Association of Hsp70 locus polymorphism with thermotolerance and ailment occurrence in Gulf Creole cattle within intensive systems

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ABSTRACT

Objective: To estimate thermotolerance by analyzing physiological constants and ailment occurrence in Gulf Creole bovine cattle (GCB) and to relate them to Hsp70 locus polymorphism under an intensive production system.

Design/Methodology/Approach: Using a 440 bp fragment, we genotyped leukocyte DNA from 60 BCG through PCR-RFLP (Fok I). Physiological variables were estimated at 7-day intervals for four months during the hottest season. The variables considered were respiratory rate (RR) and layer temperature (LT). Environmental variables —temperature and humidity— were also recorded to determine thermal comfort. Using the production system database, we categorized the animals' ailments during the studied period.

Results: The Hsp70 gene in GCB is polymorphic. The frequency of the AB heterozygous genotype was 0.77; for the AA homozygous genotype, it was 0.23. We observed a predominance of the A allele (0.61). Data analysis allowed us to find differences in RR in GCB with AA and AB genotypes ($p<0.05$). LT showed no differences ($p>0.05$). The genotype did not affect ailment occurrence in GCB ($p>0.05$).

Study limitations/implications: Since the intensive system is dynamic, and the GCB stay period is short, few animals were available for the study.

Findings/Conclusions: The Hsp70 gene present in GCB is polymorphic, and the animals are thermotolerant. Their performance regarding the occurrence of clinical conditions is favorable.

Key words: Gulf Creole bovine cattle, THI, Heat stress, Ailment, Hsp70, Polymorphism.

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INTRODUCTION

Environmental factors such as temperature, humidity, precipitation, atmospheric pressure, wind speed, among others, can affect the productive behavior of cattle (Mader

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et al., 2010). In tropical and subtropical regions, high environmental temperatures have a negative effect on cattle (Molina-Coto, 2017). Temperature and the temperature-humidity index (THI) determine the thermal comfort of animals and are, therefore, the most studied indicators to measure environmental effects on cattle (Jia *et al.*, 2012).

Numerous genes participating in thermotolerance have been described for different bovine breeds and genetic groups (Sosa *et al.*, 2022; Habimana *et al.*, 2023). Among these genes, the ones producing heat shock proteins (HSP) stand out. Said proteins have cytoprotective functions in the face of stressful changes due to heat (Abbas *et al.*, 2020).

The Hsp70 gene regulates the Hsp70 subfamily synthesis. This sensitive and conserved gene is related to thermotolerance and ailment susceptibility in cattle. These characteristics place it as a candidate gene for selecting animals resilient to adverse climates, with better immune response and greater productive performance (Hassan *et al.*, 2019; Badri *et al.*, 2021). The response to thermal stress can vary significantly between breeds and genetic groups. This variability must be considered when selecting animals for their reproductive and productive performance (meat and milk). Some breeds of Creole cattle, such as the Romosinuano (*Bos taurus*), show resistance to heat stress compared to other taurine breeds not adapted to tropical environments (Hernández-Cerón *et al.*, 2004). Silva *et al.* (2013) suggest that this higher resistance reported in different genetic groups is due to the high expression of genes that are protective against heat shock.

Gulf Creole bovines (GCB) constitute a genetic group originating in the Iberian breeds and located in the Gulf of Mexico. They have considerable rusticity, resilience, and reproductive efficiency. GCB are considered a source of genetic diversity that can contribute to forming herds with better adaptability to the climate of tropical areas (Hernández *et al.*, 2015). However, molecular tools have not been used to assess the adaptation of GCB to the adverse conditions of elevated temperatures derived from variations associated with climate change. Hence, this study aims to estimate thermotolerance by analyzing physiological constants and clinical ailment occurrence in Gulf Creole bovine cattle (GCB) and to relate them to Hsp70 locus polymorphism under an intensive production system.

MATERIALS AND METHODS

Ethics statement

All handling, immobilization, and sampling procedures conducted on the GCB within the livestock production unit (LPU) by the veterinary services were evaluated and approved by the Bioethics Committee with registration number COBIBA012/2022 (School of Veterinary Medicine and Animal Sciences, Veracruz University).

Location and description of the study area

We conducted this study from September to December 2022 in an intensive fattening bovine system located in the municipality of Veracruz, in the state of Veracruz, Mexico, with the following geographic coordinates: 19° 10' 01.9" N and 96° 17' 03.0" W, at an altitude

of 10 masl, in a warm subhumid climate (AW_2), with an average annual temperature of >18 °C. The average annual precipitation is ~1600 mm with rains in summer (Köppen-Geiger; Beck *et al.*, 2018; Vidal-Zepeda, 2005).

Sampling conditions and experimental animals

The experiment encompassed 60 bovines that met the criteria of belonging to the GCB genetic group (n=35 males and n=25 females), having an average weight of 203±6.5 kg (mean ± SD), coming from various regions of the Gulf and south-southeastern Mexico, and being destined for meat production.

Considering the management conditions of the production unit, we conducted a first selection process when animals from different herds entered the LPU. This first selection aimed to separate the bovines with the highest genotype of taurine breeds meeting the inclusion criteria of phaneroptic characteristics (coat color, coat type, and horns), indicated in the method proposed by Sponenberg (1992) and modified by Perezgrovas (2011). This first selection allowed us to characterize the differences between *Bos taurus* and *Bos indicus* by assigning scores (on a scale from 0 to 100) to specific characteristics of the animals, stipulating high values for individuals belonging to the *Bos taurus* genetic group and low values to individuals of the *Bos indicus* group.

The group of bovines with taurine characteristics (>75 points) underwent a second selection to ensure they presented the phaneroptic characteristics reported by Hernández *et al.* (2015) for GCB cattle regarding coat color, skin appendages, direction and position of horns, ear size, and head shape. This second selection allowed us to obtain a population of n=60 (GCB).

The selected GCB were housed along with other cattle from different genetic groups in pens ~37 m wide and ~40 long, with an average capacity of 80 individuals (18.5 m^2) and 2.4 m^2 of shade per animal. Each pen had concrete flooring for the entire area, a 3 m wide and ~28 m long feeding trough, and a 1.60 m wide and 4 m long concrete drinking trough, both covered with a concrete roof.

Obtaining meteorological and physiological variables

Respiratory rate (RR) and layer temperature (LT) were measured individually, in a resting state, during a four-month period (September-December 2022), in 7-day intervals from 1:00 p.m. to 3:00 p.m. To measure RR (breaths/min), we resorted to direct observation of inspiratory movements in the animals' flank region (right or left) (Romo *et al.*, 2022). LT (°C) was obtained using an infrared digital thermometer (Steren, model HER-427) directed toward the frontal region of the animal's head (Dorota *et al.*, 2019).

To establish a relationship between physiological constants and climatic variability, the environmental temperature (°C) and relative humidity (%) values were recorded on clinical evaluation days. Data were obtained from the meteorological station located 10.3 km from the LPU in a straight line (Meteorological Station #309-692 of the Veracruz International Airport, Heriberto Jara Corona). We thus estimated the livestock climatic safety index, known as the Temperature and Humidity Index (THI),

which determines the animals' thermal comfort using the following equation (Valtorta and Gallardo, 2004):

$$THI = (1.8 \times T) + 32 - (0.55 - 0.55 \times HR/100) \times (1.8 \times T - 26)$$

The THI is a number (in units) used to indicate the lack of comfort caused by the combined effects of air temperature and humidity. Hahn *et al.* (1999) and Nienaber and Hahn (2007) use the results of this equation and consider four categories of THI to evaluate thermal environmental conditions and their impact on animal welfare. THI values ≤74 are considered comfortable, 75-78 indicate alert, 79-83 indicate danger, and ≥84 indicate an emergency (Saizi *et al.*, 2019).

Determination of Hsp70 gene polymorphism

To genotype the Hsp70 gene, we used leukocyte DNA extracted from whole blood per individual and implemented the salt-precipitation method, according to the instructions provided by the manufacturer (Wizard® Genomic DNA Purification Kit Promega®). Blood was drawn by jugular venipuncture using vacuum tubes with anticoagulant (EDTA 7.2 mg), which were refrigerated at 8 °C until processing. The genotyping technique was Polymerase Chain Reaction - Restriction Fragment Length Polymorphism with restriction enzyme Fok I (PCR-RFLP/Fok I). The amplification was conducted with the commercial kit GoTaq® Green Master Mix (Promega®), 15 μL of Green GoTaq® Reaction Buffer (Taq DNA Polymerase, dNTPs [400 mM dATP; 400 mM dGTP; 400 mM dCTP; 400 mM dTTP], 3 mM MgCl2), and 1 μL/10 pM of each primer commercially designed at UNAM's Institute of Biotechnology (Biotecnologías UNAM®) (Forward 5'-CCGGCCTACTTCAACGACTC-3' and Reverse 5'-CAAGCTCCCGTAGCTGAAGA-3') (Grosz *et al.*, 1994) for a fragment of 440 base pairs (bp) (Lamb *et al.*, 2006), 5.0 μL of DNA, and nuclease-free water to complete the final volume of 35 μL (Lamb *et al.*, 2007). The oligonucleotides sequence corresponds to the Hsp70 gene in band 22 of bovine chromosome 23 (Gene Bank U09861.1, National Center for Biotechnology Information - NCBI). A negative control was used with each assay consisting of the PCR reagent mix without DNA. The PCR amplification protocol was as follows: initial denaturation 94 °C/2 minutes, followed by 35 cycles of 94 °C/30 seconds, 56 °C/30 seconds, 72 °C/30 seconds, and a final extension of 72 °C /10 minutes. The amplification and size of the amplicon was verified by electrophoresis in 2% agarose gel/1X Tris-Borate-EDTA (44.5 mM Tris-HCl, pH 8.0; 44.5 mM boric acid; 1mM EDTA) (Sambrook and Russel, 2001), stained with SYBR Safe Invitrogen® (0.8:1,0000), and molecular weight marker pBR322 DNA-MspI Digest (NEB®) (Grosz *et al.*, 1994; Lamb *et al.*, 2007). PCR amplification was performed in an MJ Mini thermocycler (Bio-Rad®).

Digestion of the 440 bp amplicon was performed with the restriction enzyme Fok I to identify the RFLPs of the Hsp70 gene. According to the amplification and digestion protocol proposed by Lamb *et al.* (2006), the size of the expected fragments is 440 bp for the non-allelic gene fragment (bp) and 171 and 269 bp for restriction sites fragments.

The expected genotypes were homozygous AA of 171 and 269 bp, genotype BB of 440 bp —undigested fragment—, and heterozygous genotype AB of 440, 171, and 269 bp. The digested product was analyzed in 2.5% non-denaturing agarose gel stained with ethidium bromide (0.5 mg/ml), molecular weight marker pBR322 DNA-MspI Digest (Promega®). To visualize the expected fragments, a UV transilluminator was used (Ultra-Lum Spectroline®, 260 nm).

Categorization of clinical conditions

To quantify and categorize the animals' ailments, we used the information provided by the LPU system database, in which clinical conditions are classified according to the main organ affected —respiratory (BRC), locomotive (LC), and digestive (DG) (Estima-Silva *et al.*, 2020). The four months of the evaluation were considered to determine the clinical conditions present in each studied animal to establish a relationship with the THI of the period and the polymorphism of the Hsp70 gene.

Statistic analysis

The Hardy-Weinberg Equilibrium test was used to determine the allele frequency. We conducted statistical analyses with the STATISTICA v.7 software (StatSoft, 2008) using main effects variance analyses considering the genotype, gender, and THI categories (Vega-Murillo *et al.*, 2023). Multiple comparisons of means of the clinical variables (RR and LT) were performed using the Tukey test ($p<0.05$). The statistical model used was the following:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \delta_k + \varepsilon_{ijk}$$

Y_{ijk}=clinical response variables (RR and LT); μ=general mean; α_i=genotype effect (AA, AB, BB); β_j=gender effect (male and female); δ_k=THI category effect (comfort, alert, danger, and emergency); ε_{ijk}=random error of *i*-th genotype, the *j*-th gender, and the *k*-th THI category.

To analyze the association of genotype, gender, and ailments, we used 2^k contingency tables and the χi^2 distribution of the non-parametric module of the Statistica v7 software (2004). Regarding the combined effects of genotype and gender, logistic regression was used to estimate the odds ratios and identify whether the latter are risk factors for the occurrence of any clinical condition (Zavaleta-Martínez *et al.*, 2024).

The logistic regression equation was:

$$\log\left(\frac{p}{1-p}\right) = \beta_0 + \beta_1 * x_1 + \beta_2 * x_2 + \beta_n * x_n$$

Where "*p*" is the probability of the event occurring (clinical condition: BRC, DG, LC); the independent variables are represented with the letter *x*; the absence of the genotype

(AA, AB, BB)=0, and the presence=1; the THI that considers thermal comfort and the coefficients associated with each variable are represented with the letter "β". The figures were edited with the Sigma Plot V11 software (Systat, 2008).

RESULTS AND DISCUSSION

Hsp70 gene polymorphism

The Hsp70 gene was polymorphic in all the specimens evaluated (Figure 1). Two genotypes were identified: homozygous AA and heterozygous AB; the presence of homozygous BB was not reported.

In the analyzed animals (Nn60), the frequency of the A allele was higher (0.89), with expression of heterozygosity for the AB genotype (0.78) and homozygosity for the AA genotype (0.22) (Table 1). These results concur with those reported by Bhat *et al.* (2016) and Li *et al.* (2011) on the presence and frequency of alleles in studies conducted on Tharpakar and Chinese Holstein cattle. The Hardy-Weinberg test indicated that the evaluated population is in equilibrium, with theoretical χi^2 values of 3.84 and experimental χi^2 values of 0.885. These values may be due to random crossbreeding with other bovine genetic groups, mainly of zebu origin, with little difference between the observed and expected values of the genotypes. However, we must consider that the animals came from various locations in the Gulf and southern Mexico, which may also lead to said results.

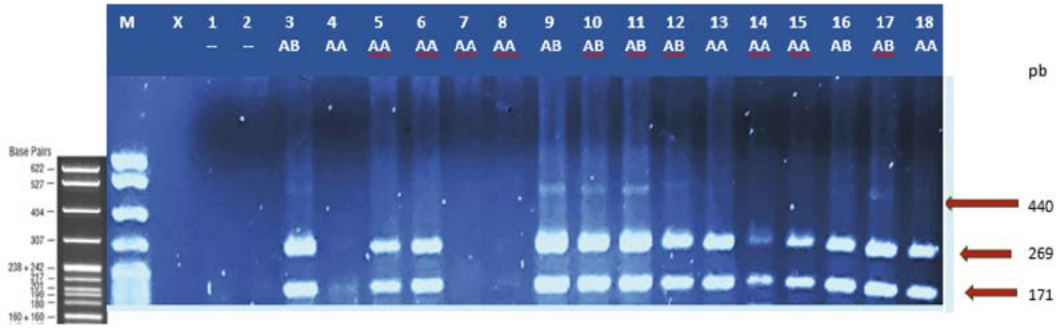

Figure 1. Electrophoresis in 2.5% agarose gel stained with EtBr (0.5mg/ml). The fragments of the Fok I digestion belong to the Hsp70 locus (440 bp). The order of the lanes is as follows: Lane M=Low molecular weight marker. Lane X=Negative control. Lanes 1 and 2, no result. Lanes 3-18 correspond to samples AA: 269 and 171 bp; AB: 440, 269, and 171 bp; BB, undigested amplicon, 440 bp not observed.

Table 1. Genotypic and allelic frequencies obtained for the Hsp70 gene in Gulf Creole bovine cattle.

Gene frequencies		Allele frequencies	
A	0.89	AA	0.22
B	0.11	AB	0.78
		BB	0.00

N=60; 1 gL; χi^2 0.8856

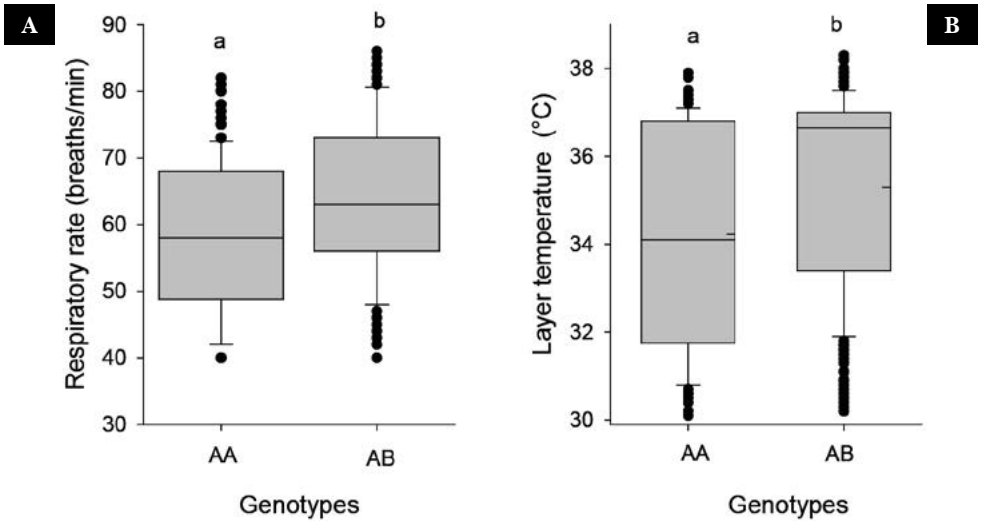

Figure 2. Box and whisker diagram showing the differences between the clinical variables RR (A) and LT (B) among the GCB genotypes studied. The box shows Q1 (bottom line) and Q3 (top line), the dashed line shows the median, and the wireframe, maximum and minimum non-outlier values. The letters a and b indicate differences between clinical variables after the Tukey post hoc test, indicating statistically significant differences ($p<0.05$). ANDEVA values for RR were $F(1, 958)=46.324$, $p=0.0001$; for LT, $F(1, 958)=35.963$, $p=0.0001$.

Environmental and physiological variables

When analyzing the comparison of means, we observed an effect ($p<0.05$) of the genotypes of the Hsp70 gene on RR (Figure 2A). GCBs with genotype AA presented low RR values compared to those with genotype AB. Likewise, the AA and AB genotypes showed an effect on LT ($p<0.05$) (Figure 2B).

Previous studies have shown an association between various polymorphisms in the Hsp70 gene locus and thermotolerance in Chinese Holstein and Tharpakar cattle (Li *et al.*, 2010; Basírico *et al.*, 2011; Liu *et al.*, 2011; Sodhi *et al.*, 2013). When evaluating Tharpakar cattle, Bhat *et al.* (2016) noted that animals with the AA genotype presented low values in RR and LT, followed by the AB and BB genotypes. Their results establish an association between the Hsp70 polymorphism and thermotolerance parameters. Likewise, the A allele positively affected thermotolerance, and the AA genotype was superior, coinciding with the results of our study.

Moreover, when studying zebu cattle (Tharpakar) and their crosses with Creole cattle (Karan), Maibam *et al.* (2017), identified a positive correlation ($r=0.86$) between layer temperature and the expression of the Hsp70 gene, in addition to finding that skin layer temperature showed changes depending on the season evaluated. However, a study conducted by Prasanna *et al.* (2022) on Sahwal cows and their crosses with Holstein Friesian reached different results: they found no difference ($p>0.05$) in the physiological parameters between the AA and AB genotypes and did not report the presence of the BB genotype. In this regard, we must point out that heterozygosity is generally greater in organisms living in limiting environments than in those living in favorable environments. A heterozygosity greater than predicted based on the effective

population size can be expected in small populations at the limit of a species' natural distribution due to selection (Liu, 1999). Higher than expected heterozygosity at some loci may indicate selection or an artifact widely observed in *Bos taurus* cattle in temperate regions (Falchi *et al.*, 2023). Small populations maintaining heterozygosity may be under intense selection pressure for heterozygous loci, or the population has recently become small and has not yet lost heterozygosity. In any case, conservation efforts should focus on increasing population size (Mitton, 2000). In local breeds with small population sizes, one of the most relevant problems is the increase in the inbreeding coefficient. High levels of inbreeding lead to reduced genetic diversity and inbreeding depression. Most local livestock breed result from a particular adaptation to environmentally conditioned production systems, and in many cases, no other breed could survive in the same habitat if the local breed became extinct. These local populations may harbor specific genetic variants that must be conserved and used to recover the loss of genetic diversity occurring in major breeds due to intensive selection for production traits (Mastrangelo *et al.*, 2016).

To establish the environmental effects on the GBC, we quantified the degree of comfort in the studied period. The THI was charted as a function of time (sampling weeks), as shown in Figure 3. With these results, we proceeded to analyze the effect of the THI on the physiological constants (RR and LT) in GBC (Figure 3B and 3C). We can see that, as the THI goes from the category of comfort to that of danger, the clinical constants RR and LT increase. Furthermore, the AB genotype shows greater increases in physiological constants than the AA genotype ($p<0.05$) due to a response mechanism that aims to dissipate the heat absorbed from the environment and to maintain a balanced body temperature (Samara *et al.*, 2016; Hansen, 2020).

A respiratory rate increase in animals is a crucial factor in the thermoregulatory response to heat stress since it helps dissipate heat through evaporative cooling. A lower respiratory rate may indicate better thermotolerance (Das *et al.*, 2016). For its part, an increase in LT is related to vasodilation of the capillary bed of the skin, which facilitates heat dissipation by increasing blood flow on the skin surface (Katiyatiya *et al.*, 2017; Madhusoodan *et al.*, 2019). Since the skin is an organ that comes into direct contact with the external and internal environment, it is also influenced by the characteristics of the coat, such as color, density, and size (Romanello *et al.*, 2018).

Aliments

One of the most salient factors in beef production is animal health since it influences the productive performance of animals and may cause their removal from the feedlots, which impacts profitability (Zhukov *et al.*, 2020). Among the main clinical conditions in bovines at the LPU were respiratory, digestive, and locomotor ailments that cause high rates of morbidity and mortality (Estima-Silva *et al.*, 2020). The etiology of these conditions is multifactorial: pathogens, age, individual susceptibility, and environmental stressors, among others. Stress is a direct contributor, especially in respiratory conditions (Wisnieski *et al.*, 2021), due to the suppression of the immune system induced by high cortisol (Bagath *et al.*, 2019).

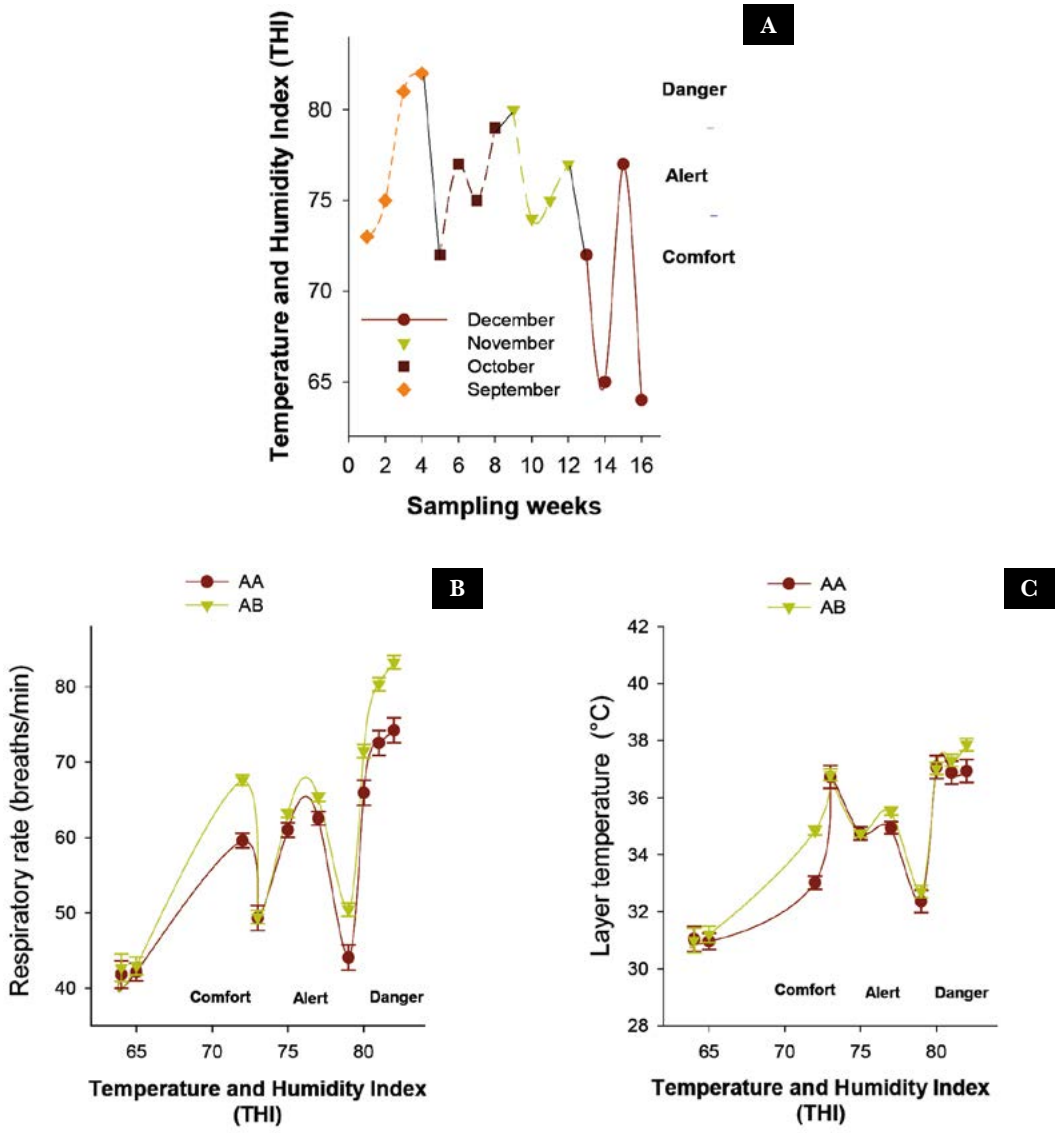

Figure 3. Climatic variables recorded in the LPU, and physiological variables analyzed in GBC concerning the THI present on the evaluation day. A. Description of the THI depending on the study period. B. Respiratory rate (breaths/min) as a function of the change in the THI and categories. C. Layer temperature (°C) as a function of the change in the THI and categories. The horizontal dashed lines in A denote the change of the THI categories. Dashed vertical lines in B and C denote changes in the THI categories.

Digestive (DG), locomotor (LC), and respiratory ailments (this last one known as Bovine Respiratory Complex, BRC) were reported in the GBC analyzed during the experimentation period at the LPU. When performing a logistic regression analysis to find associations between the effect of genotypes and the THI, only the THI showed an effect on the respiratory condition (BRC) in the GCBs evaluated ($p<0.05$) (Tables 2 and 3). The increase in environmental temperature and humidity during summer can compromise animals' thermoregulation mechanisms, causing endocrine alterations to facilitate heat loss, which in turn affects immune functions, increasing susceptibility to various pathogens (Vandana *et al.* 2018).

Table 2. Logistic regression analysis of the genotype effect on the occurrence of aliments in Gulf Creole bovines.

Indicator variables of the logistic regression equation				
n=60		**BRC**	**DG**	**LC**
Constants	β_0	β_1	β_2	β_3
Estimate	−0.93	−0.42	0.93	−0.31
Standard Error	0.74	0.36	1.41	0.8
t (56)	−12.66	−1.16	0.66	−0.38
p-level	0.01	0.24	0.5	0.69
−95%CL	−1.08	−1.13	−1.84	−1.89
+95%CL	−0.79	0.28	3.71	1.26
Wald's χi^2	160.34	1.35	0.43	0.15
p-level	0.01	0.24	0.5	0.69
Odds ratio (unit ch)	0.39	0.65	2.55	0.73
−95%CL	0.33	0.32	0.15	0.15
+95%CL	0.45	1.33	41.18	3.54
Odds ratio (range)		0.65	2.55	0.73
−95%CL		0.32	0.15	0.15
+95%CL		1.33	41.18	3.54

BRC=Bovine Respiratory Complex, DG=Digestive, LC=Locomotive.

Table 3. Logistic regression analysis of the THI effect on the occurrence of aliments in Gulf Creole bovines.

Indicator variables of the logistic regression equation				
n=60		**CRB**	**DG**	**LC**
Constants	β_0	β_1	β_2	β_3
Estimate	0.47	1.01	−0.475	−0.7
Standard Error	0.06	0.37	1.41	0.67
t (56)	6.93	2.7	−0.33	−1.03
p-level	0.01	0.006	0.73	0.3
−95%CL	0.34	0.27	−3.25	−2.02
+95%CL	0.6	1.75	2.3	0.62
Wald's χi^2	48.08	7.33	0.11	1.07
p-level	00.1	0.006	0.73	0.3
Odds ratio (unit ch)	1.6	2.76	0.62	0.49
−95%CL	1.4	1.32	0.03	0.13
+95%CL	1.84	5.76	10	1.86
Odds ratio (range)		2.76	0.62	0.49
−95%CL		1.32	0.03	0.13
+95%CL		5.76	10	1.86

CONCLUSIONS

The Hsp70 gene is polymorphic in Gulf Creole bovines, with a predominance of the A allele. We found an association of the AA genotype with lower respiratory rate values under heat stress conditions, indicative of a favorable thermotolerance genotype in this livestock. Nonetheless, the results must be complemented with more studies in a larger population and with diverse genetic groups.

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