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Hungarian indigenous Tsigai, a promising breed for excellent heat tolerance and immunity

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ABSTRACT

The adverse effects of climate change on sheep farming have become more noticeable in recent decades. Extensive efforts have been made to untangle the complex relationship between heat tolerance, animal health, and productivity, also to identify a resilient and economically suitable breed for selection that can be resilient to future climate change conditions. Using quantitative real-time polymerase chain reaction (qRT-PCR), we observed the seasonal variations in the expression of several important genes related to heat stress and immunity (*HSP70, IL10, TLR2, TLR4,* and *TLR8*) in three of the most widely kept sheep breeds in Hungary: The indigenous Tsigai, Hungarian Merino, and White Dorper. We found that the seasonal stressor affected the relative gene expression of all genes in this study. Notably, The Hungarian indigenous Tsigai was the most robust breed adapted to the Hungarian continental (hot summer, cold winter) environment, with excellent thermotolerance and immunity. Furthermore, despite suffering from heat stress in the summer, Hungarian Merino maintained their robust immune system well throughout the year.

1. Introduction

Sheep (*Ovis aries*) have been thought to be one of the most robust livestock that has inhabited a wide range of geographical areas since their domestication due to their exceptional adaptability to various types of diets and tolerance to adverse climatic conditions (Kijas et al., 2009; Sawyer and Narayan, 2019). However, in these constantly changing climate conditions, sheep are facing additional stressors (Aboul Naga et al., 2021; Mehaba et al., 2021). These challenges have, significant economic consequences as well. Farmers, of the one of the major sheep exporters, New Zealand, for example, have been facing impacts of climate change for more than a decade, which unfavourably determine the profitability of their production (Moris et al., 2009). Heat stress seriously endangers reproduction of certain sheep breeds, and thus appears as a notably risk for efficient sheep management (Van Wettere et al., 2021).

Many aspects of cellular behaviour must change in response to environmental stressors, one of them is the changes in gene expression, which are a fundamental component of stress reactions (de Nadal et al., 2011), as are changes in metabolism, cell cycle progression, protein homeostasis, cytoskeletal architecture, vesicular trafficking, and enzymatic activity modification, which requires more energy expenditure and may compensate animals' growth potential and productive capacity (Richter et al., 2010). Excessive heat exposure triggers multiple cellular responses and induces transcription and translation of several genes and modifications in protein synthesis, such as increased expression of heat shock proteins (*HSPs*), which function as intracellular chaperones in heat-stressed animals, preventing protein and cell damage (Hooper et al., 2018).

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The expression of *HSPs* is a possible sign of animal response to intense environmental stress. On the basis of their molecular size and similarity in amino acid sequence, *HSPs* are divided into multiple families (Morage, 2006). Genetic and biochemical research indicates that the 70-kDa heat shock protein (*HSP70*) family assists in the folding of proteins during translation in the cytoplasm of both prokaryotic and eukaryotic cells and is primarily found in cytosol and nucleus. There are constitutively expressed and inducible versions of *HSP70* that are activated by stress stimuli (Hansen, 2004). Its expression is a possible indicator of an animal's capacity to adjust to intense environmental stress; a strong association exists between the development of *HSPs* and the induction of thermotolerance through the inhibition of stress kinase activity (Dangi et al., 2014).

Despite the disruption in HSP cellular mechanism, the alteration in immune-related gene regulation to either compromise or enhance immune cell activity is also induced during heat shock (Rashmol et al., 2019). Interleukin-10 (*IL10*) is a major inflammatory regulator released by macrophages, regulatory T cells, dendritic cells and certain epithelial cells (Mallikarjunappa et al., 2020). In sheep, *IL10* has been linked to susceptibility to various diseases, such as Peste des petits ruminants virus (PPRV) (Wani et al., 2018), bluetongue virus (BTV) (Sánchez-Vizcaíno et al., 2015), and *Haemonchus contortus* infection (Glass, 2012). Another one is the Toll-like receptors (*TLRs*), an extensively studied immune-related gene expressed on numerous cell types, including mucosal surface cells and tissue immune cells (Cruz-Tamayo et al., 2021). TLRs are a collection of evolutionarily conserved pattern-recognition receptors (PRRs) that can detect a variety of pathogen-associated molecular patterns (PAMPs) from a wide range of microorganisms and activate innate immune responses (Janeway and Medzhitov, 2002) and are found to be essential for host resistance to gastrointestinal parasitic infection (Benavides et al., 2016) and classical Scrapie in sheep (García-Martínez et al., 2022).

With the threat of unavoidable climate change and its harmful effects, effort are constantly made to thoroughly comprehend how environmental conditions generated some coping mechanisms in the animal's body. Many studeis has reported the seasonal effect on heat-stress related gene expression in livestock (Singh et al., 2014; Archana et al., 2018; Kumar et al., 2018). Given the scarcity of research on studies of seasonal gene expression in sheep, this study aims to describe the seasonal changes of relative expression level of the following heat stress and heat stress-related genes; *HSP70, IL10, TLR2, TLR4*, and *TLR8* in three sheep breeds reared in Hungary today (Hungarian indigenous Tsigai – indigenous breed in Central-Eastern-Southern Europe; Merino – worldwide spread sheep breed, its dispersal started in the 18th century in Hungary; White Dorper – tropics origin, was introduced in Hungary 10 years ago), with the goal of observing the adaptability of the three breeds to Hungarian temperate climatic conditions, also to contribute with valuable knowledge on biological response to seasonal stressors in sheep.

2. Materials and methods

2.1. Sample collection and location

Total blood sample was initially collected from 24 animals (12 ewes and 12 rams) from three different breeds; Hungarian Merino, Hungarian indigenous Tsigai, and White Dorper, four rams and four ewes per breed. All three breeds represent the major breeds reared in Hungary. Hungarian indigenous Tsigai is a natural breed, belonging to the Tsigai group, indigenous to the Eastern and Central Europe. The Hungarian Merino is the result of Merino breeding in Hungary beginning in the late 17th century and continuing into the 18th century, when it began to be crossed with local breeds. White Dorper is a relatively new breed in Hungary imported from South Africa. All animals included in the study were relatively the same age (2 to 3 years old), body weight (ewes: 45 to 55 kg; rams: 65 to 75), and in excellent health condition with no physical and anatomical abnormalities. The animals were kept in the Kismacs Experimental Station of Animal Husbandry of the University of Debrecen, located at 127 m above sea level (47.58° N and 21.58° E). The average annual maximum and minimum ambient temperature ranges from −7.5 to 28.0 °C with annual precipitation of 550 to 600 mm. The sampling was done in four different seasons in 2019–2020; spring (April), summer (August), autumn (November), and winter (January). All animals received the same management during the experiment period. The breeds were kept and fed together throughout the year, segregated by sex in sheep shed technology, which included an enclosure. The exception was the autumn breeding season, when they were kept in harems. All animals are feed ad libitum a diet which consisted of hay + fodder 0.4 kg/sheep/day, with free access to clean water. Fodder contained 50% corn, 50% oats. During lambing, ewes receive an extra 1 kg of alfalfa hay. 4 weeks before insemination and during insemination, they were additionally given alfalfa silage 1 kg/sheep/day. Selenium lick blocks were available to the animals for 365 days.

Due to the lengthy research period, several animals had to be culled (dead or removed from the farm) from the target individuals during implementation. In spring, all 24 samples were collected, in summer 21 animals (1 Hungarian Indigenous Tsigai ewe, 1 Hungarian Indigenous Tsigai ram, and 1 Merino ram died), in autumn 17 animals (2 Merino ewes died and 2 White Dorper were removed from the farm), and in winter 15 animals (1 Merino ram died and 1 Merino ram was removed from the farm). At the end of the research period, 15 animals had completed four-season sampling; Hungarian Merino (2 ewes and 1 ram), Hungarian indigenous Tsigai (3 ewes and 3 rams), and White Dorper (2 ewes and 4 rams). In every peak season of the year, about 5 ml blood sample was obtained from the jugular vein of the same animals in Tempus™ Blood RNA Tube (Applied Biosystem) and preserved at −70 °C until further analysis.

2.2. Climatological data

The climatic conditions during the sampling day were recorded at every hour. The THI was calculated for each sampling day using the following equation by (Mader et al., 2006). The severity of heat stress in livestock is typically rated using the THI, which ranges from 0 (no stress) to > 84 (extreme heat stress), 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 (%).

2.3. Quantification of gene expression levels using qRT-PCR

The total RNA was isolated from 3 ml 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).

RNA quality and quantity assessment were done using a NanoDrop ND-1000 Spectrophotometer, (Thermo Fisher Scientific, Waltham, MA, USA). 300 ng total RNA was reverse transcribed into cDNA with specific primers (Table 1) by the qPCRBIO cDNA Synthesis Kit (PCR Biosystems, London, United Kingdom). The amount of

Table 1
Detailed list of primers for qRT-PCR analysis of target genes.

Target gene	GenBank accession	Primer	Size of amplified product (bp)
HSP70	NC_056073.1	F: GGAGTCGTACGCCTTCAACA R: CACCTTCTTCTTGTCCGCCT	85
IL10	NC_056065	F: TGATGCCACAGGCTGAGAAC R: CAGAAAACGATGACAGCGCC	110
TLR2	NM_001048231.1	F: ACTCCATCCCCTCTGGTCTC R: CAGGTCTCTGTTGCCGACAT	86
TLR4	NM_001135930.1	F: GGTGGAGCTCTATCGCCTTC R: GGTGCGTTACCCCTGCTATT	77
TLR8	NM_001135929.1	F: CAGTGAGTTGCCGTTGTTGG R: GGTGCGTTACCCCTGCTATT	77
GAPDH	NC_040254.1	F: CTGGCCAAGGTCATCCAT R: ACAGTCTTCTGGGTGGCAGT	86

cDNA equivalent to 5 ng of starting total RNA was used as template for each real-time PCR reaction. Forward and reverse primers (Table 1) were created with Primer Express v3.0.1 software (Applied Biosystems, Foster City, CA, USA) and confirmed for target Identity with Primer Blast from the National Center for Biotechnology Information (NCBI) (Ye et al., 2012). Real-Time PCR System, Roche Light Cycler96 was used for qPCR with a 3-min denaturation, followed by 50 cycles of 95 °C for 15 s, 62 °C for 20 s and 72 for 15 s. High resolution melting analysis was performed for each run.

Reactions were set up with PowerUpTM SYBR! Green Master Mix (Applied Biosystems, Foster City, CA, USA) following the manufacturers' 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 (glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*)) was amplified alongside the target genes (*IL10, TLR2, TLR4, TLR8,* and *HSP70*). Quantitative PCR was done in triplicate for each sample.

2.4. Statistical analysis

Using the Pfaffl method (Pfaffl, 2001) which requires primer efficiency for both gene of interest (GOI) and housekeeping genes using a standard curve and its cycle threshold (Ct) values and accounts for differences in primer efficiencies during the fold change calculation. 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. The standard deviation (SD) of the relative gene expression was determined by calculating the mean value of the breed population's gene expression ratio per GOI, which then use to calculate the individual deviation within the breed. LinReg PCR version 2017.0 software (Ramakers et al., 2003) was used to calculate primer efficiency. Further, because the substantial variation in relative gene expression levels makes finding statistically significant differences between groups more difficult, Ct values with less fluctuation were employed to detect statistically significant differences between groups. Ct value data were analyzed by mixed Analysis of variance (ANOVA) with a general linear model (GLM) with repeated measurement in SPSS Version 25 (IBM Corp., Armonk, NY, USA), with breed, sex, season, breed*sex, breed*season, sex*season, and breed*sex*season as the mixed design factors. 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 for breed and season as the factors. 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.

3. Results

3.1. Climatological conditions

The climatic data during the sampling day in each season of the year and the calculated temperature humidity index (THI) is presented in Table 2. The THI in all sampling days were within the thermoneutral zone, except for summer season which was 78.99.

3.2. Ct value

The gene expression of all GOI (Table 3) 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.

3.3. Relative gene expression

The relative expression was calculated using the Pfaffl method (Pfaffl, 2001) and is presented in Table 4 and visualized as Fig. 1 (A-E). The spring season was considered as a thermo-neutral season and used as a calibrator.

For *HSP70*, relative gene expression with different peak was observed; spring for Hungarian indigenous Tsigai (1.039 ± 0.326) and White Dorper (1.078 ± 0.441), summer (7.494 ± 11.932) for Hungarian Merino. In all GOI in this study, the lowest expression relative to the spring season was found in the autumn.

An increasing gene expression trend was identified for *IL10* in the summer season. The highest relative gene expression means of *IL10* were found in the summer season for White Dorper (17.3 95 ± 5.848) and Hungarian indigenous Tsigai (6.299 ± 6.412), but for Hungarian Merino, the highest expression was identified in the winter season (2.553 ± 2.128).

For the two Toll-like receptor genes, *TLR2* and *TLR8*, in spring and autumn, the gene expression tend to be low and a higher relative gene expression was observed in winter and summer. Both genes were observed to be overexpressed during the summer time in all breeds, 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 indigenous 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 indigenous 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.

4. Discussion

The health and well-being of animals are determinant factors in achieving maximum productivity. The seasonal stressor is one of the major components in achieving this optimum profit in livestock farming, as has been proven by many previous findings

Table 2
The THI in each sampling season.

Season	Date	Time	Temperature (°C)	Relative humidity (%)	THI
Spring	29/04/2020	12.00–13.30	19.31	62.66	64.94
Summer	13/08/2019	12.00–13.00	32.80	34.50	78.99
Autumn	19/11/2020	12.00–13.30	7.88	99.84	46.19
Winter	22/01/2020	12.00–13.30	−3.33	99.70	26.06

Table 3
Mean and standard deviation of CT value of *IL10, TLR2, TLR4, TLR8,* and *HSP70* of Hungarian indigenous Tsigai, Hungarian Merino, and White Dorper in each season of the year.

Gene	Season	CT-Value (Mean ± Std. Deviation)			
		Hungarian indigenous Tsigai	Hungarian Merino	White Dorper	Overall
HSP70	Summer	22.540 ± 0.473	22.500 ± 0.279	22.500 ± 0.628	22.516 ± 0.482[a,b]
	Autumn	24.835 ± 1.032	24.413 ± 1.260	23.940 ± 0.758	24.393 ± 0.992[c]
	Winter	22.582 ± 1.040	22.273 ± 0.055	21.515 ± 0.597	22.093 ± 0.875[a]
	Spring	23.037 ± 0.934	23.203 ± 0.106	22.898 ± 0.691	23.015 ± 0.705[b]
	Overall	23.248 ± 1.273[a]	23.097 ± 1.031[a]	23.7133 ± 1.086[a]	23.004 ± 1.162
IL10	Summer	32.953 ± 1.581	32.897 ± 1.055	32.122 ± 1.946	32.609 ± 1.605[c]
	Autumn	31.215 ± 0.757	30.127 ± 0.624	30.345 ± 0.441	30.649 ± 0.752[b]
	Winter	29.808 ± 1.496	28.503 ± 0.055	28.175 ± 0.444	28.894 ± 1.218[a]
	Spring	29.755 ± 0.981	29.473 ± 0.352	29.110 ± 0.527	29.441 ± 0.742[a]
	Overall	30.933 ± 1.772[b]	30.250 ± 1.791[a,b]	29.938 ± 1.802[a]	30.398 ± 1.815
TLR2	Summer	26.927 ± 1.259	26.400 ± 1.003	26.115 ± 1.283	26.497 ± 1.201[b]
	Autumn	27.528 ± 1.370	25.370 ± 0.491	25.698 ± 0.549	26.365 ± 1.340[b]
	Winter	24.195 ± 1.214	24.313 ± 1.270	23.837 ± 0.243	24.075 ± 0.906[a]
	Spring	24.453 ± 0.778	23.843 ± 0.167	24.095 ± 0.807	24.188 ± 0.716[a]
	Overall	25.759 ± 2.023[b]	24.982 ± 1.261[a,b]	24.936 ± 1.262[a]	25.281 ± 1.559
TLR4	Summer	24.617 ± 0.731	24.363 ± 0.548	23.002 ± 0.660	23.920 ± 1.000[a]
	Autumn	26.613 ± 1.076	25.640 ± 1.521	25.720 ± 0.620	26.061 ± 1.049[b]
	Winter	23.613 ± 0.275	23.787 ± 1.278	22.983 ± 0.451	23.396 ± 0.677[a]
	Spring	24.105 ± 0.767	23.837 ± 0.317	23.095 ± 0.339	23.647 ± 0.703[a]
	Overall	24.737 ± 1.367[b]	24.401 ± 1.183[b]	23.700 ± 1.292[a]	24.256 ± 1.365
TLR8	Summer	26.572 ± 1.185	25.910 ± 1.404	25.873 ± 1.529	26.160 ± 1.319[c]
	Autumn	25.952 ± 0.905	24.820 ± 1.129	24.340 ± 0.919	25.081 ± 1.162[b]
	Winter	23.610 ± 1.528	23.620 ± 1.652	23.048 ± 0.557	23.387 ± 1.190[a]
	Spring	23.282 ± 0.707	22.953 ± 0.826	22.562 ± 0.237	22.928 ± 0.638[a]
	Overall	24.854 ± 1.798[b]	24.326 ± 1.616[a,b]	23.956 ± 1.578[a]	24.389 ± 1.698
GAPDH	Summer	26.590 ± 2.218	25.013 ± 2.806	25.983 ± 3.166	26.032 ± 2.616
	Autumn	22.877 ± 1.181	21.450 ± 0.236	21.902 ± 0.387	22.201 ± 0.956
	Winter	21.833 ± 1.724	21.787 ± 1.180	21.107 ± 1.462	21.533 ± 1.467
	Spring	21.662 ± 0,707	21.710 ± 0.295	21.640 ± 0.664	21.663 ± 0.591
	Overall	23.240 ± 2.501	22.490 ± 2.011	22.658 ± 2.589	22.857 ± 2.589

SD: Standard deviation; [a-c]: different superscript showed significant difference ($p < 0.05$).

Table 4
Relative gene expression calculation with Pfaffl method [26] of *IL10, TLR2, TLR4, TLR8,* and *HSP70* of Hungarian indigenous Tsigai, Hungarian Merino, and White Dorper in each season of the year, relative to spring season as a calibrator.

Gene	Season	Relative gene expression (Mean ± SD)		
		Hungarian indigenous Tsigai	Hungarian Merino	White Dorper
HSP70	Summer	0.777 ± 0.313	7.494 ± 11.932	0.652 ± 0.219
	Autumn	0.527 ± 0.192	0.378 ± 0.193	0.381 ± 0.083
	Winter	0.810 ± 0.889	2.487 ± 2.092	0.524 ± 0.498
	Spring	1.039 ± 0.326	1.024 ± 0.265	1.078 ± 0.441
IL10	Summer	6.299 ± 6.412	1.976 ± 1.642	17.395 ± 5.848
	Autumn	0.578 ± 0.175	0.554 ± 0.156	1.202 ± 0.166
	Winter	0.838 ± 0.516	2.553 ± 2.128	4.443 ± 5.364
	Spring	1.124 ± 0.576	1.026 ± 0.294	5.949 ± 6.477
TLR2	Summer	12.053 ± 11.018	3.317 ± 2.720	14.263 ± 13.417
	Autumn	0.369 ± 0.219	0.304 ± 0.054	0.419 ± 0.116
	Winter	2.062 ± 2.126	1.838 ± 2.630	1.644 ± 1.695
	Spring	1.028 ± 0.261	1.019 ± 0.231	1.107 ± 0.420
TLR4	Summer	0.379 ± 0.100	15.204 ± 19.950	14.263 ± 13.417
	Autumn	0.428 ± 0.090	0.324 ± 0.291	0.419 ± 0.116
	Winter	2.283 ± 1.817	3.414 ± 4.913	1.644 ± 1.695
	Spring	1.021 ± 0.242	1.028 ± 0.306	1.107 ± 0.420
TLR8	Summer	5.747 ± 5.481	7.497 ± 6.507	6.910 ± 8.130
	Autumn	0.257 ± 0.085	0.842 ± 0.353	0.228 ± 0.080
	Winter	1.593 ± 1.818	6.617 ± 9.523	0.898 ± 1.296
	Spring	1.061 ± 0.406	1.271 ± 1.018	1.100 ± 0.597

SD: Standard deviation.

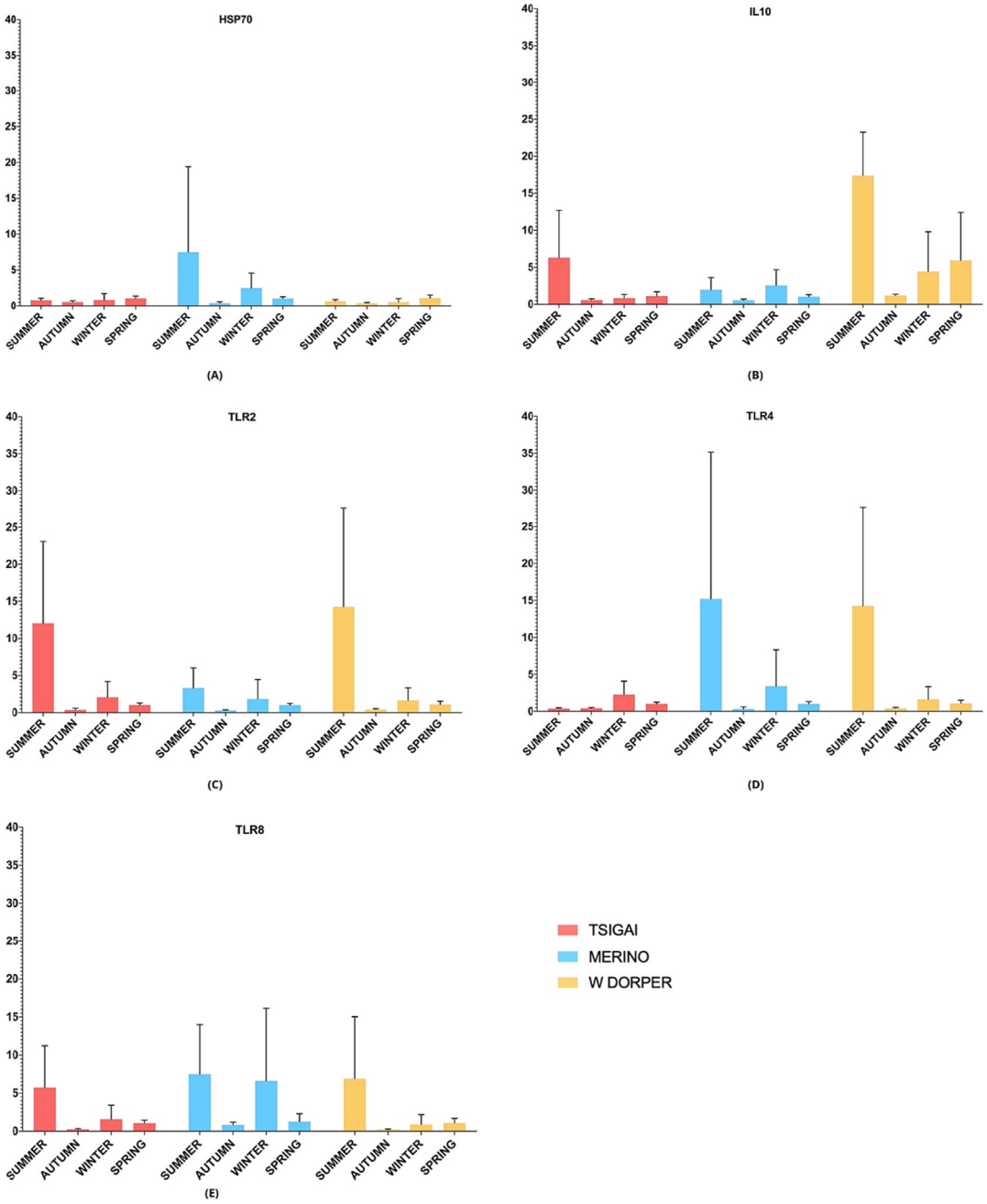

Fig. 1. Bar graph of relative gene expression of (A) *HSP70*, (B) *IL10*, (C) *TLR2*, (D) *TLR4*, and (E) *TLR8* in each season of the year with the spring season as the calibrator. The X-axis is the relative gene expression in different season of the year. The Y-axis is gene expression for each different breeds in the study. Different colours represent different breed; red for Hungarian indigenous Tsigai, blue for Hungarian Merino, and yellow for White Dorper.

(Lara and Rostagnon, 2013; Hudges et al., 2018). The seasonal variation shows temperature, humidity, rainfall, and daylight gradients across the year, which may affect the livestock's homeostasis directly through changes in physiological and biological processes, also behavior adjustment (Martelli et al., 2018; Berihulay et al., 2019). Understanding the livestock's seasonal adjustment is critical for successful and profitable livestock farming, especially in an era where climate change is the most pressing issue, exposing animals to temperatures beyond their comfort zones and compensating for their production ability.

The thermal comfort zone for sheep varies among breeds, which correlates to their morphological characteristic facilitating the body's heat exchange with the environment, for example the coat colors and characteristics or the physical characteristic of the hair/-wool (Paim et al., 2014). This morphological diversity in livestock provides a potent system for genetic dissection of traits with complex inheritance, which also demonstrates their adaptation to their environment and is an indication of natural selection driving greater diversity (Boyko et al., 2010). In this study, the three most common sheep breeds in Hungary were observed; the Hungarian indigenous Tsigai; the commercial breed with a long history of crossbreeding, the Hungarian Merino, which both are cold tolerant breeds; and the South African commercial breed, White Dorper, which is a breed with a high tolerance to heat. All of the breeds in this study have a dominating white coat colour, with both the Hungarian indigenousTsigai and Hungarian Merino having long wool and White Dorper having short wool. The aim was to see if there is any difference in adaptability to the Hungarian environ-

ment between the three different breeds, although all of them have been acclimatized to it as these breeds have been widely kept in Hungary.

Generally, sheep have an upper critical between 25 and 31 °C, depending on factors such as breed, age, and physiological state. Heat stress occurs when the effective temperature of the environment is higher than this upper critical temperature (Hopkins et al., 1978). To assess livestock productivity responses to climate change, the simplest method is to use the THI, which is calculated by combining the average temperature (°C) and relative humidity (%) of a given location (Ratchamak et al., 2021). It is undeniable, however, that THI may not entirely portray the thermal environment in which animals live because it does not take into consideration such factors as wind velocity, solar radiation, the shade provided to animals, or the availability of water. According to the THI calculation of each sampling season, almost-severe heat stress was observed in the summer season (THI = 78.99), which generated some gene expression changes inside the sheep body, explained as follows.

Thermal acclimation and adaption in small ruminants are invariably correlated with increased HSP concentrations. The HSPs have been the core of molecular responses in livestock to heat stress, but its stress tolerance mechanism is complex and not fully understood. With high HSP concentrations, cells can respond to heat stress better, while low levels of HSP make them more vulnerable (Rout et al., 2018; Joy et al., 2020). As it was observed in this study, Hungarian Merino, the cold tolerant breed with a high susceptibility to heat stress, the relative gene expression of *HSP70* was upregulated in the summertime, indicating heat-stress induced *HSP70* expression. As explained by Aleena et al. (2018) and Hooper et al. (2018), a relatively higher *HSP70* gene expression level suggests a greater need to protect the conformation of proteins on the cells from heat stress damage such as denaturation and aggregation. A previous study on goats showed *HSP70* gene is essential in goat environmental stress tolerance and adaptation, and its expression increases during heat stress (Benerjee et al., 2014; Archana et al., 2018) since it is a mechanism to protect proteins from degradation and facilitates their refolding, thereby enhancing cell survival (Dangi et al., 2014).

The expression pattern of the HSP70 gene is species- and breed-specific for goats, as shown by research by Banerjee et al. (2014), possibly due to variances in thermal tolerance and adaptability to different climatic circumstances. They also confirmed that summertime expression of *HSP70* gene is more remarkable in Indian cold-adapted goats (Gaddi and Chegu) than in heat-adapted goats (Sihori and Barbari). The spike of *HSP70* expression due to heat stress in other livestock has also been reported, e.g., cattle (Bharati et al., 2017) and buffalo (Yadav et al., 2021). In comparison, the Hungarian indigenous Tsigai and White Dorper *HSP70* expression in this study showed a small range of relative gene expression during the season of the year.

The Hungarian indigenous Tsigai, as expected, has good adaptability to the Hungarian environment (Gáspárdy et al., 2006) and Kusza et al. (2010, 2011, 2015) explained that the Hungarian indigenous Tsigai sheep is an ancient breed that has its origins traced to Asia Minor, located in modern-day Turkey. This breed was introduced to Hungary during the latter half of the 1700s. The breed has gained importance in Hungary owing to its resilience and capacity to adapt to harsh climatic and the breed has a historical significance, contributing to the country's agricultural heritage and economy. While, the White Dorper has a better thermotolerance due to the advantageous morphological characteristics such as short hair, thin skin, and a low number of hair follicles per unit area that help in combating heat stressors by facilitating heat dissipation (Gootwine, 2011) without necessitating them to make physiological regulation such as increasing heart rate and respiration rate to help to release the internal heat or reducing feed intake and body weight to decrease the endogenous heat production (Marai et al., 2007). Many findings have proven their extraordinary thermoregulation and resistance to heat stress (Almeida et al., 2013; Joy et al., 2020).

The colour and characteristics of an animal's coat can have a significant impact on its ability to acclimate to changing temperatures. Wool, not only have significant economic value, but also according to a review by Astuti et al. (2022) acts as a protective barrier that helps regulate body temperature by preventing heat loss in cold environments and blocking excessive heat gain in hot environments. Nevertheless, the thickness and type of wool can also impact an animal's ability to regulate its body temperature. For instance, wool sheep with thicker wool may have a lower thermoregulatory capacity due to the insulation effect of the wool, which inhibits body heat from escaping. In contrast, animals with thinner wool may have improved thermoregulation due to air stability within the fleece, which reduces heat loss via convection (McManus et al., 2020). The heritability of wool traits is moderate to low, as they are influenced by multiple genetic and environmental factors. The wool-growing process in the epidermis involves intricate coordination among numerous genes and cell types, such as Ubiquitin conjugating enzyme E2 E3 (*UBE2E3*) and Rhopilin Rho GTPase binding protein 2 (*RHPN2)*, which play crucial roles in keratinocyte differentiation and cell proliferation (Zhao et al., 2021), Follistatin (*FST*) genes which regulates hair follicle morphogenesis and cycling (Ma et al., 2017), also Keratin (*KRT*s) and Keratin-associated protein (*KRTAP*) which also plays important role in epithelial cell's stress response and apoptosis (Sulayman et al., 2018). The genetic and cellular mechanisms underlying the relationship between wool characteristics of sheep and their thermal adaptation remain obscure and complex. The incomplete understanding of the complexities of these biological processes contributes to a continuing lack of clarity on the subject.

Aside from its direct role in molecular protection against heat stress (intercellular function), the external *HSP70* also plays a vital role in the immune system. One is by signalling immune cells against invading pathogens via increased neutrophils and macrophages (Dybdahl et al., 2005), in collaboration with host pattern recognition molecules like toll-like receptors (*TLR*) through the pathogen-associated molecular pattern (PAMP) to activate immune response (Hassan et al., 2019). The other is through the intervention of intracellular inflammatory signalling pathways, such as in the production of interleukin-10 (*IL10*), the foremost anti-inflammatory and immunosuppressive cytokine (Borges et al., 2012).

Heat stress causes an imbalance in the immune system by shifting the adaptive immune function from the normal cell-mediated to humoral immunity, which is a significant contributor to the poor health that results from it. Heat stress has been shown to reduce sheep's immunity, making them more susceptible to disease (Sophia et al., 2016; Shi et al., 2020). It is widely thought that heat-stress-induced glucocorticoids impair immunological homeostasis via altered cytokine production. However, the specific mechanism is unknown (Bagath et al., 2019). In this study, the expression of immune-related genes (*IL10, TLR2, TLR4,* and *TLR8*) was also studied to reveal the relationship between heat stress and immune deprivation in sheep.

The expression of *IL10* was significantly different in each season and was not equivalent in each breed. In White Dorper, it was overexpressed in the summertime, as was the case in Hungarian indigenous Tsigai but with a lower peak. However, in Hungarian Merino, peak expression was seen only during the winter, and seasonal variation was minimal. The previous study on various cattle breeds; Karan Fries (Sheikh et al., 2016), Sahiwal cows (Grewal et al., 2021), and Jersey (Kim et al., 2020) showed a higher *IL10*

expression when facing heat stress, also in sheep, an increased plasma *IL10* production was reported during the hyperthermia (Caroprese et al., 2014), which was explained as a mechanism to increase immune tolerance under heat stress condition. Meanwhile, Rashamol et al. (2019) showed no significantly different expression of *IL10* in heat stress and non-heat stress Malabari goats, demonstrating strong resilience to heat stress, notably in the ability to maintain the innate immune response, which is consistent with the expression of *IL10* in Hungarian Merino in this study. Further explained by Loukovitis et al. (2022) that Merino was initially brought to Hungary in the 17th century, giving them plenty of time to acclimatize to the country's semi-arid environment and pastures, although as a imported multipurpose breed, Hungarian Merino has undergone numerous improvements programs since then by crossing them with various Merino and Merino-derived breeds. In contrast, because of its historical value, the Hungarian indigenous Tsigai has been included in the national gene conservation program in order to preserve its original qualities, particularly the traits that allowed them to thrive in a specific Hungarian environment. Nonetheless, the Hungarian Merino population has been declining recently, possibly as a result of the introduction of imported international breeds, which have become more popular among farmers in Hungary.

Pathogen identification by the highly specialized innate immune system relies heavily on Toll-like receptors (TLRs). When under stress, the immune system can either be enhanced or suppressed. In the hot summer, *TLR2, TLR4,* and *TLR8* expression was elevated in all breeds, except for *TLR4* in the Hungarian indigenous Tsigai, which showed a minimum expression fluctuation during the year with the highest expression in winter. Similarly, the *TLR2* expression in Merino also showed a minimum fluctuation. This finding is in accordance with the observation in Bengal goats (Paul et al., 2015), Malabari goats (Vandana et al., 2019), and Thapakar cattle (Bharati et al., 2017) in their case *TLR* genes were overexpressed in summertime.

TLRs are involved in the first line of defence against stress by detecting endogenous ligands such as HSPs. Researchers have already established that HSPs can stimulate the *TLR2* and *TLR4* pathways (Beg, 2022). HSPs improve antigen-presenting capacity via binding to TLR, and HSPs' exposure to these TLRs may lead to the activation of dendritic cells and macrophages and the generation of immune-enhancing cytokines critical to host infection survival (Gobert et al., 2004; Paul et al., 2015). Increased *TLR2, TLR4,* and *TLR8* expression and signalling in immune cells can in turn enhance thermo-tolerance by increasing the innate immune response to PAMPs via a distinct immune response mechanism (Zhao et al., 2007).

The Hungarian indigenous Tsigai excelled in preserving its immune response while being subjected to hyperthermic conditions, as shown by the breed's lower expression of *TLR4* compared to other breeds and the downregulation of *TLR4* in the summer. According to research conducted by Gavojdian et al. (2015), the Hungarian indigenous Tsigai is less susceptible to lameness and pneumonia than the Dorper and White Dorper. Similar results were demonstrated in the spleen of Osmanabadi goats (Sophia et al., 2016), indicating the highly adaptive nature of this breed and their improved innate immune response against heat shock proteins when exposed to heat stress. It was also noted by Bharati et al., (2017) that *TLR4* could be crucial for providing a long-term immunological response to reduce the harmful effects of heat stress (Inflammation and tissue injury) and confer thermo-tolerance under prolonged heat stress. It also identifies the Gram-negative bacteria-specific lipopolysaccharide (*LPS*) (Bulgari et al., 2017).

In this study, the Hungarian Merino was subjected to heat stress during the summer, as indicated by the relative expression of *HSP70*, but they maintained an effective immune response by maintaining high levels of *IL10* and *TLR2* gene expression throughout the year. It should come as no surprise that the indigenous Hungarian indigenous Tsigai has remarkable resilience to its natural environment and keep its resistance to seasonal stressors intact, while the immigrant White Dorper is only moderately suited to Hungarian climate. Through our research on genes for heat resistance and immunity, we confirmed that these three breeds are optimal for the Hungarian environment, given the risks posed by current and projected climate change. We recognize that our study is limited by the small and unequal sample size for each breed, which may reduce the study's statistical power and realiability, but we are confident that the results are indicative. It is essential to recognize the intricate nature of the five genes analyzed in this study, as well as their complex interactions with numerous other genes. The complexity of their functions and interrelationships is not to be disregarded, necessitating a more in-depth investigation into the relationship between sheep productivity and genetic adaptability.

5. Conclusion

This study confirmed the relative expression of genes involved in heat stress and immunity; *HSP70, IL10, TLR2, TLR4,* and *TLR8* are varied with the seasons in all three studied breeds of sheep, evidencing that seasonal stressor affects the thermo-balance and immunity of the sheep. The thermotolerance of the Hungarian Tsigai (indigenous breed) and White Dorper (tropical breed) is higher than that of the Hungarian Merino. 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.

CRediT authorship contribution statement

Putri Kusuma Astuti: Visualization, Writing – original draft. **Zoltán Bagi:** Writing – review & editing. **Lilla Bodrogi:** Methodology, Writing – review & editing. **Tímea Pintér:** Methodology, Writing – review & editing. **Gabriella Skoda:** Methodology, Writing – review & editing. **Roland Fajardo:** Writing – review & editing. **Szilvia Kusza:** Conceptualization, Methodology, Resources, Writing – review & editing, Supervision.

Declaration of Competing Interest

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

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Ethic declarations

All methods were carried out in accordance to the European Union's Animal Experimentation Directive (2010/63/EU) and ARRIVE guidelines.

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