



Intelligent multi-modeling reveals biological relationships and adaptive phenotypes for dairy cow adaptation to climate change

Robson Mateus Freitas Silveira^{a,b,*}, Angela Maria de Vasconcelos^{b,c}, Concepta McManus^d, Luiz Paulo Fávero^e, Iran José Oliveira da Silva^b

^a Department of Animal Science, “Luiz de Queiroz” Agriculture College (ESALQ), University of São Paulo (USP), 13418-900, Piracicaba, São Paulo, Brazil

^b Environment Livestock Research Group (NUPEA), “Luiz de Queiroz” Agriculture College (ESALQ), Department of Biosystems Engineering, University of São Paulo (USP), 13418-900, Piracicaba, São Paulo, Brazil

^c Department of Animal Science, State University of Vale Acaraí, Sobral, CE, Brazil

^d Center for Nuclear Energy in Agriculture (CENA), University of São Paulo (USP), Av. Centenário, 303 - São Dimas 13416-000 Piracicaba, São Paulo, Brazil

^e Faculty of Economics, Administration, Accounting and Actuarial Science, University of São Paulo, São Paulo 05508-010, Brazil

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ABSTRACT

The adaptation of animals to thermal stress involves various variables and adaptive mechanisms. These mechanisms are complex and interconnected, involving both linear and non-linear interactions and dependencies. In this study, we develop a systematic methodology with multivariate models and machine learning algorithms to (i) model complex patterns of relationships or multi-phenotypic differences between the thermal environment and thermoregulatory, hormonal, biochemical, hematological and productive responses; and (ii) identify potential associations among biological relationships that may underlie shared and specific phenotypic patterns of adaptive responses. Thirty clinically healthy multiparous lactating cows with body condition score 3–4 under the same nutritional, health and reproductive management conditions were used in the study. A simple correlation matrix revealed weak and nonexistent correlations between the variables. However, when canonical correlation analysis was used, 12 out of 15 of the canonical correlations evaluated were significant ($p < 0.05$). Moderate levels of canonical correlations ($0.300 \leq r_c \leq 0.628$) and low values of squared canonical correlation ($0.141 \leq r_c^2 \leq 0.384$) between indicators (thermal environment, thermoregulatory responses, biochemistry, hormonal profile, hematological responses and milk composition) were reported. Exceptionally, the thermal environment $\times$ biochemistry pair demonstrated notably high values ($r_c = 0.8468$ and $r_c^2 = 0.7171$). Biological analysis formed seven distinct mechanisms, each associated with specific biological functions and climate-driven effects on physiological and productive traits. 1) Blood traits were related to all milk components; 2) Lipid and energy metabolism, as well as kidney function, are related to the regulation of body temperature and milk composition; 3) Immunity and thyroid hormones are related to radiant thermal load; and 4) Homeostasis is the organic balance maintained between thermoregulatory, hormonal, hematological, productive, and biochemical functions, which are influenced by environmental variables. Applying the random forest method to classify predictions of adaptive responses based on climatic variables showed that all thermoregulatory, hormonal, biochemical, and hematological responses are important, except for urea and T_3 concentrations, which had negative importance values. We conclude that adaptation results from an integration between energy and lipid metabolism, renal function, hormonal profile, and thermoregulatory, hematological, and productive responses. Finally, we recommend the use of multi-models to reveal the complexity of adaptation mechanisms and identify biomarkers that can be used to monitor the dairy flocks, to employ strategies which mitigate the impacts of climate change in animals and promote sustainability of animal production.

* Corresponding author at: Department of Animal Science, “Luiz de Queiroz” Agriculture College (ESALQ), University of São Paulo (USP), 13418-900, Piracicaba, São Paulo, Brazil.

E-mail addresses: robsonmateusf1994@gmail.com, robsonsilveira@usp.br (R.M.F. Silveira).

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Abbreviations

AT, °C	Air temperature
ALT, U L ⁻¹	Alanine aminotransferase; albumin (g dl ⁻¹); cholesterol (mg dl ⁻¹)
AST, U L ⁻¹	Aspartate aminotransferase, creatinine (mg dl ⁻¹)
DDE,%	Defatted solids content, fat (%); glucose (mg dl ⁻¹); triglycerides (mg dl ⁻¹)
PVC,%	Hematocrit
HE, g dl ⁻¹	Hemoglobin, (%); lactose
max	Maximum
MCV, fl	Mean corpuscular volume
min	Minimum; protein (%);
RHL, W. m ⁻²	Radiant heat load
RT, °C	Rectal temperature HG, × 10 ⁶ ml ⁻¹ red blood cell
RH,%	Relative humidity
RR, breaths min ⁻¹	respiratory rate
RV	Reference values
SD	Standard deviation; thyroxine T ₄ , µg dl ⁻¹ thyroxine; total protein TP g dl ⁻¹ total protein
TS,%	Total solids T ₃ , µg dl ⁻¹ triiodothyronine UR mg dl ⁻¹ urea
WBC, × 10 ³ ml ⁻¹	White blood cell

Introduction

Increasing sustainable livestock production in the face of global climate changes is one of the most studied topics worldwide due to the growing world population [1]. Projections by the United Nations [2] estimate a global population of around 9.7 billion in 2050. Consequently, the demand for products of animal origin is expected to increase by around 70 % in the same period [3]. The intensification of production systems and genetic selection have boosted livestock productivity, but increased animals' susceptibility to thermal stress due to the reduction in genetic diversity and selection for productive traits [4]. Given this paradox, studying the adaptation of farm animals is essential for developing strategies to mitigate the effects of climate change in the coming decades [5].

The physiology of adaptation is conceptualized as an organism's biological response to environmental stress. It is the ability of organisms to respond to environmental changes through morphophysiological, behavioral, and genetic changes [6,7]. Such adaptations can occur over generations as animals are subjected to environmental challenges. The adaptive process is systematic and includes various responses that contribute to the biological balance of animals, such as morphological, behavioral, physiological, hormonal, biochemical, metabolic, molecular, and cellular responses [8,9].

Adaptive responses are seen in studies with different species of ruminants, such as Italian and Brazilian goats [10], sheep [11], and dairy cows [12]. These report that one of the primary responses to heat stress is the reduction in dry matter consumption, consequently leading to complex metabolic, hormonal, and physiological changes. Animals increase the mobilization of fat reserves through lipolysis to meet energy demands, since feed consumption is reduced, which also compromises animal production, since the reduction in feed consumption implies multifactorial responses [13].

The basic physiological mechanisms have been widely documented. However, many questions remain unanswered due to the complexity of heat stress mechanisms. Furthermore, the diverse nature of the problem is exacerbated by the influence of numerous biological variables, particularly in dairy farming, where animals have a high metabolic rate due to milk production, making them more susceptible to heat stress [14]. Therefore, the main questions which motivate this study are to understand which phenotypic biomarkers can be used in a genetic improvement program to select dairy cows with adaptive traits. Specifically, we aim to identify: *which phenotypic biomarkers are responsible*

for the shared and specific phenotypic determinisms of adaptive responses? Adaptation involving thermoregulatory, hormonal, metabolic, hematological responses and renal function? What is the relationship between adaptive responses and milk composition? From the identification of these relationships, it is possible to identify standardized biomarkers to evaluate adaptation and which can be used as selection strategies in genetic improvement programs for dairy cows to alleviate heat stress, to promote the sustainability of animal production in the face of climate change [15].

Most studies which investigate the impact of the thermal environment on thermoregulatory responses use a univariate approach, such as simple correlations to understand the relationship between physiological variables [16], but it is well known that this approach is inefficient, as shown by Stürmer et al., [17] who found few relationships when evaluating the impact of climatic variables on milk composition. However, using a multivariate approach led to the discovery of strong and significant correlations between traits. Another study by Silveira [18] clarified the specific and shared relationships between thermoregulatory responses, hormonal profile, production and carcass traits with the thermal environment for beef cattle. Here, we propose to use a multi-model approach, specifically multivariate and machine learning algorithms, the former to reveal response patterns between dependent and independent variables, and the latter to deal with large volumes of complex data, where non-linear interactions exist between variables and it is imperative to capture complex dependency patterns, in addition to identifying phenotypic biomarkers of animal adaptation and production [5,15]. Understanding these relationships is key to improve thermotolerance and sustainability in dairy systems, and this study proposes a novel multivariate framework to achieve that.

The aims of this study were (i) to model complex patterns of relationships or multi-phenotypic differences between the thermal environment and thermoregulatory, hormonal, biochemical, hematological and productive responses; and (ii) to identify potential associations among biological relationships that may underlie shared and specific phenotypic patterns of adaptive responses using multivariate models and machine learning algorithms

Hypotheses

- H₁:** Univariate approaches are not recommended for studying complex relationships;
- H₂:** Organic balance results from the synergism of thermoregulatory, hormonal, metabolic, hematological, immunological and productive responses;
- H₃:** The thermal environment influences the adaptive responses and milk composition of dairy cows.
- H₄:** A multi-model approach is capable of identifying biomarkers to study animal adaptation and production
- H₅:** Machine learning algorithms and multivariate models are efficient in determining shared phenotypic and thermal environment-specific relationships in adaptive and productive responses.

Material and methods

Regulation, study location and animal

All procedures were approved by the Animal Experimentation Ethics Committee of the Universidade Estadual Vale do Acaraú (CEUA)/UVA with protocol number 027/2012.

The experiment was performed in Limoeiro do Norte city, Ceará state, Brazil (Latitude: 05° 08' 44" S; Longitude: 38° 05' 53" W; and Altitude: 30 m above sea level). The region's climate, according to Köppen's classification, is BSw'h [19].

Thirty clinically healthy multiparous lactating cows (3 to 5 years of age) with body condition score 3–4 under the same nutritional, health and reproductive management conditions were used in the study. The

animals were in the same lactational stage when they started the experiment with a maximum difference of five days between calvings. The animals were kept throughout the day in paddocks with Tanzania grass under rotational grazing in a five-hectare area of 14 paddocks irrigated with fixed mini sprinklers, occupancy period of two days, resting period of 28 days and stocking rate of eight animals per paddock. These animals also received concentrate according to their production, with 1 kg of feed concentrate for every 3 kg of milk produced and mineralized salt available throughout the day provided in accordance with the feed management used by the farmer.

Data collection

After the morning milking, physiological data were collected, and blood samples were taken for hematological, biochemical, and hormonal analyses. Collections were conducted in March and November.

Meteorological variables

Thermal environment data [air temperature (AT), relative humidity (RH), globe black temperature (BGT), and wind speed (WS) measurements] were recorded using a TGD-400 meteorological station (Instrutherm, São Paulo, Brazil) every hour, from the beginning to the end of the collections, alongside with physiological data at 6:30 am. The meteorological station was installed in the same environment as the animals.

The radiant heat load (RHL) was calculated using the equation: $RHL = \sigma (MRT)^4$ [20]

Where:

σ = Stefan-Boltzmann constant ($5.6697 \times 10^{-8} \text{ W/m}^2/\text{K}^4$),
 MRT^4 = average radiant temperature, °K.

These variables were registered at the same time of data collection in the animals.

Physiological, hematological, biochemical and hormonal responses

Rectal temperature (RT, °C) was obtained using a digital clinical thermometer (accuracy of ± 0.1 °C; Omron Flex Temp Digital thermometer, China) introduced directly into the animal's rectum up to 3 mm until stabilization. The respiratory rate (RR) was measured using a clinical stethoscope taken directly from the animal's flank and the breaths were counted for 30 s, with the aid of a stopwatch, the value obtained was multiplied by two to obtain the measurement (mov/min).

A blood sample of approximately 5.0 mL from each animal was collected, in tubes with a vacuum system, through jugular vein puncture with 10 % disodium ethylenediaminetetraacetic acid anticoagulant. These were identified and placed in microtubes for subsequent hematological, biochemical and hormonal analysis. The samples were centrifuged at 200 rotations per minute for ten minutes and the serum was stored in Eppendorf® microtubes and frozen at -20 °C.

The analyses of biochemical metabolites were performed using specific commercial kits (in vitro) manufactured by *Vida Biotecnologia*, with measurements taken on the automatic biochemical analyzer model HumaStar80, manufactured by Human GmbH in Wiesbaden, determining serum glucose levels (GLU - mg/dl; colorimetric test enzymatic test), cholesterol (CHO - mg/dl; calorimetric enzymatic method), triglycerides (TRI - mg/dl; colorimetric enzymatic test), creatinine (CRE - mg/dl; alkaline picrate method), total proteins (TPR - g/dl; biuret method), albumin (ALB - g/dl; VBC-bromocresol green method), aspartate aminotransferase (AST- U/L; kinetic-UV method), and alanine aminotransferase (ALT - U/L; kinetic-UV method).

For hematological variables, packed cell volume (PCV, $\times 10^6 \text{ ml}^{-1}$); hemoglobin concentration (HB, g dl^{-1}); red blood cells ($\times 10^6 \text{ ml}^{-1}$); mean corpuscular volume (MCV, fl) and white blood cells (WBC, $\times 10^3$

ml^{-1}) were analyzed.

Milk composition

To evaluate composition all bottles were identified with a barcode and number, and these samples were stored at refrigeration temperature (10 °C) and transported in isothermal boxes with recyclable ice to the laboratory for composition analysis (fat, protein, lactose, total dry extract, and defatted dry extract). Milk samples were collected from each animal in individual bottles, plus a tablet of the preservative Bro-nopol® (2-Bromo-2Nitro-1,3-propanediol). Analyses of the physico-chemical composition were performed using the Combi 2500 device (Combi 2500). The samples were processed at the Milk Quality Laboratory at the Federal Rural University of Pernambuco (UFRPE), Brazil.

Statistical methods

This study used five analyses to achieve the proposed objectives (Fig. 1). First, a correlation matrix was calculated between all variables to verify the relationship between variables based on a univariate approach. Subsequently, canonical correlation analysis was performed to verify the relationship between all indicators and hierarchical cluster analysis to verify the relationship of variables according to biological mechanism. Then, a MANOVA was performed to determine the main variables which are influenced by the climate variables. Finally, random forest was used to determine and classify key variables that can be identified as phenotypic biomarkers for adaptation in dairy cows. The data were processed and analyzed using SPSS [21] and RStudio [22]

Canonical correlation analysis (CCA)

This entailed estimating the canonical correlation coefficient (CCA) to verify the extent of relationships among the indicators being studied. The attention shifts towards measures of association between two groups of variables, delineated as a linear combination of a set of dependent variables (X_p) and a linear combination of another set of independent variables (W_q).

While delineating the methodology, it is necessary to define certain statistical properties. The explanation provided will adhere to the framework outlined by Johnson and Wichern [23]. Linear combinations offer simple summary measures of sets of variables and can be elucidated as:

$$U_i = a_{i1}X_1 + a_{i2}X_2 + \dots a_{ip}X_p \quad V_i = b_{i1}W_1 + b_{i2}W_2 + \dots b_{iq}W_q$$

where: a_{ip} and b_{iq} are canonical coefficients;

U_i and V_i are the i^{th} pair of canonical variables.

The correlation r_i is defined as:

$$r_i = \text{Corr}(U_i, V_i) = \frac{\text{Cov}(U_i, V_i)}{\sqrt{\text{Var}(U_i)\text{Var}(V_i)}}$$

The first pair of canonical variables, or first canonical variate pair, is the pair of linear combinations U_1, V_1 having unit variances, which maximize the correlation ($r_i, i = 1$), as follows:

$$r_1 = \text{Corr}(U_1, V_1) = \frac{\text{Cov}(U_1, V_1)}{\sqrt{\text{Var}(U_1)\text{Var}(V_1)}}$$

The second pair of canonical variables, or second canonical variate pair, is the pair of linear combinations U_2, V_2 having unit variances, which maximize the correlation ($r_i, i = 2$) among all choices which are uncorrelated with the first pair of canonical variables. And so forth, extending to the k^{th} pair of canonical variables.

The percentage of variance explained by the first canonical variates is U_{xi}^2 e V_{wi}^2 is:

$$U_{xi}^2 = \frac{\sum_{j=1}^p a_{ij}^2}{p} \quad \text{and} \quad V_{wi}^2 = \frac{\sum_{j=1}^q b_{ij}^2}{q}$$

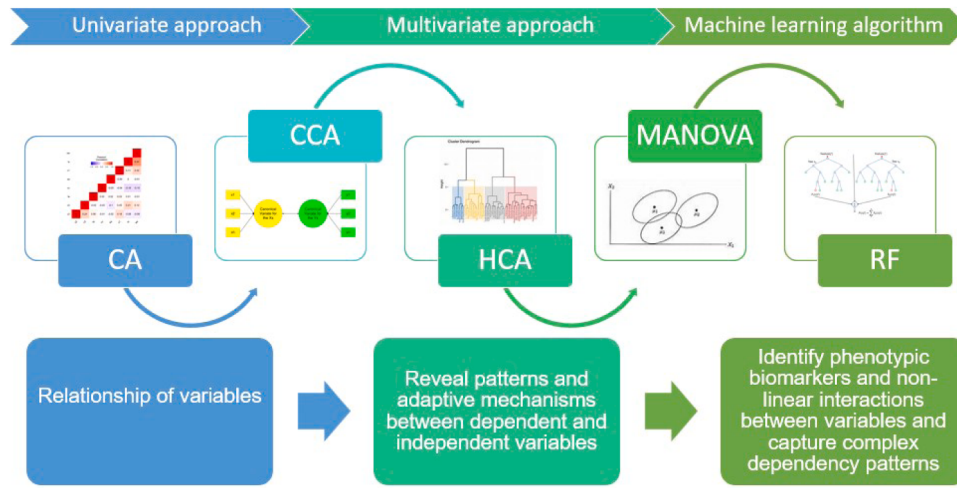


Fig. 1. Framework of the methodological proposal of the multi-model's approach.

in which:

p is number of variables of X ;

q is number of variables of W .

The number of pairs of canonical variates, each of which will be perfectly uncorrelated with the others, equals the number of variables in the smaller of the variable sets.

Finally, the C_{CA} produced a set of new variables called canonical functions (C_F), which are linear combinations of the original variables, as reported in the following equation:

$$C_F = d_1X_1 + d_2X_2 + \dots d_nX_n$$

in which:

d_n are the canonical coefficients which indicate the contribution of each variable in the composition of C_F ;

X are the scores of the n original variables.

Milk defatted dry extract (DDE) was excluded from the canonical correlation analysis to meet the assumptions. We introduced the canonical coefficients in tables and figures. In summary, while canonical correlation coefficients (r_c) measures the strength and direction of the linear association between sets of canonical variables, the coefficient of canonical determination (R_c^2) provides a more descriptive measure of the proportion of variability explained between sets of canonical variables.

Hierarchical cluster analysis (H_{CA})

The adaptive and productive responses, alongside with the climatic variables, were evaluated using a dendrogram to reveal the adaptive and productive mechanisms and the impact of climate on these mechanisms. The model used for hierarchical clustering is described in equation.

$$d[k, (i, j)] = \max [d[k, i], d(k, j)]$$

This agglomerative algorithm calculates the shortest distance between elements i and j using the distance matrix d_{ij} [24]. The elbow rule based on Ward [25] was used to decide the most appropriate number of clusters. The Euclidean distance was used as a measure of dissimilarity. The quality of clustering was assessed by the Silhouette Score

MANOVA

Multivariate Analysis of Variance (MANOVA) is a statistical analysis method used to compare mean vectors of more than one dependent variable across one or more groups. Unlike ANOVA, which analyzes the difference in means among groups for a single dependent variable,

MANOVA considers multiple dependent variables simultaneously. This approach helps understand the complex interdependencies and effects among variables, assessing whether the means of certain groups are different in a multidimensional space defined by those variables. MANOVA is particularly useful when dependent variables are correlated, allowing for a more nuanced understanding of the relationships among them.

MANOVA relies on several key assumptions, including the independence of observations, multivariate normality of dependent variables, and homogeneity of variance-covariance matrices across groups. These assumptions are crucial for the validity of the method. Different test statistics, such as Pillai's trace, Wilks' lambda, Hotelling's trace, and Roy's largest root, are used in MANOVA to determine the significance of observed differences among group means.

MANOVA, an extension of ANOVA, stands out for accommodating multiple dependent variables. In essence, it's a dependence technique which investigates significant differences between groups, taking into account these variables simultaneously [24].

Analysis of variance

$$Y_1 = X_1 + X_2 + X_3 + \dots + X_n$$

(metric) (non - metric)

Multivariate analysis of variance

$$Y_1 + Y_2 + Y_3 + \dots + Y_n = X_1 + X_2 + X_3 + \dots + X_n$$

(metric) (non - metric)

Thus, in the univariate approach, the null hypothesis evaluates the statistical significance of the means of the dependent variable among n groups, given by:

$$H_0 = \mu_1 = \mu_2 = \dots = \mu_n$$

$$H_0 = \begin{pmatrix} \mu_{11} \\ \mu_{21} \\ \vdots \\ \mu_{p1} \end{pmatrix} = \begin{pmatrix} \mu_{12} \\ \mu_{22} \\ \vdots \\ \mu_{p2} \end{pmatrix} = \dots = \begin{pmatrix} \mu_{1n} \\ \mu_{2n} \\ \vdots \\ \mu_{pn} \end{pmatrix}$$

That is, the null hypothesis that all groups have equal mean vectors is tested, as opposed to the alternative hypothesis that at least two groups have different mean vectors.

Assumptions: (1) Independence of Observations: Observations are independent of each other; (2) Multivariate Normality: Dependent variables are multivariate normally distributed within each group; (3) Homogeneity of Variance-Covariance Matrices: Variance-covariance matrices of the dependent variables are equal across groups; (4) Linearity: Relationship among dependent variables is linear and (5) No multicollinearity: Dependent variables are not perfectly linearly related.

Random forests

Random Forest (RF) is an ensemble learning method introduced by Breiman [26], widely used for classification and regression tasks. It builds multiple decision trees and aggregates their outputs, offering robustness to overfitting and effective handling of multicollinearity and high-dimensional data.

Model performance was assessed using the Out-of-Bag (OOB) error, which works as an internal cross-validation mechanism. This technique provides an unbiased estimate of prediction error by evaluating model accuracy on observations excluded from each bootstrap sample. Additionally, we used the %IncMSE (Percent increase in mean squared error) as the primary metric to quantify variable importance. This metric captures the increase in prediction error when the values of a predictor are permuted, reflecting the variable's impact on predictive accuracy. To assess model stability, we repeated the modeling procedure multiple times with different random seeds and confirmed consistency in variable rankings and error estimates.

RF models were applied to examine the influence of climatic variables on various biological parameters, including physiological, biochemical, hormonal, hematological, and milk composition traits. Three distinct analyses were conducted: 1) climatic variables versus biological responses; 2) climatic variables versus milk composition; and 3) milk composition versus biological responses. For each model, the

following steps were performed:

1. *Data preparation*: The data set was divided into predictors and response variables.
2. *Model Fitting*: Random Forest models were trained using the random forest package in R, with 500 trees built for each response variable.
3. *Variable importance extraction*: The relative contribution of each predictor variable to model performance was assessed using the %IncMSE metric. This metric was extracted and visualized to highlight the most significant predictors.
4. *Result Visualization*: Variable importance results were visualized using bar charts to provide an intuitive understanding of the most influential variables. This visualization approach facilitates comparison of variable importance between different models and response variables, offering insights into the complex interactions between climate conditions and biological processes.

Results

The descriptive statistics used to characterize the thermal environment, hormonal profile and thermoregulatory, biochemistry, hematological responses, and milk composition traits are presented in Table 1. On average, only T₃, MCV and ALT did not present values in the range for reference values for dairy cows.

Model complex patterns of relationships

A simple correlation analysis revealed weak and null correlations between the variables (Fig. 2). However, Table 2 displays the canonical correlation coefficients and the corresponding statistical significance of the canonical correlations between the pairs of indicators.

12 out of 15 of the canonical correlations evaluated between the

Table 1

Descriptive statistics for thermal environment, thermoregulatory responses, biochemistry, hormonal profile, hematological responses and, milk composition traits for dairy cows.

Indicators	Variables	Min.	Median	Mean	± SD	Max.	RV
Thermal environment	AT	23.9	27.7	28.5	2.3	31.9	–
	RH	59.3	68.5	66.7	5.2	77.5	–
	RHL	462.6	519.1	548.1	68.1	672.1	–
Thermoregulatory responses	RT	36.5	38.5	38.5	0.7	39.5	26.7–39.1
	RR	16.0	30.0	31.7	9.9	58.0	26.0–40.0
Hormones	T ₃	1.1	1.3	1.4	0.2	1.8	1.2–1.4
	T ₄	0.3	0.8	0.9	0.3	1.8	3.6–8.9
Hematological responses	PVC	26.0	38.0	38.8	7.0	54.0	24.0–46.0
	MCV	47.0	72.2	71.9	12.0	98.5	40–60
	RBC	3.3	5.5	5.5	0.9	6.8	5.0–10.0
	HG	8.4	12.3	12.5	2.2	17.4	11–17
Biochemistry	WBC	6200	10,200	11,755	444.96	22,600	4,000–12,000
	Total protein	6.20	7.80	7.78	0.08	9.20	7.00–8.50
	Albumin	3.9	5.1	5.1	0.7	6.3	2.7–3.8
	Cholesterol	89.0	157.0	155.0	38.7	240.0	80.0–180.0
	Glucose	26.0	44.0	53.9	22.0	119.0	45.0–75.0
	Triglycerides	10.0	21.0	21.0	6.5	38.0	0–14.0
	AST	46.0	77.0	78.8	15.3	118.0	78.0–132.0
	ALT	16.0	31.0	31.2	6.9	48.0	35.0–60.0
	Urea	26.0	49.0	51.2	14.9	88.0	23.0–58.0
	Creatinine	0.8	1.7	1.7	0.5	2.6	1.0–2.0
Milk composition (%)	Fat	1.4	3.6	3.7	0.9	5.8	–
	Protein	2.5	3.3	3.3	0.4	4.2	–
	Lactose	3.3	4.4	4.4	0.3	5.0	–
	TS	9.2	12.4	12.4	1.3	15.1	–
	DDE	7.4	8.7	8.7	0.6	10.0	–

Min: minimum; Max: maximum; SD: Standard deviation; RV: reference values.

Total protein (g dl⁻¹), albumin (g dl⁻¹), cholesterol (mg dl⁻¹), glucose (mg dl⁻¹), triglycerides (mg dl⁻¹), aspartate aminotransferase (AST, U L⁻¹), alanine aminotransferase (ALT, U L⁻¹), urea (mg dl⁻¹) and creatinine (mg dl⁻¹); triiodothyronine (T₃, µg dl⁻¹) and thyroxine (T₄, µg dl⁻¹); air temperature (AT, °C), relative humidity (RH, %), and radiant heat load (RHL, W. m⁻²); hematocrit (PVC), hemoglobin (HG), mean corpuscular volume (MCV, fl), red blood cell (RBC) and white blood cell (WBC, × 10³ ml⁻¹); rectal temperature (RT, °C); respiratory rate (RR, breaths min⁻¹) and defatted dry extract (DDE, %)

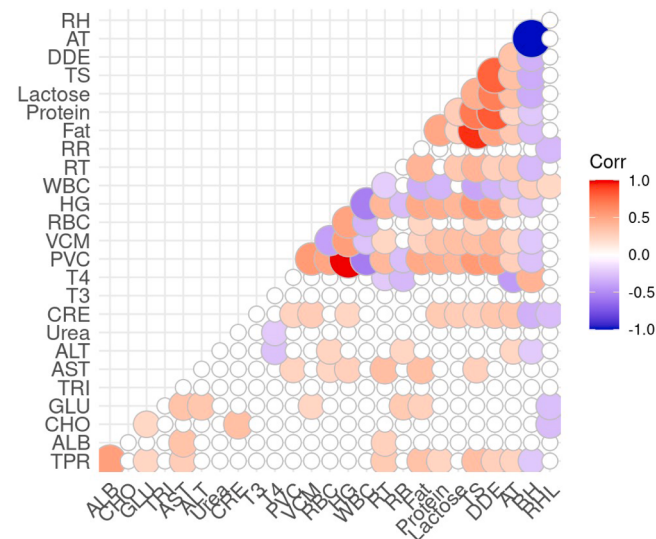


Fig. 2. Correlation matrix between the variables under study Total protein (g dl⁻¹), albumin (g dl⁻¹), cholesterol (mg dl⁻¹), glucose (mg dl⁻¹), triglycerides (mg dl⁻¹), aspartate aminotransferase (AST, U L⁻¹), alanine aminotransferase (ALT, U L⁻¹), urea (mg dl⁻¹) and creatinine (mg dl⁻¹); triiodothyronine (T₃, µg dl⁻¹) and thyroxine (T₄, µg dl⁻¹); air temperature (AT, °C), relative humidity (RH, %), and radiant heat load (RHL, W. m⁻²); hematocrit (PVC, %), hemoglobin (HG, g dl⁻¹), mean corpuscular volume (MCV, fl), red blood cell (RBC, × 10⁶ ml⁻¹) and white blood cell (WBC, × 10³ ml⁻¹); rectal temperature (RT, °C); respiratory rate (RR, breaths min⁻¹) and defatted dry extract (DDE, %).

studied indicators were considered significant ($p < 0.05$). The correlations between hematology x serum biochemistry ($p = 0.201$); milk composition x hormones ($p = 0.774$) and hormone x hematology ($p = 0.904$) were not significant. Observations revealed moderate levels of canonical correlations ($0.3007 \leq r_c \leq 0.6278$) and low values of squared canonical correlation ($0.1414 \leq r_c^2 \leq 0.3841$) between indicators (thermal environment, hormonal profile and thermoregulatory, biochemistry, hematological responses, and milk composition). Notably, the thermal environment–biochemistry pair showed the strongest association ($r_c = 0.8468$ and $r_c^2 = 0.7171$). These associations, along with the standardized canonical loads for each variable and indicator, are presented in Fig. 3 (A–L).

Thermal environment x thermoregulatory responses ($p = 0.0026$; Fig. 3A - $r_c = 0.5845$ and $r_c^2 = 0.3416$): The variables AT and RHL displayed the highest canonical loads, respectively, associated with the homeothermy indicators (RT and RR). Inversely, RH displayed low canonical loads.

Thermal environment x biochemistry responses ($p < 0.0001$; Fig. 3B - $r_c = 0.8462$ and $r_c^2 = 0.7171$): All thermoregulatory responses were mainly associated with TPR, yet the AT demonstrated a higher canonical load compared to the RH. TPR showed a high load, while CRE displayed a moderate one.

Thermal environment x hormonal profile ($p = 0.0163$; Fig. 3C - $r_c = 0.5669$ and $r_c^2 = 0.3214$). The variables RHL and RH demonstrate the highest canonical loads, respectively, with a significant association the hormone T₄ and T₃ moderately. A_T had minimal association on the hormone indicators.

Thermal environment x hematological responses ($p = 0.0053$; Fig. 3D - $r_c = 0.5362$ and $r_c^2 = 0.2876$): The variable AT displays a strong canonical load with HG. RH, and RHL present moderate canonical loads with PVC and MCV. WBC and RBC, indicators of hematological responses, display low canonical loads.

Table 2
Canonical correlations between the thermal environment and the adaptive and productive responses of dairy cows, as well as the relationships between these indicators.

Indicators	Indicators	Canonical r (r_c)	Canonical r^2 (R_c^2)	p -value
Thermal environment	Thermoregulatory responses	0.5845	0.3416	= 0.0026
Thermal environment	Biochemistry	0.8468	0.7171	< 0.0001
Thermal environment	Milk composition	0.5917	0.3501	= 0.0153
Thermal environment	Hormone	0.5669	0.3214	= 0.0163
Thermal environment	Hematological responses	0.5362	0.2876	= 0.0053
Thermoregulatory responses	Biochemistry	0.5042	0.2543	= 0.0014
Thermoregulatory responses	Milk composition	0.5247	0.2754	= 0.0029
Thermoregulatory responses	Hormone	0.3760	0.1414	= 0.0135
Thermoregulatory responses	Hematological responses	0.5606	0.3143	= 0.0005
Biochemistry	Hormone	0.5483	0.3007	= 0.0164
Biochemistry	Milk composition	0.5401	0.2918	= 0.0060
Biochemistry	Hematological responses	0.4393	0.1930	= 0.2006
Milk composition	Hormone	0.2413	0.0582	= 0.7744
Milk composition	Hematological responses	0.6278	0.3941	= 0.0118
Hormone	Hematological responses	0.2111	0.0446	= 0.9041

Note:
Milk composition: fat (%), protein (%), lactose (%), total solids (TS, %) and defatted dry extract (DDE, %)
Biochemistry: total protein (g dl⁻¹), albumin (g dl⁻¹), cholesterol (mg dl⁻¹), glucose (mg dl⁻¹), triglycerides (mg dl⁻¹), aspartate aminotransferase (AST, U L⁻¹), alanine aminotransferase (ALT, U L⁻¹), urea (mg dl⁻¹) and creatinine (mg dl⁻¹);
Hormone: triiodothyronine (T₃, µg dl⁻¹) and thyroxine (T₄, µg dl⁻¹);
Thermal environmental: air temperature (AT, °C), relative humidity (RH, %), and radiant heat load (RHL, W. m⁻²);
Hematological responses: hematocrit (PVC), hemoglobin (HG), mean corpuscular volume (MCV, fl), red blood cell (RBC) and white blood cell (WBC, × 10³ ml⁻¹);
Thermoregulatory responses: rectal temperature (RT, °C) and respiratory rate (RR, breaths min⁻¹).

Thermal environment x milk composition ($p = 0.0153$; Fig. 3E - $r_c = 0.5917$ and $r_c^2 = 0.3501$): The AT and RH showed strong canonical loads with TS and Fat. The other milk composition variables presented low canonical loads and the RHL presented low canonical load with the milk composition parameters.

Thermoregulatory responses x biochemistry ($p = 0.0014$; Fig. 3F - $r_c = 0.5042$ and $r_c^2 = 0.2543$): All thermoregulatory responses showed association with CRE, TPR and ALB, with RT exhibiting a higher canonical load compared to RR. The other biochemistry variables displayed moderate canonical loads.

Thermoregulatory responses x milk composition ($p = 0.0029$; Fig. 3G - $r_c = 0.5247$ and $r_c^2 = 0.2754$) RT presented a strong correlation with lactose and TS. The other of the milk composition variables showed low canonical loads.

Thermoregulatory responses x hormone profile ($p = 0.0135$; Fig. 3H - $r_c = 0.3760$ and $r_c^2 = 0.1414$) Each thermoregulatory response

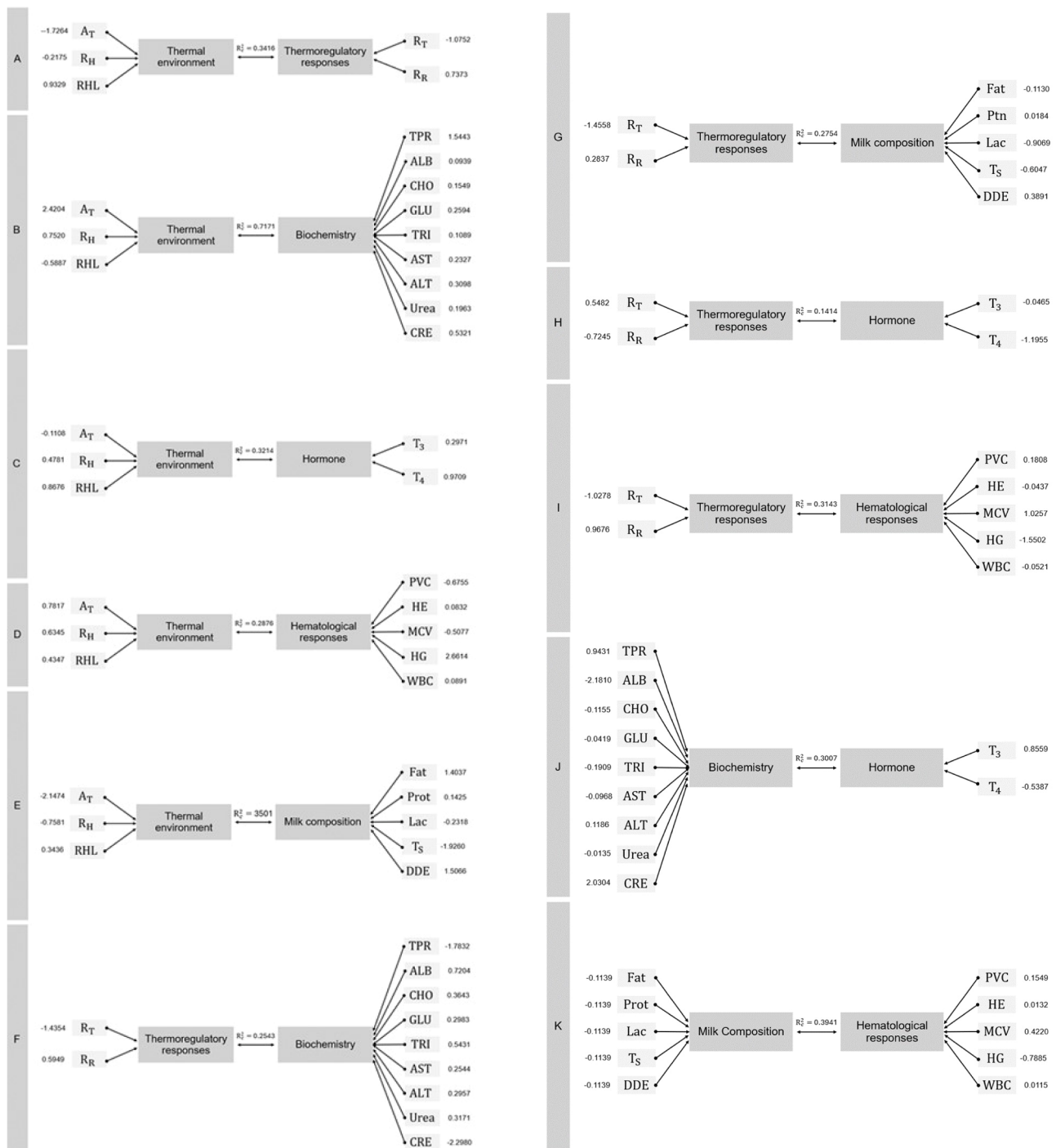


Fig. 3. Canonical relationship of the indicators under study

Note: Representation of the first canonical function for each pair of canonical variables for the relationship among the indicators under study (p -value < 0.05). Total protein (g dl⁻¹), albumin (g dl⁻¹), cholesterol (mg dl⁻¹), glucose (mg dl⁻¹), triglycerides (mg dl⁻¹), aspartate aminotransferase (AST, U L⁻¹), alanine aminotransferase (ALT, U L⁻¹), urea (mg dl⁻¹) and creatinine (mg dl⁻¹); triiodothyronine (T_3 , μ g dl⁻¹) and thyroxine (T_4 , μ g dl⁻¹); air temperature (A_T , °C), relative humidity (R_H , %), radiant heat load (RHL , W. m⁻²); hematocrit (PVC, %), hemoglobin (HG, g dl⁻¹), mean corpuscular volume (MCV, fl), red blood cell (RBC, $\times 10^6$ ml⁻¹) and white blood cell (WBC, $\times 10^3$ ml⁻¹); rectal temperature (RT, °C); respiratory rate (RR, breaths min⁻¹) and defatted dry extract (DDE, %).

displayed moderate effect on T_4 , while the hormone T_3 exhibited a little association with the thermoregulatory indicators.

Thermoregulatory responses x hematological responses ($p = 0.0005$; Fig. 3I - $r_c = 0.5606$ and $r_c^2 = 0.3143$): All thermoregulatory responses exhibited a strong correlation with HG and MCV. Inversely, the remaining hematological responses displayed weak associations with thermoregulatory variables.

Biochemistry x hormonal profile ($p = 0.0164$; Fig. 3J - $r_c = 0.5483$ and $r_c^2 = 0.3007$): ALB, CRE and TPR, as biochemistry variables, significantly associated with the hormone T_3 while they correlated the hormone T_4 moderately. The remaining biochemistry indicators had minimal association with the hormones.

Milk composition $\times$ hematological responses ($p = 0.0118$; Fig. 3K - $r_c = 0.6278$ and $r_c^2 = 0.3941$): Hematological responses exerted an equal association on the composition of milk.

Milk composition $\times$ Biochemistry ($p = 0.006$; Fig. 3L - $r_c = 0.5401$ and $r_c^2 = 0.2918$): High canonical loading coefficients for TS moderately correlated with UREA, CHO, GLU, and CRE. The other variables showed weak correlations each other.

Relationship between biological and productive systems

Hierarchical cluster analysis formed seven distinct groups, each with different biological functions and climate impact on physiological traits (Fig. 4). The relationship of these groups confirms the relationships found in the canonical correlation analysis. The main results were: 1) Blood traits (PVC, HG, and MCV) were related to all milk component; 2) Lipid and energy metabolism, as well as kidney function, are related to the regulation of body temperature; 3) Immunity and thyroid hormones are related to radiant thermal load; and 4) Homeostasis is the organic balance maintained between thermoregulatory, hormonal, hematological, productive and biochemical functions, which are influenced by environmental variables.

Phenotypic biomarkers identification

MANOVA revealed that all thermoregulatory, biochemical, hormonal and hematological responses are influenced by thermal environment variables ($p < 0.05$). However, among the productive variables, only fat showed a significant relationship ($p = 0.013$; Table 3).

Random forest ranking

1. Ranking Approach: Climatic variables $\times$ Adaptive responses.

Climatic variables and radiant thermal load moderately and strongly influence all adaptive responses, except Urea and T_3 , which present values of negative importance, which suggests that the inclusion of these

Table 3

Summary of MANOVA to evaluate the thermal environment in the adaptive and productive responses of dairy cows.

Variables	Type III Sum of Squares	F	p-value
Fat	9.40	4.52	= 0.013
Protein	0.32	0.45	= 0.640
Lactose	0.98	0.97	= 0.382
TS	19.04	2.19	= 0.116
DDE	2.14	0.65	= 0.524
RT	14.95	16.40	< 0.001
RR	1078.79	3.77	= 0.026
TPR	9.43	10.71	< 0.001
ALB	7.33	7.57	< 0.001
CHO	35,743.24	12.61	< 0.001
GLU	4937.45	2.73	= 0.069
TRI	581.23	4.56	= 0.012
AST	6613.40	10.00	< 0.001
ALT	1187.89	8.61	< 0.001
Urea	2252.51	5.14	= 0.007
CRE	8.79	21.33	= 0.001
T_3	.262	2.36	= 0.099
T_4	7.52 ^r	9.17	< 0.001
PVC	510.36	5.80	= 0.004
MCV	974,466.00	4.38	= 0.015
RBC	10.51	3.81	= 0.025
HG	42.25	4.70	= 0.011
WBC	294,188,413.78	7.16	= 0.001

Total protein (g dl⁻¹), albumin (g dl⁻¹), cholesterol (mg dl⁻¹), glucose (mg dl⁻¹), triglycerides (mg dl⁻¹), aspartate aminotransferase (AST, U L⁻¹), alanine aminotransferase (ALT, U L⁻¹), urea (mg dl⁻¹) and creatinine (mg dl⁻¹); triiodothyronine (T_3 , μ g dl⁻¹) and thyroxine (T_4 , μ g dl⁻¹); air temperature (AT, °C), relative humidity (RH, %), and radiant heat load (RHL, W. m⁻²); hematocrit (PVC, %), hemoglobin (HE, g dl⁻¹), mean corpuscular volume (MCV, fl), red blood cell (RBC, $\times 10^3$ ml⁻¹); rectal temperature (RT, °C); respiratory rate (RR, breaths min⁻¹) and defatted dry extract (DDE, %).

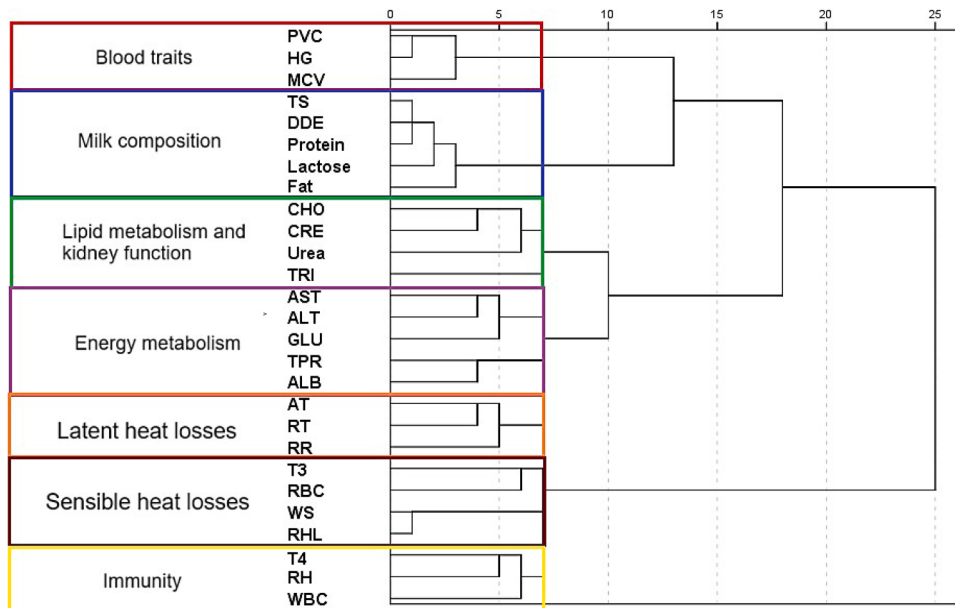


Fig. 4. Dendrogram of adaptive and productive mechanisms with environmental variables

Total protein (g dl⁻¹), albumin (g dl⁻¹), cholesterol (mg dl⁻¹), glucose (mg dl⁻¹), triglycerides (mg dl⁻¹), aspartate aminotransferase (AST, U L⁻¹), alanine aminotransferase (ALT, U L⁻¹), urea (mg dl⁻¹) and creatinine (mg dl⁻¹); triiodothyronine (T_3 , μ g dl⁻¹) and thyroxine (T_4 , μ g dl⁻¹); air temperature (AT, °C), relative humidity (RH, %), and radiant heat load (RHL, W. m⁻²); hematocrit (PVC, %), hemoglobin (HG, g dl⁻¹), mean corpuscular volume (MCV, fl), red blood cell (RBC, $\times 10^6$ ml⁻¹) and white blood cell (WBC, $\times 10^3$ ml⁻¹); rectal temperature (RT, °C); respiratory rate (RR, breaths min⁻¹) and defatted dry extract (DDE, %).

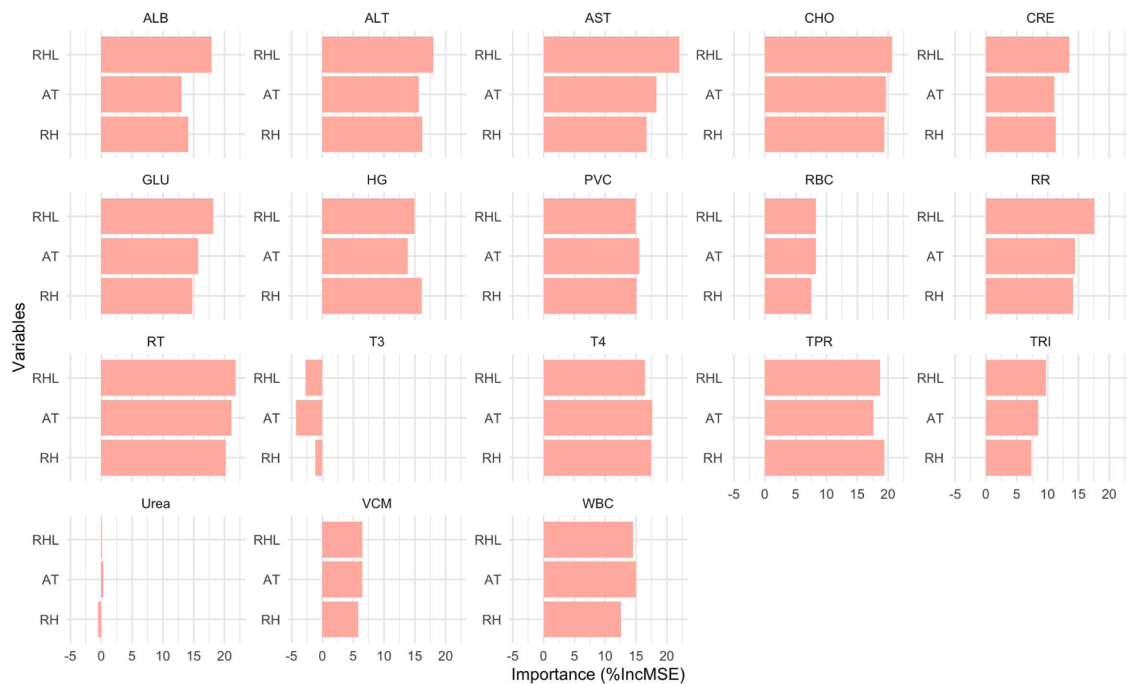


Fig. 5. Impact of environmental variables on adaptive variables
Total protein (g dl^{-1}), albumin (g dl^{-1}), cholesterol (mg dl^{-1}), glucose (mg dl^{-1}), triglycerides (mg dl^{-1}), aspartate aminotransferase (AST, U L^{-1}), alanine aminotransferase (ALT, U L^{-1}), urea (mg dl^{-1}) and creatinine (mg dl^{-1}); triiodothyronine (T3, $\mu\text{g dl}^{-1}$) and thyroxine (T4, $\mu\text{g dl}^{-1}$); air temperature (AT, $^{\circ}\text{C}$), relative humidity (RH, %), and radiant heat load (RHL, W. m^{-2}); hematocrit (PVC, %), hemoglobin (HG, g dl^{-1}), mean corpuscular volume (MCV, fl), red blood cell (RBC, $\times 10^6 \text{ ml}^{-1}$) and white blood cell (WBC, $\times 10^3 \text{ ml}^{-1}$); rectal temperature (RT, $^{\circ}\text{C}$) and respiratory rate (RR, breaths min^{-1}).

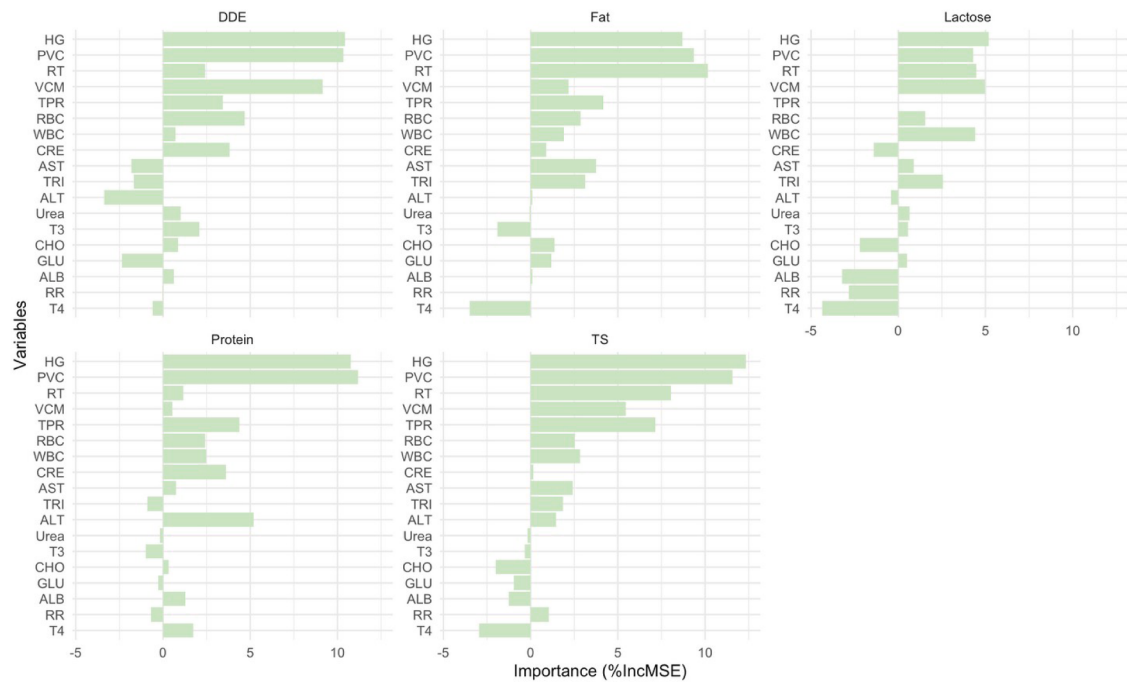


Fig. 6. Impact of adaptive variables on milk composition
Total protein (g dl^{-1}), albumin (g dl^{-1}), cholesterol (mg dl^{-1}), glucose (mg dl^{-1}), triglycerides (mg dl^{-1}), aspartate aminotransferase (AST, U L^{-1}), alanine aminotransferase (ALT, U L^{-1}), urea (mg dl^{-1}) and creatinine (mg dl^{-1}); triiodothyronine (T3, $\mu\text{g dl}^{-1}$) and thyroxine (T4, $\mu\text{g dl}^{-1}$); air temperature (AT, $^{\circ}\text{C}$), relative humidity (RH, %), and radiant heat load (RHL, W. m^{-2}); hematocrit (PVC, %), hemoglobin (HB, g dl^{-1}), mean corpuscular volume (MCV, fl), red blood cell (RBC, $\times 10^6 \text{ ml}^{-1}$) and white blood cell (WBC, $\times 10^3 \text{ ml}^{-1}$); rectal temperature (RT, $^{\circ}\text{C}$); respiratory rate (RR, breaths min^{-1}) and defatted dry extract (DDE, %).

climatic variables can slightly decrease model accuracy, possibly due to noise or low correlation (Fig. 5).

2. Ranking Approach: Adaptive responses versus Milk composition.

Variables such as PVC and HG were important for all milk composition variables (Fig. 6). While RT showed notable importance in predicting milk fat content. Serum biochemistry indicators did not present a standard response, with some being variable or not important for some specific milk components, such as TPR for milk proteins and TS. Finally, thyroid hormones were of low importance in the model.

3. Ranking Approach: Climate x Milk Composition.

Climatic variables and RHL moderately and strongly influence the composition of milk, especially fat (Fig. 7). RH and AT showed similar levels of importance for TS and lactose, with RHL having slightly lower importance.

Discussion

Overview

Adaptation involves many variables with multiple adaptive mechanisms, with these mechanisms being complex and overlapping between the variables in an interconnected way, not only through linear responses [7], but through complex interactions and patterns of dependencies [27]. The reduction in productive performance under heat stress is a final outcome [13]. In this study, we investigated which processes are interconnected and how these relationships occur. Aiming this, our findings suggest that simple correlation analysis may be insufficient to capture the complexity of adaptive mechanisms, although some studies still use this. We developed a multi-model methodology that was capable of identifying these complex linear and non-linear patterns between variables through multivariate models and machine learning algorithms; to subsequently identify associations with main phenotypic biomarkers to study adaptation in farm animals. Although dairy cows were used as the experimental model, the proposed approach is applicable across species, including both animals and plants. These findings reveal strong associations, though causal relationships cannot

be inferred given the observational design.

The combination of different methodological tools was able to answer the five proposed hypotheses. The main results found here were: (i) univariate approaches are not useful tools for studying complex relationships such as animal adaptation; (ii) thermal environment impacts adaptive and productive responses; (iii) adaptation is the integration of thermoregulatory, metabolic, hormonal, hematological, and productive responses; and (iv) hematological variables are phenotypic biomarkers to study animal adaptation to climate change. Although several additional insights were gained here, we will focus this discussion only on the main results.

Univariate approaches

Many studies have evaluated the relationship between adaptive responses using simple correlation analyses [28–33]. However, our results demonstrate that this approach is not appropriate for investigating complex biological relationships, for several reasons.

The primary limitation lies in the methodology used to calculate the Pearson correlation coefficient, which assumes linearity between variables—i.e., a straight-line relationship. When the relationship between variables is nonlinear, the Pearson coefficient may significantly underestimate or overestimate the true degree of association. Moreover, it is important to emphasize that correlation does not imply causation. Two variables may exhibit a high Pearson coefficient without there being a cause-and-effect relationship between them. In studies on animal adaptation, this is particularly critical, as multiple variables often interact in complex and non-additive ways. Adaptation is a multifactorial phenomenon that cannot be adequately explained by isolated bivariate relationships.

Simple correlation analyses fail to capture the complex response patterns underlying thermal adaptation. These limitations hinder a comprehensive understanding of the underlying biological processes [34].

Therefore, we discourage the exclusive use of simple correlation approaches in studies of animal thermal biology. We recommend the application of multivariate statistical models and machine learning algorithms, which are more appropriate for identifying and interpreting complex interactions among multiple variables.

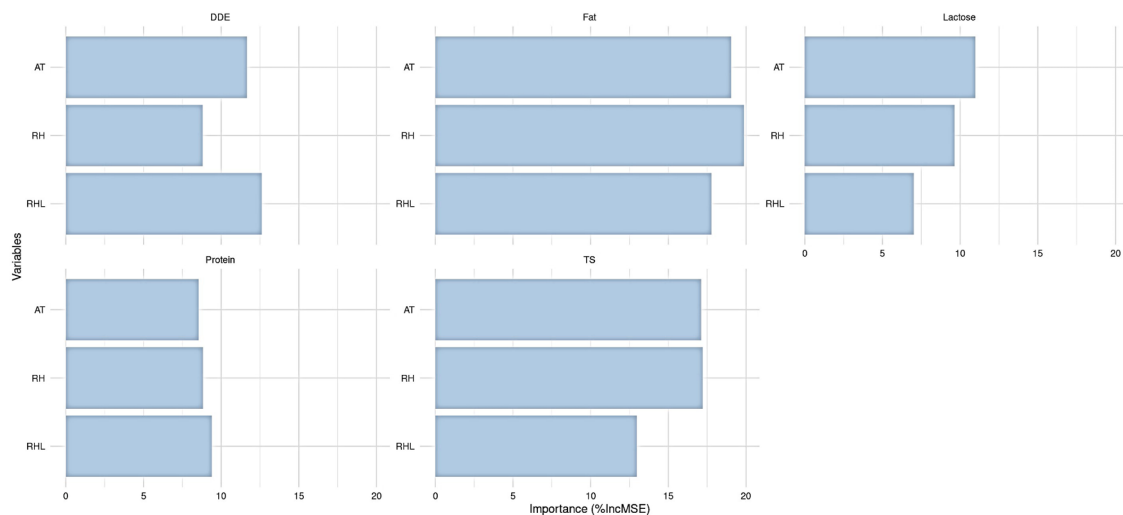


Fig. 7. Impact of environmental variables on milk composition

Total protein (g dl^{-1}), albumin (g dl^{-1}), cholesterol (mg dl^{-1}), glucose (mg dl^{-1}), triglycerides (mg dl^{-1}), aspartate aminotransferase (AST, U L^{-1}), alanine aminotransferase (ALT, U L^{-1}), urea (mg dl^{-1}) and creatinine (mg dl^{-1}); triiodothyronine (T_3 , $\mu\text{g dl}^{-1}$) and thyroxine (T_4 , $\mu\text{g dl}^{-1}$); air temperature (AT, $^{\circ}\text{C}$), relative humidity (RH, %), and radiant heat load (RHL, W m^{-2}); hematocrit (PVC, %), hemoglobin (HG, g dl^{-1}), mean corpuscular volume (MCV, fl), red blood cell (RBC, $\times 10^6 \text{ ml}^{-1}$) and white blood cell (WBC, $\times 10^3 \text{ ml}^{-1}$); rectal temperature (RT, $^{\circ}\text{C}$); respiratory rate (RR, breaths min^{-1}) and defatted dry extract (DDE, %).

Impact of thermal environment on adaptive and productive responses

Significant relationships between the thermal environment and thermoregulatory responses, serum biochemistry, hormonal profile, hematological responses, and milk composition demonstrate the relationship of the thermal environment on the adaptive and productive responses of animals, especially in a milk production system at pasture, as in this study, in which the animals were exposed to solar radiation throughout the day in a semi-arid climate. Significant canonical correlations between the thermal environment and adaptive and productive responses with cattle at pasture and sheep in confinement have already been reported [32,33]. These correlations would present greater magnitudes if data collection were carried out during the hottest periods of the day.

The high canonical coefficients of environmental temperature and radiant thermal load correlating with RT (in greater magnitude) and RR (in lesser magnitude) are explained mainly by homeostatic mechanisms between the animal and the thermal environment [35]. Once homeothermic animals are subjected to conditions outside their thermoneutrality zone, they activate the RR as a respiratory cooling mechanism that is involved in the dissipation of extra heat in the animal [36]. Some studies indicate that RR is the main physiological indicator of heat stress in dairy cattle [37,7]; especially for animals of breeds which are not adapted to the tropics. In the present study, it is incipient to evaluate this homeostatic relationship using only with RR. Information such as sweating rate and coat superficial temperature would be crucial for a better physiological understanding [38]. What can be inferred is that the animals managed to maintain homeostatic regulation, as they regulated RT, which is an indicator of animal homeostasis [35], which is within the thresholds for the bovine species.

From the relationships between the thermal environment and adaptive responses, it was observed that the thermal environment had a greater relationship on the serum biochemistry panel, especially regarding AT and total proteins (Fig. 2B). However, we cannot say directly with ALB, but possibly with globulins, as ALB had a low canonical loading, suggesting other protein fractions mediate this adaptive response. A clear interpretation of this result is difficult, as the effects of heat stress on the concentrations of metabolites in the blood vary widely between studies, making it difficult to provide a clear explanation of the animals' metabolic response to adapt to the thermal environment. It is known that these responses vary according to production systems, seasonality, diet, and physiological state [12,28,29]. As most of these factors were standardized in this study, it is believed that this result is explained by the impact of the thermal environment. Future studies can evaluate the impact of animal adaptation on proteins an isolated way, such as alpha-globulins, beta-globulins and gamma globulins to better understand how adaptation acts on these. This focus should be on alpha- and beta-globulins, as, during heat stress, the animal's body can increase the production of these acute phase proteins in an attempt to mediate the inflammatory response and repair damaged tissues [39].

Other variables in the serum biochemical panel showed weak/moderate correlations with environmental variables and that the machine learning algorithm indicated that most variables were important in adaptation. This result demonstrates the importance of including this group of variables to understand animal adaptation. Animals under heat stress reduce feed intake, and consequently there are disturbances in protein, lipid and energy metabolism, affecting the mineral balance, enzymatic reactions, hormonal levels and metabolites in the blood in an attempt by the animal to maintain homeothermy [40,7].

The relationship between thermal environment variables and thyroid hormones (especially thyroxine) is justified because these hormones are classified as calorogenic, i.e., they regulate the basal metabolic rate, influencing the amount of oxygen and energy that the body uses in biological processes [41–43]. Consequently, when the animal under heat stress, one of the first neuroendocrine mechanisms is the reduction of secretion of T_3 and T_4 in the body in an attempt to maintain

the body's vital functions. However, using the same canonical approach, Silveira et al., [18] did not observe a significant relationship between the thermal environment and thyroid hormones and cortisol in Brahman bulls. We assume that our results were significant due to the semi-arid climate (higher AT) when compared to the tropical savanna climate and because we are studying dairy cows, which are animals that are more sensitive to thermal stress due to heat production when compared to beef cattle [14].

The canonical correlation of hematological variables, especially hemoglobin, with thermal environment variables (Fig. 1D) is explained because this protein is responsible for transporting oxygen from the lungs to the body's tissues and for returning carbon dioxide from the tissues to the lungs to be exhaled, being fundamental in the processes of homeostasis playing a fundamental role in maintaining gas exchange and acid–base homeostasis, especially under increased respiratory activity during heat stress [19]. In the present study, in which the respiratory evaporative mechanism was an important heat dissipation route as discussed previously. A tendency for total hemoglobin in blood to increase with increasing environmental temperature has already been reported [9].

The significant relationship between thermal environment variables and milk composition is in line with other studies [17,43], which demonstrate that AT to the increase in fat because of the reduction in milk production caused by heat stress. However, caution is required when interpreting this relationship, as climate does not directly affect the composition of milk, but rather indirectly, influencing animal physiology. This physiological impact, through metabolic processes, changes the composition of the milk. A strong canonical correlation was observed between the animals' RT and milk lactose, indicating the effect of heat stress [17]. This suggests that the increase in temperature possibly reduced milk production and decreased the lactose concentration, while increasing the percentage of fat in the milk. The animals' feeding management was based on rotational grazing throughout the year, with irrigated paddocks and constant pasture and fertilization management. Therefore, the nutritional value of pastures possibly remained similar throughout all months of the year.

Synergism of organic balance

The significant canonical correlations between thermoregulatory responses, hematology, serum biochemistry, hormonal profile, and milk composition partially confirm our hypothesis that these factors are essential for the animal to maintain functional harmony, where all physiological systems of the body operate in an integrated manner and efficient to achieve homeostasis and maximize productive indicators.

Although some individual associations were non-significant, all canonical correlations involving thermoregulatory indicators were statistically robust and consistent. This shows the importance of including hematology, serum biochemistry and hormones when evaluating animal adaptation highlighting the necessity of a multi-systemic assessment for adaptive profiling. This is defined as a relatively stable condition that enables animals to maintain homeostasis and perform essential, regardless of the external environment [35], allowing it to reproduce and produce in a given environment [9].

The values outside the reference range for T_4 , together with the correlations of the hormonal profile with the thermal environment, thermoregulatory responses and serum biochemistry are interesting and can be justified because zebu dairy cows present lower concentrations of T_4 rather than indicating clinical hypothyroidism, aligning with previous reports of endocrine adaptation under heat stress in tropical cattle. Reference values are based on classic international physiology books, possibly analyzed from European cattle, which are the main animal model, and not for zebu animals as here. Recommendations for reassessment of thermal limits for sheep farming in Brazil, especially hair sheep, were reported by McManus et al., [44]. We suggest the same reassessment for zebu cattle. For example, average values of 6.02 µg/dL

of T_4 were obtained for Holstein cows, but for $\frac{1}{2}$ Holstein $\times$ $\frac{1}{2}$ Guzera cows, values of 1.83 $\mu\text{g/dL}$ were reported, both experiments carried out in the Brazilian semi-arid region [30,45]. Although T_4 values were below classical reference ranges, these likely reflect a thermoregulatory adjustment in Zebu breeds [45]. This pattern is corroborated by moderate canonical coefficients linking RT and RR to T_4 levels, aligning with previous findings of inverse relationships between respiratory effort and thyroid hormone concentrations under heat stress [45]. It is also noteworthy that this hormone was identified as an important predictor by the machine learning modeling to explain adaptation by the variable importance ranking in the random forest algorithm, highlighting its role in adaptive endocrine modulation.

The naming of the seven clusters formed according to the adaptive mechanisms and the composition of the milk allowed us to understand the adaptive processes more clearly, demonstrating the impact of temperature, especially on the RT and RR of the animals, which has already been discussed here. However, this dendrogram reveals something interesting which is WS together with hematocrits, T_3 and RHL in the same group, which is possibly explained by the importance of WS in hot environments, where it can be beneficial by increasing skin evaporation and convection, helping to maintain body temperature.

The relationship of RH, T_4 and WBC in the named immunity group is justified because, like environmental temperature, humidity is an essential psychometric variable in thermal exchanges between the animal and the environment, especially in conditions of high AT and high RH. In this context, the animal activates evaporative respiratory mechanisms to maintain homeothermia, as the thermal gradient between the animal and the environment is zero along with the high humidity. However, these evaporative mechanisms consume a lot of metabolic energy, which can reduce the animals' immunity through changes in the antioxidant system, increasing the number of WBC. However, two caveats are important in this discussion: 1) the 70 % RH found in this study is recommended for animals in production and 2) the animals did not have WBC.

The centrality of protein metabolism, renal function, and lipid metabolism among productive responses and homeostatic regulation in the dendrogram is justified because this set of variables are indirectly impacted by heat stress, mainly by reducing feed consumption [35], as discussed previously.

Phenotypic biomarkers of adaptation

The importance of hematological parameters in all analyzes carried out in this study first correlated with thermoregulatory responses, thermal environment and milk composition; subsequently, presented an approximation with milk composition in the dendrogram and finally, it was the main group of variables that showed importance in the adaptation and composition of milk based on the random forest partially confirms our hypothesis and is justified due to its relationship with organic balance. Furthermore, we know that blood counts, associated with thermoregulatory responses, are crucial indicators of health and disease in animals, and are used as the first diagnostic parameter before proceeding to cellular and genetic assessment [13,46].

The individual interpretation of the blood count provides information about the animals' responses to heat stress; however, the integration of hematology is important for maximum understanding. The main factors which influence these parameters are species, morphology of the skin and sweat glands [46]. These factors can influence the thermoregulatory economy of animal body fluids, causing a reduction or increase in erythrocytes [47].

The importance of these parameters in thermoregulation, as already pointed out in this study, is justified because blood is probably the most powerful medium in the body that facilitates heat dissipation during thermal stress through thermal exchanges of sensible and insensitive heat through the skin and respiratory tract [47]. The moderate canonical relationship between thermoregulatory responses and hemoglobin has

already been discussed previously in the relationship between the thermal environment and the hematological profile, but MCV also presented a moderate canonical load along with hemoglobin. This result is explained by the change in the morphology of erythrocytes, since hemoglobin and other parameters were altered to a lesser extent. These results agree with Silveira et al., [15] who, using a systematic methodology to identify sustainable animals using beef cattle as an animal model, point to MCV as one of the seven biomarkers to identify these animals, in which one of the characteristics is adaptability and animal productivity.

In addition to thermoregulation, our results also showed that hematological variables were important in the composition of milk, especially PCV and hemoglobin, which is justified because milk production is a metabolically intense activity that requires an adequate supply of oxygen to mammary tissues for synthesis of milk production. As far as we know, this is the first time that this relationship has been studied, as we have not found others reported in the literature.

In this topic we highlight the importance of hematological variables as phenotypic biomarkers for adaptation and animal production; however, it is necessary to highlight that some variables from the biochemical serum panel, especially those signaling energy and protein metabolism, can be used as biomarkers of adaptation as they are influenced by thermal environment. Caution is only necessary for this interpretation, as these variables are influenced by a series of factors that make it difficult to attribute them to adaptation and animal production.

Limitation

One of the main limitations of this study is the relatively small number of animals ($n = 30$), which may restrict the generalization of the findings to broader populations. However, it is important to emphasize that the central objective of this research was not to establish breed- or herd-specific inferences, but rather to propose and test a robust multi-modeling approach capable of revealing complex biological relationships involved in animal adaptation to heat stress. Despite the limited sample size, the study successfully integrated multivariate models and machine learning algorithms, respecting the assumptions of each analytical method, to extract structured information from high-dimensional phenotypic data, providing a comprehensive view of adaptive mechanisms.

This methodology represents a significant innovation in agricultural sciences, where many studies still rely on univariate analyses and linear correlations, such as Pearson's coefficient, which are insufficient to capture the non-linear and multi-phenotypic interactions typical of biological adaptation processes, as discussed here. Furthermore, the proposed multi-modeling framework has the potential to be applied not only in animal science, but also in plant physiology and other complex biological systems, reinforcing its interdisciplinary relevance.

A specific limitation of the dataset used in this study is the absence of daily milk yield records, which would have allowed a more direct evaluation of the relationship between adaptive traits and productive performance. Although milk composition was assessed, the lack of production data limits the interpretation of the actual impact of thermal adaptation on productivity. Future research should address these aspects by including larger sample sizes and integrating productive, economic, and molecular data to validate and expand the applicability of the proposed modeling approach.

It is important to note that the statistical approaches used in this study, including CCA and RF, allow the identification of patterns and associations, but do not establish causal relationships. Therefore, the interpretations presented here should be understood as indicative of biological interactions, not as evidence of causation.

Final remarks, implications and future research

Univariate approaches are not accurate for studying complex

relationships between variables, as is the case with animal adaptation. Therefore, we discourage researchers from using this approach in future studies. Thus, the development of a multi-modeling approach that integrates machine learning and multivariate models is a useful tool for studying animal adaptation. This approach captures a comprehensive view of the linear relationships as well as complex interactions between climate variables and adaptive and productive responses, revealing significant patterns in and between adaptive and productive responses to climate variables. These multi-phenotypic interactions suggest that adaptation may emerge from the synergy among metabolic, hormonal, thermoregulatory, hematological, renal, and productive functions. Furthermore, these responses act synergistically with different biological functions, confirming the complexity of adaptive responses. Hematology, in particular, has been identified as the main phenotypic biomarker of adaptation and milk composition.

We recommend the use of multi-models to reveal the complexity of adaptation mechanisms and identify biomarkers that can be used to monitor adaptation in dairy flocks. This will allow the implementation of strategies that mitigate the impacts of heat stress on animals due to climate change, promoting animal sustainability. Finally, technicians and geneticists are encouraged to incorporate these findings into selection indices aimed at improving thermotolerance and herd resilience under projected future climates.

Future research should utilize a multi-model approach integrating genetic and phenotypic data to better understand the relationship of thermoregulatory, metabolic, productive, hormonal, and hematological responses with and between molecular, cellular, and genetic responses (heat shock proteins and thermal shock factors). The integration of these responses will be crucial for a better biological understanding of adaptive mechanisms in farm animals. Given the versatility of the multi-modeling framework, its application can be extended to other domains, such as plant physiology, environmental physiology, and precision livestock farming.

Declarations

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Declaration of generative AI and AI-assisted technologies

During the preparation of this work, the authors utilized ChatGPT to improve readability in specific sections of the manuscript. After using this tool, the authors carefully reviewed and edited the content as needed and take full responsibility for the publication's content

Ethical statement

All procedures were approved by the Animal Experimentation Ethics Committee of the Universidade Estadual Vale do Acaraú (CEUA)/UVA with protocol number 027/2012.

CRedit authorship contribution statement

Robson Mateus Freitas Silveira: Writing – original draft, Validation, Methodology, Formal analysis, Data curation, Conceptualization, Investigation, Visualization. **Angela Maria de Vasconcelos:** Writing – review & editing, Visualization, Funding acquisition, Conceptualization, Investigation, Methodology, Project administration, Resources, Supervision, Validation. **Concepta McManus:** Writing – review & editing, Visualization, Validation, Supervision, Conceptualization, Investigation. **Luiz Paulo Fávero:** Writing – review & editing, Visualization, Conceptualization, Methodology, Validation. **Iran José Oliveira da**

Silva: Writing – review & editing, Visualization, Validation, Supervision, Project administration, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no conflicts of interest.

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None

Data availability

Data will be made available on request.

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