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Expression levels of *HSP70* and *CPT-1* in three local breeds of chickens reared under normal or heat stress conditions after the introduction of the naked neck gene

A. Galal, Lamiaa M. Radwan, Habiba H. Rezik, H. Ayoub

Poultry Production Department, Faculty of Agriculture, Ain Shams University, Cairo, Egypt

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ABSTRACT

The naked neck gene was introduced by crossbreeding into Egyptian breeds to improve body weight. Expression levels of *HSP70* and *CPT-1* were used to assess the heat tolerance of three Egyptian local breeds (Fayoumi, Dandarawi and Sinai) with and without the naked neck gene and under normal and heat stress conditions.

There were two genotypes from each breed that had the same genetic origin (the naked neck and normal plumage genotypes). For each genotype, chicks were divided into two groups, a control group and a treated group. Chicks in the treated group were subjected to heat stress (40 °C) for four hours when they were between 3 and 5 days old.

This treatment was associated with a highly significant increase in *HSP70* and *CPT-1* gene expression for the Dandarawi breed compared to the levels in the Fayoumi and Sinai breeds.

Moreover, the introduction of the naked neck gene into these local breeds caused marked increases in *CPT-1* gene expression, but these increases did not significantly differ among different naked neck genotypes.

Therefore, it could be concluded that the Dandarawi breed exhibited the best heat tolerance, followed by the Sinai breed, whereas the Fayoumi breed was inferior in this respect. Furthermore, the naked neck gene improved heat tolerance by increasing *HSP70* gene expression rather than only by reducing feather cover.

The results obtained recommended using the Sinia naked neck chicken as a male line in commercial parent stock to produce broiler chicks adapted to the hot and warm climates.

1. Introduction

Temperature is regarded as one of the most important environmental factors affecting the rate of poultry production, and elevated temperatures in recent years have led to an increase in research directed at introducing genes responsible for heat tolerance, such as the naked neck gene, into flocks to improve adaptability, production, and immunity in the tropics and hot climate (Melesse, 2011; Melesse et al., 2012; Radwan et al., 2015). When birds are subjected to heat stress, a rapid response occurs that involves the formation of heat shock proteins (HSP) that protect organs and cells from the negative effects of elevated temperatures (Rivera et al., 2005). Liu et al. (2014) used different techniques to improve heat tolerance and found that birds that better tolerated heat stress produced greater quantities of HSP70. Dridi et al. (2013) detected an increase in the expression of genes responsible for the formation of HSP70 in different tissues (heart, liver and kidney) after birds were exposed to heat stress. They also observed that blood vessels expanded after birds were subjected to heat stress. Gan et al. (2013) demonstrated that in chickens, the expression of *HSP70* differed across various tissues and organs and also across different strains or breeds. Amrutkar et al. (2014) reported that *HSP70* gene expression was highly significant in broiler flocks. Moreover, they found that the highest *HSP70* gene expression was associated with the naked neck frizzled genotype (Na-F), followed by the naked neck genotype (Na-ff), whereas birds with normal plumage (nanaff) exhibited the lowest *HSP70* gene expression.

mRNA expression of the carnitine palmitoyltransferase-1 *(CPT-1)* gene has been determined using bird livers. This gene was strongly expressed and played an important role in many vital processes, such as fat metabolism and providing supporting energy via increased feed consumption (Nilchian et al., 2013; Sojeong et al., 2013). The supporting energy required for certain muscles, such as the muscles responsible for panting, is extremely important because it improves birds' heat tolerance. Fang et al. (2014) reported that in birds, the liver is the chief organ responsible for fatty acid formation. Fatty acid formation is also controlled by specialized genes such as *SREBP-1c, ACC, PPARa,*

E-mail address: Lamia_radwan@agr.asu.edu.eg (L.M. Radwan).

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CPTI and *ACOX1*.

The main aim of this study was to estimate heat tolerance with and without the introduction by crossbreeding of the naked neck gene in Egyptian breeds (Fayoumi, Dandarawi and Sinai) reared under normal or heat stress conditions.

2. Materials and methods

The authors complied with all national legislation concerning animal welfare and followed the guidelines of the relevant ethics committee. This work was approved by the director of the Poultry Production department of the Faculty of Agriculture, Ain Shams University. A total of six hundred chicks (one day old) from three local breeds (Fayoumi, Dandarawi and Sinai) were used in this study (200 chicks per breed). Also, total of six hundred chicks (one day old) from Fayoumi naked neck, Dandarawi naked neck and Sinai naked neck were used in this study. These chicks were produced by artificially inseminating females (Sinai, Fayoumi and Dandarawi) with sperm from naked neck cocks (Nana). Two genotypes from each breed were obtained, the naked neck (Nana) and normal plumage (nana) genotypes (pure breeds). For each genotype of each local breed, chicks were divided into two groups. Identified sexed for chicks at one days of age using method vent sexing. The first group was the control group content of 100 male chicks per genotype per each local breed, which reared under normal program temperature brooded 33°C at one day of age was reduces gradually temperature till of room temperature (22–24 °C). Whereas the second group (100 male chicks per genotype per each local breed) was subjected to heat stress (40 °C for four hours when chicks were between 3 and 5 days of age), the chicks from this group were reared at 33–35.5 °C from 6 days of age until the end of experimental period. All chicks were reared under the same environmental, managerial and hygiene conditions until the end of experimental period, which continued until 14 weeks of age. Individual body weight was recorded from male only at one-day of age, 2 weeks, 4 weeks and 6 weeks of age for males in both groups (50 male birds per genotypes per breed per groups). Respiratory rate and rectal temperature were measured in both groups (the control and heat-treated groups) at 3 h after heat exposure. Birds were chosen randomly after heat exposure (30 male birds per genotypes per breed per groups). The respiratory rate was measured by counting panting breaths for 1 min. Rectal temperature was obtained by introducing a digital thermometer into the cloaca of each bird until the reading stabilized.

2.1. RNA isolation and gene expression analysis

Liver samples were obtained for each breed, genotype and treatment. RNA was extracted from these samples and used to estimate *HSP70* and *CPT-1* gene expression. Thirty six liver samples (Three different liver samples/ treatment / genotypes) were collected when chickens were 6 days of age, washed with ice-cold physiological saline, and stored at −70 °C. Approximately 0.5 g of each tissue sample was then ground in liquid nitrogen with a mortar and pestle. Total RNA was extracted from the ground tissue using TRIzol reagent (Invitrogen, USA) in accordance with the manufacturer's instructions. The primers used are listed in Table 1.

Table 1
The primers used for the different studied genes.

Gene	Sequence (5′→3′)
Heat shock protein *(HSP70)*	F - AACCGCACCACACCCAGCTATG R - CTGGGAGTCGTTGAAGTAAGCG
Carnitine palmitoyltransferase − 1 *(CPT − 1)*	F - AAGGGTACAGCAAAGAAGATCCA R - CCACAGGTGTCCAACAATAGGAG
α-Actin	F - GGAAGTTACTCGCCTCTG R- AAGACACTTGTTGGGTTAC

2.2. Relative quantification of samples via SYBR Green I-based real-time RT-PCR

SYBR Green I-based one-step real-time quantitative RT-PCR amplification was performed using an iCycler iQ Real-Time PCR Detection System (Bio-Rad, USA). Test samples were assayed in 25 µl reaction mixtures containing 5.4 µl of reaction mix, 1 µl of SYBR Green I, 0.5 µl of each forward and reverse primer, 2 µl of RNA, 0.5 µl of reverse transcriptase, and 15.6 µl of nuclease-free water. A control template was also included in these assays. The thermal profile for SYBR Green I-based one-step real-time RT-PCR consisted of 50 min of reverse transcription at 42 °C, one 3 min cycle of Taq DNA polymerase activation at 95 °C, and 40 cycles of PCR at 94 °C for 30 s (denaturation), 58 °C for 30 s (annealing), and 72 °C for 30 s (extension). Following amplification, a melting curve analysis was performed to verify the authenticity of the amplified product based on its specific melting temperature (Tm). In each run, a dilution series of in vitro-transcribed standard RNA was included in addition to clinical RNA samples. Ct values for each sample were determined using one-step real-time RT-PCR, and the copy numbers of the samples were obtained from standard curves for *HSP70* and carnitine palmitoyltransferase-1 *(CPT-1)* mRNA and α-Actin mRNA.

2.3. Scale of HSP70 and (CPT-1) by Western blotting test

Forty-eight liver samples were collected at 6 days of age for measured of level HSP70 by Western blotting test. HSP70 and CPT-1 were extracted and analysis from the liver as described by Guerreiro et al. (2004); Nguyen et al. (2015).

A protein extraction reagent was used to extract protein, and the protein concentration was determined using the Bradford method. The samples were subjected to SDS-PAGE and electrotransferred to a polyvinylidene fluoride membrane for 2 h. After transfer, the membrane was incubated with an anti-Hsp70 antibody and anti- CPT-1antibody (1:200, Cell Signaling Technology Co. USA) overnight at 4 °C and then with a secondary antibody for 1 h, followed by film exposure and development. The film was scanned immediately. The Western blot results were evaluated using Image-Pro Plus image analysis software.

2.4. Statistical analysis

Data were subjected to two-way ANOVA with Genotype, heat treatment (control, heat) as main effects and interaction between them was estimated using the General Linear Models (GLM) procedure of JMP Ver. 11 (SAS Institute, 2013. Duncan's multiple range tests were used to separate means when separation was relevant.

$$Y_{ijk} = \mu + G_i + T_x + (G^* T)_{ix} + e_{ixk}$$

Where; μ = Overall mean, G_i = Genotype effect, T_x = Heat treatment effect, $(G^*T)_{ix}$ = Interaction between Genotype and Heat treatment, e_{ijk} = Experimental error.

3. Results and discussion

Table 2 shows the effects of genotype, treatment and their interaction on body weight. The genotype effect was significant at all ages in this study. The Sinai breed was significantly heavier than the Fayoumi and Dandarawi breeds at two weeks of age. The results for body weight at two weeks of age indicated that the lightest and heaviest breeds were Dandarawi and Sinai, respectively. These results are in agreement with those obtained by Farahat et al. (2009). Egypt has only three pure breeds (Fayoumi, Dandarawi and Sinai). Notably, heat treatment induced a significant decrease in body weight only at 2 weeks. Moreover, the negative effect of heat treatment was greater in pure Egyptian breeds than in Egyptian crossbreeds with the naked neck gene. These results reflect the fact that the naked neck gene reduces birds' feather cover and enhances their heat tolerance and heat loss via radiation.

Table 2
Effects of genotype (G), treatment (T) and their interaction on body weight (g).

Genotype (G)	Treatment (T)		Overall	Prob.		
	Control	Heat		G	T	G × T
		one-day of age				
Fayoumi	30.01 ± 0.44	30.96 ± 0.54	30.49	NS	NS	NS
Dandarawi	30.21 ± 0.31	30.95 ± 0.35	30.58			
Sinai	30.01 ± 0.37	30.18 ± 0.22	30.10			
Nana Fayoumi	30.73 ± 0.15	30.80 ± 0.17	30.77			
Nana Dandarawi	30.90 ± 0.23	31.03 ± 0.15	30.97			
Nana Sinai	30.50 ± 0.69	30.32 ± 0.53	30.41			
Overall	30.39	30.71				
		2 weeks				
Fayoumi	89.27 ± 3.05	77.56 ± 3.12	83.42^{c}	0.0001	0.001	NS
Dandarawi	89.12 ± 2.80	81.57 ± 2.91	85.35^{cd}			
Sinai	103.71 ± 2.12	81.04 ± 2.40	92.38^{bc}			
Nana Fayoumi	102.00 ± 3.91	92.50 ± 2.08	97.25^{b}			
Nana Dandarawi	93.67 ± 3.87	80.80 ± 4.45	87.24^{c}			
Nana Sinai	113.00 ± 2.51	98.90 ± 3.81	105.95^{a}			
Overall	98.46^{a}	85.40^{b}				
		4 weeks				
Fayoumi	247.83 ± 5.63	229.25 ± 6.39	238.54^{b}	0.001	NS	NS
Dandarawi	231.50 ± 5.95	212.57 ± 2.42	222.04^{c}			
Sinai	268.22 ± 7.75	236.82 ± 4.89	252.52^{b}			
Nana Fayoumi	397.67 ± 3.56	287.66 ± 5.17	342.67^{a}			
Nana Dandarawi	331.33 ± 7.43	309.66 ± 3.87	320.49^{a}			
Nana Sinai	335.00 ± 3.28	354.50 ± 4.86	344.75^{a}			
Overall	301.93	271.74				
6 weeks						
Fayoumi	366.08 ± 9.12	337.54 ± 6.54	351.81^{cd}	0.0001	NS	NS
Dandarawi	319.64 ± 8.98	330.42 ± 8.80	325.03^{c}			
Sinai	386.18 ± 7.54	377.67 ± 4.61	381.93^{d}			
Nana Fayoumi	406.67 ± 8.98	417.66 ± 6.98	412.17^{b}			
Nana Dandarawi	411.71 ± 7.98	396.32 ± 8.31	404.02^{b}			
Nana Sinai	439.50 ± 8.06	458.62 ± 7.76	449.06^{a}			
Overall	388.30	386.37				

Means within the same row followed by different letters are significantly different. The threshold for significance was $P \leq 0.05$,NS, not significant.

Moreover, Mahrous et al. (2018) reported that the naked neck gene played an important role in improving weight gain and feed conversion.

Table 3 shows that rectal temperature and respiration rate were significantly higher for the group exposed to heat stress than for the control group. Additionally, rectal temperature and respiratory rate were significantly higher for Sinai naked neck chicks than for chicks of other genotypes. Notably, the introduction of the naked neck gene into Egyptian breeds caused increases in rectal temperature and respiratory rate under heat stress that reflect greater tolerance to heat stress. These results are in agreement with those of Chen et al. (2013), who observed that more heat-tolerant strains showed higher rectal temperature and respiratory rate under heat stress than other strains with low resistance or tolerance to heat stress. On the other hand, poultry subjected to high environmental temperatures reportedly exhibit many behavioural and physiological changes that lead to functional acclimatization and adaptation to these conditions (Mustafa et al., 2009; Mack et al., 2013).

Table 4 indicated that the HSP70 level was significantly increased ($P = 0.001$) for birds subjected to heat stress than for their counterparts

Table 3
Effects of genotype (G), treatment (T) and their interaction on rectal temperature and respiratory rate.

Genotype (G)	Treatment (T)		Overall	Prob.		
	Control	Heat		G	T	G × T
Rectal temperature, ˚C						
Fayoumi	40.08 ± 0.13	40.36 ± 0.19	40.22^{d}	0.0001	0.05	NS
Dandarawi	40.77 ± 0.14	40.88 ± 0.13	40.83^{C}			
Sinai	40.20 ± 0.071	40.40 ± 0.24	40.30^{d}			
Nana Fayoumi	41.40 ± 0.15	41.57 ± 0.19	41.49^{b}			
Nana Dandarawi	41.20 ± 0.06	41.50 ± 0.05	41.35^{b}			
Nana Sinai	41.60 ± 0.12	42.13 ± 0.09	41.87^{a}			
Overall	40.88^{b}	41.14^{a}				
Respiratory rate, breaths. min^{-1}						
Fayoumi	89.40 ± 6.39	108.00 ± 6.36	98.70^{ab}	0.01	0.0001	NS
Dandarawi	94.50 ± 3.97	98.40 ± 7.90	96.45^{ab}			
Sinai	84.60 ± 5.48	96.60 ± 0.23	90.60^{ab}			
Nana Fayoumi	87.26 ± 0.12	87.40 ± 0.15	87.33^{b}			
Nana Dandarawi	84.43 ± 0.09	120.27 ± 0.09	102.35^{a}			
Nana Sinai	87.26 ± 0.15	114.10 ± 0.06	100.68^{a}			
Overall	87.91^{b}	104.13^{a}				

Means within the same row followed by different letters are significantly different. The threshold for significance was $P \leq 0.05$,NS, not significant.

Table 4
Effects of genotype (G), treatment (T) and their interaction on Hsp70 levels (ng Hsp70 μg total protein-1) in the liver.

Genotype (G)	Treatment (T)		Overall	Prob.		
	Control	Heat		G	T	G×T
Hsp70 levels (ng Hsp70 μg total protein-1)						
Fayoumi	2.08 ± 0.01	4.19 ± 0.09	3.14^{e}	0.001	0.001	NS
Dandarawi	2.31 ± 0.04	5.49 ± 0.07	3.90^{d}			
Sinai	2.20 ± 0.03	4.83 ± 0.10	3.52^{d}			
Nana Fayoumi	3.17 ± 0.01	5.79 ± 0.08	4.48^{c}			
Nana Dandarawi	3.36 ± 0.06	6.48 ± 0.10	4.92^{b}			
Nana Sinai	3.59 ± 0.02	7.79 ± 0.08	5.69^{a}			
Overall	2.79^{b}	5.76^{a}				

Means within the same row followed by different letters are significantly different. The threshold for significance was $P \leq 0.05$,NS, not significant.

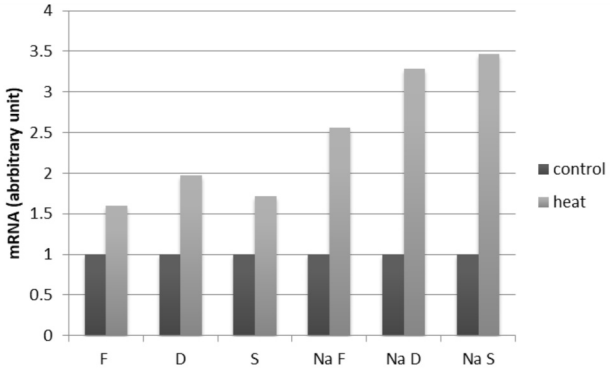
reared under normal conditions (the control group). This increase was significantly higher ($P = 0.001$) for the breeds after introduced naked neck genes compared pure breeds. Guerreiro et al. (2004) observed change level HSP70 in brain and liver during gradual heat exposure. HSP70 play the main role in the adaptive response to heat stress in chickens by improving the antioxidant capacity, inhibiting lipid peroxidation and increasing the activity of digestive enzyme activity (Hao et al., 2012; Gu et al., 2012; Varasteh et al., 2015).

As shown in Fig. 1, *HSP70* gene expression was greater for birds subjected to heat stress than for their counterparts reared under normal conditions (the control group). This increase was significantly higher ($P = 0.001$) for the Dandarawi breed than the Sinai breed, whereas the Fayoumi breed was inferior to its counterparts in this respect. The introduction of the naked neck gene led to a remarkable increase in *HSP70* gene expression in the three local breeds examined in this study. With respect to comparisons between genotypes, highly significant differences in gene expression were clearly observed between the naked neck Dandarawi and naked neck Sinai genotypes and the naked neck Fayoumi genotype. This result may reflect interactions between the environment and the genetic structure of each breed and the effect of this factor on improving adaptability and heat tolerance for local breeds. Thus, it could be concluded that the environment affected adaptability and heat tolerance via the effects of *HSP70* gene expression and that the best heat tolerance was exhibited by the Dandarawi breed, followed by the Sinai breed, with the Fayoumi breed inferior to the other tested breeds. These results are in agreement with those of Arad and Marder (1982), who reported that the desert-inhabiting Sinai breed was significantly more heat-resistant than the Leghorn breed. This superiority was indicated by the former breed's longer survival time, efficient regulation of body temperature and high lethal body temperature. The superior heat resistance of the Sinai breed might reflect physiological adaptations to the extreme conditions in its ecological habitat. Radwan and Mahrou (2018) recorded that the heat tolerance is inherited of the different generation in Sinai breed.

These findings are consistent with those reported by Moraa et al. (2015), who studied the effects of environmental conditions on the genetic background of the Kinia local breed. Mahmoud (2000) measured *HSP70* gene expression for strains that were more and less tolerant to high ambient temperature. He studied the sequences of *HSP70* alleles and found that the more heat-tolerant strains had two different alleles for that gene. Mazzi et al. (2003) analysed the promoter region and the beginning of the coding region of *HSP70* in chickens with various heat tolerance capabilities and found 2 relevant single nucleotide polymorphisms (SNPs), A + 258 G and C + 276 G, both of which were silent mutations.

Table 5 indicated that the CPT-1 level was significantly increased ($P = 0.001$) for birds subjected to heat stress than for their counterparts reared under normal conditions (the control group). This increase was significantly higher ($P = 0.001$) for the breeds after introduced naked neck genes compared pure breeds. But, these increases did not significantly differ among different naked neck genotypes. Coble et al. (2014); Huang et al. (2015) recorded metabolic of the liver were changed in response to heat condition and impairs mitochondrial functions reflected to impairing growth. While, Fayoumi breed had high adaptation to heat condition but not observed this phenomenon (Lan et al., 2016). With respect to *CPT-1* gene expression, Fig. 2 illustrates that the Dandarawi breed was superior, followed by the Fayoumi and Sinai breeds. The differences among breeds were highly significant ($P = 0.001$). The introduction of the naked neck gene resulted in high *CPT-1* gene expression for all breeds, but the differences between

a



	F	D	S	Nana F	Nana D	Nana S	Prob.
Control	1±0.053	1±0.003	1±0.007	1±0.003	1±0.002	1±0.011	NS
Heat	1.600±0.011	1.973±0.041	1.721±0.020	2.561±0.006	3.290±0.021	3.464±0.031	0.001

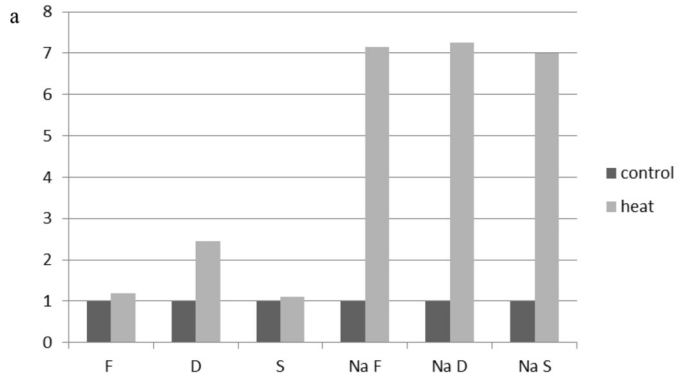
b

Fig. 1. mRNA abundances for the *HSP70* gene in the livers of Fayoumi (F), Dandarawi (D), Sinai (S), Fayoumi naked neck heterozygous (Nana F), Dandarawi naked neck heterozygous (Nana D) and Sinai naked neck heterozygous (Nana S) chickens at 7 days of age. The threshold for significance was $P \leq 0.05$, NS, not significant.

Table 5
Effects of genotype (G), treatment (T) and their interaction on CPT-1 levels (ng CPT-1 μg total protein-1) in the liver.

Genotype (G)	Treatment (T)		Overall	Prob.		
	Control	Heat		G	T	G×T
CPT-1 levels (ng CPT-1 μg total protein-1)						
Fayoumi	0.21 ± 0.02	0.49 ± 0.03	0.35[d]	0.0001	0.0001	NS
Dandarawi	0.25 ± 0.04	0.68 ± 0.05	0.47[b]			
Sinai	0.23 ± 0.01	0.52 ± 0.07	0.38[c]			
Nana Fayoumi	0.32 ± 0.03	0.77 ± 0.04	0.55[a]			
Nana Dandarawi	0.36 ± 0.06	0.79 ± 0.05	0.58[a]			
Nana Sinai	0.34 ± 0.03	0.78 ± 0.02	0.56[a]			
Overall	0.29[b]	0.67[a]				

Means within the same row followed by different letters are significantly different. The threshold for significance was $P \leq 0.05$,NS, not significant.



	F	D	S	Nana F	Nana D	Nana S	Prob.
Control	1±0.001	1±0.003	1±0.004	1±0.002	1±0.005	1±0.004	NS
Heat	1.200±0.005	2.450±0.006	1.115±0.017	7.140±0.044	7.264±0.031	7.005±0.022	0.001

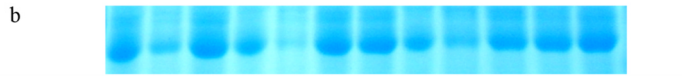


Fig. 2. mRNA abundances for the *CPT-1* gene in the livers of Fayoumi (F), Dandarawi (D) and Sinai (S), Sinai naked neck heterozygous (Nana F), Dandarawi naked neck heterozygous (Nana D) and Sinai naked neck heterozygous (Nana S) chickens at 7 days of age. The threshold for significance was $P \leq 0.05$., NS, not significant.

breeds were not significant. *CPT-1* is responsible for regulating the oxidation of fatty acids and producing energy needed for cells (Nilchian et al., 2013; Lei and Lixian, 2012) and muscles, particularly those responsible for the panting process, which results in improved heat tolerance. On the other hand the CPT1 expression was responsible that regulates energy homeostasis and appetite, which underlies the genetic establishment for our variable reactions that drive changed behaviours under heat condition (Ka et al., 2013).

4. Conclusion

Environmental conditions affected the genetic backgrounds of local breeds. The expression of genes responsible for heat tolerance was increased in breeds that inhabit desert or warm climates compared to that in breeds that inhabit moderate climates. Moreover, the naked neck gene plays an important role in improving body weight and heat tolerance by increasing *HSP70* gene expression rather than only by reducing feather cover. The results obtained recommended using the Sinia naked neck chicken as a male line in commercial parent stock to produce broiler chicks adapted to the hot and warm climates.

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