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Research article

Exploring candidate genes for heat tolerance in ovine through liver gene expression

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ABSTRACT

Thermotolerance has become an essential factor in the prevention of the adverse effects of heat stress, but it varies among animals. Identifying genes related to heat adaptability traits is important for improving thermotolerance and for selecting more productive animals in hot environments. The primary objective of this research was to find candidate genes in the liver that play a crucial role in the heat stress response of Santa Ines sheep, which exhibit varying levels of heat tolerance. To achieve this goal, 80 sheep were selected based on their thermotolerance and placed in a climate chamber for 10 days, during which the average temperature was maintained at 36 °C from 10 a.m. to 4 p.m. and 28 °C from 4 p.m. to 10 a.m. A subset of 14 extreme animals, with seven thermotolerant and seven non-thermotolerant animals based on heat loss (rectal temperature), were selected for liver sampling. RNA sequencing and differential gene expression analysis were performed. Thermotolerant sheep showed higher expression of genes G*Px3, RGS6*, *GPAT3, VLDLR*, *LOC101108817,* and *EVC.* These genes were mainly related to the Hedgehog signaling pathway, glutathione metabolism, glycerolipid metabolism, and thyroid hormone synthesis. These enhanced pathways in thermotolerant animals could potentially mitigate the negative effects of heat stress, conferring greater heat resistance.

1. Introduction

The production of small ruminants in tropical regions is heavily influenced by the animals' production capacity and ability to adjust to the local environment. Native breeds are crucial due to their ability to resist and adapt to environmental conditions, which helps them reduce heat absorption from their surroundings and increase heat loss through evaporation [1]. Enhancing the productivity and

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reproductive performance of sheep typically leads to improved economic and biological efficiency in sheep production. Conducting trials that integrate management and genetic advancements to enhance animal output are crucial [2–4].

However, greater exposure of animals to high temperatures causes a series of changes that affect their overall performance [5]. Physiological and hormonal heat stress responses have been well studied [6–8]; however, heat stress regulation at the cellular level and gene expression patterns are still poorly understood [9].

Heat stress can lead to cellular-level consequences in animals, resulting in suppression of DNA synthesis, transcription, translation, denaturation, and increased protein degradation [10,11]. Alterations in gene expression due to heat occur both during hyperthermia and upon returning to homeothermic conditions [12].

Different genes related to heat stress response have been reported as tolerance markers in sheep exposed to high temperatures [13–15]. Transcriptomic analyses have been used to identify differentially expressed genes (DEGs) in sheep exposed to heat stress conditions [16,17]. In addition, the transcriptome is becoming a powerful tool for analyzing an animal's response to heat stress, given that it fluctuates based on developmental phases, physiological states, and exposure to external factors, [18].

To identify and select animals with higher productive potential in hot climates, a good understanding of their adaptive responses to thermal challenges is needed. Current studies indicate variations in the individual responses of animals to heat stress, even within the same group, breed, and environment, indicating selective improvement opportunities [19,20]. The discovery of genes exhibiting differential expression in the liver, an organ central to various metabolic processes linked to the heat stress response [21,22], has the potential to unveil the critical metabolic pathways crucial for thermoregulation. The main goal of this study was to determine which genes in the liver of Santa Ines sheep were responsible for their varying levels of heat tolerance during heat stress.

2. Material and methods

2.1. Ethical approval

The Ethics Committee on Animal Experimentation at Faculdade de Zootecnia e Engenharia de Alimentos/USP reviewed and approved the study design and all procedures, considering the legal and ethical implications of the interventions. This approval was granted through Declaration 7498130919.

2.2. Location and animals

Eighty black-coated, non-pregnant Santa Ines ewes aged 4.5 ± 0.5 years with similar score 3 body condition (1–5 scale) were used. Initially, the animals were housed in paddocks with unrestricted access to artificial shade provided by a white cement roof (1 m^2/animal) on *Panicum maximum* cv. Aruana pasture supplemented with corn silage [23]. The trial was conducted at the Biometeorology and Ethology Laboratory at the Faculdade de Zootecnia e Engenharia de Alimentos, Pirassununga, São Paulo, Brazil.

The four groups, each comprising 20 ewes, were housed in a climatic chamber (VRA/FMVZ) at the University of São Paulo to evaluate thermotolerance. The climate chamber had a space of 56 m^2 and was completely surrounded by brick walls and slabs. It is equipped with a cemented floor, external temperature and humidity control systems, internal thermostats, and an exhaust fan [24]. The animals were fed corn silage *ad libitum* with free access to water and mineral supplements. During the trial, there was no difference in the duration of feed intake per hour between animals, with an average of 20.71 ± 1.054 min.

2.3. Experimental design

The animals underwent a ten-day heat stress period in a climatic chamber, with two-day adaptation to the new environment and eight days of heat treatment. During the heat treatment, the temperature was maintained at 36 °C from 10 a.m. to 4 p.m., and a maintenance temperature of 28 °C was maintained between 4 p.m. and 10 a.m. The relative humidity was set to 60 ± 2%. The outdoor environment, where the animals were kept before entering the climatic chamber, was similar during the entire experiment in summer, with an average air temperature of 23 ± 5 °C and relative humidity of 60 ± 12%. Rectal temperature data were collected every 3 h from 1 p.m. on Day 9–10 a.m. on Day 10.

Rectal temperature, which serves as an indicator of heat stress response, was analyzed using the restricted maximum likelihood method (REML) in a mixed model. The model considered fixed effects, such as the evaluation cycle as a block (four cycles with 20 animals each, adjusted to the same baseline), the time effect within the evaluation cycle (two cycles, 10 a.m.–7 p.m. for heat gain and 10 p.m. –7 a.m. for heat loss), and the animal as a random effect. The best unbiased linear prediction (BLUP) estimates, without considering the genetic relationship matrix, were calculated for each ewe to quantify individual heat stress responses. The effect of the second evaluation cycle from 10 a.m. to 7 a.m. (21h) was used to assess each individual's ability to dissipate heat after a heat-stress period, allowing the ranking of ewes from the most heat-tolerant to the least heat-tolerant [24].

The BLUP values ranged from −0.57 to +0.68. Animals with values between −0.57 and −0.01 were identified as thermotolerant (TT), representing 54% of the group, indicating a higher reduction in rectal temperature. Conversely, positive values from +0.02 to +0.68 categorized 46% of the animals as non-thermotolerant (NTT), suggesting a lower reduction in cumulative heat. The average rectal temperature for selected sheep in the TT group was 38.77 ± 0.019 °C, while it was 39.10 ± 0.021 °C for the NTT group ($P < 0.01$) [24].

Between day 10 and day 11, air temperature was maintained at 36 °C and at 7 a.m. on day 11; 14 sheep, seven considered the most thermotolerant and seven considered the most non-thermotolerant, were euthanized (cerebral concussion followed by bleeding) to

collect liver samples for RNA sequencing analysis to obtain information on heat stress-related target genes. Samples were stored in RNAlater solution (Invitrogen, EUA) at - 20 °C for subsequent RNA extraction.

2.4. Total mRNA extraction, library preparation, and sequencing

RNA sequencing data were obtained from the Genomics Center at ESALQ, Piracicaba, São Paulo, Brazil. The tissue was extracted using the TRIzol reagent (Life Technologies, Carlsbad, CA, USA), and the RNA quality was assessed using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). For library preparation, 2 μg of mRNA from each sample was utilized following the TruSeq RNA Sample Preparation Kit v2 manual (Illumina, San Diego, CA, USA). The library size was determined using an Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA), and the quantification was done using the KAPA Library Quantification Kit (KAPA Biosystems, Foster City, CA, USA). The samples were then diluted and combined into a thermotolerant group. The libraries were sequenced using an Illumina HiSeq2500 instrument (Illumina, San Diego, CA, USA) with a TruSeq SBS Kit v3-HS (200 cycles) following the manufacturer's instructions [24].

2.5. Bioinformatics analysis

The quality of the sequence data was assessed using the FASTQC tool version 0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Following this, reads were subjected to quality control (QC) using the TRIM Galore software version 0.6.6 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to eliminate sequence adapters, low-quality reads (QPhred <30), and short reads (<70 bp). The resulting clean reads were subsequently mapped to the sheep reference genome (*Ovis aries*, assembly GCA_016772045.1) available in the NCBI database (www.ncbi.nlm.nih.gov) using STAR 2.7.3a software [25].

mRNA abundance was quantified as Counts Per Million (CPM), and only genes with at least one CPM in a minimum of 30% of the samples were included in the analysis. Differential expression analysis between high- and low-heat-tolerance groups was conducted using the EdgeR package [26]. The significance threshold for identifying differentially expressed (DE) genes was set using False Discovery Rate (FDR) correction with a threshold of ≤0.05. Genes were classified as upregulated or downregulated based on positive or negative log2 fold-change (Log2FC) in the thermotolerant group compared to the non-thermotolerant group after FDR correction for multiple testing.

2.6. Gene ontology (GO) and functional enrichment analyses

The WebGestalt tool (WEB-based Gene SeT Analysis Toolkit) was used to perform functional enrichment and GO analyses of differentially expressed genes [27] in the liver of sheep. The KEGG pathway functional database was utilized, focusing on genes associated with *Homo sapiens*, as *Ovis aries* genes are not available on any platform [24].

3. Results

3.1. RNA sequencing data and differential gene expression analysis for liver samples

The liver samples were sequenced, generating an average of 15.14 million paired-end reads per sample (2 × 100 bp), with around 14.6 million reads remaining after quality control. Of these reads, approximately 85.42% were successfully mapped to the sheep reference genome. The Multi-Dimensional Scaling plot revealed a similarity between the seven animals with low heat tolerance, whereas the seven animals in the high heat tolerance group exhibited greater dispersion.

A total of 14,491 genes were expressed in sheep liver samples (Supplemental File 1), of which seven genes were differentially expressed (FDR <0.05) between the two groups (Table 1). Six of the seven DE genes were upregulated and one was downregulated in the thermotolerant group compared to those in the non-thermotolerant group (Fig. 1).

Hierarchical clustering analysis of the seven DE genes showed clear differences in expression patterns between samples within groups (Fig. 2). *GPX3*, *LOC101108817 (IGHG1)*, *VLDLR*, *EVC, GPAT3*, and *RGS6* were expressed at significantly higher levels

Table 1
Differentially expressed genes (FDR ≤0.05) in liver samples from sheep classified as thermotolerant (TT) and non-thermotolerant (NTT).

Gene Symbol	logFC2	logCPM2	P-value	FDR
LOC101108817	2.747135	6.272352	2.49E+07	0.003606
VLDLR	1.690921	2.520775	1.36E+08	0.009819
LOC106990580	−1.747148	1.652942	2.66E+08	0.012848
RGS6	3.092116	1.005398	6.37E+08	0.023075
GPX3	1.900299	8.102792	9.93E+08	0.028786
EVC	2.171116	2.821362	1.59E+09	0.038482
GPAT3	2.157949	1.254656	1.91E+09	0.039471

[1] Log(2) Fold Change thermotolerant: non-thermotolerant.
[2] Log(2) values of counts per million.
[3] p-value adjusted by False Discovery Rate (Benjamin-Hochberg 1995).

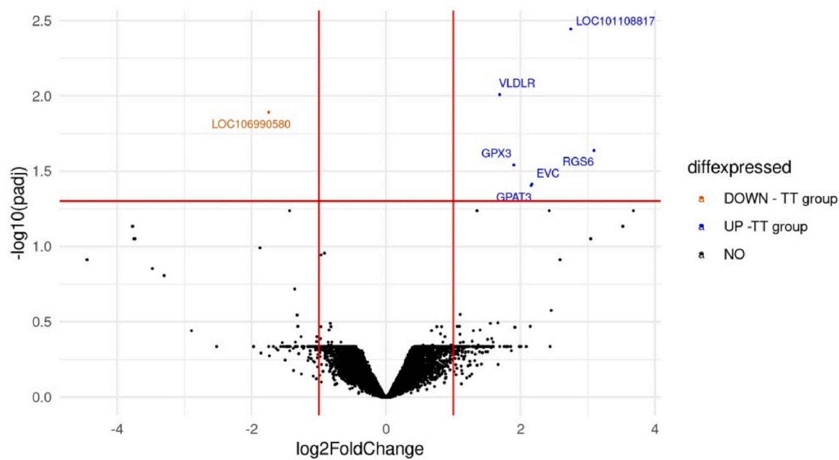

Fig. 1. Volcano plot of log2FoldChange (x-axis) versus -log10 p-value adjusted (FDR≤0.05, y-axis) of thermotolerant (TT) compared to non-thermotolerant (NTT) sheep liver samples.

(FDR≤0.5) in the liver of the TT group than in that of the NTT group when exposed to a heat stress challenge (36 °C) in a climatic chamber. LOC106990580 (*COL1A1*) was expressed at higher levels in the livers of the NTT group than in the TT group (FDR≤0.5).

3.2. Functional enrichment analysis

Functional enrichment analysis was performed to understand the biological functions of differentially expressed genes. The results showed that the following pathways were enriched with differentially expressed genes: Hedgehog signaling pathway, glutathione metabolism, glycerolipid metabolism, and thyroid hormone synthesis (Table 2).

3.3. Gene ontology

Results from the gene ontology analysis showed that the differentially expressed genes were mainly involved in the biological processes of low-density lipoprotein receptor particle metabolism and catabolic process, response to hydroperoxide, hydrogen peroxide catabolic process, and positive regulation of the smoothened signaling pathway (Table 3).

4. Discussion

Thermoregulation has an economic impact on sheep production; however, this effect is not fully understood. In this study, differences in gene expression were identified in the livers of Santa Ines sheep subjected to heat stress. Genes differentially expressed in this study were involved in pathways related to heat stress and other biological processes, such as the hedgehog signaling pathway; however, their role in thermotolerance of Santa Ines sheep remains unclear.

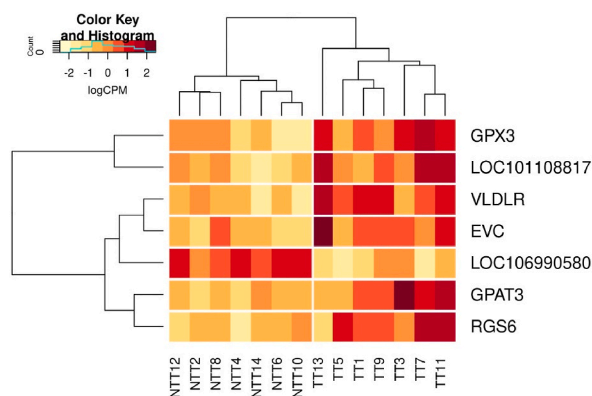

Fig. 2. Heatmap of seven differentially expressed genes (DEGs) from sheep liver samples between thermotolerant (TT) and non-thermotolerant (NTT) animals. The expression of genes is depicted through lines and columns, which organize them into a hierarchical structure. In the column for samples, thermotolerant animals are represented in red for their upregulated genes and in yellow for their downregulated genes.

Table 2
Pathways of differentially expressed genes related to heat adaptability in Santa Ines sheep.

Description	Enrichment Ratio	P Value
Hedgehog signaling pathway	51.567	0.019270
Glutathione metabolism	43.280	0.022931
Glycerolipid metabolism	39.732	0.024961
Arachidonic acid metabolism	38.471	0.025773
Thyroid hormone synthesis	32.752	0.030227
Glycerophospholipid metabolism	24.986	0.039496
Metabolic pathways	18.572	0.44763

Table 3
Gene ontology of heat adaptability differentially expressed genes in liver of Santa Ines sheep.

Description	Enrichment Ratio	P Value
Reelin-mediated signaling pathway	555.47	0.0017992
Low-density lipoprotein particle receptor catabolic process	256.37	0.0038950
Very-low-density lipoprotein particle clearance	370.31	0.0026978
CDP-diacylglycerol biosynthetic process	256.37	0.0038950
CDP-diacylglycerol metabolic process	238.06	0.0041941
Response to hydroperoxide	175.41	0.0056886
low-density lipoprotein receptor particle metabolic process	151.49	0.0065844
Hydrogen peroxide catabolic process	111.09	0.0089702
Positive regulation of smoothened signaling pathway	104.15	0.0095659
Receptor catabolic process	100.99	0.0098636

We explored liver gene expression in groups of animals that diverged in terms of heat tolerance using internal temperature. Cellular damage and a negative impact on the immune response are common responses to heat stress [28]. Overproduction of reactive oxygen species (ROS) is considered toxic to cells [29,30]. In addition, excessive accumulation of ROS causes oxidative stress [31].

Reactive oxygen species such as superoxide (O2 −), hydroxyl radicals (OH −), and hydrogen peroxide (H2O2) are naturally produced during oxygen metabolism and result from normal cellular functions [32,33]. Although small concentrations of ROS are used in cell signaling [34], unchecked growth in the levels of reactive oxygen species (ROS) can lead to harmful effects on vital cellular components, such as lipids, proteins, and nucleic acids, causing severe molecular damage and triggering apoptosis [35,36] and lipid peroxidation.

GPx3, a gene that is highly expressed in thermotolerant animals, GPx3 mRNA is typically found in the kidney, but it can also be present in the liver [37]. This gene encodes glutathione peroxidase, which is induced in response to heat shock [38]. Therefore, the higher expression in this group may be viewed as a way to protect cells from heat damage.

When an animal is subjected to a stressor, its body uses various strategies to counteract its effects. Upon stress detection, the cells translate signals and initiate appropriate countermeasures. The stress response is activated and involves altering gene expression profiles and boosting repair and defense capabilities. This adaptation is crucial for organisms to survive and thrive under stressful conditions [39,40]. Thus, increased cellular protection may be one of the reasons why thermotolerant animals suffer less under heat stress conditions. According to a study by Ref. [41], *GPx3* gene expression differs between stress-tolerant and stress-susceptible species. Living organisms trigger the transcription of genes that defend against cellular damage and demise to promote the survival and expansion of cells during periods of extreme heat. [42], which may have occurred in thermotolerant animals in the present study.

In contrast, cellular damage caused by thermal and oxidative stress can induce HSP expression. The ultimate defense mechanism utilized by a cell to safeguard itself against the consequences of heat stress is widely regarded as a defining characteristic of acclimation [43]. The function of these proteins is to counteract the harmful consequences of maintaining cellular balance and stability in response to environmental challenges [44]. Increased HSP70 expression can increase lipogenesis [45–47]. Lipids also play an important role in the heat stress response in animals, as they are an important energy source during periods of high energy demand [48], such as heat stress. The reaction of adipose tissue to this can lead to a greater storage of lipids, which suggests that it may play a significant role in the body's ability to cope with heat stress [49].

The *GPAT3* (Glycerol-3-Phosphate Acyltransferase 3) gene plays an important role in heat stress response in animals. This gene is involved in the synthesis of lipids, including triglycerides and fatty acids, which are energy sources [50]. *GPAT3* is highly expressed in thermotolerant animals because it may be able to better utilize dietary lipids, which are essential for metabolic homeostasis because they are easily digested and generate lower metabolic heat. In addition, these animals have greater thermolysis capacity, resulting in a lower energy expenditure requirement, leading to increased lipid storage for future use throughout the body [51]. Additionally, during periods of stress, an increase in lipogenesis may occur owing to the increased expression of *HSP70* protein, which results in increased levels of fatty acids and triglycerides. Therefore, very low-density lipoprotein (VLDL) may be higher in this group of thermotolerant animals to prevent fat accumulation in the liver because VLDL transports triglycerides from the liver to the peripheral tissues of the body [52]. When circulating fatty acids increase, VLDLs tend to be produced in greater quantities by the liver to carry greater amounts of lipids [53]. This was observed in thermotolerant animals that showed higher expression of the very low-density lipoprotein receptor (VLDLR) gene [54,55].

Recent studies have shown that the *VLDLR* gene also plays an important role in heat stress response in animals [56]. The study by Álvarez et al. (2020) aimed to assess how sheep adapted to the challenging West African environment and discovered the genes responsible for lipid metabolism and temperature stress responses, including *VLDLR*. Furthermore, it is believed that *VLDLR* may be involved in body temperature regulation in animals. A study in mice showed that *VLDLR* can regulate thermogenesis in brown adipocytes, suggesting its important role in body temperature regulation [57].

HSF1 expression may also be involved in the transcriptional regulation of some Regulator of G-protein signaling (*RGS*) genes that have as their main function the negative regulation in G proteins that are involved in cellular transduction [58]. Thus, the higher expression of *RGS6* in thermotolerant animals may have favored cellular signaling and responses to heat stress when *RGS6* traffic occurs from the nuclear sites to the nucleoli. The protein is believed to be involved in signaling pathways or cellular functions related to stress, and the movement of RGS proteins may prevent their negative regulatory interactions with G proteins and influence their specialized functions within the nucleolus [59]. The ribosomal biosynthesis and nucleolar biogenesis of ribosomal DNA (rDNA) occur at the nucleolus [60]. rDNA encodes ribosomal RNA, which is required for mRNA translation [61]. Higher expression of *RGS6* may also be involved in protecting rDNA during heat stress.

Hormonal changes in heat stress occur through the activation of the hypothalamic-pituitary-adrenal axis, which causes the release of corticotrophin-releasing hormone, and in the adenohypophysis (anterior pituitary), where the adrenocorticotropic hormone is secreted, and stimulates the adrenal cortex to produce and secrete cortisol [62]. Cortisol is responsible for recruiting energy during stressful situations, and since it is a steroid hormone synthesized from cholesterol, lipids may be involved in the hormone increase.

During heat stress, the concentrations of the hormones triiodothyronine (T3) and thyroxine (T4) are reduced [63]. They are produced by the thyroid via anabolic and catabolic pathways. Consequently, their release generates endogenous heat; therefore, the reduction in T3 and T4 observed in animals subjected to heat stress is justified by the attempt to decrease the production and accumulation of body heat [64,65]. GPx3 plays a key role in the biosynthesis of hormones in the thyroid gland. This gene also plays a protective role in cells by protecting them from damage caused by hydrogen peroxide (H_2O_2), which is essential for the synthesis of T3 and T4 [66].

The increase in cortisol and decreased food intake as a consequence of stress can cause alterations in the immune function of an animal, characterized by chronic heat stress [67,68] and inhibition of immunoglobulins [69,70]. A reduction in immunoglobulin levels was observed by Ref. [71], who showed that passive immunity was lower in animals under heat stress conditions. In contrast, thermotolerant animals express higher levels of Ig gamma (*LOC101108817*), which is responsible for immunoglobulin synthesis and linked to greater immunity [72,73]. It is possible that the adverse impact of heat stress on the immune system was experienced by the non-thermotolerant group, which showed a reduced expression of Ig-gamma.

Therefore, our results suggest that the immunity of thermotolerant animals may not be affected by heat stress, and the protective and signaling genes that were also highly expressed in this group may have contributed to reducing the negative effects of heat stress. Perhaps the high expression of IgG in this study facilitates the alleviation of the harmful effects of heat stress in sheep, and we believe that it is necessary to better understand the type of stimulus that may or may not inhibit genes related to the immune system.

The mutations in EVC cause the chondrodysplasia Ellis-van Creveld syndrome in humans, but EVC also participates as positive regulator of the hedgehog signaling pathway (HH) [74]. The mechanism by which EVC regulates HH signaling remains unknown. According to Ref. [75] Yang et al. (2012), the EVC gene acts downstream of Smoothened (Smo) to promote activation of the GLI transcription factor.

Smo is a key component of the HH signaling pathway [76]. The Hedgehog (HH) signaling pathway is a vital component in the development of intercellular communication and is essential for the formation of mammalian organs as well as for the regeneration and maintenance of balance within the organism [77]. In some contexts, the actions of these organisms are essential for the development, organization, and transformation of various areas of the body, such as vertebrates, insects, and possibly other invertebrates [78]. However, its relationship with thermotolerance in sheep remains unclear.

According to the enrichment analysis outcomes, these genes were discovered to participate in numerous pathways and biological processes. This suggests that the TT and NTT groups have dissimilar thermotolerances. In our study, thermotolerant animals exhibited higher expression of genes associated with protection from cellular damage, lipid synthesis (potentially serving as an energy source for thermoregulation), and increased expression of VLDL, which could be crucial for preventing fat accumulation in the liver as a consequence of heat stress. Additionally, some genes are involved in the synthesis of T3, which is linked to metabolism and can be influenced by increased air temperature. Although it is clear that more research is needed to identify the specific genes and pathways involved in regulating body temperature, further investigation is necessary to elucidate these mechanisms.

These findings could lead to the identification of animals with superior abilities to resist the harmful impacts of rising temperatures caused by climate change. Moreover, identification of genes that foster elevated heat tolerance can significantly assist in genetic improvement programs.

5. Conclusion

The elevated expression of genes linked to immune function and lipid metabolism in thermotolerant sheep suggests a crucial role in countering heat-induced cellular damage. These findings offer insights into breeding strategies to enhance thermotolerance in livestock and to promote sustainable production in changing climates. Future research should delve deeper into the mechanistic aspects of these gene pathways and explore their broader applicability across different breeds. Our work underscores the potential of genomic advancements to drive agricultural resilience, making a meaningful impact on animal well-being and productivity.

Ethics approval

The experiment was approved by the Ethics Committee on Animal Experimentation (CEUA/FZEA/USP Declaration 7498130919) considering the legal and ethical issues of the interventions performed.

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CRediT authorship contribution statement

Messy Hannear de Andrade Pantoja: Writing – original draft, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Francisco José de Novais:** Writing – original draft, Formal analysis, Data curation. **Gerson Barreto Mourão:** Writing – original draft, Methodology, Conceptualization. **Raluca G. Mateescu:** Writing – original draft, Methodology. **Mirele Daiana Poleti:** Formal analysis. **Mariane Beline:** Writing – original draft, Investigation. **Camylla Pedrosa Monteiro:** Investigation. **Heidge Fukumasu:** Methodology, Formal analysis, Conceptualization. **Cristiane Gonçalves Titto:** Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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This article has been published as part of the first author's Ph.D. thesis [79].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e25692.

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