-dependent differences in RT become particularly evident under more severe environmental pressure (THI > 79), where the demand for physiological adaptation is greater.

This genotype–environment interactions support the notion that these genes contribute to key adaptive mechanisms, potentially involving metabolic regulation, cellular stress signaling, and immune response pathways. Altogether, these findings provide strong evidence that *GRM8*, *SMAD3*, and *TLR4* are functionally relevant candidate genes associated with thermotolerance in dairy cattle as cows with higher amounts were able to maintain higher DMY and lower RT under higher HS conditions, and the slope of these changes was more favorable than those with lower genomic counts.

A similar study reported marker SNPs associated with milk yield in Holstein cows exposed to two THI thresholds, one corresponding to the comfort zone (59 units) and the other to moderate stress (72 units). Effects of these SNPs did not differ between THI thresholds and the authors recommend evaluating the relationship between climate and genotype in cows exposed to a higher THI [34]. When cows are exposed to more intense HS, the body is expected to activate key genes, as well as several major biological processes and pathways, which are associated with an adaptive response to thermal stress.

Holstein cows carrying candidate thermo-tolerance genes exhibit greater resilience to HS, enabling them to maintain milk yield even when THI > 79 units. Mechanistically, these genes support elevated expression of heat shock proteins (HSPs), which help stabilize and repair proteins damaged by thermal stress, while improving antioxidant responses and cellular protection against oxidative damage [22,35]. Cows with these favorable alleles often show lower increases in core body temperature and respiration rates under HS due to enhanced thermoregulatory capacity, such as improved vasodilation and metabolic adjustments [36]. Furthermore, genetically resilient cows can maintain feed intake and metabolic efficiency better than susceptible individuals, partially mitigating the reduction in milk synthesis despite elevated THI [37].

The slope-based analysis provided strong evidence of the differential impact of HS on productive and physiological responses as a function of genomic load. The progressive reduction in DMY slopes as genomic load declined indicates that cows carrying fewer favorable SNP genotypes are more susceptible to production losses as thermal stress intensifies. In contrast, the attenuated slopes observed in cows carrying one or more favorable genotypes suggest a mitigating effect, where the presence of favorable alleles from *GRM8*, *SMAD3*, and *TLR4* genes contributes to sustaining productive performance under increasing thermal load.

A consistent pattern was observed for RT, where slopes decreased as genomic load increased, indicating improved thermoregulatory efficiency. Notably, under severe HS con-

ditions ($\text{THI} > 79$), RT slopes increased slightly in cows lacking favorable genotypes, which may reflect a physiological limitation in their ability to further regulate body temperature. Conversely, the marked reduction in RT slopes with increasing genomic load suggests a more effective activation of adaptive mechanisms in cows carrying favorable alleles. Collectively, these results highlighted the cumulative contribution of these candidate genes to both productive resilience and thermal homeostasis, reinforcing their functional relevance as key determinants of thermotolerance in dairy cattle.

The present results are supported by previous studies describing similar productive and physiological responses to increasing THI in dairy cattle. West et al. [38] reported that milk yield in Holstein cows decreased by 0.69 kg for every unit increase in THI, confirming the high sensitivity of dairy cattle productivity to heat stress conditions. Similarly, Wen [39] observed significant increases in rectal temperature as THI increased, with RT rising by up to 0.106 °C per THI unit during early lactation. These studies support the slope patterns observed in the present work, where increasing THI was associated with reduced DMY and increased RT under heat stress conditions.

In a similar study, slopes for milk production and quality traits were evaluated in Tai-Holstein crossbred cows with different genetic compositions under THI values of 76 and 80 [40]. The rate of decline in slopes was more evident as the THI increased and was particularly pronounced in cows with lower tropical genetic contribution reflecting greater sensitivity to HS. These findings emphasize the role of genetic composition in modulating performance decline and support the use of slope-based analyses to assess genetic thermotolerance. Cows originating from tropical regions have evolved traits such as lighter hair coats, more efficient sweat glands, and behaviors that facilitate heat dissipation, enabling them to cope more effectively with thermal stress [41,42]. These characteristics are the result of long-term natural selection in environments with persistent high temperatures, allowing tropical cows to maintain homeostasis under HS conditions. A similar pattern may have occurred in our study, where slopes for daily milk yield (DMY) and rectal temperature (RT) were less pronounced in cows with higher genomic load, suggesting that greater genetic resilience mitigated the impact of HS.

The candidate genes *GRM8*, *SMAD3* and *TLR4* evaluated in this study demonstrated a clear beneficial effect on key phenotypic traits, including DMY and RT, with both traits showing improvement as genomic load increased. This pattern suggests that the cumulative presence of favorable alleles enhances the cows' capacity to maintain productive performance and physiological homeostasis under HS conditions. Notably, the effect was particularly pronounced when the *TLR4* gene was present, indicating that this gene may play a pivotal role in modulating thermotolerance. Increased *TLR4* expression supports a more effective immune response and promotes adaptive endocrine and metabolic adjustments, particularly in lipid metabolism, reducing internal heat load [43,44]. Under HS conditions, cows with favorable *TLR4* genotypes maintain greater lipolytic capacity and metabolic efficiency, which supports sustained milk synthesis [45].

Regarding to the glutamate metabotropic receptor-8 (*GMR8*) gene, it plays an important role in HS tolerance by regulating glutamate-mediated neuronal and metabolic signaling [21]. Cows carrying a favorable *GRM8* genotype exhibit enhanced gene expression that supports the maintenance of glutamatergic pathways under HS. This response facilitates metabolic adaptations by promoting carbohydrate and amino acid metabolism, thereby increasing glucose availability to meet elevated energy demands when lipolysis is limited [46,47]. Similarly, the *SMAD3* gene is a key mediator of the $\text{TGF}\beta$ signaling pathway and plays an important role in metabolic adaptation to HS. Cows carrying a favorable *SMAD3* genotype exhibit enhanced *SMAD3* expression, which contributes to improved thermo tolerance by regulating adipogenesis and lipid mobilization [48,49]. Un-

der HS, *SMAD3* acts as a negative regulator of adipocyte synthesis, limiting internal heat production while maintaining metabolic efficiency [22].

Gene expression analyses further supported the functional relevance of the genes markers under study. The differential expression of the genes *GRM8*, *SMAD3*, and *TLR4* under HS conditions in cows carrying favorable genotypes, together with expression patterns comparable to *HSP70* gene, suggests that these genes are actively involved in the adaptive response to thermal stress. The inclusion of *HSP70* as a control gene was particularly informative, as its well-established role in cellular protection against heat damage confirmed that all animals experienced HS and activated a general stress response. Interestingly, cows lacking favorable genotypes did not show differential expression of the candidate genes between thermoneutral and HS conditions, despite exhibiting a significant *HSP70* response. This indicates that, although these animals can establish a general stress response, they may lack the ability to activate more specific molecular pathways associated with thermotolerance. Together, these results highlight the potential of the genes *GRM8*, *SMAD3*, and *TLR4* not only as genetic markers but also as key functional components in the physiological adaptation to HS in Holstein cows.

The observed associations between gene expression and phenotypic traits further reinforce this interpretation. The positive relationship between gene expression and DMY, along with the inverse relationship with RT under HS conditions, indicates that higher expression of these genes is linked to improved production and thermal regulation. The reduced associations under thermoneutral conditions highlighted that these mechanisms seem to be primarily activated under high HS, emphasizing their relevance for resilience rather than baseline performance. Therefore, the inclusion of the identified SNP markers in breeding programs represents a promising tool for improving resilience to HS through genetic selection. Overall, the results demonstrate that the combined analysis of genomic load, physiological responses, and gene expression provides a comprehensive understanding of thermotolerance in dairy cattle.

5. Conclusions

The present study provides strong evidence that genomic load, defined by the number of favorable SNP genotypes in the genes *GRM8*, *SMAD3*, and *TLR4*, plays a key role in modulating productive and physiological responses of Holstein cows under HS. Slope analyses revealed that cows with higher genomic load exhibited attenuated declines in DMY and a more controlled increase in RT, indicating improved resilience to thermal stress. The progressive increase in THI during summer allowed the identification of genotype-by-environment interactions, particularly above the threshold of 79 THI units, where differences in thermotolerance became more evident. Gene expression results further supported the functional relevance of these markers, showing differential activation patterns consistent with adaptive responses. Collectively, these findings highlight the potential of integrating genomic and expression-based approaches to identify and select animals with enhanced thermotolerance, contributing to the sustainability and productivity of dairy systems under increasingly challenging climatic conditions.

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Data Availability Statement: The data that support the findings of this study are available from the corresponding author, PLN, upon reasonable request.

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Conflicts of Interest: Author Mario R. Mondaca-Duarte is employed by the company VES-Artex. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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