Theses of Doctoral (Ph.D.) Dissertation

COPING WITH CLIMATE CHANGE: UNDERSTANDING THE GENETIC MAKEUP FOR ADAPTATION IN SHEEP UNDER DIFFERENT CLIMATE CONDITIONS

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1. INTRODUCTION

Over the last several decades, increasing livestock population and production has primarily meant increasing output to keep up with the world's ever-increasing demand for food. Genetic selection and the introduction of internationally proven breeds with better genetics are our primary strategies to increase production, complemented by the development of optimal management systems to maximize genetic potential. However, in recent years, there has been a shift in the livestock production system's emphasis towards a stress-free system, driven by heightened awareness of animal welfare. Simultaneously, the persistent trajectory of global climate change adds new challenges to animal welfare issues making objectives of livestock production have become more complex.

The direct effects of heat stress (HS), as the main aspect of climate change, on livestock biochemical regulation include changes in feed intake and hormone levels as well as alterations in energy metabolism and utilization and the accumulation of reactive oxygen species. This impairs the reproductive cycle, immunity, growth rate, milk quality and quantity, and production in the long run. Some of the indirect effects of climate change on livestock farming resources include changes in pasture quality and quantity as a result of altered seasonal patterns, disruptions to ecosystems, the rapid spread of parasites and diseases, and reduced water availability.

A potential long-term solution to the problem of livestock adaptation could be to breed and select for animals that are more resistant to the effects of climate change. Thus, understanding the genetic architecture and molecular basis of adaptation traits is crucial for breeding and selecting animals with heat resistance. These traits are regulated in complex mechanism involving extensive gene networks. The objective of this doctoral research was to use polymorphism and transcriptomic study approach to contribute to the comprehension of genetic architecture of HS, an adverse consequence of climate change in sheep *(Ovis aries).*

This doctoral research is conducted in three distinct phases with the following objectives for each phase.

i. To study polymorphism of ovine genes associated with heat resistance traits across various sheep breeds from different climatic condition using single nucleotide polymorphisms (SNPs) markers.
ii. To detect seasonal relative expression levels of heat stress and immunity associated genes in different sheep breeds reared in Hungary’s environment using quantitative real time transcription polymerase chain reaction (qRT-PCR) method;

iii. To investigate the molecular mechanisms underlying thermoregulation in white-coated and black-coated Hortobágyi Racka sheep using RNA sequencing (RNA seq) method.

2. MATERIALS AND METHODS

The study was approved and carried out in accordance with the local ethics committee's guidelines of the University of Debrecen under the registration number 19/2023/DEMÁB with all procedures were conducted in compliance with the European Union's Animal Experimentation Directive (Directive 2010/63/EU) and the ARRIVE guidelines.

2.1. Polymorphism study of heat resistance related genes across various sheep breeds

2.1.1. Sample collection and genomic DNA extraction

Blood samples were taken from 720 sheep belonging to17 breeds adapted to different environmental conditions, originating from 4 countries; Hungary (Suffolk (n=30), Bábolna Tetra (n=36), Ile de France (n=33), Hungarian Indigenous Tsigai (n=41), Hungarian Racka (n=48), Hungarian Merino (n=35), and Hungarian Awassi (n=40)), Bosnia and Herzegovina (Pramenka (n=37)), Morocco (Béni Guil (n=30), D'man (n=30), Timahdite (n=30), and Sardi (n=30)), and Romania (Botosani Karakul (n=58), Romanian Racka (n=62), Transylvanian Merino (n=60), Romanian Tsigai (n=60), and Turcana (n=60)). The majority of the breeds examined are native to their country of origin, with a few exotic types included to assess whether their acclimatization contributed to their adaption. The breed characteristic (hot or cold tolerance) was assessed based on the origin and developmental history of each breed within the respective country, with the environmental circumstances present during sample collection. The FAO/IAEA recommended method for hair follicle extraction and the ZSOLNAI and ORBÁN method (1999) for blood were employed for genomic DNA isolation. The DNA was stored at −20 °C prior to analysis. The DNA concentration was measured using a NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA, USA). All samples were diluted to an uniform concentration, and genotyping was conducted using 50 ng of DNA per sample.

2.1.2. Selection of SNPs

A panel of 51 SNPs representing 30 HS-related genes (*LEP, SOCS3, PPARG, ASIP, CSN1S1, CSN2, ABCG1, ABCG2, IGF1, ESR1, GNRH1, DIO2, BTNL2, BTN1A1, GHR, STAT1, HSP90AB1, HSP90AA1, HSPA8, HSPA12A, HSPA4, IL1R1, IL2, TR, IL33, HSP90AB1, STAT_PIAS3, HCRT, USP19,* and *STAT3*), spread over 18 chromosomes, was chosen based on the results of prior marker-assisted selection and genome-wide association studies (GWAS) in sheep (SINGH et al., 2017; CAVALCANTI et al., 2017; LI et al., 2019b; AL-THUWAINI et al., 2020; YOUNIS et al., 2020). The Ovis SNP data was obtained from

the Single Nucleotide Polymorphism Database (dbSNP), which is managed by either Ensembl or the National Center for Biotechnology (NCBI).

2.1.3. Genotyping and ensuring product quality

With the help of Kompetitive Allele Specific PCR (KASP^{TM}, LGC Genomics, Teddington, Middlesex, UK), the 51 SNPs that were chosen for bi-allelic discrimination were tested. For viewing the results, we utilized SNP Viewer software, version 1.99 (Hoddesdon, UK) and from there, the genotype data was exported for the purpose of statistical analysis. Only SNPs that appeared in at least 50% of the breeds were considered. Only SNPs that present in at least 50% of the breeds were evaluated. Data quality control of genotyped data comprised eliminating animals having a call rate of less than 50% and the SNPs with call rates of less than 50%. This resulted in disparities in either the quantity of animals per breed or the count of SNPs per animal.

2.1.4. Data analysis

The raw allele calls acquired from LGC Genomics were examined utilizing LGC Genomics' KlusterCaller software. Gene diversity, allele and genotype frequencies, and adherence to or deviation from the Hardy–Weinberg equilibrium were assessed using POPGENE software version 1.31 (YEH et al., 1999).

PCA was conducted utilizing the FactoMineR (LÊ et al., 2008) and ggplot2 (WICKHAM, 2016) packages from the R Program (R CORE TEAM, 2020) to illustrate the genetic diversity among sheep breeds categorized by their climatic adaptations which was determined based on breed's origin, breed history/formation and sampling location: cold-tolerant breeds (Babolna Tetra, Hungarian Merino, Hungarian Racka, Hungarian Tsigai, Ile de France, Pramenka, Romanian Racka, Suffolk, and Turcana), heat-tolerant breeds from Morocco (Béni Guil, D'Man, Timahdite, and Sardi), and heat-tolerant breeds raised in Europe (Hungarian Awassi, Botosani Karakul, Transylvanian Merino, and Romanian Tsigai).

2.2. Relative expression levels of heat stress-related genes in sheep in different seasons

2.2.1. Sample collection and sampling location

A total blood sample was initially obtained from 24 animals (12 ewes and 12 rams) representing three distinct breeds: Hungarian Merino, Hungarian Tsigai, and White Dorper. The Hungarian Tsigai has been a part of Hungarian culture since the 1800s. Although Merino breeding began in Hungary in the late 17th century, it was not until the 18th century that

indigenous breeds were crossed with Merino to create what is now known as the Hungarian Merino. The White Dorper is an imported breed from South Africa that is still rather new to Hungary.

All animals involved in the study were of comparable age (2 to 3 years), body weight (ewes: 45 to 55 kg; rams: 65 to 75 kg), and in optimal health, exhibiting no physical or anatomical abnormalities. The animals were kept in the Kismacs Experimental Station of Animal Husbandry at the University of Debrecen, situated 127 meters above sea level (47.58° N and 21.58° E). The typical annual maximum and minimum ambient temperatures range from -7.5 to 28.0°C, with annual precipitation between 550 and 600 mm. The sample was done in four different seasons in 2019-2020: spring (April), summer (August), autumn (November), and winter (January). All animals were subjected to identical management during the experimental period. The breeds were maintained and fed collectively throughout the year, separated by sex within the sheep shed system, which featured an enclosure. During the autumn breeding season, they were maintained in harems. All animals are provided ad libitum with a diet of hay and fodder at a rate of 0.4 kg per sheep per day, along with unrestricted access to clean water. Fodder had 50% corn, 50% oats. During lambing, ewes are provided with an additional 1 kilogram of alfalfa hay. Four weeks prior to insemination and during the insemination process, they were provided with 1 kg of alfalfa silage per sheep each day. Selenium lick blocks were accessible to the animals for a duration of 365 days.

As a result of the long research duration, several animals were excluded from the target population during implementation, so decreasing the total sample size to 15 animals: Hungarian Merino (2 ewes and 1 ram), Hungarian indigenous Tsigai (3 ewes and 3 rams), and White Dorper (2 ewes and 4 rams). During each peak season, about 5 ml blood samples were collected from the jugular vein of the same animals using Tempus™ Blood RNA Tubes (Applied Biosystems) and stored at -70°C until subsequent analysis.

2.2.2. Climatological data

The climatic conditions on the sampling day were documented hourly. The THI was calculated on each sampling day utilizing the equation developed by MADER et al. (2006). The intensity of HS in animals is assessed with the THI, which ranges from 0 (no stress) to >84 (extreme HS), categorized as follows; no stress (≤ 67), mild (68–74), moderate (75–78), severe (79–83) and extreme (≥ 84) (LEWIS BAIDA et al., 2021).

$$THI = (0.8 \times T_{db}) + \left[\left(\frac{RH}{100}\right) \times (T_{db} - 14.4)\right] + 46.4$$

T_{db}- Dry bulb temperature (°C), RH- Relative humidity (%)

2.2.3. Quantification of gene expression levels using qRT-PCR

The total RNA was isolated from 3ml of total blood using Tempus Spin RNA Isolation Kit (Applied Biosystems, USA) following the manufacturer's instructions and treated with DNase (Quiagen, catalog number: 79256).

The quality and quantity of RNA were evaluated using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Three hundred nanograms of total RNA were reverse transcribed into complementary DNA using specific primers with the qPCRBIO cDNA Synthesis Kit (PCR Biosystems, London, United Kingdom). The amount of cDNA equivalent to 5 ng of beginning total RNA was used as template for each real-time PCR experiment. Forward and reverse primers were designed using Primer Express v3.0.1 software (Applied Biosystems, Foster City, CA, USA) and validated for target identity with Primer Blast from NCBI (YE et al., 2012). The Roche Light Cycler 96 Real-Time PCR System was employed for qPCR, consisting of a 3-minute denaturation phase, followed by 50 cycles at 95°C for 15 seconds, 62°C for 20 seconds, and 72°C for 15 seconds. High-resolution melting analysis was conducted for each run (*GAPDH*= 83.0°C, *IL10*= 87.0°C, *TLR2*= 82.5°C, *TLR4*= 80.5°C, *TLR8*= 80.0°C, and *HSP70*= 84.0°C).

Reactions were set up with PowerUpTM SYBR! Green Master Mix (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The amount of cDNA equivalent to 5 ng of starting total RNA was used as template for each real-time PCR reaction.

For relative gene expression studies, one housekeeping gene (*GAPDH*) was amplified alongside the target genes (*IL10, TLR2, TLR4, TLR8,* and *HSP70*). Quantitative PCR was done in triplicate for each sample.

2.2.4. Statistical analysis

Using the Pfaffl method (PFAFFL, 2001), the relative quantification of the target gene was determined by comparing the expression levels of GAPDH as a reference gene and GOI of *HSP70, IL10, TLR2, TLR4,* and *TLR8* with Spring season value as the calibrator. LinReg PCR version 2017.0 software (RAMAKERS et al., 2003) was used to calculate primer efficiency. Data were analyzed with a mixed Analysis of variance (ANOVA) with a general linear model (GLM) with repeated measurement in SPSS Version 25 (IBM Corp., Armonk, NY, USA).

Mauchly's test was used to decide the assumption of sphericity in the repeated measurement, and Levene's test was based on the median for the equality of error variance within season measurement. Further, a post-hoc multiple comparison test was done using the Tukey test. Visualization of the data was done using GraphPad Prism version 8.0.0 for macOS (GraphPad Software, San Diego, CA, USA). The results are shown as the mean ± SD. A difference with $p<0.05$ was determined as statistically significant.

2.3. Transcriptomic study in white-coated and black-coated sheep using RNA-Sequence method

2.3.1. Sample collection

This study used a total of 10 Hungarian Racka ewes; 5 individuals with white-coated and 5 individuals with black-coated. All the sheep involved in the study were of similar age, ranging from 1.5 to 2 years old, and had a body weight between 50 to 60 kg. All animals were raised under identical environmental circumstances and feeding at Hortobágyi Nonprofit Kft. Skin samples were collected from the back of the animal's neck immediately following the slaughtering process, within a maximum of one hour post-mortem and were preserved in RNAlater™ Solution. Sampling was done in summertime (30 May 2023) with the average temperature during the sampling period (09.00-13.00) ranging between 17.1 – 22.5°C. Sample was stored temporarily in ice box with 4 °C during the transportation to the laboratory and subsequently stored in a freezer at -70 °C until RNA isolation.

2.3.2. RNA extraction, library preparation and sequencing

Total RNA was extracted from skin sample with Direct-zolTM-96 MagBead RNA Kit (Zymo Research, Orange, CA, USA) according to the manufacturer's instructions. RNA libraries were generated using the QuantSeq 3' mRNA-Seq Library Prep Kit FWD for Illumina (Lexogen, Vienna, Austria). Briefly, 100 ng of total RNA was used as input for first strand cDNA generation using oligodT primer followed by RNA removing. Thereafter, the second strand synthesis is initiated by random priming and the products were purified with magnetic beads. Finally, the libraries were amplified and barcoded using PCR. Each libraries were assessed on the Agilent 4200 TapeStation (Agilent Technologies, Palo Alto, CA, USA) to examine if adapter dimers formed during PCR. The QuantSeq libraries were sequenced using the Illumina NovaSeq 6000 platform (Illumina, San Diego, CA, USA) with 2 × 151 run configuration.

2.3.3. Bioinformatics analysis

Binary Base Call (BCL) files generated by the sequencing instrument were base called, demultiplexed and translated into FASTQ files using bcl2fastq v2.20.0.422 software (Illumina Inc.). Reads were subsequently trimmed to 1x75 bp (minimum length: 40bp) and quality-trimmed to Q30 using the Phred algorithm with BBDuk from the BBTools suite v38.86 (BUSHNELL et al., 2017).

The processed reads were aligned against the domestic sheep (*Ovis aries*, Rambouillet breed, OAR_USU_Benz2616 strain) reference genome downloaded from NCBI (ARS-UI_Ramb v30 assembly; Jul 20, 2023; RefSeq: GCF_016772045.2) with STAR v2.7.6a (DOBIN et al., 2013). The number of reads aligned within each gene was counted using HTSeq Python library v0.11.1 (ANDERS et al., 2015). Gene count data were normalized using the trimmed mean of M values (TMM) normalization method of the edgeR R/Bioconductor package (v3.28, R v3.6.0, Bioconductor v3.9). Data were further log transformed using the Voom approach for statistical evaluation in the limma package (RITCHIE et al., 2015). Fold change (FC) values between the compared groups and moderated t-test p-values were calculated by the limma package. Normalized counts in exploratory data analysis and visualization were represented as transcripts per million (TPM) values.

2.3.4. Function enrichment analysis

The differentially expressed genes (DEGs) underwent gene ontology (GO) and KEGG pathway enrichment analysis using the DAVID Database (https://david.ncifcrf.gov/tools.jsp; DENNIS et al., 2003) for three primary GO categories: biological process, molecular function, and cellular component. GO keywords or KEGG pathways were considered significant if their corrected P-values (based on false discovery rate, FDR) were below 0.05. The bubble plot for the KEGG pathway enrichment was constructed using the ggplot2 package (WICKHAM, 2016) in R. Protein-protein interaction (PPI) networks for the DEGs were built in STRING Database (https://string-db.org).

2.3.5. Validation of differentially expressed genes

As a result of this study, a total of 10 genes were chosen for further investigation and comparison of gene expression in skin tissue of black coated and white coated Hortobágyi Racka sheep using qRT-PCR. These genes were selected based on their significant differential

expression in the RNA-Seq analysis and has been reported to be significantly associated with immunity, metabolism pathways, and skin pigmentation.

The total RNA sample, including 300 ng was converted into complementary DNA (cDNA) using particular primers which were designed using Primer Express v3.0.1 software (Applied Biosystems, Foster City, CA, USA) and verified for target specificity using Primer Blast from the National Centre for Biotechnology Information (NCBI) (YE et al., 2012). The qPCRBIO cDNA Synthesis Kit (PCR Biosystems in London, United Kingdom) was used for this process. For each real-time PCR experiment, the template consisted of a quantity of cDNA equivalent to 5 ng of the initial total RNA. The Roche Light Cycler96, a Real-Time PCR System, was utilized for qPCR. The process involved a 3-minute denaturation step, followed by 50 cycles of 95 °C for 15 seconds, 62 °C for 20 seconds, and 72 °C for 15 seconds. Each run underwent high resolution melting analysis.

The reactions were prepared using PowerUpTM SYBR! Green Master Mix (Applied Biosystems, Foster City, CA, USA) according to the directions provided by the manufacturer. For each real-time PCR reaction, the template consisted of an amount of cDNA that was comparable to 5 ng of the initial total RNA.

In order to conduct relative gene expression investigations, the amplification of a housekeeping gene, *GAPDH*, was performed in conjunction with the 10 target genes*: IRF4, PMEL, GPNMB, TYRP1, PLXNC1, VNN1, TYR, PLP1, SLC24A5, and TRPM1*. Each sample was subjected to quantitative PCR in triplicate. Changes in gene expression (CT value) were calculated using the $2^{-\Delta\Delta Ct}$ (DDCT) method, the fold changes were analysed with Independent Sample T-Test.

3. RESULTS

3.1. Polymorphism study of heat resistance traits related genes across various sheep breeds

3.1.1. Allele and genotype frequency

In light of the potential increase in global temperatures due to climate change, the genetic basis of thermotolerance adaption in sheep is of major relevance for current doctoral research. The purpose of this phase of the doctoral research was to examine the genetic basis of thermotolerance adaptability of different breeds from different geographical areas.

This study's use of the KASP-PCR assay did not produce a high success rate. Out of a total of 720 animals, 601 (83.47%) were successfully genotyped, 17 of these were identified as *HSPA12A, HSP90AA1, IL33, DIO2, BTNL2, CSN2, ABCG1, CSN1S1, GHR, HSPA8, STAT3*, and *HCRT*. Allelic and genotypic frequencies are presented in Appendix Table 1, were different from one population to another.

Four SNPs were successfully genotyped in all breeds: rs161504783-*HSP12A*, rs588145625-*HSPA8*, rs588498137-*STAT3* and rs602521720-*HCRT*. However, rs588498137-*STAT3* and rs602521720-*HCRT* did not show any trends in genotypic or allelic frequencies, thus the attention was focused on *HSPA12A* and *HSPA8*, two SNPs that showed patterns of allelic and genotypic frequencies in relation to climatic characteristic.

The heterozygote *TC* for rs161504783-*HSPA12A* was dominant, except for Hungarian Racka, Transylvanian Merino, Hungarian Merino, Botosani Karakul and Sardi. The *T* and *C* allele frequencies were almost equally frequent in most breeds, except in Sardi (*T* allele = 0.107 and *C* alleles= 0.828) and Botosani Karakul (*T* allele = 0.735 and *C* alleles= 0.265).

The heterozygote *GA* for SNP rs588145625-*HSPA8* was absent in the heat tolerance breeds, except the Transylvanian Merino, and in breeds with high cold tolerance (Hungarian Racka, Babolna Tetra, Hungarian Tsigai, Romanian Racka, Pramenka and Turcana). The homozygote *GG* was present in all heat-tolerant breeds and some cold-tolerant breeds (Suffolk, Ile de France, and Hungarian Merino). The *G* allele was dominant in all breeds, ranging from 0.760 to 1, and the *A* allele has only appeared in cold-tolerant breeds and Transylvanian Merino with frequency ranging from 0 to 0.308.

The *GG* genotype and *G* allele were dominant in all breeds for SNP rs588498137-*STAT3*, with *G* allelic frequency varying from 0.750 to 1. Similarly, the *CC* genotype and *C* allele for SNP rs602521720-*HCRT* was dominant in all breeds, with *C* allelic frequency varying from 0.546 to 1.

The HWE was investigated with Chi-square (x^2) test using allelic frequencies, observed and expected genotypes, and P-value of the polymorphic genes are summarized in Appendix Table 1. The breed with the highest number of SNPs that deviated from HWE (P≤0.05) was determined to be the Romanian Botosani Karakul; rs416259751-*IL33*, rs411181557-*DIO2*, rs414917134-*BTNL2* and rs420611298-*ABCG1*.

3.1.2. Genetic diversity and interrelationship between SNPs

PCA was performed using SNPs data to show how the breeds (Figure 1a) and climatic characteristics (Figure 1b) differed. Both Figure 10a and 10b show that of the overall variation in the 17 breeds, 11.21% is accounted for by the PC1, and 9.98% by the PC2. Even after we separated the heat-tolerant breeds raised in the European Union and Morocco, neither PC1 nor PC2 could distinguish between the breeds or the climate regions. All breeds were mostly concentrated in -2.50<PC1<1.12; cold tolerant breeds were outspread in PC1 score of -5.593 to -5.013 and PC2 score of -4.149 to 4.020, heat-tolerant breeds were outspread in PC1 score of -0.452 to 0.460 and PC2 score of -0.157 to 1.183, while heat tolerant breeds reared in Europe had PC1 and PC2 ranging from -0.881 to 0.686 and -3.987 to 2.931, respectively.

This could be because hot-tolerant breeds kept in the EU have adapted to the subtropical climate, and because genetic admixture has happened due to reproductive technologies.

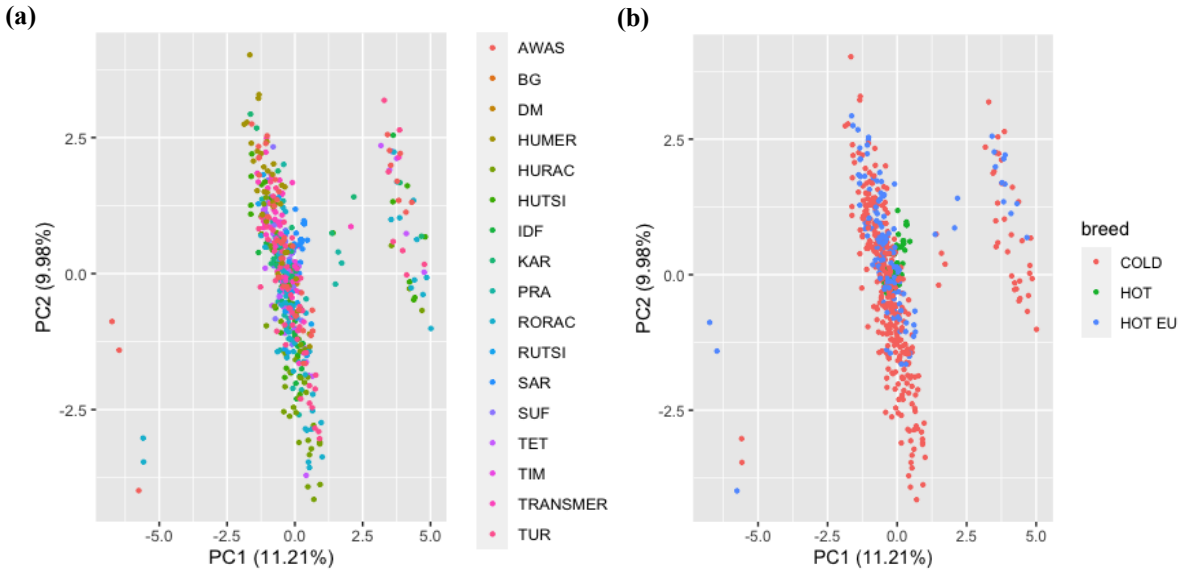

Figure1. Score biplot of Principal Component Analysis of the 17 SNPs and 601 animals.

(a) Individuals of different breeds are differently colored; (AWA: Hungarian Awassi, BG: Béni Guil, BTET: Babolna Tetra, DM: D'Man, HUME: Hungarian Merino, HUTS: Hungarian Tsigai, IDF: I de France, KAR: Botosani Karakul, PRA: Pramenka, ROME: Transylvanian Merino, RORA: Romanian Racka, ROTSI: Romanian Tsigai, SAR: Sardi, SUF: Suffolk, TIM: Timahdite and TUR: Turcana). (b) Breeds grouped by climatic characteristics; COLD: cold-tolerant breeds (Suffolk, Babolna Tetra, Ile de France, Hungarian Tsigai, Hungarian Racka, Hungarian Merino, Pramenka, Romanian Racka, and Turcana), HOT: heat tolerant breeds

originated from Morocco (Béni Guil, D'Man, Timahdite and Sardi), HOT EU: Heat tolerant breeds reared in Europe (Hungarian Awassi, Botosani Karakul, Transylvanian Merino, and Romanian Tsigai).

The highest contributions to the principal component were by rs397514117-*HSP90AA1* (c) and rs397514272-*HSP90AA1* (e), whereas rs410259751-*IL33* (g) also had a positive contribution (Figure 2).

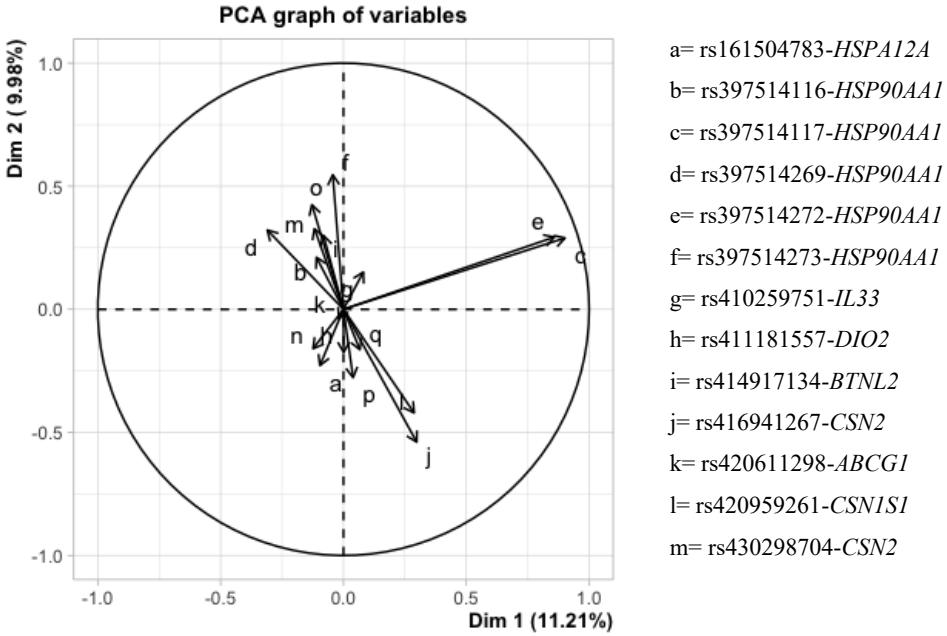

Figure 2. Loading biplot of principal component analysis of the 17 SNPs for 17 sheep breeds.

3.2. Relative expression levels of heat stress-related genes in sheep in different seasons

3.2.1. Climatological conditions

The THI in all sampling days were within the thermoneutral zone, except for summer season, which was 78.99, which was nearly severe heat stress condition. Sheep typically have an upper critical temperature ranging from 25 to 31 °C; however, this can vary greatly by breed, age, and physiological condition.

3.2.1. Relative gene expression

The relative expression was calculated using the Pfaffl method (PFAFFL, 2001) and is visualized as Figure 3 (A-E). The spring season was considered as a thermo-neutral season and used as a calibrator. In all gene of interest (GOI) in this study, the lowest expression relative to the spring season was found in the autumn.

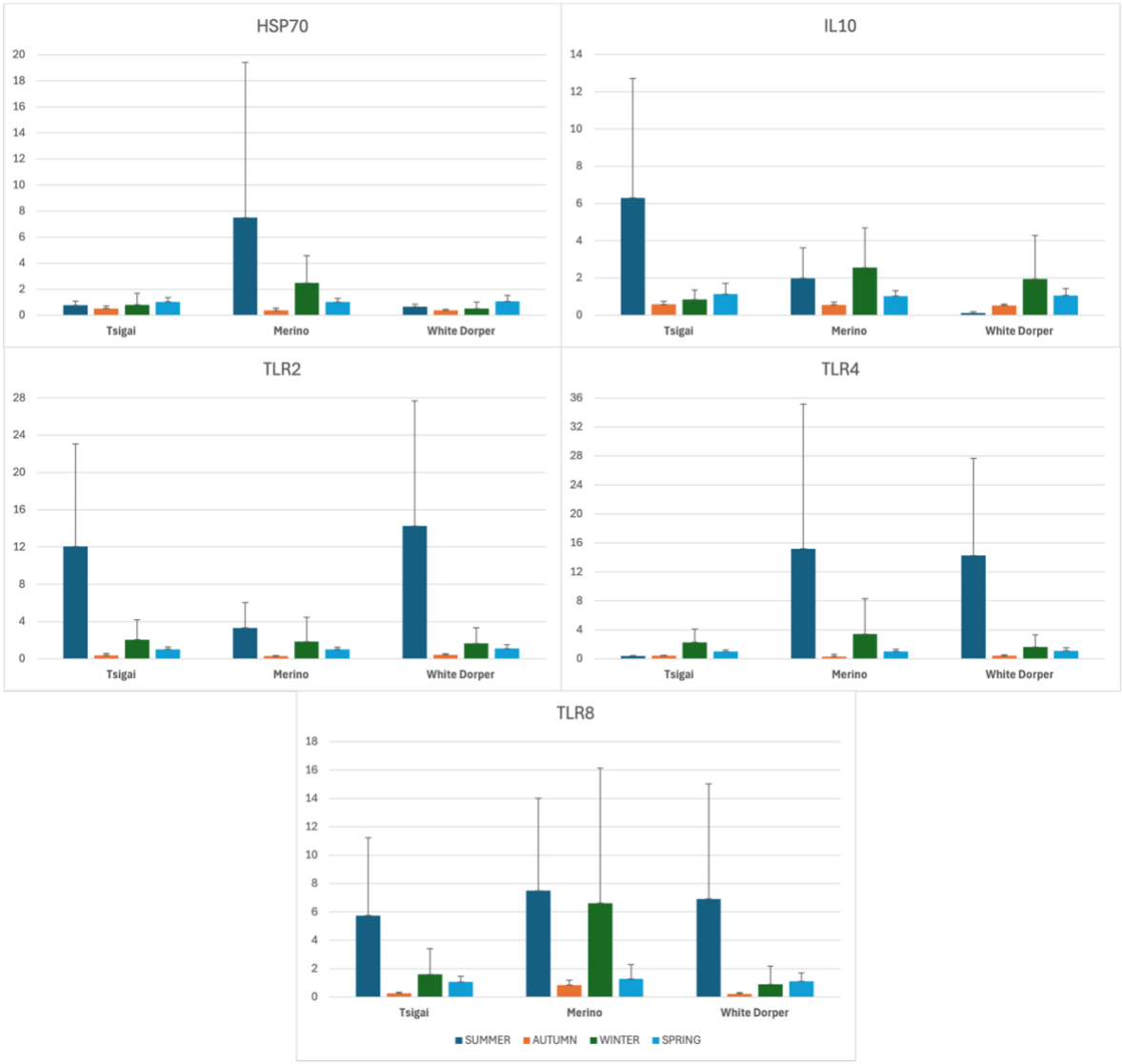

Figure 3. Bar graph of relative gene expression of (A) *HSP70*, (B) *IL10*, (C) *TLR2*, (D) *TLR4*, and (E) *TLR8* in each season of the year with the spring season as the calibrator.

The X-axis is the relative gene expression in different season of the year. The Y-axis is gene expression for each different breeds in the study. Different colours represent different breed; red for Hungarian Tsigai, blue for Hungarian Merino, and yellow for White Dorper.

Increased HSP concentrations are consistently linked to thermal acclimation and adaptation in small ruminants. Although HSPs have been at the center of cellular responses in sheep, the process by which they tolerate heat stress is complicated and yet not completely understood. In this study, for *HSP70*, relative gene expression with different peak was observed; spring for Hungarian Tsigai (1.039±0.326) and White Dorper (1.078±0.441), summer (7.494±11.932) for Hungarian Merino.

In the summer, the relative gene expression of HSP70 was elevated, suggesting that heat stress induced *HSP70* expression. This was observed in the cold tolerant Hungarian Merino

breed, which is highly susceptible to heat stress This study found that there was only a slight seasonal variation in the relative gene expression of *HSP70* between the Hungarian indigenous Tsigai and the White Dorper.

The *IL10* gene showed an increase in expression during the summer for Hungarian Tsigai (6.299±6.412), but for Hungarian Merino and White Dorper, the highest expression was identified in the winter season, accounted for 2.553±2.128 and 1.935±2.336, respectively.

The Hungarian Merino in this study, showed remarkable resilience to heat stress, particularly in terms of their capacity to sustain the innate immune response

This study found that relative gene expression of *TLR2* and *TLR8* is lowest in the spring and fall and highest in the winter and summer. All breeds showed an increase in expression of both genes over the summer, with the mean relative gene expression value of 12.053±11.018, 3.317±2.720, and 14.263±13.417 for *TLR2* in Hungarian Tsigai, Hungarian Merino, and White Dorper, respectively. While for *TLR8* were 5.747±5.481, 7.497±6.507, and 6.910±8.130, respectively.

Besides that, for *TLR4*, the highest expression was observed in the summer for Hungarian Merino (15.204±19.950) and White Dorper (14.263±13.417), but not for Hungarian Tsigai, which was observed in the winter (2.283±1.817). But still, a lower expression was observed during the thermoneutral seasons, spring and autumn. The Hungarian indigenous Tsigai was great at maintaining its immunological response in spite of hyperthermic conditions; this was demonstrated by the fact that this breed has lower *TLR4* expression than other breeds and that this expression was downregulated during the summer.

The gene expression of all GOI in this study were significantly different ($p<0.05$) in each season, showing the dynamic gene expression according to seasonal variation. The interaction between the season, breed, and sex were identified in *IL10* gene expression ($p<0.05$), indicating the changes in gene expression in each season were not equivalent in each breed and sex group. According to the between-group test, it was found that there were significant differences in gene expression ($p< 0.05$) in each breed group across the season for *IL10* and *TLR4* genes. Seasonal dynamics showed that Hungarian Merino sheep experienced heat stress in the summer (as shown by their relative expression of *HSP70*), but they kept up an excellent immunological response all year round (as evidenced by their consistently high levels of *IL10* and *TLR2* gene expression). Immigrant White Dorper was only moderately suited to the Hungarian climate, in comparison to the Hungarian indigenous Tsigai, which demonstrated extraordinary resilience to its natural environment and maintains its resistance to seasonal stresses. Given the risks

presented by both present and future climate change, our study on heat resistance and immune genes proved that these three breeds are most adapted to the Hungarian climate.

3.3. Transcriptomic study in white-coated and black-coated Hortobágyi Racka sheep using RNA-sequence method

3.3.1. Aligned RNA seq data

This work aims to examine the skin transcriptome generated by RNA-seq analysis of black-coated and white-coated Hortobágyi Racka sheep (*Ovis aries strepsiceros hungaricus*). The breed has two predominant coat color phenotypes: completely white and completely black, rendering it very appropriates for the investigation of coat pigmentation.

A total of 5,525,285 (approximately 613,921 reads per sample) reads were generated from the Illumina NovaSeq 6000 platform sequencer. The reads were successfully annotated to 21,328 genes from *Ovies aries* genome (ARS-UI_Ramb v30). The mapping percentages varied between samples.

3.3.2. Differentially expressed genes between black coated and white coated Hortobágyi Racka Sheep

Based on the specifications of FC>1.5 and p-value<0.05, a total of 108 genes demonstrated differential expression of black coated in comparison to white coated sheep (Figure 4). Among these, 25 genes were identified as downregulated, whereas 83 genes were classified as upregulated (Figure 5).

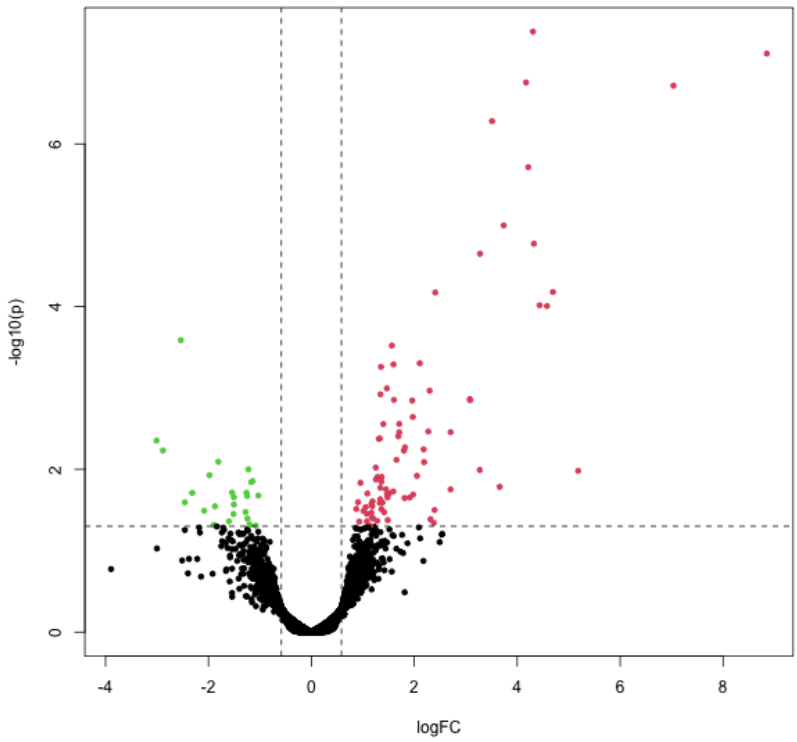

Figure 4. A volcano plot displaying the relationship between log fold change (FC) and log counts per million (CPM).

The green dots and left from dashed lines represent genes that were significantly downregulated, while the red dots above and right from dashed lines represent genes that were significantly upregulated in the comparison of the skin of black and white Hortobágyi Racka sheep. The parameters set are FC>1.5 and P-value<0.05.

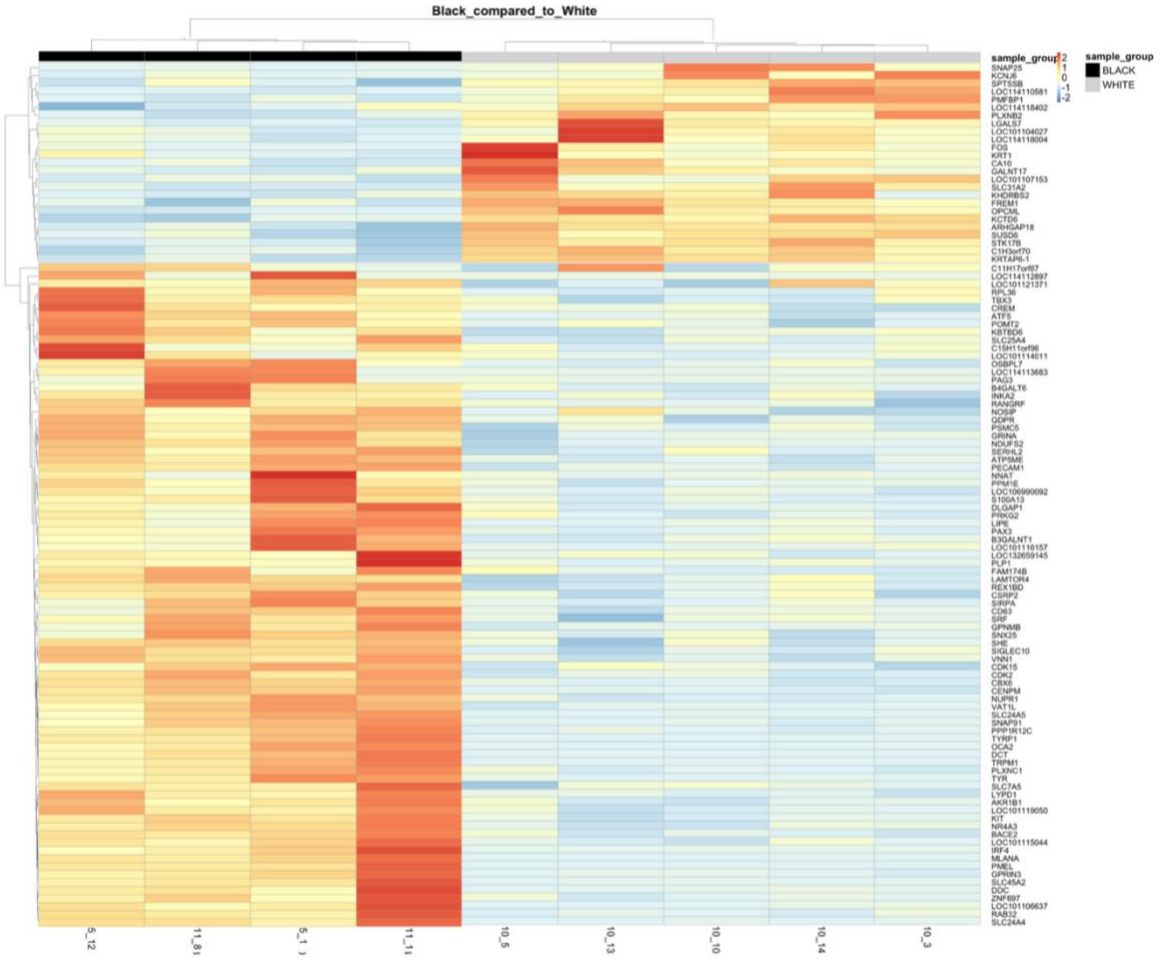

Figure 5. The heat map of the total read per million (TPM) value of 108 differentially expressed genes between the black (n=4) and white (n=5) coated Hortobágyi Racka sheep's skin.

Genes with increased expression are shown in red, while those with decreased expression are shown in blue. The color intensity shows the expression degree.

The result indicated that the quantity of upregulated genes (n= 83) exceeds that of downregulated genes (n= 25) when comparing black-coated to white-coated Hortobágyi Racka sheep. The black coat transcriptome was characterized by the overexpression of genes related to pigmentation and melanogenesis, with log fold changes ranging from 3.28 to 8.85 and an adjusted p-value of less than 0.05.

The most significantly downregulated gene was keratin associated protein 6-1 (*KRTAP6-1*) (adj.P.Val= 0.192), while the most significantly upregulated genes (adj.P.Val<0.05) included tyrosinase related protein 1 (*TYRP1*), premelanosome protein (*PMEL*), transient receptor potential cation channel subfamily M member 1 (*TRPM1*), melan-A (*MLANA*), solute carrier family 24 member 5 (*SLC24A5*), solute carrier family 24 member 4 (*SLC24A4*), solute

carrier family 45 member 2 (*SLC45A2*), tyrosinase (*TYR*), interferon regulatory factor 4 (*IRF4*), dopachrome tautomerase (*DCT*), OCA2 melanosomal transmembrane protein (*OCA2*), and dopa decarboxylase (*DDC*).

3.3.3. Enrichment and pathway analysis

The 108 DEGs between black coated and white coated Hortobágyi Racka sheep were highly enriched in 28 functional annotations, while the 83 upregulated DEGs were significantly enriched in 18 functional annotations, and the 25 downregulated DEGs were significantly enriched in 2 functional annotations. All are categorized as either CC, BP, or MF.

The combination of upregulated and downregulated DEGs yields 18 significant functional annotations (P.Val<0.05) from the DAVID website. Upon independent analysis of the upregulated (n=83) and downregulated (n=25) genes, 16 significant functional annotations (P.Val<0.05) were identified. Result of the combined analysis omitted GO:0031424~keratinization (GO BP), while the separate up/downregulated DEGs analysis excluded GO:0016310~phosphorylation (GO BP), GO:0031902~late endosome membrane (GO CC), and GO:0032993~protein-DNA complex (GO CC).

Similar differences were also seen in KEGG pathways analysis; the pathway analysis indicated six highly enriched pathways when all DEGs were evaluated, including oxidative phosphorylation, tyrosine metabolism, metabolic pathways, thermogenesis, melanogenesis, and Alzheimer's disease. Simultaneously, the separation of upregulated DEGs results in the absence of the oxidative phosphorylation and Alzheimer disease pathways, while the downregulated DEGs did not shows any significant enriched pathways (Figure 6).

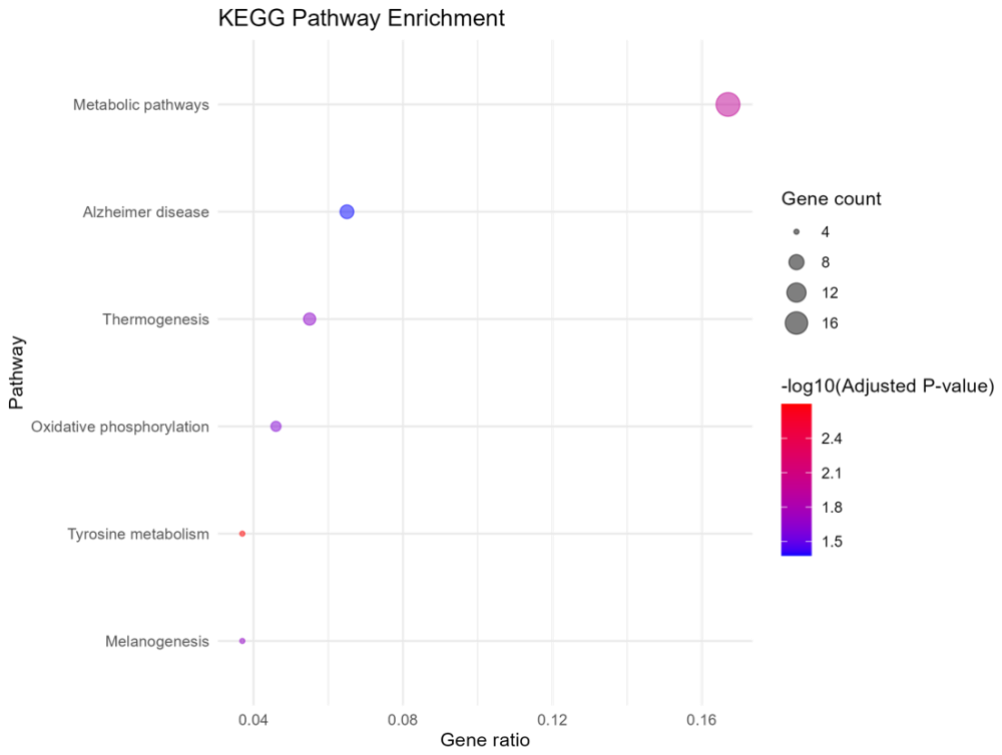

Figure 6. The bubble plot for the KEGG pathway enrichment from the 108 DEGs between black coated and white coated Hortobágyi Racka sheep

Additionally, the STRING database was used to visualize the protein-protein interactions (PPI) of the up-regulated DEGs in the black coated Hortobágyi Racka sheep. Figure 7 shows the interaction network of pigmentation and melanin synthesis (*IRF4, PAX3, SLC24A4, DCT, KIT, TRPM1, MLANA, OCA2, SLC24A5, SLC45A2, TYRP1*, and *TYR*) and immune response and inflammation (*PECAM1* and *GPNMB*) ribosomal proteins.

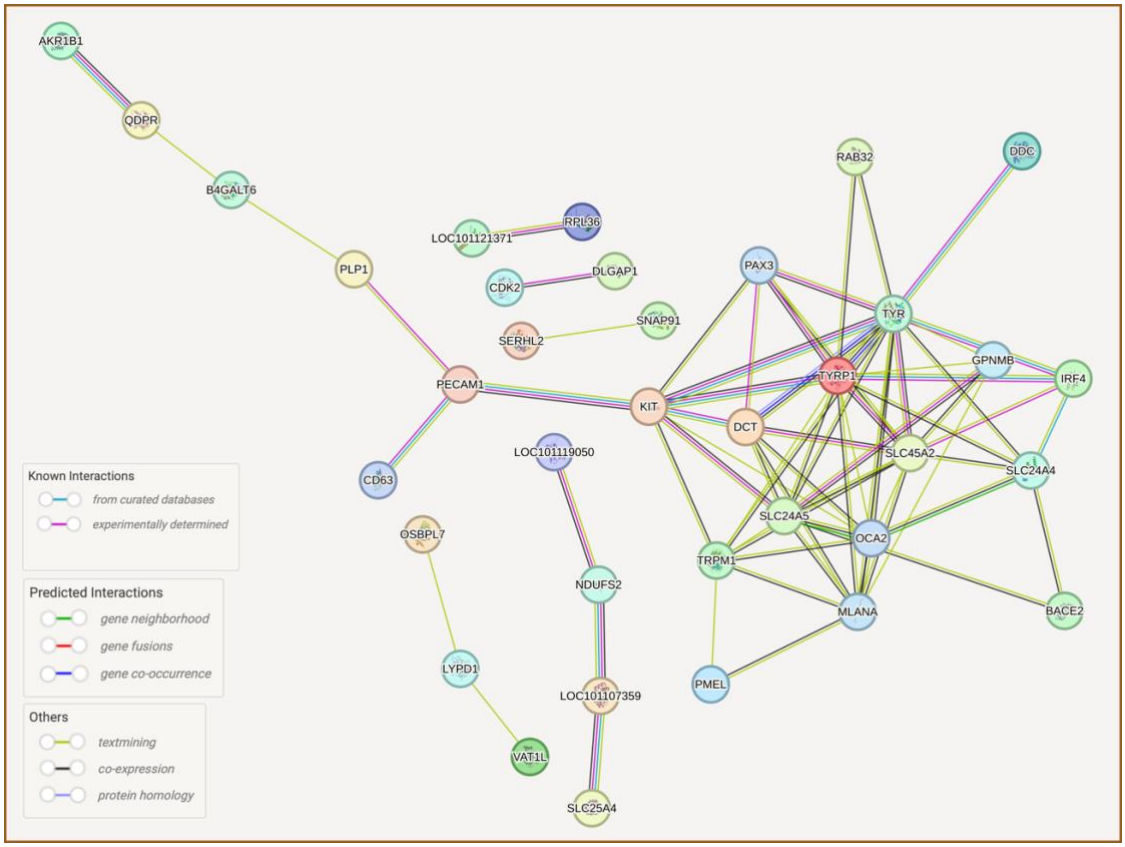

Figure 7. Database search utilizing the STRING database for gene interaction networks using upregulated DEGs.

Each gene is represented as a node, and the connections between them are represented by edges. To improve the display, we eliminate the nodes that are not part of the network. The sort of interaction is indicated by the color of the edge.

3.3.4. RNA-seq data validation with RT-qPCR

RT-qPCR was employed to quantify the expression of 10 randomly chosen from DEG list in the skin of black and white Hortobágyi Racka sheep in order to verify the RNA-Seq result. Known genes associated with immunity (*IRF4, PLXCN1, GPNMB*), metabolic pathways (*TRPM1, VNN1, PLP1*), and ruminant skin pigmentation *(TYRP1, SLC24A5, PMEL, TYR*) were among those found to be 108 differently expressed in white and black sheep skin according to transcriptome sequencing result. In line with the transcriptome sequencing data, 8 out of 10 chosen genes exhibited significantly increased expression in black sheep skin compared to white sheep skin, according to real-time PCR results. When comparing the expression of genes in sheep with black and white skin, *TYRP1* displayed the most differential expression (Figure 8).

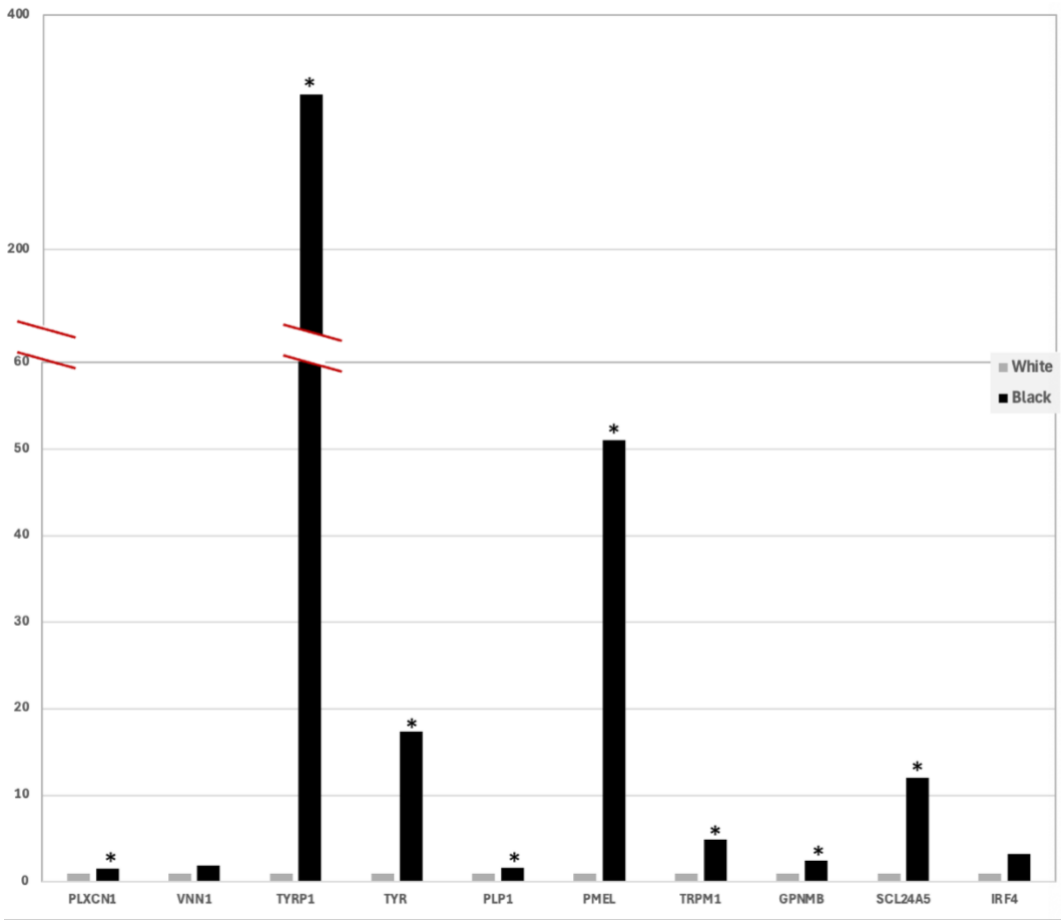

Figure 8. RT-qPCR validation of 10 differentially expressed genes in white and black coated Hortobágyi Racka sheep skin.

The expression level of target genes was normalized relative to that of the of GAPDH *gene. The bars in each panel represent the mean and *P < 0.05.*

4. CONCLUSION

Investigating polymorphisms in various sheep breeds is made feasible by the KASP-PCR technology as shown in the first part of the research. Furthermore, *HSPA12A* and *HSPA8* SNPs are prospective markers for thermotolerance adaption in sheep based on allele and genotype frequency. Principal component analysis indicated that *HSP90AA1* and *IL33* SNPs are the key probable candidates. Insights into the genetic diversity of SNPs involved in thermotolerance adaption in sheep are enhanced by these findings.

On the second research, the three sheep breeds that were analyzed show seasonal variation in the relative expression of heat stress and immunity genes, including *HSP70, IL10*, *TLR2, TLR4,* and *TLR8*. This suggests that seasonal stressors impact the thermo-balance and immunity of the sheep. Hungarian Merino has lower thermotolerance than Hungarian indigenous Tsigai and exotic White Dorper breeds. However, the Hungarian indigenous Tsigai and, secondarily, the Hungarian Merino appear to have advantages over the White Dorper in terms of strength to maintain immunity under heat stress conditions.

The third research validated the association between black coat pigmentation in Hortobágyi Racka sheep and the genes *TYRP1, PMEL, TRPM1, MLANA, SLC24A5, SLC24A4, SLC45A2, TYR, IRF4, DCT, OCA2*, and *DDC*. Furthermore, in addition to its potential influence on wool characteristics, our findings suggest a broader role for *KRTAP6-1*. The roles of these genes in adaptation occur through pleiotropic effects. It seems that animals with darker coats possess more effective mechanisms for managing high heat absorption and reducing adverse effects, whereas animals with lighter coats tend to absorb less heat by default. Their black coat may either assist in thermoregulation during hot weather or serve as an adaptive response to heat through their dark skin coat. The question of which came first remains a causality dilemma.

5. NEW SCIENTIFIC RESULTS

1. rs161504783-*HSPA12A,* rs588145625-*HSPA8,* rs397514117-*HSP90AA1,* rs397514272-*HSP90AA1*, and rs410259751-*IL33* are identified as potential candidate SNP markers for thermotolerance adaptation in sheep.
2. Sheep's thermoregulation and immunity are affected by seasonal stressors, and can be observed through the seasonal expression dynamics of *HSP70, IL10, TLR2*, *TLR4*, and *TLR8* genes. Among the three breeds studied, the Hungarian indigenous Tsigai has exceptional thermotolerance and immunity that are suited to the continental climate of Hungary.
3. This study also successfully identified 108 differentially expressed genes by comparing the skin of black- and white-coated Hortobágyi Racka sheep using the RNA-seq method. Additionally, the genes *TYRP1, PMEL, TRPM1, MLANA, SLC24A5, SLC24A4, SLC45A2, TYR, IRF4, DCT, OCA2,* and *DDC* are associated with black coat pigmentation in Hortobágyi Racka sheep. The *KRTAP6-1* gene was found to be a promising factor in sheep immunity beyond merely influencing wool properties. The pigmentation-related genes in sheep may also influence the animal's adaptability to heat stress, albeit through a pleiotropic mechanism. Also, black-coated and white-coated might have a different heat stress adaptation mechanism.

6. PRACTICAL RESULTS

1. These research on genetic diversity demonstrated that our sheep population still have a robust genetic pool that can serve as a foundation, offering potential for ongoing adaptive improvement to ensure climate-resilience livestock production. Especially the indigenous sheep breeds potentials despite the increasing popularity of international breeds. By maintaining and improving these breeds, we can secure a more sustainable and resilient livestock sector that advantages both local populations and global biodiversity.
2. The SNP markers identified in this work (rs161504783-*HSPA12A,* rs588145625-*HSPA8,* rs397514117-*HSP90AA1,* rs397514272-*HSP90AA1*, rs410259751-*IL33*) can be implemented into breeding strategies that improve heat and disease tolerance in sheep.
3. Examining how homeothermic sheep are influenced by seasonal stressors and how their morphological features (e.g., coat color) significantly impact their responses necessitates the integration of improved management practices in both housing and breeding to mitigate the adverse effects of climate change. Variations in transcript levels of pigmentation-related genes across animals of different coat colors can elucidate the molecular mechanisms governing pigmentation. It can assist in identifying markers for selective breeding related to coat color, adaptability, immunity, and the promotion of animal welfare in the context of heat stress challenges.

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8. PUBLICATIONS IN THE FIELD OF RESEARCH

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Subject: PhD Publication List

Candidate: Putri Kusuma Astuti
Doctoral School: Doctoral School of Animal Husbandry
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List of publications related to the dissertation

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