Heat shock protein 70 polymorphism associated with physio-biochemical parameters of Awassi and Arabi Iraqi sheep

Tahreer M. Al-Thuwaini[1], Mohammed Baqur S. Al-Shuhaib[2], Zainab Manji Hussein [3]

[1,2,3]Department of Animal Production, College of Agriculture, Al-Qasim Green University, Al-Qasim, Babil 51001, Iraq
[1]E-mail address: tahrearmohammed@agre.uoqasim.edu.iq; tahreeralthuwaini@yahoo.com
ORCID ID orcid.org/0000-0003-4534-2963
[2]E-mail address: mohammed79@agre.uoqasim.edu.iq, baquralhilly_79@yahoo.com
ORCID ID.org/0000-0002-6458-2068
[3]E-mail address: zainb.ma86@yahoo.com

ABSTRACT.

Heat shock proteins (HSPs) are a group of proteins that provide thermotolerance in cell and protect cells against environmental stress. Polymorphisms of the *HSP70* gene associated with thermotolerance in farm animals. Thus, the current study aimed to evaluate the association of polymorphism in the *HSP70* gene on physio-biochemical parameters in Awassi and Arabi sheep. Two breeds of sexually mature and healthy sheep that aged between 2 to 3 years were involved in this study, including 75 animals of Awassi (22 male: 53 female) and 75 animals of Arrabi (15 male: 60 female). Genomic DNA was extracted from whole blood then genotyping analysis and sequencing were performed for each genotype. In the present study, single strand conformation polymorphism (SSCP) analysis reveals two genotypes (TT and TG) in *HSP70* (exon 4) of sheep. One missense SNPs c.33163685T>G was identified in exon 4 *HSP70* gene that was responsible on the observed heterogeneity in both breeds. Hematological analysis indicate that the amount of RBC, Hb, PCV% and MCHC were significantly higher ($P<0.05$) in awassi than Arabi, in summer than winter and in TT than TG genotype. Comparison of analysis of leukocyte profile demonstrated that WBC, lymphocyte, lymphocyte % and granulocyte % mean were higher in awassi breed, winter season and TT genotype. Biochemical analysis refer that the levels of T3 and T4 were found to be extremely highly expressed in the Arabi than awassi, in the winter season and TG genotype. In conclusion, TT genotype was high tolerance of to heat stress, higher frequency of this genotype in Arabi breed make this breed better thermos-tolerance with heat stress conditions.

Keywords: *HSP70*, polymorphism, blood parameters, sheep

التباين الوراثي لبروتينات الصدمة الحرارية HSP 70 المرتبطة بالمعايير الفسلجية-الكيموحيوية في الاغنام العرابي والعواسي العراقية

زينب منجي حسين[3] تحرير محمد الثويني[1] محمد باقر صاحب[2]

قسم الانتاج الحيواني / كلية الزراعة / جامعة القاسم الخضراء/ القاسم/ بابل/ العراق.

الملخص :

بروتينات الصدمة الحرارية (HSPs) هي مجموعة من البروتينات التي تجهز التحمل الحراري للخلية وتحمي الخلايا من الإجهاد البيئي. التباين الوراثي لجين *HSP70* ارتبط بالتحمل الحراري في حيوانات المزرعة. لذلك ، تهدف الدراسة الحالية إلى تقييم ارتباط التباين الوراثي لجين *HSP70* مع المعايير الفسلجية-الكيموحيوية في الأغنام العواسي والعرابي. تضمنت هذه

الدراسة سلالتان من الأغنام الناضجة جنسياً وصحيا والتي تراوح عمرها بين 2 إلى 3 سنوات ، بما في ذلك 75 حيوانًا من العواسي (22 ذكور: 53 أنثى) و 75 حيوانًا من العرابي (15 ذكور: 60 أنثى). تم استخلاص الحمض النووي الجيني من الدم الكامل ثم تم إجراء تحليل النمط الوراثي والتسلسل لكل تركيب وراثي. في الدراسة الحالية ، اوضح تحليل تعدد الأشكال الأحادي للشريط المفرد (SSCP) عن وجود اثنين من التراكيب الوراثية (TT و TG) في *HSP70* (إكسون 4) في الأغنام. تم تحديد طفرة نقطية واحدة c.33163685T>G في إلاكسون 4 لجين *HSP70* التي سببت عدم التجانس الملاحظ في كلا السلالتين. يشير التحليل الدموي إلى أن كمية كريات الدم الحمراء , تركيز الهيموغلوبين, وحجم الكريات المضغوط , قياس تركيز الهيموكلوبين كانت أعلى بكثير ($P<0.05$) في العواسي من العرابي ، في الصيف من الشتاء وفي التركيب الوراثيTT من TG. أظهرت تحاليل المقارنة لصورة خلايا الدم البيضاء أن ، كريات الدم البيضاء, الخلايا اللمفية, النسبة المئوية للخلايا اللمفية والنسبة المئوية للخلايا المحببة كانت أعلى في العواسي ، الشتاء وفي التركيب الوراثي TT. يشير التحليل الكيموحيوي إلى أن مستويات T3 و T4 قد تم التعبير عنها بشكل كبير للغاية في العرابي مقارنة بالعواسي ، في فصل الشتاء والنمط الوراثي TG. في الاستنتاج ، كان النمط الوراثي TT يتحمل درجة عالية من الإجهاد الحراري ، وتكرار هذا النمط الوراثي في سلالة العرابي جعل هذا الصنف أفضل تحمل حراري مع ظروف الإجهاد الحراري.

الكلمات المفتاحية :بروتين الصدمة الحرارية 70 ,التباين الوراثي ,معايير الدم ,الاغنام

البحث مستل من رسالة ماجستير للباحث الاول

Introduction

Heat Stress (HS) represents the response of the body to stimuli that disturb homeostasis(1) and the physio-biochemical traits of sheep and goat (2), (3). When the farm animals are exposed to environmental stress, several proteins, which preferentially are expressed under these conditions like heat shock proteins (HSPs) (4) (5) (6). HSPs are a group of proteins that provide thermotolerance in cell and protect cells against oxidative stress (7). HSPs play significant roles in the selection of resistant animals and represent one of the major physiological parameters, which will focus on farm animals (8). Based on the molecular weight and biological functions, HSPs is classified as HSP 110, HSP100, HSP90, HSP70, HSP60, HSP40, HSP10, and small HSP families, of which thermo-tolerance development is mainly correlated with HSP70 and HSP90 in livestock species (9)(10) (11). Heat shock protein 70 (HSP70) is produced by the *HSP70* gene and includes a family of HSPs which range size from 68 to 73 kilo Dalton (12). HSP70 plays a protective role in reaction to hyperthermia as well as other stress conditions work as a molecular chaperone (13) (14) , and providing a balance between synthesis and degradation of cellular proteins (15). The HSP70 concentration in blood was also identified as a reliable indicator of chronic stress in feedlot cattle (16). Association of polymorphisms of the *Hsp70* gene with thermotolerance in farm animals take more attention.(17) studied the association of heat stress protein 90 and 70 gene polymorphism with adaptability traits in Indian sheep (*Ovis aries*). (18) denoted that the AC genotypic of dairy cows showed higher expressions of *HSP70* mRNA and lower ratio of apoptosis. Besides, the presence of SNPs (g895 C/- and g1128 G/T) in the 5′-UTR region of inducible *Hsp70.1* ameliorates heat stress response and tolerance to heat in Holstein lactating cows (19). Similarly, in the 5' UTR region, 43 SNPs and three indels were revealed in of the *HSP70.1* gene in Holstein Friesian cattle breeds (20). One single-nucleotide polymorphism with G > T substitution was found at a position 149th in Tharparkar cattle ,in which genotype AA

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represent the most thermotolerant genotype with the highest adaptability traits (13). Based on the above consideration, no research yet on the association of the *HSP70* gene with the physio-biochemical parameters have been reported in Awassi and Arabi sheep. Thus, the current studies aimed to evaluate the association of SNPs in the *HSP70* gene on physio-biochemical parameters in Awassi and Arabi sheep.

Materials and Methods

Animals, Blood collection and hematological examination

This study was conducted at the College of Agriculture /AL-Qasim Green University/department of Animal Resources for the period from January 2018 to August 2018 on Awassi and Arabi sheep. Two breeds of sexually mature and healthy sheep that aged between 2 to 3 years were involved in this study, including 75 animals of Awassi (22 male: 53 female) and 75 animals of Arrabi (15 male: 60 female). Animals were collected randomly from three Station for raising sheep (Babylon, Karbala, Kufa,). Animals were kept on natural pasture during summer, while in winter; animals were kept indoors and fed about 2.5% of their live body weight daily, comprising a mixture of barely (59%), bran (40%), and salt (1%) concentrates.

Blood samples were collected from the sheep, using vacutainer tubes with EDTA. Heamotology analyser (vet.18, mythic company) measured hematological parameters. These parameters included hematocrit (Hct), hemoglobin, total red blood cell count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total platelet count, and total white blood cell count. Plasma was separated from blood by centrifugation at 3,000 rpm at room temperature for 15 min where it was kept frozen at –20°C to determine hormonal assay.

Hormonal assay.

Tri-iodothyronine (T3) and thyroxine (T4) were measured using Bioassay Technology Laboratory company ELISA kit (sheep tri-iodothyronine T3 Elisa kit catalog number E0063Sh and sheep thyroxine T4 Elisa kit catalog number E0001Sh). The concentrations of the T3 and T4 in the plasma were determined using the standard curve.

Genomic DNA extraction, PCR and genotyping analysis

Venous jugular blood samples (2-3 ml per sheep) were collected from Awassi and Arabi sheep. Genomic DNA was extracted from whole blood by a salting out method (21). One pair of specific polymerase chain reaction (PCR) oligonucleotides were designed of the *HSP70* gene (GenBank accession No. NC_019472.2) using NCBI Primer Blast online server (22). The sequence of the primer used in this study as follows:

Table 1:primer used in PCR for dellcting with amplify protocol

Set	Primer code	Primer sequence (5′ → 3′)	Product size	Annealing temp.
1	HSP,exon 4-F	CTGTTTGTGATAACTCAGCTTTGA	205 bp	57.8 °C
	HSP,exon 4-R	ACTGTTACCAACGCTGTTGTC		

PCR experiments were conducted using *AccuPower*® PCR PreMix (Bioneer, Korea), and initiated by denaturation for 5 min, followed by 30 cycles for 30 min of denaturation (95°C), annealing (57.8 °C), and extension (72°C), with final extension

(72°C) for 5 min. The specificity of PCR amplicons was confirmed by agarose gel electrophoresis then submitted to single-strand conformation polymorphism (SSCP) protocols. For SSCP analysis, 10 µL of each amplification product was mixed with 10 µL of denaturing buffer (98 % formamide, 0.025 % bromophenol blue, 0.025 % xylene cyanol FF, 10 mmol=LEDTA (at pH 8.0) and 2 % glycerol), heated for 7 min at 95 ◦C and then cooled on ice for 7 min. Denatured PCR products were subjected to 8 % non-denaturing polyacrylamide gel electrophoresis at 200 V for the first 5 min and then 120 V cm-1 for 5 h. SSCP patterns on the gels were visualized by silver staining according to the protocol of(23). For each genotype, the PCR products were sent for purification and sequencing of multiple sequence alignment program, according to DNA Star, EditSeq. / ClustalW, with the sequences published in the GenBank database taken as a reference to identify the polymorphisms. The observed mutations were visualized and annotated by SnapGene Viewer, ver. 4.0.4. (GSL. Biotech. LLC).

Statistical analyses

The allele and genotype frequencies ,observed heterozygosity (*Ho*), and expected heterozygosity (*He*), were analyzed using PopGen32 software, v. 1.31 (24). The general linear model was carried out to analyze significant effect of breed, sex, and genotype on the various parameters studied with statistical paekage for social scinca software version 23.0:

$$Y_{ijkl} = \mu + B_i + S_j + G_k + e_{ijkl}$$

where Y_{ijkl} = phenotypic traits, μ = overall mean, B_i = fixed effect of i^{th} breed (i = Awassi, Arabi), S_j = fixed effect of j^{th} sex (j = male, female), G_k = fixed effect of k^{th} genotype, and e_{ijkl} = random error associated with Y_{ijkl} observation and assumed to be NID (0, $\sigma^2 e$). Means were compared using Tukey-Krammer test with a significance level of ($P<0.05$). The effect of factor interaction, age, season and station did not have a significant effect on phenotypic traits, so are not included in the general linear model.

Results and Discussion

The genetic polymorphism

Genotyping with SSCP was performed to identify possible unknown variation(s). In the present study, SSCP analysis reveals two genotypes (TT and TG) in *HSP70* (exon 4) of sheep (Figure 1). The overall ratio of the genotypes TG was the highest (77 and 54 %) in Awassi and Arabi sheep respectively (Table 2). One missense SNPs c.33163685T>G was identified in exon 4 *HSP70* gene that was responsible on the observed heterogeneity in both breeds. According to the value of Chi-square, the population under study was not in Hardy–Weinberg equilibrium (HWE), which was statistically significant at ($P < 0.05$).

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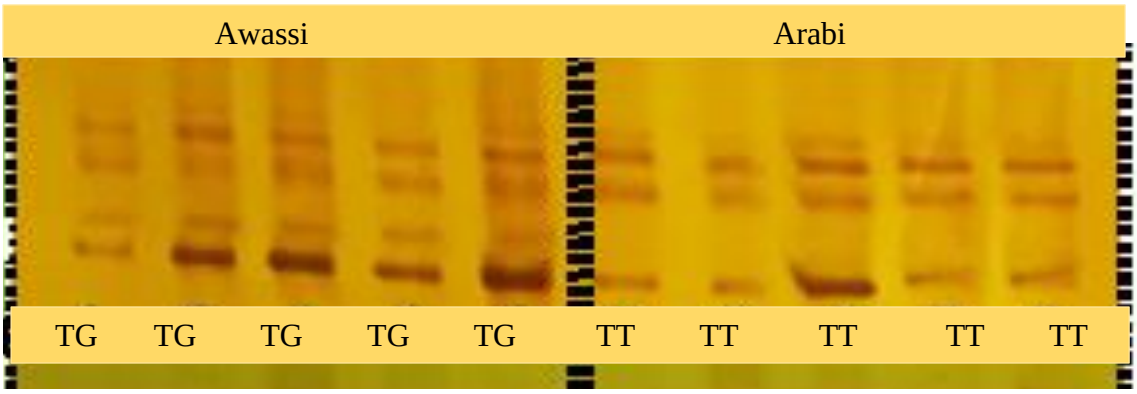

Figure (1): SSCP non-denaturing polyacrylamide gel electrophoresis of the *HSP70 gene* (exon 4) PCR fragments showed two genotypes (TT and TG). Electrophoresis conditions: Polyacrylamide gel concentration 8 %, power applied: 200V (7.5V/cm) – 100mA, time to run: 5 hr. Staining method; Silver nitrate.

Table 2. Genotype and allele frequencies and genetic diversity parameters for c.33163685T>G SNP in the *HSP70* gene (exon 4) in Awassi and Arrabi breeds.

	Genotype frequencies (n)		**Allele frequencies**		***Ho***	***He***	***χ2***
	TT(n)	TG(n)	T	G			
Awassi	0.23 (34)	0.77 (116)	0.61	0.39	0.7733	0.4759	59.030
Arabi	0.46 (69)	0.54 (81)	0.73	0.27	0.5400	0.3955	20.233

Abbreviations: (n) refers to the number of samples, χ2 – chi-square, *Ho* – observed heterozygosity, *He* – Expected heterozygosity, All Chi-square tests have one degree of freedom and within the significance level P<0.05.

Association analysis

Association analysis refer to numerous physio-biochemical changes occurs in this study. Table 3 shows the least square means of erythrocyte constituents and platelets as affected by breed, season and genotype. The amount of RBC, Hb, PCV% and MCHC were significantly higher ($p<0.05$) in awassi than Arabi, in summer than winter and in TT than TG genotype but there were no significant difference ($p>0.05$) for other parameters. The numbers of erythrocytes, Hb, PCV% and MCHC were higher in awassi (9.597×10^6/µl), (9.979), (30.334%) and (32.390) respectively than Arabi. The same pattern was seen in summer than winter and TT than TG genotype for the same parameters. Comparison of analysis of leukocyte profile among breed, season and genotypes demonstrated that WBC, lymphocyte, lymphocyte% and granulocyte % mean were higher in awassi, winter and TT genotype, while no statistically significant difference was observed for the other leukocyte profile ($P>0.05$) (Table 4).

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Table (3): Least square Mean ± SE of erythrocyte constituents and platelets for the breed, season and genotype effects.

Indices		RBC ($\times 10^6$/µl)	Hb (g/dl)	PCV (%)	MCV (fl)	MCH (pg)	MCHC (g/dl)	PLT ($\times 10^3$/µl)
Breed	Awassi	9.597± 0.482^b	9.979± 0.716^b	30.334± 1.461^b	34.850± 0.942^a	10.190± 0.792^a	32.390± 2.375^b	537.611± 6.899^a
	Arabi	8.454± 0.340^a	8.522± 0.429^a	27.503± 1.031^a	34.349± 0.665^a	9.441± 0.559^a	28.669± 1.676^a	494.092± 7.683^a
Season	Summer	9.445± 0.597^b	9.175± 0.753^b	33.377± 1.809^b	34.989± 1.166^a	9.917± 0.981^a	30.403± 2.941^b	515.536± 8.579^a
	Winter	8.830± 0.312^a	8.313± 0.393^a	30.428± 0.944^a	34.370± 0.609^a	9.692± 0.512^a	26.590± 1.535^a	519.398± 6.675^a
Genotype	TT	9.400± 0.331^b	9.120± 0.810^b	30.660± 1.941^b	36.680± 1.821^a	10.580± 0.508^a	30.260± 1.884^b	501.160± 7.093^a
	TG	8.656± 0.223^a	8.555± 0.546^a	27.391± 1.380^a	32.691± 1.227^a	10.082± 0.342^a	27.255± 1.270^a	491.164± 8.677^a

RBC, red blood cell; Hb, the concentration of hemoglobin; PCV, packed cell volume; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelets. Different superscript in the same column within each classification indicate significant differences (P <0.05).

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Table (4): Least square Mean ± SE of the constituents of white blood cell, Lymphocytes, Monocytes, Granulocytes count for the breed, season and genotype effects.

Indices		WBCs ($\times10^3$/µl)	Lymphocytes ($\times10^3$/µl)	Monocytes ($\times10^3$/µl)	Granulocytes ($\times10^3$/µl)	Lymphocytes (%)	Monocytes (%)	Granulocytes (%)
Breed	**Awassi**	14.181± 2.947^b	9.433± 2.554^b	1.284± 0.118^a	4.508± 0.468^a	57.636± 4.753^b	10.027± 0.604^a	36.633± 2.612^b
	Arabi	11.655± 2.081^a	5.797± 0.803^a	1.234± 0.167^a	4.499± 0.663^a	49.318± 3.355^a	9.027± 0.855^a	27.237± 3.700^a
Season	**Summer**	10.740± 2.649^a	5.966± 0.162^a	1.171± 0.207^a	3.898± 0.821^a	48.502± 5.886^a	10.130± 1.059^a	30.035± 4.581^a
	Winter	14.167± 1.905^b	6.307± 0.651^b	1.319± 0.108^a	4.181± 0.428^a	52.498± 3.072^b	10.017± 0.553^a	33.186± 2.391^b
Genotype	**TT**	11.120± 3.991^b	8.600± 1.344^b	1.240± 0.022^a	4.640± 0.096^a	56.920± 5.955^b	9.120± 0.964^a	32.960± 2.288^b
	TG	10.082± 2.691^a	6.455± 1.255^a	1.245± 0.015^a	4.355± 0.065^a	52.691± 4.028^a	9.400± 0.650^a	30.909± 3.565^a

WBC, white blood cell; Different superscript in the same column within each classification indicate significant differences (P <0.05).

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The hematological blood indicators are the main determinant of the animals' environmental adaptation and these parameters can be used to evaluate animal stress (25).The present study denoted that the amount of RBC, Hb, PCV% and MCHC were significantly higher (P<0.05) in awassi than Arabi means that Arabi breed was better thermotolerant than Awassi.(26) showed that the breed causes the difference in haematological parameters of goats. Regarding to the effect of hot season, the amount of RBC, Hb, PCV% and MCHC were significantly higher (P<0.05) in summer than winter. This increase of hemoglobin and PCV levels could be due to decreases voluntary intake under heat stress (27). Heat stress significantly alters the levels of hemoglobin (Hb), packed cell volume (PCV) level in the blood. (28) denoted that both Hb and PCV increased significantly in goats during exposure to severe thermal stress. The increased PCV during heat stress condition could be attributed to severe dehydration of these animals (29),or may be related to elevated loss of body fluid through heat stress induced evaporative heat loss (30)(31). However, in the case of dehydration, the hematocrit value is observed to increase in barn-housed cows. The increase in MCHC may also result from dehydration of the body or hemolysis of the analysed material (25). While in cold season, decrease in RBC has been reported in animals exposed to extreme cold (32).Genotypes or animals that show least deviation in their physio-biochemical traits between hot season and cold seasons are more adaptable to the heat stress.(17) that indicates the superiority of T allele over the G allele in terms of adaptability to heat stress. This is in agreement with the finding of present study in which TT genotype was better theromotolerance than TG genotype.

Physiological changes in blood cellular components as well as endocrine system have been used as important parameters to evaluate the adaptation of animals. This may help in the selection of thermos-tolerant animals that are capable of producing satisfactorily in harsh environments (33). Comparison of analysis of leukocyte profile among breed, season and genotypes demonstrated that WBC, lymphocyte, lymphocyte percentage and granulocyte percentage mean were higher in awassi, winter and TT genotype (Table 4). Lymphocyte function was significantly lower concentrations in cows exposed to hot environments. The reduction of lymphocyte during high temperature means that exposure to heat stress can decrease the number of viable cells and reduce their responsiveness to mitogens (34).

In this study, the levels of T3 and T4 in the serum of awassi and Arabi breeds were determined and they were found to be extremely highly expressed in the Arabi than awassi (Table 5). Regarding the effects of season on T3 and T4 concentration, the highest level of T3 and T4 were obtained in the winter and the lowest value was observed in the summer. Analyzing the influence of genotype on the thyroid hormones concentrations showed that TG genotype had significantly higher (P<0.05) T3 and T4 concentration than TT genotype.

Table (5): Least square Mean ± SE of T3 and T4 concentration for the breed, season and genotype effects.

Indices		Triiodothyronine (T3)	Thyroxine (T4)
Breed	**Awassi**	1.440± 0.017[b]	5.977± 0.034[b]
	Arabi	1.719± 0.027[a]	10.007± 0.060[a]
Season	**Summer**	1.349± 0.034[b]	6.565± 0.077[b]
	Winter	1.988± 0.015[a]	9.066± 0.345[a]
Genotype	**TT**	1.870± 0.044[b]	4.889± 0.042[b]
	TG	2.803± 0.030[a]	5.872± 0.028[a]

Different superscript in the same column within each classification indicate significant differences (P <0.05)**.**

It is well recognized physiological and hematochemical parameters are influenced by several factors including breed, age, and stress (35). In this study, the highest level of T3 and T4 were obtained in Arabi breed, in the winter season and in the TG genotype. The lower concentration of T3 and T4 observed during the summer season that may be due to direct effect of heat stress on thyroid gland activity as well as due to reduced feed intake to avoid extra metabolic (35). The reduction in serum concentration of T4 and T3 during the summer could reduce metabolism and heat generation to prevent a rise in body temperature (36). (37) reported that the changes in the ambient temperature suppresses the activity of thyroid hormone in blood level and also identified these hormones to be the stress indicators for assessing the heat tolerance in the farm animals. Analyzing the influence of genotype on the thyroid hormones concentrations showed that TG genotype had significantly higher T3 and T4 concentration than TT genotype(38). This may be due to that the thyroid metabolic hormone was affected by both season and genotype. Karacabey Merinos genotype displayed both T3 and T4 levels were seen to be lower than in the Karya and Kivircik genotype in Turkey sheep indicators that the Merinos genotype is highly adaptation to the seasonal environmental conditions (39).

Conclusion :

Genotype TT was high tolerance to heat stress, higher frequency of this genotype in Arabi breed make this breed better thermos-tolerance with heat stress conditions.

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