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Molecular investigations of the effect of thermal manipulation during embryogenesis on muscle heat shock protein 70 and thermotolerance in broiler chickens

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ABSTRACT - The objective of this study was to elucidate the optimum protocol timing of thermal manipulation (TM) during embryogenesis, which underline genetic improvement of muscle thermotolerance acquisition. For the present study, 1,440 fertile eggs were divided randomly and equally into control (37.8 °C with 56% relative humidity) and four thermally manipulated groups (TM1, TM2, TM3, and TM4) subjected to 39 °C for 18 h with 65% relative humidity daily during different embryonic periods. Then, at day 35 post-hatch, all groups were subjected to thermal challenge at 43 °C for 6 h to identify the level of thermotolerance acquisition differences between them. Hsp70 mRNA expression was evaluated by using a relative quantitatively RT-qPCR. Single nucleotide polymorphisms sequence of the Hsp70 gene was evaluated by Sanger's sequencing method. Pectoral and thigh muscles samples were subjected to immunohistochemistry to detect Hsp70. Among TM conditions that were investigated, TM1 (39 °C for 18 h during embryonic days (ED) 7–11) induced a significant improvement in thermotolerance parameters (body temperature and T3 levels) during thermal challenge combined with an increase in the levels of Hsp70 mRNA and its protein with a high stability of nucleotide sequences in both pectoral and thigh muscles. The partial DNA sequence of Hsp70 gene in TM1 was reported, and nucleotide sequences were deposited in NCBI GenBank database with the accession numbers (MK852579) and (MK852580). Thigh muscle thermotolerance acquisition was higher than pectoral muscle during thermal challenge at 43 °C for 6 h. Thus, TM during ED7–11 may improve thermotolerance acquisition without adversely affecting performance.

Keywords: broiler, embryogenesis, heat shock protein 70, immunohistochemistry, thermal manipulation, triiodothyronine

1. Introduction

Chicken meat production costs globally are relatively high due to the expense of controlling the temperature in chicken houses under extremely hot weather conditions. Moreover, high temperatures in chicken houses cause heat-related stress illnesses leading to a high mortality rate (Wasti et al., 2020; Nawaz et al., 2021). Therefore, a thermal manipulation strategy during embryogenesis is considered one of the most effective and low-cost methods for focusing on the genetic improvement of thermotolerance acquisition in broilers to overcome the negative impact of heat stress during their post-hatch life (Al-Zghoul et al., 2015; Narinç et al., 2016; Dalab and Ali, 2019; Costa et al., 2020).

The combination of thermal manipulation and the genetic improvement of broiler chicken heat shock protein 70 (Hsp70) modulation has been very important due to biological functions of Hsp70, such as the binding, stabilizing, folding, refolding, and preserving of denatured proteins with an enhancement in thermotolerance acquisition (Liang et al., 2016; Al-Zghoul, 2018; Shehata et al., 2020; Goel et al., 2021). Genetic enhancement of Hsp70 can produce genetic polymorphisms that may result in different heat tolerance levels (Mazzi et al., 2003; Tamzil et al., 2013; Hassan et al., 2019; Galal and Radwan, 2020; Perini et al., 2021).

Therefore, the objective of this study was to investigate the effects of thermal manipulation during early, mid, late, and long-lasting broiler embryogenesis on an mRNA gene expression of Hsp70 in both pectoral and thigh muscles of embryos and during the subsequent thermal challenge (TC) on day 35 post-hatch. We also investigated the effect of thermal manipulation during embryogenesis on Hsp70 genetic polymorphisms and the physiological responses on day 35 post-hatch. Thus, the results of this research may provide a means for improving heat tolerance levels that can contribute to the selection of genetically heat-resistant broilers.

2. Material and Methods

2.1. Pre-egg incubation management

All sampling and experimental incubating and hatching management conditions was conducted with approval from the institutional Animal Care and Use Committee, Al-Ahsa, Saudi Arabia (25°21'52.45" N and 49°33'55.15" E). One thousand and seven hundred Ross 708 broiler eggs were supplied from a 36-week-old broiler flock (Al-Ahsa, Saudi Arabia). Broken as well as abnormally small and large eggs were excluded (58 g < eggs < 65 g) before the first incubation day (1,550 uniformly sized eggs remained).

2.2. Egg incubation and thermal manipulation management

The 1,550 uniformly sized eggs were incubated in semi-commercial incubators (type OVA-Easy 380 Advance Series II, Brinsea, Sandford, UK) at 37.8 °C with 56% relative humidity (RH) up to embryonic day 7 (ED7), then egg candling was performed at ED7 to exclude any dead embryos and infertile eggs, resulting in 1,440 fertile eggs. These fertile eggs were distributed randomly into five treatment groups; the first was the untreated control group that remained incubated at 37.8 °C with 56% RH, whereas TM1, TM2, TM3, and TM4 were thermally subjected to 39 °C for 18 h with 65% RH daily during ED7–11, ED11–15, ED15–18, and ED7–18, respectively (288 each).

2.3. Hatching and thermal challenge management

All one-day-old hatched chicks were recorded, and water and feed were supplied *ad libitum* to the chicks, and they were kept brooding at an initial house temperature of 31±1 °C, which was reduced by an average of 0.2–0.3 °C per day to achieve a final house temperature of 22±1 °C by day 24 post-hatch. On day 35 post-hatch, chicks from each group were randomly and equally divided into two subgroups, naïve (N) and TC groups (100 each). On day 35 post-hatch, thermal challenge (43.0 °C at 1-, 3-, and 6-h intervals) was applied to the chicks of each TC subgroup, including the TC subgroup of the control, while the naïve chicks of each subgroup were kept under regular conditions (22±1 °C and 50–60% RH) in a separated room.

2.4. Sampling management

Pectoral and thigh muscle samples from five control embryos and from each thermally manipulated group were collected at the end of ED11, ED15, and ED18 for total RNA isolation of the embryonic Hsp70 (75 embryos, n = 5). On day 35 post-hatch, humanely manual cervical dislocation euthanasia

was applied and confirmed by loss of any reflexes and musculoskeletal movements; pectoral and thigh muscle samples were collected from five chicks from each TC subgroup (at 1-, 3-, and 6-h intervals of TC) and naïve chicks for total RNA isolation and immunohistochemistry (100 chicks, n = 5). The body temperature of 10 chicks per treatment group was measured and blood from the jugular vein was drawn at the beginning (0 h) and after 1, 3, and 6 h of TC exposure (200 chicks, n = 10) for measurement of serum triiodothyronine (T3) levels.

2.5. Relative quantitative RT-qPCR

2.5.1. RNA extraction and cDNA synthesis

Hsp70 mRNA expression levels during embryogenesis and during the subsequent TC on day 35 post-hatch were investigated and evaluated using a relative quantitative RT-qPCR analysis. The pectoral and thigh muscles were homogenized by Bead Ruptor (24 Bead Mill Homogenizer, OMNI, USA) and total RNA was extracted using the PureZOL TM RNA isolation method (BIO-RAD, Hercules, CA, USA). DNA was removed using a DNase I kit (Ambion), and the RNA samples were checked for their concentration and purity (260:280 nm absorbency) using a Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader (Bio-Tek, Winooski, Vermont, USA). RNA (2 μg) was reverse transcribed to cDNA in a reaction mixture using an iScriptc DNA synthesis kit (BIO-RAD, Hercules, CA, USA).

2.5.2. Relative quantification protocol

Relative quantitative CFX96 Touch™ real time qPCR analysis (BIO-RAD, Hercules, CA, USA) was performed using the ssoAdvanced™ SYBR Green Supermix kit (BIO-RAD, Hercules, CA, USA). The 20 μL reaction mix was prepared from 10 μL of the master mix, 2 μL of the forward primer (pm/μL), 2 μL of the reverse primer (pm/μL) (Table 1), 2 μL of cDNA from the sample, and 4 μL of nuclease-free water. Cycling parameters were 95 °C for 1 min, 40 cycles at 95 °C for 10 s, followed by 30 s at 60 °C, and 72 °C for 10 s with a final melting at 95 °C for 20 s. Triplicates from each cDNA were analyzed, fluorescence emission was detected, and relative quantification was calculated automatically according to the internal housekeeping control genes GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and ACTIN-1 (Actin, alpha) to normalize the threshold cycle (Ct) values of the other transcripts.

Table 1 - Real time-qPCR primer sequences

Primer	Primer sequence	GenBank reference
Hsp70	F-5-GACAAGAGTACAGGGAAGGAGAAC-3	FJ_217667.1
	R-5-CTGGTCACTGATCTTTCCCTTCAG-3	
GAPDH	F-5-GTGTTATCATCTCAGCTCCCTCAG-3	FJ_217667
	R-5-GGTCATAAGACCCTCCACAATG-3	
ACTA1	F-5-CCATCGGCAATGAGCGTTTC-3	NM_001031063.1
	R-5-GCATGCGGTCAGCAATACCT-3	

2.6. Single nucleotide polymorphisms (SNP)

The chicken Hsp70 gene sequence deposited in GenBank under the accession number J02579 was used as a reference sequence in our study. Primers were designed based on the identification of the single nucleotide polymorphisms (SNP) sequence of the Hsp70 gene to amplify several separate fragments at a special coding region (Table 2). The amplification of the Hsp70 gene was carried out using 25 μL of PCR reaction mixture as follows: 12.5 μL of GoTag Green Master Mix (Promega), 2 μL of 50 ng DNA, and 2 μL each of 10 pm/μL forward and reverse primers, and the final dilution was made by adding 6.5 μL of nuclease free water. The PCR program for F1-R1 was as follows: initial denaturing at 95 °C for 2 min

Table 2 - Polymerase chain reaction primer design for chicken Hsp70

Primer sequence	Position on reference sequence
F1- 5'GATTGGTCCTTAGCGTTCTGGC 3'	208
R1- 5'TGATCTCCACTTTGCCATGCTG 3'	479
F2- 5'TCATCATGTCTGGCAAAGGG 3'	387
R2- 5'CACTTGGTTCTTGGCAGCATC 3'	571
F3- 5'AACCGCACCACACCCAGCTATG 3'	497
R3- 5'CTGGGAGTCGTTGAAGTAAGCG 3'	856
F4- 5'CAACAGAGATAGGGTGGGAG 3'	1993
R4- 5'TGCCTTTATACACACCCAACAG 3'	2426

and 35 cycles at 95 °C for 1 min (denaturing), 65 °C for 1 min (primer annealing), and 72 °C for 1 min and 30 s (extension). The other reactions used similar PCR programs, but the annealing temperatures were 64.5, 64.5, and 61.5 °C, respectively. Horizontal (1.5%) agarose gel electrophoresis was used to check the amplification (Nucleic Acid Gel Electrophoresis, Bio-Rad, USA). The following regions of the Hsp70 gene were amplified: F1-R1 (271 bp), F2-R2 (184 bp), F3-R3 (359 bp), and F4-R4 (433 bp). All bands were purified and sequenced using Sanger's sequencing method from the forward direction to detect any SNP for each sample.

2.7. Immunohistochemistry (IHC-P) evaluation of Hsp70

Approximately 1 cm of pectoral and thigh muscle tissue was taken from five chicks in each TC subgroup at the end of 6 h of TC and from the naïve chicks on day 35 post-hatch and then fixed overnight in 4% paraformaldehyde. All the fixed samples were processed for immune-histochemical examination by dehydration, clearing, infiltration, and embedding. Tissue samples were sectioned at 5 μm using a microtome (Leica, Germany) and were deparaffinized by passing the Thermo Scientific™ SuperFrost™ Plus charged slides through xylene. Two changes were made for 5 min each followed by the hydration of the sections by dipping them for 30 s in degraded alcohol (100%, 100%, 95%, 80%, 70%). The slides were then washed for 2 × 5 min in tris-buffered saline (1X TBS) plus 0.025% triton X100 with gentle agitation. The slides were then blocked in 10% normal serum with 1% bovine serum albumin (BSA) in 1X TBS for 2 h at room temperature. The slides were left to drain for a few seconds and the sections were then wiped with tissue paper. A mouse monoclonal anti-Hsp70 antibody (ab2787) and a mouse- and rabbit-specific HRP/DAB (ABC) detection IHC kit (ab64264) were used to detect Hsp70. Slides were counterstained by immersing the tissue sections in Mayer's hematoxylin for 5 min and washing them under tap water for 10 min. Then, sections were dehydrated by dipping them in graded alcohol (70%, 95%, 100%, 100%) for a few seconds, and clearing them by using xylene—2 changes for 5 min each. Finally, the tissue sections were mounted by using DPX with cover slides (22 × 40 mm), and the staining was observed by Leica ICC50 W light microscopy under 10X and 40X magnification powers, Wi-Fi-capable digital camera detector, and Leica AirLab App software. Reaction reactivity was taken using image processing and analyzed using an imageJ 1.52a analyzer (Wayne Rasband, National Institute of Health, USA, http://imagej.nih.gov/ij).

2.8. Enzyme-linked immunosorbent assay (ELISA)

Chick's response to the TC was evaluated through the determination of serum T3 at the beginning of the TC (0 h) and after 1, 3, and 6 h of TC in the naïve and TC subgroups. The serum was isolated by the centrifugation of blood samples at 3,000 rpm for 10 min, then stored at −20 °C until further analysis. A commercial ELISA kit was used for the determination of the total T3 (MBS733878) (Mybiosource, California, San Diego, USA) on the ELISA reader (Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader, Bio-Tek. Winooski, Vermont, USA). The resulting concentrations of T3 were calculated according to the manufacturer's instruction protocols.

2.9. Statistical analysis

The original data were arranged using Excel 2007 software (Microsoft Corporation, Redmond, WA, USA). Data for body temperature (T^b), T3 concentrations, and Hps70 mRNA gene expressions were expressed as means±SE. The relative quantitative expression results were calculated using comparative ct–($2^{-\Delta\Delta Ct}$) method according to Livak and Schmittgen (2001). A two-way ANOVA followed by an all-pairs Bonferroni test were applied to compare the different parameters in each treatment group using IBM SPSS Statistics 20 software (IBM, Chicago, USA). Differences were considered significant at P<0.05.

3. Results

3.1. Relative quantification of muscle Hsp70 mRNA during embrogenesis

In ED11, the thermal manipulation of TM1 and TM4 from ED7 to ED11 resulted in a highly significant increase in the expression of Hsp70 in both the pectoral and thigh muscles when compared with those of the control (P<0.05) (Table 3). On ED15, thermal manipulation of the TM2 group from ED11 to ED15 resulted in a significant increase in the mRNA expression of Hsp70 in both the pectoral and thigh muscles when compared with all other treatment groups and those of the control (P<0.05) (Table 3). On ED18, the thermal manipulation of TM3 and TM4 groups from ED15 to ED18 showed that the pectoral mRNA expression of Hsp70 was significantly higher when compared with those of the control (P<0.05). However, in the thigh muscle, there was no significant difference between all the treatment groups and those of the control group (Table 3).

Furthermore, a comparative gene study was also performed on the relative normalized expression of Hsp70 mRNA levels in both the pectoral and thigh muscles from ED11, ED15, and ED18 (Table 4). The

Table 3 - Relative quantification of muscle Hsp70 mRNA during embrogenesis

Hsp70	ED11		ED15		ED18	
	Expression (fold)		Expression (fold)		Expression (fold)	
	Pectoral	Thigh	Pectoral	Thigh	Pectoral	Thigh
Control[1]	1.0±0.7c	1.0±0.4c	1.0±0.1b	1.0±0.2b	1.0±0.3c	1.0±0.2a
TM1	5.29±0.4a	4.02±0.9b	0.61±0.2b	0.26±0.06c	2.19±0.4a	0.70±0.08a
TM2	1.42±0.8c	1.39±0.3c	7.38±0.1a	11.87±0.2a	0.76±0.3c	0.79±0.1a
TM3	1.34±1.3c	1.47±0.6c	0.68±0.2b	0.63±0.1b	1.98±0.3b	1.08±0.2a
TM4	4.04±1.0b	5.05±0.9a	0.76±0.3b	0.38±0.07c	1.88±0.5b	1.19±0.3a

ED - embryonic day.
Control - 37.8 °C; TM1 - thermal manipulation from ED7–11 at 39 °C for 18 h; TM2 - thermal manipulation from ED11–15 at 39 °C for 18 h; TM3 - thermal manipulation from ED15–18 at 39 °C for 18 h; TM4 - thermal manipulation from ED7–18 at 39 °C for 18 h.
[1] Compared with the control of each embryonic day.
a-c - Within the same day, means±SD with different letters differ significantly (P<0.05).

Table 4 - Comparative gene study of relative quantification of muscle Hsp70 mRNA during embrogenesis

Hsp70	ED11		ED15		ED18	
	Expression (fold)		Expression (fold)		Expression (fold)	
	Pectoral	Thigh	Pectoral	Thigh	Pectoral	Thigh
Control[1]	1.0±0.7	1.0±0.4	3.01±0.3	1.18±0.2	0.25±0.3	0.19±0.06
TM1	5.29±0.4	4.02±0.9	2.89±0.4	0.40±0.1	0.51±0.1	0.13±0.01
TM2	1.42±0.8	1.39±0.3	11.11±0.6	30.85±2.3	0.18±0.01	0.14±0.02
TM3	1.34±1.3	1.47±0.6	1.73±0.3	0.97±0.2	0.51±0.08	0.20±0.04
TM4	4.04±1.0	5.05±0.9	1.62±0.3	0.57±0.1	0.51±0.1	0.22±0.07

ED - embryonic day.
Control - 37.8 °C; TM1 - thermal manipulation from ED7–11 at 39 °C for 18 h; TM2 - thermal manipulation from ED11–15 at 39 °C for 18 h; TM3 - thermal manipulation from ED15–18 at 39 °C for 18 h; TM4 - thermal manipulation from ED7–18 at 39 °C for 18 h.
[1] Compared to the control of pectoral and thigh muscle of ED11—pectoral to pectoral and thigh to thigh.
a-c - Within the same day, means±SD with different letters differ significantly (P<0.05).

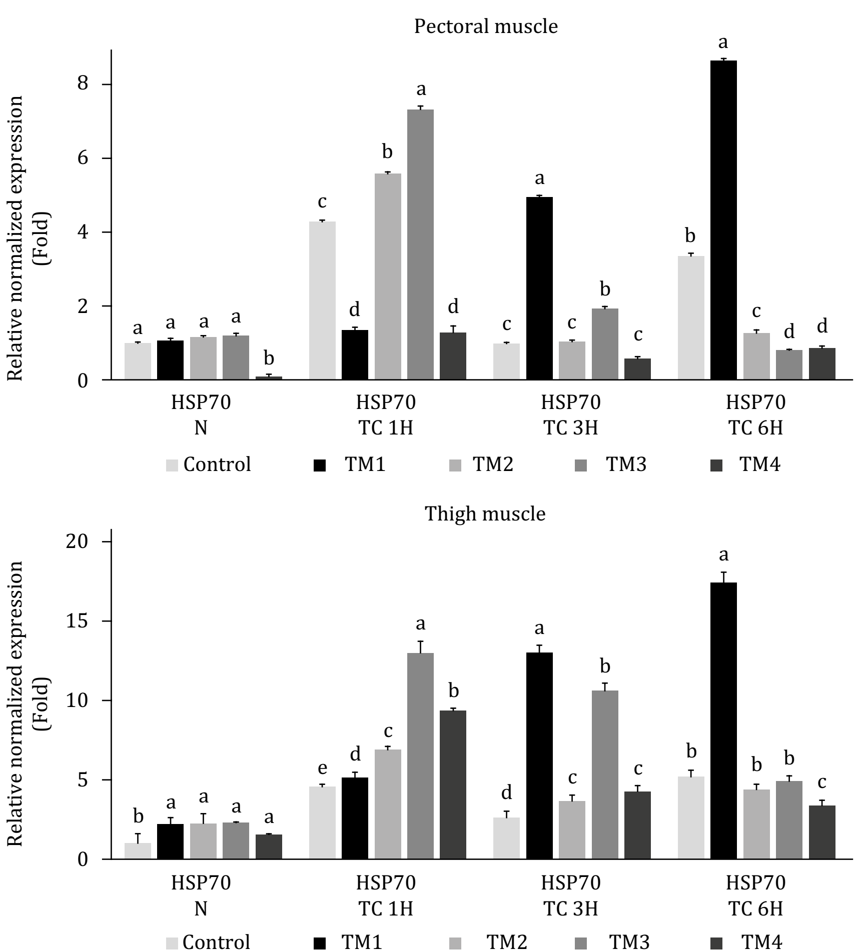
results obtained from this study showed that the highest gene expressions (up-regulations) were found to be in early embryogenesis, and the lowest gene expression was observed from ED15 to ED18. In the early and middle thermal manipulation of TM1 (ED7–ED11), TM2 (ED11–ED15), and TM4 (ED7 up to ED11), the results showed that there was up-regulated expression (significantly increased expression) of Hsp70 in both the pectoral and thigh muscles. However, the results obtained for the control and TM3 showed that there were down-regulation expressions (significantly lowered expression) of Hsp70 in both the pectoral and thigh muscles.

3.2. Relative quantification of muscle Hsp70 mRNA at day 35 post-hatch

In the pectoral muscle of the post-hatched chicks at day 35 post-hatch, embryonic thermal manipulation made no significant difference in Hsp70 mRNA expression in TM1, TM2, and TM3 when compared with the control, while it showed a lower significant Hsp70 mRNA expression in TM4 when compared with the control ($P<0.05$). In the thigh muscle, it was observed that Hsp70 mRNA expression was highly significant in all treatment groups when compared with the control ($P<0.05$) (Figure 1).

3.3. Relative quantification of muscle Hsp70 mRNA during thermal challenge at day 35 post-hatch

The results showed that a TC of 1 h at day 35 post-hatch changed the mRNA expression of Hsp70 in the pectoral muscles of the control (3-fold), TM2 (4.5-fold), and TM3 (6.5-fold) more than in those of



N - naïve chicks.
a-e - Folds with different letters differ significantly ($P<0.05$).

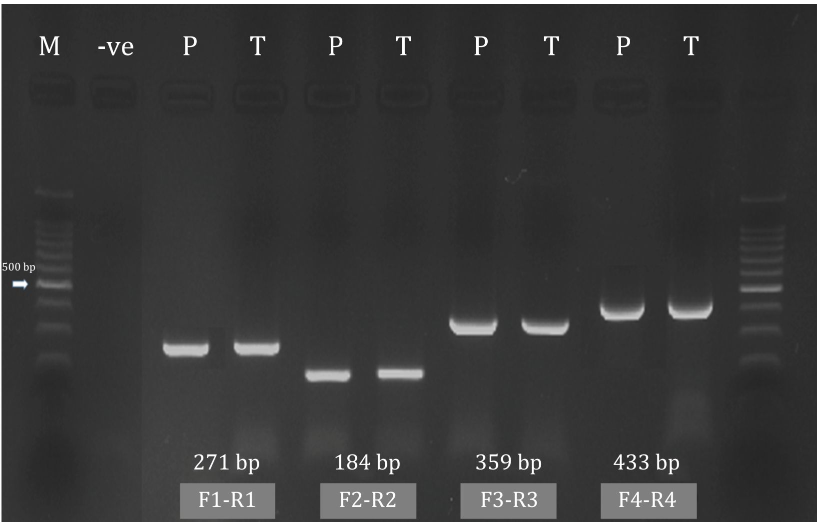
Figure 1 - Effect of thermal challenge (TC) on the mRNA level of Hsp70 in pectoral and thigh muscles in chicks subjected to TC at 43 °C for 1 h, 3 h, and 6 h at day 35 post-hatch.

TM1 and TM4, which were found to be at the same levels as in the naïve chicks (P<0.05) (Figure 1). In the thigh muscles, 1 h of TC changed the Hsp70 mRNA expression of the controls (3-fold), TM1 (4-fold), TM2 (5.5-fold), TM3 (12.5-fold), and TM4 (8.5-fold) more than in the naïve chicks (P<0.05) (Figure 1). After 3 h of TC, Hsp70 mRNA expression in the pectoral muscles of TM1 started to change at a more highly significant level (4-fold) compared with the 3 h of TC in the control, TM2, and TM4 (P<0.05). The TM1 was three times higher than TM3. In the case of TM1, when comparing 3 h of TC with 1 h of TC, it was noticed that 3 h of TC increased the mRNA expression of Hsp70 4.5 times more than 1 h of TC in TM1 and the naïve chicks (P<0.05). In thigh muscles, after 3 h of TC change, the Hsp70 mRNA expression of TM1 was 10.5 times higher than that of the control (3 h of TC), TM2 (9.5-fold), TM3 (2-fold), and TM4 (8.5-fold) (P<0.05). Moreover, 3 h of TC also induced eight times as much mRNA expression of Hsp70 than that of TM1 (1 h TC) and 12 times as much as the level of the TM1 naïve chicks (P<0.05). Six hours of TC influenced and changed the Hsp70 mRNA expression in the pectoral muscles of TM1 at a higher significant level that was four times as much as in the 6 h TC control (P<0.05). At the same time, TM1 was higher (6.5-fold) than TM2, TM3, and TM4 (P<0.05).

Furthermore, the Hsp70 mRNA expression of TM1 (6 h of TC) was found to be 2.5 times higher than the expression level of TM1 after 3 h of TC and 7.5 times higher than TM1 after 1 h of TC and the TM1 naïve chicks (P<0.05) (Figure 1). In thigh muscles, after 6 h of TC change, the Hsp70 mRNA expression of TM1 was 13 times higher than in the control, TM2, and TM3 and 14 times higher in TM4 (P<0.05). Moreover, 6 h of TC also induced five times as much Hsp70 mRNA expression as in TM1 (3 h TC), 13 times higher than the level of TM1 (1 h TC) and 15 times higher than the naïve chicks (P<0.05).

3.4. Polymerase chain reaction (PCR)

The results of the PCR amplification of the Hsp70 gene were obtained by horizontal (1.5%) agarose gel electrophoresis (Figure 2). In both the pectoral and thigh muscles, the PCR results were positive in all beginning and ending coding regions: F1-R1 (271 bp), F2-R2 (184 bp), F3-R3 (359 bp), and F4-R4 (433 bp) during ED 11, ED15, and ED 18 and also at day 35 post-hatch.



F1-R1: forward and reverse primers of coding region 1 (271 bp); F2-R2: forward and reverse primers of coding region 2 (184 bp); F3-R3: forward and reverse primers of coding region 3 (359 bp); F4-R4: forward and reverse primers of coding region 4 (433 bp); Lane M represent DNA Ladder (100 bp) and lane –ve represent negative control of Hsp70 DNA.

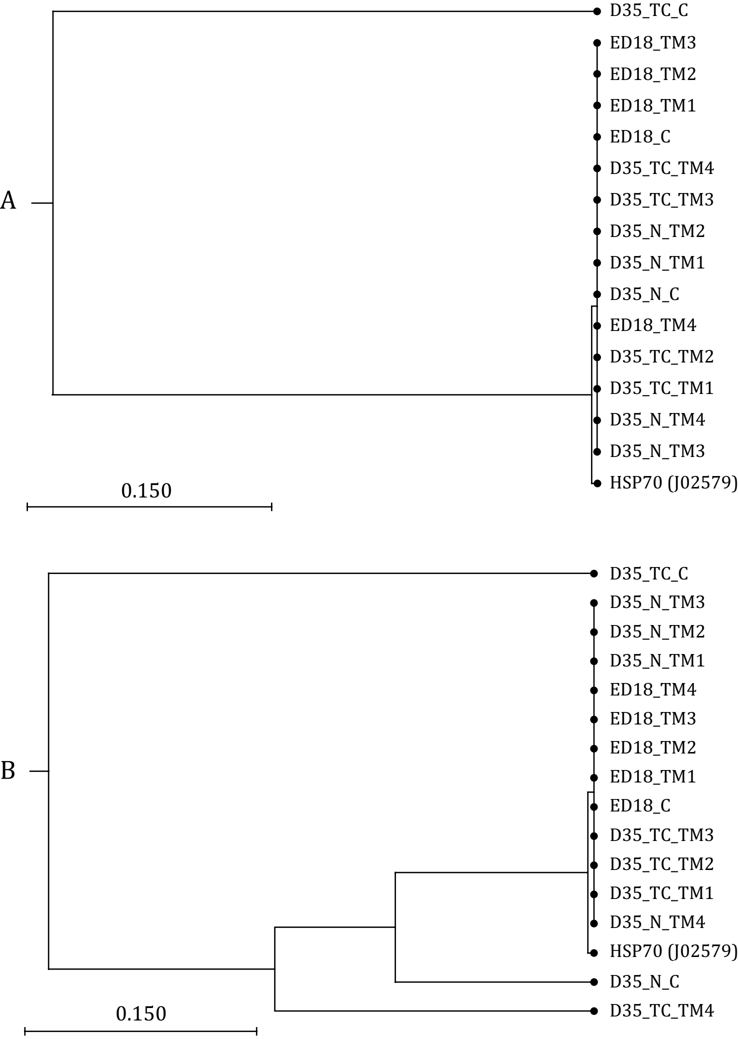
Figure 2 - Polymerase chain reaction amplifications of the Hsp70 gene in 1.5% agarose gel electrophoresis in pectoral (P) and thigh (T) muscles.

3.4.1. Hsp70 single nucleotide polymorphisms (SNP)

The results of the first nucleotide sequence alignment of Hsp70, assembling coding region 1 (F1 + F2 + F3) of the pectoral and thigh muscle pooled samples, started from the alignment position of 391 up to 839, and the second nucleotide sequence alignment of Hsp70 coding region 2 (F5) of the same samples started from alignment position 2044 up to 2295. These alignments were carried out according to the reference chicken embryo Hsp70 GenBank gene under accession number J02579. The first starting codon (ATG) in all groups establishes the reading frame, and the backbone sequence is defined by contiguous triplets and extends to the termination codon, TAA.

In embryonic and post-hatched muscles, the nucleotide sequence data showed 99% alignment similarity in the control of ED18 and the naïve D35 and all treatment groups when compared with the Hsp70 reference gene (Figure 3A). The 1% of alignment difference contained some polymorphisms, such as the transition of nucleotides (A–G), (G–C), (C–A), and (C–G) at alignment positions 649, 812, 816, and 2138, respectively. Nucleotide (A) at position 468 in the 6 h TC control was deleted (Table 5).

The transition of nucleotides (A–G) (TCA + TCG: serine) produced a silent SNP in the naïve TM2 and TM4 groups and in the 6 h of TC of the TM2, TM3, and TM4 groups at day 35 post-hatch when compared



A - nucleotide similarity alignment; B - amino acid similarity alignment.

Figure 3 - Phylogenetic tree of Hsp70 gene from embryonic and post-hatched muscles compared with Hsp70 reference gene (J02579).

with the control, TM1, TM3, and the Hsp70 reference gene (Table 5). However, the transition of the nucleotide in coding region 1 (G–C and C–A) and in coding region 2 (C–G) produced three missense mutations in the sequence of the amino acid translation—glutamic acid (E) to glutamine (Q), threonine (T) to asparagine (N), and serine (S) to tryptophan (W)—in the control and all treatment groups when compared with the Hsp70 reference gene. Nucleotide A was deleted at position 468 in the backbone of the control group (6 h of TC), which produced a nonsense nonsynonymous frameshift mutation in coding region 1 when compared to the Hsp70 reference gene. The frameshift mutation sequence produced five premature stop codons (TGA) in the TC control (6 h of TC) (Figure 3B). This will be translated to a low quality or incorrect Hsp70 protein in the muscles of this group when compared with the better conserved sequence of the other thermal manipulated groups and the Hsp70 reference gene.

The translation of the coding nucleotides of the control and all treatment groups during embryonic day 18 showed minor differences in the length of amino acid backbone sequences when compared with the Hsp70 reference gene, while in the control group (6 h TC) at day 35 post-hatch, the amino acid backbone sequences had major differences in length and numbers when compared with the Hsp70 reference gene. Our sequence data from assembling coding region 1 (F2 + F3 + F4) and coding region 2 (F5) were submitted to the GenBank database under accession numbers (MK852579) and (MK852580), respectively, and the protein sequence data for coding region 1 and coding region 2 were submitted to the GenBank database under accession numbers (QCO90626) and (QCO90627), respectively.

Table 5 - Single nucleotide polymorphisms (SNP) of Hsp70 coding region 1 (F1-F4) and coding region 2 (F5) from alignment position 391 up to 839 and 2044 up to 2295 according to GenBank chicken HSP70 reference gene (J02579)

<table>
<tr><th rowspan="3">Information</th><th colspan="6">Nucleotide SNP</th></tr>
<tr><th colspan="4">SNP alignment position</th><th colspan="2">Nonsynonymous SNP count</th></tr>
<tr><th>A</th><th>C</th><th>G</th><th>T</th><th>Missense
(Different amino acid) / total amino acid counts</th><th>Nonsense
(Premature stop codon) / total amino acid counts</th></tr>
<tr><td>CHK HSP 70 REF</td><td>N/A</td><td>N/A</td><td>N/A</td><td>N/A</td><td>N/A</td><td>N/A</td></tr>
<tr><td>ED18_C</td><td rowspan="7">N/A</td><td rowspan="10">C to A
816
C to G
2138</td><td rowspan="15">G to C
812</td><td rowspan="15">N/A</td><td rowspan="10">3/232
(1.29%)</td><td rowspan="10">N/A</td></tr>
<tr><td>ED18_TM1</td></tr>
<tr><td>ED18_TM2</td></tr>
<tr><td>ED18_TM3</td></tr>
<tr><td>ED18_TM4</td></tr>
<tr><td>D35_N_C</td></tr>
<tr><td>D35_N_TM1</td></tr>
<tr><td>D35_N_TM2</td><td>A to G (648)
synonymous</td></tr>
<tr><td>D35_N_TM3</td><td>N/A</td></tr>
<tr><td>D35_N_TM4</td><td>A to G (649)
synonymous</td></tr>
<tr><td>D35_TC_C</td><td>Deletion
468</td><td>C to A
816</td><td>111/232
(47.84%)</td><td>5/232
(2.15%)</td></tr>
<tr><td>D35_TC_TM1</td><td>N/A</td><td rowspan="4">C to A
816
C to G
2138</td><td rowspan="4">3/232
(1.29%)</td><td rowspan="4">N/A</td></tr>
<tr><td>D35_TC_TM2</td><td>A to G (648)
synonymous</td></tr>
<tr><td>D35_TC_TM3</td><td>A to G (648)
synonymous</td></tr>
<tr><td>D35_TC_TM4</td><td>A to G (648)
synonymous</td></tr>
</table>

ED - embryonic day; D35 - day 35 post-hatch; N - naïve; TC - thermal challenge; A - adenine; C - cytosine; G - guanine; T - thymine.
Control - 37.8 °C; TM1 - thermal manipulation from ED7–11 at 39 °C for 18 h; TM2 - thermal manipulation from ED11–15 at 39 °C for 18 h; TM3 - thermal manipulation from ED15–18 at 39 °C for 18 h; TM4 - thermal manipulation from ED7–18 at 39 °C for 18 h.

3.5. Immunohistological detection of the Hsp70 antibody at day 35 post-hatch

In the pectoral muscles, the cross sections of the naïve chicks showed very weak reactivity to the anti-Hsp70 antibody of the control group when compared with the weak reactivity in TM2, TM3, and TM4 and the moderate reactivity in TM1. Moreover, the longitudinal sections of the pectoral muscles of the naïve chicks showed a weak reactivity to the anti-Hsp70 antibody in the control, TM2, TM3, and TM4 groups when compared with the moderate reactivity in TM1. Thus, TM1 showed higher anti-Hsp70 antibody reactivity labeling in both cross and longitudinal sections when compared with other treatment groups and the control (Table 6). Thigh muscles of the naïve chicks showed weak reactivity to the anti-Hsp70 antibody in both cross and longitudinal sections of the control and TM3 group when compared with the moderate reactivity in TM1, TM2, and TM4 (Table 6).

However, in TC chicks, the pectoral muscles showed intense reactivity to the anti-Hsp70 antibody in both cross and longitudinal sections of the control group when compared with the moderate reactivity in TM1, TM2, and TM3 and the weak reactivity in TM4 (Table 6). However, the thigh muscles of the TC chicks showed intense reactivity to the anti-Hsp70 antibody in both cross and longitudinal sections of the TM1 and TM4 groups compared with the moderate reactivity in TM2 and TM3 and the weak reactivity in the control.

In the present study, the expression of Hsp70 was detected in the nucleus and cytoplasm of myocyte cells. It was demonstrated that the Hsp70 positive reaction in the nuclei of the muscle tissues was particularly intense in the myofiber nucleus of TM1 in addition to the cytoplasm of the TC groups (Figure 4).

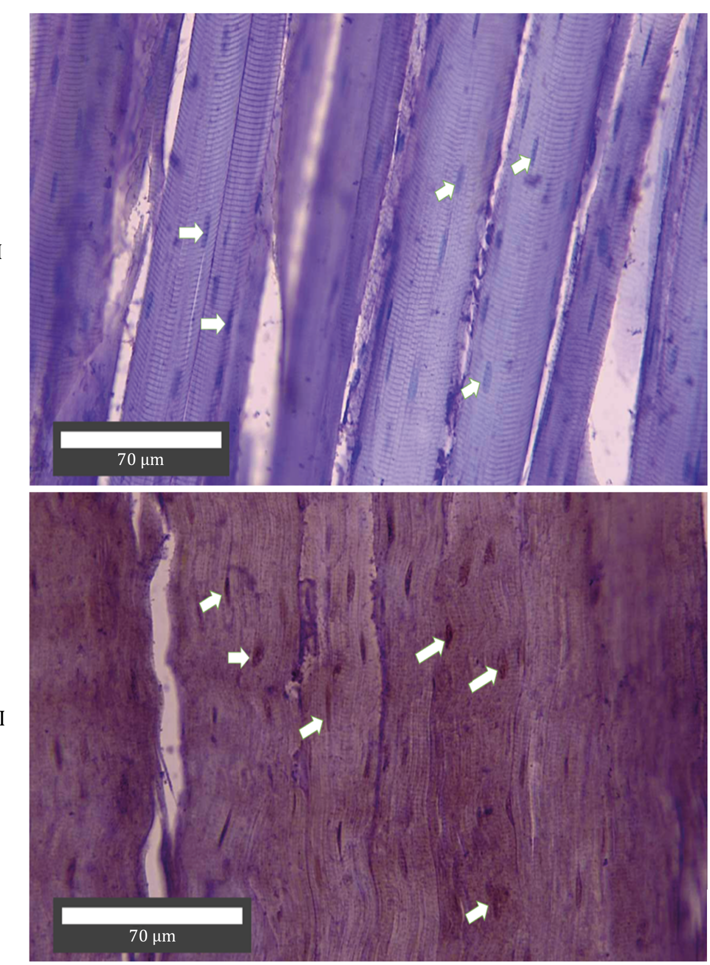
Table 6 - Semi-quantitatively immunohistochemical labelling patterns of Hsp70 on day 35 post-hatch

	Mouse monoclonal anti-Hsp70 antibody				
	Control	TM1	TM2	TM3	TM4
Pectoral muscle					
Naïve cross section	+	+ + +	+ +	+ +	+ +
(6 h) TC cross section	+ + + +	+ + +	+ + +	+ + +	+ +
Naïve longitudinal section	+ +	+ + +	+ +	+ +	+ +
(6 h) TC longitudinal section	+ + + +	+ + +	+ + +	+ + +	+ +
Thigh muscle					
Naïve cross section	+ +	+ + +	+ + +	+ +	+ + +
(6 h) TC cross section	+ +	+ + + +	+ + +	+ + +	+ + + +
Naïve longitudinal section	+ +	+ + +	+ + +	+ +	+ + +
(6 h) TC longitudinal section	+ +	+ + + +	+ + +	+ + +	+ + + +

TC - thermal challenge.
Control - 37.8 °C; TM1 - thermal manipulation from ED7–11 at 39 °C for 18 h; TM2 - thermal manipulation from ED11–15 at 39 °C for 18 h; TM3 - thermal manipulation from ED15–18 at 39 °C for 18 h; TM4 - thermal manipulation from ED7–18 at 39 °C for 18 h.
(+) = very weak reactivity; (++) = weak reactivity; (+++) = moderate reactivity; (++++) = intense reactivity.
Myofibers reactivity to mouse monoclonal anti-Hsp70 antibody (ab2787) in naïve and TC chicken subjected to thermal challenge at 43 °C for 6 h (TC) during day 35 post-hatch.

3.6. Effect of thermal challenge on T^b and T3 of naïve and thermal-challenged chickens

Embryonic thermal manipulation resulted in no significant differences in T^b between the control and the TM naïve groups. In the TC groups, the first and third hour of TC resulted in significant increases in T^b in the control and all treatment groups when compared with their naïve groups ($P<0.05$). The highest T^b was recorded in the control after 3 h of TC (43 °C) when compared with all treatment groups at 1 h and 3 h of TC. However, TM1 at 6 h of TC showed significantly lower T^b (41.9 °C) and no mortality when compared with the control, TM2, TM3, and TM4, which showed a 20–30% mortality rate (Table 7).



Hsp70 overexpression is stained by DAB/chromogen and counter stained with hematoxylin under magnification power 40X.
I - negative control of Hsp70 in longitudinal section; II - positive reaction of Hsp70 in longitudinal section shows strong cytoplasmic positivity in myocytes in both muscle fiber and its nucleus.

Figure 4 - Chromogenic immunohistochemistry of Hsp70 in longitudinal muscle section of chicks subjected to thermal challenge at 43 °C for 6 h during day 35 post-hatch.

Table 7 - Effect of thermal challenge (TC) at 43 °C for 6 h at day 35 on body temperature (T^b) and serum triiodothyronine (T3) levels after 1, 3, and 6 h of TC (n = 10)

	Control	TM1	TM2	TM3	TM4
T^b (°C)					
Naïve	41.4±0.2ax	41.2±0.2ax	41.3±0.2ax	41.4±0.2ax	41.4±0.4ax
TC 1 h	43.7±0.4ay	42.9±0.1by	42.8±0.1by	43.8±0.2ay	43.6±0.1ay
TC 3 h	44.3±0.6ay	42.6±0.3by	42.7±0.3by	43.9±0.3cy	42.9±0.6by
TC 6 h	42.5±0.4ay	41.9±0.2bx	42.3±0.4ay	42.9±0.5cy	42.3±0.3ay
T3 (pg/mL)					
Naïve	89.92±8.8aw	16.21±4.8bw	590.91±6.9cw	571.29±9.4dw	427.78±9.2ew
TC 1 h	109.56±8.2ax	16.69±4.85bw	149.93±7.8cx	55.47±3.3dx	114.11±7.9ex
TC 3 h	112.23±11.1ax	64.45±5.8bx	181.34±6.9cy	148.75±8.4dy	339.85±10.5ey
TC 6 h	133.46±10.4ay	53.89±5.9by	59.02±3.41cz	325.88±10.4dz	289.22±6.16ez
Mortality (%)	30	0	20	30	20

Control - 37.8 °C; TM1 - thermal manipulation from ED7–11 at 39 °C for 18 h; TM2 - thermal manipulation from ED11–15 at 39 °C for 18 h; TM3 - thermal manipulation from ED15–18 at 39 °C for 18 h; TM4 - thermal manipulation from ED7–18 at 39 °C for 18 h.
a-e - Within rows, means±SD with different letters differ significantly (P<0.05).
x-z - Between naïve and TC chicks within a group, means±SD with different letters differ significantly (P<0.05).

Embryonic thermal manipulation resulted in a significant reduction in T3 levels (16.21 pg/mL) in TM1 chicks when compared with the control (89.92 pg/mL) and TM2, TM3, and TM4 (427.78–590.91 pg/mL) (P<0.05). One hour of TC caused a significant reduction in T3 levels in TM2, TM3, and TM4 when compared with those of the naïve groups, and there were no significant effects on TM1 (P<0.05). Moreover, 3 h of TC resulted in a significant increase in T3 levels in TM1, TM2, TM3, and TM4 when compared with 1h of TC groups, and there were no significant effects on the control group (P<0.05) (Table 7). The control group, however, showed a significant increase in T3 levels from 1 h to 6 h of TC when compared with those of the control naïve group (P<0.05). However, TM1 and TM2 at 6 h of TC showed significantly lower T3 (53.89 pg/mL and 59.02 pg/mL) when compared with the control (133.46 pg/mL) and TM3 and TM4 (289.22–325.88 pg/mL).

4. Discussion

Tissues of thermally manipulated animals have been shown to improve stability during hyperthermia. It has been suggested that this is regulated by Hsp70 (Wang and Edens, 1998; Bodine et al., 2001; Al-Zghoul et al., 2013; Al-Zghoul et al., 2015). In this study, we examined the effect of thermal manipulation during broiler-chicken embryogenesis in Hsp70 mRNA expression in the pectoral and thigh muscles during early TM1 (ED7–11), middle TM2 (ED11–15), late TM3 (ED15–18), and long-lasting TM4 (ED7–18). Thermal manipulation resulted in a significant increase in Hsp70 mRNA expression in the pectoral and thigh muscles in TM1 at ED11, TM2 at ED15, TM3 at ED18, and TM4 at ED18 when compared with the control. This expression correlated with the initial duration of thermal manipulation (ED7–11, ED11–15, ED15–18, and ED7–18).

It appears that thermal manipulation during embryogenesis may be associated with increased Hsp70 in pectoral and thigh muscles. There is considerable agreement in the literature about the role of Hsp70 in thermotolerance development, and its expression has been correlated with playing an important role in heat stress recovery, as previously reported by Gong and Golic (2006) and Al-Aqil and Zulkifli (2009).

Heat stress can influence many biological processes, including the formation of memory (Foster et al., 2015), which is a vital process that enables an individual to adapt its behavior to current or future conditions (Teskey et al., 2012). Memory formation is dynamic, and environmental stressors can change the way an animal is able to learn and form memory, either by enhancing or blocking memory formation, depending on the nature of the stress and timing relative to the learning period (Teskey et al., 2012).

To examine whether thermal manipulation during embryogenesis induces Hsp70 expression, thus enhancing thermal memory formation later than day 35 post-hatch, we subjected TC groups to a thermally stressful condition (43 °C at 6-h intervals) at day 35 post-hatch. However, our findings showed that TC significantly increased Hsp70 mRNA expression in the pectoral muscles in the control (3.29-fold), TM2 (4.5-fold), and TM3 (6.29-fold) during the first hour of exposure when compared with their counterpart naïve groups with no effect in TM1 and TM4. However, in the thigh muscles, our data showed significant increases in Hsp70 expression in the control and all treatment groups after 1 h of TC when compared with their counterpart naïve groups.

This possibly means that the pre-thermally manipulated groups during early embryogenesis (TM1, ED7–11) suffered less stress during the first hour of TC exposure than the control and other groups. This lower response in the pre-thermally manipulated group might be due to heat stress acclimation, lasting up to 35 days, and could be indicative of acquired thermotolerance; thus, the Hsp70 response would be considered a cellular thermometer (Craig and Gross, 1991). In addition, the different responses of both the pectoral and thigh muscles to TC after 1 h may mean that the thigh muscles suffered more stress than the pectoral muscles.

At 3 h and 6 h of TC, there was up-regulation of Hsp70 expression, particularly in TM1 (4-fold and 7-fold, respectively), when compared with their counterpart naïve groups. The observed increase in the synthesis of Hsp70 in TM1, following exposure at 3 h and 6 h of TC, suggests that 1 h of TC was

unable to induce HSP70, but it was induced over longer periods (3 h and 6 h of TC), which may indicate that the time of exposure was not long enough or certain adaptations had already occurred in these pre-thermally manipulated birds (TM1, ED7–11). In either case, this may confirm that Hsp70 may play a role in the long-term memory formation of thermotolerance in relation to thermal stimulus.

Our results indicate a long-term (five to six weeks after termination of TM) induction of the expression of Hsp70 in the thermally manipulated chicks (TM1, ED7–11), improved their thermotolerance acquisition.

Enhanced Hsp70 expression may be a response to stressful environments, and it may improve cell survival by protecting the proteins from degradation and facilitating their refolding (Pratt, 1993; Hartl, 1996). In the present study, it was observed that an Hsp70-positive reaction was found in the nuclei and cytoplasm of both pectoral and thigh muscle tissues of the naïve and TC chicks in the control and all treatment groups. In addition, it was found that an Hsp70-positive reaction was more intense in the TC groups when compared with their counterpart naïve chicks. This may confirm that Hsp70 plays a role in the long-term protection of heat stress in muscles, which induces thermotolerance.

The present findings highlight the importance of Hsp70 in myocyte protection and suggests an association between Hsp70 in myocytes and thermotolerance after a thermal challenge. The data pertaining to protein synthesis and mRNA transcription are similar to the findings of previous studies (Wang and Edens, 1993; Gabriel et al., 1996). Together, both mRNA expression and Hsp70-protein detection in our findings on the pectoral and thigh muscles suggest that there is a constant relationship between them in response to thermal challenges, and this response was higher in TM chicks, particularly in TM1 (ED 7–11), compared with the control.

The present study was the first extensive study in Saudi Arabia that generated substantial data for understanding the types and distribution of naturally evolved nucleotides and their related amino acid polymorphisms in Hsp70 in broiler muscles. Moreover, this study was designed to detect the sequence variation of chicken Hsp70. Mazzi et al. (2003) reported a natural genetic polymorphism of Hsp70 in three chicken breeds (Hubbard-Pettersen, Naked Neck Label Rouge, and PP1). They also found three different alleles when compared with the Hsp70 reference gene (J02579).

Over the last decade, many studies have reported certain numbers of SNP in Hsp70 that may be associated with heat tolerance, such as the 2473 T/C human HSPA1L (Singh et al., 2006) and the 2895C insertion-deletion in the promoter region of bovine Hsp70.1, and their role in the regulation of mRNA expression for summer heat tolerance (Deb et al., 2013). These data provide clues to understanding the mechanism for the heat response.

The results of this study showed that the deletion change in the nucleotide sequence of the Hsp70 coding region 1 of the control after the 6 h of TC revealed a new amino acid sequence that is different from the reference Hsp70 gene with five premature stopping codons. The results of this frameshift mutation produced non-functional proteins and, subsequently, non-functional heat tolerance during heat stress, while TM1, TM2, and TM3 were the most similar to the reference Hsp70 both during embryonic day 18 and day 35 post-hatch. Our data demonstrates that Hsp70 in TM1, TM2, and TM3 was associated with active Hsp70 during heat stress and that the stress did not interfere with the mRNA expression, transcription, and translation processes. This allele seems to have a protective role against heat stress when compared with the allele of the control TC group, which resulted in a low-quality Hsp70 or non-functional role after the mutation in their gene sequence. More research is needed to elucidate whether Hsp70 may be used as a good indicator of thermotolerance. Thus, further investigation of the role of Hsp70 in thermal memory formation is warranted.

The body temperature of domestic chickens is maintained within a relatively narrow range that is usually reflected by the upper and lower limits of a circadian rhythm in their deep body temperature; the upper limit of the circadian rhythm is usually about 41.5 °C and the lower limit is about 40.5 °C (Daghir, 1995; Aengwanich, 2008). At day 35 post-hatch, our findings showed that thermal manipulation during embryogenesis produces no significant differences in T^b between the control (naïve) and that

of the TM (naïve) groups, which ranged between 41.2 and 41.4 °C. Previous studies (Piestun et al., 2008; Piestun et al., 2009; Piestun et al., 2011) have reported that chicks treated at 39.5 °C at ED7–16 had significantly lower T^b compared with the controls. Similarly, the findings of Al-Zghoul et al. (2013) indicated that thermal manipulation during embryogenesis was able to affect the thermoregulatory events of broiler chickens as detected by a lowered body temperature during the first two weeks of age.

After the birds are exposed to a high ambient temperature, their body temperature rises above their normal value (Aengwanich, 2008). When investigating thermal tolerance, a strong hyperthermia is needed to demonstrate any differences between the treatments applied. At day 35 post-hatch, our findings showed that chicken's exposure to TC (43 °C) for 1-3 h resulted in a significant increase in T^b in the control and in all treatment groups when compared with their naïve groups, with significant hyperthermia recorded in the control (44.3 °C) and TM3 (ED15–18; 43.9 °C) after 3 h of TC. These results indicate that TM during early and middle embryogenesis positively improves long-term thermotolerance acquisition in chicks when compared with the control and TM at late embryogenesis.

However, in contrast to the chicks' responses in the current study, Collin et al. (2007) reported a significant hyperthermia in all treated groups following TC at day 42, with higher mortality rates in treated groups when compared with the control. These results established by the authors show that TM at 39.5 °C for 3 h during early and/or late embryogenesis fails to improve long-term thermotolerance acquisition.

However, in this study, TM1 at 6 h of TC showed a significantly lower T^b (41.9 °C) and no mortality when compared with the control, TM2, TM3, and TM4, all of which showed a T^b of 42.3–42.9 °C and mortality rate of 20–30%. These results also indicated that early embryogenesis (ED7–11) was the best time to apply TM to improve long-term thermotolerance.

Our findings in TM1 were supported by Walstra et al. (2010), in which, instead of maintaining a constant temperature throughout incubation, increasing the temperature during certain periods of embryonic development might stimulate the development of different physiological control systems and body functions of the embryo, which might thereby increase the adaptation capacity of chicks at a later age.

Therefore, we hypothesized that thermal manipulation at 39 °C during early embryogenesis in broilers could induce epigenetic (temperature) adaptation and affect the adaptation capacity and performance at a later age. The differences in response to the thermal challenge between the TM and control chicks at day 35 post-hatch indicate that the thermoregulatory system has been affected by the TM applied (TM at ED7–11 at 39 °C for 18 h).

Chickens can improve their thermotolerance and adaptability by enhancing their chemical regulation, such as changing the levels of plasma hormones (Tankson et al., 2001). The importance of thyroid-gland hormones in the adaptation to heat stress is related to the central role that thyroid hormones play in the regulation of the metabolic rate of birds. Triiodothyronine is mainly involved in increasing metabolism by decreasing the rate of glucose oxidation and increasing the amount of the metabolic heat produced (McNabb, 1988; Tao et al., 2006). The current findings show that embryonic thermal manipulation results in a significant reduction in T3 levels (16.21 pg/mL) in TM1 (naïve) chicks when compared with the naïve control (89.92 pg/mL) at day 35 post-hatch. This indicated that TM at 39 °C for 18 h during embryogenesis (ED7–11) was able to affect the thermoregulatory events of the broiler chickens, which resulted in a lowered T3 in the TM1 chicks compared with the control.

At day 35 post-hatch, our findings showed that the chicken's exposure to a thermal challenge (43 °C) for 1 h caused a significant reduction in T3 levels in TM2, TM3, and TM4 when compared with those of the naïve groups and with no significant effects on TM1. Consistent with the present study, TC (41 °C/6 h at days 3, 7, and 42) caused a reduction in T3 levels in the TC chicks compared with the naïve control chicks (Al-Zghoul et al., 2013). The current findings in the control group showed a significant increase in T3 levels from 1 h up to 6 h of TC when compared with those of the control naïve group. This finding agrees with previous reports with regard to T3. Interestingly, the decline in serum T3 hormone levels in TM1 coincided with lowered T^b of the same chicks. A reduction in serum

T3 concentration during the TC in TM1 chicks suggests a lowered metabolic rate, which, in turn, leads to improved thermotolerance acquisition, as confirmed by previous studies (Iqbal et al., 1990; Yahav and McMurtry, 2001; Yahav et al., 2004a,b; Walstra et al., 2010; Loyau et al., 2013; Loyau et al., 2014; Loyau et al., 2015; Piestun et al., 2015; Loyau et al., 2016).

This study was undertaken to fill the gap in the studies that investigated the effect of TM on early, mid, and late broiler embryogenesis in relation to the thermotolerance acquisition of pectoral and thigh muscles of the Ross broiler chicken in Saudi Arabia. It can be concluded that, out of the TM conditions that were investigated, the TM1 treatment resulted in a significant improvement in thermotolerance acquisition. This was supported by a lower T^b and T3 and a higher induction of better conserved Hsp70 mRNA expression during the TC in both the pectoral and thigh muscles in both naïve and thermally challenged broiler chickens of the TM1 group at day 35 post-hatch when compared with those of the control without adversely affecting performance. The outcome of this research may provide the means for improving broiler thermotolerance efficiency. This may contribute to the increased adaptation of higher environmental heat stress in broiler chickens without negatively impacting their bodies' performance.

5. Conclusions

Thermal manipulation (39 °C for 18 h) daily during embryonic days 7–11 significantly improves thermotolerance acquisition parameters (body temperature and triiodothyronine) during thermal challenge. Moreover, it induces an increase in the levels of Hsp70 protein and mRNA in both pectoral and thigh muscles in thermal challenged broiler chickens more than in the control chickens. Thus, Hsp70 nucleotide and amino acid sequences are more stable in thermal manipulated chicks (39 °C for 18 h) in embryonic and post-hatch life (naïve and thermal challenge), which indicates more conserved mRNA transcription and amino acid translation during heat stress.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: A.M. Ali. Data curation: K.M. Alkhodair. Formal analysis: A.M. Ali and S.Y. Al-Ramadan. Funding acquisition: A.S. Dalab. Investigation: A.M. Ali, A.S. Dalab, T.A. Althnaian and S.Y. Al-Ramadan. Methodology: A.S. Dalab, T.A. Althnaian and S.Y. Al-Ramadan. Project administration: A.M. Ali. Resources: A.S. Dalab. Software: A.S. Dalab. Supervision: K.M. Alkhodair. Validation: A.S. Dalab. Visualization: A.S. Dalab and T.A. Althnaian. Writing-original draft: S.Y. Al-Ramadan. Writing-review & editing: A.S. Dalab.

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References

Aengwanich, W. 2008. Effects of high environmental temperature on the body temperature of Thai indigenous, Thai indigenous crossbred and broiler chickens. Asian Journal of Poultry Science 2:48-52. https://doi.org/10.3923/ajpsaj.2008.48.52

Al-Aqil, A. and Zulkifli, I. 2009. Changes in heat shock protein 70 expression and blood characteristics in transported broiler chickens as affected by housing and early age feed restriction. Poultry Science 88:1358-1364. https://doi.org/10.3382/ps.2008-00554

Al-Zghoul, M. B. 2018. Thermal manipulation during broiler chicken embryogenesis increases basal mRNA levels and alters production dynamics of heat shock proteins 70 and 60 and heat shock factors 3 and 4 during thermal stress. Poultry Science 97:3661-3670. https://doi.org/10.3382/ps/pey225

Al-Zghoul, M. B.; Dalab, A. E.; Ababneh, M. M.; Jawasreh, K. I.; Al Busadah, K. A. and Ismail, Z. B. 2013. Thermal manipulation during chicken embryogenesis results in enhanced Hsp70 gene expression and the acquisition of thermotolerance. Research in Veterinary Science 95:502-507. https://doi.org/10.1016/j.rvsc.2013.05.012

Al-Zghoul, M. B.; Dalab, A. E.; Yahya, I. E.; Althnaian, T. A.; Al-Ramadan, S. Y.; Ali, A. M.; Albokhadaim, I. F.; El-Bahr, S. M.; Al Busadah, K. A. and Hannon, K. M. 2015. Thermal manipulation during broiler chicken embryogenesis: Effect on mRNA expressions of Hsp108, Hsp70, Hsp47 and Hsf-3 during subsequent post-hatch thermal challenge. Research in Veterinary Science 103:211-217. https://doi.org/10.1016/j.rvsc.2015.10.015

Bodine, S. C.; Latres, E.; Baumhueter, S.; Lai, V. K. M.; Nunez, L.; Clarke, B. A.; Poueymirou, W. T.; Panaro, F. J.; Na, E. Q.; Dharmarajan, K.; Pan, Z. Q.; Valenzuela, D. M.; DeChiara, T. M.; Stitt, T. N; Yancopoulos, G. D. and Glass, D. J. 2001. Identification of ubiquitin ligases required for skeletal muscle atrophy. Science 294:1704-1708. https://doi.org/10.1126/science.1065874

Collin, A.; Berri, C.; Tesseraud, S.; Requena Rodón, F. E.; Skiba-Cassy, S.; Crochet, S.; Duclos, M. J.; Rideau, N.; Tona, K.; Buyse, J.; Bruggeman, V.; Decuypere, E.; Picard, M. and Yahav, S. 2007. Effects of thermal manipulation during early and late embryogenesis on thermotolerance and breast muscle characteristics in broiler chickens. Poultry Science 86:795-800. https://doi.org/10.1093/ps/86.5.795

Costa, B. T. A.; Lopes, T. S. B.; Mesquita, M. A.; Lara, L. J. C. and Araújo, I. C. S. 2020. Thermal manipulations of birds during embryogenesis. World's Poultry Science Journal 76:843-851. https://doi.org/10.1080/00439339.2020.1823302

Craig, E. A. and Gross, C. A. 1991. Is hsp70 the cellular thermometer? Trends in Biochemical Sciences 16:135-140. https://doi.org/10.1016/0968-0004(91)90055-Z

Daghir, N. J. 1995. Poultry production in hot climates. CAB International, Wallingford, UK.

Dalab, A. S. and Ali, A. M. 2019. Morphological investigations of the effect of thermal manipulation during embryogenesis on body performance and structure of pectoral and thigh muscle of Ross broiler chicken. Brazilian Journal of Poultry Science 21:eRBCA-2019-1100. https://doi.org/10.1590/1806-9061-2019-1100

Deb, R.; Sajjanar, B.; Singh, U.; Kumar, S.; Brahmane, M. P.; Singh, R.; Sengar, G. and Sharma, A. 2013. Promoter variants at AP2 box region of Hsp70.1 affect thermal stress response and milk production traits in Frieswal cross bred cattle. Gene 532:230-235. https://doi.org/10.1016/j.gene.2013.09.037

Foster, N. L.; Lukowiak, L. and Henry, T. B. 2015. Time-related expression profiles for heat shock protein gene transcripts (HSP40, HSP70) in the central nervous system of *Lymnaea stagnalis* exposed to thermal stress. Communicative & Integrative Biology 8:e1040954. https://doi.org/10.1080/19420889.2015.1040954

Gabriel, J. E.; Ferro, J. A.; Stefani, R. M. P.; Ferro, M. I. T.; Gomes, S. L. and Macari, M. 1996. Effect of acute heat stress on heat shock protein 70 messenger RNA and on heat shock protein expression in the liver of broilers. British Poultry Science 37:443-449. https://doi.org/10.1080/00071669608417875

Galal, A. and Radwan, L. M. 2020. Identification of single nucleotide polymorphism in heat shock protein HSP70 and HSP90 after four selection generations in two lines of chickens. Annals of Agricultural Sciences 65:124-128. https://doi.org/10.1016/j.aoas.2020.07.002

Goel, A.; Ncho, C. M. and Choi, Y. H. 2021. Regulation of gene expression in chickens by heat stress. Journal of Animal Science and Biotechnology 12:11. https://doi.org/10.1186/s40104-020-00523-5

Gong, W. J. and Golic, K. G. 2006. Loss of Hsp70 in drosophila is pleiotropic, with effects on thermotolerance, recovery from heat shock and neurodegeneration. Genetics 172:275-286. https://doi.org/10.1534/genetics.105.048793

Hartl, F. U. 1996. Molecular chaperones in cellular protein folding. Nature 381:571-580. https://doi.org/10.1038/381571a0

Hassan, F. U.; Nawaz, A.; Rehman, M. S.; Ali, M. A.; Dilshad, S. M. R. and Yang, C. 2019. Prospects of HSP70 as a genetic marker for thermo-tolerance and immuno-modulation in animals under climate change scenario. Animal Nutrition 5:340-350. https://doi.org/10.1016/j.aninu.2019.06.005

Iqbal, A.; Decuypere, E.; Abd El Azim, A. and Kuhn, E. R. 1990. Pre- and post- hatch high temperature exposure affects the thyroid hormones and corticosterone response to acute heat stress in growing chicken (*Gallus domesticus*). Journal of Thermal Biology 15:149-153. https://doi.org/10.1016/0306-4565(90)90032-D

Liang, H. M.; Lin, D. Y.; Hsuuw, Y. D.; Huang, T. P.; Chang, H. L.; Lin, C. Y.; Wu, H. H. and Hung, K. H. 2016. Association of heat shock protein 70 gene polymorphisms with acute thermal tolerance, growth, and egg production traits of native chickens in Taiwan. Archives Animal Breeding 59:173-181. https://doi.org/10.5194/aab-59-173-2016

Livak, K. J. and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. Methods 25:402-408. https://doi.org/10.1006/meth.2001.1262

Loyau, T.; Bedrani, L.; Berri, C.; Métayer-Coustard, S.; Praud, C.; Coustham, V.; Mignon-Grasteau, S.; Duclos, M. J.; Tesseraud, S.; Rideau, N.; Hennequet-Antier, C.; Everaert, N.; Yahav, S. and Collin, A. 2015. Cyclic variations in incubation conditions induce adaptive responses to later heat exposure in chickens: a review. Animal 9:76-85. https://doi.org/10.1017/S1751731114001931

Loyau, T.; Berri, C.; Bedrani, L.; Métayer-Coustard, S.; Praud, C.; Duclos, M. J.; Tesseraud, S.; Rideau, N.; Everaert, N.; Yahav, S.; Mignon-Grasteau, S. and Collin, A. 2013. Thermal manipulation of the embryo modifies the physiology and body composition of broiler chickens reared in floor pens without affecting breast meat processing quality. Journal of Animal Science 91:3674-3685. https://doi.org/10.2527/jas.2013-6445

Loyau, T.; Hennequet-Antier, C.; Coustham, V.; Berri, C.; Leduc, M.; Crochet, S.; Sannier, M.; Duclos, M. J.; Mignon-Grasteau, S.; Tesseraud, S.; Brionne, A.; Métayer-Coustard, S.; Moroldo, M.; Lecardonnel, J.; Martin, P.; Lagarrigue, S.; Yahav, S. and Collin, A. 2016. Thermal manipulation of the chicken embryo triggers differential gene expression in response to a later heat challenge. BMC Genomics 17:329. https://doi.org/10.1186/s12864-016-2661-y

Loyau, T.; Métayer-Coustard, S.; Berri, C.; Crochet, S.; Cailleau-Audouin, E.; Sannier, M.; Chartrin, P.; Praud, C.; Hennequet-Antier, C.; Rideau, N.; Courousse, N.; Mignon-Grasteau, S.; Everaert, N.; Duclos, M. J.; Yahav, S.; Tesseraud, S. and Collin, A. 2014. Thermal manipulation during embryogenesis has long-term effects on muscle and liver metabolism in fast-growing chickens. PLoS ONE 9:e105339. https://doi.org/10.1371/journal.pone.0105339

Mazzi, C. M.; Ferro, J. A.; Ferro, M. I. T.; Savino, V. J. M.; Coelho, A. A. D. and Macari, M. 2003. Polymorphism analysis of the hsp70 stress gene in broiler chickens (*Gallus gallus*) of different breeds. Genetics and Molecular Biology 26:275-281. https://doi.org/10.1590/S1415-47572003000300010

McNabb, F. M. A. 1988. Peripheral thyroid hormone dynamics in precocial and altricial avian development. American Zoologist 28:427-440. https://doi.org/10.1093/icb/28.2.427

Narinç, D.; Erdoğan, S.; Tahtabiçen, E. and Aksoy, T. 2016. Effects of thermal manipulations during embryogenesis of broiler chickens on developmental stability, hatchability and chick quality. Animal 10:1328-1335. https://doi.org/10.1017/S1751731116000276

Nawaz, A. H.; Amoah, K.; Leng, Q. Y.; Zheng, J. H.; Zhang, W. L. and Zhang, L. 2021. Poultry response to heat stress: its physiological, metabolic, and genetic implications on meat production and quality including strategies to improve broiler production in a warming world. Frontiers in Veterinary Science 8:699081. https://doi.org/10.3389/fvets.2021.699081

Perini, F.; Cendron, F.; Rovelli, G.; Castellini, C.; Cassandro, M. and Lasagna, E. 2021. Emerging genetic tools to investigate molecular pathways related to heat stress in chickens: A review. Animals 11:46. https://doi.org/10.3390/ani11010046

Piestun, Y.; Halevy, O.; Shinder, D.; Ruzal, M.; Druyan, S. and Yahav, S. 2011. Thermal manipulations during broiler embryogenesis improves post-hatch performance under hot conditions. Journal of Thermal Biology 36:469-474. https://doi.org/10.1016/j.jtherbio.2011.08.003

Piestun, Y.; Harel, M.; Barak, M.; Yahav, S. and Halevy, O. 2009. Thermal manipulations in late-term chick embryos have immediate and longer term effects on myoblast proliferation and skeletal muscle hypertrophy. Journal of Applied Physiology 106:233-240. https://doi.org/10.1152/japplphysiol.91090.2008

Piestun, Y.; Shinder, D.; Ruzal, M.; Halevy, O.; Brake, J. and Yahav, S. 2008. Thermal manipulations during broiler embryogenesis: Effect on the acquisition of thermotolerance. Poultry Science 87:1516-1525. https://doi.org/10.3382/ps.2008-00030

Piestun, Y.; Zimmerman, I. and Yahav, S. 2015. Thermal manipulations of turkey embryos: The effect on thermoregulation and development during embryogenesis. Poultry Science 94:273-280. https://doi.org/10.3382/ps/peu047

Pratt, W. B. 1993. The role of heat shock proteins in regulating the function, folding, and trafficking of the glucocorticoid receptor. Journal of Biological Chemistry 268:21455-21458.

Singh, R.; Kolvraa, S.; Bross, P.; Jensen, U. B.; Gregersen, N.; Tan, Q. H.; Knudsen, C. and Rattan, S. I. S. 2006. Reduced heat shock response in human mononuclear cells during aging and its association with polymorphisms in HSP70 genes. Cell Stress and Chaperones 11:208-215.

Shehata, A. M.; Saadeldin, I. M.; Tukur, H. A. and Habashy, W. S. 2020. Modulation of heat-shock proteins mediates chicken cell survival against thermal stress. Animals 10:2407. https://doi.org/10.3390/ani10122407

Tamzil, M. H.; Noor, R. R.; Hardjosworo, P. S.; Manalu, W. and Sumantri, C. 2013. Acute heat stress responses of three lines of chickens with different heat shock protein (HSP)-70 genotypes. International Journal of Poultry Science 12:264-272. https://doi.org/10.3923/ijps.2013.264.272

Tankson, J. D.; Vizzier-Thaxton, Y.; Thaxton, J. P.; May, J. D. and Cameron, J. A. 2001. Stress and nutritional quality of broilers. Poultry Science 80:1384-1389. https://doi.org/10.1093/ps/80.9.1384

Tao, X.; Zhang, Z. Y.; Dong, H.; Zhang, H. and Xin, H. 2006. Responses of thyroid hormones of market-size broilers to thermoneutral constant and warm cyclic temperatures. Poultry Science 85:1520-1528. https://doi.org/10.1093/ps/85.9.1520

Teskey, M. L.; Lukowiak, K. S.; Riaz, H.; Dalesman, S. and Lukowiak, K. 2012. What's hot: the enhancing effects of thermal stress on long-term memory formation in *Lymnaea stagnalis*. Journal of Experimental Biology 215:4322-4329. https://doi.org/10.1242/jeb.075960

Walstra, I.; ten Napel, J.; Kemp, B. and van den Brand, H. 2010. Temperature manipulation during layer chick embryogenesis. Poultry Science 89:1502-1508. https://doi.org/10.3382/ps.2009-00568

Wang, S. and Edens, F. W. 1993. Stress-induced heat-shock protein synthesis in peripheral leukocytes of turkeys, *Meleagris gallopavo*. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry 106:621-628. https://doi.org/10.1016/0305-0491(93)90139-V

Wang, S. and Edens, F. W. 1998. Heat conditioning induces heat shock proteins in broiler chickens and turkey poults. Poultry Science 77:1636-1645. https://doi.org/10.1093/ps/77.11.1636

Wasti, S.; Sah, N. and Mishra, B. 2020. Impact of heat stress on poultry health and performances, and potential mitigation strategies. Animals 10:1266. https://doi.org/10.3390/ani10081266

Yahav, S.; Collin, A.; Shinder, D. and Picard, M. 2004a. Thermal manipulations during broiler chick embryogenesis: effects of timing and temperature. Poultry Science. 83:1959-1963. https://doi.org/10.1093/ps/83.12.1959

Yahav, S. and McMurtry, J. P. 2001. Thermotolerance acquisition in broiler chickens by temperature conditioning early in life - The effect of timing and ambient temperature. Poultry Science 80:1662-1666. https://doi.org/10.1093/ps/80.12.1662

Yahav, S.; Rath, R. S. and Shinder, D. 2004b. The effect of thermal manipulations during embryogenesis of broiler chicks (*Gallus domesticus*) on hatchability, body weight and thermoregulation after hatch. Journal of Thermal Biology 29:245-250. https://doi.org/10.1016/j.jtherbio.2004.03.002