UNIVERSITY OF SÃO PAULO
FACULTY OF ANIMAL SCIENCE AND FOOD ENGINEERING

MESSY HANNEAR DE ANDRADE PANTOJA

Thermoregulation responses in sheep: a cellular approach

Respostas de termorregulação em ovinos: abordagem celular

Pirassununga

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Thesis presented to the Faculty of Animal Science and Food Engineering of the University of São Paulo in partial fulfillment of the requirements for the degree of Doctor of Science.

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Epigraph

“Learning is the only thing the mind never exhausts, never fears, and never regrets.”

Leonardo da Vinci

Resumo

PANTOJA, M. H. A. **Resposta de termorregulação em ovinos: abordagem celular**. 2022. 97f. Tese (Doutorado) – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2022.

O objetivo deste estudo é a identificação de diferenças fisiológicas e metabólicas entre ovinos mais e menos tolerantes ao calor, por meio da caracterização das alterações morfológicas, endócrinas e moleculares durante o estresse por calor. O trabalho foi composto por dois experimentos que foram conduzidos no *Campus* Fernando Costa (FZEA) da Universidade de São Paulo, Brasil. No primeiro experimento foram utilizadas 24 ovelhas da raça Santa Inês (pelagem preta) prenhes e 18 não prenhes, os animais foram avaliados durante duas ondas de calor consecutivas e em condições de termoneutralidade. Durante experimento foram registrados dados meteorológicos (temperatura do ar, umidade relativa e radiação solar), variáveis fisiológicas (temperatura retal, timpânica e frequência respiratória) e hormonais (triiodotironina e prolactina). No segundo experimento foram utilizadas 80 ovelhas da raça Santa Inês (pelagem preta), distribuídas aleatoriamente em 4 grupos experimentais (20 animais) alojados em piquetes com capim Aruana. Posteriormente, cada grupo foi mantido 7 dias na Câmara climática com temperatura média de 36°C (10h00 às 16h00) e com redução de no termostato para 26 °C a partir das 16h00 às 10h00. Ao final de cada ciclo foram realizadas amostragens de temperatura retal e frequência respiratória, temperatura da superfície ocular por termografia infravermelho e sangue para análise de concentração de T3 às 13h, 16h, 19h, 21h, 1h, 4h, 7h, 10h. Também, foram colhidas amostras de pele para exame histológico, de forma a avaliar a estrutura e a morfologia das glândulas sudoríparas. Neste momento foram colhidos microfragmentos de pele para a realização da transcriptoma para avaliar a expressão gênica de possíveis genes envolvidos na tolerância ao calor. Foram utilizados os parâmetros fisiológicos para selecionar animais com alta tolerância ao calor (HHT) e animais com baixa tolerância ao calor (LHT), e apenas nestes foram avaliadas as concentrações de cortisol e a expressão gênica. As análises de dados foram realizadas com auxílio do programa estatístico *Statistical Analysis System* (SAS, 2017). No experimento 1, as variáveis fisiológicas de animais gestantes e não gestantes foram maiores nas condições de onda de calor e foram relacionadas à ativação do sistema termorregulador para manutenção da homeotermia. A temperatura corporal central foi maior durante as ondas de calor sucessivas, assim como a temperatura timpânica, ambas afetadas por mudanças na temperatura do ar. No experimento 2, ovelhas de LHT apresentaram temperaturas retais e antímeros direitos mais elevados ao longo do dia. Animais com LHT acumularam mais calor e precisaram de mais tempo para dissipar o excesso de calor do que ovelhas com HHT e por fim, foram encontrados 15 genes diferencialmente expressos entre animais de baixa e alta tolerância ao calor. Os cinco genes diferencialmente expressos (DE) foram upregulated e 10 genes DE foram downregulated no HHT em comparação com o grupo LHT. Foi possível identificar individualidade em relação à capacidade de termorregulação e tolerância ao calor em ovinos Santa Inês no grupo estudado. Estudos mais aprofundados são necessários para entender como a regulação genica se comporta em ovinos deslanados.

Palavras-chave: Aclimatação. *Ovis aries*. Termólise. Transcriptoma.

Abstract

PANTOJA, M. H. A. **Thermoregulation responses in sheep: a cellular approach**. 2022. 97f. Thesis (Doctoral) – Faculty of Animal Science and Food Engineering, University of Sao Paulo, Pirassununga, 2022.

The overall objective of the study is to characterize the physiological versatility of sheep breed, through thermoregulatory and cellular responses, in biomarkers and morphological and endocrine changes during heat stress. The work was composed of two experiments that were carried out at the Fernando Costa *Campus* (FZEA) at the University of São Paulo, Brazil. In the first experiment, twenty-four non-pregnant and 18 pregnant Santa Inês ewes (black coat) were used, the animals were evaluated during two consecutive heat waves and in thermoneutrality conditions. During the experiment, weather variables (air temperature, relative humidity and solar radiation), physiological variables (rectal temperature, tympanic temperature and respiratory rate) and hormonal variables (triiodothyronine and prolactin) were recorded. In the second experiment, 80 Santa Inês sheep (black coat) were used, divided randomly in 4 experimental groups (20 animals) housed in paddocks with Aruana grass. Afterwards, each group was kept for seven days in the climatic chamber with a temperature of 36°C (10:00 am to 4:00 pm) and with a reduction of the thermostat to 26 °C from 4:00 pm to 10:00 am. At the end of each cycle, samples were taken of rectal temperature and respiratory rate, ocular surface temperature by infrared thermography and blood for T3 and insulin concentration analysis at 1:00 pm, 4:00 pm, 7:00 pm, 9:00 pm, 1:00 am, 4:00 am, 7:00 am, 10:00 am. Also, skin samples were collected for histological examination in order to evaluate the structure and morphology of the sweat glands. At this moment, microfragments were collected from skin to perform the transcriptome to evaluate the gene expression of possible genes involved in heat tolerance. Physiological parameters were used to select animals high heat tolerant (HHT) and low heat tolerant (LHT), and only these will be evaluated for T3 and insulin concentrations and gene expression. Data analyzed were performed using the statistical program Statistical Analysis System (SAS, 2017). In experiment 1, the physiological variables of pregnant and non-pregnant animals were higher under heat wave conditions and were related to the activation of the thermoregulatory system to maintaining homeothermy. The core body temperature was higher during successive heat waves, as was tympanic temperature, both affected by changes in air temperature. In experiment 2, LHT sheep had higher rectal temperature and right antimere throughout the day. LHT Animals accumulated more heat and needed more time to dissipate excess heat than HHT sheep and finally, 15 genes were found differentially expressed between animals with low and high heat tolerance. The five differentially expressed (DE) genes were upregulated and 10 DE genes were downregulated in the HHT compared with the LHT group. It was possible to identify individuality in relation to thermoregulation capacity and heat tolerance in Santa Inês sheep in the studied group. Further studies are needed to understand how gene regulation behaves in hair sheep.

Keywords: Acclimation. *Ovis aries*. Thermolysis. Transcriptome.

Sumário

1. INTRODUCTION

Climate change poses a significant threat to the planet and has negatively affected food production and increased the frequency of extreme events such as heat waves (SKUCE et al., 2013). In sheep, exposure to high temperatures can cause heat stress and compromise well-being (JOHNSON, 2018), resulting in reduced milk production (HERBUT et al., 2018), meat quality (RANA et al., 2014), and wool growth (THWAITES, 1967).

To ensure animal welfare, enhance the productive capacity of animal farms, and facilitate adaptation to adverse climatic conditions, it is important to monitor thermoregulatory mechanisms that aim to maintain thermoneutrality (LUZ et al., 2016). Such control can be achieved by monitoring physiological variables such as sweating rate, respiration rate, rectal temperature (CARDOSO et al., 2015), body surface temperature (JOY et al., 2022), hormonal profiles of triiodothyronine (T_3), insulin, and prolactin (TODINI et al., 2007a; WHEELOCK et al., 2010; OUELLET et al., 2021), as well as animal behavior (LI et al., 2018). All these variables can be affected by heat stress, either alone or in combination and at different relative intensities depending on the heat tolerance of animals (BEATTY et al., 2006).

Heat tolerance is the ability of an animal to balance heat production and dissipation under high air temperature conditions (HASSAN et al., 2019). This trait is known to be influenced by coat color and type, age, species, and origin (hot or cold climate) (OSEI-AMPONSAH et al., 2019; DIKMEN et al., 2014). However, it is still unclear why sheep of the same breed and under the same environmental and management conditions differ in their thermoregulatory responses to heat stress.

It is necessary to understand such differences between animals and how they allow each individual to overcome thermal challenges in a different manner. Therefore, studies evaluating physiological, hormonal, and behavioral responses during thermal challenges using omics data are needed to help elucidate the genetic mechanisms underlying animal responses to heat stress and the factors that might explain why some animals are more thermotolerant than others (CARABAÑO et al., 2019). This knowledge can support the selection of animals with superior genetic potential for adaptation to tropical climates as an attempt to improve herd productivity. Furthermore, selection of heat-tolerant sheep can represent a strategy to minimize the negative effects of climate change on livestock production.

1.1.OBJETIVE

1.1.1. GENERAL OBJECTIVE

- The general objective of this study is to identify skin-associated genes related to heat tolerance in Santa Inês sheep with higher or lower levels of susceptibility to heat stress, previously characterized by differences in physiological, hormonal, behavioral, and morphological responses of the skin.

1.1.2. SPECIFIC OBJECTIVES

- Identify differences in sheep thermoregulatory responses to heat stress, connecting evaporative heat loss routes and energy accumulation cycles;
- Identify the effects of acclimatation on physiological responses and endocrine patterns under heat stress;
- Histologically characterize the sweat glands and epidermis of Santa Inês sheep under heat stress; and
- Characterize cellular responses by RNA sequencing and identify potential candidate genes associated with heat stress responses in sheep with different levels of heat tolerance in skin tissues.

2. LITERATURE REVIEW

2.1.HEAT WAVE

Climate change is occurring at a global level, causing an increase in the frequency of extreme events, such as the number of extremely hot days or heat waves (PASQUI; DI GIUSEPPE, 2019). A heat wave is defined as a period of extreme temperatures (VITALI et al., 2015) lasting from three (BROWN-BRANDL et al., 2005) to nine consecutive days (REIS et al., 2019). This change in environmental temperature can affect both human and non-human animals.

In humans, heat waves mostly affect people aged 60 years or older (REY et al., 2007). In addition to the elderly, people with obesity, hypertension, lung diseases, cardiovascular diseases, or diabetes are at a higher risk for heat-related conditions such as cramps, heat exhaustion, and heatstroke (KENNY et al., 2010). In a study by Guirguis et al. (2014), the rate of hospitalization was found to increase during the peak of a heat wave.

In animals, high temperatures can affect key factors of livestock production, such as animal productivity, reproduction, and health (ROJAS-DOWNING et al., 2017). Temperatures above the critical threshold for animal homeothermy decrease productivity because nutrients are diverted from product synthesis to body temperature regulation, resulting in losses in productive parameters, including milk yield, animal growth, reproduction, and carcass traits (BAUMGARD et al., 2012). Another aspect to be considered is animal health: high temperatures can lead to immunosuppression, resulting in increased susceptibility to infectious diseases (AL-BUSAIDI et al., 2008).

Extreme events can also impact food availability, stemming from a decrease in agricultural production (MENDOZA et al., 2020) caused by high temperatures, variations in rainfall (ROJAS-DOWNING et al., 2017), and death of animals that failed to dissipate excess heat (VITALI et al., 2015; MORIGNAT et al., 2014). Given these effects, livestock systems are expected to be increasingly affected by climate change (BERNABUCCI, 2019).

Increased heat tolerance may be a strategy to improve animal performance under adverse climate conditions (BERNABUCCI et al., 2010; SCHARF et al., 2010). This information can guide the selection of animals capable of overcoming thermal challenges (HUEY et al., 2012) and maintaining high production levels under a wide variety of environmental conditions (MENDES et al., 2014).

2.2.THERMAL REGULATION

2.2.1. BEHAVIORAL RESPONSE

When the ambient temperature increases, animals try to maintain their internal body temperature within the thermoneutral zone. For this, animals might increase their respiration and sweating rates in an attempt to facilitate heat loss (WEST, 2003), alter their behaviors by seeking shade and water and reducing feed intake (SHILJA et al., 2016), extend their legs to increase the exchange surface area, and search for cool ground (SILVA et al., 2009).

The reduction in feed intake constitutes an adaptive behavior aimed at reducing thermogenesis. Peripheral and central thermosensors send information to the hypothalamus, which coordinates a response to reduce internal heat production through reduced feed intake. This response, triggered by the neural pathway, is initially transient and, in the short term, strictly dependent on heat dissipation capacity (PEREIRA et al., 2019). Several studies reported reduced feed intake in response to heat stress in sheep. For instance, a study found that animals showed a 17.5% decrease in feed intake and a 19% increase in water intake when subjected to temperatures of 33 to 45 °C in environmental chambers as compared with thermoneutral conditions (22.2 °C) (MAHJOUBI et al., 2015).

Another factor that can contribute to reducing feed intake in animals under heat stress is the increase in respiration rate, which, although being an important and efficient mechanism of heat loss, can be damaging to animal health when maintained for a long time. Prolonged panting reduces rumination and produces endogenous heat from muscle activity, consuming energy that would be used in other metabolic and productive processes to maintain homeothermy. From a certain point on, panting becomes an inefficient mechanism of heat loss (SOUZA et al., 2005).

A study carried out by Monty et al. (1991) showed that feed intake decreases significantly in sheep exposed to high temperatures. When under heat stress, ruminants that are normally active during the day and rest at night decrease their grazing activity, tend to lie down to reduce locomotion, and spend more time in the shade (SILANIKOVE, 2000). These behavioral changes can impact herd productivity due to reduced grazing time (VAN WETTERE et al., 2021).

High water intake is a typical response of sheep in warm environments compared with cold environments (SAVAGE et al., 2008). The increased need for water results from increased respiration and sweating rates (CHEDID et al., 2014). These mechanisms dissipate heat by evaporating water through respiration or from the skin surface. To avoid dehydration, animals increase their water intake.

In addition to contributing to evaporative exchanges, water immediately cools the body when ingested (PEREIRA et al., 2019). Heat exchange occurs according to a thermal gradient between the temperature of the animal body and that of water. Given that the body temperature is higher than the water temperature, ingested water has a cooling effect (SOUZA et al., 2008).

2.2.2. PHYSIOLOGICAL RESPONSE

In principle, a heat-tolerant animal is able to maintain homeothermy under high temperature conditions, which is determined by the animal's ability to balance thermogenesis and thermolysis. Different physiological parameters have been used as criteria to identify heat-tolerant animals, such as internal body temperature, respiration rate, and sweating rate, as they are directly related to physiological responses to the thermal environment (CARABAÑO et al., 2019) (Figure 1). As the ambient temperature rises, there is a reduction in heat loss by non-evaporative pathways (conduction, convection, and radiation), triggering the activation of evaporative pathways and increasing heat loss through increased respiration and sweating (SOUZA et al., 2008).

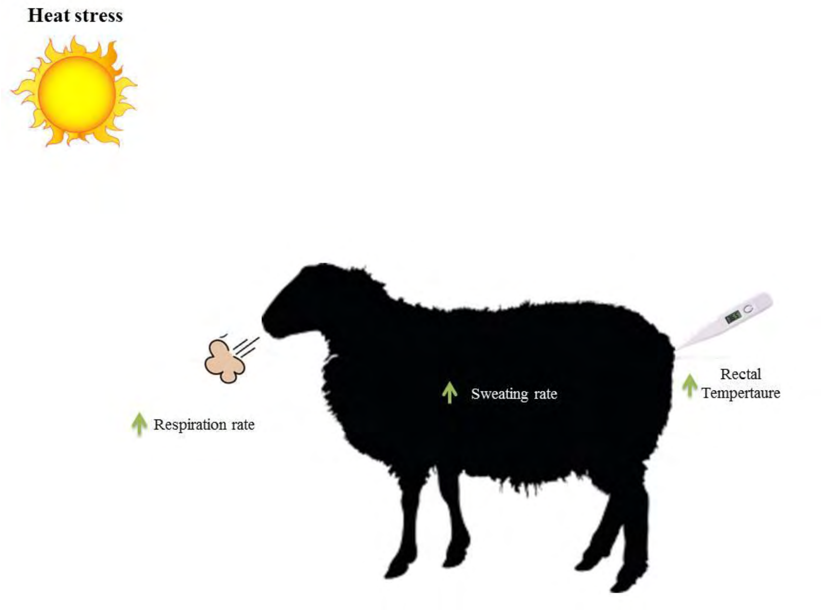

Figure 1. Physiological responses to heat stress.

The respiration rate of animals may change to compensate for variations in environmental conditions (MCMANUS et al., 2016). In sheep, respiration exchanges constitute the main mechanism of heat loss under unfavorable climate conditions (STARLING et al., 2002) and serve as a highly sensitive indicator of heat stress (LUCY; SAFRANSKI, 2017). In addition to respiration exchanges, sweating is triggered as the internal body temperature increases to prevent excessive heat accumulation (FERREIRA et al., 2009). Heat loss is achieved via evaporation of water from the skin surface (GEBREMEDHIN et al., 2008).

Sweating is an autonomic response of animals to heat stress (COLLIER; GEBREMEDHIN, 2015), being quite efficient under conditions of high temperature and low humidity (LUZ et al., 2014). Although often neglected, this mechanism is very effective, as it requires less energy input for thermoregulation and is responsible for about 63% of total evaporation (SILVA; STARLING, 2003). Titto et al. (2016) showed that sweating significantly contributes to thermoregulation in sheep.

However, if animals are unable to dissipate excess heat through thermolytic mechanisms, i.e., heat loss is not greater than heat gain, heat is stored, resulting in an increase in body temperature, as observed by an increase in rectal temperature (BROSH et al., 1998; KAUFMAN et al., 2018). Kahwage et al. (2017) reported that Santa Inês sheep increased their respiration rate by more than 300%, from 31 mov/min to 126 mov/min, when the air temperature increased from 25.7 to 29.8 °C. Respiration exchange was not sufficient to dissipate excess heat, causing a 0.6 °C increase in rectal temperature. This finding demonstrates that rectal temperature can be used as an indicator of thermal comfort or stress imposed by the environment in which the animal is inserted (AL-HAIDARY, 2004).

2.2.3. ***HORMONAL RESPONSE***

Triiodothyronine (T_3)

T_3 is a hormone produced in the follicles of the thyroid gland (ZIMMERMANN, 2009). The hormone is of great importance for adapting to heat stress and improving performance (LEITE et al., 2018b) because it is responsible for the regulation of thermogenesis (SILVA, 2006). T_3 acts on several tissues by promoting heat production in all cells (TODINI, 2007b) through metabolic acceleration and ATP turnover. It is noteworthy that not all energy from ATP hydrolysis is used in enzymatic reactions, a part can be lost in the form of heat. Thus, any reaction that results in ATP hydrolysis also promotes thermogenesis (BIANCO, 2000).

There are different mechanisms by which thyroid hormones enhance the use of ATP for heat production, such as by promoting an increase in lipolysis, lipogenesis, proteolysis, protein synthesis, glucose oxidation, and gluconeogenesis (SILVA, 2005). Another mechanism is the stimulation of uncoupling protein (UCP) expression and activity. UCPs occur in the inner mitochondrial membrane (REIS FILHO, 2013), and their function is to uncouple oxidative phosphorylation, thereby reducing ATP synthesis and producing heat (COLLIN et al., 2005).

T_3 is therefore a stimulator of thermogenesis. Its release is controlled by the hypothalamus by means of the thyrotropin-releasing hormone. When the air temperature rises, T_3 concentrations decrease (KAHL et al., 2015), a strategy of the animal body to decrease heat production and accumulation and normalize internal body temperature when under heat stress (BRAGANÇA et al., 1998; GARCIA, 2013). The degree of T_3 reduction is related to the ability of breeds to adapt to heat stress; T_3 reduction is lower in more heat-tolerant breeds (PEREIRA et al., 2008).

Insulin

Insulin, a hormone produced by pancreatic β-cells, is responsible mainly for the maintenance of blood glucose levels, cell growth, and regulation of carbohydrate, lipid, and protein metabolism (RAHMAN et al., 2021; WILCOX, 2005). When animals ingest feed, there is an increase in insulin production to maintain glucose concentrations within the normal range for organism functioning (KAUFMAN et al., 2015). Given that feed intake decreases under heat stress, insulin levels would be expected to decrease as well. However, it has been shown that under stress situations, insulin levels are elevated (O'BRIEN et al., 2010; WHEELOCK et al., 2010). It is not yet clear why such an increase occurs in animals subjected to high temperatures. This phenomenon may be related to increased intestinal permeability caused by stress, allowing the passage of endotoxins such as lipopolysaccharides from the intestinal lumen into the blood (LAMBERT, 2009). Thus, these responses may induce an increase in insulin concentrations in heat-stressed animals (BANKS et al., 2008).

Another possibility is that, during stress, the sympathetic nervous system releases catecholamines, which may be responsible for stimulating α-adrenergic receptors to increase insulin secretion (ALVAREZ et al., 1989). Tao et al. (2012) showed that animals under heat stress may use glucose in peripheral tissues, possibly promoting insulin sensitivity. Insulin resistance may be passed onto the next generation, being expressed in progeny of mothers who underwent heat stress during gestation (MONTEIRO et al., 2016). However, direct effects of heat stress on insulin concentration are not always evident (TITTO et al., 2017). Indirect effects, on the other hand, were observed by Min et al. (2015); the authors found a change in insulin concentration related to changes in feed intake in animals under heat stress.

Prolactin

Prolactin is a polypeptide hormone synthesized and secreted by the pituitary gland. It plays an important role in thermoregulation, lactation, and reproduction (FREEMAN et al., 2000), and, together with thyroid hormones, is essential for adaptation of animals to heat stress (BEED; COLLIER, 1986). Prolactin secretion follows a circannual rhythm and is influenced by season, with higher levels during warmer months (FOITZIK et al., 2009; LINCOLN et al., 2006; ALAMER, 2011).

Previous studies have shown that ambient temperature stimulates prolactin release (RONCHI et al., 2001; HOOLEY et al., 1979) during adverse environmental conditions. Sheep subjected to heat stress showed an increase in prolactin concentration compared with sheep in thermoneutral environments (JOY et al., 2020). Elevation of prolactin levels during heat stress might be related to the fact that the hormone is responsible for regulating water levels in the body, being particularly important for evaporative heat loss (ALAMER, 2011). Furthermore, prolactin acts on hair growth and seasonal shedding (ALONSO; ROSENFIELD, 2003). The coat may favor heat loss and serve as an indicator of the tolerance of sheep to heat. Of note, unlike heat-susceptible animals, heat-tolerant animals do not exhibit an increase in prolactin production under high air temperature conditions compared with thermoneutral conditions; thus, prolactin can be a good indicator of heat tolerance (SCHARF et al., 2010).

2.3.TRANSCRIPTOMICS AND SINGLE-NUCLEOTIDE POLYMORPHISMS

The transcriptome is the complete set of transcripts of an organism, organ, tissue, or cell line. Important information can be obtained by quantifying the expression levels of each transcript under different conditions (WANG, GERSTEIN; SNYDER, 2009). Although the emergence of new sequencing technologies has contributed greatly to the development of transcriptomics, it is still necessary to convert RNA to DNA for sequencing (ALBERTS et al., 2017).

During sequencing, RNA is isolated and reverse-transcribed into cDNA for subsequent creation of an RNA-seq library (WANG; GERSTEIN; SNYDER, 2009; KUKURBA; MONTGOMERY, 2015). After construction of the library, it is possible to identify sequence variations in transcribed regions, such as single-nucleotide polymorphisms (SNPs) (CLOONAN et al., 2008).

SNPs represent changes in only one base of the DNA sequence, i.e., mutations in the nitrogenous bases of the genome sequence (CAETANO, 2009). There has been an increasing use of SNPs in different areas of research, such as human psychiatric disorders (CHAN et al., 2017), milk protein (LI et al., 2019), meat quality (MAGALHÃES et al., 2016), and heat tolerance (DIKMEN et al., 2015). Therefore, SNPs can be used as a selection tool for adaptation to adverse climate conditions (e.g., high temperatures), contributing to minimizing problems related to heat stress in tropical regions (BAENA et al., 2018).

2.4.GENES RELATED TO HEAT TOLERANCE

Heat stress is defined as an increase in body temperature to above the set point, caused by environmental factors (HANSEN, 2009). Stress can reduce herd performance (MARAI et al., 2007), decrease seminal quality (MOURA et al., 2018), impair spermatogonium mitosis, cause chromatin defects, affect the ability of sperm to fertilize oocytes (MALAMA et al., 2013), increase cortisol secretion (SEVI et al., 2002), and impair the immune response of sheep breeds with low ability to overcome thermal challenges (CAROPRESE et al., 2012).

The response to heat stress is integrated with physiological responses and should be considered as a component of a system-wide gene network coordinated by a variety of cells and tissues to minimize the effects of the thermal environment on cellular functions (COLLIER et al., 2008). Of note, the physiological aspects of heat stress are generally better understood than molecular and genetic aspects (McMANUS et al., 2020).

Increasing attempts have been made to understand the molecular and genetic effects of heat stress by investigating specific genes associated with heat tolerance and stress sensitivity (RENAUDEAU et al., 2012). Previous studies have identified genes that are related to heat stress and associated with other biological functions in sheep (Table 1).

Table 1. Genes associated with heat stress in sheep.

Function	**Genes**	**Breed**	**Reference**
Regulation of body temperature	*TRPM8*	Santa Inês	GOUVEIA et al. (2017)
	HTR4	Hu	LU et al. (2019)
Height variation	*DIS3L2*, *PLAG1*, *NIPBL*	Santa Inês	GOUVEIA et al. (2017)
Spermatogenesis	*SPEF2*, *SPAG6*	Santa Inês	GOUVEIA et al. (2017)
Coat color	*MCIR*, *ASIP*, *TYRP1*	Crioula	CAVALCANTI et al. (2017)
Immunoreaction	*IL1R1*, *IL1R2*, *HSPA2*	Hu	LU et al. (2019)
Thermotolerance (melanogenesis)	*FGF2*, *GNAI3*, *PLCB1*	Barki	KIM et al. (2016)
Energy and digestive metabolism	*MYH*, *TRHDE*, *ALDH1A3*	Barki	KIM et al. (2016)
Nervous and autoimmune responses	*GRIA1*, *IL2*, *IL7*, *IL21*, *IL1R1*	Barki	KIM et al. (2016)

The genetic basis for variations in the response of sheep to changes in environmental temperature (RASHAMOL et al., 2018), as well as its relationship with differences in physiological, endocrine, behavioral, and morphological variables, may be a decisive factor in the expression of genes linked to heat stress (SINGH et al., 2017).

2.5.ANIMAL SUSCEPTIBILITY TO HEAT STRESS

Meteorological conditions can cause heat stress in animals, triggering a series of physiological, biochemical, hematological, and hormonal alterations to maintain homeothermy (RIBEIRO et al., 2018). However, each individual may respond differently to environmental conditions, and such a variation in response allows the classification of animals as more or less susceptible to heat stress (BROWN-BRANDL, 2018). Individual susceptibility is influenced by traits such as coat color, age, species, previous acclimatization, and health status (BROWN-BRANDL; JONES, 2011).

Several studies have identified factors that can increase the susceptibility of animals to heat stress, including differences between genotypes. In sheep, hair breeds have lower rectal temperature and respiration rate when subjected to direct solar radiation than woolly breeds, demonstrating that woolly breeds are more vulnerable to heat stress than hair breeds (PANTOJA et al., 2017).

Light-fleeced sheep generally suffer less from heat stress than dark-fleeced sheep because they exhibit higher reflectivity and absorb less radiation (McMANUS et al., 2011). However, there are individual variations between animals of the same breed and under the same environmental and management conditions, such as differences in respiration rate for thermoregulation (BROWN-BRANDL, 2013). For instance, in a group of animals exposed to an air temperature of 32.9 °C, respiration rates ranged from 78 to 167 mov/min (BROWN-BRANDL, 2013)

Therefore, it is still necessary to understand animal individuality, the variables influencing susceptibility to stress, factors involved in the variation of the response of animals of the same group and breed, and how this information can be used for the selection of animals with better thermolytic and productive performance.

REFERENCES

ALAMER, M. The role of prolactin in thermoregulation and water balance during heat stress in domestic ruminants. **Asian Journal of Animal and Veterinary Advances**, v.6, p.1153-1169, 2011. https://doi.org/10.3923/ajava.2011.1153.1169.

ALBERTS et al. **Biologia Molecular da Célula**. 6. ed. Porto Alegre: Artmed, 2017.

AL-BUSAIDI, R.; JOHNSON, E. H.; MAHGOUB, O. Seasonal variations of phagocytic response, immunoglobulin G (IgG) and plasma cortisol levels in Dhofari goats. **Small Ruminant Research**, v.79, n.2, p.118-123, 2008.

AL-HAIDARY, A. A. Physiological responses of Naimey sheep to heat stress challenge under semi-arid environments. **International Journal of Agriculture & Biology**, v.6, n.2, p.307-309, 2004

ALONSO, L. C.; ROSENFIELD, R. L. Molecular Genetic and Endocrine Mechanisms of Hair Growth. **Hormone Research in Paediatrics**, v.60, n.1, p.1–13, 2003. doi:10.1159/000070821

ALVAREZ, M.A.; PORTILLA, L.; GONZALEZ, R.; EZCURRA, E. Insulin response to a short stress period. **Psychoneuroendocrinology**, v.14, n.3, p.241-4, 1989. https://doi.org/10.1016/0306-4530(89)90022-x.

BAENA, M.M.; TIZIOTO, P.C.; MEIRELLES, S.L.C.; REGITANO, L.C.A. HSF1 and HSPA6 as functional candidate genes associated with heat tolerance in Angus cattle. **Revista Brasileira de Zootecnia**, v.47, p.e20160390, 2018. doi.org/10.1590/rbz4720160390

BANKS, W.A.; DOHGU, S.; LYNCH, J.L.; FLEEGAL-DEMOTTA, M.A.; ERICKSON, M.A.; NAKAOKE, R.; VO, T.Q. Nitric Oxide Isoenzymes Regulate Lipopolysaccharide-Enhanced Insulin Transport across the Blood-Brain Barrier. **Endocrinology**, v.149, n.4, p.1514 –1523, 2008.

BAUMGARD, L.H.; RHOADS, R.P.; RHOADS, M.L.; GABLER, N.K.; ROSS, J.W.; KEATING, A.F.; BODDICKER, B.L.; LENKA, S.; SEJIAN, V. Impact of climate change on livestock production. *In*: SEJIAN, V.; NAQVI, S.M.K.; EZEJI, T.; LAKRITZ, J.; LA, R. (eds.). **Environmental Stress and Amelioration in Livestock Production**. New York: Springer, 2012. pp. 413–468.

BEATTY, D.T., BARNES, A.; TAYLOR, E.; PETHICK, D.; MCCARTHY, M.; MALONEY, S.K. Physiological responses of *Bos taurus* and *Bos indicus* cattle to prolonged, continuous heat and humidity. **Journal of Animal Science**, v.84, n.4, p.972-985, 2006.

BEEDE, D. K.; COLLIER, R. J. Potential Nutritional Strategies for Intensively Managed Cattle during Thermal Stress. **Journal of Animal Science**, v.62, n.2, p.543–554, 1986. doi:10.2527/jas1986.622543x

BERNABUCCI, U. Climate change: impact on livestock and how can we adapt. **Animal Frontiers**, v. 9, n.1, p.3-5, 2019. https://doi.org/10.1093/af/vfy039.

BERNABUCCI, U.; LACETERA, N.; BAUMGARD, L. H.; RHOADS, R. P.; RONCHI, B.; NARDONE, A. Metabolic and hormonal acclimation to heat stress in domesticated ruminants. **Animal**, v.4, n.7, p.1167-1183, 2010

BIANCO, A.C. Hormônios Tireóideos, UCPs e Termogênese. **Arquivos Brasileiros de Endocrinologia e Metabologia**, v.44, n.4, p.281-289, 2000.

BRAGANÇA, M.M.; MOUNIER, A. M.; PRUNIER, A. Does Feed Restriction Mimic the Effects of Increased Ambient Temperature in Lactating Sows? **Journal of Animal Science**, v.76, p.2017–2024, 1998.

BROSH, A.; AHARONI, Y.; DEGEN, A. A.; WRIGHT, V.; YOUNG, B. A. Effects of solar radiation, dietary energy, and time of feeding on thermoregulatory responses and energy balance in cattle in a hot environment. **Journal of Animal Science**, n.76, p.2671-2677, 1998.

BROWN-BRANDL, T. M. Understanding heat stress in beef cattle. **Revista Brasileira de Zootecnia**, v.47, p.e20160414, 2018. https://doi.org/10.1590/rbz4720160414.

BROWN-BRANDL, T. M. Managing thermal stress in feedlot cattle: environment, animal susceptibility and management options from a US perspective. **Livestock Housing**, p.189–208, 2013. doi:10.3920/978-90-8686-771-4_10.

BROWN-BRANDL, T. M.; JONES, D. D. Feedlot Cattle Susceptibility to Heat Stress: An Animal-Specific Model. **Transactions of the ASABE**, v.54, n.2, p.583-598, 2011.

BROWN–BRANDL, T.M.; EIGENBERG, R.A.; HAHN, G.L.; NIENABER, J.A.; MADER, T.L.; SPIERS, D.E.; PARKHURST, A.M. Analyses of thermoregulatory responses of feeder cattle exposed to simulated heat waves**. International Journal of Biometeorology**, v.49, p.285–296, 2005. https://doi.org/10.1007/s00484-004-0250-2

CAETANO, A.R. Marcadores SNP: conceitos básicos, aplicações no manejo e no melhoramento animal e perspectivas para o futuro. **Revista Brasileira de Zootecnia**, v.38, p.64-71, 2009 (supl. especial)

CARABAÑO, M.J.; RAMÓN, M.; MENÉNDEZ-BUXADERA, A.; MOLINA, A.; DÍAZ, C. Selecting for heat tolerance. **Animal Frontiers**, v.9, n.1, 2019. doi: 10.1093/af/vfy033

CARDOSO, C.C.; PERIPOLLIA, V.; AMADORA, S.A.; BRANDÃO, E.G.; ESTEVES, G.I.F.; SOUSAA, C.M.Z.; FRANÇA, M.F.M.S.; GONÇALVESA, F.G.; BARBOSAA, F.A.; MONTALVÃO, T.C.; MARTINS, C.F.; FONSECA NETO, A.M.; MCMANUS, C. Physiological and thermographic response to heat stress in zebu cattle. **Livestock Science**, v.182, p.83–92, 2015. https://doi.org/10.1016/j.livsci.2015.10.022.

CAROPRESE, M.; ALBENZIO, M.; BRUNO, A.; ANNICCHIARICO, G.; MARINO, R.; SEVI, A. Effects of shade and flaxseed supplementation on the welfare of lactating ewes under high ambient temperatures. **Small Ruminant Research**, v.102, n.2, p.177e85, 2012.

CAVALCANTI, L.C.G.; MORAES, J.C.F.; FARIA, D.A.; MCMANUS, C.M.; NEPOMUCENO, A.R.; SOUZA, C.J.H.; CAETANO, A.R.; PAIVA, S.R. Genetic characterization of coat color genes in Brazilian Crioula sheep from a conservation nucleus. Genetic characterization of coat color genes in Brazilian Crioula sheep from a conservation nucleus. **Pesquisa Agropecuária Brasileira**, v.52, n.8, p.615-622, 2017. https://doi.org/10.1590/S0100-204X2017000800007

CHAN, K.; COOPER, J.D.; HEILMANN-HEIMBACH, S.; FRANK, J.; WITT, S.H.; NÖTHEN, M.M., STEINER, J.; RIETSCHEL, M.; BAHN, S. Associations between SNPs and immune-related circulating proteins in schizophrenia. **Scientific Reports**, v.7, n.12586, 2017. https://doi.org/10.1038/s41598-017-12986-0

CHEDID, M.; JABER, L.S.; GIGER-REVERDIN, S.; DUVAUX-PONTER, C.; HAMADEH, S.K. Review: Water stress in sheep raised under arid conditions. **Canadian Journal of Animal Science**, v.94, p.243257, 2014. https://doi.org/10.4141/CJAS2013-188

CLOONAN, N.; FORREST, A.R.R.; KOLLE, G.; GARDINER, B.B.A.; FAULKNER, J.G.; BROWN, M.K.; TAYLOR, D.F.; STEPTOE, A.L.; WANI, S.; BETHEL, G.; ROBERTSON,

A.J.; PERKINS, A.C.; BRUCE, S.J.; LEE, C.C.; RANADE, S.S.; PECKHAM, H.E.; MANNING, J.M.; MCKERNAN, K.J.; GRIMMOND, S.M. Stem cell transcriptome profiling via massive-scale mRNA sequencing. **Nature methods**, v.5, n.7, 2008.

COLLIER, R.J.; COLLIER, J.L.; RHOADS, R.P.; BAUMGARD, L.H. Invited review: Genes involved in the bovine heat stress response. **Journal of Dairy Science**, 91, 445–454, 2008.

COLLIER, R.J.; GEBREMEDHIN, K.G. Thermal Biology of Domestic Animals. **Annual Review of Animal Biosciences**, v.3, p.513–532, 2015.

COLLIN, A.; CASSY, S.; BUYSE, J.; DECUYPERE, E.; DAMON, M. Potential involvement of mammalian and avian uncoupling proteins in the thermogenic effect of thyroid hormones. **Domestic Animal Endocrinology**, v.29, p78–87, 2005.

DIKMEN, S.; KHAN, F.A.; HUSON, H.J.; SONSTEGARD, T.S.; MOSS, J.I.; DAHL, G.E.; HANSEN, P.J. The SLICK hair locus derived from Senepol cattle confers thermotolerance to intensively managed lactating Holstein cows. **Journal of Dairy Science**, v.97, n.9, p.5508-5520, 2014. https://doi.org/10.3168/jds.2014-8087.

DIKMEN, S.; WANG, X.Z.; ORTEGA, M.S.; COLE, J.B.; NULL, D.J.; HANSEN, P.J. Single nucleotide polymorphisms associated with thermoregulation in lactating dairy cows exposed to heat stress. **Journal of Animal Breeding and Genetics**, v.132, n.6, p.409–419, 2015. doi:10.1111/jbg.12176.

FERREIRA, F.; CAMPOS, W. E.; CARVALHO, A. U.; PIRES, M. F. A.; MARTINEZ, M.L.; SILVA, M. V. G. B.; VERNEQUE, R. S.; SILVA, P. F. Taxa de sudação e parâmetros histológicos de bovinos submetidos ao estresse calórico. **Arquivo Brasileiro de Medicina Veterinária e Zootecni**a, v.61, n.4, p.763-768, 2009.

FOITZIK, K.; LANGAN, E.A.; PAUS, R. Prolactin and the skin: a dermatological perspective on an ancient pleiotropic peptide hormone. **Journal of Investigative Dermatology**, v.129, n.5, p.1071-1087, 2009.

FREEMAN, M.E.; KANYICSKA, B.; LERANT, A.; NAGY, G. R. Prolactin: Structure, Function, and Regulation of Secretion. **Physiological Reviews**, v.80, n. 4, 2000.

GARCIA, A.R. Thermal comfort on reproductive processes of water buffaloes raised under tropical conditions. **Revista Brasileira de Reprodução Animal**, v.37, p.121-130, 2013.

GEBREMEDHIN, K. G.; HILLMAN, P. E.; LEE, C. N.; COLLIER, R. J.; WILLARD, S. T.; ARTHINGTON, J. D.; BROWN-BRANDL, T. M. Sweating rates of dairy cows and beef heifers in hot conditions. **American Society of Agricultural and Biological Engineers**, v. 51, n.6, p.2167-2178, 2008.

GOUVEIA, J.J.S.; PAIVA, S.R.; McMANUS, C.M.; CAETANO, A.R.; KIJAS, J.W.; FACÓ, O.; AZEVEDO, H.C.; ARAÚJO, A.M.; SOUZA, C.J.H.; YAMAGISHI, M.E.B.; CAMEIRO, P.L.S.; LÔBO, R.N.B.; OLIVEIRA, S.M.P.; SILVA, M.V.G.B. Genome-wide search for signatures of selection in three major Brazilian locally adapted sheep breeds. **Livestock Science**, v.197, p.36–45, 2017. https://doi.org/10.1016/j.livsci.2017.01.006

GUIRGUIS, K; GERSHUNOV, A.; TARDY, A.; BASU, R. The Impact of Recent Heat Waves on Human Health in California. **Journal of Applied Meteorology and Climatology**, v.53, p.3-15, 2014. https://doi.org/10.1175/JAMC-D-13-0130.1.

HANSEN, P.J. Effects of heat stress on mammalian reproduction. **Philosophical Transactions of the Royal Society B: Biological Sciences**, v.364, p.3341-3350, 2009.

HASSAN, F.; NAWAZ, A.; REHMAN, M.S.; ALI, M.A.; DILSHAD, S.M.R.; YANG, C. Prospects of HSP70 as a genetic marker for thermo-tolerance and immuno-modulation in animals under climate change scenario. **Animal Nutrition**, v.5, n.4, p.340-350, 2019. https://doi.org/10.1016/j.aninu.2019.06.005.

HERBUT, P.; ANGRECKA, S.; WALCZAK, J. Environmental parameters to assessing of heat stress in dairy cattle: a review. **International Journal of Biometeorology**, v.62, P.2089–2097, 2018.

HOOLEY, R.D.; FINDLAY, J.K.; STEPHENSON, R. G. A. Effect of Heat Stress on Plasma Concentrations of Prolactin and Luteinizing Hormone in Ewes. **Australian Journal of Biological Science**, v.32, p.231-5, 1979.

HUEY, R. B.; KEARNEY, M. R.; KROCKENBERGER, A.; HOLTUM, J. A. M.; JESS, M.; WILLIAMS, S. E. Predicting organismal vulnerability to climate warming: roles of behaviour, physiology and adaptation. **Philosophical Transactions of the Royal Society B**, v.367, p.1665-1679, 2012.

JOHNSON, J. S. Heat stress: impact on livestock well-being and productivity and mitigation strategies to alleviate the negative effects. **Animal Production Science**, v.58, p.1404–1413, 2018. https://doi.org/10.1071/AN17725

JOY, A.; DUNSHEA, F.R.; LEURY, B.J.; DIGIACOMO, K.; CLARKE, I.J.; ZHANG, M.H.; ABHIJITH, A.; OSEI-AMPONSA, R.; CHAUHAN, S.S. Comparative Assessment of Thermotolerance in Dorper and Second-Cross (Poll Dorset/Merino × Border Leicester) Lambs. **Animals**, v.10, p.2441, 2010. https://doi.org/10.3390/ani10122441

JOY, A.; TAHERI, S.; DUNSHEA, F.R.; LEURY, B.J.; DIGIACOMO, K.; OSEI-AMPONSAH, R.; BRODIE, G.; CHAUHAN, S.S. Non-invasive measure of heat stress in sheep using machine learning techniques and infrared thermography. **Small Ruminant Research**, v.207, p.106592, 2022. https://doi.org/10.1016/j.smallrumres.2021.106592.

KAHL, S.; ELSASSER, T.H.; RHOADS, R.P.; COLLIER, R.J.; BAUMGARD, L.H. Environmental heat stress modulates thyroid status and its response to repeated endotoxin challenge in steers. **Domestic Animal Endocrinology**, v.52, p.43-50, 2015. https://doi.org/10.1016/j.domaniend.2015.02.001.

KAHWAGE, P.R.; ESTEVES, S.N.; JACINTO, M.A.C.; BARIONI JUNIOR, W.; PEZZOPANE, J.R.M.; PANTOJA, M.H.A.; BOSI, C.; MIGUEL, M.C.V.; MAHLMEISTER, K.; GARCIA, A.R. High systemic and testicular thermolytic efficiency during heat tolerance test reflects better semen quality in rams of tropical breeds. **International Journal of Biometeorology**, v.61, n.10, p.1819-1829, 2017. doi: 10.1007/s00484-017-1367-4.

KAUFMAN, B. A.; LI, C.; SOLEIMANPOUR, S. A. Mitochondrial regulation of β-cell function: maintaining the momentum for insulin release. **Molecular Aspects of Medicine**, v.42, p.91–104, 2015. https://doi.org/10.1016/j.mam.2015.01.004.

KAUFMAN, J. D.; SAXTON, A. M.; RÍU, A. G. Short communication: Relationships among temperature-humidity index with rectal, udder surface, and vaginal temperatures in lactating dairy cows experiencing heat stress. **Journal of Dairy Science**, v.101, n.7, p.6424–6429, 2018.

KENNY, G.P.; YARDLEY, J.; BROWN, C.; SIGAL, R.J.; JAY, O. Heat stress in older individuals and patients with common chronic diseases. **Canadian Medical Association Journal**, v.182, n.10, p. 1053-1060, 2010.

KIM, E-S.; ELBELTAGY, A.R.; ABOUL-NAGA, AM; RISCHKOWSKY, B.; SAYRE, B.; MWACHARO, J.M.; ROTHSCHILD, M.F. Multiple genomic signatures of selection in goats and sheep indigenous to a hot arid environment. **Heredity**, v.116, p.255–264, 2016.

KUKURBA, K.R.; MONTGOMERY, S.B. RNA Sequencing and Analysis. **Cold Spring Harbor Protocols**, *v.*11, p.951–969, 2015. https://doi.org/10.1101/pdb.top084970

LAMBERT, G.P. Stress-induced gastrointestinal barrier dysfunction and its inflammatory effects. **Journal of Animal Science**, v. 87, p.E101-E108, 2009.

LEITE, J.H.G.M.; ASENSIO, L.A.B.; SILVA, W.S.T.; SILVA, W.E.; CHAVES, D.F.; FACÓ, O.; COSTA, W.P.; FAÇANHA, D.A.E. Locally adapted Brazilian sheep: a model of adaptation to Semiarid region. **Semina: Ciências Agrárias**, v. 39, n. 5, p. 2261-2272, 2018b.

LI, C.; WANG, M.; CAI, W.; LIU, S.; ZHOU, C.; YIN, H.; SUN, D.; ZHANG, S. Genetic Analyses Confirm SNPs in HSPA8 and ERBB2 are Associated with Milk Protein Concentration in Chinese Holstein Cattle. **Genes**, v.10, n.2, 2019. https://doi.org/10.3390/genes10020104.

LI, F. K.; YANG, Y.; JENNA, K.; XIA, C.H.; LV, S.J.; WEI, W.H. Effect of heat stress on the behavioral and physiological patterns of Small-tail Han sheep housed indoors. **Tropical Animal Health and Production**, v.50, p.1893–1901, 2018. https://doi.org/10.1007/s11250-018-1642-3

LINCOLN, G.A.; CLARKE, I.J.; HUT, R.A.; HAZLERIGG, D.G. Characterizing a mammalian circannual pacemaker. **Science**, v.314, n.5807, p.1941–1944, 2006.

LU, Z.; CHU, M.; LI, Q.; JIN, M.; FEI, X.; MA, L.; ZHANG, L.; WEI, C. Transcriptomic Analysis Provides Novel Insights into Heat Stress Responses in Sheep. **Animals**, v.9, p. 387, 2019. https://doi.org/10.3390/ani9060387.

LUCY, M.C., SAFRANSKI, T.J. Heat stress in pregnant sows: Thermal responses and subsequent performance of sows and their offspring. **Molecular Reproduction and Development**, v.84, n.9, p.946-956, 2017. https://doi.org/10.1002/mrd.22844.

LUZ, C. S. M.; FONSECA, W. J. L.; BARROS JUNIOR, C. P.; SOUSA, G. G. T.; AMORIM, R. B.; SILVA, L. A.; LIMA, L. A.; SOUSA JÚNIOR, S. C.; SANTOS, K. R.

Estimativas de características termorreguladoras de ovinos em período seco e chuvoso criados na região do vale do Gurguéia, sul do estado do Piauí. **Acta Veterinaria Brasilica**, v.8, n.1, p.19-24, 2014.

LUZ, C.S.M.; FONSECA, W.J.L.; VOGADO, G.M.S.; FONSECA, W.L.; OLIVEIRA, M.R.A.; SOUSA, G.G.T.; FARIAS, L.A.; SOUSA JÚNIOR, S.C. Adaptative thermal traits in farm animals. **Journal Animal Behavior Biometeorology**, v.4, n.1, p.6-11, 2016.

MAGALHÃES, A.F.B.; CAMARGO, G.M.F.; FERNANDES JUNIOR, G.A.; GORDO, D.G.M.; TONUSSI, R.L.; COSTA, R.B.; ESPIGOLAN, R.; SILVA, R.M.O.; BRESOLIN, T.; ANDRADE, H.B.F.; TAKADA, L.; FEITOSA, F.L.B.; BALDI, F.; CARVALHEIRO, R.; CHARDULO, L.A.L.; ALBUQUERQUE, L.G. Genome-Wide Association Study of Meat Quality Traits in Nellore Cattle. **Plos One**, v.11, n.6, p.e0157845, 2016. https://doi.org/10.1371/journal.pone.0157845.

MAHJOUBI, E.; HOSSEIN YAZDI, M.; AGHAZIARATI, N.; NOORI, G. R.; AFSARIAN, O.; BAUMGARD, L. H. The effect of cyclical and severe heat stress on growth performance and metabolism in Afshari lambs. **Journal of Animal Science,** v.93, p.1632–1640, 2015. https://doi.org/10.2527/jas2014-8641

MALAMA, E.; BOLLWEIN, H.; TAITZOGLOU, I.A.; THEODOSIOU, T.; BOSCOS, C.M.; KIOSSIS, E. Chromatin integrity of ram spermatozoa. Relationships to annual fluctuations of scrotal surface temperature and temperature-humidity index. **Theriogenology**, v.80, p.533–541, 2013.

MARAI, I. F. M., EL-DARAWANY, A. A., FADIEL, A., & ABDEL-HAFEZ, M. A. M. Physiological traits as affected by heat stress in sheep—A review. **Small Ruminant Research**, p.71, p.1–12, 2007.

McMANUS M.; FARIA, D.A.; LUCCI, C.M.; LOUVANDINI, H.; PEREIRA, S.A.; PAIVA, S.R. Heat stress effects on sheep: Are hair sheep more heat resistant? Concepta. **Theriogenology**, v.155, p.157e167, 2020.

McMANUS, C.; LOUVANDINI, H.; PAIM, T. P.; MARTINS, R. S.; BARCELLOS, J. O. J.; CARDOSO, C.; GUIMARÃES, R. F.; SANTANA, O. A. The challenge of sheep farming in the tropics: aspects related to heat tolerance. **Revista Brasileira de Zootecnia**, v.40, p.107120, 2011.

McMANUS, C.; TANURE, C.B.; PERIPOLLI, V.; SEIXAS, L.; FISCHER, V. ; GABBI, A.M.; MENEGASSI, S.R.O.; STUMPF, M.T.; KOLLING, G.J.; DIAS, E.; COSTA JR, J.B.G. Infrared thermography in animal production: An overview. **Computers and Electronics in Agriculture**, v.123, p. 10–16, 2016.

MENDES, A. M. P.; AZEVEDO, M. A.; LOPES, P. M. O.; MOURA, G. B. A. Zoneamento bioclimático para a raça ovina Dorper no Estado de Pernambuco. **Pesquisa Agropecuária Brasileira**, v.49, n.12, p.986-993, 2014.

MENDOZA, B.S.; VILLALVA, S.F.; HERNÁNDEZ, E.R.; ESCALERA, A.M.A.; CONTRERAS, E.A.C. Causes and consequences of climate change in livestock production and animal health. Review. **Revista Mexicana de Ciencias Pecuarias**, v.11(Supl.2), p.126-145, 2020.

MIN, L.; CHENG, J. B.; SHI, B. L.; YANG, H. J.; ZHENG, N.; WANG, J. Q. Effects of heat stress on serum insulin, adipokines, AMP-activated protein kinase, and heat shock signal molecules in dairy cows. **Journal of Zhejiang University. Science. B**, v.16, n.6, p.541–548, 2015. https://doi.org/10.1631/jzus.B1400341

MONTEIRO, A.P.A.; GUO, J.R.; WENG, X.S.; AHMED, B.M.; HAYEN, M.J.; DAHL, .G.E.; BERNARD, J.K.; TAO, S. Effect of maternal heat stress during the dry period on growth and metabolism of calves. **Journal of Dairy Science**, v.99, n.5, p.3896-3907, 2016. https://doi.org/ 10.3168/jds.2015-10699.

MONTY, D.E.; KELLY, L.M.; RICE, W.R. Acclimatization of St. Croix, Karakul and Rambouillet sheep to intense and dry summer heat. **Small Ruminant Research**, v.4, p.379–392, 1991.

MORIGNAT, E.; PERRIN, J.; GAY, E.; VINARD, J.; CALAVAS, D.; HE ´NAUX, V. Assessment of the Impact of the 2003 and 2006 Heat Waves on Cattle Mortality in France. **Plos One**, v.9, n.3, p. e93176, 2014.

MOURA, A.B.B.; BRANDÃO, F.Z.; ESTEVES, S.N.; SOUZA, G. N.; FONSECA, J. F.; PANTOJA, M.H.A.; ROMANELLO, N.; BOTTA, D.; GIRO, A.; GARCIA, A.R. Differences in the thermal sensitivity and seminal quality of distinct ovine genotypes raised in tropical conditions. **Theriogenology**, v. 123, p. 123-131, 2018.

O'BRIEN, M.D.; RHOADS, R.P.; SANDERS, S.R.; DUFF, G.C.; BAUMGARD, L.H. Metabolic adaptations to heat stress in growing cattle. **Domestic Animal Endocrinology**, v.38, v.2, p.86-94, 2010.

OSEI-AMPONSAH, R.; CHAUHAN, S. S.; LEURY, B. J.; CHENG, L.; CULLEN, B.; CLARKE, I. J.; DUNSHEA, F. R. Genetic Selection for Thermotolerance in Ruminants. **Animals**, v.9, n.11, p.948, 2019. https://doi.org/10.3390/ani9110948.

OUELLET, V.; NEGRAO, J.; SKIBIEL, A.L.; LANTIGUA, V.A.; FABRIS, T.F.; MARRERO, M.G.; DADO-SENN, B.; LAPORTA, J.; DAHL, G.E. Endocrine Signals Altered by Heat Stress Impact Dairy Cow Mammary Cellular Processes at Different Stages of the Dry Period. **Animals**, v.11, p.563, 2021. https://doi.org/10.3390/ani11020563

PANTOJA, M.H.A.; ESTEVES, S.N.; JACINTO, M.A.C.; PEZZOPANE, J.R.M.; PAZ, C.C.P.; SILVA, J.A.R.; LOURENÇO JUNIOR, J.B.; BRANDÃO, F.Z.; MOURA, A.B.B.; ROMANELLO, N.; BOTTA, D.; GARCIA, A.R. Thermoregulation of male sheep of indigenous or exotic breeds in a tropical environment. **Journal of Thermal Biology**, v. 69, p.302–310, 2017.

PASQUI, M.; DI GIUSEPPE, E. Climate change, future warming and adaptation in Europe. **Animal Frontiers**, v.9, n.1, p.6–11, 2019.

PEREIRA, A.M.; BACCARI, F. JR; TITTO, E.A.; ALMEIDA, J.A. Effect of thermal stress on physiological parameters, feed intake and plasma thyroid hormones concentration in Alentejana, Mertolenga, Frisian and Limousine cattle breeds. **International Journal of Biometeorology**, v.52, n.3, p.199-208, 2008. https://doi.org/10.1007/s00484-007-0111-x.

PEREIRA, A.M.F., TITTO, E.A.L., ALMEIDA, J.A.A. **Adaptação dos ruminantes aos climas quentes**. Curitiba: Appris Editora, 2019.

RAHMAN, M.S.; HOSSAIN, K.S.; DAS, S.; KUNDU, S.; ADEGOKE, E.O.; RAHMAN, M.A.; HANNAN, M.A.; UDDIN, M.J.; PANG, M.G. Role of Insulin in Health and Disease: An Update. **International Journal of Molecular Sciences**, v.22, n.12, p.6403, 2021. https://doi.org/10.3390/ijms22126403.

RANA, M.S.; HASHEM, M.A.; AKHTER, S.; HABIBULLAH, M.; ISLAM, M.H.; BISWAS, R.C. Effect of heat stress on carcass and meat quality of indigenous sheep of Bangladesh. **Bangladesh Journal of Animal Science**, v.43, n.2, p.147- 153, 2014.

RASHAMOL, V.P.; SEJIAN, V.; BAGATH, M.; KRISHNAN, G.; ARCHANA, P.R.; BHATTA, R. Physiological adaptability of livestock to heat stress: an updated review. **Journal of Animal Behaviour and Biometeorology**, v.6, p.62-71, 2018.

REIS FILHO, A.D.; VIEIRA JUNIOR, R.C.; VOLTARELLI, F.A. Exercício físico, proteína desacopladora-3 e termogênese. **Revista Brasileira de Prescrição e Fisiologia do Exercício**, São Paulo, v.7, n.38, p.176-183, 2013.

REIS, N.C.S.; BOIASKI, N.T.; FERRAZ, S.E.T. Characterization and spatial coverage of heat waves in subtropical Brazil. **Atmosphere**, v.10, p.284, 2019. https://doi.org/10.3390/atmos10050284

RENAUDEAU, D.; COLLIN, A.; YAHAV, S.; BASILIO, V.; GOURDINE, J. L.; COLLIER, R. J. Adaptation to hot climate and strategies to alleviate heat stress in Livestock Production. **Animal**, v.6, n.5, p.707-728, 2012.

REY, G.; JOUGLA, E.; FOUILLET, A; PAVILLON, G.; BESSEMOULIN, P.; FRAYSSINET, P.; CLAVEL, J.; HÉMON, D. The impact of major heat waves on all-cause and cause-specific mortality in France from 1971 to 2003. **International Archives of Occupational and Environmental Health**, v.80, n.7, p.615–26, 2007.

RIBEIRO, M.N.; RIBEIRO, N.L.; BOZZI, R.; COSTA, R.G. Physiological and biochemical blood variables of goats subjected to heat stress – a review. **Journal of Applied Animal Research**, v. 46, n.1, p. 1036-1041, 2018.

ROJAS-DOWNING, M.M.; NEJADHASHEMI, A.P.; HARRIGAN, T.; WOZNICKI, S.A. Climate change and livestock: Impacts, adaptation, and mitigation. **Climate Risk Management**, v.16, p.145-163, 2017.

RONCHI, B.; STRADAIOLI, G.; VERINI SUPPLIZI, A.; BERNABUCCI, U.; LACETERA, N.; ACCORSI, P.A.; NARDONE, A.; SEREN, E. Influence of heat stress or feed restriction on plasma progesterone, oestradiol-17β, LH, FSH, prolactin and cortisol in Holstein heifers. **Livestock Production Science**, v.68, n.2–3, p.231-241, 2001.

SAVAGE, D. B.; NOLAN, J. V.; GODWIN, I. R.; MAYER, D. G.; AOETPA, A.; NGUYEN, T.; BAILLIE, N. D.; RHEINBERGER, T. E.; LAWLOR, C. Water and feed intake responses of sheep to drinking water temperature in hot conditions. **Australian Journal of Experimental Agriculture**, v.48, p.1044-1047, 2008.

SCHARF, B.; CARROLL, J.A.; RILEY, D.G.; CHASE, C.C. JR.; COLEMAN, S.W.; KEISLER, D.H.; WEABER, R.L.; SPIERS, D.E. Evaluation of physiological and blood serum differences in heat-tolerant (Romosinuano) and heat-susceptible (Angus) *Bos taurus* cattle during controlled heat challenge. **Journal of Animal Science**, v.88, p.2321-2336, 2010. https://doi.org/10.2527/jas.2009-2551.

SEVI, A.; ALBENZIO, M.; ANNICCHIARICO, G.; CAROPRESE, M.; TAIBI, L. Effects of ventilation regimen on the welfare and performance of lactating ewes in summer. **Journal of animal Science**, v.80, p.2349e61, 2002.

SHILJA, S.; SEJIAN, V.; BAGATH, M.; MECH, A.; DAVID, C. G.; KURIEN, E. K.; VARMA, G.; BHATTA, R. Adaptive capability as indicated by behavioral and physiological responses, plasma HSP70 level, and PBMC HSP70 mRNA expression in Osmanabadi goats subjected to combined (heat and nutritional) stressors. **International Journal of Biometeorology**, v.60, p.1311–1323, 2016. https://doi.org/10.1007/s00484-015-1124-5.

SILANIKOVE, N. Effects of heat stress on the welfare of extensively managed domestic ruminants. **Livestock Production Science**, v.67, p.1–18, 2000.

SILVA, B.A.N.; OLIVEIRA, R.F.M.; DONZELE, J.L.; FERNANDES, H.C.; LIMA, A.L.; RENAUDEAU, D.; NOBLET, J. Effect of floor cooling and dietary amino acids content on performance and behaviour of lactating primiparous sows during summer**. Livestock Science**, v.120, p.25-34, 2009.

SILVA, J.E. Thermogenic Mechanisms and Their Hormonal Regulation. **Physiological Reviews**, v.86, p.435–464, 2006. https://doi.org/10.1152/physrev.00009.2005.

SILVA, J.E. Thermogenic mechanisms and their hormonal regulation. **Physiological Reviews**, v.86, p.435–464, 2006. https://doi.org/10.1152/physrev.00009.2005.

SILVA, R. G.; STARLING, J. M. C. Evaporação cutânea e respiratória em ovinos sob altas temperaturas ambientes. **Revista Brasileira de Zootecnia**, v.32, n.6, p.1956-1961, 2003.

SINGH, K. M.; SINGH, S.; GANGULY, I.; NACHIAPPAN, R. K.; GANGULY, A.; VENKATARAMANAN, R.; CHOPRA, A.; NARULA, H. K. Association of heat stress protein 90 and 70 gene polymorphism with adaptability traits in Indian sheep (*Ovis aries*). **Cell Stress Chaperones**, v. 22, n.5, p.675–684, 2017

SKUCE, P.J.; MORGAN, E.R.; VAN DIJK, J.; MITCHELL, M. Animal health aspects of adaptation to climate change: beating the heat and parasites in a warming Europe. **Animal**, v.7, n.s2, p.333–345, 2013.

SOUZA, B. B.; SOUZA, E. D.; CEZAR, M. F.; SOUZA, W. H.; SANTOS, J. R. S.; BENICIO, T. M. A. Temperatura superficial e índice de tolerância ao calor de caprinos de diferentes grupos raciais no Semi-árido nordestino. **Ciência e Agrotecnologia**, v.32, n.1, p.275-280, 2008.

SOUZA, E. D.; SOUZA, B. B.; SOUZA, W. H.; CEZAR, M. F.; SANTOS, J. R. S.; TAVARES, G. P. Determinação dos parâmetros fisiológicos e gradiente térmico de diferentes grupos genéticos de caprinos no Semi-árido. **Ciência e Agrotecnologia**, v.29, n.1, p.177-184, 2005.

STARLING, J. M. C.; SILVA, R. G.; CERÓN-MUÑOZ, M.; BARBOSA, G. S. C.; COSTA, M. J. R. P. Análise de algumas variáveis fisiológicas para avaliação do grau de adaptação de ovinos submetidos ao estresse por calor. **Revista Brasileira de Zootecnia**, v.31, n.5, p.2070-2077, 2002.

TAO, S.; THOMPSON, I.M.; MONTEIRO, A.P.A.; HAYEN, M.J.; YOUNG, L.J.; DAHL, G.E. Effect of cooling heat-stressed dairy cows during the dry period on insulin response. **Journal of Dairy Science**, v.95, n.9, p.5035-5046, 2012. https://doi.org/10.3168/jds.2012-5405.

THWAITES, C. Prolonged Heat Stress and Wool Growth in Sheep. **International Journal of Biometeorology**, v.11, n.3, p. 297-300, 1967.

TITTO, C. G.; VERÍSSIMO, C. J.; PEREIRA, A. M. F.; GERALDO, A. M.; KATIKI, L. M.; TITTO, E. A. L. Thermoregulatory response in hair sheep and shorn wool sheep. **Small Ruminant Research**, v.144, p.341–345, 2016.

TITTO, C.G.; NEGRÃO, J.A.; CANAES, T.S.; TITTO, R.M.; LEME-DOS SANTOS, T.M.C.; HENRIQUE, F.L.; CALVIELLO, R.F.; PEREIRA, A.M.F.; TITTO, E.A.L. Heat stress and ACTH administration on cortisol and insulin-like growth factor I (IGFI) levels in lactating Holstein cows. **Journal of Applied Animal Research**, v.45, n.1, p.1-7, 2017. https://doi.org/10.1080/09712119.2015.1091326

TODINI, L. Thyroid hormones in small ruminants: effects of endogenous, environmental and nutritional factors. **Animal**, v.1, n.7, p.997–1008, 2007b.

TODINI, L.; MALFATTI, A.; VALBONESI, A.; TRABALZA-MARINUCCI, M.; DEBENEDETTI, A. Plasma total T3 and T4 concentrations in goats at different physiological stages, as affected by the energy intake. **Small Ruminant Research**, v.68, n.3, p.285-290, 2007a.

VAN WETTERE, W.; KIND, K. L.; GATFORD, K. L.; SWINBOURNE, A. M.; LEU, S. T.; HAYMAN, P. T.; KELLY, J. M.; WEAVER, A. C.; KLEEMANN, D. O.; WALKER, S. K. Review of the impact of heat stress on reproductive performance of sheep. **Journal of animal science and biotechnology**, v.12, n.1, p.26, 2021. https://doi.org/10.1186/s40104-020-00537-z

VITALI, A.; FELICI, A.; ESPOSITO, S.; BERNABUCCI, U.; BERTOCCHI, L.; MARESCA, C.; NARDONE, A.; LACETERA, N. The effect of heat waves on dairy cow mortality. **Journal of Dairy Science**, v. 98, n.7, p. 4572-4579, 2015.

WANG, Z.; GERSTEIN, M.; SNYDER, M. RNA-Seq: a revolutionary tool for transcriptomics. **Nature Reviews Genetics**, v.10, n.1, p. 57-63, 2009. https://doi.org/10.1038 / nrg2484.

WEST, J.W. Effects of heat stress on production in Dairy cattle. **Journal of Dairy Science**, v.86, p.2131-2144, 2003.

WHEELOCK, J.B.; RHOADS, R.P.; VAN BAALE, M.J.; SANDERS, S.R.; BAUMGARD, L.H. Effects of heat stress on energetic metabolism in lactating Holstein cows. **Journal of Dairy Science**, v.93, n.2, p.644-655, 2010

WILCOX, G. Insulin and insulin resistance. **The Clinical biochemist Reviews**, v. 26, n.2, p.19-39, 2005.

ZIMMERMANN, M.B. Iodine deficiency. **Endocrine Reviews**, v.30, n.4, p.376–408, 2009. https://doi.org/10.1210/er.2009-0011.

3. Influence of successive heat waves on the thermoregulatory responses of pregnant and non-pregnant ewes

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Abstract: The frequency of heat waves has increased over the last years, with an impact on animal production and health, including the death of animals. Therefore, the aim of this study was to evaluate the dynamics of thermoregulation and hormonal responses in non-pregnant and pregnant ewes exposed to successive heat waves. Twenty-four non-pregnant and 18 pregnant Santa Inês ewes with black coat color (live weight: 55±9.03 kg; age: 60 months) were used. Weather variables such air temperature, relative humidity, and solar radiation were continuously recorded. The rectal and tympanic temperatures and respiratory rate were measured daily. Serum triiodothyronine (T3) and prolactin were evaluated during the heat wave and thermoneutral periods. The physiological variables of pregnant and non-pregnant animals were higher under the heat wave conditions and were related to the activation of the thermoregulatory system for maintaining homeothermy ($P < 0.05$). The core body temperature was higher during successive heat waves ($P < 0.05$), as was the tympanic temperature, which are both affected by changes in air temperature ($P < 0.05$). Pregnant sheep had higher T3 levels in the thermoneutral environment ($P < 0.05$). Prolactin was not affected by temperature. The results indicate that the Santa Inês breed overcomes the thermal challenge during a heat wave without showing severe signs of thermal stress regardless of being pregnant or not.

Keywords: heat stress, thermoregulation, sheep, thermoneutrality

3.1.Introduction

The current scenario of climate changes has increased the frequency and intensity of heat waves in different regions of the world (Sejian et al., 2013). A heat wave is defined as an extreme temperature period (Vitali et al., 2015) that can last three (Brown-Brandl et al., 2005) to nine consecutive days (Reis et al., 2019).

The increase in heat waves can affect animal health and production and can cause the death of animals because these waves occur suddenly and change the thermal environment without prior conditioning of the animals (Bishop-Williams et al., 2015; Pereira et al., 2020). Effective thermoregulatory responses to minimize the impact of rapid changes in the thermal environment on animals show the acclimation capacity (Collier et al., 2019).

Indigenous tropical breeds such as Santa Inês sheep exhibit characteristics related to heat tolerance (Lv et al., 2014). Under stressful environmental conditions, the animals increase their respiration rate, sweating rate and rectal temperature, in addition to modifications in hormonal concentrations (Morais et al., 2008; Titto et al., 2016) in order to avoid sudden death. However, even when acclimated to heat, a sudden change in the environmental temperature caused by a heat wave can represent a real challenge for sheep in terms of activation of their thermoregulatory system and coping with heat loss (Morais et al., 2008; Titto et al., 2016).

Heat waves have resulted in economic losses in different parts of the world. Despite the availability of studies on simulated heat waves and their effects on animal production, the thermoregulatory responses of sheep to sudden temperature changes, such as heat waves, are not sufficiently understood. Therefore, the aim of this study was to evaluate the dynamics of thermoregulation and hormonal responses in non-pregnant and pregnant ewes exposed to two successive heat waves during winter in a tropical climate.

3.2.Material and methods

Ethical approval

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

3.2.1. Animals

Twenty-four non-pregnant and 18 pregnant Santa Inês ewes with a black coat color (live weight: 55 ± 8.6 kg; body condition score: 2.5 on a scale from 1 to 5; age: 60 months) were used. Pregnancy was confirmed by ultrasound. During the experiment, the ewes had a mean of 90 days of gestation (mid-gestation) considering a mean duration of pregnancy of 150 days. The animals were kept on Aruana pasture (*Panicum maximum* cv. Aruana), with free access to artificial shade under an asbestos-cement roof painted white on the upper side (1 m^2/animal).

3.2.2. **Experimental design**

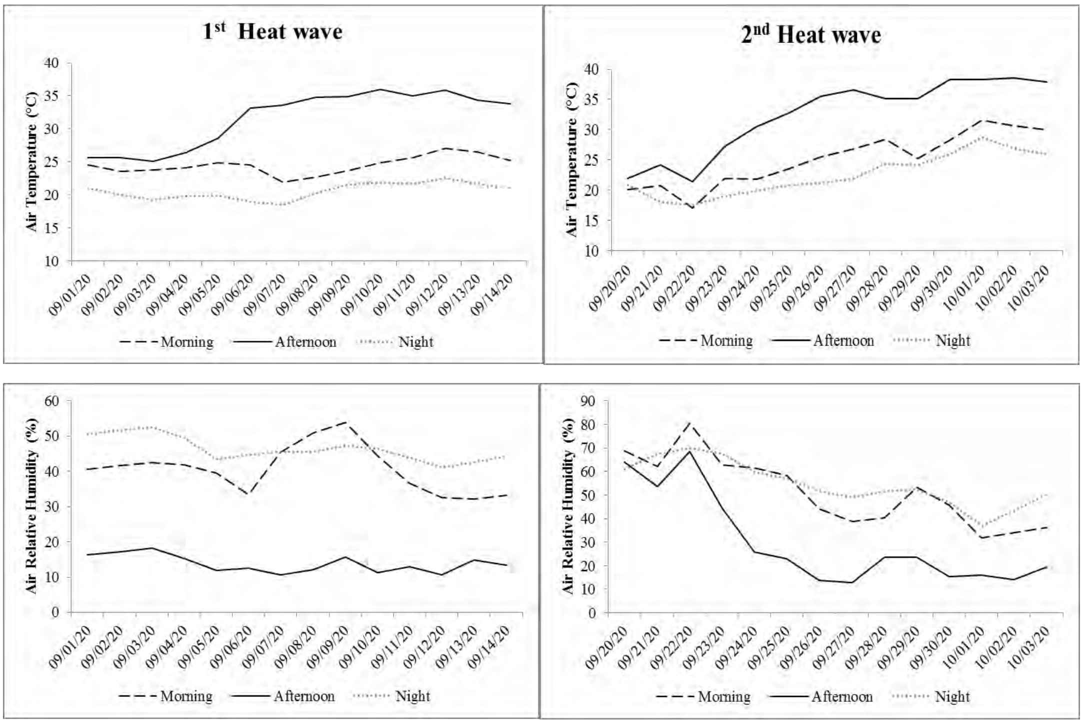
The experiment was designed to assess the thermoregulatory response of animals under different outdoor environmental conditions and was carried out in the winter of 2020 in southeastern Brazil.

Data were collected during successive heat waves, with the interval being characterized by low temperatures that occurred at the end of winter (September). The 1st heat wave occurred over four consecutive days with temperature above 35 °C, reaching a maximum temperature of 37.2 °C, and the 2nd heat wave occurred on seven consecutive days with air temperatures above 37.5 °C, reaching a maximum temperature of 39.8 °C. The interval between the 1st and 2nd heat wave was 14 days.

The animals were also evaluated in winter in the absence of a heat wave, which was called the thermoneutral period (no stress). The data were collected at a mean ambient temperature of 24.7 °C and the maximum temperature recorded on the day was 27.2 °C. The mean maximum air temperature during winter is 28 °C in Pirassununga-SP.

3.2.3. **Meteorological variables**

The air temperature (°C), air relative humidity (%), and solar radiation (W/m^2) were continuously recorded by an automatic weather station (Davis, Vantage Pro 2, Arizona, USA). The station was programmed to take readings every 5 minutes.



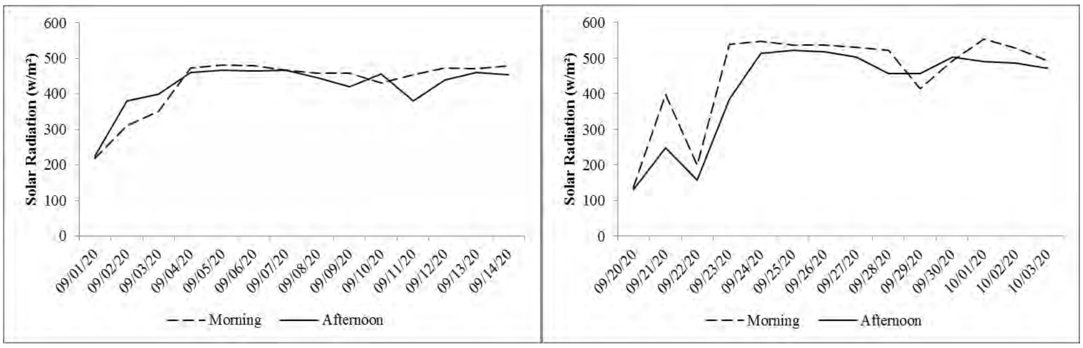


Fig. 1: Maximum values of air temperature (A), air relative humidity (B), and solar radiation (C) during successive heat waves: morning (6 a.m.-12 p.m.), afternoon (12 p.m.-6 p.m.), and night (6 p.m.-6 a.m.).

3.2.4. Physiological variables

The respiratory rate (breaths per minute), rectal temperature (°C) and tympanic temperature (°C) were measured in the afternoon (4 p.m.). The respiratory rate was obtained by observation of thoracoabdominal movements, the rectal temperature was measured with a digital clinical thermometer, and the tympanic temperature was measured with an ear thermometer (TCI100, Incoterm) inserted into the animal's ear canal.

3.2.5. Triiodothyronine (T3) and prolactin measurement

On the penultimate day of each heat wave, a blood sample was collected from each animal by external jugular vein puncture into vacuum tubes without anticoagulant. The samples were centrifuged at 3000 rpm for 15 min and refrigerated at -20 °C. Triiodothyronine and prolactin were measured by enzyme immunoassay (EIA) using commercial kits according to the manufacturer's instructions (Monobind, Lake Forest, CA, USA). Both kits were validated by demonstrating parallel curves between standard concentrations and serially diluted serum samples. The intra- and interassay coefficients of variation were, respectively, 3.8 and 6.3% for T3 and 5.5 and 7.3% for prolactin.

3.2.6. Statistical analysis

The variables were measured at three time points: during the two successive heat waves and during a period without heat stress (thermoneutrality). The response variables were analyzed using a mixed model, considering a Poisson distribution for respiratory rate and a normal distribution for the other variables. The model included the random effect of sheep, considering a specific correlation matrix of better fit because of the repeated measures in the same animal, as well as the fixed effects of physiological state (pregnant or non-pregnant) and stress condition/heat wave (no stress, wave 1, and wave 2) and the interaction between these effects. Significance was set at 5% and 5% to 7% indicated a trend.

3.3.Results

The animals had higher mean respiratory rates ($P < 0.05$; **Table 1**) in the 1st and 2nd heat waves when compared to the thermoneutral period. The rectal temperature was significantly higher ($P < 0.05$) in the 1st and 2nd heat waves than in the thermoneutral environment. Additionally, there was not difference ($P > 0.05$) in rectal temperature between non-pregnant and pregnant ewes.

Santa Inês sheep had higher tympanic temperatures ($P < 0.05$; **Table 1**) in the 1st and 2nd heat waves. There was not difference ($P > 0.05$) in tympanic temperature between non-pregnant and pregnant ewes.

Table 1: Mean and standard error values of the physiological variables of non-pregnant and pregnant Santa Inês ewes measured during successive heat waves and in the thermoneutral environment.

	Non-pregnant	Pregnant
Respiratory rate (breaths per minute)		
Thermoneutral	45.50 ± 3.22 Ca	49.11 ± 3.86 Ca
1st Heat wave	89.83 ± 4.52 Ba	78.22 ± 4.87 Bb
2nd Heat wave	124.83 ± 5.33 Aa	104.18 ± 5.77 Aa
Rectal temperature (°C)		
Thermoneutral	38.99 ± 0.08 Ba	38.90 ± 0.09 Ba
1st Heat wave	39.86 ± 0.11 Aa	39.67 ± 0.13 Aa
2nd Heat wave	39.90 ± 0.71 Aa	39.72 ± 0.08 Aa
Tympanic temperature (°C)		
Thermoneutral	34.61 ± 0.35 Ca	34.46 ± 0.41 Ca
1st Heat wave	38.94 ± 0.12 Aa	38.67 ± 0.14 Aa
2nd Heat wave	37.04 ± 0.22 Ba	37.05 ± 0.27 Ba

A,B different capital letters indicate a significant difference in the columns ($P < 0.05$).

a,b different lowercase letters indicate a significant difference in the rows ($P < 0.05$).

Pregnant ($P < 0.05$) and non-pregnant ($P = 0.065$) animals exhibited higher T3 levels in the thermoneutral environment when compared to the 2nd heat wave (**Table 2**).

Table 2: Mean and standard error values of triiodothyronine (ng/mL) in non-pregnant and pregnant Santa Inês ewes measured during successive heat waves and in the thermoneutral environment.

	Non-pregnant	Pregnant
Thermoneutral	2.67 ± 0.163 Aa	2.58 ± 0.163 Aa
1st Heat wave	2.55 ± 0.163 ABa	2.20 ± 0.163 Aba
2nd Heat wave	2.37 ± 0.163 Ba	2.22 ± 0.166 Ba

A,B different capital letters indicate a significant difference in the columns (non-pregnant: $P = 0.065$; pregnant: $P < 0.05$).

a,b different lowercase letters indicate a significant difference in the rows ($P < 0.05$).

Prolactin levels did not differ between non-pregnant and pregnant ewes (**Fig. 2**), nor were they influenced by successive heat waves ($P > 0.05$).

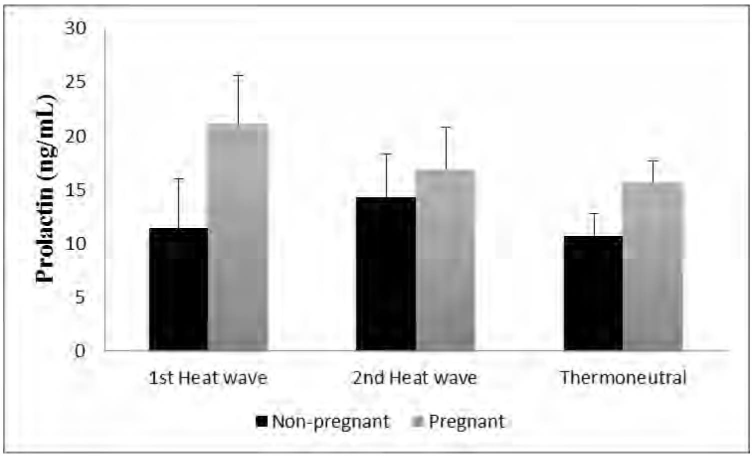


Fig. 2: Mean prolactin levels in non-pregnant and pregnant Santa Inês ewes recorded during successive heat waves and in the thermoneutral environment.

3.4.Discussion

The average air temperatures during winter in southeastern Brazil are generally within the thermoneutral range for wool sheep, with temperatures below 31 °C. According to Van Wettere et al. (2021), thermal stress causes an impact on the performance and welfare of sheep when temperatures fall below 12 °C (lower critical temperature) or rise above 25 to 31

°C (upper critical temperature), situations that trigger the mechanisms of thermoregulation and challenge the capacity of sheep to maintain homeothermy.

During the successive heat waves in winter, the rapid change in ambient temperature exceeded the upper critical limit for sheep. The temperatures reached a maximum of 37.2 °C in the 1st heat wave and of 39.8 °C in the 2nd heat wave. Therefore, the challenge for sheep to regulate their body temperature was different between the 1st and 2nd heat waves. In both heat waves, the animals exhibited a substantial increase of evaporative heat loss by significantly increasing the respiratory rate in an attempt to minimize the tendency for increases in body temperature (Souza et al., 2008). The maximum temperature recorded was higher during the 2nd heat wave than during the 1st wave and therefore triggered a greater effort of thermoregulation by increasing the respiratory rate to dissipate excessive heat.

The increase in the respiratory rate during the two heat waves was important to maintain the thermal balance. The high respiratory rates limited increases in body temperature and therefore minimized the potential consequences of thermal stress. Even the respiratory rate is high but the animal was efficient in dissipating heat, maintaining homeothermy, some consequences of thermal stress may not occur (Eustaquio Filho et al., 2011), a situation observed in the present study. Although the sheep showed an increase in rectal temperature of 1.1 to 1.5 °C during the heat wave compared to the thermoneutral environment, which indicates relevant levels of heat storage, this temperature was below the maximum limit for the species, which is 39.9 °C (Liu et al., 2012). Thus, the thermoregulatory responses associated with the preconditioning of the animals to the 1st heat wave reduced the impact of heat stress on the animal, which was able to maintain the core body temperature in the appropriate range. Another factor that may have contributed to maintaining the rectal temperature within the limits for sheep was sweating. Although not measured in the present study, heat loss by sweating was probably important to maintain homeothermy, especially during the 2nd heat wave that was more challenging. This was demonstrated by Pulido-Rodríguez et al. (2021) who observed that sheep activate heat loss mechanisms (respiratory rate and sweating) to maintain a stable body temperature when subjected to high solar radiation.

Another important parameter was the tympanic temperature that increased during the heat wave, demonstrating the influence of ambient temperature on this variable and its rapid response to changes in air temperature (Guidryr and McDowell, 1966). In fact, the tympanic temperature more faithfully reflects the temperature of the hypothalamus and has a lower thermal inertia than the rectal temperature. Despite the increase in tympanic temperature during the heat wave, this variable remained below the rectal temperature values (Boere et al., 2003). This difference in rectal and tympanic temperatures is to be expected and only tends to vary in terms of its magnitude. Whenever sheep utilize high respiratory rates, there is the side effect of selective cooling of the brain. Countercurrent heat exchange occurs between venous blood cooled at the level of the nasal turbinates and blood of the carotid artery at the level of the circle of Willis (Wang et al., 2016). This heat exchange allows the blood supply to the brain and thus to the tympanum to occur at a lower temperature than the body temperature. This can be demonstrated by the greater differences between rectal and tympanic temperatures in the 2nd heat wave, when the highest respiratory rates were also recorded (Meiners and Dabbs, 1977), resulting in low temperatures in the brain and in the tympanum.

The brain temperature is usually a priority in the cooling of the body and therefore oscillates little, whereas in animals with selective cooling of the brain the body temperature frequently increases beyond the reference values when the animals are exposed to adverse climate conditions (Eustaquio Filho et al., 2011; Maloney et al., 2001). Furthermore, the animals may have been preconditioned to the high temperature during the 1st heat wave. Thus, even under more severe heat stress conditions during the 2nd heat wave, the thermoregulatory mechanisms responded better due to preconditioning, eliciting a more intense and prolonged response characterized by a greater capacity to limit the increases in body temperature (Yadav et al., 2019).

In addition to changes in physiological parameters, hormonal alterations may occur during heat stress. Lower plasma T3 concentrations were observed, which may be an attempt by the animals to reduce the production and accumulation of body heat (Garcia, 2013; Koluman and Daskiran, 2011) in order to maintain the thermal balance. The levels of T3 are higher in the thermoneutral environment because the animal does not need to reduce heat production. Although not significant, it is important to point out that there was a downward trend in T3 compared to thermoneutrality, which was more marked in the 2nd wave, a finding that is probably related to the duration of the heat wave.

Regarding prolactin, some authors have shown that the ambient temperature is responsible for stimulating the release of this hormone under adverse environmental conditions (Alamer, 2011; Pereira et al., 2019). However, there were no differences in prolactin levels in response to the temperature increase caused by the successive heat waves between pregnant and non-pregnant ewes. This finding can be explained by the fact that Santa Inês sheep are considered a naturalized breed and are therefore better adapted to overcome the thermal challenges imposed by the environment, showing a superior thermolytic performance due to specific adaptive morphological and physiological characteristics and thus being more tolerant to heat (Titto et al., 2016; Pantoja et al., 2017). Scharf et al. (2010) also reported the lack of a prolactin response to heat stress in heat-tolerant breeds. In that study, prolactin production was not increased in heat-tolerant animals, while animals that were more susceptible to heat increased the production of prolactin in the environment with high air temperature compared to the thermoneutral environment.

The heat wave during winter demanded more from the thermoregulatory system of the animals; however, there was no difference in thermoregulation between pregnant and non-pregnant Santa Inês ewes even at high temperatures. This finding may be related to the fact that pregnant ewes were in mid-gestation. According to McConn et al. (2021), the differences in the thermoregulatory responses between non-pregnant animals and animals in mid-gestation are minimal. Animals are more sensitive to heat in late gestation because of the metabolic activity and heat production related to fetal growth (He et al., 2019); thus, a difference exists in the additional heat generated by the fetus in the last third of gestation when compared to the mid-gestation fetus.

3.5.Conclusions

The present results showed similar thermoregulatory responses in pregnant and non-pregnant Santa Inês sheep, even during successive heat waves. The thermoregulatory response of sheep in the 2nd heat wave indicates an additional capacity for heat loss, permitting the maintenance of body temperature stability even under more severe heat conditions. During the heat waves, we did not observe endocrine changes that would indicate acclimation. Despite differences in the extent of evaporative heat loss, both groups of sheep were efficient and were able to overcome the thermal challenge posed by the two heat waves.

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Declarations of interest: none

References

Alamer, M., 2011. The role of prolactin in thermoregulation and water balance during heat stress in domestic ruminants. AJAVA. 6, 1153-1169. https://doi.org/10.3923/ajava.2011.1153.1169.

Bishop-Williams, K.E., Berke, O., Pearl, D.L., Hand, K., Kelton, D.F., 2015. Heat stress related dairy cow mortality during heat waves and control periods in rural Southern Ontario from 2010–2012. BMC Vet. Res. 11, 291. https://doi.org/10.1186/s12917-015-0607-2.

Brown–Brandl, T.M., Eigenberg, R.A., Hahn, G.L., Nienaber, J.A., Mader, T.L., Spiers, D.E., Parkhurst, A.M., 2005. Analyses of thermoregulatory responses of feeder cattle exposed to simulated heat waves. Int. J. Biometeorol. 49, 285–296. https://doi.org/10.1007/s00484-004-0250-2.

Boere, V., Silva, I.O., Canale, G., Pianta, T., Tomaz, C., 2003 Correlation between tympanic and rectal temperature in marmosets (Callithrix penicillata) under acute stress. Braz. J. Vet. Res. Anim. Sci. 40 (supl 2), 90-95. https://doi.org/10.1590/S1413-95962003000800002

Collier, R.J., Baumgard, L.H., Zimbelman, R.B., Xiao, Y., 2019. Heat stress: physiology of acclimation and adaptation. Anim. Front. 9, 12–19. https://doi.org/10.1093/af/vfy031.

Eustáquio Filho, A., Teodoro, S.M., Chaves, M.A., Santos, P.E.F., Silva, M.W.R., Murta, R.M., Carvalho, G.G.P.; Souza, L.E.B., 2011. Thermal comfort zone of Santa Inês sheep based on physiological responses. R. Bras. Zootec. 40, 1807-1814. https://doi.org/10.1590/S1516-35982011000800026.

Garcia, A.R., 2013. Thermal comfort on reproductive processes of water buffaloes raised under tropical conditions. Rev. Bras. Reprod. Anim. 37, 121-130.

Guidryr, A.J., McDowell, E., 1966. Tympanic membrane temperature for indicating rapid changes in body temperature. J. Dairy Sci. 74-77.

He, J., Guo, H., Zheng, W., Xue, Y., Zhao, R., Yao, W., 2019. Heat stress affects fecal microbial and metabolic alterations of primiparous sows during late gestation. J. Anim. Sci. Biotechnol. 10,1–12. https://doi.org/10.1186/s40104-019-0391-0

Koluman, N., Daskiran, I. 2011. Effects of ventilation of the sheep house on heat stress, growth and thyroid hormones of lambs. Trop. Anim. Health Prod. 43, 1123-1127. https://doi.org/10.1007/s11250-011-29811-7.

Liu, H. W., Cao, Y., Zhou, D. W., 2012. Effects of shade on welfare and meat quality of grazing sheep under high ambient temperature. J. Anim. Sci. 90, 4764-4770. https://doi.org/10.2527/jas.2012-5361.

Lv, F., Agha, S., Kantanen, J., Colli, L., Stucki, S., Joost, J.W.K.S., Li, M., Marsan, P.A., 2014. Adaptations to climate-mediated selective pressures in sheep. Mol. Biol. Evol. 31, 3324–3343. https://doi.org/10.1093/molbev/msu264

Maloney, S.K., Fuller, A., Mitchell, G., Mitchell, D., 2001. Rectal temperature measurement results in artifactual evidence of selective brain cooling. Am J Physiol Regul Integr Comp Physiol. 281, R108-R114.

McConn, B.R., Gaskill, B.N., Schinckel, A.P., Green-Miller, A.R., Lay, D.C., Jr., Johnson, J.S., 2021. Thermoregulatory and physiological responses of nonpregnant, mid-gestation, and late-gestation sows exposed to incrementally increasing dry bulb temperature. J. Anim. Sci. 99, 1-8. https://doi.org/10.1093/jas/skab181

Meiners, M. L., Dabbs, J.M., 1977. Ear temperature and brain blood flow: Laterality effects. Bull. Psychon. Soc. 10, 194–196.

Pantoja, M.H.A., Esteves, S.N., Jacinto, M.A.C., Pezzopane, J.R.M., Paz, C.C.P., Silva, J.A.R., Lourenço Junior, J.B., Brandão, F.Z., Moura, A.B.B., Romanello, N., Botta, D., Garcia, A.R., 2017. Thermoregulation of male sheep of indigenous or exotic breeds in a tropical environment. J. Therm. Biol. 69, 302–310. https://doi.org/10.1016/j.jtherbio.2017.09.002.

Pereira, A.M.F., Titto, E.A.L., Almeida, J.A.A., 2019. Adaptação dos ruminantes aos climas quentes, first ed. Appris editora, Curitiba.

Pereira, A.M.F., Vilela, R.A., Titto, C.G., Leme-dos-Santos, T.M.C., Geraldo, A.C.M.; Balieiro, J.C.C., Calviello, R.F., Birgel Junior, E.H., Titto, E.A.L., 2020. Thermoregulatory responses of heat acclimatized buffaloes to simulated heat waves. Anim. 10, 756. https://doi.org/10.3390/ani10050756.

Pulido-Rodríguez, L. F., Titto, C.G., Bruni, G. A., Froge, G. A., Fuloni, M. F., Payan-Carrera, R., Henrique, F. L., Geraldo, A. C. A. P. M., Pereira, A. M. F., 2021. Effect of solar radiation on thermoregulatory responses of Santa Inês sheep and their crosses with wool and hair Dorper sheep. Small Rumin. Res. 202, 106470. https://doi.org/10.1016/j.smallrumres.2021.106470.

Reis N.C.S., Boiaski N.T., Ferraz, S.E.T., 2019. Characterization and spatial coverage of heat waves in subtropical Brazil. Atmosphere. 10, 284. https://doi.org/10.3390/atmos10050284

Scharf, B., Carroll, J.A., Riley, D.G., Chase, C.C. Jr., Coleman, S.W., Keisler, D.H., Weaber, R.L., Spiers, D.E., 2010. Evaluation of physiological and blood serum differences in heat-tolerant (Romosinuano) and heat-susceptible (Angus) Bos taurus cattle during controlled heat challenge. J Anim Sci. 88, 2321-2336. https://doi.org/ 10.2527/jas.2009-2551.

Sejian, V., Maurya, V.P., Kumar, K., Naqvi, S.M.K., 2013. Effect of multiple stresses on growth and adaptive capability of Malpura ewes under semi-arid tropical environment. Trop. Anim. Health Prod. 45, 107–116. https://doi.org/10.1007/s11250-012-0180-7

Souza, B. B., Souza, E. D., Cezar, M. F., Souza, W. H., Santos, J. R. S., Benicio, T. M. A., 2008. Temperatura superficial e índice de tolerância ao calor de caprinos de diferentes grupos raciais no Semi-árido nordestino. Ciênc. Agrotec. 32, 275-280.

Titto, C.G., Veríssimo, C.J., Pereira, A.M.F., Geraldo, A.M., Katiki, L.M., Titto, E.A.L., 2016. Thermoregulatory response in hair sheep and shorn wool sheep. Small Rumin. Res. 144, 341–345. https://doi.org/10.1016/j.smallrumres.2016.10.015.

Van Wettere, W.H.E.J., Kind, K.L., Gatford, K.L., Swinbourne, A.M., Leu, S.T., Hayman, P.T., Kelly, J.M., Weaver, A.C., Kleemann, D.O., Walker, S.K., 2021. Review of the impact of heat stress on reproductive performance of sheep. J. Anim. Sci. Biotechnol. 12, 26. https://doi.org/10.1186/s40104-020-00537-z.

Vitali, A., Felici, A., Esposito, S., Bernabucci, U., Bertocchi, L., Maresca, C., Nardone, A., Lacetera, N., 2015. The effect of heat waves on dairy cow mortality. J. Dairy Sci. 98, 4572–4579. https://doi.org/10.3168/jds.2015-9331.

Yadav, B., Singh, G., Wankar, A., 2019. Acclimatization dynamics to extreme heat stress in crossbred cattle. Biol. Rhythm Res. 524-534. https://doi.org/10.1080/09291016.2019.1610627.

Wang, W., Kim, M, Normoyle, K.P., Llano, D., 2016. Thermal regulation of the brain—An anatomical and physiological review for clinical neuroscientists. Front. Neurosci. 9, 528. https://doi.org/10.3389/fnins.2015.00528

4. Circadian rhythms and heat tolerance prediction in Santa Inês sheep

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Abstract: This study was to identify Santa Inês sheep with different degrees of thermoregulatory responses to thermal stress in a climatic chamber, evaluating circadian rhythm of evaporative heat loss routes and energy accumulation to classify heat tolerance. Eighty black coat Santa Inês ewes, not pregnant, were used in this study. Sheep were chosen from different Brazilian regions (Sao Paulo, Minas Gerais, Bahia), to use animals from different populations. Sheep were subjected to heat stress in a climatic chamber for eight days, the 80 animals were divided in four cycles of 20 each, each group were kept for seven days in the climatic chamber with a temperature of 36 °C (10:00 to 16:00) and with maintenance of 26 °C from 16:00 to 10:00. At the end of each cycle (days 7 and 8), samplings Rectal temperature (RT), Respiratory rate, tympanic temperature, sweating rate and body surface temperature at 13h, 16h, 19h, 21h, 1h, 4h, 7h, 10h. The BLUP prediction of the reduction of rectal temperature between 01:00 to 7:00 after the accumulation of heat between 10:00 to 21:00 was used to classify the animals as high heat tolerant (HHT) and low heat tolerant (LHT). RT, respiratory rate, tympanic temperature, sweating rate and body surface temperature presented the effect of heat tolerance degree ($P>0.05$) and hour of sampling ($P<0.05$). Higher means of all physiological variables were observed for LHT sheep ($P<0.05$). The heat storage methodology proved to be adequate in characterizing the degree of heat tolerance of Santa Inês sheep kept in a climatic chamber.

Keywords: Heat stress, Thermolysis, Ovis aries

4.1.Introduction

Heat stress negatively influences the physiology and metabolism of animals, reducing productivity and making the animal more susceptible to disease (HAFEZ, 1973). Raising sheep in tropical regions shows the concern with choosing animals with greater tolerance to heat. It can be evaluated by the animal's ability to adjust to these adverse environmental conditions, with minimal loss of performance (McDOWELL, 1989).

To assess physiological adaptation and heat tolerance, mainly related through changes in thermal balance, measurements of respiration and body temperature are already well-established criteria (ABI SAAB; SLEIMAN, 1995; BIANCA; KUNZ, 1978). Rectal temperature can be taken as an index of adaptability when values are closer to the normality of the species (HOPKINS; KNIGHTS; LEFEURE, 1978). Similarly, animals that present a lower increase in rectal temperature and lower respiratory rate are considered more heat tolerant (BACCARI Jr., 1986).

In sheep, in addition to rectal temperature and respiratory rate, sweating rate also plays an important role in thermoregulation (SIQUEIRA; FERNANDES; MARIA, 1993). Skin evaporation in sheep occurs on the surface layer of the epidermis, by sweating (SILVA, 2000; SILVA; STARLING 2003; GEBREMEDHIN et al., 2008), and it is the most efficient way of thermolysis, as the animal spends less energy for its thermoregulation (SILVA, 2000). In sheep, sweating is often neglected due to the existence of the physical barrier of the wool, which prevents the evaporation of sweat (BERNABUCCI et al., 2010), however, in fur animals, stimulation and sweat discharges occur frequently when the animal is subjected to high air temperatures (McMANUS et al., 2011, TITTO et al., 2016).

During a period of high temperatures, an acclimation period allows sheep to stabilize, as heat acclimation is the ability of an organism to perform activities in a normal way and increase heat dissipation (MOSELEY, 1997). This process is caused by the constant elevation of the body's core temperature, which is maintained for long periods, therefore, with the ability to reduce physiological tensions, such as increased respiratory rate and rectal temperature, promoting animal comfort (McCLUNG et al., 2008). On the other hand, acclimatized animals maintain a greater heat transfer to the skin, increasing its heat dissipation capacity, therefore, the organisms become more tolerant to high air temperatures (MOSELEY, 1997).

In order for a production to be exploited to the fullest, it is necessary to know the physiology and behavior of the animal and the climatic conditions that these animals will be subject to, in addition to understanding the individual responses that may be linked to the genetic profile (PAIVA et al., 2005). However, each individual may respond differently to high ambient temperatures. Although all animals could present heat stress, some of them may be more or less susceptible to this environment and remain in homeostasis for a longer time (PULIDO-RODRÍGUEZ et al., 2021).

The genetic improvement carried out in Brazilian sheep breeds and the crossing with other breeds introduced in the country has generated a positive advance in production, but it is still necessary to understand the functional relationship between pure breed animals and their environment, based on the knowledge of thermoregulatory responses in the face of environmental stress. Based on this, the objective of this study was to identify Santa Inês sheep with different degrees of thermoregulatory responses to thermal stress in a climatic chamber, evaluating circadian rhythm of evaporative heat loss routes and energy accumulation to classify heat tolerance.

4.2.Material and Methods

The study was approved by Comissão de Ética no Uso de Animais da Faculdade de Zootecnia e Engenharia de Alimentos under nº 7498130919.

4.2.1. **Location and facilities**

Experimental period was done in the summer of 2021 (january-february) in the climatic chamber of the Department of Animal Reproduction, FVMZ/USP, *Campus* Fernando Cost, Universidade de São Paulo, Pirassununga-SP, Brazil. The climatic chamber has an area of 56 m2 and is fully enclosed by brick walls, cement floor, and a slab. Each chamber is equipped with an external temperature and humidity controller, internal thermostats, and an exhaust fan. Mineral salt and corn silage (0.7% live weight) were provided daily. The animals were fed once a day (at 7:30 am) and had ad libitum access to water and the feed trough.

The animals were obtained from the Biometeorology, Ethology and Animal Welfare Research Facility of the Laboratory of Biometeorology and Ethology, Department of Animal Science, Faculty of Animal Science and Food Engineering, University of São Paulo, Fernando Costa Campus, Brazil. Sheep were housed in pens with artificial shade provided by white-painted fiber cement tiles (1 m^2 per animal) and access to a pasture of Panicum maximum cv Aruana and mineral and corn silage supplementation, as well as water ad libitum.

4.2.2. **Animals and meteorological variables**

Eighty black coat Santa Inês ewes, not pregnant, were used in this study. Sheep were chosen from different Brazilian regions (Sao Paulo, Minas Gerais, Bahia), to use animals from different populations.

During the experimental period, a data logger (HOBO® U12-013 Data logger) was positioned inside the climatic chamber to register air temperature and relative humidity (Figure 1).

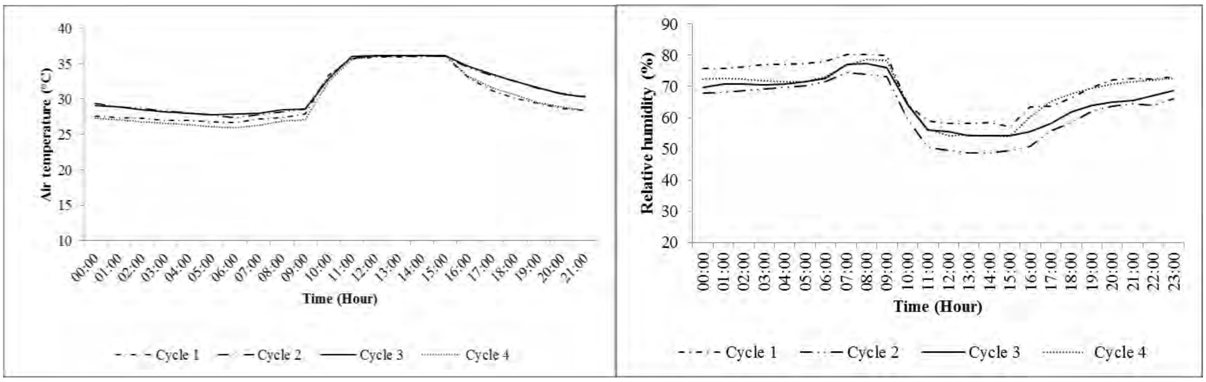

Figure 1: Mean air temperature and relative humidity during thermal challenge in a climatic chamber

4.2.3. **Experimental design**

Sheep were subjected to heat stress in a climatic chamber for eight days, preceded by two days of adaptation. In the first two adaptation days, the temperature of the climatic chamber was maintained equal to the current temperature of the external environment (around 26°C). From the 3rd to the 10th, the thermal treatment (stressor factor) was carried out, and the heat treatment in the climatic chamber with a temperature of 36°C started at 10:00 until 16:00, with a reduction of the thermostat temperature to 26 °C from 16:00 to 10:00.

The 80 animals were divided in four cycles of 20 each, as we used $5m^2$/animal to avoid discomfort. Between days 9 and 10 physiological variables were evaluated at 13:00, 16:00, 19:00, 21:00, 01:00, 04:00, 7:00 e 10:00. Rectal temperature (RT),was measured using a digital clinical thermometer (Geratherm® rapid GT-195-1, Germain, ±0,2°C), Respiratory rate (breaths min−1) was measured by observing the thoracic-abdominal movements of sheep for one min, tympanic temperature (°C) was measured using an ear thermometer (TCI100, Incoterm, ±0.2 °C) placed in the animal’s ear canal, sweating rate using Schleger and Turner method (1964) and body surface temperature was measured using an infrared thermometer (G-Tech Premium, Incoterm, ±0.2 °C).

4.2.4. **Statistical analysis**

Rectal temperature was used as the response variable, and analyzed by the restricted maximum likelihood method (REML) under a mixed model. This included the fixed effects of the assessment cycle (4 cycles with 20animals each), equivalent to the group of contemporaries, and the time effect within the assessment cycle and, as a random, the animal effect. The BLUP predictions obtained for each ewe, which quantify the individual heat stress response, were used to rank ewes from most heat tolerant to least tolerant.

Physiological variables (rectal temperature, respiratory rate, tympanic temperature, sweating rate, body surface temperature) were analyzed by ANOVA with fixed effect of heat tolerance degree (HHT or LHT) and hour of sampling and their interaction, and animal and cycles as aleatory effect. Means were compared by Tukey-Kramer at 5% of significance. All results are reported as the mean ± standard error of the mean. The SAS for Windows 9.4 software (2018) was used for the statistical analyses.

4.3.Results and Discussion

The BLUP prediction of the reduction of rectal temperature between 01:00 to 7:00 after the accumulation of heat between 10:00 to 21:00 was used to classify the animals as high heat tolerant (HHT) and low heat tolerant (LHT). Negative values from -0.57 to -0.01 explain a higher reduction of rectal temperature and 54 % of the animals were called HHT. Positive values from 0.02 to 0.68 showed a lower reduction and 46% of the animals were called LHT.

There was variation between the rectal temperature samples in the two groups of sheep, high and low heat tolerant, observed by the maximum and minimum values (Table 1). Minimum values of RT were similar between HHT and LHT at 7h, 10h and 13h ($P>0.05$), but lower minimum values were observed for HHT on the other samples ($P<0.05$). Maximum values were always lower for HHT sheep ($P<0.05$), which shows a better capacity to maintain rectal temperature above 39.0°C. Body core temperature can be used as an indicator of stress due to excess heat or cold since its variation among animals is relatively small in a given environment (SALLES et al., 2016). This small variation in HHT sheep can be also explained by better thermoregulation using the respiratory system (JOHNSON, 1989).

The methodology used in this study to classify sheep by heat tolerance presented a good result, as we could divide them into two distinguishable groups and also observed by the minimum and maximum results of rectal temperature that there are differences between HHT and LWT related to heat storage.

Some other studies used heat storage as a methodology to understand the heat loss efficiency (FISHER et al., 2008; HILLMAN et al., 2009; HOOPER et al., 2018) and our methodology seems to be an easy way to classify sheep as high heat tolerant and low heat tolerant using eight measurements of rectal temperature within a three hours interval.

Table 1. Minimum and maximum values of rectal temperature of high heat tolerant and low heat tolerant sheep along the day inside climatic chamber with air temperature between 36°C to 26°

Hour	Minimum HHT	Minimum LHT	Maximum HHT	Maximum LHT
13	38.1 a	38.2 a	38.9 b	39.4 a
16	38.0 b	38.9 a	38.9 b	39.6 a
19	38.3 b	38.5 a	39.0 b	39.9 a
21	38.4 b	38.7 a	38.9 b	39.4 a
1	38.1 b	38.5 a	38.9 b	39.4 a
4	37.7 b	38.1 a	38.7 b	39.3 a
7	38.0 a	38.2 a	38.9 b	39.4 a
10	38.1 a	38.1 a	38.9 b	39.5 a

Different letters in the same line between HHT and LHT differs by Tukey-Kramer (5%)

Rectal temperature, respiratory rate, tympanic temperature, sweating rate and body surface temperature presented the effect of heat tolerance degree ($P>0.05$; Table 2) and hour of sampling ($P>0.05$; Table 3), but not an interaction ($P>0.05$).

Table 2. Means and standard error of thermoregulatory responses of high heat tolerant (HHT) sheep and low heat tolerant (LHT) sheep during heat stress in a climatic chamber

	HHT	LHT	P value
Rectal Temperature (°C)	38.75±0.018	39.13±0.020	0.001
Respiratory Rate (breaths.min^{-1})	48±0.9	51±1.0	0.0129
Tympanic temperature (°C)	36.89±0.048	37.10±0.052	0.0144
Sweating rate (g.m^{-2}.h^{-1})	345.68±8.645	372.64±9.448	0.0356
Body surface temperature	36.76±0.029	36.99±0.032	0.001

Higher means of all physiological variables were observed for LHT sheep ($P<0.05$). Despite mean values of physiological variables being near the expected variation of sheep under heat stress, LHT animals always presented higher means which showed less adaptive responses to the hot ambient.

Table 3. Means of thermoregulatory responses of sheep during heat stress in a climatic chamber

Hour	Rectal Temperature	Respiratory Rate	Tympanic Temperature	Sweating Rate	Body Surface temperature
13	39.08 a	66 a	37.98 a	402.1 a	37.6 a
16	39.18 a	64 a	37.50 b	388.9 a	37.5 a
19	39.22 a	56 b	37.27 b	380.7 a	37.0 b
21	39.09 a	52 b	36.72 c	378.4 a	36.7 bc
1	38.90 b	41 c	36.30 c	286.1 b	36.5 c
4	38.82 bc	38 c	36.33 c	296.7 b	36.4 cd
7	38.55 d	37 c	36.45 c	343.7 ab	36.3 d
10	38.68 cd	44 c	37.31 b	396.8 a	37.0 b
Standard error	0.038	1.968	0.100	18.091	0.061

Respiratory rate (breaths.min-^{1}); rectal temperature, tympanic temperature and body surface temperature (°C); Sweating rate (g.m^{-2}.h^{-1}). Means with different letters in the same line differ by Tukey-Kramer ($P<0,05$).

At 10h, in the beginning of the heat treatment, sweating rate was able to maintain rectal temperature near basal levels, as the air temperature before 10h was around 26°C. As the air temperature increases between 10 and 16h, rectal temperature increases at 13 and 16h ($P<0.05$), and at 19h and 21h it is still similar to the hottest hours of the day ($P>0.05$).

Throughout the day, sweating rates were higher than those found for Santa Ines sheep in heat stress, 230.2 g.m-^{2}.h-^{1} (TITTO et al., 2016), showing that animals were usi Inês ng this thermoregulation system all day.

At 13h and 16h, higher means of respiratory rate and body surface were observed ($P<0.05$). Although RR was high compared to 1h to 10h, Santa Inês sheep did not increase this heat dissipation mechanism as wool breeds (WOJTAS et al., 2014). The respiratory evaporation is a physiological mechanism used in intense responses for shorter periods of the day (SILVA; STARLING, 2003) and it is very important for thermoregulation in wool sheep (BROCKWAY et al., 1965, MCMANUS et al., 2009, SILVA et al., 2002). On the other hand, sweating rate is more used by hair breeds like Santa Inês, as it plays a crucial role in thermoregulation (TITTO et al., 2016), and sweating rates, remarkably high, can be considered as a thermoregulatory response aiming for the stability of body temperature (MARAI et al., 2007).

Tympanic temperature varied 1.68°C throughout the day, with the highest temperature observed at 13h ($P<0.05$). In a study in a thermoneutral environment, higher values of tympanic temperature were around 33.86°C (SOUZA et al., 2015), lower compared to our results. Tympanic temperature in sheep is related to brain temperature and is affected by RR as vasodilation inside the nose reflects in reduction of heat (PEREIRA et al., 2019).

The lower RT was found at 7h, and differs from the other ones ($P<0.05$). It was expected to find lower values of temperature during dawn, however, inside the climatic chamber, the temperature drop does not seem to exert the same thermoregulation function as in the open environment, since there is no natural ventilation.

4.4.Conclusion

The heat storage methodology proved to be adequate in characterizing the degree of heat tolerance of Santa Inês sheep kept in a climatic chamber. The circadian rhythm of physiological variables linked to thermoregulation are affected by the variation of air temperature throughout the day and heat storage is different between animals with high and low heat tolerance.

References

ABI SAAB, S.; SLEIMAN, F.T. Physiological responses to stress of filial crosses compared to local Awassi sheep. **Small Ruminant Research**, v. 16, n. 1, p. 55-59, 1995.

BACCARI JÚNIOR, F.; POLASTRE, R.; FRÉ, C. A., ASSIS, P. S. Um novo índice de tolerância ao calor para bubalinos: correlação com o ganho de peso. In: REUNIÃO ANUAL DA SOCIEDADE BRASILEIRA DE ZOOTECNIA, 23., 1986, Campo Grande. **Anais...** Campo Grande: Sociedade Brasileira de Zootecnia, 1986. p. 316.

BERNABUCCI, U; LACETERA, N.; BAUMGARD, L.; RHOADS, R.; RONCHI, B.; NARDONE, A. Metabolic and hormonal acclimation to heat stress in domesticated ruminants. **Animal**, v. 4, n. 7, p. 1167–1183, 2010.

BIANCA, W.; KUNZ, P. Physiological reactions of three breeds of goats to cold, heat and high altitude. **Livestock Production Science**, v. 5, n.1, p.57-9, 1978.

BROCKWAY, J.M.; McDONALD, J.D.; PULLAR, J.D. Evaporative heat-loss mechanisms in sheep. **Journal of Physiology**, v.179, p.554-568, 1965.

FISHER, A.D.; MORTON, R.; DEMPSEY, J.M.A.; HENSHALL, J.M.; HILL, J.R. Evaluation of a new approach for the estimation of the time of the LH surge in dairy cows using vaginal temperature and electrodeless conductivity measurements. **Theriogenology**, v.70, p.1065–1074, 2008. https://doi.org/10.1016/j.theriogenology.2008.06.023.

GEBREMEDHIN, K.G.; HILLMAN, P.E.; LEE, C.N.; COLLIER, R.J.; WILLARD, S.T.; ARTHINGTON, J.D.; BROWN-BRANDL, T.M. Sweating rates of dairy cows and beef heifers in hot conditions. **Transactions of the ASABE**, v. 51, p. 2167-2178, 2008.

HAFEZ, E. **Adaptación de los animales domésticos**. Barcelona: Labor, 1973.

HILLMAN, P.E.; GEBREMEDHIN, K.G.; WILLARD, S.T.; LEE, C.N.; KENNEDY, A.D. Continuous measurements of vaginal temperature of female cattle using a data logger encased in a plastic anchor. **Applied Engineering in Agriculture**, v.25, n.2, p.291–296, 2009.

HOOPER, H.B.; TITTO, C.G.; GONELLA-DIAZA, A.M.; HENRIQUE, F.L.; PULIDO-RODRÍGUEZ, L.F.; LONGO, A.L.S.; LEME- DOS-SANTOS, T.M.C.; GERALDO, A.C.A.P.M.; PEREIRA, A.M.F.; BINELLI, M.; BALIEIRO, J.C.C.; TITTO, E.A.L. Heat loss efficiency and HSPs gene expression of Nellore cows in tropical climate conditions. **International Journal of Biometeorology**, v. 63, n.11, p.1475-1486, 2018. doi: 10.1007/s00484-018-1576-5.

HOPKINS, P.S.; KNIGHTS, G. I.; LEFEURE, A.S. Studies of the environmental physiology of tropical Merinos. **Australian Journal Agriculture Research**, East Medelaine, v. 29, n. 1, p. 61-71, 1978.

JOHNSON, H.D. Depressed chemical thermogenesis and hormonal function in heat. In: Horvarth, S.M., Yousef, M.K. (Eds.), **Environmental physiology aging, heat and attitude**, Elsevier Publishers, North Holland, NY, 1980.

MARAI, I.F.M.; EL-DARAWANY, A.A.; FADIEL, A.; ABDEL-HAFEZ, M.A.M. Physiological traits as affected by heat stress in sheep—a review. **Small Ruminant Research** v.71, p.1-12, 2007. doi:10.1016/j.smallrumres.2006.10.003

MCCLUNG, J.P.; HASDAY, J.; HE, J.; MONTAIN, S.; CHEUVRONT, S.; SAWKA, M.; SINGH, I. Exercise-heat acclimation in humans alters baseline levels ande x vivo heat inducibility of HSP72 and HSP90 in peripheral blood mononuclear cells. American Journal of Physiology – Regulatory**, Integrative and Comparative Physiology**. v. 294, p. 185-191, 2008.

McDOWELL, R. E. **Bases biológicas de la producción animal en zonas tropicales**. São Paulo: Ícone, 1989.

McMANUS, C.; LOUVANDINI, H.; GUGEL, R.; SASAKI, L.C.; BIANCHINI, E.; BERNAL, F.; PAIVA, S.R.; PAIM, T.P. Skin and coat traits in sheep in Brazil and their relation with heat tolerance. **Tropical Animal Health and Production**, v. 43, p. 121–126, 2011.

McMANUS, C.; PALUDO, G.R.; LOUVANDINI, H.; GUGEL, R.; SASAKI, L.C.B.; PAIVA, S.R. Heat tolerance in Brazilian sheep: Physiological and blood parameters. **Tropical Animal Health and Production**, v.41, p.95-101, 2009.

MOSELEY, P. L. Heat shock proteins and heat adaptation of the whole organism. **Journal of Applied Physiology**. v. 83, n. 5, p. 1413-1417, 1997.

PAIVA, S. E.; SILVÉRIO, V. C.; EGITO, A.A.; McMANUS, C.; FARIA, D. A.; MARIANTE, A. S.; CASTRO, S. R.; ALBUQUERQUE, M. S.M.; DERGAM, J. A. Genetic variability of the Brazilian hair sheep breeds. **Pesquisa Agropecuária Brasileira**, v. 40, n. 9, p.887-893, 2005.

PEREIRA, A.M.F., TITTO, E.A.L., ALMEIDA, J.A.A. **Adaptação dos ruminantes aos climas quentes**. Appris editor, Curitiba, 2019.

PULIDO-RODRÍGUES, L.F.; TITTO, C.G. ; BRUNI, G.A.; FROGE, G.A.; FULONI, M.F.; PAYAN-CARRERA, R.; HENRIQUE, F.L.; GERALDO, A.C.A.P.M.; PEREIRA, A.M.F. Effect of solar radiation on thermoregulatory responses of Santa Inês sheep and their crosses with wool and hair Dorper sheep. **Small Ruminant Research**, v. 202, p. 106470, 2021.

SALLES, M.S.V.; SILVA, S.C.; SALLES, F.A.; ROMA, L.C.; EL FARO, L.; MAC LEAN, P.A.B.; OLIVEIRA, C.E.L.; MARTELLO, L.S. Mapping the body surface temperature of cattle by infrared thermography. **Journal of Thermal Biology**, v. 62, p. 63-69, 2016.

SCHLEGER, A.; TURNER, H. Sweating rates of cattle in the field and their reaction to diurnal and seasonal changes. **Australian Journal of Agricultural Research**, v.16, 92, 1965. doi:10.1071/AR9650092

SILVA, I. J. O.; PANDORFI, H.; ACARARO JUNIOR., I.; PIEDADE, S. M. S.; MOURA, D. J. Efeitos da climatização do curral de espera na produção de leite de vacas Holandesas. **Revista Brasileira de Zootecnia**, v.31, n.5, p.2036-2042, 2002.

SILVA, R.; STARLING, J. Evaporação Cutânea e Respiratória em Ovinos sob Altas Temperaturas Ambientes. **Revista Brasileira de Zootecnia**, v. 32, n. 6, p. 1956-1961, 2003.

SILVA, R.G. **Introdução à bioclimatologia animal**. 1.ed. São Paulo: Nobel, 2000. 286p.

SIQUEIRA, E. R.; FERNANDES, S.; MARIA, G. A. Efecto de la lana y del sol sobre algunos parâmetros fisiológicos en ovejas de razas Merino Australiano, Corriedale, Romney Marsh e Ile de France. **ITEA**, Zaragoza, v. 89, n. 2, p. 124-131, 1993.

SOUZA, B.B.; DANTAS, N.L.B.; SILVA, I.J.O.; SUSIN, I.; GILAVERTE, S.; COSTA, F.S.; OLTRAMARI, C.E.; CASTRO, A.C. Tympanic, surface and rectal temperatures and respiratory rate of Santa Inês sheep and their crossbreds with Ile de France and Suffolk in Piracicaba, Brazil. **Journal of Animal Behavior and Biometeorology**, v.3, n.3, p.92-96, 2015. http://dx.doi.org/10.14269/2318-1265/jabb.v3n3p92-96.

TITTO, C. G.; VERÍSSIMO, C. J.; PEREIRA, A. M. F.; GERALDO, A. M.; KATIKI, L. M.; TITTO, E. A. L. Thermoregulatory response in hair sheep and shorn wool sheep. **Small Ruminant Research**, v.144, p.341–345, 2016.

WOJTAS, K.; CWYNAR, P.; KOŁACZ, R. Effect of thermal stress on physiological and blood parameters in Merino sheep. **Bulletin of the Veterinary Institute in Pulawy**, v.58, p.283-288, 2014.

5. Influence of heat stress on the physiological, hormonal, and behavioral responses of high and low heat-tolerant Santa Inês sheep

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Abstract: This study aimed to assess differences in physiological, hormonal, and behavioral responses between high and low heat-tolerant sheep during heat stress. Twenty-four sheep were used in the study, the 12 most heat-tolerant and 12 less heat-tolerant individuals identified in a thermotolerance assessment of 80 sheep. Animals were maintained for 10 days in a climatic chamber at an average temperature of 36 °C (10:00 am to 04:00 pm) and a maintenance temperature of 28 °C (0400 pm to 10:00 am). Rectal temperature, respiratory rate, sweat rate, ocular surface temperature, right antimere temperature, tympanic temperature, triiodothyronine, and insulin were analyzed at 01:00 pm, 04:00 pm, 07:00 pm, 09:00 pm, 1:00 am, 4:00 am, 7:00 am, and 10:00 am. Skin samples were collected for histological analysis after prolonged stress. High low-tolerant sheep had higher rectal and right antimere temperatures throughout the day. Although no differences in skin morphology were observed between groups, low heat-tolerant sheep continued to sweat for a longer period after the end of the thermal challenge in an attempt to lose heat. Low heat-tolerant animals accumulated more heat and needed more time to dissipate excess heat than high heat-tolerant sheep and cannot dissipate heat as efficiently as high heat-tolerant individuals. These findings show that there is ample variation in thermoregulatory response between animals of the same breed maintained under the same environmental conditions.

Keywords: Santa Inês breed, behavior, endocrine response, physiology

5.1.Introduction

In tropical countries, heat stress is one of the major factors influencing animal productivity, reproduction, health, immunity, and survival (Mandal et al. 2021). The impacts of heat stress are easily noticeable because they cause alterations in physiological parameters, behavioral responses, and hormonal profile, leading to reduced feed intake and, consequently, decreased growth rate (Mahjoubi et al. 2015; Kahl et al. 2015; Carabaño et al. 2019).

Sheep might be increasingly exposed to the deleterious effects of heat stress as a result of climate change and rising air temperatures; therefore, it is crucial to develop strategies to minimize production losses and maintain herd performance under adverse environmental conditions (Renaudeau et al. 2012). Heat tolerance has been studied in different species and breeds for the genetic selection of animals with better responses to high-temperature environments and improved production performance in tropical regions (Titto et al. 2016a; Sejian et al. 2021). However, little is known about individual differences in thermoregulatory response in woolless sheep or the mechanisms underlying how animals from the same breed, group, and environment respond differently to heat stress (McManus et al. 2010; Brown-Brandl 2013). Such information can be decisive for the selection of more productive animals in tropical climate countries as well as for designing effective approaches to reduce the impacts of climate change based on individual responses of sheep to heat stress (Saizi et al. 2019).

There is a gap in our knowledge about what makes one animal more susceptible to heat stress than another. Therefore, it is important to understand the effects of heat stress on individual thermoregulatory responses, mainly in tropical conditions. Considering these observations, this study aimed to identify differences in physiological, endocrine, and behavioral responses between high heat-tolerant and low heat-tolerant sheep exposed to heat stress in a climatic chamber.

5.2.Material and methods

5.2.1. Ethical approval

All experimental procedures were approved by the Animal Ethics Committee at the Faculty of Animal Science and Food Engineering of the University of São Paulo (CEUA/FZEA/USP protocol no. 7498130919).

5.2.2. Animals

This study used 80 black-coat, non-pregnant, Santa Inês sheep aged between 4 and 5 years with homogeneous body condition (score 3 in a scale from 1 to 5). Animals were chosen from different populations in different regions and climatic environments in Brazil.

5.2.3. General experimental procedures

The animals were obtained from the Biometeorology, Ethology and Animal Welfare Research Facility of the Laboratory of Biometeorology and Ethology, Department of Animal Science, Faculty of Animal Science and Food Engineering, University of São Paulo, Fernando Costa Campus, Brazil. Sheep were housed in pens with artificial shade provided by white-painted fiber cement tiles (1 m^2 per animal) and access to a pasture of *Panicum* *maximum* cv Aruana. For the experiment, the animals were taken to a climatic chamber at the Department of Animal Reproduction of the same university.

The climatic chamber has an area of 56 m^2 and is fully enclosed by brick walls, cement floor, and a slab. Each chamber is equipped with an external temperature and humidity controller, internal thermostats, and an exhaust fan.

Mineral salt and corn silage (0.7% live weight) were provided daily. The animals were fed once a day (at 7:30 am) and had ad libitum access to water and the feed trough.

5.2.4. **Experimental design and thermotolerance assessment**

Before the beginning of the experimental phase, the rectal temperature of animals was measured in a thermoneutral environment (average air temperature of 24.7 °C). High heat-tolerant (HHT) and low heat-tolerant (LHT) sheep had a mean rectal temperature of 38.4 ± 0.51 and 38.6 ± 0.46 °C, respectively.

The experiment was divided into two phases. In phase 1, sheep were subjected to heat stress in a climatic chamber for eight days, preceded by two days of adaptation. The climatic chamber was kept at 36 °C from 10:00 am to 0400 pm and at 26 °C from 04:00 pm to 10:00 am. Between day nine and 10, rectal temperature was recorded at 01:00 pm, 04:00 pm, 07:00 pm, 21:00 pm, 01:00 am, 04:00, 07:00 am, and 10:00 am. From the classification of animals in relation to heat management, the 12 top-ranking (high heat tolerant) and 12 bottom-ranking (low heat-tolerant) individuals were selected for use in the second phase of the experiment. Data analysis was performed using a restricted maximum likelihood (REML) mixed model. The model included contemporary and time effects as fixed effects and animal and residual effects as a random. BLUP values quantifying individual responses to heat stress were used to rank the sheep according to heat tolerance.

In phase 2, both groups were placed together in the climatic chamber, which was maintained at 36 °C from 10:00 am to 04:00 pm and at 28 °C from 04:00 pm to 10:00 am. The latter temperature represents a 2 °C increment in relation to that of phase 1. The aim was to subject sheep to a new thermal challenge and thus ensure that the acclimatization mechanisms activated in phase 1 did not interfere with the results of phase 2. During the experimental period, sheep were evaluated for feeding behavior, physiological variables, surface temperatures, and blood parameters at 01:00 pm, 04:00 pm, 07:00 pm, 21:00 pm, 01:00 am, 04:00, 07:00 am, and 10:00 am.

5.2.5. Meteorological variables

During the experimental period, air temperature and relative humidity within the climate chamber were recorded (Fig. 1) using data loggers (HOBO® U12-013).

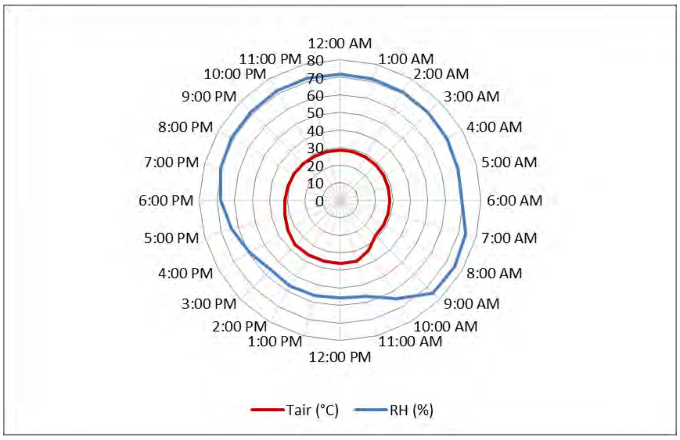

Fig. 1 Mean air temperature (T_{air}) and relative humidity (RH) during thermal challenge in a climatic chamber

5.2.6. Physiological variables

Respiratory rate (breaths min^{-1}) was measured by observing the thoracic-abdominal movements of sheep for one min. Rectal temperature (°C) was measured using a digital clinical thermometer (TH150, G-Tech, ±0.2 °C). Sweat rate (g m^{-2} h^{-1}) was determined by the method of Schleger and Turner (1964). Tympanic temperature (°C) was measured using an ear thermometer (TCI100, Incoterm, ±0.2 °C) placed in the animal's ear canal.

5.2.7. Surface temperature measurements

The surface temperature of the ocular region (°C) was measured by infrared thermography using a manual focus infrared camera (875-2i, Testo, Germany) with a thermal sensitivity (NETD) of <50 mK. The camera was maintained at the level of the ocular region at a distance of about 0.5 m. The emissivity was 0.98. The surface temperature of the right antimere (°C) was measured using an infrared thermometer (G-Tech Premium, Incoterm, ±0.2 °C).

5.2.8. Triiodothyronine (T_3) and insulin measurement

Blood samples were collected into 10 mL vacuum tubes by puncture of the external jugular vein. Samples were centrifuged at 3,000 rpm for 20 min, and the serum was immediately frozen at −20 °C until determination of Triiodothyronine (T_3) and insulin levels.

These hormones were measured by an enzyme immunoassay using commercial kits according to the manufacturer's instructions (Monobind, Lake Forest, CA, USA). Both kits were validated by parallel curves between standard concentrations and serially diluted serum samples. The intra- and inter-assay coefficients of variation were 3.8% and 6.3%, respectively, for T_3 and 4.5% and 6.2%, respectively, for insulin.

5.2.9. Skin morphology

A biopsy was performed on the right side of the animal, in the middle dorsal region. Before collection of the biopsy, the area was shaved, disinfected, and anesthetized by application of 1 mL of local anesthetic without vasoconstrictor (2% lidocaine hydrochloride). Then, micro fragments of skin tissue were excised using an 8 mm diameter punch. Specimens were immediately fixed in 10% buffered formalin for 24 h and stored in 70% alcohol until histological analysis.

Each fragment was cut into 4 µm thick sections and stained with hematoxylin and eosin for histopathological evaluation. Sections were examined under an optical microscope (Leica® DM500) at 40× magnification. One image was acquired per section. Images were analyzed using ImageJ software version 1.52a (National Institutes of Health, USA).

The parameters described below were determined in each section. For epidermal and dermal thicknesses (µm), 30 measurements per slide were made at different sections of the epidermis and dermis. Sweat gland density (number of sweat glands per linear micrometer) was determined by counting sweat glands with the aid of the multi-point tool and dividing the number by the length of the epidermal surface. Glandular area (μm^2) was measured using the freehand selection tool, which allowed tracing the area of each sweat gland. Distance of sweat gland from the epidermis (µm) was measured from the most distant edge of sweat glands (in relation to the epidermis) to the beginning of the epidermis. The arithmetic mean of parameters was used for statistical analysis, except that of sweat gland density.

5.3.0. Behavior

Animal behavior was observed during three consecutive days. Feeding time (min) and water drinking events were analyzed continuously and individually by the animal focal sampling technique. Evaluations were carried out from 8:00 to 18:00 by a trained observer (Martin and Bateson 1993; Titto et al. 2011).

5.3.1. **Statistical analysis**

For the Phase 1 data, as already presented, the model included contemporary and time effects as fixed effects and animal and residual effects as a random.

For the Phase 2 data, to model the response traits as a function of the covariates, a specific distribution under GLMM with a better link function was used. The better link function ensures good fitted values, and one specific distribution is typically used for each trait. Fixed factor of tolerance group (categorical with two levels high and low) and day time (continuous). The interaction terms are tolerance group × day time (like a cubic regression). To incorporate the dependency among observations of the same animal, we used nested as animal random intercept. For feed intake behavior, the model included the random effect of sheep as well as the fixed effects heat tolerance and observed hours 8 to 17h) and the interaction between these effects.

5.3.Results

Low heat-tolerant sheep had higher rectal temperature (Table 1) than high heat-tolerant sheep (*p* = 0.0011). However, respiratory rate, tympanic temperature, and sweat rate did not differ between groups (*p* > 0.05). Right antimere temperature was higher in low heat-tolerant animals (*p* = 0.0282; Table 1). No difference in ocular temperature was observed between groups (*p* > 0.05). There were no significant differences (*p* > 0.05) in T_3 and insulin levels between high and low heat-tolerant sheep.

Table 1 Means and standard error of respiratory rate, rectal temperature, tympanic temperature, sweat rate, right antimere temperature, ocular temperature, triiodothyronine (T_3), and insulin between high heat-tolerant and low heat-tolerant sheep

Variable	High heat-tolerant sheep	Low heat-tolerant sheep	*P*-value
Respiratory Rate (breaths min^{-1})	42.5 ± 2.63	43.3 ± 2.65	0.8189
Rectal Temperature (°C)	38.6 ± 0.063	38.9 ± 0.063	0.0011
Tympanic temperature (°C)	36.5 ± 0.083	36.6 ± 0.083	0.2762
Sweat rate (g/m^2/h)	360.7 ± 17.63	356.6 ± 17.63	0.8682
Right antimere temperature (°C)	36.9 ± 0.083	37.1 ± 0.083	0.0282
Ocular temperature (°C)	38.8 ± 0.094	39.0 ± 0.094	0.2629
T3 (ng/mL)	3.0 ± 0.311	2.9 ± 0.311	0.9045
Insulin (µIU/mL)	18.6 ± 2.458	15.8 ± 2.431	0.1823

Skin morphology did not differ between groups (*p* > 0.05) (Table 2).

Table 2 Skin morphological characteristics of high heat-tolerant and low heat-tolerant sheep

Variable	High heat-tolerant sheep	Low heat-tolerant sheep	*P*-value
Sweat gland density (glands μm^{-1})	0.01 ± 0.0011	0.01 ± 0.0011	0.4618
Glandular area (μm^{2})	11453 ± 1015.1	10680 ± 1015.1	0.5956
Distance of sweat glands[a] (μm)	1016 ± 81.2	1101 ± 81.1	0.4670
Epidermal thickness (μm)	45.90 ± 5.595	42.44 ± 5.595	0.6663
Dermal thickness (μm)	2194 ± 75.5	2279 ± 75.5	0.4318

[a] Distance of sweat glands from the epidermis

There was no difference in feed intake between high heat-tolerant and low heat-tolerant sheep ($p > 0.05$). However, air temperature influenced feed intake ($p < 0.05$; Table 3). There was no difference in water intake between groups ($p = 0.429$).

Table 3 Timing of feed intake of high heat-tolerant and low heat-tolerant sheep during thermal challenge in a climatic chamber

Hour	High heat-tolerant sheep	Low heat-tolerant sheep	*P*-value
08:00	32.92 ± 3.27 a	38.58 ± 3.27 a	0.9996
09:00	11.62 ± 4.01 b	15.00 ± 4.28 b	1.0000
10:00	25.14 ± 4.28 abc	17.27 ± 3.42 b	0.9966
11:00	21.71 ± 4.28 abc	22.00 ± 3.78 ab	1.0000
12:00	16.20 ± 3.58 bc	23.09 ± 3.42 ab	0.9977
13:00	34.00 ± 4.28 ac	16.33 ± 3.78 b	0.1976
14:00	22.14 ± 4.28 abc	26.50 ± 4.01 ab	1.0000
15:00	13.60 ± 3.58 b	26.37 ± 4.01 ab	0.6775
16:00	24.17 ± 3.27 abc	27.64 ± 3.42 ab	1.0000
17:00	22.73 ± 3.42 abc	13.33 ± 3.27 b	0.9037

Means in columns followed by different letters are significantly different ($p < 0.05$)

It was possible to observe an increase in physiological variables (respiratory rate, rectal temperature, and tympanic temperature), mainly between 10:00 and 16:00, when the thermal challenge was applied (Fig. 2). After this period, these physiological variables decreased in both high heat-tolerant and low heat-tolerant animals. Respiratory rate and tympanic temperature decreased more rapidly with decreasing temperatures than sweat rate. The sweat rate of high heat-tolerant animals did not vary much over time, but that of low heat-tolerant animals increased after the end of heat treatment. The rectal temperature of low heat-tolerant animals was higher throughout the day.

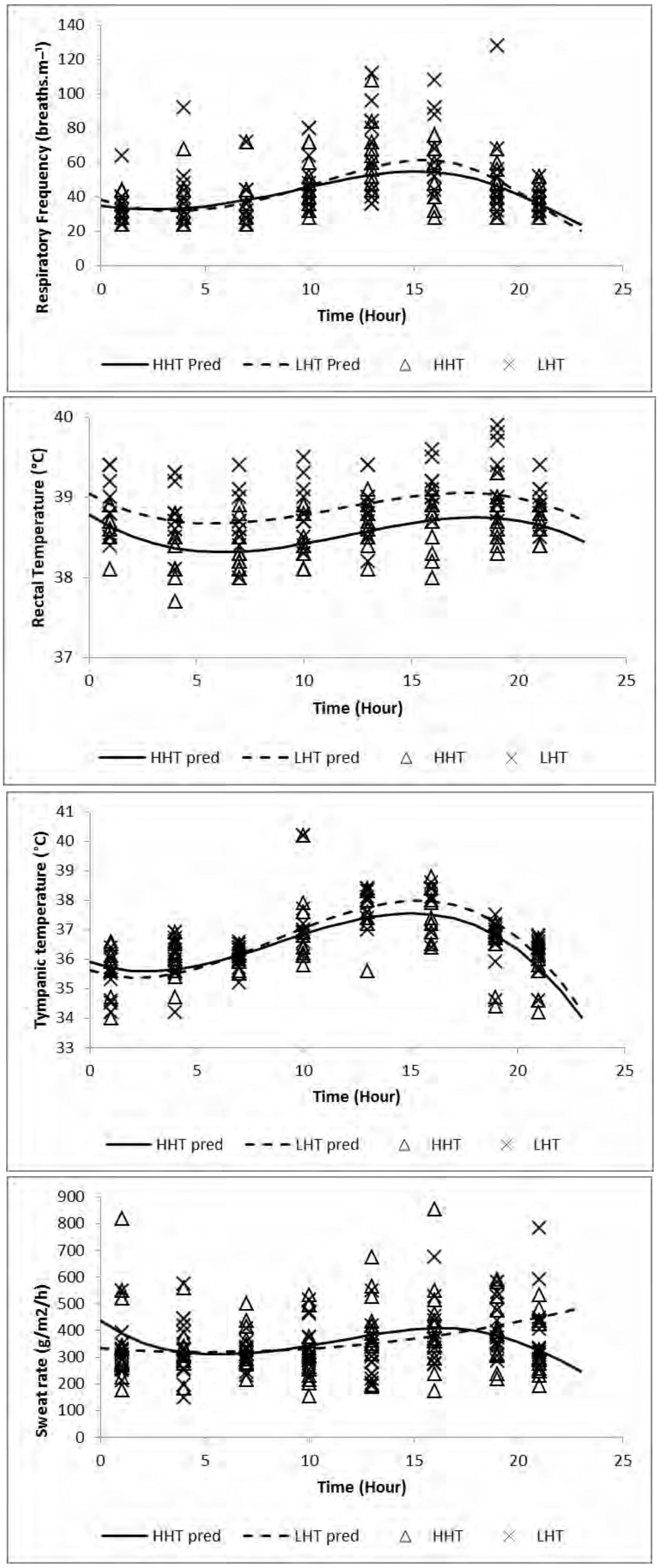

Fig. 2 Predicted (pred) and observed (Δ and X) physiological variables of high heat-tolerant (HHT) and low heat-tolerant (LHT) sheep throughout the day

Right antimere temperature was higher in low heat-tolerant than in heat-tolerant animals throughout the day, peaking between 10:00 and 16:00, when the environmental temperature was highest (Fig. 3). Ocular temperatures of low heat-tolerant animals remained higher for most of the day.

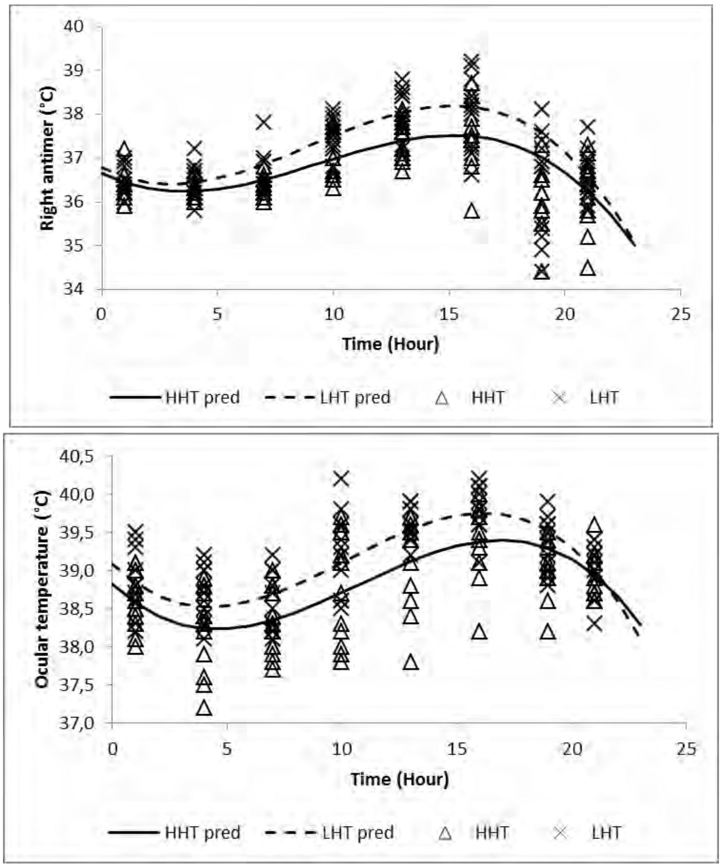

Fig. 3 Predicted (pred) and observed (Δ and X) surface temperatures of high heat-tolerant (HHT) and low heat-tolerant (LHT) sheep throughout the day

Insulin concentration increased during the thermal challenge in low heat-tolerant sheep (Fig. 4), demonstrating the influence of the circadian cycle on this hormone. By contrast, the variation of T_3 concentration with time was similar between high heat-tolerant and low heat-tolerant sheep, but the curve for high heat-tolerant animals had a more pronounced inversion.

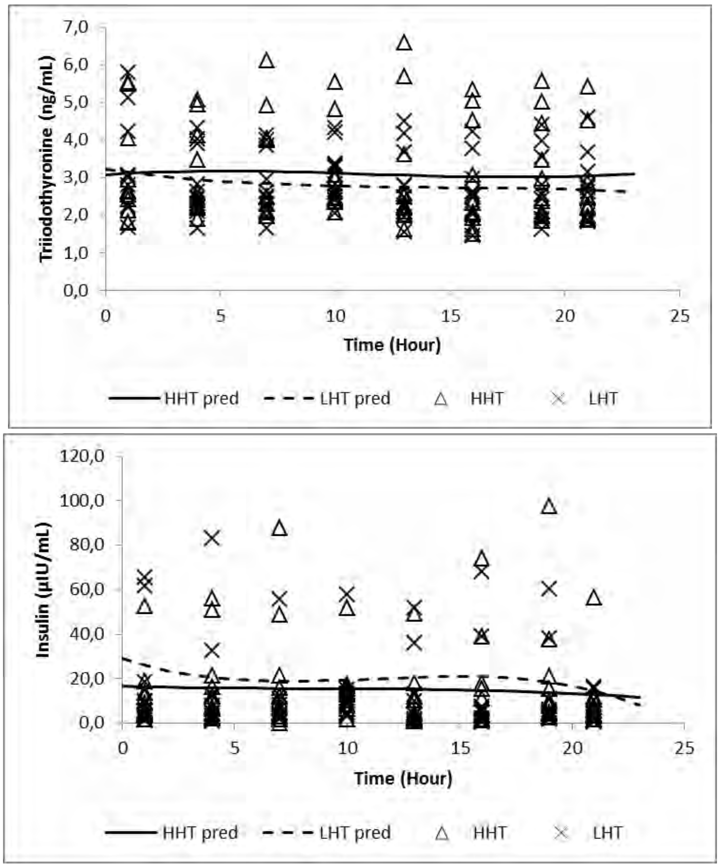

Fig. 4 Predicted (pred) and observed (Δ and X) triiodothyronine and insulin levels in high heat-tolerant (HHT) and low heat-tolerant (LHT) sheep throughout the day

5.4.Discussion

Physiological response of high heat-tolerant and low heat-tolerant sheep

Santa Inês sheep are considered to be well adapted to high temperature conditions, with an upper critical limit of 31 °C (Van Wettere et al. 2021). In the current study, the air temperature reached 36 °C, outside the thermoneutrality zone of sheep. Thus, sheep were challenged to maintain homeothermy during hot periods, requiring activation of latent heat loss mechanisms.

The use of such mechanisms was similar in both high heat-tolerant and low heat-tolerant sheep, as demonstrated by the similar changes in physiological, hormonal, and behavioral variables. High heat-tolerant animals were expected to use heat loss mechanisms more intensely. We observed that animals of the same breed under the same environmental conditions did not show the same heat loss efficiencies, despite using thermoregulatory mechanisms in a similar manner. For instance, rectal temperature was higher in low heat-tolerant animals. Sheep with low heat tolerance were not able to efficiently dissipate heat accumulated over the experimental period; even during hours of reduced air temperature, low heat-tolerant animals did not reach the basal rectal temperature of 38.6 °C. By contrast, the high heat-tolerant group was able to dissipate heat more efficiently, showing rectal temperatures of 38.3 °C between 3:00 and 10:00. The basal rectal temperature of high heat-tolerant animals was 38.4 °C.

Unlike rectal temperature, ocular temperature did not differ between high heat-tolerant and low heat-tolerant animals, even though the variable is sensitive to thermoregulatory changes associated with heat stress (Schaefer et al. 2007) and has a good correlation with rectal temperature (Barros et al. 2016). Low heat-tolerant sheep did however have higher right antimere temperatures; these animals probably attempted to dissipate excess heat via vasodilation, which increases blood flow to the skin surface (Morrison and Nakamura 2011; Pezeshki et al. 2011; Mota-Rojas et al. 2021), ultimately leading to increased skin temperature. Such changes facilitate heat dissipation by non-evaporative mechanisms (Gesualdi Junior et al. 2014). This heat dissipation mechanism might have been responsible for the higher skin temperature in low heat-tolerant sheep.

The use of surface heat exchange by low heat-tolerant sheep likely occurred because these individuals required more time and effort to dissipate excess heat. Evaporative mechanisms were likely not sufficient to eliminate accumulated heat, particularly after the end of the thermal challenge, when the main heat exchange mechanism (i.e., panting) is reduced (Starling et al. 2002). Furthermore, morphological characteristics of the skin did not differ between groups. Although low heat-tolerant animals had a greater need to dissipate heat, the thermal challenge was not sufficient to increase sweat production. It is known that sweat gland activation contributes to heat loss via cutaneous routes. The area occupied by the secretory part of sweat glands determines heat dissipation capacity via sweating (Bianchini et al. 2006). Gland-to-epidermis distance also did not differ between animals. The distance of sweat glands from the epidermis is related to the functional activity of glands: distant sweat glands have reduced functional activity (Ferreira et al. 2009).

Overall, high heat-tolerant and low heat-tolerant sheep did not differ in skin morphological characteristics, resulting in a similar sweat rate. However, low heat-tolerant animals continued to sweat after the period of thermal challenge, probably in an attempt to dissipate excess heat. The sweat rate remained high after 16:00 (end of thermal challenge), until night time, when the air temperature was markedly lower, demonstrating that sweating was important for the reestablishment of thermal balance in the low heat-tolerant group. Similar findings were reported by Titto (2016b), who observed persistent sweating in sheep even after the air temperature had decreased and panting had ceased. Despite the use of sweating to dissipate heat, these animals still needed to exchange heat with the environment through peripheral vasodilation.

Influence of air temperature on thermoregulatory response

Changes in the body temperature of domestic animals are influenced by environmental temperature (Terrien et al. 2011). In the current study, we observed that rectal temperature had rhythmic oscillations during the day, particularly when the ambient temperature was high (from 10:00 to 16:00). Oscillations surpassed the upper critical limit as a result of the increase in rectal temperature. High ambient temperatures may also increase surface temperatures, given that heat exchange occurs in the skin even in the absence of solar radiation. In sheep, the circadian rhythmicity of respiratory rate favors heat dissipation. This adaptive strategy aims to maintain the internal body temperature within the ideal range (De et al. 2017). Circadian oscillation of rectal temperature, respiratory rate, and surface temperature is influenced by the suprachiasmatic nucleus of the hypothalamus, whose main function is to regulate oscillations of the internal medium to modulate the setpoint (Rodrigues and Rodrigues 2007). These physiological adjustments are essential to prevent hyperthermia, especially in tropical regions where the ambient temperature varies significantly between day and night.

Although there were no differences in feed intake between high heat-tolerant and low heat-tolerant groups, the parameters varied throughout the day. This response can be seen as a transient adaptive behavior aimed at reducing thermogenesis, being, in the short term, strictly dependent on heat dissipation capacity (Pereira et al. 2019). Therefore, the changes in feed intake in low heat-tolerant animals may be linked to heat gain and loss. Sheep likely resumed feeding when they were able to dissipate heat and reduce their internal temperature for short periods. By contrast, high heat-tolerant animals were able to maintain feed intake during periods of the day when the temperature was below 36 °C.

The absence of solar radiation in the current experiment might also have contributed to the variation in feed intake in Santa Inês sheep. Feed intake behavior is different in animals raised in the field. Under pasture conditions, the air temperature directly influences feed intake and may change daily feeding patterns (6:00–12:00 and 12:00–18:00), shifting consumption to night time (Portugal et al. 2000). Although feed intake varied with temperature, this relationship did not influence insulin level, which increased during heat stress (O'brien et al. 2010; Wheelock et al. 2010). During stress, the sympathetic nervous system releases catecholamines that are responsible for stimulating α-adrenergic receptors to increase insulin secretion (Alvarez et al. 1989). On the other hand, T_3 concentration was not influenced by the increase in air temperature. It was expected that T_3 levels would be lower during periods of warmer temperatures to ensure a reduction in heat production (Bragança et al. 1998; Garcia 2013). Nevertheless, low heat-tolerant animals had lower T_3 levels than high heat-tolerant animals throughout the day, probably because of their greater heat accumulation. Thus, the degree of T_3 reduction is related to the ability to adapt to heat stress; more tolerant animals have a lower T_3 decrease in stress situations (Pereira et al. 2008).

5.5.Conclusion

Low heat-tolerant sheep accumulated more heat during the thermal challenge and required more time to dissipate excess heat. Furthermore, these animals continued to sweat even after the air temperature had decreased. The findings demonstrate that low heat-tolerant Santa Inês sheep cannot dissipate heat with the same efficiency as high heat-tolerant individuals. This information is important for understanding variations in the thermoregulatory response of animals of the same breed under the same environmental and management conditions and for selecting animals with better thermoregulatory performance under high temperature conditions.

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References

Alvarez MA, Portilla L, Gonzalez R, Ezcurra E (1989) Insulin response to a short stress period. Psychoneuroendocrinology 14:241-4. https://doi.org/10.1016/0306-4530(89)90022-x.

Barros DV, Silva LKX, Kahwage PR, Lourenço Júnior JB, Sousa JS, Silva AGM, Franco IM, Martorano LG, Garcia AR (2016) Assessment of surface temperatures of buffalo bulls (*Bubalus bubalis*) raised under tropical conditions using infrared thermography. Arq. Bras. Med. Vet. Zootec. 68: 422-430.

Bianchini E, McManus C, Lucci CM, Fernandes MCB, Prescott E, Mariante AS, Egito AA (2006) Características corporais associadas com a adaptação ao calor em bovinos naturalizados brasileiros. Pesq Agropec Bras 41:1443-1448.

Bragança MM, Mounier AM, Prunier A (1998) Does Feed Restriction Mimic the Effects of Increased Ambient Temperature in Lactating Sows? J Anim Sci 76:2017–2024, 1998.

Brown-Brandl TM (2013) Managing thermal stress in feedlot cattle: environment, animal susceptibility and management options from a US perspective. Livest Housing 189–208. https://doi.org/10.3920/978-90-8686-771-4_10.

Carabaño MJ, Ramón M, Menéndez-Buxadera A, Molina A, Díaz C (2019) Selecting for heat tolerance. Anim Front 9:1. https://doi.org/10.1093/af/vfy033

Chaefer AL, Cook NJ, Church JS, Basarab J, Perry B, Miller C, Tong AK, (2007) The use of infrared thermography as an early indicator of bovine respiratory disease complex in calves. Res Vet Sci 83: 376-384

De K, Kumar D, Saxena VK, Naqvi SM (2017) Study of circadian rhythmicity of physiological response and skin temperature of sheep during summer and winter in semi-arid tropical environment. Physiol Behav 169:16-21. https://doi.org/10.1016/j.physbeh.2016.11.010

Ferreira F, Campos WE, Carvalho AU, Pires MFA, Martinez ML, Silva MVGB, Verneque RS, Silva PF (2009) Taxa de sudação e parâmetros histológicos de bovinos submetidos ao estresse calórico. Arq Bras Med Vet Zootec 61:763-768.

Garcia AR (2013) Thermal comfort on reproductive processes of water buffaloes raised under tropical conditions. Rev Bras Reprod Anim 37:121-130.

Gesualdi Júnior A, Sales ESV, Freitas RS, Henry FC, Oliveira VPS, Gesualdi ACLS (2014) Effects of heat stress on the physiological parameters and productivity of hair sheep in tropical and coastal environments. R Bras Zootec 43(10):556-560. http://dx.doi.org/10.1590/S1516-35982014001000008.

Kahl S, Elsasser TH, Rhoads RP, Collier RJ, Baumgard LH (2015) Environmental heat stress modulates thyroid status and its response to repeated endotoxin challenge in steers. Domest Anim Endocrinol 52:43-50. https://doi.org/10.1016/j.domaniend.2015.02.001.

Mahjoubi E, Hossein Yazdi M, Aghaziarati N, Noori GR, Afsarian O, Baumgard LH (2015) The effect of cyclical and severe heat stress on growth performance and metabolism in Afshari lambs. J Anim Sci 93:1632–1640. https://doi.org/10.2527/jas2014-8641

Mandal DK, Bhakat C, Dutta TK (2021) Impact of environmental factors on physiological adaptability, thermo-tolerance indices, and productivity in Jersey crossbred cows. Int J Biometeorol 65:1999–2009.

McManus C, Paiva SR, Araújo RO (2010) Genetics and breeding of sheep in Brazil. R. Bras. Zootec 39:236-246 (supl. especial).

Martin P, Bateson P (1993) Measuring behaviour: An introductory guide. Cambridge University Press, Cambridge.

Morrison SF, Nakamura K (2011) Central neural pathways for thermoregulation. Front Biosci 16:74-104.

Mota-Rojas D, Titto CG, Orihuela A, Martínez-Burnes J, Gómez-Prado J, Torres-Bernal F, Flores-Padilla K, Carvajal-de la Fuente V, Wang D (2021) Physiological and Behavioral Mechanisms of Thermoregulation in Mammals. Animals 11(6):1733. https://doi.org/10.3390/ani11061733

O’Brien MD, Rhoads RP, Sanders SR, Duff GC, Baumgard LH (2010) Metabolic adaptations to heat stress in growing cattle. Domest Anim Endocrinol 38:86-94

Pereira AM, Baccari Jr F, Titto EA, Almeida JA (2008) Effect of thermal stress on physiological parameters, feed intake and plasma thyroid hormones concentration in Alentejana, Mertolenga, Frisian and Limousine cattle breeds. Int J Biometeorol 52:199-208. doi: 10.1007/s00484-007-0111-x.

Pereira AMF, Titto EAL, Almeida JAA (2019) Adaptação dos ruminantes aos climas quentes. Appris editor, Curitiba.

Pezeshki A, Stordeur P, Wallemacq H, Schynts F, Stevens M, Boutet P, Peelman LJ, Spiegeleer B, Duchateau L, Bureau F, Burvenich C (2011) Variation of inflammatory dynamics and mediators in primiparous cows after intramammary challenge with *Escherichia coli*. Vet Res 42:1-10.

Portugal JAB, Pires MFA, Durães MC (2000) Efeito da temperatura ambiente e da umidade relativa do ar sobre a frequência de ingestão de alimentos e de água e de ruminação em vacas da raça Holandesa. Arq Bras Med Vet Zootec 52(2). https://doi.org/10.1590/S0102-09352000000200013

Renaudeau D, Collin A, Yahav S, De Basilio V, Gourdine J, Collier R (2012) Adaptation to hot climate and strategies to alleviate heat stress in livestock production. Animal 6:707-728. https://doi.org/10.1017/S1751731111002448.

Rodrigues YT, Rodrigues PPB. Maturação dos fenômenos cronobiológicos na infância (2007) In: Jansen JM, Lopes AJ, Jansen U, Capone D, Maeda TY, Noronha A, Magalhães G (orgs) Medicina da noite: da cronobiologia à prática clínica, 1rd edn. FIOCRUZ, Rio de Janeiro, pp. 81-86.

Saizi T, Mpayipheli M, Idowu PA (2019) Heat tolerance level in dairy herds: a review on coping strategies to heat stress and ways of measuring heat tolerance. J Anim Behav Biometeorol 7:39-51.

Sejian V, Silpa MV, Reshma NMR, Devaraj C, Krishnan G, Bagath M, Chauhan SS, Suganthi RU, Fonseca VFC, König S, Gaughan JB, Dunshea FR, Bhatta R (2021) Heat Stress and Goat Welfare: Adaptation and Production Considerations. *Animals* 11:1021. https://doi.org/10.3390/ani11041021.

Schleger A, Turner H (1965) Sweating rates of cattle in the field and their reaction to diurnal and seasonal changes. Aust J Agric Res 16: 92. https://doi.org/10.1071/AR9650092.

Starling JMC, Silva RG, Cerón-Muñoz M, Barbosa GSC, Costa MJRP (2002) Análise de algumas variáveis fisiológicas para avaliação do grau de adaptação de ovinos submetidos ao estresse por calor. Rev Bras Zootec 31:2070-2077.

Terrien J, Perret M, Aujard F (2011) Behavioral thermoregulation in mammals: a review. Front Biosci (Landmark Ed) 16(4):1428-44. https://doi.org/10.2741/3797.

Titto CG, Veríssimo CJ, Pereira AMF, Geraldo AM, Katiki LM, Titto EAL (2016a) Thermoregulatory response in hair sheep and shorn wool sheep. Small Rumin Res 144:341–345.

Titto, CG (2016b) Relações entre termorregulação e comportamento em ovinos. Dissertation, Universidade de São Paulo.

Titto CG, Titto EAL, Titto RM, Mourão GB (2011) Heat tolerance and the effects of shade on the behavior of Simmental bulls on pasture. Anim Sci J 82:591–600.

Van Wettere W, Kind KL, Gatford KL, Swinbourne AM, Leu ST, Hayman PT, Kelly JM, Weaver AC, Kleemann DO, Walker SK (2021) Review of the impact of heat stress on reproductive performance of sheep. J Anim Sci biotechnol 12:1-26, 2021. https://doi.org/10.1186/s40104-020-00537-z.

Wheelock JB, Rhoads RP, Van Baale MJ, Sanders SR, Baumgard LH (2010) Effects of heat stress on energetic metabolism in lactating Holstein cows. J Dairy Sci 93(2):644-655.

6. Skin differential gene expression indicate candidate genes for ovine heat stress tolerance

Manuscript in the article structure of Animal (not yet submitted)

Abstract: Our objective is to identify potential candidate genes of the heat stress response in the skin of Santa Inês sheep of different heat tolerance levels. Twenty-four sheep were used in the study, the 12 most heat-tolerant and 12 less heat-tolerant individuals identified in a thermotolerance assessment of 80 sheep. Animals were maintained for 10 days in a climatic chamber at an average temperature of 36 °C (10:00 am to 04:00 pm) and a maintenance temperature of 28 °C (0400 pm to 10:00 am). From 24 animals, 14 were chosen to do the skin sample. The seven high heat tolerant (HHT) animals and the seven low heat tolerant (LHT) ones were chosen as two contrasting groups. A micro fragment of skin tissue of each sheep were excised using an 8 mm diameter punch and stored in RNA later solution (Invitrogen) in a freezer at -20°C for further RNA extraction. A total of 15,989 genes were found expressed in the sheep skin samples, of which 15 genes were differentially expressed (DE; FDR < 0.05) between the two groups. The five DE genes were upregulated and 10 DE genes downregulated in the HHT compared with the LHT group. These genes are involved in cellular protection against stress (HSPA1A e HSPA6), protein synthesis process (28S, 18S, and 5S ribossomal RNA) and immunologic responses (IGHG4, GNLY, CXCL1, CAPN14 e Serum amyloid A-4). The results indicated that High heat tolerance sheep showed up regulation for the cell protection genes and immune response. Probably the expression of these genes is important to ensure greater tolerance to heat because animals can better protect the body from damage caused by heat stress in cells and for immunity, even in adverse situations

Keywords: transcriptomic, gene, thermotolerance, sheep

6.1.Introduction

The skin is the organ that works as a barrier between intern and extern ambient, protecting the body from mechanical damage, chemical damage, ultraviolet radiation and allows to maintain homeostasis (Singh et al., 2014). The skin contributes to homeostasis by detecting thermal changes and triggering defense responses (Romanovsky, 2014). This occurs through cutaneous heat and cold receptors that are located in the skin and send thermal sensory information to the preoptic area, where the afferent signals of cold and heat are processed, generating a differentiated response to changes in skin temperatures, which is characterized by vasodilation in response to heat and by vasoconstriction in response to cold weather (Morrison and Nakamura, 2011).

The skin contains components such as hair, sebaceous glands and sweat glands (Kobielak et al., 2015). In this organ, sweating occurs, which is triggered as the internal body temperature increases, and prevent excessive accumulation of heat in the body (Ferreira et al., 2009), allowing the heat transport from deep tissues to the skin (Johnson and Kellogg Jr, 2010), where the heat lost by evaporation of moisture from the surface of the skin occurs (Gebremedhin et al., 2008). This mechanism is an autonomic response of animals under heat stress (Collier and Gebremedhin, 2015).

Given the importance of the skin in thermoregulation, this tissue has been studied to identify genes associated with heat stress. Expression of Hsp70 was reported in the upper epidermal layer in rat skin, around blood vessels, hair follicles, and sebaceous glands (Souil et al., 2001), in humans, epidermal keratinocytes were found to provide a natural barrier against possible stressful environmental attacks (Zhou et al. 1998; Jonak et al. 2009), and significant number of genes (HSP70, MMPs, iNOS, Caspase and Bcl-2 family) in bovine and buffalo dermal fibroblasts (Singh et al., 2014).

Thus, characterizing the cellular response by RNA sequencing is important to understand the differences in the stress responses of sheep subjected to high temperature, and can also be a tool for the selection of more productive animals in tropical environments. Therefore, our objective is to identify potential candidate genes of the heat stress response in the skin of Santa Inês sheep of different heat tolerance levels.

6.2.Material and methods

6.2.1. Ethical approval

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

6.2.2. Local and Animals

Eighty black coat Santa Inês sheep were used. All ewes were not pregnant, had body condition score of 3 (scale from1 to 5) and 4,3±0,7 years. The animals were obtained from the Biometeorology, Ethology and Animal Welfare Research Facility of the Laboratory of

Biometeorology and Ethology, Department of Animal Science, FZEA/USP, Brazil. Sheep were housed during five months in pens with artificial shade provided by white-painted fiber cement tiles (1 m^2 per animal) and access to a pasture of *Panicum maximum* cv Aruana, and corn silage supplementation. For the experiment, the animals were taken to a climatic chamber at the Department of Animal Reproduction of the same university (FVMZ/USP). The climate chamber has an area of 56 m^2 fully enclosed with brick walls and ceiling slabs, with cement flooring, temperature and humidity controller in a external area, internal thermostats and an exhaust fan. The animals had free access to water, corn silage and mineral supplementation.

6.2.3. Experimental design e Heat tolerance test

The experiment was divided into two phases. In phase 1, the animals were subjected to heat stress in a climatic chamber for ten days, two days of adaptation to the new environment and 8 days of heat treatment (stressor factor). The heat treatment was set at a temperature of 36°C, starting from 10:00 to 16:00, maintained at 26 °C from 16:00 to 10:00. On the 9th and 10th days, rectal temperature data were collected at 1:00 pm, 4:00 pm, 7:00 pm, 9:00 pm, 1:00 am, 4:00 am, 7:00 am and 10:00 am. Rectal temperature was used as the response variable, and analyzed by the restricted maximum likelihood method (REML) under a mixed model. This included the fixed effects of the assessment cycle (4 cycles with 20animals each), equivalent to the group of contemporaries, and the time effect within the assessment cycle and, as a random, the animal effect. The BLUP predictions obtained for each ewe, which quantify the individual heat stress response, were used to rank ewes from most heat tolerant to least tolerant. After the end of phase 1, 12 high heat tolerant (HHT) and 12 low heat tolerant (LHT) animals were selected from this classification to phase 2.

After seven days, the second phase was done during ten days inside the climatic chamber, with temperature of 36°C from 10:00 to 16:00, and reduced to 28 °C from 16:00 to 10:00, 2 °C above heat treatment in phase 1 to avoid acclimatation.

6.2.4. Collection of skin samples

From 24 animals, 14 were chosen to do the skin sample. Data analysis was performed similar to phase 1. The seven high heat tolerant (HHT) animals and the seven low heat tolerant (LHT) ones were chosen as two contrasting groups.

Inside the climatic chamber, with air temperature of 36ºC after 10 hours of heat stress, a biopsy was performed on the right side of the animal, in the middle dorsal region. Before collection of the biopsy, the area was shaved, disinfected, and anesthetized by application of 1 mL of local anesthetic without vasoconstrictor (2% lidocaine hydrochloride). A micro fragment of skin tissue of each sheep were excised using an 8 mm diameter punch and stored in RNA later solution (Invitrogen) in a freezer at -20°C for further RNA extraction. Sequencing analyzes will be performed at the Genomics Center of ESALQ, Piracicaba, São Paulo, Brazil.

6.2.5. Bioinformatics analysis

Quality control, read mapping, and differential expression (DE) analysis

Sequence data statistics and quality were estimated using the FASTQC tool version 0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). The quality control of the reads (QC) was performed using TRIM Galore software version 0.6.6 (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to remove sequence adapters, low quality reads (QPhred<30), and short reads (<70pb). The clean reads were mapped against the sheep reference genome (Ovis aries, assembly GCA_016772045.1) available from the NCBI database (www.ncbi.nlm.nih.gov) using the STAR 2.7.3a software1.

The abundance (read counts) of mRNAs for all annotated genes was calculated using Counts Per Million (CPM) and were kept only genes that presented at least one CPM in at least 30% of samples. The EdgeR package2 implemented in R language was used to identify differentially expressed genes (GDE) from high and low heat tolerance groups. The significance threshold for DE genes was set at a False Discovery Rate (FDR) ≤0.05 after multiple correction tests3. Genes were considered upregulated and downregulated according to FDR and the positives and negatives log2 fold-change (Log2FC), respectively, in the high heat tolerance (HHT) group compared to the low heat tolerance (LHT) group.

6.3.Results

RNA sequencing data and differential gene expression analysis for skin samples

The sequencing of the skin samples generated an average of 11,6 million paired-end reads per sample (2 x 100 bp), remaining about 11,2 million after the QC. Approximately, 82.46 % of the reads were mapped against the sheep reference genome (ATTACHMENT A). The Multi-Dimensional Scaling (MDS) plot showed a lack of clustering among animals belonging to the same experimental group (ATTACHMENT B).

A total of 15,989 genes were found expressed in the sheep skin samples, of which 15 genes were differentially expressed (FDR < 0.05) between the two groups (ATTACHMENT C). The five DE genes were upregulated and 10 DE genes downregulated in the HHT compared with the LHT group (Fig 1).

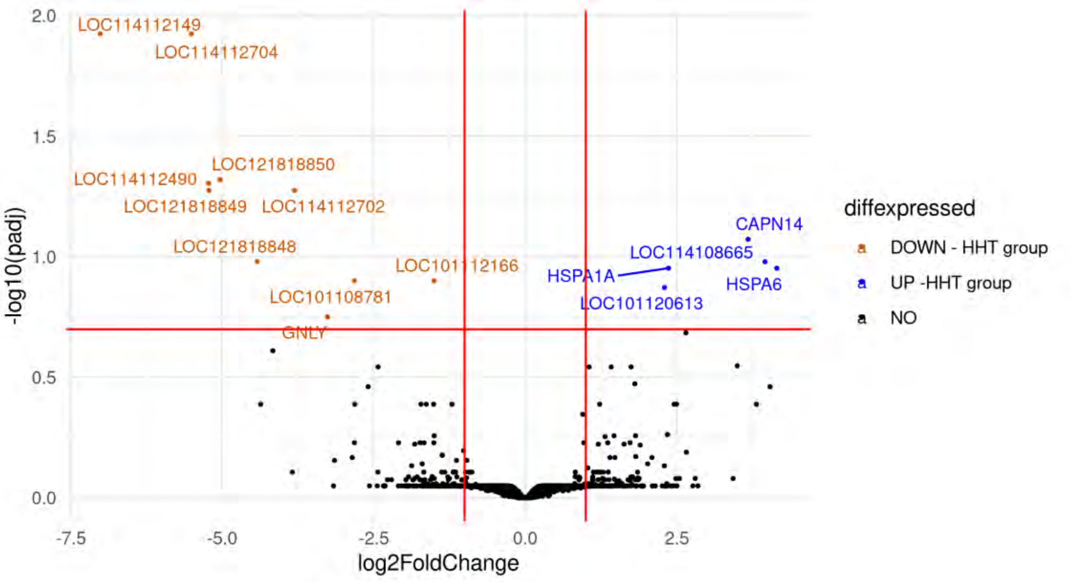

Fig 1. Volcano plot of log2FoldChange (x-axis) versus -log10 p value adjusted (FDR≤0.20, y-axis) of high and low heat tolerance sheep skin samples.

Hierarchical clustering analysis of the 15 DE genes showed a similar expression pattern among some samples of the groups (Fig 2).

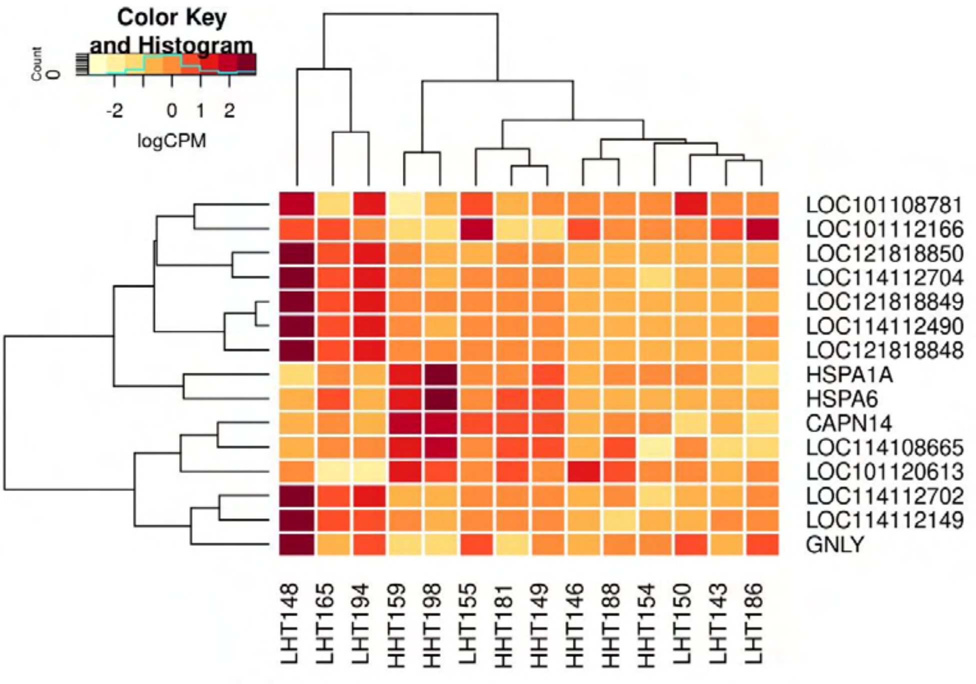

Fig 2. Heatmap with 15 differentially expressed genes between skin samples from high heat tolerance (HHT 146; 149; 154; 159; 181; 188; 198) and low heat tolerance (LHT 143; 148; 150; 155; 165; 186; 194) sheep. Each gene expression is shown in the lines and the samples in columns, resulting in a hierarchical group of genes and samples. In red are the upregulated and in yellow are the downregulated genes in the high heat tolerance animals

6.4.Discussion

10 genes were identified as differential expression in sheep classified as high heat tolerant (HHT) and low heat tolerant (LHT) under high air temperatures. These genes are involved in cellular protection against stress (HSPA1A e HSPA6), protein synthesis process (28S , 18S , e 5S ribossomal RNA) and immunologic responses (IGHG4, GNLY, CXCL1, CAPN14 e Serum amyloid A-4).

HSPA1A and HSPA6

The heat shock protein family is a group of chaperones involved in the folding, stabilizing, and transport functions of proteins throughout the cell. The main isoform is Hsp70 which is encoded by the HspA1A gene (Borges et al., 2012) and HSPA6 (Hsp70B'), which is also a member of the HSPA family but receives little attention compared to HSPA1A, which is more widely studied (Hsp70-1) (Deane and Brown, 2018).

HSPA1A and HSPA6 were more expressed in high heat tolerance animals. This increased expression in the skin during heat challenge may be because members of this protein family are induced by several types of stressors, including heat stress which is capable of activating heat shock transcription factors., e.g., HSPA1 (HSP70) and HSPA6 (Hsp70B') (Su et al., 2021). Furthermore, these proteins are involved in mitigating the deleterious effect of heat stress, so as to maintain cellular integrity and skin homeostasis against environmental stressors (Maibam et al., 2017), playing an important role in inhibiting apoptosis, ensuring cell survival (Noonan et al., 2007). In addition, the variation in the expression of HSPs from groups classified as high and low heat tolerance may be related to heat resistance and adaptation to different climatic conditions (Soghi et al., 2013; Raza et al., 2021) showing that, despite the animals were from the same race and under the same environmental conditions, they respond differently to the increasing in air temperature.

CAPN14

The relationship between calpain and heat stress is still unclear, but studies with buffaloes have found this gene to be more expressed during heat stress (Singh et al., 2014). They are calcium dependent proteins (Tizioto et al., 2013) and animals suffering from heat stress have reduced circulating calcium and this could reduce calpain synthesis. During heat stress, with lower food intake, there is a reduction in the availability of circulating calcium (Kadzere et al., 2002). The HHT group is likely to be able to overcome the effects of high temperature and therefore has a higher expression of CAPN14, with calcium mobilization.

The higher amount of calpain may be related to better meat quality (Scheffler, 2022). It has been seen that the highest expression of CAPN1 is related to the tenderness of the meat (Avilés et al., 2013). On the other hand, CAPN14 expression may be linked to immunity, since CAPN14 can be triggered by Th2-associated signaling, through interleukins (IL) such as IL-13 and IL-4 (Davis et al., 2016). Th2 cells secrete IL-4, IL-5 and IL-13 and are responsible for immunity, and it has already been identified in the activation of mast cells in immune responses against helminths (Zenobia and Hajishengallis, 2015). In addition, capains are involved in several functions, including restructuring of cytoskeletal and membrane proteins, signal transduction, and inactivating enzymes involved in cell cycle progression, gene expression, and apoptosis (Chakraborti et al., 2012).

Thus, the higher expression of CAPN14 in animals more heat tolerant may be a response of this group to the negative effects of stress. HHT animals may have higher expression of calpain in the skin as a way to improve immunity and it may be that this contributes to the better ability to handle heat stress compared to LHT animals.

LOC114108665 (growth-regulated alpha protein-CXCL1)

Growth-regulated alpha protein (CXCL1) is an important component of cellular responses to harmful agents. For this reason, intercellular signaling involving CXCL1 must be very rapid (Korbecki et al., 2022). CXCL1 is induced in normal cells by exogenous stimuli, such as microbial products and/or inflammatory cytokines. In response to bacterial endotoxin, IL-1 and TNF-α are potent inducers of CXCL chemokines in mononuclear phagocytes, epithelial cells and structural mesenchymal cells (Chensue, 2006).

CXCL1 may also have its production stimulated by IL-17. IL-17A has as its main function protection against extracellular bacteria and fungi, due to its ability to recruit neutrophils to infected areas (Normanton and Marti, 2013). IL-17 needs to be combined with specific immunostimulatory cytokines such as IL-6 and IL-21 and tumor growth factor-β to drive its development, although the IL-17-producing T cell may be involved in potent inflammatory responses, a subset of Th17 regulatory cells (rTh17) that express the anti-inflammatory cytokine IL-10 has been identified (Zenobia and Hajishengallis, 2015).

Stress can stimulate the production of IL-17 and IL-6 (Ganesan et al., 2017; Kim et al., 2021), probably this fact is responsible for the higher expression of CXCL1 in animals under stress. And just like CAPN14, the higher expression of CXCL1 in HHT sheep may be related to greater protection of the animal in response to heat stress, presenting a good immune response in adverse climatic conditions in which the immune system is suppressed.

LOC101108781 (immunoglobulin heavy constant gamma 4-like-IGHG4)

The immunoglobulin heavy constant gamma 4 (IGHG4) genes are responsible for encoding the immunoglobulin (Braga et al., 2019). Immunoglobulins can be expressed in the epidermis and are involved in skin immunity (Jiang et al., 2015), being proteins induced in response to antigen exposure.

It is known that chronic heat stress is responsible for affecting the immune system of the animal's organism (Jin et al., 2011; Marrero et al., 2021). During stress conditions, after the initiation of the immune response by immunoglobulins, pro- and anti-inflammatory cytokines are secreted and play a key role in determining the immune status of an organism (Arango-Duque and Descoteaux, 2014). Some cytokines enhance inflammatory responses and are called pro-inflammatory cytokines, while other cytokines suppress inflammatory responses and are called anti-inflammatory cytokines (Dinarello, 2000).

Thus, interleukin-10 (IL-10) is an anti-inflammatory cytokine involved in the inflammatory response (Siddiqui et al., 2020). Thus, the lower immunoglobulin response in HHT may have been due to the increase in IL-10 in this group as a result of the higher expression of HSPs that are able to regulate the immune response and the activation of regulatory T cells (Spienrings and Eden, 2017). Furthermore, induction of IL-10-producing T cells is a feature of HSPs immunization (Wendling et al., 2000).

LOC101120613 (Serum amyloid A-4 protein)

Serum amyloid A (SAA) increases its concentration in response to inflammation (Al-Dawood et al., 2017). This response is predominantly driven by the endogenous cytokines interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α, induced in macrophages that bind to toll-like receptors (TLR). Several functions have been described for Serum amyloid A, and pro- and anti-inflammatory activities can be highlighted. Pro-inflammatory activities are more prominent, requiring a small concentration of SAA, and are also related to leukocyte migration through their chemokine-inducing capacity and chemotactic effect (Buck et al., 2016).

The higher expression of Serum amyloid A may be due to heat stress, which increases the production of IL-6 (Ganesan et al., 2017) and influences the production of SAA. Thus, heat stress has already been proven to increase plasma concentrations of amyloid A (Ríus et al., 2022). Another factor that may explain the higher expression of Serum amyloid A is the higher expression of HSP70 in this group (Ather et al., 2013; Mallick et al., 2021). Responses to extracellular HSPs appear to involve a variety of cell surface receptors that may determine their influence on tissue immune reactions (Calderwood et al., 2016). Thus, the higher Serum amyloid A expression in HHT ewes may once again be related to higher heat tolerance.

GNLY

Granulysin protein (GNLY) of the saposin family is involved in protective immunity and is released by natural killer (NK) cells, NKT cells, γδ T cells, and cytotoxic T lymphocytes (Thuong et al., 2016). The main receptors of NK cells and T cells are known as killer immunoglobulin-like receptors (KIR) which are designated by the number of immunoglobulin domains (D) (Almeida-Oliveira and Diamond, 2008). On the other hand, according to Ruiz et al. (1996), immunoglobulin can inhibit the activity of cells and reduce their toxicity and probably can affect the release of the protein granulysin.

As for the protective action of granulysin, it occurs when NK cells and T lymphocytes identify an infected cell, enzymes are retained in the cytotoxic granule by binding to a granular protein called serglycine. Cytotoxic cell granules contain two types of effector molecules, pore-forming proteins that disrupt cell membranes (perforin, granulysin) and proteases (granule granzymes) (Lieberman, 2016) that induce apoptosis in infected mammalian cells.

Bacterial cell apoptosis mediated by these proteins causes damage to critical processes necessary for survival, causing DNA damage, inducing reactive oxygen species, disrupting the mitochondrial outer membrane, interfering with RNA splicing and protein translation, providing mechanisms for kill diverse bacterial strains that grow under varying conditions (Walch et al., 2014). These molecules rapidly mobilize the immune system to respond to infection and tissue injury to mediate an inflammatory response. Activated T cells and NK cells release GNLY, which activates monocytes, therefore, GNLY may be a mediator of pro-inflammatory responses (Tewary et al., 2010).

28S, 18S, and 5S ribosomal RNA

Ribosomal RNA (rRNA; 28S, 18S, 5.8S, and 5S in eukaryotes) are encoded by many copies of ribosomal DNA throughout the genome, which exhibit tissue-specific expression patterns (Parks et al., 2018). The 28s, 18s and 5s genes identified in the skin were less expressed in high heat tolerance animals. These genes had not yet been found in sheep and, therefore, it is still unclear what the function of these genes would be in the skin and how temperature might influence their expression. Thus, the effect of heat stress on RNA metabolism in mammalian cells is not well characterized. Evidence suggests that processing and transport pathways for 5s RNA, rRNA and mRNA may be sensitive to hyperthermia. Furthermore, the synthesis of 18s and 28s rRNAs in the cytoplasm can be affected by heat stress (Sadis et al., 1988).

The lower expression of 28s, 18s and 5s ribosomal RNA in high heat tolerance sheep in our study suggests that this group suffers less under stressful conditions and therefore does not require ribosomal modifications to the same degree as low heat tolerance animals. LHTs probably need to adjust RNA structure/function to try to combat the negative effects of heat stress, so greater ribosomal changes are necessary for specific transcripts to be translated in response to different environmental conditions (Baldridge and Contreras, 2014) thus controlling the gene expression in an attempt to combat the effects of heat stress. This process is termed methylation, however, what are the effects of each of these methylation events, which specific transcripts are translated, and what are the functional consequences of most rRNA modifications are still unclear (Liberman et al., 2020).

LOC101112166 **(**putative SEC14-like protein 6-SEC14L6**)**

Sec14 domain proteins interface with many cellular activities (Bankaitis et al. 2012) such as signal transduction, transport and organelle biology, where they integrate lipid metabolism with other biochemical processes (Saito et al., 2007).

Lipid metabolism involves the synthesis of structural and functional lipids (such as phospholipids, glycolipids, cholesterol, prostaglandins, etc.) that are characteristic of individual tissues (Gyamfi et al., 2019). This gene may be important during the negative effect of stress for energy production, which is important because thermoregulation requires a high energy cost (Zhang and Dong, 2021) being responsible for the consumption of energy that would be used in other metabolic and productive processes to maintain homeothermy (Souza et al., 2005). and may also be related to energy reserve and thermal insulation.

This gene may be responsible for the production of prostaglandins that play an important role in the inflammatory response. Prostaglandin production is generally low in non-inflamed tissues, but increases immediately during inflammation and before leukocyte and immune cell recruitment (Ricciotti and FitzGerald, 2011). Therefore, SEC14 may be directly and indirectly involved in several functions that are linked to heat stress, the lower expression in HHT animals may be due to a greater ability to deal with stress and to suffer less at high temperatures compared to LHT.

6.5.Conclusion

High heat tolerance sheep showed up regulation for the cell protection genes (HSPA1A and HSPA6) and immune response (CXCL1, CAPN14 and Serum amyloid A-4, IGHG4). Probably the expression of these genes is important to ensure greater tolerance to heat because animals can better protect the body from damage caused by heat stress in cells and for immunity, even in adverse situations. Further studies should be carried out to better understand the other genes addressed in this article.

Declarations of interest: none

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References

Al-Dawood, A., 2017. Acute phase proteins as indicators of stress in Baladi goats from Jordan. Acta Agriculturae Scandinavica, Section A - Animal Science 67, 58 - 65. https://doi.org/10.1080/09064702.2017.1363815.

Almeida-Oliveira, A., Diamond, H.R., 2008. The relevance of natural killer (NK) cells and killer immunoglobulin-like receptors (KIR) in hematopoietic stem cell transplantation (HSCT). Revista Brasileira de Hematologia e Hemoterapia 30, 320-329.

Arango-Duque, G., Descoteaux, A. 2014. Macrophage cytokines: involvement in immunity and infectious diseases. Frontiers in Immunology 5, 491. https://doi.org/10.3389/fimmu.2014.00491

Ather, J.L., Fortner, K.A., Budd, R.C., Anathy, V., Poynter, M.E., 2013. Serum amyloid A inhibits dendritic cell apoptosis to induce glucocorticoid resistance in CD4 þ T cells. Cell Death and Disease 4, e786. https://doi.org/10.1038/cddis.2013.327.

Avilés, C., Juárez, M., Peña, F., Domenech, V., Clemente, I., Molina, A., 2013. Association of single nucleotide polymorphisms in CAPN1 and CAST genes with beef tenderness from Spanish commercial feedlots. Czech Journal of Animal Science 58, 479–487.

Baldridge, K.C., Contreras, L.M., 2014. Functional implications of ribosomal RNA methylation in response to environmental stress. Biochemistry and Molecular Biology 49, 69-89. https://doi.org/ 10.3109/10409238.2013.859229.

Bankaitis, V.A., Ile, K.E., Nile, A. H., Ren, J., Ghosh, R., Schaaf, G., 2012. Thoughts on Sec14-like nanoreactors and phosphoinositide signaling. Advances in biological regulation 52, 115–121. https://doi.org/10.1016/j.jbior.2011.11.001

Borges, T.J., Wieten, L., Van Herwijnen, M.J., Broere, F., Van der Zee, R., Bonorino, C., Van Eden, W., 2012.The anti-inflammatory mechanisms of Hsp70. Frontiers in Immunology 4, 95. https://doi.org/10.3389/fimmu.2012.00095.

Braga, D., Barcella, M., Herpain, A., Aletti, F., Kistler, E.B., Pinto, B.B., Bendjeli, K., Barlassina, C., 2019. A longitudinal study highlights shared aspects of the transcriptomic response to cardiogenic and septic shock. Critical Care 23, 414 https://doi.org/10.1186/s13054-019-2670-8.

Calderwood, S.K., Gong, J., Murshid, A., 2016. Extracellular HSPs: The Complicated Roles of Extracellular HSPs in Immunity. Frontiers in Immunology 7, 159. https://doi.org/10.3389/fimmu.2016.00159.

Chakraborti, S., Alam, M.N., Paik, D., Shaikh, s., Chakraborti, T., 2012. Implications of calpains in health and diseases. Indian Journal of Biochemistry & Biophysics 49, 316–328.

Chensue, S.W., 2006. Chemokines, CXC | CXCL1 (GRO1)–CXCL3 (GRO3). In Encyclopedia of Respiratory Medicine (Ed(s) Laurent, G.J., Shapiro, S.D.). Academic Press, Massachusetts, EUA, pp. 407-410. https://doi.org/10.1016/B0-12-370879-6/00469-5.

Collier, R.J., Gebremedhin, K.G., 2015. Thermal Biology of Domestic Animals. Annual Review of Animal Biosciences 3, 513–532.

Gyamfi, D., Awuah, E.O., Owusu, S. 2019. Lipid Metabolism: An Overview. In The Molecular Nutrition of Fats (Ed. Patel, V.B.). Academic Press, Massachusetts, EUA, pp. 17-32. https://doi.org/10.1016/B978-0-12-811297-7.00002-0.

Davis, B.P., Stucke, E.M., Khorki, M.E., Litosh, V.A., Rymer, J.K., Rochman, M., Travers, J., Kottyan, L.C., Rothenberg, M.E., 2016. Eosinophilic esophagitis-linked calpain 14 is an IL-13-induced protease that mediates esophageal epithelial barrier impairment. JCI Insight 1, e86355. https://doi.org/10.1172/jci.insight.86355.

De Buck, M., Gouwy, M., Wang, J. M., Van Snick, J., Opdenakker, G., Struyf, S., Van Damme, J., 2016. Structure and Expression of Different Serum Amyloid A (SAA) Variants and their Concentration-Dependent Functions During Host Insults. Current medicinal chemistry 23, 1725–1755. https://doi.org/10.2174/0929867323666160418114600

Deane, C.A.S., Brown, I.R., 2018. Knockdown of Heat Shock Proteins HSPA6 (Hsp70B') and HSPA1A (Hsp70-1) Sensitizes Differentiated Human Neuronal Cells to Cellular Stress. Neurochemical Research 43, 340-350. https://doi.org/10.1007/s11064-017-2429-z.

Dear, T.N., Meier, N.T., Hunn, M., Boehm, T., 2000. Gene Structure, Chromosomal Localization, and Expression Pattern of Capn12, a New Member of the Calpain Large Subunit Gene Family. Genomics 68, 152–160. https://doi.org/10.1006/geno.2000.6289.

Dinarello, C. A., 2000. Proinflammatory cytokines. Chest Journal 118, 503–508.

Dobin, A., Davis, C.A., Schlesinger, F., et al. 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29, 15-21. https://doi.org/10.1093/BIOINFORMATICS/BTS635

Ferreira, F., Campos, W.E., Carvalho, A.U., Pires, M.F.A., Martinez, M.L., Silva, M.V.G.B., Verneque, R.S., Silva, P.F., 2009. Taxa de sudação e parâmetros histológicos de bovinos submetidos ao estresse calórico. Arquivo Brasileiro de Medicina Veterinária e Zootecnia 61, 763-768.

Ganesan, S., Volodina, O., Pearce, S.C., Gabler, N.K., Baumgard, L.H., Rhoads, R.P., Selsby, J.T., 2017.Acute heat stress activated inflammatory signaling inporcine oxidative skeletal muscle 5, e13397.https://doi.org/10.14814/phy2.13397

Gebremedhin, K.G., Hillman, P.E., Lee, C.N., Collier, R.J., Willard, S.T., Arthington, J.D., Brown-Brandl, T.M., 2008. Sweating rates of dairy cows and beef heifers in hot conditions. American Society of Agricultural and Biological Engineers 51, 2167-2178.

Jiang, D., Ge, J., Liao, Q., Ma, J., Liu, Y., Huang, J., Wang, C., Xu, W., Zheng, J., Shao, W., Lee, G., Qiu, X., 2015. IgG and IgA with potential microbial-binding activity are expressed by normal human skin epidermal cells. International Journal of Molecular Sciences 23, 2574-90. https://doi.org/10.3390/ijms16022574.

Jin, Y., Hu, Y., Han, D., Wang, M., 2011. Chronic heat stress weakened the innate immunity and increased the virulence of highly pathogenic avian influenza virus H5N1 in mice. Journal of Biomedicine and Biotechnology 2011:367846. https://doi.org/10.1155/2011/367846.

Johnson, J.M., Kellogg Jr., D.L., 2010. Thermoregulatory and thermal control in the human cutaneous circulation. Frontiers in Bioscience, v.2, p.825-853.

Jonak, C., Klosner, G., Trautinger, F. 2009. Significance of heat shock proteins in the skin upon UV exposure. Frontiers in Bioscience 14, 4758-4768.

Kadzere, C. T., Murphy, M. R., Silanikove, N., & Maltz, E. 2002. Heat stress in lactating dairy cows: a review. Livestock Production Science. 77, 59-91.

Kim, J., Suh, Y., Chang, K., 2021. Interleukin-17 induced by cumulative mild stress promoted depression-like behaviors in young adult mice. Molecular Brain 14, 11. https://doi.org/10.1186/s13041-020-00726-x.

Kobielak, K., Kandyba, E., Leung, Y., 2015. Skin and Skin Appendage Regeneration. In Translational Regenerative Medicine (Ed(s) Anthony Atala, Julie G. Allickson,). Academic Press, Massachusetts, EUA, pp. 269-292, https://doi.org/10.1016/B978-0-12-410396-2.00022-0.

Korbecki, J., Barczak, K., Gutowska, I., Chlubek, D., Baranowska-Bosiacka, I., 2022. CXCL1: Gene, Promoter, Regulation of Expression, mRNA Stability, Regulation of Activity in the Intercellular Space. International Journal of Molecular Sciences 23, 792. https://doi.org/10.3390/ijms23020792.

Liberman, N., O’Brown, Z.K., Earl, A.S., Boulias, K., Gerashchenko, M.V., Wang, S.Y., Fritsche, C., Fady, P., Dong, A., Gladyshev, V.N., Gree, E.L., 2020. N6-adenosine methylation of ribosomal RNA affects lipid oxidation and stress resistance. Science Advance 6 : eaaz4370.

Lieberman, J., 2016. Cytotoxic Lymphocytes. In Encyclopedia of Immunobiology (Ed Ratcliffe, M.J.H.). Academic Press, Massachusetts, EUA, pp. 363-373, https://doi.org/10.1016/B978-0-12-374279-7.08021-8.

Maibama, U., Hoodaa, O.K., Sharmab, P.S., Mohantyc, A.K., Singha, S.V., Upadhyay, R.C., 2017. Expression of HSP70 genes in skin of zebu (Tharparkar) and crossbred (Karan Fries) cattle during different seasons under tropical climatic conditions. Journal of Thermal Biology 63, 58–64

Mallick, S., Subramaniam, S., Biswal, J.K., Ranjan, R., Mohapatra, J.K., Sahoo, A.P., 2021. Short communication: preliminary observations on the serum levels of HSP70 and its correlation with serum cortisol, thyroid hormones, and acute-phase protein concentration in cattle naturally infected with foot-and-mouth disease virus. Tropical Animal Health and Production 53: 408

Marrero, M.G., Dado-Senn, B., Field, S.L., Yang, G., Driver, J.P., Laporta, J., 2021. Chronic heat stress delays immune system development and alters serotonin signaling in pre-weaned dairy calves. PLoS ONE 16, e0252474. https://doi. org/10.1371/journal.pone.0252474

Morrison, S.F.; Nakamura, K., 2011. Central neural pathways for thermoregulation. Frontiers Bioscience 16, 74-104.

Noonan, E.J., Place, R.F., Giardina, C., Hightower, L.E., 2007. Hsp70B' regulation and function. Cell Stress Chaperones. Winter 12, 393-402. https://doi. org/10.1379/csc-278e.1.

Normanton, M., Marti, L.C., 2013. Current data on IL-17 and Th17 cells and implications for graft versus host disease. Einstein 11, 237-46.

Parks, M.M., Kurylo, C.M., Dass, R.A., Bojmar, L., Lyden, D., Vincent, C.T., Blanchard, S.C., 2018. Variant ribosomal RNA alleles are conserved and exhibit tissue-specific expression. Science Advances 4, eaao0665. https://doi. org/10.1126/sciadv.aao0665.

Raza, S.H.A., Hassanin, A.A., Dhshan, A.I.M., Dhshan, A.I.M., Abdelnour, S.A., Khan, R., Mei, C., Zan, L., 2021.In silico genomic and proteomic analyses of three heat shock proteins (HSP70, HSP90-a, and HSP90-b) in even-toed ungulates. Electron Journal of Biotechnology 53, 61–70.

Ricciotti, E., FitzGerald, G.A., 2011. Prostaglandins and inflammation. Arteriosclerosis, thrombosis, and vascular biology, 31, 986–1000. https://doi.org/10.1161/ATVBAHA.110.207449.

Ríus, A.G., Kaufman, J. D., Li, M. M., Hanigan, M. D., Ipharraguerre, I. R., 2022. Physiological responses of Holstein calves to heat stress and dietary supplementation with a postbiotic from Aspergillus oryzae. Scientifc Reports 12, 1587. https://doi.org/10.1038/s41598-022-05505-3.

Romanovsky, A.A., 2014. Skin temperature: its role in thermoregulation. Acta physiologica 210, 498–507. https://doi.org/10.1111/apha.12231.

Ruiz, J.E., Kwak, J.Y.H., Baum, L., Gilman-Sachs, A., Beaman, K.D., Kim, Y.B., Beer, A.E., 1996. Effect of intravenous immunoglobulin G on natural killer cell cytotoxicity in vitro in women with recurrent spontaneous abortion. Journal of Reproductive Immunology 31, 125–141. https://doi.org/10.1016/0165-0378(96)00969-2.

Sadis, S., Hickey, E., Weber, L.A., 1988. Effect of heat shock on RNA metabolism in HeLa cells. Journal of Cellular Physiology 135, 377-386. https://doi.org/ 10.1002/jcp.1041350304.

Saito, k., Tautz, L., Mustelin, T., 2007.The lipid-binding SEC14 domain. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1771, 719-726. https://doi.org/10.1016/j.bbalip.2007.02.010.

Scheffler, T.L., 2022. Connecting Heat Tolerance and Tenderness in Bos indicus Influenced Cattle. Animals, 12, 220. https://doi.org/10.3390/ani12030220

Siddiqui, S.H., Kang, D., Park, J., Khan, M., Shim, K., 2020. Chronic heat stress regulates the relation between heat shock protein and immunity in broiler small intestine. Scientific Reports 10, 18872.

Singh, A.K., Upadhyay, R.C., Malakar, D., Singh, S.V., Kumar, S., Devi, R., 2014. Role of Animal Skin in Thermoregulation. Climate Resilient Livestock & Production System 50-61.

Sodhi, M., Mukesh, M., Kishore, A., Mishra, B.P., Kataria, R.S., Joshi, B.K., 2013. Novel polymorphisms in UTR and coding region of inducible heat shock protein 70.1 gene in tropically adapted Indian zebu cattle (Bos indicus) and riverine buffalo (Bubalus bubalis). Gene 527, 606-615. https://doi.org/10.1016/j.gene.2013.05.078.

Souil, E., Capon, A., Mordon, S., Dinh-Xuan, A.T., Polla, B.S., Bachelet, M., 2001.Treatment with 815-nm diode laser induces long-lasting expression of 72-kDa heat shock protein in normal rat skin. British Journal of Dermatology 144, 260-266.

Souza, E. D., Souza, B.B., Souza, W.H., Cezar, M.F., Santos, J.R.S., Tavares, G.P., 2005. Determinação dos parâmetros fisiológicos e gradiente térmico de diferentes grupos genéticos de caprinos no Semi-árido. Ciência e Agrotecnologia 29, 177-184.

Spierings, J., Van Eden, W., 2017. Heat shock proteins and their immunomodulatory role in inflammatory arthritis. Rheumatology 56, 98208. https://doi.org/10.1093/rheumatology/kew266.

Su, Y.S., Hwang, L.H., Chen, C.J., 2021. Heat Shock Protein A6, a Novel HSP70, Is Induced During Enterovirus A71 Infection to Facilitate Internal Ribosomal Entry Site-Mediated Translation. Frontiers in Microbiology 12, 664955. https://doi.org/10.3389/fmicb.2021.664955.

Tewary, P., Yang, D., de la Rosa, G., Li, Y., Finn, M.W., Krensky, A.M., Clayberger, C., Oppenheim, J.J., 2010. Granulysin activates antigen-presenting cells through TLR4 and acts as an immune alarmin. Blood 116, 3465-3474. https://doi.org/10.1182/blood-2010-03-273953.

Thuong, P.H., Tam, D.B., Sakurada, S., Hang, N.T.L., Hijikata, M., Hong, L.T., Ngoc, P.T.M., Anh, P.T., Cuong, V.C., Matsushita, I., Lien, L.T., Keicho, N., 2016. Circulating granulysin levels in healthcare workers and latent tuberculosis infection estimated using interferon-gamma release assays. BMC Infectious Diseases 16, 580. https://doi.org/10.1186/s12879-016-1911-6

Tizioto, P.C., Decker, E., Taylor, J.F., Schnabel, R.D., Mudadu, M.A., Silva, F. L., Mourão, G. B., Coutinho, L.L., Tholon, P., Sonstegard, T.S., Rosa, A.N., Alencar, M.M., Tullio, R.R., Medeiros, S.R., Nassu, R.T., Feijó, G.L.D., Silva, L.O.C., Torres, R.A., Siqueira, F., Higa, R.H., Regitano, L.C.A., 2013. Genome scan for meat quality traits in Nelore beef cattle. Physiological Genomics 45, 1012–1020.

Walch, M., Dotiwala, F., Mulik, S., Thiery, J., Kirchhausen, T., Clayberger, C., Krensky, A.M., Martinvalet, D., Lieberman, J., 2014. Cytotoxic Cells Kill Intracellular Bacteria through Granulysin-Mediated Delivery of Granzymes. Cell 157, 1309–1323.

Wendling, U., Paul, L., Van der, Z.R., Prakken, B., Singh, M., Van Eden, W., 2000. A conserved mycobacterial heat shock protein (hsp) 70 sequence prevents adjuvant arthritis upon nasal administration and induces IL-10-producing T cells that cross-react with the mammalian self-hsp70 homologue. Journal of Immunology 164, 2711–2717.

Zenobia, C., Hajishengallis, G., 2015. Basic biology and role of interleukin-17 in immunity and inflammation. Periodontology 2000, 69, 142–159. https://doi.org/10.1111/prd.12083.

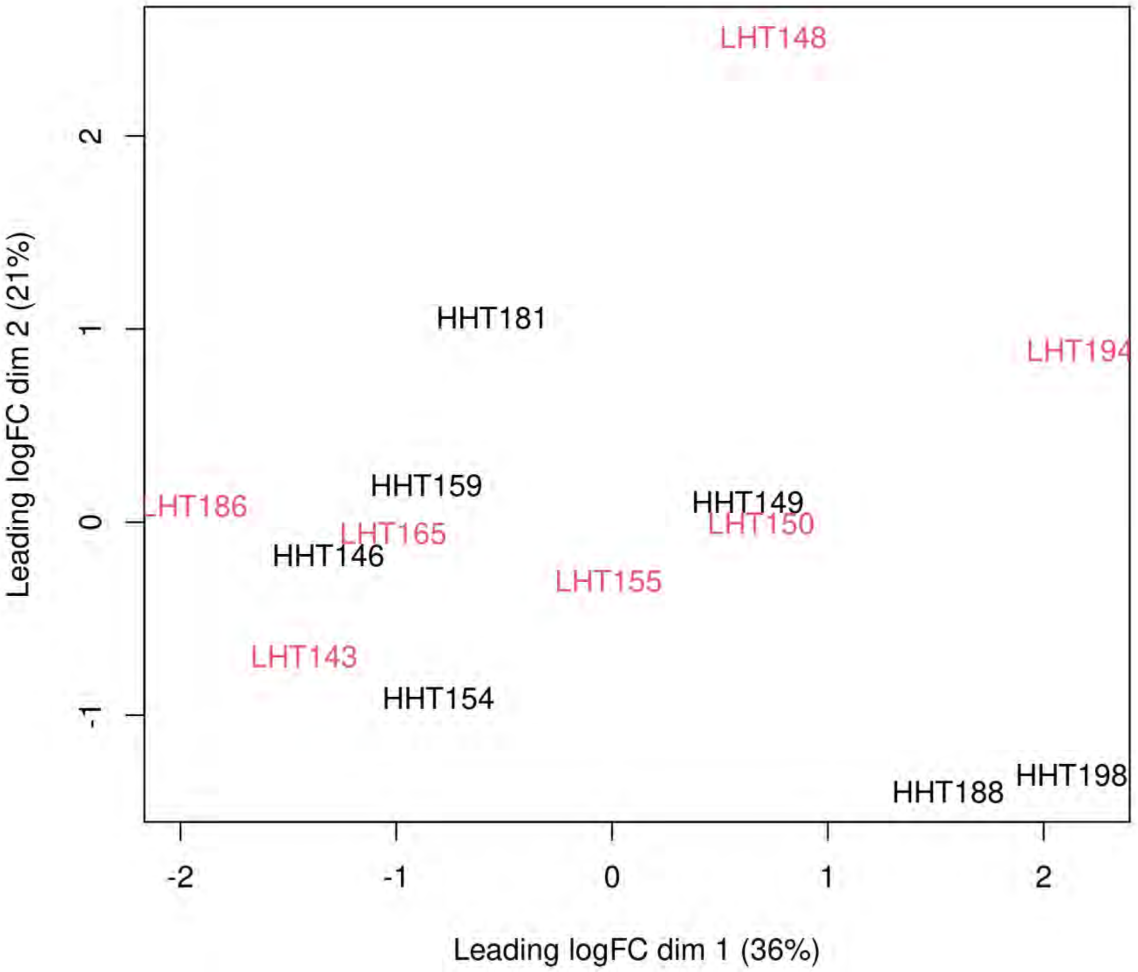
Zhang, W., Dong, Y., 2021. Membrane lipid metabolism, heat shock response and energy costs mediate the interaction between acclimatization and heat-hardening response in the razor clam Sinonovacula constricta. Journal of Experimental Biology 224, jeb243031. https://doi.org/10.1242/jeb.243031.

Zhou, X., Tron, V.A., Li, G., Trotter, M.J., 1998. Heat Shock Transcription Factor-1 Regulates Heat Shock Protein-72 Expression in Human Keratinocytes Exposed to Ultraviolet B Light. The Journal of Investigative Dermatology 3, 194-198.

ATTACHMENT A – Number of reads per sample before and after quality control analysis for sheep skin samples

Samples	Input Read Pairs (million)	Pair Reads after QC (million)	Pair mapped reads after QC (million)	Pair mapped reads after QC (percentage)
143	13885339	13307659	11447908	86.02%
146	13438037	13035136	11321520	86.85%
148	11752185	11467806	6839980	59.65%
149	11945962	11697834	10626529	90.84%
150	10897524	10385676	8460079	81.46%
154	11954216	11447075	9781361	85.45%
155	10594118	10064905	7909237	78.58%
159	11455108	11023329	9456302	85.78%
165	10899364	10479880	8786644	83.84%
181	11306668	10866579	9346649	86.01%
186	11688296	11371042	10037809	88.28%
188	10564597	10149352	8353187	82.30%
194	12274355	11764747	9142578	77.71%
198	10618638	10068141	8223356	81.68%

ATTACHMENT B – Multidimensional scale plot to visualize the clustering among animals



ATTACHMENT C –The differentially expressed genes (FDR <0.20) between High and Low heat tolerance groups are in bold

Gene Symbol	logFC	logCPM	PValue	FDR
LOC114112149	**-7,01268**	**6,215439**	**1,38E-06**	**0,011903**
LOC114112704	**-5,50822**	**8,359267**	**1,49E-06**	**0,011903**
LOC121818850	**-5,03427**	**9,235533**	**9,00E-06**	**0,047964**
LOC114112490	**-5,22442**	**11,48877**	**1,24E-05**	**0,049621**
LOC114112702	**-3,80641**	**3,939383**	**1,85E-05**	**0,053079**
LOC121818849	**-5,21762**	**11,86924**	**1,99E-05**	**0,053079**
CAPN14	**3,678007**	**4,129129**	**3,71E-05**	**0,084688**
LOC121818848	**-4,41965**	**9,975605**	**5,25E-05**	**0,104931**
LOC114108665	**3,957593**	**3,992829**	**5,91E-05**	**0,104944**
HSPA6	**4,149938**	**7,724497**	**7,26E-05**	**0,111541**
HSPA1A	**2,36498**	**8,241096**	**7,67E-05**	**0,111541**
LOC101108781	**-2,81799**	**8,678852**	**9,63E-05**	**0,125813**
LOC101112166	**-1,50208**	**6,127239**	**0,000102**	**0,125813**
LOC101120613	**2,29924**	**1,45648**	**0,000117**	**0,134129**
GNLY	**-3,26188**	**1,40785**	**0,000167**	**0,177703**
PTGS2	2,652206	4,465429	0,000207	0,206772
LOC114112489	-4,16313	12,11477	0,000261	0,245407