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Effect of thermal manipulation during embryogenesis on gene expression of myogenic upstream activation factors pre- and post-hatch in broilers

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ABSTRACT

This study aimed to determine the optimum timing of embryonic thermal manipulation (TM) that may result in improvement of mRNA expression of myogenic upstream activation factors viz. Wnt family member-3 (Wnt-3), sonic hedgehog gene (Shh), proliferating cell nuclear antigen (PCNA) and paired-box transcription factor-7 (Pax-7) during development and histogenesis of broiler muscle. Fertile eggs (1440) were divided randomly and equally into 5 treatment groups including control (no TM) and four treatment groups (TM1, TM2 TM3 and TM4) that were daily subjected to 39℃ for 18 h with 65% relative humidity during embryonic days ED 7–11, ED 11–15, ED 15–18 and ED 7–18 respectively. Pectoral and thigh muscle mRNA expressions of myogenic upstream activation factors were evaluated by semi-quantitative real-time RT-PCR. Out of TM conditions that were investigated, TM1 resulted in a significant improvement of Wnt-3, Shh, PCNA and Pax-7 expressions in broiler pectoral and thigh muscles during embryonic and post-hatch life when compared to the control. Thus, thermal manipulation during early embryongenesis (embryonic days ED 7-11) enhance broiler skeletal muscle myoblast proliferation by triggering and inducing transcription factors that regulates myogenesis and subsequently may lead to improve cell number and size of skeletal muscle. The outcome of this study indicates that TM during ED7-11 improved muscle response to heat stress, was safe to the pectoral and thigh muscles and this method may enhance myogenesis and muscle growth in a positive manner.

Keywords: Broiler, Myogenesis, Paired-box transcription factor-7, Sonic hedgehog, Thermal Manipulation, Wnt family member-3

Thermal manipulation (TM) of chicken embryos at critical phases of organs' system development has been conducted as a way of improving long-term functions of these systems (Piestun *et al*. 2008, Al-Zghoul *et al*. 2013). One of these systems is the musculoskeletal system. Recent studies revealed that applying thermal manipulation during broiler chicken embryogenesis may enhance the growth of the skeletal muscles, hence, the body weight (Al-Rukibat *et al*. 2017, Dalab and Ali 2019).

Development and histogenesis of skeletal muscle proceed from early embryogenesis through adulthood (Piestun *et al.* 2009, Almada and Wagers 2016). This development determined by the number and size of skeletal muscle cells in addition to the rate of skeletal muscle protein synthesis and/or degradation (Braun and Gautel 2011, Mukund and Subramaniam 2020). The first phase of myofibre growth occurs early in life, and it is characterized by a high level of satellite cell mitotic activity (Yablonka-Reuveni 2011). In this respect, the skeletal muscle myoblasts proliferation and myocytes differentiation are regulated directly by cellular structure and signal pathways which regulates the number, size and strength of skeletal muscle myocytes (Yu *et al.* 2021). While, in post-hatch life, muscle growth occurs exclusively through an increase in myofibre size by an increase in the volume of cytoplasm without an increase in myofibre number (Mozdziak *et al*. 2000, Yin *et al*. 2013). The proliferation and differentiation of cells results from the complex sequential action of different secreted signals, and through activation of specific transcription factors (Hernandez *et al*. 2017). The molecular pathways such as concentration of myogenic upstream activation factors, myoblast surface receptors and intracellular helix-loop-helix (bHLH) transduction signaling pathways are considered very important in determination of skeletal muscle protein synthesis and/or degradation rates (Weintraub 1993, Floss *et al*. 1997, Clase *et al*. 2000, Edom-Vovard *et al*. 2001). Thus, the rationale of this research was to understand the effect of thermal manipulation on the expression of some myogenic upstream activation factors (Wnt-3, Shh, PCNA and Pax-7) in broiler pectoral and thigh muscles as indicator of satellite cell mitotic activity

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and muscle growth that proceed from early embryogenesis through marketing day at post-hatch day 35.

MATERIALS AND METHODS

Pre-egg incubation management: All experimental procedures including experimental incubation and hatching management conditions were approved by King Faisal University's Animal Care and Use Committee (KFU-ACUC), session (5) date 27/4/2017. A total of 1,700 Ross 708 broiler fresh eggs were sourced from a 36-week-old broiler flock (from Al-Ahsa, Saudi Arabia) and stored at 24°C for 12 h in a separate room free of dust and any source of contamination. The broken as well as abnormally small (58 g) and large eggs (65 g) were excluded before the first day of incubation. After discarding these eggs, 1550 eggs with uniform weight were taken for experiment.

Egg incubation and thermal manipulation management: Eggs (1,550) with uniform weight were pre-heated in semi-commercial incubators (type OVA-Easy 380 Advance Series II, Brinsea, Sandford, UK; each incubator has 384 hens eggs capacity) at 27°C for 8 h. Further, all eggs were incubated at 37.8°C with 56% relative humidity (RH). The eggs in all incubators were turned through 45° every hour up to ED18. At embryonic day 7, egg candling was performed to exclude any dead embryos and infertile eggs, ending up with 1,440 fertile eggs. These fertile eggs were distributed randomly into five treatment groups (288 eggs/incubator); the first group remained incubated at 37.8°C with 56% RH normal temperature (control group). 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. The incubation temperature and RH were raised from day 7 to 11 (TM1) and 11-15d (TM2), 15-18d (TM3) and 7-18d (TM4).

Hatching management: From ED18-ED20, fertile eggs of all groups were incubated at 36.6°C and 70% RH, till ED 21 temperature was lowered to 36.1°C. Upon hatching and full feather drying (approximately 2 h post hatch); chickens (no sex separation) were recorded and transferred to the Agricultural and Veterinary Research and Training Station at King Faisal University where the field study was conducted. Water and feed (ARASCO, Riyadh, Saudi Arabia) were supplied *ad lib*. to the chicks, and they were kept for 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 24post-hatch. The light system design was 24 h of light in thefirst 3 days, 20 h of light and 4 h of darkness per day fromday 3 to day 7, then 16 h of light and 8 h of darkness per day up to day 35.

Sampling management: Pectoral and thigh muscle samples from embryos of all five groups were taken at the end of ED11, ED15, ED18 and 35d post hatch. The samples were subjected to RNA isolation (75 embryos, n=5) and further gene expression studies were conducted for Wnt-3, Shh, PCNA and Pax-7.

RNA isolation and semi-quantitative real time RT-PCR analysis: Wnt-3, Shh, PCNA and Pax-7 mRNA expression levels at embryonic day 11, 15 and 18 and post- hatch day 35 were analysed and quantified using a semi- quantitative real-time RT-PCR. The pectoral and thigh muscles were homogenized by Bead Ruptor (24 Bead MillHomogenizer, OMNI, USA) and total RNA was extracted using the PureZOL™ RNA isolation method (BIO-RAD, Catalog #732-6890, Hercules, CA, USA). DNA was removed using a DNase I kit (Ambion).

The purity and concentration of RNA was estimated by measuring the ratio of absorbances (260/280) using a Synergy™ Mx Monochromator-Based Multi-Mode Microplate Reader (Bio-Tek, USA). The RNA (2 µg) was reverse transcribed to cDNA in a reaction mixture using an iScriptc DNA synthesis kit (BIO-RAD, Hercules, CA, USA). A semi-quantitative Real-Time PCR analysis (BIO-RAD, Hercules, CA, USA) was performed using the ssoAdvanced™ SYBR Green Supermix kit (BIO-RAD, Hercules, CA, USA). A total of 20 µl reaction mixture was prepared using 10 µl of the master mix, 2 µl of the forward primer pm/µl, 2 µl of the reverse primer pm/µl, 2 µl of cDNA from the sample, and 4 µl of nuclease-free water. PCR conditions consisted of an initial denaturation step of 95°C for 1 min, followed by 40 cycles of 95°C for 10 sec, 60°C for 30 sec and 72°C for 10 sec, with a final melting temperature at 95°C for 20 sec. Samples were run in triplicates. GAPDH and Actin 1

Table 1. Oligonucleotides for gene expression analysis by Real time-PCR amplification

Primer	Sequence	GenBank Reference
Wnt-3	F-5'-AAGTTTTGGCAACTCCGGTTT-3' R-5'-GAATCGTAGCTGCTTGGGGA-3'	NM_001171601.1
Shh	F-5'-CCTGTCTTGCTAGGGATCGG-3' R-5'-CAAGTCAGCCCAGAGGAGAC-3'	NM_204821.1
PCNA	F-5'-ACGCATTTGTAGAGACCTCAGCCA-3' R-5'-AGTCAGCTGGACTGGCTCATTCAT-3'	NM_204214.1
Pax-7	F-5'-ACTGTGCCCTCAGTGAGTTCGATT-3' R-5'-ATTCGACATCGGAGCCTTCATCCA-3'	AF019621.1
GAPDH	F-5'-GTGTTATCATCTCAGCTCCCTCAG-3' R-5'-GGTCATAAGACCCTCCACAATG-3'	FJ_217667
ACTA1	F-5'-CCATCGGCAATGAGCGTTTC-3' R-5'-GCATGCGGTCAGCAATACCT-3'	NM_001031063.1

Table 2. Relative normalized expression of mRNA levels of Wnt-3, Shh, PCNA and Pax-7 in pectoral and thigh muscles at embryonic day 11

	Target (ED11)							
	Wnt-3		Shh		PCNA		Pax-7	
	Expression (fold)		Expression (fold)		Expression (fold)		Expression (fold)	
	Pectoral Muscle	Thigh Muscle	Pectoral Muscle	Thigh Muscle	Pectoral Muscle	Thigh Muscle	Pectoral Muscle	Thigh Muscle
Control	1.0±0.9[a]	1.0±0.7[a]	1.0±0.5[a]	1.0±0.3[a]	1.0±0.7[a]	1.0±0.3[a]	1.0±0.7[a]	1.0±0.3[a]
TM1	4.51±0.1[b]	3.21±0.2[b]	8.70±0.2[b]	2.96±0.4[b]	4.63±0.7[b]	2.77±0.3[b]	4.63±0.6[b]	1.25±0.5[a]

Control, 37.8°C; TM1, Thermal manipulation from ED 7-11 at 39°C for 18 h; [a–b]Within the same day, means±SD with different superscripts differ significantly (P<0.05).

Table 3. Relative normalized expression of mRNA levels of Wnt-3, Shh, PCNA and Pax-7 in pectoral and thigh muscles at embryonic day15

	Target (ED15)							
	Wnt-3		Shh		PCNA		Pax-7	
	Expression (fold)		Expression (fold)		Expression (fold)		Expression (fold)	
	Pectoral	Thigh	Pectoral	Thigh	Pectoral	Thigh	Pectoral	Thigh
Control	1.0±0.1[a]	1.0±0.01[a]	1.0±0.3[a]	1.0±0.1[a]	1.0±0.3[a]	1.0±0.3[a]	1.0±0.2[a]	1.0±0.2[a]
TM2	7.68±0.2[b]	4.09±0.2[b]	9.17±0.4[b]	8.44±0.4[b]	0.90±0.3[a]	4.21±0.3[b]	0.86±0.1[a]	5.78±0.1[b]

Control, 37.8°C; TM2, Thermal manipulation from ED 11-15 at 39°C for 18 h; [a–b]Within the same day, means±SD with different superscripts differ significantly (P<0.05).

were kept as housekeeping genes.

Statistical analyses: The original data were arranged using Excel 2007 software (Microsoft Corporation, Redmond, WA, USA). The Wnt-3, Shh, PCNA and Pax-7 mRNA gene expressions data was 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 $p<0.05$.

RESULTS AND DISCUSSION

In TM1 and TM2, thermal manipulation in TM1 from ED7 to ED11 resulted in a high significant increase ($p<0.05$) in the mRNA expressions of Wnt-3, Shh, PCNA and Pax-7 in pectoral and thigh muscles when compared to the control (Tables 2 and 3). It was observed that relative fold change expression of these expressions were higher in pectoral muscle more than thigh muscle. In TM3, it was observed that relative fold change expression of Wnt-3 and Shh gene in pectoral muscle increased with no significant differences in thigh muscle when compared to the control as shown in Table 4. Moreover, TM3 has no significant differences in mRNA expression of PCNA and Pax-7 in both pectoral and thigh muscles when compared to the control (Table 4). The relative fold change expression of Wnt-3 and Shh of TM4 group decreased significantly in both pectoral and thigh muscles when compared to the control (Table 4). In addition, relative fold change expression of PCNA and Pax-7 in TM4 group was also not significant in both pectoral and thigh muscles when compared to the control (Table 4). However, mRNA expression of Wnt-3, Shh, PCNA and Pax-7 was found significantly higher in TM1 in both pectoral and thigh muscles when compared to

Table 4. Relative normalized expression of mRNA levels of Wnt-3, Shh, PCNA and Pax-7 in pectoral and thigh muscles at embryonic day 18

	Target (ED18)							
	Wnt-3		Shh		PCNA		Pax-7	
	Expression (fold)		Expression (fold)		Expression (fold)		Expression (fold)	
	Pectoral	Thigh	Pectoral	Thigh	Pectoral	Thigh	Pectoral	Thigh
Control	1.0±0.6[a]	1.0±0.3[a]	1.0±0.4[a]	1.0±0.2[a]	1.0±0.3[a]	1.0±0.2[a]	1.0±0.3[a]	1.0±0.3[a]
TM1	9.30±6.5[b]	5.19±0.1[b]	9.06±5.1[b]	3.57 ±0.1[b]	1.96±0.4[b]	1.94±0.4[b]	1.95±0.5[b]	0.93±0.2[a]
TM2	3.57±0.5[c]	0.97±0[a]	4.16±2.3[c]	0.62 ±0.1[a]	1.37±0.3[a]	1.79±0.4[b]	1.34±0.3[a]	1.99 ±0.1[b]
TM3	3.54±3.1[c]	0.67±0[a]	3.52±3.6[c]	0.78±0.08[a]	0.73±0.2[a]	1.24±0.2[a]	0.62±0.2[a]	0.81±0.1[a]
TM4	N/A	N/A	0.46±0.2[d]	0.32±0.05[c]	0.87±0.1[a]	1.47±0.2[a]	0.72±0.3[a]	0.73 ±0.5[a]

Control, 37.8°C; TM1, Thermal manipulation from ED 7-11 at 39°C for 18 h; TM2, Thermal manipulation from ED 11-15 at 39°C for 18 h; TM3, Thermal manipulation from ED 15-18 at 39°C for 18 h.TM4, Thermal manipulation from ED 7-18 at 39°C for 18 h. [a–d] Within the same day, means±SD with different superscripts differ significantly (P<0.05).

the control and TM2, TM3 and TM4 (Table 4).

Furthermore, a comparative gene study was also performed on the relative normalized expression of Wnt-3, Shh, PCNA and Pax-7 mRNA levels in both pectoral and thigh muscles collected at ED 11 to ED18. The results revealed that the gene expression of Wnt-3, Shh, PCNA and Pax-7 were upregulated during early embryogenesis (ED7-ED11) and was downregulated at ED15 up to ED18 (Fig. 1).

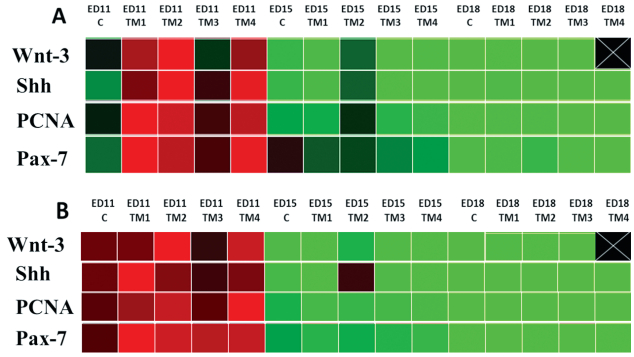

Fig. 1. Clustergram of samples and targets from ED11-ED18. (A) Pectoral muscle, (B) Thigh muscle. The data colour indicates greater expression (up regulated-red colour), lower expression (down regulated-green colour) and no change in expression (no change in regulation -black colour). The lighter the colour, the greater the expression level. by CFX96 Touch TM Real time PCR. Control, 37.8°C; TM1, Thermal manipulation from ED 7-11 at 39°C for 18 h; TM2, Thermal manipulation from ED 11-15 at 39°C for 18 h; TM3, Thermal manipulation from ED 15-18 at 39°C for 18 h. TM4, Thermal manipulation from ED 7-18 at 39°C for 18 h.

The effect of TM during embryogenesis on Wnt-3, Shh, PCNA and Pax-7 mRNA expressions at post-hatched day35 are given in Fig. 2 and Fig. 3. In pectoral muscle of post-hatched chicks at day 35, TM1 group showed higher significant (P<0.05) mRNA expression of Wnt-3, Shh, PCNA and Pax-7 when compared to the control. While in TM2, it showed higher significant mRNA expression of Wnt-3, Shh without any significant changes (P<0.05) in PCNA and Pax-7 compared to the control. In TM3 and TM4, embryonic thermal manipulation did not cause significant difference (p<0.05) of Wnt-3, Shh, PCNA and Pax-7 mRNA expressions compared to the control as shown in Fig. 2.

In thigh muscle, the expression of Wnt-3, Shh, PCNA and Pax-7 was significantly greater (p<0.05) in 35d chicks of TM1 group when compared to rest of the groups (Fig. 3). Moreover, thigh muscle of TM2, TM3 and TM4 showed no significant difference in Wnt-3, PCNA and Pax-7 mRNA expression compared to the control (P<0.05). On the other hand, TM2, TM3 and TM4 showed up regulated mRNA expression of Shh as well as TM1 when compared to the control (Fig. 3).

In the present study, the results showed that thermal manipulation at 39°C for 18 h, at early age ED7-11, prolongs stimulatory effect on muscular mRNA gene

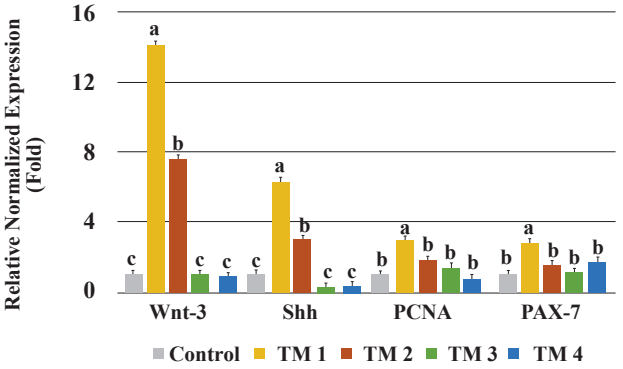

Fig. 2. The effect of thermal manipulation (TM) during embryogenesis on the mRNA level of Wnt-3, Shh, PCNA and Pax-7 in pectoral muscle at post-hatch day 35. [a-c]Folds with different superscripts differ significantly (P<0.05).

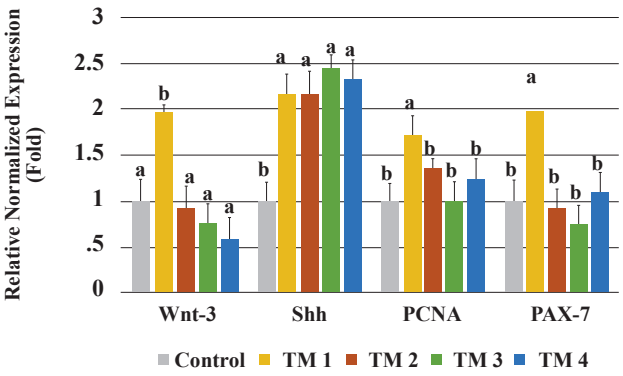

Fig. 3. The effect of thermal manipulation (TM) during embryogenesis on the mRNA level of Wnt-3, Shh, PCNA and Pax-7 in thigh muscle at post-hatch day 35. [a-b]Folds with different superscripts differ significantly (P<0.05).

expression of myogenic upstream activation factors viz. Wnt family member-3 (Wnt-3), sonic hedgehog gene (Shh), proliferating cell nuclear antigen (PCNA) and paired-box transcription factor-7 (Pax-7) as compared to the control. This is in accordance with Halevy *et al*. (2001) who reported that mild heat exposure at an early age (post-hatch day 3) results in the acceleration of myogenesis mediated by specific local myogenic regulatory factors. Myogenesis is a process of formation and maturation of skeletal muscle throughout animal development, from embryo to the adult life (von Maltzahn *et al*. 2012). This process begins by determination and proliferation of somatic cells and ends with differentiation into mature muscle cells that receive intercellular signals from pathways such as the Wnt or sonic Hedgehog (Shh) signaling pathways (Munsterberg *et al*. 1995). Moreover, any dysregulation of this signaling pathway can lead to myogenic developmental defect and perturbation of myogenic homeostasis as von Maltzahn *et al*. (2012) reported. This study demonstrates that Wnt family member-3 (Wnt-3) and sonic hedgehog (Shh) mimic pectoral and thigh muscles inducing activity. When compared to the control, the results showed that thermal manipulation at 39°C for 18 h, at early age ED7-11 or middle age ED11-15 has a positive stimulatory effect

on embryonic myogenesis on both pectoral and thigh muscles compared to late (TM3) and long lasting (TM4) thermal manipulated groups as well as the control. This is indicated by immediate significant enhancement of mRNA gene expression of myogenic upstream activation factors Wnt-3 and Shh in both pectoral and thigh muscles of early and middle thermal manipulated groups (TM1 and TM2) compared to the control. On the other hand, Gabriel *et al*. (2003) suggested that myogenic proliferation and differentiation events are compromised by variation in environmental temperature during avian embryogenesis; they observed that chicken embryos that subjected to TM at 44°C after ED4 for 1 h had delayed myofibre formation. The discrepancies between our finding and the previous study as reported by Gabriel *et al*. (2003) can be explained by the differences in incubation temperature and duration. Therefore, myogenic upstream activation factors Wnt-3 and Shh induced by early TM (39°C at ED 7-11 for 18 h) indicates that early TM was safe to the bird pectoral and thigh muscles and it may enhance myogenesis and muscle growth in a positive manner. The significant increase in gene expression of myogenic upstream activation factors Wnt-3, Shh, PCNA and Pax-7 at 39°C in TM1 in the present study indicates that small increase in incubation temperature changes the expression level of the genes positively instead of reduction in the expression, as mentioned by Gabriel *et al.* (2003).

Our finding on post-hatch days 35, showed a significant increase in Wnt-3 and Shh expression in both pectoral and thigh muscle of TM1 group (thermal manipulation at ED7-11 at 39°C for 18h) when compared to the control. It seems that early TM at ED7-11 has long effect on the expression of myogenic upstream activation factors at later age post hatch 35d which can be explained by persistent myogenesis (satellite cells differentiation) and they have a role in the maintenance of skeletal muscle homeostasis in later life (von Maltzahn *et al*. 2012). This was in agreement with Dalab and Ali (2019) who reported a significant positive effect observed in body, skinned carcass, breast and thigh muscle weights in boilers that were exposed early to thermal manipulation at ED7-11 at 39°C for 18 h when compared to the control. Taken together, these results suggest that the timing of thermal manipulation during ED7-11 is crucial for maximal expression of Wnt-3 and Shh, which is manifested in the induction of development and growth of both pectoral and thigh muscles.

Proliferating cell nuclear antigen (PCNA), considered as processivity factor that play an important role in DNA synthesis during replication and repair (Strzalka and Ziemienowicz 2011). Satellite cells proliferation during myogenesis undergo a certain number of divisions, during this process the genetic material may be damaged and which might result in uncontrolled proliferation or cell death (Strzalka and Ziemienowicz 2011). Nevertheless, satellite cells are capable of reentering the cell cycle in response to various muscular stresses and undergo proliferation (Piestun *et al*. 2009). Thermal manipulation (heat stress) during myogenesis may act as muscular stresses and it may trigger PCNA expression to play an important role in satellite cell DNA synthesis during replication and repair. Therefore, PCNA expressions considered as marker for satellite cell response during myogenesis (Johnson and Allen 1993). Our finding in TM1 group (TM during ED7-11 at 39°C for 18 h) showed a significant increase in mRNA gene expression of PCNA in both pectoral and thigh muscle at early embryonic age (ED11) and it persist higher up to ED18. The increased mRNA gene expression of PCNA also persist higher even at post-hatch day35 in this group (TM1). While the other thermal manipulated groups were comparable to the control. This was in agreement with Al-Zghoul *et al*. (2016) who reported that TM during ED14-18 at 39°C for 18 h has a significant induction effect on mRNA gene expression of PCNA during embryogenesis.

A recent study by Al-Zghoul and El-Bahr (2019) reported that TM during ED12-18 at 39°C for 18 h has a significant reduction effect on mRNA gene expression of Pax-7 during embryogenesis. This was compatible with our finding in late (ED15-18) and long-lasting (ED7-18) TM at 39°C for 18 h. On the contrary, our finding in TM1 (TM during ED7-11 at 39°C for 18 h) showed a significant increase of mRNA gene expression of Pax-7 during embryogenesis and post-hatch day35 in both pectoral and thigh muscle with a highest value in pectoral muscle. This was supported by Piestun *et al*. (2009) who observed a higher satellite cells proliferation and enhanced muscle fibre diameter after embryonic thermal manipulation at 39.5°C which manifest themselves later during post-hated life by increased the size of the pectoralis muscles, without deteriorating their quality. Moreover, Dalab and Ali (2019) also observed that early TM during ED7-11 at 39°C for 18 h enhance muscle fibre diameter which is manifested by increase in the breast and thigh muscle weight.

It can be concluded that early thermal manipulation during embryonic day 7 to 11 is the best and safe for thermal manipulated protocols that may be applied for commercial broiler which may induce a significant improvement of mRNA expression of upstream myogenic activation factors namely Wnt family member-3 (Wnt-3), sonic hedgehog gene (Shh), proliferating cell nuclear antigen (PCNA) and paired-box transcription factor-7 (Pax-7) during embryogenesis and at day 35 of post-hatch. This indicates that thermal manipulation may enhance the signaling pathways and the transcription factors that regulates myoblast proliferation; thus, early thermal manipulation (ED 7–11) my improve meat productivity rates of broilers.

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