



Molecular Investigations of the Effect of Thermal Manipulation During Embryogenesis on the Enzymatic Activity of Creatine Kinase and Lactate Dehydrogenase in Broiler Muscle

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ABSTRACT

Incubating temperature and timing or duration is critical to determine the optimum protocol of thermal manipulation (TM), which underlines muscle growth improvement. Therefore, the aim of the present study is to determine the optimum period of embryonic TM that may result in the improvement of pectoral and thigh muscle myogenesis. This is done by investigating the level of mRNA expression of creatine kinase (CK) and lactate dehydrogenase (LDH). An additional goal is measuring the blood levels of CK and LDH as a biomarker of muscle injury due to the experimental thermal challenge on post-hatch day 35. The study was conducted on 1,440 fertile eggs (Ross broilers) that were divided randomly and equally into a control group and four treatment groups (TM1, TM2, TM3, and TM4). The treatment groups were daily subjected to TM at 39 °C for 18h with 65% relative humidity (RH) during embryonic days (EDs) 7–11, 11–15, 15–18, and 7–18, respectively. Among the thermally manipulated groups that were investigated, TM1 (ED 7–11) resulted in significant improvement of mRNA expression and enzymatic concentration of CK and LDH in muscle during embryogenesis, as compared to the control. Six hours of TC showed the highest significant CK and LDH expression and concentration levels in the control as compared to TM groups. Thus, the results of this study indicate that TM during ED 7–11 improves pectoral and thigh muscles' response to heat stress without adversely affecting their performance. This finding could be used by commercial breeders to enhance local broiler production.

INTRODUCTION

During embryogenesis, CK is expressed at a high level in developing embryonic striated muscle. It has been used as an early marker for skeletal myogenesis in chickens by maintaining a high ATP/ADP ratio (Iyengar *et al.*, 1983, Lyons *et al.*, 1991). CK plays a role in cellular energy metabolism: it catalyzes the reversible transfer of high-energy phosphate from ATP to creatine, facilitating the storage of energy in the form of phosphocreatine (Hettling & van Beek 2011). Another proposed function of CK is the transportation of energy from ATP-producing to ATP-consuming sites via a shuttle mechanism involving a mitochondrial and a myofibrillar isoform of CK (Hettling & van Beek 2011). During embryogenesis, there is a high level of CK expression in the striated skeletal muscle, which is considered an early marker of striated skeletal muscle myogenesis in chickens (Lyons *et al.*, 1991). Later authors proposed a biological role for CK in maintaining the ATP/ADP ratio. Thus, relatively high CK was thought to enhance ATP-demanding processes and reduce ADP-dependent functions (Iyengar *et al.*, 1983). LDH is a glycolytic enzyme that is found in almost every tissue, especially in skeletal muscle. It is involved in the final steps of glycolysis



and glyconeogenesis, mediating the breakdown of pyruvate into lactate in the absence of oxygen (Wanda *et al.*, 2013).

Pech-Waffenschmidt (1992) and Hsiao-Mei *et al.* (2016) reported that the abnormal appearance of CK and LDH in blood plasma could hint at specific muscle or organ damage. Abnormally increased plasma levels of CK and LDH were a consequence of the disruption of the function and permeability of the muscle cell membrane (Brancaccio *et al.*, 2007). Additionally, Tang *et al.* (2013) reported that broiler exposure to high ambient temperatures significantly increases their plasma CK levels. Therefore, this study aims to investigate the effect of thermal manipulation during different durations (early, mid, late, and long-lasting) of broiler embryogenesis on mRNA gene expression of CK and LDH in both the pectoral and thigh muscles of embryos, as well as during the subsequent thermal challenge (TC) on post-hatch day 35. Additionally, it investigates the effect of thermal manipulation during embryogenesis on the plasma levels of CK and LDH released into the bloodstream after 1h, 3h, and 6h of TC on post-hatch day 35. The results of this research can be used in clinical biochemistry as a tool to assess the physiological response of broiler muscle to heat stress.

MATERIALS AND METHODS

All sampling, experimental incubation, and hatching management conditions were approved by King Faisal University's Animal Care and Use Committee (KFU-ACUC).

Pre-egg Incubation Management

A total of 1,700 Ross 708 broiler eggs were obtained from a local 36-week-old broiler flock (from Al-Ahsa, Saudi Arabia). The broken or abnormally small or large eggs (those weighing less than 58g or more than 65g, respectively) were excluded before the first incubation day. 1,550 uniformly sized eggs remained.

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% RH up to ED 7; egg candling was performed at ED 7 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 being the untreated control group that remained incubated at 37.8 °C with 56% RH, whereas TM1, TM2,

TM3, and TM4 (with 288 eggs each) were subjected to 39 °C for 18h with 65% RH daily during ED 7–11, ED 11–15, ED 15–18, and ED 7–18, respectively.

Hatching and Thermal Challenge Management

All one-day-old hatched chicks were recorded and the field study was conducted at the Agricultural and Veterinary Research and Training Station at King Faisal University. Water and feed (ARASCO, Riyadh, Saudi Arabia) were supplied *ad libitum* to the chicks, and they were kept brooding at an initial house temperature of 31 ± 1 °C, which was reduced at an average of 0.2–0.3 °C per day to achieve a final house temperature of 22 ± 1 °C by post-hatch day 24. On post-hatch day 35, chicks from each group were randomly and equally divided into two subgroups, naïve (N) and thermal challenge (TC) (100 chicks per group). Thermal challenge (43.0 °C at 1h, 3h, and 6h intervals) was applied to the chicks of each TC subgroup, including the TC control subgroup, on post-hatch day 35, while the naïve chicks of each subgroup were kept under regular conditions (22 ± 1 °C and 50–60% RH) in a separate room.

Sampling Management

Pectoral and thigh muscle samples from five control embryos and from each thermally manipulated group were collected at the end of ED 11, ED 15, and ED 18 for total RNA isolation of the embryonic CK and LDH (75 embryos, $n = 5$). On post-hatch day 35, humanely manual cervical dislocation (CD) euthanasia was conducted 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 1h, 3h, and 6h intervals of TC) and naïve chicks for total RNA isolation (100 chicks, $n = 5$). Blood from the jugular vein from 10 chicks per group (control and treatment) was drawn at the beginning (0h) and after 1h, 3h, and 6h of TC exposure (200 chicks, $n = 10$) for measurement of blood plasma CK and LDH levels.

RNA Isolation and Semi-quantitative Real-time RT-PCR Analyses

mRNA expression levels of CK and LDH during embryogenesis and during subsequent thermal challenge (TC) at day 35 post-hatch were investigated and evaluated through semi-quantitative real-time RT-PCR analyses. Pectoral and thigh muscles were homogenized using a Bead Ruptor (24 Bead Mill Homogenizer OMNI, USA) and total RNA was extracted using the PureZOL TM RNA isolation method (BIO-RAD,



Catalog #732-6890, Hercules, CA, USA). DNA was removed using DNase I kit (Ambion), and RNA samples were checked for concentration and purity (260:280nm absorbency) (Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader Bio-Tek. USA). RNA (2µg) was reverse-transcribed to cDNA in a reaction mixture using the iScriptc DNA synthesis kit (BIO-RAD, Catalog #170-8890, Hercules, CA, USA).

The semi-quantitative real-time CFX96 Touch™ Real-time PCR (BIO-RAD, Hercules, CA, USA) was performed using the ssoAdvanced™ SYBR Green Supermix kit (BIO-RAD, Catalog #170-5270, Hercules, CA, USA). The 20µl reaction mix was prepared from 10µl of master mix; 2µl forward primer pM/µl; 2µl reverse primer pm/µl (Table 1); 2µl cDNA of the sample; and 4µl of nuclease-free water. Cycling parameters were 95°C for 1m and 40 cycles of 95°C for 10s, followed by 30s at 60°C and 72°C for 10s, with final melting at 95°C for 20s. Triplicates from each cDNA were analyzed, fluorescence emission was detected, and relative quantification was calculated automatically according to GAPDH and ACTIN-1 Housekeeping genes.

Biochemical Analysis of CK and LDH

Chicks' responses to TC were evaluated by determination of plasma CK and LDH at the beginning of TC (0h) and after 1h, 3h, and 6h of TC in naïve and TC subgroups. Plasma was isolated through centrifugation of blood samples at 3,000rpm for 10m and was then stored at -20 °C until further analysis. A commercial ELISA kit was used for the determination of CK (MBS269755) and LDH (MBS263022) (Mybiosource, California, San Diego, USA) on ELISA reader (Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader Bio-Tek. USA). The concentration results of CK and LDH were calculated according to the manufacturer's instruction protocols.

Statistical Analysis

The data for mRNA gene expressions and concentrations of CK and LDH was expressed as means ± SE.

Table 1 – Oligonucleotides for gene expression analysis.

Primers	Sequence	PCR Product Size	GenBank Reference
CK	F-5-GGAGTTCAAGGGCCGCTATT-3 R-5-ATTCACCCACACCAGGAACG-3	190 bp	NM_205507.1
LDH	F-5-GCCGGCAGTACACCATGTC -3 R-5-GGCACAAGCCATTCCAAGT-3	122 bp	NM_205284.1
GAPDH	F-5-GTGTATCATCTCAGCTCCCTCAG-3 R-5-GGTCATAAGACCCTCCACAATG-3	176 bp	FJ_217667
ACTA1	F-5-CCATCGGCAATGAGCGTTTC-3 R-5-GCATGCGGTGTCAGCAATACCT-3	195 bp	NM_001031063.1

A two-way ANOVA test and an all-pairs Bonferroni test were applied to compare the different parameters in each treatment group using the IBM SPSS Statistics 20 software (IBM, Chicago, USA). Differences were considered significant at $p < 0.05$.

RESULTS

CK and LDH mRNA Expression of Pectoral and Thigh Muscle at Embryonic Days (EDs) 11, 15, and 18

On ED 11, the thermal manipulation of TM1 and TM4 from ED 7 to ED 11 resulted in a higher significant increase in mRNA expression of CK and LDH in both pectoral and thigh muscles as compared to the control group. The highest significant expression values of CK and LDH were observed in the pectoral muscles of TM1 and TM4 rather than the thigh muscles (Table 2).

On ED 15, the thermal manipulation of TM2 from ED 11 to ED 15 resulted in significant increase in mRNA expressions of CK and LDH in pectoral muscles and thigh muscles as compared to the control (Table 2). On the other hand, TM1 and TM4, which were thermally manipulated during ED 7–11, maintained increased mRNA expression of CK with no significant increases in mRNA expression of LDH in pectoral and thigh muscles in comparison to the control (Table 2).

On ED 18, the thermal manipulation of TM3 from ED 15 to ED 18 was found to increase pectoral and thigh muscles' mRNA expression of CK and LDH in comparison to control. Additionally, the pectoral mRNA expressions of CK values for TM1, TM2, and TM3 on ED 18 were significantly higher when compared to those of TM4 and the control. For thigh muscles, there were no significant differences in mRNA expression of CK values among TM1, TM2, and the control group, whereas there was significantly higher mRNA expression of LDH values in all treatment groups as compared to the control group.

The results of the relative normalized comparative gene study of mRNA levels of CK and LDH in both pectoral and thigh muscles during embryogenesis are shown in Table 3. The results show that the highest gene expressions (upregulations) were observed in early embryogenesis, while the lowest gene expression was observed in ED 15–18. Hence, in the early and middle thermal manipulation for TM1 (ED 7–11), TM2 (ED 11–15), and TM4 (ED 7–11), the results show that there was upregulation expression (significantly increased expression) of CK



Table 2 – Relative normalized expression of mRNA levels of CK and LDH in pectoral and thigh muscles during embryogenesis.

Target	Time (Age)								
	ED 11		ED 15		ED 18				
	Sample	Expression (Fold)		Sample	Expression (Fold)		Sample	Expression (Fold)	
	Pectoral Muscle	Thigh Muscle		Pectoral Muscle	Thigh Muscle		Pectoral Muscle	Thigh Muscle	
CK	Control*	1.0 ± 0.4a	1.0 ± 0.3a	Control*	1.0 ± 0.1a	1.0 ± 0.2a	Control*	1.0 ± 0.3a	1.0 ± 0.2a
	TM ₁	16.64 ± 2.5b	2.36 ± 0.2b	TM ₁	2.15 ± 0.1b	1.96 ± 0.4a	TM ₁	1.92 ± 0.2b	0.47 ± 0.1a
	TM ₂	-----	-----	TM ₂	2.71 ± 0.9b	1.89 ± 0.6b	TM ₂	1.73 ± 0.7b	0.91 ± 0.1a
	TM ₃	-----	-----	TM ₃	-----	-----	TM ₃	2.73 ± 0.3c	1.77 ± 0.3a
	TM ₄	14.64 ± 3.0c	2.84 ± 0.4b	TM ₄	2.55 ± 0.4b	1.79 ± 0.1b	TM ₄	1.52 ± 0.4a	1.63 ± 0.4b
LDH	Control*	1.0 ± 0.5a	1.0 ± 0.2a	Control*	1.0 ± 0.1	1.0 ± 0.2	Control*	1.0 ± 0.3a	1.0 ± 0.2a
	TM ₁	1.86 ± 0.2b	2.0 ± 0.1b	TM ₁	0.72 ± 0.1a	0.34 ± 0.09b	TM ₁	1.05 ± 0.1a	2.60 ± 0.2b
	TM ₂	-----	-----	TM ₂	1.83 ± 0.3a	1.73 ± 0.2a	TM ₂	1.20 ± 0.6a	2.00 ± 0.3b
	TM ₃	-----	-----	TM ₃	-----	-----	TM ₃	2.73 ± 0.4b	1.62 ± 0.3c
	TM ₄	1.89 ± 0.3a	2.08 ± 0.7b	TM ₄	0.80 ± 0.1a	0.30 ± 0.03b	TM ₄	1.01 ± 0.3a	2.22 ± 0.5b

Control = 37.8°C; TM1 = thermal manipulation during ED 7–11 at 39°C for 18h; TM2 = thermal manipulation during ED 11–15 at 39°C for 18h; TM3 = thermal manipulation during ED 15–18 at 39°C for 18h; TM4 = thermal manipulation during ED 7–18 at 39°C for 18h; a–c within the same day means that ± SD with different superscripts differ significantly ($p < 0.05$). (*Compared to the control of each embryonic day).

in pectoral muscles more than in thigh muscles when compared to the control (Table 3). Moreover, the results obtained from this study show that the highest gene expressions (upregulations) of LDH were found in early embryogenesis only, and the lowest gene expression was observed for ED 11–18. Results from the control show downregulation expressions (significantly lowered expression) of CK in both pectoral and thigh muscles when compared to TM1.

Effect of Thermal Challenge (TC) on the Expression of CK and LDH in Naïve and Chicken Subjected to 43°C for 1h, 3h, and 6h Intervals on Post-hatch Day 35

On post-hatch day 35, CK mRNA expression in the pectoral muscle of naïve chicks was significantly higher in TM1, TM2, and TM3 as compared to TM4 and the control. In naïve thigh muscle, CK mRNA expression

Table 3 – Gene study of relative normalized expression of mRNA of CK and LDH in pectoral and thigh muscles during embryogenesis.

Target	Time (Age)								
	ED 11		ED 15		ED 18				
	Sample	Expression (Fold)		Sample	Expression (Fold)		Sample	Expression (Fold)	
	Pectoral Muscle	Thigh Muscle		Pectoral Muscle	Thigh Muscle		Pectoral Muscle	Thigh Muscle	
CK	Control*	1.0 ± 0.4	1.0 ± 0.3	Control	4.77 ± 0.6	0.93 ± 0.6	Control	2.90 ± 0.8	0.86 ± 0.2
	TM ₁	16.64 ± 2.5	2.36 ± 0.2	TM ₁	11.79 ± 0.4	1.82 ± 0.3	TM ₁	5.75 ± 0.6	0.40 ± 0.1
	TM ₂	1.40 ± 0.8	1.04 ± 0.2	TM ₂	7.81 ± 0.4	1.11 ± 0.5	TM ₂	4.77 ± 0.2	0.74 ± 0.1
	TM ₃	1.34 ± 0.5	1.24 ± 0.4	TM ₃	6.40 ± 0.6	1.91 ± 0.3	TM ₃	6.36 ± 0.8	1.31 ± 0.2
	TM ₄	14.64 ± 3.0	2.84 ± 0.4	TM ₄	12.49 ± 0.8	1.73 ± 0.1	TM ₄	3.81 ± 0.8	1.41 ± 0.3
LDH	Control*	1.0 ± 0.5	1.0 ± 0.2	Control	0.32 ± 0.02	0.23 ± 0.02	Control	0.04 ± 0.01	0.05 ± 0.01
	TM ₁	1.86 ± 0.2	2.0 ± 0.1	TM ₁	0.26 ± 0.09	0.09 ± 0.01	TM ₁	0.12 ± 0.02	0.13 ± 0.01
	TM ₂	0.92 ± 0.2	0.75 ± 0.2	TM ₂	0.29 ± 0.09	0.20 ± 0.03	TM ₂	0.04 ± 0.01	0.09 ± 0.01
	TM ₃	0.41 ± 0.1	0.88 ± 0.2	TM ₃	0.18 ± 0.02	0.04 ± 0.02	TM ₃	0.06 ± 0.01	0.10 ± 0.01
	TM ₄	1.89 ± 0.3	2.08 ± 0.7	TM ₄	0.02 ± 0.01	0.07 ± 0.01	TM ₄	0.06 ± 0.01	0.11 ± 0.03

Control = 37.8°C; TM1 = thermal manipulation during ED 7–11 at 39°C for 18h; TM2 = thermal manipulation during ED 11–15 at 39°C for 18h; TM3 = thermal manipulation during ED 15–18 at 39°C for 18h; TM4 = thermal manipulation during ED 7–18 at 39°C for 18h; a–c within the same day means that ± SD with different superscripts differ significantly ($p < 0.05$). (*As compared to the control of pectoral and thigh muscle of ED 11 pectoral-to-pectoral and thigh-to-thigh).



was significantly lower for TM1, TM2, and TM3 as compared to TM4 and the control (Figure 1). Moreover, in pectoral muscle of naïve chickens, the highest significant expression of LDH mRNA expression was observed in TM4 only when compared to the control and the other treatment groups (Figure 2).

1 hour of TC produced no significant differences in mRNA expression of CK in pectoral muscle for all treatment groups when compared to the 1h-TC control group, whereas mRNA expression after 3 hours was significantly higher in all TC groups when compared to the 3h-TC control group; while 6h of TC induced mRNA expression of CK in TM1 only when compared to the other TC groups and the 6h-TC control group (Figure 1).

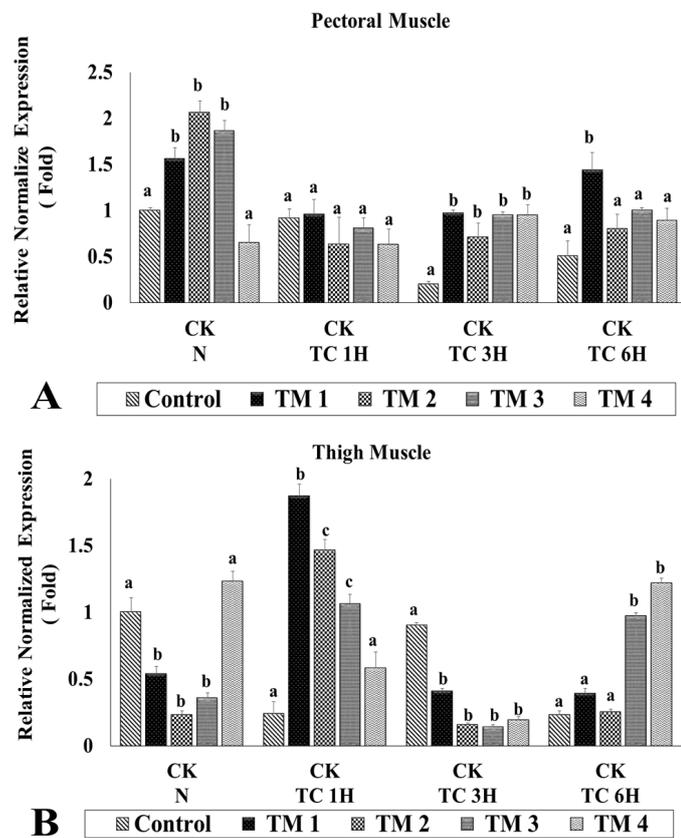


Figure 1 – The effect of thermal challenge (TC) on the mRNA level of CK in (A) pectoral and (B) thigh muscles in TC chicks subjected to thermal challenge at 43°C for 1h, 3h and 6 hours at post-hatch day 35. N: Naïve chicks. a-c folds with different superscripts differ significantly ($p < 0.05$).

In thigh muscle, 1h of thermal challenge resulted in significantly higher mRNA expression of CK in TM1, TM2, and TM3 than in control and TM4; 3h of thermal challenge resulted in higher levels of CK mRNA expression in the control group than all treatment groups (Figure 1), and 6h of thermal challenge changed the mRNA expression of CK only in TM3 and TM4 when compared to the control, TM1, and TM2. Moreover, in TM4, the highest mRNA expression of CK

was found after 6h of TC as compared to 1h and 3h of TC (Figure 1).

In pectoral muscle, 1h, 3h, and 6h of TC had no significant effect on mRNA expression of LDH in all treatment groups when compared to the control (1h, 3h, and 6h of TC) (Figure 1). In thigh muscle, only in TM2 did the 1h and 3h of thermal challenge induce mRNA expression of LDH higher than those of the 1h- and 3h-TC control group, and more than the other treatment groups; it also did so in TM2 naïve chicks. Six hours of thermal challenge influenced the mRNA expression of LDH in the control, TM2, and TM3 at a higher significant expression level than those of the 6h-TC TM1 group. Moreover, 6h of TC showed a higher significant difference in mRNA expression of LDH in the control, TM2, TM3, and TM4 when compared to those of the control (naïve) and 1h and 3h of TC (Figure 2).

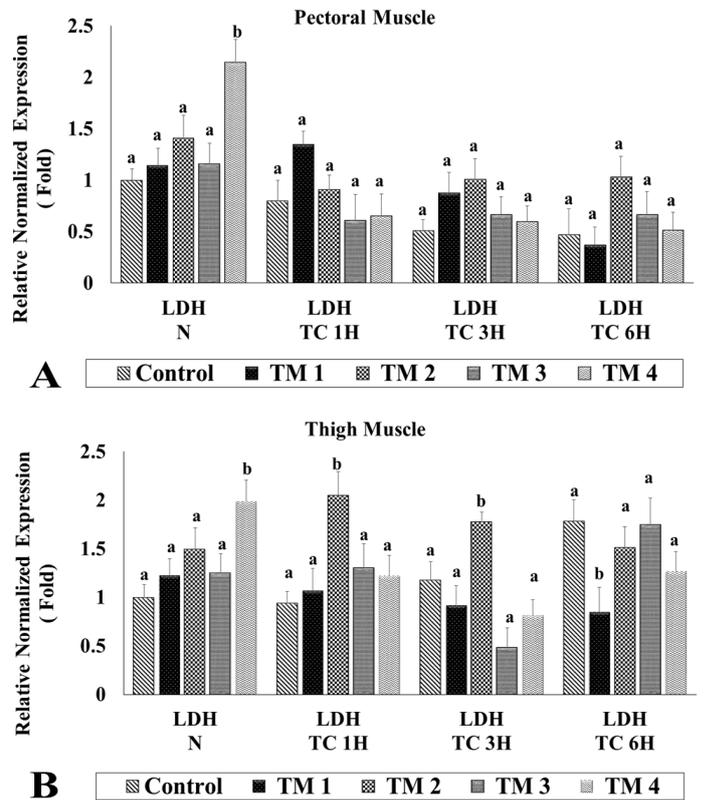


Figure 2 – The effect of thermal challenge (TC) on the mRNA level of LDH in (A) pectoral and (B) thigh muscles in TC chicks subjected to thermal challenge at 43°C for 1h, 3h and 6 hours at post-hatch day 35. N: Naïve chicks. a-c folds with different superscripts differ significantly ($p < 0.05$).

Effect of Thermal Challenge (TC) at 43°C on Plasma Levels of CK and LDH

On post-hatch day 35, 1h and 3h of TC caused significant increases in plasma levels of CK in the control, TM1, TM2, and TM3 as compared to those of naïve groups with a higher significant value in TM1 (Table 4). Meanwhile, 1h and 3h of TC caused



significant reduction in CK levels in TM4 as compared to those of the TM4 naïve group. Additionally, the control group showed a significant increase in CK levels from 1h of TC to 6h when compared to those of the control naïve group. Furthermore, TC groups showed no significant differences in CK levels after 6 hours of TC in all treatment groups that ranged between 50.04–56.66ng/ml, while the highest value was recorded in the control (62.98 ng/ml) after 6h of TC (Table 4).

Furthermore, 1h of TC caused a significant reduction in LDH levels in TM2, TM3, and TM4 as compared to those of the naïve groups. Moreover, TC during the

first hour had no significant differences in LDH levels for the control and TM1 as compared to their naïve groups (Table 4), and 3h of TC influenced LDH levels, showing significant increases in the control, TM3, and TM4 as compared to those obtained with 3h of TC for TM1 and TM2. After 3h of TC, TM1 and TM2 showed no significant difference in LDH levels when compared to their 1h-TC groups. Moreover, the observation after 6h of TC showed a significant reduction in LDH levels in TM1 when compared to the other treatment groups after 6h of TC. The highest value was recorded in the control after 6h of TC as compared to the other treatment groups (Table 4).

Table 4 – The effect of TC at 43°C for 6 hours on post-hatch day 35 on plasma levels of CK and LDH (n = 10).

CK (ng/ml)	Control	TM ₁	TM ₂	TM ₃	TM ₄
N	45.19 ± 3.07 ^{aw}	50.58 ± 0.93 ^{bw}	51.87 ± 4.61 ^{bw}	49.98 ± 2.42 ^{bw}	64.32 ± 3.54 ^{cw}
TC 1h	54.27 ± 4.65 ^{ax}	78.98 ± 4.68 ^{bx}	61.04 ± 4.83 ^{cx}	56.83 ± 5.76 ^{ax}	59.89 ± 4.49 ^{dx}
TC 3h	57.93 ± 4.14 ^{ax}	70.07 ± 1.96 ^{by}	61.28 ± 1.87 ^{cx}	57.17 ± 3.12 ^{ax}	58.91 ± 2.92 ^{ax}
TC 6h	62.98 ± 3.36 ^{ay}	54.50 ± 4.57 ^{bz}	50.04 ± 5.01 ^{bw}	50.10 ± 2.49 ^{by}	56.66 ± 5.76 ^{bx}
LDH (ng/ml)	Control	TM ₁	TM ₂	TM ₃	TM ₄
N	3.76 ± 0.31 ^{aw}	2.82 ± 0.46 ^{bw}	3.75 ± 0.52 ^{aw}	3.70 ± 0.59 ^{aw}	3.60 ± 0.34 ^{aw}
TC 1h	3.45 ± 0.05 ^{aw}	2.77 ± 0.54 ^{bw}	3.18 ± 0.46 ^{bx}	2.48 ± 0.08 ^{bx}	2.83 ± 0.24 ^{bx}
TC 3h	5.13 ± 0.04 ^{ax}	2.68 ± 0.46 ^{bw}	3.17 ± 0.44 ^{bx}	4.09 ± 0.47 ^{dw}	4.28 ± 0.64 ^{dy}
TC 6h	3.45 ± 0.52 ^{aw}	2.14 ± 0.20 ^{bx}	2.82 ± 0.45 ^{cx}	2.86 ± 0.11 ^{cy}	2.95 ± 0.53 ^{cx}

Control = 37.8°C; TM1 = thermal manipulation during ED 7–11 at 39°C for 18h; TM2 = thermal manipulation during ED 11–15 at 39°C for 18h; TM3 = thermal manipulation during ED 15–18 at 39°C for 18h; TM4 = thermal manipulation during ED 7–18 at 39°C for 18h; TC = thermal challenge; a–e within rows means that ± SD with different superscripts differ significantly (p < 0.05). x–z between naïve and TC chicks within a group means that ± SD with different superscripts differ significantly (p < 0.05).

DISCUSSION

This study shows that embryonic thermal manipulation has influenced the expression of CK for pectoral muscles more than for thigh muscles in TM1 and TM4 (ED 7–11). This level of expression might be related to the high level of ATP demand during pectoral myogenesis in comparison to that of thigh muscle. In the current study on post-hatch day 35, the highest significant mRNA expression of CK was in pectoral muscle in TM1, TM2, and TM3 when compared to TM4 and the control. Moreover, our results show that the mRNA expression of CK was higher in pectoral muscles than in thigh muscles (only in TM4 was there higher expression in the thigh). This may indicate that mRNA expressions of CK on post-hatch day 35 play a pivotal role in the temporal buffering of changes in ATP and ADP during pectoral muscle development more than thigh muscle development, thereby maintaining ATP homeostasis. Likewise, the data from Hettling & van Beek (2011) emphasized the role of CK in energy metabolism. Later authors suggested a catalytic function for CK in the transferring of high-energy phosphate from ATP to creatine.

On the other hand, TC at 43°C for 6h significantly lowered mRNA expression of CK in pectoral muscle for all treatment groups and even the control, as compared to their counterpart naïve groups. For thigh muscle, the mRNA expression of CK with 6h of TC was significantly higher in TM3 and TM4. Additionally, thermal challenge significantly changed the CK enzyme activities in the chickens' serum from the beginning of TC until 6h of exposure.

Exposure of broiler chickens to environmental stressors can lead to the release of CK into the circulation in response to changes in permeability of sarcolemma (Mitchell & Sandercock 1994). Publications about the effect of high environmental temperatures on CK activity are not consistent, possibly due to the large variability of the enzymes (Melesse *et al.*, 2011). Zheng *et al.* (2020) reported that serum CK was not significantly affected by the stress during broiler transportation. This was in agreement with previous study by Al-Zghoul, *et al.* (2015) that reported no significant effect on CK levels in broilers that were thermal manipulated during their embryogenesis. On the other hand, Sandercock *et al.* (2001) observed a significant increase in CK activity in broilers exposed to



acute type of heat stress reflecting heat stress-induced myopathy. Similarly, Yalçin *et al.* (2009) reported that broiler exposure to daily heat stress can lead to increase in plasma CK activity. Additionally, Tang *et al.* (2018) reported an increase in CK levels following broiler exposure to heat stress (42 ± 1 °C for (0, 1, 3, 5, and 10h). This was in agreement with our finding for TC groups. TC caused a significant increase in serum CK level in the control, TM1, TM2, and TM3 in the first and third hours of TC as compared to naïve groups with a higher value in TM1 (CK: 70–78ng/ml). Meanwhile, 6h of TC showed significant differences in serum CK levels in all treatment groups with a higher value in the control (CK: 62.98ng/ml) as compared to those of TM1, TM2, TM3, and TM4. In other words, we observed lower mRNA expression in TC groups after 6h, especially in the control group with higher serum CK levels. This means that the control group has more stress after 6h of TC when compared to all treatment groups. The increased serum CK levels in the control after 6h of TC may be indicative of skeletal muscle damage or a consequence of a disruption in the function and permeability of the muscle cell membrane, as reported by Brancaccio *et al.* (2007).

Together, the plasma and mRNA gene expression of CK in our findings suggest that there was the re-establishment of homeostasis in response to TC in embryonic thermally manipulated chicks (ED 7–11 at 39°C for 18h) when compared to the control. Additionally, their body requires reprogramming of metabolism and gene expression to shunt energy sources from growth-related biosynthetic processes to defense, acclimatization, or adaptation to heat stress. Therefore, the thermotolerance acquisition induced by TM1 was safe for broiler muscle tissues. More significantly, growth performance in TM1 (ED 7–11 to 39°C for 18h) was actually significantly improved over other treatment conditions compared to the control.

Jia *et al.* (2016) reported that embryonic development is directly affected by the environment, since fertilized eggs are independent from the maternal system. Moreover, Lindy & Rajasalmi (1966) reported that incubation of chick embryos in a hypoxic environment causes an increase in the proportion of tissue LDH, whereas incubation in aerobic conditions decreases the proportional amount of LDH. This study shows that embryonic thermal manipulation increases mRNA expression of LDH in pectoral and/or thigh muscle in all treatment groups as compared to the control. This means that the LDH expressed may be related to the incubation of the embryos in a hypoxic

environment condition during thermal manipulation (such as elevated incubation temperature 39°C for 18h and high humidity rate (65%)) which significantly lowers the oxygen conductance of the eggshell during these hypoxic environment conditions and subsequently causes an increase in the proportion of tissue LDH in pectoral and thigh muscles in TM groups as compared to the control.

Consequently, it may be that the adaptation of TM1 to the high incubation temperature of 39°C for 18h and high humidity rate (65%) in early development (ED 7–11) is associated with low metabolic oxygen demand in the tissue. Jia *et al.* (2016) reported that the adaptation of Tibetan chicken embryos to high altitude (low oxygen) may be associated with higher quantities of yolk in the egg and a low metabolic oxygen demand in the tissue, which illuminates the reasons why Tibetan chicken have higher hatchability with lower oxygen transport ability.

On post-hatch day 35, the highest significant mRNA expression of LDH was only in the pectoral and thigh muscles of TM4 as compared to the control. Moreover, our findings show that serum LDH levels were significantly higher in the control, TM2, TM3, and TM4 when compared to TM1. Together, mRNA gene expression and serum levels of LDH on post-hatch day 35 indicate that there was myonecrosis in both pectoral and thigh muscles of these groups as compared to TM1. On the other hand, TC at 43°C for 6h significantly lowers the mRNA expression of LDH in pectoral muscle for all treatment groups and even the control when compared to their counterpart naïve groups. In the thigh, mRNA expression of LDH was significantly higher in TM2, TM3, TM4, and even the control when compared with TM1 after 6h of TC. Additionally, TC caused a significant decline in serum LDH levels in TM1 when compared to the control and other treatment groups. This decline started from the first hour of TC until the end of the exposure after 6h.

It seems that the control group did not cope with stress after 6h of TC when compared to all treatment groups. The lower mRNA expression and lower plasma LDH levels in TM1 after 6h of TC signify that TM1 adapted for a second time to the higher environmental heat stress (43°C for 6h), and that it can cope with heat stress without a negative impact on body performance.

According to Ward & Peterson (1973), the increase in enzyme activity may be partly attributed to cellular damage as a direct consequence of heat stress. Therefore, plasma enzyme activities that are highly correlated with the ability to withstand heat stress



may serve as genetic markers for the selection and development of a chicken more resistant to heat stress (Melesse *et al.*, 2011).

It can be concluded that, among the various TM conditions that were investigated, TM1 treatment resulted in significant improvement of muscle physiological response to heat stress by lower CK and LDH during TC on post-hatch day 35 as compared to the control. The outcome of this research may improve broiler production efficiency and contribute to increased broiler chicken adaptation to environmental heat stress without negative impact on body performance.

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REFERENCES

- Al-Zghoul MB, El-Bahr SM, Al-Rukibat RK, Dalab AES, Althnaian TA, Al-Ramadan SY. Biochemical and molecular investigation of thermal manipulation protocols during broiler embryogenesis and subsequent thermal challenge. *BMC Veterinary Research* 2015;11:292-301.
- Balaban J, Wierzbicki M, Zielińska M, Szczepaniak J, Sosnowska M, Daniluk K, *et al.* Effects of graphene oxide nanofilm and chicken embryo muscle extract on muscle progenitor cell differentiation and contraction. *Molecules* 2020;25(8).
- Brancaccio P, Maffulli N, Limongelli FM. Creatine kinase monitoring in sport medicine. *British Medical Bulletin* 2007;81:209–230.
- Hettling H, van Beek JH. Analyzing the functional properties of the creatine kinase system with multiscale 'sloppy' modeling. *PLOS Computational Biology* 2011;7(8):e1002130.
- Hsiao-Mei L, Der-Yuh L, Yan-Derh, Tsung-Pingh, Hsiu-Luan C, Cheng-Yung L, *et al.* Association of heat shock protein 70 gene polymorphisms with acute thermal tolerance, growth, and egg production traits of native chickens in Taiwan. *Archives of Animal Breeding* 2016;59:173–181.
- Iyengar MR, Iyengar CW, Chen HY, Brinster RL, Bornslaeger E, Schultz RM. Expression of creatine kinase isoenzyme during oogenesis and embryogenesis in the mouse. *Developmental Biology* 1983;96(1):263–268.
- Jia CL, He LJ, Li PC, Liu HY, Wei ZH. Effect of egg composition and oxidoreductase on adaptation of Tibetan chicken to high altitude. *Poultry Science* 2016;95(7):1660–1665.
- Kongas O, and van Beek J. Creatine kinase in energy metabolic signaling in muscle. *Nature Precedings*; 2007. Available from: <https://www.nature.com/articles/npre.2007.1317.1>.
- Lindy S, Rajasalmi M. Lactate dehydrogenase isozymes of chick embryo: response to variations of ambient oxygen tension. *Science* 1966;153(3742):1401-1403.
- Lyons GE, Muhlebach S, Moser A, Masood R, Paterson BM, Buckingham ME, *et al.* Developmental regulation of creatine kinase gene expression by myogenic factors in embryonic mouse and chick skeletal muscle. *Development* 1991;113(3):1017–1029.

- Melesse A, Maak S, Schmidt R, von Lengerken G. Effect of long-term heat stress on key enzyme activities and T3 levels in commercial layer hens. *International Journal of Livestock Production* 2011;2(7):107-116.
- Mitchell MA, Sandercock DA. Age dependent changes in plasma creatine kinase activity in broiler. *Proceedings of 9th European Poultry Conference Glasgow; 1994; Scotland. Andover : World's Poultry Science Association; 1994. p.266-267.*
- Pech-Waffenschmidt V. The effect of heat stress conditions on performance, physiology and blood chemistry of laying hens of different feathering types and nutritional considerations to improve the heat resistance [dissertation]. Berlin (DE): Technical University; 1992.
- Sandercock DA, Hunter RR, Nute GR, Mitchell MA, Hocking PM. Acute heat stress-induced alterations in blood acid-base status and skeletal muscle membrane integrity in broiler chickens at two ages: implications for meat quality. *Poultry Science* 2001;80(4):418-425.
- Tang S, Yin B, Xu J, Bao E. Rosemary Reduces Heat Stress by Inducing CRYAB and HSP70 Expression in Broiler Chickens. *Oxidative Medicine and Cellular Longevity* 2018;7014126.
- Tang S, Yu J, Zhang M, Bao E. Effects of different heat stress periods on various blood and meat quality parameters in young Arbor Acer broiler chickens. *Canadian Journal of Animal Science* 2013;93:453– 460.
- Wanda MH, Colin GR. *Toxicologic pathology: an introduction*. 3rd ed. London: Academic Press; 2013. p.1–9.
- Ward MA, Peterson RA. The effect of heat exposure on plasma uric acid, lactate dehydrogenase, chloride, total protein and zinc of the broiler. *Poultry Science* 1973;52(4):1671-1673.
- Yalçın S, Bruggeman V, Buyse J, Decuypere E, Cabuk M, Siegel P. B. Acclimation to heat during incubation:4. Blood hormones and metabolites in broilers exposed to daily high temperatures. *Poultry Science* 2009;88(9):2006-2013.
- Zhang M, Bao E. Effects of different heat stress periods on various blood and meat quality parameters in young Arbor Acer broiler chickens. *Canadian Journal of Animal Science* 2013;93:453–460.

LIST OF ABBREVIATIONS

- CK: Creatine kinase
LDH: Lactate dehydrogenase
TM: Thermal manipulation
TM1: Thermal manipulation group 1
TM2: Thermal manipulation group 2
TM3: Thermal manipulation group 3
TM4: Thermal manipulation group 4
ED: Embryonic day
°C: Degrees Celsius
mRNA: Messenger ribonucleic acid
µl: Microliter
RH: Relative humidity
RT-PCR: Reverse transcription PCR
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
ACTA1: Alpha (α)-actin
ANOVA: Analysis of variance