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Thermal Manipulation Mid-term Broiler Chicken Embryogenesis: Effect on Muscle Growth Factors and Muscle Marker Genes.

ABSTRACT

Thermal manipulation (TM) during broiler chicken embryogenesis has been shown to promote muscle development and growth. However, the molecular bases of promoting broiler muscle development and growth are not fully understood. The aim of this study was to investigate the molecular bases of muscle growth and development in broiler chickens subjected to TM. This included the investigating of the changes in mRNA expression levels of muscle marker genes, namely MyoD, myogenin, paired box transcription factor (Pax7) and proliferating cell nuclear antigen (PCNA), and muscle growth factors namely insulin-like growth factor 1 (IGF-1), myostatin and growth hormone (GH) during embryogenesis and on posthatch days 10 and 28. Fertile Cobb eggs (n=1500) were divided into four groups. Eggs in the first group (control) were incubated at 37.8°C and 56% RH, whereas, eggs in the second group (TM₁), third group (TM₂) and fourth group (TM₃) were subjected to 39 °C and 65% RH daily during embryonic days (ED) 12–18 for 9, 12, and 18 hours, respectively. Body weight (BW) during embryogenesis and posthatch days (1, 3, 5, 7, 14, 21, 28 and 35) was recorded. mRNA expression levels of muscle marker genes and muscle growth factor genes during ED 12, 14, 16 and 18 and on posthatch days 10 and 28 were analyzed using real-time RT-PCR. TM upregulated the mRNA expressions of muscle marker and growth factors genes. This upregulation was accompanied by improvement of body weight near and at market age.

INTRODUCTION

Agricultural scientists and farmers have long been concerned with the improvement to the muscle mass of meat-producing livestock (Bass *et al.*, 1999). The productivity of these animals is directly related to the cellular and molecular mechanisms regulating skeletal muscle myoblasts growth and development of muscle mass (Koohmaraie *et al.*, 2002).

Embryonic muscle-precursor cells, originating from multipotential mesodermal cells, undergo myogenic determination and give rise to myoblasts (Ordahl *et al.*, 2000). In muscle cells, as in a variety of cell types, a balance of opposing cellular signals controls proliferation and differentiation. At birth, a high number of proliferating satellite cells are found in skeletal muscle nuclei; however, at the end of the growth phase, their numbers are reduced to less than 5% of total muscle fiber nuclei and most have become quiescent (Hawke and Garry, 2001). The muscle-specific basic helix-loop-helix (bHLH), a family of transcription factors, regulates the proliferation and differentiation of myoblasts (Weintraub, 1993). Upon satellite cell activation, these transcription factors are expressed in a sequential pattern. Initially, Myf5 and MyoD are expressed in the proliferating progeny, and then myogenin is



expressed as the cells enter differentiation (Cornelison and Wold, 1997).

Previously, it has been shown that incubation conditions may affect chicken embryo development (Lourens *et al.*, 2005; Lourens *et al.*, 2007). Temperature is considered as the most important environmental factor during chicken embryo incubation (Decuypere and Michels, 1992). Incubation temperature has a significant effect on hatchability rate, chick quality, and posthatch growth performance parameters (Decuypere & Michels, 1992; Lourens *et al.*, 2005). Thermal manipulation, in which the embryos are exposed to high or low temperatures during susceptible periods of broiler embryogenesis, has been reported to enhance muscle growth and to improve the acquisition of thermotolerance of broiler chickens (Yalcin & Siegel 2003; Yahav *et al.*, 2004; Piestun *et al.*, 2008a; Piestun *et al.*, 2008b; Al-Zhgoul *et al.*, 2013; Al-Zghoul *et al.*, 2015; Piestun *et al.*, 2015). However, the molecular bases for promoting muscle development and growth are not fully understood (Decuypere & Michels, 1992; Piestun *et al.*, 2009; Al-Zhgoul *et al.*, 2013). The aim of this study was to investigate the molecular bases of muscle growth and development of broiler chickens subjected to thermal manipulation (39° C and 65% for 9, 12 or 18 h) daily on embryonic days (ED)12–18. Changes in mRNA expression levels of muscle marker genes, namely MyoD, myogenin, paired box transcription factor (Pax7) and proliferating cell nuclear antigen (PCNA), and muscle growth factors, namely insulin-like growth factor 1 (IGF-1), myostatin, and growth hormone (GH) were investigated during embryogenesis and on posthatch days 10 and 28. Results of this study provide an insight into the molecular bases of muscle development and growth of thermally-manipulated broilers.

MATERIALS AND METHODS

Egg incubation and hatching management

All experimental procedures and management conditions used in this study were approved by the King Faisal University Animal Care and Use Committee (KFU-ACUC).

Fertile Cobb eggs (n = 1700) were obtained from one breeder flock (Riyadh, Saudi Arabia) and incubated in semi-commercial incubators (type OVA-Easy380AdvanceSeries II, Brinsea, Sandford, UK). Small (<55g) and large (>75g) eggs were rejected. One thousand and five hundred (1,500) normal weight eggs were selected, with an average weight

of 62±2g. The selected eggs were divided into four incubation treatment groups (375 each). Eggs in the control group were maintained at 37°C and 56% relative humidity (RH) throughout the incubation period; eggs in the TM₁ group were subjected to TM of 39°C for 9 h and 65% RH daily on ED 12–18; eggs in the TM₂ group were subjected to TM of 39°C for 12 h and 65% RH daily on ED 12–18; and eggs in the TM₃ group were subjected to TM of 39°C for 18 h and 65% RH daily on ED 12–18. After the TM application periods, eggs were maintained at 37.8°C and 56% relative humidity (RH).

At hatch, the number of hatched chicks was recorded. After feather drying, the one-day-old chicks were transferred to King Faisal University Agricultural Research Station, Department of Anatomy, where the field experiment was conducted. As recommended in the Cobb Broiler Management Guide (Cobb-Vantress 2013), chicks were distributed in floor pens (10 birds/m²) at 33°C, and the temperature was gradually decreased to 24°C on posthatch day 21. From posthatch day 24 until day 35, the temperature was maintained at 21°C. Birds were fed a commercial feed for five weeks. Feed and water were provided *ad libitum*. Birds were individually weighed daily during the first week posthatch and at days 10, 14, 21, 28 and 35 of age, using 0.01-g precision scale to determine body weight (BW).

Sample collection

Pectoral muscle mRNA expression levels of muscle marker genes (MyoD, myogenin, Pax7 and PCNA) and muscle growth factors (IGF-1, GH and myostatin) were evaluated using semi-quantitative real-time polymerase chain reaction (RT-PCR) analyses using a real-time thermal cycler (CFX96 Touch TM Real time PCR, BIO-RAD, California, USA). One hundred milligram of Pectoral muscle samples were collected from 80 embryos (five embryos per treatment group per day) on ED 12, 14, 16 and 18, and from 40 chicks (five chicks per treatment group per day) on posthatch days 10 and 28.

RNA isolation and semi-quantitative RT-PCR analyses

Total RNA was extracted from homogenated muscle tissue samples using the TRIzol/chloroform/isopropanol method, followed by the removal of supernatants. The RNA pellet was then dissolved in diethylpyrocarbonate (DEPC)-treated RNase-free water (Ambion, Austin,



TX). DNA was removed using DNase I kit (Ambion, Austin, TX), and the RNA samples were checked for concentration and purity (260:280 nm absorbency). Two μg of RNA were reverse transcribed to cDNA in a reaction mixture using iScript cDNA Synthesis Kit (BIO-RAD, California, USA). The cDNA was then used for semi-quantitative RT-PCR (CFX96 Touch TM Real time PCR, BIO-RAD, California, USA). The semi-quantitative RT-PCR was performed using the ssoAdvanced TM SYBR Green Supermix kit (BIO-RAD, California, USA). Briefly, the 20 μL reaction mix was prepared from 10 μL of master mix; 2 μL forward primer (10 pmol); 2 μL reverse primer (10 pmol); 2 μL cDNA of the sample and 4 μL of nuclease-free water. Cycling parameters were 50°C for 2 min, 95°C for 15 min, 40 cycles of 95°C for 10 s, followed by 30 s at 55°C and 72°C for 10 s with final melting at 95°C for 20 s. Duplicates from each cDNA were analyzed, fluorescence emission was detected, and relative quantification was calculated automatically using CFX managerTM software V3.1 (BioRad, Hercules, CA, USA).

Primers design

Sequences of broiler chicken MyoD, myogenin, Pax7, PCNA, IGF-1, GH, myostatin, and GAPDH were obtained from the gene bank (accession number: (NM_001004384.2, NM_204359.2, AF019621.1, NM_204214.1, FJ882411.1, NM_205065.1, NM_204170.2 and NM_204305.1)). Using these sequences, primers were designed for each gene for the semi-quantitative RT-PCR analyses. All primers were designed using IDT Primerquest software ([http://eu.idtdna.com/PrimerQuest /Home/Index](http://eu.idtdna.com/PrimerQuest/Home/Index)). The sequences of the primers are listed in (Table 1).

Table 1 - Oligonucleotides used for expression analysis of chicken genes and the annealing temperatures (AT).

Gene	Primer sequences	AT
cMyoD	(F) 5'TACCCAGTGCTGGAGCACTA3' (R) 5'GTCTTGGAGCTTGGCTG AACT3'	57°C
cMyogenin	(F) 5'AGCAGCCTCAACCAGCAGGA3' (R) 5'TCTGCCTGGTCATCGCTCAG3'	58°C
cPax 7	(F) 5'ACTGTGCCCTCAGTGAGTTTCGATT3' (R) 5'ATTCGACATCGGAGCCTTCATCCA3'	66.8°C
cPCNA	(F) 5'ACGCATTGTAGAGACCTCAGCCA3' (R) 5'AGTCAGCTGGAGCTGGCTCATTCAT3'	64.9°C
cMyostatin	(F) 5'ATGCAAAAAGCTAGCAG TC3' (R) 5'TCATGAGCACCCGCAACGAT3'	52°C
cIGF-I	(F) 5' CGTACAGAGCGTGCAGATT3' (R) 5' CGTAC AGAGCGTGCAGATT3'	55°C
cGH	(F) 5' GACCAGAGGTACACCAACAAA3' (R) 5'GACTGGATGAGAACCAGTGAAA3'	57°C
cGAPDH	(F) 5' GGTGGCCATCAATGA3' (R) 5'CAGTGGTCATAAGACCC 3'	52 °C

AT: Annealing Temperature

Statistical analysis

Body weight, relative expression of mRNA levels of MyoD, myogenin, Pax7, PCNA, IGF-1, GH, and myostatin were expressed as means \pm standard error (SE). One-way analysis of variance, followed by all-pairs Bonferroni test, was used to compare different parameters among treatment groups (control vs. TM groups) using IBM SPSS Statistics 23 Software (IBM software, Chicago, USA). Differences were considered significant at $p < 0.05$.

RESULTS

Effect of thermal manipulation on embryonic and posthatch body weight

The effect of TM on embryo BW is shown in Figure 1. On ED 12, TM₂ and TM₃ embryos were significantly heavier than the control and TM₁ embryos ($p < 0.05$). However, on ED 14 and ED16, there were no significant differences in BW among the treatment groups ($p > 0.05$). On ED 18, TM significantly increased embryonic BW when compared with the control ($p < 0.05$).

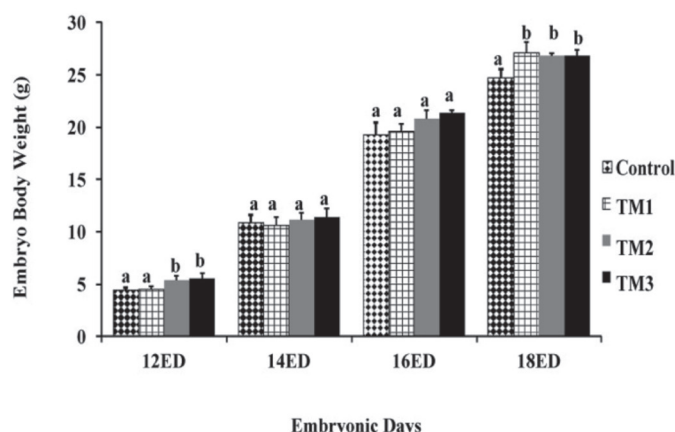


Figure 1 – The effect of thermal manipulation (TM) on the body weight of broiler embryos subjected to different thermal manipulation treatments during embryogenesis) $n = 5$). Control= 37.8°C; TM1= Thermal manipulation at 39°C for 9 h; TM2= Thermal manipulation at 39°C for 12h; TM3= Thermal manipulation at 39°C for 18 h. a,b Within the same ED, means \pm SD with different superscripts differ significantly ($p < 0.05$).

Body weight of control and TM chicks during the posthatch period (first week of age, and 14, 21, 28, and 35 of age) are shown in Figure 2. During the first three weeks of age, except for day 1 (TM₁: 53.9, TM₂: 53.7, TM₃: 54.7 vs. control:49.1) and day 3 (TM₁: 62.3, TM₂: 62.3, TM₃: 62.8 vs. control:58.9), no significant changes in BW were observed among the treatment groups ($p > 0.05$). However, on posthatch day 28, the BW of all TM groups were significantly higher compared with the control group ($p < 0.05$). However, on posthatch day 35, the BW of TM₂ and TM₃ groups were significantly higher compared with those of the control and TM₁ groups ($p < 0.05$).

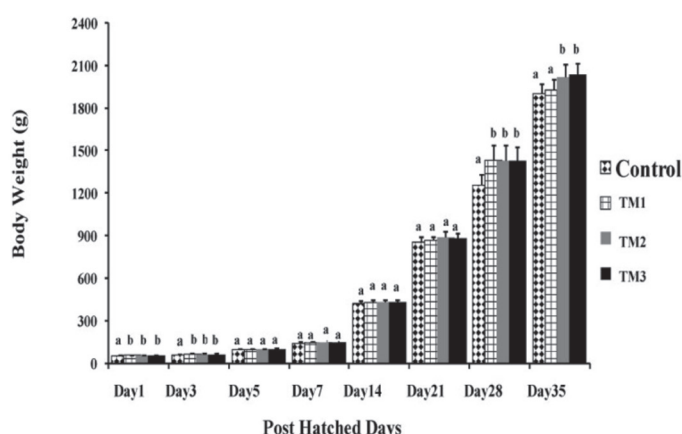


Figure 2 – The effect of thermal manipulation (TM) during embryogenesis on posthatch body weight of broilers from day 1 to day 35 (n = 20). Control= 37.8°C; TM1= Thermal manipulation at 39°C for 9 h; TM2= Thermal manipulation at 39°C for 12h; TM3= Thermal manipulation at 39°C for 18 h. a–c Within the same day, means \pm SD with different superscripts differ significantly (p<0.05).

Effects of thermal manipulation on mRNA expression levels of muscle marker genes (MyoD, myogenin, Pax7 and PCNA)

The effects of embryonic thermal manipulation on MyoD mRNA expression on ED 12, 14, 16, and 18, and on posthatch days 10 and 28 are shown in Figure 3 A-B. MyoD mRNA expression was significantly higher in groups TM₂ and TM₃ on ED 12 and in all TM groups on ED 14 (Figure 3A) (p<0.05). However, on ED 16, MyoD mRNA expression in the TM₃ group was significantly higher than the control, TM₁, and TM₂ groups, which remained comparable (Figure 3A) (p<0.05). Similarly, on ED 18, there were no significant differences in MyoD

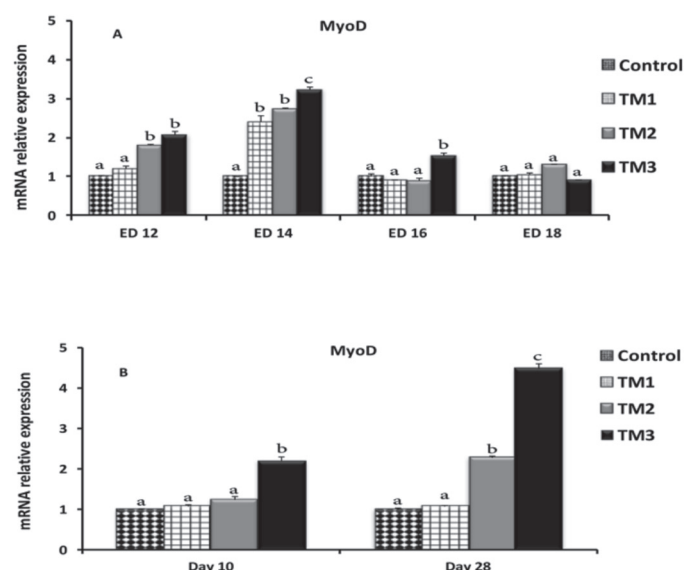


Figure 3 – The effect of thermal manipulation (TM) during embryogenesis on MyoD gene expression during embryonic days 12-18 (A) and posthatching days 10 and 28 (B) (n = 20). Control= 37.8°C; TM1= Thermal manipulation at 39°C for 9 h; TM2= Thermal manipulation at 39°C for 12h; TM3= Thermal manipulation at 39°C for 18 h. a–c Within the same day, means \pm SD with different superscripts differ significantly (p<0.05).

mRNA expression among the treatment groups (Figure 3A) (p>0.05). On day 10 posthatch, TM significantly increased MyoD mRNA expression in the TM₃ group compared with the control, TM₁ and TM₂ groups, which remained comparable (Figure 3B) (p<0.05). However, on day 28 posthatch, higher expression of MyoD was only observed in TM₂ and TM₃ groups compared with the control and TM₁ groups, which remained comparable (Figure 3B) (p<0.01).

The effects of embryo thermal manipulation on myogenin mRNA expression on ED 12, 14, 16, and 18, and on posthatch days 10 and 28 are shown in Figure 4 A-B. On ED 12, mRNA expression of myogenin was significantly lower in the TM₁ and TM₂ groups and significantly higher in the TM₃ group relative to the control group (Figure 4A) (p<0.05). On ED 14, all TM groups presented significantly higher myogenin mRNA expression compared with the control group (p<0.05), and myogenin mRNA expression was higher noted in the TM₁ and TM₃ groups compared with TM₂ (Figure 4A) (p<0.05). On ED 16 and 18, TM promoted significantly higher myogenin mRNA expression in the TM₂ and TM₃ groups compared with the control and TM₁ groups, which remained comparable (Figure 4A) (p<0.05). At day 10 of age, TM resulted in significantly higher in myogenin mRNA expression compared to the control with time-dependent manner (Figure 4B) (p<0.05). However, on day 28, the TM3 group presented significantly higher myogenin mRNA expression compared with the control, TM₁ and TM₂ groups, which remained comparable (Figure 4B) (p<0.05).

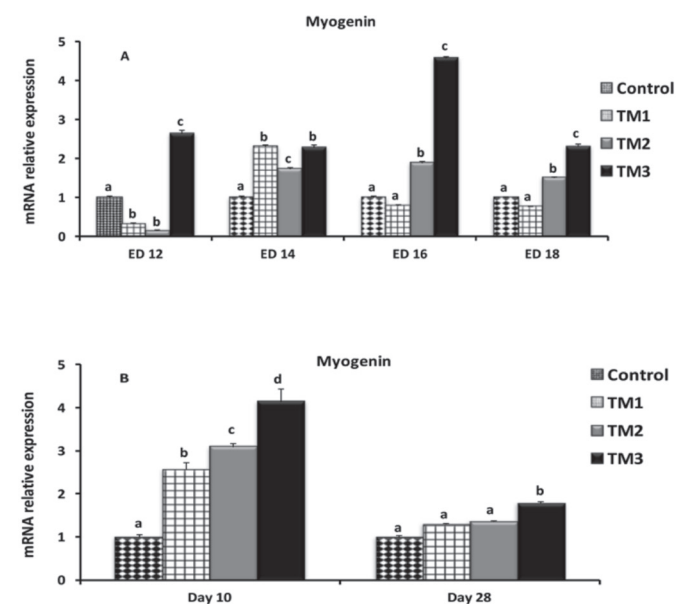


Figure 4 – The effect of thermal manipulation (TM) during embryogenesis on myogenin gene expression during embryonic days 12-18 (A) and posthatching days 10 and 28 (B) (n = 20). Control= 37.8°C; TM1= Thermal manipulation at 39°C for 9 h; TM2= Thermal manipulation at 39°C for 12h; TM3= Thermal manipulation at 39°C for 18 h. a–d Within the same day, means \pm SD with different superscripts differ significantly (p<0.05).



The effects of embryo thermal manipulation on PCNA mRNA expression on embryonic days 12, 14, 16, and 18, and on posthatch days 10 and 28 are shown in Figure 5 A-B. On ED 12, there were no significant differences in PCNA mRNA expression among treatment groups (Figure 5A) ($p>0.05$). However, on ED 14 and 16, PCNA mRNA expression was significantly higher in the TM₃ group compared with the other treatment groups (Figure 5A) ($p<0.05$). On ED 18, PCNA mRNA expression in the TM₂ and TM₃ groups were significantly higher than the control and TM₁ groups, which remained comparable (Figure 5A) ($p<0.05$). On posthatch days 10 and 28, TM groups presented significantly higher PCNA mRNA expression compared with the controls ($p<0.05$), with the highest expression observed in TM₂ and TM₃ groups in a time-dependent manner (Figure 5B) ($p<0.05$).

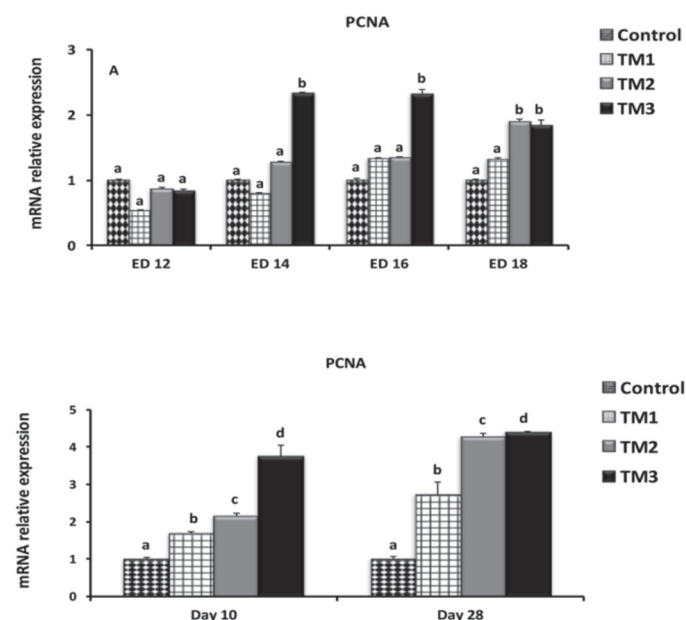


Figure 5 – The effect of thermal manipulation (TM) during embryogenesis on PCNA gene expression during embryonic days 12-18 (A) and posthatch days 10 and 28 (B) (n = 20). Control= 37.8°C; TM1= Thermal manipulation at 39°C for 9 h; TM2= Thermal manipulation at 39°C for 12h; TM3= Thermal manipulation at 39°C for 18 h. a–d Within the same day, means \pm SD with different superscripts differ significantly ($p<0.05$).

The effects of embryo thermal manipulation on Pax7 mRNA expression on ED 12, 14, 16, and 18, and on posthatch days 10 and 28 are shown in Figure 6 A-B. On ED 12 and 14, Pax7 mRNA expression of all TM groups was significantly higher relative to the control (Figure 6A) ($p<0.05$). However, on ED 16, higher Pax7 expression was observed only in the TM₃ group ($p<0.05$), while on ED 18, Pax7 expression was higher in the TM₂ and TM₃ groups compared with the TM₁ and control groups, which remained comparable (Figure 6A) ($p<0.05$). On day 10 posthatch, except for TM₁, TM groups presented significantly higher Pax7

mRNA expression compared with the control, with the highest expression observed in the TM₃ group (Figure 6B) ($p<0.05$). On day 28 posthatch, TM significantly increased in Pax7 mRNA expression compared with the control, with the highest expression observed in the TM₂ and TM₃ groups (Figure 6B) ($p<0.05$).

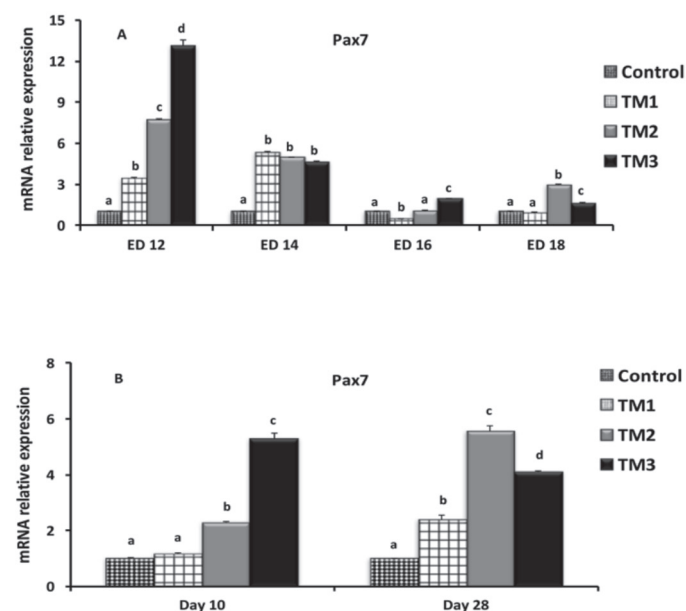


Figure 6 – The effect of thermal manipulation (TM) during embryogenesis on Pax7 gene expression during embryonic days 12-18 (A) and posthatch days 10 and 28 (B) (n = 20). Control= 37.8°C; TM1= Thermal manipulation at 39°C for 9 h; TM2= Thermal manipulation at 39°C for 12h; TM3= Thermal manipulation at 39°C for 18 h. a–d Within the same day, means \pm SD with different superscripts differ significantly ($p<0.05$).

Effects of thermal manipulation on mRNA expression levels of muscle growth factors (IGF-1, GH and myostatin).

The effects of embryo thermal manipulation on IGF-1 mRNA expression on embryonic days 12, 14, 16, and 18, and on posthatch days 10 and 28 are shown in Figure 7 A-B. From ED 12 to ED 16, TM significantly increased IGF-1 mRNA expression compared with the control (Figure 7A) ($p<0.05$). On ED 18, IGF-1 mRNA expression of TM₂ and TM₃ groups was significantly higher compared with the control and TM₁ groups, which remained comparable (Figure 7A) ($p<0.05$). The highest IGF-1 mRNA expression was observed in the TM₂ group (15-fold increase) on ED 14 (Figure 7A) ($p<0.05$). On posthatch days 10 and 28, TM resulted in significant increases of IGF-1 expression compared with the control (Figure 7B) ($p<0.05$). At 10 days of age, the highest IGF-1 mRNA expression was observed in the TM₂ and TM₃ groups ($p<0.05$), while at 28 days of age, TM₁ and TM₂ groups presented higher IGF-1 mRNA expression compared with the other treatment groups (Figure 7B) ($p<0.05$).

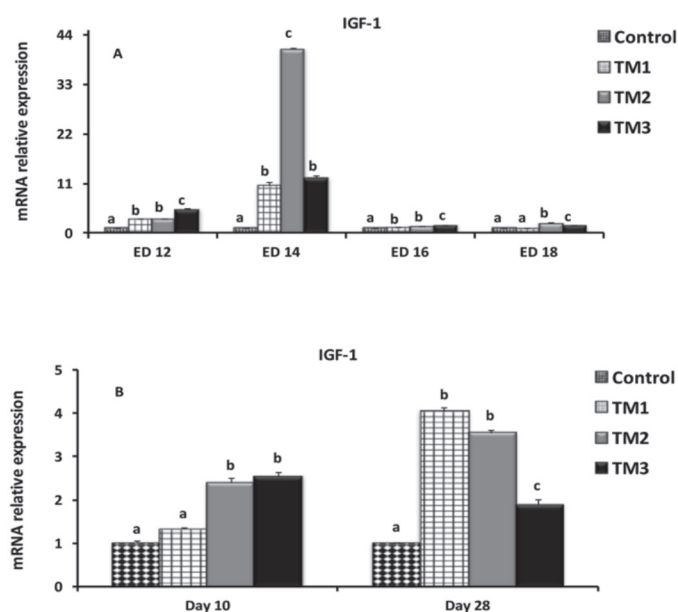


Figure 7 – The effect of thermal manipulation (TM) during embryogenesis on IGF-1 gene expression during embryonic days 12-18 (A) and posthatch days 10 and 28 (B) (n = 20). Control= 37.8°C; TM1= Thermal manipulation at 39°C for 9 h; TM2= Thermal manipulation at 39°C for 12h; TM3= Thermal manipulation at 39°C for 18 h. a–d Within the same day, means \pm SD with different superscripts differ significantly ($p < 0.05$).

The effects of embryo thermal manipulation on GH mRNA expression on embryonic days 12, 14, 16, and 18, and on posthatch days 10 and 28 are shown in figure 8 A-B. On ED 12, GH mRNA expression was significantly higher in the TM₂ group ($p < 0.05$)

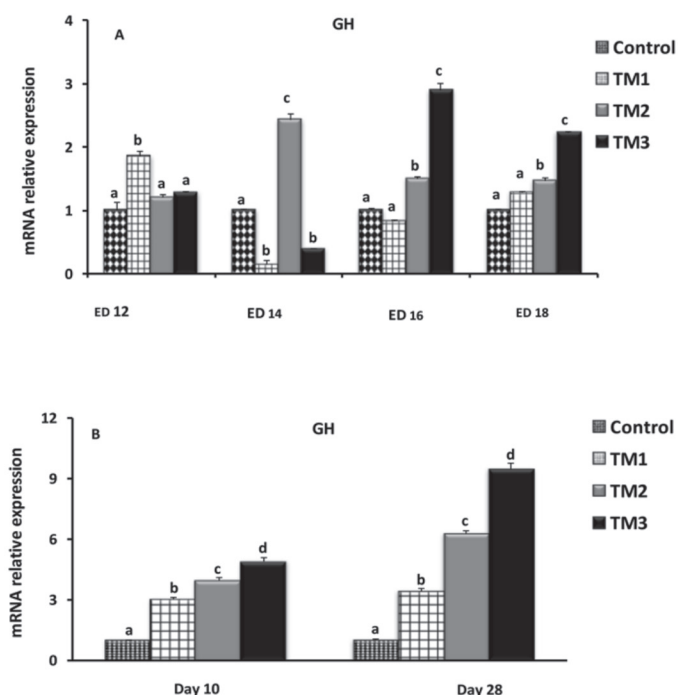


Figure 8 – The effect of thermal manipulation (TM) during embryogenesis on GH gene expression during embryonic days 12-18 (A) and posthatch days 10 and 28 (B) (n = 20). Control= 37.8°C; TM1= Thermal manipulation at 39°C for 9 h; TM2= Thermal manipulation at 39°C for 12h; TM3= Thermal manipulation at 39°C for 18 h. a–c Within the same day, means \pm SD with different superscripts differ significantly ($p < 0.05$).

compared with other groups, which remained comparable (Figure 8A) ($p > 0.05$). However, on ED 14, GH mRNA expression was significantly higher in the TM₂ group and significantly lower in TM₁ and TM₃ groups ($p < 0.05$) compared with the control (Figure 8A). On ED 16 and 18 of age, TM significantly increased mRNA expression of GH in TM₂ and TM₃ ($p < 0.05$) compared with the TM₁ and control groups, which remained comparable (Figure 8A) ($p > 0.05$). On posthatch days 10 and 28, TM significantly increased GH mRNA expression compared to control in a time-dependent manner (Figure 8B) ($p < 0.05$).

The effects of embryo thermal manipulation on myostatin mRNA expression on ED 12, 14, 16, and 18, and on posthatch days 10 and 28 are shown in figure 9 A-B. On ED 12, 14, and 16, TM significantly decreased myostatin mRNA expression relative to the control (Figure 9A) ($p < 0.05$). However, on ED 18, TM significantly increased myostatin mRNA expression compared with the control (Figure 8A) ($p < 0.05$). On posthatch day 10, except for the TM₁ group, TM significantly increased myostatin mRNA expression compared with the control (Figure 9B) ($p < 0.05$). On posthatch day 28, TM significantly increased myostatin mRNA expression compared with the control; however, the increase was more pronounced in the TM₁ group followed by the TM₂ group, and finally by the TM₃ group (Figure 9B) ($p < 0.05$).

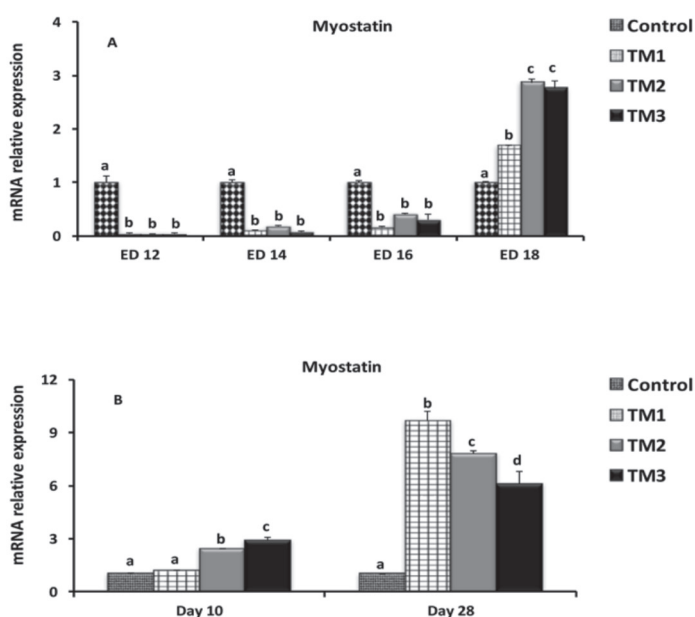


Figure 9 – The effect of thermal manipulation (TM) during embryogenesis on myostatin gene expression during embryonic days 12-18 (A) and posthatch days 10 and 28 (B) (n = 20). Control= 37.8°C; TM1= Thermal manipulation at 39°C for 9 h; TM2= Thermal manipulation at 39°C for 12h; TM3= Thermal manipulation at 39°C for 18 h. a–d Within the same day, means \pm SD with different superscripts differ significantly ($p < 0.05$).



DISCUSSION

Due to genetic selection, broiler chicken growth rates have increased, and as a result, the production cycle time has decreased by 60% over the last 40 years (Hulet, 2007; Baghbanzadeh & Decuypere, 2008). Thus, the incubation period accounts for a larger proportion of the broiler chicken life span (Havenstein *et al.*, 2003). Studies of incubation conditions (Lourens *et al.*, 2005; Lourens *et al.*, 2007) demonstrate the importance of temperature on embryo and chick development (Decuypere & Michels, 1992), particularly on hatchability, chick quality, and subsequent live performance (Decuypere & Michels, 1992; Lourens *et al.*, 2005).

Thermal manipulation at mid-term or at late-term broiler embryogenesis has been shown to enhance muscle hypertrophy as a result of increasing proliferation and differentiation of both fetal and adult myoblasts, leading to an increased myogenic cell pool in the embryo and posthatch broilers (Piestun *et al.*, 2009; Piestun *et al.*, 2015). The aim of this study was to investigate the molecular basis of muscle growth and development in broiler chickens subjected to daily thermal manipulation (39°C and 65% for 9, 12, or 18 h) during ED 12-18.

In this study, TM at 39°C on ED 12-18 resulted in a slight improvement in embryonic BW of all TM groups on ED 18 when compared with the controls. However, during the first three weeks of age, chicks in all treatment groups had similar body weights ($p>0.05$). However, on posthatch days 28 and 35, the BW of TM₂ and TM₃ chicks was significantly higher when compared with those of TM₁ and controls ($p<0.05$).

Halevy *et al.* (2001) reported that mild heat exposure of broilers at an early age resulted in the acceleration of satellite cell myogenesis mediated by specific local growth factor expression. Furthermore, embryos subjected to TM (39.5°C on ED 16-18 for 6 h daily), during satellite cell population expansion, resulted in larger myofiber diameter and increased absolute muscle growth in treated chicks relative to controls until day 35 of age (Piestun *et al.*, 2009). Similarly, TM during this period at 38.5°C for 3 h resulted in significantly higher absolute pectoral muscle weight in 42-d-old broilers relative to the controls (Hartley *et al.*, 1992; Halevy *et al.*, 2006; Collin *et al.*, 2007). The increase of muscle growth following late-term embryonic TM is thought to have both immediate and long-lasting effects on myogenic cell (satellite cells) proliferation and differentiation, and subsequent

hypertrophy (Halevy *et al.*, 2006; Piestun *et al.*, 2009; Piestun *et al.*, 2015).

Myoblast proliferation and differentiation are controlled by the muscle-specific basic helix-loop-helix (bHLH) family of transcription factors (Weintraub, 1993). The MyoD family, containing four basic helix-loop-helix (bHLH) transcription factors (MyoD, myogenic factor-5 (Myf5), myogenin, and myogenic regulatory factor-4 (MRF4)), positively regulates myogenesis (Weintraub, 1993). These factors are expressed in a sequential pattern when satellite cells are activated. Initially, Myf5 and MyoD are expressed in the proliferating progeny, and then myogenin is expressed as the cells begin to differentiate (Beauchamp *et al.*, 2000).

Our data showed that TM resulted in immediate stimulation of muscle MyoD mRNA expression on ED 12 and 14, with subsequent decline on ED 16 and 18. The highest expression was detected on ED 12 and 14 in embryos exposed to 18 h of thermal manipulation ($p<0.05$). On posthatch days 10 and 28, the only increase in the MyoD expression was observed in the TM₂ and TM₃ groups ($p<0.05$). It seems that TM has an immediate (ED 12 and 14) and late-term (posthatch days 10 and 28) effects on myoblasts proliferation as indicated by the increase of MyoD mRNA expression.

In this study, TM resulted in immediate stimulation of muscle myogenin mRNA expression on ED 12, 14, 16 and 18 in the TM₃ group, whereas in the other treatment groups, fluctuating myogenin expression was noted. On posthatch day 10, TM stimulated muscle myogenin mRNA expression compared with the controls ($p<0.05$), while on posthatch day 28, higher expression of myogenin was observed only in the TM₃ group ($p<0.05$). Similarly, it was reported that TM (39.5°C on ED 16-18 for 3 and 6 h) resulted in the enhancement of cell differentiation, as indicated by myogenin expression levels in the muscle (Piestun *et al.*, 2009). Our results indicate that, out of the TM conditions evaluated (39°C for 9, 12 or 18h), TM₃ (39°C for 18h) resulted in immediate stimulation (early-term effect) of myogenin mRNA expression during embryogenesis and a delayed stimulation on posthatch days 10 and 28 (late-term effect). These results are in contrast with those of Piestun *et al.* (2009) who reported that similar increase of muscle hypertrophy in heat-treated embryos, regardless TM length (3h vs. 6h).

In this study, TM (39°C for 18h) significantly increased muscle PCNA mRNA expression on ED 14, 16 and 18 ($p<0.05$), whereas TM (39°C for 12h) significantly increased muscle PCNA mRNA expression



on ED 18 ($p < 0.05$). On posthatch days 10 and 28, TM significantly increased PCNA expression ($p < 0.05$). Interestingly, the magnitude of the expression was time – and duration – dependent. The highest expression was detected on ED 14 and 16 in embryos exposed to 18h of thermal manipulation (TM_3) ($p < 0.05$). Furthermore, on posthatch days 10 and 28, the highest PCNA expression was observed in embryos exposed to 18 h of thermal manipulation (TM_3) ($p < 0.05$).

The paired-box transcription factor Pax7 is selectively expressed in quiescent and proliferating satellite cells and is thought to play a role in their self-renewal (Oustanina *et al.*, 2004; Zammit *et al.*, 2004). Pax7 has been suggested as an early marker of myogenesis during posthatch muscle growth, and its expression is sustained by satellite cells in the adult chicken muscle (Halevy *et al.*, 2004; Allouh *et al.*, 2008). Pax7 plays a key role in the formation of adult mouse skeletal muscle (Seale *et al.*, 2000). Furthermore, Pax7 is expressed during myoblast proliferation and declines during differentiation (Halevy *et al.*, 2004).

Our data showed that TM resulted in immediate stimulation of muscle Pax7 mRNA expression on ED 12 and 14 ($p < 0.05$). On ED 16, an increase in the Pax7 expression was only detected in TM_3 , whereas on ED 18, higher expression of Pax7 was detected in the TM_2 and TM_3 groups ($p < 0.05$). Furthermore, Pax7 expression was significantly increased on posthatch day 10 and remained high on posthatch day 28 ($p < 0.05$). Interestingly, the magnitude of the expression was time – and duration – dependent.

TM had early-term (ED 12-18) and late-term (posthatch days 10 and 28) effects in elevating myoblast proliferation relative to controls. This was shown by the higher PCNA and Pax7 mRNA expressions in the pectoral muscle of TM chicks compared with the controls. Previously, Piestun *et al.* (2016) reported that TM (39.5°C on ED 16-18) resulted in immediate (ED17) and later effect (up to 2 wks posthatch) on myoblast proliferation, as shown by higher DNA incorporation of thymidine, higher number of muscle cells expressing PCNA in intact muscle, and higher Pax7 protein levels. Those authors suggested that TM enhanced muscle hypertrophy by stimulating myogenic progeny cells (satellite cells) proliferation.

In mammals and birds, muscle cell proliferation and differentiation are controlled by growth factors and hormones that play a pivotal role in promoting the growth of skeletal muscle. The proliferation of satellite cells is regulated by several growth factors and hormones including: insulin growth factor-1 (IGF-1)

(Adams & McCue, 1998; Adams *et al.*, 1999), fibroblast growth factor (Florini *et al.*, 1996), growth hormone (Sadowski *et al.*, 2001) and hepatocyte growth factor (Gal-Levi *et al.*, 1998).

Insulin-like growth factors (IGF-1 and IGF-2) comprise a family of polypeptide hormones related in its structure to insulin, and have multifunctional metabolic and anabolic properties. IGF-1 is produced and secreted in the liver, and has an endocrine function in many tissues, but it is also produced in many other cell types to provide paracrine and autocrine functions, such as in muscle tissues (Bhaumick & Bala, 1991; Baker *et al.*, 1993, Russell *et al.*, 1998). IGF-I is the main local growth factor, playing a role in both muscle growth and hypertrophy via its effect on satellite cells (Adams & McCue, 1998; Adams *et al.*, 1999).

In broilers, the crucial proliferation and differentiation of satellite cells occurs during the first days posthatch, after which satellite cell population declines dramatically (Halevy *et al.*, 2001). Previously, it was shown that TM resulted in a rapid induction of IGF-1 gene expression in the breast muscle derived from thermally-manipulated broiler embryos, concomitantly with the rise in satellite cell proliferation. IGF-1 has been also shown to stimulate primary satellite cell proliferation in rats (Allen & Boxhorn, 1989) and in chickens (Duclos *et al.*, 1991). In the present study, TM resulted in immediate stimulation of muscle IGF-1 mRNA expression during embryogenesis and on posthatch days 10 and 28 ($p < 0.05$). Interestingly, the magnitude of the expression was time – and duration – dependent. The highest expression was detected on ED 14 in embryos exposed to 12 h of thermal manipulation ($p < 0.05$). Furthermore, IGF-1 increased significantly on posthatch day 10 and remained high on posthatch day 28 ($p < 0.05$). Previously, high IGF-1 gene expression was observed in chicken breast muscle tissues in response to early age thermal manipulation (24 h at 3 days of age) (Halevy *et al.*, 2001), TM at 38.5 °C on ED 16-18 (Halevy *et al.*, 2006), and TM at 39.5 °C on ED 16-18 (Piestun *et al.*, 2009). This increase of IGF-1 expression was associated with significant increases in the BW of thermally-manipulated chicks on posthatch days 28 and 35 ($p < 0.05$).

Our data also indicate that TM resulted in immediate stimulation of muscle GH mRNA expression during embryogenesis and on posthatch days 10 and 28. Interestingly, the magnitude of the expression was time – and duration – dependent. The highest expression was detected on ED 16 and 18 in embryos exposed to 18 h of thermal manipulation (TM_3) ($p < 0.05$).



Furthermore, GH significantly increased on posthatch day 10 and remained high on posthatch day 28, with highest expression was again observed in TM₃ group ($p<0.05$).

The growth hormone plays a pivotal role in promoting the growth of mammalian skeletal muscle. It has been shown that administration of GH to humans, ruminants, and pigs enhanced their skeletal muscle growth (Chung *et al.*, 1985; Campbell *et al.*, 1989; Grant *et al.*, 1991; Brameld *et al.*, 1996; Tavares *et al.*, 2013; Jiang & Ge, 2014). Furthermore, IGF-I has been suggested to mediate the effect of GH on muscle growth. Isgaard *et al.* (1989) showed that, following hypophysectomy, IGF-I mRNA expression decreased and the replacement therapy with human GH restored those levels in the heart and skeletal muscles. IGF-I mRNA levels are also stimulated in liver tissue and skeletal muscle as a result of GH administration (Grant *et al.*, 1991).

Finally, TM resulted in an immediate decline of muscle myostatin mRNA expression on embryonic days 12, 14 and 16. However, starting from ED 18 and on posthatch days 10 and 28, TM significantly increased muscle myostatin mRNA expression ($p<0.05$).

Myostatin, known as growth/differentiation factor-8, is a member of the transforming growth factor- β (TGF- β) family of secreted regulatory factors (McPherron and Lee, 1997). Growth factors, such as myostatin, have also been identified as key peptides in the regulation of vertebrate's myogenesis (McPherron & Lee, 1997; Bass *et al.*, 1999). Myostatin peptide acts as a negative regulator of muscle growth (McPherron & Lee, 1997; Bass *et al.*, 1999). A myostatin mutation has been found in "double-musled" cattle, which have, on average, 20% more muscle mass than normal cattle (Bass *et al.*, 1999). *In-vitro* studies demonstrate that recombinant myostatin inhibits the proliferation of C2C12 myoblasts and bovine myoblasts derived from 160-day-old fetuses (Thomas *et al.*, 2000). Also, the inhibitory effect of myostatin was shown to be reversible, as myoblasts maintained their ability to proliferate when myostatin was removed (Kocamis & Killefer, 2002). Furthermore, satellite cell proliferation and differentiation are inhibited by myostatin, which maintains them in their quiescent state and down regulates the expression of MyoD family members (Halevy *et al.*, 2006).

Together, these findings suggest a differential effect of various periods of TM on broiler muscle growth and development. It seems that embryo thermal manipulation at 39 °C for 18h on ED 12-18 was

optimal for the enhancement of muscle growth and development. This was evidenced by the significant increases in the embryonic BW and the posthatch BW at days 28 and 35 of age ($p<0.05$). The improvement of the body weight was associated with immediate and long-lasting effects of TM on the expression of muscle growth factors (IGF-1 and GH) and muscle marker genes (MyoD, myogenin, and Pax7).

CONCLUSIONS

The results of the present study demonstrated that TM, by altering incubation temperature and relative humidity to 39°C and 65% RH daily during embryonic days 12-18 leads to changes in the mRNA expression of muscle marker genes (MyoD, myogenin and Pax7) and muscle growth factors (IGF-1 and GH), which were associated with body weight improvement during embryogenesis and posthatch. It seems that the out of the three different TM durations (9, 12 or 18 h), 18 hours at 39°C promoted the best performance parameter (body weight).

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